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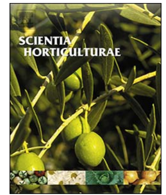

Molecular characterization of *Pistacia atlantica* Desf. subsp. *atlantica* (Anacardiaceae) in Algeria: Genome size determination, chromosome count and genetic diversity analysis using SSR markers

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ABSTRACT

The current study aims at the molecular characterization of *Pistacia atlantica* Desf. Subsp *atlantica* in Northwestern Algeria. The genome size estimation was performed for the first time for the species using flow cytometry on 97 samples. The flow cytometry analysis led to estimate a very small genome (2C = 1.21 ± 0.02 pg). Meristematic somatic cells of male floral buds showed the best prometaphase which on ($2n = 2x = 30$) chromosome complement was observed. Two chromosomes bearing satellites showed evident and large secondary constrictions. sixty-one genotypes were characterized using 6 SSR microsatellites. A total of 26 alleles were amplified. Allele frequencies ranged from 0.008 to 0.68. Eight rare alleles (30.8%) were observed. The observed heterozygosity (Ho) ranged from 0.35 to 0.67, while the expected heterozygosity (He) ranged from 0.48 to 0.77. The loci Pislen 526 and Pislen 114 were the most polymorphic with 7 and 6 observed alleles respectively. Pislen 526 and PKATG014 were the most informative markers. Cluster analysis based on the euclidean distance using (UPGMA) led to observe divergences between genotypes within site and between sites. The current study is the first molecular characterization of the subspecies; it indicated an evident diversity due to a genetic flow between populations. More genetic diversity studies are required to learn about the structure and the evolution of natural *P. atlantica* subsp. *atlantica* using more SSR markers and more accessions from other areas.

1. Introduction

The Anacardiaceae is a virtually cosmopolitan family in the Sapindales (Gadek et al., 1996) that comprises about 70 genera and over 600 species (Mitchell and Mori, 1987). Several members of the family such as cashew (*Anacardium occidentale* L.), mango (*Mangifera indica* L.) or pistachio (*Pistacia vera* L.) are cultivated for their edible fruits. Sumac (*Rhus coriaria* L.) produces small red fruits wildly used as a spice mainly in the Middle East. Other species produce edible fruits but are still underutilized; among them, we can include *Pistacia atlantica* Desf. that produces energetic fruits. Monjauze (1982) supposed that Atlas pistachio fruit interested prehistoric man in North Africa. In Algeria, the fruit knows many vernacularly names. It is called in the East 'El khodiri' and in the West 'goddim, khoddir, for the green fruit or "kohhil" for the black one (El Zerey-Belaskri, 2016). Zohary (1952) considered the genus *Pistacia* to comprise eleven species. Subsequently, although many other taxa were described [more than 55 names figure in the 'International Plant Name Index' list (IPNI, 2010)], most of them are not recognized and the taxonomic position within the genus remains discussed for several taxa (Al-Saghir, 2006; Xie et al., 2014). Except the North American species *P. texana* and *P. mexicana*, *Pistacia* species are distributed mainly within the Mediterranean region, Western and Central Asia and the Middle East. *Pistacia atlantica* is one of the most widely distributed wild species of the genus. It occurs from the Canary Islands to Pamir Mountains (Zohary, 1952). In Algeria, Atlas pistachio trees are characterized by their hemispherical silhouettes,

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vigorous trunks and their dense foliage (Mojauze, 1980; El Zerey-Belaskri, 2016). Desfontaines (1799) was the first who described the species in his '*Flora atlantica*' while numerous keys were afterwards proposed (Zohary, 1952; Quezel and Santa, 1963; Monjauze, 1968; Al Yafi, 1978). Recently, El Zerey-Belaskri and Benhassaini (2016) updated *P. atlantica* subsp. *atlantica* key describing more morphometric and morphological traits and new features. The leaves, reaching 24.5 cm long and 21.9 cm wide, are composed of one to nine leaflet pairs (El Zerey-Belaskri and Benhassaini, 2016). Atlas pistachio populations occur under different climatic conditions ranging from humid to arid climate (*sensu* De Martonne) and from sub-humid to arid bioclimate (*sensu* Emberger) (El Zerey-Belaskri and Benhassaini, 2016). The species is known for its plasticity and its distinctive xerophytic character (Boudy, 1950; Monjauze 1980; Zohary 1996). The taxon developed several adaptive strategies which have been subject of interest for macro and micro-morphological studies mainly in Algeria (Belhadj et al., 2008; Ait Said et al., 2011; El Zerey-Belaskri and Benhassaini, 2016).The morphological variability of *P. atlantica* in Algeria is a long-standing issue. Zohary (1952) suspected the presence (in the Northwest and the Northeast of Algeria) of a variety in which the leaves and the fruits are larger and look similar to those of the var. *latifolia* Zoh. Monjauze (1982) reported that the specimen of *Desfontaines herbarium* (DF-58/17; DF-58/20; see Bruns-Balogh, 1991) showed a microphyllous variety which is different from some specimens observed in the country. Monjauze (1982) reported also the possibility to have a different variety with round and black fruits referring to a certain resemblance to *P. atlantica* subsp. *kurdika* Zoh. (= *Pistacia eurycarpa* Yalt.) This resemblance was recently mentioned by El Zerey-Belaskri and Benhassaini (2016) in the region of Tlemcen (Nothwest Algeria). Thus, in the current study, natural populations of *P. atlantica* subsp. *atlantica* in a large area in Northwestern Algeria, where some populations had induced the curiosity of famous botanists (Zohary, 1952; Monjauze, 1982), were investigated through molecular characterization and intraspecific genetic diversity.

According to Gaston (2010), one of the most basic measures of genetic diversity is perhaps genome size. Genome size is a highly important factor in plant biodiversity, especially in systematics, evolutionary biology, physiology, and ecology (Ohri, 1998, 2005). Flow cytometry is a laser-based, biophysical technology employed to estimate by comparing the fluorescence emitted by an intercalating DNA fluorochrome of a sample together with a reference standard with known genome size. It is used in various molecular biology studies and it is considered as the method of choice for genome size determination studies (Catrice et al., 2006; Doležel et al., 2007; Loureiro et al., 2010). Furthermore, it is considered as a fast and relatively cheap alternative to other molecular tools (D'Hondt et al., 2011).

