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Molecular Characterization of Pomegranate (*Punica granatum* L.) Genotypes with SSR Markers

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Abstract: Pomegranate is known to be a fruit grown in tropical and subtropical climate zone belonging to Lythraceae family. Within the scope of the study, 10 kind of pomegranates and a pomegranate from a small private garden have been used for molecular analyzes from the Western Mediterranean Agricultural Research Institute in the Antalya Region. To accomplish this objective, samples of genotypes were taken under appropriate conditions and molecular analyzes were performed. As a result of analysis with SSR markers, pomegranate genotypes were separated into two main groups with a 65% similarity using UPGMA clustering method. The first main group consisted of 4 sub-groups. The genotypes in the first sub-group were Hicaz, in the second subgroup Aşınar, Batem Onur, Ernar, Batem Hicaz, the third sub-group Beynarı and the fourth sub-group Batem Esin, Batem Yılmaz and Ekşilik. The second main group is divided into 2 sub-groups where Katırbaşı and Fellahyemez were in different branches. Batem Onur and Ernar grouped together due to lack of polymorphic markers. Hicaz, Beynarı, Ekşilik, Katırbaşı and Fellahyemez types formed a separate subgroup. In the study, a close genetic similarity between Aşınar and Batem Onur-Ernar, Batem Esin and Batem Yılmaz was evident. SSR finding of pomegranate genotypes is expected to aid determining the genotypes most suitable for future breeding and conservation efforts.

***Key words**:* Marker, Molecular characterization, SSRs, Pomegranate

Nar (*Punica granatum* L.) Çeşitlerinin SSR Markörleri ile Moleküler Karakterizasyonu

Özet: Nar, Lythraceae familyasına ait tropik ve subtropik iklim kuşağında yetiştirilen bir meyve olarak bilinmektedir. Çalışma kapsamında Akdeniz Bölgesi Antalya ilinde yer alan Batı Akdeniz Tarımsal Araştırma Enstitüsü'nden 10 çeşit nar ve küçük özel mülk bahçesinden 1 çeşit nar alınarak moleküler analizler için kullanılmıştır. Bu amaçla genotiplere ait numuneler uygun koşullarda alındıktan sonra moleküler analizleri gerçekleştirilmiştir. SSR markörleri ile yapılan analizler sonucunda UPGMA kümeleme metoduna göre nar çeşitleri arasında iki ana grup ortaya çıkmış ve %65 oranında benzerlik gösterdiği belirlenmiştir. İlk ana grup kendi içinde 4 alt gruptan meydana gelmiştir. İlk alt grupta Hicaz, ikinci alt grupta Aşınar, Batem Onur, Ernar, Batem Hicaz, üçüncü alt grupta Beynarı ve dördüncü alt grupta Batem Esin, Batem Yılmaz ve Ekşilik yer almıştır. İkinci ana grup 2 alt gruba ayrılmış olup Katırbaşı ile Fellahyemez farklı gruplarda yer almaktadır. Batem Onur ve Ernar çeşitlerini birbirlerinden ayırt edecek polimorfizmler üretilemediğinden bu iki çeşit bir arada gruplanmıştır. Hicaz, Beynarı, Ekşilik, Katırbaşı ve Fellahyemez çeşitleri tek başına bir alt grup oluşturmuştur.

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Çalışmada Aşınar ve Batem Onur-Ernar, Batem Esin ve Batem Yılmaz arasında yakın genetik benzerlik olduğu gözlemlenmiştir. Nar türüne ait SSR bulgularının, gelecekteki ıslah ve koruma çalışmalarında kullanılabilecek en uygun genotiplerin belirlenmesine yardımcı olacağı beklenmektedir.

Anahtar kelimeler: Markör, Moleküler karakterizasyon, SSR, Nar

1. Introduction

Pomegranate (*Punica granatum* L.) is a perennial shrub form of the genus Punica of the Lythraceae family and is known as a fruit species that has an important place in cultural life as well as its commercial value. The cultural history of the pomegranate goes back to ancient times and various sources indicate that the breeding history dates back to 5000 years [1].

