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Evaluation of the genetic diversity of pomegranate accessions from Turkey using new microsatellite markers

Oğuzhan ÇALIŞKAN[1,*], Safder BAYAZIT[1], Mert ÖKTEM[2], Ali ERGÜL[3]

[1]Department of Horticulture, Faculty of Agriculture, Mustafa Kemal University, Antakya, Hatay, Turkey
[2]Department of Biotechnology, Institute of Science, Mersin University, Mersin, Turkey
[3]Biotechnology Institute, Ankara University, Ankara, Turkey

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Abstract: Turkey has many valuable genetic resources for pomegranate; however, there have not been many studies on the identification and characterization of these important genetic resources. New microsatellite markers were used to characterize a set of 78 pomegranate accessions from Turkey. Using six SSR primers, a total of 41 alleles were characterized with an average of 4.6 alleles per locus and mean probability of identity (PI) value of 0.366. These data indicated a high level of polymorphism in pomegranate germplasm. Five synonymous groups could be detected among 30 accessions. This microsatellite-based key is a first step towards a database for marker-assisted identification of pomegranate accessions in Turkey. The present study provides essential information to devise a pomegranate core germplasm collection without duplication of plant material, to sustainably manage pomegranate breeding programs, and to establish conservation strategies for preserving local pomegranate genetic resources.

Key words: Genetic diversity, new microsatellites, pomegranate, synonymous group

1. Introduction

Horticultural plants have been an indispensable part of human life for ages. Since ancient times, their fruits, seeds, and even roots and branches have been used to meet personal and social needs such as food, medicine, and decoration (Ercisli, 2009; Erturk et al., 2010; Hricova et al., 2016; Sarıdaş et al., 2016; Yazıcı and Şahin, 2016). Pomegranate (*Punica granatum* L.; 2n = 2x = 18) is a diploid, perennial, woody plant and belongs to the monogeneric family *Lythraceae*, which possesses two species, *Punica granatum* L. and *Punica protopunica* Balf. The genome size of pomegranate has been ascertained to be 704 Mbp, about six times the size of *Arabidopsis thaliana* (http://data.kew.org/cvalues/).

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits and has been used in the folk medicine of various countries (Holland et al., 2009). The center of origin of pomegranate is considered to be Central Asia, especially parts of modern-day Iran, Turkey, Azerbaijan, and Georgia in the Transcaucasia-Caspian region, from where it has spread to the rest of the world (Levin, 2006). Recently, pomegranate has garnered increasing interest, especially because of its high nutritional value (Seeram et al., 2006) and the phytochemical and medicinal properties of its juice (Mena et al., 2011; Caliskan and Bayazit, 2012). Precise identification of genotypes and determination of the genetic relationships among them will be necessary for conserving its genetic diversity. Such data will also facilitate improved selection of genotypes with traits preferred by consumers.

Turkey is one of the main pomegranate producers among the Mediterranean countries. The majority of its pomegranate orchards are located in the Mediterranean, Aegean, and southeastern regions of the country. Annual pomegranate production in Turkey was about 445.75 t in 2015 and production has rapidly increased from year to year. Fresh pomegranate exports from Turkey were 3.591 t in 2000 and 151.174 t in 2015 (http://www.ffv.org.tr/home.aspx). The Mediterranean region of Turkey has the most suitable ecological conditions for pomegranate cultivation and includes 60% of the country's total pomegranate production. This region also has rich pomegranate genetic diversity useful for breeding programs. In recent years, pomegranate resources have suffered genetic erosion due to vulnerability to biotic and abiotic stresses and loss of agricultural land to intensive urbanization in the country (Caliskan and Bayazit, 2013).

* Correspondence: ocaliskan@mku.edu.tr

Local pomegranate accessions are numerous and well adapted to different ecological regions of Turkey. These accessions have been cultivated using traditional methods since ancient times and can be useful for their adapted characteristics such as resistance to diseases, pests, cold, drought, and other biotic and abiotic stresses. Some morphopomological studies have been performed (Drogoudi et al., 2005; Ferrara et al., 2011; Zaouay and Mars, 2011; Caliskan and Bayazit, 2013), as have studies of phytochemical characteristics (Tehranifar et al., 2010; Mena et al., 2011; Caliskan and Bayazit, 2012) in pomegranate germplasm. Characterization based on morphological traits has commonly been used to resolve duplication problems within pomegranate germplasm (Zaouay and Mars, 2011). Although morphological parameters are generally significantly influenced by environmental conditions and agronomic practices, their characterization is a highly recommended first step for germplasm evaluation (Berinyuy et al., 2002). Nevertheless, morphological differences are often too limited to allow closely related accessions to be distinguished. Furthermore, the expression of these characteristics is not always consistent, so morphological information alone is inadequate for evaluating the genetic diversity of collections.

