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Molecular and morphometric analyses reveal discrete grouping of pomegranate (*Punica granatum*) genebank accessions away from cultivars

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ABSTRACT

Management of germplasm in the field genebanks is greatly assisted by genetic analysis. Estimation of genetic diversity and assessment of genetic relationships among 45 accessions of pomegranate (*Punica granatum* L.) that included cultivars and germplasm collections were carried out using nine morphometric, 241 ISSR and six SSR markers. The average genetic distance values based on ANOVA sum of squares (Ward) were 3.94 and 5.10 for morphometric and DNA markers respectively. Hierarchical clustering based on genetic distances grouped the accessions into at least three distinct clusters; the two-way clustering showing the contribution of individual markers in genetic grouping. Discrete grouping of field genebank accessions (with IC numbers) away from cultivars was evident based both on quantitative traits data as well as DNA marker data. The findings suggested the possibility of broadening genetic base of cultivated varieties by augmenting the breeding programmes in India with diverse as well as trait-specific pomegranate germplasm.

Key words: Genetic analysis, ISSR, JMP, Morphometric analysis, Pomegranate, SSR

Pomegranate (*Punica granatum* L.), a woody perennial shrub belonging to family Lythraceae, is one of the oldest known edible fruits (Damania 2005). In India, pomegranate grows wild in western Himalayan regions that include Himachal Pradesh, Jammu and Kashmir, and Uttarakhand (Misra *et al*. 1983, Pandey *et al*. 2008). Historical evidence reveals that pomegranate's primary centre of origin is Iran and that from there it reached India, possibly through ancient trade routes (Simmonds 1976, Levin 1994). Pomegranate is considered to be one of the first five domesticated edible fruit crops along with fig, date palm, grape and olive. It is estimated that pomegranate was domesticated in the Middle East about 5000 years ago. Despite being utilized since ancient times for fruit, pharmaceutical and nutraceutical values, cultivation of pomegranate was limited and it has been considered as a minor fruit crop in several countries including India. Globally, India is the top producer of pomegranate with an area of 1.27 lakh ha producing 8.28 lakh tonnes annually (Chandra *et al*. 2010). Maharashtra produces about 85% of the total Indian production followed by Karnataka and Andhra Pradesh. However, national productivity is very low, and consequently India has the lowest share (3.0%) in trade among pomegranate-exporting countries. A systematic approach is needed for increasing area under pomegranate by its diversification in suitable non-traditional areas of the country. Moreover, it is an ideal crop for the sustainability of small holdings in arid and semi-arid regions.

Genetic analysis of Indian pomegranate germplasm thus assumes great significance because (i) Indian pomegranate breeders aim to address multi-genic traits like red rind and red aril colour, soft and small seeds, bold arils, large size fruits, resistance to bacterial blight and fruit cracking; (ii) Search for new source of genes requires understanding the nature and extent of diversity amongst Indian cultivars and germplasm; and (iii) Under the existing plant variety protection regime in India, all the new and extant varieties of pomegranate have to be registered to ensure that the rights of breeders and farmers are protected. The aforementioned objectives are achieved by combining inventorization, phenotypic characterization, biochemical markers and DNA marker analysis.

Among the various DNA markers, PCR-based RAPD, ISSR, and AFLP markers have become popular because their application does not need any prior information about

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the target sequences on the genome (Archak *et al*. 2003a and Verma *et al*. 2010) and can be used to fingerprint horticultural germplasm (Archak *et al*. 2003b). Application of DNA markers to determine the diversity level and genetic relationships among pomegranate genotypes has been demonstrated using RAPD markers (Sarkhosh *et al*. 2006, Zamani *et al*. 2007, Ercisli *et al*. 2007, Sheidai *et al*. 2008, Hasnaoui *et al*. 2010), SPAR markers (Ranade *et al*. 2009), ISSR markers (Ghobadi *et al*. 2005, Narzary *et al*. 2009), AFLP markers (Yuan *et al*. 2007, Jbir *et al*. 2008) and SSR markers (Koohi-Dehkordi *et al*. 2007, Pirseyedi *et al*. 2010, Curro *et al*. 2010, Parvaresh *et al*. 2012).

In view of the extensive utility of DNA markers in pomegranate genetic analysis, the present study was undertaken to study a set of the pomegranate field genebank collections comprising of varieties and germplasm accessions employing morphometric markers and microsatellite based DNA markers (ISSR and SSR). The results are reported here and the implications on pomegranate germplasm management are discussed.

