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GENETIC DIVERSITY OF WILD AND CULTIVATED MANGO GENOTYPES OF PAKISTAN USING SSR MARKERS

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Pakistan is blessed with a wide range of indigenous mango germplasm. Wild mango genotypes, growing at Azad Jammu and Kashmir (AJK) and its vicinity are valuable resource for unique genetic diversity. The DNA fingerprints of this available germplasm have never been worked out. Hence, the aim of this study was to develop DNA profiles of 31 wild and 13 cultivated genotypes of the country to determine the population structure. Number of alleles per locus of the 51 Simple sequence repeat (SSR) markers ranged from 3 to 9 and a total of 296 alleles with an average of 5.80 alleles per locus. The average polymorphism information content value was 0.764. The expected and observed heterozygosity values were 0.805 and 0.720, respectively, which exhibited high level of genetic diversity in the wild and cultivated mango germplasm. The Bayesian cluster, principal coordinate and hierarchical clustering analyses divided the collected genotypes into three groups *i.e.* A, B and C. Members of group A and B consisted of wild genotypes entirely, while all commercial genotypes were clustered in group C. The obtained results highlighted genetic diversity encompassed by wild mango genotypes of AJK which can be considered as distinct genotypes for further evaluations in the framework of breeding programs and new cultivar identification in mango.
Keywords: Tropical fruits, mango cultivars, population structure, genetic resources, germplasm, genetic markers

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most important fruit crops cultivated in tropical and subtropical regions of the world. It is commercially cultivated in about 103 countries of the world. Global production of mango has been estimated to be 40 million tonnes, out of which Pakistan shares 4.5% (FAOSTAT, 2015). Pakistan is ranked sixth in the world after India, China, Thailand, Indonesia and Mexico (FAOSTAT, 2015). Pakistani mangoes have gained popularity in the world due to their high nutritive value, attractive colour, smooth texture, excellent flavour and fine aroma (Ullah *et al*., 2012). Being the center of origin and diversity, Indo-Pak subcontinent has broad history (more than 4000 years) of mango cultivation (Ravishankar *et al*., 2000). There are almost 260 mango varieties reported in Pakistan, among them 'Samar Bahisht Chaunsa', 'Sufaid Chuansa', 'Kala Chaunsa', 'Anwar Ratole', 'Fajri', 'Sindhri', 'Dusehri', 'Faiz Kareem' and 'Langra' are commercially important (Amin and Hanif, 2002). Currently mango cultivars, 'Sindhri', 'Samar Bahisht Chuansa' and 'Sufaid Chaunsa' are being exported to Middle East, Southeast Asia, EU and USA (Nafees *et al*., 2013). These mango genotypes are heavily threatened by poor orchard management practices, alternate bearing, various diseases, insect pests and physiological disorders, which affects production and export volume (Rajwana *et al*., 2008). Rich diversity present in indigenous wild germplasm offers a scope to find promising varieties having regular bearing with long shelf-life. But, this indigenous mango germplasm is depleting in their natural habitats and there is a dire need to collect, conserve and utilize this enriched source of diversity. It is imperative for the maintenance of genetic variability, the resistance to genetic erosion (Cunha *et al*., 2009) and introgression of economically important traits to sustain productivity and survival under changing climatic conditions. Characterization and selection from existing germplasm also offers a plausible way of improving genotypes with desirable traits.

Azad Jammu and Kashmir region of the country is blessed with wide range of indigenous seedling mango germplasm. This germplasm varies in fruit size, fruit colour, bearing habits, flavour, taste, juiciness, ripening time, and texture (Khan *et al*., 2015; Khan *et al*., 2016). These seedling mango trees with desirable traits can be served as a novel source for future crop improvement and sustainable mango production (Singh and Jawanda, 1962; Ravishankar *et al*., 2000). Moreover, wild mango germplasm is not only a source of genetic variation but also possesses tolerance against different pathogens. It can also be used to widen the genetic base of cultivated mango varieties along with offering a scope to extract desirable genes and their utilization in mango improvement and breeding programmes. So, compiling the DNA fingerprints to analyze and document the genetic landmarks can probe the evolutionary relationship between wild and cultivated populations.

Previously genetic variability among mango varieties has been estimated based on morphological and biochemical characters (Zaied *et al*., 2007; Rajwana *et al*., 2011; Begum *et al*., 2014; Azmat *et al*., 2016; Khan *et al*., 2016). These markers have limited features to identify crops and can vary with environment (Karihalo *et al*., 2003). Microsatellites or SSR are the most common DNA markers which have become the most appropriate and suitable choice for the analysis of genetic diversity and fingerprinting in mango due to their co-dominance nature, large allelic diversity, reproducibility, polymorphism and amenable to high throughput screening (Viruel *et al*., 2005; Schnell *et al*., 2006; Dillon *et al*., 2013).

Wild mango germplasm of the country is still unexplored and has not been properly documented. In addition, its relationship with commercial mango cultivars of the country yet to be studied. So, the present work aims to determine the population structure of 44 wild and cultivated mango genotypes using 55 SSR markers. The objectives of this work were to distinguish the collected mango genotypes, to determine the genetic diversity and relationship among them and to provide useful information for the conservation and utilization of valuable traits in future mango improvement and breeding programme.

