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SSR-BASED GENETIC DIVERSITY OF DATE PALM IN MAKRAN (PAKISTAN)

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Date palm is an important fruit crop of Pakistan with rich economic, social and nutritional values. The Baluchistan province of Pakistan has been well known for date palm cultivation since long. In addition to its long history of date palm cultivation, Baluchistan's geo-political position with Iran and Oman also highlights its position as a rich source of genetic diversity. The present study was aimed to estimate the genetic diversity and population structure of date palm in Makran division (Baluchistan), which is considered as a major groove for production of high quality date palm. A set of 52 microsatellite markers were utilized to analyze the genetic relationship among 61 date palm genotypes. High polymorphism was found with an average polymorphic Information content (PIC) of 0.67 which indicates significant genetic variation in date palm accession under study. A total 387 alleles were scored with an average of 8.23 alleles for each locus. According to the Bayesian clustering approach, the entire population was partitioned into two clusters i.e., Turbat and Panjgur with few admixture lines. The presence of two subgroups corresponded to distinct adaptive behavior of genotypes to different ecological environment. Analysis of molecular variance revealed 80% of the variation within individuals, while 16% was due to differences among individuals and 4% variation was found among populations. Principal coordinate analysis and neighbor joining tree exhibited the same clusters as illustrated in STRUCTURE V.2.2 software. The variation detected through SSR markers supports that Baluchistan region may be another center of diversity for date palm. The genetic diversity information from this study can also be used for future breeding programs, association mapping, gene cloning and germplasm conservation.
Keywords: Date palm, genetic diversity, origin, DNA fingerprinting, microsatellites.

INTRODUCTION

Phoenix dactylifera (Date Palm L., 2n= 2x=36) is a member of family *Arecaceae,* a monocotyledonous, perennial and dioecious fruit tree (Barrow, 1998, 1999). It is the ancient cultivated fruit tree which is considered as the first plant pollinated by the human (Zohary and Spiegel-Roy, 1975). Date palm can fairly tolerate desert and semi- arid conditions with high temperature and minimum rainfall and humidity (Barrow, 1998). Arabian Peninsula, Iran, Africa, and Pakistan are the famous regions where date palm is abundantly grown for its edible sweet and tasty fruit with high nutritional value. In many developing areas, date palm has been used for livelihood to reduce food uncertainties. Several uses along with high profitability and productivity make date palm a preference for both small and medium growers (Al-Shahib and Marshall, 2003). It is believed that date palm cultivation started at least 7000 years ago and its domestication traces back to 5000 years ago (Wrigley, 1995). Date palm is reported to be first planted in Mesopotamia and considered to be cultivated in southern Mesopotamia since the 5th millennium BC (Hanachi *et al*., 1998; Zohary and Hopf, 2000). It is widely spread crop tree in Middle East and North Africa (Zohary and Hopf, 2000). Historically, date palm is distributed from west (Mauritania) to north Western India and East (which is Pakistan) (Pintaud *et al*., 2013). Its cultivation had extended not only to Sub-Saharan Africa but also to other countries including Australia, California, South America, Namibia, Peru and Afghanistan (Nixon, 1951; Barrow, 1998; Zaid and de Wet, 2002a; McCubbin, 2007). Pakistan is recognized for its diverse agro-climatic conditions and stands third in list of date palm producing countries after Middle East and Africa. Baluchistan province of Pakistan is gifted with a distinctive environment which is suitable to produce quality date palms. Over 130 varieties of dates have been recorded in this province. According to one estimate Makran produces 0.5 million tons of dates every year (https://www.dawn.com/news/294012). Presence of two carbonized seeds of *Phoenix* species, Shahi Tump and Miri Qalat, has also been reported from two regions of Baluchistan (Tengberg, 1998, 1999b). The Baluchistan region is not only known for ancient history of date cultivation but also for strong system of trade, cultural norms and beliefs, movement of people, exchange of gifts with middle east countries including Iran and Oman. DNA fingerprinting is the most precise and reliable method for cataloging of cultivars as

DNA content of cell is not confounded to growth stage, environmental conditions and status of the cell and plant organ **(**Ainsworth *et al*., 1996). Furthermore, DNA markers do not depend on epistatic and pleiotropic effects and fairly overcome the discriminatory power of morphological phenotyping (Yusuf *et al*., 2015). Microsatellite (Simple Sequence Repeat) markers have been recognized as an excellent platform for plant genome analysis because of their reproducibility, high polymorphism, co-dominance and locus specificity (Hamwieh *et al*., 2011). The effectiveness of SSR markers for measuring the genetic diversity and relations among date palm varieties is reported in various studies like Tunisia (Zehdi *et al*., 2004; Zehdi *et al*., 2012; Hamza *et al*., 2013), Sudan (Elshibli and Korpelainen, 2008), Oman (Al-Ruqaishi *et al*., 2008), Qatar (Ahmed and Al-Qaradawi, 2009; Elmeer *et al*., 2011; Ahmed and Al- Hadidy, 2014), Iraq (Khierallah *et al*., 2011), Libya (Al-Racchi *et al*., 2014), Iran (Arabnezhad *et al*., 2012) and UAE (Chaluvadi *et al*., 2014). The major objective of the current study was to unravel the genetic diversity in date palm genotypes of Baluchistan to explore this region's position as a date palm center of diversity.

MATERIALS AND METHODS

***Plant material*:** Date palm leaf samples of sixty-one genotypes were collected from mature trees. Thirty-seven of these genotypes were selected from Turbat district and twenty four genotypes from Panjgur district as listed in Table 1. Geographical coordinates of Baluchistan genotypes were recorded using the geographical position system (GPS) device (GARMIN) ®, USA (Fig 1). Leaves were collected and stored at -80°C till DNA extraction.

***DNA extraction*:** Frozen leaves were grounded into fine powder using liquid nitrogen. DNA was extracted from leaf powder by modified CTAB method (Doyle and Doyle, 1987). Spectrophotometer (NanoDrop^{TM} 2000c, Thermo scientific Inc, USA) was used to assess the purity and concentration of DNA. DNA quality was evaluated by visualization under UV on 0.8% agarose gels (Sambrook *et al*., 1989).

