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RAPD MARKERS BASED CHARACTERIZATION OF *PUNICA GRANATUM* L. GERMPLASM

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ABSTRACT

Eleven germplasm lines of pomegranate (Punica granatum L.) representing each flavanoid group made from forty one lines were characterized by random amplified polymorphic DNA (RAPD) markers. Out of 32 random decamer primers, 15 were found more polymorphic (91.5%). The polymorphic information content among these primers was 0.27 to 0.48. Jaccard's similarity co-efficient among these pomegranate lines was found to be ranged from 0.28 to 1.0. Phylogentic tree constructed based on RAPD profile revealed that there are seven minor clusters formed within two major groups indicates high diversity exists among the germplasm. This information on germplasm lines is very useful in breeding programme for improvement of this crop.

Keywords: *RAPD marker; Punica granatum; characterization*

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the ancient fruit crop and is favourite table fruits of tropical and subtropical region of the world, valued for its medicinal properties. In the recent years pomegranates cultivation has became an economically viable preposition. Pomegranate is widely cultivated throughout the drier parts of India due to its hardly nature, high yield, good keeping quality and demand in market. A large area is under pomegranate cultivation in Western Rajasthan, where there is a cool winter and a hot dry summer, a climate highly suitable for pomegranate cultivation. The major problem faced by the grower is the selection of suitable variety for micro-climatic zones. With the identification of superior varieties like Ganesh, Mridula, Arka Ruby and Bhagawa, the area under pomegranate is increasing fast. More than 500 cultivars of pomegranate have been named but evidently have considerable synonymy in which the same genotype is named differently across regions of the world. Varietal identification of these cultivars is based mostly on fruit characters. These at times become highly overlapping and hence pose problems.

Morphological and anatomical characteristics of plant have been extensively exploited in plant systematic. However, at intra-specific level, particularly horticultural crops, these parameters become limiting since varieties/ cultivars are distinguished from each other on the basis of one or two floral or fruit characters. These at time are so overlapping that it becomes difficult to identify the cultivars. Pomegranate genotypes have been identified/ developed for specific purpose. In this species too, the morphological and anatomical parameters are insufficient to clearly distinguish the cultivars. Moreover, majority of cultivars have come up either due to natural selection or through deliberate breeding. The genetic variability once generated has been fixed an account of vegetative propagation, which is commonly practiced in this taxon. Hence, the phylogenetic kinship between the cultivars is not certain, which is an utmost requirement in a breeding programme.

For breeding and commercialization of promising pomegranate cultivars a precise determination and discrimination of the genotype is required. Zamani (1990) characterized some pomegranate by using morphological traits. However, due to the effects of environmental factors on this attributes, their use could be ambiguous. Therefore, markers independent from the environment are necessary for reliable identification and discrimination of genotypes and cultivars.

The value of markers depend on their heritability and on the level of polymorphism they can reveal (Porter and smith; 1982) DNA markers are independent from environmental interactions (Zhao and Pan, 2004) unlimited in number and show high level of polymorphism, therefore they are considered invaluable tool for determining genetic relationship/diversity. Among the various types of DNA markers, RAPD gained importance due to their simplicity, efficiency, the relative ease to perform the assay and non-requirement of DNA sequence information (Khanuja *et al.*, 1998). Study of the genetic diversity, as well as cultivar identification based on various molecular markers including RAPD (Random Amplified Polymorphic DNA), has been performed for several plant species including pomegranate (Ercisli *et al.*, 2007). Although pomegranate cultivars have been studied by various workers (Mars and Marrakchi, 1999; Talebi *et al*., 2003; Sarkhosh *et al*., 2006; Zamani *et al*., 2007), there are no reports available on the assessment of genetic diversity in germplasm lines of pomegranates in India. Therefore, here we report on assessment of genetic variability among the germplasm lines of pomegranate by molecular markers.

MATERIAL AND METHODS

Plant samples

Pest and disease free, fresh fully grown second/third leaves of forty-one pomegranate, were collected from different region of Rajasthan. All the samples were kept at – 20^{o} C until used. These were subjected first to foliar flavonoid analysis (data not shown here) and 11 genotypes (Table 2) representing each group were selected for RAPD analysis.

Genomic DNA extraction

Total genomic DNA was extracted from 100 mg young emerging leaves from each accession separately using QIAGEN DNA isolation kit as per manufacturer's instructions (Genetix Biotech Asia Pvt. Ltd., India). The extracted DNA was stored at -20^{o}C. DNA quality and concentration were measured by spectrophotometrically.

