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MORPHO-MOLECULAR CHARACTERIZATION AND PHYLOGENETIC RELATIONSHIP IN POMEGRANATE GERMPLASM OF PAKISTAN

Muhammad Nafees[1,2], Muhammad Jafar Jaskani[2,*], Saeed Ahmed[2] and Faisal Saeed Awan[3]

[1,2]University College of Agriculture & Environmental Sciences, The Islamia University of Bahawalpur, Pakistan; [2]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan; [3]Centre of Agricultural Biochemistry & Biotechnology, University of Agriculture, Faisalabad, Pakistan.
[*]Corresponding author's e-mail: jjaskani@uaf.edu.pk

Pomegranate is an important fruit crop for human health. Plant genetic resources are vigorously used in tree fruit improvement programs. Although Pakistan is rich in pomegranate genetic resources, yet it is a minor fruit crop in the country. Keeping in view the breeding objectives, morphological and molecular diversity was estimated in 42 pomegranate accessions using using 13 morphological traits of fruits and 29 SSR markers. Principal Component Analysis (PCA) of fruit length (mm), fruit diameter (mm), crown length (mm), hull thickness (mm), arils and seeds dimensions (mm) explained 93.9% of the total morphological diversity in first six principal components. Fruit weight and its dimensions was highly diverse and correlated with each other in all accessions. Genetic analysis indicated average value of MAF, GD, HZ and PIC as 0.5981, 0.497 0.404 and 0.425, respectively, in genomic DNA of selected accessions; moreover, primer POM_AAC1 proved highly polymorphic with maximum PIC value of 0.550. Minimum genetic similarity matrix value of 23.41 and 21.76% was measured in accessions of Chakwal and Muzaffargarh (CD2-MAN3), and Bahawalpur and Muzaffargarh (BR2-MAN3), respectively. Polygenetic tree successfully clustered all the accessions and proved accession 'BR2' as highly diverse and it did not cluster with any of the studied accessions. Most of the accessions having similar morphological traits, showed dissimilarity for molecular relationship that stressed the use of molecular markers for germplasm characterization. Moreover, morpho-genetic diversity in selected pomegranate accessions was very high which could be efficiently used in pomegranate breeding programs.

Keywords: Genetic resources, morphological diversity, PCA, SSR markers, fruit traits
***Abbreviations*:** PCA: principal component analysis, MAF: major allele frequency, GD: genetic diversity, HZ: heterozygosity, PIC: Polymorphic information content (PIC)

INTRODUCTION

Cultivated pomegranate (*Punica granatum* L. family Punicaceae, *2n=2x=16 & 18*) is a deciduous tree or shrub, rich in polyphenols, organic acids and used as ornamental and fruit tree with ecological, economical and sociological benefits. Chronological evidence indicates that pomegranate originates in Central Asia to northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe (Stover and Mercure, 2007). It had become a popular and economically important fruit crop around the world due to better understanding of its nutritional, medicinal and pharmaceutical importance as well as developments in production, postharvest techniques and food technology (Yazici *et al*., 2005). However, Food and Agricultural Organization has not declared data regarding its area and production, which might be due to its restricted cultivation and generally stated as minor fruit in various parts of the world.

There is a great variability in pomegranate regarding its tree habit, mode of pollination, leaf size-shape and various flower characteristics. A high genetic diversity exists in Iranian genotypes due to long historical cultivation and various environmental conditions. Variations exist in wild and cultivated genotypes for various fruit characters like its color, weight, size, shape, density, hull weight and thickness and aril color, juice content, acidity, sweetness and wood portion index etc. (Ercisli *et al*., 2007). Morphological and biochemical studies (Hasnaoui *et al*., 2011a) are considered a key source of germplasm diversity estimation. However, molecular markers have overcome the limitations of morphological and biochemical markers due to the influence of environment on the performance of genotypes. SSR markers has successfully used for genetic diversity estimation in litchi (Clyde *et al*., 2005) and grape fruits (Wu *et al*., 2009). A wide range of molecular markers have been used to assess the genetic diversity in pomegranate as RAPD markers have provided reliable and highly polymorphic information to discriminate different cultivars (Hasnaoui *et al*., 2010a; Narzary *et al*., 2009). AFLPs also has been used to evaluate the genetic diversity in Chinese (Yuan *et al*., 2007) and Tunisian pomegranate populations (Jbir *et al*., 2008). There are ≥137 microsatellite loci (Soriano *et al*., 2011; Curro *et al*., 2010; Hasnaoui *et al*., 2010b) and various

ISSR markers in pomegranate genomes, showing high molecular diversity in its populations. Pakistan is considered as the second source of origin of pomegranate as wild in the warm temperate Himalayan range and successfully grown in warm tropical to subtropical, arid and desert zones. Its production in the country is 54.3 thousand tones on an area of 13.4 thousand hectares (Anonymous, 2013) which is quite low to meet increasing market demand. There are large to medium size fruit with high juice percentage, sweet in taste and exceptionally white to pink aril color; moreover, these genotypes have potential to be introduced in international market but patency and limited production are major obstacles. It is still a minor and ignored fruit crop with minimum number of registered cultivars in the country. There is no research work done on pomegranate germplasm collection, characterization and conservation. Thus, there is no repository which could provide basic information for its diversity estimation, variety improvement and diversifying its production.

