Ann. For. Sci. 64 (2007) 845–853
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DOI: 10.1051/forest:2007061

Available online at:
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Original article

Genetic variation of Tunisian *Myrtus communis* L. (Myrtaceae) populations assessed by isozymes and RAPDs

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(Received 21 September 2006; accepted 16 July 2007)

Abstract – The genetic variation of six Tunisian *Myrtus communis* L. (Myrtaceae) populations was assessed using nine isozymes coding for 17 putative loci and 79 RAPD markers, amplified by five decamer random primers. The analysed populations belonged to three bioclimatic zones (lower humid, sub-humid and upper semi-arid). A high genetic diversity within populations was detected both by isozymes and RAPDs. The level of variation differed according to bioclimate. Populations collected from sub-humid bioclimate showed more polymorphism than those grown in the upper semi-arid zone. For all populations, the genetic diversity revealed by RAPDs was more pronounced than that detected with isozymes. A high differentiation among populations related to bioclimate and geographic distance was revealed by both methods. Population's structure based on RAPD markers was more concordant with bioclimatic zones in comparison with isozymes. Differentiation between ecological groups was higher than that revealed within groups. Conservation programs should take into account the level of genetic diversity within population revealed by the two complementary classes of markers according to bioclimate.

***Myrtus communis* / RAPDs / isozymes / genetic diversity / Tunisia**

Résumé – Variabilité génétique des populations tunisiennes de *Myrtus communis* L. (Myrtaceae) estimée par des marqueurs isoenzymatiques et moléculaires (RAPD). La variabilité génétique de six populations tunisiennes de *Myrtus communis* L. (Myrtaceae) a été estimée à l'aide de neuf systèmes isoenzymatiques contrôlés par 17 loci et 79 marqueurs RAPD amplifiés par cinq amorces. Les populations analysées appartiennent à trois étages bioclimatiques différents : humide inférieur, sub-humide et semi-aride supérieur. Une diversité génétique intrapopulation importante a été détectée. Le niveau de polymorphisme varie selon le bioclimat. Les populations du sub-humide sont plus polymorphes. Pour l'ensemble des populations, la diversité génétique révélée par les RAPDs est plus importante que celle détectée par les isozymes. Une forte différenciation entre les populations, selon le bioclimat et l'éloignement géographique, a été révélée par les deux méthodes. La structuration des populations selon les marqueurs RAPD concorde mieux avec le bioclimat. La différenciation entre les populations appartenant à des groupes écologiques différents est plus importante que celle entre populations d'un même groupe. Les programmes de conservation de l'espèce doivent tenir compte aussi bien du degré de la diversité génétique intrapopulation révélé par les deux types de marqueurs que du bioclimat.

***Myrtus communis* / RAPDs / isozymes / diversité génétique / Tunisie**

1. INTRODUCTION

Myrtus communis L. (myrtle) (Myrtaceae) is an evergreen shrub which grows mainly in Mediterranean *Quercus suber* L. and *Quercus faginea* Lamk. forests [36, 41], reproducing both by seeds and suckers [4]. The species is exploited in traditional medicine as astringent, antiseptic and balsamic [5, 10]. Leaves and fruits were used against respiratory and urinary diseases [5, 9]. Essential oil extracted from these organs is rich in α-pinene and 1,8-cineole [33,47]. The demand for this plant is increasing in pharmaceutic, perfumery and food industries [34, 35]. In Sardinia, myrtle berries and leaves are largely used for liquor production [2, 15].

In Tunisia, the species grows wild in different bioclimatic zones extending from the upper semi-arid to the lower humid, in three geographic areas [33]. In the North West of the country, populations grow on acid soils with an annual rainfall ranging from 1000 to 1500 mm. The associated species are mainly *Quercus suber* L., *Q. faginea* Lamk., *Arbutus unedo* L., *Erica arborea* L., *Rubus ulmifolius* Schott., *Hedera helix* L., *Halimium halimifolium* (L.) Willk., *Pistacia lentiscus* L., *Smilax aspera* L., *Cistus monspeliensis* L. and *Cytisus triflorus* L'Hérit. In the Cap-Bon, the species occurs in isolated populations with a low size, growing on calcareous or acid soils with an annual rainfall ranging from 500 to 600 mm. Main species associated to *Myrtus communis* L. are *Pinus halepensis* Mill., *Quercus coccifera* L., *Ceratonia siliqua* L., *Juniperus phoenicea* L. *Phillyrea angustifolia* L., *Pistacia lentiscus* L., *Cistus monspeliensis* L., *C. salviifolius* L. and *Rubus ulmifolius* Schott. In the Tunisian Dorsal, the species is endangered and represented by scattered individuals growing on

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calcareous soil, along profound ravines and ephemeral rivers. The annual rainfall varies from 350 to 450 mm. *Myrtus communis* L. is accompanied by *Pistacia lentiscus* L., *Ceratonia siliqua* L., *Phillyrea angustifolia* L., *Rosmarinus officinalis* L., *Nerium oleander* L., *Rubus ulmifolius* Schott. and *Calycotome villosa* (Poiret) Link.

