Molecular Characterization of Turkish Cactus Pear (*Opuntia* spp.) by RAPD Markers

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ABSTRACT

Opuntia ficus-indica Mill. commonly known as cactus pear is the most agronomically important species in Cactaceae. Turkey has important genetic resources of *Opuntia* spp. which should be characterized for further breeding strategies. In this study, molecular characterization of plant materials collected from different regions of Turkey in which *Opuntia* species grown naturally, was performed by using Random Amplified Polymorphic DNA (RAPD) markers. DNA was successfully amplified by 50 RAPD primers. Among 250 bands generated by the RAPD primers, 180 were polymorphic. The number of bands detected by a single primer set ranged from one to 12 (average of five bands/primer). The percentage of polymorphism was 72% based on RAPD data. All data were scored as discrete characters and unweighted pair group method with arithmetic mean (UPGMA) dendrogram and principle coordinate analysis (PCoA) were performed. Based on the results, *Opuntia* genotypes showed high genetic diversity and we showed that RAPD markers are powerful tool to discriminate Turkish *Opuntia* genotypes. The high genetic diversity existing in the Turkish germplasm suggests that it would be beneficial to utilize this pool in *Opuntia* breeding programs and germplasm management activities.

***Keywords*:** Barbary fig, prickly pear, PCR, diversity, similarity.

INTRODUCTION

The *Opuntia* species is known as prickly pear, cactus pear, barbary fig, tuna etc. in different countries and the name is slowly evolving into cactus pear (Saenz, 2013). Cactus pear has become an important crop in semiarid and arid lands in world. Cactus pear which is dicotyledonous perennial plant is characterized by jointed flattened stem, cylindrical or conical succulent and ephemeral leaves on young stem; fruit with thick rinds; and comparatively covered by hard, bony, light-coloured arils (Weniger, 1984; Mondragon-Jacobo and Bordelon, 1996). It is cultivated mainly for its fruits and tender pads, which are consumed as vegetable and known to be polyploid. Cultivated varieties and forms have the highest chromosome number (2n=2x=66 and 2n=8x=88), contrasting

with wild ones, which normally have 2n=2x=22 and 2n=2x=44 (Pinkava *et al*. 1992; Munoz *et al*., 1995; Pimienta, 1995; Mondragon-Jacobo and Bordelon, 1996). The genus presents mainly in Africa, Mediterranean countries, South-Western United States, all Mexico and other area (Hegwood, 1990; Matthäus and Özcan, 2011). Whereas it is originated from Mexico, wild types distribute in six continent except Antarctica, due to its well adaptive behaviour. It is cultivated in over 20 countries at present (Inglese *et al*. 2002). However there are few updated official statistics and information about growing areas, fruit production and usage. Basile (2001) reported that Mexico ranked 1st among the cactus pear producer countries with more than 300,000 tones fruit product from nearly 75,000 ha cultivated area at the beginning of 1990s. According to data from Valdez – Cepeda *et al*. (2013), vegetable production of cactus pear, an estimated more than 12,000 ha are cultivated in Mexico at the end of 2009. At present, cactus pear production in Mexico is about 812,558 tonnes (SIAP, 2016).

Turkey has no commercial plantation or commercial variety of cactus pear. Wild plants naturally grow in bushy areas and/or it is cultivated as hedge plant as an individual plant or groups. Fruits are collected to sell in local bazaars in Mediterranean and Aegean parts of the country. Fruits are consumed as fresh or used to make jam, marmalade and ice cream, but it is not used as vegetable. Efforts are currently under way to develop the cactus pear production (Toplu *et al*. 2009).

