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Evaluation of Iranian pomegranate collection using simple sequence repeat and morphological traits

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ABSTRACT

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Pomegranate, *Punica granatum* L., is one of the oldest cultivated fruit species. This study used morphological data and a set of simple sequence repeat markers to investigategenetic diversity among 202 Iranian pomegranate accessions during the 2010 and 2011 growing seasons at Saveh Research Station, Saveh, Iran. Principal component analysis showed that leaf traits were predominant in the first and second component during both years, indicating that these traits are not only useful in assessing genetic diversity, but also for characterizing pomegranate germplasm. There was high correlation between the length of style and flower shape, implying that these traits are directly associated with tree performance. There was also close correlation between leaf length with leaf width, and total leaf length as well as and flower traits such as flower diameter and width. Twenty-three alleles (ranging from two to nine per locus) were detected using seven SSR markers with ABRII-MO26 showing the highest level of polymorphism. The average expected heterozygosity and mean PIC values were 0.36 and 0.34, respectively.Cluster analysis showed a simple matching coefficient ranging from 0.24 to 1 indicating high genetic diversity. *Punica* microsatellite markers and morphological characters revealed a relatively high genetic diversity among 202 pomegranate accessions. This great variation in the pomegranate collection of Saveh Research Station ensures the future of pomegranate breeding programs in Iran. Strategic research on the base collection and characterization of accessions provides useful information to breeding programs and will enhance the development of core collections.

Keywords*: genetic diversity, heterozygosity, molecular markers, morphological characters, pomegranate*

INTRODUCTION

Pomegranate, *Punica granatum* L., is a deciduous fruit tree thought to be indigenous to the Iran region (Stover and Mercure, 2007), where edible pomegranates were cultivated as early as 3000 BC (Anarinco, 2006). It is also thought to be native to Turkey (Ercisli *et al.*, 2007) and early data indicates that it soon spread tothe Mediterranean countries (Awamleh *et al.*, 2009). Cultivation of pomegranate has greatly expanded in recent years and it is currently grown on about 60,000 hectares (MAGRAMA, 2014). There are 764 varieties and genotypes of *P. granatum* in the Iranian national pomegranate collection, which are grown and maintained mainly in the Saveh and Yazd Research Stations.

Genetic diversity of plant germplasm is an important basis for conservation biology and genetic improvement (Zabeau *et al.*, 1993). Although classical phenotypic features are extremely useful, phenotypic identification efficiency may be reduced by several factors such as age, development stage, and environmental factors. To overcome these limitations, a large panel of PCR-based methods –such as AFLP, RAPD, ISSR, and SSR –with a wide range of complexity has been developed to examine the genetic diversity between and within fruit species.

Several molecular markers such as AFLP (Jubrael *et al.*, 2005; Awamleh *et al.*, 2009), RAPD (Sarkhosh *et al.*, 2006; Ercisli *et al.*, 2007; Zamani *et al.*, 2007; Durgac *et al.*, 2008) and 18s- 28s rDNA intergenic spacer RFLP (Melgarejo *et al.*, 2009) have been used to study pomegranate genetic diversity. SSRs are multi-allelic and thus have high potential for use in evolutionary studies

(Schloetterer *et al.,* 1991; Chao *et al.,* 2007) and studies on genetic diversity (Salem *et al.,* 2008). Microsatellites are currently one of the most promising molecular marker types able to identify or differentiate genotypes within species. Their codominant inheritance, high level of polymorphism, and easy handling make them extremely useful for many different applications (Devos *et al.,* 1995; Prasad *et al.,* 2000). This study used SSR markers and morphological data to reveal the extent and distribution of genetic diversity among 202 accessions of *P. granatum* held in the national pomegranate collectionat Saveh Research Station, Iran.

MATERIALS AND METHODS

This study was conducted during 2010 and 2011 on the pomegranate collection at the Saveh Research Station and the Agricultural Biotechnology Research Institute (ABRII), Karaj, Iran.

Plant material

A total of 202 pomegranate accessions were selected from the Iranian national pomegranate collection maintained at Saveh Research Station. This collection was established in 1986 by the vegetative propagation of pomegranate accessions collected from different regions in Iran (Fig. 1). Names and codes of the accession are given in Table 1; accessions are labeled according to their origin province number, material number, and taste.

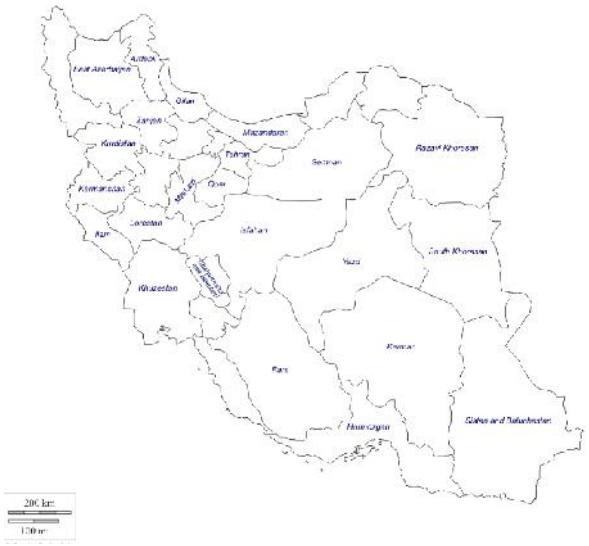

Fig. 1.Twenty provinces in of Iran from where 202 pomegranate accessions were collected.

Evaluation of morphological characteristics

In accordance with pomegranate descriptors (Melgarejo *et al.*, 1997), 21 morphological traits (8 qualitative and 13 quantitative) were evaluated on all accessions. Traits included; general shape of flower, shape of petals, number of petals, petal length, petal width, petal length: width ratio, length of style, leaf shape, leaf apex shape, leaf border color, leaf length, leaf width, leaf length: width ratio, total leaf length (leaf length with petiole), petiole margin color, petiole length, wing length, number of sepals, flower diameter, flower length, flower length: diameter ratio.

Microsatellite analysis

Fully-grown fresh leaves, free of pestsand disease symptoms, were washed with distilled water, dried, then tightly wrapped in polyethylene film. All samples were labeled and kept at -80˚C. Genomic DNA was extracted from frozen leaf samples using the GMO DNA Extraction Kit (BioNEER) following the manufacturer's instructions. Quality and quantity of DNA in the extracted sample solutions were measured using a Nano Drop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, Delaware) and electrophoretic separation through a 0.8% (w/v) agarose gel. Extracted genomic DNA was PCR-amplified using ten pairs of primers flanking SSR sequences that were previously developed for pomegranate (Pirseyedi *et al*., 2010). However, only seven of these pairs showed polymorphism. Table 2 gives the general information of these seven microsatellite markers such as locus name, repeat motif, and annealing temperature. PCR was performed using a Bio-Rad thermo-cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplification reaction products were separated on 5% denaturing polyacrylamide gel using a Sequi-Gen GT Sequencing Cell 50 cm gel apparatus (Bio-Rad Laboratories Inc.). The resulting images were manually scored.