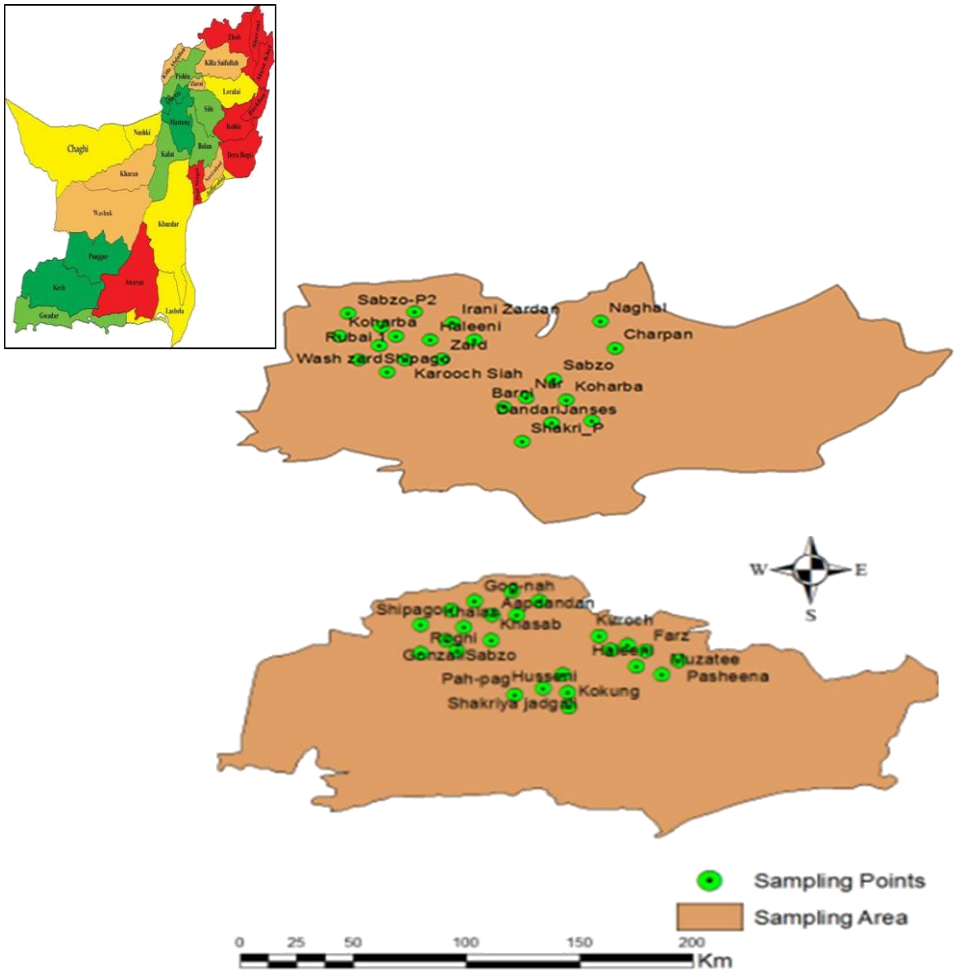

Figure 1. GPS based position of sixty one genotypes collected from two districts of Baluchistan

Table 1. List of sixty-one genotypes used for diversity analysis.

S.No.	Genotypes	Genotypes	District	Longitude(DD)	Latitude (DD)
1	B1	Pashpag	Turbat	63.043	26.006
2	B2	Shakriya Jadgali	-	63.043	26.006
3	B3	Kokung	-	63.044	26.007
4	B4	Hussaini	-	63.044	26.007
5	B5	Begum Jangi	-	63.044	26.007
6	B6	Karooch	-	63.044	26.008
7	B7	Haleni Gon	-	63.045	26.008
8	B8	Chapshuk	-	63.045	26.008
9	B9	Haleni	-	63.052	26.016
10	B10	Farz	-	63.052	26.016
11	B11	Mozati	-	63.052	25.016
12	B12	Pasheena	-	63.052	26.016
13	B13	Karooch #2	-	63.052	26.015
14	B14	Konzenabad	-	63.052	26.016
15	B15	Gush male	-	63.052	26.015
16	B16	Aapdandan	-	63.052	26.015
17	B17	Goknah	-	63.052	26.016
18	B18	Zard begum jangi	-	63.052	26.016
19	B19	Khalas	-	63.053	25.988
20	B20	Khasab	-	63.051	25.988
21	B21	Sabzo	-	63.050	25.990
22	B22	Gonzali	-	63.051	25.990
23	B23	Shipago	-	63.043	26.008
24	B24	Rogini	-	63.043	26.009
25	B25	Dishtari	-	63.043	26.009
26	B26	Johana	-	63.035	26.008
27	B27	Kaneezi	-	63.034	26.008
28	B28	Zardabdandan	-	63.030	26.008
29	B29	Misrishakar	-	63.030	26.009
30	B30	Pul	-	63.028	26.009
31	B31	Gunsh	-	63.028	26.009
32	B32	Konzenabad	-	63.052	26.017
33	B33	Shakar	-	63.052	26.016
34	B34	Shakri	-	63.052	26.016
35	B35	Karooch	-	63.052	26.016
36	B36	Mozati	-	63.051	25.991
37	B37	Rogini 2	-	63.051	25.992
38	B38	Berni	Panjgur	64.1044	26.9776
39	B39	Nar	-	64.1062	26.9780
40	B40	Sabzo_P	-	64.1056	26.9786
41	B41	Zard Karooch	-	64.1069	26.9780
42	B42	Janses	-	64.1008	26.9776
43	B43	Shakri_P	-	64.1081	26.9766
44	B44	Mozzawati-P	-	64.1087	26.9738
45	B45	Karoochzard 2	-	64.1093	26.9730
46	B46	Haleni	-	64.1086	26.9727
47	B47	Zard	-	64.1083	26.9738
48	B48	Karooch Siah	-	64.1073	26.9693
49	B49	Rubai 1	-	64.1072	26.9698
50	B50	Kaharaba	-	64.1071	26.9697
51	B51	Wash zard	-	64.1080	29.9677
52	B52	Khashkeench	-	64.1081	29.9680
53	B53	Shipago-P	-	64.1082	29.9680
54	B54	IraniZardan	-	64.1079	26.9670
55	B55	Dandari	-	64.1079	26.9672
56	B56	Kaharaba-P	-	64.1079	26.9674
57	B57	Rubai-P2	-	64.1097	26.9609
58	B58	Sabzo-P2	-	64.1097	26.9610
59	B59	Naghal	-	64.1097	26.9611
60	B60	Charpan	-	64.1103	26.9619
61	B61	Jesh	-	64.1103	26.9620

