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Morphological Traits and DNA Fingerprinting among Traditional and Commercial Indian Pomegranate (*Punica granatum* L.) Cultivars

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ABSTRACT

In the present study, the genetic relationships among forty six genotypes of pomegranate (*Punica granatum* L.) obtained from diverse locations of India were analysed by morphological and RAPD (Randomly Amplified Polymorphic DNA) markers. The morphological data was obtained for their reproductive characters. PCR-amplifiable DNA was isolated using the CTAB method and 123 amplified fragments were obtained using 8 random primers. The genetic dissimilarity matrix which was calculated based on Euclidian Distances revealed a maximum genetic distance of 22% between genotypes, 'Alah' and 'Gulsha Red' and the minimum genetic distance (2%) was between the genotypes 'Shirin Anar' and 'Kazakaki Anar' and 'Gulsha Red' and 'Kalishirin'. The Ward's method of cluster analysis grouped all the individuals on the dendrogram into two major clusters 'A' and 'B' at 54 linkage distance units with two sub-clusters each. Sub-cluster 'A_1' consisted of 21 genotypes in two groups with very hard seed, low Total Soluble Sugar (TSS) value and few fruits in group I and genotypes with high TSS value and many fruits in group II. Sub-cluster 'A_2' consisted of nine genotypes predominantly with few fruits with very hard seeds. Cluster B with 16 genotypes comprised of morphological characters predominantly showing light aril colour, soft seeds, low acidity, high TSS value and higher fruit yield. The present study showed low genetic diversity among the cultivars of pomegranate. RAPD combined with morphological analysis proved to be a quick, simple and significant testing method to assess genetic diversity among pomegranate.

Keywords: genetic diversity, morphological markers, RAPD analysis, STATISTICA, dissimilarity matrix

INTRODUCTION

Pomegranate (*Punica granatum* L.) is considered an excellent fruit crop for growing in arid and semi-arid areas and for valorising marginal lands and saline waters without special requirements (Tous and Ferguson 1996). It is native to Persia and perhaps some surrounding areas. It was cultivated in ancient Egypt and early in Greece and Italy. In the meantime it was spread into Asia (Turkmenistan, Afghanistan, India, China, etc.), North Africa and Mediterranean Europe. The domestication process took place independently in various regions and not only in the Mediterranean region (Evreinoff 1949; Zukovskij 1950; Melgarejo and Martinez 1992). It is cultivated in central Asia and to some extent in the USA (California), Russia, China, India and Japan for fruit production and is also developed as an ornamental tree in East Asia (Mars 1996; Tous and Ferguson 1996).

Botanically, the pomegranate is included in the family of Punicaceae with 2n = 16 or 18. The genus *Punica* is known to include two species *P. protpounica* and *P. granatum*. *P. protpounica* is endemic to the Socotra island (Yemen) and is the only relative of the cultivated pomegranate (Moriguchi *et al*. 1987; Guarino *et al*. 1990). The fruit has been utilised from ancient times. It was frequently used as a mystical emblem in adorning the capitals of Assyrian (Layard 1853; Bonavia 1894) and Egyptians columns. The Bible tells us that in the building of Solomon's temple the capitals of the columns were decorated with a network of pomegranate; also that the high priest's robes were adorned with imitations of pomegranates and that pomegranate was one of the three fruits brought to Moses by the men that he sent to spy out the promised land (Harris 1824). The Babylonians regarded the seeds as an agent of resurrection, the Persians as conferring invincibility on the battlefield and for ancient Chinese alchemical adepts, the bright red juice was a myth poetically regarded as a 'soul concentrate', homologous to human blood, and capable of conferring on a person longevity or even immortality (Mahdihassan 1984).

In China the pomegranate is widely represented in ceramic art symbolising fertility, abundance, posterity and virtuous offspring, and the seeds symbolized longevity and immortality (Cooper 1995). The alkaloids in the hulls of the fruit and the roots have shown anthelmintic, antimalarial, antifungal and antimicrobial properties (Naovi *et al*. 1991; Reddy *et al*. 2007). The root extract, when applied both orally and intravaginally, prevent fertility (Gujral *et al*. 1960; Jochle 1971), cause abortion and ameliorate assorted gynecological problems (Goh *et al*. 1984). In folk medicine, a decoction of pomegranate rind is used for dysentery and also for stomatitis (Cáceres *et al*. 1987; Nagaraju and Rao 1990). The seed extract is said to be useful in the advanced stage of diarrhoea (Das *et al*. 1999). The most famous usage worldwide has been as a vermifugal (Kapoor 2000), wound healing (Murthy *et al*. 2004), free radical scavenging (Rout and Banerjee 2007), anti-diabetic agent (Huang *et al*. 2005), and as an antioxidant (Ricci *et al*. 2006). It is also well known that the dried pomegranate seeds contain oil, which has been shown to contain not only the steroidal estrogen estrone (Heftman *et al*. 1966; Dean *et al*. 1971), but also the isoflavonic phytoestrogens genistein and daidzein (Moneam *et al*. 1988). Studies have proven the effect of extracts from

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the hulls on *Herpes simplex virus* (Zhang 1995), as a human immunodeficiency virus inhibitor (Neurath *et al*. 2005), against dental plaque (Menezes *et al*. 2006) and anti-tumour activity for prostate cancer (Malik *et al*. 2005), breast cancer (Jeune *et al*. 2005) and lung cancer (Khan *et al*. 2007).

Fruits produced in traditional orchards are not appropriate for new markets. Many old plantations are removed and a few local varieties are propagated in commercial nurseries and used in the new plantations. Thus, the problem of genetic erosion in pomegranate germplasm became real (Mars and Marrakchi 1998). Local genetic resources need new approaches, techniques and further collaborative efforts to be preserved and integrated in sustainable rural development programs. It is necessary to emphasize more on *in situ* preservation of gene-pools. Many local types of pomegranate were inventoried and described (Mars and Gaaliche 1993; Mars *et al*. 1994). Conservation of native land-races could be easier through the development of novel uses bringing them up as performing cultivars. However this is not a guarantee unless it is integrated in a larger sustainable agriculture development system. Such activities can not be efficiently initiated and implemented without good financial support. Hence, a molecular approach has been taken to evaluate the associations among the available genotypes and to determine the overall degree of polymorphism among the genotypes.

