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Molecular Analyses of Genetic Diversity in Indian Pomegranates using RAPD, DAMD and ISSR

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ABSTRACT

Genetic diversity and cultivar relationships of Indian pomegranates were studied for 87 accessions representing 28 cultivars and a closely related taxon (*Lagerstroemia speciosa*) as an out-group. Three different markers namely, randomly amplified polymorphic DNA (RAPD), directed amplification of minisatellite DNA (DAMD) and inter simple sequence repeats (ISSRs) were used and the data generated by these markers were analyzed individually and then in combination using different statistical methods. RAPD (21) primers revealed 92.35% polymorphism with average polymorphic information content (PIC) = 0.17, whereas DAMD (5) primers showed 98.52% polymorphism with average PIC = 0.26. ISSR (17) primers resulted in 76.50% polymorphism with average PIC = 0.16. Cumulative data analysis of all three markers showed 88.83% average polymorphism across different accessions of cultivated pomegranates. Jaccard's coefficient of similarity ranged from 0.18 to 0.55. The UPGMA dendrogram showed a clustering pattern of different accessions of pomegranate cultivars. The comparative analysis of the three markers (RAPD, DAMD and ISSR) showed that DAMD is more powerful than RAPD and ISSR in assessment of genetic diversity in pomegranates. Furthermore, implications of molecular markers in breeding and diversity analyses are also discussed in the paper.

Keywords: cultivar, molecular marker, SPAR methods

INTRODUCTION

Pomegranate is an economically important plant and chiefly cultivated for its fruits and also has innumerable medicinal and therapeutic properties ranging from lowering of blood pressure to treatment of Alzheimer's disease and AIDS (Aviram and Dornfeld 2001; Jassim and Naji 2003; Ajaikumar *et al*. 2005; Ricci *et al*. 2006; Seeram *et al*. 2006; Lansky and Newman 2007; Duke 2008). Pomegranates belong to a monogeneric family Punicaceae, and are represented by two species (*Punica granatum* L. and *P. protopunica* Balf. f.), however, Melgarejo and Martínez (1992) considered ornamental dwarf pomegranate (*P. nana* L.) as a distinct species. Pomegranates are widely cultivated in many countries like India, Iran, Afghanistan, Tunisia, Turkey, Egypt, Spain, Morocco, USA, China, Japan and Russia (LaRue 1980; Morton 1987; Mars 1994). Apart from cultivation, pomegranates are also found in the wild in the lower ranges of the Western Himalayan region of India.

Until 1985, pomegranate was considered a minor fruit crop, cultivated only in the states of Maharashtra, Gujarat, Karnataka, Andhra Pradesh, Tamil Nadu and Rajasthan, but it is now being cultivated on a large scale and has attained the status of a major fruit crop under cultivation in India. Total area under this fruit crop is now 88,600 ha with production of 518,700 tons and Maharashtra accounts for 85% of the pomegranate growing area of the country where it has great socio-economic importance among rural people (Ravindran *et al*. 2007). However, increasing demand both within the country and overseas has meant new pomegranate plantations coming up in other regions of the country.

In the early days, pomegranate cultivation in India was of seedling origin from 'Desi' or 'Local' types with inferior quality in comparison to the imported varieties from neighbouring countries like Iran, Afghanistan and Pakistan. The introduced exotic varieties, 'Bedana', 'Kabul', 'Selimi', 'Roman Chokab', 'Suffami', 'Wellissi', 'Ras-el-Baghi', 'Muskati' and 'Kandhari' from Iraq, Palestine and other Mediterranean countries however failed to establish in the Indian climate. Similarly, introductions like 'Gulsha Rose Pink', 'Shirin Anar', and 'Gulsha Red' from the former USSR also showed poor performance owing to their deciduous habit. Since the exotic varieties introduced from neighbouring countries grew unsuccessfully and produced fruits of undesirable quality, varietal improvement in pomegranate has been attempted by selection of promising types from indigenous ones. Seedling selection work from amongst the local types collected from Alandi in Poona district and Dholka in Gujarat State was carried out (Cheema *et al*. 1954; Choudhari and Shirsath 1976). Later on, cultivar improvement through controlled hybridization between indigenous and imported varieties was attempted. Some selections were found as results of such breeding programs. However, concerted efforts have been in place only over the last two decades.

The history of pomegranate cultivation is long and some 60 cultivars, both from indigenous and exotic sources, are presently available, of which only a few cultivars are commercially grown in India. These include 'Bassein Seedless', 'Jyothi' and 'Madhugiri' in Karnataka; 'Dholka' in Gujarat; 'Jalore Seedless', 'Jodhpur Red' and 'Jodhpur White' in Rajasthan; 'Chawla', 'Nadha' and 'Country Large Red' in Haryana; 'Kabul Red', 'Vellodu', 'Yercaud-1' and 'CO-1' in Tamil Nadu, and 'Ganesh', 'Bhagua', 'Muscat', 'Sindhuri', 'G-137', 'P-13', 'P-23', 'P-26' and 'Mridula' in Maharashtra. These cultivars provide a wide range of variability with respect to fruit size, shape, softness of seeds, aril colour, rind colour, rind thickness, sweetness and acidity.

Characterization of Indian pomegranates have been attempted time to time by many workers on the basis of various morphological traits like, flower and fruiting habit, fruit size and colour, aril colour, taste and juiciness, and

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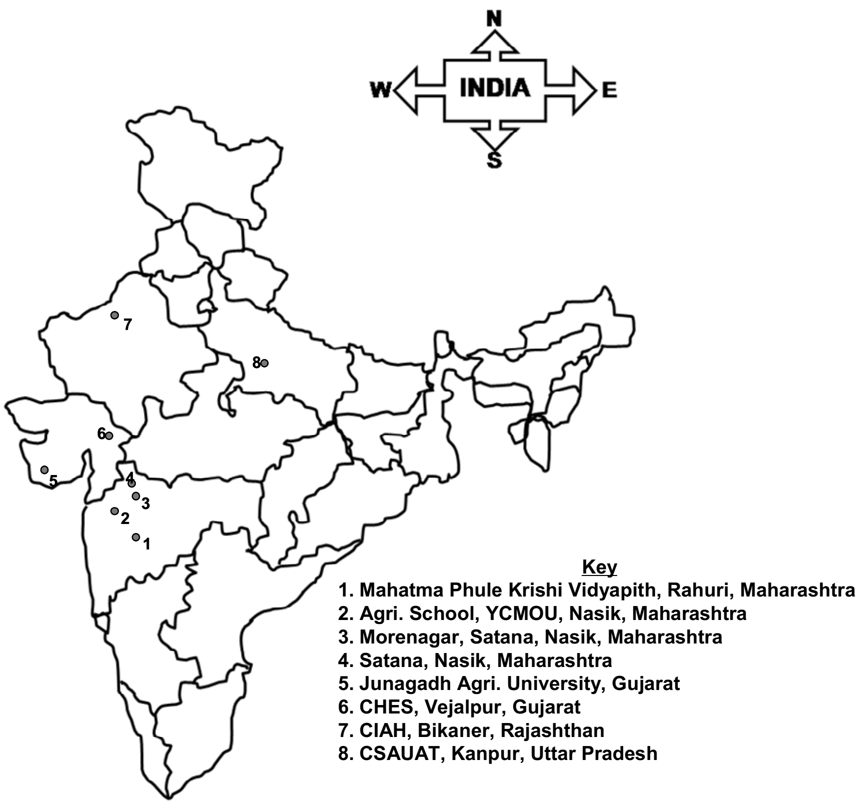

Fig. 1 Map of India indicating the sampling sites of cultivated pomegranate. The details of accessions collected from different localities are given in **Table 1**.

seed softness, as well as some chemical properties like sugar content and acidity of aril, and tannin content in rind (Nath and Randhawa 1959; Patil and Sanghavi 1977; Karale *et al*. 1979; Patil and Sanghavi 1980; Pundir and Pathak 1981; Malhotra *et al*. 1983a; Misra *et al*. 1983; Purohit 1985; Godara *et al*. 1989; Jagtap *et al*. 1992a, 1992b; Jalikop and Kumar 1998).

Nath and Randhawa (1959) reported that no single character could be reliable to establish the identity of any pomegranate variety, but a combination of several characters was more useful in this direction. Classical phenotypic characters, such as morphological traits, are still extremely useful but they can sometimes be influenced by environmental conditions. Therefore, during the past decade, classical strategies of evaluating genetic variability such as comparative anatomy, morphology, embryology and physiology have been increasingly complemented by molecular techniques. These include the analysis of chemical constituents (e.g., plant secondary compounds) and, most importantly, the characterization of macromolecules. The development of molecular markers, which are based on polymorphisms found in proteins or DNA, has greatly facilitated research in a variety of disciplines such as taxonomy, phylogeny, ecology, genetics, and plant breeding (Weising *et al*. 1995).

DNA markers are more popular now-a-days, and have achieved wide applicability in biological sciences for genetic diversity study because these markers are neutral to environmental effects and are much more preferable than the morphological and biochemical methods in the genetic diversity assessment of plants. With the advancement of technology, a number of DNA markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSRs), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs), directed amplification of minisatellite DNA (DAMD), etc. are now available. However, these markers also have their own utility and limitations. The single primer amplification reaction (SPAR) methods like RAPD (Williams *et al.* 1990), DAMD (Heath *et al.* 1993; Somers *et al*. 1996) and ISSR (Provost and Wilkinson 1999) have been used successfully for the assessment of genetic diversity in many crop plants, including pomegranates (Sarkhosh *et al*. 2006; Ercisli *et al*. 2007; Yuan *et al*. 2007; Jbir *et al*. 2008; Narzary *et al*. 2009; Ranade *et al.* 2009; Narzary *et al*. 2010). In the present study these markers were employed to unravel the genetic variability in 87 accessions of 28 different pomegranate cultivars grown in India. To our knowledge, ours is the maiden attempt to unravel the genetic diversity in cultivated pomegranates in India, using SPAR methods.

MATERIALS AND METHODS

Sampling of plant material

Most of the pomegranate cultivars developments in India are seedling origin and the denomination of these cultivars are on the basis of either colour or taste of the fruit, or locality of the occurrence. In order to procure authentic materials, different Agricultural Universities and Institutes were visited during the course of investigation so as to procure maximum representations of the well known cultivars of pomegranate from different localities in India.

About 110 accessions of pomegranate cultivars were collected from Arid Zone Horticulture, Mahatma Phule Krishi Vidyapeeth, Rahuri (Maharashtra); Agriculture Research Station, Yashbantarao Chauhan Maharastra Open University, Nasik (Maharashtra); Central Horticultural Experiment Station, Vejalpur (Gujarat); Junagadh Agriculture University, Junagadh (Gujarat); Central Institute for Arid Horticulture, Bikaner (Rajasthan); Central Arid Zone Research Institute, Jodhpur (Rajasthan), as well as from the farmers (mainly in Maharashtra). The localities of the sampling sites have been shown in the map of India in **Fig. 1**. Finally, 87 accessions representing 28 different cultivars were considered for the genetic diversity studies on the basis of their DNA quality and reproducibility in PCR reactions. The list of cultivated accessions along with their names, voucher numbers and localities has been presented in **Table 1**. A close relative of pomegranate, *Lagerstroemia speciosa* (family Lythraceae) was used as an out-group taxon in the present study (Graham *et al.* 1993).

Collected plant materials were processed in the herbarium fol-

Table 1 List of cultivated pomegranate accessions considered for the present study.

