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Morphological and Molecular Evaluation of Genetic Diversity of Wild Tunisian Oregano, *Origanum vulgare* L. subsp. *glandulosum* Desf. Ietswaart

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MK and FW designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KS and CA managed the analyses of the study. Author JW managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The objective of this work was the search for morphological and molecular markers useful for the analysis of genetic diversity of *Origanum vulgare* L. subsp. *glandulosum* in the northern region of Tunisia.
Study Design: The study of genetic diversity of *Origanum vulgare* L. subsp. *glandulosum* was assessed using RAPD- PCR, sequence analysis of the internal transcribed spacer, and eleven quantitative traits.

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Place and Duration of Study: Five oregano populations were identified and collected in four governorates of Tunisia, Plant specimens of *Origanum vulgare* L. subsp. *glandulosum* were collected during the full flowering period in 2015 in their natural habitats.
Methodology: The five Tunisian *Origanum vulgare* L. subsp. *glandulosum* populations were first characterized and evaluated based on phenotypic characteristic and RAPD- PCR. We carried out PCR amplifications of the ITS1 region of the total cellular DNA extracted either from the seeds or fresh leaves of *Origanum vulgare* L. subsp. *glandulosum*.
Results: The studied populations were highly variable in all evaluated traits ($P < 0.05$). The dendrogram estimated for the morphological traits revealed two main clusters. In total, 30 individuals from 5 *Origanum* wild populations were assessed using RAPD - PCR method coupled with sequence analysis of the internal transcribed spacer (ITS1) and ITS (ITS1 + 5.8S + ITS2) rDNA regions. The separation of amplification products from the total ITS region shows a single band of 700 bp in the oregano populations. This result shows that all the Tunisian populations of *Origanum vulgare* L. subsp. *glandulosum* studied have a common genetic basis and they all belong to the same subspecies. The Principal components analysis and the dendrogram using RAPD markers grouped *Origanum vulgare* L. subsp. *glandulosum* populations into 2 main clusters. This classification brings together the geographically closest populations.
Conclusion: Tunisian *Origanum vulgare* L. subsp. *glandulosum* is growing wild in the mountains of North Africa. Therefore, it has been shown that it is possible to discriminate Tunisian oregano populations on the basis of their morphological and molecular characteristics that can be used as identification tools in breeding and biodiversity conservation programs.

Keywords: Origanum vulgare L; glandulosum; morphological; molecular; ITS region; RAPD-PCR.

1. INTRODUCTION

Origanum vulgare is a perennial aromatic and medicinal herb belonging to the *Lamiaceae* family. The species is naturally distributed widely in Eurasia and North Africa [1]. According to Ietswaart's classification based of morphological characters, *Origanum vulgare* is subdivided into six subspecies, i.e. *vulgare, gracile* (Koch) Ietswaart, *hirtum* (Link) Iestswaart, *viridulum* (Martrin-Donos) Nyman, *virens* (Hoffmannsegg & Link) Ietswaart and *glandulosum* (Desfontaines) Ietswaart [2].

The medicinal significance of members of the genus *Origanum* has been reviewed by many researchers (e.g. [3]). Plant preparations from this genus have important biological activities and act against different kinds of human diseases. Oregano is also important and well-known for culinary uses. Furthermore, it is used as a feed additive, particularly in honeybee keeping [4]. *Origanum vulgare* designed as Oregano without discriminating specific subspecies has a great importance in industry for the preparation of spices [1], as a natural food preservative [5] and for phytotherapy and pharmacy in general. It has been shown to possess antifungal, antimicrobial [5-7] and antioxidant activities [8,9].

Many studies have demonstrated the particular importance of the subspecies *glandulosum*. It is known to have natural antioxidant [10-13] antifungal [14], antimicrobial [15-18] and insecticidal activities [19,20]. The essential oil of *Origanum vulgare* subsp. *Glandulosum* can be considered as an antidiabetic agent [13].

The subspecies *glandulosum* is endemic to Algeria and Tunisia [1,21]. It has not been described as a subject of cultivation like other *Origanum vulgare* subspecies. As an endangered taxon a program of conservation and multiplication of natural *Origanum vulgare* L. subsp. *glandulosum* populations is needed. However, a suitable strategy for conservation of the genetic resources requires a prior description of genetic variability in current populations as extensively as possible.

Molecular markers have been used widely for genetic diversity analysis in many plant species [22]. Random amplified polymorphic DNA (RAPD) is considered as a simpler marker, of lower cost, and faster than other marker systems [23]. However, RAPD are a dominant marker, it is not possible to differentiate heterozygous and homozygous loci [24].

In addition to RAPD analysis, a variety of internal transcribed spacer (ITS) regions of *Origanum vulgare subsp glandulosum* plants were

screened and compared with the known plant ITS1 sequences. The ITS1 region of the 18S–5.8S –26S nuclear ribosomal cistron is advantageous as it monitors the genetic diversity including biparental inheritance, simplicity and intergenomic variability [25].

The morphological and molecular diversity of the subspecies *glandulosum* has not been studied as extensively as the other subspecies of *Origanum vulgare* until now. For this purpose, an analysis of morphological and molecular variability is fundamental. The description of polymorphism in a given species is usually based on comparative observations of distinctive morphological characteristics performed on individuals in several populations.

