

# Imen Ben El Hadj Ali, Arbi Guetat, Mohamed Boussaid

# Genetic diversity of wild *Thymus capitatus* (Lamiaceae) in Tunisia using molecular markers

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Abstract: In Tunisia, Thymus capitatus L. populations are severely destroyed due to deforestation and over-collecting. The species occurs in small scattered populations decreasing progressively in size. Yet, no conservation or improvement programs are attempted to preserve and promote the potential value of this resource. In this work, we assessed the genetic diversity of nine Tunisian populations of Thymus capitatus L. from different bioclimates, using 103 polymorphic randomly amplified polymorphic DNA (RAPD) loci. The analysis of the genetic variation within and among populations is primordial to elaborate conservation and improvement programs. The species showed a low diversity within populations (0.276<H'<0.327) due to genetic drift caused by the habitat fragmentation. A high genetic differentiation ( $G_{sT}$ =0.359 and  $\Phi_{sT}$ =0.284) and a low gene flow (0.435<Nm<0.970) among populations, indicating a short seed and/or pollen dispersal distance, were revealed. The absence of isolation by distance and the high level of differentiation among populations suggested the existence of important historical gene flow between them. The UPGMA dendrogram analysis based on  $\Phi_{sT}$  distance matrix showed that most populations clustered independently to bioclimate or geographical distance indicating that genetic differentiation mainly occurs at local space scale due to genetic drift. Given the high proportion of the within-population diversity and the high amount of among populations variation, the ex-situ conservation should be made by the collection of seeds/cuttings rather within than among populations. The in-situ conservation should be made appropriately according to populations in their bioclimate.

Additional key words: Habitat fragmentation, conservation strategies, genetic structure.

Address: I. Ben El Hadj Ali, A. Guetat, M. Boussaid, National Institute of Applied Sciences and Technology. Laboratory of Plant Biotechnology B.P.676, 1080 Tunis Cedex, Tunisia, e-mail: imenbenelhadjali@yahoo.fr

## Introduction

*Thymus* L. genus comprises more than 350 species growing in wild throughout the world (Mabberley 1997). Species can be diploids (2n=2x=24, 26, 28, 30, 32), tetraploids (2n=4x=52, 54, 56, 58, 60) or hexaploids (2n=6x=42, 48, 84, 90) (Mártonfi and Mártonfiová 1996; Morales 1996). A growing interest in the use of these species has been recently reported for industrial, pharmaceutical and chemical fields (Hazzit et al. 2009). However, the used material often

comes from natural populations that are decreasing progressively in number and size. Studies on thyme conservation strategies and population's genetic structure in relationship with the ploidy level, geographical origin and floral biology, remain rather scarce in contrast to those assessing their chemical composition and biological properties (Ehlers and Thompson 2004; Lopez-Pujol et al. 2004).

In Tunisia, the genus *Thymus* L. is represented by four species: *Thymus capitatus* (L.) Hoffm. et Link., *Thymus numidicus* Poir., *Thymus hirtus* Willd. ssp.

algeriensis Boiss. et Reut. var. cinerescens Murb. and Thymus vulgaris L. (Pottier-Alapetite 1981). Species can be sympatric in a wide part of the country (Ben El Hadj Ali et al. 2010; Ben El Hadj Ali et al. 2012). Thymus capitatus (L.) Hoffm. et Link. [Synonym Thymbra capitata (L.) Cav., Corydothymus capitatus Rechenb. f.] (Figueiredo et al. 2008), is a diploid (2n=2x=30)(Morales 1996) and outcrossing shrub predominantly bee-pollinated. It's a typical plant with the Mediterranean region and it's used as medicinal herbs. It is consists of perennial and ornamental plant (Le Floc'h 1983). The species exhibits both vegetative propagation and sexual reproduction (Petanidou and Vokou 1993). However, natural regeneration of genets from seeds is extremely rare because of the difficulty of seedling survival, mainly consequent to the low habitat quality (Pérez-Garcia et al. 2003).