There are limited researches on cactus pear in Turkey and researches are generally focused on the assessments of pomological traits. There are a number of problems to identify and characterize cactus pear phenotypes, due to its growing environmental conditions, polyploidy and interspecific hybridization (Scheinvar, 1999; Caruso *et al*. 2010; Saenz, 2013). Therefore, advanced morphological and botanical studies on cactus pear species were carried out in order to overcome identification and characterization problems (Wang *et al*. 1998; Gutiérrez-Acosta *et al*. 2000; Malainine *et al*., 2003; Trifa *et al*. 2007). However, understanding variability considering phenotypical characteristics, which are commonly effected from environmental factors, is difficult at the genetic level (Trifa *et al*. 2007).

Nowadays, studies on genetic diversity, awareness on identification of cactus pear genotypes, establishing cactus pear germplasm collection in order to breed new high quality cactus pear variety are increased. Studies related to the genetic diversity of the genus *Opuntia* have already been conducted, using chloroplast simple sequence repeats (cpSSR) and amplified fragment length polymorphism (AFLPs) (Labra *et al*. 2003), nuclear ribosomal internal transcribed spacer (nrITS) (Griffith, 2004; Srikanth and Whang, 2015), RAPDs (Zoghlami *et al*. 2007; Bendhifi *et al*. 2013), inter simple sequence repeats (ISSRs) (Valadez-Moctezuma *et al*. 2015a; Ganopoulos *et al*. 2015), microsatellites (Caruso *et al*. 2010; Bendhifi-Zarroug *et al*. 2015; Samah *et al*. 2016) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Valadez-Moctezuma *et al*. 2015b). The present study is the first molecular research over *Opuntia* spp. in Turkey in order to present genetic diversity for further breeding researches.

There are many molecular marker systems available for plant scientists to characterize genetic resources and cultivars (Staub, 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 RAPD, they have been widely used as they were proven to be effective (Durgaç *et al*. 2008). The main objective of the present study was to determine molecular diversity among the cactus pear genotypes selected from the Eastern Mediterranean region of Turkey.

MATERIALS AND METHODS

This study was carried out at Department of Horticulture, Faculty of Agriculture, University of Çukurova, Adana, Turkey.

Plant materials

Plant materials were collected from Adana province of Turkey. A total of thirty one *Opuntia* genotypes were collected from twenty one different locations in Adana. Selected plants were labelled with coding system like Op-X. Firstly, we gave abbreviation of genus *Opuntia* (Op) and selected plant number (X) respectively.

DNA isolation

Cladodes from all samples were collected and immediately frozen in liquid nitrogen and stored at -80˚C. High molecular weight genomic DNA was extracted from the cladode of each sample following the CTAB (cetyltrimethyl ammonium bromide) protocol for minipreps (Edwards *et al*. 1991). DNA concentration was measured using a NanoDrop (ND 100) spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and gel electrophoresis. DNA was diluted in water to a final concentration of 50 ng/µL and stored at -20˚C.

RAPD analysis

Fifty RAPD 10-mer primers (Operon Technologies, Almeda, CA, USA) were used to amplify all 31 genotypes studied. Forty six primers found to be polymorphic (Table 1). Amplification reactions were performed in 9 µL volumes containing 2X PCR Mastermix (Fermentas K0171, Waltham, MA, USA), 1 U Taq DNA polymerase (Fermentas EP0402), 25 mM $MgCl_2$, 30 ng of the primer and 15 ng of prickly pear DNA. Mixtures were assembled at 0˚C, transferred to a thermal cycler then pre-cooled to 4˚C. The amplification was carried out in a model Master Gradient thermal cycler (Eppendorf, Hauppauge, NY, USA) using an optimized in-house program consisting of an initial denaturation step of 2 min at 94˚C, and then 45 cycles of 2 min at 94˚C, 1 min at 37˚C, 2 min at 72˚C, followed by a 10-min elongation step at 72˚C. PCR products were stored at 4˚C before analysis. Amplification products were separated by electrophoresis on 1.5% agarose gels and 0.5 g/mL ethidium bromide in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for 3 h at 70 V. The fragment patterns were photographed under UV light for further analysis. A 1-kb DNA ladder (Fermentas) was used to determine fragment size.

All PCR reactions and electrophoresis analyses were repeated two times and only reproducible DNA profiles were scored.

