Indian J. Hort. 70(4), December 2013: 469-474

Molecular marker-based characterization and genetic diversity of pomegranate genotypes

Dimpy Raina*, W.S. Dhillon and P.P.S. Gill**
Department of Fruit Science, Punjab Agricultural University, Ludhiana 141004

ABSTRACT

Genetic diversity in pomegranate genotypes was assessed using 47 SSR markers. A total of 3,154 alleles were amplified with an average of 105 alleles per genotype. Number of bands amplified ranged from as low as 92 in Russian Seedling to as high as114 in Shirin Anar. The number of alleles detected for 47 SSR primers ranged from 1 to 4 with an average of 2.15 alleles per primer pair. The polymorphism information content (PIC) of SSR markers ranged between 0.0 and 0.66 with an average value of 0.43 per locus. Genetic similarities among 30 pomegranate genotypes varied from 0.78 to 0.95. The UPGMA dendrogram cluster analysis grouped the pomegranate genotypes into three major clusters (I, II and III). Maximum number of (21) genotypes were clubbed in cluster III and further subdivided into three different sub-clusters IIIA, IIIB and IIIC. UPGMA dendrogram generated showed some genotypes from same province clustered in one group like genotypes Kali Shirin, Anar Shirin, Shirin Anar, Anar Shirin Mohamad Ali Anar Moherab Shirin and Panipat Selection collected from Hisar (India) clustered in cluster IIIA and genotypes Moga Local, P-26, Khog, Ps-75-K3 and Kandhari Ganga Nagari grouped in cluster II were collected from Punjab. SSR markers as an excellent genetic marker system revealed a considerable variation and provided accurate genetic information for allelic profile of genotypes in pomegranate germplasm.

Key words: Pomegranate, SSR marker, genetic diversity, UPGMA dendrogram.

INTRODUCTION

Pomegranate (*P. granatum* L.) belongs to the monogeneric family Punicaceae, which consists of only two species: pomegranate and *P. protopunica* Balf. f., an endangered species from Socotraand is one of the oldest known edible fruits (Damania, 3). It is native to central Asia and has been cultivated for centuries in several regions with different environmental conditions. Hence, due to its multipurpose medicinal uses it is also known as “Super fruit” in the global functional food industry (Poyrazog *et al.*, 12). Characterizations of pomegranate genotypes have been evaluated with respect to many morphological traits and morphometric criteria. A high phenotypic variability characterized pomegranate genotypes regarding their leaf, flower and fruit characteristics. However, these analyses are less rewarding since they were based on parameters limited in number and highly influenced by the environmental conditions. Molecular markers have proved to be a powerful tool for assessing genetic variation, and phylogenetic and genetic relationships, as well as for studying relatedness among cultivars of many species. Molecular studies of the pomegranate have been restricted to examinations of randomly-amplified polymorphic DNA (RAPD) by (Durgac *et al*., 4), inter simple sequence repeats (ISSR) by and amplified fragment length polymorphism (AFLP) by (Jbir *et al*., 7). Microsatellite markers (SSRs) have proven to be very useful for cultivar identification, pedigree analysis, evaluation of genetic distance among organisms and genetic diversity studies in a wide range of plants due to their high polymorphism, abundance and co-dominant inheritance (Gupta *et al*., 6). In Punjab, only two pomegranate cultivars, *i.e.*, Ganesh and Kandhari are recommended for cultivation. PAU-Ludhiana, is maintaining germplasm of several pomegranate varieties introduced from other parts of the countries in order to broaden the genetic base. Therefore, microsatellite markers were used to examine the level and structure of genetic diversity and develop an identification key for this fruit crop.

