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The genetic diversity of Iranian pistachio (*Pistacia vera* L.) cultivars revealed by ISSR markers

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Abstract

Iran has a rich pistachio germplasm, thereby, the diversity and number of Iranian pistachio cultivars is unique in the world. In this study, genetic relationships among thirty one cultivars was assessed by using six inter simple sequence repeat (ISSR) primers. During the ISSR screening in this study, good amplification products were obtained from primers based on GA, CA and GAA repeats. Since, primers based on CT, GT and CAA repeats produced few large separate bands which were eliminated for the final analysis. The total of 28 bands of which 13 (46/42%) were polymorphic were amplified by the three primers, an average of 9.3 bands per primer. The total number of amplified fragments was between 7 to 12 and the number of polymorphic fragments ranged from three to five. The range of genetic similarity was from 0/84 to 1 and the constructed unweighted pair group method with arithmetic averages (UPGMA) dendrogram classified the tested genotypes into 11 main clusters. The present study showed that there is low genetic diversity among tested cultivars and the ISSR-PCR analysis produces sufficient polymorphisms for large-scale DNA fingerprinting. This study reports the first application of the ISSR technique in characterization of Iranian pistachio cultivars.

Key words: Pistachio, ISSR, Genetic relationships, Clustering

1. Introduction

Pistachio (*Pistacia vera* L.) is the only cultivated and commercially important species in the genus *Pistacia*, consisting of a deciduous, dioeciously and wind-pollinated at least 11 tree species (Whitehouse, 1957). *Pistacia vera* L., is a diploid (2n=30) member of the Anacardiaceae family (Zohary, 1952; Whitehouse, 1957). *Pistacia vera* is native to north Afghanistan, northeast Iran, and central Asian republics (Browiez, 1988; Kafkas, 2006). Among the nut tree crops, pistachio tree ranks sixth in world production behind almond, walnut, Cashew, hazelnut and chestnut (Mehlenbacher, 2003). Iran is the main world producer with more than 400,000 tons followed by Turkey, USA and Syria (Faostat, 2006). The main cultivars grown in Iran are Ohady, Kaleh ghochi, Ahmad Aghai, Badami Zarand, Rezaii and Pust piazi (Esmailpour, 2001). Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. Among them, RAPD (Williams et al., 1990) has been the most commonly used method in pistachio cultivars characterization (Hormaza et al, 1994 & 1998; Kafkas et al, 2002; Katsiotis et al, 2003; Golan-Gpldhirsh et al, 2004; Mirzaei et al, 2005). AFLP and SSR techniques have been also used in pistachio to study genetic relationship among *Pistacia* species and cultivars (Golan- Goldhirsh et al, 2004; Katsiotis et al, 2003; Ibrahim Basha et al, 2007 ;Ahmad et al, 2003; Ahmad et al, 2005; Ahmadi Afzadi et al, 2007). Each marker technique has its own advantages and disadvantages. RAPD markers are very quick and easy to develop (because of the arbitrary sequence of the primers) but lack reproducibility (Karp et al, 1997; Hansen et al, 1998; Jones et al, 1999; Virk et al, 2000). AFLP has medium reproducibility but is labour intensive and has high operational and development costs (Karp et al, 1997). Microsatellites are specific and highly polymorphous (Karp et al, 1997; Jones et al, 1999), but they require knowledge of the genomic sequence to design specific primers and, thus, are limited primarily to economically important species. Since 1994, a new molecular marker technique called inter sequence repeat (ISSR) has been