Among DNA-based molecular markers, Random Amplified Polymorphic DNAs (RAPDs) provide an excellent tool to study genetic diversity and genetic relationships (Williams *et al*. 1990). They are simple, versatile, relatively inexpensive and can detect slight genetic differences and help to identify duplicates in the population (Teixeira da Silva *et al*. 2005). RAPD markers have been used successfully to study genetic diversity and relatedness among perennial fruit crops such as mango (Ravishankar *et al*. 2000; Hemanthkumar *et al*. 2001), guava (Prakash *et al*. 2002), cashew (Dhanraj *et al*. 2002) and jackfruit (Simon *et al*. 2007). In the present study, RAPD markers were used to estimate genetic diversity and assess relationships among 46 pomegranate accessions collected from different parts of India.

MATERIALS AND METHODS

Plant materials

Pomegranate genotypes (46) including traditional and commercial varieties were collected throughout India: 'Alah', 'Boseka Linski', 'Patna-5', 'Yercaud', 'A.K. Anar', 'Shirin Anar', 'Gulsha', 'P-13', 'Kazakaki Anar', 'Gulsha Rose Pink', 'Gulsha Red', 'Damani', 'Yercaud HRS', 'Mridula', 'Khog', 'Kalishirin', 'Spin Sakarin', 'Co-White', 'Kabul IIHR', 'Dholka', 'P-26', 'Bassein Seedless', 'Jellore Seedless', 'Surk Anar', 'Kabul', 'Alandi', 'Bedana Suri', 'Sural Anar', 'Sur Sakkar', 'Agah', 'Ruby', 'Jyothi', 'Achik Dana', 'Kabul Conoor', 'Siah Shirin', 'P-16', 'Malta', 'G-137', 'Kandhari', 'Yercaud Local', 'Ganesh', 'P-23', 'Muscat', 'Jodhpur Red', 'Speen Sakarin' and 'Bedana Sadana'. Approximately, 50 g of recently matured leaves (15-20 days old) were collected, washed using distilled water, wiped with 70% (v/v) ethanol, then dried in oven at 30-35°C for 20 hours and powered by using a 'Remi' mixer for 45 to 60 seconds, prior to storage at room temperature in sealed plastic bags.

Table 1 Morphological data of *Punica granatum* genotypes.

S. №	Cultivars	Aril colour	Mellowness of seeds	TSS (%)	Acidity (%)	Average fruit yield		
						№ per plant	Weight per fruit (g)	Yield per plant (Kg)
1	Achik Dana	Pinkish white	Medium hard	9.2	1.34	22	65	1.21
2	Alandi	Pink	Very hard	14.0	0.50	25	130	2.80
3	Bassein Seedless*	Light pink	Soft	13.6	0.39	20	150	3.60
4	Bedana Sadana	Pink	Very hard	7.9	2.92	18	83	1.20
5	Bedana Suri	Light pink	Very hard	12.2	0.37	5	104	0.45
6	Boseka Linski	Cream white	Hard	11.2	2.31	6	48	0.32
7	Co-White	Light pink	Very hard	17.2	0.28	39	136	5.25
8	Dholka*	Light pink	Soft	12.3	0.37	20	132	2.45
9	Ganesh*	Pink	Soft	17.6	0.41	42	118	4.30
10	G-137	Pink	Soft	17.6	0.35	28	144	3.70
11	Gulsha	Blood red	Very hard	8.6	1.78	11	76	0.93
12	Gulsha Red	Blood red	Very hard	9.0	1.48	6	62	0.30
13	Gulsha Rose Pink	Dark red	Hard	11.5	1.27	9	58	0.42
14	Jellore Seedless	Light pink	Soft	13.3	0.44	10	112	1.00
15	Jodhpur Red	Red	Very hard	13.9	0.45	14	161	2.25
16	Jyothi*	Light pink	Soft	16.7	0.52	26	136	3.30
17	Kabul*	Pink	Hard	16.2	0.44	42	137	6.25
18	Kabul IIHR	Light pink	Soft	16.8	0.24	32	115	4.05
19	Kabul Conoor	Red	Medium hard	12.3	1.62	NA	NA	NA
20	Kalishirin	Cream white	Very hard	10.4	1.48	31	104	2.80
21	Kandhari	Light pink	Soft	16.2	0.55	12	93	1.20
22	Kazakaki Anar	Light pink	Soft	12.2	2.45	14	48	0.59
23	Khog	Pink	Very hard	16.7	0.38	4	83	0.37
24	Malta	Whitish pink	Very hard	10.3	0.56	6	42	0.26
25	Muscat	Cream white	Soft	14.8	0.64	25	157	3.65
26	P-13	Light pink	Soft	18.0	0.49	17	212	3.30
27	P-16	Light pink	Soft	13.6	0.28	13	192	2.60
28	P-23	Light pink	Soft	16.0	0.42	16	310	5.00
29	P-26	Pink	Soft	15.6	0.35	20	167	3.60
30	Shirin Anar	Light Pink	Very Hard	10.3	1.65	12	57	0.63
31	Spin Sakarin	Red	Very Hard	12.6	1.09	34	138	5.00
32	Sur Sakkar	Dark Red	Very Hard	12.2	1.60	12	438	5.35
33	Surk Anar	Whitish Pink	Very Hard	9.2	1.40	13	425	6.00
34	Yercaud*	Pink	Very Hard	15.2	0.52	31	123	4.03
35	Yercaud HRS	Cream White	Very Hard	17.0	0.56	32	170	5.00
36	Yercaud Local	Whitish Pink	Very Hard	12.8	1.40	36	127	4.23

NA, Data not available
* Commercial cultivars

Morphological characters

The morphological data was recorded for the available 36 genotypes for the characters such as aril colour, mellowness of seeds, TSS (%), acidity (%), number of fruits per plant, weight per fruit (g) and total yield per plant (Kg). The mean average of 10 to 15 individuals of each genotype was selected to determination the morphological character (**Table 1**).

DNA extraction and purification

DNA was extracted from the dried leaf powder of pomegranate by the cetyl trimethyl ammonium bromide (CTAB) method according to a modified protocol of Porebski *et al*. (1997). Two and half g of leaf powder was mixed with 20 ml extraction buffer, preheated to 65°C, which contained 100 mM Tris-Base, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 3% (w/v) CTAB, 2% polyvinyl pyrrolidone and 1% β-mercaptoethanol, then incubated at 65°C for 60 minutes with gentle shaking. The mixture was cooled to room temperature, 10 ml cold 24:1 (v/v) chloroform:isoamyl alcohol was added, and the contents were mixed well. After centrifugation at 6000 × *g* for 20 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform:isoamyl alcohol step was repeated until a clear supernatant was obtained. 5 M NaCl was added to the supernatant (0.5 v/v) and mixed gently followed by the addition of equal volume of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C, and then centrifuged at 8000 × *g* for 25 min. The resulting pellet was washed with 70% (v/v) ethanol, air dried, and dissolved in 300 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Three µg RNase (bovine pancreatic ribonuclease, Bangalore Genei, Bangalore, India) was added to each sample which was incubated overnight at 37°C, mixed with an equal volume of phenol and centrifuged at 6000 × *g* for 20 min at room temperature. This step was followed by a wash with an equal volume (1:1 (v/v)) of phenol:chloroform then with chloroform alone. DNA was precipitated overnight at 4°C with 0.5 vol of 5 M NaCl and 1 vol cold isopropanol, and the resulting pellet obtained after centrifugation was dissolved in TE buffer, analysed on an agarose gel and quantified using a spectrophotometer (Nano Drop Technologies, Wilmington, USA).

