Genetic diversity of Iranian soft-seed pomegranate genotypes as revealed by fluorescent-AFLP markers

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Abstract The amplified fragment length polymorphism (AFLP) technique was used to examine the genetic relationships among 21 Iranian soft-seeded pomegranate (*Punica granatum* L.) genotypes. Out of 72 fluorescent-AFLP primer combinations screened, 31 were selected to produce the 503 polymorphic markers used in this study. Genetic similarity estimates between genotypes, calculated by the Jaccard's similarity coefficient, ranged from 0.17 to 1.00, while the cophenetic correlation coefficient between the genetic similarities and the unweighted pair group method of arithmetic averages (UPGMA) dendrogram was 0.98. The AFLP-based UPGMA dendrogram revealed two groups within the genotypes at 0.33 similarity coefficient, which reflect fruit traits such as peel and aril color, and seed firmness, as well as region of origin. Our study shows that the use of molecular markers is essential during all steps of germplasm management to avoid genotype redundancy and mislabeling. The present study will be used as a reliable reference to discriminate among these genotypes, to aid

management of germplasm collections used to breed new varieties for the Iranian pomegranate industry.

Keywords Genetic discrimination · Seed softness · Polymorphism · *Punica granatum* L.

Introduction

Pomegranate (*Punica granatum* L., Punicaceae) has been cultivated in Iran since ancient times and wild pomegranates still grow in parts of Iran (Goor and Libeman 1956; Levin 1994). Some of this genetic diversity is maintained in three major collections in Iran, encapsulating more than 1,200 genotypes from different regions (Behzadi Shahrabaki 1997). These genotypes represent a rich resource for the future development of the pomegranate industry and exhibit variation in agronomically important fruit traits such as size, color, taste, seed firmness and ripening time, as well as disease resistance. However, in some cases duplication of genotypes is suspected.

Pomegranates have been used as fresh fruit from long ago and continue to serve the food industry for production of juice, soft and alcoholic beverages and seed oil (Holland et al. 2009). The medicinal properties of pomegranate have been utilized in traditional medicine for many years and recent research has identified bioactive phytochemical substances, including antimicrobial and antioxidant components. These have been demonstrated to be beneficial in combating high blood pressure and other serious diseases such as diabetes and various cancers (Shishodia et al. 2006). Because of these positive discoveries about the health benefits of pomegranate, commercial cultivation has risen slowly over the past few years. However, the overall global area under planting is still considerably smaller than

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for apples or major stone fruits (Holland et al. 2009). Today, cost-effective mechanical harvesting of arils (seeds) is available, making the seed oil more readily accessible. Improved softer seeded cultivars are being developed to provide for a greater fresh fruit market demand (Holland et al. 2009). Pomegranate genotypes can be divided into four groups based on seed hardness phenotype: soft, semi-soft, semi-hard and hard seeded (Zamani 1990). Commercial pomegranate cultivars are often semi-hard or hard-seeded which is a distinct disadvantage, as soft-seeded fruit are the consumer's fruit of choice. Softness or absence of seeds (parthenocarpy) is a desirable economic trait in many fruit species, improving the market quality of fruit. However, parthenocarpy is not found in pomegranate and only the degree of softness of the seeds can be manipulated (Mars 2000). According to Levin (1994), completely soft-seeded pomegranates are restricted to a few narrow ecological regions. From the 760 genotypes that form the largest Iranian pomegranate collection in Yazd, Iran, 21 genotypes have local names containing the words Bihaste, Bidane or Bitolf, all meaning soft-seeded in Farsi. These genotypes represent an important genetic resource for improving the seed softness trait in commercial pomegranate varieties, and form the basis of this study.

Discrimination of genotypes is the first step for effective management of germplasm and its use in the breeding of new commercial varieties. To date, the most effective and reliable tool for genotype and cultivar characterization is the use of molecular markers (Kumar 1999; Collard et al. 2005) and the amplified fragment length polymorphism (AFLP) technique is one of the preferred DNA marker technologies for this purpose. The reliability of AFLP markers, as well as the wide range of polymorphism exhibited, without the need for prior sequence information, are factors that make AFLP analysis a desirable tool for genotype discrimination of plants possessing a minimal background of investigation at the DNA-sequence level (Kumar 1999; Gupta and Rustgi 2004).

