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Original article

Genetic structure of Tunisian natural carob tree (*Ceratonia siliqua* L.) populations inferred from RAPD markers

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Abstract

• Seven RAPD markers were used to assess the genetic diversity and structure of ten Tunisian natural *Ceratonia siliqua* L. populations from different geographic and bioclimatic zones.

• The species maintain a high diversity within population as estimated by the percentage of polymorphic loci and Shannon's index ($P\% = 76.31$, $\overline{H}'_{pop} = 0.569$). The range of variation between populations was large. Populations from the upper semi-arid bioclimates, with more continuous distribution area showed the highest level of variation.

• A high genetic differentiation among populations ($\Phi_{ST} = 0.250$ and $\overline{G}_{ST} = 0.347$), as a result of population isolation was revealed. Nevertheless, the genetic structure is in accordance with bioclimate indicating that ecological factors also should influence differentiation. Populations from the sub-humid, upper semi-arid and mean semi-arid zones clustered together and were distinct from those of the lower semi-arid ones.

• Conservation strategy should be made according to the level of polymorphism within population and bioclimate.

Mots-clés :
diversité génétique / RAPD / *Ceratonia siliqua* / populations naturelles / bioclimat / conservation

Résumé – Structure génétique des populations naturelles du caroubier (*Ceratonia siliqua* L.) en Tunisie estimée par les marqueurs RAPD.

• Sept marqueurs RAPD ont été utilisés pour analyser la diversité et la structure génétique de dix populations tunisiennes de caroubier (*Ceratonia siliqua* L.) prospectées dans différentes zones géographiques et bioclimatiques.

• L'espèce maintient une diversité génétique intra population importante ($P\% = 76.31$; $\overline{H}'_{pop} = 0.569$) et les niveaux de variation diffèrent selon les populations. Celles du subhumide et du semi-aride supérieur, à aire de distribution plus continue, ont montré la variation la plus importante.

• Une forte différenciation ($\Phi_{ST} = 0.250$ et $\overline{G}'_{ST} = 0.347$) entre les populations, due à leur fragmentation récente, est observée. Toutefois, cette différenciation concorde avec le bioclimat des sites témoignant l'influence des facteurs écologiques dans cette différenciation. Les populations du subhumide, du semi-aride supérieur et du semi-aride moyen constituent un groupe distinct de celui représenté par les populations du semi-aride inférieur.

• La conservation des populations doit tenir compte de leur niveau de diversité génétique intra population et du bioclimat.

1. INTRODUCTION

Ceratonia siliqua L. (Caesalpinioideae) is a diploid forest (or semi forest) tree ($2n = 2x = 24$) native from the Mediterranean basin (Battle, 1997; Mitrakos, 1968; Talhouk et al., 2005). It has been cultivated for a long time in many countries (i.e. Spain, Italy, Turkey, Greece, Morocco, Tunisia, USA, Australia, etc), for human and animal consumption (Albanell et al., 1996; Calixto and Canellas, 1982), and for restoration of degraded arid areas (Battle and Tous, 1997; Correia and Martin-Louçào 2005). At present, the demand for this plant (mainly for seed products) is increasing for pharmaceutical

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(Khair et al., 2001) dietetic, food and cosmetic uses (Albanell et al., 1991; 1996; Corsi et al., 2002; Roukas, 1994). However, most of the material used by these industries comes from wild populations which are more and more destroyed, and replaced with more profitable species (i.e. *Olea europea*, *Vitis vinifera*, *Ficus carica*). The number of carob orchards, mainly in North Africa, is small, they are abandoned and pods are frequently left on trees. In most Mediterranean countries (except Spain) a lack of selected cultivars was observed (Battle and Tous, 1997).

In Tunisia, Carob populations grew in uninterrupted expanses in the Northern (Mogods) and Central (Tunisian Dorsal) areas, and in the Northern coast (Cap Bon) under a rainfall ranging between 350 and 800 mm/year (Rejeb, 1989). Populations are associated with *Pistacia lentiscus* and/or *Olea europea* and grow on calcareous, sandy or low-clayey soils at altitudes varying from 300 to 970 m. Forest clearing for the cultivation of olive trees, cereals and vine, and for charcoal production have led to an extensive carob habitat destruction (mainly in the coast region). The species, at present, is mainly represented by fragmented populations with scattered individuals (Afif et al., 2006; Boussaïd et al., 1998; Le Houerou and Le Floc'h, 1995). Populations, except for those preserved within forests of Zaghouan and Bargou Jbel Mountains, are facing high degradation and isolation with an unknown impact on their genetic diversity and structure. The capacity of populations maintenance is dependent on their adaptive potential to environmental changes, which determined by their genetic diversity level.

Assessing the genetic diversity (via morphological, isozymic and molecular markers) within and among populations, according to their ecological distribution, would be required to ensure the in situ maintenance of populations and to draw up conservation and improvement strategies.

Studies addressing genetic diversity and differentiation among natural carob populations in the Mediterranean countries are very limited. Work using isozyme markers reported that Tunisian carob populations maintain a high variation within populations, and their genetic structure is concordant with bioclimate (Afif et al., 2006). Based on RAPDs, Talhouk et al. (2005) showed that Lebanese semi-natural carob populations exhibit significant genetic variation within and among populations. The populations are not clustered according to their geographic proximity. Analyses, using isozymes, performed on carob cultivars from different origins have shown low polymorphism between accessions indicating that selection has been performed from a narrow genetic base (Tous et al., 1992).

This study aimed to assess the genetic variation in Tunisian carob populations using random amplified polymorphic DNA (RAPD) to improve current understanding of the species genetic diversity and population structure. This work is an extension of that initiated on Tunisian carob population genetic structure based on isozymes for the same populations (Afif et al., 2006).