***Microsatellite analysis*:** Fifty-two public SSR markers of date palm (Billotte *et al*., 2004; Akkak *et al*., 2009; Ludena *et al*., 2011; Aberlenc-Bertossi, 2014) were used for evaluating genetic diversity (Table 2). PCR reactions were accomplished using 15 ng DNA, 10X PCR buffer, 0.2 mM of each deoxynucleotide, 1.5mM of $MgCl_2$, 0.1mM of each forward and reverse primer and 1unit of *Taq polymerase* (Fermentas, USA) to make a total volume of 20 μL. Thermocycler (Bio-Rad T-100) was used to conduct amplification by setting initial denaturation at 95°C for 4 min, followed by denaturation at 95°C for 30 s, annealing temperature for 30s (specific for every oligo pair), extension for 1min at 72°C (35 cycles) and a final elongation of 8 min at 72°C. 2.5% agarose gels prepared in 1x TAE buffer and stained with ethidium bromide were visualized to check amplification. Gels were illuminated with UV light using Gel documentation system (Bio-Rad Chemi-doc™ XRS+, USA). Successfully amplified samples were resolved on 6.5% denaturing polyacrylamide gels stained with ethidium bromide. The size of alleles across genotypes was confirmed on gels using 50bp DNA Ladder (Thermo Scientific, USA). The DNA bands were visualized on a UV trans- illuminator and gel documentation system (Bio-Rad Chemi-doc™ XRS+, USA) was used for recording data.

***Data analysis*:** A binary matrix was created for PCR results and each band was treated as a distinct allele and scored for presence or absence. The presence and absence of each single band was recorded as 1 and 0, respectively. Power Marker 3.25 program (Liu and Muse, 2005) was used to compute the total number of alleles (NA), total number of genotypes (NG), gene diversity, the major allele frequency and the polymorphic information content (PIC) at each locus as formulated by Roy *et al*., 2002. The microsatellite tool kit was used to calculate the parameters of observed (H_o) and expected heterozygosity (H_e). The values of inbreeding coefficient (FIS), the genetic differentiation (FST and Gst) were calculated using the software GenAlEx v6.5 (Peakall and Smouse, 2012). STRUCTURE v2.2 software designed by Pritchard *et al*., 2000 was used to determine the population structure. This software uses combination of related allelic frequencies and admixture without any prior information about the geographic origin of the genotypes. Structure Harvester by Earl and von Holdt, 2011 was used to locate the optimum number of K and the statistical test to count the allelic difference among different groups was conducted in R using FPTestR as reported by Fu *et al*., 2003. GenALEx 6.5 programming (Peakall and Smouse, 2012) was used to recognize the proportion of differentiation among individuals of two regions by performing analysis of molecular variance (AMOVA). Principal Coordinate analysis and cluster analysis based on Nei's genetic distance matrix (Nei, 1972) were performed using DARwin v. 5.0.157 software (Perried and Jacquemound-Collet, 2006).

Table 2. List of fifty two Primers used for diversity analysis.