In the three areas, populations are more and more disturbed and fragmented as a result of an increasing clearing, overgrazing and overexploitation. Myrtle habitat fragmentation reduced genetic diversity of the species, increased inbreeding level and led to a rapid differentiation between populations. The loss of genetic diversity affected population's evolution and reduced their future adaptation to environmental changes [8, 11, 17]. Knowledge of the level of genetic diversity within and among populations constitutes the first step to understand their subsequent evolution and to elaborate adequate preservation programs [22, 50].

Isozymes are powerful tools for genetic diversity analysis and conservation biology [19, 49]. They are codominant, and assumed to be neutral. However, this assumption is not always held [7, 26]. Yet nowadays, molecular markers such as RAPDs, RFLPs, AFLPs, SSRs are used to assess population genetic variation. RAPD markers (Random Amplified Polymorphic DNA) are selectively neutral, involve a large number of loci and cover a larger part of the genome. They also provide more valuable information into population differentiation and help to elaborate efficient conservation strategies [16, 18, 23, 42]. However, most RAPD loci are assumed to possess only two alleles and segregate as dominant markers, leading to an underestimation of the genetic diversity [30, 45].

Because isozymes and RAPDs, each has advantages and limitations to assess genetic variations their joint use is necessary to better estimatethe genetic diversity and population structure in order to avoid wrong conclusions for rational use of species [28, 46].

In this paper, we investigate the genetic variation of six natural Tunisian myrtle populations growing in different bioclimatic regions, using both isozymic and RAPD markers. This study constitutes a complementary to that previously reported on Tunisian myrtle population's structure based on isozymes [32], for further global information in order to elaborate improvement and conservation programs.

We address the following questions: (1) What is the pattern of genetic variation within and among populations based on each marker? (2) Is population differentiation related to bioclimate? (3) Do isozymic andRAPD markers provide similar conclusions? (4) What is the implication of the detected genetic variation for conservation strategies?

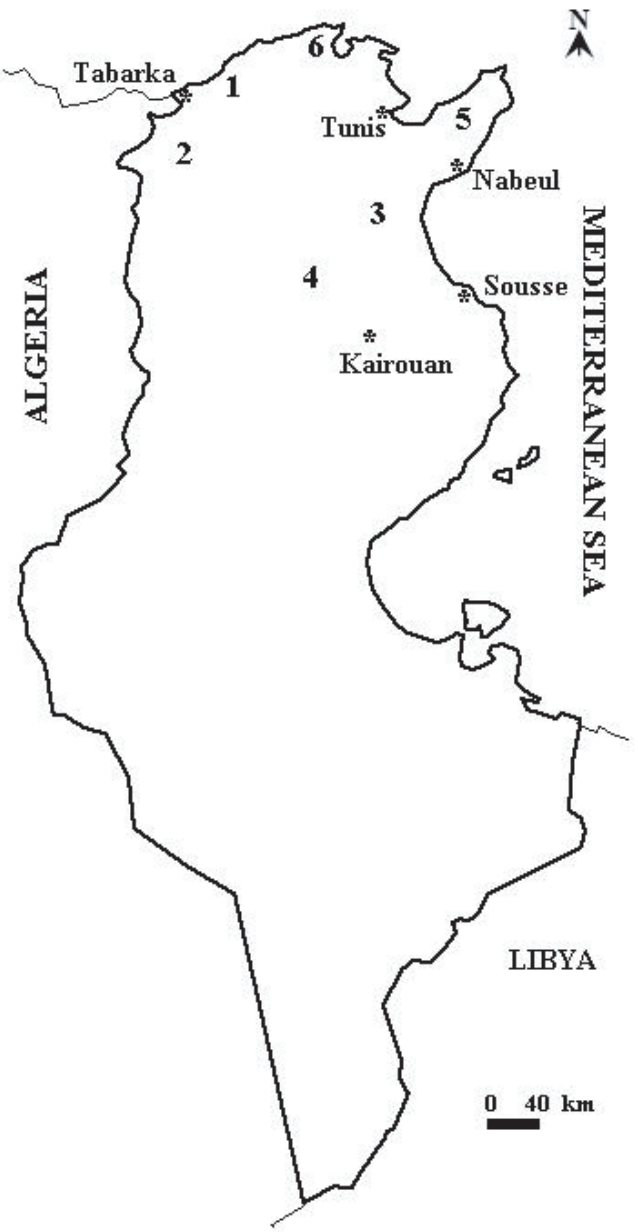

Figure 1. Map of Tunisia: Geographic location of the six populations of *Myrtus communis* analysed. 1, 2, 3, 4, 5 and 6: populations. * Great towns.

2. MATERIALS AND METHODS

2.1. Surveyed populations and sampling

Six Tunisian *Myrtus communis* populations (Fig. 1 and Tab. I) were sampled to assess their genetic variation. These populations belonged to lower humid, sub-humid and upper semi-arid bioclimatic zones [12, 36]. Plants, in each population, were sampled at distances exceeding 20 m to avoid multiple sampling from the same parent. Twenty and seven to eight plants per population were used for isozyme and RAPD analyses, respectively. Analysed organs were leaves collected from each individual.

