MOLECULAR CHARACTERIZATION OF PLUM CULTIVARS BY AFLP MARKERS

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ABSTRACT

A total of fourteen plum cultivars (1 cultivar from European and 13 cultivars from Japanese groups) were sampled from Kahramanmaras province located in Mediterranean region of Turkey and were studied using four pairs of amplified fragment length polymorphism (AFLP) markers. A total of 145 bands were scored and among them 89 (60.85%) were polymorphic. Resolving powers of the AFLP primers ranged from 0.577 to 1.347 and polymorphism information content ranged from 0.412 to 0.829 with an average of 0.601. Fourteen plum cultivars grown in worldwide could be classified into the seven major clusters by UPGMA analysis and European plum cv. President was found the most distinct group among them. The highest similarity was observed between Globe Sun and October Sun cultivars, while the lowest one was between President and Queen Rosa cultivars. The results suggested that AFLP is a good method to determine genetic relatedness among plum cultivars.

Keywords: Plum, molecular markers, AFLP, UPGMA, genetic relationship

Introduction

Plum is cultivated for over 2000 years throughout the world and it has attractive colored fruits which are consumed both in fresh and processed form. Plum puree, paste, sauce, juice concentrate and prunes are some of the common processed products. The fruits are also dried and in developed countries, 50% of the produce is utilized for processing, whereas commercial utilization of plums in the developing countries is insignificant (1).

Plum belonging to subgenus *Prunophora* is considered to be important for *Prunus* evolution, because they include more than 20 species with the abundant variations in their morphology. Although the basic chromosome number of *Prunus* species is *x*=8, some species within subgenus *Prunophora* are triploid, tetraploid, and hexaploid. According to the derivative systems of these polyploids, *Prunus domestica* L. (6*x*), one of the European plums, is considered to be derived from natural cross between *Prunus spinosa* L. (4*x*) and *Prunus cerasifera* Ehrh (2*x*). The term Japanese plum was applied originally for *Prunus salicina* Lindl. (2*x*) (12).

Plum species and cultivars are quite diverse in fruit characteristics such as size, shape, color, texture, aroma and quality. Plant characteristics are also very diverse, ranging from shrubs to large trees, spreading to upright, thick to thin leaves, and early to late blooming (16).

Cultivar identification of plums has traditionally been based on morphological traits, such as leaf and fruit shape, color, size, freedom of seed, plant growth habits etc. (2, 13, 14). However, as in many out crossing crops, most of the plum cultivars, in particular Japanese group, is highly heterozygous with most of its morphological, physiological and biochemical traits showing continuous variation and high plasticity. Similarly, most morphological traits are influenced by environmental factors, plant age and phenology. Since objectivity is crucial to accurate morphological typing, the above factors render the use of such traits in plant identification and discernment of genetic relationships difficult (8). The introduction of DNA-based markers provides an opportunity for genetic characterization that allows direct comparison of different genetic material independent of environmental influences. In the past two decades, molecular markers have become a fundamental tool for plant scientists that is useful for fingerprinting varieties, establishing phylogenies, tagging desirable genes, determining similarities among in breeding materials and mapping plant genomes (21).

Among PCR-based molecular markers, AFLPs are highly reproducible multi-locus marker system developed by Vos et al. (22). This method has been extensively used for a wide range of species including fruit trees. High levels of polymorphism and high degrees of discriminative capacity are the main advantages of AFLPs for closely related accessions. Standard AFLP methods based on two cutting enzymes requires labeling of selective primers, which necessitates the use of isotopes or fluorescent dyes.

Although the AFLP method has been used to identify genetic variability of many different plant species, the use of this powerful and reliable method in plums has been very limited (3, 7). One of these studies only compared cherry plum accessions belonging to *Prunus cerasifera* (3). The objective of this study was to characterize a total of 14 cultivars grown in Turkey by AFLP markers and to determine whether AFLP

markers are appropriate for taxonomical relationships among these accessions.

Materials and Methods

Plant material

For molecular analysis, a total of 14 cultivars (13 cultivars from *Prunus salicina* and 1 cultivar from *Prunus domestica*) were used. The cultivars were found together in a collection at Sekamer Research Station belonging to Sutcu Imam University, Kahramanmaras, Turkey.

