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Full Length Research Paper

Genetic diversity of natural Tunisian *Lavandula multifida* L. (Lamiaceae) populations assessed by allozymes and random amplification of polymorphic DNA (RAPD)

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We compared the genetic diversity estimated from allozymes and from random amplified polymorphic DNA (RAPDs) in a sample of seven *Lavandula multifida* L. populations from three bioclimates in Tunisia. Seven isozymes coding for 14 putative loci and 97 RAPD markers, amplified by seven decamer random primers, were used. A high genetic diversity within populations was detected both by isozymes (P = 73.8%; Ap = 2.0 and He = 0.231) and RAPDs (61.86 < P < 80.41% and 0.308 < H < 0.459). The populations from the lower semi-arid bioclimate showed relatively higher polymorphism. A high genetic differentiation among all populations and a limited gene flow at all space scales were detected as a result of habitat fragmentation, low size of populations and genetic drift. However, the level of differentiation among populations revealed by RAPDs (Φ_{ST} = 0.370; G_{ST} = 0.372) was higher than that of allozymes (F_{ST} = 0.244). The unweighted pair group method with arithmetic mean (UPGMA) dendrograms based on matrices of F_{ST} and Φ_{ST} were not concordant, and there is no significant correlation between the two data sets. The cluster from allozymes revealed higher separation among most populations. The dendrogram from RAPDs separated populations into three distinct subclusters. The groupings of populations in both dendrograms did not reflect spatial geographic or bioclimatic patterns, indicating particular adaptation of populations to local environments. The dendrogram, based on combined data had led to similar population groupings to that probed by RAPDs. The high differentiation among all populations implies that collection of seeds for *ex situ* conservation should be done from all populations from all bioclimatic zones even at a low geographic distance.

Key words: *Lavandula multifida*, genetic diversity, population structure, isozymes, random amplified polymorphic DNA (RAPDs).

INTRODUCTION

The genus *Lavandula* (Lamiaceae) includes over 34 species, self-grown in the Mediterranean basin (Miller, 1985). It has been subdivided into 6 sections mainly based on morphological, cytogenetic and reproductive traits (Upson et al., 2000; Lis-Balchin, 2002). The species (annuals or perennials) reproduce through seeds or vegetatively through sprouts (Chograni et al., 2008). *Lavandula* species are used in traditional medicine and in pharmaceutical and cosmetic industries endowed with biological activities (that is, antidepressant, antioxidant, anti-inflammatory, hypoglycemic properties) (Gàmez et al., 1987; Cavanagh and Wilkinson, 2002).

In Tunisia, the genus *Lavandula* includes three species, *Lavandula stoechas* L. and *Lavandula multifida*, growing in distinct geographical areas separated by the Tunisian

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Dorsal mountain range (Pottier-Alapetite, 1979), and *Lavandula dentata* found in Tunisian Dorsal mountain. *L. multifida,* a short-lived perennial, diploid (2n = 2x = 22) and outcrossing species belongs to the Pterostoechas section (Larsen, 1960; Chograni et al., 2008). It is widely distributed in the Central and the South of the country in a range of habitats such as rocky exposed ridges and slopes, along rock fissures lines, and garrigues derived from the destruction of *Pinus halepensis* L. and *Juniperus phœnicea* L. forests (Nabli, 1995). The species is exploited in traditional medicine to treat rheumatism and colds (El-Hilaly et al., 2003). It has hypoglycemic and anti-inflammatory properties (Gàmez et al., 1987; Sosa et al., 2005). The demand for this plant is increasing for industrial (pharmaceutic, perfumery and cosmetic) and ornamental purposes (Tucker and Hensen, 1985; Lis-Balchin, 2002). The bulk material comes from natural populations severely affected by human activities (that is, clearing, overgrazing, overharvesting).

The dynamics of *L. multifida* populations was influenced by the dispersion of pollen and seeds with unknown information about their clonal reproduction rate and seeds/pollen distance migration (Chograni et al., 2008). The species tends to occur in small scattered populations, often with a low size. The fragmentation of populations and their disturbance are main factors causing random genetic drift which enhances genetic erosion, and reduces the adaptability of populations to future environmental changes (Ellstrand and Elam, 1993; Young et al., 1996). Thus, knowledge of genetic diversity and genetic structure among *L. multifida* populations is required for the development of appropriate conservation and improvement programs.

Biochemical (that is, allozymes) and molecular [that is, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), inter-simple sequence repeat (ISSR)] markers, used separately, are often insufficient in establishing efficiently the whole range of genetic diversity and the relationships among plant populations.

Allozymes provide useful insights into the genetic variation of populations (Torres et al., 2003; Liu et al., 2006). They exhibit simple mendelian inheritance, codominant expression and are neutral or nearly neutral (Hamrick and Godt, 1989). However, these markers can lead for biased estimates of the genetic diversity, because the variation is detected only at coding loci, and reproducibility may vary according to both organs and development stage (Weeden and Wendel, 1989).

Random amplified polymorphic DNA (RAPD) has advantages such as speed, low cost, use of small amounts of plant material, detection of a great number of polymorphic loci, and revelation of variation in coding and non-coding regions of the genome (Liu et al., 2006; Nybom and Bartish, 2000; Allnutt et al., 2003). Their major limitation lies with their dominant property that can bias estimation of the genetic diversity and differentiation among populations (Nybom and Bartish, 2000; Allnutt et al., 2003).

