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RESEARCH ARTICLE

Analysis of Genetic Diversity in Pomegranate using SRAP (Sequence-Related Amplified Polymorphism) Markers

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Sequence-related amplified polymorphism (SRAP) was used to assess the genetic diversity of 114 pomegranate accessions sourced from all over the world and maintained in the field gene bank at ICAR-Indian Institute of Horticultural Research, Hesaraghatta, Bangalore. Totally 10 SRAP combinations were used for the study. Genetic parameters such as effective alleles (Ne), Nei genetic diversity (H), Shannon index (I), and polymorphic information content (PIC) were calculated based on molecular data. The number of alleles per locus ranged from 1.66 to 4.00 with an average of 2.03. The average polymorphic information content (PIC) value was 0.24. The expected heterozygosity varied from 0.215 to 0.445 with an average of 0.306. Cluster analysis was performed using R software. All the 114 accessions were grouped into four major clusters. There was no clear grouping based on origin. The analysis of molecular variance (AMOVA) indicated insignificant genetic variation (p=0.31) between pomegranate accessions from different geographical locations. Overall genetic variation among the population groups was low, while 99% of variation was due to within group differences. These results confirmed that SRAP markers could be a powerful and an effective marker system for determining the genetic diversity and population genetic structure of the pomegranate.

Key Words: Pomegranate; Genetic Diversity; SRAP marker

Introduction

Pomegranate (*Punica granatum* L.) is an important ancient fruit crop which belongs to family Punicaeceae (syn. Lythraceae) under order Myrtales. The present scientific name of pomegranate was derived from the Latin name *Malus granatum*, which corresponds to 'seeded apple' (Holland *et al*., 2007). It is one of the favourite fruits of tropical and subtropical regions. Having originated in Iran, it is now widely cultivated all over the world including countries like Spain, Morocco, Egypt, Afghanistan and Baluchistan. In India, pomegranate is commercially cultivated in Maharashtra, followed by Andhra Pradesh, Uttar Pradesh, Tamil Nadu, Karnataka, Gujarat, Rajasthan, Punjab and Haryana (NHB database) http://nhb.gov.in/report_files/pomegranate/POMEGRANATE.htm

The genus, *Punica* is a small genus of fruit-bearing deciduous shrub or small tree, having both cultivated (*Punica granatum* L.) and wild types (*Punica protopunica* Balf.). *Punica protopunica* is endemic to island of Socotra (part of Yemen) and is the only relative of cultivated pomegranate. In almost all the countries where pomegranate is commercially grown, despite the availability of large number of local varieties only few are commercially utilized. The names of the cultivars originate frequently either from the place of cultivation or from colour of the fruit. Varieties are often classified as sweet, sweet-sour and sour, early, mid- season and late, juicy and table fruit, soft seeded and hard seeded. Several cultivars grown today are the result of human selection from naturally occurring variation. There is a great variability in pomegranate regarding its tree habit, mode of pollination, leaf size-shape and various flower characteristics (Mars and Marrakchi, 1999). Pomegranate accessions have been mainly evaluated based on the morphological characters and show a wide range of variation in size of fruits, sweetness, time of ripening, juiciness, and proportion of seeds to flesh. Therefore, breeding for useful traits has gained importance in this crop for which determination of genetic relationships and precise identification of accessions to conserve

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its genetic diversity is required. However, these traits are affected by environment and cultivation conditions and do not result in a clear discrimination among them (Kumar, 1999). The molecular or DNA based markers are more suitable for an accurate discrimination of the accessions and cultivars.

The Sequence Related Amplified Polymorphism (SRAP) technique is a relatively simple and highly reproducible DNA marker technique useful for both mapping and gene tagging in plants (Li and Quiros, 2001). SRAP has been shown to be more informative than other PCR-based techniques in detecting genetic diversity (Budak *et al*. 2004) and has been successfully used to study the genetic diversity of, and relationships among, several species (Castonguay *et al*., 2010; Talebi *et al*., 2011; Abedian *et al*., 2012). The present work was undertaken to demonstrate the usefulness of SRAP markers for diversity studies and for differentiating the accessions in the diverse collection of pomegranates sourced from different regions of the world.

Materials and Methods

The genetic analysis was carried out on 114 pomegranate accessions which included accessions from Turkmenistan, Japan, USA, Albania, former Soviet Union and wild types of unknown origin (Table 1, Fig.1). These accessions were obtained from the USDA National Germplasm Repository in Davis (CA, USA) and maintained at Indian Institute of Horticultural Research, Hesaraghatta, Bengaluru. The plants are in juvenile stage, therefore, the pomological traits recorded for accessions available at the website of USDA National Germplasm Repository was taken into consideration for interpretation of the clustering.

