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Study on genetic diversity of some of Iranian pistachio (*Pistacia vera* L.) cultivars using RAPD, ISSR and SSR markers: A comparison study

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ABSTRACT

Iran has a rich and diverse pistachio germplasm and thereby the diversity and number of Iranian pistachio cultivars is unique in the world. In this study 31 pistachio cultivars and genotypes were characterized by RAPD, ISSR and SSR markers. The general dendrogram constructed using the combined data of the three sets of molecular markers was to some extent similar to those obtained separately with each marker. The overall Principle coordinate analysis (PCA) based on genetic similarity matrices showed that the first three eigenvectors accounted for 28.46% of the total molecular variation. Therefore, the PCA results confirmed the results of cluster analysis. In SSR population analysis; the four primers produced 11 alleles among 31 pistachio genotypes with an average value of 2.75 alleles. 100% Polymorphism was observed at all of these loci. The low average polymorphic information content value 0.4374 indicated the presence of high genetic similarity among genotypes and entails development of additional polymorphic SSR primers for effective characterization of Iranian pistachio cultivars/genotypes. According to the effective multiplex ratio and assay efficiency index, it was showed that RAPD markers were the most powerful to differentiate the genotypes followed by ISSR and SSR markers, respectively. © 2010 Trade Science Inc. - INDIA

KEYWORDS

Pistacia vera;
Genetic diversity;
Clustering;
Population parameters.

INTRODUCTION

Pistachio (*Pistacia vera* L.), a deciduous, dioecious, and wind-pollinated tree species, is a diploid (2n = 30)[13,32] member of the Anacardiaceae family and consists of at least 11 species[28,32]. Pistachio (*Pistacia vera* L.) is the only cultivated and commercially important species in the genus *Pistacia*[28]. *Pistacia vera* is native to north Afghanistan, northeast Iran, and central Asian republics[5,13,15,16]. Among the nut tree crops, pistachio tree ranks sixth in world production behind almond, walnut, Cashew, hazelnut and chestnut[18]. The main world producer is Iran with more than 400,000 tons followed by Turkey, USA and Syria[8]. The main

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TABLE 1 : List of pistachio cultivars examined for genetic relatedness using RAPD, ISSR and SSR marker systems in this study

Code	Sex	Genotypes
P1	F	Sirizi
P2	F	Badami Ravar
P3	F	Ghafori Rafsanjan
P4	F	Hasan Zadeh
P5	F	Ravar No.2
P6	F	Gholamrezaei
P7	F	Badami Zarand
P8	F	Harati
P9	F	Behesht Abadi
P10	F	Khanjari Ravar
P11	F	Ravar No. 3
P12	F	Pust Piazi
P13	F	Shahpasand
P14	F	Mohseni
P15	F	Lahijani
P16	F	Pust Khormaei
P17	F	Ghazvini
P18	F	Fandoghi 48
P19	F	Javad Aghaei
P20	F	Badami Dishkalaghi
P21	M	Paye Nar
P22	F	Vahedi
P23	F	Ohadi
P24	F	Shasti
P25	F	Khanjari Damghan
P26	F	Ebrahimi
P27	F	Saiffodini
P28	F	Kaleghochi
P29	F	Italiaei
P30	F	Ahmad Agaei
P31	F	Ravare No.1

nF = female; M = male

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

Primer	5′-3′sequence	Total number of bands	Number of polymorphic bands
NO.398	CAGTGCTCTT	7	4
NO.378	GACAACAGGA	11	6
NO.394	TCACGCAGTT	12	6
NO.400	GCCCTGATAT	8	4
NO.390	TCACTCAGAG	12	8
F	CCCACTCACG	14	9
J	CCTCACCTGT	5	3
UB1	CCTGGGCTTC	3	2
UB6	CCTGGGCCTA	6	3
UB53	CTCCCTGAGC	6	4
ISS2	5′-(GA)5GC -3′	7	3
ISS3	5′-(GA)5GT -3′	9	5
ISS5	5′-(GAA)5 -3′	12	5

