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Full Length Research Paper

Using ISSR markers to study genetic polymorphism of pistachio (*Pistacia vera* L.) in Algeria

KEBOUR Djamila[1]*, BOUTEKRABT Ammar[2] and MEFTI Med[3]

Laboratory of Biotechnology of University Saad Dahlab BLIDA, Algeria.

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***Pistacia vera* L. is a widely represented tree in Algerian semi-arid regions. It is potentially usable to restore degraded ecosystems. Genetic relationship among the 10 variety was assessed by using six inter simple sequence repeat (ISSR) primers. During the ISSR screening, good amplification products were obtained from primers based on guanine-adenine (GA), cytosine-adenine (CA) and GAA repeats. But primers based on cytosine-thyrosine (CT), and CAA repeats produced few large separate bands, so these primers were not selected for the final analysis. The total of 111 bands of which 60 (54.04 %) were polymorphic was amplified by the six primers, with an average of seven bands per primer. The total number of amplified fragments was between five to ten, and the number of polymorphic fragments ranged from four to seven. 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 2 main clusters. The present study showed that there is low genetic diversity among tested cultivars. The present study also showed that ISSR-PCR analysis is quick, reliable and produces sufficient polymorphisms for large-scale DNA fingerprinting purposes. This study reports the first application of the ISSR technique in characterization of Algerian pistachio cultivars original from Syria.**

Key words: Pistachio; *Pistacia vera* L.; Genetic relationships; DNA extraction; ISSR; Clustering.

INTRODUCTION

The pistachio tree, although grown for centuries in the Mediterranean area, has been introduced in Algeria in the mid-twentieth century by ACSAD (Syria).

The genus *Pistacia* is a diploid (2n=30) (Zohary 1952; Ila et al., 2003) member of the Anacardiaceae family, contains 13 or more species, among which *Pistacia vera* L. (Whitehouse, 1957). It produces commercially valuable edible nuts. The pistachio (*Pistacia vera*. Linnaeus, 1753), is a shrub native to the Middle East, it is a dioecious tree (Kafkas and Perl-Treves, 2006), measuring 3-8 meters. It is a deciduous tree and up to 12 cm leaves, with 3-7 leaflets. Its fruit has a dimension of 1 to 3.5 cm (Zohary 1952). Pistachio flowers have no petal and show perfect dioecy and maturity of pistachio seedlings takes between 5 and 8 years. Female flowers have no trace of stamens and mature male flowers lack any evidence of female structures (Zohary 1952). Therefore, there is no honey bee-attraction to facilitate indirect pollination. Thus, pollination usually performs by wind. Among the nut tree crops, pistachio tree ranks sixth in world production behind almond, walnut, cashew, hazelnut and chestnut (Mehlenbacher, 2003). Its use is recommended for the safe guarding of the pastoral potential and restoration of degraded ecosystems.

Although the number of varieties constituting the species *Pistacia vera* L. is considerable, inventory and identification are facing problems of taxonomic confusion. Early work on classification and identification of varieties of pistachio back to the nineteenth century. Zohary (1952).

The necessity to overcome the difficulties encountered in the morphological characterization, our study is

Corresponding author. Email: djakeb@hotmail.com.

Table 1. Algerian *Pistacia vera* L. varieties used in this study

Variety name	Label	Origin
Adjmi	1	Syria (A.C.S.A.D)
Ashouri	2	Syria (A.C.S.A.D)
Batouri	3	Syria(A.C.S.A.D)
Bayadhi	4	Syria(A.C.S.A.D)
Jalab ahmer	5	Syria(A.C.S.A.D)
Lazwardi	6	Syria(A.C.S.A.D)
Nab djamel	7	Syria(A.C.S.A.D)
Marawhi	8	Syria(A.C.S.A.D)
Oleimi	9	Syria(A.C.S.A.D)
Boundouki	10	Syria(A.C.S.A.D)

conducted in the laboratory of biotechnology (Algeria) in order to undertake further studies on varietal identification based on genetic markers (molecular markers) to determine the polymorphism breeders of this species in order to find the best ecotypes adapted to contrasting environmental conditions.