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available (Zietkiewicz et al, 1994). ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of nonanchored primers also has been called microsatellite-primed PCR, or MP-PCR, (Meyer et al, 1993). Each band corresponds to a DNA sequence delimited by two inverted microsatellites. Like RAPDs, ISSRs markers are quick and easy to handle, but they seem to have the reproducibility of SSR markers because of the longer length of their primers. Amplification in this technique does not require genome sequence information and leads to multilocus and highly polymorphous patterns (Zietkiewicz et al, 1994a; Tsumara et al, 1996; Nagaoka, 1997) and involves longer (16-18 nucleotides) primers encoding microsatellite elements that amplify DNA segments Intramicrosatellite repeats (Gupta, 1994; Zietkiewicz, 1994). ISSR is a dominant marker like RAPD (scored using presence or absence of band at a locus) but with greater robustness in repeatability and extremely high variability. These features make ISSR better than other readily available marker systems in investigating the genetic variation among very closely related individuals and in crop cultivar classification (Fang & Roose, 1997; Nagaoka & Ogihara, 1997). Recently, this marker technique has been used to detect DNA polymorphism and genetic diversity in a wide pistachio germplasm originating from seven countries accompanied with AFLP and RAPD markers (Kafkas et al, 2006). The objectives of the study were 1) to assess genetic diversity and relationships among some of Irainian pistachio cultivars 2) to set up and use first inter simple sequence repeats (ISSR) technique in pistachio cultivar identification in Iran.

2. Materials and methods

2.1. Plant materials amd DNA extraction

In this study, leaf samples of 31 pistachio genotypes (30 females and 1 male) were collected from the Rafsanjan Pistachio Germplasm Collection located in Rafsanjan city, Iran (Table 1). Total genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) with minor modifications. DNA quantity and quality were estimated both using an UV spectrophotometer by measuring absorbencies at A260 and A280 and 0.8% agarose gel electrophoresis by comparing band intensity with λ DNA of known concentrations. DNA samples were diluted to 10 ng/µl for ISSR reactions.

Table 1: List of pistachio cultivars examined for genetic relatedness using ISSR marker system in this study.

Code	genotypes	sex	Code	genotypes	sex
P1	Sirizi	F	P16	Pust Khormaei	F
P2	Badami Ravar	F	P17	Ghazvini	F
P3	Ghafori Rafsanjan	F	P18	Fandoghi 48	F
P4	Hasan Zadeh	F	P19	Javad Aghaei	F
P5	Ravar No.2	F	P20	Badami Dishkalaghi	F
P6	Gholamrezaei	F	P2 1	Paye Nar	M
P7	Badami Zarand	F	P22	Vahedi	F
P8	Harati	F	P23	Ohadi	F
P9	Behesht Abadi	F	P24	Shasti	F
P10	khanjari Ravar	F	P25	Khanjari Damghan	F
P11	Ravar No. 3	F	P26	Ebrahimi	F
P12	Pust Piazi	F	P27	Saiffodini	F
P13	Shahpasand	F	P28	Kaleghochi	F
P14	Mohseni	F	P29	Italiaei	F
P15	Lahijani	F	P30	Ahmad Agaei	F
			P31	Ravare No.1	F

(F=female; M=male)

2.2. ISSR reactions

ISSR reactions were done according to (Zietkiewicz et al, 1994) with minor modifications (Table 2). Amplification reactions were done in a 25-µL volume containing 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 200 µM each of dATP, dGTP, dCTP and dTTP, 10 pmol of a given primer, 1 unit of Taq DNA polymerase (Fermentas, Lithuania) and 10 ng of genomic DNA. PCR amplification were performed in a gradient thermal cycler (Eppendorf, Hamburg, Germany)...In ISSR, the program included 1 cycle of 4 min at 94°C, followed by 40 cycles of 45 s at 94°c, 1 min at 42 to 52°C (depending upon primer), and 2 min at 72°C, followed by a final extention for 6 min at 72°C. ISSR amplification products were analyzed by gel electrophoresis in 1.8% agarose in 1x TBE buffer, stained with ethidium bromide and digitally photographed under ultraviolet light. Reproducibility of the patterns was checked by running the reactions in duplicates.