Accession Code	Cultivar name	Voucher No.*	Locality
Pcv01	Arakhta	228732	Morenagar, Satana, Nasik, Maharashtra
Pcv02	Arakhta	228733	Morenagar, Satana, Nasik, Maharashtra
Pcv03	Arakhta	228777	AZH, MPKV, Rahuri, Maharashtra
Pcv04	Arakhta	228778	AZH, MPKV, Rahuri, Maharashtra
Pcv05	Asthagandha	228719	Satana, Nasik, Maharashtra
Pcv06	Asthagandha	228720	Satana, Nasik, Maharashtra
Pcv07	Asthagandha	228721	Satana, Nasik, Maharashtra
Pcv08	Bedana	228759	AZH, MPKV, Rahuri, Maharashtra
Pcv09	Bhagua	228771	AZH, MPKV, Rahuri, Maharashtra
Pcv10	Bhagua	228772	AZH, MPKV, Rahuri, Maharashtra
Pcv11	Darsha Malas	247827	CHES (ICAR), Vejalpur, Gujarat
Pcv12	Darsha Malas	247828	CHES (ICAR), Vejalpur, Gujarat
Pcv13	Darsha Malas	247829	CHES (ICAR), Vejalpur, Gujarat
Pcv14	Devanhalli Seedless	247820	CHES (ICAR), Vejalpur, Gujarat
Pcv15	Devanhalli Seedless	247821	CHES (ICAR), Vejalpur, Gujarat
Pcv16	Devanhalli Seedless	247822	CHES (ICAR), Vejalpur, Gujarat
Pcv17	Dholka	247852	Junagadh Agri. University, Gujarat
Pcv18	Dholka	247853	Junagadh Agri. University, Gujarat
Pcv19	Dholka	247854	Junagadh Agri. University, Gujarat
Pcv20	Dholka	228760	AZH, MPKV, Rahuri, Maharashtra
Pcv21	G-137	228706	Agri. School, YCMOU, Nasik, Maharashtra
Pcv22	G-137	228707	Agri. School, YCMOU, Nasik, Maharashtra
Pcv23	G-137	228768	AZH, MPKV, Rahuri, Maharashtra
Pcv24	Ganesh	247858	Junagadh Agri. University, Gujarat
Pcv25	Ganesh	226787	CSAUAT, Kanpur, Uttar Pradesh
Pcv26	Ganesh	228767	AZH, MPKV, Rahuri, Maharashtra
Pcv27	Ganesh	228770	AZH, MPKV, Rahuri, Maharashtra
Pcv28	Gulsha Red	247805	CHES (ICAR), Vejalpur, Gujarat
Pcv29	Gulsha Red	247806	CHES (ICAR), Vejalpur, Gujarat
Pcv30	Gulsha Red	228743	AZH, MPKV, Rahuri, Maharashtra
Pcv31	Gulsha Red	228753	AZH, MPKV, Rahuri, Maharashtra
Pcv32	Gulsha Rose	247801	CHES (ICAR), Vejalpur, Gujarat
Pcv33	Gulsha Rose	247802	CHES (ICAR), Vejalpur, Gujarat
Pcv34	Gulsha Rose	247803	CHES (ICAR), Vejalpur, Gujarat
Pcv35	Jalore Seedless	247830	CHES (ICAR), Vejalpur, Gujarat
Pcv36	Jalore Seedless	247831	CHES (ICAR), Vejalpur, Gujarat
Pcv37	Jalore Seedless	227882	CIAH, Bikaner, Rajashthan
Pcv38	Jalore Seedless	228746	AZH, MPKV, Rahuri, Maharashtra
Pcv39	Jyothi	247824	CHES (ICAR), Vejalpur, Gujarat
Pcv40	Jyothi	247825	CHES (ICAR), Vejalpur, Gujarat
Pcv41	Jyothi	228761	AZH, MPKV, Rahuri, Maharashtra
Pcv42	Jyothi	228762	AZH, MPKV, Rahuri, Maharashtra
Pcv43	Kabul	247814	CHES (ICAR), Vejalpur, Gujarat
Pcv44	Kabul	247815	CHES (ICAR), Vejalpur, Gujarat
Pcv45	Kandhari	228765	AZH, MPKV, Rahuri, Maharashtra
Pcv46	Khog	247845	CHES (ICAR), Vejalpur, Gujarat
Pcv47	Khog	247846	CHES (ICAR), Vejalpur, Gujarat
Pcv48	Khog	247847	CHES (ICAR), Vejalpur, Gujarat
Pcv49	Malta	247833	CHES (ICAR), Vejalpur, Gujarat
Pcv50	Malta	247834	CHES (ICAR), Vejalpur, Gujarat
Pcv51	Malta	247835	CHES (ICAR), Vejalpur, Gujarat
Pcv52	Mridula	228702	Agri. School, YCMOU, Nasik, Maharshtra
Pcv53	Mridula	228703	Agri. School, YCMOU, Nasik, Maharshtra
Pcv54	Mridula	228748	AZH, MPKV, Rahuri, Maharashtra
Pcv55	Mridula	228752	AZH, MPKV, Rahuri, Maharashtra
Pcv56	Muscat	228754	AZH, MPKV, Rahuri Maharashtra
Pcv57	Muscat	247807	CHES (ICAR), Vejalpur, Gujarat
Pcv58	Muscat	247808	CHES (ICAR), Vejalpur, Gujarat
Pcv59	Muscat	247809	CHES (ICAR), Vejalpur, Gujarat
Pcv60	Ornamental	247859	Junagadh Agri. University, Gujarat
Pcv61	Ornamental	247860	Junagadh Agri. University, Gujarat
Pcv62	Ornamental	247861	Junagadh Agri. University, Gujarat
Pcv63	P-16	247837	CHES (ICAR), Vejalpur, Gujarat
Pcv64	P-16	247838	CHES (ICAR), Vejalpur, Gujarat
Pcv65	P-16	228755	AZH, MPKV, Rahuri, Maharashtra
Pcv66	P-16	228764	AZH, MPKV, Rahuri, Maharashtra
Pcv67	P-23	247840	CHES (ICAR), Vejalpur, Gujarat
Pcv68	P-23	247841	CHES (ICAR), Vejalpur, Gujarat
Pcv69	P-23	228758	AZH, MPKV, Rahuri, Maharashtra
Pcv70	P-23	228774	AZH, MPKV, Rahuri, Maharashtra
Pcv71	P-26	247842	CHES (ICAR), Vejalpur, Gujarat
Pcv72	P-26	247843	CHES (ICAR), Vejalpur, Gujarat

Table 1 (Cont.)

Accession Code	Cultivar name	Voucher No.*	Locality
Pcv73	P-26	247844	CHES (ICAR), Vejalpur, Gujarat
Pcv74	Ramnagaram	247816	CHES (ICAR), Vejalpur, Gujarat
Pcv75	Ramnagaram	247817	CHES (ICAR), Vejalpur, Gujarat
Pcv76	Ramnagaram	247818	CHES (ICAR), Vejalpur, Gujarat
Pcv77	Sindhuri	228727	Satana, Nasik, Maharashtra
Pcv78	Sindhuri	228728	Satana, Nasik, Maharashtra
Pcv79	Sindhuri	228731	Satana, Nasik, Maharashtra
Pcv80	Surkh Anar	247813	CHES (ICAR), Vejalpur, Gujarat
Pcv81	Uthkal	247810	CHES (ICAR), Vejalpur, Gujarat
Pcv82	Uthkal	247811	CHES (ICAR), Vejalpur, Gujarat
Pcv83	Uthkal	247812	CHES (ICAR), Vejalpur, Gujarat
Pcv84	Yercaud	247849	CHES (ICAR), Vejalpur, Gujarat
Pcv85	Yercaud	247850	CHES (ICAR), Vejalpur, Gujarat
Pcv86	Yercaud	247851	CHES (ICAR), Vejalpur, Gujarat
Pcv87	Yercaud	228766	AZH, MPKV, Rahuri, Maharashtra
Og88	*L. speciosa*	247863	NBRI Garden, Lucknow, Uttar Pradesh

Abbreviations:
Arid Zone Horticulture, Mahatma Phule Krishi Vidyapeeth (AZH, MPKV)
Central Horticultural Experiment Station (CHES)
Central Institute for Arid Horticulture (CIAH)
Chandra Sekhar Azad University of Agriculture & Technology (CSAUAT)
Indian Council of Agricultural Research (ICAR)
National Botanical Research Institute (NBRI)
Yashbantarao Chauhan Maharastra Open University (YCMOU)
* Voucher specimens deposited in the herbarium of the National Botanical Research Institute, Lucknow (LWG), India.

lowing standard herbarium procedure (Jain and Rao 1977). Voucher specimens were prepared for all collected plants for future records. In addition to the plant specimens, tissue samples were also collected bearing the same voucher specimen number for DNA studies, following the Chase and Hills method (1991).

Genomic DNA isolation

Total genomic DNA was extracted from the dried leaves using the cetyl trimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with minor modifications. For accessions in which tissue samples were < 1 g, DNA was isolated by using the commercial DNeasy Plant Mini Kit (Qiagen, USA).

Molecular methods used in genetic diversity analyses of pomegranates

Single primer amplification reactions (SPAR) methods like RAPD, DAMD and ISSR are supposed to be the best markers for genetic diversity study of a plant whose genome sequences are unknown. Employing such markers genetic information can be assessed easily throughout the whole genome of the plant, because these primers bind arbitrarily to any complementary sequences present in the genome and amplify those regions where complementary bases are available at an amplifiable distance in the two template stands in reverse orientation (Agarwal *et al*. 2008). Therefore, in the present study RAPD, DAMD and ISSR markers were employed to study the extent of genetic diversity amongst the different accessions of pomegranate cultivars. The band data obtained from these three markers were analysed individually as well as in combination for comparison.

RAPD

The RAPD primers used in the present study were procured from Operon Tech. Inc. (Alameda, CA, USA). Initially, 120 primers belonging to OP-B, OP-G, OP-H, OP-M, OP-N, and OP-U (20 primers in each kit) were screened with pomegranate DNAs as template. All the primers did not give consistent profiles in the reactions. Therefore, only those primers which gave consistent profiles across all the cultivated accessions were considered. Finally, 21 decamer primers were considered for the RAPD analysis in the present investigation.

The reactions for RAPD-PCR were carried out in 25 µl and contained 50 ng of template DNA, 0.4 µM RAPD primer, 200 µM each dNTP, 2.5 mM $MgCl_2$ in suitable 1X assay buffer supplied along with the enzyme and 1 U of thermostable *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). To minimize pipetting errors, one large master mix including all the PCR components, except DNA template was prepared for each primer. Equal volume of master mix was distributed into the labelled reaction vials (0.2 ml PCR tubes). Optimum quantity of template DNA of each sample was added to the respective labelled tubes and one negative control was used where distilled water was added instead of DNA. All the PCR tubes were spun briefly prior to putting on the heating block of thermal cycler. Samples to be compared were amplified simultaneously in a Thermal Cycler (PTC 200, MJ Research, Inc., USA), which was programmed to include predenaturation at 94°C for 1 min, followed by 45 cycles of denaturetion at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min. The final cycle allowed an additional 5 min period of extension at 72°C.

DAMD

Six DAMD primers *viz*. M13 (Lorenz *et al*. 1995), HVA (Tourmente *et al*. 1994), HVY (Anderson and Nilsson-Tillgren 1997), HVR (-) (Winberg *et al*. 1993), 33.6 and HBV (Nakamura *et al*. 1987) were custom synthesized from Bangalore Genei. The primer HVR (-) failed to produce distinct scorable bands and was thus not used further.

The DAMD-PCR conditions used for profile generation contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.8 µM primer, 1 U *Taq* DNA polymerase (Bangalore Genei) and 60 ng genomic DNA in 25 µl reaction volume. The amplification reactions were performed in a thermal cycler (PTC 200) programmed for initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The last cycle allowed an additional extension at 72°C for 5 min.

ISSR

A set of 100 anchored microsatellite primers procured from the University of British Columbia (Canada) were screened with template DNA. Seventeen ISSR primers that produced distinct well separated fragments were selected for further profiling of pomegranate accessions.

ISSR-PCR was also carried out in a 25 µl reaction volume containing 20 ng template DNA, 10 mM Tris-HCl pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primer, and 0.9 U *Taq* DNA polymerase (Bangalore Genei). The amplification was done in a PTC-200 Thermal Cycler. The reaction cycle consisted of an initial denaturation at 94°C for 4 min, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C, 2 min extension at 72°C, followed by a final step of 7 min extension at 72°C.

Gel electrophoresis

The amplified PCR products were resolved on 1.5% agarose (Bangalore Genei) gel using 0.5X TBE buffer at a constant voltage of 5 V/cm. Before loading on the gel, 2 µl of loading dye (6X) was added to each sample and mixed thoroughly. A low range DNA ruler (Bangalore Genei) of known molecular weights was loaded in the first well of each gel to compare the size of amplified products. DNA bands were visualized by ethidium bromide (0.5 µg/ml) staining and archived using Uvitec Gel Documentation System (UK) at 302 nm. The gel images were photographed and stored as digital pictures for further processing.

Data analysis

Data analysis was carried out only for those genotypes that resulted in consistent and reproducible profiles. For each primer, the molecular sizes of each fragment were estimated on the basis of the corresponding marker lane. Distinct and well separated bands were coded in a binary form by denoting '0' for absence and '1' for presence of the bands in each genotype and binary data generated was used as input for further calculations. In order to estimate genetic diversity and relationships among the accessions, RAPD, DAMD and ISSR were analyzed separately and then in combination using the following statistical methods.

1) The polymorphic information content (PIC) was calculated according to Botstein *et al*. (1980) for each primer, and theoretically, PIC values can range from 0 to 1. At a PIC of 0, the marker has only one allele. At a PIC of 1, the marker would have an infinite number of alleles. However, a gene or marker with only two alleles has a maximum PIC of 0.375 (Hildebrand *et al*. 1992). Since RAPD, DAMD and ISSR are dominant markers, the PIC value calculations can be considered as bi-allelic markers for presence (1) and absence (0) of bands.

2) The pairwise distances between the accessions were computed by using Jaccard's coefficient for neighbour joining (NJ) method in FreeTree program (version 0.9.1.5) (Pavlicek *et al*. 1999). The NTSYS pc software ver. 2.02e (Rohlf 1998) was used to estimate genetic similarities with the Jaccard's coefficient. The unweighted pair-group method with arithmetic mean (UPGMA) was used to generate the dendrogram based on Jaccard's similarity coefficient in the sequential, agglomerative, hierarchical, and nested (SAHN) clustering module of NTSYS program. The Mantel test (Mantel 1967) was also carried out in MXCOMP module to compute the matrix correlation (r) between the similarity matrices generated from different markers which test the goodness of fit.

3) POPGENE software 1.32 (Yeh *et al*. 1999) was used for cultivar relationships and group analysis of the accessions of a cultivar.

4) In order to determine the utility of each of the marker systems, diversity index (DI), effective multiplex ratio (E) and marker index (MI) were calculated according to Powell *et al*. (1996).

RESULTS

The main objective of the present study was to estimate the genetic diversity and relationships in pomegranate cultivars using three different PCR-based methods *viz*. RAPD, DAMD and ISSR. A total of 87 pomegranate accessions representing 28 cultivars and a closely related taxon (*Lagerstroemia speciosa*) as out-group were considered in the present investigation (**Table 1**). The data generated by RAPD, DAMD and ISSR markers were analyzed individually as well as in combination.

RAPD analysis

The gel profiles of cultivated pomegranate accessions were generated with 21 RAPD primers and the data generated with all these primers were considered cumulatively for the genetic diversity study amongst the different genotypes. The typical gel profiles obtained with RAPD primers are shown in **Fig. 2A**.

A total of 327 discrete bands were scored from all the gel profiles of which 302 bands were polymorphic across the cultivars of pomegranates. The size of the band varied from 150 to 2500 bp across different accessions. Primers OP-N13 produced the highest (24) numbers of bands, while the primer OP-G14 and OP-M03 produced the lowest (10) number of bands (**Table 2**). Almost all the primers, except OP-H08 (77%), showed more than 80% polymorphic bands with an average of 92.35% polymorphism across the accessions. Primers OP-G04, OP-H20, OP-M07, OP-N07, OP-N13 and OP-U18 showed 100% polymorphic bands.

