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Assessment of Wild and Cultivated *Punica granatum* L. using Random Amplified Polymorphic DNA (RAPD) Markers

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ABSTRACT

In *Punica granatum* L. genetic variability was investigated among wild and five pomegranate cultivars ('Ganesh', 'G-137', 'Mridula', 'Musket', 'Kandhari Kabuli') growing in Western Himalaya, India. Random amplified polymorphic DNA analysis was studied using 21 arbitary decamer primers to observe genetic homogenity/polymorphism among wild and cultivated genotypes. The percentage of polymorphism was 76.26%. Jaccard's coefficient and UPGMA-based analysis showed 0.62 to 0.83 similarities among these genotypes. In the dendrogram, there was one main cluster and 4 subclusters. Wild pomegranate was found to be distant from the cultivars and grouped alone at the 69% similarity level, while Kandhari Kabuli and Mridula showed maximum similarity (0.83). Musket and Ganesh formed one cluster, while was G-137 clustered alone. The lowest value (0.62) was found between Ganesh and G-137, whereas the maximum value (0.83) was found between Kandhari Kabuli and Musket, suggesting a common origin of all cultivars from the same parent.

Keywords: cluster analysis, genetic diversity, pomegranate, stability and variability
Abbreviations: RAPD, Random Amplified Polymorphic DNA

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family Punicaceae. It is thought to have originated from a region in Iran according to Eveinoff (1949) and from there it spread to Mediterranean countries. It is an important fruit crop of tropical and subtropical regions of the world. The tree requires a hot and dry climate during the period of fruit development and ripening. High quality fruits can be produced where there are cool winters and hot summers. So, the mid-hills of Western Himalayas provide a congenial climate for pomegranate cultivation (Kumar 2005).

Pomegranate fruits are a good source of sugar and vitamin C and a fair source of iron. Seed with arils are sun-dried and commercially marketed as condiments. A wide range of variability present in a species always provides a better chance of selecting a desirable type. The variation among pomegranate (*Punica granatum* L.) cultivars was noted earlier (Kumar 2005), by using morphological parameters. Molecular characterization of germplasm using molecular markers helps to determine the similarities and differences in genetic makeup of plants. Recently, most suitable markers used to access the genetic variability in pomegranate cultivars are multi-loci marker such as Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphic DNA (AFLP). The interspecific variability of fatty acid composition and RAPD profile was used to examine biochemical and genetic relationships among six pomegranate cultivars that dominate pomegranate production in Southern Anatolia Region of Turkey (Ercisli *et al*. 2007). Similarly, genetic diversity among eighty five cultivars of Chinese pomegranate was studied by means of fluorescent-AFLP markers (Yuan *et al*. 2007).

MATERIALS AND METHODS

Plant material

Five popular cultivars ('Ganesh', 'G-137', 'Mridula', 'Musket' and 'Kandhari Kabuli'), growing in the fields of Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, India and one wild genotype of pomegranate were used for DNA extraction and RAPD amplification.

DNA extraction

DNA was extracted from fresh green leaves by CTAB method as described by Doyle and Doyle (1987) with some modifications. The upper most unfolded leaves from six year-old trees were collected in March-April when the trees are in their vegetative stage. Two grams of leaves sample were ground to a fine powder in liquid nitrogen using a pre-cooled pestle and mortar and the powder was transferred to 15 ml of pre-warmed (65°C) extraction buffer (100 mM Tris HCl (pH 8.0) (Sisco Research Laboratories Pvt. Ltd., Mumbai, India), 20 mM EDTA (Sisco Research Laboratories), 1.4 M NaCl (Sisco Research Laboratories), 2% CTAB (*N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide) (Genei Pvt. Ltd., Bangalore, India), 0.2% v/v β-mercaptoethanol (Sisco Research Laboratories) in a 50 ml capacity polycarbonate centrifuge tube (Tarsons Products Pvt. Ltd., Calcutta, India). The mixture was thoroughly stirred and incubated at 65°C for 1 h and then kept at room temperature for 10-15 min. The suspension was extracted with 15 ml of chloroform: isoamylalcohol (24:1 v/v) (Sisco Research Laboratories) to denature proteins and facilitate phase separation. After centrifugation at 14,000 rpm for 10 min at 20°C, the upper aqueous phase was pipetted out carefully into a fresh centrifuge tube. The DNA was precipitated by adding 2/3 volume of isopropanol (Sisco Research Laboratories) at room temperature. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The pellet was washed in 5 ml ethanol (70%) (Sisco Research Laboratories), and air dried overnight. The pellet was redissolved in sterile distilled water. Single stranded RNA was digested with RNase A (Sigma, USA) (10 µg/ml) for 30 min at 37°C, extracted