Chromosome count is still an important element in evaluation relationships and deducing phylogenetic sequences in Angiosperms. In spite of cytological and evolutionary importance of chromosome number (Raven, 1975; Stuessy, 1990), these kinds of studies are scarce in the genus *Pistacia* and most of the works have been carried out in *P. vera*. The first chromosome investigation in the genus was done by Zohary (1952) who reported three chromosome counts ($2n = 24$ in *P. lentiscus*), ($2n = 28$ in *P. atlantica*), and ($2n = 30$ in *P. vera*). Later, chromosome counts were performed by others researchers showing sometimes different chromosome numbers in the same species ($2n = 30$ in *P. atlantica*; Ila et al., 2003). Often, poor protocols are used and are faced with difficulties such as the small chromosome size but also to the germination and root development constraints, a circumstance that also prevents building karyotypes (Vogt and Aparicio, 1999; Fasihi-Harandi and Ghaffari, 2001; Ila et al., 2003).

Intraspecific genetic diversity can be assessed using morphological and molecular markers. However, morphological markers are influenced by environmental conditions (El Zerey-Belaskri and Benhassaini, 2016) which limit their use in genetic diversity studies whereas DNA molecular markers are independent of environmental conditions or developmental stage and show a high level of polymorphism (Ainsworth et al., 1996). Thus, molecular markers are useful tools for analyzing genetic variation and provide efficient means to link phenotypic and genotypic variation. These markers play a key role in the studies of genetic variability and diversity and can be used to select traits that are difficult to measure using phenotypic assays (Varshney et al., 2005; Appleby et al., 2009; Kalendar et al., 2011). Among the genetic marker systems, microsatellites (Litt and Luty, 1989) or Simple Sequence Repeats (SSRs) (Jacob et al., 1991), have been the preferred choice for various applications, such as variety identification, genetic diversity evaluation, phylogenetic analyses, genetic map construction, marker-assisted selection and comparative mapping (Beckmann and Soller, 1990; Gupta and Varshney, 2000; Parida et al., 2010). SSR markers have been used as an effective tool to evaluate genetic diversity and to throw light on the phylogenetic relationships in the *Pistacia* genus (Albaladejo et al., 2008; Kafkas et al., 2009; Pazouki et al., 2009, 2010; Vendramin et al., 2009, 2010; Arabnezhad et al., 2011). However, no such reports on genetic diversity using molecular markers were available at the intraspecific level in *Pistacia atlantica* in North Africa where the subspecies *Pistacia atlantica* subsp. *atlantica* occurs.

In the current study, the estimation of the genome size of *Pistacia atlantica* subsp. *atlantica* checking intra-specific variability in the nuclear DNA amount, the chromosome counts and the evaluation of the genetic diversity and genetic relationships in natural populations of *Pistacia atlantica* subsp. *atlantica* in Northwest Algeria have been performed. These studies complement previous reports of morphological (El Zerey-Belaskri and Benhassaini, 2016) and chemical (El Zerey-Belaskri et al., 2017) characterizations in the same study area.

2. Material and methods

2.1. Plant material

The study area is located in Northwest Algeria. Natural populations were investigated along a northwest-northeast transect of more than 230 km, stretching from the extreme North-West of Algeria (the region of Maghnia) to the region of Mascara. Seventeen sites were prospected: Bétaim (BTM), Chigguer (CH), Ourit (OR), Ain Fezza (AF1 + AF2), Ouled Mimoun (OM), Tallout (TA); Ben Badis (BB), Sidi Bel Abbes (SBA), Mustpha Ben Brahim (MBB1 + MBB2), Sfisef (SF1 + SF2), Beni Tala (BT), Graia(GR), Bouhanifia (BH), Trois rivières (3R). These sites were previously considered for morphological variability (El Zerey-Belaskri and Benhassaini 2016; see also data about geographical and bioclimatic description) and chemical diversity in the same species (El Zerey-Belaskri et al., 2017).

For genome size determination, young, healthy leaves were collected from male and female trees totaling 97 samples (3–8 samples per site) and kept in hermetic plastic bags at 4 °C. For chromosome studies, male and female floral buds were collected from SBA population in the early flower stage (first March). Young floral buds were fixed in absolute ethanol: acetic acid (3:1). Samples were maintained at 4 °C until use (up to two days). For the genetic diversity analysis, young woody shoots were collected from 2 to 6 genotypes per site (male and female trees). The samples were kept in sterile hermetic plastic bags and maintained at 4 °C until use. Voucher specimens (coded from N° Ana125 to N° Ana216) are deposited in the herbarium of the (Laboratoire de Biodiversité Végétale: Conservation and Valorisation) Department of Environmental Sciences (University of Sidi Bel Abbes, Algeria).

2.2. Genome size estimation

Genome size was estimated using flow cytometry following the method of Galbraith et al. (1983). In a glass Petri dish, approximately 50 mg of young leaves of the sample species and of the internal reference standard were chopped with a razor blade in 1 ml of WPB buffer

Table 1
Used Microsatellites: SRR name, primer sequence, repeat motif, annealing temperature, reference.

SSR name	F: Forward primer sequence (5′ → 3′) R: Reverse primer sequence (5′ → 3′)	Repeat motif	Ta°C	Reference
PKATG014	F: AGCATCTGCCTTAGCACTAGAC R: CGTCAACACCGCAAGTATC	$(ATC)_5$–NN–$(ATC)_3$	60	Arabnezhad et al., 2011
PKATG016	F: CCTTGTTGAGGACATGATGAT R: GAGTTCCTCCAAACCAGACG	$(AGC)_6$	60	Arabnezhad et al., 2011
Pislen R05	F: GGATTTTCCTCTACCATCCT R: GAAAACGAGGTTATTGGTCA	$(CCG)_5CTG(CCG)_2$	50	Albaladejo et al., 2008
Pislen 526	F: CAGTGAGGGTAAAAATGGAA R: ATTACCATTTTGAGGGAACC	$(GGT)_2GCT(GGC)_6$	50	Albaladejo et al., 2008
Pislen 114	F: GTGACTTTGGTTGGTGTTTT R: CTGCTTTGACTGGATTTGAT	$(GTT)_4$GCTGTTGCT	50	Albaladejo et al., 2008
EPVM 039	F: AAGCCCGATCAGTACAGTGG R: TGATCCAGGGAATGAGGTTC	$(ACA)_5$	54	Vendramin et al., 2010

(0.2 M Tris-HCl, 4 mM $MgCl_2.6H_2O$, 1% Triton X-100, 2 mM EDTA Na_2. $2H_2O$, 86 mMNaCl, 10 mM metabisulfite, 1% PVP-10, pH adjusted to 7.5 and stored at 4 °C; Loureiro et al., 2007). Nuclear suspensions were then filtered through a 50 µm nylon filter and 50 µg.mL-1 of propidium iodide (PI, Fluka, Buchs, Switzerland) and 50 $\mu g.mL^{-1}$ of RNAse (Fluka, Buchs, Switzerland) were added to sample tubes to stain the DNA and avoid staining of double stranded RNA, respectively.