RAPD markers are an appreciable tool for population genetic studies (Besse et al., 2004; Fischer et al., 2000; Mariette et al., 2007; Sudupak et al., 2002; Williams et al., 1990) particularly in woody plants (Lee et al., 2002). These markers can reveal a high number of loci, providing a more representative sample of the genome than is possible with isozymes. They are able of detecting variation in non-coding regions of the genome. However, RAPDs, as isozymes, have some limitations. The most one is their dominant expression that can bias the genetic diversity and differentiation among populations (Allnutt et al., 2003; Nybom and Bartish 2000; Wu et al., 1999).

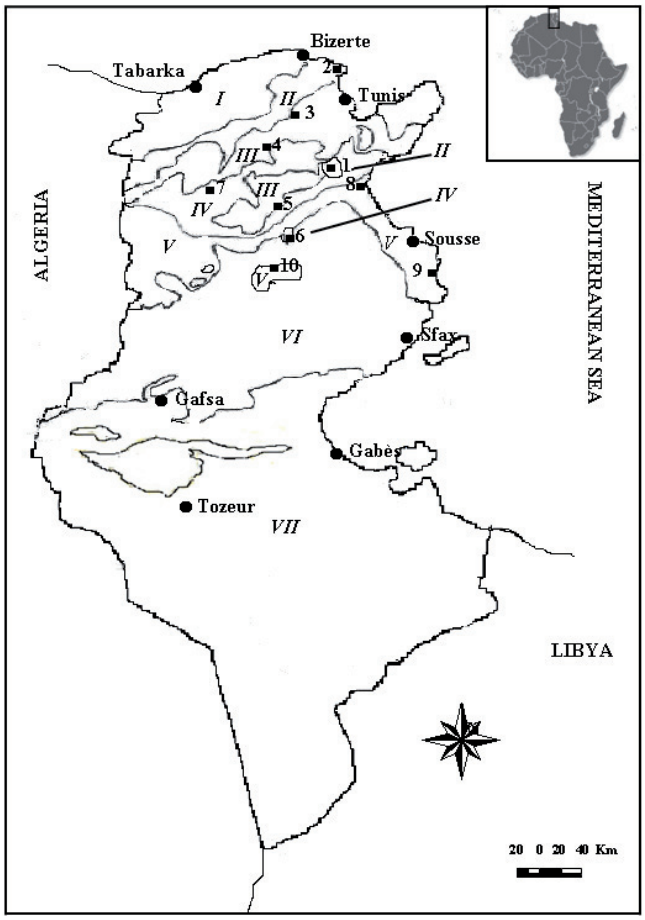

Figure 1. Map of Tunisia: Geographical distribution of the 10; *Ceratonia siliqua* populations analysed. ■1, 2,. . . ,10: populations *I*. . . *VII*: Bioclimatic zones;*I*: Lower humid, *II*: Sub-humid, *III*: Upper semi-arid, *IV*: Mean semi-arid, *V*: Lower semi-arid, *VI*: Lower arid, *VII*: Saharian. ffl Greatest cites.

2. MATERIAL AND METHODS

2.1. Analysed populations

Ten populations from different areas extending from the North to the Center of the country were assessed (Fig. 1 and Tab. I). They belong to sub-humid (populations 1 and 2), upper semi-arid (populations 3, 4 and 5), mean semi-arid (populations 6 and 7) and lower semi-arid (populations 8, 9 and 10) bioclimates according to Emberger's (1966) pluviothermic coefficient (Q_2). The geographic

Table I. Main ecological characteristics for the 10 Tunisian *Ceratonia siliqua* populations analysed.

Population	Code	Altitude (m)	Latitude	Longitude	Rainfall (mm/year)	*Q_2 coefficient	Bioclimatic zone[b] (ecological group)
Zaghouan Jb. Mt[a]	1	970	36°23' N	10°08' E	500–600	65.47	Sub-humid
Ghar El Melh	2	420	37° 20' N	10° 14' E	500–600	88.70	
Lansarine Jb. Mt	3	480	37° 05' N	09° 44'E	500–600	52.39	Upper semi-arid
El Morra Jb. Mt	4	580	36° 35' N	09° 37' E	500–600	62.64	
Bargou Jb. Mt	5	865	36° 05' N	09° 30'E	400–500	45.72	
Ksar El Lamsa	6	610	35° 58' N	09° 37' E	400–500	42.68	Mean semi-arid
Aïn Toungua	7	475	36° 34' N	09° 34'E	400–500	40.35	
Jradou	8	565	36° 17' N	10° 19' E	300-400	37.52	Lower semi-arid
Khnis	9	300	35° 45' N	10° 49' E	350–500	36.75	
Oueslet Jb. Mt	10	887	35° 53' N	09° 44' E	400–500	37.46	

[a] Jb. Mt = Jbel Mountain.

[b] Bioclimatic zones were defined according to Emberger's (1966) Q_2 pluviothermic coefficient $Q_2 = 2000P/M^2-m^2$ where P is the average of annual rainfall (mm), M is the mean of maximal temperature (K: Kelvin) for the warmest month (July) and m is the average of minimal temperature (K) for the coldest month (February).

* Q_2 was calculated for each site using P, M and m average values for the period 1953–2003 from data provided by the Tunisian National Institute of Meteorology.

distribution of populations generally matches that of their bioclimatic zones. Altitudes of sites ranged from 300 to 970 m. The average annual rainfall varied from 300 to 600 mm. Populations, except for those from Zaghouan (1), El Morra (4) and Bargou (5) Jbel Mountains and Ksar Lamsa (6) are represented by scattered individuals.

Ten individuals in each population were collected with a minimum of 200 m between trees (the number of samples is limited because of the small size of most populations). From each individual, branches with young leaves were taken, placed on ice in plastic bags and transported to the laboratory for molecular analyses.

