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Studies on genetic divergence in pomegranate (*Punica granatum* L.) using SRAP markers

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ABSTRACT

Pomegranate genotypes have been characterized mainly on the basis of morphological traits; but, these traits are affected to a large extent by environmental and cultivation conditions, resulting in their ambiguous discrimination. Molecular markers are more suited for accurate discrimination of genotypes and cultivars. Sequence-Related Amplified Polymorphism (SRAP) markers were used in the present study to analyze polymorphism among the important pomegranate genotypes grown in India. The total number of bands generated by 30 SRAP primers for 12 genotypes was 1448, with an average of 48.3 bands per primer. Polymorphism varied from 2.7 to 73.9, with an average of 40.95%. Similarity-value based on Jaccard's Coefficient ranged from 0.63 (between cvs. Naina and Amlidana) to 0.95 (between cvs. Kabul Yellow and Jalore Seedless). UPGMA (un-weighted pair group method with arithmetic mean) analysis was performed and a dendrogram was constructed using Jaccard's similarity matrix. The 12 genotypes used grouped into 5 clusters. SRAP markers were found suitable for determining variability among the pomegranate genotypes studied.

Key words: Pomegranate, molecular markers, SRAP, genetic diversity

INTRODUCTION

Pomegranate (*Punica granatum* L.) is commercially cultivated in Iran, Afghanistan, India and the Mediterranean countries. Iran is believed to be its primary centre of origin. In India, pomegranate occurs naturally just in the Western Himalayan regions of the states of Jammu and Kashmir, Himachal Pradesh and Uttarakhand. In recent years, the area under this crop has increased substantially, mainly because of its versatility, adaptability, drought resistance, low maintenance-costs and a steady high yield (Narzary *et al*, 2010). In the global food industry, pomegranate figures among a novel category of exotic plant sources termed as 'super fruit'. Extracts from this plant (juices, seed-oil and peel) have been reported as exhibiting a strong antioxidant activity helpful in preventing cancer and cardiovascular diseases (Shishodia *et al*, 2006). Therefore, breeding for useful traits in this crop has gained importance. To do this, determination of genetic relationships and precise identification of the genotypes (to conserve genetic diversity) is required. Pomegranate genotypes have been mainly evaluated in the past based on morphological characters; but, these traits are affected by the environment and cultivation conditions, and do not result in a clear discrimination (Kumar, 1999). Molecular or DNA-based markers are more suitable for accurate discrimination between genotypes and cultivars. As per literature, most of the studies on pomegranate diversity have been made using molecular markers such as randomly amplified polymorphic DNA (RAPD) (Sarkhosh *et al*, 2009; Hasnaoui *et al*, 2010), amplified fragment length polymorphism (AFLP) (Jbir *et al*, 2008, 2009), and, simple sequence repeat microsatellites (SSR) (Pirseyedi *et al*, 2010; Ebrahimi *et al*, 2010), but not using SRAP. SRAP (Sequence-Related Amplified Polymorphism) is a PCR-based marker developed by Li and Qurios (2001). The technique of SRAP consists of preferential amplification of open reading frames (ORFs) using PCR. For this purpose, a combination of two types of primers is employed. The first type (forward primer) is 17 bp long, and consists of a fixed sequence of 14 nucleotides, rich in C and G, with three selective bases at the 3' end.