The fruits of pomegranate are used in many fields such as industry, food, cleaning, health and landscape. This fruit, which has such an important place today, needs to be developed and produced higher yielding varieties in order to meet the increasing demand. Compared to the classical methods used in the past, modern methods used today save time and labor and enable the work to be completed in a shorter time. Since the PCR-based markers are highly polymorphic [2] and simple to process, they result in the generation of relatively large amounts of data per unit of time [3]. Microsatellites, or SSRs, are extending of DNA consisting of tandemly repeated short units of 1–6 base pairs in length. The high levels of variability and reproducibility associated with SSR markers will allow them to serve as anchor markers between different genetic maps within a specific crop [4]. In this study, 11 pomegranate genotypes were used and genetic identification was performed with 16 SSR (Simple sequence repeats) primers. It was aimed to determine the genetic similarities within the population and DNA identity information (allele data) related to the degree of relationship. SSR findings of pomegranate species can be used as a step for future breeding studies in the region as well as to determine the distribution areas of pomegranate genotypes and to compare genetic collections. In recent years, the development of new PCR-based marker systems has played a strategic role in molecular breeding, which will be carried out in pomegranate fruits as in many other fruit species.

2. Material and Method

2.1 Plant material

In this study, 11 genotypes belonging to pomegranate genetic resources of Turkey were used. These genotypes were obtained from the Western Mediterranean Agricultural Research Institute (Batem, Antalya, Turkey). These genotypes are Hicaz, Katırbaşı, Aşınar, Batem Esin, Batem Hicaz, Batem Yılmaz, Batem Onur, Ekşilik, Ernar, Fellahyemez and Beynarı. Molecular analyzes were carried out in Isparta University of Applied Sciences, Agricultural Biotechnology Laboratory.

2.2 DNA isolation

For the isolation of DNA, fresh leaves free from diseases and pests were used. DNA was isolated from 50-60 mg of leaf material using the CTAB extraction protocol [5]. The 500 µl of DNA isolation buffer (1M Tris-HCl pH 8.0, 0.5 M EDTA, 5 M NaCl, 20 g CTAB) and 0.8 g of PVP, 100 µl of β-mercaptoethanol were added to the samples and

the samples were ground in buffer solution. Homogenized samples were incubated for 1 hour at 55 °C and after incubation, 500 µl of chloroform was added to the samples and the tubes were mixed slowly and centrifuged at 16 rcf for 7 minutes. Then, the supernatant was transferred to new eppendorf tubes, 0.08 volumes of cold 7.5 M ammonium acetate and 0.54 volumes of cold isopropanol was added and incubated on ice for 30-40 min. The solution was centrifuged at 16 rcf for 3 minutes to remove the supernatant. The pellet was then mixed by adding 700 µl of 70% cold ethanol, then centrifuged at 16 rcf for 1 minute and discarded. 700 µl 95% cold EtOH was added and centrifuged at 16 rcf for 1 minute to remove the supernatant. DNA was dissolved at room temperature by adding 50 µl of TE buffer and DNA quality and concentration were checked by comparison with standard λ-DNAs run on 1.2% agarose gel electrophoresis of each sample and reading at 260 to 280 nm wavelengths on the spectrophotometer.

2.3 SSR analyses

In this study, 16 SSR primer pairs selected from SSR primers which were used successfully in the previous studies (Table 1).