2.2. Isozyme electrophoresis

Proteins were extracted from 350 mg young leaves ground in 1 ml extraction buffer with liquid nitrogen. The extraction method, the composition of gel and electrode buffers were reported in Messaoud et al. [32]. We used horizontal 13% starch gel electrophoresis

Table I. Main ecological traits for the six Tunisian *Myrtus communis* populations analyzed.

Population code	Location	Latitude	Longitude	Bioclimatic zone*	Geographic region	Q_2	Winter variant	Altitude (m)
1	Tamra	37° 05' N	8° 65' E	Lower Humid	North West	144.24	Cool 1< m < 3° C	440
2	Fernena	36° 42' N	8° 48' E	Lower Humid	North West	144.24	Temperate 3 < m < 4.5° C	300
3	Zaghouan Jebel mountain	36° 26' N	10° 46' E	Upper Semi-arid	Tunisian Dorsal	55.44	Temperate 3 < m < 4.5° C	300
4	Bargou Jebel mountain	36° 05' N	9° 30' E	Upper Semi-arid	Tunisian Dorsal	45.70	Temperate 3 < m < 4.5° C	865
5	Abdrrahman Jebel mountain	36° 40' N	10° 46' E	Sub-humid	Cap Bon	65.61	Temperate 3 < m < 4.5° C	420
6	Bizerte	37° 15' N	9° 48' E	Sub-humid	North East	88.70	Hot 7 < m < 10.5° C	50

* Bioclimatic zones were defined according to Emberger's Q_2 pluviothermic coefficient [12]. $Q_2 = 2000\ P/(M^2\text{-}m^2)$ 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). Q_2 values were calculated for each site using P, M and m values for the period 1953–2003. Data have been provided by the Tunisian National Institute of Meteorology.

to screen 9 enzyme systems: Leucine aminopeptidase (Lap, E.C. 3.4.11.1), Alcohol dehydrogenase (Adh, E.C. 1.1.1.1), Glutamate oxaloacetate transaminase (Got, E.C. 2.6.1.1), Esterase (Est, E.C. 3.1.1.), Isocitrate dehydrogenase (Idh, E.C. 1.1.1.42), Malate dehydrogenase (Mdh, E.C. 1.1.1.37), Phospho-glucomutase (Pgm, E.C. 2.7.5.1), 6-Phosphogluconate dehydrogenase (6-Pgd, E.C. 1.1.1.44) and Phosphoglucoisomerase (Pgi, E.C. 5.3.1.9). Electrophoresis was carried out at 4 °C for 8 h (50 mA). Staining isozymes protocols followed standard methods as reported by Goodman et al. [20] and Weeden and Wendel [51].

2.3. RAPD procedure

2.3.1. DNA extraction

Young leaves (0.5 g) were powdered in liquid nitrogen, mixed with 700 µL CTAB extraction buffer and 100 mg PVP 40000. 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. [29]. The quality of the DNA was estimated on an agarose gel (0.8 %) stained with ethidium bromide.

2.3.2. Primers and PCR conditions

Twenty RAPD primers (kit OPJ, Genset Oligos, Promega) were tested. After optimising the PCR conditions, five RAPD primers (OPJ04, OPJ08, OPJ10, OPJ12 and OPJ20) were selected on the basis of the reproducibility and the polymorphism of the generated bands. The PCR reaction was performed in 25 µL reaction volume containing 50 ng DNA template, 2.5 µL of 10 X reaction buffer, 40 pmoles of primer, 200 µM of each dNTP, 2.5 mM $MgCl_2$ and 1.5 U Taq polymerase (Promega). Mixture was overlaid with 1 drop of mineral oil and amplified in a Programmable Stuart Thermal Cycler (MaxiGene) under the following conditions: 94 °C for 2 min, followed by 45 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 for final polymerase reaction. PCR products were separated by electrophoresis in 1.5% agarose gel at 120 V for 2 h in 1 X TAE buffer (pH 8). Gel was stained with ethidium bromide, visualized under UV light and photographed with DOC PRINT Photo Documentation System. Molecular weights were estimated using a 200 pb DNA Promega ladder.

2.4. Data analysis

2.4.1. Isozyme analysis

Genetic variation within populations and within ecological groups (each group including populations from the same bioclimate) was assessed using the number of alleles per locus (A), the effective number of alleles per locus (Ae), the percentage of polymorphic loci (P_i) and the observed (Ho) and expected (He) heterozygosities. Calculation of A, P_i, Ho and He parameters were performed using BIOSYS software package [44]. The POPGENE program [56] was used to estimate Ae values. The comparison of A, Ae, He and Ho between ecological groups was performed using a variance analysis (ANOVA procedure) over all loci.

Departure from Hardy-Weinberg equilibrium was evaluated by Wright's inbreeding coefficient (F_{IS}) [54] estimated over all loci according to Weir and Cockerham's estimates [52]. Significance of deficit or excess of heterozygotes were performed by randomizing alleles among individuals.