Nevertheless, this disadvantage could be overcome by using several statistical methods such as the analysis of the molecular variance (Excoffier and Smouse, 1992; Holsinger and Wallace, 2004). The combined use of allozymes and RAPDs may provide more realiable information about genetic population structure by minimizing bias, when compared to the use of only one marker system (Messaoud et al., 2007; Tian et al., 2008). The aim of this study was: (1) To determine the pattern and extent of genetic diversity within and among Tunisian *L. multifida* populations from different bioclimatic zones, using isozymes and RAPDs; (2) to compare the level of genetic population structure revealed by each marker with that obtained by combined data and; (3) to apply these informations for the development of appropriate conservation strategies.

MATERIALS AND METHODS

Surveyed populations and sampling

L. multifida populations were collected from seven sites belonging to the lower semi-arid, upper semi-arid and upper arid bioclimatic zones (Figure 1) (Emberger, 1966; Nabli, 1995). The main ecological traits of sites were reported in Table 1. Twenty plants from each population were sampled at distances exceeding 50. Samples were placed on ice in plastic bags and transported to the laboratory for genetic analyses.

Isozyme electrophoresis

Five hundred milligrams of young fresh leaves from each individual in each population were ground in liquid nitrogen. After grinding, 2 ml of Tris-HCl (0.1 M, pH 7.5) mixed with 1% (v/v) β-mercaptoethanol, 1.5% (w/v) bovine serum albumin (BSA), 10 µL EDTA and 50 mg polyvinylpyrrolidone (PVP 40000) were added. The homogenate was centrifuged for 25 min at 12000 rpm and 4°C. Two gel-buffer systems using 13% starch gels were used to assay seven enzyme systems. Phosphoglucomutase (PGM, E.C.2.75.1), phosphoglucoisomerase (PGI, E.C. 5.3.1.9), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44) and isocitrate dehydrogenase (IDH, EC. 1.1.1.42) were assayed using histidine-citrate gel and electrode buffer system at pH 6.5 and a gel buffer dilution of 1:3 (0.065 mol/L L-histidine and 0.02 mol/L citric acid). Glutamate oxaloacetate transaminase (GOT, E.C. 2.6.1.1), leucine aminopeptidase (LAP, E.C. 3.4.11.1), and alcohol dehydrogenase (ADH; EC 1.1.1.1) were assayed using lithium-borate gel. Electrophoresis was performed at 4°C and continued until the migration indicator reached the far end of the gels. The revelation of isozymes and the genetic interpretation of zymograms (that is, isozyme loci, number of alleles within each locus) were made according to Wendel and Weeden (1989).

Random amplified polymorphic DNA (RAPD) assay

DNA extraction

Young leaves (0.5 g) from each plant were ground with liquid nitrogen, and DNA was extracted using 2 ml cetyl trimethyl-

