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Genetic diversity of North African *Thymus algeriensis* in Tunisia: Population structure and implication for conservation

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Abstract : The genetic diversity within and among nine natural populations of *Thymus hirtus* Willd. subsp. *algeriensis* (Boiss. et Reut.) Murb. from different geographical and bioclimatic zones were assessed using Random Amplified Polymorphic DNA data. A total of 154 bands were generated from seven selected primers. 141 bands were polymorphic (P=91.56%). The genetic diversity within a population based on Shannon's index which was high (H'_{pop}=0.307) and varied according to bioclimatic zones. A high genetic differentiation among populations (G_{ST}=0.335 and Φ_{ST}=0.296) was revealed, suggesting a population isolation and a low level of gene flow among them. The major proportion of the variation was attributable to individual differences within populations. The genetic structure is in accordance with geography distances. The Neighbour-joining tree based on Nei's and Li's genetic distance among individuals showed that individuals from each population clustered together. The UPGMA dendrogram based on Φ_{ST} values revealed three clusters each of them includes populations closuring to their geographical origin. The high genetic structure of populations resulted from genetic drift caused both by the habitat fragmentation and the low size of most populations. The in situ conservation should interest all populations. The ex situ conservation should be based on the collection of seeds rather within than among populations because of the maximum of variation was revealed within populations.

Additional key words: Genetic differentiation, molecular markers, natural populations.

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Introduction

The genus Thymus L. belonging to the Lamiaceae family comprises more than 350 species native to Mediterranean regions, Europe and the Western Asia (Mabberley 1997). *Thymus* species can be diploids, tetraploids or hexaploids grouped in eight sections (Mártonfi and Mártonfiová 1996; Jalas 1971). The systematic of the genus remains difficult because of the interspecific hybridization, polyploidy levels and morphological similarities among species (Morales 1996; Tzakou and Constantinidis 2005). In the Mediterranean region, some *Thymus* species from a special type of bushy vegetation, not more than 50 cm high, were well adapted to hot and dry summer weather (Stahl-Biskup and Saez 2002). In Tunisian flora, the genus is represented by four species sympatric in a wide part of their distribution area, among them is *Thymus hirtus* Willd. subsp. *algeriensis* (Boiss. et Reut.) Murb.

Thymus algeriensis Boiss. et Reut., known in Tunisia as "Mezoukesh", is a perennial and diploid species

(2n=2x=30) belonging to the Hyphodromi section and the Subbracteati subsection (Stahl-Biskup and Saez 2002). It is an endemic plant of Morocco, Algeria, Tunisia and Libya (Le Floc'h 2008; Pottier-Alapetite 1981) and it is a gynodioecious shrub (Ben ElHadj Ali et al. 2010; Morales 1996). It reproduces via seeds (20–50 cm in height) and exhibits also potential of vegetative propagation. Hermaphrodite (male and female fertile) and female (male sterile) plants can occur in the same population (Ben ElHadj Ali et al. 2010). Flowering takes place between April and June. It is belived to be outcrosser and often pollinated by bees (Orellana et al. 2005; Tarayre and Thompson 1997). However self pollination may occur in hermaphrodites (Thompson et al. 2002). It is used fresh or dried as a culinary herb and in folk medicine for its antiseptic, antispasmodic and antifungal properties (Giordani et al. 2008; Hazzit et al. 2009). The demand for essential oils from these species is increasing for perfumery, cosmetic and medicinal uses. However, the bulk material comes from natural populations which are severely affected by the anthropic pressures.

Previous work on *Thymus* species samples showed that the genetic structure and variability exhibit significant differences within and among populations in relationship to ploidy level, geographical origin and floral biology (Thompson et al. 2002; Lopez-Pujol et al. 2004; Trindade et al. 2008). Our work on the genetic diversity of Tunisian *Thymus algeriensis* populations based on isozymes and chemical markers, revealed a moderate genetic variation within population and a high differentiation among them (Bel Hadj Ali et al. 2008; Ben ElHadj Ali at al. 2010). This may lead to an increasing gene drift impeding the in situ maintenance of populations.

In Tunisia, *Thymus algeriensis* populations grow wild in different bioclimatic zones extending from the sub-humid to the lower arid, in five isolated areas: the Northwestern part of the country, the Cap Bon, the Tunisian Dorsal mountain, the Sahel and the arid areas, where the species becomes scarce. Populations are mainly associated to *Thymus vulgaris* L., *Thymus capitatus* L., *Teucrium polium* L., *Olea europaea* L., *Quercus coccifera* L. and *Lavandula multifida* L. and grow on sandy and often on rocky soils under a rainfall ranging between 150 and 1000 mm/year (Nabli 1995; Pottier-Alapetite 1981).

Tunisian *T. algeriensis* occurs in small scattered populations showing high population genetic differentiation coupled with a low level of gene flow (Bel Hadj Ali et al. 2008; Ben El Hadj Ali 2010). The species occurs as mosaic of local populations in little patches along the landscape. The populations have been severely depleted and fragmented due to overcollecting and habitat destruction caused by overgrazing, clearing, low soil quality and irregularity of rainfall. Populations, except for those preserved within forests of Bargou and Chaambi Jbel Mountains, are facing dramatic fluctuations with unknown impact on their genetic diversity and structure. The habitat fragmentation and the spatial isolation of populations increase genetic drift and differentiation between populations, and reduce their future adaptation to environmental changes (Ellstrand and Elam 1993). Therefore, the analysis of genetic variation within and among populations is crucial to evaluate the present status of these populations, to understand their future maintenance and to develop an improvement and conservation programs.

