Original Research Article

Morphological and molecular evaluation of genetic diversity of wild Tunisian Oregano, *Origanum vulgare* L. subsp. *glandulosum* Desf. letswaart

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.

Place and duration of Study: Five oregano populations were identified and collected in four governorates of Tunisia, Plant specimens of *Origanum glandulosum* were collected during the full flowering period in 2015 in their natural habitats.

Methodology: The five Tunisian *Origanum glandulosum* Desf. 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 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 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 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 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]; [6]; [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]; [11]; [12], [13]) antifungal [14], antimicrobial [15]; [16]; [17]; [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 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 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* Desf.) 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

Five oregano populations (*Origanum vulgare* L. subsp. *glandulosum* (Desf.) Ietswaart) were identified and collected in four governorates of Tunisia (Figure 1). The studied populations have different areas of origin characterized by different geographical and ecological characteristics (Table1). 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.

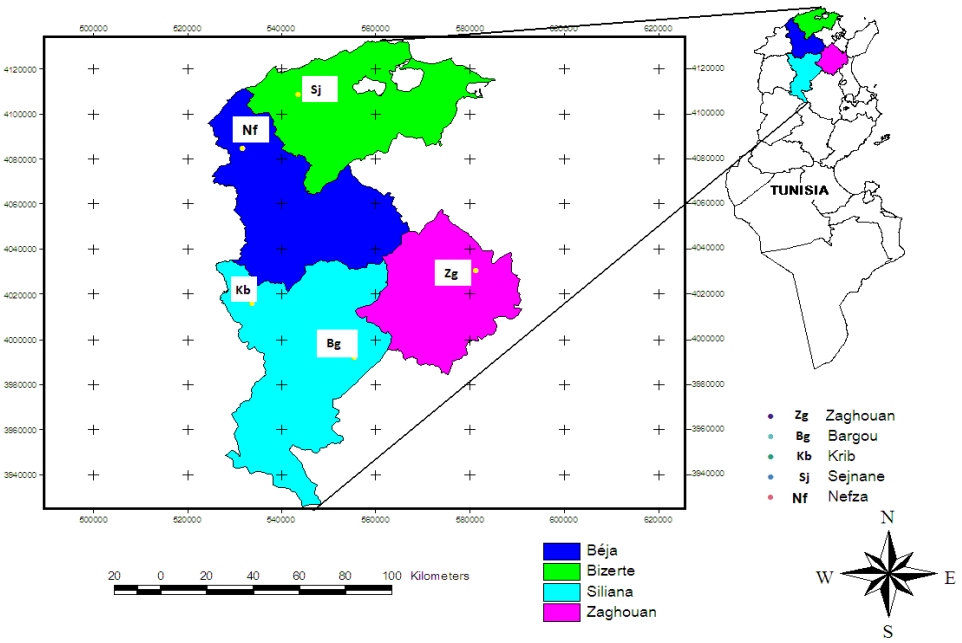


Fig. 1. Geographical localization of the five populations of *Origanum vulgare* L. subsp. *glandulosum* (Desf.) Ietswaart

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

2.2. Morphological analysis

The five Tunisian *Origanum glandulosum* Desf. populations were first characterized and evaluated based on phenotypic characteristic. Plant specimens of *Origanum 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 extraction process applied is that proposed by [26], with slight modifications. They themselves used the modified [27] protocol.

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, we took 1 µl of the DNA extracted from each and we added 8 µl of sterile water and 2 µl of charge blue, which will serve as an indicator of the migration front. We mixed the mixture well by eppendum and put 9 µl in each well.

In order to mark the weight of the different fragments, another sample was prepared which comprises 3µl of molecular weight marker. The marker used is the SMART Ladder (Eurogentec). It has a size of 100 to 1000 base pairs. When loading the solution into the wells of the gel, contamination between the wells must be avoided.

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. Followed by a hybridization phase of 30 seconds at 39°C 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. The marker used is the SMART Ladder (Eurogentec).

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. Statistical analysis of amplification profiles by RAPD

When the band size for each track is obtained, we first make a matrix of 0 and 1, 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.

2.4.4. ITS (Internal Transcribed Spacer) amplification and DNA sequencing

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

2.4.5. RAPD data analysis

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.

