International Journal of Bioinformatics and Biomedical Engineering
Vol. 1, No. 2, 2015, pp. 64-69
http://www.aiscience.org/journal/ijbbe

Analysis of Plum (*Prunus domestica* L.) Genotypes of Duhok City Using AFLP Markers

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Abstract

A plum or gage is a stone fruit tree in the genus *Prunus*, subgenus *Prunus*. The subgenus is distinguished from other subgenera (peaches, cherries, bird cherries, etc) in the shoots having a terminal bud and the side buds solitary (not clustered).The samples were analyzed by using AFLP markers. Three primer combinations generated a total of 106 bands and among them86 were polymorphic (81.1%), while 20 (18.9%) were monomorphic. A relatively low genetic diversity has been observed by using AFLP. Genetic similarity values ranging from 0.2464 the lowest genetic distance found between achas yabani thahabi and achas bathengani, whereas the highest genetic distance 0.5415 between achas no.1 and achas maryana. Using UPGMA clustering analysis method based on the similarity coefficient, cultivars were separated into two major genetic clusters within first cluster there is two sub-groups consists of achas no.1, achas samari, achas aswad daymi, achas bathangani no.2 and achas yabani thahabi and a second cluster consist of achas maryana and achas bathangani. The results suggested that AFLP is a good method to determine genetic relatedness among plum genotypes in Duhok city.

Keyword

Prunus domestica L., AFLP, Genetic Diversity

Received: June 14, 2015 / Accepted: June 27, 2015 / Published online: July 13, 2015

1. Introduction

A plum or gage is a stone fruit tree in the family Rosaseae of Prunus genus, subgenus *Prunophora* which is considered to be an important for *Prunus* evolution, the genus Prunus L. (Rosaceae) is naturally distributed in the temperate regions of the Northern Hemisphere with some species occurring in the tropical and subtropical regions (Mabberley, 2008). The genus includes around 200 species and are traditionally divided into five subgenera: Amygdalus (L.) Focke, Cerasus Pers., Laurocerasus Koehne, Padus (Moench) Koehne, and Prunus. The subgenus Prunus, consists of three sections: Armeniaca (Lam.) Koch. (Apricots), Prunocerasus Koehne (North American plums), and Prunus (Eurasian plums) (Rehder, 1940). 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. (6x), one of the European plums, is considered to be derived from natural cross between *Prunus spinosa* L. (4x) and *Prunus cerasifera* Ehrh (2x). The term Japanese plum was applied originally for *Prunus salicina* Lindl. (2x) (Okie and Weinberger, 1996).

Plum has been cultivated for over 2000 years ago throughout the world and has an 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 operated for processing, whereas commercial utilization of plums in the developing countries is minor (Ahmed *et al*., 2004).

Plum fruit tastes sweet and/or tart; the skin may be particularly tart. It is juicy and can be eaten fresh or used in jam-making or other recipes. Plum juice can be fermented

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into plum wine; when distilled, this produces a brandy known in Eastern Europe as Slivovitz, Rakia, Ţuică or Pálinka. In central England, a cider-like alcoholic beverage known as plum jerkum is made from plums.

Dried plums (or prunes) are also sweet and juicy and contain several antioxidants. Plums and prunes are known for their laxative effect. This effect has been attributed to various compounds present in the fruits, such as dietary fiber, sorbitol (Jason, 2007) and isatin. Prunes and prune juice are often used to help regulate the functioning of the digestive system.

In plum, varieties with dark purple colored skin showed 200% higher total phenolic than others (Rupasinghe et al., 2006). The plum cvs. Black Beauty and Angeleno were especially rich in phenolics (Tomás Barberán et al., 2001).

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 (Ramming and Cociu, 1990). Mature plum fruit may have a dusty-white coating that gives them a glaucous appearance and is easily rubbed off. This is an epicuticle wax coating and is known as "wax bloom". Dried plum fruits are called dried plums or prunes, although prunes are a distinct type of plum, and may have pre-dated the fruits now commonly known as plums.