Due to the limitations of morphological markers, DNA markers are increasingly being used to evaluate germplasm diversity (Soriano et al., 2011). DNA markers are independent of environmental conditions, are potentially unlimited in number, and can show a high level of polymorphism. Therefore, they are invaluable tools for determining genetic relationships, evaluating diversity, performing selection during plant breeding, and genome mapping (Currò et al., 2010; Zaouay and Mars, 2011). In recent years, RAPD (Sarkhosh et al., 2006; Zamani et al., 2013), SRAP (Ranade et al., 2009; Soleimani et al., 2012), ISSR (Zhao, 2011), AFLP (Moslemi et al., 2010; Ercisli et al., 2011b; Nemati et al., 2012), and SNP (Ophir et al., 2014) markers have been used to evaluate the diversity of pomegranate genetic resources. However, despite the popularity of these markers, they can suffer from poor reproducibility and can be insufficiently accurate for genotype identification. To overcome the potential drawbacks of certain molecular markers, other markers based on nonspecific primers, microsatellites, or simple sequence repeats (SSR) including specific primers, which can anneal the flanking region of SSR repeat units, can be advantageous due to their abundance, high polymorphism, reproducibility, codominant pattern of inheritance, and extensive genome coverage (Varshney et al., 2005; Hasnaoui et al., 2012). The advantages of microsatellite markers for plant germplasm characterization relative to other PCR-based markers have been demonstrated in many fruit crop species (Ergül et al., 2002; Sánchez-Pérez et al., 2005; Boz et al., 2011; Caliskan et al., 2012). Diverse SSR primer pairs have been published for pomegranate (Curró et al., 2010; Hasnaoui et al., 2010; Soriano et al., 2011). However, little is currently known about the level of molecular genetic diversity available in pomegranate germplasm in Turkey.

In the present study, a number of novel SSR markers have been developed to investigate the potential of such markers for assessing the genetic diversity of pomegranate accessions from Turkey. This is also the first study using microsatellite markers to evaluate the genetic diversity of pomegranate accessions from the eastern Mediterranean region of Turkey. These results will improve our understanding of the level of diversity of pomegranate germplasm in Turkey and will be helpful for devising an effective strategy for the conservation, management, and use of these genetic resources in pomegranate breeding programs.

2. Materials and methods

2.1. Plant material and DNA extraction

The present study included 78 Turkish pomegranate accessions and control cultivars listed in Table 1. Young leaves were collected from each accession in Hatay Province, which is located in the eastern Mediterranean region of Turkey. The standard cultivars Hicaznar and Katırbaşı were used as the control group. A pomegranate descriptor was used for evaluation of some fruit quality characteristics (Bellini and Giordani, 1998). Fruit size was evaluated using a scale ranging from very small (<100 g) to very large (>375 g). A color scale ranging from greenish-yellow to dark purplish-red was used to evaluate peel color. In addition, fruit taste was investigated as sweet, sweet-sour, or sour.

Genomic DNA was extracted from leaves using the procedure described by Lefort et al. (1998). DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide (Caliskan et al., 2012). The concentration and purity of the extracted DNA were analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

2.2. Primers and SSR analysis

Detection of microsatellite polymorphisms was performed using six SSR markers, among which the PgAERB3 locus was described by Ergül and Bakır (2013). Five of them were novel SSR markers named PgAER121, PgAER138, PgAER154, PgAER194, and PgAERB7 loci (A Ergül, personal communication), and three SSR markers were previously characterized by Pirseyedi et al. (2010). Six SSR loci were developed from a CA-enriched library.

SSR-PCR amplifications were carried out in reaction volumes of 11.1 μL containing 0.5 U (0.07 μL) of GoTaq

Table 1. Origin and some fruit quality characteristics of Turkish pomegranate accessions collected in the eastern Mediterranean region of Turkey.