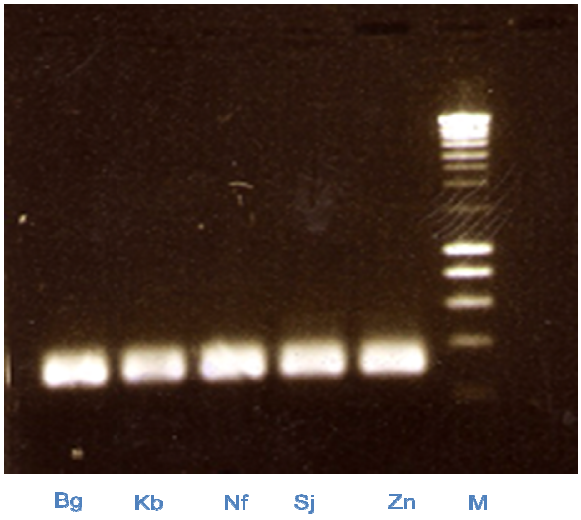
3. Results

3.1. Molecular study

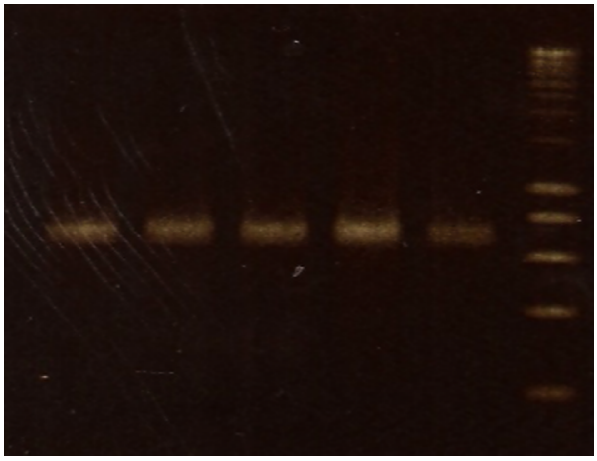
3.1.1. ITS amplification

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.



(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 glandulosum* with the primers ITS1 and ITS2. The letters Bg, Kb, Nf, Sj and Zn indicate respectively the populations of Bargou, Krib, Nefza, Sejnane and Zaghouan; M indicates the molecular marker (200 bp)

Table 4: Alignment of ITS1and total ITS region.

Alignment ITS region: ITS1 + 5.8S + ITS2

ITS-seq195-Kb
GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG

ITS-seq197-Sj
GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG

ITS-seq194-Bg
GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG

ITS-seq196-Nf
GTTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG

ITS-seq198-Zn
TTTAACATCATGGGGGACGGTGCGGGGGCAACCCTCTGCCGTAACCCATCTCCTGCGGG

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ITS-seq195-Kb
CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA

ITS-seq197-Sj
CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA

ITS-seq194-Bg
CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA

ITS-seq196-Nf
CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA

ITS-seq198-Zn
CGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGAACCCCGGCGCGGAATGCGTCAAGGA

****

ITS-seq195-Kb
AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC

ITS-seq197-Sj
AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC

ITS-seq194-Bg
AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC

ITS-seq196-Nf
AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC

ITS-seq198-Zn
AAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTCCGCGGAGCGTGTTGGGGGATCGAAC

****

ITS-seq195-Kb
GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG

ITS-seq197-Sj
GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG

ITS-seq194-Bg
GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG

ITS-seq196-Nf
GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG

ITS-seq198-Zn
GTCTATCAAATGTCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAG

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ITS-seq195-Kb
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT

ITS-seq197-Sj
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT

ITS-seq194-Bg
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT

ITS-seq196-Nf
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT

ITS-seq198-Zn
AACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTT

****

ITS-seq195-Kb
GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT

ITS-seq197-Sj
GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT

ITS-seq194-Bg
GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT

ITS-seq196-Nf
GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT

ITS-seq198-Zn
GAACGCAAGTTGCGCCCGAAGCCATTAGGCTGAGGGCACGTCTGCCTGGGCGTCACGCAT

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                                                          ITS-seq195-Kb
CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC

                                                          ITS-seq197-Sj
CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC

                                                          ITS-seq194-Bg
CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC

                                                          ITS-seq196-Nf
CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC

                                                          ITS-seq198-Zn
CGCGTCGCCCCCCTTCCCCGCGCTCAAAGCCGGGTGTTAGGGGGCGGACATTGGCCTCCC

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                                                          ITS-seq195-Kb
GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG

                                                          ITS-seq197-Sj
GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG

                                                          ITS-seq194-Bg
GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG

                                                          ITS-seq196-Nf
GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG

                                                          ITS-seq198-Zn
GTGTACTTCGGTGTGCGGCTGGCCCAAATGCGATCCCCGGGCGACTAGCGTCACGACAAG

                                                          **********

                                                          ITS-seq195-Kb
 TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC

                                                          ITS-seq197-Sj
 TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC

                                                          ITS-seq194-Bg
 TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC

                                                          ITS-seq196-Nf
 TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGGGCAC

                                                          ITS-seq198-Zn
  TGGTGGTTGAACATCTCAATCTCTCTCGTAGTCGTGCAGCTGTGTCGTCATTACGG----

                                                          **********

                                                          ITS-seq195-Kb
AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC
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                                                ITS-seq197-Sj
AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC

                                                ITS-seq194-Bg
AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC

                                                ITS-seq196-Nf
AATCACAAATGACCCAACGGTGTCGGTGCGTAACTGCACCCCATCTTCGACCGCGACCCC

                        ITS-seq198-Zn   ----------------------------------------------

                                                ITS-seq195-Kb
          AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCCGGAGGAA

 ITS-seq197-Sj    AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAA----------

                                                ITS-seq194-Bg
          AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAA

                                                ITS-seq196-Nf
          AGGTCAGGCGGGATTACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA-

                        ITS-seq198-Zn   ----------------------------------------------


Alignment ITS1

                                                ITS1-Seq1-Bg
GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                                                ITS1-seq2-Kb
GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                                                ITS1-seq3-Nf
GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                                                ITS1-seq5-Zn
GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                                                ITS1-seq4-Sj
GACTTTAAGTAGACCGCGAACACGTGTTTAACATCATGGGGGACGGTGCGGGGGCAACCC
                        ************************************************************
                                                ITS1-Seq1-Bg
TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                                                ITS1-seq2-Kb
TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                                                ITS1-seq3-Nf
TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                                                ITS1-seq5-Zn
TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                                                ITS1-seq4-Sj
TCTGCCGTAACCCATCTCCTGCGGGCGTGTATCTTCGGGTCACGTCTTGCGGGCTAACGA
                        ************************************************************
                                                ITS1-Seq1-Bg
ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
                                                ITS1-seq2-Kb
ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
                                                ITS1-seq3-Nf
ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
                                                ITS1-seq5-Zn
ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
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                                                    ITS1-seq4-Sj
ACCCCGGCGCGGAATGCGTCAAGGAAAACTAAACGAAGCGTTTCCCCCCAGCATCCCGTC
                          ************************************************************
                                                    ITS1-Seq1-Bg
CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
                                                    ITS1-seq2-Kb
CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
                                                    ITS1-seq3-Nf
CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
                                                    ITS1-seq5-Zn
CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
                                                    ITS1-seq4-Sj
CGCGGAGCGTGTTGGGGGATCGAACGTCTATCAAATGTCAAAACGACTCTCGGCAACGGA
                          ************************************************************
                ITS1-Seq1-Bg    TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
                ITS1-seq2-Kb    TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
                ITS1-seq3-Nf    TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
                ITS1-seq5-Zn    TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
                ITS1-seq4-Sj    TATCTCGGCTCTCGCATCGATGAAGAACGCAGCA
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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 Zarghouan and Sejnane.

Table 5: Distance matrix between the 5 populations of *Origanum 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

A dendrogram based on UPGMA analysis, using Jaccard similarity coefficients (Figure 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.

