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Research article

Genetic differentiation and gene flow of some Tunisian pistachio (*Pistacia vera* L.) varieties using chloroplastic DNA.

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Abstract

Genetic diversity of *Pistacia vera* sampled in three traditional areas was studied. The sequences variation of the chloroplastic gene: *trn*L (UAA) *trn*F (GAA) intergenic spacer, among fifteen pistachio varieties, was performed. The obtained analyses revealed 9 different haplotypes and three geographic groups. The cytoplasmic diversity of the *NJ* tree seems to be structured with a biogeography repartition of the variability. The overall estimate of genetic divergence (F_{ST}) revealed significant genetic differentiation between all population pairs. N_m value was high between Sidi-Bouzid and Gafsa's populations indicated high connectivity between them, exchange of varieties by human, wind dispersal of pollen and seeds by insects may be the reasons for these observations. On another hand, N_m values were very low among El-Guetar and the others populations. The surrounding geography of El-Guetar, such as Gafsa Mountains, makes it an isolated population. Finally and overall, Tunisian pistachio seems to be in mutation / derive equilibrium characteristic of a demographic stable population. Revealed cytoplasmic DNA markers are reliable to elaborate a molecular database to conduct management and breeding programs on local pistachio germplasm.

Key words: *Pistacia vera*, Chloroplastic DNA, Genetic diversity, Tunisia.

1. Introduction

The *Pistacia* genus is a member of the Anacardiaceae family. It consists of 11 species (Zohary 1952). The pistachio, *Pistacia vera* L., is the only cultivated and commercially-grown *Pistacia* genus (Zohary 1996). It is a dioecious wind pollinated tree, diploid (2n = 30) and native of the north east of Iran, north Afghanistan and middle Asian republics, (Zohary & Hopf 1994). *P. vera* has been long spread for nuts all over the Middle East and Mediterranean basin (Badenes & Byrne 2012). In the world, there are several importants varieties; Matteur (Tunisia), Aegina, Ajamy, Batoury, Ashoury and Kerman. This last variety of Iranian origin is the main variety cultivated in California and grafted on *Pistacia atlantica*. Aegina also presents the main cultivated variety in Greece (Oukabli, 2005).

Numerous reports suggest that the Romans were accountable for the propagation of this species within the Mediterranean regions (Hormaza et al., 1994).

In Tunisia, pistachio is cultivated since Carthaginians time (2800 years ago). The most important pistachio producing regions are Gafsa, Sidi Bouzid and Kasserine. The commonly cultivated pistachio variety in Tunisia is "Mateur", which resembles the Syrian variety "Achoury" (Ghorbel et al., 2001). The Sfax variety has practically disappeared, it is grown in California and has recently been reintroduced into the Gafsa region. The introduced Iranian (Ohadi, Kerman, Razzi) or Syrian (Red Aleppo) varieties were tested for their performance against local varieties. They are currently little used.

There is 44,000 ha grown pistachio areas (Abidi 2016) and *the national production* in 2014 reached 1200 tones (FAOSTAT, 2016).

Moreover, protandry is one of the mains constraints for growing Tunisian pistachio (Ghorbel & Kchouk 1998). Such problems were however overcome by artificial pollination practiced by farmers. Other major problem of the cultivation of this plant is its long juvenile phase and poor fruit setting ability. One of the most appropriate actions is to improve vegetative multiplication.

On another hand, little attention has been directed toward the conservation and evolution of Pistachio's genetic resources. Despite the increased importance of this crop, local Pistachio germplasm is far from being adequately studied and used. In this scoop, the genetic diversity of pistachio trees using Morphological marker was developed by Ghrab et al., 2012.

Several molecular markers such as randomly amplified polymorphic DNA (RAPD) (Kafkas & Perl-Treves 2001a; Kafkas et al., 2001b), simple sequence repeat (SSR) (Topcu et al., 2016), sequence-related amplified polymorphism (SRAP) (Ahmad et al., 2005), amplified fragment length polymorphism (AFLP) (Turkeli & Kafkas 2013), inter-simple sequence repeats (ISSR) (Farès et al., 2009), selectively amplified microsatellite polymorphic loci (SAMPL) (Karimi & Kafkas 2011) and single nucleotide polymorphism (SNP) (Kafkas et al., 2015) have been used to assess the genetic diversity, fingerprinting, phylogenetic relationships, germplasm characterization, sex determination, and genetic linkage mapping in cultivated and wild *Pistacia* species.