Since the mid 1980s, genome identification and selection have progressed rapidly with the help of PCR technology.

The first ISSR (inter simple sequence repeat) studies were published in 1994 and focused on cultivated species (Wolfe and Liston, 1998). These studies demonstrated the hyper variable nature of ISSR markers.

The genetic variability between and within the specific study mainly concerns non-coding regions of the genome that are characterized by the abundance of highly repetitive sequences within which the mutations are quite frequent. This variability has been studied by the technique of ISSR.

ISSR scored using presence or absence of a band at 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 *et al*., 1997; Nagaoka and Ogihara, 1997). Recently, this marker technique has been used to detect DNA polymorphism and genetic diversity of pistachio germplasm (Kafkas *et al*., 2006).

One aspect of this study concerns the determination of genetic polymorphism of a collection of *Pistacia vera* L. based on genetic markers.

The objectives of the study are:

1) To assess genetic diversity and relationships among some Algerian pistachio cultivars,
2) To set up and use first ISSR technique in pistachio cultivar identification in Algeria.

MATERIALS AND METHODS

Plant material and DNA extraction

A set of 10 *Pistacia vera* L. varieties that are listed in Table 1 was investigated. These were chosen for their good fruit quality and are the most common genotypes in the main Algerian plantation.

All these varieties, are recently introduced (1998) from Syria A.C.S.A.D (The Arab Center for the Studies of Arid Zones and Dry lands)) were included in the study. The plant material consists of young leaves provided from 400 trees (one for each genotype) were randomly chosen and sampled directly from a collection maintained in culture of pastoretum I.T.A.F.V (State institution) and other different regions of Algeria.

DNA preparation

Total genomic DNA was extracted from frozen young leaves of adult trees were kept in liquid nitrogen tanks for the purpose of DNA extraction and ISSR analyses according to the protocol Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990) with minor modifications. After purification, DNA concentrations were determined using a Gene Quant spectrometer, and its integrity was checked with agarose minigel electrophoresis according to Sambrook et al. (1989).

DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with some modifications. Young leaves tissues (100 mg) were ground to make fine powder in liquid nitrogen in 1.5 ml centrifuge tubes and mixed with 0.5 ml of CTAB extraction buffer (100 mM TRIS-HC1, 1.4 M NaC1, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% mercaptoethanol, 0.1% NaHSO3). The sample was incubated at 65 °C for 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 5 min in a desktop centrifuge. The aqueous phase was recovered and mixed with equal volume of isopropanol to precipitate the DNA.

The nucleic acid pellet was washed with 1 ml of 10 mM ammonium acetate in 76% ethanol, dried overnight and resuspended in 100 µl modified TE buffer (10 mM TRIS-HCl, 0. l mM EDTA).

DNA was extracted separately from each individual plant. In all cases, extracted DNA (25 ng per 20 µl reaction mix) was subjected to polymerase chain reaction (PCR) amplification. 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 2. ISSR primer sequences used for analysis of *Pistacia vera* L. with primer annealling temperatures, number of bands amplified, and number of polymorphic bands amplified

Primer	5'- 3' sequence	Annealing températures	Total Bands amplified	Number of polymorphic bands	Polymorphism (%)
1*	5`- $(AG)_8C$ -3`	52°C	6	6	100
2*	5`- $(GA)_8T$ -3`	54°C	7	5	71.42
3*	5`- $(TGGA)_4$ -3`	45°C	10	0	0
4*	5`- $(CA)_7AG$ -3`	56°C	10	9	90
5*	5`- $(GA)_8CG$-3`	56°C	18	16	89
6*	5`- $(ACTG)_4$ -3`	48 °C	10	2	20
7*	5`- $CCAG(GT)_7$-3`	56°C	10	9	90
8*	5`- $(GACAC)_4$-3`	45°C	11	2	18
9*	5`- $(AC)_8T$-3`	55°C	12	10	83.33
10*	5`- $(TG)_8TT$-3	45°C	7	1	14.26
TOTAL			111	60	

60*100/111= 54.04% (R = purines: **G** or A ; Y = pyrimidines: C or T)

PCR Amplification:

A total of 10 primers were tested to amplify the isolated DNA. These primers listed in Table 2, and their composition has been arbitrarily established.

