Original Research Article

Molecular Studies Ascertaining the Phylogenetic Relationships in Pomegranate (*Punica granatum* L.) Cultivars Using RAPD Markers

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A B S T R A C T

Random Amplified Polymorphic DNA (RAPD) markers were used to evaluate genetic similarity and phylogenetic relationship among ten pomegranate cultivars. 14 decamer primers of OPF series were selected, produced a total of 114 bands; some species or cultivar specific bands were also obtained using RAPD markers. The number of amplified DNA bands varied between 3 (primer OPF-12) and 11 (primer OPF 3 and OPF 6). The size of the amplified band ranged between 300 and 6000 base pairs. These bands could serve as a useful aid for the varietal identification as well as are helpful for ascertaining the phylogenetic relationship among the cultivars studied. Genetic similarity was assessed using the Jaccard similarity coefficient. UPGMA cluster analysis revealed the dendrogram clearly separating cultivars Bhagwa and Phule Arakta from rest of the cultivars at a similarity coefficient of 0.85. The highest genetic similarity value coefficient was observed between genotypes Jalore seedless and Ganesh was 0.96. Cultivars G-137 and Phule Arakta were found to be genetically most diverse.

Introduction

Pomegranate (*Punica granatum* L.) family *Punicaceae* commonly known as Anar is one of the ancient edible and favorite table fruit of the world. It is believed thatthe origin is in Central Asia especially the Transcaucasia-Caspian region in Iran. It is cultivated extensively in tropical, subtropical and arid regions. The scientific name of Pomegranate is derived fromwords; *Pomum* (apple) and *granatus* (grainy) or seeded apple.

Pomegranate is native to Persia and possibly also to some surrounding areas, from where it spread to the rest of the world (6, 22, 11, 24)

According to (1), there are three mega-centers (primary, secondary and tertiary) and five macro-centers (Middle Eastern, Mediterranean, Eastern Asian, American and South African) of origin and genetic diversity of pomegranate. It was cultivated in ancient Egypt and early in Greece, Italy and Iraq. Later, it spread to Asian countries like Turkmenistan, Afghanistan, Iran, India, China, North Africa and Mediterranean Europe (14) and to some extent in the drier parts of South East Asia, Malaya, the East Indies and tropical Africa, USA, China, Japan and Russia (10,13).

Pomegranate family has a single genus *Punica* with two species *viz., P. granatum L.* and *P. protopunica* Balf. However, *P.nana* L. is an ornamental dwarf pomegranate and has been considered as a distinct species by some authors (14). It prefers a semi-arid and mild temperate to sub-tropical climate and is naturally adapted to hot summers and cool winters. A humid climate adversely affects the formation of fruit. It is rounded shrub or small deciduous tree with a narrow lance shaped leaves which persist on tree. The attractive scarlet, white, or variegated flowers are over an inch across and have 5 to 8 crumpled petals and a red, fleshy, tubular calyx. The tough, leathery skin or rind is typically yellow overlaid with light or deep pink or rich red. The interior is separated by membranous walls and white, spongy, bitter tissue into compartments packed with sacs filled with sweet, acidic, juicy, red/pink or whitish pulp or aril. It is a drought hardy plant. Due to availability of proper soil moisture and mild temperature, flowering during August–September month is suitable under arid region, hence in the fragile agro-ecosystem, this fruit occupies a major share of horticultural produce.

Due to its hardy nature, high yield potential, good keeping quality and demand in market. The arils are dried and used as condiment and acidulate in many Indian curries, chutneys (2) and in various industries like tanning, dying and colouring. Amlidana, a hybrid variety of pomegranate is highly acidic (16-18%) and marketed as condiment for culinary preparation (8).

The morphological and anatomical parameters are insufficient to clearly distinguish the cultivars. However, at infra-specific level, particularly in horticultural crops, these parameters became limiting since varieties /cultivars are distinguished from each other's on the basis of one or two floral or fruit characters. These at times are so overlapping that it becomes difficult to identify 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 on account of vegetative propagation. Hence, the phylogenetic kinship between the cultivars is not certain.