2.2. DNA extraction

DNA was isolated according to a modified CTAB method (Lodhi et al., 1994) by the use of NaCl (5 M) to remove polysaccharides and PVP to eliminate polyphenols during DNA purification. Five hundred milligrams of leaves from each plant were grounded to fine powder in liquid nitrogen and mixed with 2 ml of preheated CTAB extraction buffer added a 1% PVP (40000). Extraction was performed using equal volume of chloroform-isoamyl alcohol (24:1). Cold ethanol 95% was employed to precipitate the DNA which was washed with 10 mM ammonium acetate in 76% ethanol, and dried. The DNA pellet was re-suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). After total DNA dissolution, the RNA was eliminated by adding 2 µL of Rnase-Dnase free solution (10 µg/mL). DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm.

2.3. Random amplification and sampling primers

Reactions were standardized and all PCR reactions were run on the same thermal cycler (Mark Maximum-Gene). For every 25 µL of volume reaction, 50 ng of DNA, 2.5 µL of 10 X Taq polymerase buffer, 40 pM of primer, 2.5 mM of MgCl2, 200 µM of dNTP and 1.5 U of Taq polymerase were included. Each reaction was overlaid with an equal volume of mineral oil.

The PCR program was set as follow: 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.

Amplification products were separated by electrophoresis in 1.5% agarose gels made with 1X TAE buffer (pH 8), stained with ethidium bromide and visualized under UV light. As a size marker, a 200 Pb DNA Ladder (Promega) was run in each gel.

Seven out of 20-tested RAPD primers (from Operon Technologies) were selected according to consistency, reproducibility and polymorphism level of electrophoretic bands to amplify DNAs. Primers used are: OPJ04 (5'CCGAACACGG3'), OPJ06 (5'TCGTTCCGCA3'), OPJ08 (5'CATACCGTGG3'), OPJ12 (5'GTCCCGTGGT3'), OPJ13 (5'CCACACTAC-C3'), OPJ14 (5'CACCCGGATG3') and OPJ20 (5'AAGC-GGCCTC3') (for examples, see addendum online only).

2.4. Data analysis

RAPD markers were scored as presence (1) or absence (0) on a band. A data matrix based on all the observed bands was constructed. In each population and ecological group (populations from the same bioclimate), the genetic diversity was estimated by the percentage of polymorphic bands ($P\%$), which was calculated by dividing the number of polymorphic bands at population (or ecological group) and species levels by the total number of bands surveyed.

A binary matrix of RAPD's phenotypes was used to calculate frequencies of markers within population and species. The Shannon's index for each RAPD locus was calculated for each population as: $H'_j = -\Sigma p_i \log_2 p_i$; where p_i is the frequency of the presence or absence of a RAPD band in a population. The average diversity over all populations was calculated for each locus as: $H'_{pop} = -1/n\Sigma H'_j$;

where n is the number of populations. The species diversity was estimated for each locus as: $H'_{sp} = -\Sigma p_s \log_2 p_s$; where p_s is the frequency of presence or absence of the RAPD in the whole sample. To estimate the level of polymorphism for each primer, each calculated index (H'_j, H'_{pop} and H'_{sp}) for each locus was averaged across primer. We partitioned the species diversity (H'_{sp}) into within and among population components, thus for each locus, the component of diversity within populations is H'_{pop}/H'_{sp} and the component between populations is G'_{ST} ($G'_{ST} = (H'_{sp}–H'_{pop})/H'_{sp}$). For all loci and for each population we have calculated the average genetic diversity index ($\overline{H}'_j$), thus for all loci, we have determined $\overline{H}'_{pop}$, $\overline{H}'_{sp}$, $\overline{H}'_{pop}/\overline{H}'_{sp}$ and $\overline{G}'_{ST}$. The same Shannon indices were calculated according the ecological groups ($\overline{H}'_{jg}$, $\overline{H}'_{grp}$, $\overline{H}'_{grp}/\overline{SH}'_{sp}$ and $\overline{G}'_{STg}$).

The relationship among RAPD phenotypes also was visualized by a principal coordinate analysis (PCO) using the program MVSP version 3.1 (Kovach, 1999) and by a neighbour-joining tree (Saitou and Nei, 1987) constructed with MEGA program version 2.0 (Kumar et al., 2001), based on Jaccard's (GS) similarity coefficients (see addendum online only). The genetic distances between individuals were calculated as GD = 1 – GS (Christoph et al., 2005; Gower and Legendre, 1986).

An analysis of molecular variance using the program WINAMOVA 1.55 (Excoffier et al., 1992; Stewart and Excoffier, 1996) was performed on the matrix of genetic distances for the estimation of the distribution of genetic variability within and among populations and ecological groups. The program also extracts analogs of F-statistics (so-called Φ-statistics: Φ_{ST} (differentiation among populations), Φ_{CT} (differentiation among ecological groups) and Φ_{SC} (differentiation among populations within groups)). Homogeneity of molecular variance between pairs of populations was tested using Bartlett tests (Bartlett, 1937). Pairwise genetic distances (Φ_{ST}) among the 10 populations allow the estimation of gene flow as the number of individuals migrating among populations per generation, using Wright's (1951) migrate number ($Nm = 1/4[1/\Phi_{ST} - 1]$). To test the significance of the variance components we used 1000 independent permutations runs.

A Mantel test (Mantel, 1967) was used to determine whether the matrix of genetic distances (Φ_{ST}) was correlated with those of geographic distances, Emberger's pluviothermic coefficients (Q_2) and altitudes using the TFPGA 1.3 program (Miller, 1997). The significance of the correlation was tested after 999 permutations.

A dendrogram, using the program MEGA version 2.0 (Kumar et al., 2001) based on the matrix of Φ_{ST}, was constructed to estimate the genetic divergence between populations.

