SPAR profiles and genetic diversity amongst pomegranate (*Punica granatum* L.) genotypes

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ABSTRACT

We are interested in studying the distribution and range of diversity amongst the pomegranates in India. Single Primer Amplification Reaction (SPAR) profiling using Random Amplified Polymorphic DNA (RAPD) and Directed Amplification of Minisatellite DNA (DAMD) methods enabled the determination of the genetic diversity amongst a total of 64 Indian pomegranate genotypes including 15 wild, 34 semi-wild and 14 cultivated types. SPAR profile data were scored for the computation of pairwise distances as well as a Neighbour Joining (NJ) tree of all the genotypes. Eight RAPD and four DAMD primers showed discrete polymorphic patterns amongst these genotypes. From the profiles obtained with all the 12 primers considered together, 259 bands were scored. The NJ tree generated after a 1000 bootstrap test using Jaccard coefficient showed separation of *Lagerstroemia speciosa* used as the out-group taxon, while the pomegranate genotypes were resolved into distinct genetic lineages such that all the cultivated (except CBd70), and wild genotypes (except W101) clearly separated from other genotypes in distinct sub clusters while the semi-wild genotypes were resolved into three sub-clusters. The greatest and least distances detected between genotypes were 0.94 and 0.12, 0.97 and 0.24 and 0.95 and 0.38, amongst the cultivated, semi-wild and the wild genotypes respectively. The results indicate the high levels of genetic diversity present amongst the genotypes. Significantly, the wild genotypes also have a reasonably good range of diversity. A good germplasm collection, especially including the wild genotypes will enable a better pomegranate improvement program. Both SPAR methods, RAPD and DAMD, are found to be useful for studying the genetic diversity of pomegranate. [Physiol. Mol. Biol. Plants 2009; 15(1) : 61-70] E-mail : shirishranade@yahoo.com

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INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the Punicaceae family and is one of the oldest known edible fruits (Damania, 2005). This fruit is cultivated extensively in India, where it is also found in wild state in the Western Himalayan phytogeographic region that includes Himachal Pradesh, Jammu and Kashmir and Uttarakhand states of India (Misra *et al.*, 1983; Pandey *et al.*, 2007; Holland and Bar-Yaakov, 2008). Pomegranate cultivation is common in Iran, Afghanistan, Mediterranean countries (Tunisia, Turkey, Egypt, Spain and Morocco) and to some extent in the U.S.A. (California), China, Japan and Russia (LaRue, 1980; Mars,

1994) amongst countries other than India. The primary centre of origin of pomegranates is believed to be in Iran, from where it has spread to other areas by human intervention (Simmonds, 1976; Levin, 1994). The information about genetic diversity among and between the different cultivars, wild genotypes and germplasm holdings in India is very scanty, though the country ranks high amongst the pomegranate exporting countries of the world (Anon., 2006; Holland and Bar-Yaakov, 2008). Elsewhere in the world, diversity of pomegranate germplasm in Tunisia based on fruit characteristics has been studied by Mars & Marrakchi (1999), who found that the geographical origin of the cultivars did not determine their clustering on the basis of morphological characters. The pomegranate genetic resources from Turkey, another of the possible centers of origin of this plant and a leading producer of the crop, have been studied by Muradoglu *et al.* (2006) for several