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once with phenol (Genei): chloroform: isoamylalcohol (25:24:1 v/v/v), precipitated by adding 1/10 volume of ammonium acetate (Sisco Research Laboratories) and 2 volume of chilled ethanol, washed with ethanol (70%) and dissolved in sterile Milli Q water. DNA samples were electrophoresed in 0.4% agarose (Central Drug House Pvt. Ltd., New Delhi, India) in TBE buffer to check the quality of DNA and quantified using a Bio Rad Smartspec TM3000 and purity was also determined using the 260/280 ratio. DNA was diluted to 25 ng/µl for PCR analysis.

PCR amplification

Six genotypes of pomegranate using 21 random decamer primers were characterized. Arbitrary decamer primers from Operon Technologies, USA (OPA-05, OPA-09, OPA-10, OPA12, OPA-17 and OPA-20 from Kit A); (OPB-05, OPB-11, OPB-18 and OPB-20 from Kit B); (OPC-01,and OPC-13 from Kit C); (OPD-13, OPD-18 and OPD-18 from Kit D) and (OPE-04, OPE-06, OPE-12, OPE-13, OPE-14 and OPE-19 from Kit E) were used for amplification reactions. PCR was performed in a reaction volume of 13 µl containing 0.5 U Taq DNA polymerase (Genei); 1× Taq Buffer (10×: 50 mM KCl, 10 mM Tris-HCl, 15 mM gelatin; Genei); 200 µM of each dNTP, 25 pM of primer and 50 ng of genomic DNA. The amplification was carried out in a Perkin Elmer Gene Amp 9700 PCR system version 3.03 with the following amplification parameters: denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 1 min, primer annealing at 35.5°C for 1 min and amplification at 72°C for 2 min. Finally, an amplification step was carried out at 72°C for 10 min.

Amplified DNA was electrophoresed in 1.8% agarose gel under submerged conditions. 1×TAE buffer was used as gel and tray buffer. Three ml of 6× loading dye (bromophenol blue) was added to each PCR amplified sample and loaded into the gel. 10,000 bp DNA ladder marker (Fermentas Life Sciences, Glen Burnie, MD) was used as standard. The amplified DNA was viewed under a UV trans-illuminator and photographs were taken by a Flour S^{TM} Multi imager and images were saved on a Bio Rad Computer.

Data analysis

Each amplified product was scored across all six pomegranate genotypes for 21 primers for its presence or absence. Co-migrating bands were considered to represent the same locus and thus treated as the same band while scoring. The presence of an amplified product was designated as '1' and absence was marked as '0'. Intensity of the products was not taken into account while scoring. NTSYS-pc, version 2.02 (Numerical Taxonomy System, Exeter Software) was used to perform cluster analysis of the complete RAPD data. A pair-wise similarity matrix was constructed by using the Dice similarity index SD = 2a/ (2a+b+c) Dice (1945), where a is the number of bands present in a pair of plants 'i' and 'j', b is the number of bands present in plant 'i' and absent in plant 'j' and 'c' the number of bands present in plant 'j' and absent in plant 'i'. Similarity estimates were analyzed by the unweighted pair group method with arithmetic averages (UPGMA) and the resulting clusters were represented as dendrograms. Dendrogram was constructed using Numerical Taxonomy System, Exeter Software.

