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Full Length Research Paper

Molecular and pomological diversity among pomegranate (*Punica granatum* L.) cultivars in Eastern Mediterranean region of Turkey

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Pomegranate (*Punica granatum* L.) is an important fruit species for Turkey where many cultivars are being cultivated. In this study, we determined the fruit characteristics and RAPD band patterns of six local cultivars from Hatay, Turkey. Our results demonstrated that there is a great level of morphological variation. The principle component analysis of 18 quantitative fruit characteristics revealed that fruit weight, aril number/fruit, peel color and soluble solids/acidity ratio are important traits for discriminating the cultivars tested. The UPGMA cluster of fruit characteristics indicated that 'Katırbaşı' and 'Kan narı' were similar to each other and they were separated from rest of the cultivars. Twenty-two RAPD primers generated total of 106 reproducible bands 22% of which were polymorphic. The UPGMA dendrogram of RAPD data showed that 'Tatlı nar' and 'Şerife' were very closely related while 'İncekabuk' is distinct from the other cultivars. As a result, discrepancies were detected between morphological and molecular data. Therefore, we confirmed that diversity among the fruit characteristics were not good indication of genetic relatedness while molecular tools are valuable to study such similarities.

Key words: Genetic resource, genetic variability, pomegranate, *Punica granatum*, RAPD.

INTRODUCTION

Pomegranate, *Punica granatum* L., is one of the oldest cultivated species among the fruits. It belongs to the subclass Rosidae and believed to be native to the region between Iran to northern India (Stover and Mercure, 2007). Currently, it is an important fruit species for India, Iran, USA and Mediterranean countries like Greece, Spain, Tunisia. The fruit is consumed as a table fruit; additionally, it can be processed into juice, syrup, jams and wine (Poyrazoğlu et al., 2002). Although the chemical composition of the fruit is affected from cultivar, growing region, climate, maturity, cultural practice and storage (Cemeroğlu et al., 1988; Ünal et al., 1995; Melgarejo et al., 2000), pomegranate is known to have rich sources of organic acids, phenolic compounds, sugar, water-soluble vitamins and minerals. In recent years, there has been an increasing interest in determining antioxidant properties of red fruits, due to their rich dietary sources of antioxidant phenolics and anthocyanins (Ozgen et al., 2007, 2008). Pomegranate is one of these fruit species; and its popularity is increasing worldwide.

Turkey is one of the important pomegranate growing countries. The total pomegranate production of Turkey is expected to exceed 100,000 tons in 2007. It has been estimated that there are more than 2.5 million trees in Turkey and most of them are located in Mediterranean, Aegean and South-East Anatolia regions where they are significant productions (Anonymous, 1996). Indeed, 52 of the 80 provinces have pomegranate production in Turkey (Özgüven and Yılmaz, 2000). As Turkey has many

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ecological regions, there are many pomegranate cultivars adapted to these regions with different consumer preferences. There has been number of studies to characterize the local Turkish pomegranate genotypes for possible utilization in breeding studies (Onur, 1988).

There are many molecular marker systems available for plant scientists to characterize genetic resources and cultivars (Staub et al., 1996). These systems have advantages and disadvantages for each study depending on several factors such as its objectives and crop studied (Hokanson, 2001; Luby and Shaw, 2001). Although there are some questions on reliability and repeatability of Randomly Amplified Polymorphic DNA (RAPD), they have been widely used as they were proven to be effective. Some of the recent examples include utilization in date palm (Trifi et al., 2000), loquat (Badenes et al., 2004), mulberry (Orhan et al., 2007) and olive (Belaj et al., 2003; Ganino et al., 2007; Gemas et al., 2004; Rotondi et al., 2003; Sanz-Cortes et al., 2001; Taamalli et al., 2006). On a two recent study RAPDs along with fruit characteristics (Sarkhosh et al., 2006; Zamani et al., 2007) and fatty acid composition (Ercisli et al., 2007) were used to assess genetic variation among pomegranate accessions.