In a previous study, Sarkhosh et al. (2009) used phenotype data for 36 fruit traits together with banding profiles derived from use of 14 randomly amplified polymorphic DNA (RAPD) primers to characterize 21 genotypes of pomegranate. However, it was found that grouping by fruit traits did not always correlate with RAPD banding profile grouping. AFLP and RAPD markers have also been used by several other researchers, some of whom have reported high polymorphism among pomegranate genotypes (Sarkhosh et al. 2006; Jbir et al. 2007; Zamani et al. 2007), whilst others have reported a high degree of similarity (Talebi Baddaf et al. 2003; Ercisli et al. 2007).

For this study, fluorescently labeled AFLP markers were used to investigate the genetic diversity and relationships among 21 genotypes of Iranian pomegranate. The AFLP primer combinations identified will be used for future

improvements in the management of pomegranate germplasm collections used for new cultivar improvement.

Material and methods

Plant material

Young leaves of 21 pomegranate genotypes (Table 1), originally classified as soft-seeded in comparison with other genotypes at their place of origin, were collected from mature trees in the pomegranate collection at the Agricultural Research Center, Yazd, Iran. The leaf samples were snap-frozen in liquid nitrogen, freeze dried and kept at -60°C until used. Genomic DNA was extracted from the freeze-dried leaf samples using the cetyltrimethylammonium bromide (CTAB) based protocol described by Murray and Thompson (1980).

AFLP markers

The AFLP analysis was performed according the protocol of Vos et al. (1995) with minor modification. Genomic DNA (250 ng) was digested by incubation for 3 hours at 37°C with the following reagents: 1U of T4 DNA ligase (Applied Biosystem, Foster City, CA, USA), 1X ligase buffer, 50 mM NaCl, 0.1 mg bovine serum albumin (BSA), $2.5\text{ }\mu\text{M}$ *MseI* adaptor, $0.5\text{ }\mu\text{M}$ *EcoRI* adaptor 2.5 U *MseI* and 5 U *EcoRI* (Applied Biosystem, Foster City, CA, USA) in total volume of $15\text{ }\mu\text{l}$, and then held at 65°C for 10 min.

Pre-amplification reactions were performed using *EcoRI* and *MseI* primers with one additional nucleotide at the 3' in $15\text{-}\mu\text{l}$ volumes containing: 1 X PCR buffer, $1\text{ }\mu\text{M}$ dNTPs, 1.5 mM MgCl_2 , $0.3\text{ }\mu\text{M}$ *MseI* primer, $0.3\text{ }\mu\text{M}$ *EcoRI* primer, 1 unit Platinum™ Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and $3.5\text{ }\mu\text{l}$ of the diluted restricted and ligated DNA template. The amplification was carried out in an Applied Biosystems 9,800 Fast Thermo Cycler (Applied Biosystem, Foster City, CA, USA) under the following conditions: 95°C for 3 min, followed by 20 cycles of 94°C for 30 s, 56°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min. The PCR products were diluted with $85\text{ }\mu\text{l}$ ddH₂O and an aliquot run on a 0.9 % agarose gel to check amplification. Selective amplification was performed using *EcoRI* and *MseI* primers with three additional nucleotides at the 3' end and $3.5\text{ }\mu\text{l}$ of the pre-selective PCR diluted as "template". *EcoRI* primer was labeled with one of the following fluorescent dyes: FAM, NED or VIC (Applied Biosystem, Foster City, CA, USA). Each selective reaction was the same as the pre-selective reaction, apart from primers and "template". The PCR program for this step consisted of 95°C for 4 min, followed by ten cycles of 94°C for 10 sec, 65°C for 30 sec, and 72°C for 2 min, followed by 24 cycles

