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Full Length Research Paper

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

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***Pistacia vera* L. is a widely represented plant in Algerian semi-arid regions. It is potentially used to restore degraded ecosystems. Genetic relationships among the 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 guanine-adenine (GA), cytosine-adenine (CA) and guanine-adenine-adenine (GAA) repeats. Primers based on cytosine-tyrosine (CT) and CAA repeats produced few large separate bands, so these primers were not selected for the final analysis (eliminated for the final analysis). This study shows that ISSR-PCR analysis is quick, reliable and produces sufficient polymorphisms for large-scale DNA fingerprinting purposes. The total of 111 bands of which 60 were polymorphic, (with 54.04%) was amplified by the six primers, 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 two main clusters. This study shows that there was low genetic diversity among the tested cultivars and the ISSR-PCR analysis produced sufficient polymorphisms for large-scale DNA fingerprinting. This study reports the first application of the ISSR technique in characterization of Algerian pistachio cultivars original from Syria.**

Key words: *Pistacia vera* L., genetic relationships, DNA extraction, ISSR, clustering.

INTRODUCTION

The pistachio fruit, although grown for centuries in the Mediterranean area, was introduced in Algeria in the mid-twentieth century by ACSAD (Syria). Demonstration orchards have been established in different regions to develop its culture. Its current area covers 400 ha, and spread over different climatic stages. Recognizing the potential interest for the development of many regions, the Ministry of Agriculture and relevant department have planned during the late eighties, the extension of this culture to approximately 2000 ha. This objective has been achieved due to several constraints including those linked to the nature of the case, ignorance of technology and especially his leadership in safe condition experienced by our country in this period. In recent years there has been an expansion of the cultivation of pistachio through Algeria, thus meeting the development objectives of the arid and semi-arid and the conservation of soil against erosion.

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), produces commercially valuable edible nuts. The pistachio (*P. vera*. Linnaeus, 1753), is a shrub native to the Middle East. It is a dioecious tree (Kafkas et al., 2006), measuring 3 to 8 m. It is also a deciduous tree and up to 12 cm, with 3 to 7 leaflets. Its fruits have a dimension of 1 to 3.5 cm (Zohary 1952). Pistachio flowers has no petal and shows perfect dioecy

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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; instead pollination is usually performed by wind. Among the nut tree crops, pistachio tree ranks sixth in the 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 *P. vera* L. is considerable, inventory and identification are facing problems of taxonomic confusion.

Earlier work on classification and identification of varieties of pistachio dates back to the nineteenth century. However, Zohary (1952) was the first to use the various organs of pistachio trees (leaf, fruit and endocarp) to characterize and classify the varieties of this species. Since then, various studies on varietal identifycation have been developed in Iran, Syria, Turkey and Italy from the combination of morphological, agronomic and phonological (Faostat, 2006). Due to the need to overcome the difficulties encountered in the morphological characterization, our study was 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 has progressed rapidly with the help of polymerase chain reaction (PCR) technology. Among them, random amplified polymorphic DNA (RAPD) (Williams et al., 1990) has been the most commonly used method in pistachio cultivars characterization (Hormaza et al., 1994, 1998; Kafkas and Perl-Treves, 2002; Katsiotis et al., 2003; Golan-Gpldhirsh et al., 2004; Mirzaei et al., 2005). Amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) techniques have also been used in pistachio to study genetic relationship among *Pistacia* species and cultivars (Ibrahim Basha et al., 2007; 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 (Virk et al., 2000). AFLP has medium reproducibility but is labour-intensive and has high operational and development costs (Hansen et al., 1998). On the other hand, microsatellites are specific and highly polymorphous (Jones et al., 1997), but they require knowledge of the genomic sequence to design specific primers and, thus, are limited primarily to economically important species. The first ISSR studies were published in 1994 and focused on cultivated species (Wolfe and Liston, 1998). These studies demonstrated the hyper variable nature of ISSR markers.

Microsatellites or simple sequence repeats (SSRs), are polymorphic loci present in nuclear DNA and organellar DNA that consist of repeating units of 1 to 4 base pairs in length (Zietkiewicz et al., 1994). Inter simple sequence repeat (ISSRs) are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Amplification in the presence of non-anchored primers also has been called microsatellite-primed PCR (Karp et al., 1997). Each band corresponds to a DNA sequence delimited by two inverted micro-satellites. 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., 1994), and involves longer (16-18 nucleotides) primers encoding microsatellite elements that amplify DNA segments Intramicrosatellite repeats (Zietkiewicz et al., 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. The genetic variability between and within 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 inter simple sequence repeat.