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Table 1. Twelve genotypes of pomegranate used in this study and their description

Sl. No.	Genotype	Description
1	Amlidana	F1 hybrid of a cross (Ganesh x Nana); grows well under tropical climate; with quality fruit attributes, is superior to the sour variety Daru, whose trees come up naturally in the temperate regions of North India; fruits provide a more acidic (16.18%) *anardana* and higher fruit yield/tree; short-statured trees suitable for high-density planting, increased fruit yield/unit area
2	Ruby	Early maturing, thin skin, red aril, sweet, soft-seeded; selection resulting from progeny of <(Ganesh x Kabul) x Yercaud-F2 x (Ganesh x Gul Shah Red)- F2>
3	Bhagwa	Larger fruit size; sweet, bold and attractive aril; glossy, very attractive; saffron-coloured, thick skin makes it suitable for distant markets; less susceptible to fruit spot or thrips compared to other pomegranate varieties; considering all these attributes, 'Bhagawa' is recommended for cultivation in pomegranate-growing regions of Maharashtra.
4	Ganesh	A selection from 'Alandi', developed by Dr. Cheema at Pune; soft seeded with pinkish flesh and juice of agreeable taste; heavy bearer.
5	Jodhpur Red	Medium-size fruit, with hard rind; fleshy aril, light pink, sweet, juicy; seed moderately-hard; a favourite cultivar in Rajasthan, the plant is erect, bearing medium-size fruits (150g to 170g each); seeds hard, red in colour; juice slightly acidic with TSS 14 to 16%; reported as highly susceptible to cracking
6	Jalore Seedless	Known cultivar from Rajasthan and Gujarat; bears small-sized (90g to 110g), round fruits; seeds normally small and soft; juice agreeable in taste and red in colour.
7	Kabul Yellow	This plant has yellow pigmentation in petiole base, leaf margins, flower buds and fruit rind
8	Naina	This is an exotic variety from Sri Lanka
9	Daya	This is an exotic variety from Sri Lanka.
10	Daru	It is a sour pomegranate, which grows wild on Himalayan foothills. Though its sugar content is over 10 per cent, yet it tastes sour due to high acid content. Its seeds are sundried to make ANARDANA. It can withstand cold and dry conditions and is considered to be important source of resistance to diseases and pests
11	Muscat	This is a favourite variety of kohlar (Maharashtra) dueto its large and sweet fruits with soft seeds. It is highly suitable for Pune and Sholapur areas of Maharashtra and some parts of Karnataka.
12	Nana	This is miniature plant and is grown for ornamental purposes. It is believed to be drought tolerant and does not yield fruits/ flowers.

This primer preferentially amplifies exonic regions (which are generally rich in these two nucleotides). The second type of primer (reverse) of 18 bp contains a sequence of 15 nucleotides rich in A and T, and three selective bases at the 3' end. This primer preferentially amplifies intronic regions and the regions with promoters (as these are rich in the nucleotides A and T) (Ferriol *et al*, 2003). Therefore, this method is widely used for assessing genetic diversity and species diversity (Budak *et al*, 2004). With this unique primer design, SRAP markers are known to be more reproducible, stable and less-complex than the other molecular markers. We used SRAP markers in our study to analyze the diversity in 12 important pomegranate genotypes found in India.

MATERIAL AND METHODS

The study was conducted at ICAR-Indian Institute of Horticultural Research, Bengaluru, India. Twelve cultivars/ hybrids were selected for this study from the available pomegranate germplasm collection at the institute. The plant material used was young leaves, sampled from adult trees. Total DNA was extracted from the leaf using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Kanupriya *et al*, 2013). Integrity and quality of the DNA so isolated was determined by agarose gel electrophoresis (0.8%). DNA quantification was done using GeneQuant UVSpectrophotometer (GE Healthcare BioSciences Ltd., England), and diluted as per standard procedure. A set of 30 SRAP primer combinations was used for amplifying DNA from 12 genotypes (Table 1) of pomegranate. Additional information on primers is presented in Table 2. PCR was carried out with an initial denaturing temperature of 94°C for 5min, followed by 5 cycles at 94°C of 1min each, 35°C for 1min, 72°C for 1min, followed by 35 cycles at 94°C for 1min, 50°C for 1min, 72°C for 1min; and, a final extension at 72°C for 5min (Li and Quiros, 2001). PCR products were separated on 2% agarose gel. The size of all the amplification products was estimated from comparison with a standard molecular-weight DNA ladder (1kb). Bands were scored as discrete variables, using "1" to indicate presence and "0" to indicate absence of any particular band. The data was then statistically analyzed by Winboot software to obtain Jaccard's similarity matrix, and, the corresponding dendrogram was drawn for obtaining clusters using NTSYS PC 2.11 software (Rohlf, 2000)