Table 2: Primers used for the ISSR analysis and number of DNA polymorphic bands produced

Primer	**5´-3´sequence**	**Total number of bands**	**Number of polymorphic bands**
ISS2	5`-(GA)5GC -3`	7	3
ISS3	5`-(CA)5GT -3`	9	5
ISS5	5`-(GAA)5 -3`	12	5

2.3. Data analysis

The amplified bands were scored manually as 1 (present) and 0 (absent). Only the clearest and strongest reproducible bands were scored and used for cluster analysis. Genetic similarities (GS) between samples for the three methods were calculated using the DICE (equivalent to Nei and Li) algorithems, described by(Sneath & Sokal, 1973). Based on the GS matrices dendrograms were constructed using the clustering methods of the Unweighted Pair Group Method of Arithmetic averages (UPGMA). Also Principle Coordinate Analysis (PCA) was estimated. NTSYS -pc. 2.02i (Rohlf, 1998) was used to perform all the analyses. To determine the efficiency of this marker type in detecting genetic variation, the assay efficiency index, AEI (Pejic et al, 1998) (AEI=BP/T, where BP is the total number of polymorphic fragments detected and T is the number of polymorphic primer pairs) ,percentage of polymorphic (PP) fragments and effective multiplex ratio(EMR) were also calculated (Powell et al, 1996). EMR is defined as the number of bands (n) analyzed per primer.

3. Results and discussion

3.1. Leves of polymorphism and discriminating of the assay

The results of this molecular assay in fingerprinting of the 31 pistachio genotypes are presented in table 3. In ISSR, according to the reported results of (Kafkas et al, 2006), first six primers were used and after initial screening three out of them primers eventually selected for the final analysis. A total of 28 bands were amplified by the three primers, an average of 9.3 bands per primer of which 13 (46/42%) were polymorphic. The total number of amplified fragments was between seven to 12 and the number of polymorphic fragments ranged from three to five. Figure 1 shows the results of amplification with primer ISSR5 (GAA) 5 on agarose 1.8% with 16 lanes gel tray. During the ISSR screening in this study, good amplification products were obtained from primers based on GA, CA and GAA repeats. But primers based on CT, GT and CAA repeats produced few large separate bands which finally were eliminated for the final analysis. (Kafkas et al, 2006) using 20 primers obtained a total of 156 bands, an average of 7.7 bands per primer, of which 73(46.2%) were polymorphic which is similar to our results in this study.

Table 3: The results of ISSR marker assay in fingerprinting of 31 pistachio genotypes

	Acronym	ISSR
Primer (no)	NP	3
Total bands (no.)	NB	28
Bnds per assay(no.)	NBA	9.3
Polymorphic bands	NPB	13
Assay efficiency index	AEA	4.3
Monomorphic bands	NMB	15
Polymorphism (%)	PP	46.42
Effective multiplex ratio	EMR	4.32

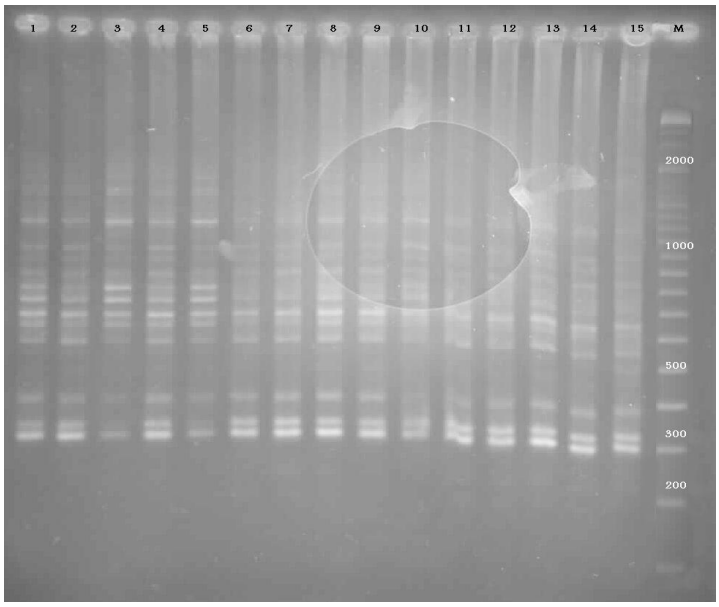

Figure1. Results of amplification with primer ISSR5 (GAA) 5 on agarose 1.8% with 16 lanes gel tray. M= molecular weight; P1-p15 individuals of *P.vera.*