Variation of cytoplasmic genome offers important opportunities to evaluate the level and the organization of molecular polymorphism in several bio-resources (Ohsako & Ohnishi 2000, 2001; Yamane et al., 2003). In fact, the organization of its genes and its nucleotide order, which are perfectly conserved through the evolution process, make it an ideal target for plant genetics, phylogeny and biogeography studies (Clegg et al., 1991; Palmer et al., 1988).

Several results indicate that chloroplastic non-coding DNA regions, such as the intron *trn*L (UAA) and the intergenic spacer between the *trn*L (UAA) 3'exon and the *trn*F (GAA) gene can be used to address questions concerning relation-ships among closely related species or genera (Van Ham et al., 1994; Gielly & Taberlet 1994).

The intron and the intergenic spacer are useful because they hold a much higher mutation rate (Gielly & Taberlet 1994; Baker et al., 1999; Tsai et al., 2006; Geleta et al., 2010; Rhouma-Chatti et al., 2014; Choulak et al., 2015).

The intergenic spacer *trn*L-*trn*F evolved faster than codon region in the chloroplast genome (Gielly & Taberlet 1994) and was frequently used in relationships studding among genus (Plunkett et al., 1997) and among species (Kajita et al., 1998).

This work aims to find out the level of chloroplast DNA sequence variation in Tunisian pistachio and to establish relationships among a set of cultivars using *trn*L-*trn*F intergenic spacer.

2. Material and methods

2.1. Plant material

Traditional pistachio cultivation zones have been prospected in the Centre and South of Tunisia. A total of 15 varieties of *P. vera* trees collected from 3 sites were considered in this study (Fig. 1). Plant materiel consists of young leaves sampled from adult trees. Samples were frozen at -80°C until use for DNA purification.

Fig. 1. Tunisian map showing the sampling sites of *P. vera*

2.2. DNA extraction and amplification

DNA extraction was carried out using the QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instruction. DNA integrity was checked in Agarose mini-gel electrophoresis as described by Sambrook et al. (1989). Total genomic DNA was preserved at -20 °C until processing.

PCR amplification protocol for the *trn*L-*trn*F spacer, non-coding region of chloroplast DNA, was previously described by Taberlet et al. (1991). This region was amplified using forward and reverse primers namely; 'e' (5'GGTTCAAGTCCCTCTATCCC-3') and 'f' (5'ATTTGAACTGGTGACACGAG-3'). PCR reactions were carried out in 25 µL reaction volume, with 1X buffer, 2 mM MgCl2, 0.2 mM dNTP, 2 µM of each primer, 1U Taq polymerase and 3 µL of DNA. PCR was conducted using the following program: initial denaturation 4 min at 94°C; 35 cycles each one consisting of denaturation 1 min at 94°C, primer annealing for 1 min at 50°C, extension for 2 min at 72°C, and final elongation with 10 min at 72°C. Agarose-gel electrophoresis (1.5%) and ethidium bromide staining were used to check the PCR products.

2.3. DNA sequencing

After amplification, excess of primers and dNTPs were removed by purification using the Wizard SV Gel PCR Clean-up system Kit according to the manufacture's instruction (Promega, WI, USA). The purified PCR products for the *trn*L-*trn*F were sequenced according to the automated Sanger method (Sanger et al., 1977) using sequencer ABI PRISMTM310 Genetic Analyser

(Applied Biosystems). PCR products were sequenced using Forward primer.

2.4. Analysis

Sequences were aligned using ClustalW (Thompson et al., 1994) implemented in Bioedit (Hall 1999). Nucleotide composition and the observed mutational event ratio (*R*) were calculated with MEGA4 program (Tamura et al., 2007).

Phylogenetic reconstruction was performed using *Neighbor Joining* (*NJ*) in MEGA4 program (Tamura et al., 2007). Support for the nodes was obtained using a bootstrapping procedure. The *NJ* tree was rooted using published sequences of *Mangifera indica* L. (GenBank Accession: JX185679).