cultivars grown in Iran are Ohady, Kaleh ghochi, Ahmad Aghai, Badami Zarand, Rezaii and Pust piazi[7]. Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. Among them, RAPD[29] has been the most commonly used method in pistachio cultivars characterization[9-11,13,15-17,19]. AFLP technique was used previously in pistachio to study genetic relationship among *Pistacia* species and cultivars[9,12,17]. Recently, SSR technique has been used to identify 17 pistachio cultivars using their nuts collected from the markets in the U.S. and in Europe[1,2], and in another study, SSR markers were used to analyze four commercially important pistachio rootstocks grown in California[1,2]. Since 1994, a new molecular marker technique called inter sequence repeat (ISSR) has been available[31]. Amplification in this technique does not require genome sequence information and leads to multilocus and highly polymorphous patterns[31,20,27]. 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[13,15,16]. The objectives of the study were 1) to assess genetic diversity and relationships among some of Iranian pistachio cultivars, 2) to compare the three molecular marker techniques in the discrimination of pistachio genotypes and 3) to set up and use first ISSR technique in pistachio cultivar identification in Iran.

MATERIALS AND METHODS

Plant materials and 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[6] with minor modifications. DNA quantity and quality were estimated both using an UV

TABLE 3 : Primer sequences, number of putative alleles and their size range revealed by 4 applied microsatellites in pistachio cultivars (Ahmad et al., 2003)

Primer	Primer sequence 5′-3′	Loci (no.)	Alleles (no.)	Allele size	Annealing temp(°C)
Ptms14	GGGAAACACAAACATGCAAA GGCCTCTGGAGAACATGGT	1	3	124-132	55
Ptms31	GGAAGCACACACATGCAAAC AGAAGAGGGGAACAGGGAGA	1	3	131-145	55
Ptms41	AGAAGAGGGGAACAGGGAGA CTGAGGACTGGGCAGAATGT	1	3	241-263	60
Ptms42	AAACAGGTGTTCCCGTTCAG ACCGACAGGATTGGATGATGG	1	2	152-164	55

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 10µg/µl for RAPD and ISSR and 20µg/µl for SSR reactions.

RAPD and ISSR reactions

RAPD reactions were performed according to Williams et al.[29] and ISSR reactions were done according to Zietkiewicz et al.[31] with minor modifications (TABLE 2). Amplification reactions in both techniques were done in a 25µL volume containing 10mM Tris-HCl, pH 8.0, 50mM KCl, 1.5mM MgCl2, 200µM each of dATP, dGTP, dCTP and dTTP, 10 pmol of a given primer, 1 unit of Taq DNA polymerase (Fermentas, Lithuania) and 10µg of genomic DNA. PCR amplification were performed in a gradient thermal cycler (Eppendorf, Hamburg, Germany). The RAPD program included 1 cycle of 4 min at 94°C, followed by 40 cycles of 45 s at 94°C, 1 min at 35°C, and 2 min at 72°C, followed by a final extension for 6 min at 72°C.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 extension for 6 min at 72°C. RAPD and 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.

SSR analysis

SSR analyses were performed in 25µl reaction mixtures containing 20µg/µl genomic DNA, 1x Fermentas PCR buffer (10mM Tris-HCl, pH 8.0; 50mM KCl; 0.1% (v/v) Triton X-100), 1.5mM MgCl2, 200µM of each dNTP, 10 pmol of each primer and 1 U *Taq* DNA polymerase (Fermentas, Lithuania). A total of four previously developed primers[1,2] were tested (TABLE 3). Reactions were performed using a Touchdown PCR program of 5 minutes denaturation at 94°C, followed by 10 cycles of 45 seconds at 94°C, 45 seconds at 63°C, decreasing with 0.8°C every cycle and 1 minute at 72°C. This was followed by 25 cycles of 45 seconds at 94°C, 45 seconds at primer set annealing temperature, 1 minute at 72°C and a final extension time of 7 minutes at 72°C. PCR amplification was confirmed by running 10µl of PCR product on 2% agarose gels. Then, the amplification products were detected on 6% non denaturing polyacrylamide gels followed by ethidium bromide staining and digitally photographed under ultraviolet light.