Accession name	Location	Fruit size	Peel color	Taste	Accession name	Location	Fruit size	Peel color	Taste
Cınzar	Altınözü	Large	Red	Sour	Ekşi10	Hassa	Very large	GY	Sour
Ekşiyeşil kabuk	Altınözü	Large	GY	Sour	Ekşi11	Hassa	Large	GY	SS
Katırbaşı 2	Altınözü	Very large	Y	SS	Tatlı9	Hassa	Very large	RY	Sweet
Kırmızı Lifani	Altınözü	Very large	Y	SS	Fellahnarı	İskenderun	Very large	GY	Sour
Lifani4	Altınözü	Very large	GY	SS	Katırdişi	İskenderun	Very large	Y	SS
Lifani5	Altınözü	Very large	GY	SS	Kızılgöbek	İskenderun	Very large	RY	SS
Ekşi16	Altınözü	Very large	GY	SS	Kızılgöbek Ekşi	İskenderun	Very large	RY	Sour
Tatlı6	Altınözü	Large	Y	Sweet	Payasnarı	İskenderun	Very large	GY	SS
Kara Ekşi	Antakya	Medium	Red	SS	Lifani1	İskenderun	Very large	Y	SS
Katırbaşı1	Antakya	Very large	RY	Sweet	Kırmızı Kabuk	Kırıkhan	Very large	Y	Sour
Lüfeni6	Antakya	Large	Y	SS	Kara Mehmet1	Kırıkhan	Very large	GY	SS
Mayhoşnar1	Antakya	Very large	RY	SS	Kara Mehmet2	Kırıkhan	Large	GY	SS
Mayhoşnar2	Antakya	Very large	RY	SS	Ekşi13	Kırıkhan	Very large	RY	SS
Mayhoşnar4	Antakya	Very large	Y	SS	Tatlı5	Kırıkhan	Very large	Y	Sweet
Sayfi	Antakya	Very large	Red	Sweet	Tatlı12	Kırıkhan	Very large	Y	Sweet
Ekşi8	Antakya	Medium	RY	SS	Büğleknarı	Samandağ	Very large	RY	SS
Ekşi9	Antakya	Very large	RY	SS	Tatlı10	Samandağ	Very large	GY	Sweet
Ekşi12	Antakya	Large	Red	Sour	Ekşiliknar	Yayladağı	Very large	Y	SS
Tatlı7	Antakya	Large	Y	Sweet	Gavur Güzeli	Yayladağı	Medium	Red	Sour
Tatlı8	Antakya	Large	RY	Sweet	Kara Ahmet	Yayladağı	Very large	RY	SS
Tatlı11	Antakya	Very large	GY	SS	Kandıl	Yayladağı	Very large	RY	SS
Tatlı13	Antakya	Large	Y	Sweet	Lüfeni	Yayladağı	Very large	RY	SS
Tatlı14	Antakya	Large	Red	Sweet	Yeşil Tatlı	Yayladağı	Large	GY	Sweet
Tatlı15	Antakya	Large	Y	Sweet	Kırmızı Tatlı1	Yayladağı	Very large	RY	Sweet
Tatlı16	Antakya	Large	GY	Sweet	Nifani1	Yayladağı	Very large	RY	SS
Antepnarı	Belen	Large	Red	Sweet	Nifani2	Yayladağı	Very large	RY	SS
Ekşi Lifani	Belen	Very large	GY	Sour	Nifani3	Yayladağı	Very large	Red	SS
Körnar	Belen	Very large	GY	Sour	Ekşi1	Yayladağı	Large	Y	Sour
Tatlı Lifani	Belen	Very large	GY	SS	Ekşi2	Yayladağı	Large	GY	SS
Lifani2	Belen	Large	RY	Sour	Ekşi3	Yayladağı	Large	Y	Sour
Lifani3	Belen	Very large	RY	SS	Ekşi4	Yayladağı	Very large	RY	SS
Ekşi7	Belen	Large	GY	SS	Ekşi5	Yayladağı	Large	Red	Sour
Mayhoşnar3	Dörtyol	Very large	GY	Sweet	Ekşi6	Yayladağı	Large	RY	SS
Nuznarı2	Dörtyol	Very large	Y	SS	Tatlı1	Yayladağı	Large	RY	Sweet
Ekşi14	Dörtyol	Very large	Y	SS	Tatlı2	Yayladağı	Very large	Red	Sweet
Ekşi15	Dörtyol	Medium	GY	Sour	Tatlı3	Yayladağı	Very large	Y	Sweet
Gıcıknar	Hassa	Very small	GY	Sour	Tatlı4	Yayladağı	Very large	RY	Sweet
Nuznarı1	Hassa	Large	GY	Sour	Katırbaşı	Mersin	Very large	RY	SS
Kırmızı Tatlı2	Hassa	Large	Red	Sweet	Hicaz	Mersin	Very large	Red	SS

GY: Greenish-yellow, Y: yellowish, RY: reddish-yellow, SS: sweet-sour.

Flexi DNA polymerase (Promega, Madison, WI, USA), 15 ng (in 6 µL) of template DNA, 0.5 pmol of each forward and reverse primer, 0.5 mM of each dNTP (1 µL), 25 mM $MgCl_2$ (1 µL), and 5X PCR buffer (2 µL) (Caliskan et al., 2012). Temperature cycling conditions were performed using a thermocycler (Biometra, Göttingen, Germany). The SSR amplification conditions were 1 cycle, 94 °C for 3 min; 35 cycles, 94 °C for 1 min, 50–60 °C for 1 min, and 72 °C for 2 min; and a final cycle at 72 °C for 10 min.

PCR amplification was performed separately for each SSR locus. Forward primers for each pair were covalently labeled with D2 (black), D3 (green), or D4 (blue) WellRED fluorescent dye (Proligo, Paris, France) so that amplicons could be distinguished according to fluorescent dye tags when the PCR products were run and separated in the same lane.

Initially, a sample of each PCR product was run at 100 V for 40 min on 2% (w/v) agarose gels in 1X TBE buffer and stained with 10 mg mL^{-1} ethidium bromide. PCR products were then diluted in 20 µL of sample loading solution together with 0.5 µL of GenomeLab DNA Standard-400 standards. A total of 78 samples were loaded for electrophoresis on a CEQ 8800XL capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA) in three groups depending on fragment size and fluorescent dye. All reactions were repeated at least twice to confirm reproducibility. Allele sizes for each microsatellite locus were determined using Beckman CEQ DNA Analysis Software (version 8.0).

2.3. Data analysis

IDENTITY software version 1.0 was used to determine identical genotypes for each locus, as well as the number of alleles (n), allele frequency, expected (H_e) and observed (H_o) heterozygosity, estimated frequency of null alleles (r), and the probability of identity (calculated as $PI = \Sigma(p_i)^4 - \Sigma\Sigma(2p_ip_j)^2$, where p_i is the frequency of the ith allele) (Paetkau et al., 1995). The proportion of shared alleles (ps) was calculated using MicroSat version 1.5 (Minch et al., 1995) with the ps option [option 1 – (ps)] (Bowcock et al., 1994) to assess genetic distances between individuals (Tangolar et al., 2009). After converting data to a similarity matrix, NTSYS-pc version 2.0 (Rohlf, 1998) was used to perform cluster analysis according to the unweighted pair-group with arithmetic mean (UPGMA) method (Sneath and Sokal, 1973).