Thymus capitatus L., known in Tunisia under the vernacular name of "Zaater", is used as herbal teas, condiment and as a food additive. It's an aromatic plant very important for the horticultural and medicinal industry and it's the most important and expensive of all commercial origanum oils (Bauer et al. 1997; Hedhili et al. 2005). In Mediterranean cooking, it's extensively used for culinary purposes, for its use in flavouring sauces, stews and soups. In Tunisian folk medicine, the species is used as decoction in case of colic, ulcer and arterial hypertension (Bouraoui 2000). It's mainly known for its antibacterial, antioxiantimycotic and spasmolytic activities dant. (Bounatirou et al. 2007). Infusion and decoction of leaves and flowers were used against diarrhoea, digestive and respiratory system disorders (Stahl-Biskup and Sáez 2002).

In Tunisia, the species grows mainly in dry and sunny garrigues issued from the degradation of Pinus halepensis L. and Juniperus phoenicea L. primary forests. Populations are located in different bioclimatic zones (extending from the sub-humid to the upper arid) on sandy and often on rocky soils, under a rainfall varying from 300 to 1000 mm/year and at altitudes ranging from 150 to 500 m (Nabli 1995). The species occurs as mosaic of local populations in little patches along the landscape. The populations have been sedepleted verely and fragmented due to overexploitation and habitat destruction caused by grazing, clearing, low soil quality and irregularity of rainfall. The habitat fragmentation and the spatial isolation of populations increase genetic drift and differentiation between populations, and reduce their adaptation to environmental changes future (Ellstrand and Elam 1993). Thus, understanding the patterns of the genetic variation within and among populations is crucial to evaluate the present status of these populations and develop conservation programs. The aim of this study is to assess the fragmentation and the extent of genetic diversity within and

among Tunisian populations of *Thymus capitatus* from different bioclimatic zones using RAPDs (Random Amplified Polymorphic DNA) (Williams et al. 1993), for information on the genetic variation of the species in order to work out the conservation strategy and improvement programs.

RAPDs are extensively applied to assess the genetic diversity and to address conservation and protection purposes in woody plant populations (Monteleone et al. 2006; Solouki et al. 2008; Zheng et al. 2008). They involve the analysis of a large number of loci, thus providing a more complete evaluation of the genome as compared with other biochemical and molecular markers (Monteleone et al. 2006). RAPDs are selectively neutral and able to detect high variation both in coding and non-coding DNA regions without prior knowledge of the genome (Allnutt et al. 2003; Nybom and Bartish 2000). Nevertheless, RAPDs have same disadvantages, the most significant being 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 (Nybom 2004). However, these disadvantages could be overcome by the use of appropriate statistical methods such as the analysis of the molecular variance (Nybom and Bartish 2000). Besides, the detection of a high number of polymorphic loci, the analysis of bands with frequency higher than 3/N (N was the number of the analysed plants) and the reproducibility of experiments could buffer significantly the bias of estimation of the genetic variation (Hovmalm et al. 2004; Nybom and Bartish 2000).

# Material and Methods

#### Surveyed populations and sampling

Nine Thymus capitatus L. populations in the whole distribution area of the species in Tunisia were chosen on the basis of previous work using isozyme and chemical markers (Bel Hadj Ali et al. 2006; Ben El Hadj Ali 2010; Ben El Hadj Ali et al. 2012). They have been collected from different bioclimatic zones (sub-humid, upper semi-arid, mean semi-arid, lower semi-arid and upper arid) according to Emberger's  $Q_2$ coffecient (1966)  $[Q_2 = 2000 \text{ P/(M^2-m^2)}, \text{ where P is}$ the mean of annual rainfall (mm), M (K) is the mean of maximal temperatures for the warmest month (June) and m (K) is the mean of minimum temperatures for the coldest month (February)]. The altitudes of sites varied from 100 m to 600 m. The average of the annual rainfall ranged from 125 mm to 550 mm (Table 1). The soil is calcareous or marno-calcareous. Ten plants, from each population, were randomly sampled at distances exceeding 20 m to avoid