RESULTS AND DISCUSSION

RAPD analysis was used to assay six pomegranate genotypes (**Figs. 1-3**) growing in the Western Himalayas. The sequences of 21 random primers used in this study and the number of RAPD markers generated for each are given in **Table 1**. A total of 198 bands ranging from 217 to 2893 bp were obtained of which 23.74% were monomorphic, 76.26% were polymorphic shared by at least 2 genotypes (**Table 2**). This high degree of polymorphism detected with

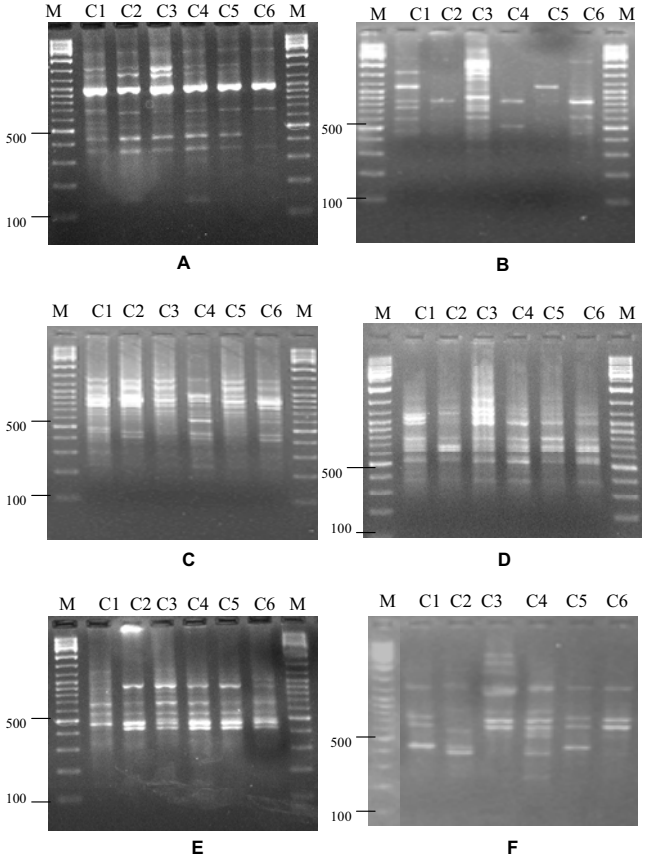

Fig. 1 RAPD patterns of *Punica granatum* L. genotypes using 10-mer primers. (**A**) OPD-16, (**B**) OPD-17, (**C**) OPD-18, (**D**) OPE-04, (**E**) OPE-06 and (**F**) OPE-12. Lanes: M, Gene Ruler^{TM} (10,000 bp molecular marker); C1, 'Daru' (wild pomegranate); C2, 'Ganesh'; C3, 'G-137'; C4, 'Mridula'; C5, 'Musket'; C6, 'Kandhari Kabuli'.