RAPDs provides a useful tool to evaluate the genetic diversity and differentiation issues in plants populations (Boulila et al. 2010; Rajeb et al. 2010; Mariette et al. 2007; Solouki et al. 2008; Trindade et al. 2008; Zheng et al. 2008). They are assumed to be selectively neutral, involve a large number of loci and cover a large part of the genome. They are able to detect variation both in coding and non-coding regions of the genome (Katsiotis et al. 2009; Lin et al. 2009). RAPDs have also other advantages such as their potential for detecting polymorphism without need for prior knowledge of the genome, low coast, rapidity and requiring little genomic DNA as template. Nevertheless, RAPDs have some disadvantages, the most significant being from their dominant allelic expression and their low level of reproducibility. Thus, allelic frequencies estimated for loci are less accurate than those obtained with codominant markers allowing to bias in the evaluation of the genetic diversity (Lynch and Milligan 1994). These problems can be partially overcome by rigid laboratory protocols. In addition, the use of appropriate statistical methods such as the Analysis of the Molecular Variance (AMOVA) can also overcame the problem of RAPDs reputability (Excoffier et al. 1992) and analysed only reproducible RAPD bands with frequencies higher than 3/N [N is the number of the analysed plants per population] (Lynch and Milligam 1994) also buffer the bias of estimation of the genetic variation.

The aim of this study is to assess the variation of RAPDs within and among Tunisian *Thymus algeriensis* populations from different ecological and geographical zones. It can provide crucial informations to develop genetic improvement programs and elaborate conservation strategies.

Material and methods

Surveyed populations and sampling

Nine Tunisian *Thymus algeriensis* Boiss. et Reut. populations previously assessed for their essential oil and isozymic variation were analyzed (Bel Hadj Ali et al. 2008; Ben El Hadj Ali 2010). Populations belong

Table 1. Ecological traits of nine Tunisian *Thymus algeriensis* populations

Population code	Population	Bioclimatic zone*	Latitude	Longitude	Q_2 coefficient	Altitude (m)	Rainfall (mm/year)
1	Sabbah Jeb. Mt. **	Sh	36°46'N	9°1'E	83.5	460	600–700
2	Bahra	Usa	36°14'N	8°36'E	49.77	450	400–500
3	Mansour Jeb. Mt.		36°17'N	9°36'E	45.72	600	400–500
4	Essers	Msa	36°76'N	9°40'E	44.24	604	400–500
5	Chaambi Jeb. Mt.		35°11'N	8°45'E	44.9	1010	400–00
6	Chrechira Jeb. Mt.	Lsa	35°8'N	9°29'E	35.3	560	300–400
7	Toujene	Ua	33°27'N	9°58'E	29.19	600	100–150
8	Ouled Bou Saad	La	34°27'N	8°35'E	18.43	350	150–200
9	Douaou Dj. Mt.		34°38'N	9'30'E	28.83	650	150–200

Sh: Sub-humid, Usa: Upper semi-arid, Msa: Mean semi-arid, Lsa: Lower semi-arid, Ua: Upper arid, La: Lower arid.
*Bioclimatic zones were defined according to Emberger's (1966) pluviothermic quotient $Q_2 = 2000P/M^2 - m^2$, where P is the average of annual rainfall (mm). M is average of maximal temperature (K: Kelvin degree) for the hottest month (June) and m the mean of minimal temperature (K) for the coldest month (February). Q_2 was calculated for each site using P, M and m values for the period from 1953 to 2009 (Tunisian National Institute of Meteorology)
**Jeb. Mt.: Jebel Mountain

to the sub-humid, upper semi-arid, mean semi-arid, lower semi-arid, upper arid and the lower arid bioclimatic zones according to Emberger's (1966) pluviothermic coefficient (Q_2) (Table 1 and Fig. 1). Ten plants from each population were sampled at random. Samples were collected at a distance exceeding 20 m from each other to avoid collecting multiple plants from the same parent.

RAPD procedure

DNA extraction

DNA extraction was made with 600 mg of young leaves ground in liquid nitrogen. The obtained powder was mixed with 2 ml of a CTAB extraction buffer (250 mM NaCl, 200 mM Tris-HCl pH 7.0, 10 mM 2-mercaptoethanol and 20 mM EDTA) and 50 mg PVP 40000. Samples were then incubated at 65°C for 1 hour with slow shaking every 5 min. Subsequently the mixture was treated twice with 600 μL chloroform-isoamyl alcohol (24:1) and centrifuged for 10 min at 12000 rpm. DNA precipitation was performed following the method described by Lodhi et al. (1994). The quality of the DNA was determined by electrophoresis on 0.8% agarose gel stained with ethidium bromide.

Primers and PCR conditions

RAPD amplification was performed in Programmable Stuart Thermal Cycler (Maxi-Gene) in 25 μL reaction volume containing 50 ng DNA template, 2.5 μL of 5 X reaction buffer, 40 pmoles of primer, 200 μM of each dNTP, 2.5 mM $MgCl_2$ and 1.5 U Taq polymerase (Promega). The mixture was overlaid with one drop of mineral oil. The amplification program was as follows: 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 36°C for 1 min and 72°C for 2 min.