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morphological or agronomic characters such as fruit weight, fruit dimensions, and fruit quality such as total soluble solids. In order to characterize Iranian germplasm of pomegranates at the molecular level, Talebi Baddaf *et al.* (2003) applied 13 RAPD primers on 28 pomegranate DNAs belonging to the Yazd collection in Iran and observed a low level of polymorphism and explained that the cultivars were clonally or vegetative propagated. More RAPD analysis in case of the Iranian genotypes was reported by Sarkhosh *et al.* (2006) with mostly similar conclusions. Very recently, AFLP analysis of Tunisian genotypes has been reported by Jbir *et al.* (2008). In this case, they report the clustering of the genotypes independent of their geographical origins, leading to the assumption of a continuous distribution of diversity in these regions (Jbir *et al.*, 2008). Genetic relationships amongst 24 Iranian pomegranate genotypes relative to several fruit characteristics were studied using RAPD markers by Zamani *et al.* (2007). However, the authors did not get any significant correlation and therefore, inferred that information based on fruit characteristics was not sufficient for genetic discrimination in pomegranate; though the RAPD markers were useful to study genetic diversity in pomegranate. Besides these studies on molecular assessment of genetic diversity, other reports of indirect evidences of diversity based on their phytochemical constituents, botanical characteristics, fruit properties, and post-harvest biology and biochemistry of the fruits (Kader *et al.*, 1984; Elyatem and Kader, 1984; Gil *et al.*, 2000; Hess-Pierce and Kader, 2003; Kader 2004; Al-Said *et al.*, 2009) are also available. However, the wild and cultivated accessions have not been compared well in many of the above studies so that a true assessment of diversity of characters that determine the economic importance of the plant is not available for large collections.

The use of PCR-based profiling methods is a common practice, for analyzing diversity amongst any collection of genotypes. A group of such methods using a single primer in the amplification reaction is collectively described as Single Primer Amplification Reaction (SPAR) method. SPAR methods generate markers unaffected by the environment, with a high throughput for analysis and high degree of reliability and rapidity for identification and discrimination of genotypes and cultivars. These methods require no prior knowledge of DNA sequence and are almost universally applicable. Such methods include Random Amplified Polymorphic DNAs (RAPDs; Williams *et al.*, 1990), Directed Amplification of Minisatellite DNA (DAMD; Heath *et al.*, 1993) and Inter Simple Sequence Repeats (ISSRs;

Provost & Wilkinson, 1999). RAPD analysis utilizes single arbitrary sequence of ten bases as oligonucleotide primers to amplify discrete fragments of DNA in low-stringency polymerase chain reactions (Williams *et al.*, 1990; Welsh & McClelland, 1990), whereas DAMD technique involves the primers from minisatellite core sequences. Minisatellites (Jeffreys *et al.*, 1985) are regions of a genome containing hyper variable regions (HVR) or variable number of tandem repeats (VNTR). These are tandem repetitions of a 10 to 60 bp DNA sequence motif known as the "Core" sequence, also known to occur in many diverse species of plants and animals and can be effective as PCR primers at relatively high stringencies in a wide range of organisms (Heath *et al.*, 1993). These two methods based on different principles and experimental rationales generate different extent and nature of polymorphism that can be scored. SPAR methods, RAPD and DAMD, have been used together for biodiversity and biosystematic studies in plants (Verma *et al.*, 2004; Bhattacharya *et al.*, 2005; Saxena *et al.*, 2005; Srivastava *et al.*, 2005; Ranade *et al.*, 2006; Srivastava *et al.*, 2007).

In the present study, we have used these two SPAR methods to assess diversity in pomegranate genotypes collected in India from both wild as well as cultivated plants. Our study shows that these methods enable identification of genetically diverse genotypes suitable for further work on selection and breeding.

MATERIALS AND METHODS

Plant material

Pomegranate genotypes were collected from seven different states of India as shown in Figure 1. A total of 63 pomegranate genotypes were used in this study (Table 1, Figure 1). These include 14 cultivated, 34 semi-wild and 15 wild genotypes. In addition, *Lagerstroemia speciosa* (L.) Pers. (Lythraceae) was used as the out-group for comparison. Leaf samples were collected from plants growing in different locations as listed in Table 1 and stored dry over silica gel at the time of first collection.

DNA extraction

Total genomic DNA was extracted from pomegranate leaves using QIAGEN DNeasy Plant Kit (Qiagen, USA) according to manufacturer's protocol. Generally three to five different DNA preparations were made for each genotype. The quantity and quality of isolated total genomic DNA was determined using 0.8 % agarose gel

Fig. 1. Schematic outline map (not to scale) of India and the few states where the plant material were collected from. The individual state outlines show the schematic and relative positions of the locales where the actual collections were made from. (Details as in Table 1).

electrophoresis in TBE 0.5 X buffer for comparison of mobility relative to that of known concentrations of lambda DNA, double digested with *EcoRI* and *HindIII*.