Table 1 Fruit traits for the 21 pomegranate genotypes studied

No.	Genotype ^a	Peel color	Aril color	Taste	Seed hardness
1	'Bihaste Neiriz'	Yellow	White	Sweet	Semi-soft
2	'Bihaste Najaf Abad'	Yellow	White	Sweet	Semi-soft
3	'Bihaste Ladiz'	Yellow	White	Sweet	Semi-soft
4	'Bihaste Dane Sefide Ravar'	Yellow	White	Sweet	Soft
5	'Bihaste Sistan va Baloochestan'	Yellow	White	Sweet	Semi-soft
6	'Bihaste Porbar Shirin'	Yellow	White	Sweet	Semi-soft
7	'Bibaste Shirin Najaf Abad'	Yellow	White	Sweet	Semi-soft
8	'Bitolf Dane Ghermez'	Red	Pink	Sweet-Sour	Semi-hard
9	'Bihaste Khafre Jahrom'	Yellow	White	Sour	Semi-soft
10	'Bihaste Sangan'	Yellow	White	Sweet	Soft
11	'Bihaste Shirin Khabre Baft'	Red	Red	Sweet	Hard
12	'Bidane Kashmar'	Red	Pink	Sweet-Sour	Semi-hard
13	'Bihaste Ghasrodasht'	Yellow	White	Sweet	Semi-soft
14	'Bihaste Shirin Kambar'	Yellow	White	Sweet	Semi-soft
15	'Bihaste Ardestan'	Red	Red	Sweet-Sour	Semi-hard
16	'Bitolf Dane Sefid'	Red	Pink	Sour	Semi-hard
17	'Bihaste Shirin Saravan'	Yellow	White	Sweet	Soft
18	'Bidane Darjazin'	Yellow	White	Sweet	Semi-soft
19	'Bihaste Chenche'	Yellow	White	Sweet	Semi-soft
20	'Bihaste Dane Ghermez Kerman'	Yellow	White	Sweet	Semi-hard
21	'Bihaste Hajiabad'	Yellow	White	Sweet	Soft

^a Bihaste, Bidane and Bitolf all mean 'soft-seeded' in Farsi. Pomegranate genotypes are often named after their place of origin.

of 94 °C for 10 sec, 56 °C for 30 sec, and 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Fluorescently labeled amplification products were mixed together in an equal ratio. Aliquots (2 µl) were mixed with 4 µl ddH₂O, 2 µl loading dye, 2 µl de-ionized formamide and 1 µl ET-500 Rox internal size standard (Pharmacia Amersham, Freiburg, Germany), then denatured at 95 °C for 5 min. The denatured samples were loaded onto an ABI 377 sequencer (Applied Biosystem, Foster City, CA, USA). Results were scored and analyzed using Gel Processor (version 1.0; ABI 377) and Peak Scanner™ software (versions 1.0; Applied Biosystem 2006).

Data analysis

NTSYS software (version 2.02; Rohlf 1998) was used to estimate genetic similarities using Jaccard's similarity coefficient. The similarity matrix developed was used with SPSS software (version 11.5; Norusis 1994) to generate a dendrogram by the UPGMA method using bootstrap analysis. Pattern robustness of the dendrogram was tested using 1,000 resampling permutations.

Results and discussion

After screening 72 AFLP primer combinations on DNA of 21 pomegranate genotypes, 31 primers were found to yield

reproducible and clear polymorphic profiles (Table 2). The remaining 41 primers produced few or no polymorphic peaks or consistent amplicons. Amplified product peaks ranged between 100 and 500 bp for all primers tested. In total, the 31 primers produced 1,290 discrete DNA peaks, which exhibited 503 polymorphisms between two or more pomegranate genotypes.

The genetic similarity coefficient based on AFLP data varied widely from 0.17 to 1.00 across all 21 genotypes (Table 3), with a mean of 0.46. Analysis of the similarity matrix (Table 3) demonstrated that the lowest similarity (0.17) was between 'Bihaste Neiriz' (No. 1) and the genotypes 'Bitolf Dane Ghermez' (No. 8), 'Bitolf Dane Sefid' (No. 16), 'Bihaste Chenche' (No. 19), 'Bihaste Dane Ghermez Kerman' (No. 20) respectively. 'Bihaste Neiriz' from western Iran (No. 1) exhibits similar fruit traits including yellow peel, white aril, sweet taste and semi-soft seed (Table 1) to 'Bihaste Chenche' (No. 19) but differs from the other three genotypes with respect to fruit traits. The fruit traits may provide an instrumental platform for differentiating genotypes relationships in cases such as these; however, AFLP markers should theoretically provide a more accurate and reliable assessment of genotype relationships than fruit traits, as AFLPs relate to DNA sequence differences (Poehlman 1987; Joshi et al. 1999).