Data analysis

Morphological data analysis was performed using SPSS 17.0.0 (SPSS, 2007). Principal component analysis (PCA) was performed for each year of morphological evaluation and loading values greater than 0.55 were considered significant. To show the relationships among the traits, a correlation analysis was performed on the complete set of data (202 samples and 21 variables) using the mean values of two years of evaluation. The parametric Pearson correlation and the non-parametric Spearman correlation were used for quantitative traits and qualitative traits, respectively. To establish the overall relationships among accessions, cluster analysis was performed on morphological data, based on co-efficient and un-weighted pair group

Table 1. Names and codes of the 202 pomegranate accessions studied during 2010-2011 growing seasons.

Name	Code	Name	Code
Poost-Nazok-Ardal	1-1-N	Shahvar-Kashmar	12-102-W
Poost-Ghermez-Dareh-Hourand	2-2-S	Ghand-Kashmar	12-103-W
Dane rize-Dare hourand	2-3-W	Bi daneh-Kashmar	12-104-W
Nar shirin-Dareh-Hourand	2-4-W	Garche-Shahvar	12-105-N
Poost-Nazok-Dareh-Hourand	2-5-WS	Ghandi-Poost-Sefid- Bejeston	12-106-W
Meikhosh-Dareh-Hourand	2-6-W	Torsh-Shooshtar	13-107-S
Binam-Kouhestan-Dareh Hourand	2-7-N	Meikhosh-Behbahan	13-108- WS
Poost-Ghermez-Dareh- Hourand	2-8-W	Malas-Behbahan	13-109-WS
Shirin riz-Dareh-Hourand	2-9-W	Daneh ghermez-A lot-Baneh	14-110-S
Shirin-Sourati-Dareh Hourand	2-10-W	Abbasi-Kordestan	14-111-N
Poost-Sefid-Dareh Hourand	2-11-W	Dane Ghermez-Lorestan	15-112-W
Dane Dorosht-Dareh-Hourand	2-12-S	Khoramabad-Lorestan	15-113-N
Zoodres-Dareh-Hourand	2-13-W	Jafari-Shei-Nesha-Lorestan	15-114-WS
Shekarnar-Tasuj-Shabestar	2-14-W	Ghermez-Poost-Koloft-Tang Seab	15-115-W
Dane Sefid-Mehran	3-15-W	Soz-Poost-Koloft-Lorestan	15-116-W
Dane-Ghermez-Mehran-Ilam	3-16-S	Poost-sorkh- tang seab	15-117-W
Sabz-Shirin-Kalam-Ilam	3-17-W	Soz-Lori-Shi-Nesha-Lorestan	15-118-S
Malas-Charmak-Ilam	3-18-W	Bavasi-Poost-Sefid-Lorestan	15-119-WS
Binam-Salehabad-Mehran	3-19-W	Ghermez-Shirin-Kouhdasht-Lorestan	15-120-W
Sefid- Ilam	3-20-W	Zard-Mahali gerab-Lorestan	15-121-W
Sabz-Charmak- Ilam	3-21-S	Gol-Khoramabad	15-122-N
Kadro-Poost-Koloft-Kazeron-Fars	4-22-W	Shirin-Nami-Khoramabad	15-123-W
Abdorahomkhani	4-23-N	Poost-Sefid-Khoramabad	15-124-S
Torbat-Sefid-Shiraz	4-24-W	Dane Sefid-Lorestan	15-125-W
Atabaki-Shiraz	4-25-WS	Meikhosh-Poost-Koloft-Lorestan	15-126-WS
Shirin-Shahbar-Shiraz	4-26-W	Binam-Lori-Khoramabad-Lorestan	15-127-W
Shirin-Sabz-Shiraz	4-27-W	Meikhosh-Bavasi-Shei-Nesha-Lorestan	15-128-WS
Khoram rize-Shiraz	4-28-W	Shirin-Lori-Khoramabad-Lorestan	15-129-W
Berit-Mamoli-Kazeron	4-29-S	Gav damagh-Kouhdasht	15-130-S
Berit-Mamoli-Kazeron	4-30-W	Abbasi-Khoramabad	15-131-W
Ghojagh-Ghom	5-31-WS	Bi daneh-Saveh	16-132-WS
Jangali-Talesh-Rasht	6-32-S	Meikhosh-Saveh	16-133-W
Dareh-Loushan	6-33-S	Malas-Saveh	16-134-WS
Hajiabad-Bandar abbas	7-34-W	Shirin seah-Saveh	16-135-W
Minab-Bandar abbas	7-35-W	Alak-Parand-Saveh	16-136-W
Meikhosh-Pish Ras-Kouhpayeh	8-36-WS	Malas-Torsh-Saveh	16-137-W
Poost-Nazok-Natanz	8-37-W	Alak-Shirin-Saveh	16-138-N
Bi name-Dastjerd	8-38-S	Tabestani-Saveh	16-139-W
Poost-Ghermez-Kouhpayeh	8-39-W	Dane dorosht-Shahsavar	17-140-W
Bihasteh-Najafabad	8-40-W	Shirin-Behshahr	17-141-W
Malas-Mortazavi	8-41-WS	Ardestani-Daneh-Sorkh-Semnan	18-142-S
Zaghi-Kouhpayeh	8-42-S	Torsh-Zabol	19-143-S
Damagh baste-Kouhpayeh	8-43-N	Meikhosh-Zahedan	19-144-WS
Khatooni-Poost-Sefid-Natanz	8-44-W	Poost-Sabz-Shirin-Zahedan	19-145-W
Pish ras-Najafabad	8-45-W	Shirin-Zabol	19-146-W
Daneh-Ghermez-Natanz	8-46-WS	Torsh-Poost-Sabz-Zahedan	19-147-S
Malas-Isfahan	8-47-WS	Vahshi-Tamin-Khash	19-148-W
Dane-Sefid-Kouhpayeh	8-48-W	Bi daneh-Pishva	20-149-W
Sabz-Dane-Ghermez-Zavare-Ardestan	8-49-W	Marsel-Shouravi-Varamin	20-150-WS
Sar barik-Kouhpayeh	8-50-WS	Rabab-Ghermez-Pishva	20-151-WS
Anbari-Poost-Koloft-Kashan	8-51-S	Torki-Pishva	20-152-WS
Poost-Sefid-Yaran	8-52-S	Gouzal-Shouravi-Varamin	20-153-W
Narak-Kouhpaye-Isfahan	8-53-W	Ghojagh-Pishva	20-154-WS
Malas-Shirin-Dastjerd	8-54-W	Togh-Pishva	20-155-WS
Khodroo-Vahshi-Najafabad	8-55-W	Piyazi-Ghermez-Pishva	20-156-N
Khatooni-Natanz-Isfahan	8-56-S	Ghahve dan-Kan	20-157-WS
Shomare yek-Kashan	8-57-S	Ghiyasin-Shirin-Kan	20-158-W
Poost-Ghermez-Natanz	8-58-W	Ghiyasin-Zati-kan	20-159-WS
Aban mahi-Isfahan	8-59-N	talghid-kan	20-160-W
Torsh-Mar mar	8-60-S	Poost-Keremi-Pishva	20-161-W
Poost-Sefid-Najafabad	8-61-W	Tokhm-Save dar-Kan	20-162-N
Dane seah-Isfahan	8-62-N	Maroof be ghomi-Kan	20-163-WS
Shirin Gar-Najafabad-Isfahan	8-63-W	Malas-Kan	20-164-WS
Mamuli-Kouhpayeh-Isfahan	8-64-WS	Ghaojagh-Shahpar-Varamin	20-165-WS
Bihaste-Isfahan	8-65-N	Shahpar-Pishva-Varami	20-166-WS
Bezi-Isfahan	8-66-W	Poost-Nazok-Saghand	21-167-W
Torsh-Isfahan	8-67-S	Poost-Koloft-Saghand	21-168-W
Haste riz-Najafabad	8-68-W	Daneh-Ghermez-Saghand-Yazd	21-169-WS
Bi haste-Shirin-Khabar	9-69-W	Bafti-Poost-Koloft-Saghand	21-170-N
Dande dar-Khabar-Baft	9-70-W	Tafti-Marvest-Yazd	21-171-S
Haste dar-Khabar-Baft	9-71-W	Bafti-Poost-Nazok-Saghand	21-172-W
Kam bar-Khabar-Baft	9-72-N	Karche-Tafti-Torsh	21-173-S
Poost-Sefid-Khabar	9-73-W	Se-anbeli-Taft-Yazd	21-174-S
Shahvar-Poost-Nazok-Baft	9-74-WS	Zagh-Poost-Ghermez-Saghand	21-175-W