MATERIALS AND METHODS

Plant materials**:** A total of 44 genotypes (31 wild and 13 cultivated) collected from AJK and adjoining plain areas of Punjab-Pakistan were analyzed in this study. The sampling area lies at an altitude of 200 to 900 m above the sea level. The topography of sampling area is moderately hilly with valleys and stretches of plains. The climate of AJK ranges from temperate to subtropical, while mango grows mainly in subtropical regions. The average temperature in AJK and Northern Punjab districts ranges from 25°C to 35°C, while in Multan from 25°C to 40°C in Multan. Sampling of wild germplasm was done from subtropical region of AJK [Bhimber (22), Kotli (2) and Mirpur (4) districts], and Northern Punjab [Sialkot (2) and Gujrat (1) districts]. Thirteen commercially grown varietal voucher samples were collected from germplasm unit located at Mango Research Station, Shujabad, Multan. The geographical position of each sampled tree was recorded using a hand-held global positioning system (GPS map 76CS X, Garmin, Taipei, Taiwan) along with location information and local names of the surveyed trees (Table 1). Young and tender leaf tissues were collected, washed thoroughly with distilled water, dried, packed in zipper bags and stored at -80°C before DNA extraction.

DNA extraction**:** Leaf samples were ground to powder form and genomic DNA was extracted following a modified CTAB method (Azmat *et al*., 2012). DNA concentration and purity were assessed using gel electrophoresis and comparison with Lambda Hind III marker (Fermentas, Vilnius, Lithuania). DNA samples were subsequently diluted to a working stock with final concentration of 10 ng μL^{-1} and stored at 4°C.

PCR condition and PCR product analysis**:** Fifty-five polymorphic SSR markers were initially screened and selected based on PIC values adapted from previously reported studies (Table 2). Forward primers were tagged with fluorescent compounds *i.e.*, FAM or HEX, and PCR was conducted for all 44 samples. PCR reaction mixture (15 µL final volume) contained 10 ng/µL template DNA, 2 µL of 10X *Taq* buffer (pH 8.3), 2.5 m*M* $MgCl_2$, 2.5 m*M* dNTPs, 1U of *Taq* DNA polymerase (MBI, Fermentas, Vilnius, Lithuania) and 10 pmol of each of the forward and reverse primers. SSR markers were amplified by using Bio-Rad C-1000 thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 45 s at 92°C, 45 s at 48 to 56°C and 1 min at 72°C. The program ended with one additional final extension at 72°C for 10 min. PCR amplification conditions for annealing temperature and $MgCl_2$ were optimized for all SSR markers. Fifty-one primers were successfully amplified with desired allele sizes and selected for their high reproducibility. Allele sizes were resolved by using an automated ABI 3130 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Raw data were analyzed using GeneMapper v4.1 (Applied Biosystems, Foster City, CA, USA) software to score genotypes.

Data analysis**:** Population genetic parameters for each marker and genotype such as number of alleles per locus (Na), number of effective alleles (Ne), observed heterozygosity (H_o), expected heterozygosity (H_e), and polymorphic information content (PIC) for each marker locus were estimated using "GenAlEx 6.5" (Peakall and Smouse, 2012). Multilocus matching was also performed to identify duplicates in the data set, using "GenAlEx 6.5" (Peakall and Smouse, 2012). Genotypes with different names but genetically identical at all 51 loci were considered duplicates. Pair-wise genetic distances were computed using the DISTANCE procedure implemented in GenAlEx 6.5 (Peakall and Smouse, 2012).

To assess ability of the markers to infer genetic diversity, distribution and relationship between cultivated and wild genotypes, multivariate approaches *i.e.* principle coordinate analysis (PCoA), hierarchical clustering (Ward method), were conducted using DARWin6 (Perrier *et al*., 2006) and Bayesian clustering analysis was determined using STRUCTURE (Pritchard *et al*., 2000). A burn-in of 500,000 Markov Chain Monte Carlo (MCMC) iterations with a subsequent 250,000 data generating iterations and range of cluster number (K) from one to ten was used with 10 replicates. Evanno's approach (Evanno *et al*., 2005) was used to determine the most appropriate number of genetic clusters (K). The genetic variation among individuals within and between populations was further investigated through an Analysis of Molecular Variance (AMOVA) using GenAlEx

Table 1. List of names, collection place, origin and GPS values of 31 wild and 13 cultivated *Mangifera indica* genotypes.