3. RESULTS

3.1. Level of genetic diversity

The seven selected primers generated a total of 114 bands. The number of bands varied from 14 (OPJ06, OPJ12 and OPJ20) to 20 (OPJ08 and OPJ14) (Tab. II). The amplified products size ranged from 200 to 3000 bp. The percentage of polymorphism (P%) over all populations varied from 70 (OPJ14) to 85.71% (OPJ20) according to primer, with an average of 76.31% (Tab. II). For all assessed primers, the highest P% value (64.91%) was observed for population 5 (Bargou Jbel Mountain), whereas the lowest one (44.74%) is scored for the population 10 (Oueslet Jbel Mountain). According to ecological groups, the percentage of polymorphism varied from 49.30% (lower semi-arid) to 61.51% (upper semi-arid) (Tab. II).

All populations showed a high level of genetic diversity (Tab. III). The Bargou Jbel Mountain population exhibited the highest variation ($\overline{H}'_j = 0.687$), the lowest variation ($\overline{H}'_j = 0.458$) was detected for Jradou population. The average of diversity using all primers at population ($\overline{H}'_{pop} = 0.569$) and species ($\overline{H}'_{sp} = 0.871$) levels were high. Within ecological groups, the highest Shannon's diversity index ($\overline{H}'_{jg}$) was observed for populations belonging to the upper semi-arid ($\overline{H}'_{jg} = 0.763$); the lowest values ($\overline{H}'_{jg} = 0.606$) were recorded for those from the lower semi-arid zone. The most of the variation accrued within population and within ecological group (respectively $\overline{H}'_{pop}/\overline{H}'_{sp} = 65.3\%$, $\overline{H}'_{grp}/\overline{H}'_{sp} = 80.1\ \%$). Variations between populations ($\overline{G}'_{ST} = 34.7\%$) and between ecological groups ($\overline{G}'_{STg} = 20\%$) were substantial.

3.2. Genetic structure

The average of genetic similarity based on Jaccard's coefficients (GS) was 50.3%. The highest value of GS (65.4%) was observed among individuals belonging to population of Khnis. The Bargou Jbel Mountain population showed the lowest value (GS = 52.5%).

Neighbour-joining analysis produced tow distinct groups (Fig. 2). The first one is constituted by individuals belonging to populations from the sub-humid and the upper semi-arid bioclimates (populations 1, 2, 3, 4 and 5), the second includes individuals from the mean semi-arid and lower semi-arid populations (populations 6, 7, 8, 9 and 10). Several individuals from different populations (in the same bioclimate) were located in a same subcluster.

The plot of the Principal Coordinates Analysis (PCO), according to axes 1 and 2 (described 17.2% of the total variation) (Plot not shown), revealed a similar population groupings than those showed by the dendrogram (Fig. 3).

All pairs of populations showed significant differentiation ($P < 0.001$; after 1000 permutations). The average of Nm was 1.462 indicating a low gene flow between populations. Zaghouan (1) and Bargou (5) populations were not significantly differentiated ($\Phi_{ST} = 0.047$; $P > 0.05$) (Tab. IV). The highest Φ_{ST} (0.361) and the lowest level of gene flow (Nm = 0.442) were observed between populations of Zaghouan and Oueslet Jbel Mountains (populations 1 and 10) belonging to the sub-humid and the lower semi-arid bioclimates respectively. The distance between these populations is 75 Km. The lowest differentiation ($\Phi_{ST} = 0.040$, Nm = 5.862) was scored among Jradou (8) and Khnis (9) populations, both belonging to the lower semi-arid, and geographically distant by 62 km.

The relationships among Φ_{ST} and geographic distance (Mantel's test; $r^2 = 0.091$; $P < 0.05$ after 999 permutations) or among Φ_{ST} and Emberger's Q_2 matrices ($r^2 = 0.111$;

Table II. Percentage of polymorphic loci (*P%*) per primer in each population and bioclimatic zone.

Bioclimate	Population code	Primer	OPJ04	OPJ06	OPJ08	OPJ12	OPJ13	OPJ14	OPJ20
		tnb	17	14	20	14	15	20	14
Sub-humid	1		47.05 (8)	64.28 (9)	80.00 (16)	50.00 (7)	66.66 (10)	60.00 (12)	42.86 (6)
	2		58.82 (10)	50.00 (7)	65.00 (13)	42.86 (6)	53.33 (8)	55.00 (11)	64.29 (9)
Average			52.94	57.14	72.50	46.43	60.00	57.50	53.57
Upper Semi-arid	3		64.70 (11)	57.14 (8)	55.00 (11)	64.29 (9)	60.00 (9)	60.00 (12)	42.86 (6)
	4		53.00 (9)	64.28 (9)	70.00 (14)	71.43 (10)	73.33 (11)	60.00 (12)	42.86 (6)
	5		58.82 (10)	71.42 (10)	80.00 (16)	64.29 (9)	73.33 (11)	55.00 (11)	50.00 (7)
Average			58.82	64.28	68.33	66.66	68.88	58.33	45.23
Mean Semi-arid	6		70.58 (12)	50.00 (7)	65.00 (13)	64.29 (9)	73.33 (11)	50.00 (10)	57.14 (8)
	7		35.29 (6)	42.85 (6)	60.00 (12)	50.00 (7)	66.66 (10)	55.00 (11)	50.00 (7)
Average			52.94	46.42	62.50	57.14	70.00	52.50	53.57
Lower Semi-arid	8		47.05 (8)	35.71 (5)	55.00 (11)	28.57 (4)	73.33 (11)	40.00 (8)	57.14 (8)
	9		47.05 (8)	50.00 (7)	75.00 (15)	35.71 (5)	60.00 (9)	45.00 (9)	42.86 (6)
	10		47.05 (8)	21.42 (3)	65.00 (13)	42.86 (6)	53.00 (8)	30.00 (6)	50.00 (7)
Average			47.05	35.71	65.00	35.71	73.33	38.33	50.00
	Over all populations		70.58 (12)	78.57 (11)	80.00 (16)	71.42 (10)	80.00 (12)	70.00 (14)	85.71 (12)

tnb: Total number of bands revealed; number of polymorphic bands are given in parentheses.