Table 1. Continued

Name	Code	Name	Code
Khodroo-Vahshi-Baft	9-75-N	Togh-Gardan-Torsh-Yazd	21-176-N
Daneh-Ghermez-Ravar	9-76-S	Meikhosh-Ardekan	21-177-W
Vahshi-Narak-Shahdad	9-77-W	Mamulii-Saghand-Yazd	21-178-W
Togh-Ravari-Malas	9-78-WS	Poost-Sefid-Chak chak-Ardekan	21-179-W
Dopayeh-Rize-Ravar	9-79-WS	Malas-Torsh-Yazd	21-180-WS
Meikhosh-Haste-Rize-Shahdad	9-80-WS	Teloz-Shirin-Yazd	21-181-W
Meikhosh-Soorati-Rafsanjan	9-81-W	Aban mahi-Torsh-Yazd	21-182-S
Daneh-Ghermez-Sirjan	9-82-WS	Zagh-Karche-Torsh-Yazd	21-183-S
Golabi-Poost-Ghermez-Ravar-Torsh	9-83-W	Zood ras-Yazd	21-184-N
Haste-Rize-Baft	9-84-W	Garche-Shabar-Shirin-Yazd	21-185-W
Golnar-Farsi-Shahdad	9-85-N	Gabri-Yazd	21-186-W
Bihasteh-Chenje-Rijab	10-86-W	Malas-Ardekan	21-187-WS
Poost-Nazok-Rijab	10-87-WS	Poost-Seah-Yazd	21-188-W
Ghomi-Poost-Nazok-Rijab	10-88-N	Torsh-Yazd	21-189-S
Maroof be sheryan-Ghasre shirin	10-89-W	Garche-Dadashi-Poost-Nazok-Ashkzar	21-190-W
Poost-Sfid-Ghasre shirin	10-90-N	Shour-Poost-Koloft-Saghand	21-191-W
Shahvar-Ghasre shirin	10-91-N	Zagh-Ardekan	21-192-W
Ghomi-Poost-Ghermez	10-92-W	Shahvar-Dadashi-Daraje Yek-Ashkzar	21-193-W
Shahrbani-Torsh-Rijab-Bakhtaran	10-93-S	Zagh-poost-Sefid-Ashkzar	21-194-W
Poost-Sefid-Rijab	10-94-S	Koohi-Siri-Tabas-Torsh	21-195-S
Poost-Koloft-Rijab	10-95-W	Koohi-Siri-Tabas	21-196-WS
Poost-Koloft-Rijab-Bakhtaran	10-96-S	Nabati-Poost-Sefid-Ashkzar	21-197-W
Razhnar-Ravansar-Paveh	10-97-S	Ratki-Daneh-Sefid-Bafgh	21-198-W
Shirin Paveh	10-98-W	Dadash-Peivandi-Ashkzar	21-199-WS
Shirin-Nar-Paveh	10-99-W	Kartchi-Por Bar-Bafgh	21-200-WS
Mamoli-Birjand	11-100-N	Poost-Nazok-Zanjan	22-201-WS
Malas-Sabzevar	12-101-WS	Shahvar-Miveh-Dorosht-Zanjan	22-202-W

Province code: 1 = Chahar-Mahall-va-Bakhtiari; 2 = East-Azarbayejan; 3 = Ilam; 4 = Fars; 5 = Ghom; 6 = Gilan; 7 = Hormozgan; 8 = Isfahan; 9 = Kerman; 10 = Kermanshah; 11 =Khorasan-Gonubi; 12 = Khorasan-Razavi; 13 = Khuzestan; 14 = Kordestan; 15 = Lorestan; 16 = Markazi; 17 = Mazandaran; 18 = Semnan; 19 = Sistan-baluchestan; 20 = Tehran; 21 = Yazd; 22 =Zanjan.

Taste code: S =sour; W =sweet; WS =sweet-sour; N = Unknown

Table 2. Locus name, repeat motif, PCR annealing temperatures (Ta), number of alleles detected (*Na*), number of effective alleles (*Ae*), major allele frequency, observed heterozygosity (*Ho*), expected heterozygosity (*He*), PIC and FIS values for seven polymorphic nuclear microsatellite loci in pomegranate.

