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Genetic Diversity of Pistachio Tree using Inter-Simple Sequence Repeat Markers ISSR Supported by Morphological and Chemical Markers

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Abstract: The identification and the characterization of some Pistachio cultivars trees in South of Tunisia revealed a remarkable diversity between different cultivars, thanks to molecular markers (ISSR) supported by morphological markers (length sheets, leaf area, length of fruit, forms of final leaflet, their length and by chemical markers (content of vitamins). For the whole of the studied cultivars, the length of fruits is between 16.3 and 20.4 cm, the length of the final leaflet is 5.88 to 8.42 cm its form oscillates from elliptic to round, the average leaf area varies between 17.13 to 34.05 cm^2 in all varieties. The analysis of chemical variability allows to distinguish in different cultivars studied a variability on the level of the composition of vitamin B1, B2, B6 and VC. The content of vitamin B1 is going from 0 to 1 mg g^{-1} in the varieties Kermizi, Meknessy 1, Mumtez, Mateur 2 and Mateur 3 and 0.019 mg g^{-1} in Lybie rouge then the range of B2 is going from 0.001 mg g^{-1} at the variety Mateur 3, with 0.07 mg g^{-1} in Lybie rouge, the content of vitamin B6 is very variable from 0. 0016 mg g^{-1} at Mateur 3, with 0.01643 mg g^{-1} in Red Aleppo and the VC is going from 0.013 mg g^{-1} in Meknessy 1 to 0.09 mg g^{-1} in Lybie blanc. The PCR amplification of Pistachio varieties revealed a high percentage of the polymorphic fragments (26 fragments are polymorphs), the varieties Mumtez, El Guettar and Mateur 1 are characterized by a high percentage of the polymorphic locus (38.46%) whereas the varieties Lybie blanc, Kermizi, Lovy, Meknessy 1, Lybie rouge, Brise vent, Mateur 2, Mateur 3, Mateur 4, Meknessy 2, Kerman, Red Aleppo have an average polymorphism (between 26.92 and 34.61%). The variety Meknessy I (rate = 3.84%) and Lybie blanc (rate = 15.38%) have a low polymorphism. Several varieties have a coefficient of similarity (ds) close to 1 they are considered genetically very similar. Among these varieties Brise vent and Mateur 3 whose a coefficient of similarity equal to 0.667 (dg = 0.333), Lovy and Mumtez whose coefficient is equal to 0.632(dg = 0.368), Lybie rouge and El Guettar whose coefficient is equal to 0.632 (dg = 0.368), Kermezi and Kerman whose coefficient of similarity is equal to 0.750 (dg = 0.250), Meknessy 2 and Red Aleppo whose coefficient of similarity is about 0.857 (dg = 0.143). Certain varieties are considered genetically very distant between them, the genetic distance which separate them is large (dg = 1) it's the case of the variety Mateur 4 with Meknessy1 (dg = 1) and Meknessy 1 with Mumtez and Mateur 3. Other varieties can be considered distant like Lybie Blanc with Brise vent and Mateur 3 (dg = 0.846), Meknessy 1 and El Guettar (dg = 0.82). The use of ISSR markers for amplification PCR of different Pistachio cultivars revealed a high percentage of the polymorphic fragments (26 bands).

Key words: *Pistacia vera*, genetic diversity, morphological traits, ISSR markers, chemical composition

INTRODUCTION

The main world producers of Pistachio nuts are Iran, USA, Turkey and Syria. Commercial exploitation of Pistachio commenced in the 1930s in Iran, which still remains the largest producer (Chang, 1990), providing 56.10% of the world's production. The second largest Pistachio producer is USA, where kerman is the most commonly grown cultivar. It covers over 90% of the total country production of Pistachios. In both Iran and USA, Pistachio plantations are irrigated whereas in Turkey there is no irrigation yet in place for this crop.

Within the *Pistacia* genus, *Pistacia* genus, *Pistacia vera* (also called Green Gold Tree) is the only edible and worldwide marketable species.

In the World 571,150 Mt or 1.2 billion pounds. Pistachios are produced commercially in 18 countries on 1.1 million acres. Worldwide average yields are 1050 lbs/acre. The top 10 countries percentage of world production: Iran (52%), USA (24%), Syria (9%), Turkey (7%), China (5%), Greece (2%), Afghanistan (<1%), Italy (<1%), Uzbekistan (<1%), Tunisia (<1%). United States (2004 USDA) - 158,182 MT or 348 million pounds. Virtually all Pistachio production is in California [Statistics are

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for California only]. Small acreages exist in Arizona, New Mexico and western Texas. The industry value is $438 million. Bearing acreage is 93,000. Yield is 1700 to 3300 lbs/acre, depending on "on" or "off" status of the crop. Price paid to growers was $1.26/lb.

In Tunisia, there were no selected female varieties before the introduction of the cultivar Mateur. The two other local varieties from this country are Sfax and El Guettar. It has been reported that var. Sfax introduced to the USA from Algeria is in fact a clone of the local Tunisian cultivar Sfax.

At present the most commonly cultivated variety in Tunisia is Mateur, which resembles the Syrian variety Achoury (Jacquy, 1973); this variety includes three main genotypes: male precocious 25 A, male late 40 A and female 11 D (Ghorbel and Kchouk, 1996).

In Tunisia, *Pistacia vera* is propagated by grafting on seedlings of *Pistacia vera*. This species is adapted to marginal climatic and edaphically conditions such as drought, cold, calcareous and rocky soils. Such proprieties have led farmers to pursue its cultivated in the marginal and arid zones where olive and almond trees cannot grew successfully. The Tunisian cultivars Mateur, Sfax and El Guettar are adapted to low altitudes and can perform well also in temperate zones near the coast.

Currently. There are 44000 ha planted with Pistachio, corresponding to about 2730 million trees. Pistachio trees represent about 11% of the country's total area planted with stone fruit trees (excluding date palm and olive trees). Irrigated areas cover about 2000 ha while non-irrigated orchards consist of 42000 ha.

The most important Pistachio producing zones are Gafsa, Sidi Bouzid, Kasserine, Sfax and Kairouan: as a whole they contribute to 80% of the total national production. The area of Kasserine (the largest concentration of Pistachio orchard in the country) contributes with 29% to the national Pistachio production, Sidi Bouzid (22) and Gafsa (17%).

A recent increase in interest for this horticulture crop has contributed to expansion of its cultivated area, which has risen from 9300 ha in 1984 to 44000 ha in 1997.

However, production and profitability are still relatively low due to drought, late bearing (7-10 years after establishment) and controlled pollination (Twey, 1998).

During the past 10 years, the Pistachio production in Tunisia has increased greatly from 1982 (188 Tonnes) to 1997 (1141 t). This is due to the new young plantings that have entered their productive cycle and to the fact that greater attention was given to the management of orchards (pruning, ploughing, fertilizing, disease and pest control) and to pollination techniques improvement (Twey, 1998). However, this production is fluctuating from season to season due to alternate bearing and to severe climatic conditions.