Table III. Percentage of polymorphic loci (*P%*) in each population and each ecological group, Shannon's index and ratio of genetic diversity.

	Bioclimate zones	Sh		Usa			Msa		Lsa		
	Population code	1	2	3	4	5	6	7	8	9	10
Population	*P%*	59.65	56.14	57.89	62.28	64.91	61.40	51.75	50.00	51.75	44.74
	$\overline{H}'_j$	0.593	0.576	0.597	0.667	0.687	0.627	0.517	0.458	0.502	0.469
Ecological group	*P%*	57.15		61.51			56.44		49.30		
	$\overline{H}'_{jg}$	0.678		0.763			0.739		0.606		

$\overline{H}'_{pop}$	0.569	$\overline{H}'_{grp}$	0.696
$\overline{H}'_{sp}$	0.871	$\overline{H}'_{grp}/\overline{H}'_{sp}$	0.801
$\overline{H}'_{pop}/\overline{H}'_{sp}$	0.653	$\overline{G}'_{STg}$	0.199
$\overline{G}'_{ST}$	0.347		

Sh, Sub-humid; Usa, Upper semi-arid; Msa, Mean semi-arid; Lsa, Lower semi-arid. $\overline{H}'_j$, $\overline{H}'_{pop}$, $\overline{H}'_{sp}$, $\overline{H}'_{pop}/\overline{H}'_{sp}$ and $\overline{G}'_{ST}$ are the average per primer values of genetic diversity for each primer within each population (H'j), 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. $\overline{H}'_{jg}$, $\overline{H}'_{grp}$, $\overline{H}'_{grp}/\overline{H}'_{sp}$ and $\overline{G}'_{STg}$ are the average per primer values of genetic diversity for each primer within each ecological group ($H'_{j)}$, over all groups (H'_{grp}), and their partition within-(H'_{grp}/H'_p) and between-groups (G'_{STg} respectively, calculated for all primers.

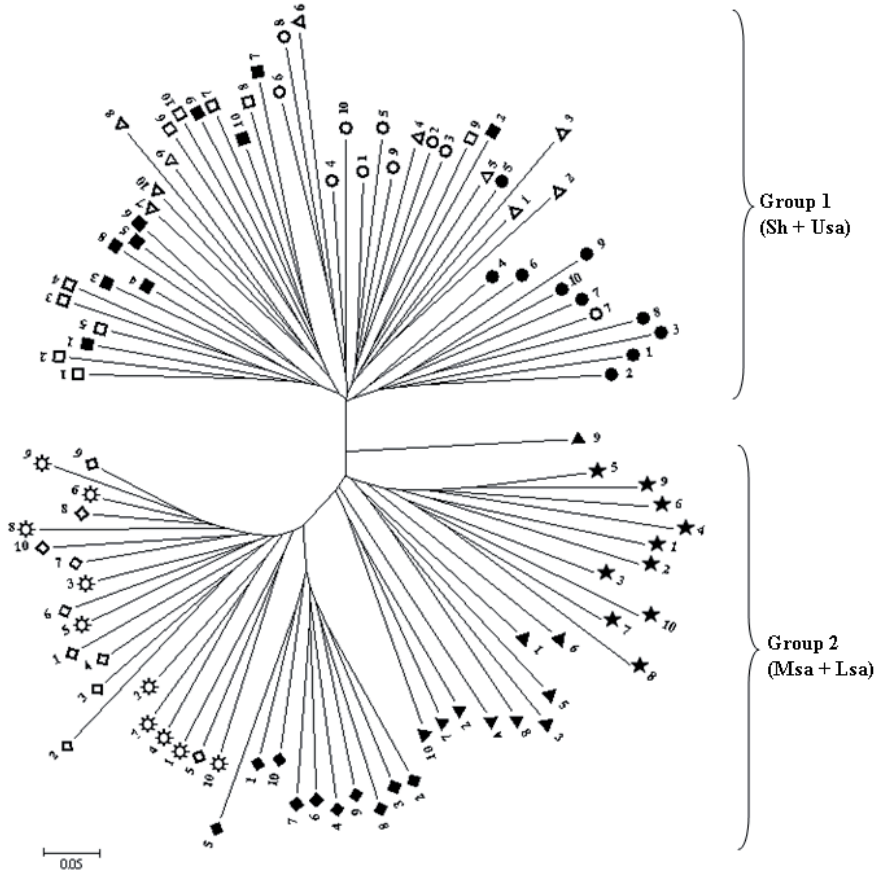

Figure 2. Neighbour-joining dendrogram generated from Jaccard similarity matrix for all individuals of *Ceratonia siliqua* analysed. Sh: sub-humid; Usa: upper semi-arid; Msa: mean semi-arid; Lsa: lower semi-arid. Symbols indicate populations; individuals from the same population are numbered 1,2,... 10. ◯ Zaghouan Jbel Mountain; • Ghar El Melh; □ Lansarine Jbel Mountain; ■ El Morra Jbel Mountain; ⊿ Bargou Jbel Mountain; ▼ Ksar Lamsa; ★ Aïn Tounga; ☼ Jradou; ◇ Khnis; ♦ Oueslet Jbel Mountain.