Sr.	Primer name	primer sequence	Reference
1	mPdCIR10	5'-ACCCCGGACGTGAGGTG-3' 5'-CGTCGATCTCCTCCTTTGTCTC-3'	Biollete *et al.* (2004)
2	mPdCIR13	5'-GCGGAGACAGGAGATGGTAA-3' 5'-CTTGACTGCTTCTGCTGCTG-3'	-d0-
3	mPdCIR15	5'-AGCTGGCTCCTCCCTTCTTA-3' 5'-GCTCGGTTGGACTTGTTCT-3'	-d0-
4	mPdCIR16	5'-AGCGGGAAATGAAAAGGTAT-3' 5'-ATGAAAACGTGCCAAATGTC-3'	-d0-
5	mPdCIR25	5'-CAAATCTTTGCCGTGAG-3' 5'-GGTGTGGAGTAATCATGTAGTAG-3'	-d0-
6	mPdCIR31	5'-GCAGGTGGACTGCAAAATCT-3' 5'-CTATTGGGGTGCTGATCCAT-3'	-do-
7	mPdCIR32	5'-CAAGACCCAAGGCTAAC-3' 5'-GACAAGAGGGAAGGGGAGAG-3'	-d0-
8	mPdCIR33	5'-GGAGCATACAGTGGGTTTGC-3' 5'-CAGCCTGGGAATGAGGATAG-3'	-do-
9	mPdCIR35	5'-ACAAACGGCGATGGGATTAC-3' 5'-CCGCAGCTCACCTCTTCTAT-3'	-d0-
10	mPdCIR40	5'-GAGAGATGCGTCAGGGAATC-3' 5'-CCAGAATCTTCCAAGCAAGC-3'	-d0-
11	mPdCIR50	5'-CTGCCATTTCTTCTGAC-3' 5'-CACCATGCACAAAAATG-3'	-d0-
12	mPdCIR57	5'-GGTGTTTGGGCCTATTTCCT-3' 5'-GTTCTCACTCGCCCAAAAATAC-3'	-d0-
13	mPdCIR63	5'-CTTTTATGTGGTCTGAGAGA-3' 5'-TCTCTGATCTTGGGTTCTGT-3'	-d0-
14	mPdCIR70	5'-CCATTTATCATTCCCTCTCTTG-3' 5'-CTTGGTAGCTGCGTTTCTTG-3'	-d0-
15	mPdCIR78	5'-CCCCTCATTAGGATTCTAC-3' 5'-GCACGAGAAGGCTTATAGT-3'	-do-
16	mPdCIR85	5'-TGGATTTCCATTGTGAG-3' 5'-CCCGAAGAGACGCTATT-3'	-d0-
17	mPdCIR90	5'-GCAGTCAGTCCCTCATA-3' 5'-TGCTTGTAGCCCTTCAG-3'	-do-
18	mPdCIR93	5'-GAGAGAGGGTGGTGTTATT-3' 5'-TTCATCCAGAACCACAGTA-3'	-do-
19	DPALM100	5'-GCCACTATCACCATTGCTGT-3' 5'-CAATGGAGGTCGTAGTGGTG-3'	Hamwieh *et al.* (2010)
20	DPALM103	5'-TTCCATCCCTGGAGAAAGG-3' 5'-AACCCAACCTAAGCCCTACC-3'	-d0-
21	DPALM104	5'-GGAAAGTTTCGGAACATTTTGT-3' 5'-AACCAAGACATCGTCCCAAG-3'	-d0-
22	DPALM107	5'-GGAAGGCGTCAAGGTATCTC-3' 5'-ACAACACGGGGAAAGAACAT-3'	-d0-
23	DPALM110	5'-TGTCACATTATTTGAGCATAATCCA-3' 5'-ACCCTTTGTTGATGCACCTC-3'	-d0-
24	DPALM112	5'-AGCAGGTTCATGGTTTGCTT-3' 5'-AGAACCAGGGAGGATGAGGT-3'	-d0-
25	DPALM113	5'-GGTCCCGACGCCTATTTTAT-3' 5'-AGCAAAGTCCACCCCTTTTT-3'	-d0-
26	DPALM119	5'-TGCGCTAAATAGTTCCCTTCA-3' 5'-CACATTCACAAGGCCTGCTA-3'	-d0-
27	DPALM120	5'-TTCAATTCATCCCACTGCAA-3' 5'-CACCAACATGAGCAAATGGA-3'	-d0-
28	DPALM121	5'-CATATGATTGTGATGGGGACA-3' 5'-CACCTCTCCGAGAAAACCAG-3'	-d0-
29	DPALM123	5'-GGCAGGTGGATTGTTCTTGT-3' 5'-CAGGGGTATGGAGAGAGAGAGA-3'	-d0-
30	DPALM125	5'-TTATGCTGAGGCCAGGTTTT-3' 5'-CATGCTGCAGAACCTGAAGA-3'	-d0-
31	DPALM132	5'-TCAGCTCAAAGCACACAACA-3' 5'-CCGGAGATTTTGTTTCGATG-3'	-d0-
32	DPALM133	5'-CAGATGGGATCGTTTTACCTG-3' 5'-CCGTATCGGGAGAGAGAGAG-3'	-d0-
33	DPALM139	5'-TCTCGATCTCGACCTTGGTT-3' 5'-CGGATCCGGTTCTCTCATTA-3'	-d0-
34	DPALM141	5'-CATTGCTCAGAAGCATCCAA-3' 5'-CTCTCCCTCCCTCTCGTTCT-3'	-d0-
35	DPALM142	5'-CAATGGACCACCAAAATCAA-3' 5'-CTCTCCGAGAAAACCAGGTC-3'	-d0-
36	DPALM144	5'-ACACACACACACACGCGAAT-3' 5'-CTTGCAGCCATTTAGGCAAC-3'	-d0-
37	DPALM146	5'-ATGATTGAGAGGCAGGCAAA-3' 5'-GACAAGAGGGAAGGGGAGAG-3'	-d0-
38	DP151	5'-TTGCTGGTTGAAATGGTGTT-3' 5'-GCAACAGATGCTCTTGCTCA-3'	Chaluvadi *et al.* (2014)
39	DP159	5'-AGCTCCAATTTGCTGCAGAG-3' 5'-GCTGACCTGGAGTCCAAAAC-3'	-d0-
40	DP160	5'-AAGAGCGACAATCATGACCA-3' 5'-GGAAATTGAAGGGCATCTTG-3'	-d0-
41	DP168	5'-GCAGCAAAGCCCTTAGGC-3' 5'-GGTGTTATGTGCAGCCAATG-3'	-d0-
42	DP169	5'-GCATGGACTTAATGCTGGGTA-3' 5'-GGTTTTCCTGCCAACAACAT-3'	-d0-
43	DP170	5'-TCTTTGGGCTTACGACAACC-3' 5'-GTATGGCCCAAGATGCAGAT-3'	-d0-
44	DP171	5'-GTGGGAGTAGCGAGGTATGG-3' 5'-GTCCGGCACTTTAGGAAGTT-3'	-d0-
45	DP172	5'-GGTGTTTGGGCCTATTTCCT-3' 5'-GTCCTCCTCCTCCTCTGTCC-3'	-d0-
46	DP175	5'-ACACACACACACACACACACC-3' 5'-GTGGCTTCTTTTTGGCTGTC-3'	-d0-
47	PDCAT2	5'-GCCTTCTCTTCCCTAATGGG -3' 5'-AGTTTCTTGCCCCTGTTCTTTC-3'	Akkak *et al.* (2009)
48	PDCAT6	5'-AATCAGGGAAACCACAGCCA-3' 5'GTTTAAAGCCTTCTCAAGATAGCCTCAG3'	-d0-
49	PDCAT11	5'-TTAGTAGACTCCCCACCGTCC-3' 5'-TGTTTCATGGTGCTGGAGAATGAA-3'	-d0-
50	PdAG1-ssr	5'-TCTGATTTCGTTTACTTCTTAGGA-3' 5'-TTCATATTCAGTTGTCGGGTGTA-3'	Ludena *et al.* (2011)
51	PdCUC3-ssr1	5'-CGTGGACTCATGACTCGCATGTCC-3' 5'-GGTCCTTGCCGGTGGCCTTC-3'	Zehdi-Azouzi *et al.* (2015)
52	PDCUC3-ssr2	5'-ACATTGCTCTTTTGCCATGGGCT-3' 5'-CGAGCAGGTGGGGTTCGGGT-3'	-d0-

RESULTS

***SSR based polymorphism*:** Fifty-two primers were used to analyze genetic difference in sixty-one genotypes of date palm. Two primers did not amplify while three primers found to be monomorphic. Forty-seven primers were found to be polymorphic with a total of 387 alleles, showing an average of 8.23 alleles for each locus (Table 3). Each marker and genotype showed varying number of alleles. The variation in number of alleles ranged from 3 to 14, as listed in Table 3.

Table 3. Genetic diversity obtained with forty seven SSR markers in sixty one date palm genotypes.