Sr. No.	Genotypes	Area of collection/District	Origin	Longitude (DD)	Latitude (DD)	Elevation
	Cultivated					
1	Anwar Ratole	MRS, Multan, Punjab	North India	29.8787	71.3490	114 M
2	Dusehri	MRS, Multan, Punjab	North India	29.8780	71.3489	115 M
3	Faiz Kareem	MRS, Multan, Punjab	Multan, Pakistan	29.8783	71.3487	117 M
4	Fajri	MRS, Multan, Punjab	North East India	29.8782	71.3476	115 M
5	Kala Chaunsa	MRS, Multan, Punjab	Multan, Pakistan	29.8790	71.3479	116 M
6	Langra	MRS, Multan, Punjab	North India	29.8811	71.3485	114 M
7	Late Ratole No.12	MRS, Multan, Punjab	Multan, Pakistan	29.8824	71.3484	115 M
8	Late Ratole No.14	MRS, Multan, Punjab	Multan, Pakistan	29.8820	71.3480	111 M
9	Neelum	MRS, Multan, Punjab	South India	29.8827	71.3537	113 M
10	Ratole No. 3	MRS, Multan, Punjab	India	29.8781	71.3494	111 M
11	Samar Bahisht Chaunsa	MRS, Multan, Punjab	North India	29.8823	71.3483	114 M
12	Sindhri	MRS, Multan, Punjab	Sindh, Pakistan	29.8822	71.3530	115 M
13	Sufaid Chaunsa	MRS, Multan, Punjab	Multan, Pakistan	29.8819	71.3520	112 M
	Wild types					
14	BMB-38	Bhimber, AJK	Pakistan	33.0506	74.0424	610 M
15	BMB-39	Bhimber, AJK	Pakistan	33.1326	74.0435	618 M
16	BMB-61	Bhimber, AJK	Pakistan	32.9317	74.0260	314 M
17	BMB-78	Bhimber, AJK	Pakistan	32.8929	74.2817	384 M
18	BMB-80	Bhimber, AJK	Pakistan	32.8993	74.2843	387 M
19	BMB-92	Bhimber, AJK	Pakistan	32.9061	74.2168	450 M
20	BMB-119	Bhimber, AJK	Pakistan	32.8765	74.2868	345 M
21	BMB-134	Bhimber, AJK	Pakistan	32.9068	74.2882	456 M
22	BMB-135	Bhimber, AJK	Pakistan	32.9069	74.2882	454 M
23	BMB-137	Bhimber, AJK	Pakistan	32.9075	74.2889	467 M
24	BMB-138	Bhimber, AJK	Pakistan	32.9070	74.2884	463 M
25	BMB-177	Bhimber, AJK	Pakistan	32.9095	74.2175	379 M
26	BMB-179	Bhimber, AJK	Pakistan	32.9089	74.2168	378 M
27	BMB-180	Bhimber, AJK	Pakistan	32.9096	74.2174	378 M
28	BMB-213	Bhimber, AJK	Pakistan	32.9361	74.0240	314 M
29	BMB-214	Bhimber, AJK	Pakistan	32.9354	74.0237	317 M
30	BMB-215	Bhimber, AJK	Pakistan	32.9353	74.0234	317 M
31	BMB-216	Bhimber, AJK	Pakistan	32.9343	74.0262	309 M
32	BMB-219	Bhimber, AJK	Pakistan	33.0389	74.2331	832 M
33	BMB-220	Bhimber, AJK	Pakistan	33.0384	74.2040	832 M
34	BMB-222	Bhimber, AJK	Pakistan	33.0430	74.1971	849 M
35	BMB-227	Bhimber, AJK	Pakistan	33.0411	74.1986	901 M
36	GRT-185	Gujrat, Punjab	Pakistan	32.6968	74.3271	174 M
37	KTL-19	Kotli, AJK	Pakistan	33.3800	73.8768	545 M
38	KTL-27	Kotli, AJK	Pakistan	33.3800	73.8768	722 M
39	MRP-02	Mirpur, AJK	Pakistan	32.4113	73.6262	559 M
40	MRP-03	Mirpur, AJK	Pakistan	32.4109	73.6258	565 M
41	MRP-07	Mirpur, AJK	Pakistan	33.4104	73.6275	541 M
42	MRP-14	Mirpur, AJK	Pakistan	33.3710	73.6604	499 M
43	SKT-203	Sialkot, Punjab	Pakistan	32.6499	74.4831	243 M
44	SKT-211	Sialkot, Punjab	Pakistan	32.6440	74.4835	245 M

Code: Azad Jammu and Kashmir (AJK), Bhimber (BMB), Gujrat (GRT), Kotli (KTL), Mirpur (MRP), Mango Research Station (MRS), and Sialkot (SKT)

6.5 (Peakall and Smouse, 2012). Groups were defined according to clusters obtained by the Bayesian analysis.

RESULTS

Results indicated that out of 55 SSR markers used to evaluate the genetic diversity in wild and cultivated mango genotypes, 51 showed consistent high quality amplification, while three markers failed to amplify and one was monomorphic. In the subsequent screening, 44 genotypes were screened using 51 selected SSR markers (Table 3). A total of 296 alleles were found with size ranging from 99 to 344 bp. The number of alleles ranged from 3 (mMiCIR001) to 9 (MillHR-34) with an average of 5.80 alleles per locus. The observed heterozygosity (H_0) ranged from 0.364 (LMMA9) to 0.864 (MillHR-34) with a mean of 0.715.

Table 3. Salient characteristics of 51 SSR markers used for genotyping.

Sr.	Locus	Na	Ne	Ho	He	PIC
1	MiSHRS-1	6	24	0.744	0.779	0.738
2	MiSHRS-32	4	14	0.523	0.720	0.665
3	LMMA9	4	24	0.364	0.528	0.496
4	LMMA15	5	18	0.545	0.614	0.539
5	MIAC5	6	33	0.750	0.792	0.755
6	mMiCIR001	3	11	0.548	0.781	0.541
7	mMiCIR003	4	18	0.748	0.830	0.796
8	mMiCIR008	7	2	0.818	0.900	0.881
9	mMiCIR009	8	26	0.784	0.896	0.876
10	mMiCIR013	6	24	0.484	0.863	0.836
11	mMiCIR016	6	34	0.649	0.849	0.819
12	mMiCIR018	7	26	0.789	0.835	0.804
13	mMiCIR021	5	22	0.830	0.874	0.848
14	mMiCIR022	7	29	0.841	0.892	0.871
15	mMiCIR025	4	12	0.650	0.796	0.754
16	mMiCIR028	7	48	0.682	0.806	0.767
17	mMiCIR029	8	42	0.837	0.865	0.839
18	mMiCIR032	6	26	0.852	0.870	0.845
19	mMiCIR034	6	19	0.760	0.789	0.744
20	mMiCIR036	5	17	0.682	0.748	0.711
21	MiIIHR01	8	38	0.442	0.761	0.724
22	MiIIHR03	7	19	0.659	0.820	0.786
23	MiIIHR05	4	18	0.731	0.776	0.734
24	MiIIHR06	4	12	0.614	0.705	0.649
25	MiIIHR07	5	16	0.808	0.812	0.774
26	MiIIHR09	5	28	0.721	0.800	0.757
27	MiIIHR10	6	34	0.750	0.783	0.741
28	MiIIHR12	5	1	0.523	0.748	0.696
29	MiIIHR13	5	21	0.682	0.759	0.713
30	MiIIHR14	4	15	0.659	0.744	0.686
31	MiIIHR16	5	22	0.636	0.786	0.741
32	MiIIHR17	6	24	0.659	0.750	0.699
33	MiIIHR18	5	29	0.684	0.792	0.749
34	MiIIHR19	5	15	0.784	0.806	0.770
35	MiIIHR20	8	52	0.848	0.852	0.822
36	MiIIHR21	6	38	0.717	0.803	0.761
37	MillHR22	7	27	0.814	0.834	0.801
38	MiIIHR23	4	11	0.697	0.780	0.735
39	MiIIHR24	6	32	0.750	0.806	0.769
40	MiIIHR25	8	27	0.818	0.886	0.864
41	MiIIHR26	6	17	0.773	0.807	0.767
42	MiIIHR27	6	23	0.785	0.792	0.748
43	MiIIHR28	5	21	0.731	0.751	0.699
44	MiIIHR29	5	18	0.773	0.829	0.794
45	MiIIHR30	5	12	0.748	0.776	0.730
46	MiIIHR31	8	24	0.750	0.868	0.844
47	MiIIHR32	5	32	0.682	0.804	0.766
48	MiIIHR33	7	18	0.830	0.885	0.862
49	MiIIHR34	9	52	0.864	0.903	0.883
50	MiIIHR35	8	34	0.841	0.875	0.851
51	MiIIHR36	5	14	0.832	0.867	0.840