Knowledge of the genetic diversity and relationships among the cultivated species of *Prunus* is essential to distinguishing gene pools, to identifying drawback in germplasm collections, and to develop effective management and conservation strategies. Traditional classifications afford rough guidelines for species relationships, but molecular DNA-based markers provides an opportunity for genetic characterization that allows direct comparison of different genetic material independent of environmental influences and differentiation within and among taxa useful for all geneticists, plant breeders, as well as gene bank managers (Aradhya *et al*., 2004). In the other side molecular DNA-based markers also have been used for cultivar identification and quantification of genetic and phonetic diversity to overcome some of the morphological limitations. In management of gene banks, molecular markers can be used to identify duplicates or to select a percentage of the total collection containing most of the genetic diversity to create core collections.

Choice of the marker system to use for a particular application depends on its ease for using and the particular purposes of the investigation (Rafalski *et al*., 1996). Biochemical and molecular characterization of European and Japanese plums have been reported using isozymes (Byrne and Littleton, 1988) and random amplified polymorphic DNA (RAPD) analysis (Shimada *et al*., 1999). These markers provide compensations over morphological observations, but still suffer from an important problems including reproducibility and insufficient quantity of information per analyses.

Recently, markers like amplified fragment length polymorphism (AFLP) and inter-simple sequence repeats (ISSR), which sample multiple loci simultaneously has become more important. AFLP combines restriction digestion with polymerase chain reaction (PCR), (Rafalski *et al*., 1996) and results in highly informative fingerprints, due to a very high multiplex ratio.

AFLPs are highly reproducible multi-locus marker system developed by (Vos *et al*., 1995). 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. AFLP has a number of advantages over the RAPD technique: more loci analyzed per experiment and better reproducibility of banding patters resulting from the higher specificity of primer annealing to complementary adapters. Powell *et al*., 1996 found that AFLPs had a much higher multiplex ratio (number of polymorphic products per "reaction") than other molecular marker systems. Consequently, AFLPs also shows a higher marker index. These markers have been mainly used in *Prunus* for genetic mapping and molecular characterization and estimation of genetic diversity among apricot cultivars. Standard AFLP methods based on two cutting enzymes requires labeling of selective primers, which necessitates the use of isotopes or fluorescent dyes.

AFLP markers have been used to study the genetic diversity in lots of fruit crops, such as Peach (*Prunus persica* L.) (Dirlewanger *et al*., 1998), Apricot (*Prunus armenica* L.) (Geuna *et al*., 2003), Sweet cherry (*Prunus avium* L.) (Struss *et al*., 2001), and Plum (*Prunus domestica* L.) (Goulao *et al*., 2001). Considering the high reproducibility and high polymorphic nature of AFLP markers (Jones *et al*., 1997), this technique is valuable for breeders to accelerate plant crops enhancement for variety of criteria, by using molecular genetics maps to undertake marker-assisted selection and positional cloning for special characters.

The objective of this study was to identify polymorphisms among plum cultivars collected from Duhok city using AFLP. Polymorphisms discovered would provide additional evidence in supporting, granting, and enforcing individual plant variety protection to new additional strain selections.

2. Material and Methods

2.1. Samples Collection

Samples of Plum (*Prunus domestica*) leaves were collected from Duhok (Ministry of Agriculture station). The varieties of Plum

selected for this study were achas no.1, achas sameraay, achas maryana, achas aswad demi, achas yabani thahabi, achas bathengani and achas ahmer damawi.

2.2. DNA Extraction

3g of fresh tissue was grinded to fine powder using liquid nitrogen. The fine powder was dissolved in a pre-heated (60^0c) 2x CTAB extraction buffer (2x CTAB, 5M NaCl, 1M Tris-HCl, 0.5 M EDTA), and incubate at 60^0c in shaking water bath for 30 min. The mixture was extracted with an equal volume of choloroform / isoamyl alcohol (24:1, v/v) (Maniatis *et al.*, 1982). The mixture was then centrifuged (at 4000 rpm for 30 min). The aqueous phase was transferred into another tube and precipitated with 0.66 volume of isopropanol. Precipitated nucleic acids were then dissolved in TE-buffer and stored at -20°c until use.