RESULTS AND DISCUSSION

The total number of bands generated using 30 SRAP primers for the 12 genotypes was 1448, with an average of

48.3 bands per primer. The total number of SRAP loci (Table 2) are arranged in ascending order (percentage polymorphism) from the primer pair Me1+Em9 to the primer pair Me1+Em3. Highest number of bands (92) was observed in the primer pair Me7+Em7 (Fig. 1). Polymorphism per cent varied from 2.7 to 73.9, with an average of 40.95%. Similarity-value, based on Jaccard's coefficient, ranged from 0.63 (between cvs. Naina and Amlidana) to 0.95 (between cvs. Kabul Yellow and Jalore Seedless) (Table 3). UPGMA (un-weighted pair group method with arithmetic mean) analysis was made, and a dendrogram was constructed using Jaccard's similarity matrix involving data generated from 30 SRAP primers on 12 genotypes of pomegranate (Fig. 2). These genotypes grouped into four clusters. 'Amlidana' and 'Bhagwa' were placed in one cluster. 'Amlidana' is the progeny of 'Ganesh' and 'Nana', while, 'Bhagwa' is a selection of unknown parentage. It is likely that these share some common ancestors. 'Daru', found growing wild in the Himalayan foothills, separated itself from the rest of the genotypes. It is a sour pomegranate growing in the wild in Himalayan foothills. It is commercially an important genotype, since, its seeds are sun-dried to turn into *anardana*. This genotype withstands cold and dry conditions, and is considered an important source of resistance to diseases and pests. The third cluster was made up of seven phenotypically divergent genotypes, viz., Daya, Muscat, Jalore Seedless, Kabul Yellow, Nana, Ganesh and Jodhpur Red, while, the fourth and last cluster consisted of cvs. Naina and Ruby. All these genotypes are phenotypically divergent; cvs. Muscat, Jalore Seedless and Kabul Yellow are grown in dry regions of the North-West of India, while, cv. Daya is originally from Sri Lanka. 'Nana' is an ornamental pomegranate variety of very short stature. Parentage of 'Naina' is not known, and, it is likely that it shares with Ruby common ancestors.

Similarity-value based on Jaccard's coefficient clearly demonstrates that the genotype, Naina, is divergent from Amlidana; while, the genotypes Kabul Yellow and Jalore Seedless are greatly similar to each other. Overall similarity between genotypes was high, reflecting weak genetic differentiation even among genotypes belonging to various geographical regions. Apart from the genotype, the nature of the markers used in a study also has an influence on the picture emerging on genetic diversity, since, the preferential amplification-region differs with the marker used. Aradhya *et al* (2006) used AFLP analysis to assess genetic diversity within a pomegranate collection maintained at National Clonal Germplasm Repository (NCGR), Davis, California, and reported that despite the different origin of individual accessions, diversity was very low. Accessions from the same place were analyzed recently for evaluating diversity, by Parvaresh *et al* (2012) using microsatellite markers. They