Table 1. Primary pairs used in pomegranate genotypes

Primer code	Forward	Reverse	References
UDO24	GGATTTATTAAAAGCAAAACATACAAA	CAATAACAAATGAGCATGATAAGACA	[6]
UDO99	AAAAACACAACCCGTGCAAT	AAATTCCTCCAAGCCGATCT	
DCA4	CTTAACTTTGTGCTTCTCCATATCC	AGTGACAAAAGCAAAAGACTAAAGC	[7]
DCA16	TTAGGTGGGATTCTGTAGATGGTTG	TTTTAGGTGAGTTCATAGAATTAGC	
GAPU47	GATCAGCTTAGTCTCATATTCTCTCTC	CCTCGACTGATTTACACACCA	[8]
GAPU59	CCCTGCTTTGGTCTTGCTAA	CAAAGGTGCACTTTCTCTCG	
GAPU103	TGAATTTAACTTTAAACCCACACA	GCATCGCTCGATTTTATCC	
GD15	CGAAAGTGAGCAACGAACTCC	ACTCCATCATCGGGTGGTG	[9]
GD147	TCCCGCCATTTCTCTGC	AAACCGCTGCTGCTGAAC	
RiM015	CGACACCGATCAGAGCTAATTC	ATAGTTGCATTGGCAGGCTTAT	[10]
RiM019	ATTCAAGAGCTTAACTGTGGGC	CAATATGCCATCCACAGAGAAA	
RiM036	AGCAACCACCACCTCAACTAAT	CTAGCAGAATCACCTGAGGCTT	
RhM001	GGTTCGGATAGTTAATCCTCCC	CCAACTGTTGTAAATGCAGGAA	
RhM003	CCATCTCCAATTCAGTTCTTCC	AGCAGAATCGGTTCTTACAAGC	
Ch05e03	CGAATATTTTCACTCTGACTGGG	CAAGTTGTTGTACTGCTCCGAC	[11]
CHO49	TGGAGAGATGGCTCGAGGTT	TGGTTGCTGGGAATTGAACTC	[12]

PCR reaction for primer pairs UDO99, UDO24, DCA4, DCA16, RhM001, RhM003: PCR reactions were carried out in a volume of 50 µl containing 50 ng of DNA template, 0.75 pmol of each primer, 0.2 mM dNTP mix, 1 mM $MgCl_2$, 1 X Taq buffer and 0.25 unit of Taq DNA polymerase. PCR conditions were as follows: denaturation of 3 min at 95 °C; 35 cycles of 60 s at 95 °C; 1 min at 52 °C; and 1 min at 72 °C; and final extension of 10 min at 72 °C. PCR products were run in 2.2% agarose gel under a 90 volt electric current for 1 hour and 15 minutes [13].

PCR reaction for primer pairs of GAPU59, GAPU103, GAPU47, Ch05e03, GD147, GD15, RiM019, RiM020, RiM036, CHO49: The PCR reaction consisted of the following components, with a total volume of 20 µl. Amplification was performed in 20 ng of DNA template, 0.40 pmol of each primer, 0.20 mM of dNTPs, 1 X Taq buffer and 0.25 unit of Taq DNA polymerase. PCR conditions were as follows: denaturation of 3 min at 95 °C; 35 cycles of 60 s at 95 °C; 1 min at 52 °C; and 1 min at 72 °C; and final extension of 10 min at 72 °C. PCR products were run in 2.2% agarose gel under a 90 volt electric current for 1 hour and 15 minutes [13].

2.4 Evaluation of polymorphisms and data analysis

Genetic analysis of genotypes was performed as described in Bittel et al. [14]. Genetic parameters such as number of alleles (n), allele frequency, expected heterozygote (He), observed heterozygote (Ho) ratio, null allele frequency (r) and probability of detection (Probability of Identity) (PI) were determined using IDENTITY 1.0 [15] software program and the similarity index was determined using Microsat [16] program. Relationships of the pomegranate populations were estimated from the SSR data using the UPGMA clustering method on the basis of Nei's [17] unbiased genetic distance. The UPGMA tree was constructed using NTSYS-pc 1.8 programme [18].

3. Results

As a result of the SSR analysis, the total number of alleles was 195, the number of specific alleles was 71 and the band size was between 121-462 bp (Table 2). The number of alleles per loci was between 3 and 16, with an average of 12.19. Furthermore, it was found that the expected He was higher for most primer pairs than the observed Ho. The expected He was lower in the Ch05e03, GD147, CH049 and DCA16 primers than the Ho observed. The highest number of alleles was found in GD147 (16) and RhM003 (16), the highest expected heterozygous RiM019 (0.876) primer and the observed heterozygosity value was found in GD147 (0.856) primer. The polymorphic information content (PIC) varied between 0.04 and 0.83. The lowest PIC value (0.04) was obtained in GD15 primer and the highest (0.83) in UDO99 primer. The lowest detection probability (0.06) and the highest RhM003 (0.951) were determined in the GD15 primer pair.