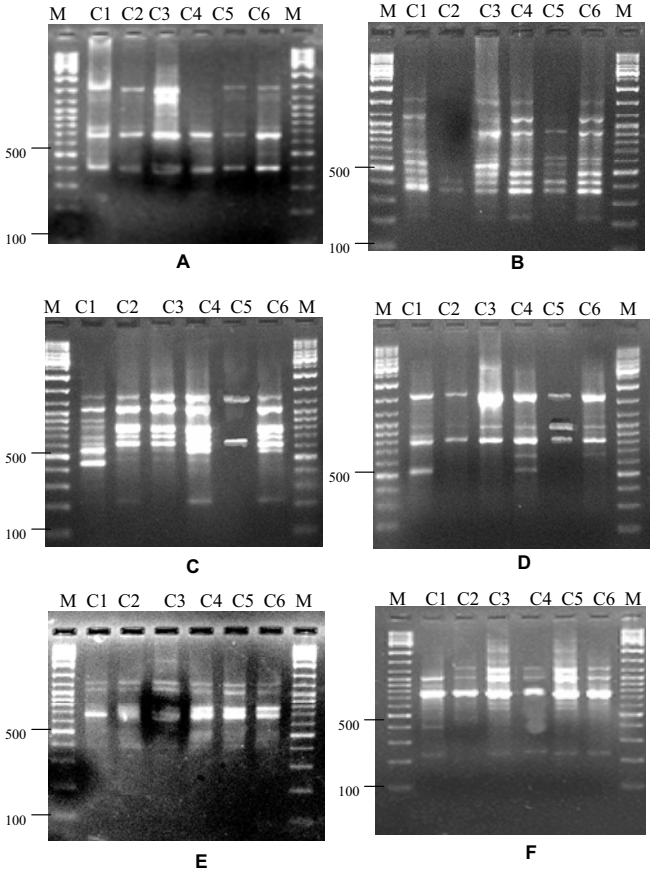

Fig. 2 RAPD patterns of *Punica granatum* L. genotypes using 10-mer primers. (**A**) OPB-05, (**B**) OPB-11, (**C**) OPB-18, (**D**) OPB-20, (**E**) OPC-01 and (**F**) OPC-13. Lanes: M, Gene Ruler^{TM} (10,000 bp molecular marker); C1, 'Daru' (wild pomegranate); C2, 'Ganesh'; C3, 'G-137'; C4, 'Mridula'; C5, 'Musket'; C6, 'Kandhari Kabuli'.

Table 1 Description of 21 decamer primers used for fingerprint analysis of six genotypes of pomegranate, *Punica granatum* L.

Sr. №	Primer name	Sequence	Scorable bands	Monomorphic bands	Polymorphic bands	Fragment size (bp)
1	OPA-05	AGGGGTCTTG	17	1	16	2356-447
2	OPA-09	GGGTAACGCC	9	2	7	2100-630
3	OPA-10	GTGATCGCAG	9	0	9	1872-479
4	OPA-12	TCGGCGATAG	9	3	6	1150-517
5	OPA-17	GACCGCTTGT	9	2	7	2623-619
6	OPA-20	GTTGCGATCC	6	2	4	1704-588
7	OPB-05	TGCGCCCTTC	11	3	8	1456-376
8	OPB-11	GTAGAGCCGT	11	2	9	1521-224
9	OPB-18	CCACAGCAGT	9	5	4	1335-218
10	OPB-20	GGACCCTTAC	8	2	6	2795-537
11	OPC-01	TTCGAGCCAG	4	3	1	1269-645
12	OPC-13	AAGCCTCGTC	8	2	6	1500-393
13	OPD-16	AGGGCGTAAG	8	5	3	1497-354
14	OPD-17	TTTCCCACGG	12	0	12	2121-520
15	OPD-18	GAGAGCCAAC	14	4	10	1563-305
16	OPE-04	GTGACATGCC	10	5	5	1583-393
17	OPE-06	AAGACCCCTC	8	2	6	1262-468
18	OPE-12	TTATCGCCCC	14	0	14	2893-238
19	OPE-13	CCCGATTCGG	8	1	7	2013-293
20	OPE-14	TGCGGCTGAC	11	1	10	1042-307
21	OPE-19	ACGGCGTATG	3	2	1	691-217

Table 2 Summary showing RAPD amplified products obtained from six genotypes of *Punica granatum* L.

Total number of primers used	21
Number of polymorphic primers	21
Total number of scorable bands amplified by polymorphic primers	198
Average number of bands per polymorphic primer	9.42
Total number of polymorphic bands identified	151
Total number of monomorphic bands identified	47
Average number of polymorphic bands per polymorphic primer	7.19
Percentage of total polymorphic bands	76.26 %

