Australian Journal of Crop Science AJCS
AJCS 6(2):268-275 (2012) ISSN:1835-2707

Genetic variation among Iranian pomegranates (*Punica granatum* L.) using RAPD, ISSR and SSR markers

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Abstract

Pomegranate (*Punica granatum* L.) is one of important horticultural plants in Iran with about 800 genotypes cultivated in several regions of country. Three molecular markers including RAPD, ISSR and SSR were used to evaluate genetic diversity of thirty six Iranian pomegranate genotypes. Genetic parameters consisted of effective alleles (Ne), Nei genetic diversity (H), Shannon index (I) and polymorphic information content (PIC) were calculated based on molecular data. SSR markers with their co-dominant nature showed the highest value of genetic parameters aforementioned except PIC value among all markers. Combined data of three markers showed higher genetic diversity than two ISSR and RAPD markers. UPGMA tree obtained from combined molecular data (total 235 amplicons) discriminated pomegranate genotypes in three major groups. Principle Component Analysis (PCA) based on the first two components confirmed clustering. The homonymous, synonymous and/or mislabeled genotypes were identified using three molecular markers. The analysis of molecular variance (AMOVA) indicated no significant genetic variation (p= 0.27) between pomegranate genotypes in different localities (seven locality groups). Only 2% of overall genetic variation was due to among locality groups differences while 98% of variation was due to within group differences.

Keywords: AMOVA, combined molecular markers, homonym, PIC, *Punica granatum* L.
Abbreviations: Analysis of Molecular Variance (AMOVA), Inter-simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), principle Component analysis (PCA), Polymorphic Information Content (PIC), Restriction Fragment Length Polymorphism (RFLP), Unweighted Paired Group Mean using Arithmetic Average (UPGMA), Neighbor Joining (NJ).

Introduction

Pomegranate (*Punica granatum* L.), is one of oldest fruit trees known to human (4000 and 3000 BC). Pomegranate fruit juice makes an excellent drink which contains potassium, phosphorous and calcium as well as micronutrients like iron, manganese, zinc and copper. The antioxidant, immune-boosting, and anti-carcinogenic properties of the pomegranate offers its multiple potential medical applications (Kaplan et al., 2001). The origin of pomegranate is considered to be in central of Asia especially the Transcaucasia- Caspian region in Iran (Harlan, 1992), from where it has spread to the rest of the world (Levin, 1994; Verma et al., 2010). Iran as a center of pomegranate growth possesses more than 800 genotypes which have been collected and maintained in Yazd and Saveh germplasm (Behzadi-Shahrebabaki, 1998). A high genetic diversity is expected to occur in pomegranate genotypes cultivated in Iran due to long historical cultivation and various environmental conditions in which, these cultivars are growing. Therefore our study is concerned with identification of genetic diversity (by using different molecular markers) in pomegranate cultivars as these data may be of further use in hybridization and selection programs. Molecular markers have been overcome limitations of morphological and biochemical markers due to the influence of environment on the performance of genotypes. A wide range of molecular markers have been used to assess genetic diversity of pomegranate cultivars as well as wild genotypes from different parts of the world. Random amplified polymorphic DNA (RAPD) markers have provided reliable and highly polymorphic information to discriminate pomegranate cultivars (Ercisli et al., 2011; Hasnaoui et al., 2010a; Narzary et al., 2009; Durgaç et al., 2008). AFLPs (Amplified Fragments Length Polymorphism) are another marker which has been used to evaluate genetic diversity within and among Chinese pomegranate populations (Yuan et al., 2007) and Tunisian cultivars (Jbir et al., 2008). Up to now, more than 137 microsatellite loci in pomegranate genome (Soriano et al., 2011; Curro et al., 2010; Hasnaoui et al., 2010b) showing different ranges of genetic polymorphism in the genotypes studied. Inter-Simple sequence Repeats (ISSR) analysis is considered as another efficient molecular marker, showing genetic variation in the wild pomegranate populations studied