Data analysis

Reproducible RAPD profiles were scored manually in the binary mode with 1 indicating the presence, and 0 indicating the absence of a band, then data were used to generate a pair-wise similarity matrix using Jaccard's coefficient (Jaccard, 1908). The un-weighted pair-group method with UPGMA was employed to create the clustering dendrogram using the NTSYS-PC program (version 2.02i) (Rohlf, 1998). The principle coordinate (PCoA) analyses were performed based on the same similarity matrix using the PAST software (Hammer *et al*., 2001). Polymorphism information content (PIC) values were calculated according to Smith *et al*. (1997), using the algorithm for all primer combinations as follows: $PIC = 1 - \Sigma fi^2$, where fi^2 is the frequency of the i^{th} allele.

RESULTS AND DISCUSSION

A total of 50 RAPD primers were screened for their ability to generate consistently amplified band patterns and to evaluate polymorphism among 31 prickly pear genotypes. Amplification was successful with 50 RAPD markers assayed. All primers produced clear and good amplification. Forty-six RAPD primers that produced polymorphic bands were used to generate RAPD markers with all genotypes (Table 1).

Among the 250 DNA bands generated by 50 selected RAPD primers, 180 were polymorphic. The number of alleles detected by a single primer set ranged from 1 to 12, with an average of 5 band per primer.

The rate of polymorphism was calculated as 72% among the 31 *Opuntia* genotypes based on RAPD data. Different primers generated various banding patterns and range of alleles were between 250 bp and 1750 bp. The highest numbers of DNA profiles were determined in OPAK-06 primer with totally 8 polymorphic of which 12 alleles.

The lowest number of DNA profile was scored in OPAD-06, OPAE-14, OPAE-17, OPAK-14, OPZ-09 and UBC-39 RAPD primers with only two bands. PIC values ranged from 0.00 (OPAE-14, OPAE-17, OPAI-15, OPAK-09) to 0.96 (OPZ-14) for RAPD data (Table 1).

PIC values of 16 primers used in RAPD analysis were higher than 0.6. The average level of stable polymorphisms was very good, demonstrating that several RAPDs were useful to discriminate all *Opuntia* genotypes.

The similarity coefficient ranged from 0.45 to 0.98 as a result of RAPD analysis. Cluster analysis (UPGMA) employing RAPD data resulted in a dendrogram with two main branches as shown in Figure 1.

Table 1. Results of amplification obtained from RAPD primers and polymorphism information content (PIC) was calculated using the algorithm for all primer combinations.

