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Comparative leaf epidermis and molecular analyses of Micromeria dalmatica Bentham and Clinopodium vulgare L. from Bulgaria

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Abstract. The volume and number of taxa within the genus *Micromeria* have changed over the past decades based on several molecular genetic studies. In the Bulgarian flora, no revision has been conducted, and the boundaries of the genus remain unclear. Molecular studies have shown that members of the section *Pseudomelissa* within the genus *Micromeria* are more closely related to the genus *Clinopodium* than to the typical section *Micromeria*. In the present study, anatomical investigations of the leaf epidermis were carried out, and the molecular genetic structure of *Micromeria dalmatica* was determined. Its phylogenetic relationship with *Clinopodium vulgare* was established based on sequences in the ITS1 and tRNA-Leu regions.

Key words: *Micromeria dalmatica*, *Clinopodium vulgare*, leaf epidermis, Bulgaria, tRNAleu, ITS1 region.

Introduction

The genus *Micromeria*, first described by Bentham in 1829, is distributed from the Macaronesian-Mediterranean region to South Africa, India, and China. It belongs to the Lamiaceae family, subfamily Nepetoideae, tribe Menthae, and subtribe *Menthinae*. The genus is part of the *Satureja* complex, and based on the morphological characters' diversity, the genus is divided into several genera: *Satureja* L., *Clinopodium* L., *Calamintha* Mill., *Acinos* Mill., and *Micromeria* Benth. (Bentham, 1848; Boissier, 1879; Ball & Getliffe, 1972; Davis, 1982). According to Harley et al. (2000), *Micromeria* is divided into four sections: Sect. *Micromeria*, Sect. *Pseudomelissa*, Sect. *Pineolentia* and Sect. *Cymularia*.

More recent studies, including cladistic analyses, show that the *Satureja* complex is not monophyletic (Prather et al., 2002; Trusty et al., 2004; Bräuchler et al., 2008). As a result of Morales's studies in 1993, the genus *Micromeria* has undergone significant changes, such as revision of the taxonomy of some American *Micromeria* species (Govaerts, 1999; Harley, 2000).

In Bulgaria, the genus *Micromeria* is represented by four species: *Micromeria juliana* (L) Bentham ex Reichenb., *Micromeria cristata* (Hampe) Griseb., *Micromeria dalmatica* Bentham ssp. *bulgarica* (Velen.) and *Micromeria frivaldszkyana* (Degen) Velen. Nevertheless, the taxonomic classification of *Micromeria dalmatica* relative to the genus *Clinopodium* remains ambiguous. In this study, we provide a comparative analysis of the morphoanatomical and molecular characteristics of *Micromeria dalmatica* and *Clinopodium*, which is of highest importance for accurate species classification.

Materials and methods

Plant Material

Micromeria dalmatica (Fig. 1) was collected during the growing season of 2022 from the floristic region of Rhodopa mountain, near the town of Krichim. A voucher specimen has been deposited in the herbarium of the Agricultural University – Plovdiv (SOA 062648).

Clinopodium vulgare (Fig. 2) was collected during the growing season of 2020 from the floristic region of Stara planina mountain, "Bulgarka" Nature Park. A voucher specimen has been deposited in the herbarium of the Agricultural University – Plovdiv (SOA 062645).

Fig. 1. *Micromeria dalmatica* in its natural habitat (author's photograph).

Fig. 2. *Clinopodium vulgare* in its natural habitat (author's photograph).

Anatomy of the Leaf Epidermis

The leaf blades were fixed in 70% ethanol, and the anatomical studies were conducted using the classical method of Metcalfe & Chalk (1950). Taking into account the data published by Mladenova et al. (2019) concerning *Micromeria frivaldszkyana* and *Clinopodium vulgare*, and analyzing both the upper and lower epidermis, the following anatomical features were examined: type of trichomes; number (per 1 mm²), type, length, and width of stomata; number (per 1 mm²), length and width of the main epidermal cells; and cuticle thickness. The values for the quantitative indicators are based on the examination of 30 visual fields. The statistical data concerning *Clinopodium vulgare* were taken from the jointly published results of Mladenova et al. (2019).