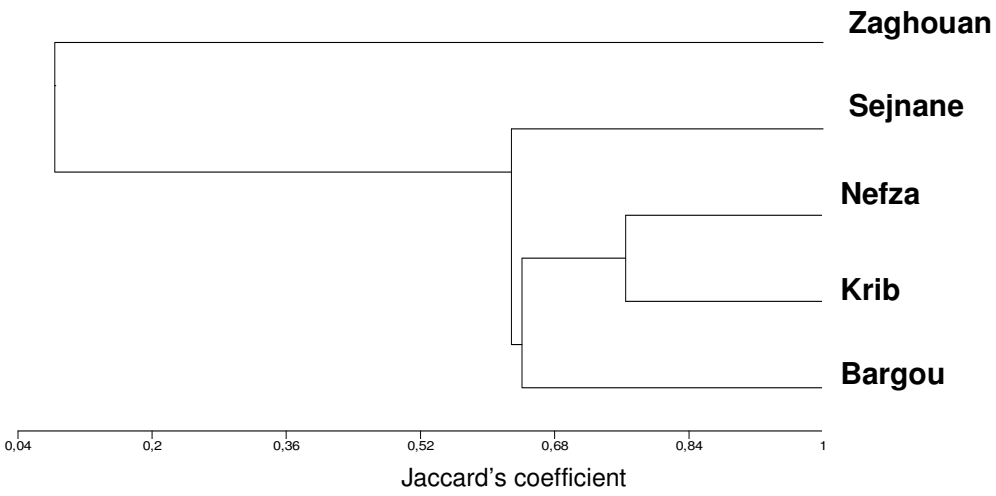
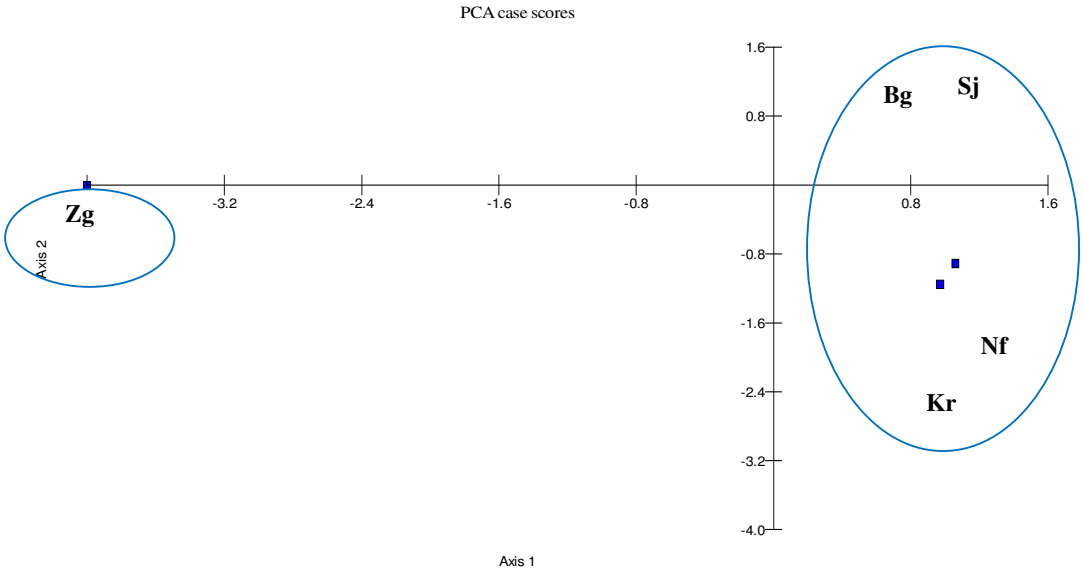


Fig.3. Dendrogram based on unweighted pair-group method with arithmetic average (UPGMA) among individuals of *Origanum glandulosum* using RAPDs

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



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378 **Fig.4.** Principal component analysis of RAPD data among individuals of *Origanum glandulosum*

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

382 **Table 6:** Genetic similarity matrix between the 5 populations of *Origanum glandulosum* using RAPD
383 markers.

384

385

	Bargou	Krib	Nefza	Sejnane	Zaghouan
386 **Bargou**	1,000				
387 **Krib**	0.641	1,000			
388 **Nefza**	0.641	0.765	1,000		
389 **Sejnane**	0.641	0.600	0.644	1,000	
390 **Zaghouan**	0.134	0.085	0.076	0.041	1,000

391 The lowest similarity (0.041) was observed between the populations Zaghouan and Sejnane. These
392 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 glandulosum* are ranging from 4.094 and 4.220 (Table 7). This result reflects a low molecular genetic diversity between these populations.