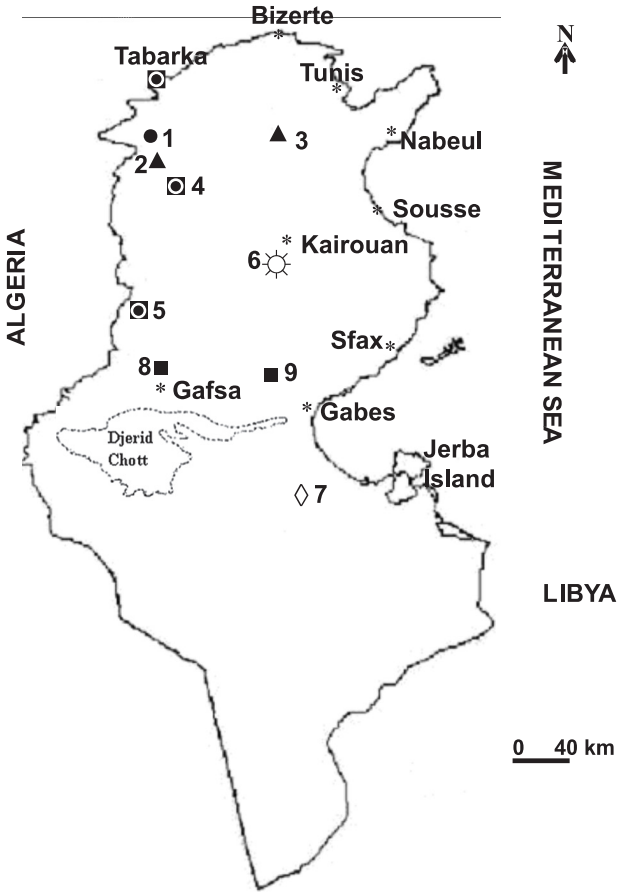

Fig. 1. Map of Tunisia: Geographic location of the nine *Thymus algeriensis* populations analysed
Numbers (1, 2,...): Population code
● sub-humid, ▲ upper semi-arid, ◙ mean semi-arid, ☼ lower semi-arid, ◊ upper arid, ■ lower arid
* Great towns

The last step was 72°C for 10 min for final polymerase reaction. Amplification products were separated on 1.5% agarose gel in TAE buffer (pH 8), stained with ethidium bromide, and photographed under UV light

using a Doc Print Photo Documentation System. Molecular weights were estimated using a 200 bp DNA Promega ladder. To ensure the reproducibility within and between runs, DNA from same two additional individuals was included and amplified twice in every PCR run. A negative control reaction, in which DNA was omitted, was included in every run. Out of the eight initially primers tested seven of them yielded polymorphic and reproducible bands. Primers selected are: OPJ-06 (5'TCGTTCCGCA3'), OPJ-08 (5'CATACCGTGG3'), OPJ-10 (5'AAGCCCGAGG3'), OPJ-12 (5'GTCCCGTGGT3'), OPJ-13 (5'CCACACT ACC3'), OPJ-14 (5'CACCCGGATG3') and OPJ-16 (5'CTGCTTAGGG3').

Data analysis

For RAPD analysis, the presence of a band was scored 1, whereas the absence of the band was coded 0. The scored RAPD markers are converted into a binomial (0/1) matrix. Since RAPD markers are dominant, it was assumed that each band represented the phenotype at a single bi-allelic locus (Williams et al. 1990). Only bands with frequency higher than 3/N (N: number of plants analysed) were considered according to Lynch and Milligam (1994).

In each population and ecological group (each ecological group includes populations from the same bioclimate), the genetic diversity was estimated using the percentage of polymorphic bands P% [(number of polymorphic bands/number of total bands)×100)] and Shannon's index for each RAPD locus (H') was calculated as: $H' = -\Sigma p_i \log_2 p_i$; where p_i is the frequency of the presence or absence of a RAPD band in a population. The correlation among Shannon's indices (H') and altitude or Emberger's Q_2 or rainfall/year matrices was evaluated by the calculation of the Spearman coefficient (*r*-Spearman) using Spearman/Kendall's rank test. Shannon's index was also used to estimate the average diversity H_{pop} over all populations [$H_{pop} = -1/n \; \Sigma \; H'$; where n is the number of populations]. The species diversity was estimated as: H_{sp} [$H_{sp} = -\Sigma p_s \log_2 p_s$; where p_s is the frequency of presence or absence of the RAPD in the whole sample].The proportion of diversity within the populations was estimated as H_{pop}/H_{sp}, and that among populations was evaluated by G_{ST} [$G_{ST} = (H_{sp} - H_{pop})/H_{sp}$]. P%, H' and G_{ST} were also estimated at the ecological group level. Calculations were made by POPGENE program version 1.31 (Yeh et al. 1999). The comparison among Shannon's indices at the population and ecological group levels was performed using a variance analysis (ANOVA procedure; SAS 1990) and Duncan's test (Dagnelie 1975).

The genetic distance between individuals was estimated using the Nei and Li's (1979) coefficient S_{xy} [$S_{xy} = 2m_{xy} / (m_x + m_y)$, where m_{xy} is the number of bands shared by samples x and y, and m_x and m_y are the number of bands in samples x and y, respectively], using the program MVSP version 3.1 (Kovach 1999). The genetic distance (D_{xy}) between individuals was estimated using the complementary value S_{xy} [$D_{xy} = 1 - S_{xy}$]. A Neighbour-joining tree (Saitou and Nei 1987), based on Nei and Li's distance matrix between individuals, was constructed to ordinate relationships among individuals and construction of phylogenetic trees using the Win95/98/NT program FreeTree (Hampl et al. 2001). Support values of the internal branches of NJ were evaluated through bootstrap method (1000 replicates) (Hampl et al. 2001).