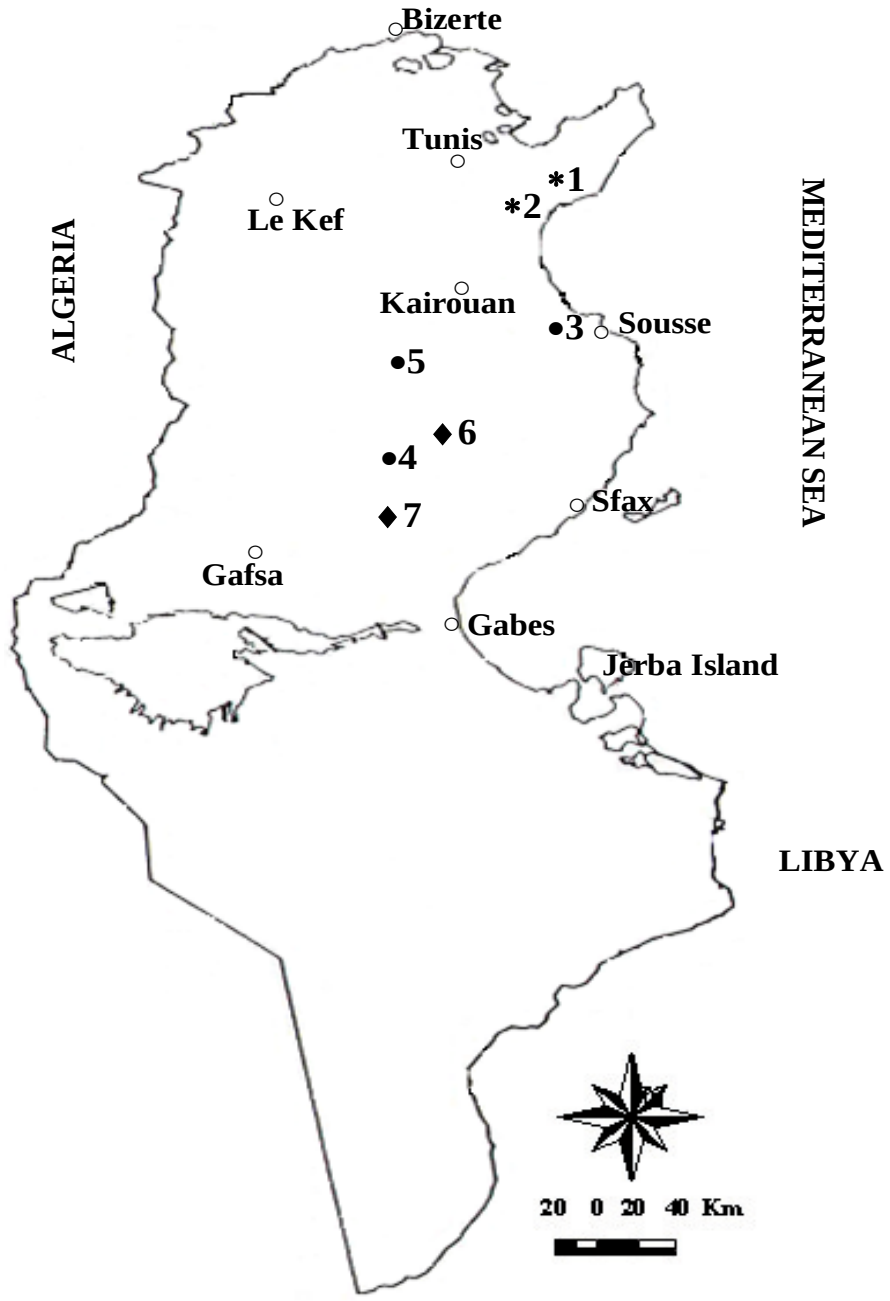

Figure 1. Map of Tunisia, geographical distribution of the analysed *Lavandula multifida* populations. 1, 2, 3, 4, 5, 6 and 7: Jedidi Jeb. Mt; Bouargoub; Khniss; El Fayedh; Ouslett Jeb. Mt; Echrarda and Goubrar Jeb. Mt populations. *,●,♦: populations belonging to the upper semi-arid, lower semi-arid and upper arid zones, respectively.

ammonium bromide (CTAB) extraction buffer (Doyle and Doyle, 1990) and 100 mg PVP 40000. The extraction buffer consisted of 250 mM NaCl, 200 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol and 20 mM ethylenediaminetetraacetic acid (EDTA). The samples were then incubated at 65°C for 30 min with slow shaking every 10 min. Subsequently, the mixture was treated twice with 700 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 estimated by agarose gel (0.8%) stained with ethidium bromide. The DNA concentration was evaluated spectrophotometrically by measuring absorbance at 260 nm.

Primers and polymerase chain reaction (PCR) conditions

Twenty (20) RAPD primers (kit OPJ, Genset Oligos, Promega) were tested. After optimizing the PCR conditions, seven RAPD primers (OPJ06, OPJ08, OPJ10, OPJ12, OPJ14, OPJ16, and OPJ18) were selected on the basis of the reproducibility and the polymorphism of

the generated bands. The reproducibility of bands was tested by repeating the PCR amplification twice under the same conditions. The PCR reaction was performed in 25 µl reaction volume containing 50 ng DNA templates, 2.5 µl of 10× reaction buffer, 40 pmoles of primer, 200 µM of each dNTP, 2.5 mM $MgCl_2$ and 1.5 U Taq polymerase. Mixture was overlaid with 10 µl of mineral oil and amplified in a Programmable Stuart Thermal Cycler (Maxi-Gene) under the following conditions: 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. The last step was 72°C for 10 min. PCR products were resolved by electrophoresis in 1.5% agarose gel at 160 V for 1 h in 1× Tris-acetate-EDTA (TAE) buffer (pH 8). The gel was stained with ethidium bromide, visualized under ultraviolet (UV) light and photographed with DOC PRINT Photo Documentation System. Molecular weights were estimated using a 200 pb DNA Promega ladder.

Data analysis

Isozyme analysis

The genetic variation within a population or within an ecological group (each group includes populations from the same bioclimate) was estimated using allele frequencies, the percentage of polymorphic loci (P) at 95% criterium, the mean number of alleles per polymorphic locus (Ap), and averages of the observed (H_o) and expected (H_e) heterozygosities. Calculations were made using Biosys software package (Swofford and Selander, 1981). For each population, departure from Hardy-Weinberg equilibrium was estimated by Wright's fixation index (F_{IS}) (Wright, 1965). The genetic divergence among populations was estimated by Nei's (1978) unbiased genetic distances. The genetic differentiation among populations or among ecological groups was estimated by Wright's F_{ST} index, according to Weir and Cockerham (1984) estimates. The significance of F_{ST} values was tested using permutations. Calculations were made using the program FSTAT versions 1.2 and 2.9.3 (Goudet, 1995, 2001). The gene flow (Nm) between populations was estimated as $Nm = [(1 / F_{ST}) - 1] /4)]$ (Wright, 1951). The correlation between the matrices of genetic (F_{ST}) and geographic distances among pairs of populations was estimated by a Mantel test using ZT program (Mantel, 1967; Bonnet and de Peer, 2002). A cluster analysis based on F_{ST} genetic distances was constructed to ordinate population's relationship using MVSP program (version 3.1) (Kovach, 1999).