The agro-morphological characterization of Pistachio that is a slow-growing tree, reaching a mean height of 8 m. Its longevity exceeds 150 years. The trunk consists of a tough resistant wood yellow to red in adult trees.

Mateur cultivar has a large crowned habit, whereas Sfax has a semi-erected one. Generally, male trees present a more erect habit than female trees. Tree vigour is highly correlated with the genotype: Mateur for example has a strong vigour, whereas Sfax shows a low vigour.

Male tree leaves are small, bright green with raised veins. Leaflets are oval (Mateur) or orbicular (Sfax). Lateral leaflets are smaller in size than the apical ones. At the juvenile stage, leaves are simple.

Little attention has been directed towards the conservation and evaluation of Pistachio genetic resources in Tunisia. Despite the increased importance of this crop, local Pistachio germplasm is far from being adequately studied and used.

Currently, Tunisian Pistachio cultivation is seriously threatened by genetic erosion as a result of increased monoculture. Mateur is the only widely employed variety (Ghorbel and Kchouk, 1998). Thus, El Guettar populations and especially Sfax are being neglected and are actually facing a serious danger of being lost.

Since the 18 Century and during the Ottoman Empire, Pistachio was the most appreciated crop by the local rules (Beys). Pistachio nuts are used as dessert and in the confectionery industry to prepare cakes and sweets. The nuts contain about 60% of fatty acids (oleic and linoleic acid) and 22% of proteins. Their energy value is twice as important as that of sugar or butter. *Pistacia vera* is also used in Tunisia as a rootstock. Wood and leaves are used as agricultural implements and fodder. The trees can be used for erosion and desertification control or as ornamental plants. For all these different uses, *Pistacia vera* can be considered a truly valuable multipurpose species.

Works on Pistachio breeding has been very scarce. Therefore, little is known on Pistachio genetics. Most of this wide genetic variability has not been exploited to solve production drawbacks. There are good prospects for obtaining outstanding cultivars, crossing superior male and female cultivars from different origins. However, Pistachio breeding faces some important limitations: it is a dioecious species and therefore the potential of male parents for any nut related character is unknown and it shows late bearing and thus long generation cycles.

In Tunisia, the identification and the characterization of the varieties of Pistachio tree was the research object many.

However, in spite of the revelation of several cultivars, the morphological characters remain fluctuating criteria, owing to the fact that same cultivar can be expressed in a very different way according to ecological conditions' (ground, climate) and present considerable morphological differences (colour, size of the fruits).

Under these conditions, the exact description of the cultivars of the Pistachio tree becomes very difficult and the problem of varietals identification becomes complicated of advantage. Thus, other techniques must be used (protein electrophoresis, technique based on the AND) which especially aim at establishing in a more precise way the characterization and classification of the cultivars of Pistachio tree. Simple Sequence Repeat (SSR) is possible to efficiently mark any portion of the genome for inheritance studies. RFLP are generally detected using low copy nuclear DNA sequences as hybridisation probes on Southern blots of restriction digested genomic DNA (Botstein *et al*., 1980), RAPDs are identified by the Polymerase Chain Reaction (PCR) using arbitrary primers, making them simpler to assay than RFLPs and can detect polymorphism in both low copy and repetitive DNA sequences (Williams *et al*., 1990; Welsh and McClelland, 1990). While most RFLP markers are co dominants and may detect may alleles at a locus, most RAPD markers are dominants and can detect only two alleles for a locus (presence or absence of the marker). Dominant RAPD markers thus provide less genetic information than RFLP markers in certain mating and may segregate in only a limited number of populations (Echt *et al*., 1993). The use of SSR loci as polymorphic DNA markers has expanded considerably over the past decade both in the number of studies and in the number of organisms, primarily due to their facility and power for population genetic analyses (Estoup and Angers, 1998).

Inter Simple Sequence Repeat (ISSR) is a dominant molecular marker revealed in mass. Under conditions of adapted amplifications, the DNA fragments were separated in agarose gel or acrylamide gel. Revealed polymorphism is primarily of presence/absence type, as for the RAPD, but corresponds sometimes to differences in lengths length of fragment, as for the microsatellites (Sylvain *et al*., 2000). These markers appeared very polymorphic.), supported by morphological and chemical descriptors.

The aims of this research are to study the genetic variability of different cultivars from Pistachio tree in the South of Tunisia in basing on molecular descriptors. Through the use of methods to DNA polymorphisms, such as Restriction Fragment Length polymorphisms (RFLPs) or Random Amplified Polymorphic DNAs (RAPDs).

MATERIALS AND METHODS

Plant material: This study was performed using 15 Tunisian Pistachio trees cultivars (Table 1). The plant material consisted of young leaves that were sampled from adult trees on the Pistachio germplasm collections maintained at IRA (Institut des Régions Arides, Medenine-Chenchou) in April.

Study of the morphological aspects: For each cultivar of the Pistachio tree we determined the length, width and the thickness of fruit, length, width, thickness, colour of almond and the same for his apex of the hull, length, width of the sheets as well as the leaf area , length, width, and numbers, form and as well as the apex of the final leaflet. The leaf area is measured by a planimeter.

Reagents: The thiamin hydrochloride was purchased from Carlo Erba (Val de Reuil, French). Takadiastase was from aspergillusorizae were from Fluka (Buchs, Switzerland). Methanol grad was and acetic acid glacial grade were purchased from Panreac Quimica SA (Barcelona, Spain). Sodium acetate trihydrate was from J.T. Baker (Holland). L-Ascorbic acid standard was Reagent grade and was obtained from MP Biomedicals (Eschwege, Germany). Orthophosphoric acid 85% was from Merck (French). The water used in HPLC and sampling was prepared with Millipore Simplicity (Millipore S.A.S, Molsheim, French).

Analytical methods: One was interested in the study of the contents of vitamin B1, B2, B6 and VC The determination is done by the HPLC.

Thiamin, riboflavin and pyridoxine contents

Extraction of B1, B2 and B6 vitamins: Twenty five milliliters of 0.1 M sulfuric acid were added to the sample (5 g of Pistachio kernel) in a 125 mL conical flask. The solution was placed in the autoclave at 121°C for 30 min.

Table 1: Pistachio tree cultivars studied with their origin

Names of the cultivars	Place of collection	Code	Origin
Lybie blanc	Chenchou-Gabès	C 1	Libya
Kermezi	Chenchou-Gabès	C 2	Turkey
Lovy	Chenchou-Gabès	C 3	Tunisia
Meknessy1	Chenchou-Gabès	C 4	Tunisia
Mumtez	Chenchou-Gabès	C 5	Iran
Lybie rouge	Chenchou-Gabès	C 6	Libya
El Guettar	Chenchou-Gabès	C 7	Tunisia
Meknessy 2	Chenchou-Gabès	C 8	Tunisia
Brise vent	Chenchou-Gabès	C 9	Turkey
Kerman	Chenchou-Gabès	C 10	USA
Red Aleppo	Chenchou-Gabès	C 11	USA
Mateur 1	Chenchou-Gabès	C 12	Tunisia
Mateur 2	Chenchou-Gabès	C 13	Tunisia
Mateur 3	Chenchou-Gabès	C 14	Tunisia
Mateur 4	Chenchou-Gabès	C 15	Tunisia

After being allowed to cool it was adjusted to pH 4.5 with 2.5 sodium acetate. Takadiastase (0.1 g) was added. The solution was incubated for a night in over at 37°C, then diluted to 50 mL with ultra pure water and filtered through a filter paper. The filtrate obtained after a second filtration through a cellulose acetate filter (0.45 µm) was used for chromatography determination of vitamins (Arella *et al*., 1996).