3. Results

Considering the fruit weights as given by the pomegranate descriptor, 65% of them were very large (>375 g) and 29% were large (225–375 g). Fruit peel color of the accessions was commonly reddish-yellow (24 accessions) and greenish-yellow (23 accessions). According to the classification suggested by Bellini and Giordani (1998), the accessions were grouped in the classes of 'sour-sweet' (39 accessions), 'sweet' (22 accessions), and 'sour' (17 accessions) by considering the acidity contents (Table 1).

Genotyping of the 78 pomegranate accessions using nine microsatellites markers generated 38 polymorphic alleles, and six markers were polymorphic (Table 2). The number of alleles per locus ranged from 1 for MP12, MP42, and MP30 to 8 for PgAER154, with an average allele number of 4.6. Mean expected heterozygosity (H_e) and observed heterozygosity (H_o) were 0.556 and 0.610, respectively. The H_o values for PgAER154, PgAER194, PgAERB3, and PgAERB7 were higher than those of other primers. The most informative locus as to the probability of identity (PI) value was PgAERB3, for which six alleles (PI: 0.591) were detected, while the least informative locus was PgAER121, for which six alleles (PI: 0.239) were detected. The frequencies of null alleles (r) at the PgAER121 and PgAER138 loci were positive. Microsatellite fragments ranged from 174 bp to 304 bp. In addition, four alleles were identified in one or two of the 78 accessions; allele 288 of PgAER154 was detected alone in Ekşiliknar and two individual alleles were found in PgAER194 (255 in Lifani3 and 261 in Nifani3) and PgAERB3 (246 in Ekşi Lifani and 252 in Tatlı8). Five synonymous groups (SGs) could be defined within the studied pomegranate accessions: SG2 and SG4 are composed of two accessions, SG5 includes three accessions, SG3 comprises six accessions, and SG1 is composed of 17 accessions. Multilocus matching generated 43 different genotypic profiles across these 78 pomegranate accessions (Table 3 and 4). As shown in Table 3, differences among genetic SGs and identical groups (IGs) based on allelic profiles were clearly detected. Individual allele 269 of PgAER194 and allele 233 of PgAERB7 was identified only in SG1 and SG5 among the SGs. In addition, allele 287 of PgAER194 was found in IG1 as one locus. Only one allele, allele 304 of PgAER154, was found in IG3 among all groups.

To elucidate the genetic relationships among these pomegranate accessions, a dendrogram was generated by UPGMA analysis of pairwise genetic distances over six SSR loci. The genetic relationships among these Turkish pomegranate accessions are shown in the Figure. These accessions are predominantly grouped into Groups A and B, which further comprise different subgroups, while the other accessions are clustered into Groups C and D. Putatively synonymous accessions (from SG1 to SG5) were found within each cluster, except for Group C.

The accessions Antepnarı, Büğleknarı, Fellahnarı, Gıcıknar, Kandıl, Kırmızı Kabuk, Mayhoşnar2, Kırmızı Tatlı1, Lifani1, Lifani2, Lifani4, Ekşi1, Ekşi3, Ekşi6, Ekşi10, Tatlı3, and Tatlı14 were closely related to each other but distantly related to other accessions. They have different fruit sizes, fruit skin and aril colors, and acidity.

Table 2. Locus name, primer sequence, detected alleles (bp), number of alleles (n), expected heterozygosity (He), observed heterozygosity (Ho), probability of identity (PI), and the frequency of the null allele (r) for polymorphic nuclear SSR loci in pomegranate.

Locus name	Primer sequence 5′-3′	Detected alleles (bp)	n	H_e	H_o	PI	r
PgAER121	F-TGATTCTCACAGCGTCTTGTC R-AAAAGAACTGTCGGCATCAAC	250, 252, 254, 256, 258, 260	6	0.708	0.602	0.239	0.061
PgAER138	F-AGGCTTTCAGAATATAAGTGCA R-CCTTTCTCCTTTCCTTGTTATC	274, 276, 278, 280, 294	5	0.610	0.423	0.347	0.116
PgAER154	F-TGCCCCTACTCGCATAAAC R-CTTCCCTTCTTGAGGATTCG	260, 262, 280, 282, 288, 298, 300, 304	8	0.669	1.000	0.259	–0.197
PgAER194	F-CCCAGGCGTAACGATAAG R-AAACCGTAAAGCAGCAGTAA	255, 261, 269, 283, 287, 289	6	0.418	0.423	0.516	–0.003
PgAERB3	F-CGTCCAGCTTTTCGGTTA R-TTGTGCTTGATCTCTCATTTG	246, 252, 260, 262, 264, 272	6	0.278	0.307	0.591	–0.023
PgAERB7	F-CGGGTTCACTCGTCTTCTTC R-CCAACAACAATCATCAAAGGTC	217, 221, 225, 227, 231, 233, 241	7	0.653	0.910	0.314	–0.154
MP12	F-TTGAGTCCCGATCATATCTC R-CAATCTGTCAGGAACAACA	270	1	0.00	0.00	0.00	0.00
MP30	F-CCCAGTTTGTAGCAAGGTA R-AAGCTGACATTCTTTGAAGC	174	1	0.00	0.00	0.00	0.00
MP42	F-GAGCAGAGCAATTCAATCTC R-AACAATTTCCCATGTTTGAC	192	1	0.00	0.00	0.00	0.00
Total			41	3.336	3.665		
Mean			4.6	0.556	0.610		

The majority of the accessions in Group B cluster into the Subgroups B1 and B2. The accessions Kara Ahmet and Gavur Güzeli clustered together, distant from all other accessions in Group B. Sayfi and Hicaznar in Subgroup B1 have a very close genetic relationship. The accessions Yeşil Tatlı and Kara Mehmet1 in Subgroup B1 were also found to be genetically similar. The accessions within Subgroup B2, Kızılgöbek, Kızılgöbek Ekşi, Lüfeni, Mayhoşnar1, Nuznarı2, and Tatlı Lifani, also have very close genetic relationships. In addition, the accessions Payasnarı and Kara Mehmet2, also in Subgroup B2, were found to be genetically similar.