Random amplified polymorphic DNA (RAPD) analysis

RAPD bands amplified by each of the five primers were numbered sequentially in decreasing order according to the molecular weight. RAPD fragments with the same mobility were scored for band presence (1) or absence (0). The genetic diversity within a population was estimated using the percentage of polymorphic bands: Pr% [(number of polymorphic bands/number of total bands) × 100)] and Shannon's diversity index H [$H = (-\Sigma pi \log_2 pi)$; pi is the frequency of a given RAPD band] (Lewontin, 1972). The comparison of Shannon's indices among populations or among ecological groups, was performed using a variance analysis (ANOVA procedure) (SAS, 1990) followed by Duncan's test (Dagnelie, 1975). Correlations between Shannon's index (H) and altitudes of sites, and between H and Emberger's Q_2 coefficients were evaluated using Kendall's test (Oiki et al., 2001). The Shannon index also was used to estimate average diversity within all populations H_{POP} [$H_{POP} = (1 / n\Sigma H_o)$ where n is the number of populations], the total diversity among all individuals within the species H_{SP} [$H_{SP} = -\Sigma p_s \log_2 p_s$, where p_s is the frequency of RAPD fragments in the entire sample]. The proportion of diversity within populations H_{pop}/H_{sp} and the proportion of diversity among populations are estimated by G_{ST} [$G_{ST} = (H_{SP} - H_{POP}) / H_{SP}$]. The components of variability at the ecological group level were estimated by H_{EG} (the diversity within a group), H_{EG}/H_{SPG} (the proportion of diversity within ecological groups), and G_{STG} (the proportion of diversity among ecological groups).

The genetic variation within and among populations or within and among ecological groups was also estimated by the analysis of the molecular variance (AMOVA) performed on the genetic distances (Φ_{ST}) between individuals, using the WinAmova program, version 1.55 (Excoffier and Smouse, 1992). Φ-statistics (Φ_{ST}: differentiation among populations, Φ_{CT}: differentiation among groups and Φ_{SC}: differentiation among populations within groups) were calculated. The significance of the variance components and that of Φ-statistics were estimated using permutations procedures. The correlation between matrices of genetic distance (Φ_{ST}) and geographic distance among population pairs was estimated by a Mantel test using ZT program (Mantel, 1967; Bonnet and de Peer, 2002). The gene flow (Nm) between populations was estimated as $Nm = [(1 / \Phi_{ST}) - 1] / 4)]$. A cluster analysis based on Φ_{ST} genetic distances was constructed to ordinate population's relationships using the MVSP program (version 3.1) (Kovach, 1999).

Combined data analysis

The relationship between allozyme and molecular genetic diversity parameters was tested by the Mantel test (1967) (at $P < 0.05$; after 1100 permutations) performed on Euclidean distances matrices calculated on both isozyme and RAPD band frequencies, using ZT program (Bonnet and de Peer, 2002). The two data sets were combined in one matrix to construct a common dendrogram. The obtained dendrogram was compared with those probed by each marker.

RESULTS

Isozyme genetic diversity and population structure

For all populations, genetic interpretation of the electrophoretic patterns showed that the seven enzymes were encoded by 14 putative loci. Six of them were polymorphic (Adh-1, Pgi-1, Pgi-2, Pgm-1, Pgd-2, Pgd-3). The genetic diversity intrapopulation was high (Table 2). The number of alleles per polymorphic locus (Ap) varied from 1.8 (populations 2 and 6) to 2.3 (populations 1) with an average of 2.01. The average of polymorphism was 73.8%. The observed heterozygositiy (H_o) ranged from 0.175 (population 2) to 0.338 (population 7). A non significant excess of heterozygosity was observed for the populations 2, 4, and 7 ($F_{IS} < 0$; $P > 0.001$). The level of genetic variation within ecological groups varied according to the bioclimate. The lower semi-arid (P = 83.3%, $H_o = 0.257$) was the most polymorphic (Table 2). Nei's (1978) genetic distance values between pairs of populations (data not shown) were low and varied from 0.004 (between populations 6 and 7 from to the upper semi-arid bioclimate) to 0.068 (between populations 2 and 5 from the upper semi-arid and the lower semi-arid zones, respectively). The average of Nei's distance over all populations was 0.026. A substantial genetic structure among the seven populations ($F_{ST} = 0.244$) and a low

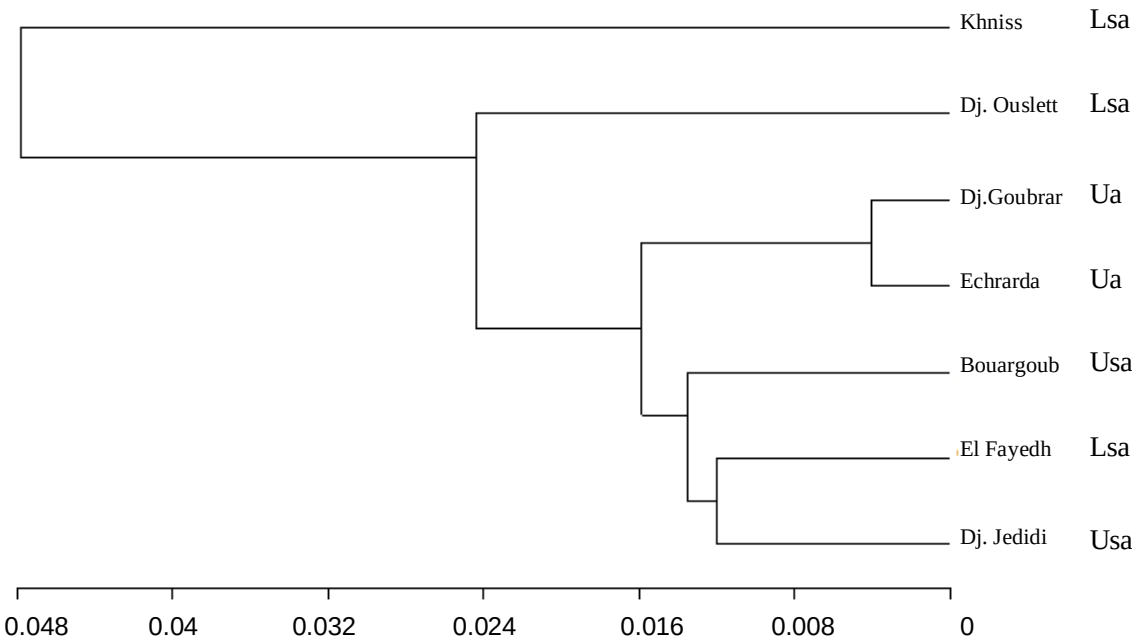

Figure 2. UPGMA dendrogram based on F_{ST} genetic distance for the 7 populations of *Lavandula multifida*. Usa: upper semi-arid, Lsa: lower semi-arid and Ua: upper arid zones.