PCR amplification

PCR amplification protocol was of Williams *et al*. (1990) with minor modifications. Of the 25 primers screened using the genotype 'Ruby', 8 showing clear and distinguishable bands were selected for RAPD-PCR analysis. Reproducibility of the primers was tested by repeating the PCR amplification three times under similar conditions. PCR reactions were carried out in a volume of 25 µl containing 25 ng template DNA, 150 µM each dNTP, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India), 5 pmol primer (OPA, OPB, OPC, OPD, OPE, OPF, OPG, OPH, OPI, OPJ and OPK series, Operon Technologies, Alameda, CA, US) in PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100). Amplifications were performed in a MJ Research PTC-100 Thermocycler (Bio-Rad Laboratories, Bangalore, India), programmed for an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, primer extension at 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were resolved in a 1.2% (w/v) agarose gel, visualised and documented using an Alpha Digidoc system (Alpha Innotech, San Leandro, CA, US).

RAPD profile analysis

Amplified fragments from each primer were manually scored for their presence (1) or absence (0) and a matrix of the different RAPD phenotypes of all eight primers was assembled for statistical analysis. The sizes of the fragments were estimated using 500 bp standard DNA markers, co-electrophoresed with the PCR products. A genetic dissimilarity matrix was developed using Euclidean Distances, which estimates all pair-wise differences in the amplification products (Sokal and Sneath 1973) and a cluster analysis was based on Ward's method using a minimum variance algorithm (Ward 1963).

RESULTS AND DISCUSSION

Information on genetic diversity among plant species is important for efficient utilization of plant genetic resources. Geographical isolation of a population may cause its genome to drift away from other populations of the same species (Biron *et al*. 2002). Hence, authentic identification of taxa is necessary both for breeders to ensure protection of intellectual property right and also for propagators and consumers. The traditional method of identifying species by morphological characters is now been accompanied by DNA profiling that is more reliable because of several limitations of their morphological data (Nayak *et al*. 2003). Therefore, organisms with a high tendency for morphological differentiation, studies considering both molecular and morphological characters are highly relevant (Yara *et al*. 2005). Evidently, RAPD technology is a rapid and sensitive technique, which can be used to estimate relationships between closely and more distantly related species of pomegranate.

Genetic studies are rare or lacking in pomegranate because they have not been subject of much scientific investigation. Studies based on morphometric criteria have been performed to determine the degree of polymorphism within pomegranate populations (Mars and Marrakchi 1998a). However, use of morphological or physiological characters in pomegranate such as fruit size, rind and juice colour, ripening date, sugars and acidity in the juice, etc. are strongly influenced by environmental factors (Shulman *et al*. 1984; El Kassas *et al*. 1992). Varieties in pomegranate are often classified based on characters such as sweetness, sweet-sour and sour, early, mid season and late, juicy and table fruit, soft-seeded and hard-seeded or major and minor. Gulick and van Sloten (1984) reported that in India there are three principal collections containing, each one, at least 30 accessions. Despite the large number of local varieties, in almost all countries, very few are commercially utilised and propagated in nurseries (Aksoy 1995; Llácer *et al*. 1995). Recently Ercisli *et al*. (2007) used RAPD to assess the interspecific variability of fatty acid composition in six commercial pomegranate cultivars from Southern Anatolia, Turkey. Similarly, genetic diversity among eighty five cultivars of Chinese pomegranate was studied by means of fluorescent-AFLP markers (Yuan *et al*. 2007).

PCR amplification was followed according to a stan-

Table 2 RAPD-PCR Primers. The sequence and level of polymorphism of primers.

S. №	Primer	Sequence (5′–3′)	№ of bands	№ of shared polymorphic bands	№ of unique polymorphic bands	№ of monomorphic bands
1	OPA 04	AATCGGGCTG	17	16	1	0
2	OPA 11	CAATCGAAGT	14	11	2	1
3	OPB 04	GGACTGGCGT	17	14	2	1
4	OPC 02	GTGAGGCGTC	12	10	1	1
5	OPC 05	GATGACCGCC	14	9	3	2
6	OPC 06	GAACGGACTC	20	17	2	1
7	OPD 19	CTGGGGACTT	14	13	1	0
8	OPG 19	GTCAGGGCAA	15	14	0	1
Total bands (percentage)			123	104 (84.5%)	12 (9.8%)	7 (5.7%)