3.2. Clustering and genetic relationships among pistachio cultivars.

In this study, the pattern of cluster analysis of based on DICE's similarity coefficient and UPGMA algorithm placed the genotypes in different clusters. The range of genetic similarity was from 0/84 to 1 and the constructed unweighted pair group method with arithmetic averages (UPGMA) dendrogram classified the tested genotypes into 11 main clusters. The first cluster contained 6 genotypes namely Sirizi (P1), Javad Aghaei (P19),Badami Nishkalaghi (P20), Ravar No.2 (P5), Badami Zarand (P7) & Saifeddini (P27). The second cluster contained 3 genotypes namely Vahedi (P22), Khanjari Damghan (P25) and Ahmad Aghaei (P30). The third cluster contained 6 genotypes namely Ravar No.3 (P11), Pust Piazi (P12), Shahpasand (P13), Mohseni (P14), Lahijani (P15) and Ebrahimi (P26). The fourth cluster consisted of 2 genotype namely Shasti (P24) and Ravar No.1 (P31). The fifth cluster consisted of 4 genotypes namely Badami Ravar (P2), Hasanzadeh (P4), Gholamrezaei (P6) and Beheshtabadi (P9).The sixth cluster consisted of 2 genotype namely Postkhormaei (P16) and Ghazvini (P17). The seventh cluster consisted of 1 genotype namely Paye Nar (P21). The eighth cluster consisted of 2 genotype namely KalehGhochi (P28) and Italiaei (P29). The nineth cluster consisted of 3 genotypes namely Harati (P8), Khanjari Ravar (P10) & Ohadi (P23). The tenth cluster contained 1 genotype of Ghafori Rafsanjan (P3) and the eleventh cluster contained one genotype namely Fandoghi 48 (P18). (Figure. 2)

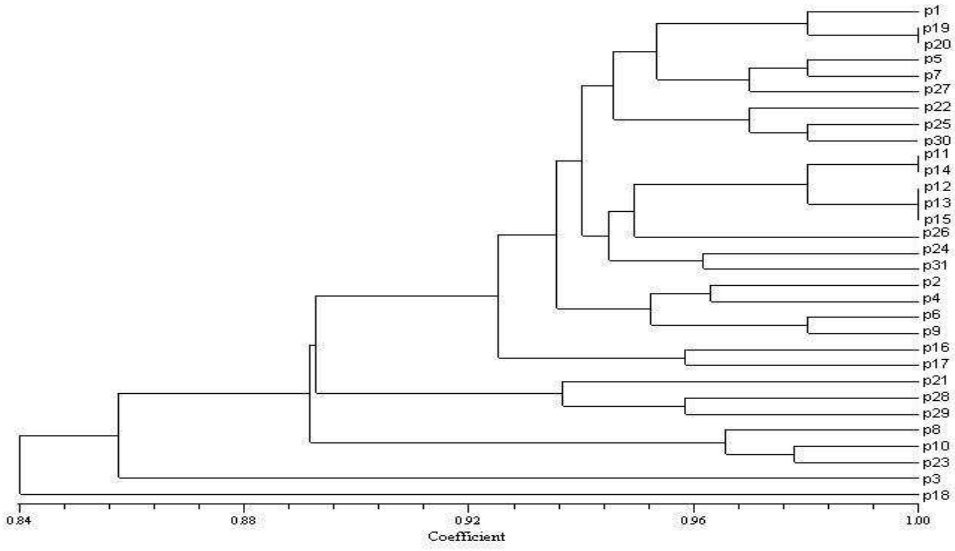

Figure2: UPGMA dendrogram based on the Dice similarity coefficient illustrating the relationships among pistachio genotypes.