The expected heterozygosity (He) varied from 0.528 for LMMA9 to 0.903 for MillHR-34, with a mean of 0.803. All SSR markers were highly polymorphic which displayed maximum PIC value 0.883 at locus (MillHR-34), while minimum PIC value 0.496 at locus LMMA9, with a mean of 0.762. Pairwise comparisons of individual genotypes did not identify any matching genotypes, which indicates absence of mislabelling or duplicates in our collected mango genotypes. The principle coordinate analysis (PCoA), hierarchical clustering (Ward method) and a model-based clustering method implemented in the program STRUCTURE clearly grouped all genotypes into three distinct groups with each group containing same set of genotypes. PCoA showed spatial distribution among genotypes into three distinct groups (Fig. 1). The maximum dissimilarity was observed in wild genotypes of group A and B. While, the minimum dissimilarity was observed in group C which mainly occupied commercial mango genotypes. The axis 1 and 2 represent 16.57 and 12.25% variation, respectively.

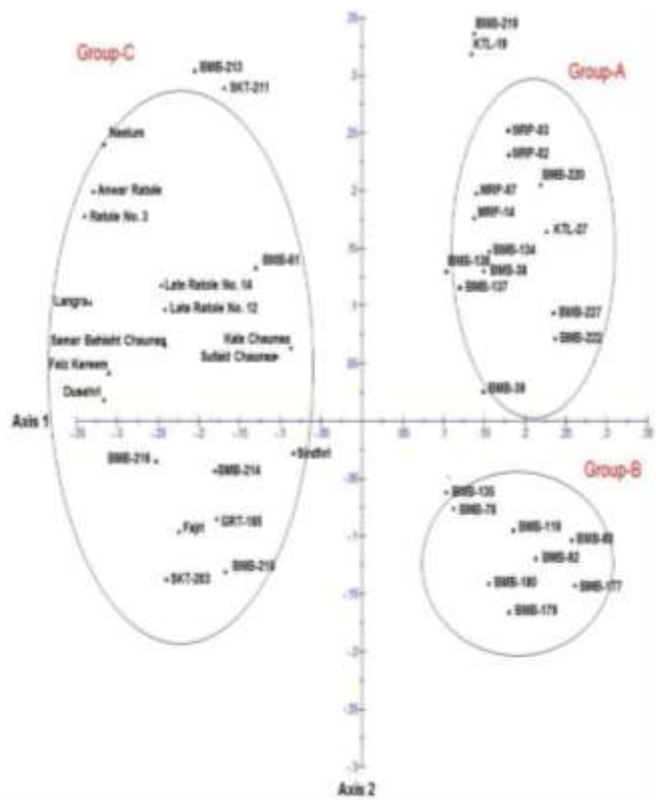

Figure 1. Principle coordinates analysis of 44 mango genotypes from AJK and Punjab with 51 SSR markers.

The Ward and UPGMA hierarchical clustering methods were used to differentiate the wild genotypes from cultivated varieties (Fig. 2). Group-A consisted of wild type genotypes from high to mid altitude areas (450-900 m) in the AJK, which was comprised of two sub-groups. Nine of mango genotypes, namely, 'BMB-137', 'BMB-138', 'BMB-134', 'BMB-38', 'KTL-27', 'MRP-14', 'MRP-07', 'MRP-03' and 'MRP-02', which are wild types, were in subgroup one. Other six wild types ('BMB-227', 'BMB-222', 'BMB-220', 'BMB-39', 'BMB-219', and 'KTL-19') were grouped together. Group-B contained eight wild genotypes viz., 'BMB-92', 'BMB-135', 'BMB-119', 'BMB-180', 'BMB-78', 'BMB-179', 'BMB-177', and 'BMB-80', from low hill regions (340-450 m) of AJK. Group-C consisted of 14 cultivated mango varieties with eight wild type genotypes of *M. indica* from northern Punjab. Indian cultivars 'Neelum' and 'Anwar Ratole' were close to each other in all three analyses. 'Samar Bahisht Chaunsa' was close to 'Faiz Kareem' in all three analyses, because former is considered as one of the parents of 'Faiz Kareem' (Rajwana *et al*., 2010). The cluster analysis indicated that genotypes grouping was according to origin and the nature type was only at major group level and not along the sub-group level that indicates significant variations/differences among the collected genotypes.