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Population code	Population	Bioclimatic zone*	Latitude	Longitude	Q <sub>2</sub> coefficient	Altitude (m)	Rainfall (mm/year)
1	El Hairech Jeb. Mt. <sup>*</sup>	Sh	36°10'N	8°4'E	64.52	440	500-600
2	Abderrahmen Jeb. Mt.	Sh	36°40'N	10°41'E	65.64	420	500-600
3	Bou Argoub	Usa	36°31'N	10°3'E	59.72	100	400-500
4	Mansour Jeb. Mt.	Usa	36°17'N	9°36'E	45.72	600	400-500
5	Essers	Msa	36°76'N	9°40'E	44.24	604	400-500
6	Goubellat Jeb. Mt.	Msa	36°60'N	9°52'E	39.75	300	400-500
7	Khenis	Lsa	35°45'N	10°49'E	34.05	300	300-400
8	Aïn Errahma	Lsa	36°13'N	10°22'E	33.24	100	300-400
9	Toujene	Ua	33°27'N	9°58'E	29.19	600	100-150

Table 1. Main ecological traits for the nine *Thymus capitatus* populations analysed

Sh: Sub-humid, Usa : Upper semi-arid, Msa: Mean semi-arid, Lsa: Lower semi-arid, Ua: Upper arid. \*Jeb. Mt.: Jebel Mountain. \*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 2008 (Tunisian National Institute of Meteorology).

the sampling of closely related individuals. Samples were stored in plastic bags at -80°C before DNA extraction.

#### **DNA** extraction

DNA was prepared by grinding 500 mg of young leaves from each plant in liquid nitrogen, 2 ml of preheated CTAB extraction buffer added with 1% PVP (40000). Samples were incubated at 65°C for 1 hour in a water bath. Subsequently, the mixture was treated twice with  $600 \,\mu$ L 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 estimated on an agarose gel (0.8%) stained with ethidium bromide.

#### RAPD assay and PCR conditions

PCR was performed in 25  $\mu$ L reaction volume containing 50 ng DNA, 5  $\mu$ L of 5 X reaction buffer, 40 pmoles of primer, 200  $\mu$ M of each dNTP, 2.5 mM MgCl<sub>2</sub> and 1.5 U Taq polymerase (Promega). The mixture was overlaid with one drop of mineral oil. Reactions were performed in Stuart Thermal Cycler (Maxi-Gene) programmed for an initial denaturation step of 94°C for 2 min, followed by 45 cycles of 30 s at 94°C, 1 min at 36°C (annealing step), and 2 min at 72°C (elongation step). An additional 10 min period for elongation at 72°C followed this cycle. To test reproducibility between and within runs, DNA from same two plants was included in every PCR run. A negative control without DNA was also used in every PCR run. Eighteen RAPD 10-mer oligonucleotide primers (kit OPJ, Promega) were screened. After optimizing the PCR conditions, seven RAPD primers were selected according to consistency, reproducibility and polymorphism level of electrophoretic bands to amplify DNAs. Primers selected are: OPJ-06 (5'TCGTTCCGCA3'), OPJ-08 (5'CATACCGTGG3'), OPJ-10 (5'AAGCCCGAGG3'), OPJ-12 (5'GTCCCG-TGGT3'), OPJ-13 (5'CCACACTACC3'), OPJ-14 (5'CACCCGGATG3') and OPJ-16 (5'CTGCTTAG-GG3'). Amplification products were separated by electrophoresis in 1.5% agarose gels in TAE buffer (pH 8), stained with ethidium bromide, and visualized under UV light using a DOC PRINT Photo Documentation System. Molecular weights were estimated using a 200 bp DNA Promega ladder.

#### Data analysis

RAPD markers were scored as presence (1) or absence (0) of bands, and then transformed into a binary matrix. Each marker band was assumed as being a single locus. For each primer the percentage of polymorphic bands was estimated for each population. The genetic diversity within a population was estimated using the percentage of polymorphic bands P% [(number of polymorphic bands/total number of bands) x 100] and Shannon's index (H') for each RAPD locus [H' = -  $\Sigma p_i \log_2 p_i$ , where  $p_i$  is the frequency of RAPD band in ith population]. Shannon's index was also used to estimate the average diversity  $H_{DOD}$  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 populations was estimated as  $H_{pop}/H_{sp}$ , that among populations was evaluated by  $G_{sT} = (H_{sp} - H_{pop})/H_{sp}$ . Shannon's diversity indices and G<sub>ST</sub> were also used to partition the variation within and among ecological groups (each ecological group includes populations from the same bioclimate). The different indices were calculated by the POPGENE computer package software (Yeh et al. 1999). The comparison among Shan-