In the present study, five Tunisian oregano populations (*Origanum vulgare* L. subsp. *glandulosum*) collected from the northern region of Tunisia were used to analyze genetic diversity by using 11 morphological traits, RAPD markers and ITS1 sequences, respectively.

2. MATERIALS AND METHODS

2.1 Plant Material

5 oregano populations (*Origanum vulgare* L. subsp. *glandulosum* Desf. Ietswaart) were identified and collected in four governorates of Tunisia (Fig. 1). The studied populations have different areas of origin characterized by different geographical and ecological characteristics (Table 1). The Laboratory of Botany, National Institute of Agriculture of Tunisia, has confirmed the identification of the species. Voucher specimens were deposited at the herbarium of National Institute of Agriculture of Tunisia and a voucher specimen of the seeds of these wild populations was deposited in the Tunisian National GeneBank.

2.2 Morphological Analysis

The five Tunisian *Origanum vulgare* L. subsp. *glandulosum* populations were first characterized and evaluated based on phenotypic characteristic. Plant specimens of

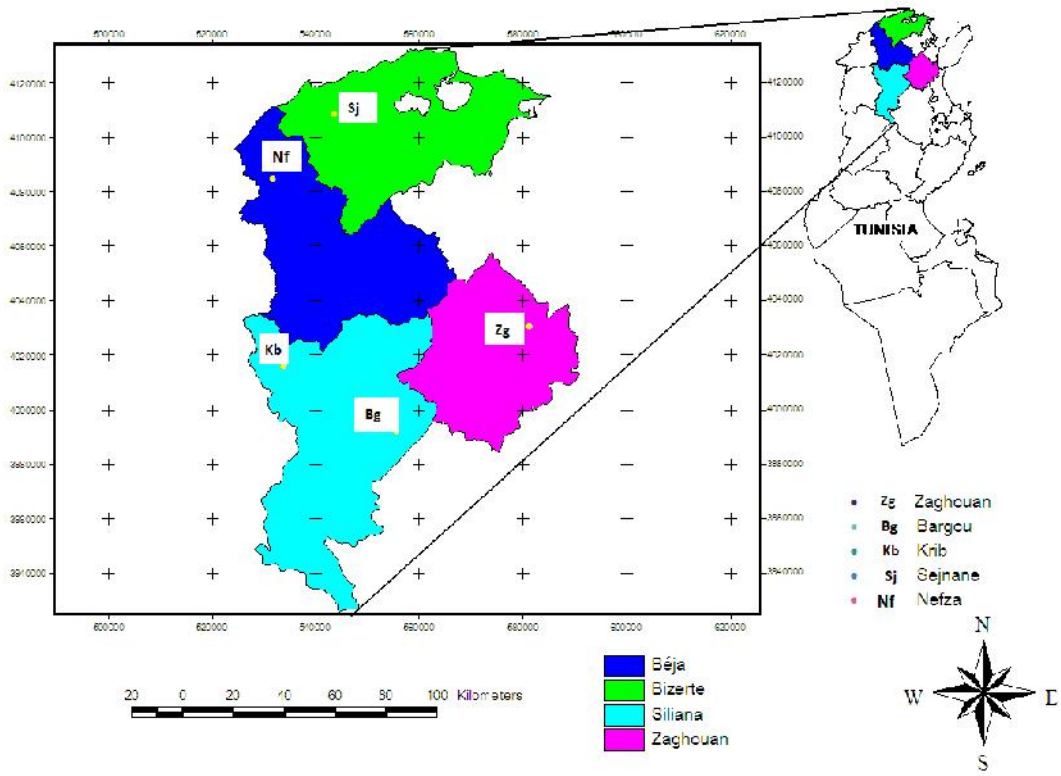


Fig. 1. Geographical localization of the five populations of *Origanum vulgare* L. subsp. *glandulosum* (Desf.) Ietswaart

Table 1. Geographic and ecological information's on localities where oregano plants were sampled

Population	Exact locality of origin	Code	Geographic coordinates	Altitude (m)	Bioclimatic stage	Understory
Bargou	Djebel el Gwèjria	Bg	36°11´ N, 9°51´ E	681	Semi arid	Superior
Krib	Manjem faj el hodoum	Kb	36°38´ N, 9°11´ E	682	Semi arid	Superior
Nefza	Djebel eddamous of Tabouba	Nf	36°87´ N, 9°09´ E	389	Humid	Inferior
Sejnane	Twajnia	Sj	36°98´ N, 9°26´ E	517	Humid	Inferior
Zaghouan	Djebel Zaghouan	Zg	36°35´ N, 10°09´ E	792	Semi arid	Superior

Origanum vulgare L. subsp. *glandulosum* were collected during the full flowering period in 2015 in their natural habitats. In total, 11 descriptors were measured (Table 2), related to vegetative and reproductive developmental stages with 30 individuals per population.

The analysis of variance (ANOVA) between populations and correlation coefficients of morphological characters were calculated using the SPSS program version 20. A principal component analysis was conducted by the Past program, to provide a better multidimensional estimate of the difference(s) between populations. To group the populations based on morphological similarity or dissimilarity, a cluster analysis was conducted on the Euclidean distance matrix with the Unweighted Pair Group Method based on Arithmetic Averages (UPGMA) using the Past program.

