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Genetic relationships among wild pomegranate (*Punica granatum*) genotypes from Coruh Valley in Turkey

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ABSTRACT. The pomegranate has been used traditionally in Coruh Valley in Turkey for a long time; fruits are harvested from wild, semi-domesticated and cultivated trees. In the valley, it occurs in general along with olive trees. We sampled 23 wild-grown pomegranate genotypes sampled from different parts of Coruh Valley and compared them using RAPD primers to determine genetic variability. Eighty-six RAPD primers were used for molecular characterizations, among which 12 gave reliable polymorphic patterns. These primers generated 145 RAPD bands of which 91% were polymorphic. The highest polymorphism ratio was observed with primers OPY06, OPY13, OPBA03, OPBB03, OPBB07, and OPBB09 (100%), while the lowest was with OPBB09 and OPBB10 (75%). The band size was between 250 and 2400 bp. There were five main clusters in the dendrogram; the highest genetic similarity was 0.24 and the lowest was 0.08.

Key words: Wild pomegranate; *Punica granatum*; RAPD

INTRODUCTION

Pomegranate is considered to be an excellent tree for growing in arid or semiarid zones in both subtropical and tropical conditions. It is widely cultivated in India, Iran and Mediterranean countries. It can be encountered in regular plantations in most of the Mediterranean basin countries including Egypt, Morocco, Spain, Tunisia, and Turkey. A high number of scattered trees on the borders of or within other fruit orchards are reported in other Mediterranean countries, including Greece, Croatia, Albania, Bosnia Herzegovina, and Montenegro, where the fruit is very popular in local markets. Pomegranate is cultivated in Central Asia and to some extent in the USA (California), Russia, China, and Japan for fruit production, and it is also grown as an ornamental tree in East Asia (Mars, 1996; Ercisli et al., 2007).

Turkey is one of the native lands of the pomegranate, which has been cultivated mainly in the Mediterranean, Aegean and Southern Anatolia and Northeast part of Turkey since ancient times. Numerous wild, semi-domesticated and cultivated pomegranate plants are well adapted to different agro-ecological conditions in the country (Ercisli, 2004).

Turkish pomegranate genotypes vary with consumer preference, preferred use, and marketing. There is great variability among pomegranate genotypes in terms of fruit characters: size, skin color (ranging from yellow to purple, with pink and red being the most common), aril color (ranging from white to red), hardness of seed, maturity, juice content and its acidity, sweetness, and astringency (Ozguven et al., 1997; Ercisli et al., 2007). These variations are more visible in wild genotypes naturally grown in different parts of Turkey. In general, these wild forms grow outside of settlements.

Pomegranate raisins (*anardana*) are dried arils of wild pomegranates that are manually separated from the rind and septa of the fruit and sun- or air-dried. Pomegranate raisins have a distinct sour or tart flavor, and are commercially available in many West and East Asian countries, where they are consumed in large quantities (Jaiswal et al., 2010).

In Turkey, pomegranate sauce (Turkish: *nar ekşisi*) is used as a salad dressing, to marinate meat, or simply to drink straight. The people believe that wild pomegranates have better quality properties for pomegranate sauce production. Pomegranate vinegar is also common in Turkey and it is made from the juice of wild pomegranates. The juice is naturally fermented in wooden barrels and blended with a special balsamic juice concentrate made using low-temperature vacuum evaporation process.

Earlier classifications and evaluations of the pomegranate were done primarily based on phenotypic expressions of the plants such as color, shape and other agronomical characters of fruits in Turkey (Ercan et al., 1992; Polat et al., 1999; Ozkan, 2005). However, information from these environmentally influenced morphological and phenotypic characteristics is not sufficient to identify pomegranate genotypes because of environmental changes. Thus, genotypic traits free from environmental effects should be used for proper identification and estimation of genetic diversity among these genotypes.

Several different molecular methods for documenting genetic information are used for horticultural plants. These methods include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), etc. (Briard et al., 2001). Among them, RAPD analysis can provide a simple and reliable method for measuring genomic variation in different fruit species (Ercisli et al., 2007, 2008).

In the literature, there are no published molecular reports on wild pomegranates in Turkey. Therefore, the aim of this study was to determine the genetic variability among wild-grown pomegranates in Turkey.

MATERIAL AND METHODS

Leaf samples from 23 wild-grown pomegranates were collected from Coruh Valley in Turkey. The leaves were stored immediately at -80°C for later DNA extraction.