Samples were kept at room temperature and were analyzed within a 5 min period in a PartecCyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) equipped with a 532 nm green solid-state laser, operating at 30 mW. Integral fluorescence and fluorescence height and width emitted from nuclei were collected through a 620 nm band-pass interference filter. Relative fluorescence intensity (FL) histograms were obtained and evaluated using FloMax software (v2.5; Partec GmbH, Münster, Germany). Furthermore, FL vs. time and FL vs. side light scatter in logarithmic scale cytograms were also obtained. In the latter cytogram, a region of interest comprising mostly the isolated nuclei was defined. The FL histogram in linear scale was gated with this region. At least 1500 nuclei in both sample's and standard's G1 peaks were analyzed per sample (Suda et al., 2007). The holoploid genome size in pg (2C; *sensu* Greilhuber et al., 2005) of each individual was estimated using the following formula: GSs = G1 s/G1r _ GSr where GSs and GSr are the genome size of sample and reference nuclei respectively, and G1 s and G1r are the mean G1 fluorescence of sample and reference nuclei respectively. The reference standard was grown from seeds of *Solanum lycopersicon* L. cv. Stupicke (2C = 1.96 pg; Doležel et al., 1992) Seeds were sown in plastic pots filed with commercial peat and pots were kept in a climate chamber operating at 20 ± 2 °C, with a photoperiod of 16 h/8 h (light/dark) and a light intensity of 530 ± 2 µE/m2/s. Descriptive statistics of genome size were calculated for each site (mean, standard deviation of the mean and coefficient of variation of the mean). Differences in genome size were assessed using a non-parametric Kruskal–Wallis one-way analysis of variance (ANOVA).

2.3. Chromosome count

Squash preparations for chromosome count studies were carried on the fixed buds according to Schwarzacher and Heslop-Harrison (2000) with some modifications. Briefly, fixed male floral buds were washed for 10 min (on slowly agitation) in 2–5 ml in 1 × Enzyme buffer, diluted from 10 x Enzyme buffer (100 mM citric acid, 100 mM tri-sodium-citrate (10 × stock, adjust to pH 4.8). Buds were washed twice to remove the fixative. Buds were transferred into a small petri dish with10 ml enzyme solution 5.5% (w/v) cellulase from *Aspergillus niger* (Sigma) or a mixture of 1.8% Calbiochem (72units/ml) and 0.6% 'Onozuka' RS cellulase and 5% (v/v) pectinase from *Aspergillus niger* (ICN Biomed)) and digested at 37 °C until the material was soft (20–30 min). Buds were stored in 1 x enzyme buffer and a bud was transferred to a pre-cleaned slide in 1 drop (10–30 µl) of 45% acetic acid. The bud tissues were dissected with needles under the stereo microscope (×10) until separation of cells. A coverslip was applied and a gentle tapping with a needle was done to disperse the material. Lastly, pressure was applied by the thumb to improve chromosome separation. The slides were checked under a phase contrast microscope (×100; ×400). Quick freezing by liquid CO_2 was used to remove the coverslip and attach the material to the slide. Selected frozen-dried slides were mounted in glycerol: water (1:1) and visualized under phase contrast. Images were collected using an AxionCam digital camera (Zeiss) controlled by AxioVision 3.0, and processed using the Adobe Photoshop 5.0 (Adobe Systems, Mountain View, CA).

2.4. Genetic diversity analysis

2.4.1. DNA extraction and SSR analysis

Genomic DNA extractions were performed according to Doyle and Doyle (1987), modified by Hormaza et al. (1998), with some additional modifications. Genomic DNA was extracted from approximately 50 mg of phloem tissue of young woody shoots, treated with liquid nitrogen and homogenized with 400 ml of extraction buffer (100 mMTris-HCl; 20 mM EDTA; 1.4 M NaCl; 2% CTAB, 1% PVP, 0.2% b-mercaptoethanol). The samples were incubated at 65 °C for 30 min to 1 h, mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 6000*g* at 4 °C for 15 min. The upper aqueous phase was recovered and mixed with 200 ml of cold isopropanol to precipitate the DNA. Then, the samples were kept at −80 °C for 30 min. The nucleic acid precipitation was recovered through centrifugation at 13,000*g* at 4 °C for 5 min and washed in 800 ml of 10 M ammonium acetate in ethanol for 30 min, dried and resuspended in 100 ml MTE buffer. DNA concentration and purity were calculated by measurement of absorbance using a spectrophotometer (INanodrop™ ND-1000).

2.4.2. SSR analysis

After many series of selection, 6 SSRs (Table 1) were finally selected since they provided the best amplification patterns with the material analyzed. PCR amplifications were performed in 15 µl vol. containing 16 mM $(NH_4)_2SO_4$, 67 mMTris-HCl pH 8.8, 0.01% Tween20, 2 mM $MgCl_2$, 0.1 mM each dNTP, 0.4 mM each primer, 25 ng genomic DNA and 0.5 unit of BioTaq™ DNA polymerase (Bioline, London, UK) on an I-cycler (Bio-Rad Laboratories, Hercules, CA, USA) thermocycler using the following temperature profile: an initial step of 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 50/54/60 °C (depending on each primer pair) and 1 min at 72 °C, and a final step at 72 °C for 5 min. The PCR products were analyzed by capillary electrophoresis in a CEQTM 8000 capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). Samples were denaturalized at 90 °C during 120 s, injected at 2.0 kV 30 s and separated at 6.0 kV during 35 min. Each reaction was repeated

twice in each run to ensure size accuracy and to minimize run-to-run variation.