Locus	Repeat motif	Ta(˚C)	*Na*	*Ae*	Major allele frequency	*Ho*	*He*	*PIC*	*FIS*
ABRII-MP26	(AG)25	55	9	2.102	0.65	02381	0.5259	0.48	0.4079
ABRII- MP30	(CT)15	55	2	1.934	0.53	07785	0.4832	0.48	-0.7590
ABRII-MP51	(GA)19	50	3	1.913	0.68	02979	0.4786	0.42	0.1354
ABRII-MP28	(GAGG)3(GA)19	55	3	1.983	0.65	03405	0.4970	0.44	-0.0585
ABRII-MP12	(CA)11	55	2	1.025	0.98	0.0251	0.0249	0.024	-0.0292
ABRII-MP07	(AT)9 (GT)8	55	2	1.010	0.99	0.0104	0.0103	0.01	-0.1664
ABRII-MP39	(GA)8(TTTTCT)2	55	2	1.999	0.40	0.4507	0.5017	0.56	-0.1083
Mean			3.28	1.709	0.70	0.3059	0.3604	0.3487	-0.0937

methods with arithmetic means (UPGMA) using NTSYS-pc Version 2.11 (Rohlf, 2002). Morphological clustering was constructed based on the mean values of two years of evaluation using Ward's method. Principal coordinate analysis via a distance matrix with data standardization for grouping of all accessions was also conducted.

Each SSR band was scored as present (1) or absent (0). For the SSR dataset, number of observed alleles per locus (Na), major allele frequencies, expected heterozygosity (He), and polymorphic information content (PIC) were computed using the Power Marker 3.25 (Liu and Muse, 2005). Based on these data, the number of effective alleles (Ae), the observed heterozygosity (Ho), and Wright's inbreeding coefficient (FIS) were calculated. The effective number of migrants per generation (an indirect estimate of gene flow between two populations) was estimated using the formula: Nm= 0.5 (1-Gst)/Gst) (Kim *et al.*, 2005).

The Ewens-Watterson test for neutrality (Manly, 1985) was conducted on the seven SSR loci using POPGEN (Yeh *et al.*, 1997). AMOVA, the analysis of molecular variance (Excoffier *et al.*, 1992), was used to estimate the component of variance attributable to differences among populations and among individuals within populations. Similarity matrix values for SSR data were generated using NTSYS, based on the Jaccard co-efficient, anda dendrogram was generated using UPGMA. Morphological and SSR data were compared by calculating the correlation between the two datasets using the Mantel test with 250 permutations in the matrix comparison (MxCOMP) program of NTSYS.

RESULTS

Morphological analysis

Correlation was observed among most of the traits (Table 3). Leaf length, width, and total leaf length were highly correlated with each other and also correlated with some flower traits such as flower diameter and petal width. Length of flower style was highly correlated with flower shape and diameter and there was also high correlation between petiole color and leaf border color. Among the traits studied, petal length showed the highest correlation with other attributes, including: number of petals, number of sepals, flower length, flower diameter, petal width, and petal length: width ratio. Descriptive statistics of morphological traits for the two years of study are shown in Table 4. In the first year, of all the traits studied, flower length, length of style, and petiole length showed the highest variance. In the second year, petiole length had the highest variance.

PCA showed that four components explained 47.61% and 50.52% of the total variation contributed by all traits for the first and second year, respectively (Table 5). In each year, leaf characteristics were predominant in the first and second components. In the first year, the first and second component presented 24.44% of the total variation, of which total leaf length, leaf length, petiole length, and leaf length: width ratio had the highest loading. In the second year, the first and second components explained 29.44% of the total variance and the same attributes – plus leaf width – had the highest loading (Table 5). For both years, flower traits had the highest loading for the third and fourth components. The morphological characters measured in the first year included: number of petals, petal length:width ratio, number of sepals, flower length, flower length: diameter ratio. In the second year they were flower diameter, flower length: diameter ratio, petal length: diameter ratio, length of style, and flower shape.

PCA divided the 202 accessions into four distinct groups (Fig. 2). Up to 75% of the total variation was explained by the first three axes. In this analysis, most accessions with a sour taste located in a separate group. Morphological cluster analysis by simple matching co-efficient and UPGMA method showed five distinct groups (Fig. 3).

Molecular analysis

A total of 23 alleles were detected and the number of alleles per locus ranged from two (for mp07, mp12, mp30, and mp39) to nine (for mp26), with an average of 3.28 alleles per locus (Table 2). Major allele frequency ranged from 0.4 to 0.99, with a mean of 0.7. PIC of the markers varied from 0.01 to 0.56 with an average of 0.34. Marker mp39 revealed the highest PIC (0.56) while marker mp07 had the lowest PIC (0.01) (Table 2).

Observed heterozygosity across the seven SSR loci ranged from 0.01 (marker mp07) to 0.76 (marker mp30), with a mean of 0.305 (Table 2). Expected heterozygosity ranged from 0.01 (locus mp07) to 0.52 (locus mp26), with an average of 0.36. Wright's inbreeding co-efficient (FIS) showed a negative average. Nm, the estimate of gene flow from Gst, was 0.56. The Ewens-Watterson test for neutrality indicated that the majority of SSR loci were neutral. AMOVA showed that the variances among the population were significant (93%), while the variance within populations accounted for 6% of the total variance (Table 6). Molecular cluster analysis by simple matching co-efficient and the UPGMA method showed five distinct groups (Fig. 4).

DISCUSSION

Knowledge about the genetic relationships of germplasm provides useful information for breeding programs and efficient management of genetic resources (Roldán-Ruiz*et al.*, 2001). In studies of genetic diversity, the combination of the necessary (and also less laborious) morphological characterization, alongside molecular markers, has led to more reliable conclusions in assessing genetic diversity (Sorkheh *et al.*, 2007; Khadivi-Khub *et al.*, 2008).

Close relationships among traits could have a positive or negative role in the transfer of traits through gene introgression, since strong selection for a desirable trait could support the presence of other trait(s) (Dicenta and Garcia, 1992). This study demonstrated the high correlation between length of style and flower shape, both of which are directly associated with pomegranate tree performance. The close correlation among leaf length, leaf width, total leaf length, and flower traits such as flower diameter and petal width indicate that leaf expansion results in flowers with greater diameter.

In pomegranate, the male flower (with a short style) drops and rarely set fruits, leaving the hermaphrodite types (with long styles) to produce the majority of the crop (Chaudhari and Desai, 1993; El Sese, 1988). This study also found significant correlation between flower diameter and leaf traits and between flower diameter and style length. Expanding the leaf and thereby increasing the flower diameter would therefore probably lead to genotypes with higher percentage of hermaphrodite flowers and a greater number offruits.