TABLE 4 : Comparison of RAPD, ISSR and SSR marker systems in fingerprinting of 31 pistachio genotypes

	Acronym	SSR	ISSR	RAPD
Primer or primer pairs(no)	NP	4	3	10
Total bands (no.)	NB	11	28	84
Bands per assay(no.)	NBA	2.75	9.3	8.4
Polymorphic bands (no.)	NPB	11	13	49
AEA (Assay efficiency index)		2.75	4.3	5
Monomorphic bands (no)	NMB	0	15	35
Polymorphism(%)	PP	100	46.42	58.33
Effective multiplex ratio	EMR	2.75	4.32	4.9

Data analysis

The amplified bands in the three marker systems 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) algorithms, described by Sneath & Sokal[26]. 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[24] was used to perform all the analyses. To determine the efficiency of each marker type in detecting genetic variation, the assay efficiency index, AEI[22] (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[23]. EMR is defined as the number of bands (n) analyzed per primer (in RAPD and ISSR) or primer pairs (in SSR analysis) multiplied by the

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percentage of polymorphic loci.

For population genetic analysis by SSR marker, POPGENE program[30] was used to calculate observed (Ho), expected (He) heterozygosity, and Hardy-Weinberg equilibrium. The percentage of observed of heterozygosity was calculated. Average expected theoretical heterozygosity from Hardy Weinberg assumptions was calculated using the formula[14]:

$$h_i = 1 - H_i = 1 - \sum_{i=1}^{n} P_i^2$$

where P_i as the its allele frequency. HET software package[21] was used to estimate Polymorphic information content (PIC) using the formula[4].

$$PIC = 1 - \left(\sum_{i=1}^{k} P_i^2\right) - \sum_{i=1}^{k-1}\sum_{j=i=1}^{k} 2P_i^2 P_j^2$$

where: P_i and P_j are frequencies of corresponding alleles. Effective number of alleles (n_e) was calculated using the formula[14]:

$$n_e = \frac{1}{\sum_{i=1}^{n} P_i^2}$$

this parameter gives an indication of the relative influence of the alleles. The Shannon information (I) index was calculated using the formula:

$$H' = -\sum_{i=1}^{n} P_i \ln P_i$$

where $P_i = \frac{n_i}{N}$

RESULTS AND DISCUSSION

The results of three molecular assays in fingerprinting the 31 pistachio genotypes are presented in TABLE 4. In RAPD analysis, the ten selected RAPD primers amplified a total of 84 scorable bands, an average of 8.4 bands per primer, of which 50 (59.52%) were polymorphic. The number of bands ranged from 3 to 14 and the number of polymorphic varied between one and 9 (average 5). Mirzaei et al.[19] reported 80% polymorphism among 22 Iranian cultivars and wild pistachio species. The difference in polymorphism reported in the current study and that of Mirzaei et al.[19] could be attributed to differences in the tested genotypes and the selected primers. Katsiotis et al.[17] obtained 82.41% polymorphism and 22.11 total and 18.2 polymorphic bands. In a study reported by Golan-Goldhirsh et al.[9] in assessing polymorphisms among 28 Mediterranean *Pistacia* accessions, twenty seven selected primers produced 259 total bands (average 9.59) and 86.1 of them were polymorphic.