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non's diversity 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 partitioning of the genetic variation within and among populations or within and among ecological groups, besides  $H_{pop}/H_{sp}$  and  $G_{ST}$  estimates, was evaluated by AMOVA using WINAMOVA program, version 1.55 (Excoffier et al. 1992).  $\Phi$ -statistics:  $\Phi_{ST}$ (differentiation among populations),  $\Phi_{CT}$  (differentiation among ecological groups) and  $\Phi_{sc}$  (differentiation among populations within groups) were calculated. The significance of variance components and that of  $\Phi$ -statistics were estimated using permutation procedures (NTSYS-pc, version 2.0, Rohlf 1998). The gene flow (Nm) between populations was estimated as Nm =  $[(1/\Phi_{ST})-1]/4$  (Wright, 1951). The correlations between the matrices of genetic distances ( $\Phi_{ST}$ ) and geographic distances (Km),  $\Phi_{ST}$  and altitudes or  $\Phi_{sT}$  and Emberger's Q<sub>2</sub> pluviothermic coefficients (Emberger 1966) was estimated by a Mantel test (Mantel 1967) using ZT program (Bonnet and Van de Peer 2002). The significance of the correlation was tested after 1000 permutations.

The genetic similarity between individuals was estimated using the Nei and Li's (1979) similarity 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,  $m_x$  and m<sub>v</sub> are the number of bands in samples x and y, respectively]. 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). A PCO based on Nei and Li's genetic similarity was performed to ordinate the relationship among all individuals using the MVSP program (version 3.1). UPGMA dendrogram analysis displaying the relationship among populations was also produced using pairwise  $\Phi_{\rm ST}$  matrix among populations.

## Results

#### The genetic diversity

The seven primers used for all populations generated 121 discernible and reproducible DNA fragments, out of which 103 (85.12 %) were polymorphic. The bands ranged in size from 200 to 2000 bp. The number of bands produced by primer varied from 13 (OPJ-06 and OPJ-13) to 22 (OPJ-08), with an average of 17.28 at the species level. The percentage of polymorphic loci per primer varied from 64.71% (OPJ-14) to 100% (OPJ-08). Specific bands were revealed according to populations and bioclimates. For example, bands 340 bp (OPJ-06), 460 bp (OPJ-08) and 400 bp (OPJ-12) were restricted to populations from the sub-humid and the mean semi-arid zones (populations 1, 2, 5 and 6). Bands 700 bp and 1700 bp (OPJ-16) were only observed in populations from the sub-humid and the upper arid bioclimates (populations 1, 2 and 9). The band 650 bp (OPJ-12), which was found to be widely represented, is not found in the populations 1 and 2 from the sub-humid area. Bands 1000 bp (OPJ-08) and 1400 bp (OPJ-16) were not observed in the lower semi-arid populations 7 and 8.

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 30.77% to 86.36%. Primers differed in their ability to distinguish individuals within and among populations. OPJ-08 followed by OPJ-10 and OPJ-14 revealed the highest percentage of polymorphism for all populations (63.64%, 58.89%) and 55.53% respectively), while OPJ-06 and OPJ-16 identified only 42.74 and 48.15% of polymorphism. The highest number of polymorphic fragments within populations was scored for OPJ-08 (populations 1, 4, 7, 8 and 9), OPJ-10 (population 6), OPJ-13 (populations 3 and 5) and OPJ-16 (population 2). The average percentage of the polymorphic loci detected with all primer ranged from 61.16% (population 1) to 50.41% (population 3) with an average of 69.26%. The percentage of polymorphic loci scored in each ecological group over all primers varied from 53.72% (Upper arid zone) to 76.86% (Sub-humid zone). OPJ-16, OPJ-12, OPJ-13, OPJ-10 and OPJ-08 are characterized by being more efficient in the detection of polymorphism according to bioclimates.