The genetic variation within and among populations or within and among ecological groups also was estimated by AMOVA performed on the genetic distances (D_{xy}) between individuals using the WINAMOVA program, version 1.55 (Excoffier et al. 1992). Φ-statistics: Φ_{ST} (differentiation among populations), Φ_{CT} (differentiation among ecological groups) and Φ_{SC} (differentiation among populations within groups) were calculated. The significance of variance components and that of Φ-statistics were estimated using permutation procedures. The number of migrants per generation (gene flow) was estimated using the equation $Nm = [(1/\Phi_{ST}) - 1]/4$ (Wright 1951). All analyses were performed using WINAMOVA program, version 1.55 (Excoffier et al. 1992).

The phylogeographic relationships and the test for isolation of populations were performed using the number of shared private and rare fragments for each population. Mantel's tests (Mantel 1967), were used to determine whether the matrix of genetic differentiation (Φ_{ST}) was correlated with those of geographic distances, altitudes, Emberger's pluviothermic coefficient Q_2 and rainfall/year, using ZT program (Bonnet and Van de Peer 2002). The significance of the correlation was tested after 1000 permutations. All analyses were performed using WINAMOVA program, version 1.55 (Excoffier et al. 1992). UPGMA tree based on pairwise Φ_{ST} was also generated to compare similarities among populations using the program MVSP version 3.

Results

RAPD genetic diversity

The seven primers used for all populations generated 154 discernible and reproducible DNA fragments, out of which 141 (91.56%) were polymorphic. The bands ranged in size from 200 to 2000 bp. The number of bands varied from 20 (OPJ-10) to 24 (OPJ-13 and OPJ-16) with an average of 22 at the species level and specific bands were revealed according to populations. For example, bands 1700 bp (OPJ-13) and 620 bp (OPJ-14) were restricted to populations from the upper semi-arid and the mean semi-arid

Table 2. Percentage of polymorphic loci (*P* %) per primer in each population and in each ecological group

Populations	Primer							Ns%	Pt%
	OPJ06	OPJ08	OPJ10	OPJ12	OPJ13	OPJ14	OPJ16		
1	72.73 (16)	71.43 (15)	60.00 (12)	52.38 (11)	37.50 (9)	45.45 (10)	45.83 (11)	7.1	54.55
2	72.73 (16)	61.90 (13)	40.00 (8)	76.19 (16)	79.17 (19)	68.18 (15)	62.50 (15)	9.1	66.23
3	59.09 (13)	52.38 (11)	40.00 (8)	52.38 (11)	66.67 (16)	68.18 (18)	50.00 (12)	2.6	55.84
4	86.36 (19)	61.90 (13)	50.00 (10)	85.71 (18)	70.83 (17)	40.91 (9)	58.33 (14)	9.7	64.94
5	59.09 (13)	52.38 (11)	50.00 (10)	61.90 (13)	70.83 (17)	59.09 (13)	58.33 (14)	5.2	59.09
6	54.55 (12)	71.43 (15)	55.00 (11)	66.67 (14)	75.00 (18)	72.73 (16)	62.50 (15)	7.1	65.58
7	68.18 (15)	71.43 (15)	45.00 (9)	47.62 (10)	54.17 (13)	45.45 (10)	45.83 (11)	7.8	53.90
8	72.73 (16)	57.14 (12)	40.00 (8)	66.67 (14)	75.00 (18)	36.36 (8)	70.83 (17)	8.4	60.39
9	68.18 (15)	71.43 (15)	55.00 (11)	42.86 (9)	66.67 (16)	45.45 (10)	50.00 (12)	4.5	57.14
Average	68.18	63.49	48.33	61.38	66.20	53.53	56.02		59.74
Bioclimatic zone									
Sub-humid	72.73 (16)	71.43 (15)	60.00 (12)	52.38 (11)	37.50 (9)	45.45 (10)	45.83 (11)		54.55
Upper semi-arid	81.82 (18)	71.43 (15)	55.00 (11)	85.71 (18)	87.50 (21)	86.36 (19)	83.33 (20)		79.22
Mean semi-arid	90.91 (20)	76.19 (16)	70.00 (14)	85.71 (18)	79.17 (19)	68.18 (15)	70.83 (17)		77.27
Lower semi-arid	54.55 (12)	71.43 (15)	55.00 (11)	66.67 (14)	75.00 (18)	72.73 (16)	62.50 (15)		65.58
Upper arid	68.18 (15)	71.43 (15)	45.00 (9)	47.62 (10)	54.17 (13)	45.45 (10)	45.83 (11)		53.90
Lower arid	86.36 (19)	90.48 (19)	70.00 (14)	76.19 (16)	83.33 (20)	54.55 (12)	75.00 (18)		76.62
Average	75.76	75.40	59.17	69.05	69.45	62.12	63.89		67.86

Number of polymorphic bands are given in parentheses; Pt%: Percentage of polymorphic loci; Ns %: Percentage of specific alleles.

zones. Bands 580 bp (OPJ-10) and 1300 bp (OPJ-16) were only observed in populations from the semi-arid bioclimate. Arid populations were characterized by a bands of 280 bp and 560 bp revealed by OPJ-08. Bands according to 220 bp and 260 bp (OPJ-06) were not found in the population 1 (sub-humid area). Bands of 460 bp, 520 bp and 650 bp, revealed by OPJ-06, were not observed in the lower semi-arid population 6 (Fig. 2).