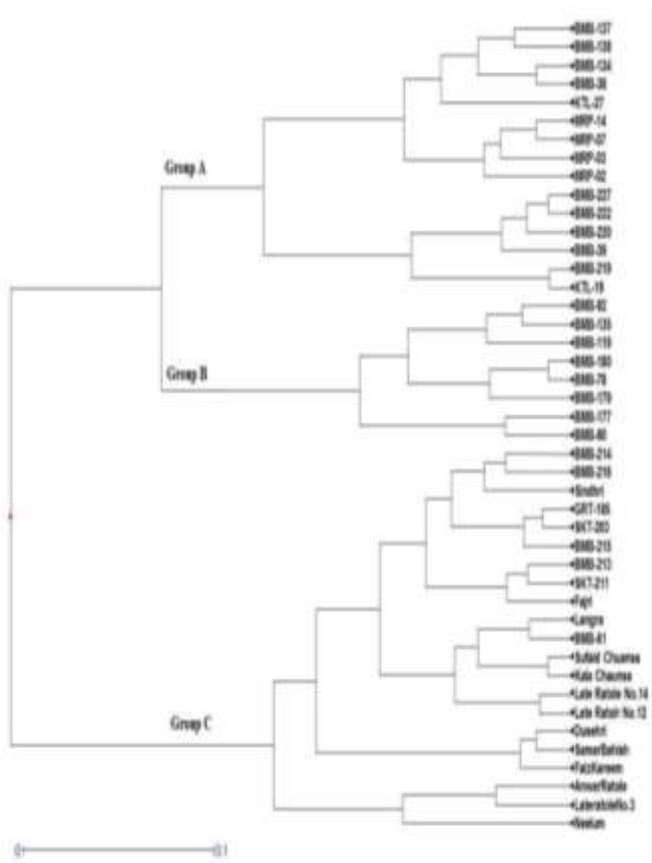

Figure 2. Dendrogram of 44 genotypes based on hierarchal cluster analysis (Ward method) using the simple dissimilarity matrix derived from 51 SSR markers.

The highest likelihood value was obtained maximum when the number of sub-populations (K) = 3 (Fig. 3). The three groups produced by STRUCTURE were similar to those determined by PCoA. Group A contained 15 wild mango genotypes, all of which originated from high to mid altitude areas of AJK. The Q-value for membership in this group was 0.80 or above for 15 genotypes. Q-values of 'Langra', 'BMB-215' and 'BMB-216' split between the group A and B. The wild genotype 'BMB-214', collected from AJK, had Q-value split between group A and C. Group B consisted of 8 wild genotypes collected from low hilly regions of AJK and were placed in this group with Q-values 0.80 or more. Group C contained all the cultivated varieties with some wild genotypes originated from areas of northern Punjab. A cultivated variety 'Neelum' originated from India had Q-value split between the group B and C. The genotypes 'Neelum', 'Langra', 'BMB-214', 'BMB-215', and 'BMB-216' having split Q-values between two groups were categorized as admixed ancestry. The analysis showed high level of variation in wild genotypes and maximum genetic similarity in cultivated varieties.

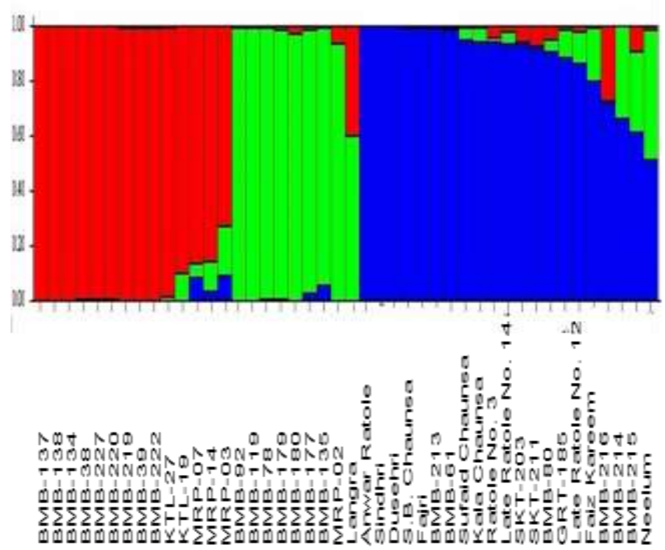

Figure 3. Estimated genetic structure of the wild and cultivated populations based on STRUCTURE analysis at K = 3.

Analysis of Molecular Variance (AMOVA) showed significant genetic differences among populations which accounted for 7%, whereas 19% variability was recorded among individuals within a population. Without considering the population boundaries, genetic variation between individual genotypes was highly significant *i.e.* 74% (Table 4). The Fst value between wild and cultivated genotypes was 0.069 ($P = 0.001$). The obtained pairwise Fst value between the populations was 0.050 ($P = 0.000$).

Table 4. AMOVA results showing the partitioning of genetic diversity among the wild and cultivated mango genotypes in Pakistan.

Source	df	SS	MS	Est. Var.	%	p-value
Among Population	1	21385	21385	408	7%	<0.001
Among Individual	42	278150	6623	1108	19%	<0.001
Within Individual	44	191681	4356	4356	74%	<0.001
Total	87	491216		5831	100%	

Table 2. List of forward and reverse primer sequences for 55 SSR used for studying genetic diversity in wild and cultivated mango genotypes.