2.4.3. Data analysis

For each SSR locus, allele size determination and the total number of alleles were determined in each accession. Putative alleles were indicated by the estimated size in bp. The following parameters were estimated for variability and genetic diversity analysis:

Number of alleles per locus (Na), effective number of alleles (Ne = 1/1 − He), observed heterozygosity (Ho) calculated as the number of heterozygous genotypes over the total number of genotypes analyzed for each locus, expected heterozygosity (He = 1-Σ p_i^2 where p_i is the frequency of the ith allele, Nei, 1973), Wright's fixation index (F = 1 − Ho/He) (Wright, 1951) and the probability of identity (PI = 1-Σ p_i^4 + ΣΣ$(2\, p_i\, p_j)^2$ where p_i and p_j are the frequency of the ith allele and j^{th} allele respectively) that measures the probability that two randomly drawn diploid genotypes will be identical assuming observed allele frequencies and random assortment (Paetkau et al., 1995). Significant deviations from Hardy-Weinberg equilibrium HWE ($p < 0.01$) at individual loci were tested using a Markov chain method. A marker index was calculated for the SSR markers to characterize the efficiency of each primer combination (PC) to detect polymorphic loci among the accessions. As such, the marker index was the sum of the polymorphism information content (PIC) values for all the selected markers produced by a particular PC. The PIC value was calculated using the formula PIC = 1-Σ p_i^2, where p_i is the frequency of the ith allele (Smith et al., 1997). Wright's fixation index for interpopulational differentiation (F_{ST}) (Wright, 1931; Malécot, 1948) is a measure of population differentiation due to genetic structure. Analysis of Molecular variance (AMOVA) was performed for the accessions using the program ARLEQUIN ver. 3.01 (Excoffier et al., 2005). F-statistics were used to estimate the proportion of genetic variability found among populations (F_{ST}), among populations within groups (F_{SC}) and among groups (F_{CT}). The AMOVA was run to estimate the genetic differentiation between populations grouped according to their geographical locations (Fig1). Significance associated with the fixation index was evaluated through random allelic permutation procedures (10,100 permutations).The program ARLEQUIN ver. 3.01 (Excoffier et al., 2005) was used to calculate Ho (observed heterozygosity), He (expected heterozygosity), allele frequencies (considering $p < 0.05$, $p > 0.9$ as rare and fixed alleles respectively), departure from HWE and *F*-statistics indexes (Wright's fixation index for interpopulational differentiation). POPGENE 1.32 software (Yeh et al., 1997) was used to calculate Na (Number of alleles per locus), Ne (effective number of alleles), and F (Wright's fixation index, Wright, 1951). Probability of identity (PI) was estimated by IDENTITY 1.0 (Wagner and Sefc, 1999). Genetic relationships among the genotypes studied were calculated using UPGMA cluster analysis of the similarity matrix obtained from the proportion of shared amplification fragments (Nei and Li, 1979) with NTSYSpc 2.11 (Exeter Software, Stauket; NY, USA). The cophenetic correlation coefficient was computed for the dendrogram after the construction of a cophenetic matrix to measure the goodness of fit between the original similarity matrix and the dendrogram. The reliability of the dendrogram groups was assessed by bootstrap resampling analysis using 2000 replications by the program Treecon 1.3b (Van de Peer and De Watchter, 1994).

3. Results

3.1. Genome size determination

The data obtained from FCM analysis permits us to estimate the genome size of *Pistacia atlantica* subsp. *atlantica* from seventeen sites in natural populations (Table 2). A good overall quality of the results is shown by the coefficient of variation (CV) values of G1 peaks and by the backround debris with mean CV values (2.08%, < 5%) observed in all the samples. The IP fluorescence intensity (Fig. 2) showed that all the analyzed samples are diploid (2*n* = 2x). The flow cytometry analysis led to estimate a very small genome (2C = 1.21 ± 0.02 pg). Among all samples, the genome size varies between 1.15 pg to 1.25 pg. The variance analysis in genome size (at α = 5%) revealed that no significant difference was observed (Between sites ANOVA: *P* = 0.06).

3.2. Chromosome count

Chromosome number was counted in several meristematic somatic cells of male floral buds since root meristematic cells were not efficient for this investigation. The results showed that *Pistacia atlantica* subsp. *atlantica* is a diploid with 2n = 30 chromosome complement (Fig. 3). Two chromosomes bearing satellites (connected with red lines) show evident and large secondary constrictions.

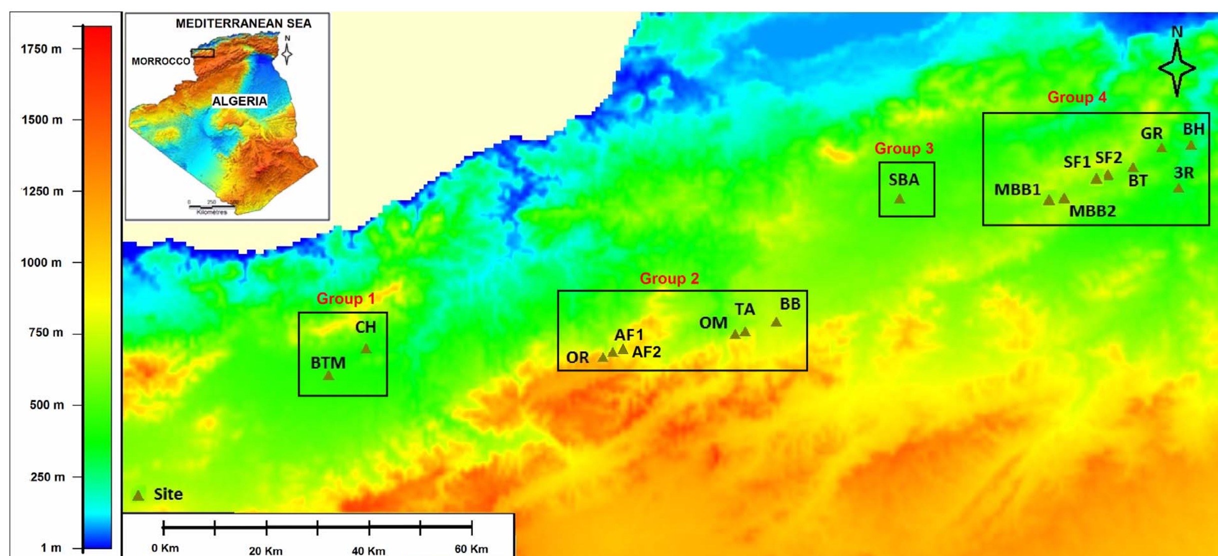

Fig. 1. *Pistacia atlantica* subsp. *atlantica* natural populations grouped into four groups according to the geographical location of the sites for Wright's fixation index for interpopulational differentiation (F_{ST}) estimation.