The number and distribution of polymorphic products detected with each primer in each population or ecological group were given in Table 2. At the population level, the percentage of polymorphic bands for each primer varied from 48.33 (OPJ-10) to 68.18% (OPJ-06). For all assessed primers and for all populations, the genetic diversity index (H') varied significantly among populations (ANOVA test, $p=0.03<0.05$). The highest genetic diversity

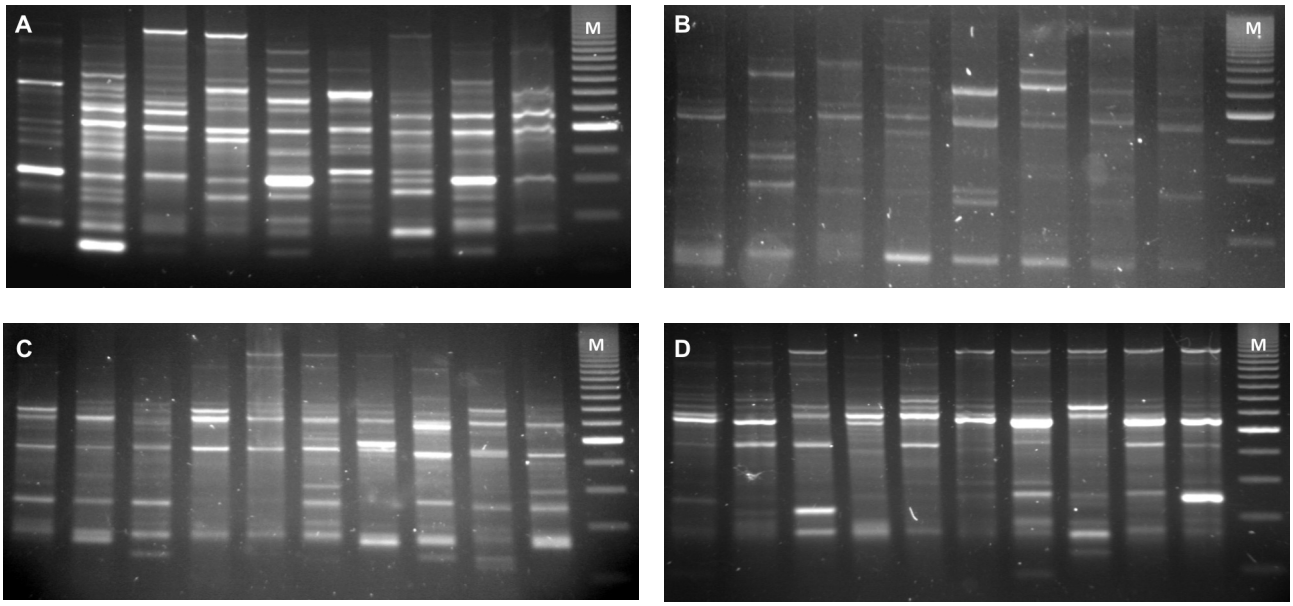

Fig. 2. Examples of DNA profiles revealed by the OPJ08 primer in four populations.
Populations: A: population 2; B: population 4; C: population 5; D: population 6.
M: Size Marker.

Table 3. Shannon's index and ratio of genetic diversity in each population and each ecological group

Population		1	2	3	4	5	6	7	8	9
H'		0.285^{ab}	0.341^{a}	0.262^{b}	0.329^{ab}	0.306^{ab}	0.354^{a}	0.261^{b}	0.314^{ab}	0.307^{ab}
H_{pop}	0.307*									
H_{sp}	0.461									
H_{pop}/H_{sp}	0.665									
G_{ST}	0.335									

Ecological group		Sh	Usa	Msa	Lsa	Ua	La
H'_{gp}		0.285^{b}	0.382^{a}	0.379^{a}	0.354^{ab}	0.261^{b}	0.387^{a}
H_{gp}	0.341^{ns}						
H_{sp}	0.460						
H_{gp}/H_{sp}	0.742						
G_{STg}	0.258						

Population codes as identified in Table 1. Sh. Sub-humid, Usa. Upper semi-arid, Msa. Mean semi-arid, Lsa. Lower semi-arid, Ua. Upper arid, La. Lower arid. Numbers with the same letter are not significantly different (Duncun's test, $p > 0.05$).

H', H_{pop}, Hsp, H_{pop}/H_{sp} and G_{ST} are the average per primer values of genetic diversity for each primer within each population (H'), over all populations (H_{pop}), whole sample (H_{sp}) and their partition within- (H_{pop}/H_{sp}) and between-populations (G_{ST}) components respectively, calculated for all primers. H'_{gp}, H_{gp}, H_{sp}, H_{gp}/H_{sp} and G_{STg} are the average per primer values of genetic diversity for each primer within each ecological group (H'gp), over all groups (H_{grp}), and their partition within- (H_{gp}/H_{sp}) and between-groups (G_{STg}) respectively, calculated for all primers.

(P=57.14–66.23% and H'=0.306–0.354) was observed for the populations in the Northwestern, the Sahel and the Tunisian Dorsal. Whereas the lowest (P= 53.90% and H'=0.262) is scored for the population Toujene in the extreme South and the population Sabbah Jbel Mountain (P=54.55% and H'=0.285) located in the North part of the country (Tables 2, 3).