Liquid Chromatography conditions: LC separation was carried out at room temperature on Eurospher C18 column, 100 A pore size, 18, 5 µm particle size, 250*4.6 mm I.D (Knauer, Germany). The mobile phase used was a solution of ultra pure water, acetic acid glacial and methanol an in ration (6/2/1). Prior to use, solvents were filtered over a 0.45- µm membrane filter and sonicated for 15 min in a Ultrasonic Cleaner Model SM 25E-MT (Branson Ultrasonics Corporation, Dambury, USA). The separation was performed at programming of flow rate:

-0.5 mL min^{-1}: 0-5 min
-1.0 mL min^{-1}: 5-10 min
-1.5 mL min^{-1}: 10-15 min
-2.1 mL min^{-1}: 15-30 min

The UV detector operates at wavelength of 254 nm. Quantification was carried out from integrated pick areas of the sample against the corresponding standard graph.

Ascorbic acid contents: To 3 g of almond, 1 mL of 2% Orthophosphoric acid was added, vortexed and the volume was adjusted to 5 mL by adding water ultra-pure. The mixture was centrifuged at 5000 rpm for 8 min at 4°C The supernatant was filtered and vitamin C level was determined by HPLC, utilizing a column (250 mm*4.6 mm i.d.,) packed with Eurospher C18 reversed-phase materiel (18, 5 µm particle size) with mobile phase (water, pH 2.2) at 1 mL min^{-1} flow rate.

Data analysis: The statistical analyses (hierarchical classification by using UPGMA (Unweighted Method Par-Group, of Arithmetic Means) were carried out by software MVSPW and SPSS version 12.0.

Extraction of ADN and realization of PCR: Genomic DNA was extracted from fresh leaves of single adult trees following the method described by Doyle and Doyle (1990) with minor modifications.

DNA concentration was determined by both spectrophotometry at 260 nm and 2% agarose gel electrophoresis.

DNA samples of the 15 individuals plants were adjusted to 20 ng μL^{-1} and used in the amplification reactions with a final volume of 25 µL containing 2.5 µL PCR buffer, 3 µL of $MgCl_2$ (25 mM), 1 µL of dNTPS (10 mM), 2 µL of primer (40 µM), 2 µL of DNA, 0.2 µL Taq DNA polymerase (5 U μL^{-1}) and 14.3 µL deionized water.

DNA amplification was carried out using a Gene Amp PCR system 9700 thermal cycler programmed with 3 min at 94°C for initial denaturation, followed by 35 cycles of 1 min at 94°C, 45 sec at 55°C, 2 min at 72°C and a final 5 min extension at 72°C.

After amplification (Fig. 1), the DNA fragments were separated by electrophoresis for about 2 h under constant voltage (60 Volts) in 2% agarose gel submersed in 1x TBE buffer. The gels were stained with ethidium bromide solution and observed under ultraviolet light. Each gel was photo documented using the image capturing system bioprint.

The Jules DNA ladder (QBiogene) was used as standard molecular weight marker.

Statistical analysis: The amplified bands were scored as 1 and 0 based on band (allele) presence and absence, respectively. Sizes of amplified bands were estimated using Gel Pro analyzer software.

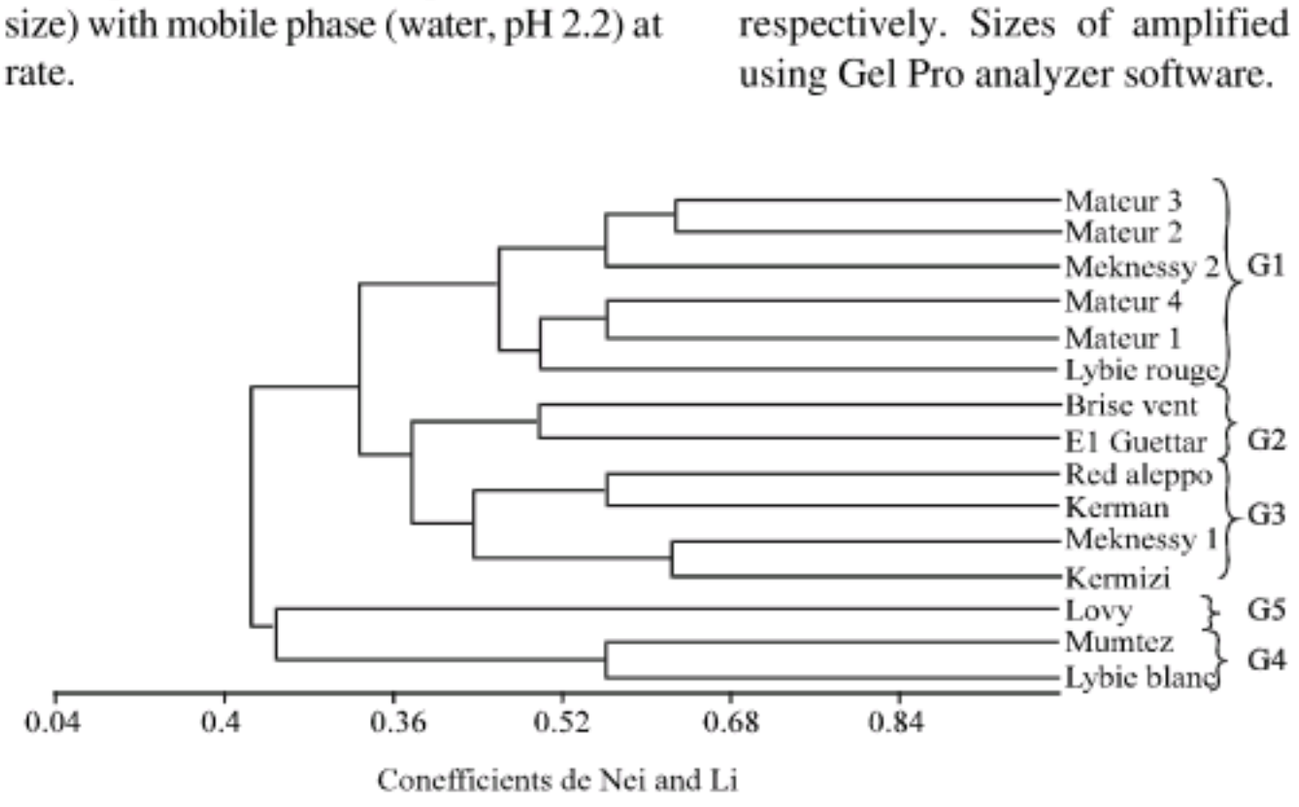


Fig. 1: Morphological clustering of 15 cultivars Pistachio trees

The similarity of all samples for all scored bands was assessed using Dice's similarity coefficient (Dice, 1945).