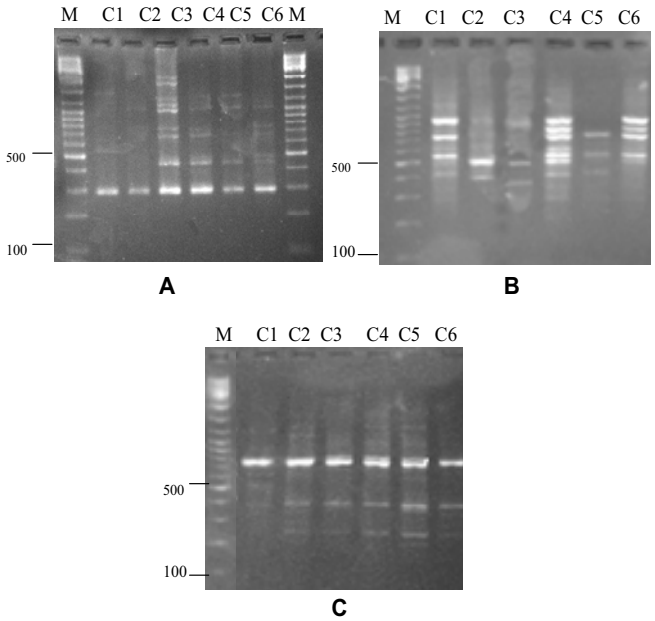

Fig. 3 RAPD patterns of *Punica granatum* L. genotypes using 10-mer primers. (**A**) OPE-13, (**B**) OPE-14 and (**C**) OPE-19. Lanes: M, Gene Ruler^{TM} (10,000 bp molecular marker); C1, 'Daru' (wild pomegranate); C2, 'Ganesh'; C3, 'G-137'; C4, 'Mridula'; C5, 'Musket'; C6, 'Kandhari Kabuli'.

21 RAPD markers indicates a high marker index. This was comparable with the results obtained by Shashidhara *et al*. (2003) in *Santalum album* (sandalwood) (156 bands from 11 primers) and Samaee *et al*. (2003) in *Olea europea* (olive) cultivars (153 polymorphic bands from 21 primers). In contrary, Sharifani and Jackson (2000) used 20 primers to characterized 16 *Pyrus* species (pear). Only 9 primers were polymorphic and yielded 66 scorable bands, of which only 18 were useful polymorphic bands. The genetic diversity among the Chinese pomegranate cultivars was studied by means of fluorescent – AFLP markers by Yuan *et al*. (2007). An average of 158.25 polymorphic loci were amplified by eight pairs of primers. The polymorphism percentage ranged from 62.5% to 86.11% although the average polymorphism percentage was 73.26%. This indicated presence of plentiful genetic diversity in pomegranate cultivars.

The average number of scorable bands per primer was 9.42 and the average number of polymorphic bands per polymorphic primer was 7.19 (**Table 2**). However, the maximum numbers of polymorphic bands were found to be 16 with primer OPA-05, whereas only one polymorphic band was obtained with primer OPC-01 and OPE-19. Each primer except OPC-1, OPE-06 and OPE-19 yielded a unique set of amplified products. RAPD markers specific for a particular genotype are described in **Table 3**.

Cluster analysis was done on basis of similarity coefficients generated from RAPD data of 198 bands. Similarity indices ranged from 0.66 to 0.83 among six genotypes. Kaushal and Kanwar (2003) observed a high range of similarity coefficient (0.86 to 0.96) among micropropagated plants of *Robinia pseudocacia* L. Soyza and Lima (2004) found a high range of similarity coefficient (0.55 to 0.98) among 40 genotypes of *Mangifera indica* (mango) by using 32 random 10-mer primers. In contrary, a low similarity value (0 to 49%) was observed by Mallikarjuna *et al*. (2005) among 32 *Arachis* accession using 29 primers. Similarly, Das *et al*. (2004) found a low value (16-60%) of genetic distance among 12 cultivars and rootstocks of *Citrus* using 10 decamer primers.

Cluster analysis based on the result of 21 polymorphic primers was used to generate a dendrogram (**Fig. 4**) grouped 6 genotypes into one major cluster at a 66% similarity level depicting their origin from the same parent, possibly a long time ago. The wild genotype formed its own cluster at the 69% similarity level. However, 'Kandhari Ka-