MATERIALS AND METHODS

Thirty pomegranate genotypes were used in this study are presented in Table 1, including local cultivars, strains and exotic accessions, which are being maintained at Punjab Agricultural University, Ludhiana. For DNA extraction young, fresh, disease and insect-free leaves from different pomegranate genotypes were used for DNA extraction. Leaf samples were collected in butter papers and placed in ice containers while transferring from field to laboratory. Subsequently, these were stored in deep freezer at -80°C for DNA isolation and SSR marker studies. DNA was isolated using CTAB (Cetyl trimethyl ammonium

*Corresponding author's E-mail: dimpy.raina@gmail.com
**Director, Punjab Horticulture Postharvest Technology Center, PAU, Ludhiana

bromide) method as modified by Saghai-Maroof *et al.* (13) with some minor modifications. Forty seven SSR primer pairs from pomegranate (Pirseyedi *et al*., 11) were used for PCR amplification of DNA. 20 µl PCR reaction mix consisted of 40-60 ng of genomic DNA, 1 unit of *Taq* polymerase (Life Technologies, USA) and 2 µl of forward and reverse primers. PCR was performed using the following temperature profile: initial denaturation for 3 min. at 94ºC followed with 1 min. at 94ºC, 1 min. at an appropriate annealing temperature and 2 min. at 72ºC, with a final elongation step of 10 min. at 72ºC. To 20 µl of amplified product, 3.0 µl of 6X loading dye was added so as to make final concentration of the loading buffer in the reaction samples to 1X. The PCR products were fractionated in 2.5 per cent agarose in 0.5X TBE buffer. The gel was

Table 1. Type and origin of the pomegranate genotypes used in the study and total number of alleles amplified using 47 SSR markers.

Genotype	Symbol	Location (Geographical-coordinates)/ Source	Type/ Parentage	No. of amplified fragments
Ganesh	G	India (Rahuri)	Clonal selection (Alandi)	103
Mridula	M	India (Rahuri)	Hybridization (Ganesh × Gul-E-Shah Red	96
Jyoti	Jyoti	Darward (Karnataka)	Selection (Bassein Seedless × Dholka)	110
Kandhari	K	Afghanistan	Introduction	104
Moga Local	ML	India	Clonal selection	104
Assam Local	AL	India	Clonal selection	105
Mallas	Mallas	Iran	Introduction	103
Russian Seedling	RussianS	Russia	Introduction	92
G-137	G-137	India (Rahuri)	Clonal selection (Ganesh)	97
P-26	P-26	India	Clonal selection	110
Khog	Khog	India	Clonal selection	99
Jodhpur White	JhW	India	Clonal selection	113
Achikdana	Achikdana	India	Clonal selection	106
Panipat Selection	PS	India	Clonal selection	112
Shirin Anar	SA	India	Clonal selection	114
Kali Shirin	KS	India	Clonal selection	110
Anar Shirin	AS	India	Clonal selection	111
$PS_{75}k_5$	$PS_{75}k_5$	India	Clonal selection	97
Kandhari Kabuli	KK	India	Clonal selection	97
Anar Shrin Mohamad Ali	ASMA	India	Clonal selection (unknown Iranian variety)	110
Chawla-I	Chawla-I	India	Clonal selection	110
Chawla-II	Chawla–II	India	Clonal selection	111
Botta-I	Bh-I	India	Clonal selection	103
Botta-II	Bh-II	India	Clonal selection	104
Botta-III	Bh-II	India	Clonal selection	100
Anardana selection-I	ADS-I	India	Clonal selection	103
Anardana selection-II	ADS-II	India	Clonal selection	110
Amalidana	Amalidana	IIHR (Bengaluru)	Hybridization (Ganesh × Nana)	106
Kandhari Ganga Nagri	KGN	India	Clonal selection	103
Anar Mohereb Shirin	AMS	India	Clonal selection	111

prepared in 0.5X TBE buffer. Ethidium bromide was added at concentration of 0.5 µg/ µl. The gel was run at 10V/cm, visualized under UV light and photographed using UVP gel documentation system (Model GDS 7600). The total number of alleles was recorded for each microsatellite marker in all the genotypes under study by giving the number to amplified alleles as 1, 2, 3 and 4. The amplified bands were recorded as 1 (band present) and 0 (band absent) in a binary matrix and the lines which did not any amplification were scored as null alleles. The data was further analyzed for numeric taxonomic and multivariate analysis with NTSYS-pc (Version 2.02e) software. A dendrogram of 30 genotypes was constructed by using UPGMA (Unweighted Pair Group Method using Arithmetic Averages) available in NTSYS based on Nei genetic identity. Similarity coefficients were estimated for alleleic data generated by 47 SSR primer pairs by NTSYS-pc (Version 2.02e) software. Polymorphic information content (PIC) values for each primer were calculated using the following formula: PIC= $1-\sum_{i=1}^{n}(P_{ij})^2$ Where P_{ij} is the frequency j allele for the i^{th} primer and summation extends over n patterns.