PCR was performed using ISSR primers and amplification reactions were carried out in an Eppendorf Master cycler gradient (Eppendorf Netheler-Hinz, Hamburg, Germany), The apparatus was programmed to execute the following conditions, 1 cycle: a denaturation step of 5 min at 94°C, followed by 35 cycles composed of 30 s at 94°C, 90 s at the annealing temperature (Table 2), and 90 s at 72°C. A final extension of 72°C for 5 min was included.

PCR reactions were carried out in a volume of 25 µl with 30 ng of genomic DNA, 2 µM primer ISSR (Table 2), 1 U of Taq polymerase, 0.2 mM dNTPs, 10 mM Tris–HCl (pH 8.3), and 2.5 Mm $MgCl_2$. After amplification, the gels were stained with 0.5 µg/ ml ethidium Bromide solution and visualized by illumination under UV light. Reproducibility of the patterns was checked by running the reactions in duplicates.

Data analysis

For each DNA sample, ISSR bands were transformed into a binary matrix where the presence of reproducible polymorphic DNA band at particularly position on gels is scored manually as 1 (present), while a 0 (absent) denotes its absence of co-migrating fragments for all accessions. Only the clearest and strongest reproducible bands across two PCR amplification replicates were used for cluster analysis. Clearly detectable amplified ISSR ranged from 200 to 2400 bp in size.

The genetic similarity matrix was constructed using Jaccard's coefficient (Jaccard, 1908).

Dendrogram was constructed by the unweighted pair-group method using arithmetic average (UPGMA) and complete linkage algorithms. In addition to cluster analysis, principal component analysis (for precise relationships between the *Pistacia vera* L. varieties) 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 2.2 (Rohlf, 1998).

RESULTS AND DISCUSSION

This study reports the first application of the ISSR technique in pistachio characterization of Algerian varieties. 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.

The results of this molecular assay in fingerprinting of the 10 pistachio genotypes are presented in Table 2. In ISSR, according to the reported results of Kafkas et al. (2006), first ten primers were used and after initial screening six out of them primers eventually selected for the final analysis. A total of 111 bands were amplified by the six primers, an average of 8 bands per primer of which 60 (54.04%) were polymorphic. The total number of amplified fragments were between 6 to 18, and the number of polymorphic fragments ranged from 0 to 16. The Figure 1 shows the results of amplification with primer ISSR ($(GA)_8$ CG**)** on aga, rose to 1.8% with 16 lanes gel tray.

From the results of Ehsanpour et al. (2008) good amplification products were obtained from primers based on AC, repeats ($(AC)_8$ CG and $(AC)_8$ TA). Since, primers based on CT, GT, CAG and CAA repeats produced few large separate bands which 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.

A total of 10 primers were screened for their ability to generate consistently amplified band patterns and to access polymorphism in the tested varieties. Among