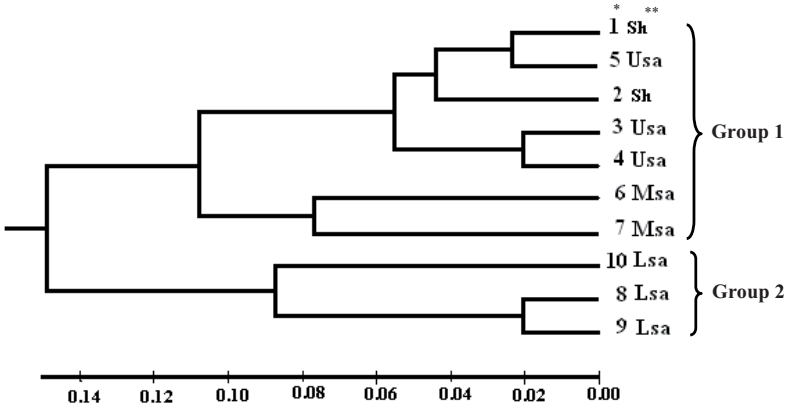

Figure 3. Dendrogram of the 10 populations of Carob studied based on the Φ_{ST} values. * Population: 1, Zaghouan Jbel Mountain; 2, Ghar El Melh; 3, Lansarine Jbel Mountain; 4, El Morra Jbel Mountain; 5, Bargou Jbel Mountain; 6, Ksar Lamsa; 7, Aïn Tounga; 8, Jradou; 9, Khnis; 10, Oueslet Jbel Mountain. ** Bioclimate: Sh: Sub-humid; Usa: Upper semi-arid; Msa : Mean semi-arid; Lsa: Lower semi-arid.

Table IV. Estimated gene flow (Nm) (above diagonal) and Φ_{ST} values (below diagonal) between pairs of populations.

Population	1	2	3	4	5	6	7	8	9	10
1		2.245	1.640	3.222	5.024	0.950	0.815	0.465	0.458	0.442
2	0.100***		1.213	1.986	3.083	1.001	0.641	0.449	0.488	0.447
3	0.132***	0.170***		5.848	1.916	1.085	0.668	0.532	0.534	0.495
4	0.072***	0.111***	0.041***		3.769	1.166	0.816	0.592	0.588	0.533
5	0.047^{ns}	0.075***	0.115***	0.062***		1.351	0.928	0.626	0.627	0.622
6	0.208***	0.199***	0.187***	0.176***	0.156***		1.371	1.253	1.504	0.793
7	0.234***	0.280***	0.272***	0.234***	0.212***	0.154***		0.601	0.676	0.624
8	0.349***	0.357***	0.319***	0.296***	0.285***	0.166***	0.293***		5.862	1.069
9	0.353***	0.338***	0.318***	0.298***	0.285***	0.142***	0.270***	0.040***		1.313
10	0.361***	0.358***	0.335***	0.319***	0.286***	0.239***	0.285***	0.189***	0.160***	

^{ns}, Not significant; ***, highly significant at $P < 0.001$ after 1000 permutations. 1, Zaghouan Jbel Mountain; 2, Ghar El Melh; 3, Lansarine Jbel Mountain; 4, El Morra Jbel Mountain; 5, Bargou Jbel Mountain ; 6, Ksar Lamsa; 7, Aïn Tounga ; 8, Jradou; 9, Khnis; 10, Oueslet Jbel Mountain.

Table V. Nested analysis of molecular variance (AMOVA) of random amplified polymorphic DNA for all individuals sampled from 10 populations belonging to 4 ecological groups.

Source of variation	df	MSD	Variance	Total variance (%)	Φ -statistics
Population					
Among populations	9	0.822	0.061	22.89***	$\Phi_{ST} = 0.250$***
Within population	90	0.207	0.207	77.11***	
Total	99				
Ecological group					
Among groups	3	1.533	0.043	15.66***	$\Phi_{CT} = 0.157$***
Among populations Within group 6	0.466	0.02	9.38***	$\Phi_{SC} = 0.111$***	
Within population	90	0.207	0.207	74.96***	
Total	99				

df, Degree of freedom; SSD, sum of squared deviation; MSD, mean squared deviation. *** Significant at $P < 0.001$ after 1000 permutations.

$P < 0.05$ after 999 permutations) were significant. The correlation between Φ_{ST} and site altitudes matrices was not significant ($r^2 = 0.002$, $P > 0.05$ after 999 permutations).

The dendrogram generated from pairwise Φ_{ST} matrix among populations showed two major groups (Fig. 3). The first one which could be subdivided into two subclusters includes populations 1, 2, 3, 4 and 5 from the sub-humid and upper semi-arid (subcluster 1) and populations 6 and 7 from the mean semi-arid bioclimate (subcluster 2). The second group includes the three populations 8, 9 and 10 belonging to the lower semi-arid. The divergence between populations based on RAPDs was similar to that based on isozymes (Afif et al., 2006).

The analysis of the molecular variance (AMOVA) attributed 77.11% ($P < 0.001$) of the total genetic diversity to differences within population, only 22.89% was attributable to the population divergence (Tab. V). The Φ_{ST} among all populations was 0.250 ($P < 0.001$) indicating a high differentiation among them. The level of AMOVA, which considered the ten populations as four groups (according to bioclimate) indicated that the majority of variation (74.96%, $P < 0.001$) resided within population. Variation observed between populations belonging to the same bioclimatic zone was lower (9.38%; $P < 0.001$) than that among the three ecological groups (15.66%; $P < 0.001$) and both showed a high differentiation ($\Phi_{CT} = 0.157$; $\Phi_{SC} = 0.11$; $P < 0.001$, respectively) (Tab. V).

4. DISCUSSION

Natural Tunisian carob populations maintain a high genetic diversity as revealed by RAPDs estimates. The level of variation was higher than that reported for Lebanon semi natural carob populations (Talhouk et al., 2005). It could be explained by the predominantly outbreeding mating system. The species is gynodicious (male and female), rarely hermaphrodite (Battle and Tous, 1997; Haselberg 1986; Tucker, 1992) and entomophilous (pollination by flies). Outcrossing species showed higher variation within than among populations (Nybom and Bartish, 2000). The gene flow via seed or pollen migration between adjacent populations might be favoured by the continuous distribution area of the species before population fragmentation.