RESULTS AND DISCUSSION

The result of SSR analysis of pomegranate genotypes is summarized in Tables 1 & 2. The 30 genotypes amplified a total 3154 of fragments with an average of 105.1 fragments for each genotype. Number of bands amplified ranged from as low as 92 in Russian Seedling to as high as 114 in Shirin Anar (Table 1). Thus, some markers did not show any amplification in certain genotypes, while others amplified more than one band in certain genotypes (Fig. 1), which could be due to presence of multiple alleles or heterozygosity. The number of alleles detected for 47 SSR primers ranged from 1 to 4 with an average of 2.15 alleles per primer pair. Among these 47 markers, 6 (Pom010, ABRII-MP28, PGCT046, PGCT088, PGCT112 and PGCT037) showed monomorphic pattern on agarose gel revealing single alleles across the genotypes. Out of 41 polymorphic SSR loci, 24 showed two alleles each and 13 primers amplified three alleles each and four alleles were amplified by three markers in pomegranate genotypes (Table 2). The variation in the number of alleles produced by SSR markers demonstrates heterozygosity in different alleles at a given locus in which the heterozygosity could reflect greatly the state of genetic variability (Elfalleh *et al*., 5). Similarly, Jian *et al*. (8) detected 18 markers resulted in amplification in 42 pomegranate accessions, with an average of 2-5 alleles (mean = 2.80) per locus. The 9 SSR loci provided 22 alleles, with the allele

Table 2. Polymorphic Information Content (PIC) value and number of alleles amplified by SSR markers.

Primer No.	No. of alleles amplified	PIC value
Pom006	3	0.49
Pom010	1	0.00
Pom021	2	0.50
Pom024	2	0.48
ABRII-MP04	2	0.40
ABRII-MP42	3	0.58
ABRII-MP46	2	0.47
ABRII-MP07	2	0.49
ABRII-MP26	3	0.58
ABRII-MP28	1	0.00
ABRII-MP30	2	0.47
POM_AAC1	2	0.31
POM_AAC14	2	0.23
POM_AGC5	2	0.38
POM_AGC11	2	0.49
PGCT001	2	0.40
PGCT005	2	0.43
PGCT015	3	0.48
PGCT016	3	0.49
PGCT017	2	0.50
PGCT021	2	0.47
PGCT022	3	0.38
PGCT023	2	0.41
PGCT025	2	0.46
PGCT028	3	0.49
PGCT030	2	0.50
PGCT031A	2	0.44
PGCT032	3	0.50
PGCT046	1	0.00
PGCT037A	1	0.00
PGCT057	3	0.47
PGCT059	4	0.50
PGCT061	3	0.58
PGCT062	2	0.49
PGCT070	2	0.48
PGCT080	2	0.50
PGCT083	2	0.47
PGCT087	3	0.59
PGCT088	1	0.00
PGCT093B	3	0.49
PGCT093	3	0.66
PGCT097	4	0.51
PGCT104	4	0.65
PGCT109	2	0.49
PGCT110	2	0.57
PGCT111	3	0.49
PGCT112	1	0.00

numbers per locus ranging from 1 to 5 among 34 pomegranate accessions (Curroo *et al.*, 2).

In present study, PIC value range from 0 (monomorphic) to 0.66 with an average value of 0.43 across 30 pomegranate genotypes (Table 2). Six out of 47 SSR markers revealed PIC value of 0 and PGCT093 had highest 0.66 PIC value among 41 polymorphic primers. Eight primers among 47 primers showed PIC value more than 0.50 and in remaining it was less than 0.5. Some primers found with two alleles had a PIC value range from 0.23 to 0.57, whereas, the primers with three alleles had PIC values ranged from 0.38 to 0.66. In many primers amplified fragments were high but PIC values were low. Soriano *et al.* (14) reported the polymorphism information content (PIC) value across all loci range between 0.09 and 0.71, with an average of 0.37 among pomegranate genotypes. The higher number of alleles amplified cannot be considered for higher PIC values. The PIC values of a primer vary with the crop and the set of the genotypes used. Lower PIC value may be the result of closely related genotypes and higher PIC values may be the result of diverse genotypes. Marker loci with an average number of alleles running at equal frequencies will have the highest PIC values. Hence, the other reason could be due to differences in medium for resolving the amplified products i.e. agarose gels vs. polyacrylamide gels.