In ISSR, according to the reported results of Kafkas et al.[13,15,16], first six primers were used and after initial screening three out of them primers eventually selected for the final analysis. a total of 28 were amplified by 3 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. Our results are similar to those of Kafkas et al.[13,15,16]. This study reports the first application of the ISSR technique in pistachio characterization of Iranian cultivars. The ISSR technique produced more reproducible bands than RAPD which is in accordance with Kafkas et al.[13,15,16]. During the ISSR screening in this study, good amplification products were obtained from primers based on GA and GAA repeats. But Primers based on CT and CAA repeats produced few large separate bands, so these primers were not selected for the final analysis. The present study showed that ISSR-PCR analysis is quick, reliable and produces sufficient polymorphisms for large-scale DNA fingerprinting purposes. The highest EMR and AEA values belonged to RAPD marker followed by ISSR and SSR marker, respectively. In this study SSR markers had the lowest number of bands per assay and RAPD markers had the highest value. The high value of the EMR and AEA for the two dominant markers (RAPD and ISSR) is a very high multiplex ratio, and highlights the distinctive nature of these markers. Although the SSR markers had the lowest values of the EMR and AEA, they had the highest level of polymorphism detected in pistachio cultivars. This state could be attributed to the lowered number of bands per assay detected by SSR markers. There were slight differences between ISSR and RAPD in the comparison of EMR and AEA.

In SSR, four specific SSR primers originally developed by Ahmad et al.[1,2] were used for assessing level of genetic diversity and relatedness of tested genotypes. Totally, the four primers produced 11 alleles among 31 pistachio genotypes (TABLE 3). The number of amplified alleles per primer varied from two for primer Ptms42 to 3 for Ptms 31, Ptms14 and Ptms41, with an average value of 2.75 alleles which compared to that of Ahmad et al.[1,2] is relatively lower. These differences could be attributed to differences in genotypes as well as the lowered number of SSR primers. However the reported average value in this study is similar to that of Ahmad et al.[1,2]. The size of the amplification bands using differ-

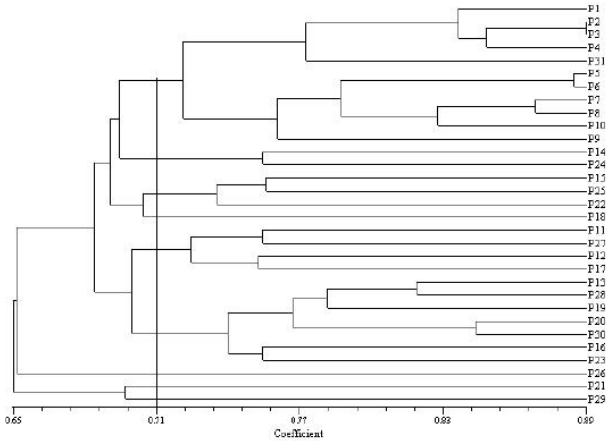

Figure 1 : Dendroram of 31 pistachio cultivars and genotypes using the unweighted pair group method with arithmetic averages (UPGMA). The database included 84 RAPD, 28 ISSR and SSR bands

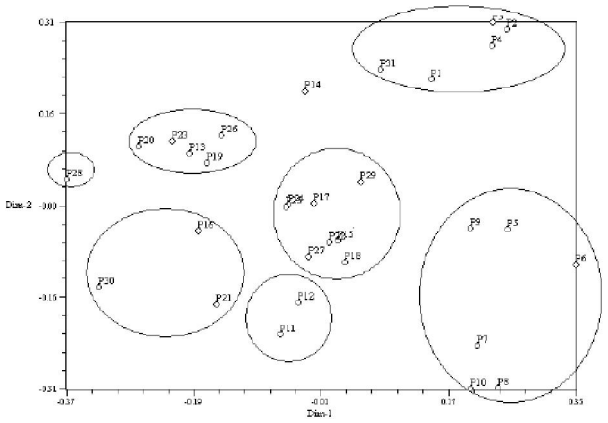

Figure 2 : Graph of the first and second principle components PC-1 and PC-2 of the variation in the pistachio population studied

ent microsatellites specific primers ranged between 124 bp (Ptms 14) to 263 bp (Ptms41). 100% Polymorphism was observed at all of these loci.