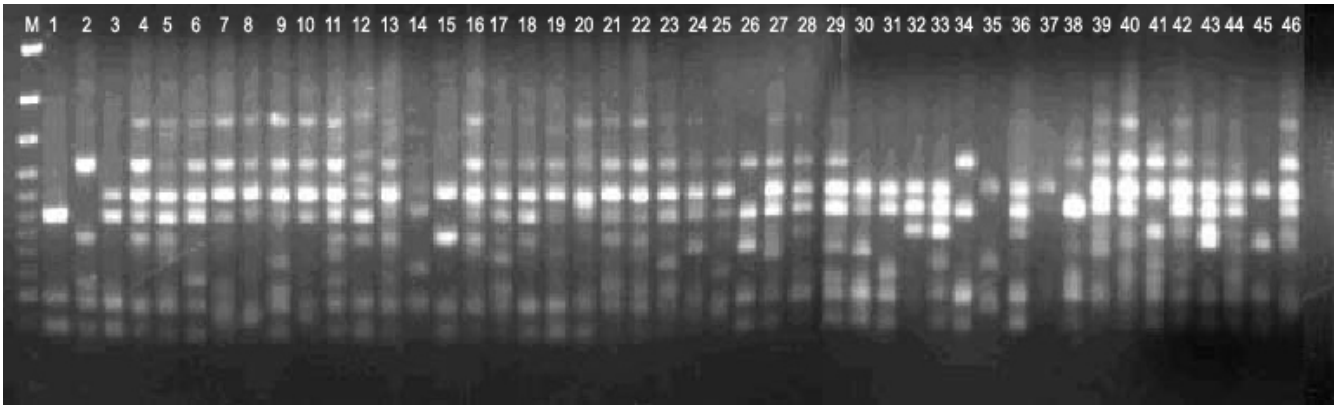

Fig. 1 Gel profile of Pomegranate using RAPD-PCR primers OPA-04. Lanes 1-46, contain the amplification profile obtained using the genotypes 'Alah', 'Boseka Linski', 'Patna-5', 'Yercaud', 'A.K. Anar', 'Shirin Anar', 'Gulsha', 'P-13', 'Kazakaki Anar', 'Gulsha Rose Pink', 'Gulsha Red', 'Damani', 'Yercaud HRS', 'Mridula', 'Khog', 'Kalishirin', 'Spin Sakarin', 'Co-White', 'Kabul IIHR', 'Dholka', 'P-26', 'Bassein Seedless', 'Jellore Seedless', 'Surk Anar', 'Kabul', 'Alandi', 'Bedana Suri', 'Sural Anar', 'Sur Sakkar', 'Agah', 'Ruby', 'Jyothi', 'Achik Dana', 'Kabul Conoor', 'Siah Shirin', 'P-16', 'Malta', 'G-137', 'Kandhari', 'Yercaud Local', 'Ganesh', 'P-23', 'Muscat', 'Jodhpur Red', 'Speen Sakarin' and 'Bedana Sadana'. Lane M, standard (100 bp) DNA markers.

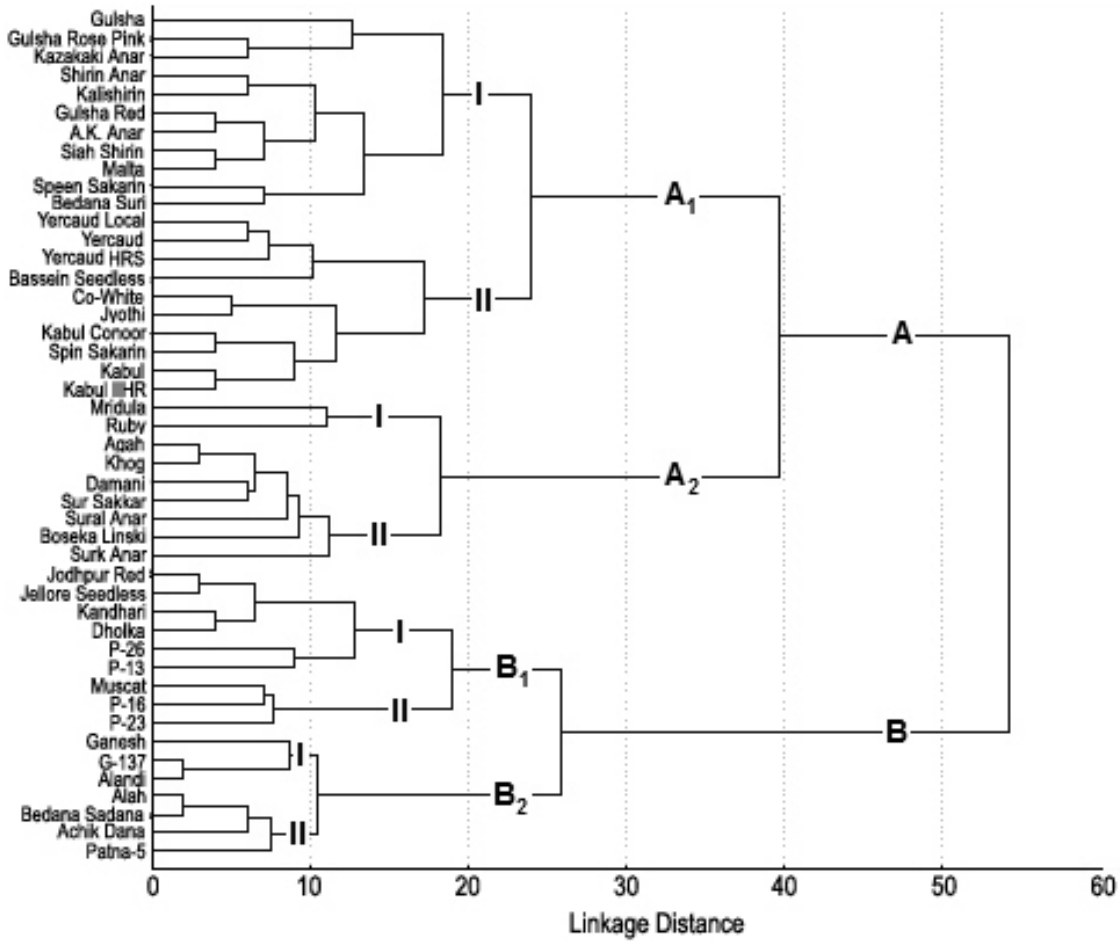

Fig. 2 Dendrogram showing RAPD-marker-based genetic relationships among 46 pomegranate genotypes.

dard protocol (Williams *et al*. 1990) with minor modifications, which produced good amplifications with 25 ng of template DNA. The amplifications using 1 unit of *Taq* DNA polymerase and 1.5 mM $MgCl_2$ produced intense and clear banding patterns. The primer-screening step resulted in 8 decamer primers which detected good polymorphisms (**Table 2**) and 17 other random primers, which did not give any amplification products. Screening is essential to save time and cost, and to reject primers not informative for the analysis (Prakash *et al*. 2002). The amplification profiles of total genomic DNA from eight pomegranate accessions with eight random primers produced a total of 123 fragments ranging in size from 250 bp to 2.5 kbp with an average of 15.4 bands per primer. Of the 123 bands, 104 (84.5%) were polymorphic and shared between at least two individuals, 7 (5.7%) were monomorphic and common to all the individuals. Twelve (9.8%) were polymorphic and unique. The number of fragments produced by a primer ranged from 12 (OPC 02) to 20 (OPC 06). Pattern of RAPD fragments produced by the random primer OPA-04 is shown in **Fig. 1**. The dissimilarity matrix obtained using Euclidian Distance (Sokal and Sneath 1973) is shown in **Table 3**. The highest genetic dissimilarity (22%) was between genotypes 'Alah' and 'Gulsha Red', while the least genetic dissimilarity (2%) was noticed between the genotypes 'Shirin Anar' and 'Kazakaki Anar' and 'Gulsha Red' and 'Kalishirin'.