The similarity coefficient of 47 genotypes is depicted from dendrogram (Fig. 2). Genetic similarity values between genotypes ranged from 0.78 to 0.95. The dendrogram generated based on UPGMA is depicted in Fig. 2. The UPGMA clustering algorithm grouped the pomegranate genotypes into three main clusters I, II, and III. The cluster III was further sub-divided into IIIA, IIIB and IIIC. The cluster I comprised of one genotypes. The cluster II contained eight genotypes. The sub-cluster IIIA contained 12 genotypes, five and four in sub-cluster IIIB and IIIC, respectively. Cluster I consisted of Russian Seedling genotype, which stands alone and far from the other genotypes. Second cluster II contained genotypes predominantly from Punjab (India) which are selections except two introductions from Afghanistan (Kandhari and Kabul Kandhari). Moga Local (ML) found sharing highest similarity coefficient (0.939) with P-26 and both shared 0.89 and 0.87% similarity coefficient with Khog and Ps-75-K3 in the same sub-group. The second highest (0.934) similarity coefficient was seen with Kabul Kandhari and Kandhari Ganga Nagari, whereas both shared 90% with Kandhari in Cluster II. Twenty one genotypes clubbed in cluster III found to be sub-divided into three different sub-clusters IIIA, IIIB and IIIC. Sub-cluster IIIA contained mostly selections except two introductions Mallas from Iran and Achikdana from Turkmenistan. Among selections, Chawla-I and Chawla-II showed maximum similarity (0.95) followed by Kali Shirin and Anar Shirin (0.948) and Shirin Anar and Anar Shirin Mohamad Ali (0.943). Anar Moherab Shirin was seen sharing similarity of 94% with Kali Shirin and Anar Shirin and Mallas with Shirin Anar and Anar Shirin Mohamad Ali at 0.92 similarity coefficient. In cluster IIIA, Jhodpur White clustered with Panipat Selection collected from two different provinces, *i.e.* Rajasthan and Panipat, respectively and sharing (0.92) similarity coefficient, whereas Achik dana found farthest from all other genotypes in Cluster IIIA, followed by Jyoti and Mallas. Such distant affinity of these genotypes with other genotypes of pomegranate indicates that they might be independent clones or may be due to the differences in their pedigree and environmental influences. Predominantly, five genotypes grouped in cluster IIIB were collected from same province Hamirpur (H.P., India) among which Bhota-I and II clubbed together at proximity of 92% similarity

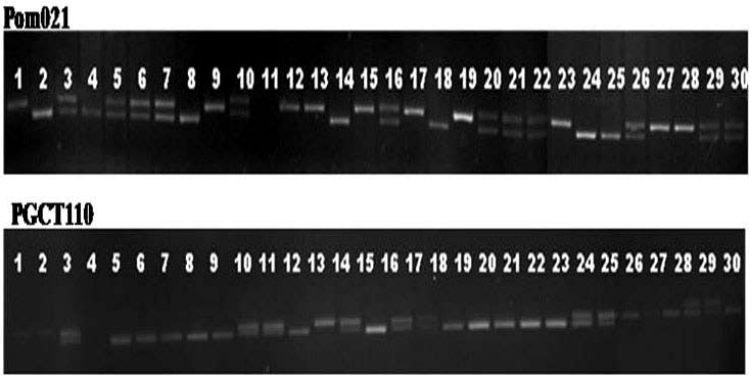

Fig. 1. *In vitro* amplification profile of 30 pomegranate genotypes for SSR markers Pom021 and PGCT110. Numbers at the top of each line are the genotypes as presented in Table 1.