Statistical Analysis

The statistical processing of the quantitative data was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The difference between the mean values of normally distributed quantitative variables in two independent groups was assessed using the Independent-samples T-test.

Light microscope images of the examined anatomical features of the leaves were taken with a Magnum T microscope equipped with a Si5000 photo documentation system at magnifications of ×100 to ×400 at the Department of Botany and Biological Education, Faculty of Biology, Paisii Hilendarski University of Plovdiv.

Plant genomic DNA isolation

Plant genomic DNA was extracted from 100 mg fresh plant material using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's requirements. DNA integrity was assessed on a 0.8% agarose gel, and quantified by UV spectrophotometry at 260 nm using an Epoh2 reader (Agilent Technologies, Inc., USA)

PCR amplification of target DNA regions and DNA sequencing

Both target sequences - the transcribed spacer region (ITS) of the nuclear ribosomal DNA and the tRNALeu region of the chloroplast DNA were amplified using the following primers:

ITS5A: 5′ccttatcatttagaggaaggag 3′ and **ITS4:** 5′ tcctccgcttattgatatgcg 3′ (Stanford et al., 2000) for the ITS1 region

trnL-c: 5′cgaaatcggtagacgctacg3′ and **trnL-b:** 5′attgaaactggtgacacgag3′ for trnLeu-trnPhe of chloroplast DNA (Taberlet et al., 1991).

All reactions were performed in a final volume of 50 µl, using Q5 High-Fidelity 2x Master Mix (New England Biolabs), according to the manufacturer's requirements.

The annealing temperature was calculated using the NEB Tm calculator. After the final extension for 2 min at 72°C, PCR products were separated on a 0.8% agarose gel. The resulting bands of approx. 650 bp for the ITS1 region and approx. 550 bp for the tRNALeu region were excised from agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN).

The purified DNA fragments were then sent to the Microsynth laboratory facility (Microsynth AG, Switzerland) for Sanger sequencing. The sequence files were inspected and edited using Snap Gene 8.0.3 (GSL Biotech LLC, USA).

For the subsequent bioinformatics analysis, MEGA 12 (https://www.megasoftware.net) software was used.

Results and Discussion

According to the study by Mladenova et al. (2019), concerning *Micromeria frivaldszkyana* and *Clinopodium vulgare*, the leaf epidermis of *Micromeria dalmatica* consists of main epidermal cells, stomata, and trichomes. The structure of the main epidermal cells is similar to that of the species examined by Mladenova et al. (2019) (Fig. 3). Based on the classifications of Sveshnikova (1970) and Aneli (1975), the cell walls of these cells can be described as curved to strongly curved or zigzag-folded.

The leaves are amphistomatic, predominantly featuring diacytic stomata and, rarely, anomocytic stomata (Fig. 4). This type of stomatal apparatus is also typical for other members of the Lamiaceae family (Haruna & Ashir, 2017; Mladenova et al., 2019; Gyuzeleva et al., 2022).

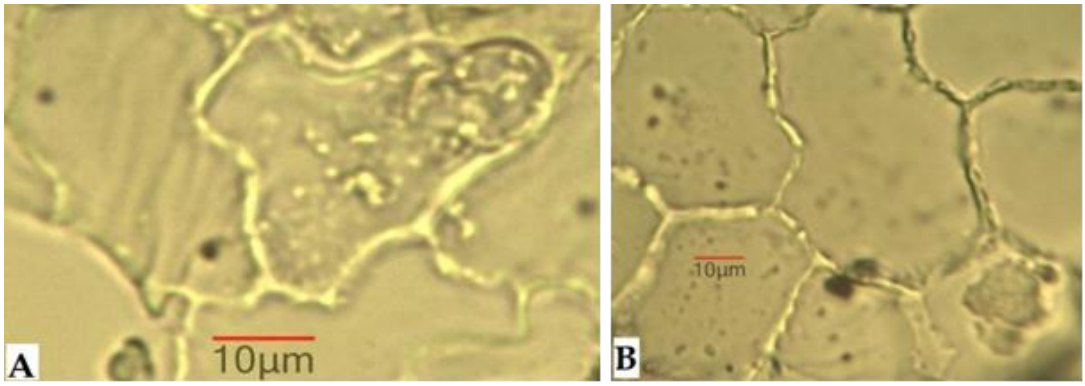

Fig. 3. Basal epidermal cells of *Micromeria dalmatica:* A – Adaxial epidermis; B – Abaxial epidermis.

