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Assessing the genetic diversity screening of pomegranate cultivars using random amplified polymorphic DNA markers

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Abstract
Pomegranate is an important fruit crops in India and other parts of the world. The high genetic diversity among its cultivars is a potential tool for crop improvement. The genetic diversity of 24 cultivars /varieties of pomegranate was assessed using 120 RAPD markers. A total of 1017 amplicons were produced out of which 504 were polymorphic levels with an average number of polymorphic amplicons levels per primer being 31.5. Among the selected 16 polymorphic markers, 12 markers scored maximum number of polymorphic amplicons levels (46-65 amplicons) and could effectively detect the polymorphism between a set of seven pomegranate varieties. The neighbour-joining clustering generated using 16 random DNA markers clearly separated varieties /cultivars into six major clusters. This study revealed that RAPD fingerprinting can be used to develop robust and reliable SCAR types and RAPD markers can effectively be used to assess on-farm diversity.

Keywords: genetic diversity, pomegranate amplified polymorphic DNA markers and RADP marker

Introduction
Pomegranate (*Punicagranatum* L.) belongs to family Punicaceae (2n = 16) is a monoecious, grown in tropical and subtropical regions of the world including India, Iran, Mediterranean, Spain, Egypt, Afghanistan, Arabia, Baluchistan, Burma, China, Japan, USA, USSR, Bulgaria and Southern Italy (Pirseyedi *et al.,* 2010) [9]. In India, total area under cultivation of pomegranate is crop 107000 ha with aproduction of around 7, 43,000 tonnes. In Karnataka, pomegranate is cultivated in 12,042 ha of area with an annual production of 1, 30,000 tonnes. (Annual Report. 2012, National Horticulture Board Ministry of Agriculture, Government of India), Pomegranate production has attracted several farmers in India for its wider adaptability, relatively low cost of cultivation, drought tolerance, good yields, and export potential. The pomegranate fruit is symbolic for its cool, refreshing juice and valued for its medicinal properties (Sturgeon and Ronnenberg, 2010) [12].
A wide genetic base of crop varieties / cultivars helps to overcome sudden outbreak of pests and diseases. High genetic diversity among the cultivated varieties of crop plants is expected to overcome effects of climate change. Differences in DNA sequence among germplasm accessions could be detected by different molecular methods. Morphometric and phytochemical criteria have recently been attended to determine the degree of polymorphism among pomegranate collections (Wunsch and Hormaza, 2002) [13].

Materials and Method
The present study was undertaken for assessing the genetic diversity and molecular fingerprinting of pomegranate varieties / cultivars like Amlidana, BasseinSeedless, Bhagwa, Daru, Daye, Ganesh, Ganesh Selection, Geps, Gulesha Pink, Gulesha Red, JalloreSeedless, Jodhpur Red, Jodhpur Collection, Jyothi, Kandahari, KY, Mana, Mridula, Musket, Nana, P-23, Ruby, Yaracuy and Arakta cultivated in different part of India.. The existing diversity among the selected genotypes was explored using RAPD markers.

Plant sample collection
A total of 24 pomegranate varieties / cultivars were used in present study collected from Indian Institute of Horticultural Research (IIHR), Bangalore, Karnataka and National Research Centre on Pomegranate (NRCP), Solapur, Maharashtra. The leaf samples of these cultivars were collected and washed three times in sterile distilled water. Further, the leaf samples were frozen in liquid nitrogen and kept at -80 °C until used.

Isolation of genomic DNA pomegranate leaf samples
The genomic DNA was isolated from leaf samples collected from 24 varieties / cultivars of pomegranate following method given by Krishna and Jawali (1997) [6] with minor modifications and DNA samples were further purified and quantified by Nano Drop spectrophotometer pedestal.

Results

A set of 24 varieties / cultivars of pomegranate being cultivated in different part of India were used in the present study. Screening for genetic diversity and DNA fingerprinting of these cultivars was done to analyse their molecular diversity using RAPD markers.