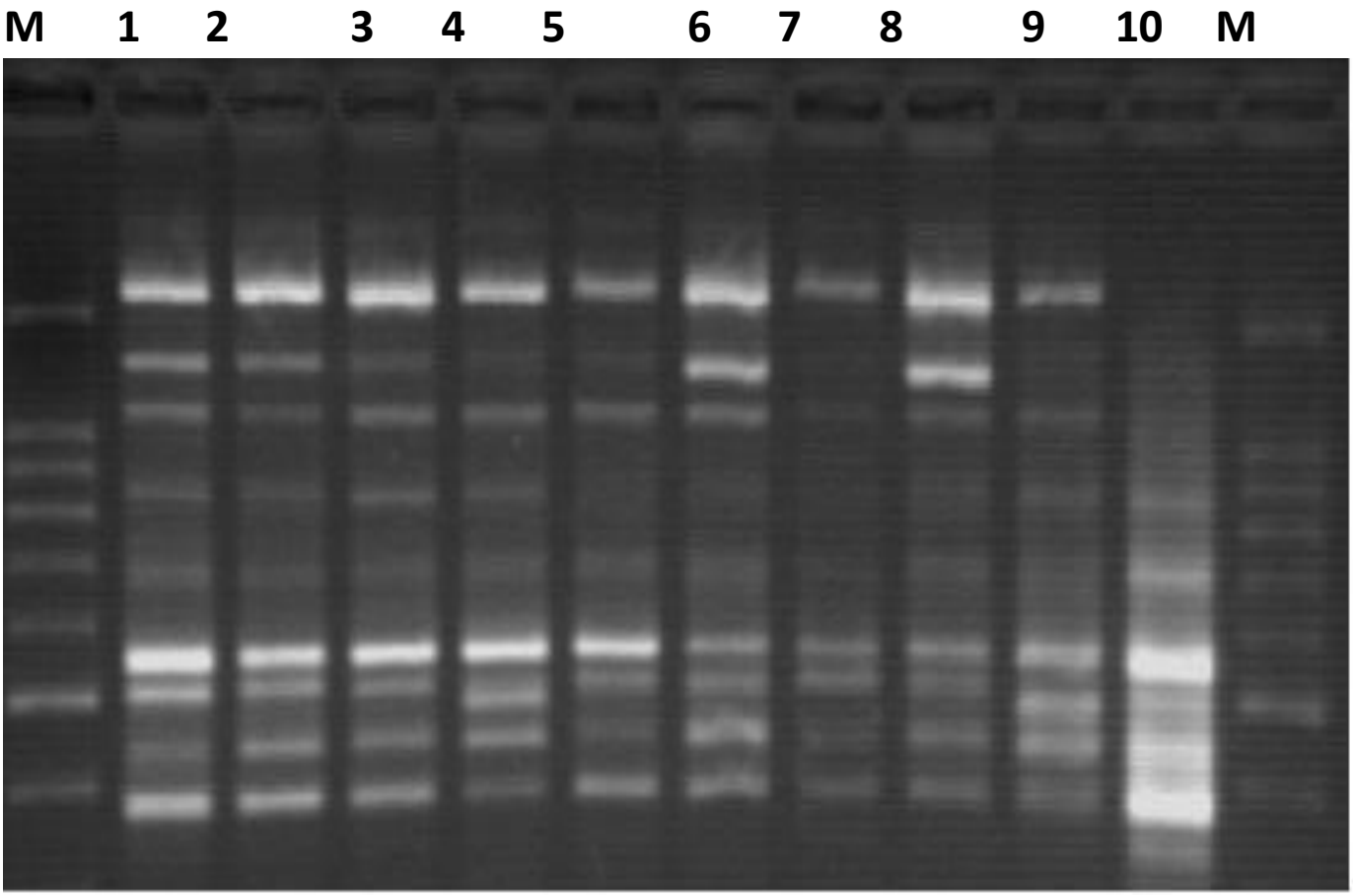

Figure 1**.** Typical Example of ISSR polymorphism banding patterns in a subset of Algerian *Pistacia vera* L. varieties using $(GA)_8$ CG primer.
M: Standard molecular size marker, sizes of molecular weight markers are indicated in kb. Lane labeled.

these primers, only 6 revealed polymorphic and unambiguously scorable bands. While smear or no amplified products were observed with the other primers.

These 6 primers generated 5 to 10 polymorphic DNA bands with a range of 200 to 2500 bp. Typical amplified products are reported in Figure 1. The polymorphic patterns obtained suggested that the ISSR procedure constitutes an alternative approach that is suitable to examine the *Pistacia vera's* genetic diversity at the DNA level. A total of 60 polymorphic ISSR products were obtained (Table 2).

