Comparison of genetic diversity in species and cultivars of pistachio (*Pistacia* sp. L.) based on Amplified Fragment Length Polymorphism (AFLP) markers

Masoud Ahmadi Afzadi[1*], Badraldin Ebrahim Sayed Tabatabaei[2], Sayed Abolghasem, Mohammadi[3], Ali Tajabadipur[1]

[1]Department of Plant Breeding, Pistachio Research Institute of Iran, Rafsanjan P.O. Box 77175-435, I.R. Iran [2]Department of Agricultural Biotechnology, Faculty of Agriculture, Isfahan University of Technology, Isfahan P.O. Box 84156, I.R. Iran [3]Department of Agronomy & Plant Breeding, Faculty of Agriculture, University of Tabriz, Tabriz P.O. Box 51664, I.R. Iran

Abstract
The genetic diversity of a large number of pistachio genotypes grown in Iran is not exactly known. Most of the studies on genetic diversity of Iranian pistachio varieties are based on morphological characteristics or isozyme markers. In the present study, the genetic diversity of selected pistachio cultivars along with some wild species were evaluated by Amplified Fragment Length Polymorphism (AFLP) markers. 13 AFLP primers were used for evaluation of 45 pistachio genotypes. Totally, 506 polymorphic bands with an average polymorphism of 95.2% were detected. Cluster analysis assigned pistachio genotypes into 4 groups. All commercially cultivated pistachios were clustered in group I and II. The wild pistachio genotypes were separated from the cultivated ones. These data demonstrate that AFLP is a reliable tool for analyzing pistachio genetic diversity.
Keywords: Pistachio; Genetic Diversity; Polymorphism; AFLP; Cluster Analysis.

INTRODUCTION

Pistachio (*Pistacia* sp. L.) belongs to a genus of the Anacardiaceae family. It is one of the most prominent horticultural plants from an economic and commercial point of view, so that vast pieces of land in Iran are allocated to the growth of this plant (Anonymous, 2001).

All pistachio species are dioecious and wind-pollinated. The genus *Pistacia* consists of eleven species which only has edible nuts and is commercially important (Zohary, 1952). In addition, there are two other wild species in Iran; *Pistacia atlantica* subsp. *mutica* and *Pistacia khinjuk*. These species are used mainly as rootstock for *Pistacia vera* and rarely for oil extraction in some countries (Kafkas and Perl-Treves, 2002b).

Molecular studies addressing the genus *Pistacia* are few. Most studies regarding the analysis of the genetic diversity of Iranian pistachios have been based on morphological characteristics (Kafkas *et al*., 2002a; Tajabadipur, 1997).

Isozyme markers have also been used to investigate the genetic diversity of pistachios (Aalami *et al*., 1996; Rovira *et al.*, 1995). Mirzaei *et al*. (2005) used Random Amplified Polymorphic DNA (RAPD) markers for analyzing the genetic diversity of 22 pistachio species in Iran. RAPD markers have also been used for studying genetic relationships of two wild pistachios (Kafkas and Perl-Treves, 2002b). Hormaza *et al*. (1994) identified a RAPD marker linked to the sex determinant gene.

Genetic relationship among native and introduced pistachio in Greece has been studied by RAPD and AFLP markers (Katsiotis *et al*. 2003). Golan-Goldhirsh *et al*. (2004) has assessed polymorphism among the *Pistacia* species of the Mediterranean basin and accessions within the same species using RAPD and AFLP markers. Ahmad *et al*. (2003) used microsatellite markers to identify Pistachio varieties grow in kerman province. These isolated microsatellite loci along with sequence-related amplified polymorphism (SRAP) markers were used for assaying genetic relationships

Correspondence to: **Masoud Ahmadi Afzadi,** M.Sc.
Tel: +98 913 1435937; Fax: +98 391 4225208
E-mail: masoud_a_a@alumni.iut.ac.ir

among pistachio species (Ahmad *et al.,* 2005).

Pistachio as an important agricultural plant has several cultivars, but there have been few studies regarding the genetic diversity of *Pistacia* using molecular markers in Iran. The objective of the present study is to investigate genetic diversity among *Pistacia* species grows in Iran. In this study, AFLP markers with 13 primers have been used to investigate the extent of diversity in 45 species of *Pistacia*.