3. PCR Amplification of AFLP-Primers

Amplified fragment length polymer-phism (AFLP) procedure was performed as described by (Vos *et al.*, 1995) with minor modification and as follows; 500ng of DNA from each sample was double digested with 5U each of the two restriction enzymes, *Tru91* (recognition site 5'T↓TAA3') and *PstI* (recognition site 5'CTGCA↓G3'). The digestion reaction was prepared in 30µl final volume containing, 1x one- phor all buffer (Pharmacia Bioteh, Uppsala, Sweden), and incubated for three hours at 37°C. DNA fragments, were then ligated to *Pst I* and *Tru91* adapters by adding 50pmol of *Tru91*-adapter, 5Upmol *PstI*-adapter in a reaction containing 1U of T4-DNAligase, 1mM rATP and 1x of one-phore-buffer and incubating for 3hr. at 37°C. After ligation, the reaction mixture was diluted to 1:5 using sterile distilled water. Preselective PCR amplification was performed in a reaction volume of 20 µl containing 50ng of each of the primers (P00, M43) corresponding to the *Tru91* and *Pst I* adapters, 2µl of template- DNA, 1U *Taq* DNA polymerase, 1x PCR buffer and 5mM dNTPs. PCR amplification was performed in WMG thermal cycler using the following program: 30 cycles of 30s at 94 ºC, 1min at 60ºC, 1min at 72 ºC. Pre-amplification products were then diluted to 1:5 and 2µl were used as template for selective amplification. Selective amplification was conducted using *Tru91* and *Pst1* selective primer combinations, (Table 1). Amplification was performed using a selective program of 36 cycles with the following profile: a 30sec. DNA denaturation step at 94ºC, 30sec. annealing step, and a 1 min extension step at 72ºC. The annealing temperature in this program varied in the first cycle where it was 65ºC and in each subsequent cycle for the next 12 cycles it was reduced by 0.7ºC (touchdown PCR). Then for the remaining 23 cycles, it was 56ºC. Selective amplification products were loaded onto 6% denaturating polyacrylamid gels, and DNA fragments were visualized by silver staining kit(Promega, Madison, Wis) as described by the supplier. Silver stained gels were scanned to capture digital images of the gels after air drying.

4. Data Analysis

Digital photographs of gels were used to score the data for AFLP analysis starting from the higher molecular weight product to lowest molecular weight product. Presence of a product was identified as (1) and absence was identified as (0). Data were scored for all genotypes, their amplification product and primers. The data then entered into NTSYS-PC (Numerical Taxonomy and multivariate Analysis System), Version 1.8 (Applied Biostatistics) program (Rohlf, 1993) using the program editor. The data were analyzed using SIMQUAL (Similarity for Qualitative Data) routine to generate genetic similarity index (Nei and Li., 1979).

5. Results and Discussion

With AFLP analysis, three primer combinations produced (86) polymorphic markers. In total, 106 AFLP products were obtained from amplification of 7 plum cultivars, of which 86 were polymorphic (81.1%), while 18.9% were monomorphic, and the number of bands produced by each primer combination ranged from 23 (P109\M289) to 45 (P109\M184) with an average of 35.33 bands (Tables 1and 2).

PCR-based molecular marker techniques play an important role in the analysis of plum varieties. AFLP markers have been previously used in the genetic analysis of plum varieties (Ayanoglu *et al.*, 2007) 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.

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 (Ilgin *et al.*, 2009).

Previously, Goulao *et al*., 2001, 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 (Ilgin *et al*., 2009).

Table 1. Represents the sequences of Pre-amplification and Selective amplification primers, used in this study.