Fig. 4. Diacytic stomatal type of *Micromeria dalmatica*: A – Adaxial epidermis; B – Abaxial epidermis.

Analysing both leaf surfaces, covering (non-glandular) and glandular trichomes were identified. The covering trichomes are multicellular and unbranched (Fig. 5).

Such a structural type was also reported by Mladenova et al. (2019) in the leaf epidermis of the taxonomically contentious *Micromeria frivaldszkyana* and *Clinopodium vulgare*. The structure of the glandular trichomes likewise corresponds to the description by Mladenova et al. (2019). These are sessile, located on both epidermises, and exhibit a multicellular structure (Fig. 6).

In contrast to the results reported by those authors, glandular trichomes with a unicellular structure were also observed on both epidermises of *Micromeria dalmatica* (Fig. 7).

The bicellular structure of glandular trichomes in the genus *Clinopodium,* noted by Al-Zubaidy et al. (2015), was not detected in the present study.

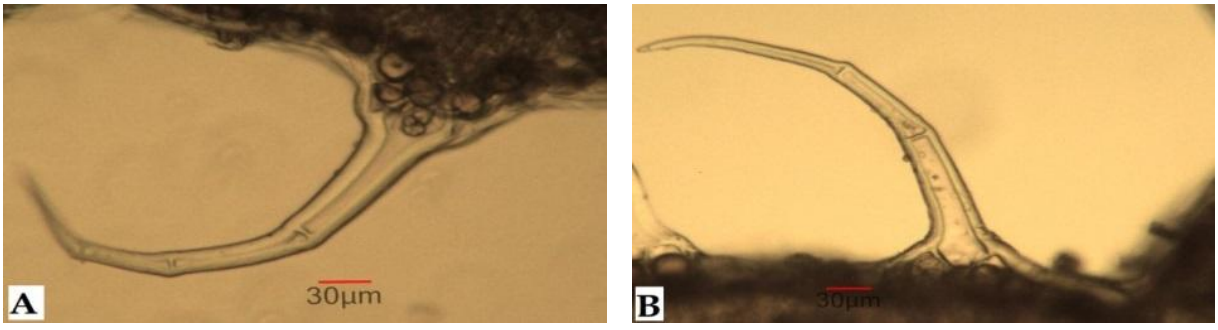

Fig. 5. Covering trichomes of *Micromeria dalmatica:* A – Adaxial epidermis; B – Abaxial epidermis.

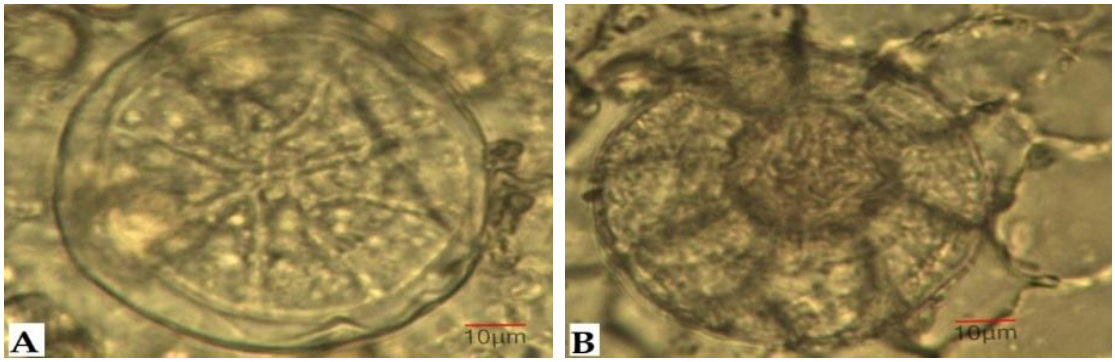

Fig. 6. Glandular trichomes with multicellular structure of *Micromeria dalmatica*: A – Adaxial epidermis; B – Abaxial epidermis.