Table 7: Shannon's information index (I)

Population	Indice (I)	Uniformité
Bargou	4.220	1,000
Krib	4.094	1,000
Nefza	4.094	1,000
Sejnane	4.094	1,000
Zaghouan	4.205	1,000

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* (Desf.) letswaart were highly variable for all evaluated morphological characters ($P < 0.05$).

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

Traits	Mean	Standard deviation	Minimum	Maximum	F	*P* > F
SD (mm)	1,757	0,514	0,400	3,100	12,760	< 0.0001
PH (cm)	44,878	12,736	8,600	78,600	20,598	< 0.0001
LRA (cm)	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 (cm)	1,921	0,403	1,073	3,010	13,504	< 0.0001
DMW (g)	0,999	0,641	0,100	2,800	43,412	< 0.0001

AWGL (mm)	11,349	3,001	6,800	17,150	15,681	< 0.0001
ALGL (mm)	16,500	4,272	9,225	24,325	40,773	< 0.0001
ALA (mm^2)	135,547	62,076	45,750	252,000	23,003	< 0.0001

411

412 By Pearson's matrix correlation (Table 9), it's shown that significant relationships exist between some
413 of the morphological traits. The majority of the characters are positively and significantly correlated at
414 the 0.01 level. For example, high positive correlations were detected between SD and PH (r = 0.647),
415 TNB and PH (r =0.465), ALIS and PH (r =0.486), NFB and LRA (r = 0.470), NFB and TNB (r =0.479),
416 PH and ALIS (r =0.486), AWGL and ALA (r =0.808), AWGL and ALGL (r =0.751), ALGL and ALA (r =
417 0.843), ALA and AWGL (r =0.808).

418 **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).											

419 **SD**: Stem diameter; **PH**: Plant height; **LRA**: Length of the reproductive axis; **TNB**: Total number of
420 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.

The UPGMA cluster tree based on genetic distances estimated for the 11 morphological traits is presented in Figure 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.

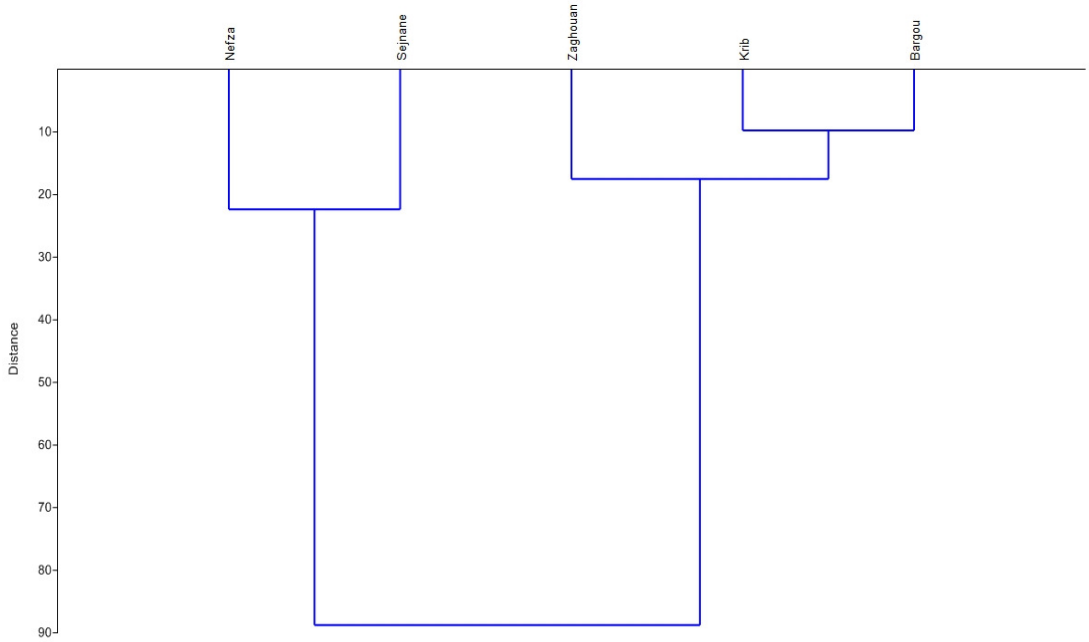

Fig.5. UPGMA Dendrogram showing the genetic relatedness between the 5 Tunisian oregano populations based on the 11 morphological traits

To define morphological relationships among the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* (Desf.) Ietswaart, we have applied a Principal Component Analysis (PCA). A clear separation of the studied populations was observed, and four main groups can be distinguished (Figure 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 (Figure 6).