No.	Pre selective primer ('5------3')		Selective primer ('5-----3')	
1	POO	GACTGCGTACATGCAG	P109	GACTGCGTACATGCAGAATG
2	M43	GATGAGTCCTGAGTAAATA	M184	GATGAGTCCTGAGTAACCGA
			M289	GATGAGTCCTGAGTAATAAA
			M237	GATGAGTCCTGAGTAAGATA

Table 2. Total number of bands, number of polymorphic bands and their percentage as amplified by the two primer combinations.

AFLP primer Combination	Number of Amplified Bands	Number of Polymorphic Bands	Percentage of Polymorphic Bands
P109/M184	45	34	75.5%
P109/M237	38	31	81.5%
P109\289	23	21	91.3%
Total	106	86	82.7

Table 3. The genetic distance values between Plum samples studied.

1	0.0000						
2	0.3636	0.0000					
3	0.5415	0.5361	0.0000				
4	0.4006	0.2747	0.3962	0.0000			
5	0.4256	0.3569	0.4258	0.3233	0.0000	0.0000	
6	0.4225	0.4838	0.3945	0.3874	0.2464	0.3006	0.0000
7	0.3906	0.3016	0.2497	0.2497	0.2700		

The AFLP technique has been reported as more reproducible than RAPD methodology (Jones *et a*l., 1997) because longer primers are used, and the annealing occurs at higher melting temperatures.

Estimation of genetic relationships will help to prevent genetic erosion within varieties by selecting a large number of different clones of each variety (Rühl, *et al.,*2000).

Identification of varieties depending on morphological traits alone is difficult and not accurate. A variety may have different names in different plantation and genetically different varieties may have the same name (Torres and Tisserat, 1980). There are several different DNA marker analysis techniques that have been used to identify and characterize fruits to determine genetic diversity (Jubrael, 2005). Each technique has its own requirements, sensitivity, and reliability. In this study AFLP technique reliably distinguished all selected varieties and have high efficiency compared to other markers like RFLPs, RAPD, so we select AFLP markers to estimate genetic diversity of plum cultivars.

In the case of identical genetic material of plum two samples within studied samples that means the genetic distance between them is zero and the genetic similarity equal 1 (100%), and the presence of high present of similarity between varieties may refer that these varieties have the same allele which comes down both of them from ancestor, upon these basis genetic relationship will be built (Esselman *et al*., 2000).

Genetic diversity among 7 cultivars of Plum ranged between 0.2464 the lowest genetic distance found between achas yabani thahabi which means a presence of high similarity between these two varieties whereas the highest genetic distance 0.5415 between achas no.1 and achas maryana means that the similarity between them is very low (Table3). As shown in the dendrogram (Figure1 and 2), the coefficient of similarities based on AFLP fragments revealed genetic diversity between the Plum genotypes under consideration.

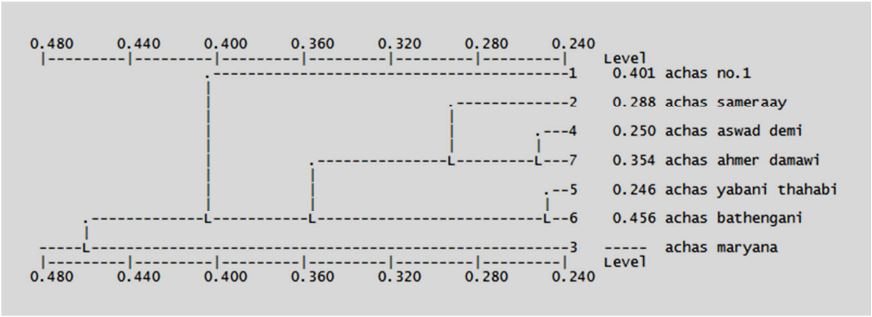
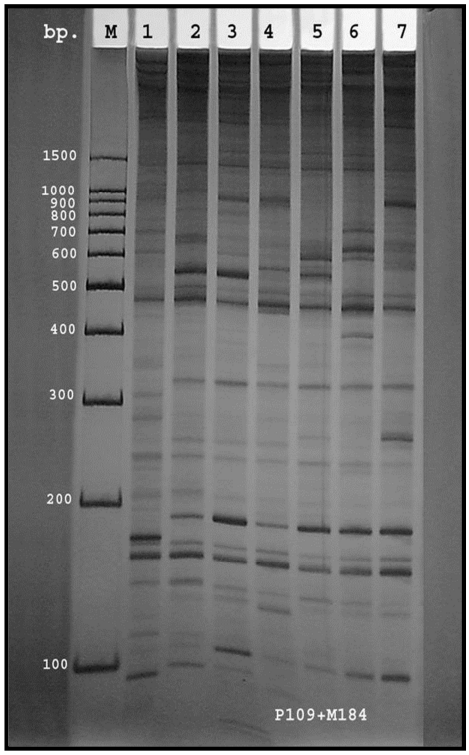