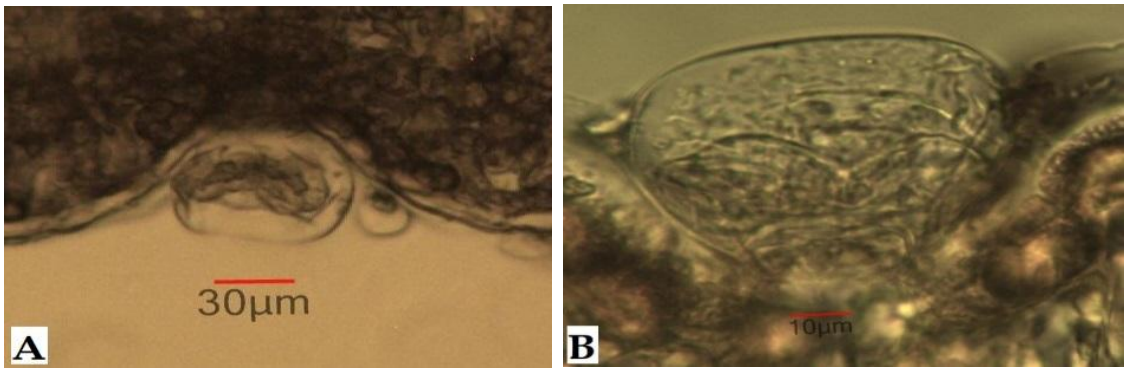

Fig. 7. Glandular trichomes with unicellular structure of *Micromeria dalmatica*: A – Adaxial epidermis; B – Abaxial epidermis.

Table 1 presents the results of the statistical processing of the studied quantitative features for the adaxial epidermis.

A statistically significant difference between the two taxa exists for all traits except for the length of the epidermal cells ($p = 0.435$). The leaf epidermis of *Micromeria dalmatica* is characterised by a lower number but larger stomata compared to that of *Clinopodium vulgare*. The species *Micromeria frivaldszkyana*, studied by Mladenova et al. (2019), also shows larger stomatal cells than those of *Clinopodium vulgare*. The number and width of the main epidermal cells are also greater in *Micromeria dalmatica*, which again aligns with the findings of Mladenova et al. (2019). The trend of a thicker cuticle on the upper epidermis in the genus *Micromeria* is also confirmed in the present study. The identified differences between the traits of the upper epidermis are graphically presented in Fig. 8.

Table 1. Comparison of the mean values of anatomical characteristics in *Micromeria dalmatica* and *Clinopodium vulgare* for adaxial epidermis.

Feature	***Micromeria dalmatica* Mean±SD**	***Clinopodium vulgare* Mean±SD**	**Independent-Samples T-test**
Stomata (Number)	29.2±14.7	37.5±16.4	p=0.009
Stomata (Length, µm)	5.3±0.1	4.3±0.1	p<0.0001
Stomata (Width, µm)	3.1±0.1	2.8±0.3	p<0.0001
Epidermal cells (Number)	1000.4±202.3	465.3±56.3	p<0.0001
Epidermal cells (Length, µm)	13.2±1.6	13±0.5	p=0.435
Epidermal cells (Width, µm)	8.2±0.7	7.9±0.23	p=0.013
Cuticle (Thickness, µm)	2.3±1.9	1.11±0.21	p<0.0001