The genetic diversity based on RAPDs was relatively higher than that revealed by isozymes for the same populations (Afif et al., 2006). These results are in agreement with those reported for forest trees comparing molecular and isozymic data (Aagaard et al., 1998; Bucci et al., 1997; Gómez et al., 2001;

Messaoud et al., 2007; Yildiray et al., 2007). RAPDs are less responsive to selection and are able to detect high variation both in coding and non-coding regions of the genome (Allnutt et al., 2003; Nybom and Bartish, 2000; Wu et al., 1999).

The range of variation between populations was large. Populations from Zaghouan Jbel Mountain (1) and Bargou Jbel Mountain (5) with a large size and more continuous distribution area (both located in Tunisian Dorsal Mountain) showed the highest diversity. Besides, these populations have shown high variation in their pod and seed sizes, number of seeds per pod and gum and sugar yields (Tous et al., 2006). Populations from Jradou, Knis and Oueslet Jbel Mountain, with a little size and scattered individuals, were less heterogeneous.

All the estimates of genetic differentiation (G'_{ST} = 34.7 % and Φ_{ST} = 0.250) indicate that population genetic structure was high in comparison with wind pollinated forest trees (Fournier et al., 2006; Godt and Hamrick, 2001; Þvingila et al., 2005). The genetic structure of plant populations reflects the interactions among different factors, including the long-term evolutionary history of the species, genetic drift, mating system, gene flow and selection (Burgarella et al., 2007; Schaal et al., 1998). Geographically disjunct populations showed higher genetic structure than populations with more continuous distributions (Hamrick and Godt 1996; Premoli et al., 2001). The fragmentation of carob populations associated probably to a limited movement of pollinators and seed dispersal are the main factors affecting gene flow and differentiation among population. No intense study on seed dispersal distance was reported in carob tree.

The population structure is significantly correlated to geographic distances, and it is in accordance with bioclimate as defined by Emberger (1966). However, isolation by distance did not sufficiently explain this differentiation. Populations from Zaghouan and Oueslet Jbel Mountains, distant by 75 km and belonging to different bioclimates, showed high level of dissimilarity and did not clustered together. Thus, the genetic structure also could be derived from adaptive RAPD differentiation in response to ecological factors (i.e. temperature, rainfall) (Kölliker et al., 1998; Nevo and Beiles, 1989).

The differentiation among ecological groups (Φ_{CT} = 0.157) and among the populations from the same group (Φ_{SC} = 0.111) is moderate and significant. The demand for *Ceratonia siliqua* L. is in increasing for its use in industrial fields and in the restoration of degraded arid zones. However, natural populations which provide the bulk materiel are decreasing in number and size as a result of diverse anthropic pressures. Limiting the species habitat destruction and coal mining and to the selection of cultivars with traits of interests to targets breeders constitute an effective strategy to preserve populations. Improvement work carried out since 1950 has led to a selection (from natural populations) of *Ceratonia siliqua* cv. Sfax (high production of pods with high sugar and gum contents, excellent flavour, etc.). This cultivar in Tunisia has been eradicated in most areas (several orchards persist in Souassy and Enfidha regions. It has been introduced in the USA (California), Australia and Spain and is considered as the most productive cultivar (Battle and Tous, 1997; Crossa-Raynaud, 1960).

The analysis of RAPD markers provides information that could be a benefit in conservation and improvement strategies. The significant variation within and among Tunisian carob populations suggests that populations constitute a valuable germplasm to conceive suitable conservation and genetic improvement programs. However, no study on the relationship between RAPDs considered as neutral (or nearly neutral) and adaptive markers has been reported. The assessment of the correlation between the tow sets of traits might help to better knowledge of adaptive potential and for future use of populations (Hansson and Richardson, 2005; Hedrick, 2001; van Tienderen, 2002).

The level of variation differed according to population and bioclimate. Population of Bargou and Zaghouan Jbel Mountains, harbouring the high diversity level (both by RAPDs and isozymes), firstly 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.

The genetic variation was due: 77.11 % within population while 28.29 % was distributed among populations (or ecological groups 15.66%), thus, ex situ conservation should be based on the collect of a maximum of individuals within populations and their multiplication via grafting in multilocal parks.

Further genetic diversity analyses combining molecular and adaptive traits are needed to more intense information of population genetic structure and to dictate appropriate decision associating conservation and management strategies of the species.