Shannon's diversity index (H') estimating the within-population variability ranged from 0.276 (population 3 from the upper semi-arid zone) to 0.327 (population 8 from the lower semi-arid zone). However, H' values did not show significant differences among populations (ANOVA test, p > 0.05). The average of H' value for all populations was 0.303 indicating a low level of the within-population genetic diversity (Table 3). At the species level, the Shannon index was moderate ( $H_{sp}=0.473$ ). The within-ecological group diversity (H'<sub>gp</sub>) varied from 0.296 (Upper arid zone) to 0.417 (Mean semi-arid zone) with an average of 0.384. The average of within-group diversity did not differ statistically among groups (ANOVA; p>0.05). The proportions  $(H_{pop}/H_{sp}=0.641)$ within-populations of and within-ecological groups ( $H_{gp}/H_{sp} = 0.791$ ) genetic diversity were high.

Denulation				Primer				D+07
Population	OPJ06	OPJ08	OPJ10	OPJ12	OPJ13	OPJ14	OPJ16	Pl%
1	53.85 (7)	86.36 (19)	50 (10)	53.33 (8)	61.54 (8)	47.06 (8)	66.67 (14)	61.16
2	53.85 (7)	59.09 (13)	45 (9)	53.33 (8)	38.46 (5)	58.82 (10)	76.19 (16)	56.20
3	38.46 (3)	40.91 (9)	60 (12)	53.33 (8)	69.23 (9)	47.06 (8)	47.62 (10)	50.41
4	46.15 (6)	72.73 (16)	35 (7)	53.33 (8)	61.54 (8)	58.82 (10)	38.10 (8)	52.07
5	53.85 (7)	63.64 (14)	50 (10)	46.67 (7)	69.23 (9)	58.82 (10)	42.86 (9)	54.55
6	38.46 (5)	54.55 (12)	85 (17)	60.00 (9)	38.46 (5)	58.82 (10)	28.57 (6)	52.89
7	23.08 (3)	68.18 (15)	60 (12)	46.67 (7)	38.46 (5)	64.71 (11)	42.86 (9)	51.24
8	30.77 (4)	59.09 (13)	80 (16)	53.33 (8)	46.15 (6)	52.94 (9)	42.86 (9)	53.72
9	46.15 (6)	68.18 (15)	65 (13)	53.33 (8)	30.77 (4)	52.94 (9)	47.62 (10)	53.72
Average	42.74	63.64	58.89	52.59	50.43	55.55	48.15	69.26
Bioclimatic zone	2							
Sh	84.62 (11)	86.36 (19)	60.00 (12)	66.67 (10)	84.62 (11)	64.71 (11)	90.48 (19)	76.86
Usa	53.85 (7)	81.82 (18)	75.00 (15)	86.67 (13)	76.92 (11)	70.59 (12)	76.19 (16)	75.21
Msa	69.23 (9)	68.18 (15)	90.00 (18)	80.00 (12)	92.31 (12)	64.71 (11)	57.14 (12)	73.55
Lsa	46.15 (6)	81.82 (18)	85.00 (17)	66.67 (10)	46.15 (6)	64.71 (11)	61.90 (13)	66.94
Ua	46.15 (6)	68.18 (15)	65.00 (13)	53.33 (8)	30.77 (4)	52.94 (9)	47.62 (10)	53.72
Average	42 74	63 64	58.89	52 59	50.43	55 55	48 15	69.26

Table 2. Percentage of polymorphic loci (Pt%) and number of polymorphic bands (in parenthesis) per primer in each population and bioclimatic zone

Number of polymorphic bands are given in parentheses; Pt%: Percentage of polymorphic loci

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'		0.321 a	0.309 a	0.276 a	0.302 a	0.314 a	0.294 a	0.291 a	0.327 a	0.296 a
	$H_{\text{pop}}$	0.303 ns									
	$H_{sp}$	0.473									
	$H_{pop}/H_{sp}$	0.641									
	G <sub>ST</sub>	0.359									
Ecological group			Sh	Usa		Msa		Lsa		Ua	
	$H'_{gp}$		0.408 a	0.412 a		0.417 a		0.389 a		0.296 a	
	$H_{\rm gp}$	0.384 ns									
	$H_{sp}$	0.486									
	$H_{gp}/H_{sp}$	0.791									
	$G_{\text{STg}}$	0.209									

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 ,  $H_{pop}$ , 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 (Hsp) and their partition within- ( $H_{pop}$ ,  $H_{sp}$ ) and between-populations ( $G_{sT}$ ) components respectively, calculated for all primers.  $H'_{sp}$ ,  $H_{sp}$ , H