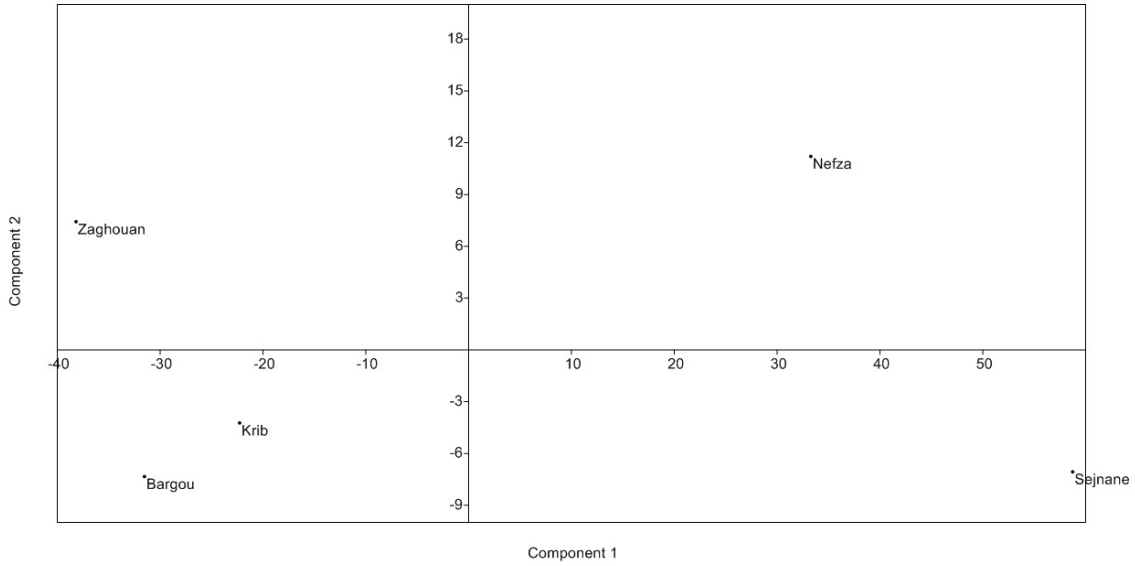

Fig.6. Principal Component's analysis of the 5 populations of *Origanum vulgare* L. subsp. *glandulosum* (Desf.) Ietswaart using morphological characters

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* (Desf.) Ietswaart.

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 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 glandulosum* is a rare and endangered medicinal plant; its conservation is

455 indispensable and very urgent. Because the species is endemic and its loss is an irreversible loss of
456 our plant heritage.
457 For most of the morphological traits, significant differences between these populations were
458 demonstrated. In fact, a substantial variation and significant heterogeneity between these populations
459 were observed for phenotypic traits. Our results are in agreement with [29] who showed that the
460 examined accessions of *Origanum vulgare* were highly variable in all morphological characters they
461 had evaluated.

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

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

471 The morphological variability of plants has been subject of numerous research projects as a
472 preliminary work for breeding and crop cultivation programs. Examples are from *Sorghum* landraces
473 [31]; *Acacia tortilis* subsp. *raddiana* (Savi) ([32]; *Pyrus mamorensis* Trab. [33]; *Cynara cardunculus* L.
474 subsp. *flavescens* Wiklund [34] and *Cicer arietinum* L. [35]. So, the morphological characterization
475 continues to be a major and necessary initial step for the classification of plants: for example, in olive
476 [36], cotton [37] or wheat [38]; [34]).

477 With regard to oregano, a number of studies have shown that a high morphological diversity exists
478 among *Origanum* species ([39], *Origanum onites* L.) and more especially in *Origanum vulgare*
479 populations ([40]; [41]; [42]; [43]; [44]; [29]; [30]; [45]).

480 The description of morphological variation is very important for the use of the material in breeding
481 programs [44]. In addition, the detection of associations between different characters is important to
482 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]).

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 glandulosum* may be due to environmental conditions.

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.

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