RAPD marker analyses

The survey for polymorphism was performed with a set of 120 random decamer DNA markers screening across 24 DNA samples of pomegranate varieties / cultivars. Among the 120 random decamer DNA markers,16 random markers produced maximum number of amplicons that were consistent, clear and also detected polymorphism when repeated for three times. Essentially, these 16 random DNA markers were selected for further analysis of genetic diversity and fingerprinting of 24 pomegranate varieties / cultivars. Details on sequence of 16 random decamer DNA primers and sequence of primer pairs of RAPD in presented in Table 1.

Table 1: Random decamer RAPD markers successfully amplified and the number of total and polymorphic amplicons amplified in pomegranate varieties / cultivars used in the present study

Marker name	Primer name	Sequence (5'-3')	Total amplicons	Number of polymorphic amplicons	Polymorphic amplicons (%)
RAPD					
	OPB-14	AAGTGCGACC	69	49	68.11
	OPK-3	GAGAACGCTG	86	46	53.48
	AC-9	AGAGCGTACC	78	48	61.53
	AC-3	CACTGGCCCA	96	47	48.95
	I-10	ACAACGCGAG	67	17	25.37
	I-14	GTGACAGGCT	57	25	43.85
	L-12	GGGCGGTACT	31	20	64.51
	M-11	GTTGGTGGCT	21	3	09.52
	N-7	CAGCCCAGAG	113	65	57.52
	N-15	CAGCGACTGT	67	29	43.28
	N-18	GGTGAGGTCA	25	13	52.00
	L-13	ACCGCCTGCT	48	21	43.75
	L-14	GTGACAGGCT	57	49	85.96
	AT-7	ACTGCGACCA	81	6	07.40
	AT-12	CTGCCTAGCC	52	8	13.46
	OPAC-4	ACGGGACCTG	72	62	86.11

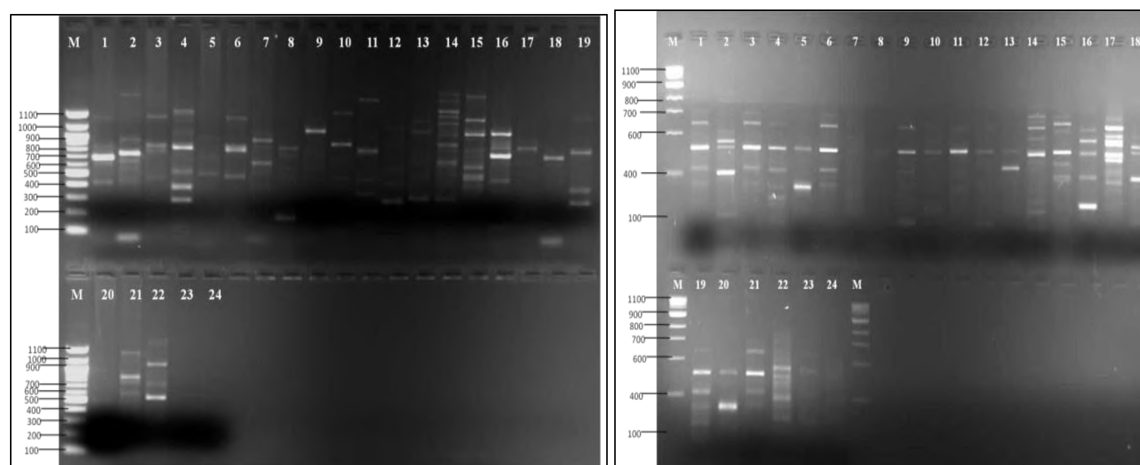

Fig 1: PCR-RAPD amplicons separated on 1.8% agarose gel with ethidium bromdestaining. 1a) PCR-RAPD amplification profile resulting from OPB-14 primer; 1b) PCR-RAPD amplification profile resulting from OPK-3primer