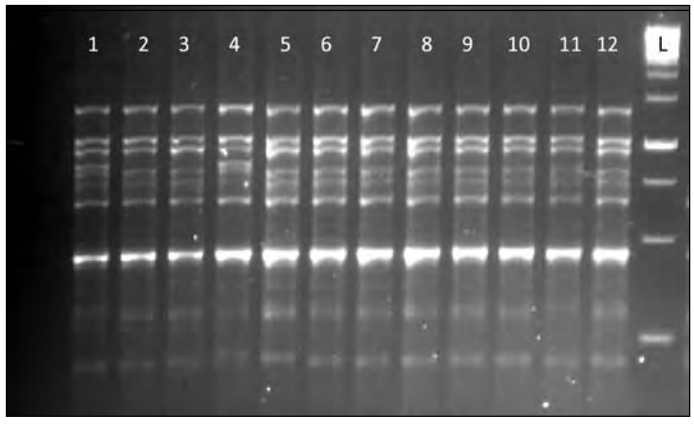

Fig. 1. Gel profile showing PCR products obtained from the SRAP primer combination Me7 + Em7 [1-12 serially represents pomegranate varieties (genotypes) Amlidana, Bhagwa, Daru, Daya, Ganesh, Jodhpur Red, Jalore Seedless, Kabul Yellow, Muscat, Nana, Naina and Ruby, respectively; L is 1kb ladder]

Table 2. Primer combinations in SRAP markers used in the study, showing their amplification and polymorphism

Sl. No.	Primer	Total number of SRAP loci	Polymorphism	Percentage
1	Me1+Em9	46	34	73.9
2	Me4+Em9	43	39	72.0
3	Me7+Em9	39	27	69.2
4	Me4+Em2	67	13	64.2
5	Me3+Em3	33	21	63.6
6	Me2+Em7	34	21	61.8
7	Me9+Em9	57	33	57.9
8	Me8+Em3	28	15	53.5
9	Me8+Em6	55	29	52.7
10	Me8+Em5	72	36	50.0
11	Me1+Em8	47	23	48.9
12	Me10+Em5	45	21	46.6
13	Me9+Em3	45	21	46.6
14	Me7+Em4	45	20	44.4
15	Me1+Em1	21	9	42.8
16	Me8+Em7	62	26	41.9
17	Me2+Em5	41	17	41.4
18	Me4+Em3	58	22	37.9
19	Me8+Em9	19	7	36.8
20	Me8+Em8	55	19	34.5
21	Me7+Em6	35	11	31.4
22	Me4+Em4	51	15	29.4
23	Me2+Em4	31	7	22.5
24	Me7+Em7	92	20	21.7
25	Me10+Em3	61	13	21.3
26	Me9+Em7	60	12	20.0
27	Me10+Em8	24	4	16.6
28	Me8+Em4	43	6	13.9
29	Me1+Em5	54	6	11.1
30	Me1+Em3	37	1	2.7

Table 3. Pair-wise genetic distance calculated using Jaccard's similarity matrix between 12 genotypes of pomegranate using SRAP markers

Genotype	Amlidana	Bhagwa	Daru	Daya	Ganesh	Jodhpur Red	Jalore Seedless	Kabul Yellow	Muscat	Nana	Naina	Ruby
Amlidana	1.00											
Bhagwa	0.89	1.00										
Daru	0.70	0.76	1.00									
Daya	0.77	0.81	0.79	1.00								
Ganesh	0.75	0.75	0.82	0.86	1.00							
Jodhpur Red	0.74	0.76	0.74	0.85	0.87	1.00						
Jalore Seedless	0.74	0.78	0.76	0.88	0.89	0.87	1.00					
Kabul Yellow	0.75	0.76	0.74	0.86	0.89	0.87	0.95	1.00				
Muscat	0.77	0.80	0.75	0.89	0.86	0.86	0.92	0.93	1.00			
Nana	0.74	0.77	0.76	0.85	0.85	0.83	0.92	0.89	0.90	1.00		
Naina	0.63	0.67	0.76	0.75	0.76	0.75	0.77	0.77	0.78	0.81	1.00	
Ruby	0.65	0.70	0.79	0.77	0.80	0.76	0.80	0.81	0.81	0.82	0.90	1.00