Genomic DNA was extracted from the leaves of each plant by using the CTAB-based method (Doyle and Doyle, 1990). The DNA quality was checked and adjusted to 50ng/µL final concentration for PCR reactions. A total of forty primer combinations (PC) based on literature were selected for the study. These primers were used for screening a panel of ten accessions from diverse origin to identify the polymorphic primers. After the initial screening, ten PCs which gave a reliable amplification product were selected for the molecular characterization of the pomegranate collection (Table 2). The PCR reaction was performed in a total volume of 15µl, containing 50–70ng template DNA, 0.2mM dNTPs, 1.6 mM $MgCl_2$, 0.3µM of each forward and reverse primer, 2.5 µl 10× PCR buffer, 1unit *Taq* DNA polymerase, and sterile double-distilled water. The amplification was conducted in a thermo-cycler programmed with the following thermal condition: initial denaturation at 94°C for 5min followed by 5 cycles of denaturation at 94°C for 1 min, 35°C for 1 min, 72°C for 1 min and annealing for 35 cycles of 94°C for 1min, 57°C for 1 min and extension at 72°C for 1 min, final extension at 72°C for 10 min and final hold at 4°C forever. The PCR product mixed with 2 µl bromophenol blue dye were analysed on 2% agarose gel in 1x TBE (Tris-borate-EDTA) buffer with 100bp DNA ladder as size marker. PCR products were visualized by ethidium bromide staining.

In order to evaluate the effectiveness of the SRAP markers, only the intense, clearly resolved PCR amplified bands were scored manually for their presence (1) and absence (0) in the matrix of SRAP data sheet. The band size was estimated by using medium range DNA ruler (100 bp) which was run along with the amplified products. The number of alleles per locus and the polymorphism information content (PIC) were calculated. The polymorphism information content (PIC) value was calculated by use of the formula: $PIC = 1 - \sum Pi^2$ where Pi is the frequency of the i^{th} allele (Smith *et al*., 1997). The genetic relatedness among the pomegranate accessions was assessed using Jaccard's co-efficient of similarity and by generating dendrogram based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using R software (R Core Team 2013). The binary data scored was used to construct a dendrogram. Analysis of molecular variance (AMOVA) was performed to estimate variance components for SRAP data, partitioning the variation into within and among populations, by use of GenAlEx 6.5 - Genetic Analysis in Excel written in Visual Basic for Applications (VBA) within Excel (Peakall and Smouse 2012)

Results and Discussion

Ten SRAP PCs which exhibited the characteristics of good stability and repeatability were selected for this study. These primer pairs produced a total of 64 bands ranging from 80 to 1500bp of which 29 (47.8%) were found to be polymorphic. The number of amplified fragments ranged from 4 (Me3F +Em4R) to 12 (Me5F +Em1R). The polymorphic information content (PIC) value ranged from 0.17 to 0.34 with an average of 0.24. (Table 3). The primer combinations Me1F +Em8R

Table 1. Pomegranate accessions used in this study with their geographical origins and description

Code No.	Accessions	Accession No	Origin	Description
1	110 Podarok	EC798803	Turkmenistan	Soft-seeded, excellent sweet and sharp taste
2	121 Lyubimyi	EC798813	Turkmenistan (Balkan)	cultivated
3	109 Medovyi Vahsha	EC798802	Turkmenistan	Soft-seeded, early, sweet.
4	87 Mae	EC798785	USA (California)	Fruit light red or pink, medium to small. deep red arils, slightly sour
6	29 King	EC798739	Unknown	Tree shrubby, somewhat shy bearer, fruit medium to large, flesh very sweet.
7	16 White Flower	EC798733	Albania	Flowers white, ripens in October.
9	90 Sour	EC798788	USA (California)	Fruit slightly striped red, yellow-green, size medium. arils large, deep red, juice sour, seeds hard, suitable for culinary use.
10	106 Gissarskii Alyi	EC798799	Turkmenistan	Soft-seeded, excellent very sweet
11	10 Nochi-Shibori	EC798728	Japan (Saitama)	
12	81 Wonderful	EC798779	Unknown	Fruit large (up to 5 inches in diameter), bright red, arils red, juice red, sweet sour. Seeds medium soft, very juicy, ripens early, good shipper, large. ripens late September and October.
13	124 Parfyanka	EC798816	Turkmenistan (Balkan)	Seeds medium hard, seed red, flavour sweet-sour
14	42 Pink	EC798744	Unknown	
17	19 Hyrdanar × kirmizy-Akbu	EC798736	Unknown	
18	45 Elf	EC798747	USA	Dwarf, fruit greenish-yellow with orange blush, shape blocky, size small to medium, arils large, translucent pink, slightly tart.
19	38 Balegal	EC798743	USA (California)	Fruit large, round, pale pink, flesh pink, very sweet.
20	61 DPUN 61	EC798761	Unknown	Seeds soft, seed red, skin reddish yellow, flavour sweet
22	139 Myagkosemyannyi Rozovyi	EC798830	Turkmenistan (Balkan)	Seeds soft, seed rose, skin yellowish, flavour sweet-sour
28	112 1/25 Rannii	EC798805	Turkmenistan	Soft-seeded, early, sweet.
30	164 Utah sweet	EC798849	USA (California)	
31	125 Ariana	EC798817	Turkmenistan (Balkan)	Seeds medium hard, seed skin red, flavour sweet-sour
32	107 Gissarskii Rozovyi	EC798800	Turkmenistan	Seeds soft, seed colour rose, skin yellowish
34	9 Ki-zakuro	EC798727	Japan (Saitama)	
37	92 Gold	EC798790	USA (California)	Fruit size medium, vivid gold, arils and juice pink, sweet, seeds medium soft.
39	15 Parfianka	EC798732	Turkmenistan	Sweet-acid, soft seeded type, ripens in October, high juice quality.
40	7 Haku-botan	EC798725	Japan (Saitama)	Ornamental white, fruits large, light yellow, almost white, rind thick, arils medium-sized, white, sour, seeds medium hard.
41	57 Rosamia	EC798757	USA (California)	Produces single crop.
42	134 Myatadzhy	EC798826	Turkmenistan ((Balkan)	Seeds soft, seed colour, skin red, flavour sweet-sour
46	88 ELF	EC798786	USA (California)	Dwarf, fruit greenish-yellow with orange blush, shape blocky, size small to medium, arils large, translucent pink.
47	101 Kukurchiaskii	EC798795	Unknown	
48	108 Desertnyi	EC798801	Turkmenistan	Soft-seeded, excellent sweet and sharp taste
50	111 Shainakskii	EC798804	Turkmenistan	Soft-seeded, excellent sweet and sharp taste
56	75 Surh-anor	EC798773	Soviet Union Former	
58	104 Hotuni Zigar	EC798797	Turkmenistan	
63	13 Sverkhranniy	EC798730	Turkmenistan	Sweet, soft seeded type, ripens in August.