Among the key factors influencing the PCR efficiency and accuracy a few important factors were optimised and then used in actual generation of data. Different concentrations of $MgCl_2$ titrated (1 mM, 2 mM and 5mM) 2 mm was found to be optimum: low concentration (1mM) reduced number of PCR products while high concentrations increased the frequency of non-specific amplicons. About 16 random decamer markers recorded the consistent amplification pattern. Amplification profiles of OPB-14, OPK-3, AC-9 and AC-3 random markers are presented in Figure 1 and 2. Among the selected 16 DNA random markers, OPB-14 recorded a total of 69 amplicon levels with over 47 of them being polymorphic, accounting to 68.11 per cent polymorphism. Another random DNA marker, OPK-3, produced 86 amplicon levels with 46 polymorphic ones, which accounted to 53.48 per cent

polymorphism. The AC-9 recorded 78 amplicon levels among which 48 were found to be discriminating the pomegranate cultivars / varieties used in the study. The summary statistics of PCR-RAPD analysis of pomegranate varieties / cultivarsrevealeda size range of amplicons to be between 50 and 1400 bp. All the amplicons were essentially consistent and produced major/brighter PCR products that totalled to 1017 levels. Of the 1017 total amplicon levels resulted from 16 random markers, 504 were polymorphic across the pomegranate varieties / cultivars studied. On an average 31.5% number of polymorphic amplicons per primer was noticed in selected pomegranate varieties / cultivars.The maximum number of amplicon levels observed was 113 and minimum level of amplicons observed was 21 (Table 2).

Table 2: Summary statistics of PCR-RAPD analysis of pomegranate varieties/ cultivars

Sl. No.	Particulars	Numbers
1	Total number of amplicon levels	1017
2	Total number of polymorphic amplicons levels	504
3	Total number of random DNA marker used	16
4	Maximum number of amplicon levels observed	113
5	Minimum number of amplicon levels observed	21
6	Average number of amplicon levels per random DNA marker	63.56
7	Average number of polymorphic amplicon per random DNA marker	31.5

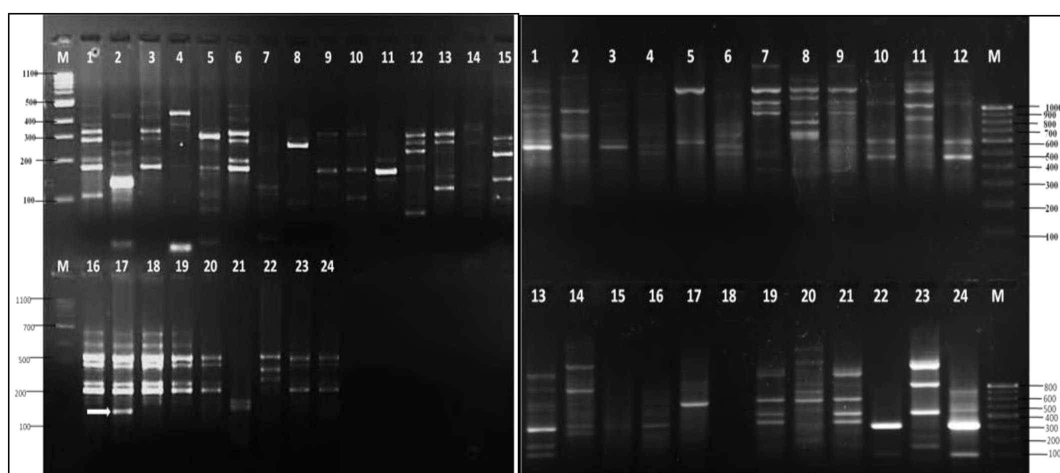

Fig 2: PCR-RAPD amplicons separated on 1.8% agarose gel with ethidium bromde staining. 2a) PCR-RAPD amplification profile resulting fromAC-9 primer, 2b) PCR-RAPD amplification profile resulting from AC-3 prime

A total of 18 nuclear RAPD markers were tested for their amplification in pomegranate. A set of four RAPD markers *viz*., FBPG-MP07, FBPg10, FBPg14 and FBPg46 revealed successful amplification in all the pomegranate varieties / cultivars but all they were monomorphic on 2.5 % agarose gels stained with ethidium bromide. Further, when tested PCR amplified products of all the four markers on 6% polyacrylamide gel electrophoresis with silver staining, again they were found to be monomorphic across all the varieties / cultivars of the pomegranate tested. In case of 4 successfully amplified nuclear RAPD markers, the amplification of expected allele was observed in all the markers and across all the varieties / cultivars tested, but, in all varieties / cultivars the allele size was monomorphic (Table 1). The RAPD decamer AC-3 had highest PIC value 0.96 and marker N-7 had 0.13 (Table 3).