ISSR is a dominant marker like RAPD, however scoring using presence or absence of 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 and Roose, 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 is concerned with the determination of genetic polymorphism of a collection of *P. vera* L. based on genetic markers. The objectives of the study are: 1) to assess genetic diversity and relationships among some Algerian pistachio cultivars and 2) to set up and use first ISSR technique in pistachio cultivar identification in Algerian.

MATERIALS AND METHODS

Plant material and DNA extraction

A set of 10 *P. vera* L. varieties listed in Table 1 were investigated. These were chosen for their good fruit quality and are the most common genotypes in the main Algerian plantation. All the varieties recently introduced (1998) from Syria; the Arab Center for the Studies of Arid Zones and Dry lands (A.C.S.A.D) were included in the study. The plant material consisting 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 others at different

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)

region of Algeria.

DNA preparation

Extracts 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 of cetyltrimethylammonium 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). Frozen tissue (0.5 to 0.75 g) was ground in a mortar and pestle in liquid nitrogen and homogenized in 5 ml of preheated (60°C). DNA was extracted according to the CTAB (hexadecyltrimethylammonium bromide) method of Doyle and Doyle (1987) with some modifications. Young leaf tissue (100 mg) was ground to fine powder in liquid nitrogen in 1.5 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 and 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 then 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, the 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 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 samples were diluted to 10 ng / µL for ISSR reactions.

Primers and ISSR assay

A total of 10 primers were tested to amplify the isolated DNA. These primers are listed in Table 2, and their composition has been arbitrarily established. For PCR amplifications, a 25-µL reaction mixture was used and it contained between 20 and 30 ng of total genomic DNA (1 µL), 60 pg of primer ISSR primers (1 µL), 2.5 µl of 10X Taq DNA polymerase reaction buffer, 1.5 unit of Taq DNA polymerase (Fermentas, Lithuania) and 200 mM of each dNTPs (DNA polymerization mix, Amersham-Pharmacia, France). Amplification reactions were done in a 25 µL volume containing: 10 mM Tris-HCl, (pH 8.0), 50 mM KCl, 1.5 Mm $MgCl_2$, 200 mM each of d'NTPs, 10 pmol of a given primer, 1 unit of Taq DNA polymerase (Fermentas, Lithuania) and 10 ng of genomic DNA.

PCR Amplification

PCR was performed using ISSR and amplification reactions were carried out in an Eppendorf Master Cycler Gradient (Eppendorf Netheler-Hinz, Hamburg, Germany). The apparatus is programmed to execute the following conditions in 1 cycle: a denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 90 s at the annealing temperature, and 90 s at 72°C. A final extension of 72°C for 5 min was included. ISSR amplification products were analyzed by gel electrophoresis in 1.8% agarose in 1x TBE buffer, stained with ethidium bromide (0.5 µg ml^{-1}) according to Sambrook et al. (1989) and digitally photographed under ultraviolet light at 300 nm. 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 metrics were constructed using Jaccard's (Jaccard, 1908).

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 (for precise relationships between the *P. 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 version 2.2 (Rohlf, 1998).

RESULTS AND DISCUSSION

This study reports the first application of the ISSR technique in pistachio characterization of Algerian varieties. This 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), the first ten primers were used and after initial screening, six out of these primers were eventually selected for the final analysis. A total of 111 bands were amplified by the six primers, an average of eight bands per primer of which 60 were polymorphic (54.04%). The total number of amplified fragments was

Table 2. ISSR primer sequences used for analysis of *Pistacia vera* L. with primer annealing temperatures, number of bands amplified, and number of polymorphic bands amplified.

Primer	5'- 3' sequence	Annealing temperature (℃)	Total band amplified	Number of polymorphic band	Polymorphism (%)
1*	5`- $(AG)_8$C -3`	52	6	6	100
2*	5`- $(GA)_8$T -3`	54	7	5	71.42
3*	5`- $(TGGA)_4$ -3`	45	10	0	0
4*	5`- $(CA)_7$AG -3`	56	10	9	90
5*	5`- $(GA)_8$CG-3`	56	18	16	89
6*	5`- $(ACTG)_4$ -3`	48	10	2	20
7*	5`- CCAG$(GT)_7$-3`	56	10	9	90
8*	5`- $(GACAC)_4$-3`	45	11	2	18
9*	5`- $(AC)_8$T-3`	55	12	10	83.33
10*	5`- $(TG)_8$TT-3	45	7	1	14.26
TOTAL			111	60	