Code No.	Accessions	Accession No	Origin	Description
64	93 Palermo	EC798791	Unknown	Fruit large to extra-large, round, medium, red-yellow rind, flavor rich-tart, slightly acid, cuttings introduced in early 1980's from Italy.
66	2 Double Red	EC798721	USA (California)	Ornamental, some fruiting, rootstocks own, fruit Medium to large, red, rind thin, arils medium sized, pink, juice white, sour, seeds medium hard.
67	149 Gulistan	EC798837	Turkmenistan (Balkan)	
68	17 Dotch legrelley	EC798734	Azerbaijan	Flowers double, red-white.
70	105 Agat	EC798798	Turkmenistan	Soft-seeded, excellent sweet and sharp taste.
71	62 Salavatski	EC798762	Soviet Union Former	
72	167 Ink	EC798850	USA (California)	
73	14 Molla Nepes	EC798731	Turkmenistan	Sweet-sour, soft seeded type. ripens in October
74	4 Orange	EC798723	Unknown	Fruit very large, reddish orange, rind medium thick, arils large, pink, juice pink, seeds medium hard
76	53 Loffani	EC798753	USA (California)	
77	30 How sweet it is	EC798740	USA (California)	
78	117 Vkusnyi	EC798810	Turkmenistan (Balkan)	Seeds soft, seed colour red, skin red, flavour sweet-sour
79	100 Koinekasyrskii Kislosladkii Krasnyi	EC798794	Unknown	
80	5 Small Leaf	EC798724	USA (California)	Fruit small
83	85 Crab	EC798783	USA (California)	Fruit bronze-red, size medium, blocky pleasant appearance, arils deep red, juice red, mild sour, large, red, sour rich red juice, heavy bearing.
84	99 DK from Shevlan	EC798793	Turkmenistan (Balkan)	
85	122 Turan	EC798814	Turkmenistan (Balkan)	
86	59 Sakerdze	EC798759	Soviet Union, Former	High yield, large fruit, thin pericarp of fruit, coarse grain, high taste
89	43 Mae	EC798745	USA (California)	Excellent taste, medium to large fruit with sweet, tangy red flesh, sweet-sour
90	35 Vina	EC798741	USA (California)	Fruit papershell type, rind pale with some pink flush.
91	28 Fleischman's	EC798738	Unknown	
92	44 Eve	EC798746	USA (California)	Fruit red, large, arils deep red, juice red, good sweet/acid balance, seeds hard, productive, rated excellent.
95	50 Eversweet	EC798750	USA (California)	Derived from common home-grown cultivar in Lebanon, plants produce 2-3 crops of mature fruit each year in southern California, beginning in mid-summer and continuing through late fall. Fruit sweet even when small and immature, flesh light pink red, excellent for wine, syrup or fresh.
99	31 Toryu shibori	EC798729	Unknown	
102	54 Loulou	EC798754	USA (California)	Fruits small, sweet.
104	37 Wonderful	EC798742	USA (California)	Seeds medium hard, seed colour very red, skin red, flavour sour, eating quality excellent.
106	48 Ambrosia	EC798749	USA (California)	Fruits large, taste tart and sweet with pleasant aroma, kernels large and thick, good for jelly, syrup, and fresh eating.
108	91 Dewey	EC798789	Unknown	Fruit medium to large, reddish-pink.
109	103 Shirin Zigar	EC798796	Turkmenistan	
113	86 Cranberry	EC798784	USA (California)	Fruit uniformly cranberry red, size medium, suitable for culinary use.
5	127 Kemine	EC798819	Turkmenistan (Balkan)	wild
8	64 DPUN 64	EC798764	Unknown	wild
15	163 Machtumkuli	EC798848	Turkmenistan	wild