level of gene flow among them (Nm = 0.774) were observed. The differentiation among population pairs were all significant (Table 4) and ranged from 0.004 (among populations 6 and 7, from the upper arid bioclimate) to 0.566 (between populations 2 and 3, belonging to the upper semi-arid bioclimatic zone). The differentiation among ecological groups was the highest for the lower semi-arid populations (F_{ST} = 0.450) (Table 3).

The correlation between F_{ST} and geographic distance matrices, estimated by the Mantel test, was not significant (r = -0.047, P > 0.05). The unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on F_{ST} genetic distances between pairs of populations showed two distinct groups (Figure 2). The first includes population 3 belonging to the lower semi-arid bioclimate. The second group is subdivided in two sub-clusters; the first sub-cluster contains population 5. Populations 1, 2, 4, 6 and 7 from the three bioclimatic zones clustered together in the second sub-cluster (Figure 2).

RAPD polymorphism and genetic structure among populations

For all individuals, a total of 97 RAPD fragments ranging in size from 180 to 1900 bp were amplified. The number of bands varied from 11 (OPJ14 and OPJ18) to 17 (OPJ08) (Table 5). The percentage of polymorphic loci per primer (P) ranged from 72.7 (OPJ14) to 100% (OPJ10), and 86.6% of the amplified bands were polymorphic. Average polymorphisms within populations ranged from 59.79% (population 5) to 80.41% (population 1), with a mean of 68.19% (Table 2).

Shannon's indices (H) ranged from 0.308 to 0.459 without significant differences among populations (P > 0.05). Shannon's H for each population did not correlate with altitudes (r = -0.43, P > 0.05) or with Emberger's Q_2 coefficients (r = 0.28, P > 0.05). The average of variation within all the populations was moderate (H_{POP} = 0.367). The total genetic diversity among all individuals was H_{SP} = 0.576. The proportion of diversity within all populations was high (H_{POP}/H_{SP} = 0.636). The genetic diversity index G_{ST} was 0.372. H indices varied significantly among ecological groups, the highest value (0.515) was noted for the upper semi-arid group and the lowest (0.489) for the upper arid one. The average of Shannon's index within all groups (H_{EG}) was 0.503. The genetic diversity across all groups (H_{SPG}) was 0.568, and the amount of variation of populations within groups was high (H_{EG}/H_{SPG} = 0.885). The level of genetic diversity among groups (G_{STG}) was 0.123.

The differentiation among population pairs, estimated by Φ_{ST} was high and varied from 0.233 (populations 3 to 4, 127 km distant) to 0.448 (populations 5 to 7, 90 km distant) (Table 4). The average among all populations was Φ_{ST} = 0.370 (Table 5). AMOVA analysis showed that 63.39% of the total variation was found within populations. The differentiation among populations within groups (Φ_{SC}) was 0.354, and the variation among bioclimates (Φ_{CT} = 0.025) was low. The level of gene flow estimated by Nm was 0.722. The Mantel test performed on geographic and Φ_{ST} distance matrices among population pairs did not revealed significant correlation