Table 2
Nuclear DNA content estimations in 97 samples of *Pistacia atlantica* subsp. *atlantica*: the values are given as mean and standard deviation of the mean of the holoploid genome size (2C, pg), the mean coefficient of variation (CV, %) of G0/G1 peaks, the supposed ploidy level (2), the total number of analyzed individuals per site (n/site).

Sites	Genome size (2C, pg)				n/site	*Pistacia atlantica* genome size estimation (2C, pg)	Ploïdy level[2]	Previous estimation
	Mean ± SD	CV	Min	Max				
BTM	1.2 ± 0.01	1.0	1.18	1.22	8	**1.21 ± 0.02**	2n = 2x	**First estimation**
CH	1.21 ± 0.02	1.4	1.15	1.22	8			
OR	1.2 ± 0.02	1.3	1.18	1.22	6			
AF1	1.2 ± 0.03	2.3	1.18	1.25	5			
AF2	1.22 ± 0.02	1.9	1.19	1.25	6			
OM	1.22 ± 0.03	2.2	1.17	1.24	6			
TA	1.21 ± 0.02	1.9	1.18	1.24	6			
BB	1.2 ± 0.03	2.4	1.15	1.22	6			
SBA	1.2 ± 0.03	2.2	1.15	1.24	6			
MBB(1 + 2)	1.23 ± 0.01	0.8	1.21	1.24	8			
SF1	1.23 ± 0.00	0.5	1.23	1.24	3			
SF2	1.2 ± 0.02	1.7	1.18	1.22	4			
BT	1.21 ± 0.03	2.2	1.18	1.24	7			
GR	1.2 ± 0.03	3.0	1.15	1.22	6			
BH	1.2 ± 0.02	1.5	0.02	1.22	6			
3R	1.21 ± 0.03	2.7	1.17	1.24	6			

Site codes: Bétaim (BTM), Chigguer (CH), Ourit (OR), Ain Fezza (AF1 + AF2), Ouled Mimoun (OM), Tallout (TA); Ben Badis (BB), Sidi Bel Abbes (SBA), Mustpha Ben Brahim (MBB1 + MBB2), Sfisef (SF1 + SF2), Beni Tala (BT), Graia(GR), Bouhanifia (BH), Trois rivières (3R).

3.3. SSR polymorphism analysis

A total of 61 genotypes of *Pistacia atlantica* subsp. *atlantica* natural populations in Northwestern Algeria, were characterized genetically using 6 SSRloci selected from available SSR loci in different species of *Pistacia,* such as *P. khinjuk* (Arabnezhad et al., 2011)*, P. vera* (Vendramin et al., 2010) and *P. lentiscus,* (Albaladejo et al., 2008).

A total of 26 alleles (4.33 alleles on average) were amplified ranging from 2 (Pislen R05) to 7 alleles (Pislen 526) for each SSR (Table 3). The loci Pislen 526 and Pislen 114 were the most polymorphic with 7 and 6 observed alleles respectively.The effective number of alleles ranged between 1.92 and 4.34 with an average of 2.41. The observed heterozygosity (Ho) ranged from 0.35 in Pislen 114–0.67 in Pislen 526 with an average of 0.49, while the expected heterozygosity (He) ranged from 0.48 in both EPV039 and Pislen R05 to 0.77 in Pislen 526 with an average of 0.55. The Wright's fixation index (F) was used to compare between the two heterozigosity parameters (Ho and He) and to measure heterozygote deficiency or excess according to Wright (1951, 1978). Except for the locus PKATG014, this index was positive for all the loci meaning a deficit of heterozygotes, with an average over all the SSRs of 0.13; however, none of the assayed loci displayed a significant heterozygote deficiency. F_{ST} index ranged from 0.06 to 0.26 with an average of 0.13. The analysis of SSR data showed through this index moderate and significant differentiation in allele frequencies between the populations by four loci (EPVM 039, Pislen R05, PKATG014 and Pislen 114) and great differentiation by two loci (Pislen 526, PKATG 016). EPVM 039 and Pilsen 114 showed significant departure from Hardy–Weinberg equilibrium (HWE), while no significant deviations ($P < 0.01$) from the Hardy–Weinberg proportions were found for the other loci. PIC values ranged between 0.48 and 0.77, of which 4/6 loci (Pislen 114, Pislen 526, PKATG014, PKATG 016) with PIC $>$ 0.5 could be classified as very informative markers (Guo and Elston, 1999). Pislen

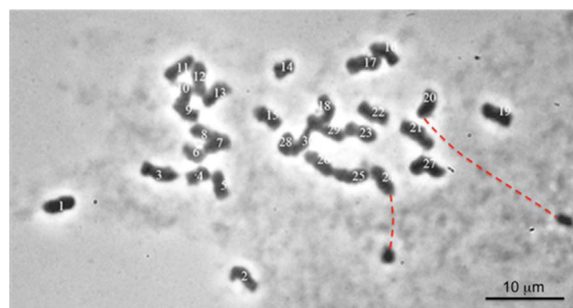

Fig. 3. Prometaphase chromosomes of *Pistacia atlantica* subsp. *atlantica* from Northwest Algeria (2*n* = 30). Red dashed lines connect the chromosome to its respective satellite through the secondary constriction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

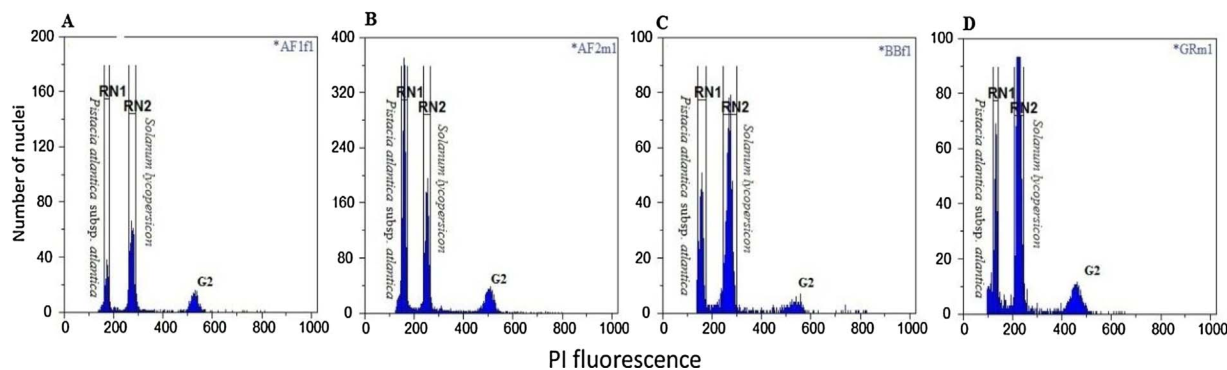

Fig. 2. Examples of flow cytometric histograms of relative Propidium Iodide (PI) fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Pistacia atlantica* subsp. *atlantica* (the G1 peak is shown by RN1, the third peak is the G2 peak) and from the internal reference standard *Solanum lycopersicon* (the peak is showed by RN2); *(tree code). The largest genome size (2C = 1.25 pg) was observed in a female individual from AF1 (A) and a male individual from AF2 (B). The smallest genome size (2C = 1.15 pg) was observed in a female individual from BB (C) and a male individual from GR (D).