This publication is supposed to be the first to estimate morphological and molecular diversity in domesticated pomegranate accessions of Pakistan. Therefore, objective of this study is to estimate the morphological and molecular diversity in pomegranate and the generated knowledge would be useful for its hybridization and germplasm improvement programs.

MATERIALS AND METHODS

***Plant materials*:** Leaves and fruit samples were collected from 42 cultivated/domesticated (some selected accessions of Chakwal, Bahawalpur, D.G. Khan were not commercially growing so named as domesticated) accessions in 10 different regions of Pakistan for morpho-genetic characterization. Detail of collected cultivated and domesticated pomegranate germplasm along with given name of accessions shown in Table 1 and details of collection sites along with GPS data on map of Pakistan shown in Figure 1.

***Morphological traits evaluation*:** Various pomegranate fruit traits of selected accessions were studied to estimate the genetic diversity. These were fruit, hull, 100 aril and 100 seed weight using weighing balance model UniBl°C, SHIMADZU (U x 320 g, Min. 0.02 g, e= 0.01 g and d=0.001 g) in grams. Whereas, fruit length and diameter, crown length, hull thickness, aril and seed dimensions were measured using digital vernier caliper (KBD-MT 0014) in millimeter.

***DNA fragment amplification and gel electrophoresis*:** Fresh leaves were collected from pomegranate plants of selected accessions for DNA extraction using Cetyle Trimethyle Bromide (CTAB). The quality and quantity of extracted DNA was assessed in Nano-Drop spectrophotometer (NANODROP 2000 Spectrophotometer, Thermo Science).

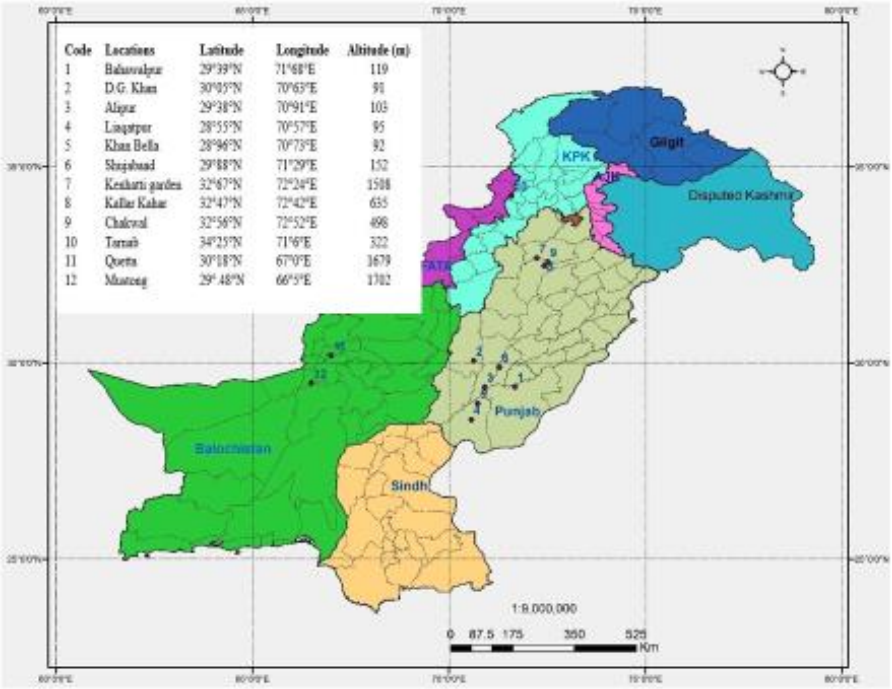

Figure 1. Map of Pakistan with collection sites for 42 pomegranate accessions from Punjab, KPK and Baluchistan. (Number indicates the regions from which samples were taken).

Highly polymorphic 29 SSR primers (Pirseyedi *et al*., 2010; Ebrahimi *et al*., 2010; Soriano *et al*., 2011; Curro *et al*., 2010) were used in PCA reaction after optimizing the PCR reagents and temperature profiles. Amplified DNA fragments were visualized and counted on high resolution agarose gel and polyacrylamide gel electrophoresis (PAGE) to determine the molecular diversity in selected accessions. Polymorphic information contents were measured following formula PIC= 1-Epi^2 where E is the total number of alleles detected for a locus of a marker and Pi the frequency of the ith alleles in the specified locus.

***Data scoring and analysis*:** Morphological data from 42 pomegranate accessions of involving 13 traits were analyzed by XLSTAT (2014) software (version 3.4). In the principal components analysis (PCA), factor loadings >0.55 were regarded as significant. Genetic similarity computing and construction of respective PCA plot were also performed.