RAPD and DAMD Primers

Forty decamers from G and H kits (Qiagen Operon Technologies Inc., Alameda, USA) were used as primers. Of these, eight RAPD primers were selected after preliminary screening since these resulted in clear and discretely banded profiles for all the genotypes. Four minisatellite core sequence primers (M13 according to Lorenz *et al.*, 1995; and 33.6, HBV and HVR according to Zhou *et al.*, 1997) were custom synthesized from Bangalore Genei, Bangalore, India. The sequences of the different RAPD as well as DAMD primers used for final analysis are listed in Table 2.

Table 1. Pomegranate genotypes and out-group taxon used

Sample Code ^a No.	LWG Herbarium Voucher No.	Place of Collection	Fig. 1 Reference (State ^b : Location ^c)
CG1	226087	MPKV ^d	MH: RAH
CG2–CG9	226779 226781 226782 226783 226784 226785 226786 226787	CSAUAT ^e	UP: KNP
Cg30	226088	MPKV ^d	MH: RAH
CBg40	226089		
CMr50	226090		
CMd60	226094	Komboikkode	TN: KBK
CBd70	227810	Barpeta Road	AS: BRP
CU201	226003	Shillong	ME: SHG
CU202	226004		
CU203	226030	Barapani	ME: BPN
CU204	226079	Jhansi	UP: JHS
CU205	226061	Lucknow NBRI ^f	UP: LKO
CU206	226091	Palayamkottai	TN: PMK
CU207	226733	Gwalior	MP: GWL
CU208	226735		
CU209–CU214	226756 226757 226758 226759 226760 226761	Lucknow	UP: LKO
CU215–CU234	227801 227802 227803 227804 227805 227806 227808 227809 227812 227813 227814 227815	Barpeta Kokrajhar Barpeta Guwahati	AS: BRP AS: KKJ AS: BRP AS: GWH

Table 1. Continued

Sample Code ^a No.	LWG Herbarium Voucher No.	Place of Collection	Fig. 1 Reference (State ^b ; Location ^c)
W101 – W115	227816	Jorhat Tezpur	AS: JHT AS: TZP
	227817		
	227818		
	227819		
	227820		
	227822	Jorhat	AS: JHT
	227832		
	227835		
	226080	Almora Ranikhet	UK: ALM UK: RNK
	226081		
	226082		
	226084	Nainital	UK: NTL
	226085		
	226086		
	227906	Dehra Dun	UK: DDN
	227907		
	227908		
	227909		
	227910		
	227911	Mussoorie	UK: MUS
	227912		
	227914		
	227915		
Ogl	ND	Lucknow NBRI ^f	UP: LKO

ND – Not deposited because it is a live plant in the NBRI Botanical Garden.

^aAbbreviations and codes used for samples: CG1 to CG9 – All Ganesh cultivars; Cg30 – G-137 cultivar; CBg40 – Bhagwa cultivar; CMr50 – Mridula cultivar; CMd60 – Madlai cultivar; CBd70 – Bedana cultivar; CU201 to CU234 – All cultivars of unknown types (semi-wild cultivars); W101 to W115 – wild genotypes; Ogl – Outgroup Taxon 1 (*Lagerstroemia speciosa*)

^bAbbreviations used for the states: AS – Assam State; ME – Meghalaya State; MH – Maharashtra State; MP – Madhya Pradesh State; TN – Tamil Nadu State; UK – Uttarakhand State; UP – Uttar Pradesh State.

^cAbbreviations used for the locations: ALM – Almora; BPN – Barapani; BRP – Barpeta; DDN – Dehra Dun; GWH – Guwahati; GWL – Gwalior; JHS – Jhansi; JHT – Jorhat; KBK – Komboikkode; KJ – Kokrajhar; KNP – Kanpur; LKO – Lucknow; LNL – Lonavala; MUS – Mussoorie; NTL – Nainital; PMK – Palayamkottai; PNE – Pune; RAH – Rahuri; RNK – Ranikhet; SHG – Shillong; TZP – Tezpur.