Nucleotide and haplotype diversities (π, *Hd*) were calculated using the DnaSP software (Librado & Rozas 2009).

Differentiation between populations was estimated with the θ estimator of F_{ST} using ARLEQUIN (Excoffier & Lischer, 2010), and the null hypothesis of no differentiation was tested by performing 10 000 permutations among individuals between populations. F_{ST} indices calculated using both genetic distances (based on the observed number of pairwise differences) and haplotypic frequencies. The historical population expansion events within *P. vera* populations were investigated by an analysis of the mismatch distributions. Since these tests were found to be conservative, we also computed Tajima's *D*, Fu's *Fs* and *R2* of Ramos-Onsins neutrality tests implemented in DnaSP (Librado & Rozas 2009).

Gene flow (N_m) was estimated with mean number of migrants per generation among populations. Values of N_m were calculated with 1000 data permutations using the software DnaSP (Librado & Rozas 2009).

3. Results

3.1. trnL-trnF spacer sequences variation

Using specific primers (e and f) of the *trn*L-*trn*F spacer, PCR products are bands of about 421 bp for all the studied cultivars. *BLAST* showed that the obtained sequences have a maximum of homologies (99%) with *P. vera* L. *trn*L-*trn*F intergenic spacer of chloroplast DNA. A high level of polymorphism was reported. In fact, 9 haplotypes were detected in the 15 studied pistachios. Overall, haplotype diversity was *Hd* = 0.933±0.001 and the nucleotide diversity was π = 0.0294 ±0.0002.

All populations presented nearly similar and height values of haplotype diversity. The nucleotide diversity was height in Sid-Bouzid and Gafsa and low in El-Guetar (Table 1). Among the 421 bp sequenced, 34 nucleotide sites were variable and 26 were parsimony informative. The nucleotide composition percentages were 30% (A), 31.9% (T), 21.2% (C) and 16.9% (G) (Fig. 2). Therewith, the percentage of GC, at this region, was 38% and 62% for AT (Fig. 2). In the other hand, a low transitional/transversional ratio (*R* = 0.592) occurred in the *trn*L-*trn*F spacer and the transition/transversion rate ratios were k_1 = 1.914 (purines) and k_2 = 2.207 (pyrimidines).

Table 1. Estimates of genetic diversities and neutrality tests of *P. vera* populations. (*P<0.05).

	Diversity indices				Neutrality tests		
	n	*Nh*	*Hd*	*Π*	*D*	*Fs*	*R2*
Sidi-Bouzid	5	3	0.800	0.0123	0.109	2.385	0.272
Gafsa	5	3	0.700	0.0137	1.490	2.608	0.276
El-Guetar	5	3	0.800	0.0028	-1.048*	-0.186*	0.266
Total	15	9	0.933	0.0294	0.259	1.575	0.171

3.2. Differentiation genetics

We considered genetic relationships among pistachio varieties by taking into account the variation observed in the *trn*L-*trn*F spacer sequences. The pairwise sequence divergence ranged from 0.000 to 0.049. We estimated zero distance between [SB4-SB5] and between [GF1-GF8] cultivars. These pistachios showed a perfect sequence homology of the *trn*L-*trn*F spacer. The highest genetic distance of 0.049 is observed between cultivars GF9 and GT3 indicating the present of nucleotide differences in the studied sequences. The rest of cultivars have intermediate genetic distances.

The overall estimate of genetic divergence using F_{ST} statistics revealed significant genetic differentiation (P < 0.05) between all population pairs. The F_{ST} estimates showed a highly significant differentiation between pairs of population with $F_{ST} \geq 0.642$ and representing incredibly high inter-population divergence (Table 2).

Table 2. Pairwise F_{ST} and *Nm* estimates based on spacer sequences among the populations of *P. verra*. (F_{ST} under the diagonal and *Nm* above the diagonal) (*P<0.05).