RAPD amplification

Initially, the PCR protocol was optimized with varying concentration of (i) template DNA (50, 100, 250 and 500 ng) (ii) *Taq* DNA polymerase (0.25, 0.5, 1.0 and 1.5U; MBI fermentas) and (iii) $MgCl_2$ (1.0, 2.0, 3.0 and 4.0 mM). 100 ng of template DNA with 1.0U of *Taq* DNA polymerase and 3.0 mM $MgCl_2$ gave reproducible and good quality bands. These concentrations were constantly used for all the reactions. First thirty-two random decamer primers belong to 1 to 16 of OPBE, 1 to 10 of OPA and 1, 3 to 7 of OPN series (Operon Technologies, Almeda, CA, USA) tested by PCR with randomly selected five germplasm lines. Finally 15 most polymorphic primers were selected and used in all the subsequent studies. The PCR reaction was performed with 25 µl of reaction mixture containing 100 ng of template DNA, 3 mM $MgCl_2$, 20pmoles primers, 1U *Taq* DNA polymerase, 0.2 mM dNTP (MBI Fermentas) on thermal cycler (Thermo scientific) with the thermal profile of initial denaturation at 94^oC for 5 min, followed by 33 cycles at 94^oC for 30 sec, 36^oC for 1 min and 72^oC for 2 min 30 sec with a final extension at 72^oC for 10 min. The amplified products was electrophoresed on 1.4% agarose gel in 1X TAE buffer pre-stained with ethidium bromide (0.5µg/ml) and photographed.

Data analysis

Only polymorphic and reproducible bands were considered for analysis. The presence of band was scored as '1' and absence as '0'.Molecular weight of the bands was estimated using 1 Kb DNA ladder (MBI Fermentas). A pair wise matrix of genetic distance between genotypes was determined using the Jaccard's similarity coefficient and phylogenetic tree was constructed using NTSYS pc-2.02e Version 2.0.1.5 software (Applied Biostatistics Inc.). The polymorphism information content (PIC) was calculated by the formula: PIC = 2 Pi (1-Pi) where, Pi is the frequency of occurrence of polymorphic bands in different primers (Bhat, 2002).

RESULTS

Out of 32 random decamer primers tested on randomly selected germplasm lines of pomegranate, 15 were found most polymorphic; OPBE01, OPBE04, OPBE06, OPBE08, OPBE12, OPBE14, OPA01, OPA03, OPA05, OPA07, OPN01, OPN04 and OPN06 (Table 1). Although all the primers yielded DNA fragments, however, the number and size of the amplicons were different. The 15 primers used in this study generated a total of 189 bands resulting in 173 polymorphic amplicons suggesting polymorphic nature of primers. Polymorphic bands were obtained in the size ranged from 200 to 3500 bp. The minimum number of bands (9) was produced by OPBE-01 and OPBE-03 and maximum number of bands (19) was produced by OPBE-12. Percent polymorphism given by these primers was ranged from 75 to 100 among the pomegranate cultivars. Primers such as OPBE-01, OPBE-02, OPBE-12, OPBE-14 and OPA-05 were given higher polymorphism compared to others. The polymorphic information content was calculated for all the 15 primers which ranged from 0.23 (OPA-03) to 0.48 (OPBE-01) with an average of 0.35 per primer (Table 1). Genetic similarity among the pomegranate cultivars was found diverse. Jaccard's similarity co-efficient among the pomegranate cultivars were ranged from 0.25 to 0.83 (Table 2). Maximum genetic similarity co-efficient was 0.83 between cultivar P-10 (Mridula) & P-7 (Khog) and between P-10 (Mridula) & P-14 (Jallore Red) followed by cultivar P-5 with P-7 and P-10, with the similarity co-efficient of 0.82. The minimum similarity co-efficient (0.25) was between cultivar P-11 (Yercaud Local) and P-22 (Coimbaiture White). Phylogenetic analysis revealed that there are two major clusters exist among the pomegranate cultivars; cluster I comprises of six cultivar P-5, P-7, P-10, P-11, P-14 and P-16 and rest of the cultivars belong to cluster II (Figure1).

DISCUSSION

It is necessary to have an authentic account of true genetic variability among various germplasm accessions, before they are put into breeding cycle. Accordingly, the present investigation was undertaken on eleven cultivars of pomegranate to assess the efficacy of RAPD markers to discriminate the phylogenetic relationship among the randomly selected genotypes of pomegranate.