RAPD primer	Sequence	Size range (bp)	Total bands number / polymorphic bands (Polymorphism (%))	PIC
OPA-11	CAATCGCCGT	300 – 1750	11/11 (100)	0.77
OPAD-05	ACCGCATGGG	510 – 900	4/4 (100)	0.53
OPAD-06	AAGTGCACGG	1100	2/2 (100)	0.24
OPAD-17	GGCAAACCCT	650 – 780	3/1 (33.33)	0.54
OPAD-18	ACGAGAGGCA	400 – 800	4/3 (75)	0.12
OPAE-01	TGAGGGCCGT	350 – 1100	7/3 (42.85)	0.66
OPAE-05	CCTGTCAGTG	350 – 1300	11/10 (90.9)	0.72
OPAE-07	GTGTCAGTGG	550 – 1100	3/2 (66.66)	0.56
OPAE-09	TGCCACGAGG	300 – 1150	4/3 (75)	0.54
OPAE-10	CTGAAGCGCA	250 – 700	7/2 (28.57)	0.48
OPAE-14	GAGAGGCTCC	400 – 650	2/0 (0)	0.00
OPAE-16	TCCGTGCTGA	500 – 1350	5/4 (80)	0.83
OPAE-17	GGCAGGTTCA	650 – 750	2/0 (0)	0.00
OPAF-03	GAAGGAGGCA	300 – 1100	5/4 (80)	0.36
OPAF-08	CTCTGCCTGA	450 – 1400	5/4(80)	0.07
OPAF-13	CCGAGGTGAC	350 – 850	6/3 (50)	0.55
OPAG-03	TGCGGGAGTG	600 – 1100	3/1 (33.33)	0.56
OPAG-06	GGTGGCCAAG	500 – 1100	5/5 (100)	0.41
OPAG-14	CTCTCGGCGA	650 – 1400	4/2 (50)	0.05
OPAI-06	TGCCGCACTT	300 – 1250	7/6 (85.71)	0.64
OPAI-15	GACACAGCCC	400 – 1200	5/0 (0)	0.00
OPAI-16	AAGGCACGAG	400 – 1000	5/3 (60)	0.64
OPAK-06	TCACGTCCCT	270 – 1250	12/8 (66.66)	0.66
OPAK-09	AGGTCGGCGT	510 – 800	3/0 (0)	0.00
OPAK-14	CTGTCATGCC	490 – 750	2/1 (50)	0.09
OPAK-19	TCGCAGCGAG	700 – 1000	4/3 (75)	0.05
OPB-02	TGATCCCTGG	500 – 1100	6/5 (83.33)	0.60
OPB-05	TGCGCCCTTC	650 – 850	3/2 (66.66)	0.57
OPB-08	GTCCACACGG	350 – 1250	9/8 (88.88)	0.69
OPB-09	TGGGGGACTC	700 – 1000	2/2 (100)	0.32
OPB-15	GGAGGGTGTT	400 – 1250	5/3 (60)	0.64
OPB-16	TTTGCCCGGA	500 – 1150	7/7 (100)	0.64
OPC-05	GATGACCGCC	350 – 1050	6/4 (66.66)	0.56
OPZ-03	CAGCACCGCA	500 – 1000	3/3 (100)	0.60
OPZ-06	GTGCCGTTCA	250 – 1100	4/3 (75)	0.13
OPZ-09	CACCCCAGTC	500 – 1150	8/7 (87.5)	0.81

RAPD primer	Sequence	Size range (bp)	Total bands number / polymorphic bands (Polymorphism (%))	PIC
OPZ-10	CCGACAAACC	550 – 750	3/2 (66.66)	0.57
OPZ-12	TCAACGGGAC	390 – 1100	4/2 (50)	0.52
OPZ-13	GACTAAGCCC	250 – 750	4/1 (25)	0.42
OPZ-14	TCGGAGGTTC	300 – 1500	6/5 (83)	0.96
OPZ-15	CAGGGCTTTC	300 – 1050	8/6 (75)	0.29
OPZ-16	TCCCCATCAC	300 – 1100	3/2 (66.66)	0.10
OPZ-17	CCTTCCCACT	250 – 1250	8/8 (100)	0.78
OPZ-19	GTGCGAGCAA	500 – 1500	5/2 (40)	0.02
UBC-09	CCTGCGCTTA	530 – 900	5/5 (100)	0.48
UBC-29	CCGGCCTTAC	300 – 650	3/2 (66.66)	0.04
UBC-39	TTAACCGGGC	650	2/2 (100)	0.30
UBC-40	TTACCTGGGC	650 – 1000	4/3 (75)	0.66
UBC-44	TTACCCCGGC	300 – 1300	7/7 (100)	0.48
UBC-52	TTCCCGGAGC	750 – 1100	4/4 (100)	0.48
	Total		250/180 (72)	