The polymorphic information contents (PIC) were cal-

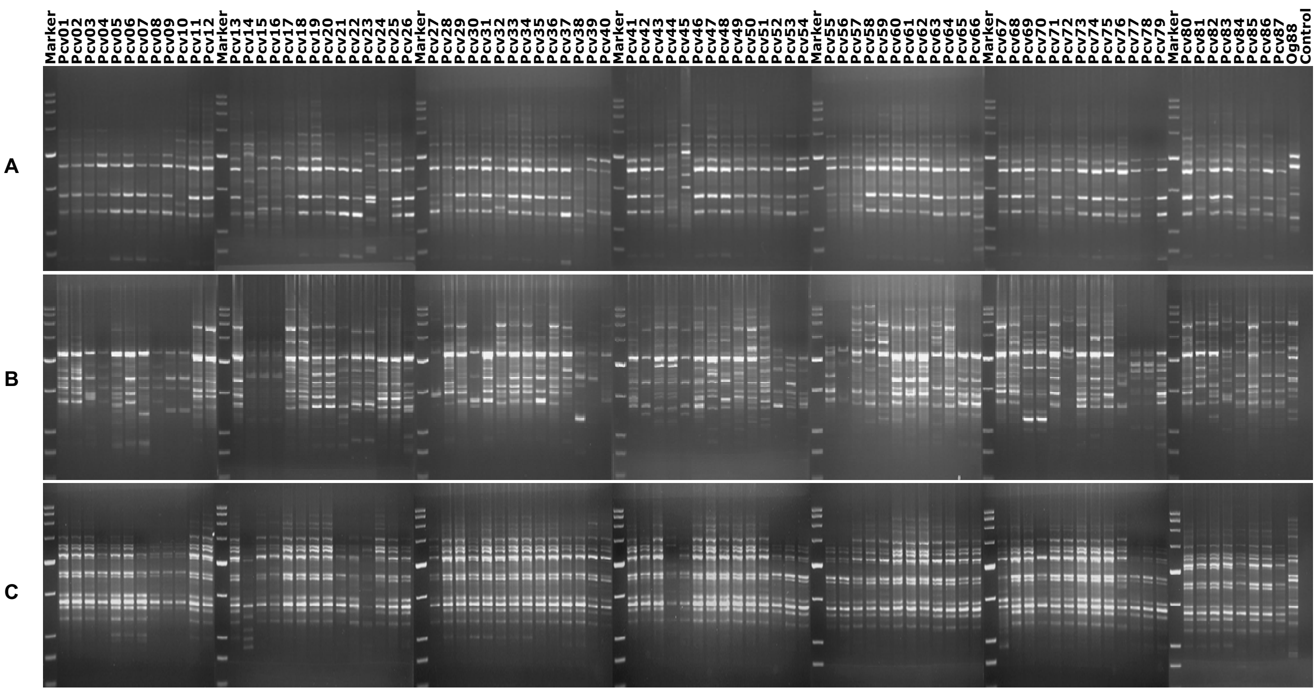

Fig. 2 Gel profiles obtained typically with (A) RAPD primer OP-G02, (B) DAMD primer M13 and (C) ISSR primer. All profiles were resolved in 1.5% agarose gels in 0.5x TBE buffer. The lanes marked as Marker contain the Low Range Ruler (Bangalore Genei, Bangalore) as DNA fragment size marker. The other lanes are marked with accession names as in **Table 1**. The last lane is the negative control without template DNA in the gel.

Table 2 RAPD band data and polymorphic information content (PIC) of primers used in cultivated pomegranate analyses.

Primer name	Sequence (5′ – 3′)	Amplified bands	Polymorphic bands	Percentage polymorphism	Approx. band size (bp)	Mean PIC
OP-B15	GGAGGGTGTT	17	16	94	200-2500	0.21
OP-G02	GGCACTGAGG	17	14	82	190-2000	0.13
OP-G03	GAGCCCTCCA	13	11	85	200-1750	0.10
OP-G04	AGCGTGTCTG	17	17	100	320-2400	0.17
OP-G05	CTGAGACGGA	13	12	92	200-1300	0.20
OP-G10	AGGGCCGTCT	16	14	88	320-1900	0.19
OP-G14	GGATGAGACC	10	09	90	280-1350	0.16
OP-G17	ACGACCGACA	15	13	87	230-1900	0.17
OP-H08	GAAACACCCC	13	10	77	250-1900	0.17
OP-H19	CTGACCAGCC	14	12	86	290-2400	0.13
OP-H20	GGGAGACATC	15	15	100	250-2400	0.18
OP-M01	GTTGGTGGCT	14	13	93	290-2500	0.16
OP-M03	GGGGGATGAG	10	08	80	220-1400	0.12
OP-M07	CCGTGACTCA	15	15	100	350-2000	0.19
OP-N07	CAGCCCAGAG	21	21	100	250-2500	0.26
OP-N13	AGCGTCACTC	24	24	100	240-2000	0.26
OP-N16	AAGCGACCTG	19	17	90	200-1750	0.19
OP-N18	GGTGAGGTCA	17	16	94	150-1600	0.15
OP-U11	AGACCCAGAG	16	15	94	220-2200	0.15
OP-U18	GAGGTCCACA	15	15	100	290-2000	0.16
OP-U20	ACAGCCCCCA	16	15	94	200-2000	0.16
Total	21	327	302	92.35	150-2500	0.17

Polymorphic Information Content (PIC)

Table 3 Jaccard's pairwise mean dissimilarity coefficient between different pomegranate cultivars as well as with *L. speciosa* (outgroup taxon) based on RAPD data. Value in parenthesis is the number of accessions of a cultivar.

	Arakhta	**Asthagandha**	**Bedana**	**Bhagua**	**Darsha Malas**	**Devanhalli Seedless**	**Dholka**	**G-137**	**Ganesh**	**Gulsha Red**	**Gulsha Rose**	**Jalore Seedless**	**Jyothi**	**Kabul**	**Kandhari**	**Khog**	**Malta**	**Mridula**	**Muscat**	**Ornamental**	**P-16**	**P-23**	**P-26**	**Ramnagaram**	**Sindhuri**	**Surkh Anar**	**Uthkal**	**Yercaud**
Arakhta (4)	0																											
Asthagandha (3)	0.39	0																										
Bedana (1)	0.45	0.40	0																									
Bhagua (2)	0.42	0.37	0.28	0																								
Darsha Malas (3)	0.33	0.43	0.55	0.53	0																							
Devanhalli Seedless (3)	0.46	0.54	0.59	0.58	0.42	0																						
Dholka (4)	0.34	0.46	0.52	0.52	0.35	0.40	0																					
G-137 (3)	0.45	0.44	0.49	0.47	0.45	0.50	0.47	0																				
Ganesh (4)	0.37	0.46	0.50	0.51	0.35	0.44	0.35	0.41	0																			
Gulsha Red (4)	0.29	0.41	0.52	0.50	0.28	0.43	0.31	0.47	0.33	0																		
Gulsha Rose (3)	0.30	0.43	0.54	0.52	0.20	0.40	0.29	0.45	0.32	0.17	0																	
Jalore Seedless (4)	0.34	0.44	0.52	0.51	0.29	0.42	0.30	0.44	0.31	0.25	0.21	0																
Jyothi (4)	0.37	0.47	0.52	0.52	0.29	0.44	0.35	0.48	0.37	0.31	0.29	0.29	0															
Kabul (2)	0.44	0.53	0.60	0.61	0.38	0.43	0.38	0.50	0.43	0.38	0.37	0.37	0.36	0														
Kandhari (1)	0.53	0.56	**0.63**	0.61	0.50	0.53	0.52	0.53	0.51	0.51	0.49	0.50	0.53	0.51	0													
Khog (3)	0.33	0.44	0.51	0.52	0.22	0.39	0.32	0.44	0.31	0.26	0.21	0.25	0.28	0.36	0.51	0												
Malta (3)	0.32	0.43	0.51	0.52	0.25	0.40	0.33	0.47	0.33	0.26	0.22	0.25	0.27	0.38	0.49	0.16	0											
Mridula (4)	0.39	0.48	0.49	0.41	0.48	0.55	0.46	0.51	0.50	0.41	0.45	0.46	0.48	0.54	0.56	0.47	0.45	0										
Muscat (4)	0.37	0.49	0.55	0.52	0.39	0.43	0.34	0.50	0.41	0.35	0.33	0.35	0.39	0.40	0.53	0.36	0.34	0.44	0									
Ornamental (3)	0.33	0.44	0.52	0.52	0.22	0.38	0.33	0.46	0.34	0.25	0.19	0.27	0.30	0.37	0.50	**0.15**	0.20	0.46	0.33	0								
P-16 (4)	0.36	0.46	0.50	0.49	0.36	0.45	0.36	0.45	0.41	0.33	0.33	0.34	0.37	0.40	0.51	0.30	0.32	0.47	0.34	0.30	0							
P-23 (4)	0.33	0.44	0.51	0.50	0.29	0.41	0.35	0.46	0.35	0.30	0.27	0.30	0.32	0.37	0.51	0.24	0.26	0.45	0.34	0.24	0.34	0						
P-26 (3)	0.34	0.44	0.51	0.51	0.29	0.38	0.31	0.45	0.33	0.30	0.26	0.29	0.29	0.37	0.51	0.22	0.24	0.47	0.34	0.24	0.33	0.22	0					
Ramnagaram (3)	0.35	0.48	0.54	0.53	0.28	0.42	0.34	0.48	0.38	0.31	0.25	0.29	0.28	0.37	0.50	0.22	0.25	0.47	0.34	0.24	0.34	0.24	0.24	0				
Sindhuri (3)	0.50	0.45	0.43	0.43	0.55	0.59	0.52	0.45	0.48	0.50	0.53	0.54	0.53	0.61	0.60	0.54	0.55	0.45	0.54	0.55	0.54	0.56	0.53	0.56	0			
Surkh Anar (1)	0.36	0.44	0.55	0.52	0.23	0.43	0.39	0.46	0.39	0.30	0.24	0.32	0.34	0.41	0.51	0.23	0.28	0.48	0.38	0.22	0.37	0.29	0.30	0.28	0.54	0		
Uthkal (3)	0.37	0.46	0.58	0.54	0.26	0.42	0.38	0.48	0.40	0.29	0.24	0.33	0.33	0.40	0.49	0.24	0.27	0.50	0.35	0.23	0.35	0.30	0.29	0.26	0.58	0.20	0	
Yercaud (4)	0.40	0.50	0.57	0.56	0.41	0.47	0.41	0.53	0.45	0.39	0.38	0.39	0.40	0.44	0.54	0.39	0.38	0.49	0.38	0.39	0.43	0.35	0.37	0.35	0.59	0.38	0.39	0
L. speciosa (1)	0.85	0.88	0.90	0.90	0.84	**0.82**	**0.82**	0.87	0.84	0.86	0.83	0.83	0.84	0.84	0.86	0.84	0.84	0.89	0.86	0.84	0.84	0.85	0.85	0.84	**0.91**	0.85	0.84	0.84

culated for each primer. The PIC value of the primers OP-M07 and OP-N07 was found maximum (0.26) with 100% polymorphism. On the other hand, OP-G03 exhibited lowest PIC value (0.10) with 85% band polymorphism (**Table 2**). The mean PIC value recorded was 0.17 over all the RAPD primers used in cultivated pomegranates.

The pair-wise distance matrix calculated by NJ method using Jaccard's coefficient (Jaccard 1908) showed a distance range of 0 to 0.70 among the accessions of different pomegranate cultivars. The maximum distance (0.70) was observed between the accessions Pcv44 ('Kabul') and Pcv78 ('Sindhuri'), whereas the accessions Pcv09 and Pcv10 both from the same cultivar 'Bhagua' showed negligible distances revealing that they are 100% similar (data not

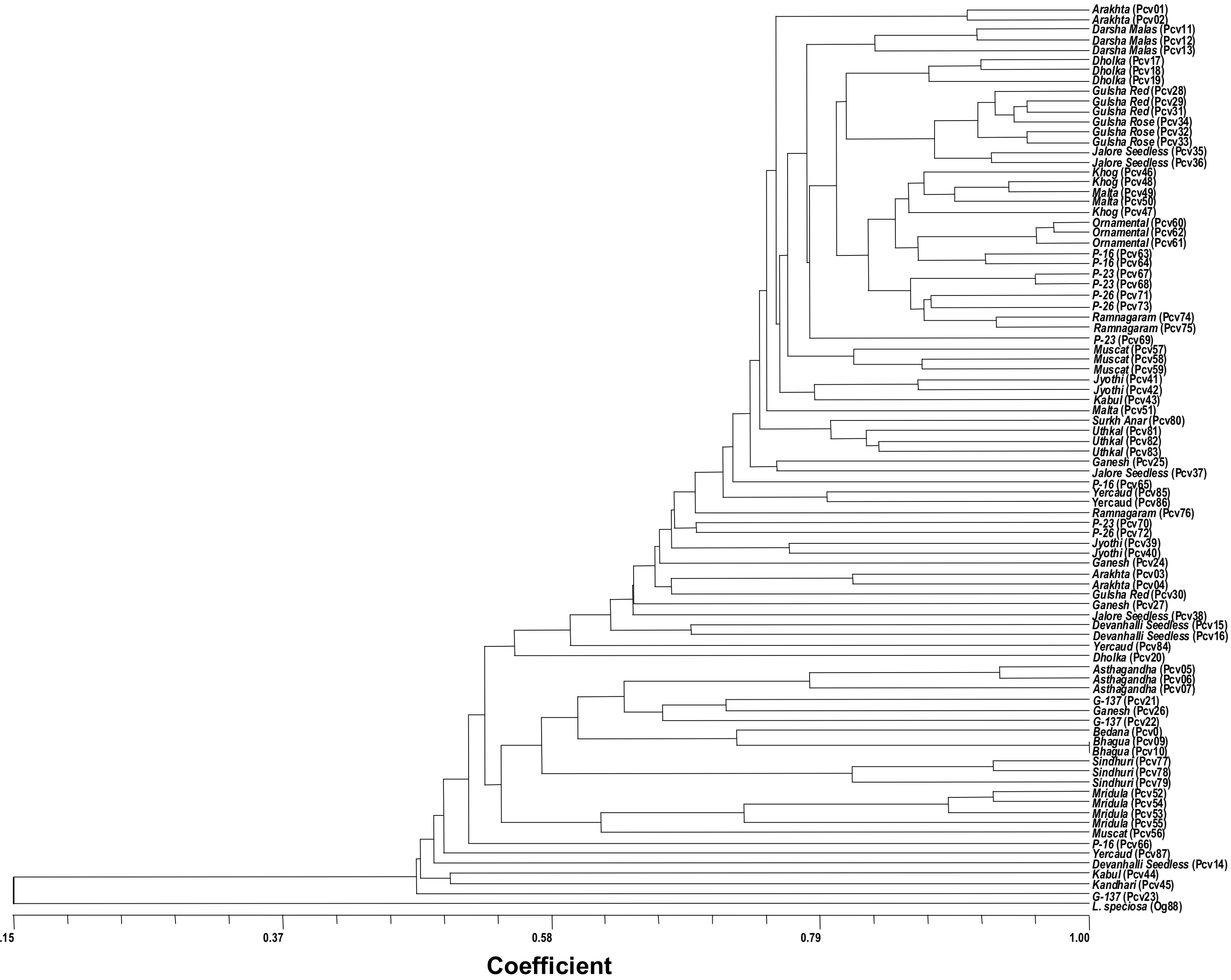

Fig. 3 UPGMA dendrogram based on RAPD data showing the relationship of the accessions representing different cultivars of pomegranates. Accessions are as given in **Table 1**. *Lagerstroemia speciosa* was used as an out group in this analysis.

shown). Again, based on the distance matrix of Jaccard's coefficient, pairwise average distances were calculated among all the cultivars of pomegranate (**Table 3**). 'Bedana' showed maximum distances (0.63) to 'Kandhari' and 'Khog' showed minimum distances (0.15) to 'Ornamental', revealing 0.39 mean genetic distances across all the cultivars.