MATERIALS AND METHODS

National Research Centre on Pomegranate, Solapur maintains 184 primary and secondary collections of pomegranate representing the best of the germplasm available in the country. The study was carried out at the experimental farm of National Research Centre on Pomegranate, Solapur. The farm is located at 17° 68' N latitude and 75° 91' E longitude, at an altitude of 457m above msl. The observations were recorded during 2010 season of a late *hasta-bahar* crop (October-November flowering). The mean monthly minimum and maximum temperatures of the area ranged between 16 °C and 41 °C during experimental period, and the average annual rainfall was 717 mm. The morphometric data were recorded from five year old trees of 45 accessions (Table 1) following standard procedures.

Leaf material of 45 germplasm accessions of pomegranate were collected on single plant basis in September 2009. The leaf samples were washed in sterile distilled water, labeled, fixed in liquid nitrogen and stored at -80°C till DNA extraction. Isolation of total DNA was carried out by modified CTAB method (Archak *et al*. 2003a) with minor modifications. The DNA was purified following RNase treatment. Integrity, purity and quantity of DNA were analyzed by running 0.8% agarose gel electrophoresis and using Nanodrop spectrophotometer 1000 (Thermo scientific). Aliquots of DNA samples were diluted to 5 ng/µl for PCR amplification and stored at 4°C.

Twenty-one 19mer or 20mer primers were heuristically optimized. Out of these, only 19 primers gave satisfactory amplification and were chosen for further analysis (Table 2). PCR amplification was performed in a total volume of 25ìl having 25ng of template DNA, 10X Taq buffer (with 1.5mM $MgCl_2$), $MgCl_2$ (concentrations for different primers were empirically optimized), 2mM dNTPs, 10µM decamer primer, 5 units of Taq DNA polymerase (New England Biolabs) and sterile distilled water. PCR reactions were carried out in a Perkin Elmer 9600 thermo-cycler. PCR conditions were as follows: (i) Initial denaturation at 94°C for 4min; (ii) 35 cycles of denaturation at 94°C for 1 min, primer annealing at T_a°C for 1 min (Table 2) and primer extension at 72°C for 2 min. (iii) Final extension step at 72°C for 5 min. The PCR products were separated on 1.5% agarose gel in 1X TBE buffer. The gel was visualized under

Table 1 Details of pomegranate germplasm analyzed

Genebank identity	Accession	Source
EC0109925	Speen Sakarin	Afghanistan
EC0109926	Bedana Sadana	Afghanistan
EC0116225	Alah	Iran
EC062813A	Gul-e-Shah Red	USSR
EC0638574	Shirin Anar	USSR
IC0318705	Germplasm line	Himachal Pradesh
IC0318720	Germplasm line	Himachal Pradesh
IC0318723	Germplasm line	Himachal Pradesh
IC0318753	Germplasm line	Himachal Pradesh
IC0318754	Germplasm line	Himachal Pradesh
IC0318779	Germplasm line	Himachal Pradesh
IC0318790	Germplasm line	Himachal Pradesh
IC0318803	Germplasm line	Himachal Pradesh
IC0418154	Ganesh	Karnataka
IC0418155	Kabuli Yellow	Maharashtra
IC0418163	Jodhpur Red	Rajasthan
IC0418164	Jalore Seedless	Rajasthan
IC0418165	Muscat	Maharashtra
IC0418166	G137	Maharashtra
IC0418167	Dholka	Maharashtra
IC0418168	P-23	Maharashtra
IC0418170	P-26	Maharashtra
IC0418172	Yercaud local	Tamil Nadu
IC0565445	Phule Arakta	Maharashtra
IC0565446	Bhagwa	Maharashtra
IC0595399	Kalpitiya	Karnataka
IC0595400	Jodhpur Collection	Rajasthan
IC0595401	KRS	Maharashtra
IC0595402	Kandhari	Maharashtra
IC0595403	Jyoti	Karnataka
IC0595404	Coimbtore white	Maharashtra
IC0595405	Tabesta	Maharashtra
IC0595406	Surat Anar	Maharashtra
IC0595407	Bassein Seedless	Karnataka
IC0595408	Bedana Suri	Maharashtra
IC0595409	Bosckalinsi	Maharashtra
IC0595410	Kabuli Canoor	Maharashtra
IC0595411	Patna -5	Maharashtra
IC0595412	Speen Danedar	Maharashtra
IC0595413	Dorsata Mallus	Maharashtra
IC0595414	Bedana Thinskin	Maharashtra
IC0595415	P-13	Maharashtra
IC0595416	Kasuri	Maharashtra
IC0595417	P-16	Maharashtra
IC0595418	Mridula	Maharashtra