The data was then used for dendrogram construction. Euclidean distance was used in Ward's method for agglomerative hierarchical clustering (AHC).

The bands of DNA fragments on SSR analysis were scored in a binary format (0 for absence, 1 for presence) for allele(s) on respective locus. Efficacy and degree of polymorphism of reported 29 SSR markers was accessed

Table 1. Details of all 42 pomegranate (*Punica granatum* L.) germplasm collection

S. No.	Accession Code	Accession given Name	Districts	Collection Site
1	BR1	Pink	Bahawalpur	Regional Agricultural Research Institute
2	BR2	Red Green		
3	BR3	Red		
4	BR4	Lalari		
5	DH1	White	D.G. Khan	Horticulture Res Station D.G. Khan
6	DH2	White Pink		
7	DH3	Lalari		
8	MAN1	Sandhora	Muzafar Garh	Alipur (Nabi Shah Walla)
9	MAN2	Sava		
10	MAN3	Sava Kagzi		
11	MAN4	Dunail		
12	MAN5	Kalahari		
13	MAN6	Sandhora khatta		
14	MAM7	Kalihari-khatta		Mullawali
15	RLA6	Lalari	R. Y. Khan	Allahaabad
16	MSA2	Dasi-Metha	Multan	Shujaabad
17	MSA3	Qabli		
18	CD1	Pink	Chakwal	District coordination officer house
19	CD2	Red		
20	CD3	Pink-Sava		
21	CD4	Sava		
22	CK1	White kandhari		KallarKahar
23	CK2	Red kandhari		
24	CB1	Pinkish green		Takht-e-Babri
25	CB2	Pink-Red		
26	KK1	Khatta	Khushab	Kanhatti
27	KK2	Sava		
28	KK3	Pink		
29	KK4	Pink green		
30	Q1	Red Kagzi	Quetta	Quetta
31	Q2	White Kagzi		
32	Q3	White		
33	MG1	Red	Mastung	Gulab Bag
34	MG2	Red Pink		
35	MG3	Pink Red		
36	MG4	White		
37	MK1	White Sour hard		Kari Kucha
38	MK2	White-pink Sour		
39	MK3	White Sour		
40	MK4	Whit-sweet soft		
41	TG	T. Gulabi	Peshawar	Agri. Res. Institute Tarnab
42	TK	T. Kandahari		

through Power Marker, Analysis of Molecular Variance (AMOVA) was performed to estimate genetic variance in GenAlEx6.1 and PCA performed in PAST for assessing genetic diversity. Cluster analysis of molecular data performed in PopGEN statistical program.

RESULTS AND DISCUSSION

***Principal component analysis of morphological traits*:** PCA placed the 13 morphological traits of fruits in to six components that explained 93.9% of total diversity (Table 2). The first component, which accounted for 41.6% of the total variation, was comprised of fruit, aril and hull weight, and fruit length and diameter. The second component, which explained 23.7% diversity, included rind thickness, crown and fruit length; however, third component, accounted 12.2% of the total variation and included aril width and length. Moreover, 4th, 5th and 6th components covered 8.5, 4.6 and 3.3% of total variability, respectively, with high factor load in seed and rind weight in PC-4 and PC-6, respectively (Table 2). Genetic diversity in each selected accession could be accessed in 2D PCA plot based on the first two components shown in Figure 2. Accessions closed to the center of axis were considered as less diverse

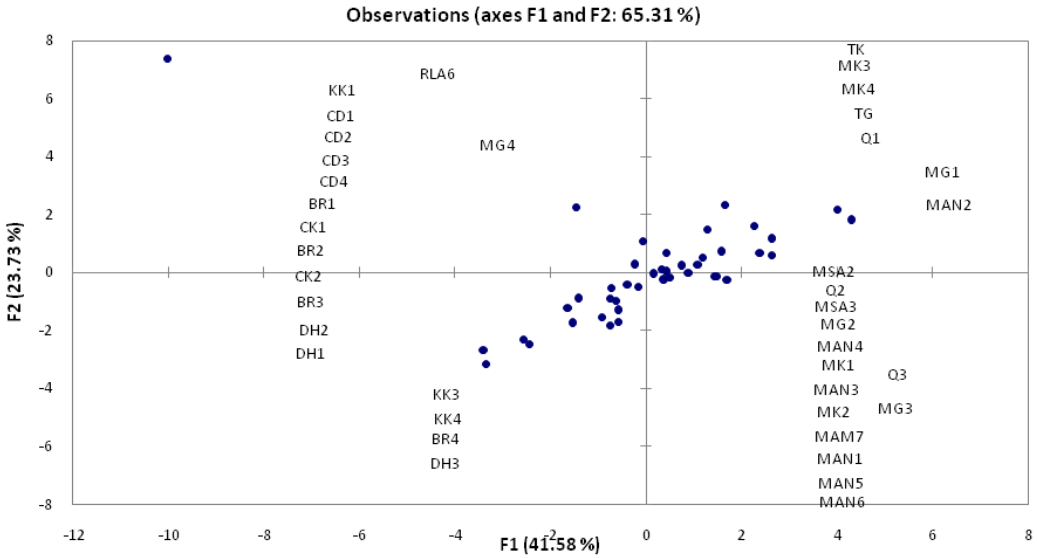

Figure 2. Assessment of morphological diversity on PCA plot in 42 pomegranate accessions