MATERIALS AND METHODS

Plant materials and DNA extraction: Plant materials (leaves) were collected from Iran's Pistachio Research Institute (IPRI) in Rafsanjan. Young leaf samples were kept in liquid nitrogen tanks for the purpose of DNA extraction and AFLP analyses. In total, 45 pistachio species were used in this study which consisted of three wild species (*Pistacia palaestina*, *Pistacia atlantica* subsp. *mutica* and Sarakhs), and 42 cultivars (Table 1). Total genomic DNA was extracted from freez-dried leaf tissue using the Cetyl Trimethyl Ammonium Bromide (CTAB) mini-extraction protocol based on the method by Hormaza *et al*. (1998), but with minor modification (Mirzaei *et al.,* 2005). Quality and quantity of DNA were determined by a spectrophotometer (Beckman DU 530) and the concentration of DNA was also confirmed by electrophoresis using 1% (v/v) agarose for 45 min at 80 V in 0.5X TAE buffer, followed by visualization under UV light after staining with ethidium bromide.

AFLP Analysis: The AFLP reactions were carried out according to the method by Vos *et al*. (1995). Two hundred nanograms of genomic DNA from each sample was digested with *Eco*RI and *Mse*I in a total volume of 20 µl at 37°C for 4 hour and then double stranded adaptors were ligated to the fragment ends as well. The digested and ligated DNA were then diluted by the addition of 125 µl ddH_2O and pre-amplified using *Eco*RI and *Mse*I primers with no additional selective nucleotide. Pre-amplification was performed in a total volume of 25 µl containing 50 ng of each primer, 0.2 mM of each dNTP (Roche), 1X PCR buffer (Roche), 1 U of Taq DNA Polymerase (Roche) and 3 µl of the diluted digested and ligated DNA.

The temperature profile for pre-amplification was as follows; denaturing for 1 min at 94°C, 1 cycle; annealing for 1 min at 56°C, extension for 1 min at 72°C, 30 cycles; 72°C for 7 min, 1 cycle. For selective amplification, the product of pre-amplification was diluted by addition of 125 µl ddH_2O. Selective amplification was carried out using thirteen primers with three selective nucleotides (Table 2). The selective amplification reactions were carried out in a total volume of 15 µl comprising 15 ng of each primer, 1X PCR buffer, 0.25 mM of each dNTP, 1 U of *Taq* DNA Polymerase and 4 µl of diluted pre-amplification product using a 'Touchdown' cycle programmed as

Table 1. List of pistachio species analyzed.

Number	Name	Number	Name
1	Akbari	24	Shahpasand
2	Ghazvini Zodras	25	Ghafouri
3	Ahmad Aghaii	26	Jandaghi
4	Badami Nishkalaghi	27	Mohseni
5	Kale Ghoochi	28	Sefid Peste Nough
6	Ohadi	29	Khanjari Ravar
7	Fandoghi Riz	30	Fandoghi 48
8	Badami Zarand	31	Momtaz tajabadi
9	Rezaii Zodras	32	Ravar 1
10	Sabz Peste Nough	33	Sirizi
11	Ebrahim Abadi	34	Ravar 3
12	Hasan Zadeh	35	Badami Ravar
13	Seyfadini	36	Amiri
14	Italiaii Zodras	37	Hasani
15	Bayazi	38	Ebrahimi
16	*P. atlantica* subsp. *mutica* (Baneh)	39	Momtaz
17	Male tree	40	Javad Aghaii
18	Lahijani	41	Mosa Abadi
19	Behesht Abadi	42	Shasti
20	Baneh Baghi	43	Harati
21	Fandoghi Ghafouri	44	*P. vera* var. *sarakhs*
22	Ravar 2	45	*P. palaestinia*
23	Poust Khormaii		

Table 2. Pairs of primers used for AFLP analysis, number of polymorphic bands and percentage polymorphism.