Marker	Major Allele. Freq	Genotye No	Allele. No	G.D	PIC	Ho	He	Fis	Fst	Gst
PDCAT2	0.18	24	15	0.87	0.86	0.93	0.87	-0.09	0.01	0.00
PDCAT6	0.21	20	13	0.87	0.86	0.98	0.88	-0.14	0.01	0.00
PDCAT 11	0.32	14	12	0.83	0.81	0.94	0.80	-0.18	0.01	0.00
DPALM119	0.81	3	5	0.32	0.29	0.10	0.09	-0.09	0.04	0.03
DPALM110	0.74	3	5	0.42	0.38	0.94	0.51	-0.93	0.00	0.00
DPALM 112	0.86	3	5	0.25	0.23	0.13	0.12	-0.07	0.00	-0.01
DPALM 139	0.36	9	9	0.74	0.70	0.33	0.69	0.31	0.30	0.28
DPALM 121	0.34	5	7	0.74	0.70	0.98	0.67	-0.63	0.06	0.06
DPALM 144	0.25	8	7	0.78	0.74	0.84	0.76	-0.20	0.04	0.03
DP 146	0.23	14	10	0.84	0.82	0.98	0.83	-0.20	0.00	0.00
DP 125	0.44	7	7	0.69	0.64	0.67	0.57	-0.23	0.01	0.00
DP 160	0.27	9	7	0.77	0.74	0.84	0.72	-0.18	0.01	0.00
DP 168	0.39	7	8	0.71	0.66	0.57	0.61	0.03	0.01	0.00
DP 120	0.30	10	7	0.78	0.75	0.83	0.73	-0.20	0.01	0.00
DP 175	0.43	8	7	0.72	0.68	0.63	0.73	0.09	0.02	0.00
DP 104	0.41	9	7	0.73	0.69	0.91	0.69	-0.38	0.01	0.00
DP 103	0.43	7	7	0.70	0.65	0.86	0.67	-0.36	0.02	0.02
DP 107	0.51	7	7	0.66	0.61	0.40	0.65	0.30	0.17	0.15
DP 123	0.42	9	7	0.73	0.69	0.77	0.69	-0.15	0.02	0.01
DP 151	0.36	9	10	0.75	0.72	0.65	0.71	-0.03	0.11	0.10
DP 141	0.48	4	7	0.64	0.56	0.72	0.51	-0.46	0.02	0.02
DP159	0.26	9	7	0.79	0.76	0.67	0.75	0.07	0.03	0.01
DP169	0.39	11	10	0.76	0.73	0.79	0.72	-0.14	0.02	0.01
DP170	0.27	9	7	0.78	0.74	0.72	0.72	-0.01	0.00	-0.01
DP171	0.32	12	9	0.79	0.76	0.96	0.75	-0.32	0.01	0.00
DP172	0.73	4	6	0.44	0.41	0.21	0.44	0.44	0.08	0.07
mPdCIR 16	0.34	17	10	0.79	0.76	0.85	0.78	-0.13	0.02	0.01
mPdCIR 35	0.47	4	7	0.66	0.60	0.98	0.62	-0.62	0.00	0.00
mPdCIR 40	0.39	4	4	0.66	0.58	0.50	0.51	0.01	0.00	-0.01
mPdCIR 32	0.30	18	12	0.84	0.83	0.93	0.82	-0.16	0.01	0.00
mPdCIR 31	0.52	7	7	0.61	0.54	0.49	0.50	0.06	0.03	0.02
mPdCIR 70	0.26	16	12	0.81	0.78	0.69	0.78	0.01	0.04	0.02
mPdCIR 25	0.22	30	16	0.87	0.86	0.93	0.87	-0.11	0.02	0.01
mPdCIR 33	0.40	4	5	0.65	0.57	0.53	0.50	-0.17	0.00	-0.01
mPdCIR 57	0.39	4	7	0.66	0.59	0.71	0.50	-0.45	0.01	0.01
mPdCIR 63	0.48	3	7	0.57	0.48	0.99	0.58	-0.73	0.01	0.01
mPdCIR 85	0.36	7	7	0.70	0.64	0.93	0.59	-0.69	0.01	0.00
mPdCIR 50	0.54	4	5	0.59	0.52	0.42	0.36	-0.17	0.00	-0.01
mPdCIR 93	0.39	9	7	0.72	0.67	0.75	0.68	-0.18	0.02	0.01
mPdCIR 78	0.54	4	5	0.59	0.52	0.42	0.36	-0.17	0.00	-0.01
mPdCIR 90	0.40	9	7	0.74	0.70	0.65	0.70	-0.02	0.06	0.05
mPdCIR 15	0.40	4	7	0.66	0.58	0.71	0.49	-0.44	0.00	0.00
mPdCIR 13	0.31	8	7	0.78	0.74	0.88	0.75	-0.19	0.01	0.00
mPdCIR 10	0.22	18	10	0.84	0.82	0.92	0.82	-0.13	0.02	0.01
PdAG1-ssr	0.21	30	16	0.88	0.87	0.94	0.88	-0.15	0.03	0.02
PDCUC3-ssr2	0.20	20	12	0.87	0.85	0.94	0.86	-0.16	0.04	0.03
PdCUC3-ssr1	0.44	6	9	0.72	0.68	0.98	0.74	-0.46	0.06	0.05
Total			387.00	33.33		34.51	30.55			

* G.D, genetic diversity; PIC, Polymorphic information content; Ho, observed heterozygosity; He, expected heterozygosity; Fis, fixation index values; Fst, Wright's analysis of hierarchical F-statistics; Gst, Genetic differentiation among groups

The highest number of alleles was detected for the markers PdAG1-ssr and mPdCIR25 and the lowest were detected for a group of markers viz., mPdCIR40, mPdCIR50, mPdCIR78, mPdCIR33, DPALM112, DPALM110 and DPALM119. The major alleles represented a profound variation in allelic frequency that varied from 0.18 (PDCAT2) to 0.86 (DPALM112). The number of genotypes for each locus ranged from 3 (DPALM 119, DPALM110, DPALM112, mPdCIR63) to 30 (PdAG1-ssr&25) with an average of 9.78.

***Genetic diversity analysis*:** The average value of PIC parameter for forty-seven loci was 0.67 (Table 3). It ranged from 0.23 (DPALM112) to 0.86 (PDCAT2). Based on high

Table 4. Analysis of Molecular Variance (AMOVA) for sixty one genotypes of Baluchistan using forty seven SSR markers

Source	df	SS	MS	Estimated Variation	%
Among populations	1	55.6	55.6	0.6	4%
Among Individuals	59	1133.75	19.21	2.7	16%
Within Individuals	61	843.5	13.82	13.8	80%
Total	121	2032.89		17.14	100%

The population level included two populations: Turbat and Panjgur; The individual level considered all the sixty one genotypes of Baluchistan.