Table 2. Distribution of morphological diversity in principal components

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	5.4	3.1	1.6	1.1	0.6	0.4
Variability (%)	41.6	23.7	12.2	8.5	4.6	3.3
Cumulative %	41.6	65.3	77.5	86.0	90.6	93.9
Factor load: Variables	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
Fruit weight	0.8	0.4	0.1	0.1	-0.2	0.2
Fruit length	0.7	0.6	0.0	0.1	-0.3	-0.1
Fruit diameter	0.9	0.1	-0.2	0.1	-0.3	0.0
Crown length	-0.5	0.7	-0.1	-0.2	-0.3	-0.2
Rind weight	0.8	0.4	0.1	0.0	0.2	0.4
Rind thickness	-0.2	0.9	0.1	-0.1	0.2	0.2
Aril weight	0.7	0.2	0.0	0.5	0.3	-0.3
Aril length	0.7	-0.2	0.6	-0.2	0.0	-0.1
Aril width	-0.1	0.0	0.9	-0.2	-0.1	-0.1
Seed weight	-0.5	0.3	0.5	0.7	0.0	0.0
Seed length	0.7	-0.5	0.3	-0.2	0.0	-0.1
Seed width	-0.7	0.6	0.2	-0.2	0.0	-0.1
Wood portion index	-0.6	-0.5	0.2	0.4	-0.3	0.2

while accessions away from it were highly diverse like accession RLA6 from Rahim Yar Khan showed the highest level of diversity. Moreover, the plot grouped the accessions based on resemblance of studied traits like accessions of Muzaffargarh (MAN1, MAN2, MAN3, MAN4, MAN5, MAN6 & MAM7), Mastung (MK1, MK2 & MG3), Multan (MSA2 & MSA3) and Quetta (Q2 & Q3) were very close to each other for having high fruit weight, diameter and aril juiciness (Fig. 2). However, various accessions of Chakwal (CD1, CD2, CD3, CD4, CK1 & CK2), D.G. Khan (DH1 & DH2), Kenhatti (KK1) and Bahawalpur (BR2) were placed very close to each other for having small fruit size, diameter and length and high wood portion index. Various accessions of Peshawar (TG, TK,), Mastung (MK3, MK4) and Quetta (Q1) were placed together for having high fruit weight, size and aril weight with less wood portion index.

These results have proved a high level of morphological diversity in selected pomegranate accessions. Hasnaoui *et al.* (2011a), and Ercisli *et al.* (2007) also reported higher variation in fruit size and rind weight, aril weight and color, seed hardness and juice content, acidity, sweetness and fruit maturity in wild and cultivated pomegranate accessions. Most of the investigated accessions are grouped on the basis of similarities of morphological traits and growing regions. Similarly Caliskan and Bayazit (2013) stated that Turkish pomegranates were grouped on the basis of morphological and biochemical properties of cultivars with little or no influence of growing regions, whereas Hasnaoui *et al.* (2011b) grouped the Tunisian pomegranate cultivars independent of growing regions. Qualitative and quantitative morphological studies of pomegranate fruits are required for accessions evaluation and provide the basis for breeding programs (Zamani, 2007).

***Molecular diversity estimation*:** DNA fragments of 42 pomegranate accessions were successfully amplified by 29 SSR primers and polymorphism by one of PGCT series primer (PGCT086b) is shown in Figure 3 while unmarked bands are of wild pomegranate accessions, need not discussed in this manuscript. Molecular data analysis indicated minimum allele frequency (0.423) amplified by PGCT089b marker while primer ABRII-MP30 amplified maximum 0.893 alleles of genomic base among 42 accessions (Table 3) which showed broad genetic base of cultivated pomegranate of Pakistan. Primer ABRII-MP30 detected minimum genetic diversity of 0.1970, whereas its maximum value (0.6240) was recorded by primer PGCT089b in all selected accessions. Minimum heterozygosity of 0.0595 was measured in SSR primer ABRII-MP28 and its maximum value (0.7976) was detected by primer PGCT089b among all the cultivated accessions. Maximum Polymorphic Information Content (PIC) value (0.5717) in all selected accessions was amplified in primer POM_AAC1 whereas its minimum value (0.1877) was detected in the primer ABRII-MP30 (Table 3).