Genomic DNA was extracted from powdered (ground in liquid nitrogen) leaf material using the modified method described by Lin et al. (2001). Approximately 10-15 mg tissue from each plant sample was snap-frozen in liquid nitrogen in 2-mL Eppendorf tubes. A volume of 1000 µL DNA extraction buffer [100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 2% SDS (w/v); 2% 2-mercaptoethanol (v/v); 1% PVP (w/v)] was added and the contents well mixed. The mixture was incubated at 65°C in a water bath for 40 min with intermittent shaking at 5-min intervals. The mixture was centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was transferred to a clean 1.5-mL tube, mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), and then centrifuged. The supernatant was collected and mixed with 1/10 volume 10% CTAB-0.7 M NaCl in a clean tube. After centrifugation, the supernatant was collected and mixed gently with an equal volume of chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 0.6 volume freezer-cold isopropanol, for 10 min at -20°C. DNA was pelleted by centrifugation (12,000 *g*, 10 min) and the isopropanol was poured off; the DNA recovered was allowed to air-dry before being dissolved in 100 µL TE buffer.

The samples were screened for RAPD variation using the standard 10-base primers supplied by Operon. A 30-µL reaction cocktail was prepared as follows: 10X 3.0 µL buffer, 1.2 µL dNTPs (10 mM), 1.2 µL $MgCl_2$ (25 mM), 2.0 µL primer (5 µM), 0.4 µL *Taq* polymerase (5 U), 19.2 µL water, and 3.0 µL sample DNA (100 ng/µL). A total of 86 RAPD primers were tested in this study, and the polymorphisms obtained with the primers are shown in Table 1.

The thermocycler (Eppendorf Company) was programmed as follows: 2 min at 95°C; 2 cycles of 30 s at 95°C, 1 min at 37°C, and 2 min at 72°C; 2 cycles of 30 s at 95°C, 1 min at 35°C, and 2 min at 72°C; 41 cycles of 30 s at 94°C, 1 min at 35°C, and 2 min at 72°C, and a final 5-min extension at 72°C, followed by cooling down to 4°C.

The markers were checked twice for their reproducibility. The polymerase chain reaction (PCR) products (27 µL) were mixed with 6X gel loading buffer (3 µL) and loaded onto an agarose (1.5%, w/v) gel in 0.5X TBE (Tris-borate-EDTA) buffer, and electrophoresis was at 70 V for 150 min. The gel was stained in an ethidium bromide solution (2 µL/100 mL 1X TBE buffer) for 40 min, and the bands were visualized under UV in a Bio Doc Image Analysis System with Uvisoft analysis package (Cambridge, UK).

The positions of scorable RAPD bands were transformed into a binary character matrix (1 for the presence and 0 for the absence of a band at a particular position), which were entered in the RAPDistance computer program (Armstrong et al., 1994). These data were used for the calculation of pairwise genetic distances between cultivars using the Jaccard coefficient. The computer program calculated the degree of genetic dissimilarity between each pair of the 23 genotypes using the simple equation: $JC = 1 - a / (a + b + c)$, where a is the number of bands shared by plant x and plant y, b is the number of bands in plant x, and c is the num-

ber of bands in plant *y*. The Jaccard coefficient ignores the absence of matches. The distance matrix was used for cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA).

RESULTS

Results of RAPD analysis of 23 wild-grown pomegranate genotypes are summarized in Table 1. A total of 86 decamer oligonucleotide primers were used to investigate 23 wild-grown pomegranate genotypes all belonging to *Punica granatum*. In total, 12 of 86 primers produced good and reproducible polymorphic bands and used for further analysis of 23 wild-grown genotypes. The 12 random primers generated a total of 145 RAPD bands and among them 135 were polymorphic (Table 1). The primer OPY13 gave the highest number of RAPD bands (16), while the OPBB04, OPBB13 and OPBB17 primers all yielded the lowest total number of bands (10). The highest polymorphism ratio was observed with primers OPY06, OPY13, OPBA03, OPBB03, and OPBB07 (100.0%), while the lowest was with OPBB09 and OPBB10 (75.0%). The band size was between 200 and 2400 bp for the primers used (Table 1).

Table 1. List of the selected primers and the degree of polymorphism obtained among 23 wild-grown pomegranate genotypes.