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

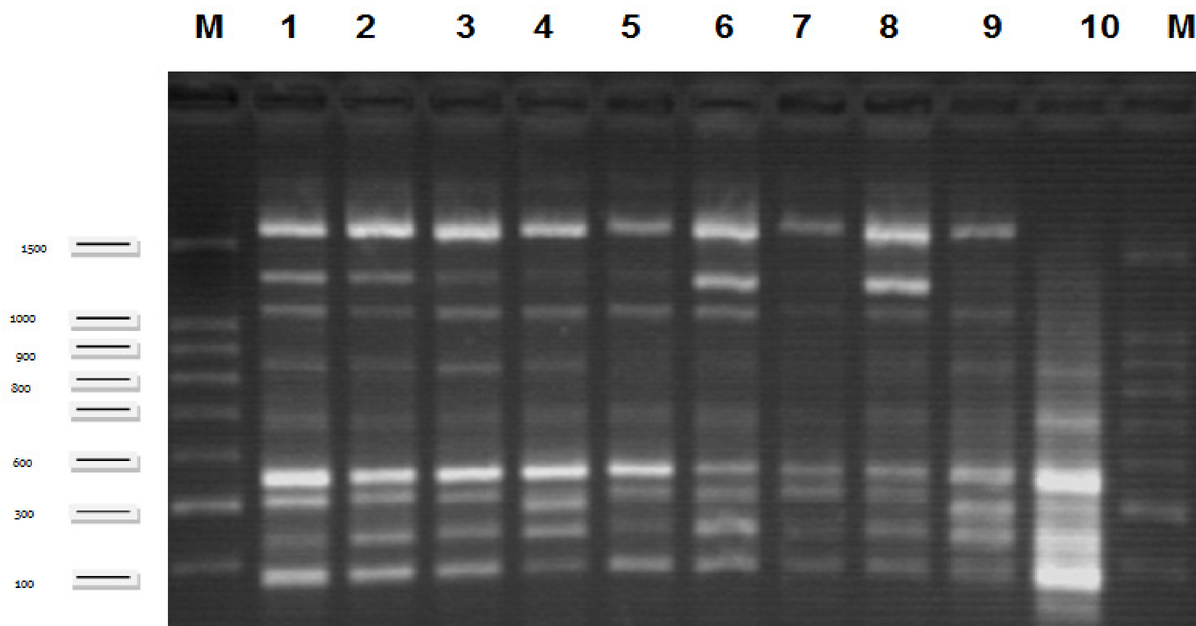

Figure 1. Typical example of ISSR polymorphism banding patterns in a subset of Algerian *Pistacia vera* L. varieties using $(GA)_8$CG primer. M, Standard molecular size marker: sizes of molecular weight markers are indicated in kb; 1, Adjmi; 2, Ashouri; 3, Batouri; 4, Bayadhi; 5, Jalab ahmer; 6, Lazwardi; 7, Nab djamel; 8, Marawhi; 9, Oleimi; 10, Boundouki.

between 6 to 18 and the number of polymorphic fragments ranged from 0 to 16. Figure 2 shows the results of amplification with primer ISSR $(GA)_8$CG) on agarose 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)8CG and (AC)8TA], 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 these primers, only six revealed polymorphic and unambiguously scorable bands while smear or no amplified products were observed with the other primers. These six primers generated five 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 suggest that the ISSR