in Western Himalaya region (Narzary et al., 2010). Similarly morphological, cytological and DNA markers (RAPD, AFLP, SSR and ISSR) have been used to evaluate the genetic variability of Iranian pomegranates. These studies showed the occurrence of high genetic diversity among Iranian genotypes studied at both cytogenetic (Sheidai and Noormohammadi, 2005) and molecular levels including RAPD (Zamani, 1990; Sarkhosh et al., 2006; Zamani et al., 2007; Sheidai et al., 2007; Noormohammadi et al., 2010), AFLP (Moslemi et al., 2010) and SSR markers (Koohi-Dehkordi et al., 2007; Pirseyedi et al., 2010; Ebrahimi et al., 2010). The present study was performed with the aim to indentify genetic diversity present in 36 (30 not reported genotypes) pomegranate cultivars of Iran by using RAPD, ISSR and SSR molecular markers and attempt to evaluate the usefulness of the three molecular markers used in combination for discriminating pomegranate genotypes as well as identification of mislabeled genotypes.

Results

RAPD analysis

Thirteen out of thirty RAPD primers as well as one combined RAPD primer produced reproducible bands across 36 pomegranate cultivars studied. The number of amplified fragments varied from 4 in the RAPD primer OPA02 to 19 in the RAPD primer OPI18. The size of RAPD bands obtained varied from 350 to 2500 bps (Table 2). The average percentage of polymorphism obtained for these molecular markers was 52.61% with OPB05 primer showing the highest percentage of polymorphism (100%) and OPC10 showing the lowest percentage (0%). The highest number of unique bands were observed in OPA10 (4), OPB05 (4) and OPI18 (4) primers. These 12 specific alleles were observed in Alakshirin cultivar. The highest mean values of effective alleles (Ne), Shannon index (I) and Nei's genetic diversity (He) were observed in OPC08 primer (1.655, 0.538 and, 0.370 respectively) while the lowest values of the same parameters occurred in OPC10 primer (Table 2). The PIC value ranged from 0.000 (OPC10) to 0.500 (OPR08).

ISSR analysis

Six out of ten ISSR primers used as well as one combined primer produced 106 reproducible fragments across 36 pomegranate genotypes studied. The number of ISSR bands obtained varied from five (UBC-810 and UBC-834) to twenty-two (GA)9C and (GA)9T. The size of fragments obtained ranged from 200 to 3000 bps (Table 2). The average percentage of polymorphism obtained in ISSR markers was 45.42% ranging from 5% in (GA)9T to 60% in UBC-810 and UBC-834. The PIC values obtained ranged from 0.272 in the ISSR primer UBC-834 to 0.494 in the primer UBC-811. The highest values of the number of effective alleles (Ne), Shannon index (I) and Nei's genetic diversity (He) occurred in UBC-811 marker (1.540, 0.414 and 0.289 respectively) while the lowest values for the same parameters were observed in (GA)9A primer (1.263, 0.206 and 0.143 respectively).

SSR analysis

Two (POM-AGC11 and ABRII-MP30) out of four pomegranate microsatellite markers used produced scorable fragments. The number of alleles obtained per locus varied from 1 (ABRII-MP30- 176bp) to 3 (POM-AGC11- 179, 181, 183 bps) with a mean value of 2.0. The value of observed heterozygosity ranged from 0.000 to 0.667 (mean value = 0.333) while the mean value of expected heterozygosity was 0.254 ranging from 0.000 to 0.508. PIC value was obtained 0.000 for ABRII-MP30 because of lack of polymorphic alleles and POM-AGC locus had PIC value of 0.405. In both SSR loci studied, the Hardy-Weinberg equilibrium test was not significant ($P < 0.05$).