In the dendrogram (**Fig. 2**), all 46 accessions were divided into two major clusters 'A' and 'B' at 54 linkage distance units. Cluster 'A' consisted of 30 genotypes in two

sub-clusters 'A_1' and 'A_2' linked at 40 distance with 21 and 9 genotypes, respectively and two groups each. Group I of 'A_1' consists of 11 genotypes linked at 19 distance units. The genotypes 'Gulsha Red' and 'A.K. Anar', and 'Siah Shirin' and 'Malta' are closely related at 4 linkage distance units and clustered with genotypes 'Shirin Anar' and 'Kalishirin' at 6 linkage distance units. The genotypes 'Speen Sakarin' and 'Bedana Suri' are closely clustered together at 7 linkage distance units. The three genotypes 'Gulsha', 'Gulsha Rose Pink' and 'Kazakaki Anar' were clustered at 13 linkage distance units. The morphological features of the group suggest that all the accessions have varied aril colour ranging from dark red to whitish pink but with very hard seeds except for the genotype 'Kazakaki Anar' (soft seed). All the genotypes showed a low TSS value ranging from 8.6 to 12.2%. The genotypes in the group predominately yielded small sized fruits of very low number (6-14) excluding 'Kalishirin' with 31 fruits.

Group II of 'A_1' consists of 10 genotypes that are clustered at 17 linked distance units and divided into two minor groups. The genotypes 'Kabul Conoor' and 'Spin Sakarin', and 'Kabul' and 'Kabul IIHR' are closely related at 4 linkage distance units and are associated with the genotypes 'Co-White' and 'Jyothi' at 12 distance units to form a minor group. The genotypes 'Yercaud Local' and 'Yercaud' are linked at 6 distance units and clustered with genotypes 'Yercaud HRS' and 'Bassein Seedless' at 10 distance units to form a minor group. Most of the varieties of the group were obtained from South Indian states; as they are clustered together they should have a common origin. The morphological characters among the group show that the genotypes predominantly had a light aril colour and varied in seed mellowness ranging from soft to very hard. All the genotypes had a very high TSS value ranging from 12.3 to 17.2% and yielded many fruits ranging from 20 ('Bassein Seedless') to 42 ('Kabul') with high yield per plant (3.3 to 6.25 Kg).

Sub-cluster 'A_2' consists of nine genotypes clustered at 18 linkage distances into two groups. Group I has two genotypes 'Mridula' and 'Ruby' grouped at 11 linkage distances. The genotypes 'Agah' and 'Khog' in group II are clustered at 3 linked distances, closely related to genotypes 'Damani' and 'Sur Sakkar' at 7 distances and subsequently with genotypes 'Sural Anar', 'Boseka Linski' and 'Surk Anar'. The morphology showed that the genotypes 'Sur Sakkar' and 'Surk Anar' had highest fruit weight of 438 and 425 g, respectively. A comparison of the morphological characters among the sub-cluster 'A_2' revealed that all the genotypes distinctly showed very hard seed and low number of fruit yield per plant (4 to 13).

Cluster 'B' consists of 16 accessions clustered into two sub-clusters 'B_1' and 'B_2' at 26 linkage distances. Sub-cluster 'B_1' with nine genotypes is grouped into two distinct groups at 19 linkage distance units. The genotypes 'Jodhpur Red' and 'Jellore Seedless' are linked at 3 distance units and are closely related to genotypes 'Kandhari' and 'Dholka' at 7 distance units. The genotypes 'P-26' and 'P-13' were clustered at 9 linkage distance units to form a discrete cluster in group I. The genotypes 'Muscat' and 'P-16' are linked at 7 distance units in group II and closely related to 'P-23' at 8 distance units. The genotypes 'P-13', 'P-16', 'P-23' and 'P-26' are selections from 'Muscat' variety, which might be a forcing factor for sheltering under the same cluster. The morphological feature of sub-cluster 'B_1' suggested that all the genotypes predominantly are comprised of a light pink-coloured aril with soft seeds except for the genotype 'Jodhpur Red', which has a red aril and very hard seeds. The genotypes also showed a high TSS value (12.3 to 16.2%), low acidity (0.28 to 0.64%) and moderate number of fruits (10 to 25).

Sub-cluster 'B_2' consists of seven genotypes distinctly clustered into two groups (I and II) at 11 linkage distance units with three and four genotypes, respectively. The genotypes 'G-137' and 'Alandi' were clustered at 2 linkage distance units and closely related to genotype 'Ganesh' in group I. The genotypes 'Alah' and 'Bedana Sadana' in group II are clustered at 2 linkage distance units and linked to genotypes 'Achik Dana' and 'Patna-5' at 6 and 8 distance units, respectively. The genotype 'Bedana Sadana' showed the lowest TSS value of 7.9% and the highest acidity of

Table 3a Genetic dissimilarity matrix of 46 pomegranate genotypes based on polymorphism of RAPD markers.

	Alah	Boseka Linski	Patna-5	Yercaud	A.K. Anar	Shirin Anar	Gulsha	P-13	Kazakaki Anar	Gulsha Rose Pink	Gulsha Red	Damani	Yercauh HRS	Mridula	Khog	Kalishirin	Spin Sakarin	Co-White	Kabul IIHR	Dholka	P-26	Bassein Seedless	Jellore Seedless	Surk Anar	Kabul
Alah	0																								
Boseka Linski	14	0																							
Patna-5	11	11	0																						
Yercaud	21	13	12	0																					
A.K. Anar	19	15	16	8	0																				
Shirin Anar	18	1	11	7	7	0																			
Gulsha	15	17	12	8	10	7	0																		
P-13	13	13	10	8	14	7	8	0																	
Kazakaki Anar	18	14	11	7	7	2	5	9	0																
Gulsha Rose Pink	16	14	11	7	9	6	7	9	4	0															
Gulsha Red	22	16	15	5	7	6	7	11	4	6	0														
Damani	19	17	14	10	12	13	18	14	15	13	13	0													
Yercauh HRS	20	10	11	5	13	12	11	11	10	10	6	15	0												
Mridula	13	9	12	12	10	11	14	12	11	9	11	12	13	0											
Khog	12	6	7	13	15	14	15	11	14	14	14	17	10	11	0										
Kalishirin	20	14	13	5	7	6	9	11	4	6	2	13	6	11	12	0									
Spin Sakarin	17	15	10	8	12	9	10	12	9	7	7	2	7	10	13	7	0								
Co-White	16	8	9	7	13	10	11	9	10	8	10	15	4	9	10	10	7	0							
Kabul IIHR	16	12	13	9	11	8	11	7	8	8	8	11	10	5	14	8	7	8	0						
Dholka	11	11	12	10	14	11	8	8	11	9	13	16	11	12	10	13	10	7	11	0					
P-26	13	9	12	8	12	9	10	6	9	7	9	16	9	10	9	9	12	7	9	6	0				
Bassein Seedless	18	10	11	3	9	10	9	9	8	6	6	13	4	11	10	6	8	4	10	7	5	0			
Jellore Seedless	12	10	9	11	13	14	11	11	12	10	12	17	8	9	6	12	9	8	12	7	9	8	0		
Surk Anar	13	9	6	8	14	11	12	8	11	11	11	12	7	6	7	11	8	5	7	8	8	7	5	0	
Kabul	14	12	11	9	13	8	7	9	6	6	8	15	10	7	12	8	9	10	6	9	9	10	8	7	0

Table 3b Genetic dissimilarity matrix of 46 pomegranate genotypes based on polymorphism of RAPD markers.