	El-Guetar	Gafsa	Sidi-Bouzid
El-Guetar	**0**	0.07*	0.07*
Gafsa	0.788*	**0**	0.14*
Sidi-Bouzid	0.789*	0.642*	**0**

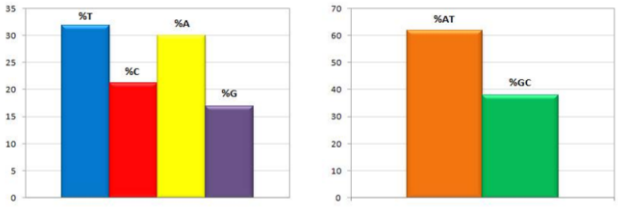

Fig. 2. Average percentage of nucleotide composition for all dataset.

Haplotypes of the *trn*L-*trn*F spacer obtained made it possible to make highly sustained phylogenetic trees (Bootstrap values between 46% and 99%). The *NJ* tree, illustrated in Fig. 3, supported the varieties organization into two main clusters. The first group was composed by cultivars collected from Gafsa and Sidi-Bouzid. All the other remaining cultivars (from

El-Guetar) were ranged in the second group. On the topology of the *NJ* tree of the *trn*L-*trn*F spacer sequences was structured according to geographical origin. Indeed, the three populations (El-Guetar, Gafsa and Sidi-Bouzid) were significantly diverged.

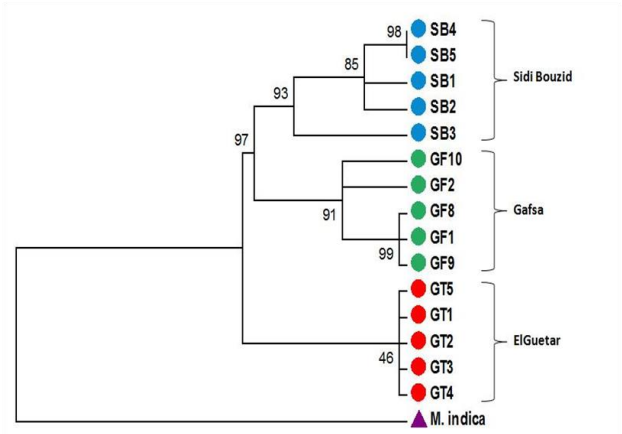

Fig. 3. *NJ* tree obtained from *P. verra* sequences. Populations names: SB = Sidi-Bouzid, GF = Gafsa, GT = El-Guetar

3.4. Demographic histories and gene flow

To test the population expansion, mismatch distributions of pairwise nucleotide differences were performed. We usually found a multi-modal distribution of differences between haplotypes in samples drawn from populations at demographic equilibrium, whereas the distribution was usually unimodal in populations that have gone through a recent demographic expansion. The mismatch frequency spectra for the populations studied are shown in Fig. 4. Overall, the *trn*L-*trn*F spacer mismatch distribution is multimodal characteristic of a stable population. The observed distribution was also multimodal for the populations of Sidi-Bouzid and Gafsa as a result of constant population size (Fig. 4). This was supported by the selective neutrality tests: *Tajima's D* and *Fu's Fs* statistic as well as *R2* tests were positive and insignificant for the total studied sample and for Gafsa and Sidi Bouzid populations (Table 1). The observed variation patterns provide evidence that pistachio trees have been undergoing stable history.

However, El-Guetar population showed a unimodal mismatch distribution suggesting that this population has been expanded in the past. The significant negative values of *Fs* and *D* supported a recent demographic expansion of this population or selective sweeps affected the studied marker.

For gene flow estimation, N_m was low among populations from South localities (Gafsa-ElGuetar) and between Sidi-Bouzid and El-Guetar (Table 1). This reflects low levels of connectivity between these population pairs. While N_m was modernly high between Gafsa and Sidi-Bouzid (Table 1), indicating that few differentiations could be established among them.

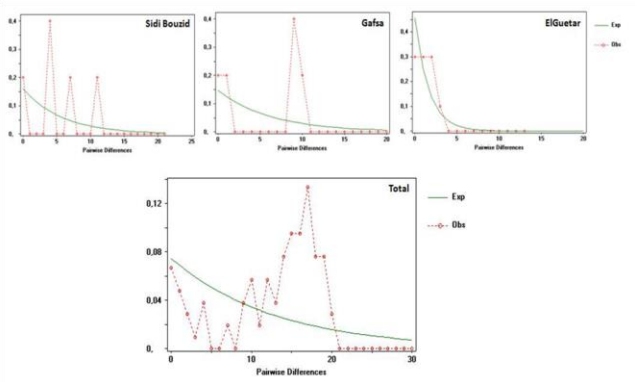

Fig. 4. Pairwise mismatch distribution of *P. verra*.