The matrice generated were analyzed with SPSS version 12 software to evaluate genetic distance.

RESULTS

Analysis of morphological variability

Variability in the morphology of the leafs, fruit:

- The leaf area is large in Lovy, Mateur 1, 2, 3 and 4, El Guettar, Meknessy 2 and Brise vent and small in Mumtez, Meknessy 1
- The shape of the final leaflet is elliptic in Lybie blanc, oval in Meknessy 1, round oval in Mumtez, round in Mateur 3 and lancelet widened in Mateur 4
- The length of fruit is also a distinctive character between the varieties which extends 16.3 cm in Lovy to 20, 4 cm in Kerman
- The length of the final leaflet is 5, 88 cm in Mumtez, 20, 83 cm in Red Aleppo

Figure 1 represents morphological clustering of 15 cultivars Pistachio trees.

There were another's parameters which do not show a variability between the varieties such as the apex of the final leaflet which is almost acuminate at all the varieties and the number of leaflet which is about 5, the apex is acute dissymmetrical in the majority of the varieties thus its colour of green to yellow-green.

Other studies of prospecting and morphological characterizations of Pistachio tree were carried out, in particular those which studied the male and female periods of flowering (Crane, 1984; Köroglu and Köksal, 1995; Alipour *et al*., 2002). The description of the female varieties showed differences which exist between them for the port, strength and the characteristics of leafs, the time and the duration of flowering and the characteristics of fruits . The port and the strength of Kerman variety were also studied by Rouskas (2002). Zakinthinos and Rousks (1995) are studied the fruit dehiscence. Crane and Iwakivi (1982), were also interested in the dehiscence of the endocarp which is a character sought in Pistachio tree.

The study of Larue (1960) made it possible to classify Iranian Pistachios in three groups: the first contains the lengthened Pistachios which resemble almonds, the dehiscence is approximately 90% and the interior of the fruit is yellow. The second group is characterized by round Pistachios which resemble hazel nuts, the dehiscence is also 90% and the interior of the fruit is also yellow drawing however slightly on the green. In the third groups Pistachios are with the smaller fruits, the interior is always of a beautiful green and the dehiscence is approximately 30%. Among the studied morphological characters others that the dehiscence, which is a selection criterion for the pistachio tree, the pollination is the criterion of fertilization. In the same way, the weight of the fruit is a selection criterion sought enough by the consumer and one of the pomological characteristics which differs from a variety to another). A work of follow-up of phenology and a pomological characterization of some Pistachio trees varieties were led to Morocco and Zohary (1952) and in which it divides the *Pistacia* into four sections and ten species. A recent study on the identification and the pomological and phenologic description of the male and female varieties of the Pistachio tree was carried out.

However, the analysis of morphological variability remains much influenced by the climatic factors. Thus, other descriptors of the content of vitamin and the molecular analysis make it possible to better apprehend this variability.

Study of genetic diversity based on chemical composition

Variability in the content of B1 vitamin: The content vitamin B1 according to Fig. 2: content of vitamin B1 of 15 cultivars of Pistachio (quantity of mg of vitamin per g of fresh matter) is very variable and going from 0 to 1 fresh matter mg g^{-1} in Kermizi, Meknessy 1, Mumtez, Mateur 2 and Mateur 3 varieties to 0.019 mg g^{-1} in Lybie rouge.

Variability in the content of B2 vitamin: We can note that the content is variable; it varies from 0.001 mg g^{-1} in the variety Mateur 3 to 0.07 mg g^{-1} in Lybie rouge (Fig. 3).

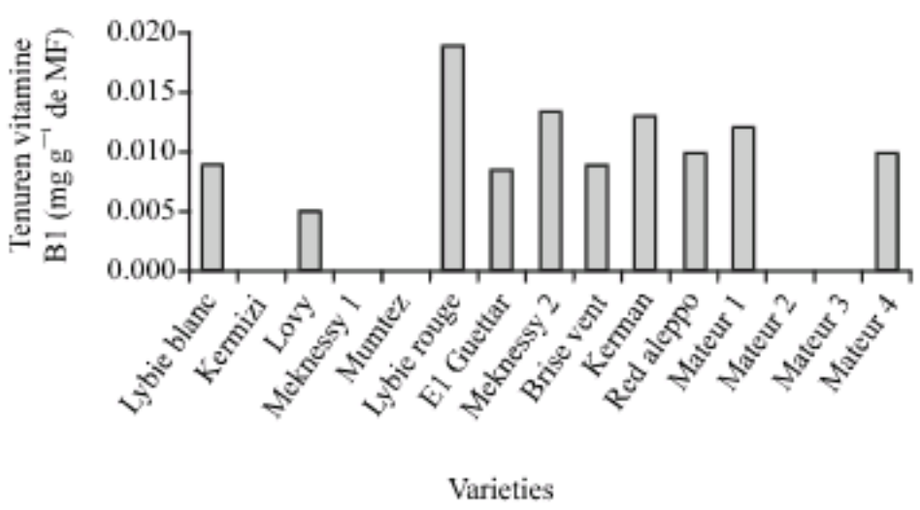


Fig. 2: Content of vitamin B1 of 15 cultivars of Pistachio

Variability in the composition of B6 vitamin: These values rather remarkable, it oscillates between 0.16 mg g^{-1} in Mateur 3 variety and 1.643 mg g^{-1} in Red Aleppo (Fig. 4).

Variability in the composition of vitamin VC: From Fig. 5 we can deduce that VC content is very variable and varies from 0. 013 mg g^{-1} in the variety Meknessy 1 to 0.09 mg g^{-1} in Lybie blanc.

The analysis of this chemical variability in combination of various vitamins (B1, B2, B6 and VC) allows differentiating some cultivars (Fig. 6):

- Brise vent, Mateur 1, Mumtez, Lybie rouge, kerman, Mateur 4 have a fairly raised content of B1, raised in B6 content, average in B2 and low VC content
- Meknessy 1, Meknessy 2, Kermizi, El Guettar, Mateur 2, Lybie blanc have a fairly to high content of B1, average content of B2 and B6 and low content of VC
- Mateur 3: low content of B1, B2, B6 and VC
- Lovy, Red Aleppo: fairly to high content of B1 and B2, very high content of B6 and low to raised of VC

Molecular polymorphism: The DNA analysis resulted in 26 bands. Across all cultivars these 26 bands are polymorphic. The list and number of ISSR primer is mentioned in Table 2 and the profile obtained from 13 primers is carried in (Fig. 7).

The analysis of these profiles allows choosing the A4 primer which gave a rate of high polymorphism.