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Table 2. Primers used in the SPAR profiling studies

SPAR method	Primer Name	Sequence 5'-3' (Length)	Length
RAPD	OP-G03	GAGCCCTCCA	10 mer
	OP-G04	AGCGTGTCTG	10 mer
	OP-G05	CTGAGACGGA	10 mer
	OP-H03	ACGCATCGCA	10 mer
	OP-H04	GGAAGTCGCC	10 mer
	OP-H05	AGTCGTCCCC	10 mer
	OP-H12	ACGCGCATGT	10 mer
	OP-H13	GACGCCACAC	10 mer
	HBV	GGTGTAGAGAGGGGT	15 mer
	HVR	GCTCCTCCCTCCT	13 mer
	M13	GAGGGTGGCGGTTCT	15 mer
	33.6	GGAGGTGGGCA	11 mer

Amplification Reactions

SPAR for RAPD primers from kits G and H (Operon Technologies Inc., Alameda, California, USA) was carried out essentially following Williams *et al.* (1990) after first optimizing most of the PCR parameters (data not shown). The SPAR with minisatellite primers was carried out essentially according to Zhou *et al.* (1997) for the primers 33.6, HBV and HVR(-) while for primer M13 the reactions were essentially according to Lorenz *et al.* (1995) after first determining the optimum annealing temperature from the range 40 °C to 55 °C for each primer. Error was minimized in the reactions by making one large batch (master mix) of all reagents (except template DNA) for each primer. Polymerase chain reactions were performed in a total volume of 25 µl containing 50 ng template DNA, 1X PCR buffer, 2.0 mM Mg²⁺, 200 mM each dNTP (Bangalore Genei, India), 10 picomoles of a single RAPD primer or 40 picomoles of the single DAMD primers and 0.6 U *Taq* DNA polymerase (Bangalore Genei, India). The reactions were performed in a thermocycler (PTC 200, MJ Research Inc., USA) programmed for initial denaturation at 94 °C for 3 min followed by 45 cycles (in case of RAPD) of denaturation at 94 °C for 45 s, annealing at 35 °C for 45 s and extension at 72 °C for 1 min in each cycle. The last cycle allowed an additional incubation for 5 min at 72 °C for completion of extension.

In case of DAMD, 35 cycles of denaturation at 92 °C, annealing at 55 °C for 2 min and extension at 72 °C for 2 min were carried out. Here also the final cycle allowed an extension duration of 5 min. Amplified products were separated in 1.5 % (w/v) agarose gels in TBE 0.5X buffer. SPAR products were visualized and photographed in UV light after staining gels with ethidium bromide. Image archiving and analysis was carried out with the help of a Gel Documentation system (Vilber Lourmat, France).

Data analysis

Data (fragment sizes of all the amplification products, estimated from the gel by comparison with standard molecular weight marker) were scored as discrete variables, using “1” to indicate presence and “0” to indicate absence of a band. Only distinct and well-separated bands were included in the analysis. A pair wise matrix of similarity between genotypes was determined for the band data using Jaccard's similarity coefficient for UPGMA method (data not shown) while the pair wise distances were computed by using Jaccard's coefficient for NJ method (data not shown) in the FreeTree program (ver. 0.9.1.5; Pavlicek *et al.*, 1999). From the pair wise similarity data, the UPGMA phenogram was computed after allowing a 1000 replicate bootstrap test using the same program. The phenogram was viewed, annotated and printed using Tree View (ver. 1.6.5; Page, 2001).