Table 2. The number of alleles, the size of bands, the observed (Ho) and expected (He) heterozygosity, the probability of identity (PI) and polymorphic information content (PIC) obtained from SSR primer combinations in pomegranate

Primer	Number of total alleles	Number of specific alleles	Allele size range, bp	Ho	He	PI	PIC
UDO99	12	4	220-441	0.682	0.703	0.080	0.830
DCA4	11	3	145-386	0.750	0.790	0.370	0.632
GAPU59	8	3	224-341	0.680	0.760	0.796	0.750
GAPU103	12	5	141-322	0.709	0.796	0.280	0.649
GAPU47	9	3	133-251	0.620	0.640	0.320	0.574
Ch05e03	13	4	161-215	0.742	0.715	0.082	0.824
GD147	16	5	121-172	0.856	0.831	0.069	0.820
GD15	3	1	142-159	0.021	0.023	0.951	0.040
RiM019	15	5	162-234	0.541	0.876	0.223	0.452
RiM036	15	6	221-363	0.641	0.849	0.162	0.424
RhM003	16	6	210-291	0.630	0.820	0.060	0.720
CHO49	11	4	172-224	0.751	0.709	0.076	0.811
DCA16	12	5	139-392	0.710	0.707	0.290	0.711
RiM020	14	6	215-376	0.529	0.853	0.215	0.461
UDO24	13	5	231-462	0.672	0.692	0.090	0.810
RhM001	15	6	207-312	0.710	0.790	0.080	0.760
Total	195	71					
Mean	12.19	4.44		0.64	0.722	0.259	0.642

Luo et al. [19] determined the genetic variation and population structure of 136 pomegranate varieties using 13 SSR markers. While the average n per loci was 6.31, genetic variation varied between 0.28, 0.16 and 0.37. The PIC varied between 0.14 and

0.29 at an average value of 0.22. In our study, the average n per locus was 12.19, which showed that the SSR primers we used were more polymorphic than some pomegranate populations from Fars using SSR markers [20]. In another study new microsatellite markers were used to determine the character of the 78 varieties of pomegranate from Turkey. Using 6 SSR primers, a total of 41 alleles were characterized by an average of 4.6 alleles per locus and the PI of 0.366 [21]. Zarei et al. [20] conducted 50 pomegranate varieties and 16 SSR markers from five regions in the Moroccan province of Iran. Each SSR marker used in the study was polymorphic and 48 fragments were produced in the studied samples. The expected and observed average He of the 16 SSR loci were 0.33 and 0.48, respectively. The polymorphic information content ranged from 0.41, 0.18 and 0.58 on average.

In order to reveal the relationship between varieties and genotypes by using dice similarity value, grouping analysis was performed with NTSYS-pc program using UPGMA method. The similarity values of the obtained groups varied between 0.77-0.88. Group analysis between pomegranate genotypes revealed two main groups (Figure 1). The first main group consists of 4 sub-groups. In the first subgroup Hicaz, in the second subgroup were Aşınar, Batem Onur, Ernar, Batem Hicaz, Beynarı in the third subgroup and Batem Esin, Batem Yılmaz and Ekşilik in the fourth subgroup. The second main group was divided into 2 subgroups. The first subgroup was Katırbaşı and the second subgroup was Fellahyemez. Batem Onur and Ernar varieties were grouped together due to lack of polymorphic marker. Hicaz, Beynarı, Ekşilik, Katırbaşı and Fellahyemez varieties formed a subgroup by itself. It was observed that there was a close correlation between Aşınar and Batem Onur-Ernar, Batem Esin and Batem Yılmaz. Dice coefficient values calculated using all genotypes are given in Table 3.