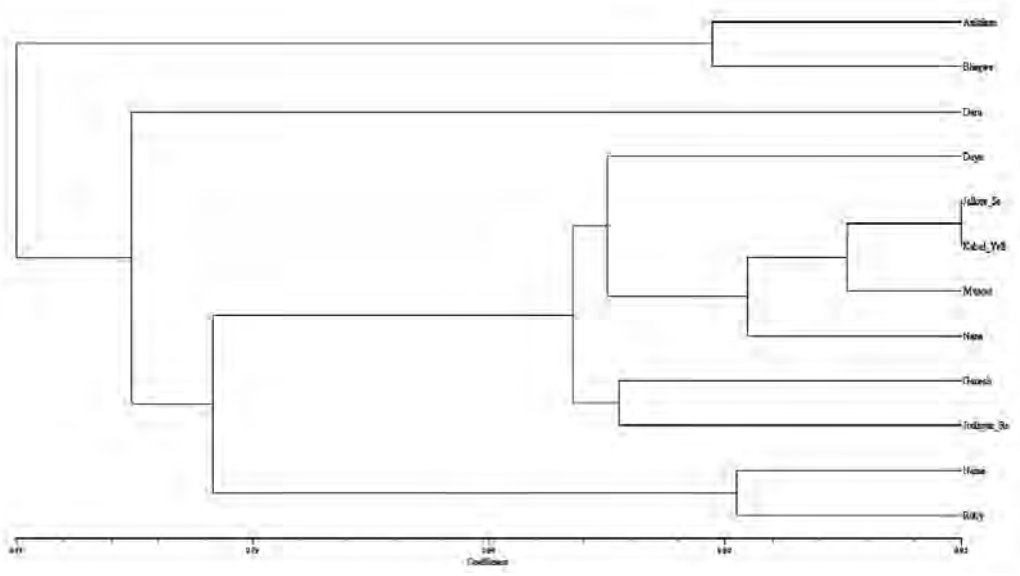

Fig. 2. Dendrogram based on UPGMA analysis by Jaccard's similarity matrix using data generated from 30 SRAP primers for 12 genotypes of pomegranate

reported a high level of genetic diversity within a group and a low level among groups. Moslemi *et al* (2010) and Yuan *et al* (2007) also reported a low diversity in Iranian and Chinese genotypes, using AFLP markers. On the other hand, Narzary *et al* (2010) reported a high genetic diversity across natural populations of the Western Himalayan region of India, based on inter-simple sequence repeat (ISSR) markers. As seen in Fig. 1, SRAP markers showed good stability, repeatability and also clear bands, which facilitated easy scoring. SRAP markers preferentially amplify the ORF regions of DNA, and have been demonstrated to be more powerful at revealing genetic diversity among closely-related cultivars than are SSR, ISSR or RAPD markers as such in other crops like buffalo grass (Budak *et al*, 2004), okra germplasm (Gulsen *et al*, 2007), *Cucurbit pepo* germplasm (Ferriol *et al*, 2003) and *Brassica* (Li and Quires, 2001).

The weak genetic differentiation seen between genotypes of pomegranate is attributed to an inherently narrow genetic base. The family Punicaceae is monogeneric, comprising just two species, *P. granatum* L. and *P. protopunica* L., the latter restricted to Island of Socotra. This narrow genetic base, along with domestication of the desirable genotypes, human selection and clonal propagation, has led to high levels of genetic uniformity in this ancient fruit crop. This is corroborated by molecular analysis carried out in various countries by researchers quoted above, and by our present work using SRAP markers. However, it may be noted that the material analyzed in this study represents a fraction of the germplasm conserved worldwide. Future studies need to explore germplasm available elsewhere and attempt to relate the diversity observed to other traits, for identifying parents for breeding programmes. These can range from agronomic and quality-related traits (such as antioxidant content), to novel uses of pomegranate, such as in aesthetics. Generally, our study confirmed the narrow genetic base reported in *P. granatum*, and emphasizes a need to widen the existing genetic diversity through further exploration. Although SRAP markers in our study were able

to determine variability among the pomegranate accessions tested, a combination of multiple molecular techniques (AFLP, ISSR and SSRs) may lead to a more accurate estimation of genetic diversity, and, relate the diversity observed to qualitative traits in future studies.