The averages within all populations (H_{pop}) and within the species (H_{sp}) were 0.307 and 0.461, respectively. Within ecological groups, the highest Shannon's diversity index was observed for populations belonging to the lower arid zone (H_{grp}=0.387). The average of within-group diversity did not differ statistically among groups (ANOVA; $p>0.05$). Besides, there was no correlation between Shannon's diversity index (H') and altitude matrices (Spearman test; r=–0.276, p=0.471>0.05) or between H' and Emberger's Q_2 matrices (Spearman test; r=–0.1, p=0.798>0.05) or between H' and rainfall/year (Spearman test; r=0.89, p=0.051> 0.05). The most of the variation occured within populations (H_{pop}/H_{sp}=66.5%) and within populations in ecological groups (H_{grp}/H_{sp}=74.2 %).

Genetic structure and divergence among populations

The level of the differentiation among populations estimated by G_{ST} (0.335) was higher than that estimated between ecological groups (G_{STg}=0.258) (Table 3). The within-population component of variance esti-

Table 4. Nested analysis of molecular variance (AMOVA) within and among *Thymus algeriensis* populations and within and among ecological groups

Source of variation	d.f.	M.s.d.	Variance component	Total variance (%)	Φ-statistics
Population					
Among populations	8	94.84	7.88	29.63	F_{ST} = 0.296**
Within populations	78	18.71	18.71	70.37	
Total	86				
Ecological group					
Among groups	5	110.82	3.11	11.58	F_{CT} = 0.116**
Among populations/group	3	68.19	5.04	18.76	F_{SC} = 0.212**
Within populations	78	18.71	18.71	69.66	
Total	86				

d.f.: Degree of freedom ; M.s. d.: Mean squared deviation, ** Significant at $p < 0.001$ after 1000 permutations.

Φ_{ST}: differentiation among populations, Φ_{CT}: differentiation among ecological groups, Φ_{SC}: differentiation among populations within groups.

mated through AMOVA accounted for 70.37% of overall variation (Table 4), while that among populations was 29.63%. The mean Φ_{ST} value among all populations was 0.296 ($p<0.001$) indicating a high differentiation among them. At the ecological group level, the AMOVA analysis revealed that 69.66% of the total genetic variance occurred among individuals within-ecological groups and only 18.76% occurred among populations within a group. The differentiation among ecological groups ($\Phi_{CT}=0.116$, $p<0.001$) or among populations within ecological groups were also significant ($\Phi_{SC}=0.212$, $p<0.05$) (Table 4).

The Neighbour-joining dendrogram based on Nei and Li's genetic distance showed that all individuals from the same population clustered together excepting one individual from the population 6 grouped with samples from the population 5 (Fig. 3). The population 7 from the extreme South of the country was more isolated from the other populations.

Pairwise Φ_{ST} values from AMOVA were all significantly different from zero ($p<0.001$ after 1000 permutations) and ranged from 0.159 (among populations 4 and 5) and 0.370 (among populations 1–7 and 1–9) (Table 5). The highest values of Nm (Nm=1.321) was observed for populations geographically close (4 and 5) and belonging to the same bioclimate (mean semi-arid area), and the lowest (Nm=0.425) was found, respectively, between populations 1 and 7, which of 375 Km distant, and between the populations 1 and 9, which of 250 Km apart. The Mantel test, performed on