A UPGMA dendrogram was generated based on Jaccard's similarity coefficient, using NTSYS program to describe the relative clustering of the accessions of cultivated pomegranates (**Fig. 3**). In the dendrogram, the out-group taxon *L. speciosa* was separated from rest of the pomegranate cultivars. UPGMA dendrogram has two major clusters showing about 47% genetic distances among them. The majority of cultivars like 'Arakhta', 'Darsha Mallas', 'Dholka', 'Gulsha Red', 'Gulsha Rose', 'Jalore Seedless', 'Khog', 'Malta', 'Ornamental', 'Muscat', 'Ramnagaram', 'Jyothi', 'Surkh Anar', 'Uthkal', 'Ganesh' and 'Yercaud' showed similarity between 53-90% and clustered together in a major cluster in the dendrogram. The ornamental accessions (Pcv60-Pcv62) clustered with 'P-16', 'P-23' and 'P-26', which are seedling selections of 'Muscat'. Another major cluster includes cultivars like 'Asthagandha', 'G-137', 'Ganesh', 'Bedana', 'Bhagua', 'Sindhuri', 'Mridula' and 'Muscat'. Accessions Pcv66 ('P-16'), Pcv87 ('Yercaud'), Pcv14 ('Devanhalli Seedless'), Pcv44 ('Kabul') and Pcv23 ('G-137') are independent of any of the major groupings showing less proximity with their respective cultivars (**Fig. 3**). The lone representative of the introduced cultivar 'Kandhari' (Pcv45) was also included in neither of the major clusters and exhibited close affinity to one of the 'Kabul' accession (Pcv44) in the dendrogram.

DAMD analysis

Another PCR-based technique known as DAMD was employed to study the genetic diversity in pomegranate cultivars. DAMD reactions were carried out for all the 87 pomegranate accessions and one out-group taxon (*L. speciosa*). Five gel profiles were generated with five DAMD primers, four of which have been given in **Fig. 2B**. A total of 136 discrete bands were scored from five gel profiles, of which 134 were polymorphic showing 98.52% polymorphism across the accessions (**Table 4**). The number of amplified bands ranged from 22 (with primer 33.6) to 33 (with primer HVA). The sizes of amplicons were in the range of 100 to 3000 bp.

The polymorphic information content (PIC) value was calculated, considering the number of amplified products in case of each DAMD primer. The PIC value of DAMD primers used in cultivated pomegranates ranged from 0.16 (in 33.6) to 0.30 (in HBV) with a mean value of 0.26 (**Table 4**). The primer 33.6 was found comparatively less informative with 96% polymorphism in comparison to other DAMD primers used in the present study.

Jaccard's pair-wise distances were calculated based on DAMD profiles for both accession-wise as well as cultivar-wise. These analyses showed a wide range of distance values revealing significant genetic variations among the accessions of cultivated pomegranates in India. The highest and lowest genetic distances ranged from 0.10 to 0.92 with a mean of 0.51 (data not shown). The smallest genetic distance of 0.10 was revealed between the accessions Pcv22 and Pcv23 (both from 'G-137') suggesting close affinities. Accessions Pcv05 ('Asthagandha') and Pcv16 ('Devanhalli

Table 4 DAMD band data and polymorphic information content (PIC) of primers used in cultivated pomegranate analyses.

Primer name	Sequence (5′ – 3′)	Amplified bands	Polymorphic bands	Percentage polymorphism	Approx. band size (bp)	Mean PIC
33.6	AGGGCTGGAGG	22	21	96	100-2400	0.19
HVA	AGGATGGAAAGGAGGC	33	33	100	150-3000	0.29
HBV	GGTGTAGAGAGGGGT	29	29	100	130-3000	0.30
M-13	GAGGGTGGCGGTTCT	25	24	96	230-3000	0.26
HVY	GCCTTTCCCGAG	27	27	100	150-2500	0.26
Total	05	136	134	98.52	100-3000	0.26

Polymorphic Information Content (PIC)

Table 5 Jaccard's pairwise mean dissimilarity coefficient between different pomegranate cultivars as well as with *L. speciosa* (outgroup taxon) based on DAMD data. Value in parenthesis is the number of accessions of a cultivar.

	Arakhta	Asthagandha	Bedana	Bhagua	Darsha Malas	Devanhalli	Dholka	G-137	Ganesh	Gulsha Red	Gulsha Rose	Jalore Seedless	Jyothi	Kabul	Kandhari	Khog	Malta	Mridula	Muscat	Ornamental	P-16	P-23	P-26	Ramnagaram	Sindhuri	Surkh Anar	Uthkal	Yercaud
Arakhta (4)	0																											
Asthagandha (3)	0.62	0																										
Bedana (1)	0.68	0.66	0																									
Bhagua (2)	0.66	0.64	0.63	0																								
Darsha Malas (3)	0.64	0.76	0.83	0.75	0																							
Devanhalli Seedless (3)	0.80	0.85	0.82	0.75	0.76	0																						
Dholka (4)	0.65	0.74	0.79	0.74	0.64	0.71	0																					
G-137 (3)	0.73	0.78	0.77	0.72	0.69	0.75	0.68	0																				
Ganesh (4)	0.68	0.76	0.71	0.76	0.61	0.78	0.73	0.70	0																			
Gulsha Red (4)	0.56	0.68	0.65	0.67	0.63	0.75	0.64	0.75	0.67	0																		
Gulsha Rose (3)	0.60	0.72	0.72	0.67	0.63	0.72	0.6	0.77	0.75	0.50	0																	
Jalore Seedless (4)	0.62	0.73	0.75	0.72	0.71	0.74	0.67	0.77	0.76	0.57	**0.43**	0																
Jyothi (4)	0.73	0.76	0.79	0.72	0.74	0.77	0.70	0.77	0.81	0.70	0.71	0.75	0															
Kabul (2)	0.69	0.79	0.79	0.75	0.64	0.76	0.63	0.76	0.68	0.62	0.64	0.66	0.74	0														
Kandhari (1)	0.72	0.76	0.78	0.72	0.68	0.75	0.67	0.75	0.72	0.67	0.65	0.71	0.71	0.57	0													
Khog (3)	0.63	0.74	0.76	0.70	0.66	0.76	0.56	0.74	0.73	0.65	0.60	0.67	0.69	0.58	0.55	0												
Malta (3)	0.67	0.74	0.77	0.69	0.59	0.78	0.57	0.74	0.71	0.62	0.57	0.65	0.70	0.52	0.62	0.54	0											
Mridula (4)	0.72	0.68	0.70	0.69	0.78	**0.86**	0.74	0.80	0.73	0.69	0.70	0.76	0.74	0.78	0.79	0.73	0.67	0										
Muscat (4)	0.72	0.78	0.76	0.69	0.74	0.82	0.72	0.82	0.77	0.69	0.69	0.70	0.77	0.74	0.82	0.70	0.70	0.64	0									
Ornamental (3)	0.64	0.73	0.71	0.70	0.68	0.79	0.66	0.76	0.72	0.56	0.57	0.61	0.73	0.67	0.70	0.59	0.63	0.67	0.61	0								
P-16 (4)	0.81	0.71	0.75	0.70	0.73	0.78	0.69	0.78	0.77	0.63	0.61	0.66	0.68	0.69	0.64	0.63	0.62	0.72	0.66	0.53	0							
P-23 (4)	0.72	0.79	0.77	0.73	0.75	0.77	0.69	0.78	0.72	0.74	0.63	0.68	0.79	0.68	0.66	0.63	0.66	0.75	0.70	0.59	0.61	0						
P-26 (3)	0.66	0.78	0.78	0.73	0.67	0.74	0.66	0.76	0.69	0.64	0.64	0.69	0.74	0.68	0.73	0.65	0.64	0.72	0.70	0.56	0.65	0.60	0					
Ramnagaram (3)	0.64	0.78	0.79	0.70	0.70	0.78	0.62	0.79	0.74	0.68	0.65	0.66	0.75	0.65	0.69	0.60	0.65	0.73	0.67	0.52	0.62	0.60	0.54	0				
Sindhuri (3)	0.72	0.71	0.69	0.68	0.84	0.85	0.81	0.79	0.77	0.77	0.80	0.76	0.80	0.85	0.82	0.79	0.79	0.73	0.78	0.71	0.71	0.75	0.76	0.74	0			
Surkh Anar (1)	0.67	0.79	0.77	0.72	0.70	0.75	0.60	0.80	0.77	0.62	0.54	0.62	0.78	0.60	0.64	0.53	0.58	0.73	0.66	0.44	0.55	0.57	0.59	0.54	0.76	0		
Uthkal (3)	0.66	0.81	0.79	0.76	0.68	0.78	0.68	0.77	0.77	0.64	0.59	0.66	0.77	0.64	0.68	0.60	0.63	0.77	0.71	0.58	0.59	0.66	0.60	0.60	0.83	0.60	0	
Yercaud (4)	0.76	0.84	0.81	0.75	0.71	0.73	0.66	0.77	0.78	0.69	0.65	0.71	0.76	0.65	0.61	0.64	0.64	0.77	0.78	0.66	0.64	0.66	0.70	0.68	0.83	0.59	0.57	0
L. speciosa (1)	0.86	0.91	0.91	0.82	0.85	0.81	0.84	0.86	0.87	0.83	**0.75**	0.81	0.91	0.8	0.86	0.82	0.76	0.86	0.83	0.83	0.84	0.79	0.80	0.84	**0.92**	0.80	0.80	0.76

Seedless'), however, seem to be the most divergent since they present the highest genetic distance value (0.92). The cultivar wise genetic distance revealed that 'Mridula' and 'Devanhalli Seedless' had maximum genetic distance value (0.86), whereas 'Jalore Seedless' and 'Gulsha Rose' had minimum distance (0.43) value (**Table 5**). DAMD markers used in the present study revealed 0.65 average distance value amongst the pomegranate cultivars. All the cultivars were also compared to the out group accession (*L. speciosa*), where 'Sindhuri' showed maximum distance value of 0.92, and 'Gulsha Rose' minimum distance value of 0.75.

The similarity data were analysed further by SAHN method using the NTSYS program to describe the relative clustering of the cultivated pomegranates. UPGMA dendrogram based on the similarity matrix is shown in **Fig. 4**. In the dendrogram, the out-group taxon (*L. speciosa*) was clearly separated from the rest of the accessions of pomegranate cultivars. The cultivated accessions formed two major clusters. All the pomegranate accessions clustered together in major cluster I except three accessions of 'Devanhalli Seedless', which formed major cluster II. The major cluster I further divided into six sub-clusters, where majority of the accessions were grouped together according to their cultivars. Cultivars like 'Arakhta', 'Gulsha Red', 'Gulsha Rose', 'Jalore Seedless' clustered together in one sub-cluster, whereas 'Dholka', 'Kabul', 'Khog', 'Malta', 'Kandhari' were in another sub-cluster. All the ornamental accessions (Pcv60, Pcv61 and Pcv62) clustered with 'P-16', 'P-23', 'P-26', 'Surkh Anar', 'Uthkal', and 'Ramnagaram'. Accessions belonging to 'Yercaud' grouped together in a separate sub-cluster. 'Darsha Malas' clustered with 'Ganesh' and 'G-137'. 'Asthagandha', 'Bedana', 'Bhagua', 'Jyothi' and two accessions of 'P-16' formed a separate sub-cluster, whereas all the accessions belonging to 'Mridula' clustered in a different sub-cluster with 'Muscat'. All the tree accessions of 'Sindhuri' formed a separate distinct sub-cluster.