The main objective of the present study was to determine the molecular and pomological diversity among the popular cultivar from the Eastern Mediterranean region of Turkey. The second objective was to relate these diversity patterns to develop strategies for further breeding studies.

MATERIALS AND METHODS

Plant materials

Six local cultivars ('İncekabuk', 'Ekşi nar', 'Kan narı', 'Katırbaşı', 'Şerife' and 'Tatlı nar') were sampled from various parts of Hatay, Turkey. Hatay is located in the eastern Mediterranean Region of Turkey with a typical Mediterranean climate. Pomegranate is one of the most important fruit species in Hatay. The fruit is usually consumed as fresh but Hatay has also tradition to use pomegranate products as well.

Pomological characterization

For each cultivar, approximately 25 kg mature fruits were randomly harvested from trees in 2007. The fruits representing the typical characteristics of each cultivar were then transported to laboratory for analysis. Fruit and aril weight were measured by using a digital balance with a sensitivity of 0.001 g (Scaltec, SPB31). Linear dimensions, length and width of fruits were measured by using a digital caliper gauge with a sensitivity of 0.01 mm. Aril width was determined by the same instrument. The red coverage on peel color was subjectively estimated in percentage. Peel color measurements were conducted by Minolta Chroma Meter CR-400 having a measuring area of 8 mm in diameter for readings of small samples without cut-off. L* (lightness), a* (green to red) and b* (blue to yellow) values were measured. Minolta a* and b* values were used to compute values for hue angle (a = tan-1b*/a*) and chroma (a*2+b*2)1/2, two parameters that are effective for describing visual color appearance (Bernalte et al., 2003). Seed firmness (1 to 9 scale 9 being hardest), juice color were determined subjectively by a team of three researchers. Aril numbers were determined at each fruit separately. All measurements were carried out in three replicates having 10 fruits in each replicate.

Molecular analysis

Young leaves were collected from a single tree for each pomegranate cultivar and immediately frozen in liquid nitrogen and stored at –80°C. High molecular weight genomic DNA was extracted from the leaf samples following the protocol for minipreps by using CTAB (Dellaporta et al., 1983). DNA concentration was measured using a NanoDrop, ND 100 spectrophotometer (NanoDrop Technologies, Inc.) and gel electrophoresis. DNA was diluted in water to a final concentration of 50 ng/µl and stored at –20°C.

A hundred RAPD primers (from sets of OPAD, OPAF, OPAG, OPAH, OPAI, OPAJ, OPB, OPD and OPX, Operon Technologies, Almeda, CA, USA) were screened initially on a sample of the accessions. Primers that produced reproducible, polymorphic bands were used to amplify the rest of the accessions. Twenty-two 10-mer primers which were found to be polymorphic were used to generate the RAPD markers. Amplification reactions were done in 10 µl volumes containing 2x PCR Mastermix (Fermantas K0171), 1 unit of Taq DNA polymerase (Fermentas EP0402), $MgCl_2$, 30 ng of the primer and 20 ng of myrtle DNA. The mixtures were assembled at 0°C, and then, transferred to thermal cycle, precooled at 4°C. The amplification was carried out in a model Master Gradient thermal cycler (Eppendorf) using a program consisting of an initial denaturation step of 2 min at 94°C, and then, 55 cycles of 2 min at 94°C, 1 min at 37°C, 2 min 72°C, followed by a 10 min elongation step at 72°C. PCR products were stored at 4°C before analysis.

The amplification products were separated by electrophoresis in 2% agarose gels and 0.5 µg/ml ethidium bromide in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0) for 3 h at 70 volts. The fragment patterns were photographed under UV light for further analysis. A 1 kb DNA ladder was used as the molecular standard in order to confirm the appropriate RAPD markers.

Statistical analysis

Fruit characteristic data were analyzed using SAS procedures (SAS, 1990). The means and standard deviations were calculated using PROC TABULATE. PROC PRINCOMP was used to conduct principle component (PCo) analysis with using 18 quantitative fruit characteristics.

