USE OF THE ISSR MARKERS FOR THE STUDY OF GENETIC POLYMORPHISM OF THE PISTACHIO FRUIT *PISTACIA VERA* L. IN ALGERIA

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Pistacia vera L. este o plantă larg răspândită în regiunile semi-aride algeriene. Este potenţial utilizabilă pentru a restabili ecosistemele degradate. Relaţiile genetice dintre soiuri au fost evaluate cu ajutorul a şase interpretări cu amorsuri simple (ISSR). În timpul de proiectărilor ISSR din acest studiu, au fost obţinute produse de bună amplificare de la amorsele bazate pe GA (guanina-adenina), CA cu repetare GAA. Dar amorsele bazate pe citozina-thyrosine (CT) şi cu repetare CAA au produs câteva benzi mari separate, astfel încât aceste amorse nu au fost selectate pentru analiza finală (eliminate pentru analiza finală). Studiul de faţă a arătat că analiza ISSR-PCR este rapidă, de încredere şi că produce suficiente polimorfisme în scopul de amprentare al ADN-ului pe scară largă. Totalul de 111 benzi, din care 60 au fost polimorfice, (cu 54.04%) a fost amplificat de către cele şase amorse, o medie de şapte benzi polimorfe. Numărul total de fragmente amplificate a fost între cinci şi zece, iar numărul de fragmente polimorfe a variat între patru si şapte. Gama de similaritate genetică a fost de 0 / 84 la 1. Studiul de faţă a arătat că există o diversitate genetică scăzută în rândul soiurilor testate şi analiza ISSR-PCR produce polimorfisme suficiente pentru amprentarea ADN-ul pe scară largă. Acest studiu arată prima aplicare a tehnicii de ISSR în caracterizarea de soiuri algeriene originare din Siria.

Pistacia vera L. is a widely presented plant in Algerian semi-arid regions. It is potentially usable to restore degraded ecosystems. Genetic relationships among the cultivars was assessed by using six inter simple repeat (ISSR) primers. During the ISSR screening in this study, good amplification products were obtained from primers based on GA (guanine-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 (eliminated 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 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

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to 1. 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 Algerian pistachio cultivars originary from Syria.

1. Introduction

The pistachio fruit, although grown for centuries in the Mediterranean area, has been introduced in Algeria in the mid-twentieth century by ACSAD (Syria). Demonstration orchards have been established in different regions to develop its culture. His current area covers 400 ha, 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) [8; 10] member of the Anacardiaceae family, contains 13 or more species, among which *Pistacia vera* L. Produces commercially valuable edible nuts. The pistachio [7], is a shrub native to the Middle East, it is a dioecious tree [13], measuring 3-8 meters It is a deciduous tree and up to 12cm, with 3-7 leaflets. Its fruits have a dimension of 1 to 3.5 cm. Pistachio flowers have no petal and shows 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 [8;10] 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.

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 varietal identification have been developed in Iran, Syria, Turkey and Italy from the combination of morphological, agronomic and phonological [6].

The need to overcome the difficulties encountered in the morphological characterization, our study is 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 PCR technology. Among them, RAPD has been the most commonly used method in pistachio cultivars characterization [6; 9; 10]. AFLP and SSR techniques have been also used in pistachio to study genetic relationship among *Pistacia* species and cultivars [1; 2; 3]

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 AFLP has medium reproducibility but is labour intensive and has high operational and development costs [4]. Microsatellites are specific and highly polymorphous [12], 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. These studies demonstrated the hypervariable 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 1-4 base pairs in length [5]. 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. 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 multi-locus and highly polymorphous patterns and involves longer (16-18 nucleotides) primers encoding microsatellite elements that amplify DNA segments of Intra-micro-satellites repeats. 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 [10; 13].

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 ISSR (Inter Simple Sequence Repeat).

ISSR is a dominant marker like RAPD (scored using presence or absence of band at a 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 [4; 8]. Recently this marker technique has been used to detect DNA polymorphism and genetic diversity of pistachio germplasm [13].

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 Algerian.

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 others different region of Algeria.

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)

2. DNA preparation

Total 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 the protocol Cetyl Trimethyl Ammonium Bromide (CTAB) method [11; 13] with minor modifications. After purification, DNA concentrations were determined using a Gene Quant spectrometer and its integrity was checked with agarose minigel electrophoresis.

Frozen tissue (0.5–0.75 g) was ground in a mortar and pestle in liquid nitrogen and homogenised 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 (100mg) 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, 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 waswashed 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. 1 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 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.

3. Primers and ISSR assay

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.

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 polymerisation mix, Amersham-Pharmacia, France).