Code No.	Accessions	Accession No	Origin	Description
16	130 Messarian	EC798822	Turkmenistan (Balkan)	wild
21	151 Sirenevyi	EC798839	Turkmenistan (Balkan)	wild
23	135 Azadi	EC798827	Turkmenistan (Balkan)	wild
24	60 DPUN 60	EC798760	Unknown	wild
25	47 DPUN 47	EC798748	Unknown	wild
26	157 Chandyr	EC798845	Turkmenistan (Balkan)	wild
27	158 Balkan	EC798846	Unknown	wild
29	131 Dahistan	EC798823	Turkmenistan (Balkan)	wild
33	147 Sumbar	EC798835	Turkmenistan (Balkan)	wild
35	80 DPUN 80	EC798778	Unknown	wild
36	115 11 / 15	EC798808	Unknown	wild
38	123 Saharnyi	EC798815	Turkmenistan (Balkan)	wild
43	56 Purple Heart	EC798756	USA (California)	wild
44	78 DPUN 78	ED798776	Unknown	wild
45	154 Chernaya roza	EC798842	Turkmenistan (Balkan)	wild
49	143 Sogdiana	EC798833	Turkmenistan (Balkan)	wild
51	136 Syunt	EC798828	Turkmenistan (Balkan)	wild
52	137 Andalib	EC798829	Turkmenistan (Balkan)	wild
53	67 DPUN 67	EC798766	Unknown	wild
54	206 WEO 50	EC798851	USA (California)	wild
55	153 Kyz-bibi	EC798841	Turkmenistan (Balkan)	wild
57	74 DPUN 74	EC798772	Unknown	wild
59	113 15/4 Pamyati rozanova	EC798806	Turkmenistan	wild
60	68 DPUN 68	EC798767	Unknown	wild
61	58 DPUN 58	EC798758	Unknown	wild
62	70 DPUN 70	EC798768	Unknown	wild
65	152 Kopetdag	EC798840	Turkmenistan (Balkan)	wild
69	66 DPUN 66	EC798765	Unknown	wild
75	140 Seidi	EC798831	Turkmenistan (Balkan)	wild
81	116 Vishnevyi	EC798809	Turkmenistan (Balkan)	wild
82	142 Anvari	EC798832	Turkmenistan (Balkan)	wild
87	126 Girkanets	EC798818	Turkmenistan (Balkan)	wild
88	133 Hvalynskii	EC798825	Turkmenistan (Balkan)	wild
93	145 Nusai	EC798834	Turkmenistan (Balkan)	wild
94	155 Kara gul	EC798843	Turkmenistan (Balkan)	wild
96	129 Nisa	EC798821	Turkmenistan (Balkan)	wild
97	150 Ovadan	EC798838	Turkmenistan (Balkan)	wild
98	160 Gulyalek	EC798847	Turkmenistan (Balkan)	wild
100	63 DPUN 63	EC798763	Unknown	wild
101	79 DPUN 79	EC798777	Unknown	wild
103	114 31/69	EC798807	Turkmenistan	wild
105	73 DPUN 73	EC798771	Unknown	wild

Code No.	Accessions	Accession No	Origin	Description
107	51 DPUN 51	EC798751	USA (California)	wild
110	120 Kubarchatyi	EC798812	Turkmenistan (Balkan)	wild
111	76 DPUN 76	EC798774	Unknown	wild
112	72 DPUN 72	EC798770	Unknown	wild
114	128 Molla Nepes	EC798820	Turkmenistan	wild