PIC value, PDCAT2 was found most informative marker. The genetic diversity ranged from 0.24 to 0.88 at loci DPALM 112 and PdAG1-ssr respectively with a mean value of 0.70. The results showed that observed and expected values of heterozygosity were high. The minimum to maximum values of observed heterozygosity (H_o) were observed from 0.12 (DPALM 119) to 0.99 (mPdCIR35) respectively. The mean H_o was 0.73. The expected heterozygosity (H_e) for DPALM 119 was 0.1and 0.87 for mPdCIR25. The mean value of expected heterozygosity was 0.65 showing a wide range of genetic diversity for Pakistani germplasm. The Fst and Gst values ranged from- 0.93 to 0.44 and -0.01to 0.28 respectively. The low Fst and Gst values indicated a high level of genetic recombination indicating prominent differences among the genotypes. It further suggested that the maximum variation is locally sustained (Table 3). Molecular variance was assessed through AMOVA which partitioned the total genetic variation into two components: variation within populations and among populations. The results showed that 80 % of the variation existed within individuals, while 16% was due to differences among individuals ($P < 0.001$). Among two populations, variation amounted to 4% (Table 4).

***Population structure and genetic clustering*:** Structure program was used for the identification of population structure among the date palm cultivars (Pritchard *et al*., 2000) to assess the genetic composition and number of subpopulations. For calculating the optimum value of K, the mean estimate log posterior probability of the delta L (K) was plotted against the given K value. STRUCTURE results were further analyzed by STRUCTURE HARVESTER (Earl and von Holdt, 2011), the first peak value of delta K was identified at $K = 2$ and second peak value was observed at $K = 5$ as shown in the graph (Fig. 2). Accordingly, the two major groups were named as G1 and G2, respectively. The G1 group, containing thirty four genotypes in total, was dominated by twenty nine genotypes collected from Turbat and five from Panjgur. The G2 group consisted of seventeen genotypes from Panjgur and four from Turbat. The genotypes with the probability of membership fraction ≥80% were assigned to corresponding subgroups while remaining genotypes with a membership probability less than 80% in any given cluster were categorized as a mixed cluster. Six genotypes were retained as admixture on the basis of membership fraction. Structure analysis further partitioned the major two groups into five sub-groups named as P1, P2, P3,P4 and P5 respectively (Fig. 3).

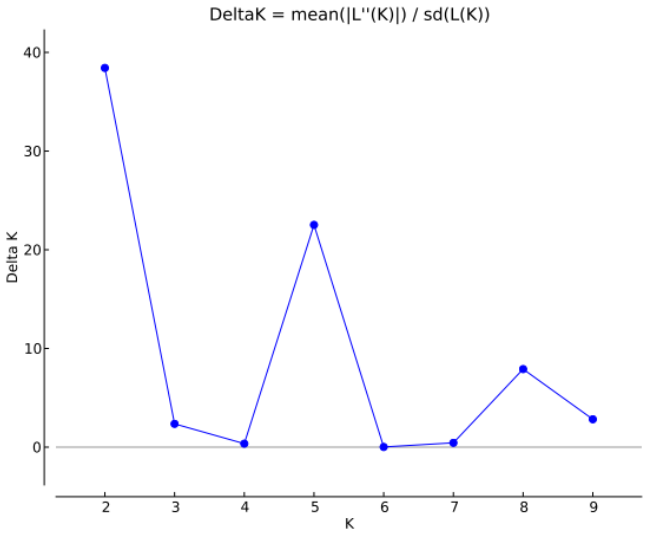

Figure 2. Graphical plot based on estimation of best K value by Structure Harvester (Earl and Vonholdt, 2012) as in Evanno *et al*., (2005) for calculatinge the optimal number of clusters.

The first two sub-groups (P1 and P2) were overwhelmed with Pangjur genotypes and contained eleven and five genotypes in each group respectively. The P3 and P5 sub-group included twenty one genotypes from Turbat while P4 consisted of six genotypes from Turbat and three from Panjgur. The remaining fifteen lines were categorized into a mixed subgroup as they had membership probabilities less than 0.80 within a specified subgroup. Moreover, genetic clustering of these genotypes was also examined through neighbor-joining tree. The number of alleles or allelic abundance is an important parameter for estimating diversity. Pairwise test of allelic abundance or richness was performed to compute differences among five STRUCTURE subpopulations. Significant allelic differences were present in all the five sub-populations. Allelic count of group 2 (P2) was significantly different with that of other groups (Fig. 4). PCoA was also performed to recognize all the subclusters in the germplasm set. A two dimensional scatter plot of PCoA represented the