Among the various phytochemical markers employed to resolve riddles, DNA based markers have gained reputation as excellent markers. RAPD markers that result from the PCR amplification of genomic DNA fragments using short oligonucleotide (usually 10-mers) of arbitrary sequence as primers (25) provide a fast and easy approach for taxonomic classification and cultivar-typing of fruit trees. RAPD markers have been employed to understand and unravel the diversity and systematics of pomegranate by different workers from time to time (Talebi Bedaf *et al.,* 2003; Sarkhosh *et al.,* 2006; Ercisli *et al.,* 2007; Jambhale *et al.,* 2007; Yang *et al.,* 2007; Zamani *et al.,* 2007; Sheidai *et al.,* 2008; Sarkhosh *et al.,* 2009; Hasnaoui *et al.,* 2010; Noormohammadi *et al.,* 2010, 2012; Ercisli *et al.,* 2011; Pitsiouni *et al.,* 2012; Zhang *et al.,* 2012; Ercisli *et al.,* 2014; Mansour *et al., 2*015). Random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been used to study genetic diversity within and among various species. Their constitution in plants is specific and is not affected by environmental factors. RAPD technique requires no knowledge of the target DNA and is simple, fast and sensitive. The technique relies upon the use of a single ten-oligonucleotide primer on random sequence. A good reproducibility of the marker is easily obtained when reaction conditions are rigorously maintained. Low expense, efficiency in developing a large number of DNA markers in a short time and requirement

for less sophisticated equipment have made the RAPD technique valuable.

Materials and Methods

Plant material

Ten pomegranate genotypes (Table 1) used in this study, were collected pomegranate germplasm repository field, Central Institute for Arid Horticultural, Bikaner, Rajasthan, India. 14 decamer primers of OPF (Table 2) series were selected for evaluation of genetic relationship between the cultivars.

Experimental site and location

The experiment was conducted at Plant Physiology laboratory of ICAR Central Institute for Arid Horticulture (C.I.A.H.) and College of Agriculture, Beechwal, Bikaner, Rajasthan. The young leaves from 10 pomegranate varieties were collected from pomegranate germplasm repository of ICAR Central Institute for Arid Horticulture, Bikaner. ICAR-Central Institute for Arid Horticulture (C.I.A.H.) is situated 10 Km away from Bikaner on NH 15 at an altitude of 234.7 m above sea level and latitude 28º01' N and longitude 73º22' E. According to "Agro-ecological region map" brought out by National Bureau of Soil Survey and Land Use Planning (NBBS&LUP), Bikaner falls under Agro ecological region No. 2 (M 9 E 1) under arid ecosystem (Hot Arid Eco-region with desert and saline soils), which is characterized by deep, sandy and course loamy, desert soils with low water holding capacity, hot and arid climate, precipitation less than 300mm. PET (Potential Evapo-transpiration) in this region ranges between 1500-2000 mm as per NARP. Bikaner falls in agro climate zone (I C), which had been carved out of original (I A). The newly created zone (I C) is known as hyper arid partially irrigated north western Plain Zone (I C). According to National Planning commission, Bikaner falls under agro climate Zone 12 (western dry region) of India.

Climate and weather conditions

Bikaner has arid climate with an annual average rainfall of about 200-300mm. More than (80%) rainfall is received with the southwest monsoon during summers, the maximum temperature may go as high as 48 ºC while in the winters it may fall as low as -2ºC. This region is prone to high wind velocity and soil erosion.

Chemicals, glasswares and polywares

All the chemicals and biochemicals used in analysis were of fine molecular and analytical grade. These were obtained from standard manufacturer *viz.,* Himedia, Qiagen, Sd fine-chem Ltd, Sigma-Aldrich, Merck, *etc.* For all the experiments, double distilled water was used. The glasswares and disposable plastic wares were of standard make obtained from Borosil, Axygene and Eppendorf. All reagents, solutions, Eppendroff tubes, microcentrifuge tubes, PCR tubes and tips were sterilized at 15 psi, 121ºC for 20 minutes in autoclave before use.