Table 2. Morphological traits used in this study

Morphological trait	Acronym
Stem diameter (mm)	SD
Plant height (cm)	PH
Length of the reproductive axis (cm)	LRA
Total number of branches	TNB
Numbers of flowered branches	NFB
Number of *nodes* per *stem*	NNS
Average length of internodes on the stem (cm)	ALIS
Dry matter weight (g)	DMW
Average width of the green leaves (mm)	AWGL
Average length of the green leaves (mm)	ALGL
Average leaf area (mm^2)	ALA

2.3 Extraction of DNA for RAPD and ITS Analyses

The DNA extraction process applied in this study is proposed by [26], with slight modifications using the modified protocol of [27].

2.4 Quantification of DNA

The quality control and amount of extracted DNA is done by electrophoresis on a 1% agarose gel by allowing migrating samples in 1 XTBE buffer. The visualization is done with Ethidium Bromide. The latter is intercalated in the DNA. Under ultraviolet, it emits a fluorescence that will be proportional to the amount of DNA.

2.4.1 Electrophoresis and visualization of bands

To prepare the samples for electrophoresis, 1 µl of the DNA extracted were took and 8 µl of sterile water and 2 µl of charge blue were added, which will serve as an indicator of the migration front. The mixture was mixed well and 9 µl in each well were putted.

In order to mark the weight of the different fragments, another sample was prepared which comprises 3 µl of molecular weight marker. The marker used is the SMART Ladder (Eurogentec). It has a size of 100 to 1000 base pairs.

2.4.2 RAPD- PCR

The amplification reactions were carried out in a reaction volume of 25 µl for each tube. The reaction volume is composed of : 1 µl of genomic DNA (50 ng), 3 µl dNTPs 400 µM, 1 µl of 5 U / µl Taq polymerase (Promega Madison WI USA), 1

µl of primer (25 pmol / µl, 0.75 µl of 50 mM Cl_2 Mg, 5 µl of 5X buffer and the rest is sterile water.

The thermal cycler employed is "BIO-RAD type, Tetrad 2"; the amplification program is 40 cycles, after incubation at 94°C for 6 min, it is initiated by a denaturation phase of 30 seconds at 94°C. Then, a hybridization phase of 30 seconds at 39°C is done and finally an extension phase of 1 min at 68°C. The last amplification cycle was always extended by 8 min at 72°C.

The primers used are oligomers of 10 bases, arbitrary sequences of kit B manufactured by Eurofins MWG Operon (Ebersberg, Germany). We selected 8 primers among the 20 of kit B, the most suitable which allow a good quality of amplification and a great capacity to produce the polymorphism (Table 3). To prepare the samples for electrophoresis, we took 15 µl of RAPD - PCR product from each and we added 5µl of charge blue. We placed 15 µl in each well. On the other hand, we put another sample that included 7µl of molecular weight marker.

Table 3. The primers used and their sequences

Oligomers names	N°of primer	Sequence (5´-> 3´)
OPB-01	Primer 1	GTTTCGCTCC
OPB-02	Primer 2	TGATCCCTGG
OPB-03	Primer 3	CATCCCCCTG
OPB-05	Primer 5	TGCGCCCTTC
OPB-06	Primer 6	TGCTCTGCCC
OPB-13	Primer 13	TTCCCCCGCT
OPB-14	Primer 14	TCCGCTCTGG
OPB-16	Primer 16	TTTGCCCGGA

2.4.3 ITS (Internal Transcribed Spacer) amplification and DNA sequencing

PCR amplifications of the ITS1 region of the total cellular DNA extracted either from the seeds or fresh leaves of *Origanum vulgare* L. subsp. *glandulosum* were carried out. The amplified DNA fragments are separated on 1% agarose gel. The ITS1 and ITS (ITS1 + 5.8S + ITS2) regions were sequenced also. The sequences obtained are examined to see their homologies as well as their alignment using the NCBI of "BLAST" program.

2.4.4 RAPD data analysis

When the band size for each track is obtained, a matrix of 0 and 1 were first made, indicating the absence or presence of each band corresponding to each population. The presence and absence of PCR-RAPD fragments were determined visually. The reading of the bands must take into consideration several anomalies of the experiment. For this purpose, only the well amplified bands are selected. Bands that cannot be read horizontally should be removed.

Amplified fragments were scored according to the presence (1) or absence (0) of the homologous bands. The data were analyzed using MVSP 3.22 (MultiVariate Statistical Package). Accordingly, Shannon's information index and distance matrix between the studied populations were determined.

A principal component analysis (PCA) test that provides a graphical representation of the RAPD relationships between individuals was demonstrated with the variance-covariance matrix calculated from marker data and the similarity matrix were performed using the software MVSP 3.22. A dendrogram was generated based on Jaccard's similarity coefficients [28] using the unweighted pair group method with calculating the arithmetic average (UPGMA) by MVSP 3.22.

3. RESULTS

3.1 Molecular Study

3.1.1 ITS amplification

A single band measuring approximately 300 bp is generated in both cases in all populations (Fig. 2a). Similarly, separation of amplification products from the total ITS region (ITS1 + 5.8S + ITS2) shows a single band of 700 bp in the five populations of oregano: Bargou, Krib, Nefza, Sejnane and Zaghouan (Fig. 2b).