Among the forty one genotypes of pomegranate cultivars varying with different morphological and flavanoid spectrum, eleven diverse genotypes were used for molecular analysis to understand their extent of their genetic diversity. Only 47 percent of the primers used in this study could reveal polymorphism among the pomegranate cultivars. Various studies reported that the range of polymorphic primers among the randomly selected primers was between 14 to 87 percent (Noormohammadi, 2008; Sarkosh *et al*., 2006; Zamani *et al*., 2007; Jambhale *et al*., 2007; Sheidai *et al*., 2008; Ercisli *et al*., 2011). Out of fifteen primers employed, nine were found new RAPD primers *viz*., OPBE-01, OPBE-04, OPBE-06, OPBE-08, OPBE-12 and OPBE-14, and OPN-01, OPN-04 and OPN-06 were also identified to give higher (80 - 100 %) polymorphism in the pomegranate cultivars. These new primers will be highly useful to identify polymorphism among pomegranate genotypes in future. Primers such as OPBE-01, OPBE-08, OPBE-12 and OPN-04 are having higher PIC value, which can be used for diversity analysis. Lower genetic similarity co-efficient between cultivars indicates these pomegranate cultivars are highly diverse. For example the genetic similarity co-efficient between cultivars Coimbatore white and Yercaud local was 0.25, these two cultivars are highly distinct. In India, genetic diversity of pomegranate cultivars was done only on four cultivars *viz.,* Ganesh, G-137, Mridulla and Phule Bhagwa in Maharashtra State (Jambhale *et al.,* 2007). Here eleven genotypes representing each flavanoid groups covering 41 genotypes in India were used. Perusal of literature reveals that although various diversity studies on pomegranate cultivars/ genotypes have been undertaken in Iran and China (Sarkosh *et al*., 2006; Yuan *et al*., 2007; Zamani *et al*., 2009), this is the first time that such large number of genotypes of pomegranate have been used in genetic diversity studies.

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Table 1 Details of primers used and their polymorphism

S. No.	Primer	5'-3' Sequence	Total No. of Bands	No. of polymorphic bands	Band range (bp-Kb)	Per cent Polymorphism	PIC
1	OPBE-1	CACTCCTGGT	9	9	200- 3.5	100.0	0.48
2	OPBE-2	ACGCCTGTAG	10	10	200- 2.0	100.0	0.35
3	OPBE-3	TGGACTCGGT	9	8	250- 1.5	88.9	0.32
4	OPBE-4	CCCAAGCGAA	12	11	200-1-9	91.7	0.36
5	OPBE-6	CAGCGGGTCA	15	12	300- 1.7	80.0	0.32
6	OPBE-8	GGGAAGCGTC	12	11	250- 2.0	91.7	0.40
7	OPBE-12	GGTTGTTCCC	19	19	230- 2.5	100.0	0.42
8	OPBE-14	CTTTGCGCAC	12	12	300- 1.2	100.0	0.38
9	OPA-01	CAGGCCCTTC	14	12	300-2.5	85.7	0.32
10	OPA-03	AGTCAGCCAC	13	12	250- 1.5	92.3	0.23
11	OPA-05	AGGGGTCTTG	17	17	250- 1.8	100.0	0.30
12	OPA-07	GAAACGGGTG	12	9	250- 2.1	75.0	0.30
13	OPN-01	CTCACGTTGG	11	10	350- 2.0	90.9	0.34
14	OPN-04	GACCGACCCA	14	13	300- 2.0	92.9	0.40
15	OPN-06	GAGACGCACA	10	8	250- 1.1	80.0	0.27

PIC: Polymorphism information content

Table 2 Jaccard's similarity co-efficient among eleven pomegranate cultivars

	P-14	P-5	P-31	P-37	P-11	P-10	P-28	P-7	P-22	P-36	P-16
P-14	1.00										
P-5	0.81	1.00									
P-31	0.38	0.37	1.00								
P-37	0.39	0.39	0.78	1.00							
P-11	0.76	0.79	0.33	0.34	1.00						
P-10	0.83	0.82	0.36	0.37	0.79	1.00					
P-28	0.36	0.37	0.7	0.61	0.3	0.36	1.00				
P-7	0.76	0.82	0.32	0.34	0.76	0.83	0.33	1.00			
P-22	0.29	0.29	0.58	0.57	0.25	0.29	0.58	0.28	1.00		
P-36	0.39	0.39	0.57	0.67	0.33	0.36	0.58	0.33	0.6	1.00	
P-16	0.78	0.77	0.38	0.39	0.68	0.75	0.34	0.73	0.28	0.38	1.00

P-14 (Jallore Red); P-5 (Bedana Suri); P-31 (Kabul Kohinoor); P-37 (P-21); P-11 (Yercaud Local); P-10 (Mridula); P-28 (Uthkal); P-7 (Khog); P-22 (Coimbaiture White); P-36 (Dholka); P-16 (P-23)

FIGURE LEGEND

Figure 1 Phylogenetic tree constructed based on eleven accessions of *P. granatum* L. germplasm analyzed by RAPD primers by UPGMA method using NTSYSpc-2.02e version 2.0.1.5 software. Vertical distance is arbitrary and horizontal distance indicates genetic distance clusters /groups among eleven genotypes are indicated by Roman letters