Figure (1). Represent the trees of genetic relationship between plum genotypes by using AFLP markers.

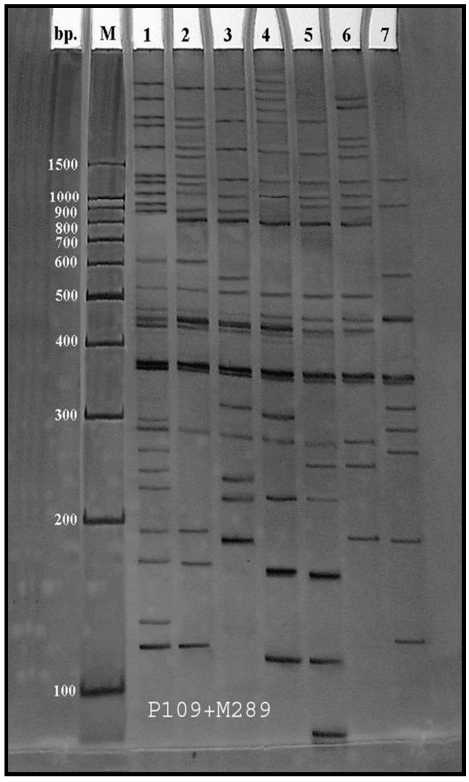
The analyzed data illustrates a good variability in the genetic pool of the common local fig making it a valuable source for incorporation into potential breeding programs for the region.

In the dendogram, the studied individuals are split up into two major clusters: within first cluster there is two sub-groups consists of achas no.1, achas samari, achas aswad daymi, achas bathangani no.2 and achas yabani thahabi, the

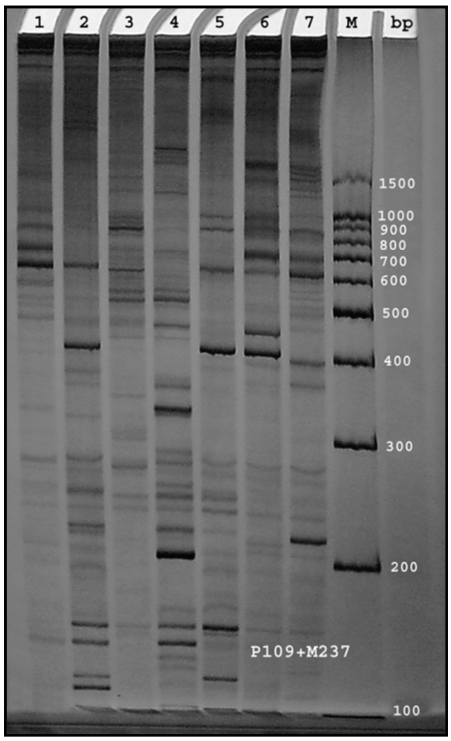
reason that makes these two varieties have come into this sub group may be attributed to the total number of amplified DNA fragments. Morphologically these two varieties have some characters that are close to each other and a second cluster consist of achas maryana and achas bathangani.



A



B



C

Figure (2). Show the result of (AFLP) banding patterns on denatured polyacrylamide gel electrophoresis (PAGE).

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