Allele length, number of alleles and PIC values of selected primers of PGCT series amplified the genomic DNA of selected pomegranate accessions were at par as reported by Soriano *et al.* (2011) in Spanish pomegranate accessions. Moreover, the selected POM_AAC series primers detected PIC value was in the range of 0.37-0.55 which was quite higher than the findings of Curro *et al.* (2010) for the pomegranate germplasm of Italy, Spain and Turkey. ABRII-MP series primers recorded a range of 0.25 to 0.467 PIC value in our selected pomegranate accessions which confirmed the findings of Basaki *et al.* (2011) as they reported 0.01-0.56 PIC with an average of 0.34 in Iranian pomegranate germplasm. Most of the primers used in this research, amplified more than one locus in all the accessions and declared as multiallelic primer which is also reported in pomegranate accessions of Spain (Soriano *et al.*, 2011).

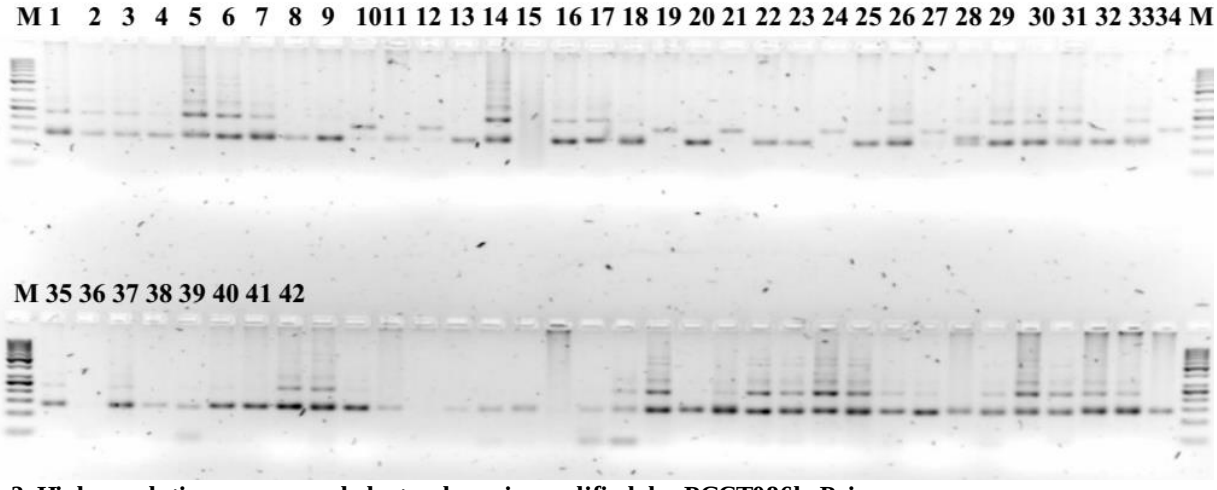

Figure 3. High resolution agarose gel electrophoresis amplified by PGCT086b Primer

Table 3. Detail of polymorphism performance of selected SSR Primers in 42 selected pomegranate accessions