Table 3: Polymorphic information contended (PIC) of RAPD markers used in the present study

Marker name	Primer name	Sequence (5'-3')	Number of polymorphic Amplicons	PIC value
RAPD				
	OPB-14	AAGTGCGACC	49	0.69
	OPK-3	GAGAACGCTG	46	0.86
	AC-9	AGAGCGTACC	48	0.78
	AC-3	CACTGGCCCA	47	0.96
	I-10	ACAACGCGAG	17	0.67
	I-14	GTGACAGGCT	25	0.57
	L-12	GGGCGGTACT	20	0.31
	M-11	GTTGGTGGCT	2	0.21
	N-7	CAGCCCAGAG	65	0.13
	N-15	CAGCGACTGT	29	0.67

	N-18	GGTGAGGTCA	13	0.25
	L-13	ACCGCCTGCT	21	0.48
	L-14	GTGACAGGCT	49	0.57
	AT-7	ACTGCGACCA	6	0.81
	AT-12	CTGCCTAGCC	7	0.52
	OPAC-4	ACGGGACCTG	62	0.72

Discussion

The genetic diversity among many pomegranate varieties has been studied through characterizing fruit traits such as rind and seed colour, taste and shape besides a few qualitative parameters. There are attempts made in the past where pomegranate germplasm was characterized using morphological traits according to the descriptor available in respective country. On the other hand, there are very few reports available indicating use of molecular methods for genetic studies in pomegranate (Mars, 2001) [7] DNA markers are useful in both basic and applied research to assess even the functional variants of relevant genes governing target trait. Diversity among organisms is a result of variations in DNA sequences and of environmental effects (Zhang, 2012) [14].

Screening a set of 120 random DNA markers using randomly selected DNA samples from 24 different pomegranate varieties / cultivars was done to identify the markers for further detailed analysis of genetic diversity and to identify the DNA fingerprints. Among the 120 markers, 16 random markers produced consistent amplicon pattern across all the 24 pomegranate varieties / cultivars. Sheidai *et al.* (2008) [11] studied RAPD and cytogenetic study of ten pomegranate varieties / cultivars. Random DNA markers owing to their arbitrary nature of primer and relatively short length (10 mer) of primer their amplification patterns in the same genotype vary which is undesirable for genetic diversity analysis (Ercisli *et al*., 2011; Hasnaoui *et al*., 2010) [3, 5] However, identification of RAPD marker which gives consistent amplification patterns can effectively overcomes this limitation (Narzary *et al*., 2009; Durgaç *et al*., 2008) [8, 2]. In the present study, specific amplicons from RAPD markers were observed in some of the pomegranate cultivars, which may be used for differentiating cultivars. Different similarity coefficients determined among the cultivars studied, showed the highest value of similarity between cultivars Bihasteh and Berit (r = 0.86) while the lowest value of similarity occurred between the cultivars Alakshirin and Bihasteh (r = 0.36). Different clustering methods showed distinctness of the pomegranate cultivars studied. The grouping of the cultivars did not correlate with the cytogenetic clustering (Sheidai *et al.,* 2008) [11]. Using sixteen random DNA markers present / absent information of PCR amplicons which are unambiguous and repeatedly reproducible revealed the genetic relationship among the 24 pomegranate varieties / cultivars. The NTSYS-pc UPGMA tree cluster analysis led to the distribution of 24 pomegranate cultivars into two major clusters labelled 1 and 2 respectively. Use of random DNA markers, as PCR-RAPD method is an effective technique to reveal genetic diversity in any crops including pomegranate accessions. Many a time higher polymorphism at phenotypic level has been recorded but at genotypic level using RAPD marker a low level of polymorphism has been recorded (Sarkhosh *et al*., 2006) [10]. The morphological differences observed among the cultivars might be resulted from the ecological or growing conditions. It is well-known fact that the environment has a great effect of expression of quantitative traits. However, several characteristics of these cultivars (peel and aril colour, juice characteristics) are stable across environments (Durgac *et al.,* 2008). Thus, it is more possible option that the amplicon patterns of random DNA markers are successful to study genetic diversity. Further, RAPD markers can very well be used for establishing marker-trait associations in crop where rare robust markers such as SSRs / SNPs are scanty. There are several successful previous efforts where RAPDs have been used to link the specific amplicon to specific monogenic traits (Ercisli *et al*., 2011; Hasnaoui *et al*., 2010) [3, 5] and to span through quantitative trait loci regions (Narzary *et al*., 2009; Durgaç *et al*., 2008) [8, 2]. Random nature and not needing any sequence information of crop under consideration is major strength of using random DNA markers. Genetic similarity coefficient matrix recorded similarities of various degrees between 24 varieties / cultivars of pomegranate. The genetic similarity ranged from 81 per cent (between KY and P-23) to 94 per cent (between Gulesha Pink and Gulesha Red). The low genetic similarity coefficient indicated the more genetic distance between KY and P-23, and similarly, lesser genetic distance between Gulesha Pink and Gulesha Red. Genetic relationships and diversity pattern observed in the present study is in agreement with reports of Glaszmann (1987) [4] where similar DNA marker type was used. RAPD markers cover the entire genome, revealing length polymorphisms in coding or non-coding and repeated or single-copy sequences. A total of 36 fingerprints were obtained on screening the 24 selected pomegranate varieties / cultivars with 16 RAPD random markers. A total of sixteen RAPD random markers amplified 504 polymorphic amplicons in 24 genotypes. The size of specific amplicon ranged from 50 to 1400 bases in their size. The marker N-15 amplified five different amplicons of varies in size (200 to 700 bases) in four different genotypes. The random decamer OPK-3 amplified 4 specific amplicons each, in four different varieties / genotypes. From this it can be said that the marker N-15 had a better capacity to distinguish twenty-four varieties / cultivars. Thus, the study provided a detailed first analysis and quantification of genetic diversity in selected varieties / cultivars of India. The data also reaffirms the power of RAPD marker to distinctly group closely related Landraces. RAPD markers provide a quick screen for DNA polymorphism. A very small amount of DNA is required for actual analyses. In addition, information on template DNA sequence is not necessary. However, with respect to RAPD markers problems of reproducibility are reported. In order to assure reproducibility, optimization of PCR reaction and also its repetition is essential. In general, among the set of accessions