Table 3. Correlation coefficients between 21 morphological traits of pomegranate accessions.

	Np	Ns	Leng F	Dia F	Leng F/ Dia F	Pl	Pw	Pl/ Pw	L	Lw	L2	L1	L1/ Lw	Leng S	Sha F	S
Np	1															
Ns	0.881**	1														
Leng F	0.125	0.106	1													
Dia F	0.052	-0.008	0.352**	1												
Leng F/ Dia F	0.026	0.091	0.459**	-0.587**	1											
Pl	0.454**	0.366**	0.407**	0.430**	-0.084	1										
Pw	0.178*	0.095	0.321**	0.475**	-0.166*	0.575**	1									
Pl/ Pw	0.214**	0.223**	0.040	-0.134	0.149*	0.315**	-0.530**	1								
L	0.044	0.071	0.053	0.128	-0.049	0.062	0.005	0.086	1							
Lw	0.132	0.180*	0.164*	0.153*	-0.040	0.172*	0.186**	-0.028	0.392**	1						
L2	0.050	0.034	0.156*	0.241**	-0.097	0.156*	0.083	0.104	0.393**	0.372**	1					
L1	-0.036	-0.009	0.003	0.067	-0.022	-0.040	-0.046	0.054	0.766**	0.406**	0.424**	1				
L1/ Lw	-0.170*	-0.192**	-0.136	-0.088	0.035	-0.203**	-0.197**	0.054	0.326**	-0.513**	0.056	0.511**	1			
Leng S	-0.147*	-0.132	-0.009	0.083	-0.064	-0.126	-0.007	-0.121	-0.037	0.023	-0.047	-0.057	-0.091	1		
Sha F	0.160*	0.164*	-0.107	-0.236**	0.107	0.009	0.004	0.046	-0.016	0.031	-0.085	0.045	0.016	-0.594**	1	
Sha P	-0.063	-0.054	0.014	0.102	-0.070	-0.071	0.352**	-0.475**	0.104	0.087	0.032	0.001	-0.065	0.000	-0.018	
Col L	0.038	0.084	-0.125	-0.081	-0.032	-0.112	-0.107	-0.070	-0.089	-0.141*	0.021	-0.114	0.061	-0.079	-0.016	
Sha L	-0.028	-0.056	0.019	-0.002	0.048	-0.107	-0.085	-0.023	-0.119	-0.255**	-0.130	-0.098	0.136	0.046	-0.122	
Sha LT	0.033	0.061	-0.005	-0.143*	0.101	-0.116	-0.035	-0.076	0.039	-0.100	-0.107	0.043	0.127	-0.056	0.034	
Petio MV	0.043	0.074	0.204**	0.059	0.113	0.040	-0.066	0.150*	0.281**	0.213**	0.417**	0.233**	0.055	-0.047	-0.016	
Col P	0.017	0.026	0.030	0.067	-0.036	0.023	-0.017	0.017	0.075	0.067	0.127	0.124	0.046	-0.048	-0.001	

Traits: Np = number of petals;Pl = petal length; Pw = petal width; Pl:Pw = petal length:width ratio; Sha P = petal shape; Ns = number of sepals;Leng F = F = flower length:diameter ratio; Sha F = flower shape; L = total leaf length; Lw = leaf width; L1 = leaf length; L1:Lw = leaf length:width ratio;Col L = le of leaf tip(Sha LT);Petio MV = petiole to middle vein;Col P = petiole color; L2 = petiole length; Leng S = style length.

Table 4.Descriptive statistics for 21 morphological traits (abbreviation and units), among 202 pomegranate accessions during the 2010 and 2011 growing

Traits	Unit	Min		Max		Mean		SD	
		First year	Second year	First year	Second year	First year	Second year	First year	Sec
Quantitative									
Np	-	3.00	4.70	7.56	8.30	6.2060	6.2079	0.56616	0
Ns	-	4.70	4.70	7.72	8.30	6.1673	6.2188	0.48993	0
Leng F	Cm	2.14	1.10	8.02	4.81	3.5696	3.5436	1.02136	0
Dia F	Cm	.52	.40	2.82	3.30	0.8778	0.9618	0.23585	0
Leng F/ Dia F	-	1.35	.33	10.33	6.50	4.1964	3.8704	1.29343	0
Pl	Cm	1.10	1.30	2.95	2.93	2.3287	2.2107	0.33842	0
Pw	Cm	0.60	1.16	2.72	2.44	1.8713	1.7366	0.30859	0
Pl/ Pw	-	0.74	0.59	2.37	1.64	1.2599	1.2866	0.17354	0
L	Cm	2.84	2.94	8.52	6.94	5.0388	5.0139	0.79887	0
Lw	Cm	0.87	0.76	2.94	2.56	1.6006	1.4734	0.31449	0
L2	Cm	0.20	0.16	0.97	1.32	.4446	0.3900	0.12695	0
L1	Cm	2.50	2.72	8.02	6.42	4.5942	4.6252	0.74799	0
L1/ Lw	-	1.54	1.75	6.47	4.81	2.9347	3.1921	0.58970	0
Qualitative									
Leng S	code(1-4)	1.00	1.00	4.00	4.00	2.2746	1.2474	1.24257	0
Sha F	code(1-3)	1.00	1.00	4.00	3.00	1.3938	2.5596	0.84185	0
Sha P	code(1-3)	1.00	1.00	4.00	2.00	1.4124	1.3782	0.75841	0
Col L	code(1-3)	1.00	1.00	2.00	2.00	1.2714	1.0594	0.44578	0
Sha L	code(1-3)	2.00	1.00	3.00	3.00	2.8458	2.4179	0.36207	0
Sha LT	code(1-3)	1.00	1.00	3.00	3.00	1.7114	2.1045	0.93076	0
Petio MV	code(3-9)	3.00	3.00	7.00	7.00	5.3267	5.1188	1.19376	1
Col P	code(1-3)	1.00	1.00	2.00	2.00	1.1018	1.4627	0.30329	0

Traits: Np = number of petals; Pl = petal length; Pw = petal width; Pl: Pw = petal length: width ratio; Sha P = petal shape; Ns = number of sepals; Leng F Dia F = flower length: diameter ratio; Sha F = flower shape; L = total leaf length; Lw = leaf width; L1 = leaf length; L1: Lw = leaf length: width ratio; Co = shape of leaf tip (Sha LT); Petio MV = petiole to middle vein; Col P = petiole color; L2 = petiole length; Leng S = style length.

Table 5. Eigenvectors of the four principle component axes from PCA analysis of the 202 pomegranate accessionsin the 2010 and 2011 growing seasons.