	Bedana Sadana	Speen Sakarin	Jodhpur Red	Muscat	P-23	Ganesh	Yercaud Local	Kandhari	G-137	Malta	P-16	Siah Shirin	Kabul Conoor	Achik Dana	Jyothi	Ruby	Agah	Sur Sakkar	Sural Anar	Bedana Suri	Alandi
Alah	16	14	9	15	11	11	13	14	9	18	14	15	15	11	13	12	14	16	14	15	9
Boseka Linski	14	10	13	11	9	9	13	14	7	14	10	13	11	13	11	10	18	10	12	9	7
Patna-5	15	13	10	10	10	10	12	11	6	13	9	10	14	6	12	11	15	11	9	8	10
Yercaud	9	13	14	12	12	12	8	11	12	11	13	12	16	12	14	13	15	7	9	6	12
A.K. Anar	13	13	14	12	16	18	12	15	18	9	13	14	14	14	12	11	11	11	13	14	18
Shirin Anar	14	12	13	9	13	13	15	10	12	11	14	13	11	13	13	10	12	8	8	9	15
Gulsha	13	15	12	14	12	10	10	9	12	13	15	12	14	12	12	11	13	11	7	8	14
P-13	11	13	12	14	8	8	14	7	8	15	15	14	14	10	12	11	13	9	7	6	10
Kazakaki Anar	12	12	13	9	13	13	13	10	11	10	14	13	11	15	13	10	14	10	10	7	15
Gulsha Rose Pink	10	12	9	13	11	13	9	12	13	6	16	11	11	11	13	8	16	10	12	7	13
Gulsha Red	8	12	13	9	11	13	11	8	15	8	12	11	11	15	13	12	14	10	12	9	15
Damani	13	13	16	14	18	18	14	17	18	13	17	14	18	14	14	13	11	13	15	14	18
Yercauh HRS	10	14	13	11	9	11	9	8	11	10	8	11	15	13	15	14	18	10	12	7	11
Mridula	11	7	8	10	10	12	14	15	10	7	13	8	6	12	8	3	8	7	11	16	10
Khog	10	10	9	7	5	7	13	10	7	12	6	7	13	9	9	10	18	10	10	9	7
Kalishirin	8	12	13	9	11	13	11	10	13	8	12	11	13	15	15	12	16	12	14	9	15
Spin Sakarin	11	12	8	10	12	14	10	11	12	7	13	8	12	14	16	11	13	9	9	10	14
Co-White	12	14	11	13	9	11	9	10	9	10	8	9	13	11	13	10	16	6	10	7	9
Kabul IIHR	10	8	11	11	11	11	15	10	11	8	16	11	9	15	11	6	8	8	10	9	13
Dholka	13	15	10	14	10	10	8	13	10	15	15	12	14	12	12	9	13	9	9	6	10
P-26	11	9	10	12	6	8	10	7	10	13	13	14	12	10	10	9	17	9	11	6	6
Bassein Seedless	8	14	13	13	9	11	5	10	11	10	10	11	15	11	13	12	18	8	12	5	9
Jellore Seedless	10	12	15	9	7	11	9	12	9	10	10	9	11	11	11	8	14	8	8	5	9
Surk Anar	11	9	8	8	8	8	10	11	6	11	9	8	12	10	10	7	11	5	7	4	8
Kabul	10	8	9	9	9	7	11	12	9	10	16	9	9	11	11	4	10	8	8	5	11
Alandi	9	9	10	10	4	6	10	9	4	17	9	12	10	10	10	11	19	9	9	8	0
Bedana Suri	9	11	10	10	8	8	8	11	6	13	13	12	12	12	12	9	15	7	7	0	
Sural Anar	12	12	9	9	9	9	13	10	7	16	12	11	11	11	9	10	10	4	0		
Sur Sakkar	10	12	9	9	9	11	11	12	9	12	10	9	9	11	9	8	10	0			
Agah	16	12	13	13	17	15	17	16	17	14	18	13	11	13	11	8	0				
Ruby	12	6	7	9	9	9	13	14	11	8	14	7	7	9	7	0					
Jyothi	13	9	10	10	8	10	16	11	12	13	11	12	10	10	0						
Achik Dana	15	13	10	14	8	8	10	11	12	13	11	10	14	0							
Kabul Conoor	11	9	8	8	8	12	16	13	10	11	13	12	0								
Siah Shirin	9	11	10	10	10	10	12	13	12	7	9	0									
P-16	10	14	11	7	7	11	11	10	9	14	0										
Malta	12	10	9	13	13	15	13	14	17	0											
G-137	11	11	10	8	6	6	12	11	0												
Kandhari	12	12	13	11	7	9	15	0													
Yercaud Local	9	15	10	14	10	10	0														
Ganesh	11	7	10	10	4	0															
P-23	7	9	6	8	0																
Muscat	9	7	8	0																	
Jodhpur Red	11	9	0																		
Speen Sakarin	12	0																			
Bedana Sadana	0																				

2.92% and the genotypes 'Ganesh' and 'G-137' showed the highest TSS value (17.6%) among all the genotypes examined. Sub-cluster 'B_2' predominantly had varieties with a pink coloured aril and a high number of fruits per plant (18-42). The comparison of morphological characters among the genotypes in cluster 'B' predominantly showed a light aril colour, soft seeds, low acidity, a high TSS value and higher fruit yield.

The pomegranate genotypes studied showed relatively low (2 to 22%) variation despite collected from all geographical locations of India. Even though various types of pollination have been described in pomegranate (Karale *et al*. 1993) but recent investigations demonstrated that cultivars 'Ganesh' and 'Kabul Yellow' are predominantly self-pollinated with a low proportion (13%) of cross-pollination (Jalikop and Kumar 1990) which could be attributed to low variation since almost all of the varieties in the region are maintained through vegetative propagation. The low number of fruits set among the accessions is due to self pollination (Karale *et al*. 1993). The locally grown cultivars may be considered as the primary gene pool, wild forms would be the secondary gene pool and the tertiary gene pool would consist of forms of the pomegranate wild relative *P. protopunica*. Disease and abiotic stress resistance could be produced in nature since, hybridisation between cultivated and wild forms are probably still taking place (Zukovskij 1950).