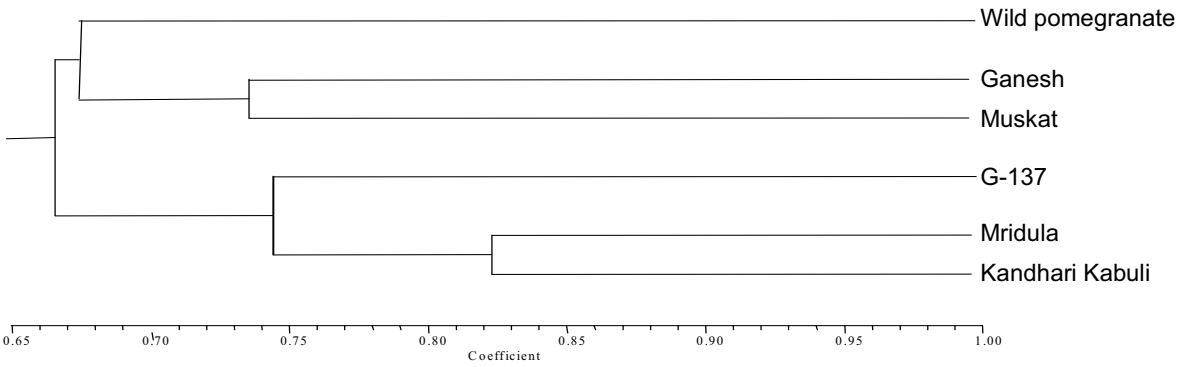

Fig. 4 UPGMA dendrogram with similarity coefficients between pomegranate genotypes (*Punica granatum* L.) based on RAPD data.

Table 3 Informative RAPD markers specific for a particular pomegranate genotype.

Primer	Size of DNA Band	Genotype
OPA-05	2042 bp	G-137
OPA-09	2100 bp	G-137
	1798 bp	G-137
OPA-10	479 bp	Wild pomegranate
OPA-12	785 bp	Ganesh
	678 bp	Mridula
OPA-17	2623 bp	G-137
	1912 bp	G-137
	973 bp	G-137
	1097 bp	Kandhari Kabuli
OPA-20	781 bp	Ganesh
	699 bp	Mridula
OPB-05	1378 bp	G-137
	1330 bp	Wild pomegranate
	1152 bp	G-137
	621 bp	Mridula
	374 bp	G-137
OPB-11	1009 bp	Wild pomegranate
	608 bp	Wild pomegranate
OPB-18	905 bp	Wild pomegranate
OPB-20	2795 bp	G-137
	860 bp	Wild pomegranate
OPC-13	1300 bp	Ganesh
OPD-16	555 bp	Wild pomegranate
OPD-17	1618 bp	Wild pomegranate
	1567 bp	G-137
	1377 bp	G-137
	1132 bp	Wild pomegranate
OPD-18	1563 bp	Musket
	1391 bp	Mridula
	485 bp	Wild pomegranate
	305 bp	Wild pomegranate
OPE-04	1583 bp	G-137
	1349 bp	Ganesh
OPE-12	2893 bp	G-137
	2315 bp	G-137
	1906 bp	G-137
	687 bp	G-137
	573 bp	Ganesh
	554 bp	Mridula
	527 bp	Mridula
OPE-13	2013 bp	G-137
	1724 bp	G-137
OPE-14	1042 bp	G-137

buli' and 'Mridula' form one cluster at the 83% similarity level. The percentage of polymorphism was 76.26%.

However, five different clusters could be obtained. UPGMA cluster analysis reveals that six genotypes of *P. granatum* clustered in four different sub-clusters. Wild pomegranate was the most diverse and grouped alone in a separate cluster. 'Ganesh' and 'Musket' formed another cluster. Distribution of 'G-137' was somewhat random. 'G-137' was clonal selection of 'Ganesh' but it alone formed third clusters. 'Mridula' and 'Kandhari Kabuli' showed higher mean similarity values (0.83) and thus grouped into a separate cluster which originates in Afghanistan and Bangalore, respectively. Out of six genotypes of *P. granatum*, wild pomegranate is found to be most diverse as it is grouped alone in a separate cluster. On the basis of available literature *P. granatum* has originated from regions of Iran and spread to different countries. Similarity values of four clusters at different levels shows that all the six genotypes are related to each other to different extents but seems to be originated from same parent, may be very long ago in the past.

This investigation confirmed that RAPD markers provide a powerful and simple tool for genetic diversity studies and for DNA finger printing of individual genotypes of different pomegranate cultivars. Since each primer yields a typical electrophoretic pattern, the number of marker bands for each genotype fingerprint could be improved simply by performing further amplifications with more primers.

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