Amplification reactions were done in a 25μl 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)
- 10 ng of genomic DNA.

4. PCR Amplification

PCR was performed using ISSR and amplification reactions were carried out in an Eppenorf Master Cycler Gradient (Eppendorf Netheler-Hinz, Hamburg, Germany).

The apparatus is programmed to execute the following conditions were 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, 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 [9] and digitally photographed under ultraviolet light at 300 nm. Reproducibility of the patterns was checked by running the reactions in duplicates.

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 temperatures	Total Bands amplified	Number of polymorphic bands	Polymorphism (%)
1*	5`- (AG)$_8$C -3`	52°C	6	6	100
2*	5`- (GA)$_8$T -3`	54°C	7	5	71.42
3*	5`- (TGGA)$_4$ -3`	45°C	10	0	0
4*	5`- (CA)$_7$AG -3`	56°C	10	9	90
5*	5`- (GA)$_8$CG-3`	C°56	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)$_8$T-3`	C°55	12	10	83.33
10*	5`- (TG)$_8$TT-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)

5. 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 metrices were constructed using Jaccard [11].

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 *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 [9; 11].

6. 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 [13] 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 were polymorphic (54.04%). The total number of amplified fragments was between 6 to 18 and the number of polymorphic fragments ranged from 0 to 16. The Fig. 2 shows the results of amplification with primer ISSR **$(GA)_8CG$** on agarose 1.8% with 16 lanes gel tray.

From the results of [3] 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.

[13], use 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 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 Fig. 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 0,5688. 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) and 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).

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).

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	**0,6119**	1								
Batouri	0,68657	0,6111	1							
Bayadhi	0,64179	0,5273	0,5345	1						
JalabAhmer	0,71642	0,4833	0,541	0,717	1					
Lazwardi	0,67164	0,5357	0,4677	0,76	0,72222	1				
NabDjamel	0,61194	0,4909	0,5	0,7143	0,64815	0,65385	1			
Marawhi	0,68657	0,4746	0,4375	0,6182	0,70909	0,71698	0,61111	1		
Oleimi	0,80597	0,5833	0,6129	0,7636	**0,82143**	0,73684	0,72727	0,72414	1	
Boundouki	0,62687	0,431	0,5172	0,6346	0,8	0,67308	0,72917	0,66038	0,7143	1

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

The Principle coordinate analysis (PCA) based on genetic similarity matrices were used to visualize the genetic relationships among genotypes (Fig. 1). 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 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.
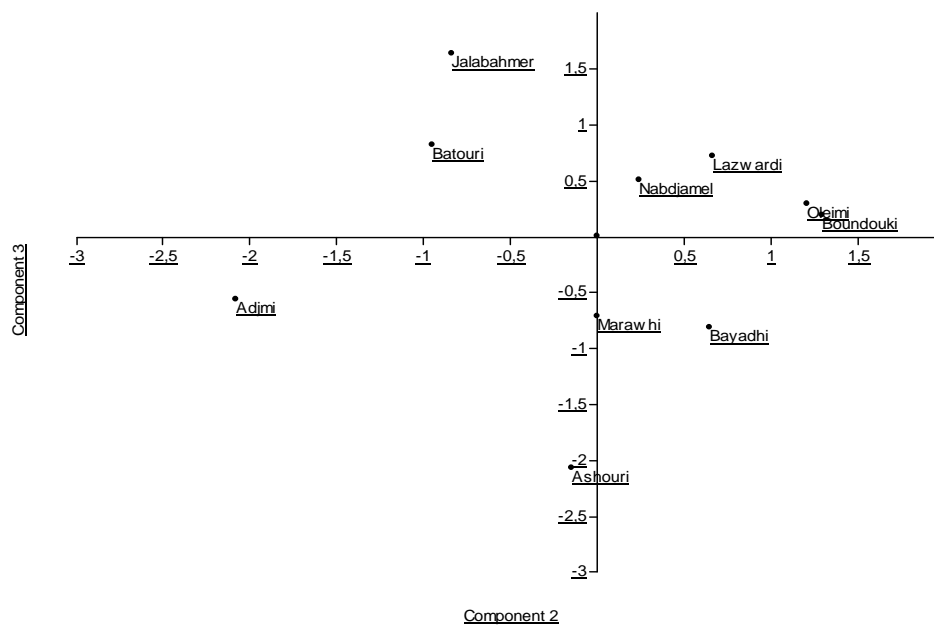

Fig.1. Relationships among the pistachio genotypes revealed by principal component analysis based on ISSR genetic similarity.

R E F E R E N C E S

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