REFERENCES

Aradhya, M. 2006. http://www.ars-grin.gov/npgs/cgc_reports/woody2006/ncgrdavis2006.html

Budak, H., Shearman, R.C., Parmaksiz, I., Gaussoin, R.E., Riordan T.P. and Dweikat, I. 2004. Molecular characterization of buffalo grass germplasm using sequence-related amplified polymorphism markers. *Theor. Appl. Genet*., **108**:328-334

Ebrahimi, S., Sayed Tabatabaei, B.E. and Sharifnabi, B. 2010. Microsatellite isolation and characterization in pomegranate (*Punica granatum* L.). *Iranian J. Biotech*., **8:**156-163

Ferriol, M., Pico, B. and Nuez, F. 2003. Genetic diversity of a germplasm collection of C*ucurbita pepo* using SRAP and AFLP Markers. *Theor. Appl. Genet*., **107:**271-282

Gulsen, O., Karagul, S. and Abak, K. 2007. Diversity and relationships among Turkish okra germplasm by SRAP and phenotypic marker polymorphism. *Biologia Bratislava*, **62**:41-45

Hasnaoui, N., Mars, M., Chibani, J. and Trifi, M. 2010. Molecular polymorphism in Tunisian pomegranate (*Punica granatum* L.) as revealed by RAPD fingerprints. *Diversity,* **2:**107-113

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. *Sci. Hortic.,* **115:**231-237

Kanupriya, C., Nischita, P. and Ravishankar, K.V. 2013. An efficient method of genomic DNA isolation from pomegranate. *Indian J. Hort.,* **70**:584-586

Kumar, L.S. 1999. DNA markers in plant improvement. *Biotech. Adv*., **17**:143-183

Li, G. and Quiros, C.F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: Its application to mapping and gene tagging in *Brassica*. *Theor. Appl. Genet*. **103**:455-461

Moslemi, M., Zahravi, M. and Bakhshi Khaniki, G. 2010. Genetic diversity and population genetic structure of pomegranate (*Punica granatum* L.) in Iran using AFLP markers. *Sci. Hortic*., **126**:441-447

Narzary, D., Rana, T.S. and Ranade, S.A. 2010. Genetic diversity in inter-simple sequence repeat profiles across natural populations of Indian pomegranate (*Punica granatum* L.). *Pl. Biol*., **12**:806-813

Parvaresh, M., Talebi, M., Ebrahim, B. and Tabatabaei, S. 2012. Molecular diversity and genetic relationship of pomegranate (*Punica granatum* L.) genotypes using microsatellite markers. *Sci. Hortic.,* **138**:244-252

Pirseyedi, S.M., Valizadehghan, S., Mardi, M., Ghaffari, M.R., Mahmoodi, M., Zahravi, M., Zeinalabedini, M. and Khayam Nekoui, S.M. 2010. Isolation and characterization of novel microsatellite markers in pomegranate (*Punica granatum* L.). *Int'l. J. Mol. Sci*., **11:**2010-2016

Rohlf, F.J. 2000. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2. Exeter Software. Setauket, New York, USA

Sarkhosh, A., Zamani, Z., Fatahi, R. and Ranjbar, H. 2009. Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions by fruit characteristics and RAPD markers. *Sci. Hortic*., **121**:313-319

Shishodia, S., Adams, L., Bhatt, I.D. and Aggarwal, B.B. 2006. Anticancer potential of pomegranate. In: Pomegranates. Ancient Roots to Modern Medicine, 1st edn. Taylor and Francis Group, Boca Raton, FL, USA, pp. 107-116

Yuan, H., 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. Genom.,* **34**:1061-1071

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