Differentiation among populations (all populations or populations within the same ecological group) or among groups was estimated by Wright's F-statistics (F_{IT}, F_{ST} and F_{IS}) [54] using the program FSTAT version 2.9.3 [21]. F_{IT} and F_{IS} are the inbreeding coefficients in all populations and within population respectively. F_{ST} indicates the level of differentiation among populations. Significance of F-statistics was tested after 1000 permutations.

Nei's unbiased genetic identity coefficients [37] were calculated for all pairs of populations. The data was then used to produce a dendrogram based on the unweighted pair group method with the arithmetic averaging algorithm (UPGMA).

2.4.2. RAPD analysis

RAPD bands amplified by each of the five primers were numbered sequentially in decreasing order according to the molecular weight.

Table II. Genetic diversity within populations and within ecological groups assessed by isozyme and RAPD markers: mean number of alleles per locus (A), effective number of alleles per locus (Ae), percentage of polymorphic loci (P_i and P_r), observed (Ho) and expected heterozygosities (He) and Shannon's diversity index (H').

	Isozyme						RAPD	
	A	Ae	P_i%	Ho	He	F_{IS}	P_r%	H'
Population								
1	1.4 (0.1)	1.31 (0.10)	41.20	0.121 (0.048)	0.179 (0.055)	0.334***	49.37	0.442
2	1.4 (0.1)	1.18 (0.08)	29.40	0.065 (0.042)	0.107 (0.045)	0.403***	56.96	0.482
3	1.5 (0.1)	1.22 (0.09)	47.10	0.074 (0.034)	0.135 (0.044)	0.446***	50.63	0.435
4	1.5 (0.1)	1.31 (0.10)	47.10	0.082 (0.042)	0.190 (0.055)	0.573***	53.16	0.470
5	1.5 (0.1)	1.30 (0.09)	52.90	0.103 (0.049)	0.178 (0.050)	0.428***	54.43	0.523
6	1.5 (0.1)	1.35 (0.11)	47.10	0.135 (0.050)	0.195 (0.057)	0.310**	46.84	0.422
Mean	**1.47 (0.02)**	**1.28 (0.03)**	**44.13**	**0.097 (0.01)**	**0.163 (0.012)**	**0.410*****	**51.90**	**0.462 (0.015)**
Bioclimatic zone								
LH	1.5 (0.1)a	1.25 (0.07)a	41.20	0.093 (0.043)a	0.150 (0.048)a	0.387**	62.03	0.507 (0.047)a
SH	1.6 (0.1)a	1.34 (0.10)a	47.10	0.125 (0.047)a	0.203 (0.056)a	0.397**	64.56	0.553 (0.046)a
USA	1.6 (0.1)a	1.28 (0.10)a	47.10	0.078 (0.034)a	0.165 (0.048)a	0.529**	60.76	0.503 (0.046)a
Mean	**1.57 (0.03)**	**1.29 (0.02)**	**45.13**	**0.099 (0.020)**	**0.173 (0.033)**	**0.433*****	**62.45**	**0.521 (0.016)**

Standard errors are in parentheses; ** significant at $P < 0.01$; *** significant at $P < 0.001$; P_i% = (polymorphic loci /total loci) ×100; P_r% = (polymorphic RAPD bands/total RAPD bands) ×100; LH: lower humid, SH: sub-humid, USA: upper semi-arid.
Values with the same letter in column are not significantly different (ANOVA, $P > 0.05$).

RAPD fragments with the same mobility were scored for band presence (1) or absence (0). Since RAPD markers are dominant, it was assumed that each band represented the phenotype at a single bi-allelic locus [24, 53].

Genetic diversity within populations was estimated using the percentage of polymorphic bands P_r% [P_r% = (polymorphic bands/total bands) × 100)] and Shannon's H' index [$H' = (-\Sigma\pi_i \log_2 \pi_i)/L$, π_i is the frequency of the ith RAPD band in that population and L is the number of loci].

To compare Shannon's H' index between the three bioclimatic zones, a variance analysis (ANOVA procedure) over loci was used.

Genetic similarity between individuals was estimated using the Nei and Li's similarity coefficient [38]: $S_{xy} = 2m_{xy}/(m_x + m_y)$, where m_{xy} is the number of bands shared by samples x and y, and m_x and m_y are the number of bands in samples x and y, respectively. Genetic distance (D) was estimated using the complementary value of similarity coefficient: $D_{xy} = 1 - S_{xy}$. A cluster analysis (UPGMA), based on the similarity matrix between individuals, was used to ordinate relationships among individuals using Multi-Variate Statistical Package MVSP [27].

The genetic variation within and among populations or within and between bioclimatic zones was also estimated by the molecular variance analysis (AMOVA) [13], based on the genetic distances between individuals. Φ-statistics: Φ_{ST} (differentiation among populations), Φ_{CT} (differentiation among ecological groups) and Φ_{SC} (differentiation among populations within groups), were calculated. Significance of variance components and of Φ-statistics were evaluated using permutations procedures. All analyses were performed using WINAMOVA program, version 1.55 [13].