Hardy-Weinberg Equilibrium: Chi square (x^z) test was used to evaluate Hardy-Weinberg equilibrium in (HWE) 11 alleles at 4 loci. Results showed that Ptms14 and Ptms31loci in this population were found to be deviating from HWE equilibrium ($p<0.05$). The maximum number of alleles (3 alleles) were observed at Ptms 14, Ptms31 and Ptms41, and the minimum number of alleles (2 alleles) was observed at Ptms42 locus. However, these deviations for each locus indicate locus-specific effects that suggest selection affecting some of these loci. It is possible that such deviations from Hardy-Weinberg equilibrium may result from population substructure and the presence of null alleles[3].

Heterozygosity

TABLE 5 shows various Genetic parameters measured for four microsatellite markers in total sample that used in this study. The Maximum value of expected heterozygosity was 0.6192 at Ptms14 locus and the minimum expected heterozygosity, 0.3728 belonged to Ptms42 locus. The highest and the lowest expected heterozygosity belonged to Ptms14 and Ptms42, respectively. These two loci had the most (3) and the least (2) observed number of alleles in this population. In others words, the loci with more alleles contain higher rate of heterozygosity In this study, The discriminative power of each SSR primer was assessed by calculating polymorphic information contents (PIC) using allele frequencies in each polymorphic microsatellite locus. The result showed that the average PIC values were 0.4374. The highest and the lowest PIC values belonged to Ptms14.locus and Ptms42 Respectively. PIC values were positively correlated with the number of amplified alleles per primer. It was found that a comparing heterozygosity with PIC, all PIC values were less than related heterozygosity. Therefore; it seems that these two parameters are closely related. The low average PIC value 0.4374 indicated the presence of high genetic similarity among genotypes and entails development of additional polymorphic SSR primers for effective characterization of Iranian pistachio *cultivars*/genotypes. This illustrated the inadequacy of the available SSR primers to scan different parts of the genome and to pinpoint genetic differences between pistachio genotypes. Hence, a wider range of informative SSR primers need to be developed for successful fingerprinting. The study of Shannon information index (I) and PIC also indicated that the least and the most diverse loci are Ptms14 and Ptms42, respectively. The effective number of alleles varied from 1.5793 for PTMS42 to 2.5593 for PTMS14. These differences between the number of effective and of observed alleles indicated the presence of rare alleles that exist in one or a few genotypes and could be used for their identification.

Clustering of pistachio genotypes

In this study, the pattern of cluster analysis of based on DICE's similarity coefficient and UPGMA algorithm in all maker systems were to some extent different and thereby genotypes placed in different cluster with respect to the maker used. In SSR assay the number of genotypes with identical genetic similarity which could not be separated was much more than the other two

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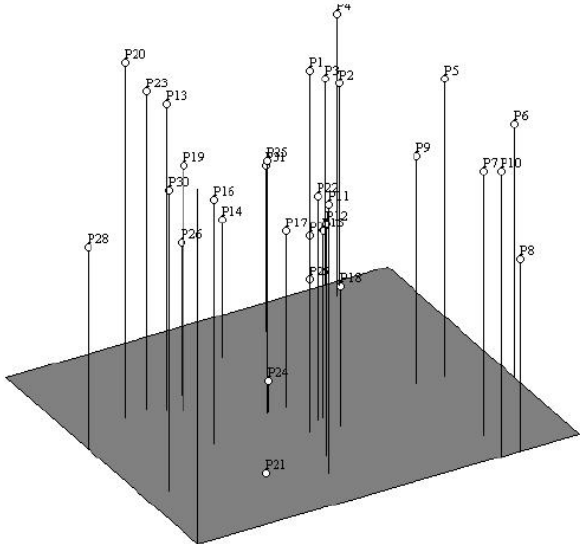