Table 3
Summary statistics of genetic diversity: number of genotypes (Nbr gnt), Number of alleles per locus (Na), effective number of alleles (Ne), size range of alleles, Polymorphic information content (PIC), observed heterozygosity (Ho), expected heterozygosity (He), Wright's fixation index (F), Hardy-Weinberg equilibrium (HWE), probability of identity (PI), Wright's fixation index for interpopulational differentiation (F_{ST}).

LOCUS	Nbr gnt	Na	Ne	Size range	PIC	Ho	He	F	HWE	PI	F_{ST}
PKATG014	58	3	2.26	220–241	0.56	0.57	0.56	-0.02	0.08^{NS}	0.44	0.10
PKATG 016	61	4	2.05	122–136	0.51	0.46	0.52	0.10	0.50^{NS}	0.58	0.26
Pislen R05	61	2	1.93	171–180	0.48	0.48	0.48	0.01	1 ^{NS}	0.62	0.08
Pislen 526	61	7	4.34	120–134	0.78	0.67	0.77	0.13	0.21^{NS}	0.15	0.16
EPVM 039	61	4	1.92	252–258	0.48	0.43	0.48	0.11	*	0.55	0.06
Pislen 114	60	6	2	158–178	0.50	0.35	0.52	0.3	**	0.34	0.15
Mean	60.3	4.3	2. 41		0.53	0.49	0.55	0.13	0.3 ^{NS}	0.45	0.13

* significant at $P < 0.05$; ** significant at $P < 0.01$

526 and PKATG014 were the most informative markers. According to the probability of identity (PI), Pislen 526 was the most informative with (PI = 0.15) and Pislen R05 was the least informative (PI = 0.62). The total PI was 4.42×10^{-3}. Allele frequencies ranged from 0.008 to 0.68. Eight alleles (30.8%) are considered as rare alleles (allele frequency < 0.05) while no fixed alleles (allele frequency > 0.9) were observed. Four population groups were generated regarding their geographical area (Fig. 1). The first group includes sites situated in the extreme West of the study area (BTM, CH). The second group contains western sites (OR, AF1, AF2, OM, TA, BB). The third group includes one site (SBA) situated in the center of the study area. The fourth group includes the sites situated farther East of the study area (MBB1, MBB2, SF1, SF2, BT, GR, BH, 3R).

The AMOVA (Table 4) demonstrated that most of the molecular variation was distributed within populations since 86.28% of the variation was derived from variability within populations rather than among populations (9.55%).A low percentage of variance was observed among groups (4.17%). Moreover, the fixation index among groups (F_{CT}) was not significant in this analysis ($F_{CT} = 0.041$, $P = 0.05$), whereas it was significant among populations within groups ($F_{SC} = 0.099$, $P < 0.01$) and within populations ($F_{ST} = 0.137$, $P < 0.01$).

3.4. Similarity relationships among genotypes

The resulting similarity coefficients (Nei and Li, 1979) were used to evaluate the relationships among the genotypes. The similarity index values ranged from 0.00 to 0.95. The lowest genetic similarities ranged between (0 and 1.53) and are observed between couples from the sites (BTM and AF2): 10/29 (0.00), 10/27 (0.13), 10/23 (0.14), the sites (BTM and BB): 10/115, the sites (BTM and OM): 10/94 (0.33), 10/93 and the sites (BTM and SBA)10/123 (0.53). The pair of accessions 51/5 showed the highest similarity (0.95) and those samples correspond to genotypes from the sites BTM and MBB2. A high similarity (0.94) was also shown by the pairs 124/125 (SBA/SBA) and 43/39 (BT/BT). Cluster analysis based on Nei and Li's similarity coefficients using UPGMA is shown in Fig. 4. The cophenetic correlation coefficient between the original similarity matrix and the cophenetic matrix derived from the UPGMA dendrogram was 0.65. The results showed a first divergent subcluster containing two genotypes from the site BTM and one genotype from the site MBB1. The two sites are geographically very distant (Fig. 1). For the rest of the clusters, and except for the two groups selected (Group I and Group II) in Fig. 4, the studied genotypes were not grouped either according to their sites of origin neither according to the geographical locations. The clusters contain genotypes from different sites even geographically distant. Although the subcluster called group I retains 6 genotypes from the sites SBA and 3 genotypes from the site 3R both sites are distant from each other by more than 50 km. However, the subcluster called group II contains genotypes from the two sites AF2 and OM which are closer (Fig. 1). The rest of the genotypes in the dendrogram were clustered with different origin intermixed.

4. Discussion

4.1. Genome size determination

Chromosome studies are often useful in suggesting taxonomic and phylogenetic relationships (Raven, 1975; Stuessy, 1990). Previous reports from cytogenetic studies on *Pistacia atlantica* were limited to chromosome numbers. In this work, the genome size in this species is reported for the first time. In the current study, the coefficient of variation (CV) values showed an acceptable percentage. The CV has been considered as a significant statistical parameter in FCM, indicating the quality of nuclei suspensions (Doležel and Bartos, 2005). The authors agreed that CVs below 5% are adequate for FCM assessments in plants. According to the genome size categories defined by Leitch et al. (1998), the species is qualified having a very small genome when 2C ≤ 2.8 pg. A small genome size was also observed by (Siljak-Yakovlev et al., 2010) in the genus *Pistacia* for *P. lentiscus* (2C = 1.19 ± 0.03 pg) and *P. therebinthus* (2C = 1.14 ± 0.02 pg). Although, the amount of DNA per chromosome set is known to be a fairly constant characteristic of a species, it will be interesting to use FCM to determine the other subspecies which occur mainly in Asia. Genome size is associated with several phenotypic, physiological and/or ecological characteristics (Bennett 1972; Loureiro et al., 2010). Very little data is available regarding the genome size in the genus *Pistacia* and in the Anacardiaceae family; further investigations may provide more information on the evolutionary histories of these taxa and their distribution in the region (Bennett 1972; Loureiro et al., 2010).