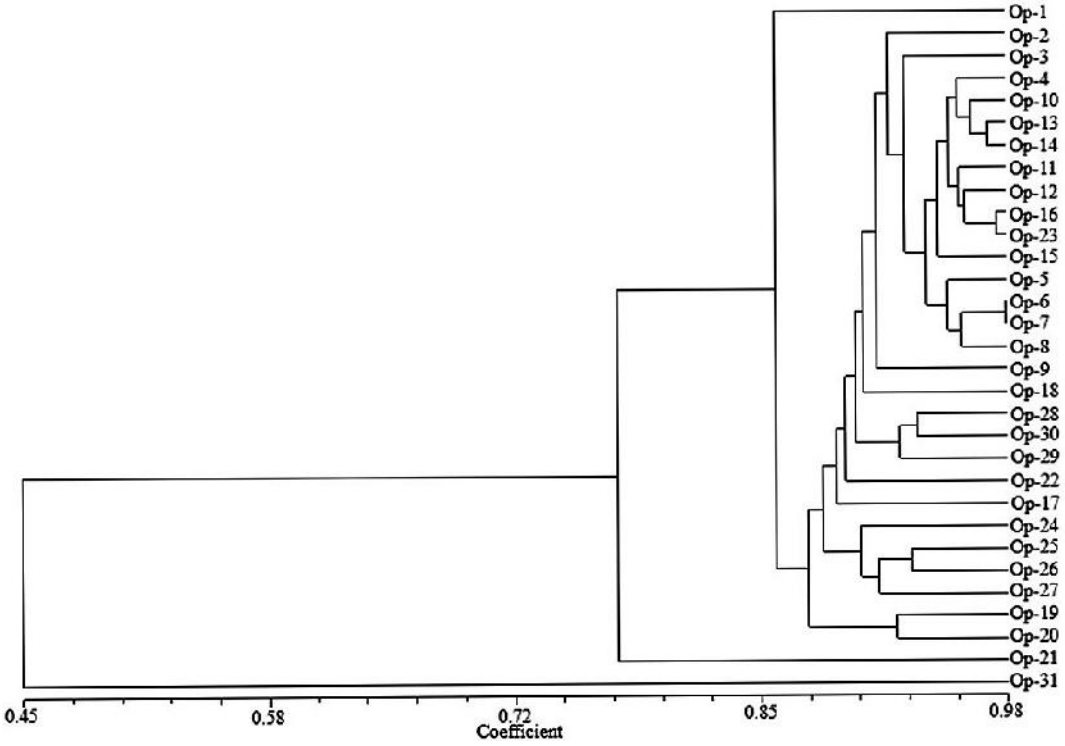

Figure 1. UPGMA dendrogram of 31 *Opuntia* genotypes from RAPD data. Similarity values are shown at the bottom of the dendrogram.

The genotypes were determined variable up to 40%. One of the primary branch of the dendrogram consisted of one genotype (Op-31). The second main branch contained all the other 30 genotypes. Principle coordinate analysis (PCoA) was also performed using the similarity matrix, and the two dimensional dendrogram corroborated UPGMA analyses (Fig. 2). While the first axis (D1) explained 28.35% of the total molecular variance, the second axis (D2) explained 10.60%.

Opuntia is one of the largest genus has over 181 species in subfamily of Opuntioidae belongs to Cactaceae family (Anderson, 2001; Labra *et al*. 2003). Members of the genus well adapted to semiarid and arid regions around world and cultivated in America, Africa, Asia, Europe, and Oceania (Felker and Inglese, 2003).