Genetic relationships

Based RAPD and ISSR data, different similarity coefficients were determined among the genotypes upon which UPGMA and NJ dendrograms were constructed. In RAPD, the highest value of cophenetic correlation ($r = 0.91$) was obtained for UPGMA dendrogram constructed from Jaccard similarity while in ISSR, the highest value of cophenetic correlation ($r = 0.90$) was obtained for UPGMA dendrogram constructed from Dice similarity matrix. Cophenetic correlation between Dice similarity and UPGMA dendrogram showed the highest value ($r = 0.86$) in SSR data. The values of similarity coefficient obtained in RAPD analysis ranged from 0.59 to 0.97 among the genotypes studied, while the same values ranged from 0.74 to 0.95 in ISSR analysis. In SSR similarity matrix, the highest and lowest similarity values were 1.00 and 0.40 respectively. The Mantel test performed among the genetic distance matrices obtained from RAPD, ISSR and SSR analyses, showed a non significant regression ($p < 0.05$) between RAPD versus ISSR ($R^2 = 0.212$), RAPD versus SSR ($R^2 = 0.005$), and also between ISSR versus SSR data ($R^2 = 0.007$). Meanwhile regression for ISSR and RAPD+ISSR+SSR combined data was slightly high ($R^2 = 0.682$, Fig. 1). The dendrograms obtained from RAPD, ISSR and SSR markers did not agree completely to each other. UPGMA dendrogram obtained from the pooled data of RAPD, ISSR and SSR analyses (total 235 amplicons) is presented in the Fig. 2. The 36 genotypes are grouped into three major clusters. Cluster I consisted of Alak-Shirin cultivar which stands alone and far from the other cultivars studied. Cluster II is comprised of two sub-clusters (IIa and IIb). Sub cluster IIa contained 29 pomegranate cultivars among which Dom-Alak-Chatrood-Shireen and Ghorsgeloo-Kootah-Bafgh as well as Abdandan-Ravar and Torsh-Nar-Tasooj-Shabestar showed the highest degree of similarity. Sub cluster IIb consisted of six cultivars. Studying clusters based on each markers in close, revealed that neither RAPD nor ISSR data discriminated two cultivars named Meykhosh-Khoosef-Birjand and Khatooni-Daneghermez-Zavareh (99% similarity) while, cluster based only SSR data could differentiate them (data not shown). Therefore, these cultivars placed separately in cluster built on combined RAPD, ISSR and SSR data (Fig. 2). Two genotypes with similar name – AghamohammadAli - in different gardens of Saveh germplasm are placed in different clusters with about 80% similarity in RAPD, ISSR and SSR clusters studied separately (Data not shown) as well as combined data (Fig. 2). The same is true for two genotypes labeled one name "Roodaki-DaneSefid-Bafgh". PCA ordination based on two first components (factors) confirmed cluster analysis (Fig. 3) using combined molecular data when Eigen value for first and second components were 20.88% and 9.45% respectively. The analysis of Molecular variance (AMOVA) using molecular data indicated no significant genetic difference ($p= 0.27$) between pomegranate genotypes cultivated in different localities (seven provinces). However, only 2% of overall genetic variation was due to among locality groups differences while 98% of variation specified

Table 1. List of Iranian pomegranate cultivars studied and their localities

No	Cultivar	Locality (province)	No	Cultivar	Locality (Province)
1	Alak-Shirin	Saveh(Markazi)	19	Tokhmooshi Taft	Taft (Yazd)
2	Poost-Sefid-Shirin	Saveh (Markazi)	20	Malas Shirin Saveh	Saveh(Markazi)
3	AghamohammadAli[a]	Saveh(Markazi)	21	Khatooni Daneghermez Zavareh	Zavareh (Isfahan)
4	Poost-Sefid-Bihaste	Mazandaran	22	Domalak Chatrood Shirin	Chatrood (Kerman)
5	Poost-Sefid-Torsh	Paveh (Kermanshah)	23	Zagh Mashbaghi Tabas Malas	Tabas (Yazd)
6	Malas Shirin	Saveh(Markazi)	24	Malas Greh Abrkooh	Abarkooh (Yazd)
7	Tabestani Torsh	Saveh(Markazi)	25	Meykhosh Khoosef Birjand	Birjand (Khorasan)
8	Malas Torsh Saveh	Saveh(Markazi)	26	AghamohammadAli[a]	Saveh(Markazi)
9	Alak Torsh	Saveh (Markazi)	27	Narak Rafsanjan	Rafsanjan (Kerman)
10	Poost Siyah Torsh	Ardakan (Yazd)	28	Sanaee Saveh	Saveh (Markazi)
11	Atabak Khafr Jahrom	Jahrom (Fars)	29	Roodaki Danehsefid Bafgh[a]	Bafgh (Yazd)
12	Abdandan Ravar Shirin	Ravar (Kerman)	30	Ghorsgeloo Kootah Bafgh	Bafgh (Yazd)
13	Torsh Nar Tasooj Shabestar	Shabestar(West Azarbayejan)	31	Kederpoost Nazok Kazeroon	Kazeroon (Fars)
14	Sagi Torsh Khasoof Birjand	Birjand (Khorasan)	32	Malas Sarvi Paveh	Paveh (Kermanshah)
15	Roodaki Danesefid Bafgh[a]	Bafgh (Yazd)	33	Shirin Bihasteh Hajabad	Hajabad (Hormozgan)
16	Poost Ghermez Ashkzar	Ashgzar (Yazd)	34	Meykhosh Tasooj Shabestar	Shabestar(West Azarbayejan)
17	Shirin Poostkoloft Bejeston	Bejeston (Khorasan)	35	Kalegavi Torbat	Torbat (Khorasan)
18	Torsh Poost Sefid Abarkooh	Abarkooh (Yazd)	36	Shahvar Dadashi darajeyek	Ashgzar (Yazd)