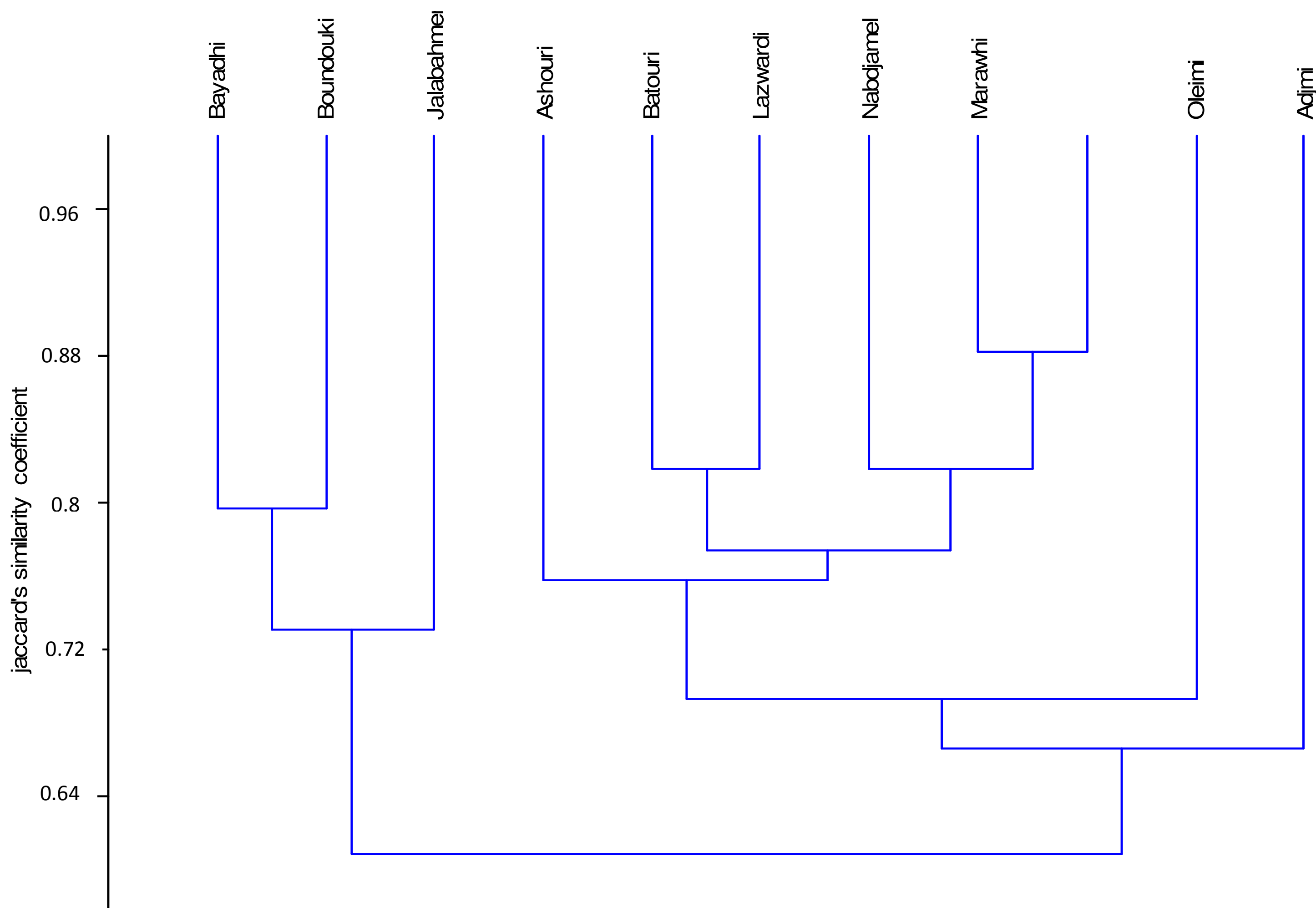

Figure 2. UPGMA dendrograms of 10 varieties of *Pistacia vera* by ISSR analysis using Jaccard's similarity matrices.

procedure constitutes an alternative approach that is suitable to examine the *P. 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 0.5688. 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 varieties indicating that these ecotypes are the most similar. The maximum distance value (0.82) suggesting high divergence was detected between marawhi and JalabAhmer varieties (Table 3).

Furthermore, the phenogram obtained (Figure 2) 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 parent plants collected from Syria (cultivars Boundouki , Bayadhi and Jalab ahmer) and cluster 'B' included cultivars with parents from Syria too (cultivars Adjmi, Ashouri, Batouri, Adjmi, Ashouri and Batouri). The cophenetic correlation (0.6627), 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 matrices were also used to visualize the genetic relationships among genotypes (Figure 3), and it confirmed the results of cluster analysis.

The results of this study show that there is a relatively low level of genetic diversity in the studied samples, which was expected in view of the dioecius and outbreeding nature of the cultivated pistachio cultivars, as well as the 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

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

	Adjmi	Ashouri	Batouri	Bayadhi	JalabAhmer	Lazwardi	NabDjamel	Marawhi	Oleimi	Boundouki
Adjmi	1									
Ashouri	**0.61194**	1								
Batouri	0.68657	0.61111	1							
Bayadhi	0.64179	0.52727	0.53448	1						
JalabAhmer	0.71642	0.48333	0.54098	0.71698	1					
Lazwardi	0.67164	0.53571	0.46774	0.76	0.72222	1				
NabDjamel	0.61194	0.49091	0.5	0.71429	0.64815	0.65385	1			
Marawhi	0.68657	0.47458	0.4375	0.61818	0.70909	0.71698	0.61111	1		
Oleimi	0.80597	0.58333	0.6129	0.76364	**0.82143**	0.73684	0.72727	0.72414	1	
Boundouki	0.62687	0,43103	0.51724	0.63462	0.8	0.67308	0.72917	0.66038	0.71429	1

1, Adjmi; 2, Ashouri; 3, Batouri; 4 , Bayadhi; 5, Jalab ahmer; 6, Lazwardi; 7, Nab djamel; 8, Marawhi; 9, Oleimi; 10, Boundouki.