Table 2 Details of the ISSR Primers employed for pomegranate genetic analysis

Primers	T_a (°C) [a]	No. of amplicons	Polymorphism (%)
Pome_$(GAAGTGGG)_2$	50	17	35.3
Pome_$(TA)_5(GT)_5$	50	12	100
Pome_$(GA)_8C$	50	15	100
Pome_$(ACTG)_4$	39	10	70
Pome_DAMD_HBV	44	18	55.6
Pome_DAMD_M13	52	14	78.6
Pome_CCA$(AG)_8$T	60	14	100
Pome_DAMD_HVR	44	13	100
Pome_$(GACA)_4$	50	13	30.8
Pome_$(TCC)_5$	50	8	25
Pome_$(GTG)_6$	60	13	100
Pome_$(TGGA)_5$	62	7	100
Pome_$(GTG)_4$	39	8	87.5
Pome_$(AGG)_6$	60	9	100
Pome_$(GA)_8$YT	52	14	14.3
Pome_$(AG)_8$YT	52	15	46.7
Pome_$(GACAC)_4$	60	12	100
Pome_CCA$(CT)_8$	60	15	33.3
Pome_DAMD_33.6	38	14	100

[a]Annealing temperature

UV-light and photographed using gel documentation system (Syngene).

Five pairs of forward and reverse primers of 20mer were screened for amplification. Out of these, only two pairs of primers that gave satisfactory amplification and band resolution were chosen for the study (Table 3). PCR amplification was performed in a total volume of 25µl having 5ng of template DNA, 10X Taq buffer, 25mM $MgCl_2$, 2mM dNTPs, 10µM decamer primer, 5U of Taq DNA polymerase (MBI Fermentas) and sterile distilled water. PCR reactions were carried out in a Biometra GeneAmp thermo-cycler. PCR conditions were as follows: (1) Initial denaturation at 94°C for 3 min. (2) 35 cycles of denaturation at 94°C for 15 sec, primer annealing at 50°C for 15 sec and primer extension at 72°C for 15 sec. (3) Final extension step at 72°C for 10 min. Electrophoresis and documentation were carried out as explained in section 2.2.

Table 3 Details of the SSR Primers employed for pomegranate genetic analysis

Primers [a]	Primer Sequence	T_a (°C)	No. of amplicons	Polymorphism (%)
PG-IRAN-F1	GCTCTCCGATAGTGATTCA	50	3	100
PG-IRAN-R1	CCGCATATAGACAAAGAAA			
PG-IRAN-F2	GGGTTTTGTGTGGGGAAGA	50	3	100
PG-IRAN-R2	ATGCCATCGTTGCCTCCCTG			

[a] Unpublished information on SSR primers was obtained through personal communication with Dr BE Sayed-Tabatabaei, Department of Biotechnology, College of Agriculture, Isfahan University of Technology, Iran.

Quantitative data were recorded as per the standard methodology. The DNA profiles were manually scored from gel photographs across the lanes in relation to molecular weight marker. Genotypes were scored for the presence (1) or absence (0) of amplicons. Both ISSR and SSR marker data were combined for the final DNA marker data analysis. Quantitative as well as binary data were prepared as rectangular data matrices and pair wise complete linkage distances were computed as —

$$D(X,Y) = \max_{x \in X} \max_{y \in Y} (d(x,y))$$

where, d(x,y) is the distance between genotypes x and y, and X and Y are two clusters. JMP Genomics version 5.1 (SAS package) was used to carry out statistical analyses and to construct dendrograms.

RESULTS AND DISCUSSION

Morphometric polymorphism

Morphometric traits (excluding fruit traits) threw up significant range of polymorphism with all of them showing coefficient of variation above 13% (Table 4). Plant height ranged from as tall as 357.7 cm in Jodhpur Red to just 181.7 cm in popular cultivar Ganesh. The maximum variability was observed in plant spread with a germplasm accession IC318754 (433.3 cm EW and 397.7 cm NS) showing the maximum spread of about 3.5 times the minimum spread of genotype Ganesh. IC318754 also had thickest stem (37.80 cm stem girth). Speen Sakarin had the largest leaves (9.70 cm^2) against small leaves of IC318790 (3.40 cm^2).