# The genetic structure and divergence among populations

The  $G_{ST}$  value estimating the genetic structure among populations was 0.359 and among groups  $(G_{STg})$  was 0.209 (Table 3). The within-population component of variance estimated through AMOVA accounted for 71.61% of overall variation, while that among populations was 28.39% (Table 4). The mean  $\Phi_{ST}$  value among all populations was 0.284, and all pairwise  $\Phi_{ST}$  values were significantly different from zero (Table 5). The highest  $\Phi_{ST}$  values were found, respectively, between populations 3 and 4 ( $\Phi_{ST}$ =0.365), which of 90 Km distant and between the populations 3 and 8 ( $\Phi_{ST}$ =0.353), which of 40 Km apart. The lowest  $\Phi_{ST}$  value (0.205) was found among the populations 7 and 8, separated by 50 km. The effective number of migrants among populations is low

Source of variation	d.f.	Msd	Variance	Total variance (%)	Φ-statistics
Population		1104	, unance		1 Stationed
Among populations	8	65.10	5.25	28.39	$\Phi_{\rm ST} = 0.284^{**}$
Within populations	80	13.23	13.23	71.61	
Total	88				
Ecological group					
Among groups	4	68.26	0.35	1.89	$\Phi_{CT}=0.019^{ns}$
Among populations/group	4	61.93	4.93	26.65	$\Phi_{SC} = 0.272^{**}$
Within populations	80	13.23	13.23	71.46	
Total	88				

Table 4. Analysis of molecular variance within and among *Thymus capitatus* populations and within and among ecological groups

d.f.: degree of freedom; Msd: Mean squared; ": not significant; \*\*: significant at p<0.001 after 1000 permutations

 $\Phi_{st}$ : differentiation among populations,  $\Phi_{ct}$ : differentiation among ecological groups,  $\Phi_{sc}$ : differentiation among populations within groups.

(0.435<Nm<0.970). The correlation coefficient, by Mantel test, did not show significant relationship between matrices of  $\Phi_{ST}$  and geographical distances (r=-0.13, p=0.29>0.05),  $\Phi_{ST}$  and Emberger's Q<sub>2</sub> coefficients (r=0.253, p=0.07>0.05) or  $\Phi_{ST}$  and altitudes (r=0.028, p=0.41>0.05). At the ecological group level, the AMOVA analysis revealed that 71.46% of the total genetic variance occurred among individuals within-ecological groups and only 26.65% occurred among populations within a group (Table 4). The differentiation among groups was low ( $\Phi_{CT}$ =0.019).

Neighbour-joining dendrogram generated from Nei and Li's genetic distances between all individuals showed that the majority of individuals from the same population grouped together and populations from the same bioclimate did not always clustered closely (Fig. 1). The PCO based on Nei and Li's similarity matrix for all individuals showed that the first three principal axes accounted for 19.9% of the total variation. The plot according to the first two PCO axes (14.26%), revealed three major population groups (Fig. 2). The first one, projected at the negative side of axes 1 and 2, is represented by individuals belonging to populations Essers (population 5, Mean semi-arid zone) and Bou Argoub (population 3, Upper semi-arid zone). The second group, situated at the positive side of the axis 2, includes individuals of Abderrahmen (population 2) and Mansour Mountains (population 4) from the sub-humid and the upper semi-arid, respectively. Individuals belonging to the Mean semi-arid population 6, the lower semi-arid populations 7 and 8 and the upper arid population 9, constitute the third group situated at the positive side of the axis 1. Individuals from the sub-humid population 1 (El Hairech Montain) were sparsely plotted near the origin of axes.

The dendrogram generated from pairwise  $\Phi_{ST}$  matrix among populations showed three distinct groups without evident relationship to bioclimate and/or geographic distance (except for populations 7 and 8) (Fig. 3). The first group (G1) includes populations 3 and 5 from the upper semi-arid and mean semi-arid bioclimates, respectively. The population 4 from the upper semi-arid zone constituted the second group (G2). The third group (G3), which could be subdivided into two subclusters includes the lower semi-arid populations 7, 8 and the upper arid population 9 (subcluster 1) and the populations 1, 2, 6 from the sub-humid and mean semi-arid bioclimates, respectively (subcluster 2).