DNA isolation

The genomic DNA was isolated from pomegranate leaf sample using DNeasy Plant Mini Kit (QIAGEN). Leaves sample material of pomegranate (100 mg fresh weight) were ground to a fine powder using mortar and pestle. Fifty milligrams of the powder was transferred to a pre-chilled micro-centrifuge tube. About 400 µl of lysis buffer AP1 and 4 µl of RNaseA (stock solution 100 mg/ml) were added to micro-centrifuge tube. The suspension was vortexed vigorously until a complete emulsion is formed and then incubated for 10 min at 65°C.The tube is

inverted 2-3 times during the incubation. 130 µl of buffer AP2 was added to the lysate and the mixture was incubated for 5 min on ice. After centrifugation at room temperature for 5 min at 14,000 rpm, the supernatant was transferred into mini columns filtration "QIAshredder spin column" placed in 2 ml collection tubes using a wide bore pipette and centrifuged for 2 min at 14,000 rpm. The flow-through fraction was transferred into clean Eppendorf tube.

To this fraction, 0.5 volume of buffer AP3 and 1 volume of ethanol (96 to 100%), *i.e.,* 1.5 volumes of buffer AP3/E was added and mixed by pipetting.

650 µl of the mixture was transferred to DNeasy mini spin column and centrifuged for 1 min at 8000 rpm. The DNeasy column was placed in a new collection tube and 500 µl of AW buffer (washing buffer) was added onto the column. After centrifugation for 1 min at 8000 rpm, the supernatant was discarded. An additional 500 µl of AW buffer was added to column and then centrifuged for 2 min at 14,000 rpm. An additional washing step with 500 µl ethanol (96 to 100%) was applied to avoid coloration of the final eluted DNA.

The column was placed in a new collection tube and span for 5 min under a vacuum in order to completely dry the column membrane. Finally, the DNeasy column was transferred to 1.5 ml microcentrifuge tube and 100 µl of preheated (65°C) buffer AE (elution buffer) was added directly onto the DNeasy column membrane. After incubation for 5 min at room temperature, the DNA was eluted in buffer AE by centrifugation for 1 min at 8000 rpm.

DNA quality was examined following electrophoresis on agarose gel. Spectrophotometric analysis was performed at 260 and 280 nm absorbance at 260 nm and the A260/A280 ratio provided an estimate of quantity and purity of extracted DNA respectively.

PCR procedure

PCR reactions were performed in a final volume of 25 µl containing 10 X Assay Buffer, 1.0 unit of Taq DNA polymerase, 200 µM each of dNTPs, 10 pmols/reaction of random primers (OPERON TECHNOLOGIES) and 50 ng of template (sample) DNA. The Polymerase Chain Reaction (PCR) was carried out in 25 µl of reaction mix. PCR reactions were followed by 45 cycles comprising 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. An additional cycle of 7 min at 72°C was used for final extension. Following the amplification, the PCR products were loaded on 1.2 per cent Agarose gel (Himedia, molecular grade), which was prepared in 0.5 X TAE buffer containing 0.5 µg/ml of the Ethidium Bromide. The amplified products were electrophoresed for 2.5 – 3.5 hrs at 100 V. After separation, the gel was viewed under UV Gel Documentation System.

Data analysis and scoring

Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivars were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using 'SIMQUAL', a subprogram of the NTSYSPC-2.02 program (numerical taxonomy and multivariate analysis system program) (18). The dendrogram was constructed by using a distance matrix using the unweighed pair group method (UPGMA).

Results and Discussion

RAPD amplification of the DNA isolated from 10 selected pomegranate cultivars yielded amplified bands. RAPD markers have been employed to understand and unravel the diversity and systematics of pomegranate by different workers from time to time. The low level of polymorphism was observed among 28 Iranian pomegranate genotypes, due to clonal or vegetative propagation of the cultivars tested (23). Random amplified polymorphic DNA (RAPD) marker was employed to determine the genetic diversity amongst 24 Iranian pomegranate genotypes. Similarly, 16 of 100 primers showed reliable polymorphic patterns. Cluster analysis using Jaccard's similarity coefficient and UPGMA revealed that the highest and lowest similarities detected between genotypes were 0.89 and 0.29, respectively. At a similarity of 60 per cent, the genotypes were divided into four sub-clusters (19).