RAPD data were recorded as 1 for the presence of a band and 0 for its absence to generate a binary matrix. Only reproducible bands were scored for all the accessions tested. The data set was used to perform Principle Coordinate (PCoA) and cluster analyses using NTSYS program (Rohlf, 1992). First, a similarity matrix was generated using Jaccard coefficients. This matrix was then, used for PCoA. For cluster analysis, the UPGMA (Unweighted Pair Group Method using Arithmetic Average) method was used to construct dendrograms. The bootstrap values for the clusters were calculated by 1000 replicates using PAUP program (Swofford, 1998). The representativeness of dendrograms was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix, using Mantel's matrix correspondence test (Mantel, 1967). The result of this test is a cophenetic correlation coefficient, *r*, indicating how well dendrogram represents similarity data.

RESULTS AND DISCUSSION

Considerable morphological variations were determined for almost all fruit characteristics (Table 1). For example,

Table 1. Several fruit characteristics of six pomegranate cultivars sampled from Hatay, Turkey. Qualitative characteristic values represent triplicate means ± standard deviation from the mean.

Cultivar	Fruit weight (g)	Fruit width (mm)	Fruit length (mm)	Width/length ratio	Peel width	Peel background color	Red coverage on peel (%)	Peel color				
								L	a	b	Chroma	Hue
İncekabuk	313 ± 4	84.5 ± 0.6	75.4 ± 1.0	1.12 ± 0.02	3.03 ± 0.24	Yellow	43.3 ± 7.1	30.3 ± 4.0	16.8 ± 4.6	7.7 ± 1.3	18.5 ± 4.6	25.2 ± 3.6
Ekşi nar	389 ± 5	89.7 ± 0.9	80.2 ± 1.0	1.12 ± 0.01	2.59 ± 0.14	Green-yellow	21.3 ± 2.1	31.1 ± 6.2	17.7 ± 8.0	8.5 ± 1.7	19.9 ± 7.3	28.9 ± 13.3
Kan narı	532 ± 15	100.0 ± 1.3	91.4 ± 0.8	1.09 ± 0.01	3.35 ± 0.08	Cream	94.0 ± 1.6	21.0 ± 7.4	18.6 ± 5.1	6.1 ± 2.2	19.6 ± 5.5	17.7 ± 1.5
Katırbaşı	610 ± 15	102.9 ± 0.5	93.7 ± 0.2	1.10 ± 0.01	3.71 ± 0.03	Green-yellow	15.9 ± 9.0	24.1 ± 4.6	12.7 ± 1.7	5.9 ± 0.5	14.0 ± 1.5	25.5 ± 4.4
Şerife	213 ± 4	75.1 ± 0.4	69.2 ± 0.4	1.09 ± 0.01	2.73 ± 0.32	Yellow	35.3 ± 3.5	34.9 ± 6.3	14.0 ± 3.1	8.8 ± 0.7	16.7 ± 2.4	32.8 ± 7.0
Tatlı nar	377 ± 5	91.8 ± 2.0	79.0 ± 2.4	1.16 ± 0.01	3.11 ± 0.33	Yellow	37.8± 14.2	36.1 ± 4.9	3.1 ± 1.6	8.6 ± 0.8	9.2 ± 0.8	70.1 ± 9.6
Mean	406 ± 136	90.7 ± 9.6	81.5 ± 8.9	1.11 ± 0.03	3.09 ± 0.33		41.3± 26.9	29.6 ± 1.3	13.8 ± 2.4	7.6 ± 0.6	16.3 ± 2.5	33.3 ± 4.3

fruit weight ranged from 213 to 610 g/fruit. 'Katırbaşı' had the heaviest fruit. The fruit width and length were also high in 'Katırbaşı'. This is not surprising as 'Katırbaşı' is known as the largest-fruited pomegranate in the region. The cultivars also had variable peel width ranging from 2.59 to 3.71 mm. Three colors were determined for peel background; yellow, cream and green-yellow. Red coverage on peel ranged from 15.9 ('Katırbaşı') to 94.0 ('Kan narı'). For aril color, L (darkness to lightness) varied between 21.0 and 36.1. Even greater variations were detected for a values (green to red), 3.1 – 18.6. Although to less extent, cultivars had different b values (5.9 – 8.8). Hue was one of the most varied measurements. The lowest means were recovered from 'Kan narı'. The differences were modest among 'İnce kabuk', 'Ekşi nar', 'Katırbaşı' and 'Şerife'. However, 'Tatlı nar' seemed to be distinct from these groups based on the H value.