Primer code	Sequence 5'→3'	Size (bp) Min-Max	Total band number	Polymorphic bands	Polymorphism ratio (%)
OPY06	AAGGCTCACC	400-2400	14	14	100
OPY13	GGGTCTCGGT	250-2000	16	16	100
OPBA03	GTGCGAGAAC	200-1800	14	14	100
OPBA06	GGACGACCGT	480-2000	12	11	92
OPBB03	TCACGTGGCT	450-1100	11	11	100
OPBB04	ACCAGGTCAC	450-1200	10	9	90
OPBB07	GAAGGCTGGG	450-2100	12	12	100
OPBB08	TCGTCGAAGG	280-1200	14	13	93
OPBB09	AGGCCGGTCA	350-2000	12	9	75
OPBB10	ACTTGCCTGG	350-1400	12	9	75
OPBB13	CTTCGGTGTG	250-950	10	8	80
OPBD17	GTTCGCTCCC	450-1100	10	9	90
Total			145	135	
Average		200-2400		135	91

The dendrogram created from the RAPD markers grouped the genotypes into five main groups. The first cluster includes WP7 and WP11; the second cluster includes WP1, WP5, WP8, WP19, WP21, and WP23; the third cluster includes WP3, WP4, WP9, WP16, and WP20; the fourth cluster includes WP10, WP12 and WP14, and the last cluster includes WP2, WP6, WP13, WP15, WP17, WP18, and WP22 (Figure 1).

The similarity matrix showed that the highest genetic similarity (0.24) was between WP3 and WP4, and WP7 and WP22 were found to be the most distinct with the lowest genetic similarity (0.08).

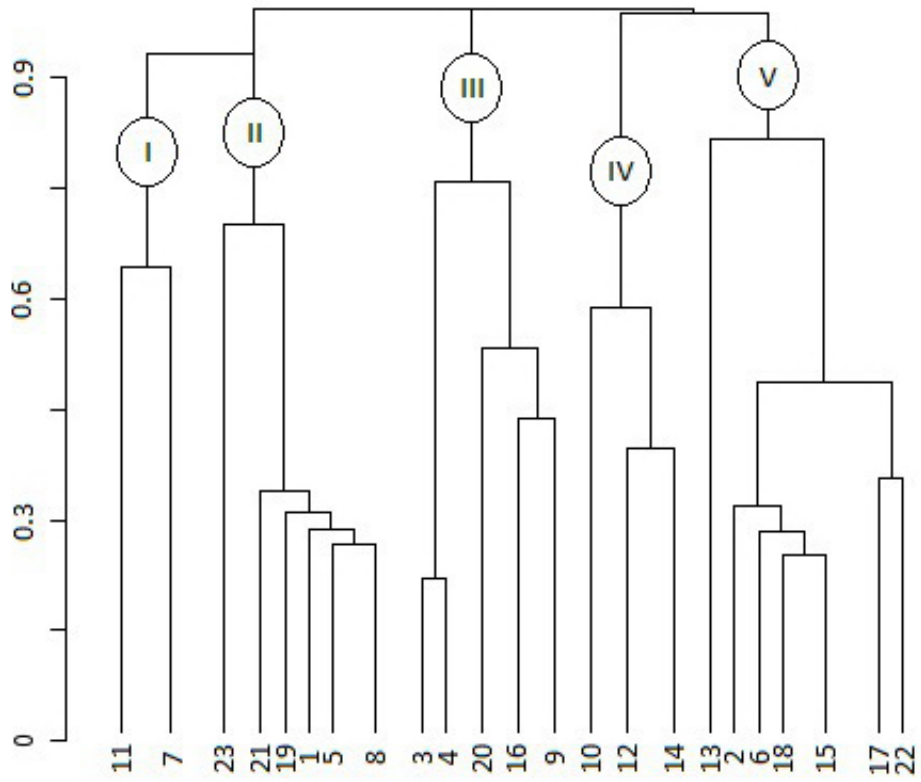

Figure 1. UPGMA dendrogram of 23 wild pomegranate genotypes based on 12 random RAPD primers.

DISCUSSION

The results indicate that there was abundant genetic polymorphism among wild-grown pomegranate genotypes in Coruh Valley in Turkey. The average genetic similarity among the genotypes was 0.32, which clearly shows significant genetic diversity among the wild-grown genotypes. Earlier studies, using RAPD markers, in pomegranate showed large genetic variations (22-85%) among different cultivars in different countries (Sarkhosh et al., 2006; Ercisli et al., 2007; Zamani et al., 2007; Durgac et al., 2008). On the other hand, Narzary et al. (2009) found about 94% genetic diversity among 49 wild-grown pomegranate genotypes in India, which is in agreement with our result. The high genetic distances present between these genotypes clearly suggest that they may have originated from genetically divergent parents or have a long history of adaptation to their respective micro-climatic regions. Moreover, continuous propagation of seeds for thousands of years and their selection by humans could have resulted in a diversity of pomegranate genotypes. This genetic diversity is an important resource that could be used to contribute to pomegranate breeding program for different aims.