DNA Marker polymorphism

Nineteen primers of ISSR generated a total of 241 bands of which about 71% were found to be polymorphic (Table 2). Minisatellite region targeting primer

Table 4 Distribution of morphometric traits among 45 accessions under study

Trait	Max	Min	Mean	SD	CV (%)
Plant height (cm)	357.70	181.70	258.41	38.16	14.8
Plant spread EW (cm)	433.30	124.00	223.32	67.45	30.2
Plant spread NS (cm)	397.70	118.00	220.62	60.72	27.5
Stem diameter (cm)	12.10	5.60	8.40	1.29	15.3
Stem girth (cm)	37.80	17.80	26.34	4.48	17.0
Thorn length (cm)	10.20	5.50	7.54	0.99	13.1
Leaf area (cm^2)	9.70	3.40	6.63	1.15	17.3
Leaf length (cm)	6.70	3.10	4.69	0.80	17.0
Leaf width (cm)	2.10	1.20	1.76	0.26	15.0

Pome_(DAMD_HBV) produced maximum number of bands (18) whereas Pome_$(TCC)_5$ and Pome_$(TGGA)_5$ produced least number of bands (7). Amplicons generated by as many as nine primers [Pome_$(GA)_8$C, Pome_CCA$(AG)_8$T, Pome_$(GTG)_6$, Pome_$(TA)_5(GT)_5$, Pome_$(GACAC)_4$, Pome_$(AGG)_6$Pome_$(TGGA)_5$, Pome_DAMD_33.6 and Pome_DAMD_HVR] were all polymorphic. On the other hand, primer Pome_$(GA)_8$YT produced 14 bands but only about 14% were polymorphic. Interestingly the primers targeting minisatellite core sequences (DAMD label) generated 15 bands per assay with 84% polymorphism against 12 bands per assay and 70% polymorphism by primers targeting microsatellite core sequences. Two pairs of SSR primers produced three bands each, which were all polymorphic across 45 genotypes. Being very few in numbers to reveal any genetic information on their own, SSR marker data were clubbed with ISSR data for further analysis.

Cluster analysis

The genetic relationships among 45 pomegranate genotypes were resolved on the basis of two-way dendrograms. Based on 247 DNA markers, all the accessions were grouped into discrete clusters (Fig 1). The mean distance (complete linkage) was found to be 17.6. Greatest distance (33.6) was observed between a local selection Gul-e-Shah Red and a germplasm accession IC318790. Indigenous collections IC318705 and IC318720 were found to be identical at all loci tested. Nine quantitative traits were sufficient to distribute the pomegranate accessions into distinct groups (Fig 2). Based on morphometric markers, in contrast, it was mainly germplasm accessions that exhibited maximum (9.6 between IC318754 and Dorsata Mallus) and minimum (0.88 between IC318790 and IC318803) pair wise distances. The germplasm accessions were clustered together based on morphometric markers (IC318754, IC318790, IC318803, IC318753, IC318779 and IC318705) and DNA markers (IC318754, IC318803, IC318753, IC318705, IC318720, and IC318779).

The outcome of any genetic conservation or breeding program depends primarily upon the existence as well as

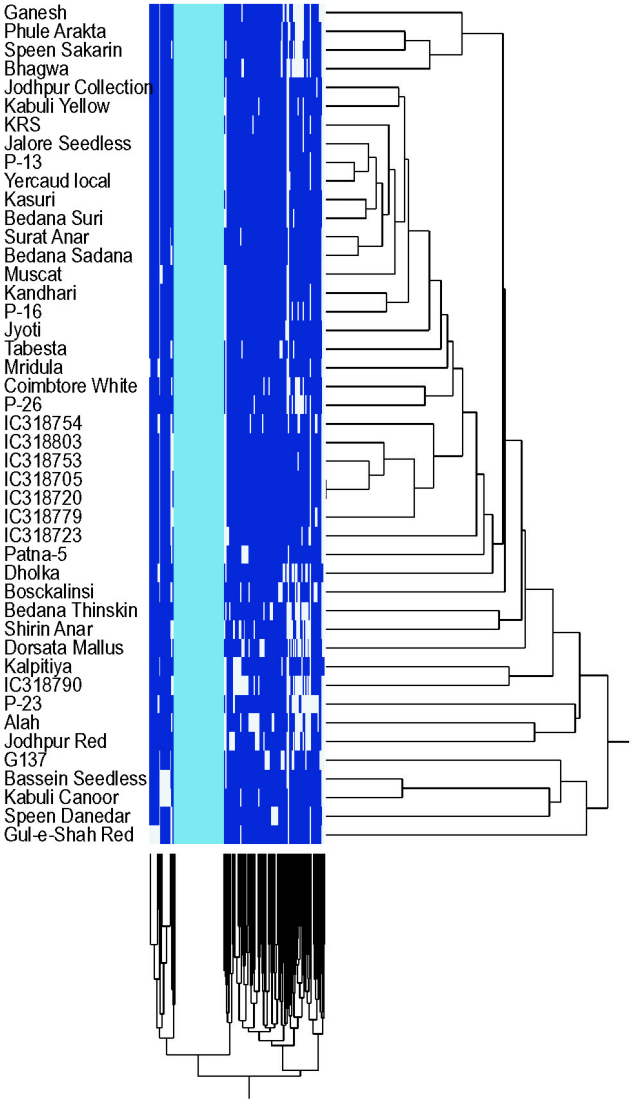

Fig 1 Two-way dendrogram of 45 pomegranate accessions based on 247 microsatellite-based markers