- The primers which I tested them are universal and the only one which gave best polymorphism is (AG) 10 T

Figure 8 shows that the polymophic DNA fragments is obtained with primer (AG)10 T

After examination of each cultivar, we can deduce that:

- The cultivars Mumtez, El Guettar and Mateur 1, are characterized by a high percentage of the polymorphic locus (38.46 %) whereas the cultivars Lybie blanc, Kermizi, Lovy, Red Aleppo, Meknessy 1, Lybie rouge, Brise vent, Mateur 2, Mateur 3, Mateur 4, Meknessy 2, Kerman, Red Aleppo have an average polymorphism (between 26.92 and 34.61%)
- The cultivar Meknessy 1 (rate = 3.84%) and Lybie blanc (rate = 15.38%) has a low polymorphism

The variation of the polymorphism in the different cultivars can be explained by the two following hypothesis:

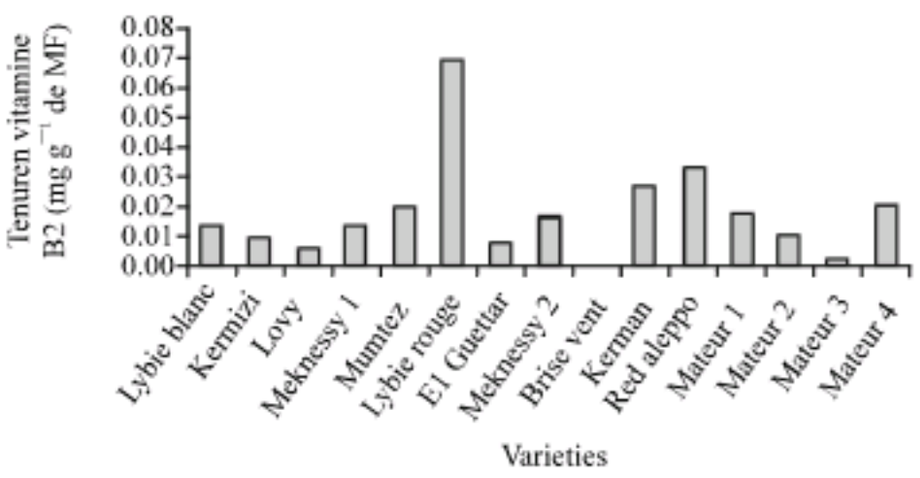


Fig. 3: Content of vitamin B2 of 15 cultivars of Pistachio

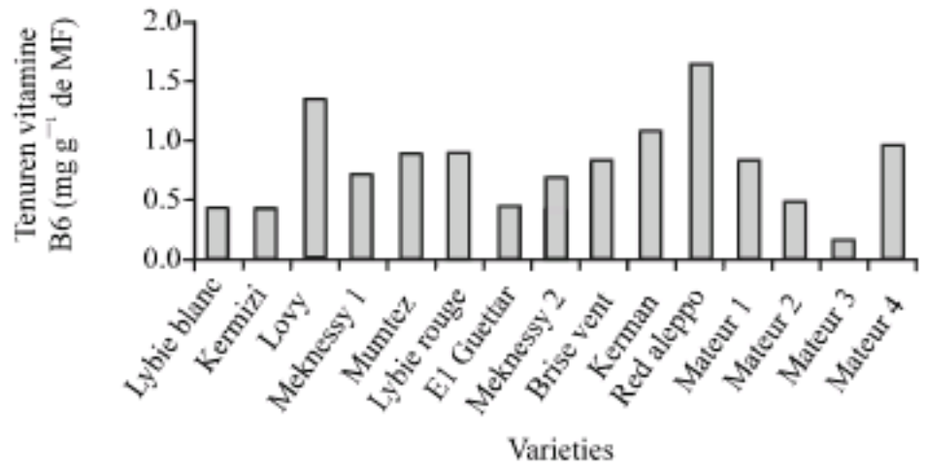


Fig. 4: Content of vitamin B6 of 15 cultivars of Pistachio

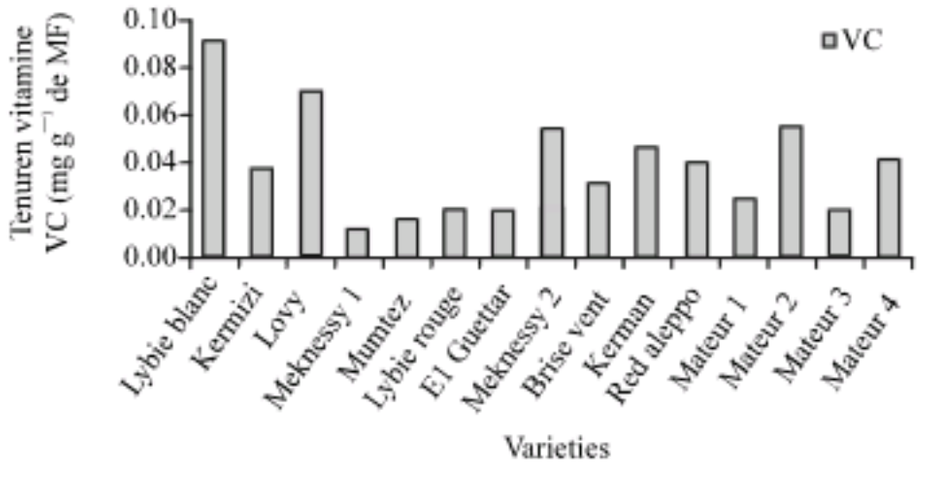


Fig. 5: Content of vitamin VC of 15 cultivars of Pistachio

Table 2: Thirteen primers were tested

Primer	Sequences
A1	(TAGGA) 5
A2	(GA) 6CC
A3	(CT) 7CAC
A4	(AG) 10T
A5	(CA) 6GT
A6	(CT) 10G
A7	(CA) 6GG
A8	CAATTCCTAT(C) 4A(C) 4TAAG
A9	GTCC(A)3TGGTGG(A)4CTACC
A10	GTGGTACCCTCACTAGCTCTCT
A11	GAGGTTGAAGCATGCAGTTC
A12	GTCATCTGAATAGTGTAGATG
A13	GGCCATGATTTATTCACTCAG