Aril characteristics of the cultivars were presented in Table 2. Similar to fruit size characteristics, the highest aril number was recovered from 'Katırbaşı' (335 aril/fruit). 'Şerife' only had 118 arils/fruit. The lightest aril weight was also measured on 'Şerife' (23.4 g/100 arils) while 'Tatlı nar' had the heaviest arils (62.7 g/100 arils). Aril/fruit ratio was similar among the cultivars (53.4 to 59.4%) except 'Tatli nar' (36.9%). The subjective seed firmness values varied between 3 – 9 and 'Kan narı' had the most favorable seed firmness. The juice color of the cultivars ranged from salmon/cream (sweet 'Tatlı nar') to dark red ('Kan narı'). The sour cultivars 'Ekşi nar' and 'Şerife' has dark pink and light red juice while the juice color in 'İnce kabuk' was red.

Pomegranate has been grown in many parts of Turkey with many different consumption purposes since ancient times; hence, due to long history of cultivation numerous pomegranate cultivars are currently available. There have been number of studies to characterize these pomegra-nate cultivars. For example, Onur (1988) made selection study in Mediterranean region and identified superior genotypes. Ozguven et al. (1997) also determined the overall fruit characteristics of Turkish pomegranate cultivars. Indeed, the characteristics of most of the Turkish pomegranates were reviewed in Ozguven and Yilmaz (2000). The fruit characteristics in this study were comparable to previous studies conducted in Turkey. Moreover, our results were also comparable to those conducted in Greece, Tunisia and Iran (Drogoudi et al., 2005; Mars and Marrakchi 1999; Zamani et al., 2007).

The results of the PCo are displayed in Table 3. The first three PC explained virtually all of the morphological variation among the cultivars tested. PC1 had 93% the variation followed by 4 and 3% for PCs 2 and 3. The highest correlations among the variables and PC1 were fruit weight and aril number/fruit. Peel color was highest positively correlated trait to PC2 while Hue of aril color was highly but negatively correlated with PC2. PC3 was positively correlated by fruit and aril weights and soluble solids/acidity ratio.

Principle component analyses were previously employed by several researchers to characterize pomegranate germplasm. For example, Mars and Marrakchi (1999) studied 30 pomegranate accessions for Tunisia and found that the discriminating characters for their germplasm were fruit size, color and juice characteristics. The same traits were found to be important in the study of Drogoudi et al. (2005) where they determined the several characteristics of 20 Greek pomegranate cultivars. Therefore, our results of PCo regarding the important discriminating traits were in agree-

Table 2. Several aril and seed characteristics for six pomegranate cultivars sampled from Hatay, Turkey. Qualitative characteristic values represent triplicate means ± standard deviation from the mean.

Cultivar	Type	Aril number/fruit	Arils weight (g/100 arils)	Aril / fruit ratio (%)	Seed Firmness (1 - 9 scale)	Juice color
İncekabuk	Sour-sweet	186 ± 4	35.9 ± 2.4	59.4 ± 2.0	5	Red
Ekşi nar	Sour	208 ± 4	47.4 ± 7.9	53.4 ± 1.6	9	Dark pink
Kan narı	Sour-sweet	287 ± 3	40.0 ± 4.2	53.9 ± 0.9	3	Dark red
Katırbaşı	Sour-sweet	335 ± 15	53.2 ± 4.0	54.9 ± 3.3	5	Light pink
Şerife	Sour	118 ± 1	23.4 ± 1.2	55.6 ± 1.5	5	Light red
Tatlı nar	Sweet	139 ± 12	62.7 ± 7.6	36.9 ± 2.9	9	Salmon/cream
Mean		212 ± 79	43.8 ± 13.7	52.4 ± 7.6		

Table 3. Coefficients and eigenvalues for the first three principle components (PC) of PCA analysis for six pomegranate cultivar using 18 quantitative fruit characteristics.