Understanding the spatial organization of genetic diversity within the plant populations is of critical importance for the development of strategies designed to preserve genetic variation (Brown and Briggs 1991; Hamrick *et al*. 1991). It has been shown that species with limited gene flow, i.e. with restricted seed and/pollen movement, have considerably more among-population variation for total amount of genetic diversity (Schoen and Brown 1991). The future of this fruit depends on the selection of high quality cultivars (Tous and Ferguson 1996). Since the *ex situ* collection cannot exceed a limited number of accessions, it is difficult to preserve the evolutionary potential of the species. Thus, conservation strategies among traditional cultivars of pomegranate should be developed with the morphological characters in mind. This study effectively revealed the use of morphological and RAPD markers in estimating genetic diversity in pomegranate, which could be the first step towards efficient germplasm storage management in these perennial crops where land, time, effort and money could be saved.

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REFERENCES

Aksoy U (1995) Present status and future prospects of underutilized fruit production in Turkey. *Cahiers Options Mediterraneennes* **13**, 97-107

Biron DG, Landry BS, Nenon JP, Coderre1 D, Boivin G (2002) Geographical origin of an introduced pest species, *Delia radicum* (Diptera: Anthomyiidae), determined by RAPD analysis and egg micromorphology. *Bulletin of Entomological Research* **90**, 23-32

Bonavia E (1894) *Flora of the Assyrian Monuments and its Outcome*, Archibald Constable and Co., Westminster, 98 pp

Brown AH, Briggs JD (1991) Sampling strategies for genetic variation in *ex situ* collections of endangered plant species. In: Falk DA, Holsinger KF (Eds) *Genetics and Conservation of Rare Plants*, Oxford University Press, New York, pp 99-119

Cáceres A, Girón LM, Alvarado SR, Torres MF (1987) Screening of antimicrobial activity of plants popularly used in Guatemala for the treatment of dermatomucosal diseases. *Journal of Ethnopharmacology* **20**, 223-237

Cooper JC (1995) *An Illustrated Encyclopaedia of Traditional Symbols*, Thames and Hudson, New York, 134 pp

Das AK, Mandal SC, Banerjee SK, Sinha S, Das J, Saha BP, Pal M (1999) Studies on antidiarrhoeal activity of *Punica granatum* seed extract in rats. *Journal of Ethnopharmacology* **68**, 205-208

Dean PDG, Exley D, Goodwin TW (1971) Steroid estrogens in plants: Re-estimation of oestrone in pomegranate seeds. *Phytochemistry* **10**, 2215-2216

Dhanraj AL, Rao EVVB, Swamy KRM, Bhat MG, Prasad T, Sondur SN (2002) Using RAPDs to assess the diversity in Indian cashew (*Anacardium occidentale* L.) germplasm. *Journal of Horticultural Science and Biotechnology* **77**, 41-47

El Kassas SE, Amen KIA, Hussein AA, Osman SM (1992) Effects of certain methods of weed control and nitrogen fertilization on the yield, fruit quality and some nutrient contents of Manfalouty pomegranate trees, Flowering and fruit setting. *Assiut Journal of Agricultural Sciences* **23 (3)**, 183-198

Ercisli S, Agar G, Orhan E, Yildirim N, 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

Evreinoff VA (1949) Le grenadier. *Fruits d'Outre-Mer* **4 (5)**, 161-170

Goh SH, Soepadmo E, Chang P, Banerjee U, Chan KC, Deverre JR, Hadi H, Loke SE, Nassrulhawq A, Oo SL, Taylor CE, Wong WH, Zakaria M (1984) *In: Proceedings of Fifth Asian Symposium on Medicinal Plants and Spices*, Han BH, Han DS, Han YN, Woo WS (Eds) Seoul, Korea, 20-24 August 1984, Vol 5, pp 473-483

Guarino L, Miller T, Baazara M, Obadi N (1990) Socotra: The island of Bliss revisited. *Diversity* **6 (3-4)**, 28-31

Gujral ML, Varma DR, Sareen KN (1960) Oral contraceptives. Part I. Preliminary observations on the antifertility effect of some indigenous drugs. *Indian Journal of Medical Research* **48**, 46-51

Gulick P, van Sloten DH (1984) Directory of germplasm collections. *Tropical and Subtropical Fruits and Tree Nuts*, IBPGR, Rome, 197 pp

Hamrick JL, Godt MJ, Murawski DA, Loveless MD (1991) Correlations between species traits and allozyme diversity: Implications for conservation biology. In: Falk DA, Holsinger KE (Eds) *Genetics and Conservation of Rare Plants*, Oxford University Press, New York, pp 75-86

Harris TM (1824) *The Natural History of the Bible*, Boston, 474 pp

Heftman E, Ko ST, Bennett RD (1966) Identification of estrone in pomegranate seeds. *Phytochemistry* **5**, 1337-1339

Hemanthkumar NV, Narayanaswamy P, Prasad TD, Mukund GK, Sondur SN (2001) Estimation of genetic diversity of commercial mango (*Mangifera indica* L.) cultivars using RAPD markers. *Journal of Horticultural Science and Biotechnology* **76**, 529-533

Huang TH, Peng G, Kota BP, Li GQ, Yamahara J, Roufogalis BD, Li Y (2005) Anti-diabetic action of *Punica granatum* flower extract: activation of PPAR-gamma and identification of an active component. *Toxicology and Applied Pharmacology* **207**, 160-169

Jalikop SH, Kumar SP (1990) Use of a gene marker to study the mode of pollination in pomegranate (*Punica granatum* L.). *Journal of Horticultural Science* **65**, 221-223

Jeune MA, Kumi-Diaka J, Brown J (2005) Anticancer activities of pomegranate extracts and genistein in human breast cancer cells. *Journal of Medical Food* **8**, 469-475

Jochle W (1971) Biology and pathology of reproduction in Greek mythology. *Contraception* **4**, 1-13

Kapoor LD (2000) *Handbook of Ayurvedic Medicinal Plants*, CRC Press Inc., Boca Raton, Florida, 424 pp

Karale AR, Supe VS, Kaulgud SN, Kale PN (1993) Pollination and fruit set studies in pomegranate. *Journal of Maharashtra Agriculture University* **18**, 364-366

Khan N, Afaq F, Kweon MH, Kim K, Mukhtar H (2007) Oral consumption of pomegranate fruit extract inhibits growth and progression of primary lung tumors in mice. *Cancer Research* **67**, 3475-3482