Traits	PC1		PC2		PC3		PC4	
	First year	Second year	First year	Second year	First year	Second year	First year	Second year
Quantitative								
Np	**0.138**	**-0.449**	**0.488**	**0.449**	**0.656**	**0.262**	**-0.162**	**0.387**
Ns	**0.125**	**-0.427**	**0.434**	**0.438**	**0.612**	**0.293**	**-0.192**	**0.377**
Leng F	**0.104**	**-0.040**	**0.310**	**0.496**	**0.156**	**0.136**	**0.835**	**-0.394**
Dia F	**0.444**	**0.164**	**0.475**	**0.151**	**-0.174**	**-0.683**	**0.020**	**-0.075**
Leng F/ Dia F	**-0.212**	**-0.144**	**-0.016**	**-0.059**	**0.239**	**0.687**	**0.770**	**-0.097**
Pl	**0.307**	**-0.355**	**0.620**	**0.462**	**0.301**	**0.103**	**0.145**	**-0.182**
Pw	**0.443**	**-0.252**	**0.521**	**0.308**	**-0.347**	**-0.531**	**0.238**	**0.269**
Pl/ Pw	**-0.197**	**-0.056**	**0.061**	**0.089**	**0.706**	**0.672**	**-0.145**	**-0.465**
L	**0.803**	**0.864**	**-0.523**	**0.295**	**0.201**	**0.169**	**0.048**	**0.258**
Lw	**0.473**	**0.168**	**0.264**	**0.702**	**-0.207**	**-0.086**	**-0.293**	**0.194**
L2	**0.658**	**0.552**	**-0.013**	**0.550**	**0.042**	**0.072**	**-0.033**	**-0.142**
L1	**0.748**	**0.834**	**-0.558**	**0.219**	**0.209**	**0.169**	**0.057**	**0.305**
L1/ Lw	**0.238**	**0.596**	**-0.676**	**-0.500**	**0.340**	**0.198**	**0.255**	**0.126**
Qualitative								
Leng S	**0.175**	**0.205**	**0.186**	**0.056**	**-0.141**	**-0.319**	**0.188**	**-0.533**
Sha F	**0.083**	**-0.142**	**-0.062**	**-0.131**	**0.214**	**0.467**	**-0.308**	**0.548**
Sha P	**0.245**	**-0.057**	**0.019**	**0.020**	**-0.531**	**-0.570**	**0.128**	**0.330**
Col L	**-0.188**	**0.185**	**-0.161**	**-0.166**	**0.077**	**-0.157**	**-0.104**	**-0.006**
Sha L	**-0.378**	**0.447**	**-0.150**	**-0.432**	**0.066**	**0.164**	**0.397**	**0.070**
Sha LT	**-0.175**	**0.180**	**-0.238**	**-0.273**	**0.182**	**-0.050**	**0.048**	**0.149**
Petio MV	**0.378**	**0.430**	**-0.092**	**0.543**	**0.090**	**0.083**	**0.154**	**-0.071**
Col P	**0.072**	**0.280**	**-0.321**	**0.254**	**-0.108**	**-0.013**	**-0.061**	**-0.118**

Traits: Np = number of petals; Pl = petal length; Pw = petal width; Pl: Pw = petal length: width ratio; Sha P = petal shape; Ns = number of sepals; Leng F = flower length; Dia F = flower diameter; Leng F: Dia F = flower length:diameter ratio; Sha F = flower shape; L = total leaf length; Lw = leaf width; L1 = leaf length; L1: Lw = leaf length: width ratio; Col L = leaf border color; Sha L = leaf shape; Sha LT = shape of leaf tip (Sha LT); Petio MV = petiole to middle vein; Col P = petiole color; L2 = petiole length; Leng S = style length.

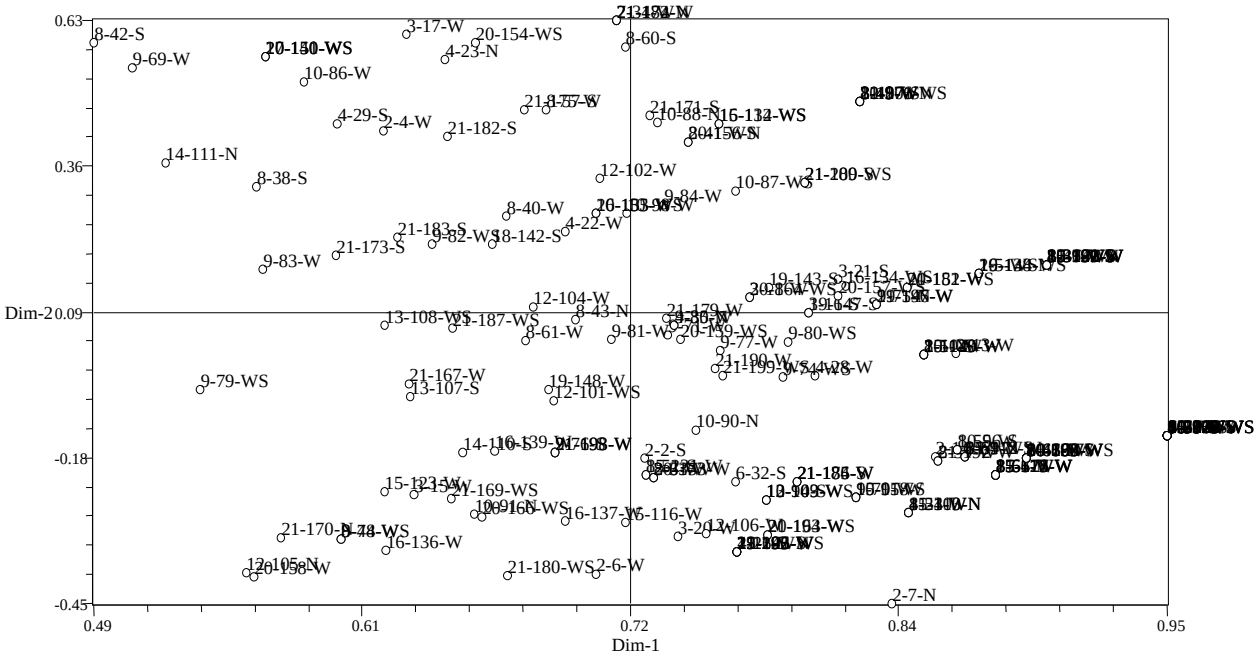

Figure 2. Principlecoordinate analysis based on a distance matrix of 202 pomegranate accessions.