In fact, no variation was observed in the ITS1 region and in the total ITS region (Table 4). The two ITS regions are identical in the five populations. Results showed that populations of oregano studied with different geographical origins showed no difference in their ITS1 and total ITS regions.

3.1.2 RAPD- PCR analysis

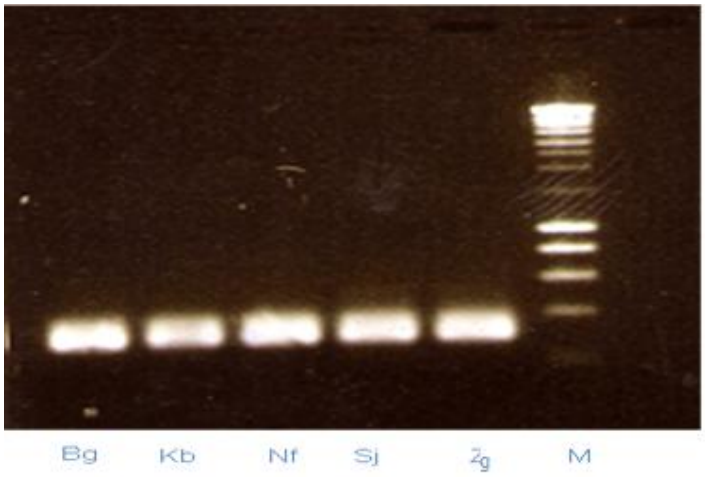
The genetic distance between the 5 populations of wild Oregano ranged between 4.00 and 10.817 with an average of 5.292 (Table 5). This indicates that these populations are

characterized by a high degree of polymorphism at DNA level. The lowest ratio was observed with the populations Nefza and Krib which indicates the low molecular similarity between these two populations. The highest similarity 10.817 was observed between the populations Zaghouan and Sejnane.

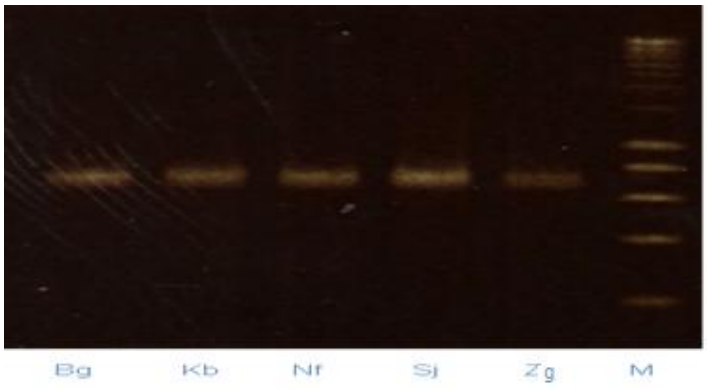
A dendrogram based on UPGMA analysis, using Jaccard similarity coefficients (Fig. 3), grouped the 5 Oregano populations into 2 main clusters, with a similarity rate of 76.5%. The dendrogram showed 2 main clusters. Cluster A has 3 subclusters. Subcluster A_1 contains only the population of Bargou, the subcluster A_2 grouped Nefza and Krib and the subcluster A_3 is presented by the population of Sejnane. The second cluster B contains only Zaghouan.

The Principal components analysis using RAPD markers grouped the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* into 2 clusters: cluster 1 represented by Bargou, Sejnane, Nefza and Krib, the cluster 2 contains only the population of Zaghouan (Fig. 4).

The application of MVSP software version 3.22 to RAPD molecular data allowed us to get the genetic similarity matrix (Table 6). The analysis of this matrix shows that similarity coefficients are ranging from 0.041and 0.765.



(a) ITS1



(b) ITS total

Fig. 2. Revelation on 1% agarose gel of PCR amplification product of the ITS1 (a) region and total ITS (b) of the ribosomal DNA of different Tunisian populations of *Origanum vulgare* L. subsp. *glandulosum* with the primers ITS1 and ITS2. The letters Bg, Kb, Nf, Sj and Zg indicate respectively the populations of Bargou, Krib, Nefza, Sejnane and Zaghouan; M indicates the molecular marker (200 bp)