Layard AH (1853) *Nineveh and its Remains* (Vol II), Gorgias Press, New York, 233 pp

Llácer G, Martínez-Valero R, Romero M, Toribio F (1995) Present status and future prospects of underutilized fruit tree crops in Spain. *Cahiers Options Méditerranéennes* **13**, 69-78

Mahdihassan S (1984) Outline of the beginnings of alchemy and its antecedents. *Cahiers Options Mediterraneennes* **12**, 32-42

Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H (2005) Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. *Proceedings of the National Academy of Sciences USA* **102**, 14813-14818

Mars M (1996) Pomegranate genetic resources in the Mediterranean region. In: *Proceedings of the First MESFIN Plant Genetic Research Meeting*, Tenerife, Spain, pp 345-354

Mars M, Carraut A, Marrakchi M, Gouiaa M, Gaaliche F (1994) Ressources genetiques fruitieres en Tunisie (poirier, oranger, figuier, grenadier). *FAO/IPGRI Plant Genetic Research Newsletter* **100**, 14-17

Mars M, Gaaliche F (1993) Les varietes de grenadier en Tunisie. Mars M, Gaaliche F (Eds) GOVPF/Alpha S.A. Tunis, 32 pp

Mars M, Marrakchi M (1998) Conservation et valorisation des ressources genetiques du grenadier (*Punica granatum* L.) en Tunisie. *FAO/IPGRI Plant Genetic Research Newsletter* **114**, 35-39

Mars M, Marrakchi M (1998a) Diversity among pomegranate (*Punica granatum* L.) germplasm in Tunisia. *Genetic Resources and Crop Evolution* **46**, 461-467

Melgarejo MP, Martínez VR (1992) *El Granado*, Ediciones Mundi Prensa, Madrid, 162 pp

Menezes SM, Cordeiro LN, Viana GS (2006) *Punica granatum* (pomegranate) extract is active against dental plaque. *Journal of Herbal Pharmacotherapy* **6**, 79-92

Moneam NMA, El Sharasky AS, Badreldin MM (1988) Oestrogen content of pomegranate seeds. *Journal of Chromatography* **438**, 438-442

Moriguchi T, Omura M, Natsuta N, Kozaki I (1987) *In vitro* adventitious shoot formation from anthers of pomegranate. *HortScience* **22**, 947-948

Murthy KN, Reddy VK, Veigas JM, Murthy UD (2004) Study on wound healing activity of *Punica granatum* peel. *Journal of Medical Food* **7**, 256-259

Nagaraju N, Rao KN (1990) A survey of plant crude drugs of Rayalaseema, Andhra Pradesh, India. *Journal of Ethnopharmacology* **29**, 137-158

Naovi SAH, Khan MSY, Vohora SB (1991) Antibacterial, antifungal and anthelmintic investigations on Indian medicinal plants. *Fitoterapia* **62**, 221-228

Nayak S, Rout GR, Das P (2003) Evaluation of the genetic variability in bamboo using RAPD markers. *Plant Soil Environment* **49**, 24-28

Neurath AR, Strick N, Li YY, Debnath AK (2005) *Punica granatum* (pomegranate) juice provides an HIV-1 entry inhibitor and candidate topical microbicide. *Annals of the New York Academy of Sciences* **1056**, 311-327

Porebski S, Bailer G, Baun BR (1997) Modification of a CTAB DNA extraction protocol for plant containing high polysaccharides and poly phenols compounds. *Plant Molecular Biology Reporter* **15**, 8-15

Prakash DP, Narayanaswamy P, Sondur SN (2002) Analysis of molecular diversity in guava using RAPD markers. *Journal of Horticultural Science and Biotechnology* **77**, 287-293

Ravishankar KV, Anand L, Dinesh MR (2000) Assessment of genetic relatedness among mango cultivers of India using RAPD markers. *Journal of Horticultural Science and Biotechnology* **75**, 198-201

Reddy MK, Gupta SK, Jacob MR, Khan SI, Ferreira D (2007) Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Medica* **73**, 461-467

Ricci D, Giamperi L, Bucchini A, Fraternale D (2006) Antioxidant activity of *Punica granatum* fruits. *Fitoterapia* **77**, 310-312

Rout S, Banerjee R (2007) Free radical scavenging, anti-glycation and tyrosinase inhibition properties of a polysaccharide fraction isolated from the rind from *Punica granatum*. *Bioresource Technology* **98**, 3159-3163

Schoen DJ, Brown AHD (1991) Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. *Proceedings of the National Academy of Sciences USA* **88**, 4494-4497

Shulman Y, Fainberstein L, Lavee S (1984) Pomegranate fruit development and maturation. *Journal of Horticultural Science* **59**, 265-274

Simon L, Shyamalamma S, Narayanaswamy P (2007) Morphological and molecular analysis of genetic diversity in jackfruit. *Journal of Horticultural Science and Biotechnology*, **82(5)**, 764-768

Sokal RR, Sneath PHA (1973) *Principles of Numerical Taxonomy*, WH Freeman and Co., San Francisco, CA, USA, 359 pp

Teixeira da Silva JA, Nhut DT, Giang DTT, Rashid SZ (2005) The phylogeny, breeding and ecology of trees using molecular markers. In: Thangadurai D, Pullaiah T, Tripathy L (Eds) *Genetic Resources and Biotechnology* (Vol III), Regency Publications, New Delhi, India, pp 279-308

Tous J, Ferguson L (1996) Mediterranean fruits. In: Janick J (Ed) *Progress in New Crops*, ASHS Press, Arlington, VA, pp 416-430

Ward JH (1963) Hierachic grouping to optimize an objective function. *Journal of the American Statistical Association* **58**, 236-239

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphism amplified by arbitary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531-6535

Yara MB, Renata SP, Janet H, Evanilde BC, Alberto JP, Sonia MAPP (2005) Morphological and molecular characterization of *Corbicula* (Mollusca, Bilvalvia) at Rosana Resevoir, Brazil. *Acta Scientiarum. Biological Sciences* **27**, 397-404

Yuan Z, Yin Y, Qu J, Zhu L, Li Y (2007) Population genetic diversity in Chinese pomegranate (*Punica granatum* L.) cultivars revealed by fluorescent-AFLP markers. *Journal of Genetics and Genomics* **34 (12)**, 1061-1071

Zhang J (1995) Antiviral activity of tannin from pericarp of *Punica granatum* against genital *Herpes simplex* virus *in vitro*. *Chinese Journal of Materia Medica* **20**, 556-558

Zukovskij PM (1950) *Punica*. In: *Cultivated Plants and Their Wild Relatives*, State Publishing House Soviet Science, Moscow, pp 60-61