ISSR analysis

Apart from RAPD and DAMD techniques, ISSR-PCR was also employed in the present study. The gel profiles for 87 accessions of pomegranates representing 28 cultivars and one accession of *L. speciosa* as an out-group taxon were generated with 17 ISSR primers, and representative gels have been presented in **Fig. 2C**. A total of 200 bands in the size of 150 to 2700 bp were scored with all the primers used, out of which 153 bands were polymorphic, revealing 76.5%

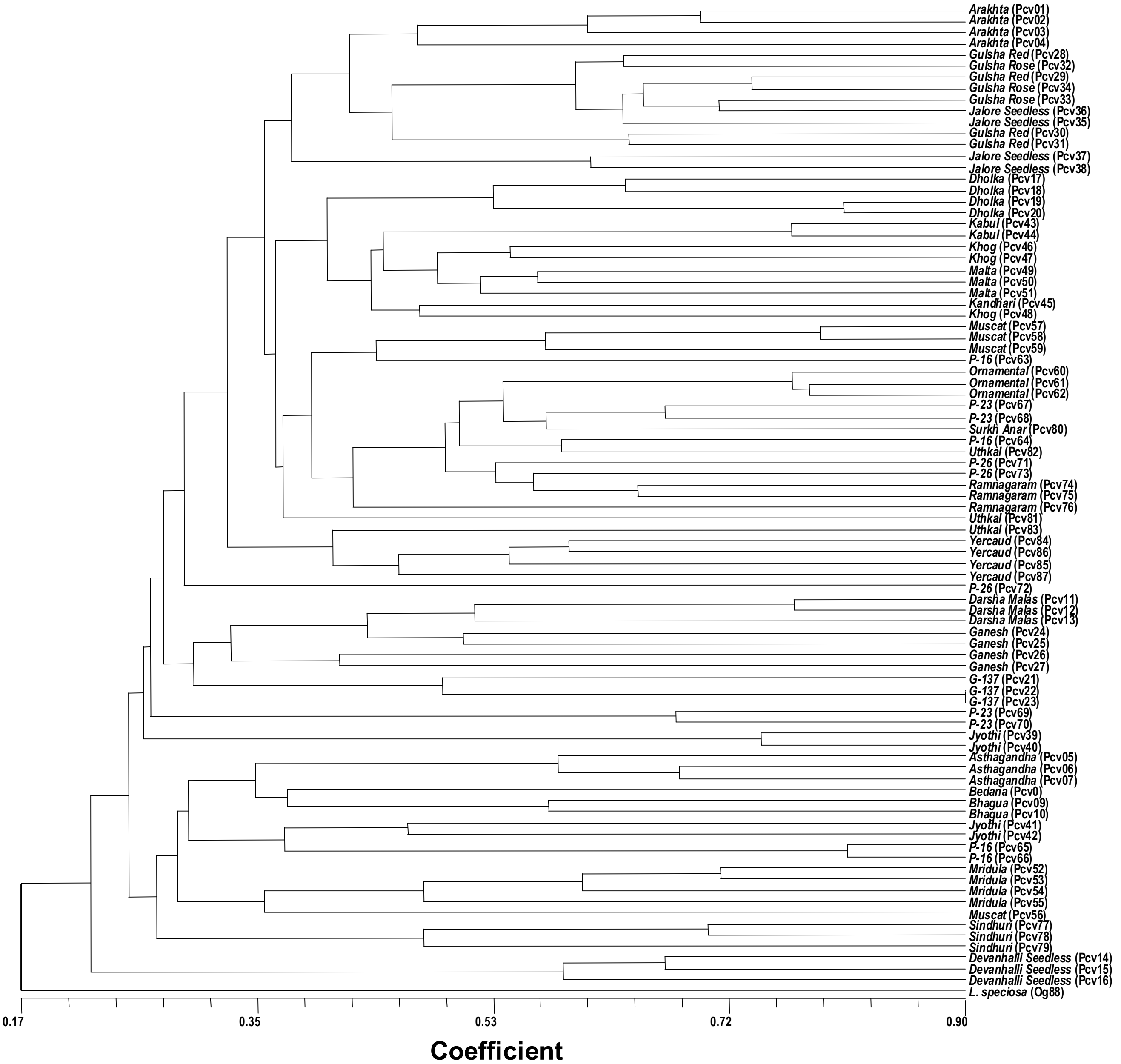

Fig. 4 UPGMA dendrogram based on DAMD data showing the relationship of the accessions representing different cultivars of pomegranates. Accessions are as given in **Table 1**. *Lagerstroemia speciosa* was used as an out group in this analysis.

average polymorphism across the accessions. The primer 810 produced minimum number of bands (8), while primers 807, 827 and 880 produced maximum number of bands (16) in each. The highest average polymorphism (94%) was revealed by the primer 827.

The polymorphic information content (PIC) value for all the ISSR primers used in the analysis were also estimated. The PIC values were maximum (0.21) in case of 827 and 880 primers, whereas primers 809, 825 and 861 revealed minimum (0.12) PIC values (**Table 6**). The average PIC value was 0.15 for all the ISSR primers used in cultivated pomegranates.

The bands obtained with all 17 primers were cumulatively considered for pair-wise analysis using Jaccard's coefficient (data not shown). The highest distance value (0.43) was between the accession pairs Pcv24-Pcv78, Pcv24-Pcv79 and Pcv78-Pcv84. The accessions showing highest genetic distances were from 'Ganesh', 'Sindhuri' and 'Yercaud'. The lowest distance value was 0.01 between the accession pairs Pcv01-Pcv02, Pcv17-Pcv18, Pcv32-Pcv33, Pcv33-Pcv34, Pcv48-Pcv50, Pcv49-Pcv50, Pcv52-Pcv53, Pcv60-Pcv62, and Pcv63-Pcv64; these accessions with low genetic distances, were mainly from 'Arakhta', 'Dholka', 'Gulsha Rose', 'Khog', 'Malta', 'Mridula', 'Ornamental' and 'P-16'.

The genetic distances were also calculated among the 28 pomegranate cultivars. The distance value varied from 0.05 to 0.39 across the cultivars (**Table 7**). The lowest genetic distance (0.05) was observed between the cultivars 'Malta' and 'Khog', whereas the highest distances (0.39) were observed between the cultivar pairs – 'Bedana' : 'Gulsha Rose', 'P-23'; 'Bhagua' : 'Darsha Malas', 'Gulsha Rose', 'Ornamental', 'P-16', 'P-23'; 'Mridula' : 'Darsha Malas'; and 'Sindhuri' : 'Uthkal', 'Yercaud'.

The distance data were further analyzed by UPGMA method using NTSYS program to describe the relative clustering of the accessions. The out-group taxon *L. speciosa* was clearly separated from rest of the pomegranate accessions (**Fig. 5**). The accessions of pomegranate cultivars grouped in two major clusters showing about 60% similarity. Majority of the accessions of cultivars like 'Arakhta', 'Darsha Malas', 'Dholka', 'Gulsha Red', 'Gulsha Rose', 'Jalore Seedless', 'Kabul', 'Khog', 'Malta', 'Muscat', 'Ornamental', 'P-16', 'P-23', 'P-26', 'Ramnagaram', 'Surkh Anar', 'Uthkal', 'Yercaud', 'Ganesh', 'Jyothi', 'Kandhari', 'Asthagandha', 'Devanhalli Seedless' and 'G-137' were

Table 6 ISSR band data and polymorphic information content (PIC) of primers used in cultivated pomegranate analyses.

Primer name	Sequence (5′ – 3′)	Amplified bands	Polymorphic bands	Percentage polymorphism	Approx. band size (bp)	Mean PIC
807	$(AG)_8T$	16	12	75	250-2100	0.13
809	$(AG)_8G$	09	06	67	450-1800	0.12
810	$(GA)_8T$	08	04	50	350-2500	0.13
811	$(GA)_8C$	10	06	60	310-2500	0.16
812	$(GA)_8A$	14	13	93	250-2500	0.13
825	$(AC)_8T$	11	08	73	380-2400	0.12
827	$(AC)_8G$	16	15	94	250-2700	0.21
834	$(AG)_8YT$	11	09	82	220-1800	0.14
835	$(AG)_8YC$	09	06	67	200-2500	0.20
836	$(AG)_8YA$	11	06	55	300-1800	0.16
840	$(GA)_8YT$	14	11	79	150-2300	0.17
841	$(GA)_8YC$	12	11	92	220-2500	0.18
842	$(GA)_8YG$	12	09	75	190-2000	0.13
861	$(ACC)_6$	09	07	78	260-2500	0.12
880	$(GGAGA)_3$	16	14	88	200-2000	0.21
886	$VDV(CT)_7$	13	10	77	220-1900	0.17
891	$HVH(TG)_7$	09	06	67	400-1700	0.18
Total	17	200	153	76.50	150-2700	0.16

Polymorphic Information Content (PIC)

Table 7 Jaccard's pairwise mean dissimilarity coefficient between different pomegranate cultivars as well as with *L. speciosa* (outgroup taxon) based on ISSR data. Value in parenthesis is the number of accessions of a cultivar.

	Arakhta	Asthagandha	Bedana	Bhagua	Darsha Malas	Devanhalli Seedless	Dholka	G-137	Ganesh	Gulsha Red	Gulsha Rose	Jalore Seedless	Jyothi	Kabul	Kandhari	Khog	Malta	Mridula	Muscat	Ornamental	P-16	P-23	P-26	Ramnagaram	Sindhuri	Surkh Anar	Uthkal	Yercaud
Arakhta (4)	0																											
Asthagandha (3)	0.20	0																										
Bedana (1)	0.28	0.22	0																									
Bhagua (2)	0.32	0.26	0.09	0																								
Darsha Malas (3)	0.19	0.25	0.38	**0.39**	0																							
Devanhalli Seedless (3)	0.26	0.28	0.35	0.36	0.26	0																						
Dholka (4)	0.18	0.25	0.34	0.34	0.18	0.24	0																					
G-137 (3)	0.26	0.26	0.25	0.25	0.31	0.26	0.24	0																				
Ganesh (4)	0.21	0.27	0.31	0.34	0.23	0.26	0.22	0.26	0																			
Gulsha Red (4)	0.21	0.27	0.36	0.37	0.16	0.29	0.21	0.32	0.24	0																		
Gulsha Rose (3)	0.21	0.27	**0.39**	**0.39**	0.10	0.27	0.19	0.29	0.22	0.11	0																	
Jalore Seedless (4)	0.21	0.26	0.35	0.37	0.17	0.26	0.2	0.27	0.22	0.15	0.11	0																
Jyothi (4)	0.22	0.27	0.37	0.38	0.19	0.27	0.24	0.30	0.24	0.19	0.16	0.17	0															
Kabul (2)	0.24	0.28	0.38	0.37	0.22	0.31	0.21	0.33	0.28	0.24	0.19	0.19	0.21	0														
Kandhari (1)	0.26	0.28	0.35	0.35	0.26	0.31	0.25	0.30	0.29	0.26	0.24	0.23	0.24	0.18	0													
Khog (3)	0.20	0.25	0.36	0.37	0.14	0.26	0.18	0.29	0.22	0.17	0.12	0.13	0.15	0.14	0.21	0												
Malta (3)	0.18	0.25	0.33	0.35	0.15	0.25	0.17	0.26	0.20	0.18	0.12	0.13	0.14	0.14	0.20	**0.05**	0											
Mridula (4)	0.33	0.30	0.27	0.28	**0.39**	0.38	0.36	0.29	0.34	0.35	0.37	0.32	0.33	0.35	0.33	0.33	0.30	0										
Muscat (4)	0.25	0.28	0.34	0.33	0.25	0.29	0.23	0.28	0.29	0.22	0.22	0.22	0.25	0.24	0.29	0.22	0.21	0.21	0									
Ornamental (3)	0.20	0.25	0.38	**0.39**	0.11	0.25	0.19	0.30	0.23	0.18	0.11	0.16	0.18	0.19	0.24	0.11	0.12	0.36	0.20	0								
P-16 (4)	0.20	0.26	0.38	**0.39**	0.16	0.28	0.19	0.31	0.25	0.18	0.14	0.15	0.18	0.18	0.22	0.13	0.12	0.33	0.19	0.11	0							
P-23 (4)	0.20	0.26	**0.39**	**0.39**	0.15	0.26	0.19	0.30	0.24	0.18	0.14	0.16	0.18	0.19	0.22	0.11	0.11	0.36	0.22	0.10	0.14	0						
P-26 (3)	0.20	0.26	0.36	0.37	0.12	0.25	0.18	0.30	0.29	0.16	0.12	0.14	0.16	0.18	0.24	0.10	0.11	0.36	0.21	0.10	0.13	0.10	0					
Ramnagaram (3)	0.21	0.25	0.35	0.35	0.15	0.24	0.18	0.28	0.24	0.18	0.13	0.15	0.18	0.21	0.25	0.13	0.13	0.34	0.19	0.12	0.15	0.11	0.08	0				
Sindhuri (3)	0.31	0.26	0.20	0.24	0.38	0.35	0.35	0.25	0.32	0.37	0.36	0.34	0.35	0.36	0.30	0.34	0.32	0.21	0.33	0.37	0.36	0.37	0.36	0.35	0			
Surkh Anar (1)	0.20	0.23	0.34	0.38	0.15	0.32	0.22	0.31	0.23	0.16	0.12	0.16	0.19	0.20	0.25	0.13	0.14	0.36	0.25	0.13	0.18	0.30	0.12	0.14	0.36	0		
Uthkal (3)	0.23	0.27	0.37	0.38	0.14	0.27	0.24	0.32	0.27	0.16	0.12	0.16	0.19	0.20	0.26	0.13	0.13	0.37	0.24	0.15	0.17	0.15	0.13	0.15	**0.39**	0.07	0	
Yercaud (4)	0.27	0.32	0.38	0.38	0.25	0.31	0.27	0.33	0.29	0.24	0.23	0.22	0.25	0.25	0.29	0.21	0.21	0.36	0.28	0.23	0.24	0.23	0.21	0.21	**0.39**	0.18	0.15	0
L. speciosa (1)	0.77	0.78	0.79	0.79	0.75	**0.80**	0.76	0.78	0.77	0.75	0.76	0.76	0.76	0.76	0.79	0.76	0.76	0.78	0.75	0.75	**0.74**	0.75	0.76	0.76	**0.80**	0.77	0.76	0.76

clustered together in major cluster I, and the accessions of 'Bedana', 'Bhagua', 'Mridula', 'Muscat' and 'Sindhuri' all together formed the major cluster II. The cultivar 'Muscat' showed close affinities with 'Ornamental' and 'P-16' in the major cluster I, whereas with 'Mridula' in the major cluster II. UPGMA tree generated with ISSR data, showed no cultivar specific groupings. It was also observed that cultivars were independent of their geographical affiliations.