The majority of the accessions within Group A possessed a diverse set of morphological characteristics, including fruit peel color, aril color, and flavor. In some cases, the cluster positions of accessions were not related to their morphological or organoleptic characteristics. For example, fruit flavor was 'sweet-sour' for 14 accessions, 'sour' for 9 accessions, and 'sweet' for 7 accessions in Group A. Most accessions in Subgroup B2, except for Ekşi5 and Tatlı5, had 'sweet-sour' fruit. The accessions Katırdişi, Tatlı11, Ekşi2, and Tatlı4 were included in Group C but were sampled from different locations and are likely be synonymous. Group D comprised 16 accessions, including the similar accessions Tatlı13 and Tatlı15; Ekşi4 and Ekşi9; and Tatlı8 and Tatlı10. The accessions Cinzar, Ekşi14, and Tatlı2 have distinct fruit peel color and flavor types, respectively, but were nonetheless found to have very close genetic relationships.

The genetic data showed that three pairs of identical accessions were detected. The accession Sayfi (Group B1) was defined to be a clone of the cultivar Hicaznar in IG1. Identical accessions Ekşi4 and Ekşi9 (IG2) and the accessions Tatlı13 and Tatlı15 (IG3) were included within Group D. In addition, IG2 had very close genetic relationships with SG5. The fruit taste of the accessions was sweet-sour, except for Tatlı2. Accessions Tatlı13 and Tatlı15 with sweet fruit taste were included in IG3. The accessions with sweet-sour fruit taste were grouped in SG2, SG3, SG4, and SG5. The accessions within SG1 had different fruit skin colors and fruit taste.

4. Discussion

Due to the single region of origin of pomegranate in Turkey and its subsequent vegetative propagation, several instances of identical, synonymous, or homonymous accessions were found in this germplasm collection. Thus, it is very important to use sensitive and accurate molecular techniques such as SSR, AFLP, or SNP to detect DNA variation and to identify pomegranate germplasm, which would be most helpful to breeders and nursery workers for selection and propagation of a cultivar (Hasnoui et al., 2012; Nemati et al., 2012; Ophir et al., 2014). In the present study, we report for the first time the application

Table 3. Multilocus SSR profiles of the five synonymous groups (SGs) and three identical groups (IGs).

Accessions	MP12	MP42	MP30	PgAER121	PgAER138	PgAER154	PgAER194	PgAERB3	PgAERB7
SG1									
Antepnarı	270	192	174	250/254	274/278	260/300	269	272	221/227
Büğleknarı	270	192	174	250/254	274/278	260/300	269	272	221/227
Gıcıknar	270	192	174	250/254	274/278	260/300	269	272	221/227
Kandıl	270	192	174	250/254	274/278	260/300	269	272	221/227
Kırmızı Kabuk	270	192	174	250/254	274/278	260/300	269	272	221/227
Mayhoşnar2	270	192	174	250/254	274/278	260/300	269	272	221/227
Kırmızı Tatlı1	270	192	174	250/254	274/278	260/300	269	272	221/227
Lifani1	270	192	174	250/254	274/278	260/300	269	272	221/227
Lifani2	270	192	174	250/254	274/278	260/300	269	272	221/227
Lifani4	270	192	174	250/254	274/278	260/300	269	272	221/227
Ekşi1	270	192	174	250/254	274/278	260/300	269	272	221/227
Ekşi3	270	192	174	250/254	274/278	260/300	269	272	221/227
Ekşi6	270	192	174	250/254	274/278	260/300	269	272	221/227
Ekşi10	270	192	174	250/254	274/278	260/300	269	272	221/227
Tatlı3	270	192	174	250/254	274/278	260/300	269	272	221/227
Tatlı14	270	192	174	250/254	274/278	260/300	269	272	221/227
SG2									
Yeşil Tatlı	270	192	174	250/256	274	260/282	269/287	272	221/233
Kara Mehmet1	270	192	174	250/256	274	260/282	269/287	272	221/233
SG3									
Kızılgöbek	270	192	174	250/258	274	260/282	269/287	272	221/227
Kızılgöbek Ekşi	270	192	174	250/258	274	260/282	269/287	272	221/227
Lüfeni	270	192	174	250/258	274	260/282	269/287	272	221/227
Mayhoşnar1	270	192	174	250/258	274	260/282	269/287	272	221/227
Nuznarı2	270	192	174	250/258	274	260/282	269/287	272	221/227
Tatlı Lifani	270	192	174	250/258	274	260/282	269/287	272	221/227
SG4									
Payasnarı	270	192	174	250/258	274	260/280	269/287	272	221/227
Kara Mehmet2	270	192	174	250/258	274	260/280	269/287	272	221/227
SG5									
Cınzar	270	192	174	256	276	260/300	269	262/272	227/233
Ekşi14	270	192	174	256	276	260/300	269	262/272	227/233
Tatlı2	270	192	174	256	276	260/300	269	262/272	227/233
IG1									
Sayfi	270	192	174	256	274	260/282	287	272	221/233
Hicaz	270	192	174	256	274	260/282	287	272	221/233
IG2									
Ekşi4	270	192	174	256	276	260/300	269	262/272	221/227
Ekşi9	270	192	174	256	276	260/300	269	262/272	221/227
IG3									
Tatlı13	270	192	174	256	278	260/304	269	262/272	221/225
Tatlı15	270	192	174	256	278	260/304	269	262/272	221/225

Table 4. Multilocus SSR alleles of the different genotypic profiles in the pomegranate accessions.