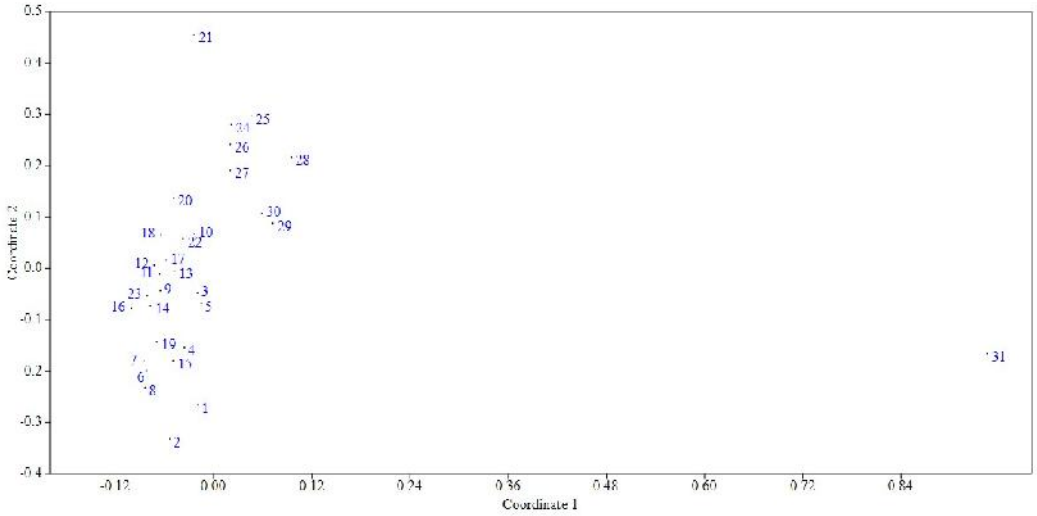

Figure 2. Biplot (the first two principle coordinate analysis) of 31 *Opuntia* genotypes generated by the data from RAPD.

In the present study, we collected different *Opuntia* spp. genotypes naturally grown in the city of Adana located Southern part of the Turkey. We demonstrated that there is a high variation among genotypes based on the RAPD data. Different molecular markers have been employed to investigate genetic diversity in *Opuntia* species. Wang *et al*. (1998) used RAPD markers and detected polymorphism rate 78% with 22 informative markers. In another study, genetic relationships of *Opuntia* species were investigated. A total of 20 markers of which 7 ones polymorphic were used and polymorphism rate was calculated as 32% (Nagaty and Rifaat, 2012).

On the other hand several different molecular marker systems in addition to RAPD were employed to determine genetic diversity among *Opuntia* genotypes. Labra *et al*. (2003) used AFLP and cpSRR markers to compare genetic relationships among two *O. ficus-indica* and four *O. megacantha* populations. Results showed that of cpSSR and AFLP markers provided a quantitative estimation of genetic relationships among several *Opuntia*

species. Trifa *et al*. (2009) used AFLP markers to characterize the cactus germplasm present in three collections in Tunisia. Fifty seven cactus accessions were analysed with 10 AFLP primer combinations. Seven out of ten generated stable and interpretable profiles with a total of 519 bands. Genetic diversity was detected and noticed a high genetic similarity within *Opuntia ficus-indica* varieties and their difference from the other *Opuntia* species was clearly revealed.

Another marker systems performed to understand genetic similarity among *Opuntia* genotypes is ISSR. A total of 175 polymorphic fragments with an average of 14 fragments per primer were scored in the 11 cactus pear clones with 11 selected ISSR markers. The grouping analysis based on molecular data evidenced low genetic variability in the three clusters (Souto *et al.* 2009).

RAPD and ISSR markers were employed in another study to investigate genetic diversity of Mexican *Opuntia* spp. varieties. In that paper, researchers reported the genetic variability of 52 *Opuntia* cultivars with agronomic and economic importance, classified into 12 different species using random amplified polymorphic DNA, and inter-simple sequence repeats markers. Ten primers, five for each marker type, were selected to assess their ability to detect polymorphisms in this plant accessions/varieties. Both marker systems generated a total of 307 bands, of which 50.8 % were polymorphic with an average of 15.6 polymorphic bands per primer. Thus, Mexican *Opuntia* varieties present broad genetic variation (Valadez-Moctezuma *et al*. 2015a).

In the present study, we calculated polymorphism rate as 72% with 50 RAPD markers. The polymorphism rate is determined relatively high comparing the previous studies. The reason of high polymorphism among *Opuntia* genotypes is that wild cactus pear genotypes as well as other *Opuntia* species were collected considering its attractiveness of fruit or other plant characteristics by people through the time. However, collected plants that grow close together in relatively small areas involved to increase gene-flow via cross-pollination among the genotypes. Therefore, it caused to appear new genetic combinations in nature (Pimienta, 1995).