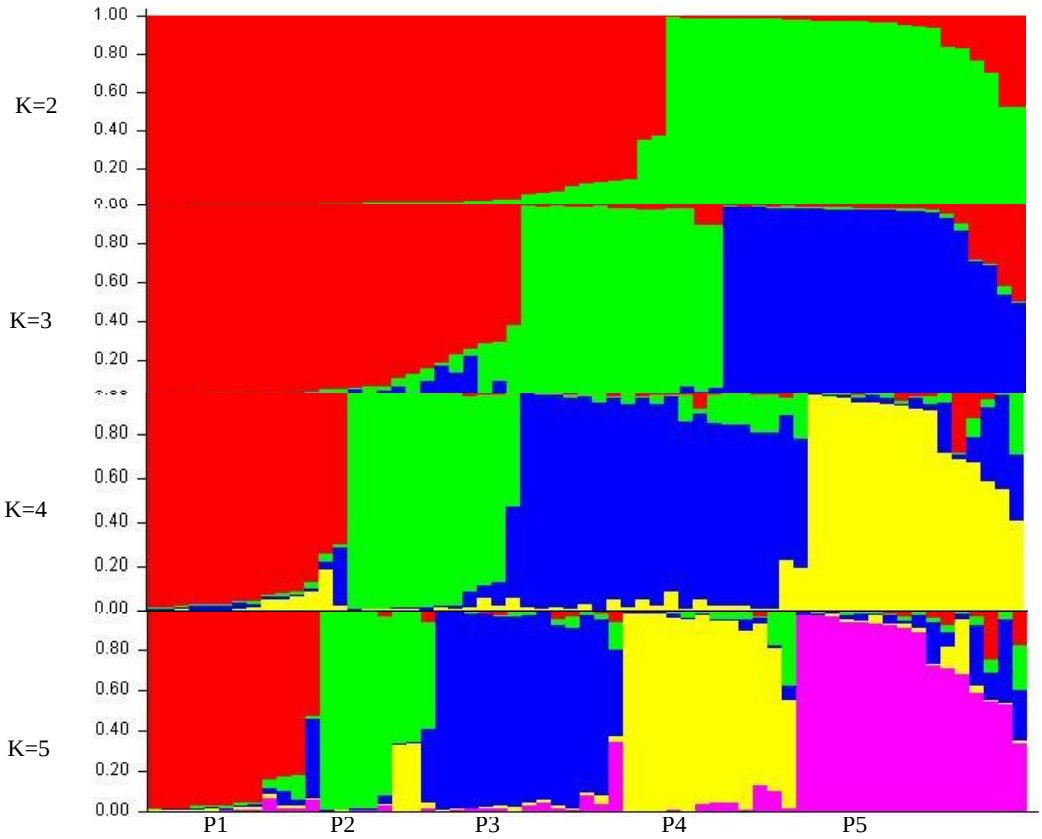

Figure 3. Populations (K=2 to K=5) obtained from STRUCTURE analyses are coded in different colors. Each colored segment represents the location of genotypes within sub-groups. At highest value of K=2, two groups were identified: P1 dominated by Panjgur genotypes and P2 dominated by Turbat genotypes. K=3, P1= Panjgur, P2=Panjgur, P3= Mixture of Turbat and Panjgur. K=4, P1= Panjgur, P2=Panjgur, P3=Turbat, P4= Mixture of Turbat and Panjgur K=5 represented five subpopulations: P1 & P2 Panjgoor; P3 &P5 Turbat; P4; Mixture of Turbat and Panjgoor genotypes.

placement of sixty one genotypes into two axes, showing 11% and 10% of the genetic deviation respectively (Fig. 5). DARwin software was used for culster analysis on the basis of Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which showed a similar pattern of clusters derived from STRUCTURE (Fig. 6).

DISCUSSION

Various studies have been performed on date palm for diversity analysis (Elshibli and Korpelainen, 2008; Khierallah *et al*., 2011; Zehdi *et al*., 2012; Al- Racchi *et al*., 2014; Zehdi-Azouzi *et al*., 2015; Elmeer *et al*., 2015). In the present study, it was tried to collect maximum number of samples from two main representative districts of Baluchistan (Pakistan) and many SSR markers were used. In the recent years, fingerprinting through SSR markers has been a dominant tool to study the genotypic diversity and structure.

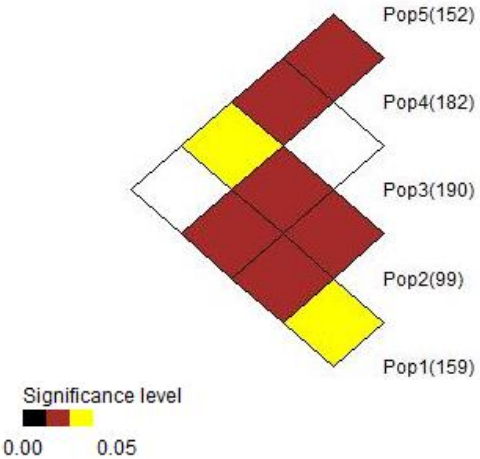

Figure 4. Pair-wise tests of allelic differences among all populations. The number of observed alleles in each group is given in the parenthesis

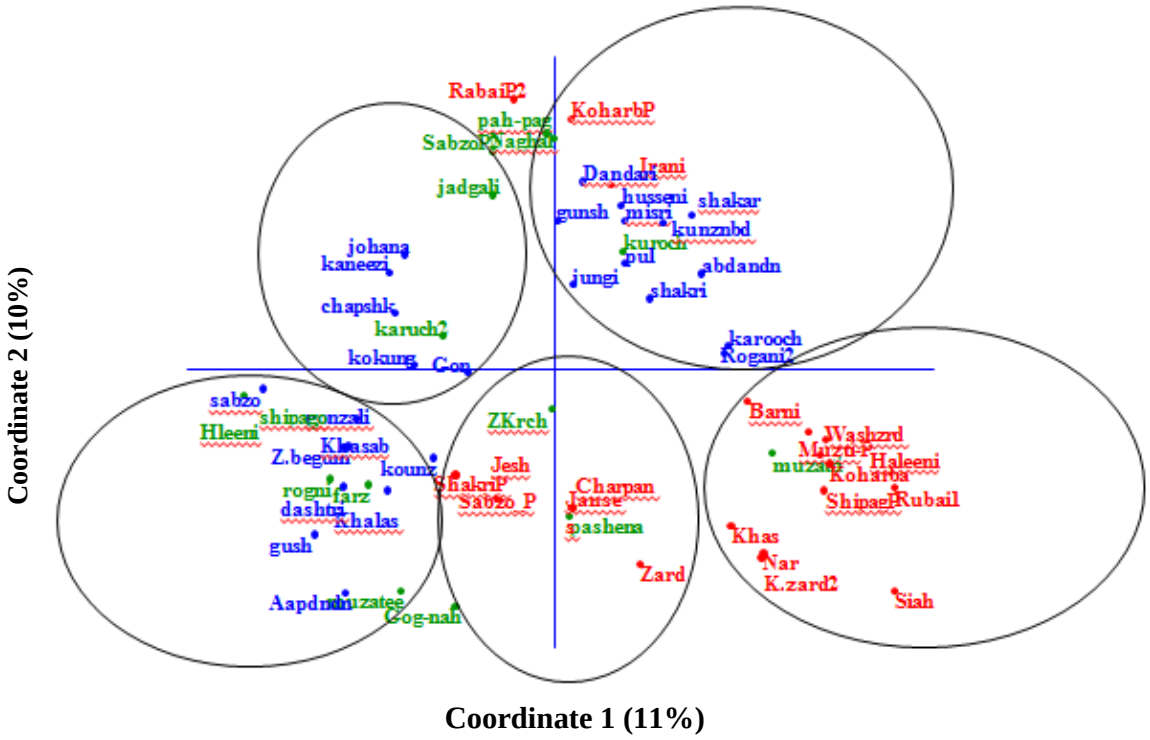

Figure 5. Principal Coordinates of 61 genotypes based on 47 SSR loci. First coordinate (Coord 1) and second coordinate (Coord 2) accounted for11% and 10 % of the genetic variation among populations.