Cumulative data analysis of RAPD, DAMD and ISSR markers

The binary matrix data generated for 87 accessions of cultivated pomegranates and an out-group taxon (*L. speciosa*), by using RAPD, DAMD and ISSR markers were combined together and a cumulative analysis was carried out. A total 663 bands resulted with 43 primers of RAPD, DAMD and ISSR were taken into consideration. Out of these, 589 bands were polymorphic revealing 88.83% average polymorphism across different accessions of cultivated pomegranates. The

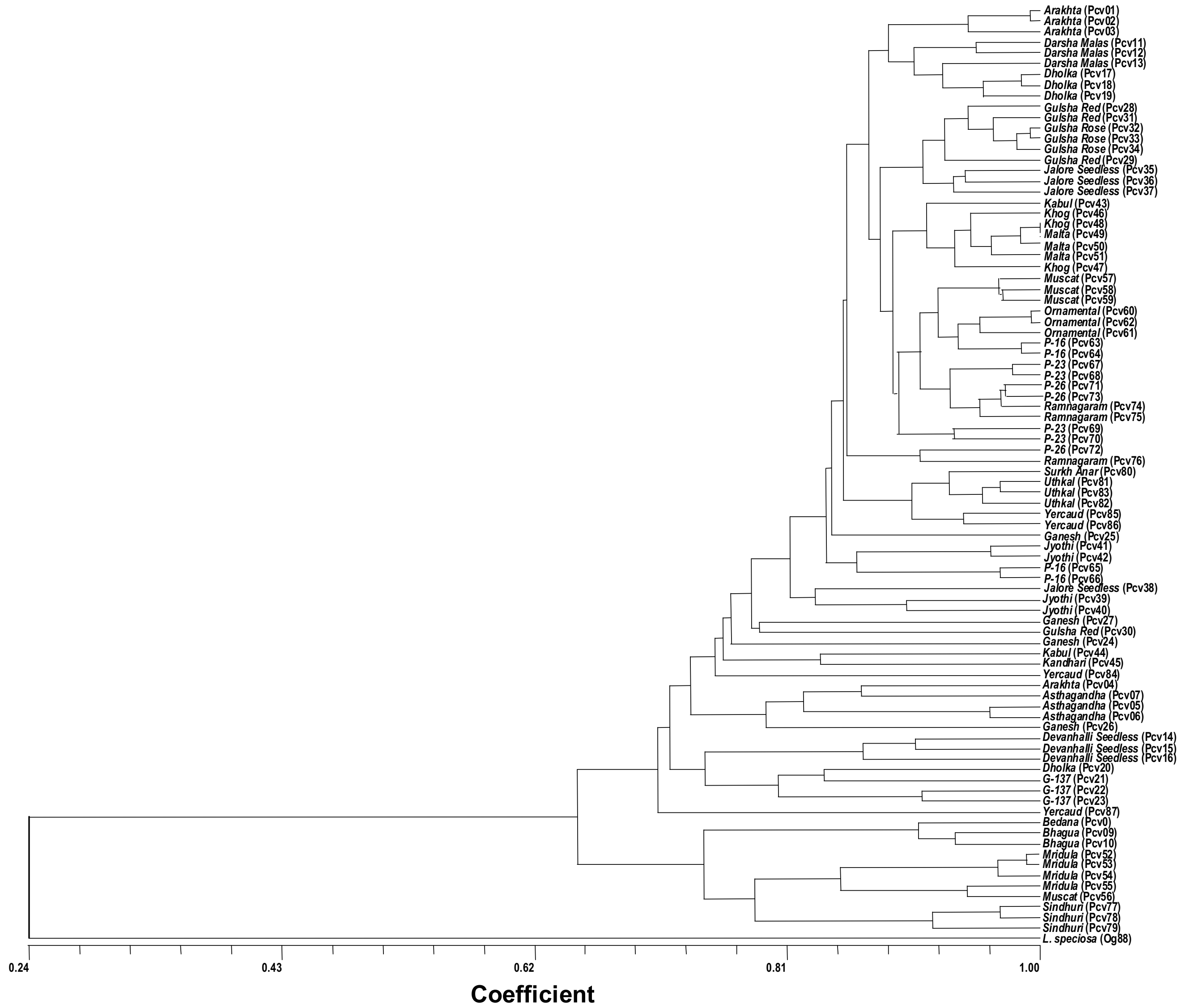

Fig. 5 UPGMA dendrogram based on ISSR data showing the relationship of the accessions representing different cultivars of pomegranates. Accessions are as given in **Table 1**. *Lagerstroemia speciosa* was used as an out group in this analysis.

cumulative data was used to compute Jaccard's distance coefficient, by NJ method in FreeTree program (data not shown). The highest distance value calculated was 0.61 between Pcv44 ('Kabul') and Pcv78 ('Sindhuri'). The lowest distance value was 0.05 between the two accessions, Pcv60 and Pcv62 of 'Ornamental'.

Among the cultivars, genetic distances ranged from 0.18 to 0.55 (**Table 8**). The highest distance value (0.55) was observed between 'Kabul' and 'Bedana', and also between 'Kabul' and 'Sindhuri'. The lowest distance value was 0.18 between 'Khog' and 'Malta', showing close affinities to each other.

Jaccard's coefficient data was further analysed by the UPGMA method using the NTSYS program to describe the clustering pattern of different accessions of pomegranate cultivars. The pomegranate accessions were grouped together in two major clusters in UPGMA dendrogram (**Fig. 6**). The major cluster I represented majority of the accessions of the pomegranate cultivars forming several sub-clusters within, for instance all the accessions of 'Arakhta' (Pcv01, Pcv02, Pcv03) were clustered together in one single sub-cluster. Similarly, the accessions of cultivars like 'Darsha Malas' (Pcv11, Pcv12, Pcv13) and 'Dholka' (Pcv17, Pcv18, Pcv19) also formed distinct sub-clusters. 'Gulsha Red' (Pcv28, Pcv29, Pcv31) and 'Gulsha Rose' (Pcv32, Pcv33, Pcv34) along with 'Jalore Seedless' (Pcv35, Pcv36) formed a single sub-cluster. The other sub-clusters of different cultivars were 'Khog' (Pcv46, Pcv47, Pcv48) and 'Malta' (Pcv49, Pcv50); 'Ornamental' (Pcv60, Pcv61, Pcv62), 'P-16' (Pcv63, Pcv64), 'P-23' (Pcv67, Pcv68), 'P-26' (Pcv71, Pcv73), 'Ramnagaram' (Pcv74, Pcv75), 'Muscat' (Pcv57, Pcv58, Pcv59); 'Surkh Anar' (Pcv80), 'Uthkal' (Pcv81, Pcv82, Pcv83), 'Malta' (Pcv51); 'Yercaud' (Pcv84, Pcv85, Pcv86) and 'Devanhalli Seedless' (Pcv14, Pcv15, Pcv16). 'Arakhta', 'Asthagandha', 'G-137', 'Ganesh', 'Bedana', 'Bhagua', 'Mridula', 'Muscat' and 'Sindhuri' formed the major cluster II except for 'Mridula' and 'Sindhuri'; the remaining cultivars were scattered throughout the dendrogram without preferential groupings to their geographical distribution. The accessions of a cultivar from the same region also showed less affinity to each other in the dendrogram.

A UPGMA dendrogram was also constructed using POPGENE to understand the relationship between 28 different cultivars. In the dendrogram the out-group accession, *L. speciosa* was distinctly separated from pomegranate cultivars. All the pomegranate cultivars except 'Kandhari' were grouped in two major clusters. 'Kandhari' was clustered separately showing distinctiveness from rest of the cultivars (**Fig. 7**). Five cultivars ('Asthagandha', 'Bedana', 'Bhagua', 'Mridula' and 'Sindhuri') formed one major cluster. On the other hand, the remaining 22 cultivars all together formed another major cluster, where the cultivars were distributed in four sub-clusters except for three ('Kabul', 'Yercaud' and 'Darsha Malas'). 'Devanhalli Seedless' and 'G-137' formed the first sub-cluster showing a sister group

Table 8 Jaccard's pairwise mean dissimilarity coefficient between different pomegranate cultivars as well as with *L. speciosa* (outgroup taxon) based on cumulative data. Value in parenthesis is the number of accessions of a cultivar.

	Arakhta	Asthagandha	Bedana	Bhagua	Darsha Malas	Devanhalli	Dholka	G-137	Ganesh	Gulsha Red	Gulsha Rose	Jalore Seedless	Jyothi	Kabul	Kandhari	Khog	Malta	Mridula	Muscat	Ornamental	P-16	P-23	P-26	Ramnagaram	Sindhuri	Surkh Anar	Uthkal	Yercaud
Arakhta (4)	0																											
Asthagandha (3)	0.35	0																										
Bedana (1)	0.41	0.35	0																									
Bhagua (2)	0.40	0.36	0.24	0																								
Darsha Malas (3)	0.38	0.40	0.51	0.50	0																							
Devanhalli Seedless (3)	0.43	0.47	0.52	0.53	0.40	0																						
Dholka (4)	0.33	0.42	0.5	0.49	0.32	0.39	0																					
G-137 (3)	0.41	0.42	0.43	0.43	0.44	0.45	0.41	0																				
Ganesh (4)	0.35	0.43	0.46	0.47	0.33	0.42	0.35	0.39	0																			
Gulsha Red (4)	0.29	0.39	0.46	0.47	0.29	0.41	0.33	0.45	0.34	0																		
Gulsha Rose (3)	0.30	0.40	0.50	0.49	0.24	0.39	0.31	0.44	0.35	0.20	0																	
Jalore Seedless (4)	0.33	0.40	0.48	0.48	0.31	0.40	0.32	0.43	0.34	0.26	0.20	0																
Jyothi (4)	0.36	0.42	0.48	0.48	0.31	0.42	0.35	0.43	0.37	0.33	0.29	0.32	0															
Kabul (2)	0.41	0.48	**0.55**	0.54	0.37	0.44	0.36	0.49	0.41	0.37	0.35	0.35	0.36	0														
Kandhari (1)	0.45	0.48	0.54	0.53	0.43	0.48	0.45	0.48	0.44	0.43	0.41	0.43	0.44	0.40	0													
Khog (3)	0.32	0.41	0.48	0.48	0.25	0.39	0.30	0.43	0.34	0.29	0.23	0.27	0.29	0.31	0.40	0												
Malta (3)	0.33	0.40	0.48	0.48	0.27	0.40	0.32	0.44	0.33	0.28	0.24	0.28	0.27	0.31	0.40	**0.18**	0											
Mridula (4)	0.41	0.44	0.42	0.40	0.49	0.53	0.47	0.47	0.45	0.42	0.45	0.45	0.44	0.51	0.50	0.44	0.42	0										
Muscat (4)	0.37	0.45	0.49	0.38	0.38	0.43	0.35	0.45	0.41	0.36	0.33	0.35	0.38	0.40	0.47	0.35	0.34	0.37	0									
Ornamental (3)	0.32	0.41	0.49	0.49	0.25	0.39	0.33	0.46	0.35	0.27	0.22	0.28	0.31	0.36	0.43	0.21	0.24	0.45	0.32	0								
P-16 (4)	0.34	0.40	0.48	0.47	0.34	0.43	0.34	0.44	0.38	0.31	0.29	0.31	0.33	0.37	0.41	0.28	0.29	0.45	0.31	0.25	0							
P-23 (4)	0.34	0.42	0.49	0.49	0.31	0.40	0.35	0.46	0.36	0.31	0.28	0.31	0.33	0.36	0.42	0.26	0.26	0.46	0.34	0.24	0.28	0						
P-26 (3)	0.34	0.41	0.49	0.48	0.29	0.39	0.32	0.45	0.35	0.29	0.27	0.29	0.30	0.36	0.43	0.24	0.26	0.46	0.34	0.24	0.30	0.23	0					
Ramnagaram (3)	0.34	0.44	0.50	0.48	0.29	0.41	0.33	0.46	0.38	0.31	0.27	0.29	0.31	0.35	0.43	0.25	0.27	0.45	0.33	0.23	0.31	0.24	0.21	0				
Sindhuri (3)	0.45	0.39	0.36	0.37	0.52	0.53	0.50	0.43	0.45	0.48	0.49	0.48	0.48	**0.55**	0.51	0.49	0.50	0.38	0.47	0.49	0.48	0.50	0.49	0.49	0			
Surkh Anar (1)	0.35	0.41	0.50	0.49	0.27	0.42	0.36	0.46	0.39	0.30	0.24	0.31	0.35	0.36	0.43	0.24	0.27	0.47	0.37	0.21	0.33	0.28	0.27	0.26	0.49	0		
Uthkal (3)	0.36	0.44	0.52	0.50	0.28	0.41	0.37	0.47	0.40	0.30	0.25	0.32	0.32	0.37	0.42	0.26	0.28	0.48	0.36	0.25	0.32	0.29	0.27	0.27	0.53	0.21	0	
Yercaud (4)	0.40	0.48	0.53	0.52	0.40	0.45	0.40	0.50	0.44	0.37	0.36	0.38	0.39	0.41	0.45	0.36	0.36	0.49	0.4	0.37	0.38	0.35	0.36	0.35	0.54	0.34	0.33	0
L. speciosa (1)	0.82	0.83	0.85	0.84	0.81	0.81	0.80	0.83	0.81	0.81	**0.79**	0.80	0.82	0.80	0.83	0.80	**0.79**	0.84	0.81	**0.79**	**0.79**	0.80	0.80	0.80	**0.86**	0.81	0.80	0.80

to cultivars 'Kabul' and 'Yercaud'. The second sub-cluster was 'Surkh Anar' and 'Uthkal' forming a sister group to 'Darsha Malas' at the deeper node. The third sub-cluster included 'P-23', 'P-26', 'Ramnagaram', 'Khog', 'Malta', 'Ornamental', 'Gulsha Red', 'Gulsha Rose' and 'Jalore Seedless', whereas the fourth sub-cluster included 'Muscat', 'P-16', 'Arakhta', 'Ganesh', 'Jyothi' and 'Dholka'. The third and fourth sub-clusters were at the terminal positions with deepest nodes in the dendrogram revealing that the cultivars in these sub-clusters are genetically closer.