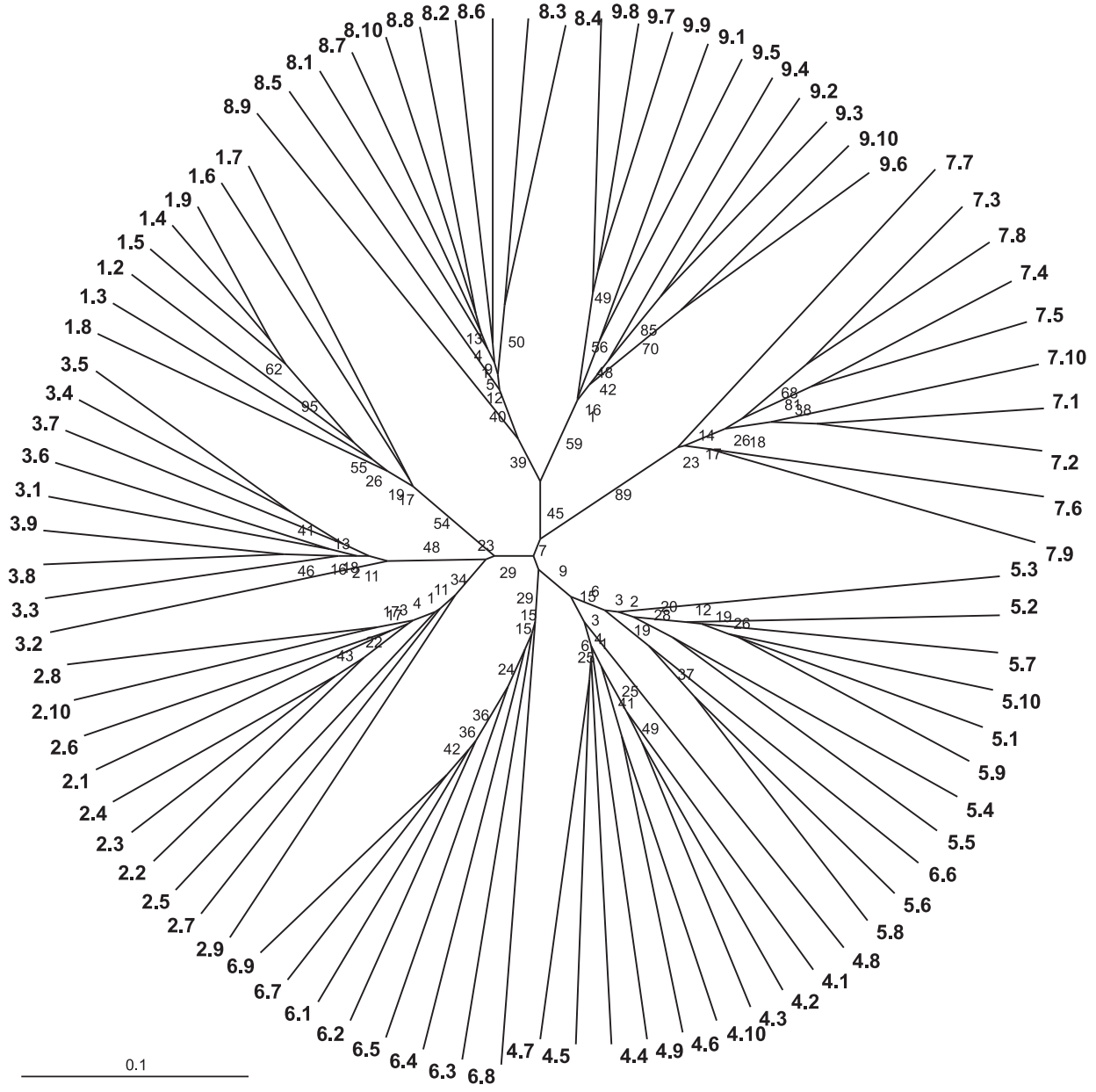

Fig. 3. Neighbour-joining dendrogram generated from Nei and Li's similarity coefficient for the 86 individuals of *Thymus algeriensis* analysed

Numbers (1, 2,...): Population code and (1.1, 1.2,...) individuals number according to population

Table 5. Matrices of genetic distance (pairwise Φ_{ST} values; below diagonal) and geographic distances (above diagonal) among the 9 populations of *Thymus algeriensis*

Population	1	2	3	4	5	6	7	8	9
1		52	82	82	184	146	375	262	250
2	0.260**		75	38	135	116	343	210	214
3	0.285**	0.233**		60	148	71	304	217	182
4	0.285**	0.258**	0.280**		102	79	302	178	170
5	0.239**	0.264**	0.300**	0.159**		108	228	79	113
6	0.302**	0.299**	0.304**	0.230**	0.194**		243	157	109
7	0.370**	0.347**	0.360**	0.326**	0.349**	0.312**		171	120
8	0.309**	0.309**	0.309**	0.251**	0.245**	0.282**	0.342**		97
9	0.370**	0.354**	0.353**	0.317**	0.321**	0.299**	0.345**	0.232**	

Population codes as identified in Table 1; **Highly significant at $p < 0.001$ (after 1000 permutations).

geographic and Φ_{ST} distance matrices among population pairs, showed a high significant correlation between the two matrices ($r=0.64$, $p=0.0009<0.001$ after 1000 permutations), indicating an isolation by distance. The correlation estimated by the Mantel test between matrices of several ecological indices (Q_2, altitudes and rainfall) and Φ_{ST} matrices is significant between matrices of Φ_{ST} and altitude ($r=-0.409$; $p=0.044< 0.05$) and between Φ_{ST} and rainfall/year ($r=0.591$; $p=0.002<0.05$).

The UPGMA dendrogram based on the Φ_{ST} distance matrix showed three population groups clearly clustered according to bioclimatic zones and geographical distances (Fig. 4). The first one (GI) is constituted by the population 7 from Toujene belonging to the upper arid bioclimate. The second group (GII), which could be subdivided into two subclusters, includes populations 6 (lower semi-arid), 4 and 5 (mean semi-arid) (subcluster 1) and populations 1, 2, 3 from the sub-humid and upper semi-arid bioclimates (subcluster 2). Populations 8 and 9 (lower arid area) constituted the third group (GIII).

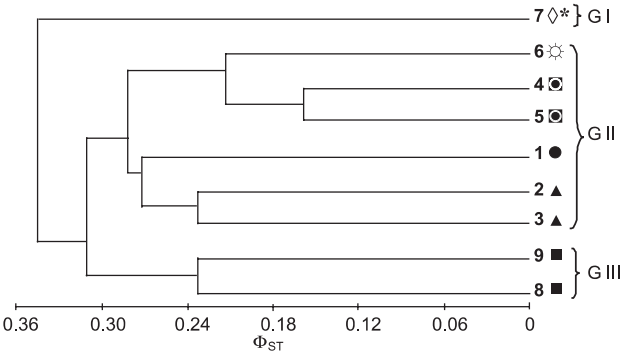

Fig. 4. Dendrogram of the 9 analysed populations based on Φ_{ST} matrix

Numbers (1, 2,…): Population code

● sub-humid, ▲ upper semi-arid, ◙ mean semi-arid, ☼ lower semi-arid, ◊ upper arid, ■ lower arid

Discussion

In our study, RAPD markers were used to assess genetic diversity of Tunisian *Thymus algeriensis* populations. The species maintained a high genetic diversity within populations ($H_{pop}/H_{sp}=0.665$) and within ecological group ($H_{grp}/H_{sp}=0.742$). The level of variation could be explained by the predominantly outbreeding mating system and the persistence of multiple individuals through generations issued from large populations before fragmentation (Hamrick and Godt 1996). Out-crossers with a large distribution area are known to exhibit a high genetic diversity within populations than in inbreeded species with restricted geographical distribution (Li and Jin 2006; Nybom 2004). However, in *Thymus algeriensis* the vegetative propagation could be responsible for a decreasing level of genetic diversity within a given population. This mode of propagation is frequent in most *Thymus* species (Lopez-Pujol et al. 2004; Tarayre and Thompson 1997).