This is one of the first attempts to use molecular markers to investigate the genetic relationships of a wide number of wild-grown pomegranate genotypes belonging to *Punica*

granatum grown in Turkey. The RAPD technique could be a useful tool for the management of plant collections. These data provide the scientific basis for future selection and management of germplasm. The results of this study indicate that, in Turkey, the level of polymorphism in pomegranate is appreciably high. Therefore, these genotypes can be used in breeding programs, and the information generated in this study would be valuable to the improvement of pomegranates through breeding.

REFERENCES

Armstrong J, Gibbs A, Peakall R and Weiller G (1994). The RAPDistance Package. Available at [http://life.anu.edu.au/molecular/software/rapd.html]. Accessed April 23, 2010.

Briard M, Le Clerc V, Mausset AE and Veret A (2001). A comparative study on the use of ISSR, microsatellites and RAPD markers for varietal identification of carrot genotypes. *Acta Hortic.* 546: 377-385.

Durgac C, Ozgen M, Simsek O, Kacar YA, et al. (2008). Molecular and pomological diversity among pomegranate (*Punica granatum* L.) cultivars in Eastern Mediterranean region of Turkey. *Afr. J. Biotechnol.* 7: 1294-1301.

Ercan N, Ozvardar S, Gonulsen N, Baldiran E, et al. (1992). Determination of Suitable Pomegranate Cultivars for Aegean Region. Proceedings of 1st National Horticultural Congress, Izmir, 553-557.

Ercisli S (2004). A short review of the fruit germplasm resources of Turkey. *Genet. Res. Crop Evol*. 51: 419-435.

Ercisli S, Agar G, Orhan E, Yildirim N, et al. (2007). Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punica granatum* L.) growing in Southern Anatolia Region in Turkey. *Biochem. Syst. Ecol.* 35: 764-769.

Ercisli S, Orhan E, Yildirim N and Agar G (2008). Comparison of sea buckthorn genotypes (*Hippophae rhamnoides* L.) based on RAPD and FAME data. *Turk. J. Agr. Forest.* 32: 363-368.

Jaiswal V, DerMarderosian A and Porter JR (2010). Anthocyanins and polyphenol oxidase from dried arils of pomegranate (*Punica granatum* L.). *Food Chem.* 118: 11-16.

Lin RC, Ding ZS, Li LB and Kuang TY (2001). A rapid and efficient DNA minipreparation suitable for screening transgenic plants. *Plant Mol. Biol. Rep.* 19: 379a-379e.

Mars M (1996). Pomegranate Genetic Resources in the Meditaerranean Region. In: First MESFIN Plant Genetic Resources Meeting, Tenerife, 345-354.

Narzary D, Kamalesh SM, Rana TS and Ranade SA (2009). Analysis of genetic diversity among wild pomegranates in Western Himalayas, using PCR methods. *Sci. Hortic.* 121: 237-242.

Ozguven AI, Tatli H, Coskun M and Dasgan Y (1997). Mediterranean and Aegean pomegranate varieties under ecological conditions of Adana, Turkey. *Acta Hortic.* 441: 345-348.

Ozkan Y (2005). Investigations on physical and chemical characteristics of some pomegranate genotypes (*Punica granatum* L.) of Tokat province in Turkey. *Asian J. Chem.* 17: 939-942.

Polat AA, Durgac C, Kamiloglu O, Mansuroglu M, et al. (1999). Studies on Determination of Pomological Characteristics of Some Pomegranate Types Grown in Kirikhan District of Hatay Province. Proceedings of 3rd National Horticultural Congress, Ankara, 746-750.

Sarkhosh A, Zamani Z, Fatahi R and Ebadi A (2006). RAPD markers reveal polymorphism among some Iranian pomegranate (*Punica granatum* L.) genotypes. *Sci. Hortic.* 111: 24-29.

Zamani Z, Sarkhosh A, Fatahi R and Ebadi A (2007). Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. *J. Hortic. Sci. Biotechnol.* 82: 11-18.