Comparison of RAPD, DAMD and ISSR markers used in cultivated pomegranates

In order to determine the utility of each of the marker system used in the genetic diversity study of pomegranates a comparative statistical analysis was carried out. The diversity index (DI), the effective multiplex ratio (EMR) and the marker index (MI) of all the three markers were computed according to the Powell *et al*. (1996). The DI values were 0.19, 0.27 and 0.21 for RAPD, DAMD and ISSR, respectively. The MI value, which reveals the predictive power of a marker system in diversity studies was found to be maximum in DAMD (7.13) followed by RAPD (2.52) and then ISSR (1.46). These values revealed that DAMD is more powerful marker than RAPD and ISSR in the assessment of diversity in pomegranates (**Table 9**). This finding corroborated the percentage of polymorphisms (P) and mean polymorphic information content (PIC) values recorded in RAPD (P = 92.35%, PIC = 0.17), DAMD (P = 98.52%, PIC = 0.26) and ISSR (P = 76.50%, PIC = 0.16) analysis (**Table 9**). However, the level of discrimination of the out-group from the pomegranate accessions was comparatively low in the UPGMA dendrogram of DAMD (**Fig. 4**) than in RAPD (**Fig. 3**) and ISSR (**Fig. 5**).

Another parameter for comparison of different profiling methods is to correlate the respective distance matrices by Mantel correlation test (Mantel 1967). It is obvious that the data (such as number of bands, similarity coefficient and the topology of dendrogram) generated by different markers may vary. Such data variability and their reliability to each other can be tested by Mantel correlation test. Therefore, Mantel's pairwise matrix correlations (r) were carried out between the genetic distances obtained from individual marker systems as well as in combination. Thus, the pairwise correlation of genetic distances was performed amongst (i) RAPD and DAMD, (ii) RAPD and ISSR, (iii) DAMD and ISSR, (iv) cumulative (RAPD, DAMD and ISSR) and RAPD, (v) cumulative and DAMD, and (vi) cumulative and ISSR (**Table 10**). The matrix correlation between RAPD and ISSR was r = 0.90, whereas r = 0.64 and r = 0.58 were found between RAPD and DAMD, and DAMD and ISSR, respectively. These values revealed that RAPD and ISSR data have good correlation and are best fit to each other. But the DAMD data has shown a weak correlation to both RAPD and ISSR data. On the other hand, the r value in case of cumulative versus RAPD, cumulative versus ISSR and cumulative versus DAMD were 0.98, 0.96 and 0.70, respectively. It is interesting that the cumulative data has shown better correlation with individual marker in comparison to the correlation amongst the markers.

DISCUSSION

Assessment of genetic diversity among cultivated pomegranates

Besides its consumption as raw fruit, pomegranate is known for its multifarious uses like home gardening to the treatment of cancer and AIDS (Lansky and Newman 2007).

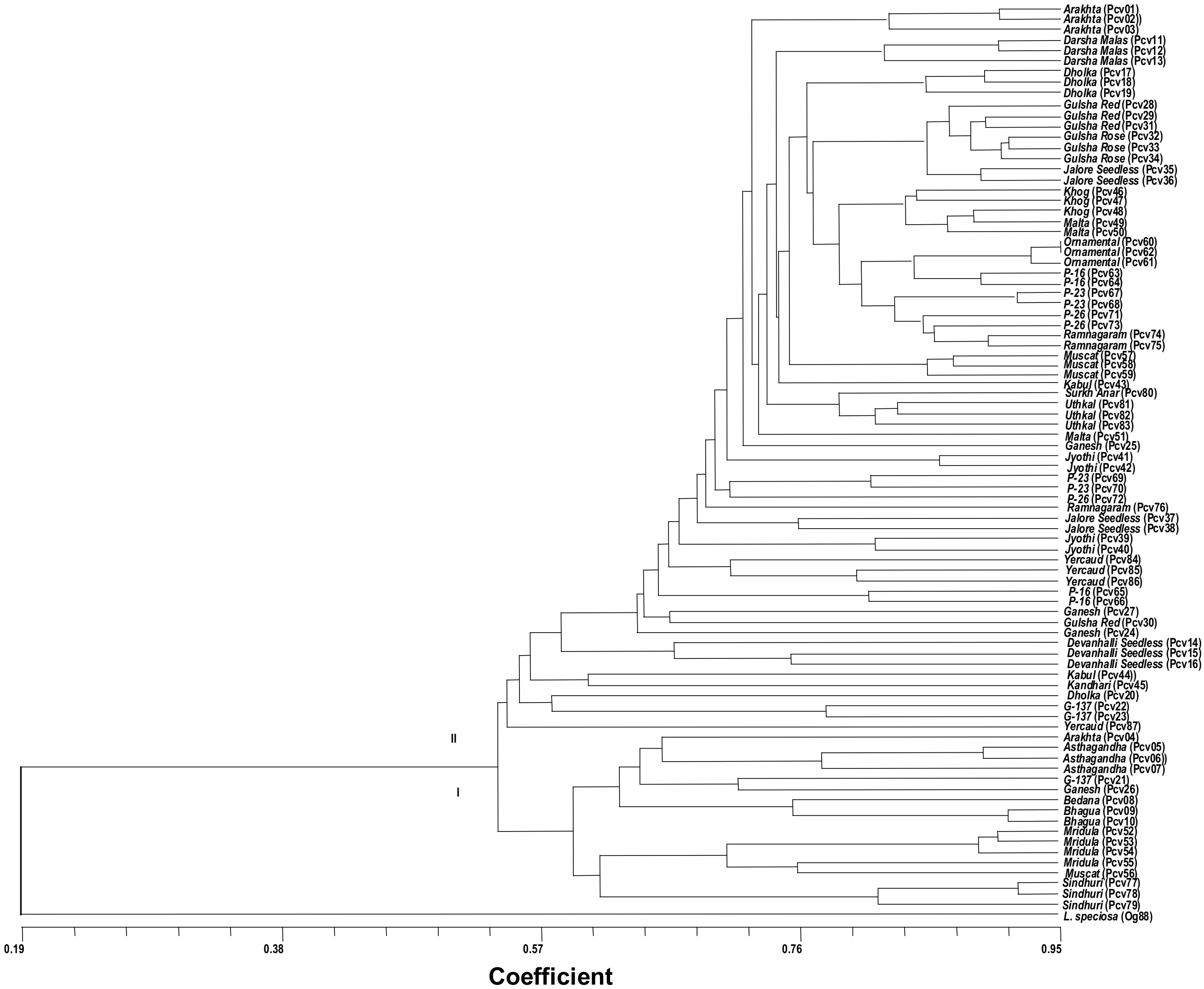

Fig. 6 UPGMA dendrogram based on Cumulative data showing the relationship of the accessions representing different cultivars of pomegranates. Accessions are as given in **Table 1**. *Lagerstroemia speciosa* was used as an out group in this analysis.

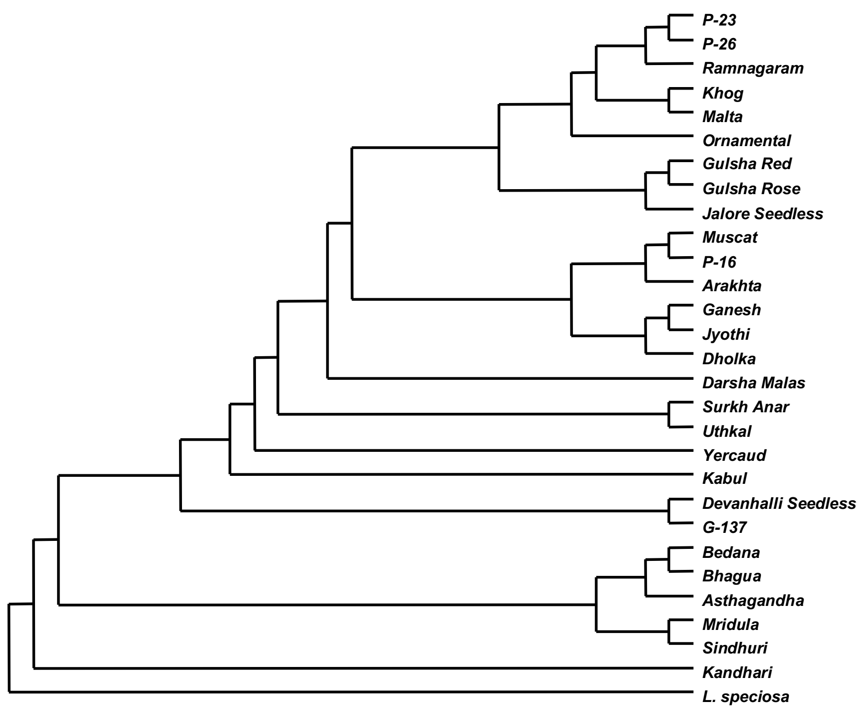

Fig. 7 Cultivar relationships of Indian pomegranates based on Cumulative data analysis. *Lagerstroemia speciosa* was used as an out group in this analysis.

Table 9 Comparison of RAPD, DAMD, ISSR and Cumulative data analyses in the cultivated set of pomegranate accessions.

Molecular Marker	RAPD	DAMD	ISSR	Cumulative[a]
Number of genotypes	87	87	87	87
No. of primers used	21	05	17	43
Total no. of bands (n)	327	136	200	663
Polymorphic bands (n_p)	302	134	153	589
Percentage polymorphism	92.35	98.52	76.50	88.83
Band size range (in bp)	150 - 2500	100 - 3000	150 - 2700	100 - 3000
Average PIC	0.17	0.26	0.16	0.20
Genetic distance range among the genotypes	0 - 0.70	0.10 - 0.92	0.01 - 0.43	0.05 - 0.61
Diversity index (DI)	0.19	0.27	0.21	0.22
Effective multiplex ratio (E)	13.28	26.40	6.89	15.52
Marker index (MI)	2.52	7.13	1.46	3.41

[a] Combined data of RAPD, DAMD and ISSR.

Table 10 Mantel correlations between the genetic distances obtained from RAPD, DAMD, ISSR and Cumulative data analyses among the cultivated accessions of pomegranate.

Marker pairs	RAPD vs. DAMD	RAPD vs. ISSR	DAMD vs. ISSR	Cumulative[a] vs. RAPD	Cumulative vs. DAMD	Cumulative vs. ISSR
Correlation coefficient (r)	0.64	0.90	0.58	0.98	0.70	0.96
p-value	0.0020	0.0020	0.0020	0.0020	0.0020	0.0020

[a] Combined data of RAPD, DAMD and ISSR.

Another important advantage of pomegranate is its hardy nature and capability to grow in marginal soils. Though, India is one of the largest pomegranate producers in the world, the quality of Indian pomegranate is not as good as the exported varieties from Iran, Afghanistan and Spain. Therefore, germplasm evaluation and systematic improvement program is necessary in India. Another scope for improvement of pomegranate in India is the occurrence of wild pomegranates in the Western Himalayan region. Wild relatives are donor for some qualitative traits like disease resistance, drought resistance and adaptability to marginal soils. Wide ranges of phenotypic variations were reported in the naturally growing pomegranates due to their cross pollination and propagation through seeds. While the cultivated pomegranates are often propagated through the seeds, and mainly through the vegetative means (hard-wood cuttings/ air-layering) to maintain their qualitative and quantitative attributes. Seed propagation and long-term human selection has given rise to certain extent of diversity among the cultivated pomegranates (**Fig. 8**). Earlier classification and evaluation of Indian pomegranates were done primarily on the basis of growth form, fruit size and colour; aril colour, size and taste; seed softness and other biochemical characteristics (Malhotra *et al*. 1983a, 1983b; Godara *et al*. 1989; Sharma and Sharma 1990; Jagtap *et al*. 1992a, 1992b; Jalikop and Kumar 1998). In some cases, they were able to identify different cultivars based on morphological or biochemical characteristics, but these environmentally influenced traits are not sufficient to unambiguously assess the genetic diversity between them.

In the present study, single primer amplification reaction methods *viz*. RAPD, DAMD and ISSR were used to assess the genetic variability among the Indian pomegranate cultivars due to their simplicity, efficiency, relative ease to perform the assay and non-requirement of DNA sequence information. These markers are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism. Furthermore, these markers are efficient in allowing multiple loci to be analyzed for each individual in a single gel run (Kosman and Leonard 2005).

The average polymorphism and genetic distance range observed among the accessions of Indian cultivated pomegranates were 92.35% and 0 to 0.70 in RAPD; 98.52% and 0.10 to 0.92 in DAMD; 76.5% and 0.01 to 0.43 in ISSR; and 88.83% and 0.05 to 0.61 in cumulative data analysis, respectively. Conversely, the polymorphism (57.30%) observed in Iranian pomegranates is of lesser extent (Sarkhosh *et al*. 2006), in comparison to Indian cultivated pomegranates, whereas, six locally grown pomegranates in Turkey showed 85.22% polymorphism across different genotypes (Ercisli *et al*. 2007). Thus RAPD analysis reveals the presence of higher genetic variability among the accessions of Indian pomegranate cultivars. The genetic diversity study in Chinese pomegranate cultivars was carried out by Yuan *et al*. (2007) using AFLP markers revealed 73.26% average polymorphism, whereas present study in cultivated Indian pomegranates revealed was significantly higher polymorphism (88.83%) than the Chinese pomegranates. Similarly, in another study of Tunisian pomegranate germplasm, Jbir *et al*. (2008) detected 94.70% polymorphism amongst different genotypes using AFLP markers. This data, however, showed high polymorphism in Tunisian germplasm, but are significantly correlated with the DAMD data obtained in the present study on Indian cultivated pomegranates.