	Primer	F/R primer	Ta (°C)*	AL*	MAF*	GD*	HZ*	PIC*
1	PGCT006	TTGAATTGATGTAACGCTTG GAGGAAAGTCGTTTGAAGTG	55	100-300	0.611	0.493	0.563	0.428
2	PGCT015	GACGCCTTTAGTTTGCTCCA CTCGGGACAGGACTTGGAAT	60	100-200	0.560	0.563	0.357	0.492
3	PGCT061	GAATAAGGCGTCCCTCTCTC CTCCTCCTCGTAATCCCAAC	58	150-200	0.560	0.548	0.440	0.483
4	PGCT066b	CGAGGAGTGGTCCAGGTTAG AACAGACGACAAGGGGAATG	59	150-430	0.631	0.453	0.254	0.373
5	PGCT075b	GGCGAGCTTCTGCTACTTCT TCTGTCCCCAGATCATCAAA	59	200-250	0.679	0.430	0.226	0.383
6	PGCT086b	TGGTGATTCGTGTTGTTTTC CAACAACCTCCTCTGCTCTC	57	150-250	0.577	0.531	0.488	0.461
7	PGCT088	TCTCTCTCTACCCCGACACC TAGCGTCAAGATTGTGAAAAGG	59	110-400	0.698	0.419	0.325	0.371
8	PGCT089b	TGCATCCTTCCCCTACTCTC AGCTCATGTAATGCGTCGTG	59	150-200	0.423	0.624	0.798	0.544
9	PGCT091b	ATCAGAATTGGAATCGGAAC ACCGAGGTCATCGAACTAAA	56	170-250	0.452	0.594	0.794	0.505
10	PGCT093A	GTAGCCACTTTAGGGCGAGA CGTCTAAAAGCGACAGCAAG	58	200-330	0.440	0.615	0.714	0.531
11	PGCT093B	GCCTTTTCCTGCTTTCCTTT CATACAGCGGACCACAACAC	60	200-250	0.518	0.561	0.607	0.470
12	PGCT096b	CAGACCCTGCGCTCGCT TTATGGAGAGCGGGAGAAAC	59	200-250	0.643	0.503	0.345	0.435
13	PGCT098b	ATCAACCAAACCGCACAGAC CCATTTCATTCTCCCCCTCT	60	150-200	0.524	0.585	0.583	0.503
14	PGCT110	GAGCCATTGTAGAGACAAGA GACTGCTGACAACTTTCTTT	52	100-300	0.500	0.609	0.298	0.533
15	PGCT111	TATCTGTCGCAGGAAGGATG GAAGCCAATTCCTCAAAGATG	58	100-300	0.661	0.470	0.333	0.407
16	POM_AAC1	GGGTCTTCCTAATTCTCTGG TACAACTTCGGACTCACTTGC	55	100-200	0.488	0.624	0.286	0.550
17	POM_AAC2	TGTTGTATCCCATCTTCTTCC TTTCCACCGCCATTTACTTC	55	100-250	0.649	0.456	0.298	0.366
18	POM_AGC5	TTCGATATTGTTTATTGTGTCG CAACGAACTAGACGACACAC	55	100-150	0.607	0.504	0.298	0.410
19	POM_AGC11	CGTCATCCCTTATGTTCTTC CTGGGGAAGTCGACGAAG	55	150	0.714	0.408	0.429	0.325
20	POM_AAC3	TGATGAAACCATGTAACTCG CTCCGATAACGTCTCCAAGC	55	100-250	0.625	0.463	0.083	0.399
21	POM_AAC7	GCCTGGACATCTAACGCTCTC GCCGAACAAAGTCCTGAAAC	55	200	0.607	0.477	0.595	0.363
22	POM_AAC13	TCTCCCGACAACAAATCAC CCCGACACAACACATACTTCAG	53	150-300	0.518	0.592	0.238	0.516
23	POM_AAC14	CGAGAACCGTTAGTCATGC AGTGACGGCAGGACAAGAAC	55	150	0.500	0.523	0.381	0.409
24	ABRII-MP07	GATTAACAGCAAAGCCTAGAGG AGTAGCTGCAACAAGATAAGG	60	150-250	0.452	0.576	0.810	0.476
25	ABRII-MP12	TTGAGTCCCGATCATATCTC TCAATCTGTCAGGAACAACA	60	100-340	0.702	0.399	0.083	0.359
26	ABRII-MP26	TTTCTCGAAGAATTGGGTAA CTGAGTAAGCTGAGGCTGAT	55	160-240	0.583	0.542	0.560	0.464
27	ABRII-MP28	ATCCTCTGTCTTTGTGTTCG TGAGTAATTCCGGTCAGAAG	56	100-300	0.708	0.387	0.060	0.348
28	ABRII-MP39	AGTCTCTGAAGTTTGTCGGA CCTGAGTAAAGCATCTCACTG	60	200	0.821	0.293	0.357	0.250
29	ABRII-MP30	CCCAGTTTGTAGCAAGGTA AAGCTGACATTCTTTGAAGC	60	200	0.893	0.197	0.119	0.188
Average					0.5981	0.497	0.404	0.425

Abbreviations: Ta: Annealing temperature, AL: Allele length, MAF: Major allele frequency, GD: Genetic diversity, Hz: Heterozygosity, PIC: Polymorphic Information Content

Table 4. Analysis of Molecular Variance (AMOVA) of selected 42 pomegranate accessions

	df	SS	MS	Est. Var.	%
Among regions	6	226.09	37.682	2.079	7
Within accessions	35	911.22	26.035	26.035	93
Total	41	1137.31		28.113	100

P-value<0.010

The lower level of cross pollination or high rate of asexual propagation in pomegranate could be the cause of low number of alleles per locus which is in accordance with the findings of Parvaresh *et al*. (2012) and Soriano *et al*. (2011). The results of present study proved that SSR markers could successfully be used in pomegranate germplasm characterization and molecular breeding (Pirseyedi *et al*., 2010; Ebrahimi *et al*., 2010; Soriano *et al*., 2011; Curro *et al*., 2010). The selected SSR markers were highly polymorphic in Pakistani pomegranate germplasm as described by Noormohammadi *et al*. (2012) that among combined data of three markers, SSR showed higher molecular diversity than the ISSR and RAPD markers in 36 Iranian pomegranate genotypes. The PIC value in selected 42 pomegranate accessions was 0.188-0.55 which showed high to low loci polymorphism as it was proved by Xie *et al*. (2010) considering PIC >0.5, 0.5> PIC >0.25 and PIC <0.25 as high, medium or low polymorphism, respectively. Average PIC value was 0.425, which indicated that selected markers could amplify high loci polymorphism in this study (Soriano *et al*., 2011; Pirseyedi *et al*., 2010; Ebrahimi *et al*., 2010).