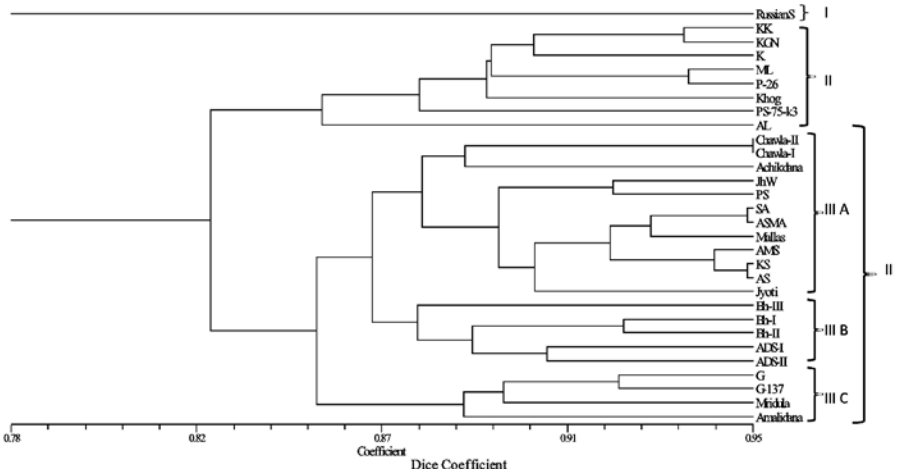

Fig. 2. Dendrogram showing genetic relatedness of 30 pomegranate genotypes. The genotypes are listed in Table 1.

and 0.90 similarity coefficient shared by Anardana Selection I and II. The sub-cluster IIIC comprised of four genotypes among which Ganesh and G-137 were closely related with 91% similarity and both sharing proximity with Mridula and Amlidana at 0.89 and 0.88 coefficient.

Thus, the UPGMA dendrogram generated showed that some genotypes from same province clustered in one group like in cluster IIIA (Kali Shirin, Anar Shirin, Shirin Anar, Anar- Shirin- Mohamad- Ali, Anar Moherab Shirin and Panipat Selection) collected from Hisar (India). Similarly, Moga Local, P-26, Khog, Ps-75-K3 and Kandhari Ganga Nagari grouped in Cluster II were collected from Punjab (India). Hence, the grouping of genotypes in different clusters showed considerable variation in pomegranate germplasm and proved that SSR markers as an excellent genetic marker system for pedigree analysis. The grouping seen in cluster IIIC contained genotypes clubbed together on the basis of parentage and pedigree, *i.e.* having Ganesh as one parent in common in their origin. G-137 is a true clone of Ganesh related at maximum proximity, whereas, Mridula is hybrid of Ganesh and Gul-E-Shah-Rose confirmed its closeness with Ganesh and similar grouping of Amilidana in subcluster IIIC, where male parent is Nana and female parent is Ganesh. Hence, SSR markers provide accurate genetic information for allelic profile of genotypes in pomegranate germplasm. Diverse genotypes placed in clusters irrespective of their origin with other genotypes showed that geographical diversity of the genotypes are not corroborating with the genetic diversity and pomegranate plants are independent of their geographical affiliations. The lack of correlation or correspondence between geographical origin of the genotypes and their genetic characters seems to be a feature for some genotypes in pomegranate (Narzary *et al.*, 10). The different pomegranate genotypes collected did not show high similarities amongst provinces, because these may not be related to each other by descent. Within species genetic exchange rather than past relationships has been emphasized as the determinant of genetic diversity or genetic structure.

The result of present study are found in concord with findings of Mars and Marrakchi (9) who revealed that the geographical origin of the cultivars did not determine their clustering on the basis of morphological characters. Similarly, in Iranian pomegranate genotypes (Alamuti *et al.*, 1) reported the clustering of the genotypes independent of their geographical origins based on SSR profiles and had further assumed a continuous distribution of diversity in the region. Similar results were reported by Yuan *et al.* (15) using SSR markers on Chinese pomegranate cultivars. The high level of genetic diversity within groups (populations) and low level of that among them may be explained by the clone propagation of pomegranate and the extensive gene flow between different localities in Iran due to material exchanges. It can be concluded that despite the low number of genotypes analyzed, these preliminary results indicate that the SSR markers showed a high level of variation in pomegranate vital, thus for genetic diversity studies as well as for cultivar identification.

ACKNOWLEDGEMENTS

Authors are very thankful for Inspire Fellowship of the DST, New Delhi for providing financial assistance and to Head, School of Agricultural Biotechnology, PAU, Ludhiana for the facilities.