Opuntia genotypes used in the present study have some differences morphologically, especially Op-8 and Op-11 have obviously variation based on fruit characteristics. Both of these genotypes are red-purple fleshed and fruit sizes are smaller than other genotypes. Ripe flesh colour of cultivars for commercial fruit production differentiate such as red-purple, yellow-orange, white-cream or greenish. Yellow-orange fruit flesh is the most preferred colour among the international markets. Recent studies shows that consumers are not unfamiliar with pink-red fruit flesh, but consumers buy pink-red fruit first due to its attractive colour (Inglese *et al*. 2002). In the present study, we especially selected different fruit fleshed for further breeding programme.

Overall results of clustering analysis show that selected genotypes from closer locations clustered together except Op-1, Op-21 and Op-31 and same results also determined in PCoA (Figure 2). Whereas these genotypes look like morphologically similar with other

genotypes, they have some differences in terms of fruit and plant characteristics or selection area. Narrow elliptical cladode shape was only major difference of Op-1, while cladode shape of 30 genotypes were wide elliptical, narrow obovate, wide obovate or circular shape (data not shown). Op-31 was genotype located individually one of the main branch in dendrogram.

In another study, Tütüncü *et al*. (2016) determined fruit characteristics of same material used in the present study evaluating eight fruit traits such as fruit shape, fruit size, fruit flesh and skin color. According to results, Op-31 is only genotype having oblong fruit shape. This result could explain the main reason of the difference of Op-31. Our survey included some "semi-wild" Opuntias that are not *O. ficus-indica* such as can be seen in Figure 4a. The next step will be to compare with RAPD data and simple measurements on Brix, fruit size and pulp percentage, to narrow down the varieties with potential for genetic improvement including only clones without spines, Brix greater than 12%, pulp percentages > 50% and fruit sizes > 90 g. Overall results may suggest that morphological differences may not reflect phylogenetic similarity and phenotypic variation in cactus pear may be influenced many other factors besides its genetic patterns (Wang *et al*. 1998).

Some of the morphological differences among genotypes given in Figure 3 and Figure 4. These results showed that Turkey has great potential for different characteristics of *Opuntia*. These great genetic resources of cactus pear could be evaluated as breeding material.

The RAPD markers were able to provide good separation of many of these clones, possibly due the great variation in the genetic material analysed that included some wild non *Opuntia ficus-indica* clones (Figure 4a). In a similar manner, Caruso *et al*. (2010), were able to distinguish *Opuntia* clones with greatly contrasting morphology but they were not able to distinguish closely related Sicilian clones with different fruit colors that were obviously different.

Caruso *et al*. (2010) hypothesized that "It is likely that the phenotypic variation was the result of somatic mutations of a few clones that occurred in the cultivated region after the 16th century when *Opuntias* started to become naturalized in the Mediterranean region and later in other warm regions of the world". If these varieties are a result of somatic mutations in only one small genetic loci, it is unreasonable to expect that SSR's, and other random primer based techniques will be able to distinguish between them and the only solution will be sequencing of the genomic DNA or possibly the transcriptomes.

In this study, we showed that RAPD markers were powerful tools to separate different *Opuntia* genotypes. This study is an initiation study for the characterization of Turkish *Opuntia*. In order to determine genetic diversity deeply especially among the same species, different marker systems can be used. Especially, SSR markers can be employed for genetic characterization of *Opuntia* species.

Figure 3. Some fruit samples from selected *Opuntia* genotypes (a: Op-1; b: Op-3; c: Op-11; d: Op-31).

Figure 4. Different cladode shapes observed among selected *Opuntia* genotypes (a: Op-10; b: Op-26; c: Op-21; d: Op-23).

Given their high polymorphism, codominant inheritance and the simplicity of the methods required for their development, microsatellite or simple-sequence repeat (SSR) markers seem to be the appropriate marker system to solve these problems (Aranzana *et al*. 2003).

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