Table 4
Analysis of Molecular variance (AMOVA) partitioning genetic variability within and among groups/populations grouped according to geographic distribution ($p < 0.01$).

Source of variation	degree of freedom	Sum of squares	Variance components	Percentage of variation	Statistics	P
Among groups	3	14.8	0.07	4.17Va	$F_{CT}=0.041$	0.05
Among populations within groups	11	28.94	0.16	9.55Vb	$F_{SC}=0.099$	0
Within populations	107	153.16	1.43	86.28Vc	$F_{ST}=0.137$	0
Total	121	196.9	1.66			

Probabilities were derived from 10,100 permutation tests. Va = 0.069; Vb = 0.158; Vc = 1.431.

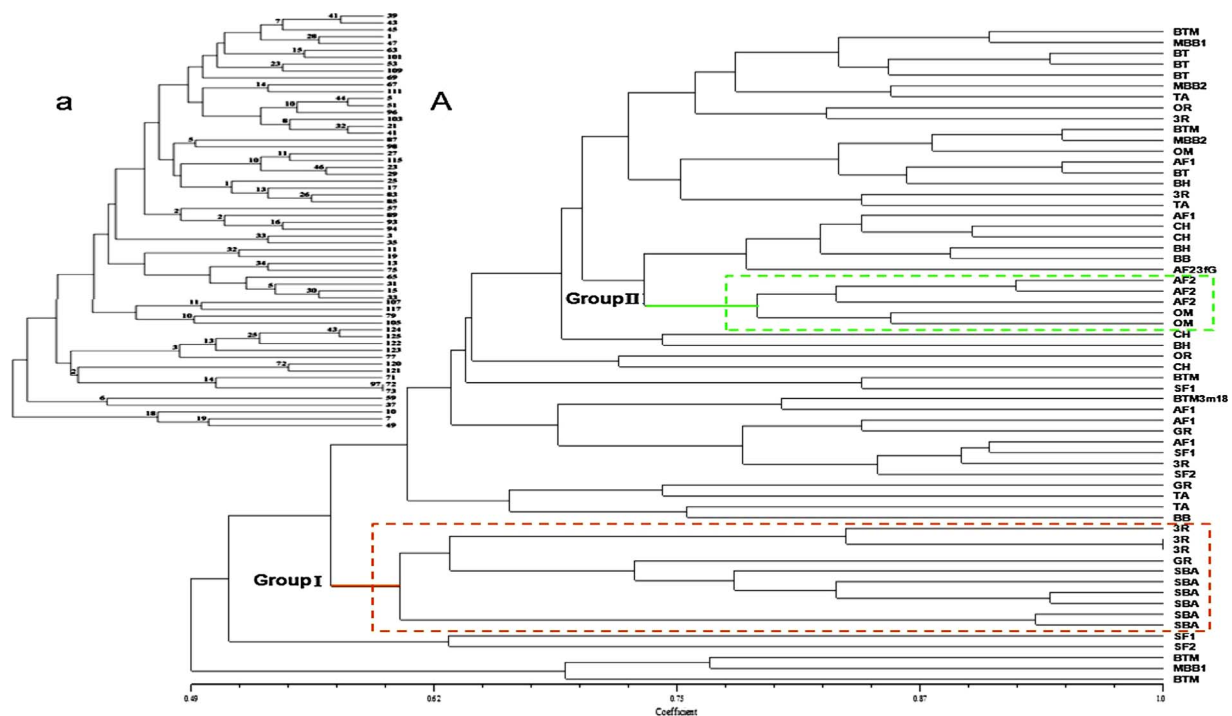

Fig. 4. Clustering of 61 accessions of *Pistacia atlantica* subsp. *atlantica* from Northwestern Algeria: (A) Dendrogram based on UPGMA analysis using the similarity matrix generated by the Nei and Li coefficient, (a) UPGMA dendrogram using the SAHN clustering based coancetred coefficients (The numbers above nodes indicate bootstrap values).

4.2. Chromosome count

For chromosome number assessment, phase contrast microscopy was useful to obtain the best contrast between chromosomes and cytoplasm. The genus *Pistacia* has been referred as diploid (Zohary, 1952). The current FCM analysis and the chromosome counting confirm the diploidy of *Pistacia atlantica* subsp. *atlantica*. However, the chromosome count observed in the present study is different from that given by Zohary (1952), Ghaffari and Fasihi-Harandi (2002) and Ghaffari et al. (2005). These authors reported 28 chromosomes (=2n) for *Pistacia atlantica* including all the varieties and/or the subspecies known for this species, except for *P. atlantica* subsp. *atlantica*. The later subspecies has never been clearly mentioned in previous chromosome studies. Nevertheless, the count (2n = 30) was previously reported in *P. atlantica* without specifying the subspecies by Vogt and Aparicio (1999) in Cyprus and by Ila et al. (2003) in Turkey. Our results agree with the later studies suggesting that (x = 15) is the basic number scince the same chromosome number (2n = 30) was also reported for *P. vera* and *P. khinjuk* and *P. eurycarpa*. The phylogenetic studies may be in favor of these results since many authors suggest that these species are the most primitive in the genus (Kafkas and Perl-Treves 2001, 2002; Karimi et al., 2009; Karimi and Kafkas, 2011). Al-Saghir (2006) argues that the reported diploid numbers of 24 and 28 probably result from chromosome reduction. According to Raven (1975), in Anacardiaceae, x = 14, 15, and 16 are common basic numbers and several genera have x = 12. Several authors (Ila et al., 2003; Al-Saghir, 2006; Ayaz and Namli, 2009) believe that the divergence in chromosome count in the genus *Pistacia* may be due to counting errors. In this genus chromosomes are small and sometimes appear to be associated; inversely, portions may detach from a chromosome and, thus, the observed chromosome number will be higher. That situation is clearly seen in Fig. 3 where two satellites lay very far away from their chromosomes. This may be the cause of the difference in chromosome numbers reported by Al Yafi (1979) for many species.

4.3. Analysis of SSR polymorphisms

From 17 *Pistacia atlantica* subsp. *atlantica* sites in natural populations (previously characterized morphologically and chemically (El Zerey-Belaskri and Benhassaini, 2016; El Zerey-Belaskri et al., 2017) a total of 61 genotypes were characterized genetically using 6 SSRs markers developed for other *Pistacia* species such as *P. vera*, *P. khinjuk* and *P. lentiscus*. A total of 26 alleles were amplified with the 6 SSRs used with an average of 4.33 allele per locus. The results obtained demonstrated a relative transferability of microsatellite markers from the cited species to *P. atlantica*.