Genetic diversity is considered as the key factor for utilization of germplasm in crop improvement. In the present study, genotyping was performed through microsatellites to evaluate the genetic diversity and relatedness in date palm genotypes of Baluchistan. Some earlier reports indicated the number of alleles per locus, gene diversity and polymorphic information content. Khierallah *et al*. (2011) identified 188 alleles in Iraqi germplasm for 12 SSR markers showing average value of 8.54 alleles per locus. Characterization of Tunisian germplasm by Zehdi *et al.* (2004, 2012) identified 100 and 134 alleles using 14 SSR markers for 49 and 101 genotypes respectively. They reported average number of 4-10 alleles per locus. DNA fingerprinting of 377 female date palm trees of central Libya Oasis generated a total of 134 alleles with an average of 6.88 alleles per locus using 16 SSR markers (Al-Racchi *et al*., 2014). Elmeer and co-workers (2015) scored 94 alleles with a mean of 6.71 alleles for 14 SSR markers in Qatar germplasm. Sixteen SSR markers were employed by Elshibli and Korpelainen (2008) and they scored 343 alleles with an average of 21.4 alleles in Sudan and Morocco germplasm. Zehdi-Azouzi and colleauges (2015) reported 237 alleles for characterization of eastern and western germplasm using 18 SSR markers. The average number of alleles per locus was 13.17 that decreased to 4.22 after removal of rare alleles. Our study depicted a total of 387 alleles with a mean value of 8.2 alleles per locus. This mean value is higher than the mean values scored by Elmeer *et al*. (2011) in Qatri date palm (7.7 alleles), and Al-Racchi *et al*. (2014) in Libyan date palm (6.88 alleles) but less than Zehdi *et al*. (2012) in Tunisian date palm (4-10 alleles) and Elshibli and Korpelainen (2008) in Sudani date palm (21.4alleles). Nevertheless, the results highlighted presence of rich allelic diversity and high variation within the studied germplasm collected from Baluchistan.

The mean value of the observed heterozygosity and the expected heterozygosity for all loci counted to 0.65 and 0.73, respectively, suggesting the Baluchistan germplasm possessed a large proportion of the genetic diversity in major date palm growing region of Pakistan and inferred that this diversity is comparable with reported diversity of Tunisia, Qatar, Sudan and UAE. The level of observed heterozygosity was close to 0.657 as reported by Zehdi *et al*. (2012). The observed heterozygosity values were higher than 0.571 as reported by Zehdi-Azouzi *et al*. (2015), 0.25 by Chaluvadi *et al*. (2014) and 0.57 by Elmeer *et al*. (2011). The suggested values of observed heterozygosity of 0.84 and 0.83 by Elshibli and Korpelainen (2008) and Zehdi *et al*. (2004) were higher than our study. Similarly, the measure of expected heterozygosity which represents the level of genetic diversity in the investigated germplasm was comparable to 0.73 as

reported by Zehdi *et al*. (2012). It was higher than reported values of 0.66 by Elmeer *et al*. (2015), 0.40 by Zehdi *et al*. (2004) and 0.576 by Zehdi-Azouzi *et al*. (2015) but lower than 0.853 that found in Sudanese date palm germplasm by Elshibli and Korpelainen, (2008). This was expected as SSRs are more variable markers than other DNA markers (RAPD, AFLP and ISSR). The variation in number and type of the genotypes and DNA markers makes it difficult to draw general assumptions. However, high level of diversity could be favorable in long-lived plants like date palm as the hybrid vigor is the main element for date palm productivity. The amount and occurrence of genetic variation in available germplasm could be used for broadening the genetic base for successful future genetic or breeding conservation programs.

In genetics, the polymorphic information content value is generally used for measuring polymorphism of a marker locus. The average PIC value of 0.66 was generated in the studied germplasm which was similar to the value of 0.65 as reported by Zehdi-Azouzi *et al*. (2015). The most informative SSR marker in this study was PDCAT2 with PIC value of 0.86 while DPALM112 was the least informative with PIC value 0.23. Markers with PIC greater than 0.5 are considered competent in differentiating genotypes and exceedingly useful in revealing the allelic variation at a certain locus (Dewoody *et al*., 1995). Our data indicated the feasibility of utilizing these beneficial markers as representatives in the documentation of Baluchistan date palm germplasm. Few researchers employed model based approach by STRUCTURE for studying population structure in date palm (Chalavadi *et al*., 2014; Zehdi-Azouzi *et al*., 2015; Hazzouri *et al*., 2015). Chalavadi *et al*. (2014) identified ten groups in the date palm germplasm collected from Egypt, Iraq, Algeria, Iran, Morocco, Saudi Arabia, Oman and Tunisia. Zehdi-Azouzi *et al*. (2015) identified two groups in their studied germplasm using 18 SSR markers and assigned the date palm genotypes into two pools; Eastern pool (Asia and Djibouti) and Western pool (Africa). Similar grouping was observed by Hazzzouri *et al*. (2015) who differentiated Middle East and African germplasm into two separate groups. Different research groups used varied levels of ancestry threshold for assigning genotypes to subgroups. In the present study, genotypes were sub-grouped at 80 % threshold level which left only six genotypes as admixtures. Population structure analysis indicated the existence of two major groups in the studied population. In the current date palm diversity panel of sixty-one genotypes, thirty four genotypes were assigned to group one (G1), dominated by Turbat genotypes and group two (G2) consisted of twenty one genotypes mainly from Panjgur. The separation of these two groups into G1 and G2 relates to their ecological difference and correspond to distinct adaptive behavior of genotypes to different ecological environment. These results of STRUCTURE grouping were in accordance with the group pattern of Principal Coordinate Analysis and Neighbor Joining Tree. The microsatellite markers used in this study are useful to understand the population structure and genetic diversity of date palm in Baluchistan region. The results showed considerable variation among date palm cultivars which could be a promising source, not only for the comparative studies, but also for successful future genetic or breeding conservation programs. The presence of sufficient diversity in the current germplasm can help in association of traits with specific markers and genetic improvement of date palm cultivars for sustainable production.

***Conclusion*:** This study gives a thorough assessment of date palm genetic diversity across its traditional area of cultivation (Baluchistan) in context of eastern gene pool and has established an understanding of genetic structure, the route of germplasm flow in Baluchistan in finding the origin of domestication. Although the high allelic richness, private (unique) alleles and genetic structure are indicative of the importance of Baluchistan region in shaping the origins of domestication but still the routes and barriers of germplasm flows and the breeding history within the cultivated area further needs to be explored.

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