The Principle coordinate analysis (PCA) based on genetic similarity matrices were used to visualize the genetic relationships among genotypes (Figure 3). The first three eigenvectors accounted for 23/11% of the total molecular variation. Therefore, the Principle Coordinate Analysis (PCA) results confirmed the results of cluster analysis. The results of this study showed that there is a relatively low level of genetic diversity in the studied samples which are expected in view of the dioecius and outbreeding nature of the cultivated pistachio cultivars and high level of heterozygosity due to the cross-pollinating nature of the plant established during the evolution and domestication processes which have been conserved by the propagation of clones through vegetative reproduction. This study reports the first application of the ISSR technique in pistachio characterization of Iranian cultivars. The present study showed that ISSR-PCR analysis is quick, reliable, produces sufficient polymorphisms for large-scale DNA fingerprinting purposes, and also showed that ISSR markers are able to reveal variability between pistachio cultivars.

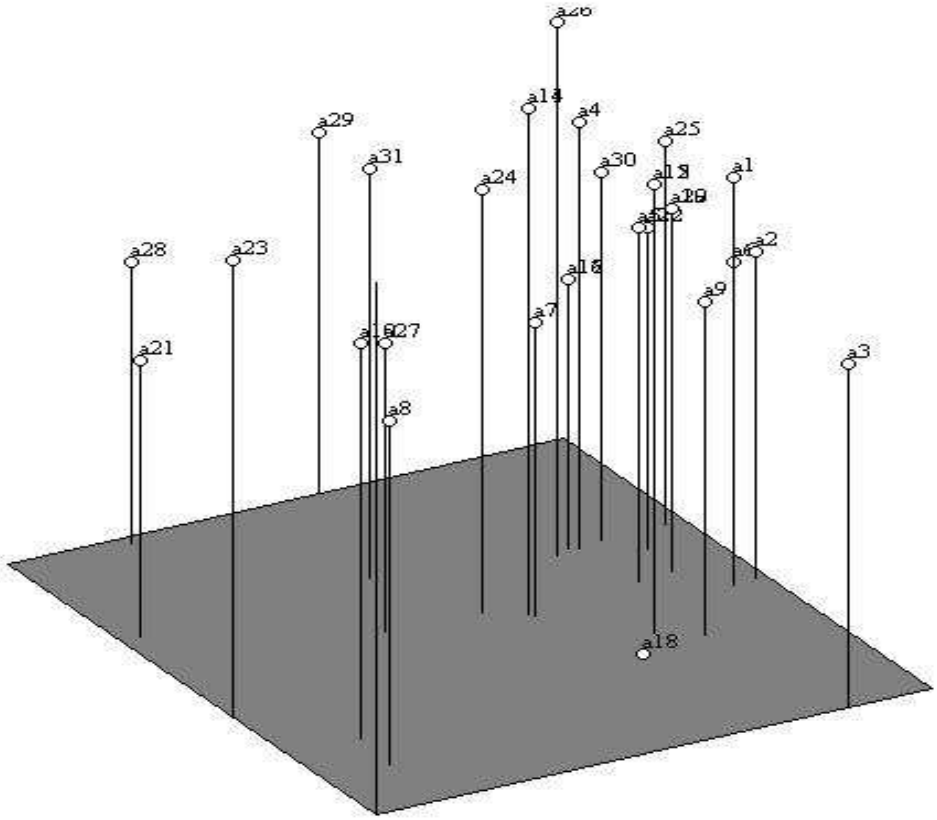

Figure3: Graph of the first and second and third principle components PC-1 and PC-2 and PC-3 of the variation in the pistachio population studied.

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