Figure 3 : Graph of the first and second and third principle components PC-1 and PC-2 and PC-3 of the variation in the pistachio population studied

marker systems. The main reason for this state is the lowered number of primers used in this study compared to Ahmad et al.[1,2]. But the ISSR and RAPD markers could separate the tested genotypes more efficiently. The general UPGMA dendrogram constructed using the combined data of the three sets of molecular markers was to some extent similar to those obtained separately with each marker (data not shown). The range of genetic similarity was from 0.65 to 0.89. The tested genotypes were classified into 9 main clusters. Genetic relationships among pistachio cultivars. The first cluster divided into two sub clusters. In the first sub-cluster contained 5 genotypes namely Sirizi (P1), Badami Ravar (P2), Ghafori Rafsanjan (P3), Hasan Zadeh (P4) and Ravar 3 (P31) and the second sub cluster contained 6 genotypes namely Ravar 2 (P5), Gholamrezaei (P6), Badami Zarand (P7), Harati (P8), Khanjari Ravar (P10) and Behesht Abadi (P9). The second cluster consisted of 2 genotypes namely Mohseni (P14) and Shasti (P24). The third cluster contained 3 genotypes namely Lahijani (P15), Khanjari Damghan (P25) and Vahedi (P22). The fourth cluster consisted of one genotype namely Fandoghi 48 (P18). The fifth cluster divided into two sub-clusters, the first sub-cluster consisted of 2 genotypes namely Ravar 3 (P11) and Saiffodini (P27) and the second sub-cluster consisted of 2 genotypes namely Post Piazi (P12) and Ghazvini (P17). The sixth cluster divided into two sub-clusters. The first sub-cluster consisted of 5 genotypes namely Shahpasand (P13), Kale Ghochi (P28), Javad Aghaei (P19), Badami Nishkalaghi (P20) and Ahmad Aghaei (P30) and the second sub-cluster contained 2 genotypes of Post Khormaei (P16) and Ohadi9 (P23). The seventh cluster consisted of one genotype namely Ebrahimi (P26), and in the eighth cluster contained one genotype namely Paye Nar (P21) and Italiaei (P29) placed in the ninth cluster alone (Figure 1).

TABLE 5 : Genetic parameters for four microsatellite markers in total sample that used in this study

Locus	Sample size	na	ne	I	He	Ho	PIC
PTMS42	62	2	1.5793	0.5533	0.3728	0.3548	0.2995
PTMS14	62	3	2.5593	1.0075	0.6192	1	0.5329
PTMS31	62	3	2.4237	0.9602	0.5970	1	0.5021
PTMS41	62	3	1.9086	0.8102	0.4839	0.6452	0.4151
Mean	62	2.7500	2.1177	0.2045	0.75	0.5182	0.4374

The first column shows locus name, number of alleles per locus (Na), effective number of alleles (Ne), Polymorphic information content (PIC), Shannon information(I), Expected heterozygosity (He) and observed heterozygosity (Ho)

The overall Principle coordinate analysis (PCA) based on genetic similarity matrices were used to visualize the genetic relationships among genotypes (Figure 2 and 3). The first three eigenvectors accounted for 28.46% of the total molecular variation. Therefore, PCA results confirmed the results of cluster analysis. The results of this study showed that there is a high level of genetic diversity in the studied samples which are expected in view of the dioeciously and out breeding 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 The results. There are several molecular techniques to assess genetic variability of plant cultivars and individuals. Reproducibility, cost, speed, and the ability to detect genetic variation between genotypes mainly determine their utility in germplasm characterization. The results presented here showed that RAPD, ISSR and SSR markers are able to reveal variability between pistachio genotypes. SSR and ISSR assays are more reliable than RAPD because of their reproducible bands, and ISSR is preferred over RAPD.

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