Accessions	MP12	MP42	MP30	PgAER121	PgAER138	PgAER154	PgAER194	PgAERB3	PgAERB7
Ekşiliknar	270	192	174	250/254	274/278	260/288	269	272	221/227
Ekşi Lifani	270	192	174	256	274	260/282	269/289	246/272	221/227
Ekşiyeşil kabuk	270	192	174	256	274/280	260/282	269/289	272	221/227
Fellahnarı	270	192	174	250-254	274/278	260/300	269	272	221/227
Gavur Güzeli	270	192	174	250-256	278	260/282	269/287	264/272	221/227
Kara Ahmet	270	192	174	250	274/294	260/282	269/289	272	221/225
Kara Ekşi	270	192	174	250/254	274/278	260/300	269	262/272	221/241
Katırbaşı1	270	192	174	256	274/278	260/282	269/287	272	221/233
Katırbaşı2	270	192	174	250	274	260/282	269/287	272	221/233
Katırdişi	270	192	174	250/258	276	260/300	269/287	272	227
Kırmızı Lifani	270	192	174	252	274/278	260/282	269	272	221/227
Körnar	270	192	174	256	276	260/300	269/287	272	227/231
Mayhoşnar3	270	192	174	250/258	274	260/282	269/287	272	221
Mayhoşnar4	270	192	174	250/258	274	260/280	269/287	260/272	221/227
Nuznarı1	270	192	174	256	274	260/300	269/287	272	221/227
Kırmızı Tatlı2	270	192	174	256	276	262/300	269	262/272	221/233
Lifani3	270	192	174	250/254	274/278	260/300	255/269	272	221/227
Lifani5	270	192	174	256	274	260/300	269	262/272	221/233
Lüfeni6	270	192	174	256/258	276	260/300	269/287	262/272	227/233
Nifani1	270	192	174	256	276	260/300	269/287	262/272	227/233
Nifani2	270	192	174	250/256	274	260/282	269/287	262/272	227
Nifani3	270	192	174	250/254	274/278	260/300	261/269	260/272	221/227
Ekşi2	270	192	174	250/256	276	260/300	287	272	225
Ekşi5	270	192	174	250/258	274	260/280	269/283	262/272	221/227
Ekşi7	270	192	174	250/254	274/278	260/282	269	272	221/227
Ekşi8	270	192	174	250/254	276	260/300	269	272	221/227
Ekşi11	270	192	174	252/260	274	260/282	269/287	272	221/227
Ekşi12	270	192	174	256	274	260/298	269/287	272	221/227
Ekşi13	270	192	174	250/254	274/278	260/300	269	262/272	221/227
Ekşi15	270	192	174	250/256	274/278	260/300	269	262/272	221/227
Ekşi16	270	192	174	250	274/278	262/300	269	272	227
Tatlı1	270	192	174	250/256	274/278	260/300	269	272	221/227
Tatlı4	270	192	174	250/256	276	260/282	287	272	221/233
Tatlı5	270	192	174	250	274	260/280	269/287	262/272	221/233
Tatlı6	270	192	174	256	274	260/282	269/287	272	221/225
Tatlı7	270	192	174	256	274/278	260/300	287	262/272	221/233
Tatlı8	270	192	174	256	278	260/304	269	252/262	225/233
Tatlı9	270	192	174	256	274/278	262/282	269	272	221/227
Tatlı10	270	192	174	256	278	260/280	269	262/272	233
Tatlı11	270	192	174	254	276	260/282	269/287	272	227/233
Tatlı12	270	192	174	256	276	260/300	269/287	272	217/221
Tatlı16	270	192	174	252/254	274/278	260/300	269	272	221/227
Katırbaşı	270	192	174	256	274	260/282	269	272	221