Number	Primers	Number of polymorphic bands	Total Bands	Polymorphism (%)
1	E(ATA)-M(CCA)*	55	56	98
2	E(ATG)-M(CCT)	23	24	95
3	E(AAC)-M(CCC)	28	30	93
4	E(AAC)-M(CGA)	31	32	96.9
5	E(AAC)-M(CAA)	26	26	100
6	E(AAG)-M(CAG)	46	47	97.9
7	E(AAG)-M(CCA)	18	22	81.8
8	E(AAG)-M(CCC)	47	49	95.9
9	E(AAG)-M(CCG)	45	47	95.7
10	E(AAG)-M(CCT)	58	61	95.1
11	E(AAG)-M(CGA)	32	34	94.1
12	E(AAG)-M(CAA)	20	21	95.2
13	E(AAG)-M(CAC)	77	78	98.7
Total		506	527	95.2

* *E: EcoRI primer: 5′-GACTGCGTACCAATTC-3′* and M: *Mse*I primer: 5′-GATGAGTCCTGAGTAA-3′.

follows; 30 s at 94ºC, 30 s at 65ºC and 60 s at 72ºC. The 65ºC annealing temperature was reduced by 0.7ºC per cycle for 12 consecutive cycles and then maintained at 56ºC for the remaining 24 cycles. The amplified products were separated on a denaturing 6% (v/v) polyacrylamide sequencing gel. After electrophoresis, the gel was stained with silver nitrate and then visualized.

Data analysis: Banding patterns were scored as presence (1) and absence (0) using the cross Checker software ver 2.91. The genetic similarity metrices were constructed using Simple Matching (Sokal and Sneath 1963), Jaccard (Jaccard 1908) and Dice coefficients (Nei and Li 1997). Dendrograms were constructed by the unweighted pair-group method using arithmetic average (UPGMA) and complete linkage algorithms. In addition to cluster analysis, principal component analysis was used to confirm the results of cluster analysis. The efficiency of clustering algorithms and their goodness of fit were determined based on the cophenetic correlation coefficient. Data analyses were performed by the NTSYS software ver 2.02.

RESULTS

AFLP profiling of 45 pistachio genotypes with 13 primer combinations revealed a total of 527 scorable bands ranging in size from 100-1000 nucleotides. A total of 506 AFLP fragments were polymorphic across all the genotypes for the 13 primer pairs. The 506 polymorphic fragments accounted for 95.2% of the total amplified fragments.

The degree of polymorphism among primers was different (Table 2). The E(AAC)-M(CAA) primers showed the highest polymorphism whereas the minimum number of bands and the lowest degree of polymorphism was observed with the E(AAG)-M(CCA) primers. However, the E(AAG)-M(CAC) primer combination showed the maximum number of bands.

The cophenetic correlation, a measure of the correlation between the similarity represented on the dendrograms and the actual degree of similarity, was calculated for each dendrogram. Among the different methods, the highest value (r= 0.8855) was observed for UPGMA based on Jaccard's coefficient (Table 3). Therefore, the dendrogram constructed based on this method was used for depicting genetic diversity of

Table 3. Comparison of different methods for constructing similarity matrices and dendrogram.

Similarity matrices	Algorithm		Cophenetic coefficient
Jaccard	UPGMA		r = 0.8855
		Complete Linkage	r = 0.8246
Dice (Nie & Li)	UPGMA		r = 0.8705
		Complete Linkage	r = 0.8096
Simple Matching	UPGMA		r = 0.8291
		Complete Linkage	r = 0.7534