4. Discussion

In this frame, the length of the *trn*L-*trn*F intergenic spacer was 421 bp for all the studied pistachio cultivars which is situated in the registered size range to several Angiosperms taxa (Borsch et al., 2003). Similar sizes of the same ADNcp region were also indicated at *Pinus* (467-471) (Chen et al., 2002) and *Ficus carica* (430-474) (Baraket et al., 2009).

The GC and AT percentages detected in our study were similar to those obtained in *Ficus carica* L. (Baraket et al., 2009) and in *Phoenix dactylifera* L. (Sakka et al., 2013; Rhouma-Chatti et al., 2014). The relatively high AT average value (62 %) in *trn*L-*trn*F spacer can explain the low proportion of transversions (ti/tv = 0.592). This result corroborates the finding in angiosperm species with ratio ti/tv below 1 for any of the examined groups (Bakker et al., 2000). In several substitution studies in chloroplast non-coding regions, it has been found that in a context of high AT content, the transversions occurred with a higher frequency than in a high GC context (Bakker et al., 2000).

NJ tree, based on cytoplasmic sequences diversity, was clearly structured with a biogeography origin. These results were in agreement with those obtained by analyzing *trn*L (UAA) region of chloroplastic DNA (Choulak et al., 2015).

The F_{ST} values support this disjunction between populations. This estimator was highly significant divergence between Sidi-Bouzid and El-Guetar, but very low between Sidi-Bouzid and Gafsa. Gene flow estimates maintain this observation, N_m value was high between this two last populations indicated high connectivity between them. In theory, high N_m values within populations associated with high levels of genetic diversity across geographic regions imply a good migratory model of random samples recruited from previous living populations and vice versa (Wade & McCauley 1988). This easy connection makes high breeding possible and gene exchanges. This could be

the result of the relative proximity of geographical sites.

Even though, the genetic diversity was higher than genetic differentiation. This observation could be maintained by gene flow since gene flow has a homogenizing effect on the included populations. Certainly, human impact on these regions may be the reasons for these finding. Actuality, in these localities, cultivars are named by the farmers after continuous selection, cloning and exchange of varieties.

Otherwise, it is widely accepted that breeding systems and pollen or seeds dispersal mechanisms in particular are associated with levels of genetic variation within and among populations (Hamrick & Godt, 1996). Although wind dispersal of pollen or seeds by insects may be highly localized allows them to disperse over long distances (kilometers). The long distance dispersal could maintain gene flow among populations, and even small amounts of gene flow may have significant consequences for the homogenization of genetic variation among populations.

Same interpretations are cited by Guenni et al., (2016) where Tunisian pistachio presented the maximum of variability within the different geographic groups (Kasserine, Gabes and Sfax). These populations showed a large genetic diversity and low level of genetic differentiation between them. These observations were justified by the high gene flow (N_m = 1.127).

Gene flow estimations N_m were very low among populations El-Guetar/Sidi-Bouzid and El-Guetar/Gafsa and vice versa. Gene flow could also be limited by the surrounding geography. The Gafsa Mountains extending to the west (1165 m average) represent a long geographic barrier that could be a major obstacle to pollen and seeds propagation. This may prevent gene flow between populations located in the both side of Gafsa Mountains.

If not, this low within-population genetic diversity between El-Guetar and the two other populations could be explained by their specific geographical location and the direction of dominant winds in the area which make it an isolated population (Job, 1992).

Populations from Sidi Bouzid and Gafsa were in mutation/drift equilibrium characteristic of a stable population. The multimodal distribution and the neutrality tests confirmed this hypothesis. This represents strength because the stability and the height genetic diversity ensure the exhaust to natural and anthropological perturbations. Investigation of genetic diversity and relationships within Tunisian pistachio cultivars are necessary to highlight the priorities for the conservation approaches of plant genetic resources.

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