Genetic studies using RAPD revealed high divergence in Tunisian collection (7). 15 out of 76 primers produced good and reproducible polymorphic bands, out of 88 fragments bands generated 13 fragments were monomorphic and 75 were polymorphic (3). Molecular studies on 25 pomegranate cultivars using RAPD markers were carried out by Yang *et al.,* (26). Twelve RAPD primers were used resulted in 110 polymorphic bands with 71.8 per cent polymorphism. The range of genetic distance between cultivars varied from 0.027 to 0.342. Zamani *et al.,* (27) studied the genetic relationships in 24 Iranian pomegranate genotypes. Among 113 random decamer primers tested, 27 showed good amplification and polymorphism, and total 158 bands were produced. Estimates of genetic relationship, using Jaccard's similarity coefficient, ranged from 0.30 to 0.88. Sheidai *et al.,* (21) studied the genetic variations in 11 pomegranate genotypes using 15 RAPD markers.

Thirteen primers were found polymorphic producing a total of 173 bands out of which 73 bands were common in all cultivars, while six bands were specific, which could be used in the cultivar discrimination. Primers OPB12 and OPA13 produced the highest number of polymorphic bands (12 bands out of 16 = 0.75% and 11 bands out of 25 = 0.44%), while primers OPR15 and OPA15 produced the least number of polymorphic bands (2 out of 12 = 0.16%). The morphological and RAPD analysis of 18 pomegranate cultivars was carried out using forty RAPD primers by Noormohammadi *et al.,* (16) produced 182 bands, out of which eight were found to be unique (cultivar-specific).

Table.1 List of plant material used in the experiment

| Name of cultivar	Symbols
Jalore Seedless	V1
Ganesh	V2
G-137	V3
Khog	V4
Mridula	V5
Bassein Seedless	V6
Bhagwa	V7
Phule Arkata	V8
GKVK-1	V9
Dholka	V10

Table.2 List of RAPD primers, total number of bands obtained by each primer and T_m value

Sr. No.	RAPDNo.	Sequence 5'- 3'	No of bands	T_m
1	OPF-1	ACGGATCCTG	7	32
2	OPF-3	CCTGATCACC	11	32
3	OPF-4	GGTGATCAGG	8	32
4	OPF-5	CCGAATTCCC	9	32
5	OPF-6	GGGAATTCGG	11	32
6	OPF-7	CCGATATCCC	10	32
7	OPF-8	GGGATATCGG	10	32
8	OPF-9	CCAAGCTTCC	10	32
9	OPF-10	GGAAGCTTGG	9	32
10	OPF-11	TTGGTACCCC	6	32
11	OPF-12	ACGGTACCAG	3	32
12	OPF-13	GGCTGCAGAA	7	32
13	OPF-14	TGCTGCAGGT	4	32
14	OPF-15	CCAGTACTCC	9	32
	Total			114

Table.3 Jaccard's similarity coefficients of ten pomegranate cultivars using UPGMA cluster analysis

DNA	JaloreSeedless	Ganesh	G-137	Khog	Mridula	BasseinSeedless	Bhagava	PhuleArakta	GKVK-1	Dholka
JaloreSeedless	1									
Ganesh	0.9622642	1								
G-137	0.8796296	0.8785047	1							
Khog	0.8928571	0.875	0.846847	1						
Mridula	0.9181818	0.9174312	0.888889	0.901786	1					
BasseinSeedless	0.9449541	0.9090909	0.863636	0.910714	0.9363636	1				
Bhagava	0.8504673	0.8490566	0.854369	0.834862	0.8773585	0.8518519	1			
PhuleArakta	0.853211	0.8518519	0.788991	0.821429	0.8454545	0.8545455	0.8627451	1		
GKVK-1	0.9166667	0.9158879	0.869159	0.866071	0.8909091	0.9	0.8571429	0.8773585	1	
Dholka	0.8888889	0.8703704	0.841122	0.855856	0.8981481	0.8727273	0.8640777	0.8148148	0.8785047	1