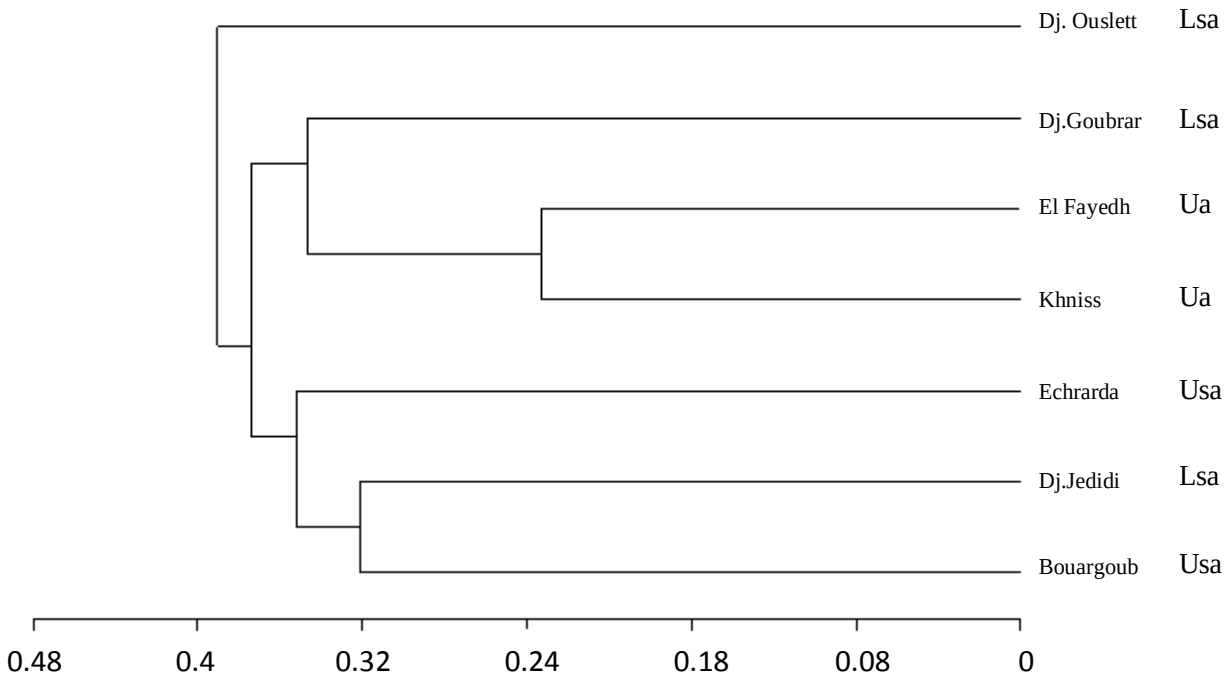

Figure 3. Dendrogram of seven analyzed *Lavandula multifida* populations based on Φ_{ST} genetic distance. Usa, Upper semi-arid; Lsa, lower semi-arid; Ua, upper arid zones.

between the two sets of data (r = −0.121, P = 0.304 > 0.05 after 1100 permutations). The cluster based on the Φ_{ST} matrix showed three population groups (Figure 3). The first group includes the populations 1, 2, and 6, from the upper semi-arid and the upper arid areas. The populations 3, 4, and 7 (from the lower semi-arid and the upper arid zones) formed the second group. The third sub cluster consists of the population 5.

Combined isozyme and RAPD data analysis

Mantel's test performed on isozyme and RAPD matrices indicated non significant correlation between the two sets of data (r = -0.547, P > 0.05). The UPGMA dendrogram based on the combined matrix data disclosed three population groups similar to those shown using the RAPDs matrix (Figure 4). The population 7 from the upper arid zone clustered separately from the other populations subdivided into two sub-clusters.

DISCUSSION

Isozyme markers used to assess the genetic diversity of Tunisian *L. multifida* showed that populations maintain a high genetic variation coupled with a non significant deficit of heterozygosity. The amount of allozyme variation was higher than that reported for other species with similar life history (Sun and Ganders, 1990; Hamrick and Godt, 1996). It could be explained by the species high outcrossing rate, and the number of initial founders that differed genetically in a population. The level of the intrapopulation variation differed according to populations. Those from the lower semi-arid zone were more polymorphic than populations of other bioclimatic stages. These populations occurred in well large spatial rea. The average F_{ST} value among all populations was high (0.244) and greater than those reported for outcrossing species dispersed by wind (Nybom and Bartish, 2000). The differentiation among population pairs was high without significant correlation to geographical distance. This pointed to a local spatial of genetic differentiation due to founding events such as time since colonization, number of initial founders in populations and genetic drift due to a low level of gene flow (Nm = 0.774) (Coleman and Abbott, 2003; Jacquemyn et al., 2004).

The dendrogram based on F_{ST} distances matrix showed that populations were separated to well defined groups. They were interspersed with each other, without association between isozyme pattern and geographic or bioclimatic origin. The paucity of genetic divergence among populations estimated by Nei's distance (0.026), and their high level of differentiation indicate a recent fragmentation of populations under anthropic pressures.

The within-population genetic diversity revealed by RAPDs was high (0.308 < H < 0.459) and did not differ significantly among populations according to altitudes. However, the upper semi-arid populations were more polymorphic. RAPDs were known to be neutral and did not reflect selective constraint variation compared to allozymes (Charlesworth, 1994; Aagaard et al., 1998; Lee