The correlation between $\log_{10}$ of gene flow (Nm) and $\log_{10}$ of geographic distance between pairs of populations was tested by the Mantel test [31] using the program zt [6]. $Nm = [(1/\Phi_{ST})\text{-}1]/4$ [54], where Φ_{ST} is the pairwise genetic distance between populations, calculated from AMOVA.

2.4.3. Combined data analysis

Population structure, based on both isozymic and RAPD markers, was estimated by an UPGMA cluster analysis performed on the similarity matrix calculated from the combination of allele (isozyme) and RAPD band frequencies data. Cluster analysis was carried out using Multi-Variate Statistical Package MVSP [27].

The relationship between isozyme and molecular genetic diversity parameters was tested by the calculation of the correlation coefficient (i) between the Shannon's index (H') and the expected heterozygosity (He) for each population using Kendall's rank test [39], (ii) between pairwise F_{ST} (isozyme) and Φ_{ST} (RAPD) values. Significance of the correlation was evaluated by the Mantel test [31].

3. RESULTS

3.1. Isozyme genetic diversity and population structure

For all populations, the nine analysed enzymes were encoded by 17 putative loci [32]. Twelve (70.59%) out of them were polymorphic (Lap-1, Pgm-1, Est-1, Got-1, Idh-1, Idh-2, Pgi-2, Adh-2, 6Pgd-2, Mdh-1, Mdh-2 and Mdh-3). Allelic frequencies varied according to bioclimate [32]. Pgd-2b and Got-1a were not detected in populations of the lower humid bioclimate. Adh-2a was not revealed in the upper semi-arid. Pgi-2a was detected only in some populations from the sub-humid and the lower humid.

Levels of genetic diversity varied according to populations (Tab. II). The average number of alleles per locus (A) was 1.47 (from 1.4 for populations 1 and 2 to 1.5 for populations 3, 4, 5 and 6). The effective number of alleles per locus (Ae) varied from 1.18 (population 2) to 1.35 (population 6). The percentage of polymorphic loci (P_i) ranged between 29.4% (population 2, Fernena) to 52.9% (population 5, Abderrahman Jebel

Table III. *F*-statistics (F_{IT}, F_{ST}, F_{IS}) calculated for all populations, populations within the same bioclimate and between bioclimates.

	F_{IT}	F_{ST}	F_{IS}
All populations (all polymorphic loci)	0.657 (0.106)***	0.414 (0.097)***	0.410 (0.138)***
Within bioclimatic zone			
LH	0.405 (0.208)***	0.077 (0.047)^{ns}	0.348 (0.205)***
SH	0.425 (0.165)***	0.093 (0.043)^{ns}	0.364 (0.169)***
USA	0.532 (0.152)***	0.032 (0.015)^{ns}	0.516 (0.157)***
Among bioclimatic zone (LH- SH-USA)	0.688 (0.102)***	0.445 (0.104)***	0.433 (0.139) ***

Standard errors are in parentheses; ns: not significant at $P > 0.05$; *** significant at $P < 0.001$.

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

Population	1	2	3	4	5	6
1	–	0.079^{ns}	0.616***	0.521***	0.047^{ns}	0.169*
2	0.017^{ns}	–	0.679***	0.593***	0.105^{ns}	0.275*
3	0.401***	0.412***	–	0.033^{ns}	0.557***	0.505***
4	0.359***	0.368***	0.033^{ns}	–	0.458***	0.405***
5	0.283***	0.275***	0.372***	0.379***	–	0.091^{ns}
6	0.324***	0.365***	0.417***	0.403***	0.119***	–

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

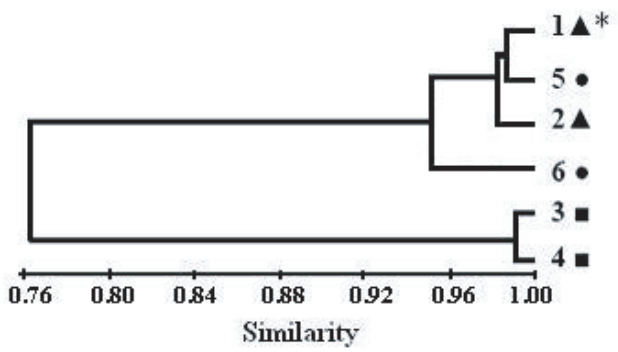

Figure 2. UPGMA dendrogram based on Nei's unbiased genetic similarity coefficients for the 6 populations of *Myrtus communis*. * Bioclimatic zone appartenance: ▲ lower humid, ● sub-humid, ■ upper semi-arid.

mountain) with a mean of 45.13%. The averages of the observed (*Ho*) and expected (*He*) heterozygosities were 0.097 and 0.163, respectively. A significant excess of homozygotes ($F_{IS} > 0$, $P < 0.01$) was observed for all populations.