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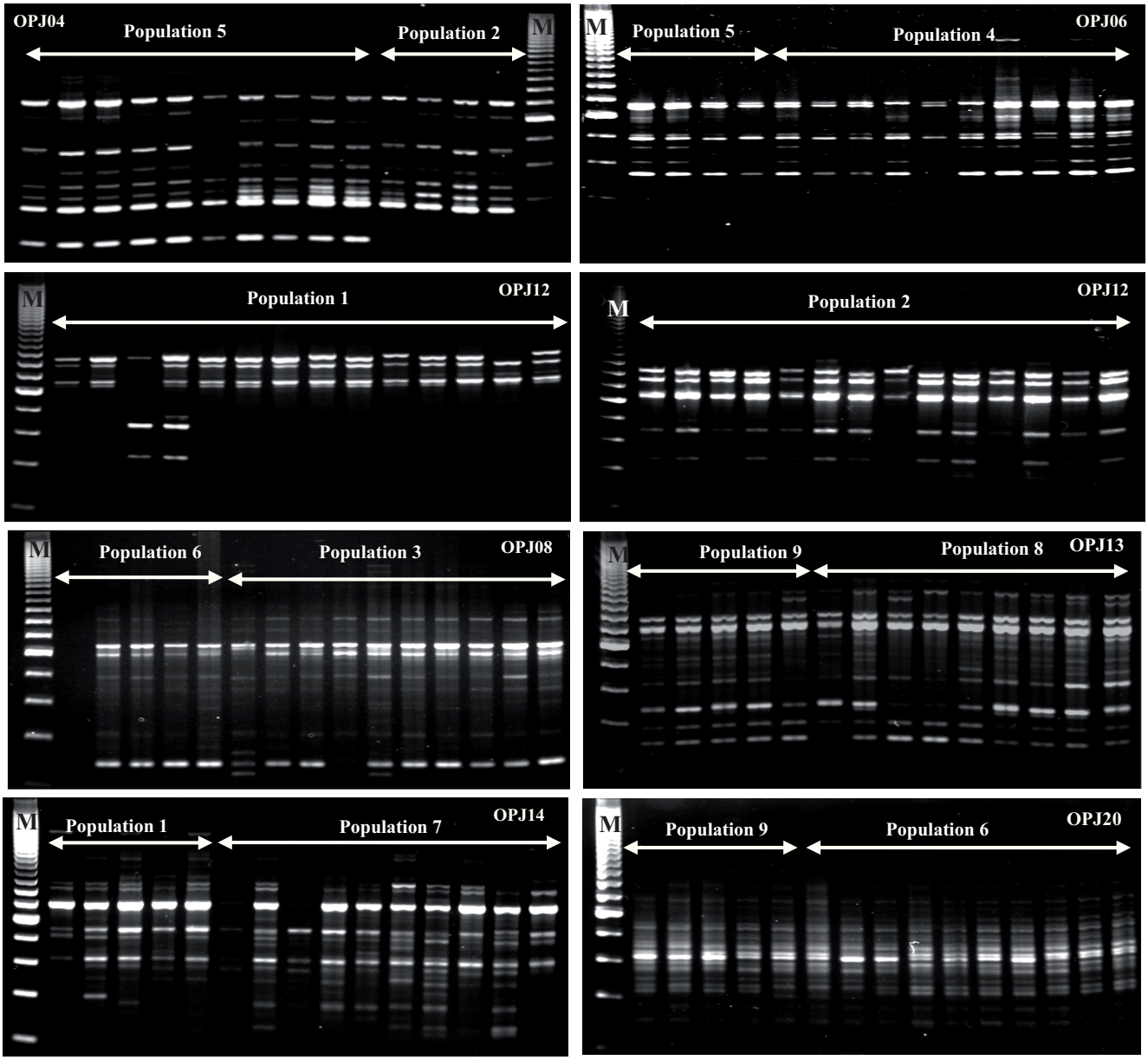
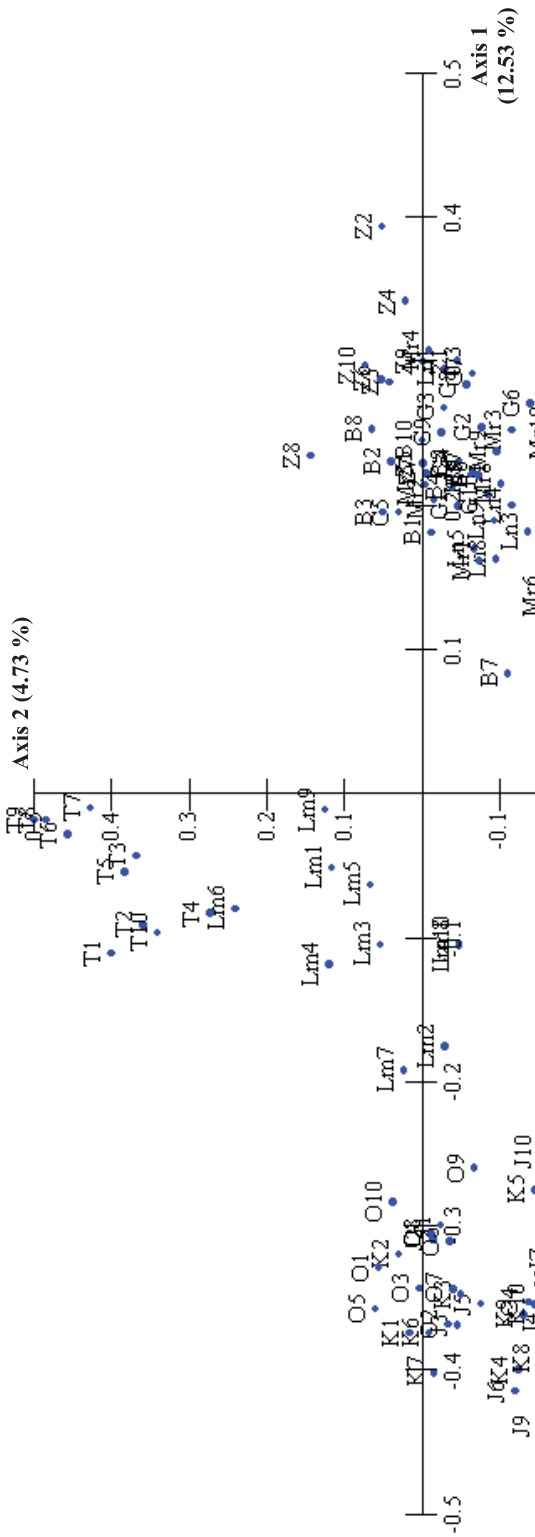
Online Material

ADDENDA



Examples of DNA profiles revealed by the 7 analyzed primers (OPJ04, OPJ06, OPJ12, OPJ08, OPJ13, OPJ14 and OPJ20) in some populations.
M: Size Marker.



Principal coordinate analysis of the 100 *Ceratonia siliqua* individuals sampled based on the pairwise Jaccard coefficients of similarity matrix.

Z, Zaghouan Jbel Mountain.; G, Ghar El Melh; Ln, Lansarine Jbel Mountain; Mr, El Morra Jbel Mountain; B, Bargou Jbel Mountain; Lm, Ksar Lamsa; T, Ain Tounga; J, Jradou; Kn, Khnis; O, Oueslet Jbel Mountain. 1,2,……,10 indicate samples from each population.