Comparing our results to those reported by Pazouki et al. (2010) using different SSR markers on different *Pistacia* species, including *P. atlantica* subsp. *kurdica (= P. eurycarpa* Yalt.*)*, we can note that Na, Ne, and He showed higher values in the current work. This may indicate that the markers used in the present study were more efficient. The comparison may indicate also that the genetic diversity of *P. atlantica* subsp. *kurdica* was relatively lower than that of *Pistacia atlantica* subsp. *atlantica*.

According to the observed alleles, and the PIC values, the loci Pislen 526 and Pislen 114 were the most polymorphic and the most informative. The loci PKATG014, PKATG 016 were more informative in the current study in *Pistacia atlantica* subsp. *atlantica* than in Arabnezhad et al. (2011)'s study. Regarding mean number of alleles and number of allele per locus, similar values to those reported in this work were reported by Arabnezhad et al. (2011), Vendramin et al. (2010) and Albaladejo et al. (2008) in different species of the genus *Pistacia*. Eight alleles were considered as rare alleles while no fixed alleles were observed, and none of the studied loci displayed a significant heterozygote deficiency. This may indicate that there is no reproductive barrier or genetically isolated groups among the studied populations. It may indicate also possible interbreeding between populations and that some genotypes are genetically related. The migratory flow of pollen or seeds leads to the homogenization of populations. Except for EPVM 039 and Pilsen 114, no significant deviations ($P < 0.01$) from the Hardy–Weinberg proportions were found for the other loci.

4.4. Similarity relationships among genotypes

AMOVA showed a highly significant ($P < 0.01$) genetic difference within populations suggesting that populations are constituted by genetically distinct individuals and normally indicating a sexual reproduction ongoing in populations (Wright, 1951). This situation is certainly favoured by the dioecy. In addition, F_{ST} is directly related to the variance in allele frequency among populations and, conversely, to the degree of resemblance among individuals within populations. Thus, the estimation of *F*-statistics assume that all current subpopulations have undergone the same process of evolution since divergence and were derived from an ancestral population that was in Hardy-Weinberg Equilibrium (HWE), and in Linkage Equilibrium (LE) (Wright, 1951). The obtained F_{ST} estimating the interpopulational genetic differentiation showed a moderate differentiation suggesting that there is no correlation between the genetic diversity and the geographical location. The AMOVA analysis did not show a significant variance between the groups established according to the geographic situation. A moderate differentiation was mentioned by Talebi et al. (2012) using SRAP data on *P. vera*, *P. atlantica* subsp. *mutica* and *P. Khinjuk*. UPGMA analysis showed that genotypes from certain sites were distributed in different clusters and individuals from different geographical regions were clustered together. The accessions from each site were not grouped together. Similar results were found by Vendramin et al. (2009) when the clustered *Pistacia* accessions were not related to geographic origin. In addition, except for the subcluster 'Group II' accessions from close sites fell into different clusters. For the two defined subclusters (GroupI and Group II) in this analysis, it is interesting to see that morphologically, genotypes from the sites AF2 and OM (Group II) showed the largest leaves and leaflets (El Zerey-Belaskri and Benhassaini, 2016). They were also retained in the same subcluster according to the leaf colour mainly for the very dark green leaf colour (El Zerey-Belaskri, 2016). For the subcluster containing genotypes from SBA and 3R (Group I), it is interesting to mention that chemically, individuals from these sites were characterized by their original essential oil composition where germacrene D and *E*-caryophyllene were originally the major compounds (El Zerey-Belaskri et al., 2017). A high rate of diversification of genes involved in plant secondary metabolism has often been observed and both structural and regulatory changes might contribute to the large variety of terpene patterns found in plants (Iijima et al., 2004; Boutanaev et al., 2015).

Due to the lack of previous works on *P. atlantica* intraspecific genetic diversity, it was not possible to compare our findings with results obtained elsewhere. However, UPGMA analysis supported the AMOVA results and the distribution of the genotypes shown by the UPGMA analysis indicated genetic diversity within populations. Although the studied populations are dispersed in more than 250 km and under different climatic conditions (El Zerey-Belaskri and Benhassaini, 2016), the results obtained may indicate a genetic flow between populations. This could have occurred mainly by two processes: pollen dissemination by wind between the relative close populations and seed dissemination by wind, human and animals between close or distant populations. We can add that our study area is subject to multiple transhumance flow that could probably contribute to a large range dissemination of seeds. Seeds are spread through animal excrement or passively transported into the wool of animals. Some of the characteristics of the genus *Pistacia* (dioecy, apetaly and anemophily) allow both inter and intra-populational gene flow (Kafkas et al., 2002) and these phenomena can explain the genus distribution (Jordano, 1989; Al-Saghir, 2006). The overall findings tend to discount the hypothesis to have other subspecies in Algeria. Our results suggest that under climatic variation, the phenotypic plasticity and the genetic diversity of *P. atlantica* are likely to play a crucial role in allowing the species to persist in its environment. The genetic diversity probably originated from long-range dispersal and the mosaic population structure is likely a result of dispersal of pollen or seeds.

5. Conclusion

The current study reports the first molecular characterization of *Pistacia atlantica* subsp. *atlantica.* Natural populations were investigated in Northwestern Algeria. The cytogenetic assessment revealed a very small genome and the chromosome complement of the subspecies. The genetic polymorphism in *P. atlantica* subsp. *atlantica* was shown by the number of alleles at a locus and their frequency of distribution in the different populations. UPGMA analysis evaluated the relationships among genotypes and populations and led to observe a high genetic diversity and divergences between genotypes within the same site and between genotypes. Molecular characterizations in the cytogenetic studies of *Pistacia atlantica* are still necessary and more extensive investigations are needed for enhanced characterization of Atlas pistachio germplasm diversity. The objective of this study is to characterize natural *Pistacia atlantica* germplasm in Northwestern Algeria at the molecular level. The natural populations are unfortunately threatened with destructive logging. In-situ and ex-situ conservation programs and protection against the loss of diversity are urgently required. The study will contribute as database to the preservation programs of biodiversity of Atlas pistachio in Algeria.

Disclosure statement

No potential conflict of interest was reported by the authors.

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