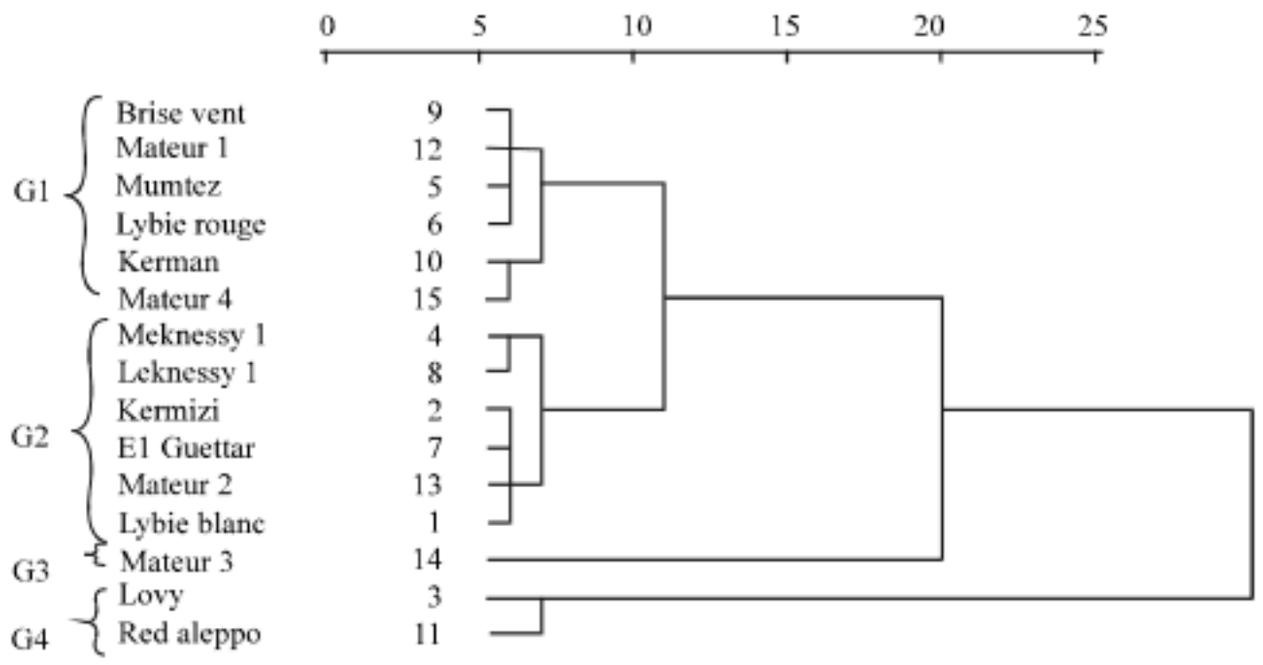
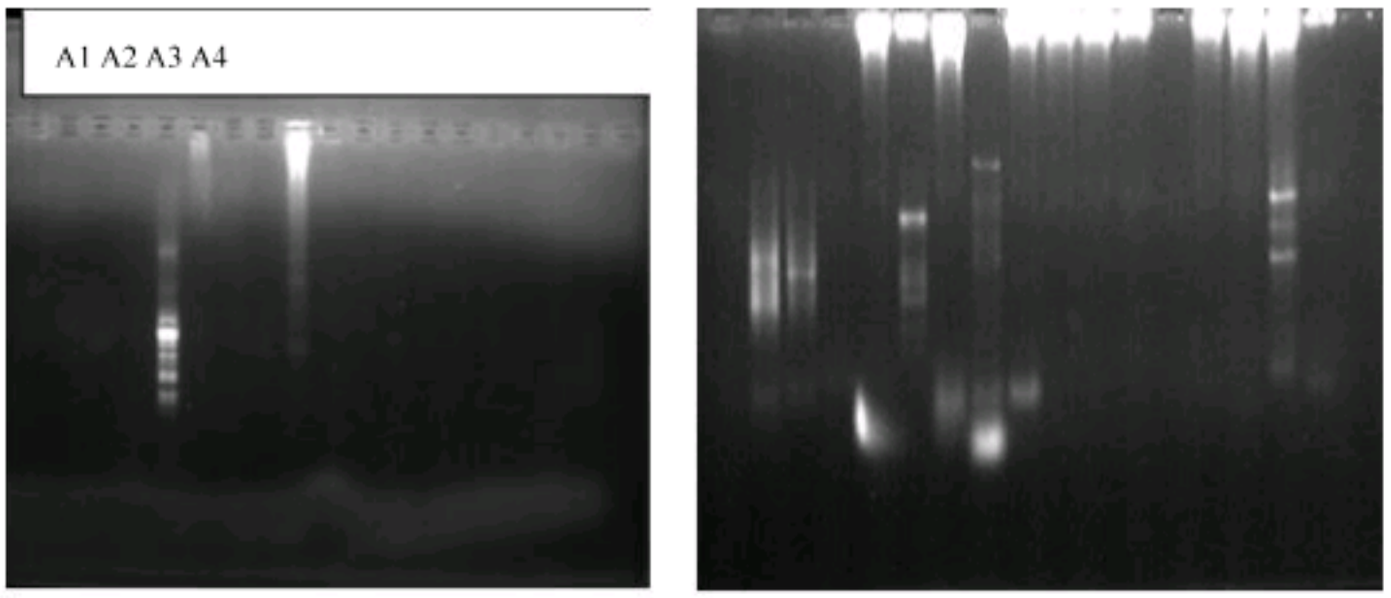


Fig. 6: Clustering of the content of vitamins (B1, B2, B6, VC) in 15 cultivars of Pistachio trees



A1, A2, A3, A4, A5, A6, A7 A8, A9, A10, A11, A12, A13

Fig. 7: Profiles of PCR obtained by the use of the various primers for amplification PCR (A1……. A13: amorces)