Table 4. Alignment of ITS1 and total ITS region

Alignment ITS region: ITS1 + 5.8S + ITS2

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ITS-seq195-Kb       GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG
ITS-seq197-Sj       GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG
ITS-seq194-Bg       GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG
ITS-seq196-Nf       GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG
 ITS-seq198-Zg      TTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG
                    ************************************************************
ITS-seq195-Kb       CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA
ITS-seq197-Sj       CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA
ITS-seq194-Bg       CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA
ITS-seq196-Nf       CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA
 ITS-seq198-Zg      CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA
                    ************************************************************
ITS-seq195-Kb       AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC
ITS-seq197-Sj       AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC
ITS-seq194-Bg       AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC
ITS-seq196-Nf       AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC
 ITS-seq198-Zg      AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC
                    ************************************************************
ITS-seq195-Kb       GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG
ITS-seq197-Sj       GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG
ITS-seq194-Bg       GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG
ITS-seq196-Nf       GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG
 ITS-seq198-Zg      GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG
                    ************************************************************
ITS-seq195-Kb       AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
ITS-seq197-Sj       AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
ITS-seq194-Bg       AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
ITS-seq196-Nf       AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
 ITS-seq198-Zg      AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT
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ITS-seq195-Kb       GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT
ITS-seq197-Sj       GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT
ITS-seq194-Bg       GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT
ITS-seq196-Nf       GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT
 ITS-seq198-Zg      GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT
                    ************************************************************
ITS-seq195-Kb       CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC
ITS-seq197-Sj       CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC
ITS-seq194-Bg       CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC
ITS-seq196-Nf       CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC
 ITS-seq198-Zg      CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC
                    ************************************************************
ITS-seq195-Kb       GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG
ITS-seq197-Sj       GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG
ITS-seq194-Bg       GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG
ITS-seq196-Nf       GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG
 ITS-seq198-Zg      GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG
                    ************************************************************
ITS-seq195-Kb       TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC
ITS-seq197-Sj       TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC
ITS-seq194-Bg       TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC
ITS-seq196-Nf       TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC
 ITS-seq198-Zg      TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGG----
                    ************************************************************
ITS-seq195-Kb       AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC
ITS-seq197-Sj       AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC
ITS-seq194-Bg       AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC
ITS-seq196-Nf       AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC
 ITS-seq198-Zg      ------------------------------------------------------------

ITS-seq195-Kb       AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCCGGAGGAA
ITS-seq197-Sj       AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAA----------
ITS-seq194-Bg       AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAA
ITS-seq196-Nf       AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA-
 ITS-seq198-Zg      ----------------------------------------------------
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Alignment ITS1

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ITS1-Seq1-Bg      GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
ITS1-seq2-Kb      GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
ITS1-seq3-Nf      GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
ITS1-seq5-Zg      GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
ITS1-seq4-Sj      GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                  ************************************************************
ITS1-Seq1-Bg      TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
ITS1-seq2-Kb      TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
ITS1-seq3-Nf      TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
ITS1-seq5-Zg      TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
ITS1-seq4-Sj      TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                  ************************************************************
ITS1-Seq1-Bg      ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
ITS1-seq2-Kb      ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
ITS1-seq3-Nf      ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
ITS1-seq5-Zg      ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
ITS1-seq4-Sj      ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
                  ************************************************************
ITS1-Seq1-Bg      CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
ITS1-seq2-Kb      CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
ITS1-seq3-Nf      CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
ITS1-seq5-Zg      CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
ITS1-seq4-Sj      CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
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ITS1-Seq1-Bg      TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
ITS1-seq2-Kb      TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
ITS1-seq3-Nf      TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
ITS1-seq5-Zg      TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
ITS1-seq4-Sj      TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
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Table 5. Distance matrix between the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* using RAPDs

	Bargou	Krib	Nefza	Sejnane	Zaghouan
Bargou	0.000				
Krib	5.292	0.000			
Nefza	5.292	4.000	0.000		
Sejnane	5.292	5.477	5.099	0.000	
Zaghouan	10.149	10.344	10.440	10.817	0.000

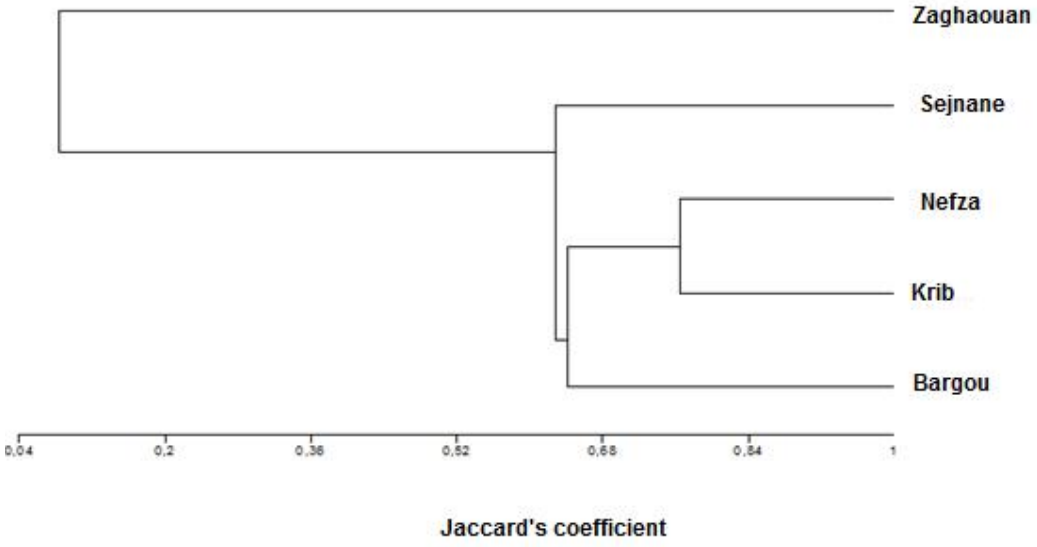

Fig. 3. Dendrogram based on unweighted pair-group method with arithmetic average (UPGMA) among individuals of *Origanum vulgare* L. subsp. *glandulosum* using RAPDs