The matrix has a genetic distance of 0.61 to 0.82 with a mean of 0.56. Thus, it may be assumed that the varieties are characterized by a high degree of genetic diversity at the DNA level. The smallest distance value of 0.61 was observed between (Adjmi - Lazwouardi) and (Ashouri - Nabdjamel) and varieties indicating that these ecotypes are the most similarity to each other. The maximum distance value (0.82) suggesting high divergence was detected between Marawhi and JalabAhmer varieties (Table 3).

The phenogram obtained (Figure 2) informs supports the varietal clustering. The cluster analysis generated a dendrogram with two main branches that clustered individuals that share the same gene pool of origin. Branch 'A' included all those cultivars whose genomic background is mainly from parents collected from Syria, and cluster 'B' included cultivars with parents from Syria too.

The cophenetic correlation (0.66), 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= 0.82) was observed for UPGMA based on Jaccard's coefficient (Table 3).

The Principle coordinate analysis (PCA) based on genetic similarity matrix was used to visualize the genetic relationships among genotypes (Figure 3). It 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

Table 3. Genetic similarity among studied *Pistacia vera* L. based on Jaccard's coefficient.

	Bayadhi1	Boundouki	MaleJaber	NabDjamel2	Batoury	Oleimy	Nabdjamel	Batouri	Achouri	Bayadhi	Malekhalifa	Adjmi	Adjmi 1	Boundouki1	AchouriBl	OleimiBl	NabDjamel1
Bayadhi1	1																
Boundouki	0,68519	1															
MaleJaber	0,7037	0,76786	1														
NabDjamel2	0,66071	0,72414	0,80357	1													
Batoury	0,7037	0,83333	0,81818	0,74138	1												
Oleimy	0,69811	0,76364	0,81481	0,73684	0,81481	1											
Nabdjamel	0,51563	0,65079	0,66667	0,68254	0,64063	0,71667	1										
Batouri	0,68333	0,8	0,84746	0,83333	0,78689	0,81356	0,78125	1									
Achouri	0,57407	0,64286	0,66071	0,74074	0,69091	0,78431	0,66102	0,7	1								
Bayadhi	0,65385	0,69091	0,70909	0,79245	0,70909	0,76923	0,59677	0,7458	0,7755	1							
Malekhalifa	0,61404	0,73684	0,85185	0,77193	0,81818	0,75	0,66667	0,7581	0,7222	0,74074	1						
Adjmi	0,66667	0,72881	0,83929	0,85714	0,74576	0,80357	0,74194	0,8361	0,7143	0,76364	0,80702	1					
Adjmi 1	0,55738	0,7	0,71667	0,65079	0,68852	0,71186	0,8	0,8065	0,6	0,59016	0,66129	0,73777	1				
Boundouki1	0,61364	0,52941	0,51923	0,48148	0,51923	0,57143	0,44828	0,4915	0,5	0,55319	0,51923	0,54717	0,4386	1			
AchouriBl	0,60377	0,67273	0,63158	0,67857	0,63158	0,65455	0,58065	0,6721	0,6226	0,67308	0,66071	0,71429	0,5738	0,6	1		
OleimiBl	0,67925	0,68421	0,7963	0,81481	0,76364	0,82692	0,61905	0,7966	0,7647	0,78431	0,73214	0,78571	0,6667	0,55102	0,63636	1	
NabDjamel1	0,68421	0,77586	0,82456	0,81034	0,85714	0,85455	0,73016	0,8833	0,7636	0,78182	0,7931	0,84483	0,7833	0,53704	0,67241	0,83636	1