a : genotypes with the same names in two garden of Saveh germplasm.

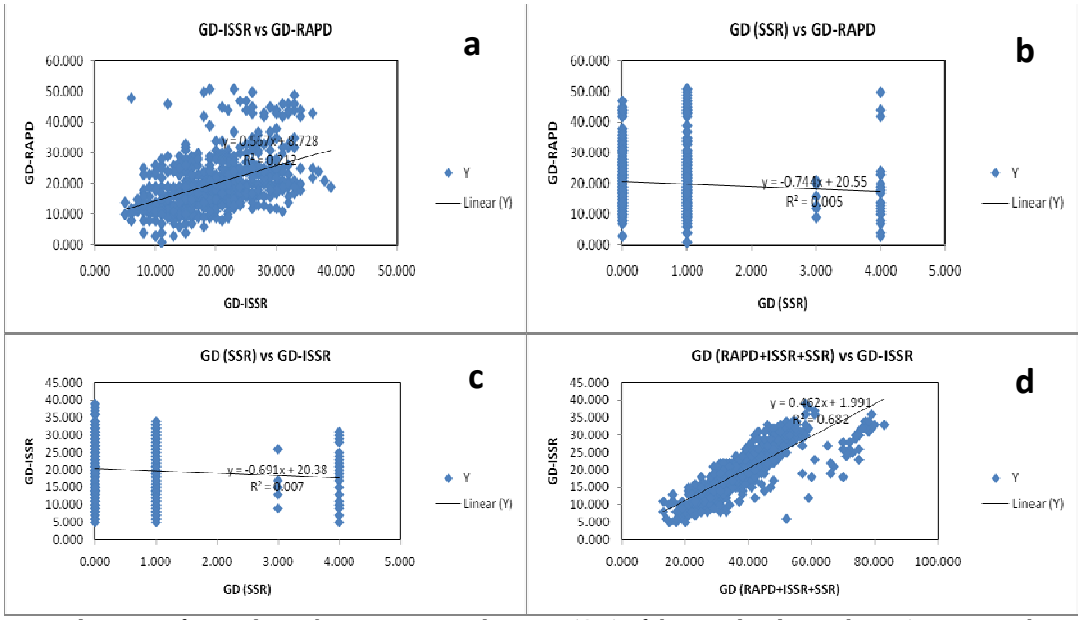

Fig 1. Regression diagrams of Mantel tests between genetic distances (GD) of three molecular markers. a) regression between RAPD (GD) and ISSR (GD) $R^2 = 0.212$; b) regression between RAPD (GD) and SSR (GD) $R^2 = 0.005$; c) regression between SSR (GD) and ISSR (GD) $R^2 = 0.007$; d) regression between RAPD+ISSR+SSR (GD) and ISSR (GD) $R^2 = 0.682$

to within group differences. The pair wise Fst analysis also showed no significant differences between all locality groups.