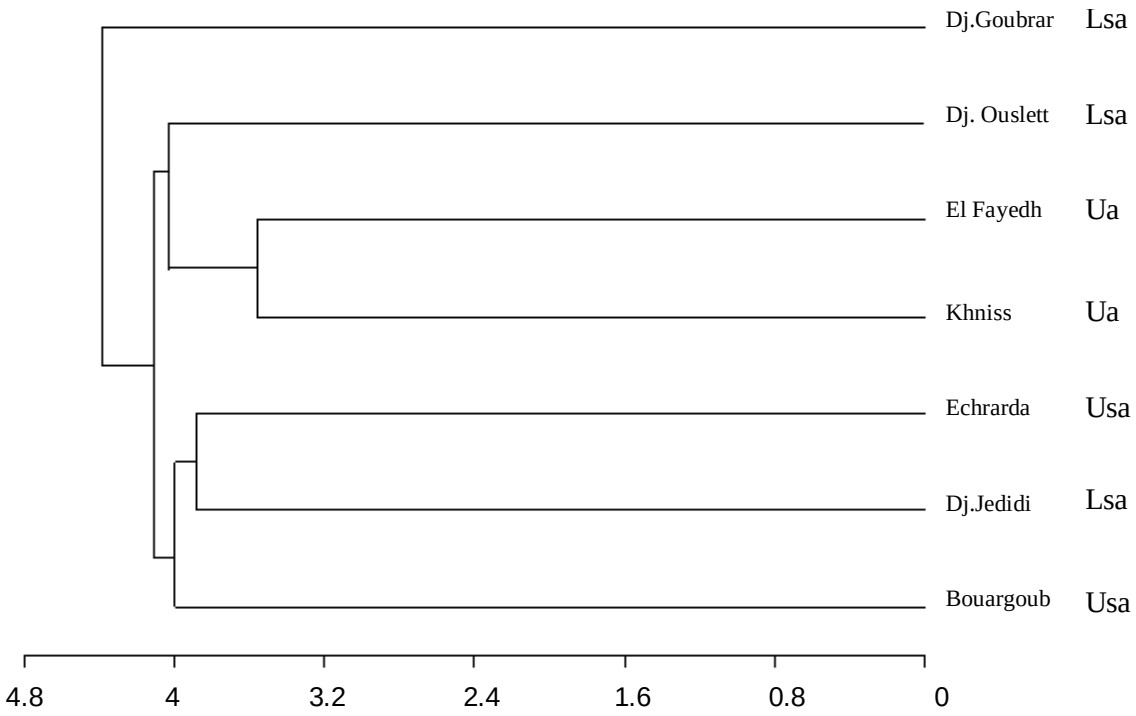

Figure 4. Dendrogram generated by genetic similarity between populations based on combined allele and RAPD band frequencies. Usa, upper semi-arid; Lsa, lower semi-arid and Ua, upper arid zones.

Table 1. Main ecological traits for the seven Tunisian *Lavandula multifida* populations analyzed.

Population code	Location	Bioclimatic zone*	Q_2	Winter variant	Altitude (m)	Latitude	Longitude	Rainfall mm/year
1	Jedidi Jeb. Mt[a]	Upper semi-arid	65.65	Temperate 3<m<4.5°C	450	36°25'N	10°28'E	450
2	Bouargoub		67.70		300	36°25'N	10°28'E	450
3	Khniss	Lower semi-arid	35.24	Cool 1<m<3°C	100	35°43'N	10°43'E	356
4	El Fayedh		34.40		550	35°10'N	9°35'E	313
5	Ouslett Jeb. Mt		34.39		500	35°47'N	9°42'E	313
6	Echrarda	Upper arid	28.83	Hot 7<m<10.5°C	400	35°9'N	10°2'E	313
7	Goubrar Jeb. Mt		33.39		600	34°50'N	9°27'E	268

[a]Djebel mountain, Mt: mountain, *bioclimatic zones were defined according to Emberger's (1966) (Q_2) pluviothermic quotient. $Q_2 = 2000P / (M^2 - m^2)$, P, mean of annual rainfall (mm); M, average of maximal temperature (°K, Kelvin degree) for the warmest month (June); m, average of minimal temperature (°K) for the coldest month (February).

et al., 2002). Thus, the high variation revealed for these populations could result from their local potential of seed dispersal. However, the level of variation observed within *L. multifida* populations was globally lower than that in outcrossing species assessed by RAPD's. This could be explained by the fragmentation of habitats as a result of human disturbance (Hunter, 1996; Nybom, 2004; Xiao et al., 2005). In fact, most *L. multifida* populations occurred in isolated and scattered populations. The genetic differentiation among populations estimated by both G_{ST} (0.372) and Φ_{ST} (0.370) was comparable to that reported by Bussel (1999) and Nybom and Bartish (2000) in outbreeding species. The differentiation among all population pairs was high and not significantly correlated with geographic distance confirming that the differentiation among populations occurs at local space scales without relationship to geographical barriers.

Tunisian *L. multifida* showed a high genetic variation within populations as estimated by both isozymic and RAPD markers. With RAPD markers, the observed

Table 2. Genetic diversity parameters according to the used marker for the 7 analyzed populations.

Genetic marker	Parameter	Value	1	2	3	4	5	6	7
	Ap		2.3(0.2)	1.8(0.3)	2.0(0.0)	2.2(0.3)	2.0(0.3)	1.8(0.3)	2.0(0.3)
	P (%)		83.3	50.0	66.7	83.3	83.3	66.7	83.3
Isozyme	Ho		0.194(0.07)	0.175(0.10)	0.247(0.16)	0.227(0.08)	0.255(0.07)	0.187(0.06)	0.338(0.09)
	He		0.233(0.05)	0.181(0.08)	0.191 (0.07)	0.202(0.06)	0.303(0.07)	0.228(0.07)	0.282(0.07)
	F_{IS}		0.180^{ns}	-0.108^{ns}	0.478^{ns}	-0.106^{ns}	0.164^{ns}	0.188^{ns}	-0.193^{ns}
	F_{ST}	0.244 (0.07)**							
	Pr%		80.41	70.10	72.16	61.86	59.79	71.13	61.86
	H		0.459(0.26)[a]	0.356(0.27)[a]	0.410(0.28)[a]	0.345(0.30)[a]	0.308(0.29)[a]	0.370(0.27)[a]	0.324(0.29)[a]
RAPD	H_{POP}	0.367(0.28)**							
	H_{SP}	0.576(0.13)							
	H_{POP}/H_{SP}	0.636							
	G_{ST}	0.372							

H_{POP}, Average diversity within all populations; H_{SP}, diversity among all individuals within the species; P, the percentage of polymorphic loci at 95% criterium; Ap, the mean number of alleles per polymorphic locus; H_o, averages of the observed heterozygosities; H_e, averages of the expected heterozygosities; F_{IS}, Wright's fixation index. Standard errors are in parentheses, **highly significant at P < 0.001, *significant at P < 0.05 and ns not significant at P > 0.05 after 1100 permutations. Numbers with the same letter are not significantly different (Duncun's test, P > 0.05).