DNA extraction and AFLP analysis

Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle (6) with minor modifications (9). Concentration of extracted DNA was estimated by comparing band intensity with λ DNA of known concentrations, after 0.8% agarose gel electrophoresis and ethidium bromide staining. DNA was diluted to 50 ng μL^{-1} for AFLP reactions.

Details of AFLP assay, adaptor and primer sequences, PCR conditions for pre selective and selective amplifications, and selective primer designation were according to Vos et al. (22) with minor modifications (10). Genomic DNA was restricted with *Eco*RI/*Mse*I enzyme combination and double-stranded adaptors specific to each site were ligated. Pre selective amplification was carried out with primers complementary to the adaptors with an extra selective base on each primer (*Eco*RI-A/*Mse*I-A). Selective amplification was performed with four primer combinations involving three *Mse*I (M) and three *Eco*RI (E) primers (E_{ACG}/M_{AAT}, E_{ACG}/M_{ACT}, E_{ACG}/M_{AGT}, E_{ACG}/M_{ATG}).

A total of 10 μl of the AFLP selective amplification product was mixed with 10 μl of loading buffer (98% formamide, 10 mM EDTA, 0.25% each of bromophenol blue and xylene cyanol FF), then denatured at 94ºC for 5 min and placed immediately on ice. Electrophoresis was performed on an EC160 standard sequencing unit (Thermo Electron Corporation, Milfort, Massachusetts). About 3 μl of mixture were loaded onto a 4.5% (w/v) polyacrylamide denaturing gel with 0.5 X TBE buffer after a pre-run electrophoresis at 60 V for 30 min, and then were run at 60 V until the loading dye reached to the bottom of the gel. The gels were dried at 80ºC for 3h. Hyperfilm-Multi Purpose (Amersham Biosciences, Buckinghamshire, England) was exposed to the gels for 2 days.

Band scoring and data analysis

The AFLP fragments were scored manually as present [1] or absent [0]. Only the clearest and strongest bands were scored and used for the analysis. The ability of the most informative primer pairs to differentiate between the genotypes was assessed by calculating their resolving power (Rp) according to Prevost and Wilkinson (15) using the formula $Rp = \sum Ib$, where $Ib=1-(2 \times | 0.5 - p |)$, and p is the proportion of the accessions containing the I band. The polymorphism information content (PIC) of each marker was calculated using $PIC = 1 - \sum Pi^2$ where Pi is the band frequency of the i^{th} allele (19). Jaccard's similarity coefficients (20) were calculated for all pair-wise comparisons among the 14 plum cultivars. A dendrogram was generated using NTSYSpc version 2.11V (Exeter Software, Setauket, NY) (17) based on the un-weighted pair-group method of arithmetic average cluster analysis (UPGMA). The representativeness of the dendrogram was evaluated by estimating cophenetic correlation for the dendrogram and comparing it with the similarity matrix using the same program. The result of this test was a cophenetic correlation coefficient, *r*, indicating how well the dendrogram represents similarity data.

Results and Discussion

Level of polymorphism and discriminating capacity of the AFLP primer pairs

The data generated by the 4 AFLP primers is summarized on **Table 1**. A total of 145 bands were generated and the number of bands produced by each primer combination ranged from 27 (E_{ACG}/M_{ACT}) to 59 (E_{ACG}/M_{ATG}) with an average of 36.3 bands. Out of 145 bands, 89 were polymorphic with yielded 60.85% polymorphism. The percentage of polymorphic bands varied considerably among the primer combinations. For example, 37 of the 59 fragments generated by E_{ACG}/M_{ATG} primer pair were polymorphic, while the E_{ACG}/M_{ACT} primer combination

TABLE 1

Number of AFLP bands , percentage of polymorphic bands, resolving power and polymorphism information content in the DNA fingerprinting of 14 plum cultivars

Primer pairs	Total bands (no.)	Polymorphic bands (no.)	Polymorphism (%)	Resolving power (Rp)	Polymorphism information content (PIC)
E_{ACG}/M_{AAT}	29	19	65.52	1.347	0.829
E_{ACG}/M_{ACT}	27	14	51.85	0.686	0.515
E_{ACG}/M_{AGT}	30	19	63.33	1.039	0.647
E_{ACG}/M_{ATG}	59	37	62.71	0.577	0.412
Total	145	89	-	3.648	-
Mean	36.3	22.3	60.85	0.912	0.601