The highest similarity (1.00) was within the pairs 'Bihaste Dane Sefide Ravar' (No. 4) and 'Bihaste Hajiabad' (No. 21); 'Bibaste Shirin Najaf Abad' (No. 7) and 'Bihaste Najaf Abad'

Table 2 Amplified fragment length polymorphism (AFLP) informative primer combinations for the 21 pomegranate genotypes studied

No.	Primer combination	Total bands	Polymorphic bands	Polymorphism (%)
1	E-ACG+M-CAA	41	13	31.71
2	E-AAC+M-CTA	53	19	35.85
3	E-AGC+M-CAC	34	14	41.18
4	E-AGG+M-CTC	36	16	44.44
5	E-ACA+M-CTA	32	13	40.63
6	E-AAG+M-CTG	37	15	40.54
7	E-AGC+M-CAA	34	16	47.06
8	E-AGG+M-CTT	48	18	37.50
9	E-AGG+M-CTA	27	10	37.04
10	E-ACT+M-CAC	36	12	33.33
11	E-AAC+M-CTG	13	5	38.46
12	E-ACA+M-CAC	19	7	36.84
13	E-ACA+M-CTT	37	16	43.24
14	E-AAG+M-CTC	35	15	42.86
15	E-ACT+M-CTC	45	16	35.56
16	E-AAG+M-CAG	47	18	38.30
17	E-ACG+M-CTA	44	14	31.82
18	E-ACG+M-CAG	43	15	34.88
19	E-AGC+M-CTA	52	18	34.62
20	E-AGC+M-CAG	36	13	36.11
21	E-AAG+M-CAC	43	16	37.21
22	E-ACT+M-CAA	66	28	42.42
23	E-ACT+M-CTT	49	18	36.73
24	E-ACT+M-CTG	52	19	36.54
25	E-ACT+M-CAG	61	26	42.62
26	E-ACC+M-CTG	33	13	39.39
27	E-ACC+M-CAT	33	15	45.45
28	E-ACC+M-CAG	67	25	37.31
29	E-ACC+M-CAC	55	24	43.64
30	E-ACC+M-CTA	34	16	47.06
31	E-ACC+M-CTC	48	20	41.67
Total		1,290	503	-
mean		41.61	16.23	38.99

(No. 2); ‘Bihaste Sangan’ (No. 10) and ‘Bihaste Shirin Saravan’ (No. 17); and ‘Bitolf Dane Ghermez’ (No. 8) and ‘Bitolf Dane Sefid’ (No. 16). The derived AFLP data indicated genotypes in each pair could originate from the same region, for instance ‘Bihaste Shirin Najaf Abad’ (No. 7) and ‘Bihaste Najaf Abad’ are from the same city (Najaf Abad, Central Iran). Interestingly, Sarkhosh et al. (2009) reported previously high degrees of genetic similarity among these genotypes when using analysis of morphological characteristics. Hence, it can be deduced that each of these pairs had a similar genetic origin. According to the local pomegranate growers and experts, the trees are propagated clonally by cuttings. This invites the hypothesis that different growers gave different names to genotypes in each pair after transferring them locally by cuttings from a single original clone.

According to the dendrogram (Fig. 1), the 21 genotypes were divided into two main groups (A and B): 13 genotypes within group (A) and eight genotypes within group (B), both groups including two sub-groups (I and II). Co-phenetic correlation coefficients indicated a correlation of $r=0.98$ between the similarity matrix and co-phenetic matrix measured from the dendrogram data, indicating good correlation between the similarity matrix and the co-phenetic matrix.