There is a correlation between some characteristics of the fruit or mature plants with some other attributes, e.g. between the anthocyanin percentage in the petiole and the fruit skin color (Zamani *et al.*, 2007). This study showed a high correlation between petiole color and leaf border color, which could be considered criteria for selection in pomegranate breeding programs.

PCA showed that – compared to other traits – leaf traits such as leaf length, leaf width, and total leaf length were predominant in the first and second components in both years, indicating that they are not only useful for assessing genetic diversity but also for pomegranate germplasm characterization. These results concur with the results of previous studies. Noormohammadi *et al.* (2010) studied 18 Iranian pomegranate landraces using RAPD marker and morphological traits and found significant

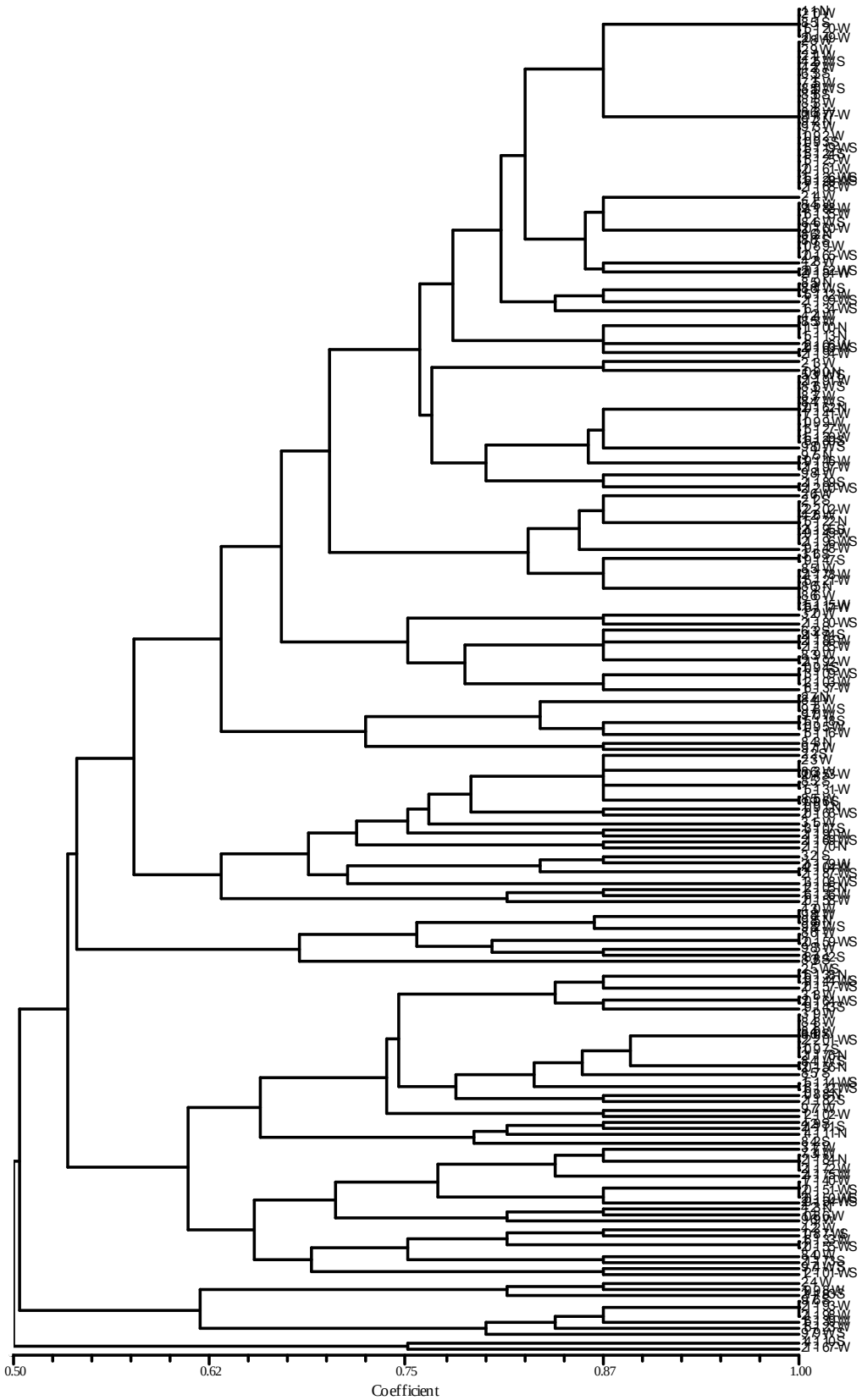

Fig. 3. A dendrogram generated from UPGMA cluster analysis on 202 pomegranate accessions based on morphological traits.

Table 6. Analysis of molecular variance (AMOVA) for 202 pomegranate accessionsfrom 22 populations, based on seven microsatellite loci.

Source	d.f.	Sum of squares	Variance components	Percentage of variation	p-Value
Among population	**21**	**109.141**	**0.5959**	**93.54**	**0.000**
Within population	**179**	**7.361**	**0.0411**	**6.46**	**0.000**
Total	**200**	**116.502**			