Table 2. List of sequence of forward and reverse SRAP primers used in this study

Sl. No.	Primer name	Sequence (3'-5')
1	Me 1F	TGA GTC CAA ACC GGA TA
2	Me 2F	TGA GTC CAA ACC GGA GC
3	Me 3F	TGA GTC CAA ACC GGA AT
4	Me 4F	TGA GTC CAA ACC GGA CC
5	Me 5F	TGA GTC CAA ACC GGA AG
6	Me 6F	TGA GTC CAA ACC GGA CA
7	Me 7F	TGA GTC CAA ACC GGA CG
8	Me 8F	TGA GTC CAA ACC GGA CT
9	Me 9F	TGA GTC CAA ACC GGA GG
10	Me 10F	TGA GTC CAA ACC GGA AA
11	Me 11F	TGA GTC CAA ACC GGA AC
12	Me 12F	TGA GTC CAA ACC GGA GA
13	Me 13F	TGA GTC CAA ACC GGA AG
14	Em 1R	GAC TGC GTA CGA ATT AAT
15	Em 2R	GAC TGC GTA CGA ATT TGC
16	Em 3R	GAC TGC GTA CGA ATT GAC
17	Em 4R	GAC TGC GTA CGA ATT TGA
18	Em 5R	GAC TGC GTA CGA ATT AAC
19	Em 6R	GAC TGC GTA CGA ATT GCA
20	Em 7R	GAC TGC GTA CGA ATT CAA
21	Em 8R	GAC TGC GTA CGA ATT CAC
22	Em 9R	GAC TGC GTA CGA ATT CAG
23	Em 10R	GAC TGC GTA CGA ATT CAT
24	Em 11R	GAC TGC GTA CGA ATT CTA
25	Em 12R	GAC TGC GTA CGA ATT CTC
26	Em 13R	GAC TGC GTA CGA ATT CTG
27	Em 14R	GAC TGC GTA CGA ATT CTT
28	Em 15R	GAC TGC GTA CGA ATT GAT
29	Em 16R	GAC TGC GTA CGA ATT GTC