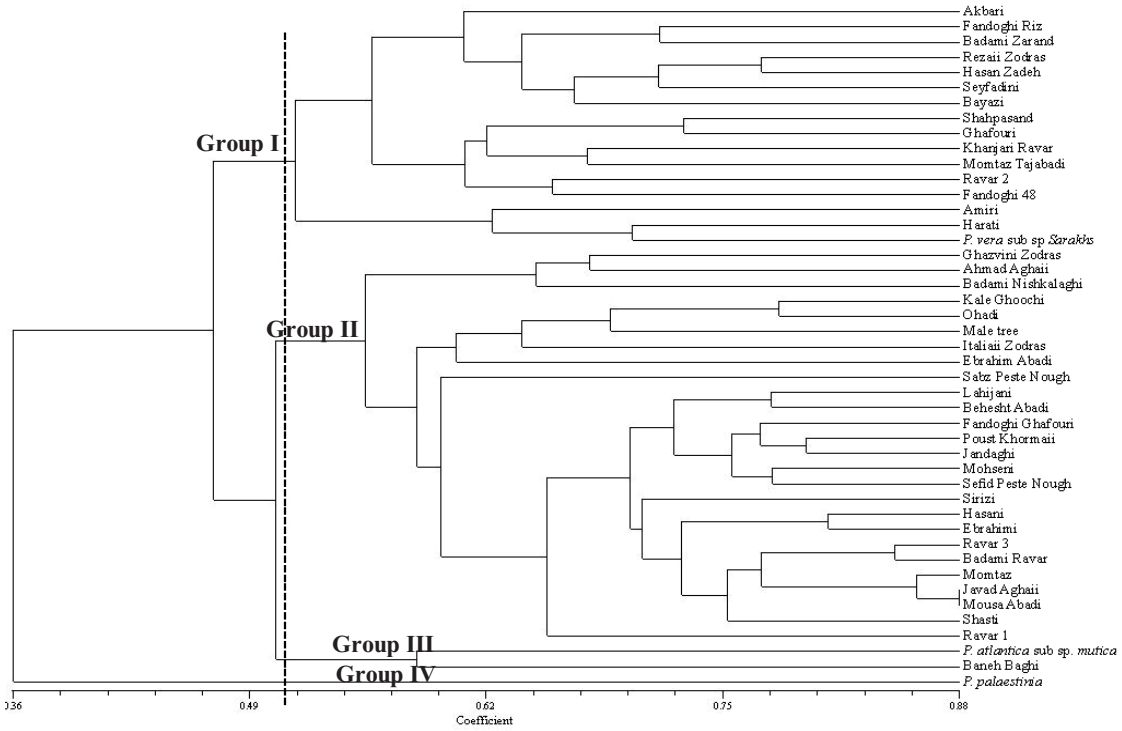

Figure 1. Dendrogram depicting the genetic relationships of pistachio species based on AFLP data, UPGMA clustering method and Jaccard's coefficient.

genotypes (Figure 1).

Cutting the dendrogram at the point with maximum distance among groups resulted into four main groups (Figure 1). All pistachio cultivars were placed in group I and II. Group III included two wild pistachios, Baneh (P. *atlantica* subsp. *mutica*) and Baneh Baghi. A wild pistachio species, *P. palaestinia* was placed in a separated cluster with very low similarity to other groups.

In group I the highest similarity value was observed between Rezaii Zodras and Hasan Zadeh cultivars. Group I was further divided into two subgroups in which Sarakhs as a wild variety of *P. vera* was placed in subgroup II. In group II, the highest similarity value was found between two cultivated pistachios; Javad Aghaii and Mosa Abadi cultivars (0.88). Comparing with group I, the pairwise similarities in group II were higher. For example, the highest observed similarity value in group I was 0.78 which was found between Rezaii Zodras and Hasan Zadeh cultivars. Cultivars in group II were clustered together at a higher similarity value. The lowest similarity values were between *P. palaestinia* and all other species.

Considering morphological characteristics, cultivars in group I had fruits with orbicular or elongated shapes except for the Harati cultivar which had an ovate-shaped fruit, however, most of the cultivars with ovate-shaped fruits were mainly clustered in group II. According to fruit ripening time, cultivars were almost separated by cluster analysis. Early ripening cultivars were placed in group I except for the Ghafouri which as a late ripening cultivar was also placed in this group. Late ripening cultivars were placed in group II which also included the early ripening cultivars of Ghazvini Zodras and Italiaii Zodras.

Principal coordinate analysis (PCA) based on genetic similarity matrices were used to visualize the genetic relationships among species. The first two eigenvectors accounted for 44.03% of the total molecular variation. Therefore, PCA results confirmed the results of cluster analysis. Based on PCA, cultivars and wild species were almost separated from each other (Figure 2).

DISCUSSION

The utility of DNA-based markers such as AFLP as a reliable technique for assaying genetic variation among plant species has widely been reported (Blears *et al.,* 1998). This technique is more informative and reproducible compared to previously used biochemi-