Within bioclimatic zones the average number of alleles per locus (*A*) was 1.57 (1.5 to 1.6), the effective number of alleles per locus (*Ae*) ranged from 1.25 to 1.34, with an average of 1.29. The mean percentage of polymorphic loci (P_i) was 45.13% (41.2% to 47.1%). The averages of the observed (*Ho*) and expected (*He*) heterozygosities were 0.099 and 0.173, respectively. *A*, *Ae*, *Ho* and *He* values did not show significant differences among bioclimatic zones ($P > 0.05$, Tab. II). All bioclimatic zones were characterized by a significant deficit of heterozygotes ($F_{IS} > 0$, $P < 0.01$).

Wright's *F* statistics revealed significantly deficiencies of heterozygotes at species ($F_{IT} = 0.657$) and population ($F_{IS} = 0.410$) levels (Tab. III). For all loci, the average F_{ST} was 0.414, indicating a high genetic differentiation among populations (Tab. III). Significant differences in F_{ST} values were observed among the majority of pairwise populations ($P < 0.05$ and $P < 0.001$ after 1000 permutations) (Tab. IV). The lowest F_{ST} value (0.033) was observed among populations 3 and 4 (60 km distant) and the highest one (0.679) was detected between populations 2 and 3 (140 km distant). Gene flow among populations was not significantly correlated with geographic distance (Mantel test, $r = -0.286$, $P = 0.178 > 0.05$).

Within each ecological group, differentiation between populations was not significant ($0.032 < F_{ST} < 0.093$, $P > 0.05$ after 1000 permutations). However, a high differentiation was observed between the three ecological groups ($F_{ST} = 0.445$, $P < 0.001$).

Nei's genetic similarity indices (*I*) between pairs of populations were high ($0.701 < I < 0.991$; data available upon request). The average genetic identity for all populations was 0.86. The dendrogram, constructed using these similarity coefficients, showed two distinct groups (Fig. 2). The first one includes populations 3 and 4 belonging to the upper semi-arid bioclimate. Populations 1, 2, 5 and 6 from the lower humid and the sub-humid zones clustered together in the second group.

3.2. RAPD genetic diversity and differentiation

For all individuals (45 over all populations), a total of 79 RAPD fragments were amplified (Tab. V). The number of bands varied from 9 (OPJ04) to 23 (OPJ20) according to the used primer. 88.61% of the amplified bands were polymorphic and 11.39% were scored for all individuals. Specific bands were revealed according to bioclimate. Bands 950 pb (OPJ04), 320 pb, 1500 pb, 1800 pb (OPJ08) and 1400 pb, 1600 pb, 1800 pb (OPJ20) were restricted to populations from the lower humid and the sub-humid. Bands 500 pb, 550 pb (OPJ10) and 320 pb, 340 pb, 360 pb, 390 pb, 650 pb, 1300 pb (OPJ20) were observed only in the upper semi-arid.

The percentage of polymorphic loci per primer (P_r) ranged from 66.67 (OPJ08) to 100% (OPJ04 and OPJ20). Average polymorphisms within populations ranged from 46.84% (population 6) to 56.96% (population 2), with a mean of 51.9%

Table V. Selected RAPD primers, number of polymorphic bands and percentages of polymorphic loci ($Pr\%$) per primer.

Primer	Sequence	Polymorphic bands	Total bands	$P_r\%$
OPJ04	5'CCGAACACGG3'	9	9	100
OPJ08	5'CATACCGTGG3	10	15	66.67
OPJ10	5'AAGCCCGAGG3'	17	20	85
OPJ12	5'GTCCCGTGGT3'	11	12	91.67
OPJ20	5'AAGCGGCCTC3'	23	23	100
Total bands and percentage of polymorphic bands		70	79	88.61

Table VI. Nested analysis of molecular variance (AMOVA) for the 45 individuals sampled from the 6 populations.

Source of variation	d.f.	M.s.	Variance	% of total variance	Φ -statistics
Within populations	39	0.098	0.0985	63.57***	$\Phi_{ST} = 0.364^{***}$
Among populations within bioclimatic zones	3	0.148	0.0067	4.32*	$\Phi_{SC} = 0.064^{*}$
Among bioclimatic zones	2	0.895	0.0497	32.11***	$\Phi_{CT} = 0.321^{***}$

d.f.: Degree of freedom. M.s.: Mean squared. * Significant at $P < 0.05$, *** significant at $P < 0.001$ (after 1000 permutations).

(Tab. II). Shannon's diversity index (H') for all populations was 0.462 ($0.422 < H' < 0.523$) and its average within ecological groups was 0.521. Populations from the sub-humid bioclimate showed the highest P_r (64.56%) and H' (0.553) values (Tab. II). Differences for H' among bioclimatic zones were not significantly different (ANOVA test, $P = 0.69 > 0.05$).