Primer	Sequence 5'-3'	Allele size range	Reference
MiSHRS-1F	TAACAGCTTTGCTTGCCTCC	189-216	Schnell *et al*. (2005)
MiSHRS-1R	TCCGCCGATAAACATCAGAC		
MiSHRS-32F	TTGATGCAACTTTCTGCC	190-203	Schnell *et al*. (2005)
MiSHRS-32R	ATGTGATTGTTAGAATGAACTT		
LMMA9-F	TTGCAACTGATAACAAATATAG	174-184	Viruel *et al*. (2005)
LMMA9-R	TTCACATGACAGATATACACTT		
LMMA15-F	AACTACTGTGGCTGACATAT	207-219	Viruel *et al*. (2005)
LMMA15-R	CTGATTAACATAATGACCATCT		
MIAC-5F	AATTATCCTATCCCTCGTATC	118-228	Honsho *et al*. (2005)
AB190348-R	AGAAACATGATGTGAACC		
mMiCIR001-F	TGAGTTGTTGTCCTGCT	191-203	Duval *et al*. (2005)
mMiCIR001-R	GGTGCTTGTTTCTCGT		
mMiCIR003-F	GATGAAACCAAAGAAGTCA	306-322	Duval *et al*. (2005)
mMiCIR003-R	CCAATAAGAACTCCAACC		
mMiCIR008-F	GACCCAACAAATCCAA	156-184	Duval *et al*. (2005)
mMiCIR008-R	ACTGTGCAAACCAAAAG		
mMiCIR009-F	AAAGATAAGATTGGGAAGAG	151-170	Duval *et al*. (2005)
mMiCIR009-R	CGTAAGAAGAGCAAAGGT		
mMiCIR013-F	GCGTAAAGCTGTTGACTA	144-160	Duval *et al*. (2005)
mMiCIR013-R	TCATCTCCCTCAGAACA		
mMiCIR016-F	TAGCTGTTTTGGCCTT	228-246	Duval *et al*. (2005)
mMiCIR016-R	ATGTGGTTTGTTGCTTC		
mMiCIR018-F	CCTCAATCTCACTCAACA	202-244	Duval *et al*. (2005)
mMiCIR018-R	ACCCCACAATCAAACTAC		
mMiCIR021-F	CCATTCTCCATCCAAA	162-184	Duval *et al*. (2005)
mMiCIR021-R	TGCATAGCAGAAAGAAGA		
mMiCIR022-F	TGTCTACCATCAAGTTCG	145-172	Duval *et al*. (2005)
mMiCIR022-R	GCTGTTGTTGCTTTACTG		
mMiCIR025-F	ATCCCCAGTAGCTTTGT	212-230	Duval *et al*. (2005)
mMiCIR025-R	TGAGAGTTGGCAGTGTT		
mMiCIR028-F	AAGAGGGAATCTTAATCAAC	175-197	Duval *et al*. (2005)
mMiCIR028-R	GTCGTTTTGCGTTAGTG		
mMiCIR029-F	GCGTGTCAATCTAGTGG	152-202	Duval *et al*. (2005)
mMiCIR029-R	GCTTTGGTAAAAGGATAAG		
mMiCIR032-F	TCATTGCTGTCCCTTTTC	118-172	Duval *et al*. (2005)
mMiCIR032-R	ATCGCTCAAACAATCC		
mMiCIR034-F	TCGGTCATTTACACCTCT	192-216	Duval *et al*. (2005)
mMiCIR034-R	TTATTGAGCTTCTTTGTGTT		
mMiCIR036-F	ACCACGAAAAGACAACTC	248-272	Duval *et al*. (2005)
mMiCIR036-R	TCATCTTTGTTAAATAGGTTAAT		
MiIIHR01-F	GGATGCACAACAACAAGCAC	237-269	Ravishankar *et al*. (2011)
MiIIHR01-R	TCAGCAAGCAATCCCTTCTT		
MiIIHR03-F	GTCGATGCCTGGAATGAAGT	223-243	Ravishankar *et al*. (2011)
MiIIHR03-R	AAGCATCGAACAGCTCCAAT		
MiIIHR05-F	CTCTCCCTCACTTGCTCCAC	181-197	Ravishankar *et al*. (2011)
MiIIHR05-R	AGACCACCGACAACGAAAAC		