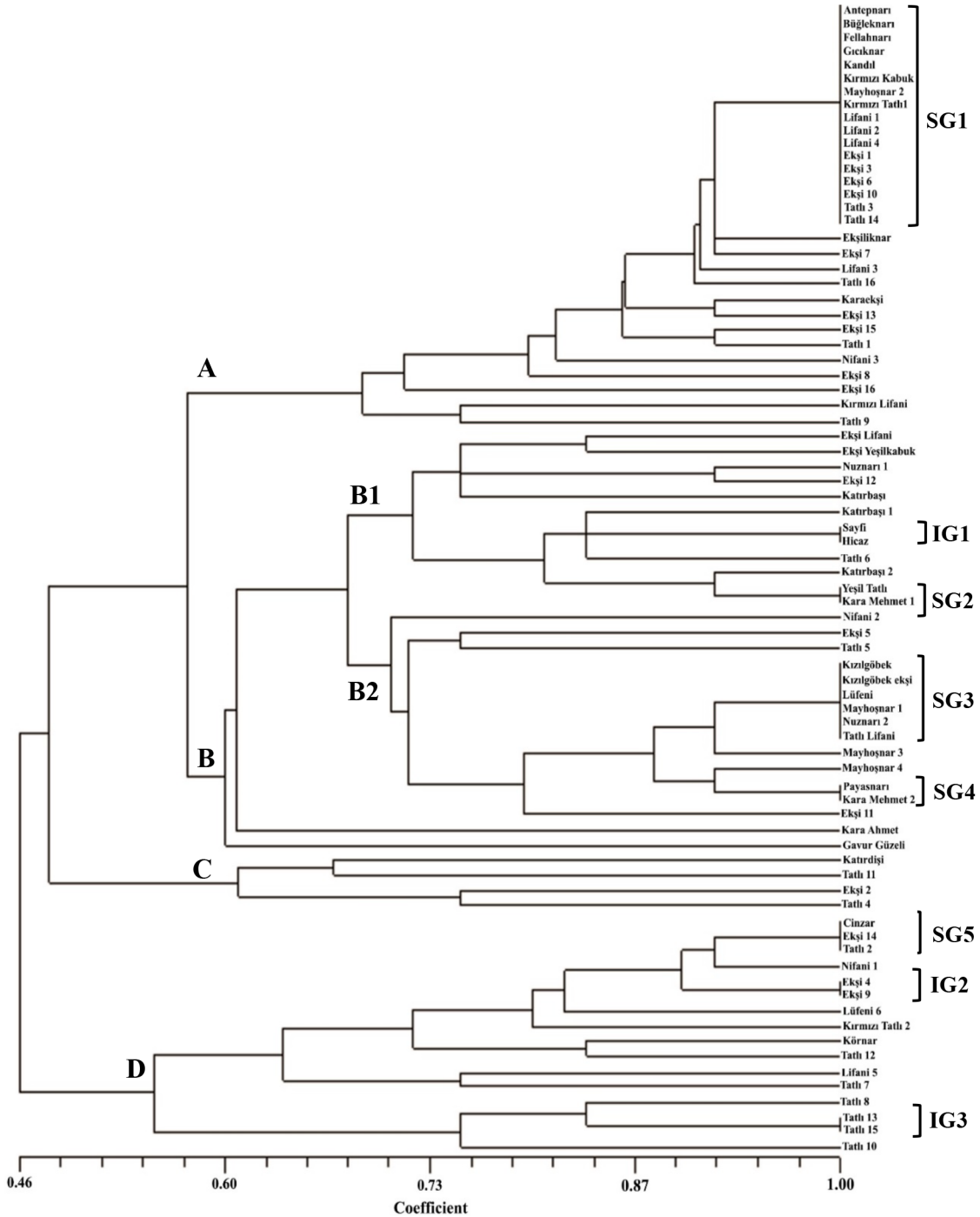

Figure. Dendrogram of genetic similarity among the analyzed 78 pomegranate accessions based on SSR markers.

of new SSR markers for assessing genetic diversity in pomegranate accessions from Turkey. These results showed that microsatellite markers could be successfully used in pomegranate germplasm characterization, in agreement with the previous results of Alamuti et al. (2012), Ferrara et al. (2014), and Ravishankar et al. (2015). The new microsatellite markers PgAER121, PgAER138, PgAER154, PgAER194, and PgAERB3 each revealed five alleles or more, while three SSR markers (MP12, MP42, and MP30) were monomorphic. Pirseyedi et al. (2010) indicated polymorphic alleles for MP12, MP30, and MP42 in genotypes from Iran, but these markers were

not polymorphic in pomegranate accessions from Turkey. These results indicate that local pomegranate resources probably have a quite narrow genetic background (Hasnaoui et al., 2010). Similar to these results, Soriano et al. (2011) previously reported 1–6 alleles per locus for pomegranate cultivars from Spain, the United States, and Turkey. Jbir et al. (2012) also obtained similar results (1–6 alleles per locus), with an average of 3.08, in a pomegranate collection from Tunisia comprising 32 accessions. In addition, allelic richness can be affected by sample size, fruit species, and marker systems (Bashalkhanov et al., 2009; Hasnaoui et al., 2010).

The mean observed heterozygosity value (0.610) for accessions from Turkey indicates substantial genetic diversity among the pomegranate accessions studied here. This value is higher than those of the genotypes from Tunisia (Hasnaoui et al., 2010) or Iran (Parvaresh et al., 2012), but is slightly lower than the value obtained by Pirseyedi et al. (2010).

A phenogram depicting the relationships among accessions based on the analysis of six SSR loci showed four major clusters. No clear separation according to the geographic origins of accessions was observed; rather, clustering followed a general pattern of shared morphopomological characteristics. Among the pomegranate accessions examined, three pairs of identical (clone) accessions were identified, whereas sets of five synonymous accessions were found. The accession Sayfi (Group B1) was found to be a clone of the cultivar Hicaznar (IG1), which is the most important cultivar in Turkey for fruit and juice consumption. The other identical accessions including the pair Ekşi4 and Ekşi9 (IG2) and the pair Tatlı13 and Tatlı15 (IG3) clustered into Group D. These accessions are grown in proximal locations (Antakya and Yayladağı) and have similar fruit parameters, such as fruit size, fruit color, and fruit flavor (Table 1).