A. DNA of ten genotypes of Pomegranate
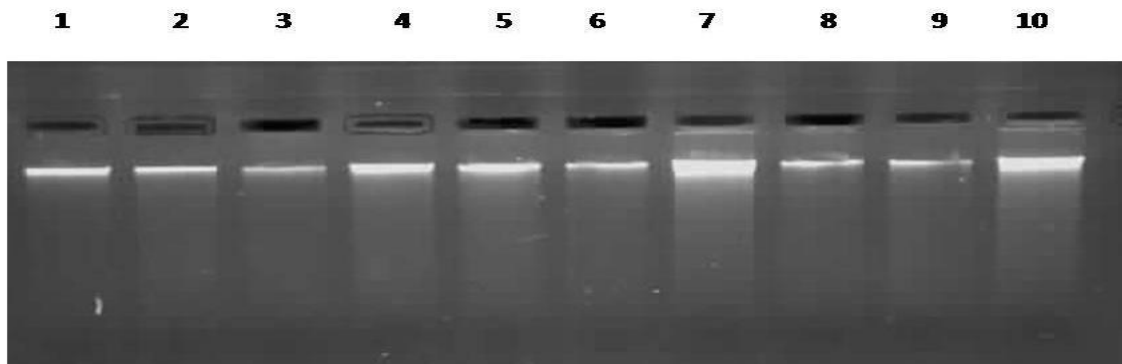
(1=Jalore Seedless,2= Ganesh,3=G-137,4= Khog,5=Mridula,6= Bassein Seedless, 7 = Bhagwa, 8= PhuleArakta, 9= GKK-1, 10= Dholka)

B. RAPD patterns of bands obtained in ten genotypes of pomegranate using primer OPF 4. Lane M = DNA size marker (1KB)
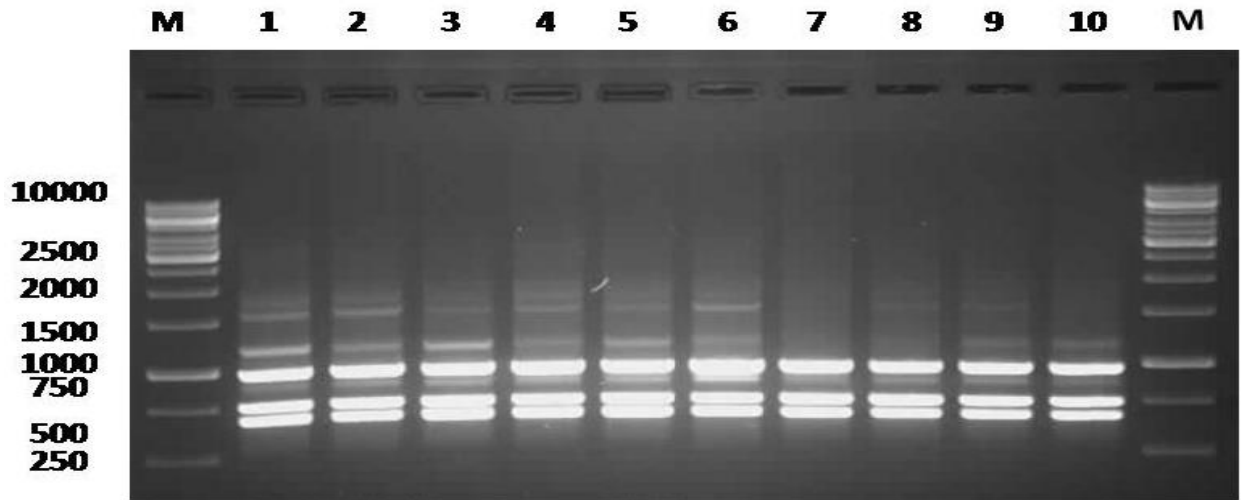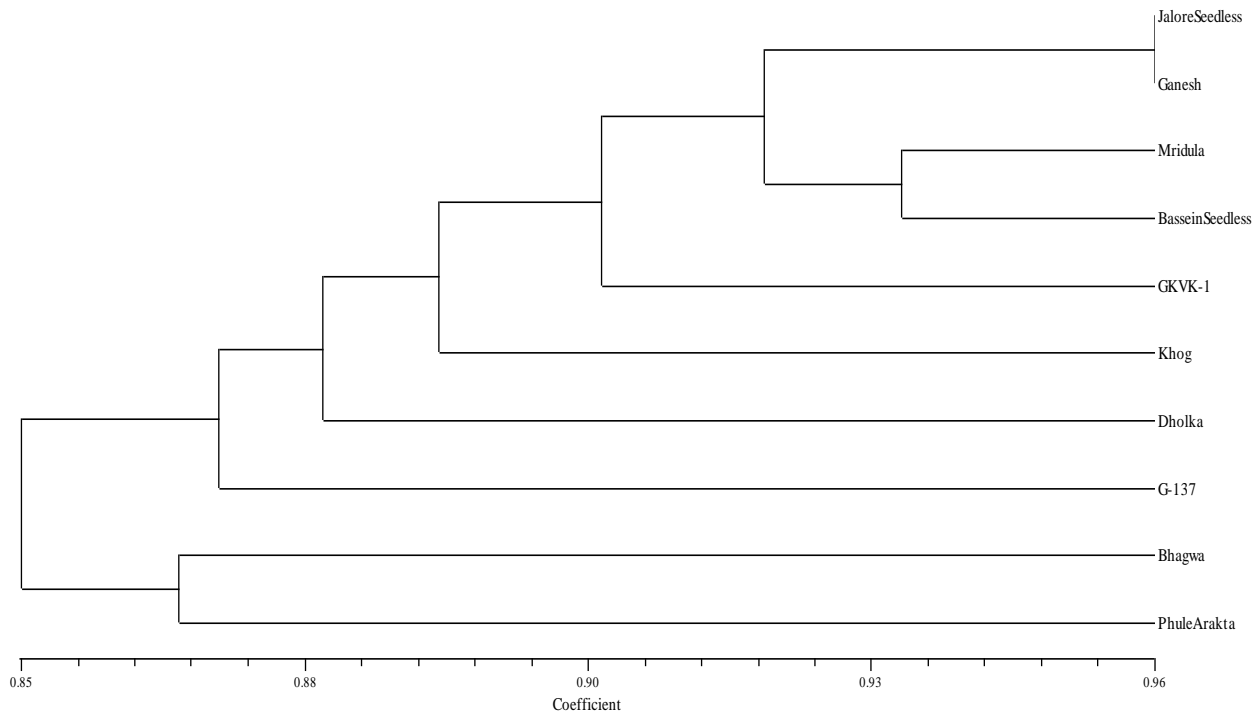