TABLE 2

The Jaccard's similarity index among 14 plum cultivars

	Globe Sun	October Sun	Original Sun	Queen Rose	Bella Di Barbiano	Fortune	Larry Ann	Autumn Giant	Angeleno	TC Sun	Black Beauty	Friar	President	Black Amber
GlobeSun	1.00													
OctoberSun	1.00	1.00												
OriginalSun	0.81	0.81	1.00											
QueenRosa	0.72	0.72	0.74	1.00										
BDiBarbiano	0.81	0.81	0.81	0.75	1.00									
Fortune	0.83	0.83	0.76	0.77	0.90	1.00								
LarryAnn	0.83	0.83	0.77	0.83	0.82	0.88	1.00							
AutumnGiant	0.81	0.81	0.81	0.79	0.86	0.85	0.84	1.00						
Angeleno	0.82	0.82	0.76	0.78	0.83	0.89	0.88	0.81	1.00					
TCSun	0.98	0.98	0.80	0.71	0.81	0.82	0.81	0.83	0.80	1.00				
BlackBeauty	0.83	0.83	0.84	0.73	0.86	0.88	0.87	0.85	0.84	0.82	1.00			
Friar	0.82	0.82	0.88	0.83	0.89	0.86	0.88	0.87	0.80	0.80	0.84	1.00		
President	0.56	0.56	0.53	0.50	0.59	0.58	0.55	0.56	0.60	0.56	0.56	0.53	1.00	
Black Amber	0.78	0.78	0.80	0.70	0.74	0.78	0.76	0.76	0.86	0.78	0.83	0.77	0.53	1.00

yielded 14 polymorphic bands. Therefore, polymorphism ratio changed between 51.85 and 65.52 among the four primer pairs (**Table 1**).

PCR-based molecular marker techniques play an important role in the analysis of genetic diversity and relatedness for crop plants, where most of the species involved are almost unknown at the genetic level. In this context, DNA profiles have been suggested as a complementary key strategy to determine cultivar identification and hence as leading to improve property rights protection (5).

AFLP markers have been previously used in the genetic analysis of plum cultivars. Ayanoglu et al. (3) studied a total of 20, of which 17 were cherry plum genotypes that belonged to *Prunus cerasifera* originating from different locations along the Mediterranean cost in Turkey and the rest of the studied 3 accessions belonging to *Prunus cerasifera* namely, Can Erik, Papaz and Havran, widely cultivated in Turkey. In their study, the number of bands obtained with each of the six primer combinations, generated 80-100 amplification products and their percentage of polymorphic bands ranged from 6 to 18% indicating lower genetic diversity among *Prunus cerasifera* accessions selected from the same region. Goulao et al. (7) characterized 24 diploid (Japanese group) and 4 hexaploid (European group) cultivars of plum. They used six AFLP primer pairs resulted in amplification of 379 products with 62.8% polymorphism ratio indicating slightly higher polymorphism ratio than our study. These could be results of different accessions and numbers used in both studies. On the other hand, the markers used in this study appeared superior to RAPD markers because Shimada et al. (18) reported low percentage of polymorphism (24.0%) among plum accessions when using RAPD. The AFLP technique is more reproducible than RAPD, because longer primers are used and the annealing occurs at higher melting temperatures.

It was well known that there is limited diversity among the plum cultivars compared to the other tree fruit species. The major Japanese plum cultivars go back to a few genotypes that were the result of hybrization between *P. salicina*, *P. simonii* and native North American species. Today's breeding programs are utilizing the best existing cultivars, thereby narrowing the genetic base further (16).

Previously, Goulao et al. (7) showed that AFLP bands separated the closely-related Japanese plum (*Prunus salicina*) accessions. Thus, our results are also in general agreement with this study. On the other hand, the accessions were most tightly clustered by their species. Previous studies on the relationships of the plum accessions conducted by RAPD and ISSR confirm the suitability of molecular markers for the diversification of plum cultivars (4, 11).

Resolving power (Rp) is an interesting tool to assess the capacity of a given primer to distinguish among various

genotypes. In our study, Rp values ranged from 0.577 (E_{ACG}/M_{ATG}) to 1.347 (E_{ACG}/M_{AAT}), with a total of 3.648. The PIC values of the primer combinations ranged from 0.412 (E_{ACG}/M_{ATG}) to 0.829 (E_{ACG}/M_{AAT}) with an average of 0.601 (**Table 1**).