Variable	PC1	PC2	PC3
Fruit weight	0.87	-0.08	0.38
Fruit width	0.06	0.00	0.08
Fruit length	0.06	0.02	0.02
Width/length ratio	0.00	0.00	0.00
Peel width	0.00	0.00	0.00
Red coverage on peel (%)	0.02	0.76	0.17
Aril number/fruit	0.49	0.13	-0.71
Aril weight	0.04	-0.18	0.31
Aril / fruit ratio	0.00	0.05	-0.25
Soluble solids	0.00	0.01	-0.01
pH	0.00	0.00	0.00
Acidity	0.00	0.01	-0.02
Soluble solid/acidity ratio	0.00	-0.10	0.35
Peel color L	-0.01	-0.15	-0.09
Peel color a	-0.01	0.33	0.08
Peel color b	0.01	-0.10	-0.02
Peel color Chroma	0.01	0.06	0.00
Peel color Hue	0.02	-0.45	-0.11
Eigen value	27586	1250	934
Difference	26336	316	913
Proportion	0.93	0.04	0.03
Cumulative	0.93	0.97	1.00

ment with previous studies.

Factor score of the cultivars generated from the analysis of these 18 quantitative measurements were plotted on the first three PCs (Figure 1). The cultivars were scattered on this figure. ‘Katırbaşı’ and ‘Kan narı’ were separated from other groups. This is not surprising as ‘Katırbaşı’ had the largest fruits and aril number/fruit. ‘Kan narı’ was separated from other cultivars mostly by PC2. ‘Kan narı’ is distinct from other cultivars for its peel, aril, and juice colors. UPGMA phenogram of the same data was presented in Figure 2. Similar results were obtained by this analysis as well: ‘Katırbaşı’ and ‘Kan narı’ were separated from the rest of the cultivars.

The primers code, their sequences, size of the repeatable bands and their polymorphism (in percentage) were presented in Table 4. Twenty-two RAPD primers generated a total of 106 bands. The sizes of the bands ranged from 250 - 2500 bp. Twenty-three of these bands were polymorphic making 22% polymorphism.

Sarkhosh et al. (2006) studied 24 Iranian pomegranate cultivars by RAPD primers. After their initial screening by 100 primers, they proceeded by 16 primers which gave 178 reportable bands. Therefore, their percentage polymorphism was 57%. When the same genotypes were

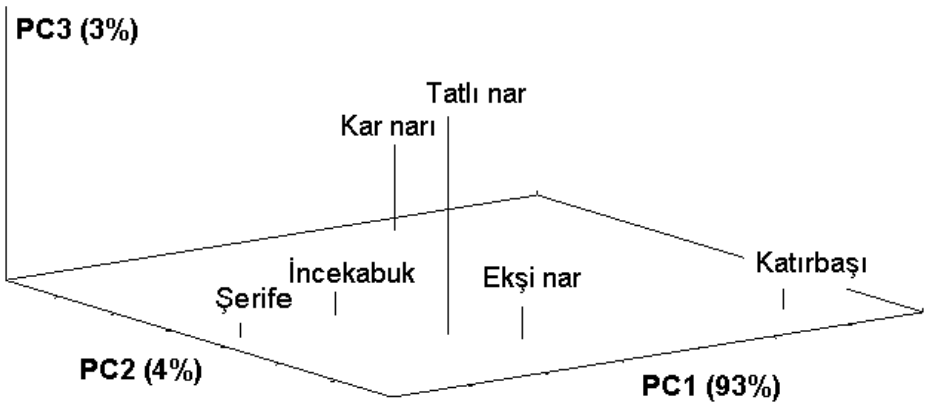

Figure 1. Factor scores for the first three principle components (PC) of 18 quantitative fruit characteristics for six pomegranate cultivars.