RESULTS

Preliminary experiments for SPAR reproducibility helped to establish the optimal amplification conditions for both SPAR methods, namely, RAPD and DAMD. Once these conditions were established, all amplification reactions for all the genotypes and all the primers were thereafter carried out as per these optimized conditions. In case of RAPD reactions, forty primers were tested with a few genotypes initially and only those primers resulting in clear unambiguous banding patterns with all genotypes tested were selected for use with the full set of genotypes. The eight RAPD primers used for the full set of 64 genotypes together resulted in a total of 111 bands while the four DAMD primers together resulted in 148 bands from all the pomegranate genotypes as well as the out-group taxon. Typical RAPD and DAMD profiles obtained with primer OP-H04 and HVR respectively are shown in Figure 2.

A data set of profile bands was scored manually from the gel profiles and included only the well separated

and distinct bands. The data were scored cumulatively first for all the primers in a SPAR method and subsequently the data sets for the two methods were combined together for the final NJ analysis. This combined data set resulted in a matrix of pair wise distances (data not shown) for the NJ method using software package FreeTree. This matrix showed that the

Fig. 2. SPAR profiles obtained typically with one RAPD primer (OP-H04) and one DAMD primer (HVR). All profiles were resolved in 1.5 % agarose gels in TBE. The lanes marked as Marker contain the Low Range Ruler (Bangalore Genei, Bangalore) as DNA fragment size marker. The other lanes are marked with sample names as in Table 1.

greatest and least distances detected between pairs of genotypes were 0.94 and 0.12, 0.97 and 0.24 and 0.95 and 0.38, amongst the cultivated, semi-wild and the wild genotypes respectively. Least distance (0.12) was between two Ganesh accessions (CG3 and CG4) while the greatest distance was between several pairs of accessions (for example CU230 with either of CG 3, 4, 8). In all three groups of genotypes the range of distance values between the above extremes is very large (0.82, 0.73 and 0.57 distance units respectively) amongst the cultivated, semi-wild and wild genotypes (Table 3). This table reveals some interesting trends. The average distance between the wild genotypes and either of the other two groups of genotypes is similar. While the range of distances within cultivated and semi-cultivated genotype groups is high, it is low in the case of wild genotypes.

The NJ tree determined from the pair wise similarities is shown in Figure 3 and 4 as a phenogram and a radial tree respectively. The tree was found to be robust with the out-group taxon *Lagerstroemia speciosa* clearly separating from the pomegranates after a 1000 replicate bootstrap analysis. The radial tree is selected for its visual clarity in demarcating the five sub clusters of the pomegranates that include one each of only the wild and cultivated genotypes and three sub clusters of the semi-wild genotypes. There does not appear to be any specific correlation between geographical provenance of the genotypes and the position within any sub cluster.

Table 3. Summary of distances computed for all the genotypes

Group (Numbers of pairs)	Distance Range (Diff. between highest and least distance)	Average distance
Within Cultivated (91)	0.12-0.94 (0.82)	0.72
Within semi-wild (561)	0.24-0.97 (0.73)	0.83
Within wild (105)	0.38-0.95 (0.57)	0.76
All genotypes (1953)	0.12-0.98 (0.86)	0.87
Between Cultivated and semi-wild (476)	ND	0.91
Between Cultivated and wild (210)	ND	0.92
Between semi-wild and wild (510)	ND	0.91

Fig. 3. NJ Tree generated from the cumulative band data from both SPAR methods after a 1000 bootstrap test. OTU names are as in Table 1. Numbers at nodes are bootstrap percentages. The branch lengths are based on the distances between genotype pairs (data not shown).

However, the clear separation of the wild genotypes as well as the cultivated genotypes that are either Ganesh cultivars or others derived from Ganesh cultivars is a significant result that shows the usefulness of the two SPAR methods to resolve relative affinities amongst the genotypes as well as the genetic diversity within and amongst the Indian genotypes.