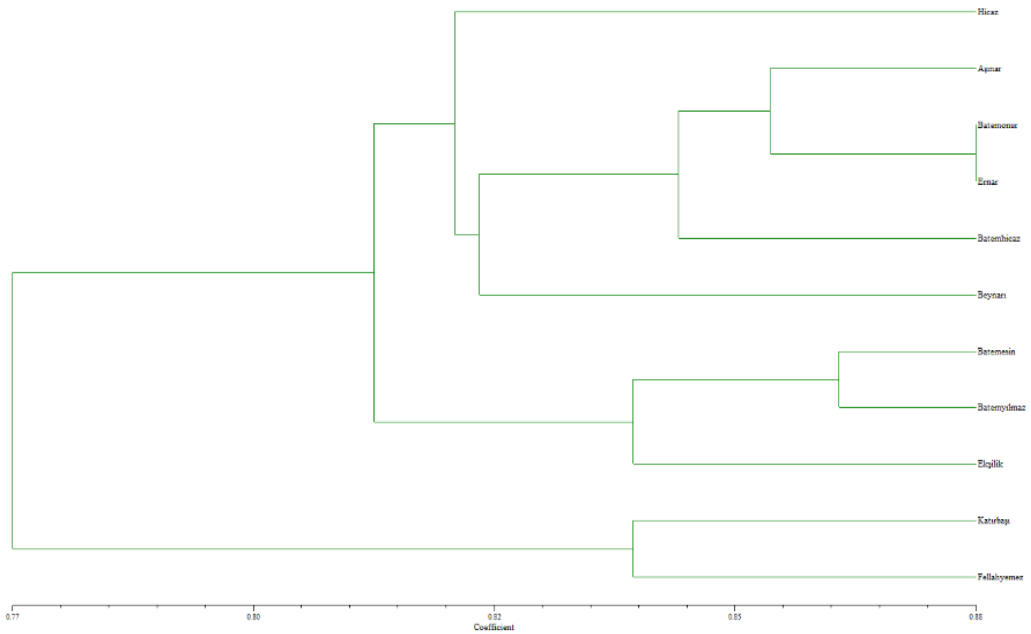

Figure 1. The classification of pomegranate genotypes based on UPGMA method with SSR primer combinations

Table 3. Similarity values of pomegranate genotypes calculated by Dice coefficient method

	Hicaz	Katırbaşı	Aşınar	Batem Esin	Batem Hicaz	Batem Yılmaz	Batem Onur	Ekşilik	Ernar	Fellah Yemez	Bey narı
Hicaz	1.000										
Katırbaşı	0.767	1.000									
Aşınar	0.858	0.742	1.000								
Batem Esin	0.775	0.808	0.800	1.000							
Batem Hicaz	0.833	0.767	0.858	0.825	1.000						
Batem Yılmaz	0.825	0.792	0.850	0.867	0.808	1.000					
Batem Onur	0.833	0.800	0.875	0.842	0.867	0.858	1.000				
Ekşilik	0.767	0.817	0.792	0.858	0.817	0.825	0.833	1.000			
Ernar	0.767	0.700	0.842	0.775	0.817	0.808	0.883	0.783	1.000		
Fellah Yemez	0.725	0.842	0.750	0.817	0.758	0.800	0.775	0.808	0.725	1.000	
Beynarı	0.808	0.725	0.833	0.783	0.792	0.833	0.842	0.808	0.825	0.717	1.000

The similarity coefficients ranged between 0.700-0.883. The lowest value was 0.700 between Ernar and Katırbaşı, two being genetically the most dissimilar. The similarity coefficient between Batem Onur and Ernar was found to be 0.883 as the highest similarity value. SSR markers were able to distinguish the genotypes and established genetic similarity index. Results may aid both breeding, variety protection, and conservation efforts.

4. Conclusion and Comment

In this study, molecular characterization of 11 pomegranate genotypes were performed by SSR technique and genetic relationship between genotypes was revealed. 11 pomegranate genotypes have genetic similarities over 70% or differences as much as 30%. When the similarity dendrogram was examined, it was found that the genotypes used in the study showed similarity at least 65%. According to the results, SSR markers are useful for identification and classification of pomegranate genotypes. The results obtained from this study can be used to determine the distribution areas of pomegranate genotypes, to compare genetic collections, to characterize pomegranate genotypes and to select parent in future breeding programs.

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