Fig.1 Dendrogram representing cluster for ten pomegranate genotype

Thirteen out of 30 RAPD primers produced reproducible bands across 36 pomegranate cultivars studied. The number of amplified fragments varied from 4 in the RAPD primer OPA 02 to 19 in the RAPD primer OPI 18.

The size of RAPD bands obtained varied from 350 to 2500 bps (15).

Pitsiouni *et al.,* (17) identified different pomegranate genotypes using the RAPD

marker, 35 primers were screened, 10 primers gave polymorphic bands. NTSYSPC-2.02e software, version 2.02 was used to estimate genetic similarities using Jaccard's algorithm and the dendrogram was constructed by UPGMA. Mansour *et al.,* (12) used RAPD markers to investigate the genetic diversity among 21 accessions of pomegranate originating from South Eastern Tunisia. In RAPD analysis, six out of 15 employed random primers showed good amplification with a 63 bands, of which 56 were polymorphic, the lowest (0.29) and highest (0.94) similarities were detected between genotypes.

In India, limited studies have been done on this aspect in pomegranate. Therefore, the present study was conducted opens scope of classifying the pomegranate cultivars on more sound footing as well as helpful in ascertaining the phylogenetic kinship among various pomegranate cultivars.14 decamer primers of OPF series were selected, primers yielded significant information to characterize and assess the genetic relatedness among the species. A total of 114 bands; some species or cultivar specific bands were obtained using RAPD markers with primers OPF 1, OPF 4, OPF 5, OPF 6, OPF 7, OPF 8 and OPF 9 OPF 10 and OPF 15) that can be considered as typical bands for the cultivars identification. The number of amplified DNA bands varied between 3 (primer OPF-12) and 11 (primer OPF 3 and OPF 6). The size of the amplified band ranged between 300 and 6000 basepairs. The genetic relatedness among various pomegranate cultivars was evaluated using Jaccard coefficient (Table 3). UPGMA cluster analysis revealed the dendrogram clearly separating cultivars Bhagwa and Phule Arakta from rest of the cultivars at a similarity coefficient of 0.85. The highest genetic similarity value coefficient was observed between genotypes Jalore seedless and Ganesh was 0.96, followed by cultivars Jalore

Seedless and Bassein Seedless which showed close relationship with similarity index value of 0.94. Cultivars Phule Arakta and Dholka also showed genetic relatedness and closer affinities are recorded with a similarity coefficient of 0.81. Cultivars G-137 and Phule Arakta were found to be genetically most diverse from rest of cultivars since they have lowest similarity index of 0.78, these cultivars showed distant relationship and are found to be less closely related.