Several RAPD loci specific to populations were revealed. However, the presence of these loci might not reflect the adaptability to ecological factors since they were not detected in all populations from the same bioclimatic zone. RAPD's are believed to be neutral and amplified mostly non coding DNA sequences, which are subject to weaker selection pressure. Their variation may not necessarily reflect the pattern of variation in adaptative genes. However, the range of variation between populations was large and the level of the within-population diversity varied significantly among populations due to the restriction of the species to small and degraded populations. Population from Chrechira Jbel Mountain (6) with a large size (>200 flowering individuals) and more continuous distribution area showed the highest diversity index ($H'= 0.354$). Populations of Toujene, Mansour and Sabbah Jbels Mountains, with a little size (50–100 flowering individuals) and scattered individuals, were

less heterogeneous (H'=0.261, H'=0.262 and H'= 0.285, respectively). The particularity of rare fragments detected in those localities could be a result of genetic drift as consequence of low size and geographic isolation.

The AMOVA analysis showed that 70.37% of the genetic variation is apportioned among individuals within populations or within populations in their corresponding group. This suggests that mating occurs mainly among individuals within a sub-population thus favouring the divergence between populations. The genetic differentiation among all populations is high (Φ_{ST}=0.296; G_{ST}=0.335) with the differentiation between ecological groups and between populations from the same group being moderate (Φ_{SC}=0.212; Φ_{CT}=0.116). The observed amount of differentiation was higher than the average for perennial outcrossing species (G_{ST}=0.22, Φ_{ST}=0.27) (Nybom, 2004) or species with a mixed mating system (G_{ST}=21.2–24.0%) (Hamrick and Godt 1996).

All the estimates of genetic differentiation G_{ST} and Φ_{ST} were similar and indicated a significant genetic structure among populations and ecological groups, coupled to a low level of gene flow (the average was 0.627). All pairwise comparison of Φ_{ST} values were significant indicating that all populations sampled were genetically different. The highest Φ_{ST} and the lowest level of gene flow were observed between population 1 (located in the northern limit of the distribution area of the species) and 7 (located in the southern limit of the distribution area of the species), and between populations 1 and 9 (located in the south). So, the population's structure could be explained by geographic isolation probably combined to ecological factors such as altitudes and rainfall influencing flowering time and longevity. The distribution of genetic variation along altitudinal gradients is known to be the result of the interplay of gene flow and genetic drift (Aradhya et al. 1993). Therefore, genetic structure was significantly affected by geographic barriers. Populations geographically distinct showed higher genetic structure (populations 1 and 7) than populations with more continuous distributions founded in the central mountain ranges. Thus, the gene flow via seed and pollen dispersion between adjacent populations, represent a critical determinant of the genetic structure of natural plant populations (Premoli et al. 2001) and might be favoured by the continuous distribution area of the species before population fragmentation. This high differentiation could be explained by genetic drift due to limited gene flow via seed and/or pollen dispersal. Thompson et al. (2002) reported that pollen and seed dispersals in thymes are highly localised, increasing the tendency for reproduction to occur within spatially localized groups.

Neighbour-joining dendrogram generated from Nei and Li's genetic distance showed that individuals from each population clustered together. The cluster analysis based on Φ_{ST} show a relationship between population groupings and geographic distances. Populations geographically near clustered together. Several populations from the central mountain ranges (populations 2, 3, 4, 5 and 6) gathered together, indicating that genetic differentiation mainly occurs at local space scale due to limited gene flow and genetic drift.

Most *Thymus algeriensis* populations in Tunisia were represented by few individuals. The long term viability of populations could be affected by increasing habitat destruction. An increase in human activities may decrease the size of populations and increase the genetic drift. Taking into account these points, efforts should be made to protect all populations and limit human impact. Given the high genetic differentiation among populations and the low level of genetic variation recorded, any *in-situ* conservation strategy should aim to include firstly populations such as Sabbah Jebel Mountain and Toujene, characterized by a high level of habitat destruction and a high genetic isolation. The long term evolutionary viability of these populations must be in doubt particularly when such low variation is coupled with the overgrazing, over-gathering and the increasing habitat destruction. Populations 2 (Bahra) and 6 (Chrechira Jbel Mountain), harbouring the high diversity level, should be more preserved. The significant differentiation among ecological groups suggests that *in-situ* conservation should be made appropriately according to bioclimate and site disturbance level. Populations exhibiting particular alleles (i.e. populations 1, 4, 7 and 8), should be preserved, and the correlation of these bands with other markers (morphological or chemical or isozymes) should be assessed to improve conclusions because RAPDs are considered selectively neutral and do not obligatory reflect ecologically variation for adaptative traits. The genetic variation was due 70.37% within population while 29.63% was distributed among populations, thus, *ex-situ* conservation should be based on the collect of seeds/or cuttings within rather than among populations. The species is outcrosser and capable of vegetative reproduction. Therefore, the prelevement of samples should take into account these factors and should be made appropriately in each bioclimate.

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