investigated, the efficiency of a molecular marker technique depends on the amount of polymorphism it can detect. In our study, RAPD fingerprinting was more efficient assay. Results of present study clearly demonstrated the ability of random DNA markers to amplify differential amplicons that could fingerprint the commercial varieties / cultivars of pomegranate. A similar effect in related crops such as mango by Srivastava *et al.* (2007) have been reported where a total of 158 amplicons ranging between 250 to 2,500bp, of which 134 (84%) have been polymorphic. The DNA fingerprints / polymorphic amplicons for varieties *viz*., Mana, Daru, Bhagwa, Amlidan, Kandahari, Bassein Seedless and Arakta were identified with arbitrary primed marker, N-18, I-12, L-13, L-14, N-15 and N-7 respectively. In case of KY variety polymorphic DNA fragment was identified with OPB-14, N-18, L-13 and AT-12. Similarly, for Ruby variety DNA fingerprint was identified with OPAC-14, N-15 random DNA markers. On other hand, OPAC-4, OPK-3 and I-12 could develop distinct polymorphic amplicon for Jodhpur variety. In case of P-23 variety, the DNA fingerprint was identified using N-7 and N-18 arbitrary markers. The OPK-3, AC-9 and N-7 detected DNA fingerprints for pomegranate genotype 'yercaud'. Random DNA markers *viz*., OPK-3, N-15 and L-13 detected polymorphic amplicons for the Ganesh variety. Whereas, in case of Jyothi variety; AC-3 and AT-7, and Gulesha Red variety I-14 decamer marker reported the polymorphic loci. In the present study, the RAPD revealed high variation at the molecular level, indicating the suitability of the RAPD for genetic clustering.

Conclusion

Assessment and monitoring of genetic diversity in different pomegranate varieties sampled from most of the pomegranate growing areas of India in the present study can be used for further assessing of on-farm diversity using more robust markers such as SNPs. The RAPD fingerprints identified in the present study can be converted into more robust and reliable SCAR types and used for assessing the high genetic diversity of pomegranate varieties / cultivars cultivated in India and elsewhere due to long historical cultivation and preference by the farmers as a potential tool for improvement of pomegranate.

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