UPGMA dendrogram was constructed to understand the clustering pattern and relationships of different pomegranate cultivars, and revealed that ornamental pomegranate cultivars did not separated out from the other cultivars in the dendrogram, and rather showed closer affinities with 'P-13' cultivar, except in DAMD analysis. The cultivars also clustered independently irrespective of their geographical origin and denomination suggesting that a common genetic base of the cultivars despite their phenotypic divergences. In addition, the accessions of cultivars like 'Arakhta', 'Dholka', 'Ganesh', 'Jalore Seedless' and 'Yercaud' did not clustered together, instead distributed randomly into different sub-clusters in the dendrograms (**Fig. 6**). Nevertheless, some cultivars like 'Asthagandha', 'Darsha Malas', 'Devanhalli Seedless', 'Gulsha Rose', 'Gulsha Red', 'Khog', 'Mridula', 'Sindhuri' and 'Uthkal', having the same denomination clustered together. Interestingly the newly developed Indian cultivars *viz*. 'Asthagandha', 'Bhagua', 'Mridula' and 'Sindhuri' were clustered more or less including a few accessions of 'Ganesh', 'G-137' and 'Muscat' cultivar in the dendrograms generated with different SPAR methods or in combination of the data. Cultivar 'G-137' is a seedling selection of 'Ganesh' (Sawant 1973) and the cultivars 'Asthagandha', 'Bhagua' and 'Sindhuri' are the sibling cultivars (unpublished source from MPKV, Rahuri). Whereas, according to Sheikh (2006), the cultivar 'Bhagua' is under cultivation by different names *viz*. 'Asthagandha', 'Sindhuri', and 'Keskar'. Such random clustering of similar cultivars into different sub-clusters and the clustering of ornamental cultivar together with other cultivars in the dendrograms were also reported by Jbir *et al*. (2008) in Tunisian pomegranates. In another study of Iranian pomegranates, the clusters were not in agreement with the morphological traits in most cases, sometimes not even with the meaning of the accession's names (Sarkhosh *et al*. 2006).

The types of pomegranates under cultivation at the early stages in India seem to be of seedling origin from the

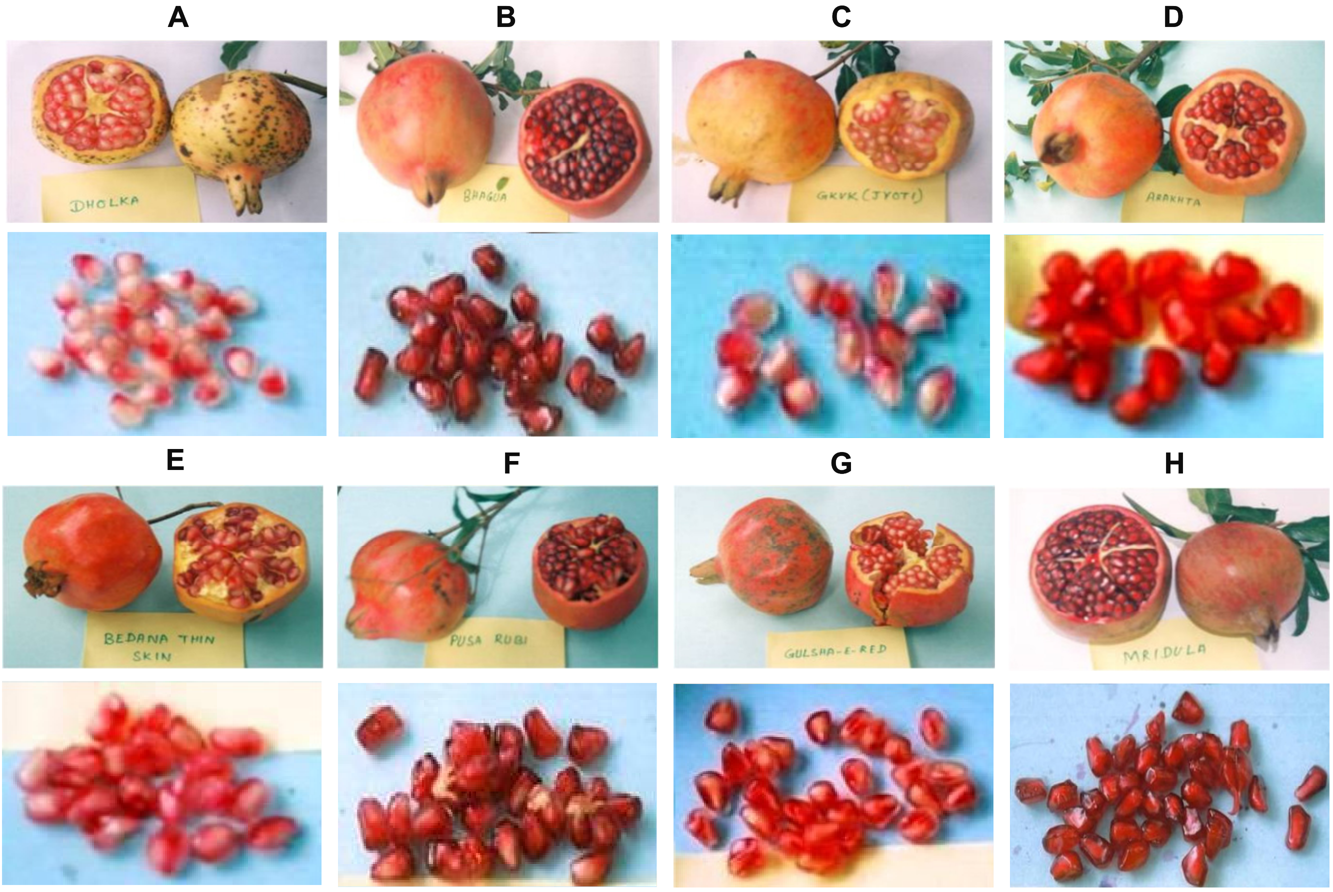

Fig. 8 Morphological diversity in fruits (top panel) and arils (bottom panel) of 8 pomegranate cultivars. (**A**) 'Dholka', (**B**) 'Bhagua', (**C**) 'GKVK Jyothi', (**D**) 'Arakhta', (**E**) 'Bedana', (**F**) 'Pusa Ruby', (**G**) 'Gulsha Red', (**H**) 'Mridula'.

varieties grown in the neighbouring countries along north-west border. Pomegranates being an ancient and widespread fruits, cultivar names often have considerable synonymy, in which the same basic genotype is known by different names in different regions. Most of them were known by the names of the new places where they were cultivated, and cannot be considered as distinct varieties as they were being largely propagated by seed resulting in seedling variations, so that the developed grove consists of a mixture of varied types going under one name (Phadnis 1974). Synonymy is further encouraged by the fact that husk and aril colour can vary markedly, when grown in different regions. A number of characteristics vary between pomegranate genotypes. 'Alandi', 'Dholka', 'Ganesh', 'Muscat', 'Jalore (Seedless)' and 'Yercaud' are the cultivars that were reported during the early stages of pomegranate improvement in India (Cheema *et al*. 1954; Sayed *et al*. 1985). Later on, these cultivars were progressively used in the breeding programs of Indian pomegranate. Random distribution and clustering of these cultivars with different cultivars in the dendrograms reveals either the contribution of these cultivars in the development of newer cultivars or their similarity in genetic make up at least partially. Another reason of clustering of these old Indian cultivars might be that it has been cultivated in different regions with different geographical conditions in India. Because, of the long selection pressure and the adaptive stress to a new locality give certain additive effects in the accumulation of genetic differences among the germplasm growing in different localities. The evolution of varieties/cultivars in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh *et al*. 1998). It is, therefore, not surprising to find significant levels of polymorphism among the cultivars of pomegranates in the present study.

Based on such studies, measures of genetic diversity could be used to identify new germplasm sources that, when crossed with existing varieties, would result in both, qualitatively as well as quantitatively enhanced yields. The extent of heterosis expressed in hybrid seedlings depends upon the origin, relationship and compatibility between the cultivars involved in different crosses. The hybrids from crosses between distantly related cultivars of more genetic diversity presumably exhibit more hybrid vigour than those from related parents or parents of same and similar origin (Karale and Desai 2000). Therefore, the present information on the genetic distances between the cultivars (**Table 8**) and their relationships (**Fig. 7**) will be of paramount significant to the pomegranate breeders as well as germplasm conservationists. The local grown pomegranates can be used as the primary gene pool and the wild forms (populations) that would have valuable attributes and features, such as high drought resistance and frost resistance, soil unpretentiousness, resistance to pests and diseases, etc. can be used as secondary gene pool in breeding programmes.

Although pomegranate is grown on a fairly large scale in India, it has not attained to positions, as it deserves in commercial cultivation. Pomegranate crop is comparatively drought resistant and has wide adaptability and it is being increasingly valued both as desert fruit and in processing of various fruit products. In fact the demand for the fruit in the country exceeds the production and extension of the area planted to this crop has not kept pace with the increase in demand. Therefore, development and propagation of the superior cultivars with high yielding potentialities for commercial cultivation in Indian condition is required.

Comparison of RAPD, DAMD and ISSR markers

In the present study, DAMD was found the most efficient marker detecting 98.52% polymorphism among the pomegranate accessions as compared to 92.35 and 76.50% polymorphism with RAPD and ISSR analysis, respectively. The order of polymorphism detected by these markers is comparable to our earlier studies on wild pomegranates (Narzary *et al*. 2009, 2010). Similar observations of higher polymorphism with RAPD markers than ISSR markers were also reported in *Jatropha curcas* (Gupta *et al*. 2008). This is in contrast to the results as obtained for several other plant species like wheat (Nagaoka and Ogihara 1997) and *Vigna* (Ajibade *et al*. 2000).

The ability to resolve genetic variation among different genotypes may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed (Gupta *et al*. 2008). The differences in clustering pattern of genotypes using RAPD, DAMD and ISSR markers may be attributed to the amplified regions and the level of polymorphism detected, reinforcing the importance of the number of loci and their coverage of the overall genome in obtaining reliable estimates of genetic relationships among cultivars (Loarce *et al*. 1996).

DAMD and ISSR markers are carried out at high annealing temperatures and therefore pose less reproducibility problem. Problems of the reliability and repeatability of RAPD markers are well known. Nagaoka and Ogihara (1997) in their studies found that ISSR primers, compared with RAPD primers produce more reliable and reproducible bands. However, once the PCR conditions were optimized and well set up, a high reproducibility for RAPD was also obtained in our study.

Nagaraju *et al*. (2001) compared three PCR-based techniques, RAPD, SSR and ISSR-PCR, as well as RFLP methods for their ability to generate useful polymorphisms in silkworm varieties. In this study, the highest level of polymorphism was detected using RFLP probes (97.77%), followed by RAPD (94.4%), SSR (86%) and ISSR (76.8%). The correlations of marker data revealed the best correspondence between RFLP and SSR (0.796), followed by SSR and ISSR-PCR (0.685) and RAPD and ISSR-PCR (0.682). Similar pattern was observed in our data where RAPD recorded 92.35% in contrast to 76.50% by ISSR marker. Better correlation of ISSR data with RAPD also supports our findings of high correlation between RAPD and ISSR data instead of DAMD data (**Table 10**). These findings are also in congruity with our earlier studies on wild pomegranates (Narzary *et al*. 2009, 2010). It implies that the RAPD and ISSR markers exhibit congruency in discrimination among the same set of genotypes, and therefore the resolution power of these two markers are comparable to each other. However, higher marker index (MI) and diversity index (DI) in ISSR-PCR than RAPD as recorded by Nagaraju *et al*. (2001) is contrary to our findings in pomegranates.

In the present study, the genetic distances generated by all three markers (RAPD, DAMD and ISSR) were compared and correlated to each other. Genetic diversity value obtained from DAMD analysis showed weak correlation to the genetic diversity obtained from RAPD and ISSR analysis, while the correlation between RAPD and ISSR data showed good correlation with moderate ‘r’ value. However, genetic distances obtained from all these three markers showed highly significant correlation to the genetic distances obtained from combined data analysis (**Table 10**).

Different markers target different portions of the genome and therefore variations generated by different markers are possible. In fact the inter-simple sequence repeats (ISSRs) are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism and offer great potential to determine intra-genomic and inter-genomic diversity as compared to other arbitrary primers, like RAPDs (Zietkiewicz *et al*. 1994). Whereas DAMD primers are the core minisatellite sequence repeats that amplify the adjacent areas of minisatellite regions at an amplifiable lengths. The decamer primers of RAPD amplify the region wherever it gets the complementary sequences to bind at an amplifiable region throughout the genome. All these three markers might detect non-coding regions, and therefore, more polymorphic DNA by exploiting the different regions of the genome. It is therefore significant that the estimates of genetic diversity with two or more techniques are necessary, because a single type of molecular marker does not provide the best estimates of genome-wide variability in organisms (Avise 1994). For the similar reason, data representations based on cumulative data have been found more appropriate to unravel the genetic diversity and relationship of pomegranate cultivars in the present study.

CONCLUSIONS

The wide range of genetic diversity estimated among pomegranate accessions in the present study corroborated the morphological variations exhibited by the pomegranates. This might be due to the banding patterns of diploids with dominant markers represent individuals’ phenotype rather than genotypes (Kosman and Leonard 2005). However, the molecular and morphological differences are apparently independent, due to diverse pressure and evolutionary factors, because the former is invisible and therefore, unselected by breeders, while the latter is subject to selection. For these reasons, the molecular analysis should be used as a complement instead of replacing the traditional morphological characterization (Lage *et al*. 2003).

Genetic diversity is in fact the result of long-term evolution, and represents the evolutionary potential of a species. Surviving in a harsh environment, a species has to change in some aspects and accumulate more genetic variation in order to adapt itself in such condition (Li *et al*. 1999). The genetic diversity study of pomegranate has found high and exceeded 85% polymorphism among the accessions. This indicated that the pomegranate possesses a high level of genetic variation and adaptability.

We can conclude that the RAPD, DAMD and ISSR markers were extremely useful for studying the genetic diversity and relationships in pomegranates. The genetic diversity and cultivar relationships presented here are significant information that could be further utilized in planning the breeding experiments, management, and evaluation of pomegranate germplasm, occurring in wild and cultivation in India.

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