Primer	Sequence 5’-3’	Allele size range	Reference
MiIIHR06-F	CGCCGAGCCTATAACCTCTA	99-113	Ravishankar *et al*. (2011)
MiIIHR06-R	ATCATGCCCTAAACGACGAC		
MiIIHR07-F	GCCACTCAGCTAAATAGCCTCT	153-177	Ravishankar *et al*. (2011)
MiIIHR07-R	TGCAGTCGGTAAAGTGATGG		
MiIIHR09-F	GTTGTGACCGAGGCCTTAAA	273-281	Ravishankar *et al*. (2011)
MiIIHR09-R	CTTTGACATCGCTGATCTGG		
MiIIHR10-F	CGATTCAAGACGGAAAGGAA	163-179	Ravishankar *et al*. (2011)
MiIIHR10-R	TTCAAGCACAGACGACCAAC		
MiIIHR12-F	GCCCCATCAATACGATTGTC	161-173	Ravishankar *et al*. (2011)
MiIIHR12-R	ATTTCCCACCATTGTCGTTG		
MiIIHR13-F	CCCAGTTCCAACATCATCAG	171-185	Ravishankar *et al*. (2011)
MiIIHR13-R	TTCCTCTGGAAGAGGGAAGA		
MiIIHR14-F	CCGAAACAACTCTTCCTCCA	332-344	Ravishankar *et al*. (2011)
MiIIHR14-R	TGCTCTCTGGCCTCTTCTTC		
MiIIHR16-F	TTTCACTTGGTTCTGGATTGC	178-186	Ravishankar *et al*. (2011)
MiIIHR16-R	ATTTCCCACCATTGTCGTTG		
MiIIHR17-F	GCTTGCTTCCAACTGAGACC	234-242	Ravishankar *et al*. (2011)
MiIIHR17-R	GCAAAATGCTCGGAGAAGAC		
MiIIHR18-F	TCTGACGTCACCTCCTTTCA	156-164	Ravishankar *et al*. (2011)
MiIIHR18-R	ATACTCGTGCCTCGTCCTGT		
MiIIHR19-F	TGATATTTTCAGGGCCCAAG	181-191	Ravishankar *et al*. (2011)
MiIIHR19-R	AAATGGCACAAGTGGGAAAG		
MiIIHR20-F	CCTAACGCGCAAGAAACATA	176-190	Ravishankar *et al*. (2011)
MiIIHR20-R	ACCCACCTTCCCAATCTTTT		
MiIIHR21-F	TTTGGCTGGGTGATTTTAGC	225-239	Ravishankar *et al*. (2011)
MiIIHR21-R	TTAATTGCAGGACTGGAGCA		
MiIIHR22-F	TGGCCGAACTAGCAAACTCT	216-228	Ravishankar *et al*. (2011)
MiIIHR22-R	CCCCATTTCGAGAAAATTCC		
MiIIHR23-F	TCTGACCCAACAAAGAACCA	136-144	Ravishankar *et al*. (2011)
MiIIHR23-R	TCCTCCTCGTCCTCATCATC		
MiIIHR24-F	GCTCAACGAACCCAACTGAT	238-248	Ravishankar *et al*. (2011)
MiIIHR24-R	TCCAGCATTCAATGAAGAAGTT		
MiIIHR25-F	TGTGAGTCTCCGTTTGTGCT	143-169	Ravishankar *et al*. (2011)
MiIIHR25-R	CCCTCTCATTTTCCCAGTCA		
MiIIHR26-F	GCGAAAGAGGAGAGTGCAAG	136-146	Ravishankar *et al*. (2011)
MiIIHR26-R	TCTATAAGTGCCCCCTCACG		
MiIIHR27-F	TGGGGATTCATCGGAGATAG	186-196	Ravishankar *et al*. (2011)
MiIIHR27-R	TGGAAGACCCATTCTCATGC		
MiIIHR28-F	GCGGTCGCAGACAAATTCTATAT	102-110	Ravishankar *et al*. (2011)
MiIIHR28-R	ACAACTCGAGATTGTCACATCTTT		
MiIIHR29-F	CGATGAGGATGGTTGGTTTT	141-155	Ravishankar *et al*. (2011)
MiIIHR29-R	CATCAACAGTCGCCATCAAT		
MiIIHR30-F	AGCTATCGCCACAGCAAATC	186-194	Ravishankar *et al*. (2011)
MiIIHR30-R	GTCTTCTTCTGGCTGCCAAC		
MiIIHR31-F	TTCTGTTAGTGGCGGTGTTG	215-233	Ravishankar *et al*. (2011)
MiIIHR31-R	CACCTCCTCCTCCTCCTCTT		
MiIIHR32-F	TGGTGGTGTTTGTTTGCAGT	172-184	Ravishankar *et al*. (2011)
MiIIHR32-R	ACCACCCGCAGTATTGAAAG		
MiIIHR33-F	GAAGCACTTGTCTCCCTTGC	162-184	Ravishankar *et al*. (2011)
MiIIHR33-R	CCTCACACTCCTCCACCTGT		
MiIIHR34-F	CTGAGTTTGGCAAGGGAGAG	223-251	Ravishankar *et al*. (2011)
MiIIHR34-R	TTGATCCTTCACCACCATCA		
MiIIHR35-F	TGGTGAAGCTTGTTGTCTGC	189-219	Ravishankar *et al*. (2011)
MiIIHR35-R	TGGCTTGACTGTTTTTCAGC		
MiIIHR36-F	TCTATAAGTGCCCCCTCACG	219-241	Ravishankar *et al*. (2011)
MiIIHR36-R	ACTGCCACCGTGGAAAGTAG		

DISCUSSION

It is important to understand the amount and structure of genetic variability present in mango germplasm for conservation, management and further improvement for various important characteristics. It was noticed that surveyed area was an enriched reservoir of genetically diversified wild mango populations. It is a well-established fact that the cultivated genotypes of mango in Indo-Pak subcontinent were mainly selected from naturally occurring chance seedlings and these selected genetic variants have been conserved and subsequently maintained through vegetative propagation. The molecular analysis revealed that cultivated and wild mango germplasm of Pakistan is genetically diverse. The wild mango germplasm has distinct genetic profile. However, it is important to further study the results of this genetic analysis for pedigree analysis and comparison with world mango germplasm to draw the conclusion.

Microsatellite markers have been used widely in *Mangifera* species for genetic mapping, genetic variation, cultivar identification and phylogenetic analysis for the improvement of mango genotypes (Dillon *et al*., 2013; Schnell *et al*., 2006; Viruel *et al*., 2005). There is enormous and valuable genetic diversity present among Pakistani mango cultivars due to diverse geo-ecological regions and climatic conditions. The present study revealed abundant allelic variation among 51 SSR loci in the characterization of 44 mango genotypes. These markers painted high level of genetic variation by producing a total of 296 alleles. This is lower in comparison to 318 bands detected in 90 mango genotypes with 106 SSR loci by Surapaneni *et al*. (2013) and higher than the 103 alleles generated in 241 mango genotypes (Singh *et al*., 2009). The existence of different SSR alleles showed genetic polymorphism in the studied mango genotypes. Moreover, the detection of specific alleles in some genotypes shows the occurrence of deletions/insertions in the DNA. The results for average number of alleles per locus (5.92) fall within the range of previously reported for microsatellite studies of different mango germplasm (Schnell *et al*., 2006; Viruel *et al*., 2005). A comparable study of diversity in Indian collection of 387 mango genotypes was carried out by Ravishankar *et al*. (2015), which revealed a mean of 24.61 alleles per SSR locus and a mean Ho of 0.624. Another study showed that a panel of 90 Indian local genotypes harboured 2.87 alleles per SSR locus and a mean Ho of 0.29 (Surapaneni *et al*., 2013). Dillon *et al*. (2013) reported an average of 12.09 alleles per SSR locus and a mean Ho of 0.69 in Australian National Mango Genebank, which mainly comprised of *M. indica* and its related species. The high level of heterozygosity in our study was attributed to cross pollination and out crossing. Different levels of genetic diversity and polymorphism have been reported in mango using various types of markers, like 90% and above by SSR markers (Dillon *et al*., 2013; Surapaneni *et al*., 2013), 85 to 99% with ISSR (Ariffin *et al*., 2015; Samal *et al*., 2012; Tomar *et al*., 2011), 73 to 100% with RAPD (Ravishankar *et al*., 2000; Karihaloo *et al*., 2003; Rahman *et al*., 2007; Rajwana *et al*., 2008; Souza *et al*., 2011), 84 to 96% by AFLP (Yamanaka *et al*., 2006; Ga´lvez-Lo´pez *et al*., 2010) and 73% by SCoT markers (Luo *et al*., 2010).