Table 5. Pairwise gene flow (N	lm) (above	diagonal) and	d $\Phi_{sT}$ values	(below diagonal)	among populations	of Thymus	capitatus
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Population	1	2	3	4	5	6	7	8	9
1		0.796	0.605	0.534	0.744	0.674	0.737	0.717	0.720
2	0.239**		0.777	0.671	0.676	0.836	0.591	0.565	0.592
3	0.292**	0.243**		0.435	0.850	0.505	0.527	0.450	0.516
4	0.319**	0.271**	0.365**		0.547	0.593	0.555	0.562	0.579
5	0.252**	0.270**	0.227**	0.314**		0.704	0.479	0.552	0.549
6	0.271**	0.230**	0.331**	0.297**	0.262**		0.681	0.802	0.816
7	0.253**	0.297**	0.322**	0.311**	0.343**	0.269**		0.970	0.710
8	0.259**	0.307**	0.353**	0.308**	0.312**	0.238**	0.205**		0.828
9	0.258**	0.297**	0.326**	0.302**	0.313**	0.235**	0.260**	0.232**	

1, 2, ...: population code; "Highly significant at p < 0.001 (after 1000 permutations).



Fig. 1. Neighbour-joining dendrogram generated for 90 individuals of *Thymus capitatus* analysed using Nei and Li's genetic distances

Population code: Numbers (1, 2, ..., 9).



Fig. 2. Principal Coordinates Analysis (PCO) generated from Nei and Li's similarity coefficient for the 90 individuals analysed of *Thymus capitatus*. Plot according to axes 1–2. *Population code*: Numbers (1, 2,..., 9).



Fig. 3. UPGMA dendrogram of pair-wise  $\Phi_{ST}$  values derived from AMOVA of RAPD profiles *Population code*: Numbers (1, 2,..., 9).

#### Discussion

Our results based on RAPD markers revealed a low level of variation within populations and high differentiation among them. The analysis of population genetic variation with RAPDs could be hampered by a loss of a part of genetic information (Kremer et al. 2005; Nybom 2004). However, this disadvantage could be buffered by the detection of a high number of loci (Aagaard et al. 1998) and the use of appropriate genetic population parameters such as  $\Phi_{ST}$  which reduces the bias in estimating the genetic variation. In our study, the seven selected primers revealed 121 loci, of which 103 were polymorphic. This large number of loci could be considered sufficient to compensate loss of genetic information content at loci.

Thymus capitatus populations maintain a moderate genetic diversity ( $H_{sp}$ =0.473). It could be explained by the outcrossing system associated probably with the number of initial founders that differed genetically in a population (Dittbrenner et al. 2005; Thompson 1999, 2002). Additionally, populations of Thymus capitatus analysed were fragmented due intensive forest clearing, grazing and occur in small scattered patches. The level of the genetic diversity may result from loss of genetic variation through inbreeding and/or genetic drift caused by the restriction of the species to small and degraded populations. However, the outbreeding mating system and the persistence of multiple individuals through generations issued from large populations before fragmentation might be the main contributing factors of this high level of intrapopulational variation ( $H_{pop}/H_{sp}$ =0.641). The level of average within-population gene diversity was relatively lower compared to values derived from RAPDs for other Lamiaceae plants with an outcrossing mating system (Boulila et al. 2009; Verma et al. 2007).

In our work several RAPD loci appear to be specific to populations according to the biocliamte (i.e. OPJ06-5, OPJ08-8, OPJ12-3, OPJ16-12 and OPJ16-21 in the Sub-humid populations 1 and 2, OPJ08-8 and OPJ12-3 in populations 5 and 6 from the Mean semi-arid, and OPJ16-12 and OPJ16-21 in the Upper arid population 9). However, the detection of these loci might not reflect adaptability to ecological factors since they were not detected in all populations from the same bioclimate. Besides, the variation of RAPDs, may not be a reliable measure of adaptive potential (Volis et al. 2005). These markers were believed to reveal variation both in coding and non-coding genes (Castro-Félix et al. 2008; Nybom and Bartish 2000).