Discussion

RAPD data showed high polymorphism (53%) among cultivars supporting the results obtained by Sarkhosh et al. (2006), Sheidai et al. (2007) and Zamani et al. (2007) in other Iranian pomegranate genotypes. Similarly, Hasnaoui et al. 2010(a) and Ersicli et al. 2011 reported high RAPD polymorphism in Tunisian cultivars (83%) and Turkish wild-grown pomegranate trees (91%). RAPD and ISSR markers are dominant molecular markers but ISSR markers showed a higher mean value for the number of effective alleles, Shannon index, Nei's genetic diversity and PIC. SSR markers with their codominant nature showed the highest value of these genetic parameters (except for PIC) among the three molecular markers used. This study represents the first attempt to used ISSR markers with GA repeated motifs beside other DNA markers to differentiate Iranian un-worked pomegranate genotypes. Inter-simple sequence repeats markers have been used in discriminating wild species as well as plant cultivars and have been introduced as a useful tool in several DNA marker studies (Zietkiewicz et al., 1993;

Table 2. Genetic parameters based on RAPD and ISSR markers.

RAPD loci	Size range	Total band	P	Unique band	Ne	I	He	PIC
OPA02	600-1450	4	25	0	1.096	0.113	0.069	0.082
OPA04	600-1500	8	50	0	1.206	0.204	0.131	0.454
OPA05	400-1400	9	22	0	1.125	0.120	0.079	0.216
OPA10	950-1800	10	90	4	1.461	0.362	0.247	0.498
OPA13	450-1100	5	20	0	1.139	0.120	0.082	0.110
OPB05	430-1600	11	100	4	1.445	0.380	0.251	0.418
OPC05	350-2500	17	59	0	1.395	0.336	0.228	0.462
OPC06	600-1450	8	25	1	1.129	0.096	0.066	0.304
OPC08	680-1600	10	80	0	1.655	0.538	0.370	0.478
OPC10	350-1520	9	0	0	1.00	0.00	0.00	0.00
OPC12	650-2400	9	44	0	1.383	0.290	0.205	0.312
OPI18	410-2500	19	89	4	1.244	0.255	0.155	0.460
OPR08	600-1200	5	80	0	1.531	0.429	0.292	0.500
ISSR loci								
(GA)9A	400-1500	17	35	0	1.263	0.206	0.143	0.338
(GA)9C	320-1650	22	50	0	1.388	0.300	0.209	0.422
(GA)9T	200-1700	22	5	0	1.311	0.252	0.173	0.402
(GA)9A/UBC834	250-1400	18	44	0	1.466	0.356	0.250	0.362
UBC811- (GA)8C	600-3000	17	64	1	1.540	0.414	0.289	0.494
UBC810-(GA)8T	480-1400	5	60	0	1.509	0.389	0.274	0.470
UBC834-(AG)8YT	400-1600	5	60	0	1.377	0.335	0.224	0.272

Polymorphism% (P); Number of effective allele (Ne); Shannon index (I); Nei's genetic diversity (He); Polymorphic Information Content (PIC)

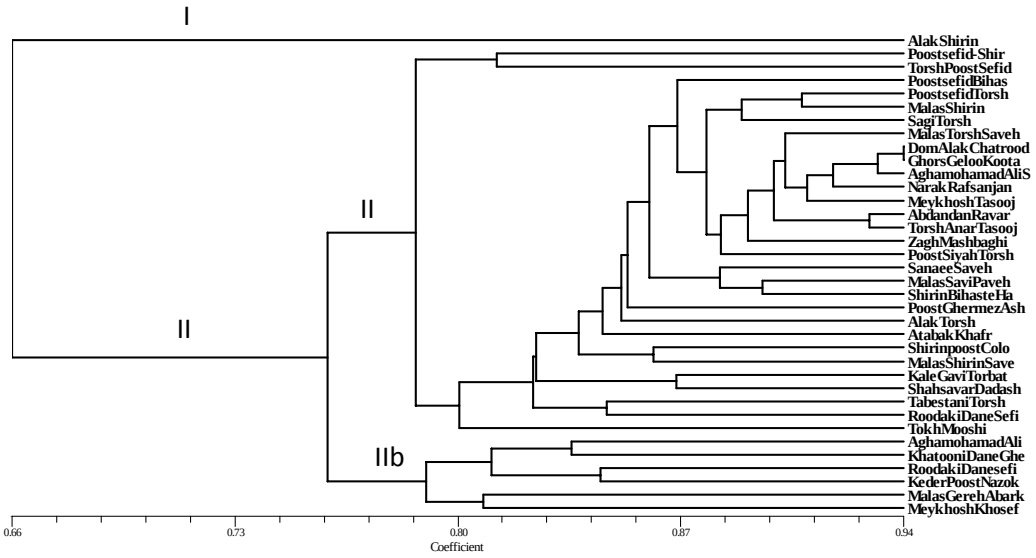

Fig 2. UPGMA dendrogram of pomegranate cultivars based RAPD, ISSR and SSR markers