***Analysis of molecular variance (AMOVA)*:** Analysis of molecular variance in molecular data of 42 pomegranate accessions amplified by selecting 29 SSR primers proved genetic diversity of 7% among the cultivated accessions growing in 7 different regions having different ecological history while 93% molecular diversity was studied within these accessions (Table 4), which is in accordance to the findings of Narzary *et al*. (2010). This might be due to the reason that these regions had a high genetic overlap as a result of relatively high gene flow and this reasoning is further strengthened by the clonal propagation of pomegranate accessions. Parvaresh *et al*. (2012) also revealed 91.52% of total genetic variation within pomegranate germplasm and only 8.48% was estimated among regions.

***Analysis of genetic similarity matrix*:** Genetic similarity matrix chart based on molecular data of all selected accessions showed number of amplified common alleles (similarity matrix chart not shown). The minimum and maximum range of genetic similarity proved the wider genetic base having potential to be selected as variety improvement programs (similarity matrix chart not shown). The summary of genetic similarity matrix chart is shown in Figure 4 which clearly indicates that accessions from D.G. Khan (DH3-DH1), Chakwal and Muzaffargarh (CD3-MAN6) had high genetic similarity of 76.68 and 76.27%, respectively, whereas its minimum value of 23.41 and 21.76% was recorded in accessions of Chakwal and Muzaffargarh (CD-2-MAN3), and Bahawalpur and Muzaffargarh (BR2-MAN3), respectively. The accessions with less genetic similarity index showed good potential to be selected for developing inbred lines instead of accessions showing high similarity index. Genetic similarity index has not only detected the range of common alleles among selected pomegranate accessions but also proved that genetic base of Pakistani pomegranate germplasm is broad. Similarly Narzary *et al*. (2009) described genetic diversity in Indian pomegranates and proved that genetic similarity coefficient varied from 0.08 to 0.79 across different pomegranate accessions. This shows that genetic diversity in Indian pomegranate accessions is broader as compared to the Pakistani pomegranates. Accessions having broad genetic base have the potential to develop the new lines for patency in new regions under changing agro-climatic scenario.

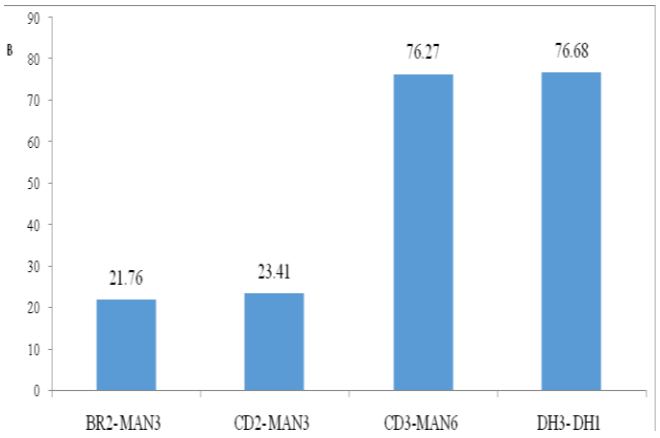

Figure 4. Variation in the range of genetic similarity in selected accessions

***Assessment of molecular diversity in principal component analysis*:** Scattered pattern of most of the selected accessions away from the center of PCA axis showed the higher molecular diversity (Fig. 5). PCA axis shared that accessions MK3, TG, KK3 and MSA3 of Mustang, Tarnab, Khushab and Multan, respectively, were highly diverse and scattered

throughout the four planes. Moreover, the accessions from Bahawalpur (BR1, BR2 and BR3) are scattered close to each other sharing most of the common alleles but were highly diverse from rest of the accessions. Accessions CD1, KK4, DH2, CD3 and MK4 are placed close to the center of axes and showed the narrow genetic base. However, rest of the accessions showed medium to high molecular diversity and may be used in breeding programs.

PCA of molecular data of 29 SSR primers confirmed the high genetic diversity which might be due to reasons that selected accessions were growing in various agro-climatic regions (warm tropical, subtropical, rainfed and desert zones). These results are supported by Soriano *et al*. (2011) who observed narrow gene pool in cultivated pomegranate accessions of a region and stated that it might be due to clonal propagation of these commercial genotypes for its distribution in different regions.