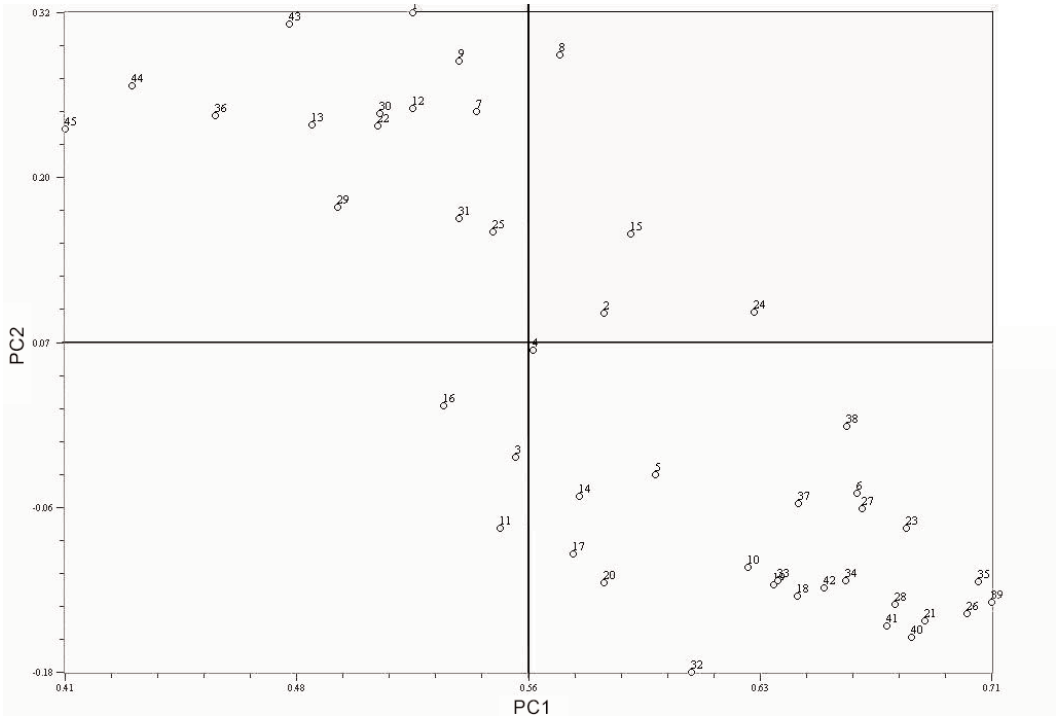

Figure 2. Relationships among the pistachio genotypes revealed by principal component analysis based on AFLP genetic similarity.

cal and molecular methods such as isozyme, and RAPD markers in detecting genetic relationships of pistachio genotypes (Golan-Goldhirsh *et al*., 2004; Katsiotis *et al*., 2003).

Cluster analysis of AFLP data could separate cultivars and wild species of pistachio. In this grouping, wild species *P. atlantica* subsp. *mutica* was closer to the cultivated pistachio than *P. palaestinia*, supporting the earlier report of Kafkas and Perl-Treves, (2002b).

All the cultivated pistachios were clustered in groups I and II. These cultivars are mostly separated according to two morphological traits; fruit shape and time of fruit ripening. Results showed that, cultivars with orbicular and elongated nut shapes are close to each other but separated from cultivars having ovate shapes. Hence, orbicular and elongated nuts are different form nuts with ovate shape, from molecular point of view. Mirzaei *et al*. (2005) who analyzed the genetic relationships of Iranian cultivated pistachio and wild species also could not separate orbicular nuts from elongated ones. It seems that primers used in the RAPD and also in the present study did not cover the genomic region(s) consisting of gene(s) controlling the orbicular and elongated nut shapes.

Based on AFLP data, the cultivars with early and late ripening fruits were distinguished from each other. Therefore, the primer used in this study could be employed in further studies to separate cultivars having these two morphological traits. It was hypothesized that regions amplified with these primers probably contain gene(s) which control(s) fruit ripening and specify ovate fruit shape. Nonetheless confirming the location of these genes and relating them to AFLP markers needs further research.

P. vera subsp. *Sarakhs* is a wild species of *Pistacia* which grows as self-grown forests in North-east of Iran. It showed the highest genetic similarity with cultivated species. The results of this study as in previous studies (Mirzaei *et al.,* 2005; Aalami *et al*., 1996) suggest that the cultivated pistachio in Iran may have originated from *P. vera* subsp. *Sarakhs*. Baneh (*P. atlantica* subsp. *mutica*) and Baneh Baghi have been placed in a single cluster. These two species are similar based on morphological characteristics such as tree height, crown shape and number of leaflets. As a result, it can be hypothesized that Baneh Baghi is a hybrid of Baneh and cultivated pistachio that has acquired certain traits from cultivars through time.

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