Thymus capitatus populations were highly differentiated ( $G_{ST}$ =0.359;  $\Phi_{ST}$ =0.284). The observed amount of differentiation was higher than the average for perennial outcrossing species ( $G_{ST}$ =0.22,  $\Phi_{ST}$ =0.27) (Hamrick and Godt 1996; Nybom 2004; Nybom and Bartish 2000). This high differentiation could be explained by genetic drift due to limited gene flow (0.435<Nm<0.970) via seed and/or pollen dispersal caused by the restriction of the species to small isolated and degraded populations. Most thymes were believed to be short distance dispersal species. Thompson (2002) reported that pollen and seed dispersals in thymes are highly localised, increasing the tendency for reproduction to occur within spatially localized groups. The differentiation among Tunisian T. capitatus populations occurs at local spatial scale without relationship to geographical distance or ecological factors (altitudes and Q<sub>2</sub> coefficients). The highest  $\Phi_{st}$  value and the lowest level of gene flow were observed between populations 3 and 4 (90 Km), both belonging to the upper semi-arid zone and between populations 3 and 8 (40 Km), belonging to the upper semi-arid and the lower semi-arid bioclimates, respectively. Besides, the highest Nm value and the lowest  $\Phi_{ST}$  value were noted between populations 7 and 8 (50 Km), belonging to the lower semi-arid bioclimate. The absence of correlation between population genetic structure and geographic distances suggests the existence of important historical gene flow between populations. Therefore, the current scattered distribution area of Tunisian *T. capitatus* and the high differentiation among populations is probably recent due mainly to anthropic pressures.

Shannon's index indicates that the genetic diversity in *T. capitatus* was distributed within rather than among populations ( $H_{pop}/H_{sp}$ =0.641). AMOVA conducted at the population and ecological group levels showed also that most of the total variation was found among individuals within populations or within populations in their corresponding group (71.61%; 71.46%). This suggests that mating occurs mainly among individuals within a sub-population thus favouring the divergence between populations.

The PCO analysis showed that populations plotted as a broad scatter, and gathered often without evident relationship to the bioclimate with the exception of populations 7 and 8 (Lower semi-arid zone). Results from the UPGMA dendrogram also revealed a differentiation among populations independently to bioclimate excepted for populations 7 and 8. Several populations from continuous bioclimates (populations 3 and 5) gathered together, and a high distinction between the two mean semi-arid populations 5 and 6 was revealed indicating that genetic differentiation mainly occurs at local space scale due to limited gene flow and genetic drift.

Thymus capitatus occurs in small scattered populations due to habitat fragmentation. The within-populations genetic diversity is low and the among-populations differentiation is high  $(G_{ST}=0.359 \text{ and}$  $\Phi_{sT}$ =0.284). These processes may increase genetic drift. Thus, efforts to conserve populations are urgently needed. Similar patterns of genetic diversity as estimated by the Shannon's index and no differences were observed between mean values of ecological groups. Therefore, the *in-situ* conservation should include all populations. It could be achieved by valuable defence favouring natural individual regeneration. However, given the similar levels of the within-population diversity, preserving several populations may lead to capture maximum of genetic diversity within the species. The Northwestern population 1 (El Hairech Montain) from the sub-humid zone growing in hard environmental conditions and exhibiting high individual's heterogeneity should be preserved. The marginal upper arid population 9 and the mean semi-arid populations (5 and 6), characterized by high genetic isolation, should be also protected according to their specific local environmental pressures (overgrazing, level of site degradation, etc.). Despite, the non significant difference of H' values among ecological groups and among populations, conservation actions should be made appropriately within and among bioclimates (i.e. populations 7 and 8 from the lower semi-arid zone or populations 1 and 2 from the sub-humid zone). Populations exhibiting specific bands (i.e. populations 1, 2, 5, 6 and 9) must be first protected, necessary for capturing most diversity.

The amount of the within-population variation is higher than that revealed among populations. This suggests that the *ex-situ* conservation should be based on the collection of seeds/or cuttings within rather than among populations. The species is outcrosser and capable of vegetative reproduction. Therefore, the collect of plants should take into account these factors. The collection of samples must interest both close and distant geographically populations because of the significant differentiation among populations at all spatial scales (i.e.  $\Phi_{ST}$  for populations 3 and 8 or populations 7 and 8). Because of the divergence among populations from the same bioclimate (e.g. populations 5 and 6 or populations 1 and 2), the collection of samples should be made appropriately within each bioclimate.

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