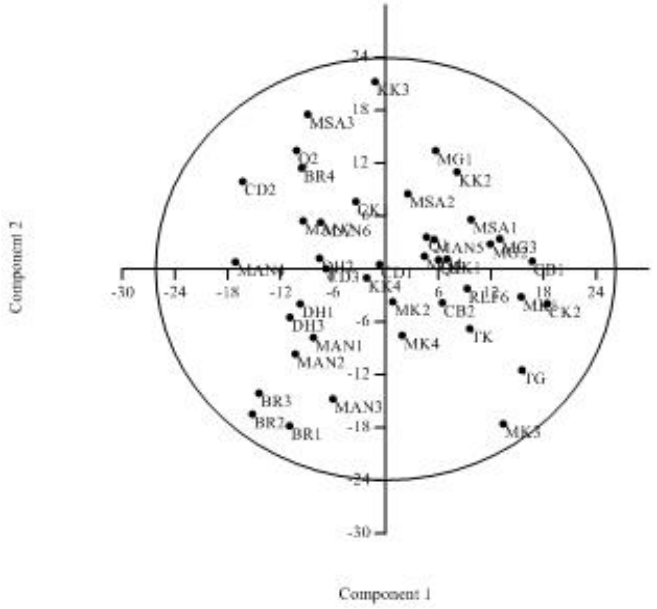

Figure 5. Molecular diversity estimation in pomegranate accessions on PCA plot

***Phylogenetic relationship among pomegranate accessions*:** The cultivated pomegranate accessions successfully clustered into three main groups, such as 12 accessions each in class A and B, and 8 accessions in class C (Fig. 6). Directly clustered cultivated accessions (BR4 & KK3) and (MSA3 & CD1) are presented in main class A, whereas two accessions from D.G. Khan (DH1 & DH3) were strongly associated and shared some alleles with MAM7. Two accession from Chakwal (CD3 & CD4) directly clustered, showing no genetic dissimilarity in class A. In class B, MSA2 & MG3, Q1 & Q2 and MAM6 & MK4, were closely clustered but shared some genetic material with MK1, MG1 and CK2, respectively (Fig. 6). Accessions MAN1 & TK and KK1 & TG were directly clustered and showed no genetic difference whereas accessions MAN5 & CB2 openly clustered and shared some alleles with CB1 which collectively shared their genetic materials with CK1. Bahawalpur (BR1) and Muzaffargarh (MAN2) accessions were openly clustered and did not resemble with any of the cultivated accessions. Moreover, accessions KK2 & MG4 openly and separately clustered and showed no common allele of rest of the accessions, whereas BR3 and MAN3 directly clustered and shared some genetic material with cultivated genotype of Muzaffargarh (MAN4). Bahawalpur accession (BR2) showed high diversity and did not cluster with any of the studied accession (Fig. 6).

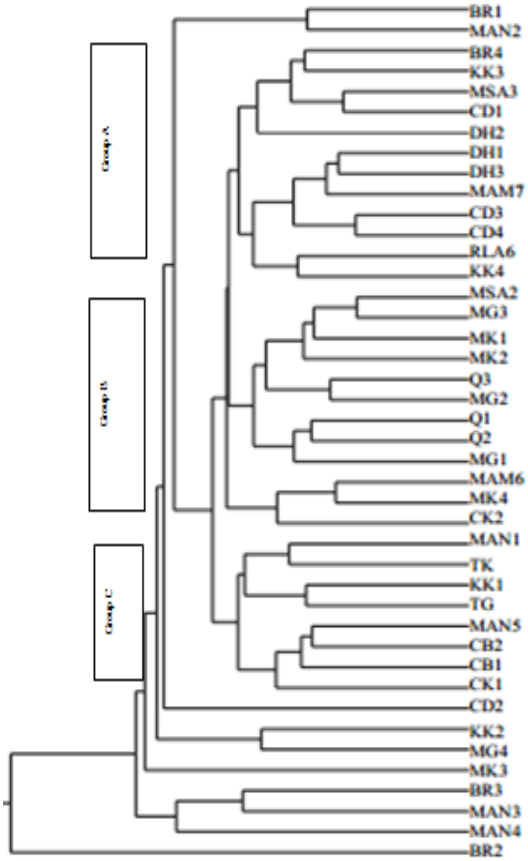

Figure 6. UPGMA phenograms of 42 accessions based on molecular data

Selected accessions were clustered with or without considering the growing regions whereas genetically similar accessions were predominantly similar for some morphological traits of fruits. Most of the accessions are similar for morphological traits, especially the accessions (Muzaffargarh and Rahim Yar Khan; Quetta, Mastung, Chakwal and Khushab; Bahawalpur and D.G. Khan) showed dissimilarity for their genetic relationship. This observation was favored by Zamani *et al*. (2013) as they reported that the

principle component analysis and UPGMA phenograms of molecular and morphological data is poorly correlated (r=0.45). Moreover, this disagreement of morphological and molecular data is supported by Gupta and Rustgi (2004) such as their discussion presented the numerous reasons including main effects of diverse agro-climatic conditions on morphological traits which did not influence genetic markers. Moreover, disagreement of morphological and molecular dendrograms of pomegranates was also in agreement with previous reports of Zamani *et al*. (2007) who observed that genetic distance matrices calculated through RAPD markers and fruit characteristics of pomegranates had no significant association. The response of other fruits for morpho pomological and molecular studies was neither similar nor related to each other (Khadivi-Khub *et al*., 2008). Estimation of morphological diversity in Pakistani pomegranate germplasm was not fully consistent with molecular diversity of the same large and extensive pomegranate germplasm because of the influence of agro-climate. This is in accordance with the findings of Zamani *et al*. (2013) as they stated that morpho-chemical characterization is used only for discrimination of germplasm.

In conclusion, morphological and molecular studies proved the broad genetic base of the pomegranate germplasm of Pakistan with predominant effect of growing region on clustering of accessions. This diversity may efficiently be used in breeding programs for genotype improvement.

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