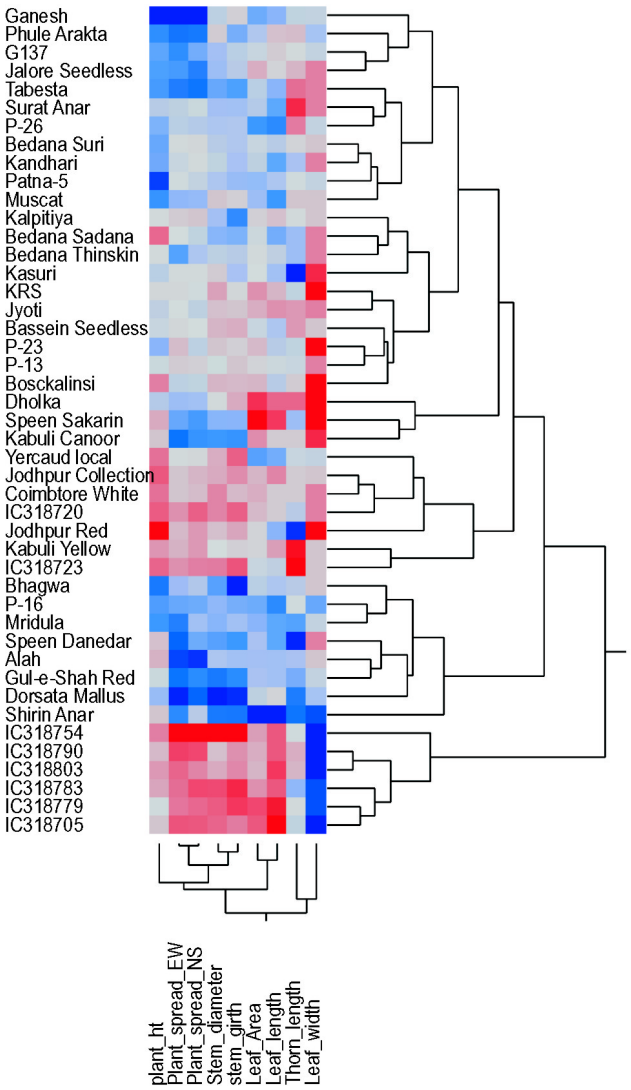

Fig 2 Two-way dendrogram of 45 pomegranate accessions based on nine quantitative morphometric traits

comprehension of the amount and distribution of the genetic variation available in the genetic pool. Our experience with genetic analysis of either indigenous (Pradeepkumar *et al*. 2003) or introduced (Archak *et al*. 2003b) germplasm, particularly in perennial horticultural crops, has clearly indicated that a more scientific management of germplasm is possible with the availability of information on genetic relationships. Identification of duplicates is an important activity in field genebank management. Shrinking availability of space and resources is resulting in cutting down on number of accessions and effective maintenance. DNA markers can help identify possible duplicates and help genebank managers in decision making on replacement. For instance, DNA markers in the current study showed that indigenous collections IC318705 and IC318720 were identical. However, morphometric markers showed differences between the same two accessions. The genebank managers can decide on maintaining both of them or replacing one with a truly different genotype.

Pomegranate has a long history of selection, cultivation and pan-continental spread. Experts attribute the variation observed in pomegranate to genetic history, eco-geographic origin and selection for desired traits by breeders. Traditionally, morphological traits were used to describe such genetic variation in fruit crops. However in pomegranate morphological traits are complex and greatly influenced by environmental factures (Sarkhosh *et al*. 2006). For instance, the observations recorded by a couple of earlier studies (Singh 2004 and Meena *et al*. 2011) were substantially different from the morphometric data recorded in the current study. This reiterates the significant role of DNA markers in genetic analysis of horticultural crops germplasm where pedigree is at best anecdotal and breeding is difficult (Archak *et al*. 2009). In the present study, remarkably, the germplasm accessions were tightly clustered together as distinct group away from elite cultivars based on morphometric markers as well as DNA markers. The observation should attract the attention of pomegranate breeders and encourage the use of germplasm lines in breeding programmes.

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