Hess et al., 2000; Zhao et al., 2008; Rout et al., 2009; Salukhe et al., 2009; Narzary et al., 2010; Lu et al., 2011). In ISSR analysis, both single and combined primers produced reproducible and informative data. Due to high PIC value obtained in majority of RAPD and ISSR markers, these markers could be used for identification and characterization of pomegranate genotypes. However, both dominant markers used (RAPD and ISSR) revealed high genetic variability among pomegranate genotypes. The reason for higher genetic diversity revealed by microsattelite markers may be their co-dominant or multiallelic nature, hypervariability, high information content and amenability to automation (Powell et al., 1996; Rafalski et al., 1996; Morgante and Olivieri, 1993). However, lower PIC value obtained by SSR markers compared to that of RAPD and ISSR markers maybe only due to low number of SSR loci studied. Similar results have been reported by other workers (Ebrahimi et al., 2010; Hasnaoui et al., 2010b; Pirseyedi et al., 2010; Soriano et al., 2011) due to the narrow gene pool of the cultivated genotypes which are usually propagated in clonal manner (Soriano et al., 2011). Pair-wise Mantel test among the three molecular markers used produced a low and no significant regression values between these markers. The main reason might be due to different genome portions being amplified by these different DNA markers. The regression for SSR and RAPD+ISSR+SSR combined data was low (0.0001) while it was highest for ISSR and RAPD+ISSR+SSR based similarities (0.69). This showed that ISSR data was more

Table 3. Means of genetic parameters based on three molecular markers studied.

Molecular markers	Na	Ne	I	He	PIC
RAPD	1.597(0.044)	1.310(0.034)	0.266(0.026)	0.178(0.018)	0.330
ISSR	1.514(0.042)	1.393(0.042)	0.308(0.031)	0.214(0.022)	0.394
SSR	2.00 (1.00)	1.516(0.516)	0.391(0.391)	0.254(0.254)	0.202
RAPD/ISSR/SSR	1.779(0.516)	1.432(0.274)	0.338(0.205)	0.224(0.134)	0.308

Number of different allele (Na); Number of effective allele (Ne); Shannon index (I); Nei's genetic diversity (He); Polymorphic Information Content (PIC). The numbers in parenthesis: Standard deviation.

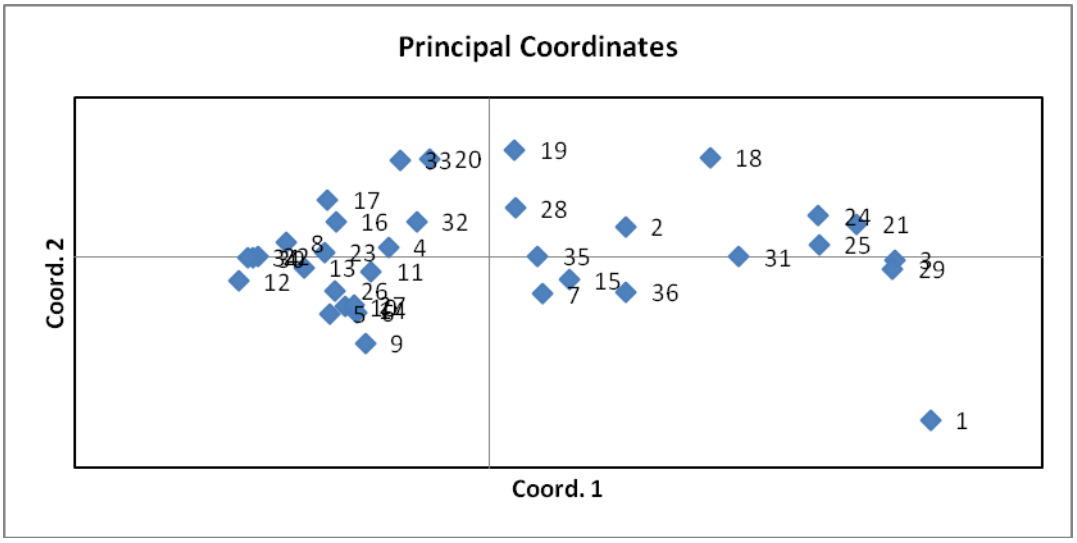

Fig 3. Principle Component Analysis (PCA) ordination based on combined RAPD, ISSR and SSR data. Numbers of cultivars are according to table 1.