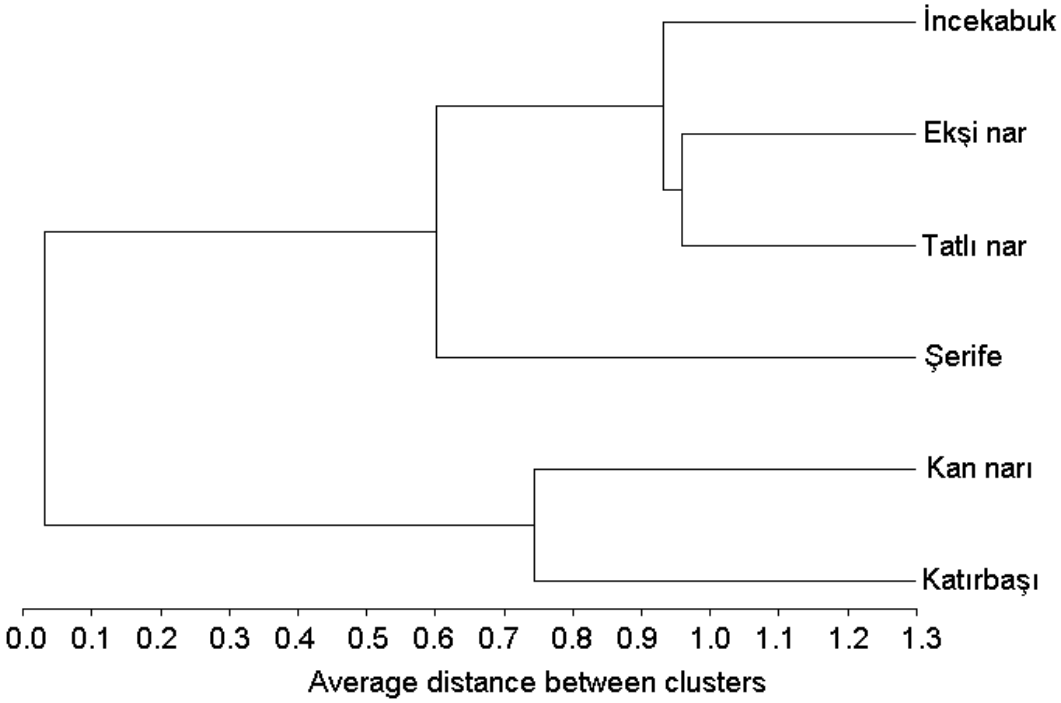

Figure 2. UPGMA phenogram of six pomegranate cultivars based on average distances among cultivar means of 18 quantitative fruit characteristics.

studied by other primer sets, 113 of 27 primers generated reproducible polymorphic bands (Zamani et al., 2007). Fifty-eight of 257 bands were found to be polymorphic (57%). It can be argued that small numbers of cultivars (6 vs. 20) resulted in lower percentage of polymorphism in our study. However, when Ercisli et al. (2007) studied the same numbers of cultivars form Southern Anatolia Region of Turkey, 15 RAPD primers generated 88 reproducible bands 85% of which were polymorphic. Therefore, it is possible that the genetic variation among the pomegranate germplasm of previous studies (Ercisli et al., 2007; Sarkhosh et al., 2006; Zamani et al., 2007) were higher than that of our cultivars tested.

Factor scores of the first three dimensions of PCoA for the RAPD data were shown in Figure 3. The Mantel test indicated that the cophenetic matrix of dendrogram was very high (r = 0.97) indicating that the dendrogram was a good representation of the similarity matrix. In this analysis, the first three dimensions explained 56, 25 and 16% of the variation making a total of 100%. The results re-

Table 4. Arbitrary oligonucleotide primers, the sizes of the amplified fragments, numbers of mono-and polymorphic bands and polymorphism studied to reveled molecular relationship among six pomegranate cultivars sampled from Hatay, Turkey.