The genotypes in sub-cluster AI are mainly from central and southern Iran and possess the same fruit traits (Table 1), except for ‘Bihaste Shirin Khabre Baft’ (No. 11), which exhibits differences in peel color, aril color and seed hardness. The genotypes ‘Bihaste Dane Sefide Ravar’ (No. 4) and ‘Bihaste Hajiabad’ (No. 21) from southern Iran and ‘Bihaste Sangan’ (No. 10), ‘Bihaste Shirin

Table 3 Genetic similarity matrix for pomegranate genotypes based on AFLP data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	1.00																				
2	0.64	1.00																			
3	0.46	0.40	1.00																		
4	0.53	0.46	0.68	1.00																	
5	0.59	0.52	0.76	0.92	1.00																
6	0.62	0.91	0.40	0.46	0.52	1.00															
7	0.64	1.00	0.40	0.46	0.52	0.91	1.00														
8	0.17	0.30	0.34	0.31	0.36	0.29	0.30	1.00													
9	0.52	0.60	0.48	0.57	0.64	0.58	0.60	0.39	1.00												
10	0.46	0.40	0.66	0.91	0.84	0.40	0.40	0.31	0.55	1.00											
11	0.41	0.59	0.54	0.47	0.54	0.55	0.59	0.53	0.68	0.45	1.00										
12	0.19	0.26	0.31	0.27	0.33	0.25	0.26	0.46	0.44	0.25	0.50	1.00									
13	0.19	0.33	0.31	0.21	0.27	0.31	0.33	0.65	0.44	0.22	0.50	0.55	1.00								
14	0.47	0.48	0.56	0.50	0.53	0.45	0.48	0.33	0.53	0.47	0.61	0.43	0.48	1.00							
15	0.25	0.39	0.36	0.32	0.38	0.37	0.39	0.62	0.57	0.33	0.66	0.53	0.60	0.54	1.00						
16	0.17	0.30	0.34	0.31	0.36	0.29	0.30	1.00	0.39	0.31	0.53	0.46	0.65	0.33	0.62	1.00					
17	0.46	0.40	0.66	0.91	0.84	0.40	0.40	0.31	0.55	1.00	0.45	0.25	0.22	0.47	0.33	0.31	1.00				
18	0.23	0.37	0.34	0.31	0.36	0.35	0.37	0.67	0.55	0.31	0.65	0.73	0.81	0.52	0.76	0.67	0.31	1.00			
19	0.17	0.24	0.34	0.20	0.25	0.23	0.24	0.45	0.33	0.20	0.35	0.47	0.53	0.42	0.53	0.45	0.20	0.57	1.00		
20	0.17	0.24	0.34	0.25	0.30	0.23	0.24	0.45	0.40	0.26	0.44	0.59	0.53	0.42	0.52	0.45	0.26	0.69	0.83	1.00	
21	0.53	0.46	0.68	1.00	0.92	0.46	0.46	0.31	0.57	0.91	0.47	0.27	0.21	0.50	0.32	0.31	0.91	0.31	0.20	0.25	1.00

Fig. 1 Dendrogram of unweighted pair group method of arithmetic averages (UPGMA) based on amplified fragment length polymorphism (AFLP) data showing genetic relationships among the 21 pomegranate genotypes using by bootstrap analysis

Saravan' (No. 17) and 'Bihaste Sistan va Baloochestan' (No. 5) from south-eastern Iran were located together in sub-cluster AII. Genotypes 4, 10, 17 and 21 had been recognized as good soft-seed genotypes based on sensory panel assessment for seed texture in a previous study (Sarkhosh et al. 2009). These genotypes exhibited high similarity coefficients, ranging from 0.91 to 1.00 based on AFLP data. It is hence highly probable that they have the same genetic background as well as geographical origin. Genotype 'Bihaste Ladiz' (No. 3), with a semi-soft character, was also located in this sub-cluster AII. Genotypes in sub-cluster AII exhibited similar taste, peel color and seed color characters. However, a high degree of similarity in fruit traits does not necessarily indicate a similar genetic background and some mutations are not recognizable by AFLP markers (Kumar 1999).