close to RAPD+ISSR+SSR combined markers. UPGMA tree of the combined data, separated the cultivars labeled identically as Aghamohammad-Ali but collected from different gardens of Saveh germplasm in different clustered, indicating their mislabeling. The genetic differences also have been detected in genotypes with similar name "Roodaki-DaneSefid-Bafgh" based on molecular data. These genetic differences in three molecular data are the reason for the separation of these accessions. Like other trees, homonymy case is one of the problems of Iranian pomegranate germplasm. Therefore, these cultivars may be considered as homonyms or mislabeling. Discrimination of homonymous cases (AghamohammadAli, Alak Torsh and Poost Sefid Bihaste genotypes) in pomegranate cultivars has also been reported by using RAPD by Sarkhosh et al. 2006. On the other hand, Meykhosh-Khoosef-Birjand and Khatooni-Daneghermez-Zavareh produced partially identical allelic profiles (99% similarity). Therefore, they may be considered as synonyms or mislabeled accessions due to morphological difficulties. Transferring genotypes from one location to other locations is probability based on morphological characteristics or coding genotypes in different collections available in Iran which might cause mistakes or mislabeling. The results obtained from AMOVA analysis and pair wise Fst test performed between locality groups (populations) showed no significant difference among the localities studied. The result of AMOVA also supported genetic relationship between genotypes by cluster analysis, where genotypes belong to different localities grouped together. Moslemi et al. 2010 also did not found significant differences between Iranian pomegranate genotypes from Markazi, Yazd and Kerman provinces by using AFLP markers. Similar result was reported by Yuan et al. 2007 using same markers on Chinese pomegranate cultivars. The high level of genetic diversity within groups (populations) and low level of that among them may be explained by the clone propagation of pomegranate and the extensive gene flow between different localities in Iran due to material exchanges. The result obtained agrees with the general observation that woody perennial outbreeding species maintain most of their variation within population (Hamrick and Godt, 1989, Bartish et al., 2000). In conclusion, using different DNA markers with different nature could help to differentiation of pomegranate genotypes and evaluate their genetic variations. Combined molecular data can bring more information and clear discrimination of genotypes. In addition, some homonymous and synonymous and or mislabeling pomegranate genotypes have been distinguished by molecular data studied.

Materials and Methods

Plant materials

Thirty-six Iranian pomegranate cultivars of the Saveh germplasm collected from seven different provinces were used for molecular studies (Table 1).

Fresh leaves were collected randomly from 3-5 plants in each genotype and dried in silica gel powder. Genomic DNA was extracted using CTAB-activated charcoal protocol (Križman et al., 2006). The extraction procedure was based on activated charcoal and Polyvenyl Pyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions, promoting high-molecular weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

RAPD and ISSR amplification

Thirty RAPD primers of (A, C, I, M and R sets) from the Operon Technologies, Calif., USA and their combination were used. RAPD reactions were conducted in 20 µL containing 50 ng of template DNA solution; 1X PCR buffer (10 mM Tris–HCl buffer at pH 8; 50 mM KCl); 1.5 mM Mg^{2+}; 200 µM dNTP mix ; 0.4 µM primer and 0.4 unit *Taq* polymerase (Bioron, Germany). Thermal program was carried out in thermocycler (Techne, UK). The profile used consisted of an initial denaturation for 3 min at 94° C, followed by 35 cycles in three segments: 1 min at 92°C, 1 min at 36°C, 1 min at 72 and final extension for 10 min at 72°C. The ISSR primers used as well as their combination were selected in a set of ten primers; UBC807, UBC810, UBC811, UBC823 UBC834 and UBC849, commercialized by UBC (the University of British Columbia, Canada) and (GA)9A, (GA)9T, (GA)9C, (CA)9GT. PCR reactions were performed in a 25 µL volume containing 10 mM Tris–HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP; 0.4 µM of a single primer; 20 ng genomic DNA and 0.4 unit of *Taq* DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (UK) with following program: 3 min initial denaturation step 94° C, 30 s at 94° C; 45 s at 52° C, 1 min at 72° C. The reaction was completed by final extension step of 5 min at 72° C. Amplification products were visualized by running on 2% agarose gel in 0.5 X TBE buffer system, followed by ethidium bromide (0.5 µg mL^{-1}) staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany).