Primer code*	Sequence (5' to 3')	Size (bp)	Number of bands		
			Monomorphic	Polymorphic	Polymorphism (%)
OPAD10	AAGAGGCCAG	250-1500	7	1	14
OPAD18	ACGAGAGGCA	350-1400	5	0	0
OPAE14	GAGAGGCTCC	250-1400	6	0	0
OPAG08	AAGAGCCCTC	800-2500	3	2	67
OPAG12	AAGAGCCCTC	250-1600	8	3	38
OPAG20	CTCCCAGGGT	750-2500	6	4	67
OPAH16	TGCGCTCCTC	350-1000	3	1	33
OPAH19	CAAGGTGGGT	600-2000	4	1	25
OPAH2	GGCAGTTCTC	500-700	2	1	50
OPAH20	CACTTCCGCT	350-1000	5	0	0
OPAI08	GGAAGGTGAG	650-1700	6	1	17
OPAI18	AAGCCCCCCA	350-1900	5	1	20
OPAJ08	TCGCGGAACC	550-1400	5	0	0
OPAJ14	GTGCTCCCTC	300-1500	5	0	0
OPAK19	ACCGATGCTG	350-1000	4	0	0
OPB1	TCGCAGCGAG	300-750	4	1	25
OPB12	TGATGGCGTC	300-1000	5	1	20
OPB2	GTTTCGCTCC	700-1600	4	0	0
OPB20	CCTTGACGCA	350-1000	5	1	20
OPD17	TGATCCCTGG	350-1100	4	0	0
OPD17	GGACCCTTAC	350-1400	4	1	25
OPX19	TTTCCCACGG	600-2000	6	4	67
Total			106	23	22

*Marker notion refers to the kit (last netter) and the primer (-number) purchased from Operon Technologies (OP).

vealed that 'Şerife' and 'Tatlı nar' were very closely related. Similar to morphological patterns, 'Katırbaşı' and 'Kan narı' were separated from other cultivars. However, substantially different from morphological results, 'İnce kabuk' was found to be distinct from rest of the cultivars tested. Similar pattern was confirmed by UPGMA phenogram generated by the RAPD data (Figure 4).

Using a total of 24 RAPD primers and 129 reproducible amplification product, we successfully revealed the genetic relationship among the six pomegranate cultivars sampled from Hatay, Turkey. Therefore, our results confirmed the previous studies (Ercisli et al., 2007; Sarkhosh et al., 2006; Zamani et al., 2007) concluding RAPD is effective technique to reveal genetic diversity among pomegranate accessions.

Despite the cultivars characterized in this study exhibited a great deal of morphological variation they seem to have a relatively limited polymorphism level of RAPD primers. They may indeed have a limited genetic differentiation given that all the cultivars tested in our study originated from a small region. If this is the case, the morphological differences observed among these cultivars might be resulted from the ecological or growing conditions. It is well-known fact that the environment has

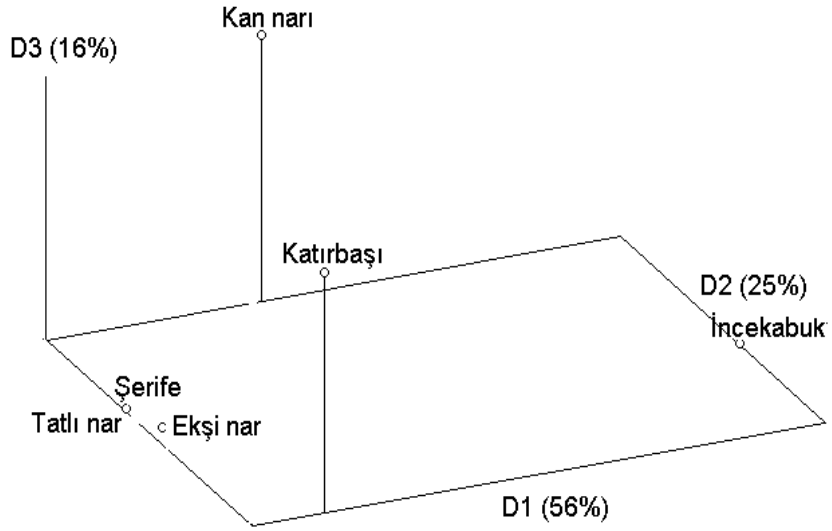

Figure 3. Factor scores for the first three principle coordinate (D) cultivar RAPD frequencies of 18 quantitative fruit characteristics for six pomegranate cultivars.