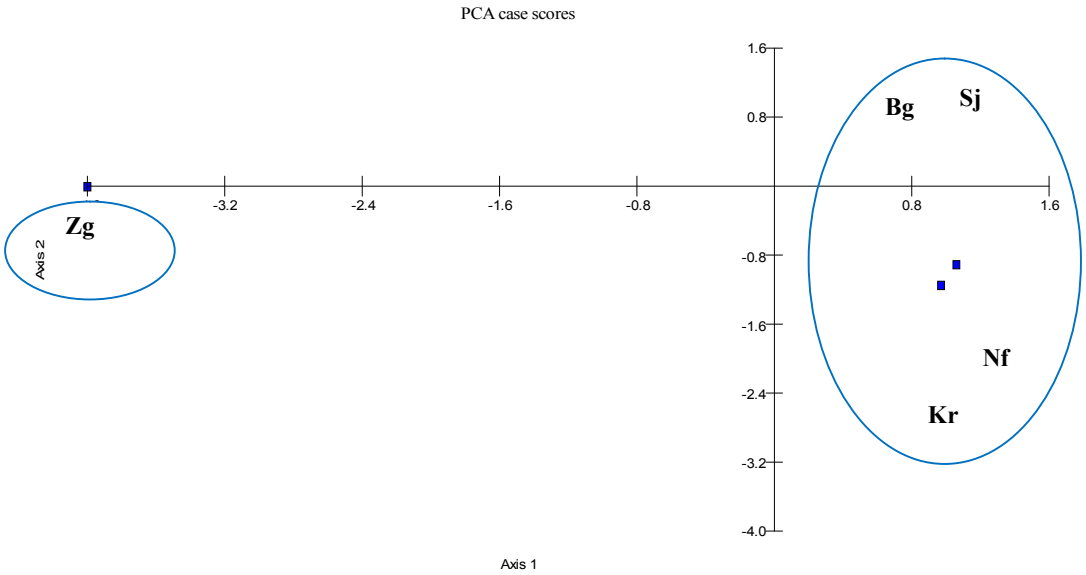

Fig. 4. Principal Component analysis of RAPD data among individuals of *Origanum vulgare* L. subsp. *glandulosum*

Table 6. Genetic similarity matrix between the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* using RAPD markers

	Bargou	Krib	Nefza	Sejnane	Zaghouan
Bargou	1.000				
Krib	0.641	1.000			
Nefza	0.641	0.765	1.000		
Sejnane	0.641	0.600	0.644	1.000	
Zaghouan	0.134	0.085	0.076	0.041	1.000

The lowest similarity (0.041) was observed between the populations Zaghouan and Sejnane. These coefficients reflect a weak molecular similarity of these populations. On the other side, the highest coefficient (0.765) was observed with the populations Nefza and Krib showing a great molecular resemblance between them.

The Shannon's information indexes (I) of the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* are ranging from 4.094 and 4.220 (Table 7). This result reflects a low molecular genetic diversity between these populations.

3.2 Morphological Study

The results of ANOVA for the 11 measured quantitative traits related to the plant morphology are presented in Table 8. It's obvious that the five accessions of *Origanum vulgare* L. subsp. *glandulosum* were highly variable for all evaluated morphological characters ($P < 0.05$).

Table 7. Shannon's information index (I)

Population	Indice (I)
Bargou	4.220
Krib	4.094
Nefza	4.094
Sejnane	4.094
Zaghouan	4.205

By Pearson's matrix correlation (Table 9), it's shown that significant relationships exist between some of the morphological traits. The majority of the characters are positively and significantly correlated at the 0.01 level. For example, high positive correlations were detected between SD and PH (r = 0.647), TNB and PH (r =0.465), ALIS and PH (r =0.486), NFB and LRA (r = 0.470), NFB and TNB (r =0.479), PH and ALIS (r =0.486), AWGL and ALA (r =0.808), AWGL and ALGL (r =0.751), ALGL and ALA (r = 0.843), ALA and AWGL (r =0.808).

Table 8. Range (maximum and minimum values), Means (SD) and ANOVA of the studied morphological traits

Traits	Means	Standard deviation	Minimum	Maximum	F	P > F
SD	1.57	0.514	0.400	3.100	12.760	< 0.0001
PH	44.878	12.736	8.600	78.600	20.598	< 0.0001
LRA	9.373	6.008	1.200	38.400	6.171	< 0.0001
TNB	30.153	5.445	20.000	42.000	6.108	< 0.0001
NFB	10.707	4.521	4.000	26.000	10.616	< 0.0001
NNS	19.000	3.217	13.000	28.000	23.773	< 0.0001
ALIS	1.921	0.403	1.073	3.010	13.504	< 0.0001
DMW	0.999	0.641	0.100	2.800	43.412	< 0.0001
AWGL	11.349	3.001	6.800	17.150	15.681	< 0.0001
ALGL	16.500	4.272	9.225	24.325	40.773	< 0.0001
ALA	135.547	62.076	45.750	252.000	23.003	< 0.0001