Most pomegranate genotypes located in the main group B are from central Iran, except 'Bidane Kashmar' (No. 12), which is from eastern Iran, and 'Bihaste Chenche' (No. 19) from western Iran. In sub-cluster BI, genotypes with similar fruit traits were grouped close to one another, for example 'Bihaste Ghasrodasht' (No. 13) and 'Bidane Darjazin' (No. 18) are semi-soft seeded genotypes, with a similarity coefficient of 0.81, while 'Bitolf Dane Ghermez' (No. 8) and 'Bitolf Dane Sefid' (No. 16) are semi-hard seeded genotypes, exhibiting a similarity coefficient of 1.00. Although genotypes 'Bidane Kashmar' (No. 12) and 'Bihaste Ardestan' (No. 15) were grouped together in the sub-cluster BI and exhibit similar fruit traits to commercial

genotypes in their growing regions with respect to seed structure, taste and peel color, they are from different geographic backgrounds, from eastern and central Iran respectively, two main areas of pomegranate production in Iran. Similarly, 'Bihaste Chenche' (No. 19) from the west grouped with 'Bihaste Dane Ghermez Kerman' (No. 20) from central Iran in sub-group BII (Fig. 1). Genotypes 19 and 20 have very similar small fruit with yellow peel color, white arils and a sweet taste, differing only in degree of seed hardness. We suggest that the genotypes 12/15 and 19/20 had been recognized as desirable and had been transported deliberately from one growing region to another.

In an earlier study (Sarkhosh et al. 2009), 36 fruit traits and 14 randomly amplified polymorphism DNA (RAPD) polymorphic primers were used to discriminate the same 21 genotypes. Based on the morphopomological traits, the genotypes were divided into three groups, at a distance of 15% on the dendrogram, on the basis of fruit juice and seed traits such as titratable acidity (TA), total soluble solids (TSS), 100 aril fresh weight, aril diameter and woody portion index. Based on RAPD data, 16 genotypes were located in one group, and five genotypes divided into three groups (Sarkhosh et al. 2009). In most cases in that study, in contrast to the current one, the grouping by RAPD profiles did not agree with the grouping by fruit traits or geographical origin of genotypes.

In order to test our previous results and to minimize possible experimental error, we have now applied more

reliable AFLP markers to discriminate the same 21 soft-seeded pomegranate genotypes. Pomegranate genotypes based on AFLP profiles clustered together with important fruit traits such as peel and aril color, seed texture, as well as geographical origin in many cases. Grouping of pistachio genotypes based on geographic distance using AFLP markers has also been reported (Karimi et al. 2009). Results from this research illustrated that the pomegranate collection may contain genetic duplication. This could have arisen through renaming of clonally propagated material by growers as discussed above, or through mislabeling, or incorrect classification. Mislabeling and duplication have already reported following molecular marker analysis in the Saveh pomegranate collection (central Iran) by Zamani et al. (2007). In the current study, such duplications can be seen in sub-cluster AI; between ‘Bihaste Shirin Najaf Abad’ (No. 7) and ‘Bihaste Najaf Abad’ (No. 2), sub-cluster AII; ‘Bihaste Dane Sefide Ravar’ (No. 4) and ‘Bihaste Hajiabad’ (No. 21), sub-cluster AII; ‘Bihaste Sangan’ (No. 10) and ‘Bihaste Shirin Saravan’ (No. 17), and sub-cluster BI; ‘Bitolf Dane Ghermez’ (No. 8) and ‘Bitolf Dane Sefid’ (No. 16). These results strongly suggest that each of these pairs could be considered as one genotype in future pomegranate breeding programs.

In conclusion, the results derived from this study demonstrate that AFLP markers can be effectively used in future germplasm management, genotype discrimination and identification of redundancy in pomegranate collections in Iran. These primer combinations are also being used in the development of a pomegranate genetic linkage map in populations segregating for key fruit characters, with the goal of enabling identification of DNA markers linked to desirable characteristics and marker assisted selection to improve the efficiency of new cultivar development in future breeding programs.

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