Microsatellite amplification

Four SSR primers specifically produced for pomegranate were used via. POM021 (Hasnaoui et al., 2010b), POM-AGC5, POM-AGC11 (Currò et al., 2010) and ABRII-MP30 (Pirseyedi et al., 2010). Amplification of microsatellites was performed in PCR reactions in a total volume 20µl, containing 20 ng genomic DNA, 1X supplied PCR buffer (Bioron, Germany), 2 mM MgCl2, 200µM of each dNTP (Bioron, Germany), 1 unit of *Taq* DNA polymerase (Bioron, Germany) and 0.2 µM of forward (fluorescently labeled) and reverse primers. PCRs were carried out on a thermal cycler (Techne, UK) programmed with a denaturation at 94ºC for 5 min, 35 cycles of 94ºC for 1 min, the annealing temperature 54 ºC for 1 min and 72ºC for 1.5 min and final extension at 72ºC for 15 min for POM-AGC5 and POM-AGC11 loci while thermal program for POM21 locus was carried out as following program: initial step of 3 min at 95_C followed by 10 touchdown cycles of 30 s at 94ºC, 40 s at 65ºC (-1ºC per cycle), 30 s at 72ºC and 25 cycles of 30 s at 94ºC, 30 s at 55ºC, 40 s at 72ºC with final extension time of 8 min at 72ºC.

The following reaction conditions were used for ABRII-MP30: 5 min at 95 °C, followed by 10 touchdown cycles of 30 s at 95 °C, 45 s at 60 °C (1 °C lower per cycle) and 40 s at 72 °C, and 25 cycles of 30 s at 95 °C, 30 s at 50 °C and 40 s at 72 °C, with a final extension step of 7 min at 72 °C.

Finally, the analysis was carried out on an automatic capillary sequencer ABI 3130 Genetic Analyzer (Applied Biosystems/HITACHI) using fluorescent dyes (FAM and HEX), and fragment sizes were determined using internal standards.

Data analysis

Reproducibility of amplified DNA fragments was examined by repeating PCR reactions as well as running on the gel for 3 times. Reproducible bands of each locus of RAPD and ISSR markers were scored as binary characters (present = 1, absent = 0). Combined data matrix obtained from all 3 molecular markers was used for further analysis. In SSR study, the detection of amplification products was carried out with an automated sequencer (ABI PRISM 3130 DNA sequencer, Applied Biosystems, Foster City, CA) at Laboratory of Kowsar Biotech Co. (Tehran, Iran). Sizing was performed using the program Peak Scanner ver. 1.0 from Applied Biosystems. For studying the informative potential of molecular markers and genetic diversity among genotypes including the effective number of alleles, percentage of polymorphic loci, effective alleles (Ne), Shannon's index (I), Heterozygosity (He), Polymorphic Information Content (PIC) and Nei's genetic diversity (H) were calculated based on frequency of alleles of each locus. POPGENE version 1.31 (Yeh et al., 1997), Cervus 2.0 (Marshall et al., 1998) and GenAlex 6.4 (Peakall and Smouse, 2006) were used for these analyses. For grouping of the cultivars, UPGMA (Unweighted Paired Group Mean using Arithmetic Average) and Neighbor Joining (NJ) clustering based on different similarity matrices (Jaccard and Simple matching coefficient) as well as ordination plot based on Principal Component Analysis (PCA) were used. Cophenetic correlation was determined to check the fit of dendrograms obtained (Podani, 2000), while Mantel test (Mantel, 1967) was performed for estimating correlation between RAPD, ISSR and SSR similarity matrices. NTSYS-pc version 2.02 (Rohlf, 1998) and GenAlex 6.4 were used for tree construction and PCA plot.

Acknowledgments

The authors gratefully acknowledge Science and Research Branch, Islamic Azad University (IAU) and Shahid Beheshti University.

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