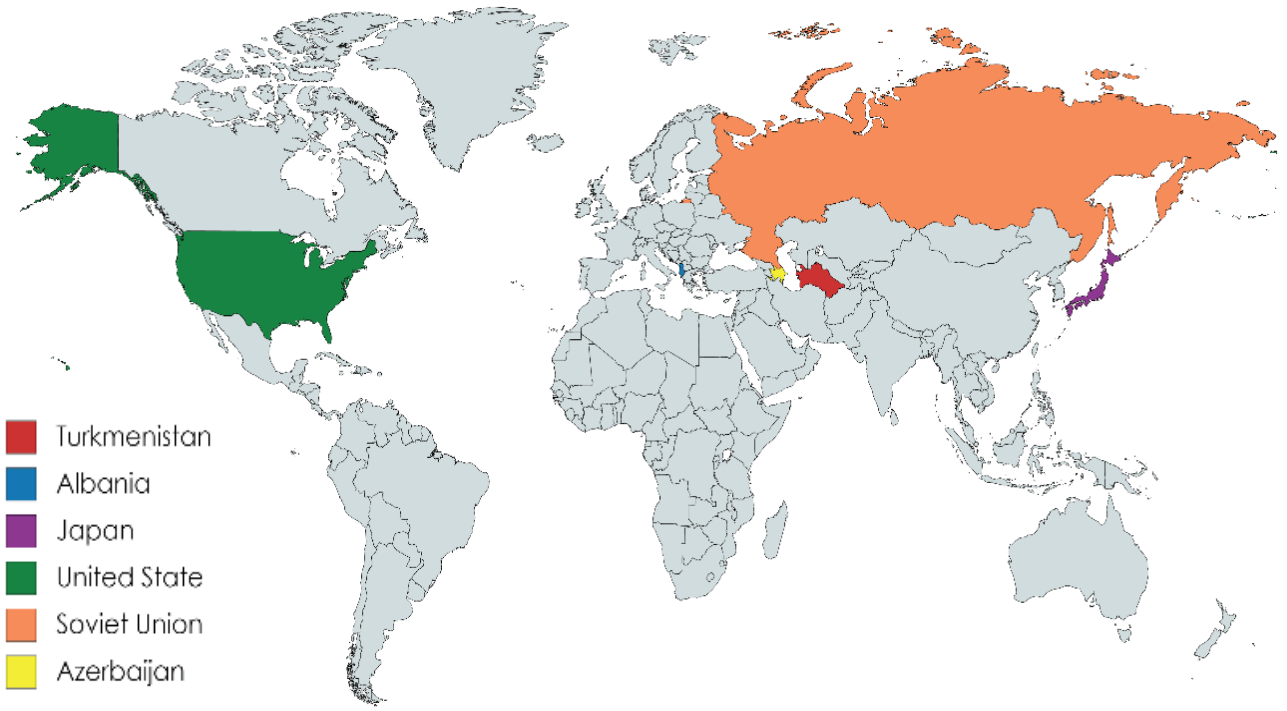

Fig. 1. Pomegranate accessions collected from different regions

and Me5F +Em1R produced maximum number of polymorphic bands (4). These primer combinations were found to be the most informative for genetic diversity studies among pomegranate accessions. The number of alleles per locus ranged from 1.66 to 4.00 with an average of 2.03. The expected heterozygosity varied from 0.215 to 0.445 with an average of 0.306. In previous studies, SRAP markers have been shown to be quite efficient in evaluating the genetic diversity in pomegranate. Substantial polymorphism (53%) was indicated by Soleimani *et al*. (2012) using SRAP marker system on accessions native to different regions of Iran. In another study by Amar and El-Zayat, (2017) this marker system was found to be superior to ISSR and IRAP markers in estimating molecular diversity among the Egyptian pomegranate varieties. Since the accessions in the germplasm have been derived from different geographical regions of the world, this investigation revealed significant polymorphism (47.8%) among the accessions and is in agreement with the result of previous workers. The PIC value of a marker is a useful measure of the efficiency of polymorphic loci in revealing genetic diversity among accessions. Botstein *et al*. (1980) reported that PIC index can be used to evaluate the level of gene variation, when PIC>0.5, the locus was of high diversity; when PIC<0.25, the locus was of low diversity and the locus was of intermediate diversity, when PIC was between 0.25 and 0.5. In this study, five PCs had PIC<0.25 and five had 0.5>PIC>0.25, with an average of 0.24 which is low signifying low variability among the accessions probably due to narrow genetic pool and monogeneric nature of this crop.

The genetic relatedness among the 114 different pomegranate accessions was assessed using Jaccard's co-efficient of similarity and by generating dendrogram based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The resulting dendrogram (Fig. 2) was highly fragmented implying that the accessions were genetically distinct and most variation was present within the clusters. All the 114 accessions clustered into 4 major groups, the inter cluster distance lying between 0.3 - 0.4. The I cluster contained 12 accessions out of which 10 (83%) were wild. The II cluster comprised of 5 accessions, all of which belonged to Turkmenistan except number 86 which originated from former Soviet Union. In this cluster there were 2 wild and 2 soft seeded accessions. The III is the largest containing 80% (91) of the accessions. The III cluster was further divided into 3 sub-clusters (III a, III b and III c) comprising of 8, 37 and 46 accessions respectively. The sub-cluster III a, consisted of 8 accessions. Two ornamental accessions available in this germplasm collection came together in this cluster. The sub-cluster III b comprised of 37 accessions out of which 25 (68%) were cultivars. Maximum number of accessions present in this cluster originated from Turkmenistan (38%) followed by USA

Table 3. Polymorphic information content (PIC) value and incidence for 10 SRAP PCs

Sl. No.	Primer combination	No. of total bands	No. PB	PPB (%)	Expected Heterozygosity	PIC
1	Me1F +Em1R	5	3	60	0.3304	0.2476
2	Me1F +Em4R	4	2	50	0.2554	0.1919
3	Me1F +Em8R	6	4	66	0.4459	0.3421
4	Me3F +Em4R	4	2	50	0.3067	0.2401
5	Me6F +Em3R	5	3	60	0.2416	0.1944
6	Me6F +Em4R	5	2	40	0.3329	0.2489
7	Me5F +Em5R	7	3	43	0.2752	0.2193
8	Me2F +Em2R	7	3	43	0.3248	0.2518
9	Me5F +Em1R	12	4	33	0.3359	0.2732
10	Me8F +Em13R	9	3	33	0.2156	0.1709
Total		64	29	-	3.06	2.38
Mean		-	-	45	0.30	0.24

PIC- Polymorphic Information Content.
PPB- Percentage of Polymorphic Bands.