A high number of mislabeled accessions and several synonymous cases were identified among the pomegranate accessions. Synonymous subgroups were found within Groups A, B, and D among these pomegranate accessions (Figure). Although they have different fruit skin colors, the accessions within SG1, including Antepnarı, Ekşi6, Kırmızı Kabuk1, and Tatlı14 (red-fruited accessions); Büğleknarı, Ekşi3, Kırmızı Kabuk1, Lifani2, and Mayhoşnar2 (pink-fruited accessions); and Ekşi1, Fellahnar, Gıcıknar, Kandıl, Lifani1, Lifani4, and Tatlı3 (greenish-yellow fruited accessions) shared the same genotypic profile at six SSR loci. The fruit sizes and tastes of the accessions are also different. Similar results were obtained from SG5. Ben-Simhon et al. (2015) indicated that mutations in anthocyanin-biosynthesis genes in pomegranate could be due to harmful environmental conditions, such as temperature and UV radiation. It is possible that some of the accessions analyzed here could be bud sports, as SSR markers are not powerful enough to discriminate true bud mutants from the original genotypes (Yamamoto et al., 2003; Ellegren, 2004; Boz et al., 2011). In addition, the apparent lack of relationship between the accessions within each group with respect to fruit skin color, aril color, and organoleptic properties might have been due to the fact that the SSR markers measured a specific aspect of genetic diversity. However, these results could also be explained partly by the analysis of an insufficient number of SSR loci. Identification of clusters using highly variable SSR markers is not always simple (Hedrick, 1999), as also noted in previous studies of pomegranate germplasm (Alamuti et al., 2012; Hasnoui et al., 2012). Some mutations and genetic changes that are easily recognizable phenotypically may not yet be detectable with molecular markers (Sarkhosh et al., 2006; Ebrahimi et al., 2010; Nafees et al., 2015). However, morphological characteristics such as fruit size, peel color, and flavor of the accessions located in SG2, SG3, and SG4 were quite similar to those obtained molecularly. These results support the occurrence of synonymous mislabeling in pomegranates, as in many other cultivated fruit trees.

These results show that the relatively low level of diversity detected here using microsatellite markers reflects a narrow genetic background for pomegranate germplasm in Turkey, despite the large degree of morphological variability. This result is in agreement with previous studies on pomegranate genotypes from Turkey using RAPD (Ercisli et al., 2011a) and AFLP (Ercisli et al., 2011b) markers. Similarly low levels of molecular diversity were also found in pomegranate germplasm from Iran (Sarkhosh et al., 2006), Pakistan (Nafees et al., 2015), and Tunisia (Hasnoui et al., 2012).

Pomegranate accessions have names that refer mainly to their geographical origin, flavor, or fruit color. Genetic erosion due to biotic and abiotic stress has also been reported for many accessions that are well adapted to local and regional conditions (Caliskan et al., 2012). It has therefore become imperative to establish strategies for preserving local pomegranate germplasm and conserving these genetic resources. Several studies have been carried out in all pomegranate-growing areas in Turkey and have allowed the collection of numerous genotypes (Onur, 1982; Caliskan and Bayazit, 2013; Orhan et al., 2014; Okatan et al., 2015). The collected genotypes have been identified and characterized to establish the regional distribution of morphological and genetic diversity of pomegranate in Turkey. These studies have also reported the morphopomological characteristics (Özgüven et al., 2009; Orhan et al., 2014; Okatan et al., 2015) and phytochemical parameters (Caliskan and Bayazit, 2012) of pomegranate genotypes. As suggested by Caliskan and Bayazit (2012), high levels of morphological variability

in fruit characteristics characterize local pomegranate resources. The fruits of pomegranate genotypes are generally classified as sweet, sweet-sour, or sour; early, midseason, or late; for juice or fresh consumption; and soft-seeded or hard-seeded. In addition, biochemical parameters have been used to discriminate among genotypes in Turkey based on compounds found in fruit (Poyrazoğlu et al., 2002). However, these analyses tend to be less useful because they depend on a few parameters that are strongly influenced by the environment. Molecular markers such as RAPD and AFLP have been used to identify and characterize pomegranate germplasm from Turkey (Ercisli et al., 2011a, 2011b; Orhan et al., 2014). In addition, Soriano et al. (2011) used microsatellite markers to show that pomegranate genotypes from Turkey are more closely related to genotypes from Spain than to those from India, Israel, or the United States. Therefore, further detailed studies of both genetic diversity and the distribution of pomegranate germplasm in Turkey are needed.

According to the results presented here, these new microsatellite markers could be used to assess the extent of genetic diversity within a pomegranate germplasm collection. The data could then be used to design sampling strategies, create core collections, eliminate duplicate materials, and establish pomegranate breeding studies. Alamuti et al. (2012) indicated that the use of core collections of a subset of accessions that contain most of the genetic variation in an entire germplasm collection can improve the management and efficient utilization of the germplasm.

The present study developed the first molecular database for eastern Mediterranean pomegranate germplasm using new SSR markers and represents a key step in gathering knowledge of the resources available for the genetic improvement of pomegranate. Knowledge of the information content of the SSR primer pairs used here to detect polymorphisms among these pomegranate accessions will allow the selection of the most efficient markers. Future studies of genetic diversity in pomegranate could be extended by using a larger number of SSR loci. The data presented here can also be integrated into future studies to investigate the genetic diversity of pomegranates from other regions. In conclusion, we have found that this rich and diverse genetic collection should be useful for pomegranate breeding programs.

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