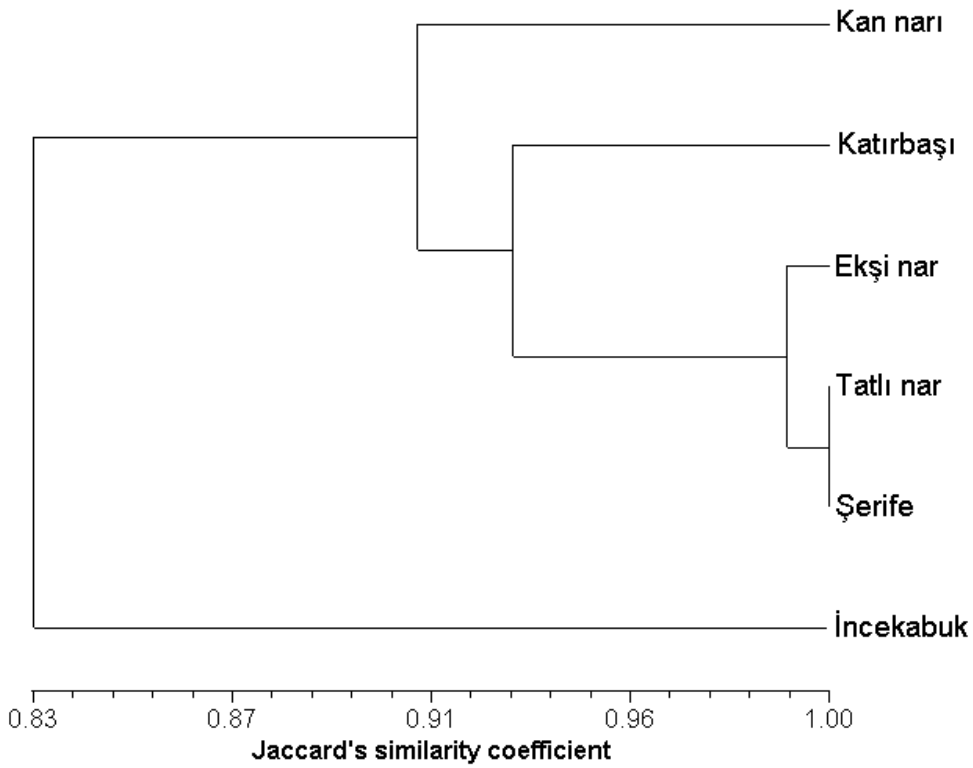

Figure 4. UPGMA phenogram of six pomegranate cultivar based on average distance among cultivar RAPD fragment frequencies.

a great effect of expression of quantitative traits. However, several characteristics of these cultivars (peel and aril color, juice characteristics) are stable across environments. Thus, it is more plausible option that the banding patterns of RAPD primers are successful to study genetic diversity but may not be well-correlated with morphological differences. Indeed both PCo vs. PCoA and UPGMA phenograms of morphologic and molecular data were found to be poorly correlated in our study (data not shown). These discrepancies were previously reported by

Zamani et al. (2007) when they compare between data from the genetic distance matrices obtained from RAPD markers and fruit characteristics. Their correlation coefficient, for comparison of morphological and RAPD data, were only 23% (Zamani et al., 2007). Therefore, we suggest utilization of different marker systems. For example, SSRs or AFLPs can be utilized when aiming to reveal genetic diversity among closely related pomegranate cultivars.

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