The average PIC value obtained in this study was 0.764 which was higher than the average value reported by earlier studies (Surapaneni *et al*., 2013; Dillon *et al*., 2013; Ravishankar *et al*., 2011; Hirano *et al*., 2010 and Schnell *et al*., 2005). The markers having higher PIC values are considered highly prospective to reveal allelic variation. The average PIC value of microsatellites generated in different studies varies during the testing of different number of genotypes and microsatellites. Microsatellites are more reliable and due to very high reproducibility it can be successfully used for the cultivar identification by using variety specific primers. The unique allelic pattern was observed mostly in wild genotypes along with two cultivated varieties 'Langra' and 'Neelum', which revealed the presence of unique genetic makeup in these genotypes. This information can be used as molecular signatures in fingerprinting studies and to determine the genetic purity of the genotypes. Furthermore, the wild genotypes may be useful in hybridization program with other cultivated commercial varieties or species to broaden the genetic base of mango genotypes (Mukherjee, 1997).

Based upon the pedigree and geographical origin, phenotypic approach is used to distribute genotypes into various groups (Schnell *et al*., 2005). However, this approach has limitation for grouping genotypes based on their genetic similarity. Therefore, an alternative Bayesian based cluster analysis approach is applied in this study by using STRUCTURE, which distributed the genotypes into three different gene pools based on allele information per locus. The cross pollination and out crossing may hold true for *M. indica* too, as the STRUCTURE analysis revealed some level of population admixture and gene exchange. Interestingly, this approach was able to distribute different genotypes based on several criteria like geographical origin and type (cultivated or wild). The highest likelihood was observed for K = 3, which separated the wild and cultivated mango genotypes into two different genetic backgrounds. The Pakistani genotypes were divided into two groups, which represents cultivated and wild separately, and showed two distinct genetic backgrounds. While, cluster of Indian varieties did not change and seem to be almost same. This analysis also confirmed that cultivated varieties of Pakistan share the genetic similarity with Indian origin, while wild genotypes from northern Punjab, which make cluster with cultivated varieties, have close relationship with them. The Indian mango genotypes have different genetic backgrounds and showed clear differences from other genotypes. Both 'Sufaid Chaunsa' and 'Kala Chaunsa', which were selected based on their superior fruit quality, are the variants of 'Samar Bahisht Chaunsa', whose origin is India (Rajwana *et al*., 2008). So, these genotypes showed a close genetic relationship with Indian germplasm. India is considered the primary center of origin for mango (Ravishankar *et al*., 2000). It is worth mentioning that both countries were united till 1947 and it is logical to conclude the similar genetic base for few Pakistani and Indian mango cultivars. But, Pakistani wild genotypes do not have Indian genetic background. So, Himalayan region of Pakistan might be center of diversity for mango germplasm in Pakistan. However, it can be confirmed by the further analysis of more wild genotypes from India and other mango growing countries.

The genetic relationship of Pakistani mango germplasm was determined by hierarchical clustering based on minimum variance ward method. Three major groups distinguished fifteen genotypes (34%) originated from AJK and second group contained fifteen genotypes (18%) from Northern Punjab with two cultivated varieties. The remaining cultivated varieties (47%) showed narrow genetic basis, which could probably be due to ancient selection of cultivars from the existing cultivars of India. Rajwana *et al*. (2008) reported similar genetic relationship among cultivated Pakistani and Indian genotypes by RAPD markers. The factorial correspondence analysis also showed similar results which separated the mango genotypes in three groups. These findings are also in accordance with both of our results obtained by the dendrogram and Bayesian cluster analyses. The genetic exchange within species rather than ancient relationships has been emphasized to determine genetic structure or genetic diversity. Although, sometimes restriction occurs in structure exchange across the species range, either limited dispersion of pollen or seed, and by geographical distribution of the genotypes (Schaal *et al*., 1989). Since, the information on the precise migration of these wild mango plants from center of diversity is still unknown, it is most probable that the wild Pakistani mangoes are either descendants of isolated plants or relics of past migrants, which planted in the past but now have been established in the wild.

***Conclusions*:** The reported results provide detailed information about the genetic makeup of new germplasm sources and genetic diversity in wild mango germplasm which could be useful to improve the yield and quality of existing varieties. Our results confirm that wild genotypes have high genetic diversity and unique structure pattern than the cultivated genotypes originated from Pakistan and India, which could be the result of the long history of mango cultivation in Pakistan. The results of the study clearly revealed that microsatellites can successfully be used to characterize mango germplasm of Pakistan.

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