Table 9. Pearson correlation coefficients between the morphological traits

	Traits										
	SD	**PH**	**LRA**	**TNB**	**NFB**	**NNS**	**ALIS**	**DMW**	**AWGL**	**ALGL**	**ALA**
SD	1										
PH	0.647**	1									
LRA	0.198*	0.348**	1								
TNB	0.378**	0.465**	0.392**	1							
NFB	0.087	0.072	0.470**	0.479**	1						
NNS	0.151	0.049	0.111	0.059	0.225**	1					
ALIS	0.295**	0.486**	0.254**	0.222**	0.256**	0.060	1				
DMW	0.055	0.121	0.025	0.093	0.012	-0.245**	-0.131	1			
AWGL	-0.177*	-0.029	-0.145	0.008	-0.162*	-0.265**	0.001	0.164*	1		
ALGL	-0.041	0.184*	-0.009	0.123	-0.073	-0.265**	0.154	0.142	0.751**	1	
ALA	-0.155	0.009	-0.093	0.032	-0.132	-0.261**	0.015	0.162*	0.808**	0.843**	1

***. Correlation is significant at the 0.01 level (bilateral).*

**. Correlation is significant at the 0.05 level (bilateral).*

SD: Stem diameter; PH: Plant height; LRA: Length of the reproductive axis; TNB: Total number of branches; NFB: Numbers of flowered branches; NNS: Number of nodes per stem; ALIS: Average length of internodes on the stem; DMW: Dry matter weight; AWGL: Average width of the green leaves; ALGL: Average length of the green leaves; ALA: Average leaf area

Table 10. Comparative table of the majority chemical compounds of oregano essential oils collected in 2009

Main compound	*Origanum vulgare* subsp. *glandulosum*				
	Krib	**Bargou**	**Sejnane**	**Nefza**	**Zaghouan**
p-Cymene	27.3	38.1	55.36	11.51	28.7
γ-Terpinene	23.5	11.2	0.2	23.97	17.1
Thymol	27.0	38.6	3.04	46.05	40.7
Carvacrol	7.7	3.1	28.38	2.94	2.7
Total (%)	**85.5**	**91**	**86.98**	**84.47**	**89.2**

The UPGMA cluster tree based on genetic distances estimated for the 11 morphological traits is presented in Fig. 5. The dendrogram shows two main clusters: The first cluster can be divided into two subgroups, where the population of Zaghouan represents the first subgroup; Bargou and Krib represent the second subgroup. The second cluster includes Sejnane and Nefza and therefore contains the geographically closest populations.

In order to define the morphological relationships among the 5 populations of *Origanum vulgare* L. subsp. *glandulosum*, we have applied a Principal Component Analysis (PCA). A clear separation of the studied populations was observed, and four main groups can be distinguished (Fig. 6). The first group positively related to the axis 2 and negatively related to the axis 1 is represented by the population of Zaghouan. The second group, including the population of Sejnane is positively

related to the axis 1 and negatively correlated to the axis 2. The third group is composed of Bargou and Krib is negatively related to the two axes. The fourth group is positively related to the two axes and is represented by the population of Nefza (Fig. 6).

4. DISCUSSION

In this study, we used morphological and molecular studies using ITS amplification and RAPD markers to assess the variation among the five Tunisian wild populations of *Origanum vulgare* L. subsp. *glandulosum*.

Therefore, these populations do not differ on the basis of their ITS1 and total ITS regions. This molecular study confirms discrimination based on the morphology of these populations. This result shows that all the Tunisian populations of *Origanum vulgare* L. subsp. *glandulosum* studied have a common genetic basis and they all belong to the same subspecies.

The molecular study revealed a low genetic diversity between the studied populations of Tunisian oregano. In fact, *Origanum vulgare* L. subsp. *glandulosum* is a rare and endangered medicinal plant; its conservation is indispensable and very urgent. Because the species is endemic and its loss is an irreversible loss of our plant heritage.

For most of the morphological traits, significant differences between these populations were demonstrated. In fact, a substantial variation and significant heterogeneity between these populations were observed for phenotypic traits. Our results are in agreement with [29] who showed that the examined accessions of *Origanum vulgare* were highly variable in all morphological characters they had evaluated.

The results of [30] show a high degree of variability of Hungarian *Origanum vulgare* populations and the phenotypic response to habitat parameters. Also, [29] showed that the matrices obtained for quantitative morphological traits and specific molecular marker data analyses were significantly correlated (r = 0.27).

In addition, Pearson's coefficients between morphological and chemotypic characteristics among 42 accessions of *Origanum vulgare* studied by [29] showed that there was a significant positive correlation between some morphological characters and the dry mass yield as well as the drug fraction. Furthermore, [29] have shown that the UPGMA clustering, inferred population structure based on quantitative morphological traits revealed a high level of polymorphisms.

The morphological variability of plants has been subject of numerous research projects as a preliminary work for breeding and crop cultivation programs. Examples are from *Sorghum* landraces [31]; *Acacia tortilis* subsp. *raddiana* (Savi) ([32]; *Pyrus mamorensis* Trab. [33]; *Cynara cardunculus* L. subsp. *flavescens* Wiklund [34] and *Cicer arietinum* L. [35]. So, the morphological characterization continues to be a