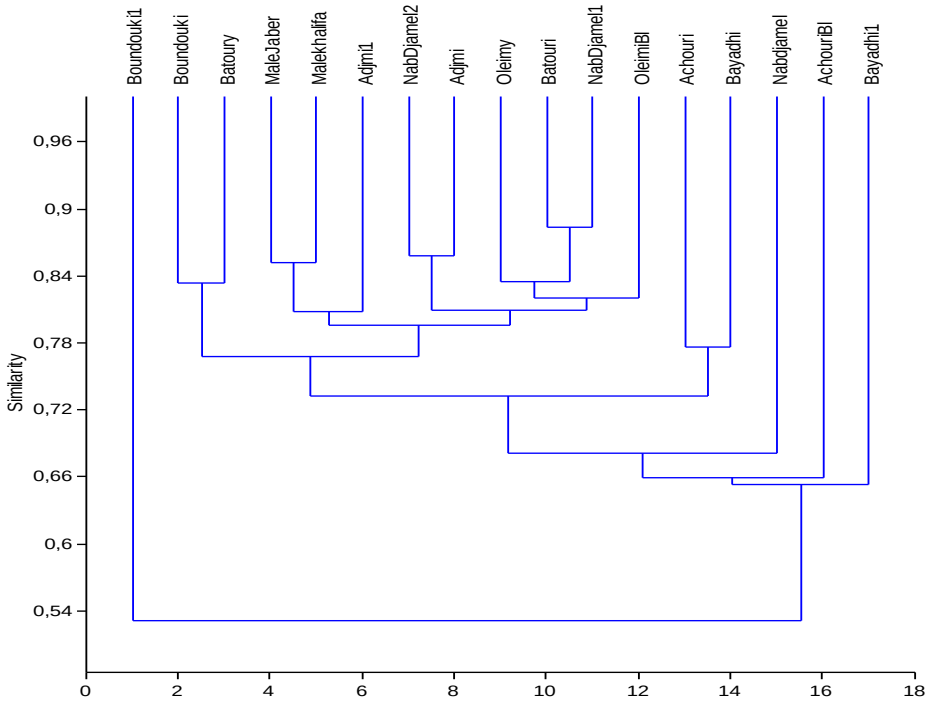

Figure 2. UPGMA denderogram of 17 varieties of *Pistacia vera* by ISSR analysis using Jaccard's similarity matrix.

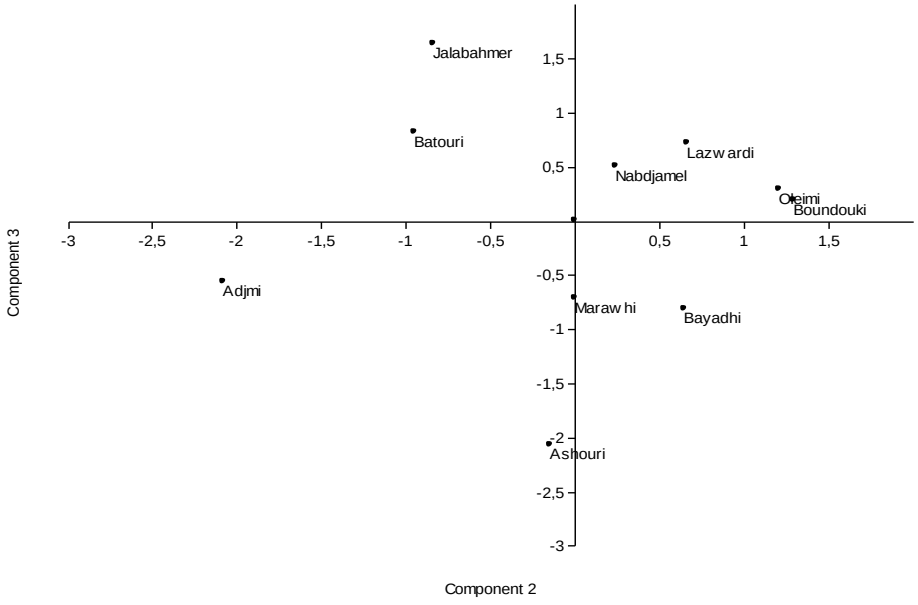

Figure 3. Relationships among pistachio genotypes revealed by principal component analysis based on ISSR genetic similarity.

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.

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