Pairwise Φ_{ST} values calculated from AMOVA ranged from 0.017 (populations 1-2, 60 km distant) and 0.417 (populations 3-6, 102 km distant) (Tab. IV). Pairwise comparison of Φ_{ST} values were all significant ($P < 0.001$) except among populations 1 and 2 (60 km distant) and populations 3 and 4 (60 km distant). A significant differentiation was observed among populations ($\Phi_{ST} = 0.364$, $P < 0.001$) or among ecological groups ($\Phi_{CT} = 0.321$, $P < 0.001$). The Mantel test showed that gene flow and populations geographic distance were significantly correlated ($r = -0.63$, $P = 0.018 < 0.05$), indicating an isolation by distance. The differentiation among populations within ecological groups was also significant ($\Phi_{SC} = 0.064$, $P < 0.05$).

AMOVA revealed that 63.57% of the total variation occurred within populations (Tab. VI). Variation among populations belonging to the same bioclimatic zone was lower (4.32%) than that among the three ecological groups (32.11%).

Nei and Li's similarity coefficients (S) between individuals were substantial ($0.407 < S < 0.911$) and the average genetic identity was 0.653. The UPGMA dendrogram showed three distinct groups (Fig. 3). Within each group, populations clustered according to their bioclimatic and geographic locations.

3.3. Combined isozyme and RAPD data analysis

The UPGMA cluster, established on the basis of the combination of isozyme and RAPD data, showed three population groups corresponding to their bioclimatic zone appartenance (Fig. 4). The cluster is comparable to that constructed through RAPD data.

Kendall's rank test performed on Shannon's diversity index (H') and gene diversity (He) matrices was not significant ($\tau = -0.333$, $P = 0.348$). However, a significant positive correlation was scored between Φ_{ST} based on RAPD and F_{ST} based on isozymes ($r = 0.778$, $P = 0.0027 < 0.05$).

4. DISCUSSION

Tunisian *Myrtus communis* showed a high genetic variation within populations as estimated by both isozymic and RAPD markers. The high level of genetic variation could be explained by the species outcrossing breeding system and the persistence of multiple individuals through generations issued from large populations before fragmentation [22, 57]. With RAPD markers, the observed genetic diversity was higher than that revealed by isozymes, as it has been reported in previous studies [3, 39, 46]. Differences could be attributed to (i) the inability of isozymes to detect variations that do not modify (or modify slightly) the amino-acids sequence, (ii) the high rate of mutation detected by RAPDs in both coding and noncoding genes [1, 55].

Isozyme data have revealed that the observed heterozygosity was lower than that expected under Hardy-Weinberg equilibrium. The deficit of heterozygotes was more important for populations belonging to the upper semi-arid bioclimate. The low size of these populations and the low rate of regenerated individuals from seeds are the main factors contributing to the deficiency of heterozygosity. This may lead to an increasing genetic drift [48].

A high genetic differentiation among populations and ecological groups was revealed either with isozymes and RAPDs. This differentiation might be the consequence of population isolation due to habitat destruction started since the last few decades [36,43]. Nevertheless, in this work, only RAPD markers showed an effect of isolation by distance in comparison