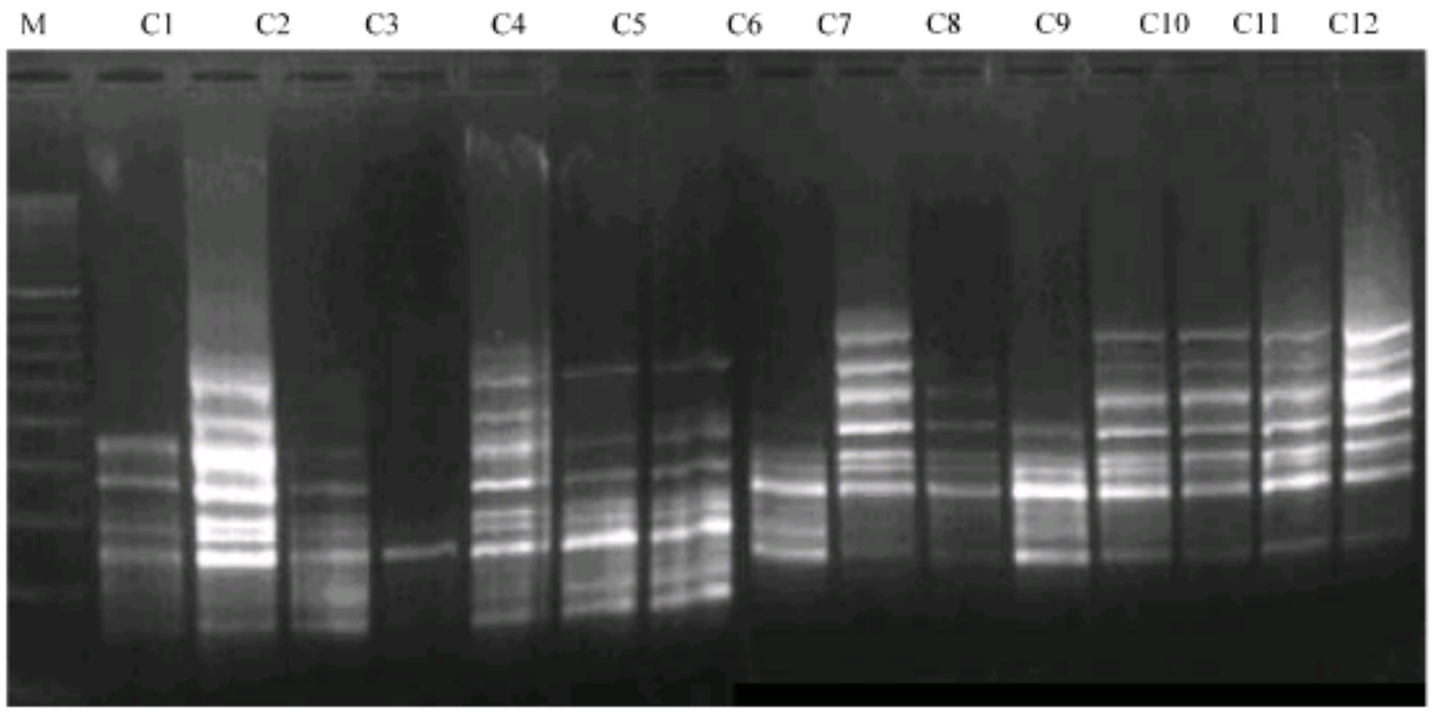


Fig. 8: Electrophoresis pattern obtained in the ISSR primer $(AG)_{10}T$ in 15 cultivars of Pistachio trees (C1…..C15 = cultivars, M is 100 bp DNA ladder)

- The microsatellites whose sequences are complementary to the primer are abundant or rare in the genome of the cultivar
- These microsatellites occupy some sites sufficiently distant not allowing the synthesis of sequences that separates them

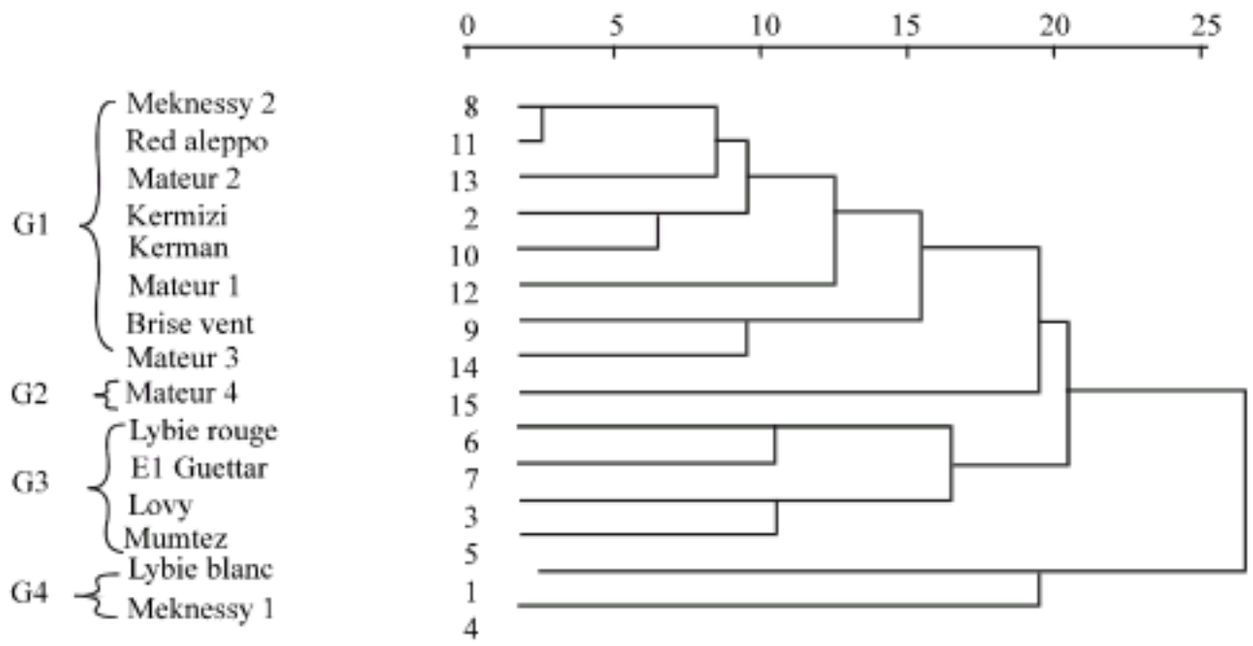


Fig. 9: Dendrogram of 15 Pistachio trees cultivars based in Dice similarity index

The method described by Doyle and Doyle (1990) with some modifications proves to be more appropriate to obtaining a good quality of DNA of Pistachio tree for the amplification by RCR.

The utilization of microsatellites markers in order to study diversity of different cultivars of Pistachio-tree revel the presence of 26 fragments.

This study provides evidence that the ISSR procedure is an informative and suitable approach to the examination of the molecular polymorphism and the phylogenic relationships in the Pistachio germplasm.

Relationships among cultivars of Pistachio tree: Four groups are representing in Fig. 9:

Group 1: Formed by Meknessy 2, Red Allepo, Mateur 2, Kermezi, Kerman, Mateur 1, Brise vent, Mateur 3 cultivars. This group is also characterized by a number of bands between (7 and 10 whose weight of these bands is between 500 and 1200 pb)

Group 2: Contained only one cultivar Mateur 4 which is genetically far away from the others and is characterized by a number of bands equal to 8 and one variable molecular weight between 500 and 1100 pb

Group 3: Contained four cultivars Lybie Rouge, El Guettar, Lovy, Mumtez, they presenting more than 8 bands between 9 and 11 and a molecular weight which ranges between 400 to 1200 pb

Group 4: Formed by two cultivars Lybie Blanc, Meknessy1 which are genetically far away from the others and are characterized by a low number of bands and a molecular weight between 600 and 811 pb

DISCUSSION

The analysis of chemical variability made it possible to distinguish at the several varieties variability in the level of the chemical composition in B1, B2, B6 and VC, so it made possible to classify the varieties in homogeneous groups and the content of vitamins are various.

The content of B1 vitamin for El Guettar, Lybie blanc, Brise vent and Mateur 4 varieties, is about 0.81 mg 100 g and 1 mg 100 g for Red Aleppo, Mateur1, Kerman, Meknessy 2, so its oscillates from 0 to 1.9 mg 100 g in Lybie rouge, this value is smaller then the content in rice its ranged from 0 to 5.34 mg 100 g whereas for the *Prunus dulcis* this value is very higher, the content of VB1 in Prunus is 0.241 mg 100 g (http:// tous-les-fruits.com/fruit-364.html). While the content of B2 vitamin for Lybie blanc, Brise vent, Mateur 4, Meknessy 1, Mumtez, Meknessy2, Mateur 1 and Mateur 2 varieties is equal to 1.60 and 7 mg 100 g for Lybie rouge, this value is very higher then the content in *Prunus dulcis* 0.811 mg 100 g and for rice is also higher the content in VB2 in rice ranged from 0.02 to 0.04 mg 100 g. For VB6 it's oscillated from 0.16 mg 100 g to 1.643 mg 100 g this value is important then the value existed in *Prunus dulcis* and more and more important than rice because the rice is poor in B6. The vitamin C for Kermizi, Meknessy 1, Mumtez, Lybie rouge, El Guettar, Brise vent, Mateur 3 and Mateur1 varieties is from 1.3 to 9 mg 100 g in Lybie blanc this values are comparable with those is published, or mentioned by De Beer *et al*. (2003). Yousfi *et al*. (2003) were interested being studied of the composition in fatty acid of the fruit oil of Pistachio tree of the Algerian Atlas. Duru *et al*. (2003), Alma *et al*. (2004), Assimopoulou *et al*. (2004) and Orhan *et al*. (2005) showed that these oils present an antifungal activity, antimicrobial, antioxidant, anti-inflammatory drug, antiviral and antibacterial.