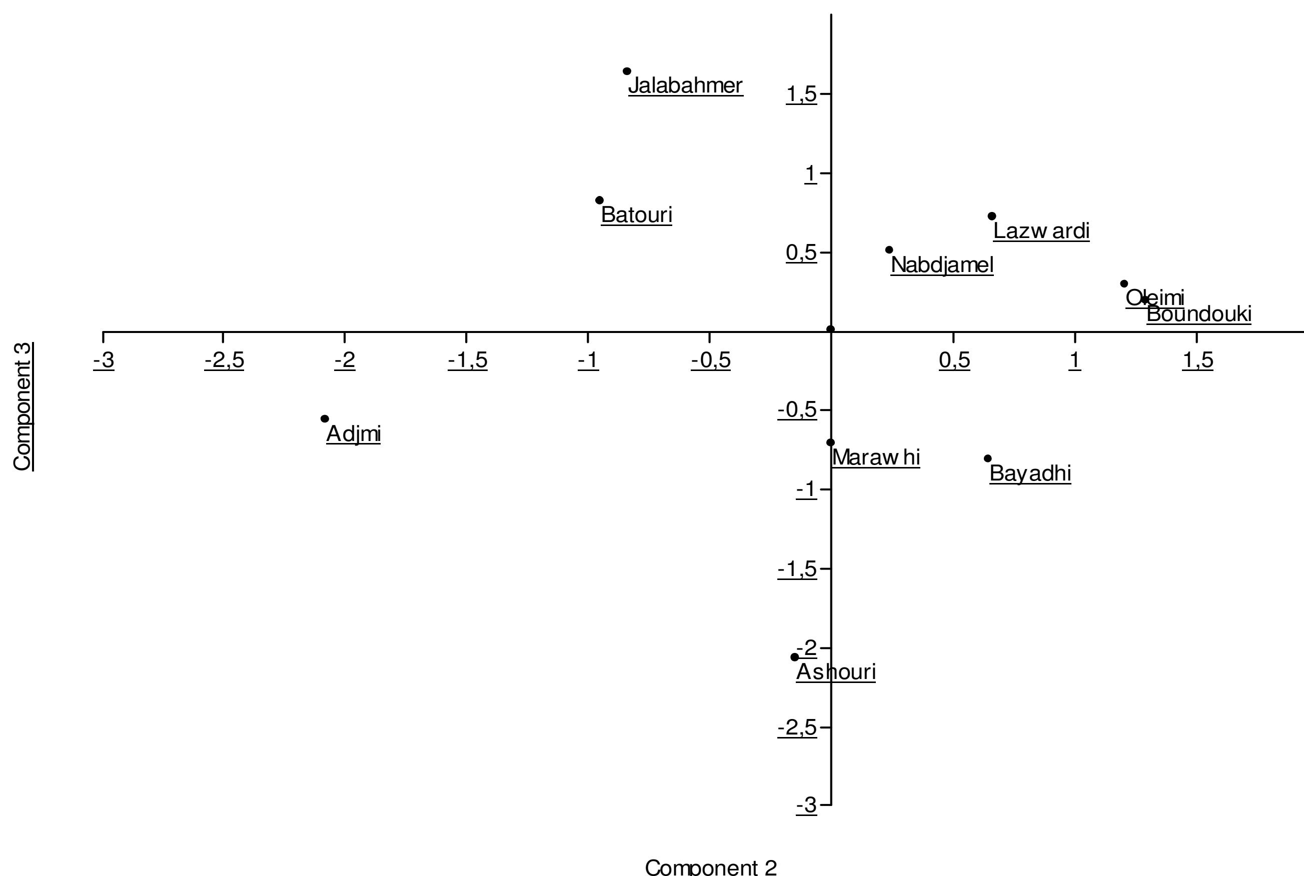

Figure 3. Relationships among the pistachio genotypes revealed by principal component analysis based on ISSR genetic similarity.

vegetative reproduction.

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