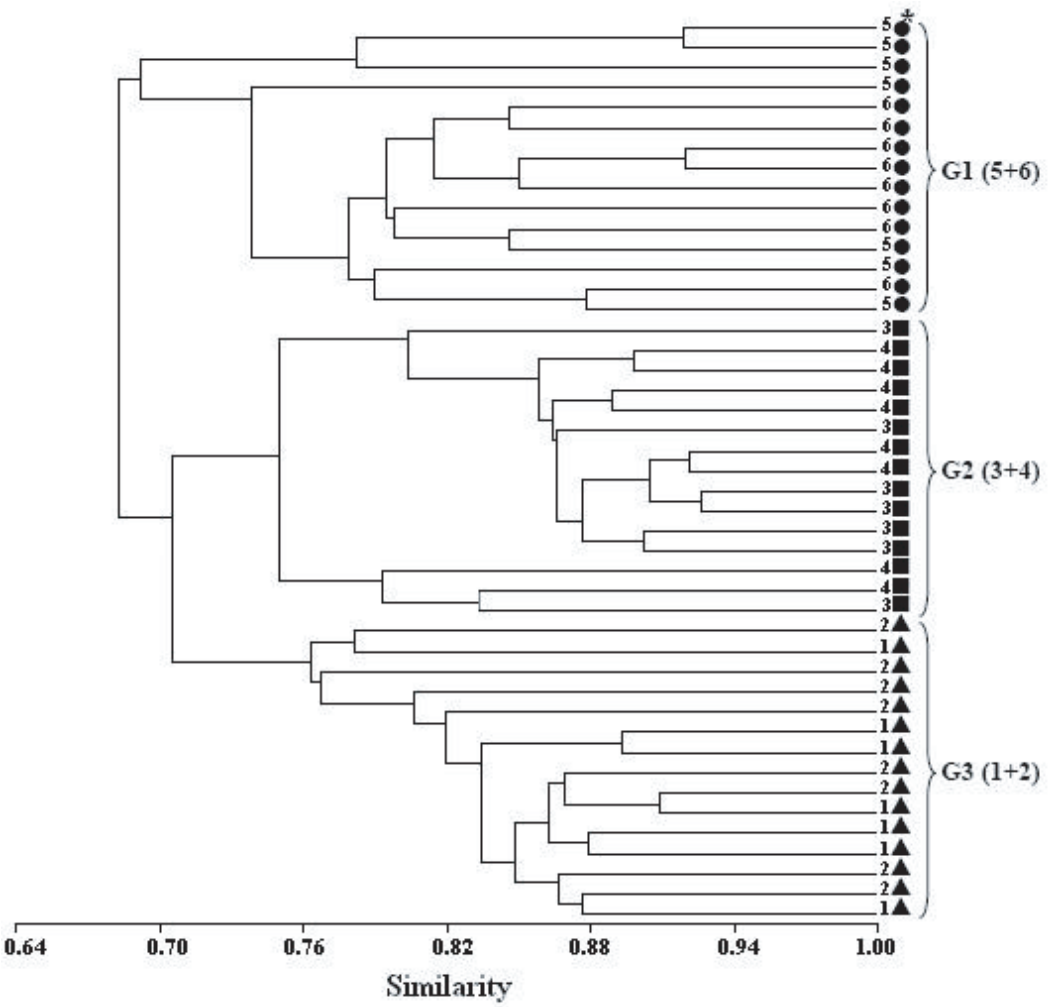

Figure 3. Dendrogram of the 45 individuals based on Nei's and Li's similarity coefficient. * Bioclimatic zone appartenance: ▲ lower humid, ● sub-humid, ■ upper semi-arid. 1, 2, 3, 4, 5 and 6: individuals belonging to populations 1, 2, 3, 4, 5 and 6, respectively G1, G2 and G3: group of populations.

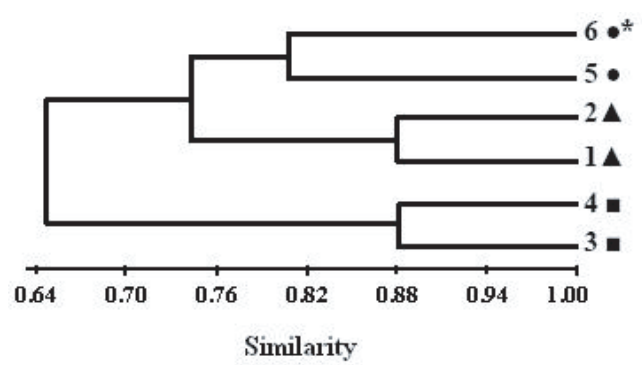

Figure 4. Dendrogram generated by genetic similarity between populations based on combined allele and RAPD band frequencies. * Population bioclimatic zone appartenance: ▲lower humid, ● sub-humid, ■ upper semi-arid.

with isozymes, although our previous study on 17 populations using isozymic markers has revealed a pattern of geographic isolation [32]. Thus, RAPD markers are more able than isozymes to reveal a more accurate effect of isolation by distance when population number is low [48].

Molecular (Φ_{ST}) and isozyme (F_{ST}) differentiation indices are significantly correlated ($r = 0.778$, $P < 0.05$). The correlation between the two data sets suggests that the variation of the two markers may result from parallel evolutionary forces. However, RAPD markers revealed more bioclimatic differentiation than allozyme traits. These results differed from earlier polymorphism studies performed on fragmented populations of perennial species [30,40,55]. However, differentiation based on allozymes could be similar or higher than that based on RAPDs [3, 25], this may be attributed to (i) the higher number of analysed individuals per population in allozyme data that allows to an increasing allozyme differentiation [40], (ii) the dominance of RAPD markers, their biallelism and the interpretation of allele frequencies indirectly from RAPD phenotypes.

According to our estimates of genetic variation analysed by isozymes and RAPDs, the species showed a high genetic diversity within rather than among populations as it has been reported for outcrossing species [16, 22]. So, efficient ex situ conservation programs may be mainly based on sampling

individuals within populations intensively, in each ecological group, to catch the greatest gene diversity revealed by both methods. In situ conservation of populations should be conducted jointly with the restoration of biotopes. Favor artificial gene flow through transplantation between sites would be avoided. This could lead to a decreased fitness, disruption of locally adapted gene combinations and reduction of local variation [11, 14].

The divergent allele frequencies and particular alleles observed by both methods according to bioclimate call to appropriate preservation management. Populations of the subhumid, containing relatively high level of heterozygosity and sharing rare alleles, should be preserved first. Populations of the upper semi-arid were less genetically different. They should be protected mainly by valuable defence favouring individual regeneration. They are located at the South limit range of the species distributing area. Their low size could decrease their fitness through inbreeding depression. However, they showed specific alleles which could be related to local environment adaptation. So, conservation decisions about these populations should include additional adaptative parameters (morphological, physiological and edaphic characters).

Acknowledgements: The authors thank the Tunisian Ministry of Scientific Research and Technology and the National Institute of Applied Science and Technology for their financial support (Research grant 99/UR/09-10). The authors are also grateful to Professor Zaatour Nejib for helpful comments on earlier versions of the manuscript.

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