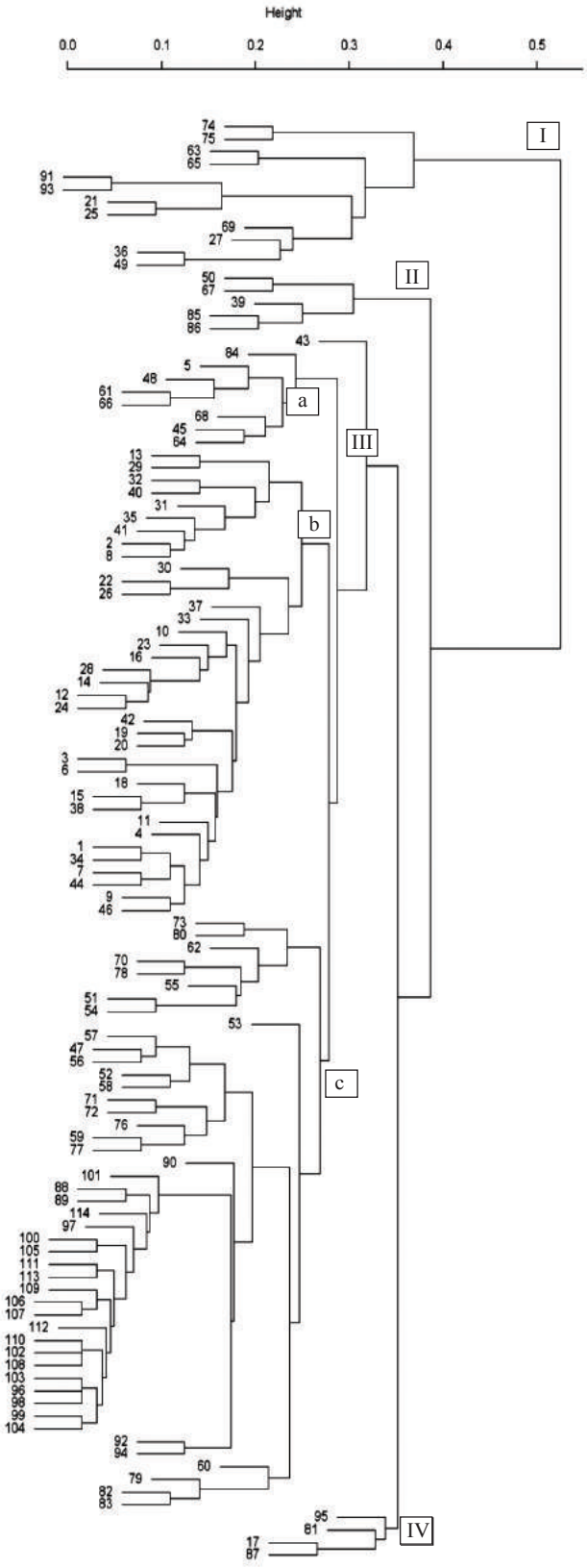

Fig. 2. Neighbour-joining dendrogram of 114 pomegranate accessions based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) using SRAP markers

(30%) and Japan (8%). This cluster contained maximum number of soft seeded cultivars (16%) and cultivars for which phenotyping data had been generated (numbers 13,22,31, 32 and 42). The sub cluster III c was found to be the largest containing a total of 46 accessions and geographically heterogeneous of all. This sub cluster had high number of accessions from Turkmenistan (43%) and USA (33%) contributing to 27 cultivated accessions. The number of soft seeded cultivars (4%) and accessions from unknown origin (17%) were less in comparison to the previous sub-cluster. The IV cluster was the smallest and consisted of 4 accessions of which, one being a cultivar and one hybrid along with two accessions of wild nature from Turkmenistan. The analysis of genetic distances showed that the distances between the 114 pomegranate accessions ranged between 0.01 and 0.84 with an average of 0.42. The largest distance (0.84) was recorded between ‘‘DPUN121 Lyubimyi’’ (2) and ‘‘DPUN115 32/30’’ (36) which are both wild types; the lowest distance was recorded between several pairs of accessions which were present in sub cluster III c. Analysis of wild pomegranates in the western Himalayas by use of RAPD, DAMD, and ISSR markers (Narzary *et al*., 2009 and 2010), Tunisian pomegranates on the basis of AFLP profiles (Jbir *et al*., 2008), and Iranian pomegranate accessions by use of RAPD and ISSR markers (Talebi *et al*., 2011) has revealed that the geographical diversity of the accessions is not correlated with genetic diversity. Similar result was obtained in the present study which provides evidence that SRAP markers are simple, informative and reproducible approach for evaluation of molecular diversity and phylogenetic relationships in pomegranate germplasm with the added advantage that they target ORFs as functional regions of the pomegranate genome.

The relationship between accessions for genetic, geographical origin and pomological traits was made clear by the clustering pattern. Wild pomegranate is known to occur in the regions from Iran to northern India, as can be observed in the forests of these areas (Stover and Mercure, 2007). These mostly have thicker rind, smaller arils, harder seeds and much higher acidity than the cultivated types. On the basis of SRAP markers, the wild pomegranates from Turkmenistan considered in this study were found to be very similar to each other and they mainly clustered in Group I (83%). The ornamental accessions clustered with cultivars in group III a. These ornamental plants are double-flower forms which, as the name implies, produce double flowers wherein

numerous stamens are modified into petals; they do not usually set fruit but, because of their beautiful flowers, are important as ornamental plants. These sterile flowers can be pollinated manually to yield fruit (Jalikop, 2007). The same result of narrow genetic variation between ornamental and cultivated types has been reported by Jbir *et al*. (2008). Pomegranate accessions can be divided into four groups based on seed hardness: soft, semi-soft, semi-hard and hard seeded (Zamani, 1990). Commercial pomegranate cultivars are often semi-hard or hard-seeded which is a distinct disadvantage, as soft-seeded fruit are the consumer's fruit of choice. According to Levin (1994), completely soft-seeded pomegranates are restricted to a few narrow ecological regions representing an important genetic resource for improving the seed softness trait in commercial pomegranate varieties. There are 8 soft seeded cultivars present in the collection and maximum number (6) clustered together in group III b (13,32,10,12,3,1). This group also comprised of 6 accessions with sweet taste (3, 13,20,28,37,95,102,19), 6 accessions with sweet-sour taste (31,22,12,10,1,42), 3 accessions with tart and watery taste (18,4,46) and 2 sour accessions (9,32). The lowest genetic distance was observed between accessions (110,102,108,103,96,98,99 and 104)) located in group III c. The SRAP data of these accessions are very similar in pattern though they have originated from different geographical areas (Turkmenistan, USA, Japan). Similar observation was made in a recent study by Giancaspro *et al*., 2017 using SSR markers the authors report that pomegranate accessions from different geographical areas appeared more similar with respect to accessions within the same country. They attributed this to the intense flow of genetic materials from Persia to different countries all over the world since ancient times which has led to genetic homogenization among these groups of accessions.