The dendrogram obtained by UPGMA clustering method revealed the genetic relationship among ten pomegranate cultivars. From the figure 1, it is evident that the dendrogram is grouped into two major clusters I and II, out of 10 genotypes that got clustered into two groups, the first major group consists of 8 cultivars (*i.e*Jalore Seedless, Ganesh, Mridula, Bassein Seedless, GKVK -1, Khog, Dholka, G-137) and second major group includes 2 cultivar (Bhagwa and Phule Arakta). The first group can further be divided into two sub-groups *viz.*, I A and I B. The group I A consists of 4 cultivars (Jalore Seedless, Ganesh, Mridula and Bassein Seedless) which shows closer affinity among each other. Jalore Seedless and Ganesh showed close relationship. Group I B consists of the remaining 4 cultivars *viz.,* GKVK-1, Khog, Dholka and G-137 shows distant relationship among all the cultivars.

Second cluster consists of two accession Bhagwa and Phule Arakta that show close relationship among themselves, but are distantly related to all other cultivars. From the above results it can be concluded from that the variability at molecular level can be successfully reflected through RAPD analysis. Based on the RAPD profiles generated using 14 primers, it was recorded that bands produced from primers OPF 11, OPF 12, were present in all taxa under study. Hence, these primers have low value for

varietal identification. However, the other primers in combination can help in varietal identification.

The present study revealed that RAPD markers represent an efficient tool for estimating the genetic variability and the genetic relationships among closely related genotypes of pomegranate. This could represent a useful tool in pomegranate improving programs.

References

1. Chandra, R., Babu, D.K., Jadhav, V.T. and Teixeira da Silva, J.A. (2010). Origin, history and domestication of pomegranate. In:Chandra, R.(Ed.), Pomegranate. *Fruit, Vegetable and Cereal Science and Biotechnology*, 2(4):1–6.

2. Chauhan, S.K., Lal, B.B. and Sharma, R. (1994). Development of instant dehydrated wild Pomegranate Chuteny. *Journal of Food Science Technology*, 31:58-59.

3. Ercisli, S., Agar, G., Orhan, E., Yildirim, N. and Hizarci, Y. (2007). Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punica granatum* L.) growing in Southern Anatolia Region in Turkey. *Biochemical Systematics and Ecology*, 35(11): 764-769.

4. Ercisli, S., Gadze, J., Agar, G., Yildirim, N. and Hizarci, Y. (2011). Genetic relationships among wild pomegranate (*Punica granatum L.*) genotypes from Coruh Valley in Turkey. *Genetic Molecular Resource*, 10(1): 459-464.

5. Ercisli, S., Orhan, E., Esitken, A. and Sengul, M. (2014). Molecular and morphological characterization of pomegranate (*Punica granatum* L.) genotypes sampled from Coruh valley in Turkey. *Genetic Molecular Research*, 13 (3): 6375-6382.

6. Harlan, J.R., (1992). Crops and Man, 2nd Edn. *American Society of Agronomy and Crop Science Society of America*, Madison, WI, Pp. 295.

7. Hasnaoui, N., Mars, M., Chibani, J. and Trifi, M. (2010). Molecular polymorphism in Tunisian pomegranate (*Punica granatum* L.) germplasm as revealed by RAPD fingerprints. *Diversity,*2(1): 107–114.

8. Jalikop, S.H., Tiwari, R.B., and Sampath Kumar, P. (2002). Amlidana: A new Pomgranate hybrid. *Indian Horticulture*, 47 (2): 22-23.

9. Jambhale, V. M., Patil, S. C., Pawar, S. V., Jadhav, A. S., Patil, H. E. and Dahake, K. D. (2007). Molecular characterization of pomegranate cultivars with RAPD markers. *Asian Journal of Horticulture*, 2(1):176-179.

10. La Rue, J.H., (1980). Growing pomegranates in California. *UC Fruit & Nut Research Informationcenter*, http://fruitsandnuts.ucdavis.edu/crops/pome granate factsheet.shtml

11. Levin, G.M.(1994). Pomegranate (*Punica granatum* L.) plant genetic resources in Turk-menistan. *Plant Genetic ResourceNewsletter*, 97: 31–36.