DISCUSSION

Pomegranate is an ancient horticultural crop believed to have originated in Iran (Simmonds, 1976; Levin, 1994). It is also amongst the most ancient fruit trees to be domesticated as inferred from historical evidences of its earliest cultivation between 4000 and 3000 BC (Damania,

Fig. 4. Radial NJ tree showing clustering of genotypes. Yellow cluster is of Ganesh cultivars; Red, Green and Pink clusters are of the semi-wild genotypes; Blue cluster is of the wild genotypes. The out-group taxon is in Purple circle. One Cultivar (Cb70) and one wild genotype (W101) that are clustered along with the semi-wild genotypes are indicated in Yellow and Blue circles respectively. Scale at the bottom is Distance Scale.

2005). In spite of ancient age, it is included amongst the Neglected and Under-utilized food resources (IPGRI, 2002), because it has not received much attention from the researchers for various aspects of its agriculture or botany. In terms of economic importance, pomegranates are well known for their medicinal uses such as for lowering of blood pressure (Aviram and Dornfeld 2001); as antiviral agents (Jassim & Naji, 2003); as anti-oxidant and anti-ulcer treatments (Ajaikumar *et al.*, 2005; Ricci *et al.*, 2006) and as a potential treatment of inflammation and cancer (Lansky & Newman, 2007); as a source of vegetable dyes besides their importance as an edible fruit. In the recent years there has been a considerably renewed interest in this ancient fruit, especially from a medicinal perspective and there is an impressive listing of potential disease targets such as coronary heart diseases, cancers (skin, breast, prostate and colon), inflammation, hyperlipidemia, diabetes, cardiac disorders, hypoxia, ischaemia, aging, brain disorders and AIDS, where therapeutic effects of pomegranate compounds and extracts have been shown (Seeram *et al.*, 2006). Pomegranate is also considered to be one of the best sources of plant estrogens that may have a role in

treatment for Alzheimers disease (Duke 2008). Botanically, the plant is of interest because of a still inconclusive taxonomic circumscription (Nath & Randhawa, 1959). Pomegranates have been variously placed in either Lythraceae family or in a separate family, Punicaceae, depending on whether morphological or molecular data are considered, and this dichotomy has still not been completely resolved (Still, 2006). Though commonly recognized as a monotypic plant (genus with just a single species), the occurrence of an endemic second species (*Punica protopunica* Balf. f.) with a very narrow and exclusive distribution on the Socotra Islands off the coast of Yemen adds to the confusion about the exact affinities and phylogeography of the pomegranates. Additionally, some workers recognize a third species of the genus as *Punica nana* L. that seems to be made up of only the ornamental tree types (Jbir *et al.*, 2008). The exact limits of species boundaries within the genus *Punica* as well as the position of the genus in two different families Lythraceae and Punicaceae have not been clearly elucidated.

Very little work has been reported on the genetic diversity of this interesting horticultural crop. In the recent study by Sarkhosh *et al.* (2006), most of the genotype clusters by UPGMA method were not in agreement with the morphological traits, with the rationale for the accession names and the geographical provenance of the accessions. Somewhat contrary to this, however, Mars & Marrakchi (1999) had previously concluded that among the fruit characteristics measured for grouping of pomegranate accessions, those related to fruit size, color and juice parameters showed the highest discriminating value. In the study of RAPD profiles by Sarkhosh *et al.* (2006) and of AFLP profiles by Jbir *et al.* (2008) the authors could not detect any correlation between provenance of the accessions and similarity or otherwise in the PCR profiles. However, in both studies diversity analysis was possible by the PCR profiling and the basic interpretation of the RAPD profile diversity in Iranian pomegranates (Sarkhosh *et al.*, 2006) or the AFLP profile diversity in Tunisian pomegranates (Jbir *et al.*, 2008) was that the accessions show a continuous distribution of the diversity. It is possible that the use of a solitary SPAR method may not successfully resolve all genotypes uniquely. In our laboratory we have used more than one SPAR method for diversity and biosystematic studies after analyzing the combined data from all the methods used. In other horticultural plants like papaya (Saxena *et al.*, 2005) and mango (Srivastava *et al.*, 2007), combined data from more than one SPAR method have enabled inferences

not only about genetic diversity but also about correlation between SPAR profiles and fruit pulp characteristics in papaya and about correlation between profiles and parentage relationships in case of mango.