To understand the genetic relationships among accessions that originated from different geographical regions, they were divided into 4 panels (Asia, North America, Europe and Unknown). Four indices of population-level genetic diversity, namely observed number of alleles (Na), expected heterozygosity (He), effective number of alleles (Ne) and Shannon's information index (*I*), were calculated as shown in (Table 4). As a whole among the four populations, the effective number (Ne) of alleles ranged from 1.29 to 1.95 with average of 1.66 the Shannon's information index (*I*) ranged from 0.53 to 0.68 with average of 0.63 and expected heterzygosity (He) ranged from 0.34 to 0.48 with average of 0.44 at the group level. The highest Shannon's information index (*I*), observed and effective number of alleles (Na and Ne), and expected heterozygosity (He) were observed in accessions from the Unknown region which constitute the wild types (Table 4). Analysis of molecular variance (AMOVA) was performed to study genetic differentiation among four population from distinct geographic regions and to estimate the percentage of intra and inter- population genetic variation (Table 5). The results from AMOVA analysis revealed that 99% of total genetic variation occurred within population and only 1% was attributed to among populations. The analysis indicated insignificant genetic variation (p = 0.31) between pomegranate accessions in different populations, low differentiation in allele frequencies (F-statistics = 0.019) and high gene flow (Nm =24.353) among the four populations (Table 5) which indicates that the genetic diversity of pomegranate is independent of their geographical origin. The mean genetic distance among groups ranged from 0.165 to

Table 4. Summary of genetic variation statistics for 10 SRAP primer combinations in four pomegranate population by AMOVA (Analysis of molecular variance)

Population	Sample size	Band frequency	Na	Ne	*I*	He
Asia minor	54	0.611	2.000	1.885	0.662	0.469
North America	25	0.760	2.000	1.296	O.678	0.485
Europe	5	0.400	2.000	1.537	0.534	0.349
Unknown	30	0.683	2.000	1.956	0.682	0.488
Total	114	2.454	8.000	6.674	2.556	1.791
Mean	-	-	2.000	1.668	0.639	0.447

Na - Observed number of alleles,
Ne - Effective number of alleles,
I - Shannon's information index,
He -Expected heterozygosity,

Table 5. Analysis of molecular variance (AMOVA) among and within the pomegranate accessions for four populations using SRAP markers

Source of variation	Degrees of freedom	Sum of Squares	Mean Square	Estimated Variance	Percent variance	*P* value	Fst	Nm
Among Pops	3	1.712	0.571	0.005	1%	0.31	0.019	24.353
Within Pops	110	49.700	0.452	0.452	99%			
Total	113	51.412		0.457	100%			

Fst- F statistics
Nm- Gene flow

0.011. The highest genetic distance (0.165) was observed between the North America and Europe population and lowest (0.011) was detected among the Asia and the Unknown population (Table 6). The result obtained from analysis of molecular variance and pair wise F-statistics test showed insignificant differences among the populations. The result of AMOVA is in agreement with genetic relationship between accessions by cluster analysis, where accessions belonging to different origins have grouped together. The low differentiation in allele frequencies among populations (Fst = 0.019) and high gene flow (Nm = 24.35) observed in this study indicated that the genetic diversity of pomegranates is independent of their geographical origin. Similar result has been reported by Giancaspro *et al*. (2017); Soleimani *et al*. (2012); Jbir *et al*. (2008) and Talebi *et al*. (2011) using different molecular markers which explains that the geographical diversity of the accessions is not correlated with genetic diversity. Pomegranate is an ancient fruit crop which has been propagated clonally by cuttings. It is adaptable to various soil and climate conditions resulting in its spread to different parts of the world. It can therefore be concluded that the higher level of genetic diversity within populations and the lower level among geographical groups is a result of movement of genetic material from the centre of origin (Central Asia) to other countries such as Japan, USA, European countries leading to high level of migration and gene flow among regions. The present investigation clearly demonstrated the efficiency of SRAP markers in detecting sufficient polymorphism to establish genetic relationships and population genetic structure of a diverse collection of cultivars and wild pomegranates sourced from different geographical regions of the world. These markers have a great potential since they target the ORFs as functional regions of the pomegranate genome which could be useful in identifying trait-marker association of interest in the marker-assisted breeding programs. Close relationship between wild and cultivated pomegranates indicates that the cultivated pomegranates have descended from wild plants and they are genetically not very diverse. Low genetic differentiation between accessions from different geographical regions of the world points to narrow genetic base, migration and gene flow in this crop since ancient times.

Table 6. Matrix of genetic distances among pomegranate populations

	Asia minor	North America	Europe	Unknown
Asia minor	0.000			
North America	0.052	0.000		
Europe	0.034	0.165	0.000	
Unknown	0.011	0.015	0.078	0.000

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