12. Mansour, E., Triki, T., Abid, A., Bachar, K. and Ferchichi, A. (2015). Evaluation of genetic diversity among South Tunisian pomegranate (*Punica granatum* L.) accessions using fruit traits and RAPD markers. *Journal of Agricultural Science and Technology*, 17(1): 109-119.

13. Mars, M. (1994). La culture du grenadier (*Punica granatum* L.) et du figuier (*Ficuscarica* L.) en Tunisia. In: First meeting CIHEAMcrop. *Research Network on underutilized Fruit Trees,* Zaragoza, Spain, Pp: 76–83.

14. Melgarejo, M.P. and Martinez, V.R. (1992). El Granado. *Ediciones Mundi-Prensa*, Madrid,S.A.,Pp:163.

15. Noormohammadi, Z., Fasihee, A., Homaee-Rashidpoor, S., Sheidai, M., Baraki, S. G., Mazooji, A. and Tabatabaee-Ardakani, S. Z. (2012). Genetic variation among Iranian pomegranates (*Punica granatum* L.) using RAPD, ISSR and SSR markers, *AustralianJournal of Crop Science*, 6(2): 268.

16. Noormohammadi, Z., Parvini, F., Sheidai, M. and Vazifeshenas, M. R. (2010). Further study of morphological and molecular diversity in 18 pomegranate landraces of Iran. *Gene Conserve*, 9 (38):189-200.

17. Pitsiouni, M., Linos, A., Hagidimitriou, M. and Drogoudi, P. (2012). Genetic diversity of greek wild and cultivated pomegranate (*Punica granatum* L.) genotypes and cultivars using molecular markers*: XXVIII International Horticultural Congress on Science and Horticulture for People*,940:193-200.

18. Rohlf, F.J. (1998) NTSYS-PC Numerical Taxonomy and Multivariate Analysis System Version 2.02 User Guide. *Applied Biostatistics. Inc.*, Setauket,New York.37pp

19. Sarkhosh, A., Zamani, Z., Fatahi, R. and Ebadi, A. (2006). RAPD markers reveal polymorphism some Iranian pomegranate (*Punica granatum L.)* genotypes. *ScientiaHorticulturae*, 111(1): 24-29.

20. Sarkhosh, A., Zamani, Z., Fatahi, R. and Ranjbar, H. (2009). Evaluation of genetic diversity among Iranian soft-seed pomegranate accessions by fruit characteristics and RAPD markers. *ScientiaHorticulturae*, 121: 313–319.

21. Sheidai, M., Noormohammadi, Z., Saneghi, A. and Shahreiyari, Z. H. (2008). RAPD analysis of eleven Iranian pomegranate (*Punica granatum* L.) cultivars.*ActaBiologicaSzegediensis*, 51(1): 61-64.

22. Smith, P.M., (1976). Minor crops. In: Simmonds, N.W. (Ed.), *Evolution of Crop Plants*, Longman, London, Pp. 301–324.

23. Talebi, B.M., Shareifi, B. and Bahar, M. (2003). Analysis of genetic diversity in pomegranates cultivars of Iran using random amplified polymorphic DNA (RAPD) markers. In: Proceedings of *3rd National Congress on Biotechnology,* volume 2, Mashad, Iran,2: 343–345.

24. Verma, N., Mohanty, A. and Lal, A. (2010). Pomegranate genetic resources and germplasm conservation: a review. In: Chandra, R.(Ed.), Pomegranate. *Fruit, Vegetable and Cereal Science and Biotechnology*, 2(4):120–125.

25. Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research,* 18(15): 6531-6535.

26. Yang, R. P., Long, W. H., Zhang, H., Xu, B. and Li, W. X. (2007). RAPD analysis of 25 *Punica granatum* germplasm resources collected in Yunnan province. *Journal of Fruit Science*, 2:226–229.

27. Zamani, Z., Sarkhosh, A., Fatahi, R. and Ebadi, A. (2007). Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. *Journal of Horticultural Science and Biotechnology*, 82(1):11-18.

28. Zhang, Y. P., Tan, H. H., Cao, S. Y., Wang, X. C., Yang, G. and Fang, J. G. (2012). A novel strategy for identification of 47 pomegranate (*Punica granatum* L.) cultivars using RAPD markers. *Genetic Molecular Research*, 11: 3032-3041.