Genetic relatedness among the plum cultivars

The dendrogram derived from an UPGMA cluster analysis of the AFLP results is shown in **Fig. 1**. Seven distinct groups were observed in the dendrogram. Group I consisted of Globe Sun, October Sun and TC Sun cultivars with a very high similarity ratio (**Table 2**). Group II composed of Original Sun, Friar and Autumn Giant cultivars. Group III included Bella Di Barbiano, Fortune and Black Beauty cultivars. The Group IV had Lary Anne and Angeleno cultivars. Group V included Black Amber. Group VI had Queen Rosa and finally Group VII included President cultivar that belongs to European plums (*Prunus domestica*).

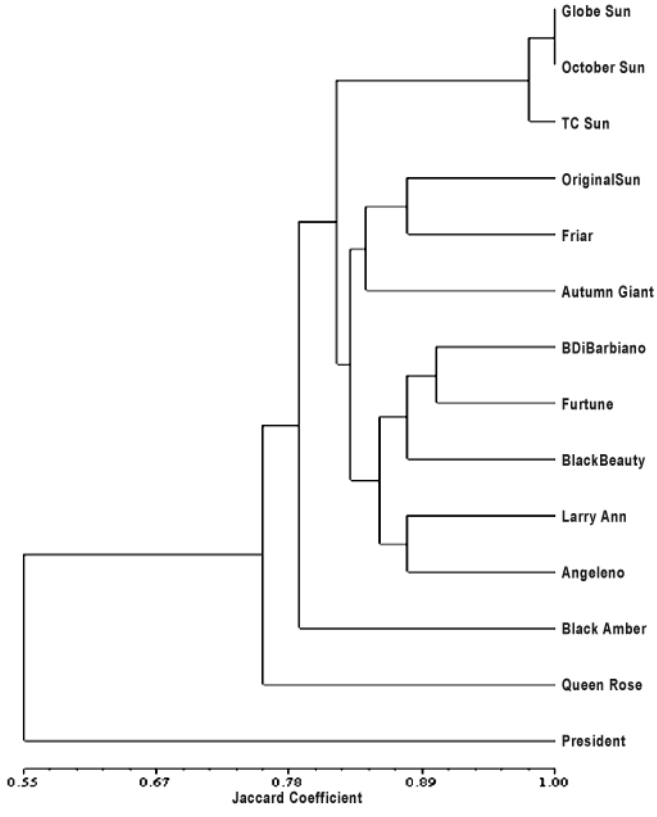

Fig. 1. Dendrogram resulting from UPGMA cluster analysis of 14 plum cultivars based on data derived from four AFLP primer combinations with 145 AFLP markers

In fact the 3 cultivars located in Group I have similar fruit characteristics as for example fruit shapes of all 3 cultivars are ellipse, flesh color is yellow, taste is sweet-sour, skin color is yellow and freestone. Group II includes Original Sun, Friar and Autumn Giant where all 3 cultivars are clingstone and their fruit shape is round. However, fruit skin color of all cultivars differs from each other. Group III included Bella Di Barbiano, Fortune and Black Beauty. The closer cultivars in this group, Bella Di Barbiano and Fortune are half-clingstone, however Black Beauty is clingstone. Flesh color of all accessions in Group III is yellow and taste is sweet-sour. The Group IV had Lary Anne and Angeleno accessions which has clingstone, sweet and round fruits. Group V included Black Amber which has distinct black skin colored fruits. The flesh color of this accession is dark yellow. Group VI had Queen Rosa and finally Group VII included President accession belonging to European plums (*Prunus domestica*) and this group was most distinct from the other groups studied.

The coefficient of cophenetic correlation between dendrogram and similarity matrix was r_{AFLP}=0.93 indicating good fit between dendrogram and similarity matrix.

Conclusions

In conclusion, the AFLP analysis was found useful for detection of genetic differences among the plum cultivars studied. The results of the present study may also benefit breeders in selecting the most diverse cultivars with similar fruit characteristics to begin crossing and selection programs. This may result in increased plum growing for fruit production.

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