REFERENCES

1. Alamuti, M.K., Zeinalabedini, M., Derazmahalleh, M.M., Shojaie, T.R., Irandoost, H.P., Zahravi, M., Vazifehshenas, M., Ebrahimi, M.A, Nekouei, S.M.K., Salekdeh, G.H. and Mardi, M. 2012. Extensive genetic diversity in Iranian pomegranate (*Punica granatum* L.) germplasm revealed by microsatellite markers. *Scientia Hort.* **17**: 104-14.

2. Curroo, S., Caruso, M., Distefano, G., Gentile, A., and Malfa, S.L. 2010. New microsatellite loci for pomegranate, *Punica granatum* (Lythraceae). *American J. Bot.* **6**: 58-60.

3. Damania, A.B. 2005. The pomegranate: its origin, folklore, and efficacious medicinal properties. **In**: Nene, Y.L. (Ed.), *Agriculture Heritage of Asia-Proc. Int. Conf., Asian Agri History Foundation*, Secunderabad, India, pp. 175-83.

4. Durgac, C., Ozgen, M., Simsek, O., Kacar, A.Y., Kıyga, Y., Celebi, S., Gündüz, K. and Serce, S. 2008. Molecular and pomological diversity among pomegranate (*Punica granatum* L.) cultivars in Eastern Mediterranean region of Turkey. *Afican. J. Biotech.* **7**: 1294-1301.

5. Elfalleh, W., Sarrai, N., Gasmi, F., Marzougui, N. and Ferchichi, A.A. 2008. Taxonomic analysis of seed storage protein in eight Tunisian pomegranate (*Punica granatum*) ecotypes. *Trends Bioinform.* **1**: 1-6.

6. Gupta, P.K. and Varshney, R.K. 2000. The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. *Euphytica*, **113**: 163-85.

7. Jbir, R., Hasnaoui, N., Mars, M., Marrakchi, M. and Trifi, M. 2008. Characterization of Tunisian pomegranate (*Punica granatum* L.) cultivars using amplified fragment length polymorphism analysis. *Scientia Hort.* **115**: 231-37.

8. Jian, Z.H., Liu, X.S., Hu, J.B., Chen, Y.H. and Feng, J.C. 2012. Mining microsatellite markers from public expressed sequence tag sequences for genetic diversity analysis in pomegranate. *J. Genet.* **91**: 1-6.

9. Mars, M. and Marrakchi, M. 1999. Diversity of pomegranate (*Punica granatum* L.) germplasm in Tunisia. *Genet. Res. Crop. Evol.* **46**: 461-67.

10. Narzary, D., Rana, T.S. and Ranade, S.A. 2010. Genetic diversity in inter-simple sequence repeats profiles across natural populations of Indian pomegranate (*Punica granatum* L.). *Plant Biol.* **12**: 806-13.

11. Pirseyedi, S.M., Valizadehghan, S., Mardi, M., Ghaffari, M.R., Mahmoodi, P., Zahravi, M., Zeinalabedini M. and Nekoui, S.M.K. 2010 Isolation and characterisation of novel microsatellite markers in pomegranate. *Int. J. Mol. Sci.* **11**: 2010-16.

12. Poyrazog, E., Gokmen, V. and Artik, N. 2002. Organic acids and phenolic compounds in pomegranates (*Punica granatum* L.) grown in Turkey. *J. Food Compos. Anal.* **15**: 567-75.

13. Saghai-Msaroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, chromosomal location, and population dynamics. *Proc. Nat. Acad. Sci.* (*USA*), **81**: 8014-18.

14. Soriano, J.M., Zuriaga, E., Rubio, P., Llacer, G., Infante, R. and Badenes, M.L. 2011. Development and characterization of microsatellite markers in pomegranate (*Punica granatum* L.). *Mol. Breed.* **27**: 119-28.

15. Yuan, Z., Yin, Y., Qu, J., Zhu, L. and Li, Y. 2007. Population genetic diversity in Chinese pomegranate (*Punica granatum* L.) cultivars revealed by fluorescent-AFLP markers. *J. Genet. Genomics*, **34**: 1061-71.

Received : December, 2012; Revised : August, 2013;
Accepted : September, 2013