In addition the studies of the wealth of vitamins C, B of the fruits interested several authors Yurena Hernandez *et al*. (2005), Cökmen *et al*. (2000) and Brause *et al*. (2003).

Recently, the technique of ISSR is used to study the genetic relations between the various Coffee species and to determine the family ties between the hybrids (Paulo *et al*., 2003). In the same way this technique appeared effective for the study of the genetic variation in *Changium smyrnioides (Apiaceae*) studied by Ying-Xiong *et al*. (2004). These same markers ISSR were used successfully for the genetic study of polymorphism (Meyer *et al*., 2004) in rice (Blair *et al*., 1999), in potato (McGregor *et al*., 2000) and in the olive-tree (Terzopoulos *et al*., 2005).

The ISSR markers were used successfully for the genetic study of polymorphism in rice (Blair *et al*., 1999), potato (Mcgregor *et al*., 2000) etc. Recently, this technique is used to study the genetic relations between the various coffee species and to determine the family ties between the hybrids (Paulo *et al*., 2003). In the same way this technique appeared effective for the study of the genetic variation at Changium smyrnioides (Apiaceae) studied by Ying-Xiong *et al*. (2004). Also Terzopoulos *et al*. (2005) identified the cultivars olive-tree by using the ISSR, but the Pistachio tree was not studied and for the reasons described in top I chose this marker. For the study of the genetic variability of Pistachio tree several other work was published and which differs by the choice from the genetic markers thus the application from the random technique of amplification from the polymorphic DNA (RAPD) was studied by Hormaza *et al*. (1994) and Kafkas (2001). Another technique which is the SCAR (Sequence Characterized Amplified Regions) was used by Yakubov *et al*. (2004) for the analysis of the genetic diversity of the Pistachio tree and the establishment of the phylogenetic relations between the studied varieties. Also study was made by Loukas and Pontikis (1979), Rovira *et al*. (1995) and Dollo (1993), which showed that there was an enzymatic polymorphism of *Pistacia vera* while supporting on the isoenzymatic method.

Synthetic approach of variability in Pistachio tree: The morphological and biochemical assay is related to molecular assay (Table 3):

Genetic markers = Morphological Markers+chemical Markers+molecular Markers two states that presents itself, homogeneity and heterogeneity.

Homogeneity on the level

Morphological level: Mateur 1, Mateur 2, Mateur 3 and Kerman, Kermizi, Red Allepo. Chemical level: Meknessy 1 with Meknessy 2

Molecular level: Mateur 1, Mateur 2, Mateur 3 and Kerman, Kermizi, Red Allepo

The combination of the whole of the three approaches (morphological, chemical and molecular) make it possible to deduce that it exists groups presenting a genetic homogeneity:

- Mateur 1, Mateur 2, Mateur 3, presents homogeneity either at the morphological level or on molecular level and is characterized by:

(i) Fairly large leaf area (>23 cm^2), short final leaflet of widened lancelet form, fruit lengthened, long almond and apex of the hull is acute dissymmetrical
(ii) A number of bands between 7 and 10 who's their sizes range between 500 and 1200 Pb

- Meknessy 1 and Meknessy 2 present homogeneity in the chemical level:

(i) Low content of B2, VC and B6
(ii) Low content with fairly high of B1

Table 3: Combination of group of Pistachio (B1, B2, B6 or of their combination)

Group according to the content of B1	Group according to the content of B2	Group according to the content of B6	Group according to the content of VC	Group according to the content of B1, B2, B6, VC
Mateur 2, Mateur 3, Kermizi, Meknessy 1, Mumtez	Lybie blanc, Meknessy 1, Mateur 2, Meknessy 2, Kermizi, El Guettar, Lovy, Brise vent, Mumtez, Mateur 1, Mateur 4	Kermizi, El Guettar, Meknessy1, Mateur 2, Meknessy 2, Lybie blanc	Kermizi, El Guettar, Meknessy1, Mateur 3, Mumtez, Lybie rouge, Brise vent, Mateur1, Mateur4, Lovy, Red Aleppo	Brisevent, Mateur1, Kerman, Mateur4, Mumtez, Lybie rouge
Lovy	Mateur 3	Mateur 3	Meknessy 2, Mateur 2, Kerman	Meknessy1, Mateur 2, Meknessy 2, Kermizi, El Guettar, Lybie blanc
Red Aleppo, Mateur 4, Lybie blanc, Brise vent, El Guettar, Meknessy2, Kerman, Mateur 1	Kerman, Red Aleppo	Mumtez, Lybie rouge, Brise vent, Mateur 1, Kerman, Mateur 4	Lybie blanc	Mateur 3
Lybie rouge	Lybie rouge	Lovy, Red Aleppo		Red Aleppo Lovy

- Red Aleppo, Kermizi and Kerman present homogeneity either in the morphological level or on the molecular level which are characterized by:

(i) Large Leaf area (>29 cm^2), long final leaflet of elliptic form, ovoid fruit, fairly long almond and apex of the hull is round
(ii) A number of bands between 7 and 10 who's their sizes range between 500 and 1200 Pb

The combination of the whole three markers (morphological, chemical and ISSR) makes it possible to deduce that the following groups present genetics homogeneity:

- Mateur 1, Mateur 2, Mateur 3, present homogeneity either in the morphological level or in the molecular level and are characterized by:

(i) Fairly large leaf area (> 23 cm^2), short final leaflet of widened lanceolet form, fruit lengthened, long almond and apex of the hull is acute dissymmetrical
(ii) A number of bands between 7 and 10 who's their sizes range between 500 and 1200 pb

- Meknessy 1 and Meknessy 2 present homogeneity in the chemical level:

(i) Low content of B2, VC and B6
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(ii) A number of bands between 7 and 10 who's their sizes range between 500 and 1200 pb

The overall results of this investigation demonstrated that all genotypes could provide valuable material for use in breeding programs.

Finally more works are necessary to enlarge the number of markers by the use of other molecular technologies in order to have a deeper insight into the molecular polymorphisms and to establish varietals identification.

At this moment only 56% references are cited from ISI web of knowledge.

For original articles (research, short communication, technical notes), at least 75% of the references must be from ISI Science Citation Index from the last decade.

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