Table 3. Genetic diversity parameters according to markers used for ecological group.

Genetic marker	Parameter	Value	USA	LSA	UA
	Ap		2.3 (0.2)	2.3 (0.2)	2.0 (0.3)
	P (%)		66.7	83.3	66.7
Isozyme	Ho		0.183 (0.075)	0.257 (0.074)	0.261 (0.075)
	He		0.210 (0.054)	0.274 (0.066)	0.254 (0.072)
	F_{IS}		0.046^{ns}	0.146**	-0.018^{ns}
	F_{ST}		0.036 (0.029)*	0.450 (0.124)**	$0.004\ (0.009)^{ns}$
	Pr (%)		94.85	90.72	86.60
	H		0.515 (0.191)[c]	0.505 (0.210)[a]	0.489 (0.224)[b]
RAPD	H_{EG}	0.503(0.208)**			
	H_{SPG}	0.568(0.124)			
	H_{EG} / H_{SPG}	0.885			
	G_{STG}	0.123			

H_{POP}, Average diversity within all populations; H_{SP}, diversity among all individuals within the species; P, the percentage of polymorphic loci at 95% criterium; Ap, the mean number of alleles per polymorphic locus; H_o, averages of the observed heterozygosities; H_e, averages of the expected heterozygosities; F_{IS}, Wright's fixation index. Standard errors are in parentheses, **highly significant at P < 0.001, *significant at P < 0.05 and ns not significant at P > 0.05 after 1100 permutations. Numbers with the same letter are not significantly different (Duncan's test, P > 0.05). Usa, Lsa, and Ua: upper semi-arid, lower semi-arid and upper arid bioclimates, respectively.

Table 4. F_{ST} (above diagonal) and Φ_{ST} (below diagonal) values between pairs of populations analyzed.

Population	1	2	3	4	5	6	7
1		0.039^{ns}	0.512*	0.021^{ns}	0.152*	0.034^{ns}	0.034^{ns}
2	0.348**		0.566*	0.041*	0.220*	0.045*	0.069*
3	0.319**	0.309**		0.559*	0.521*	0.491*	0.434*
4	0.404**	0.411**	0.233**		0.109*	0.032^{ns}	0.073*
5	0.405**	0.412**	0.361**	0.360**		0.072*	0.100*
6	0.321**	0.356**	0.337**	0.359**	0.356**		0.004^{ns}
7	0.364**	0.441**	0.356**	0.338**	0.448**	0.419**	

ns: Not significant P > 0.05; *significant at P < 0.05; *significant at P < 0.001.

Table 5. Nested analysis of molecular variance (AMOVA) for the 70 individuals sampled from the 7 populations.

Parameter	Source of variation	d.f	Variance	% of total variance	Φ-Statistics
Population	Within populations	63	13.013	63.39	Φ_{ST}=0.370***
	Among population	6	7.52	36.61	
Bioclimatic zone	Within populations	63	63	63.39	
	Among populations within bioclimatic zones	4	7.117	34.46	Φ_{SC}=0.354***
	Among bioclimatic zones	2	0.524	2.54	Φ_{CT}=0.025^{ns}

d.f: Degree of freedom, *significant at P < 0.05, ***significant at P < 0.001 (after 1100 permutations).

genetic diversity was higher than that revealed by isozymes according to previous studies (Bartish et al., 2000; Oiki et al., 2001; Torres et al., 2003). Differences could be attributed to: (i) The inability of isozymes to detect variations that do not modify (or modify slightly) the amino-acids sequence or/and; (ii) the high rate of mutation detected by RAPDs in both coding and noncoding genes (Aagaard et al., 1998; Wu et al., 1999).

The level of differentiation among *L. multifida* obtained from RAPDs was higher than that based on allozymes (F_{ST} = 0.244). This result confirms previous work in annual and perennial plant species (Krutovskii et al., 1999; Lee et al., 2002; Xiao et al., 2005). The dendrogram based on Φ_{ST} distance matrix from RAPDs was completely different from that by allozymes. It divided populations into three groups. Within each subcluster, populations did not group spatially or bioclimatically together, indicating evidence of genetic drift and low level of gene flow. The cluster analysis based on the combined matrix produced population groupings similar to that obtained using RAPDs without resolving clear geographically or bioclimatically defined populations (that is, populations 1, 5 and 6). The only homogenous group is constituted by the populations 3 and 4. The correlation between RAPDs and allozyme matrices was not significant. This finding is rarely reported for outcrossing species assessed by both markers (Peakall et al., 1995). The lack of correlation between the two markers at regional scales should be due to the high genetic divergence and the high isolation of populations.

Populations of *L. multifida* are severely affected by human activities causing a significant destruction of sites. The *in situ* maintenance of substantial population size should be initiated through fencing and controlling collection to restore the regeneration of populations. Considering the significant differentiation among populations, preservation only of several populations would be insufficient to conserve all the genetic variation in the species. Therefore, to preserve the maximum of diversity in the species, all populations must be collected even at a small geographic range. *Ex situ* conservation should aim to include firstly, populations from the lower and the upper semi-arid bioclimates harbouring relatively high genetic diversity. Further studies including the variation of the chemical composition among populations, and its relationship with the genetic diversity, are necessary to elaborate efficient improvement program.

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