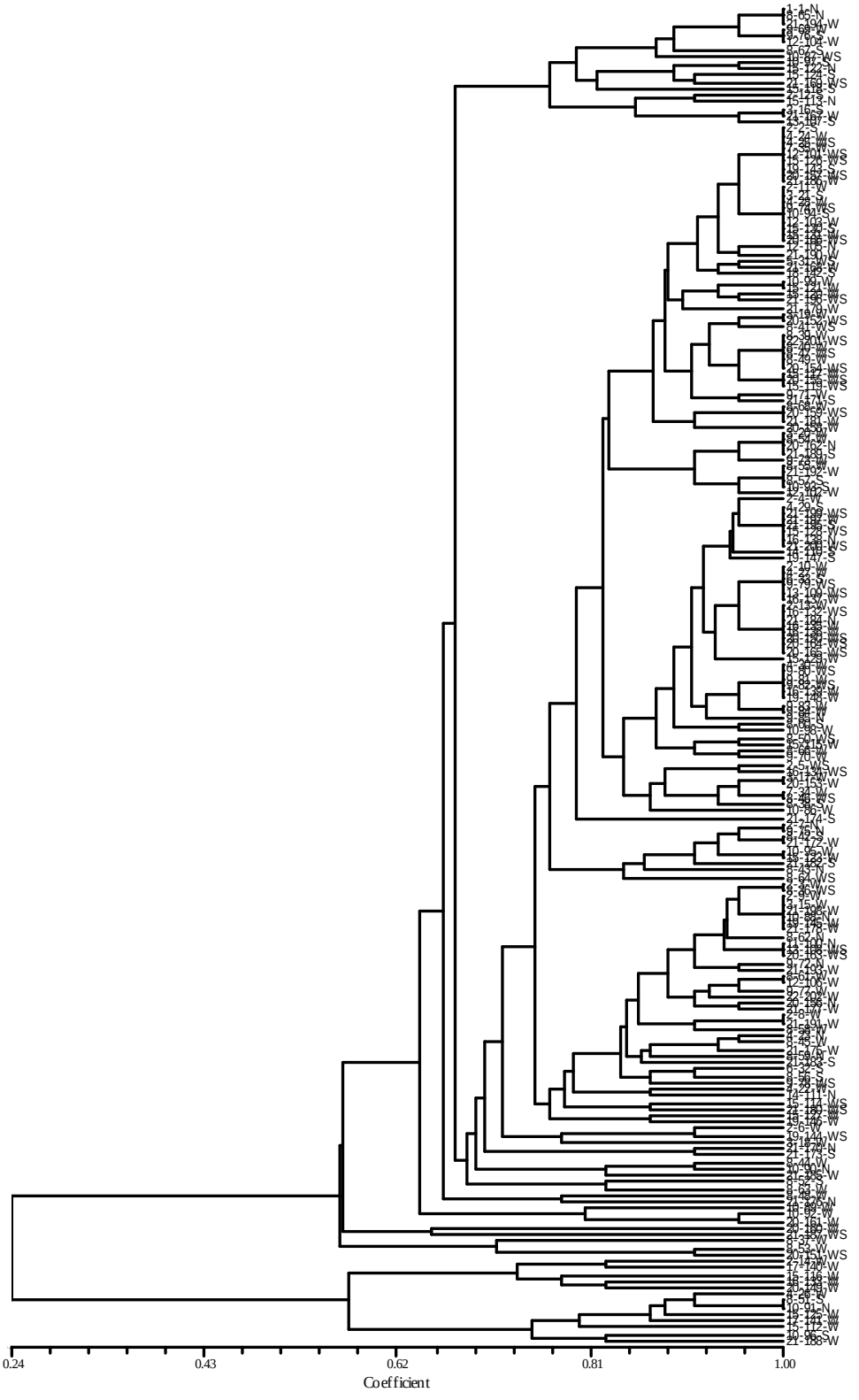

Fig. 4. A dendrogram generated from UPGMA cluster analysis on 202 pomegranate accessions based on microsatellite markers.

differences in leaf length among these genotypes, indicating the possibility of using leaf traits in discrimination between *Punica*g ermplasm during the vegetative growth period.

Previous studies have determined genetic relationships and diversity in pomegranate using different markers such as RAPD and fluorescent-AFLP (Sarkhosh *et al.*, 2006; Ercisli *et al.*, 2007; Youn *et al.*, 2007; Awamleh *et al.*, 2009). To our knowledge, this is the first study conducted on the

Iranian pomegranate germplasm using both morphological traits and microsatellite markers.

Cluster analysis using microsatellite markers and morphological traits data revealed five separated groups of genotypes, but grouping obtained from molecular dendrogram did not match those obtained by morphological dendrogram. Furthermore, neither the morphological nor molecular analysis showed completely expected grouping in both clusters. Accessions were spread in the dendrogram with a minor tendency to cluster by geographic origin. These results could be explained by the fact that pomegranate accessions might have moved to neighbor provinces with the same climatic conditions and then spread to other locations.

The correlation between matrices of morphological and molecular data sets was not significant**.** Semagn (2002) suggested two reasons for low correlation between DNA markers and morphological data as well as protein data: (a) DNA markers cover a larger proportion of the genome – including coding and noncoding regions – than the morphological markers, and (b) DNA markers are less subjected to artificial selection compared to morphological markers. Martinez *et al.* (2005) proposed that the correspondence between different methods might be improved by analyzing more morphological characters and DNA markers. However, the range of genetic distance based on SSR markers was, on average, higher than morphological traits.

FIS is a measure of deviation from the Hardy-Weinberg equilibrium in whole populations; positive values indicate heterozygote deficiency and negative values represent heterozygote frequency (Wright, 1951). A negative average FIS value in this study indicates that heterozygosity was higher than expected. The indirect estimate of gene flow (Nm) based on Gst was 0.56 meaning that the total number of migrants per generation was less than one, and therefore the level of genetic diversity maintained within a population is susceptible to genetic drift. This value is even lower than that of mixed-mating species (Nm= 0.72) (Hamrick and Godt, 1990).

A higher genetic variation within (rather than between) populations has been reported in out-crossing species such as perennial ryegrass (*Lolium perenne* L.), meadow fescue (*Festuca pratensis* Hunds), orchard grass (*Dactylis glomerata* L.), and Rhodes grass (*Chloris gayana*) (Huff, 1997; Kolliker *et al.,* 1998; Ubi *et al.,* 2003). In this study, AMOVA revealed a greater genetic variation among rather than within populations, yet our results showed that – in pomegranate – self-crossing is higher than out-crossing. This concurs with a study by Jalikop and Sampath Kumar (1990) who used marker genes to confirm that pomegranate is self-crossing species with a low level (13%) of cross-pollination.

Successful preservation of any given gene pool is largely dependent on understanding its diversity and its distribution in a given region (Zhang *et al.*,2003). In this study, *Punica* microsatellite markers and morphological characters revealed a relatively high amount of genetic diversity among 202 pomegranate genotypes. This variation within the Saveh Research Station pomegranate collection should assure the required genetic resources for future pomegranate breeding programs.

Despite the poor correlation between morphological and molecular markers, both techniques can be used effectively in pomegranate germplasm characterization and for management strategies including identification of duplicates, identification of accessions with desirable traits, and establishment of core collections. A core collection consists of a limited set of accessions derived from a germplasm collection, which would represent – with minimum repetition – the genetic diversity of a crop species and its relatives. The establishment of core collections is an effective strategy in optimizing human, material, and financial resources by providing greater efficiency in the use of germplasm collections (Spagnoletti-Zeuli and Qualset, 1993; Van Hintum *et al.*, 2000).

Germplasm preservation centers have been established to preserve the available genetic variation before it is lost due to the widespread use of improved cultivars (Brown, 1989). The Iranian national pomegranate collection at Saveh contains commercial varieties, landraces, ornamental varieties, and elite germplasm.

Understanding the extent of the diversity in this collection is essential for effectively managing and utilizingthe national germplasm collections. Strategic, research-based collection and characterization of accessions will enhance the development of core collections for pomegranate germplasm, though the use of different markers and other traits (especially economically important traits) are also an essential of achieving this goal. The use of survey data and information regarding economically important traits related to fruit quality can also be very useful for pomegranate breeders.

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