In considerable contrast to the results as in case of the Tunisian or Iranian or Moroccan pomegranates, we are able to show from our SPAR profiling analysis that the cultivated pomegranates of the Ganesh type group together while all the semi-wild cultivars are grouped clearly into three sub-clusters. While these groupings are independent of the geographical provenances of the genotypes, our results clearly show at least 5 discrete genetic lineages of the pomegranates wherein, one lineage includes all the wild genotypes, one includes Ganesh cultivar as well as other cultivars derived from Ganesh and three groups of semi-wild cultivars. This result is important and shows that very few (if not altogether none) of wild genotypes were involved in pomegranate cultivar development and improvement. The cultivar Ganesh is a chance seedling selection from cultivar “Alandi” (Cheema and Bhat, 1954; Adsule and Patil, 1995) while cultivar “Mridula” is a hybrid of Ganesh and Russian Cultivars; cultivars Bhagwa, G-137 and Madlai are selections from cultivar Ganesh. Thus, very significantly, all these genotypes group together in a single distinct cluster. The other cultivar “Bedana” is of exotic origins (from Afghanistan), though commonly cultivated in India at present because of its soft seeds. In the present study, this cultivar separates from all other “Ganesh” cultivars in the NJ tree (Figures 3, 4).

The NJ tree separates out all the wild genotypes with the exception of W101 (from Ranikhet) as a separate cluster while all other genotypes are included as one big cluster that is in turn divided into a sub cluster of all Ganesh cultivars as well as three other cultivated genotypes, Bhagwa, Mridula and Madlai; and a large sub cluster of three groups of the semi-wild genotypes along with one wild genotype (W101 from Ranikhet) and one cultivated genotype (CBd70, cultivar Bedana). This result is very interesting and shows that the semi-wild genotypes have at least three genetic lineages amongst them. Such semi-wild as well as wild material should be useful for genetic improvement of the pomegranate cultivars. The cultivar Ganesh is amongst the most commonly cultivated pomegranate in the country and has also been used for the development of other improved cultivars such as Bhagwa and Mridula. In this respect, our NJ tree clearly includes Bhagwa and Mridula genotypes along with all the Ganesh genotypes as was expected.

The occurrence of three discrete genetic lineages amongst the semi-wild cultivars suggests not only the existence of adequate diversity but may also have significance in phylogeography of this horticultural crop. Considering that the commonest belief about the center of origin and diversification of the pomegranates assigns it to Iran, it is possible that the ancient trade-routes to and from Persia (former kingdom that included the present boundaries of Iran) have resulted in the spread and localization of secondary centers of diversification of the pomegranates in regions such as Mediterranean countries, India and Afghanistan. If indeed the Indian center is only a secondary center of diversification it would mean that even the wild genotypes of the country would have limited origins from the earliest founder genotypes. Our SPAR profiling results provide a good support to this paradigm, because fourteen of the fifteen wild genotypes used in the present study group together in a single cluster. Had these genotypes been truly independent in origin and diversification, it is possible that these would have separated from all other cultivars in several discrete sub clusters or may even have been as diverged as the out-group taxon *Lagerstroemia speciosa* is. Ideally, a detailed characterization of the phylogeographics of this ancient but still interesting plant will include representative accessions and genotypes from all possible centers of origin, diversification as well as domestication, such that all samples are exhaustively analyzed by two or more SPAR as well as other PCR methods. The taxonomic ambiguities still persisting with the systematic position of the genus *Punica* also needs to be resolved and the molecular methods will be the best possible approach for this. Additionally, with renewed interest in this plant and with several countries maintaining germplasm holdings from a few to several hundreds of accessions and varieties, it becomes essential to determine duplication of collected materials, not only in terms of morphological and agronomic characters but also through a genome wide survey of diversity using molecular methods (Still, 2006). This is important in terms of understanding the crop domestication *vis-à-vis* the wild gene pool and it is only after such studies are completed that a definite conclusion can be drawn about the centres of origin or domestication of the pomegranates.

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