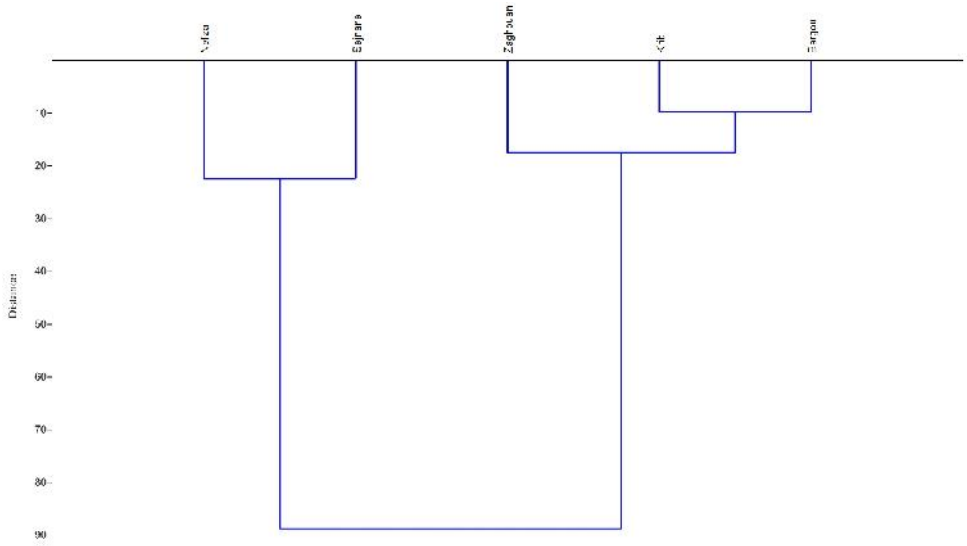

Fig. 5. UPGMA dendrogram showing the genetic relatedness between the 5 Tunisian oregano populations based on the 11 morphological traits

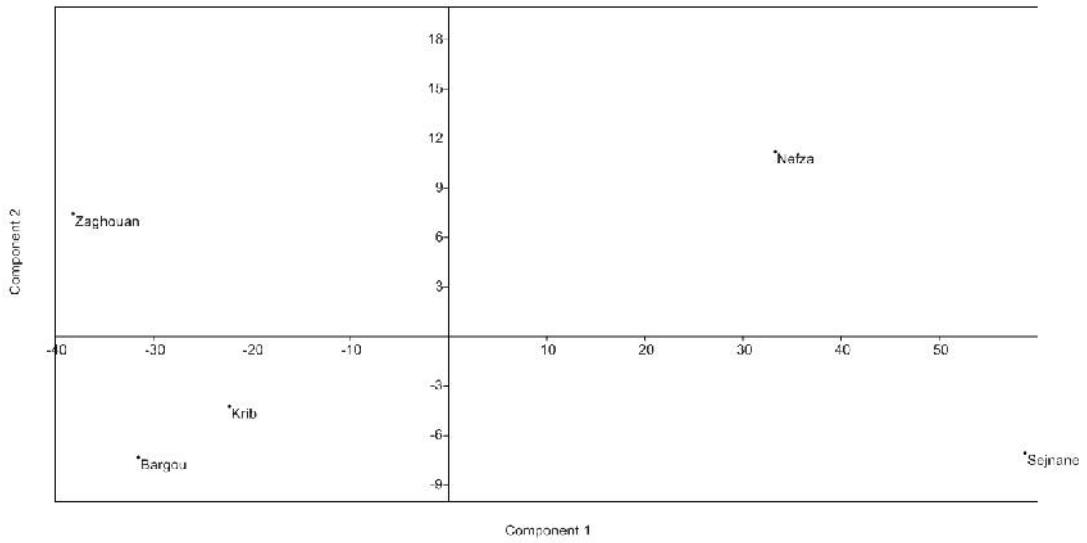

Fig. 6. Principal component's analysis of the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* using morphological characters

major and necessary initial step for the classification of plants: for example, in olive [36], cotton [37] or wheat [38,34].

With regard to oregano, a number of studies have shown that a high morphological diversity exists among *Origanum* species ([39], *Origanum onites* L.) and more especially in *Origanum vulgare* populations [40-44,29,30,45].

The description of morphological variation is very important for the use of the material in breeding programs [44]. In addition, the detection of associations between different characters is important to predict the possibility to combine these characters by "combination breeding" using sexual crosses. It is also important to estimate the production and yield of secondary metabolites in leaves and/or inflorescences of the plants which are considered the main parts of essential oil accumulation in *Origanum vulgare* [46].

The observed phenotype diversity can also be explained by seasonal effects that would alter the morphological, structural and physiological characteristics accessions over time [47]. Oregano plants grown at higher altitude were found to be shorter than those grown at lower altitude. This plant shortening effect at high altitude is proposed to be associated with the short duration of the growing period and/or with reduced temperature, as well as limited nutrient and water supply [48,49].

The observed morphological variations may be due to environmental conditions, genetic or biochemical differences. Biochemical analysis based on essential oils chemical composition of the studied populations have shown that the most close populations geographically, like Sejnane and Nefza or Krib and Bargou, are not those with the closest chemical composition (Table 10).

Studies of molecular diversity through SSR markers were made. The results of this study showed that even the closest populations for the used markers are not morphologically the closest [50]. We can therefore conclude that the morphological and molecular variations observed in the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* may be due to environmental conditions.

5. CONCLUSION

Tunisian *Origanum vulgare* is a species showing significant variation among regional populations, their classification based on morphological and molecular studies shows a close correspondence to the geographical origin of the populations. Based on the phenotypic and molecular classification, it's possible to choose suitable accessions with valuable traits that can be useful for breeding and/or biodiversity programs in this economically important medicinal plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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