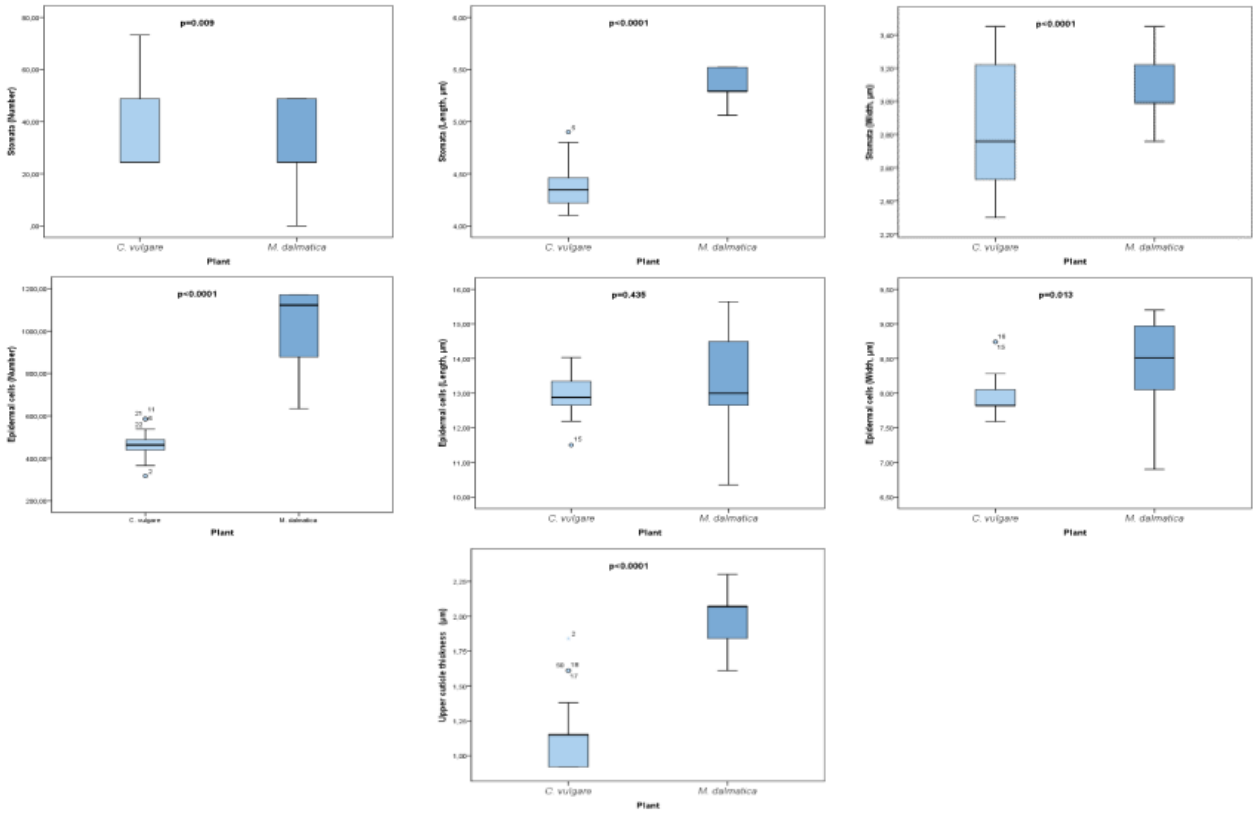

Fig. 8. Significant differences between the mean values of the leaf indicators for adaxial epidermis of *Micromeria dalmatica* and *Clinopodium vulgare*.

The results obtained for the abaxial epidermis showed a statistically significant difference ($p<0.0001$) for all studied marks (Table 2).

The variation in the mean values is consistent with the results obtained by Mladenova et al. (2019) for *Micromeria frivaldszkyana* and *Clinopodium vulgare*: *Micromeria dalmatica* shows a greater number and larger stomata compared to *Clinopodium vulgare*. The epidermal cells of *Micromeria dalmatica* are more numerous and smaller than those of *Clinopodium vulgare*. The thickness of the lower cuticle in *Micromeria dalmatica* is statistically significantly greater compared to the cuticle of *Clinopodium vulgare*.

The identified differences between the traits of the lower epidermis are graphically presented in Fig. 9.

Table 2. Comparison of the mean values of anatomical characteristics in *Micromeria dalmatica* and *Clinopodium vulgare* for abaxial epidermis.

Feature	*Micromeria dalmatica* Mean±SD	*Clinopodium vulgare* Mean±SD	Independent-Samples T-test
Stomata (Number)	170.2±29.3	100.9±24.6	p<0.0001
Stomata (Length, µm)	5.6±0.1	5.04±0.3	p<0.0001
Stomata (Width, µm)	3.9±0.2	2.8±0.2	p<0.0001
Epidermal cells (Number)	1180.9±136.3	696.1±42.7	p<0.0001
Epidermal cells (Length, µm)	8.5±1.2	18±1.8	p<0.0001
Epidermal cells (Width, µm)	3.3±0.3	5.8±0.44	p<0.0001
Cuticle (Thickness, µm)	2.3±1.8	0.9±0.2	p<0.0001

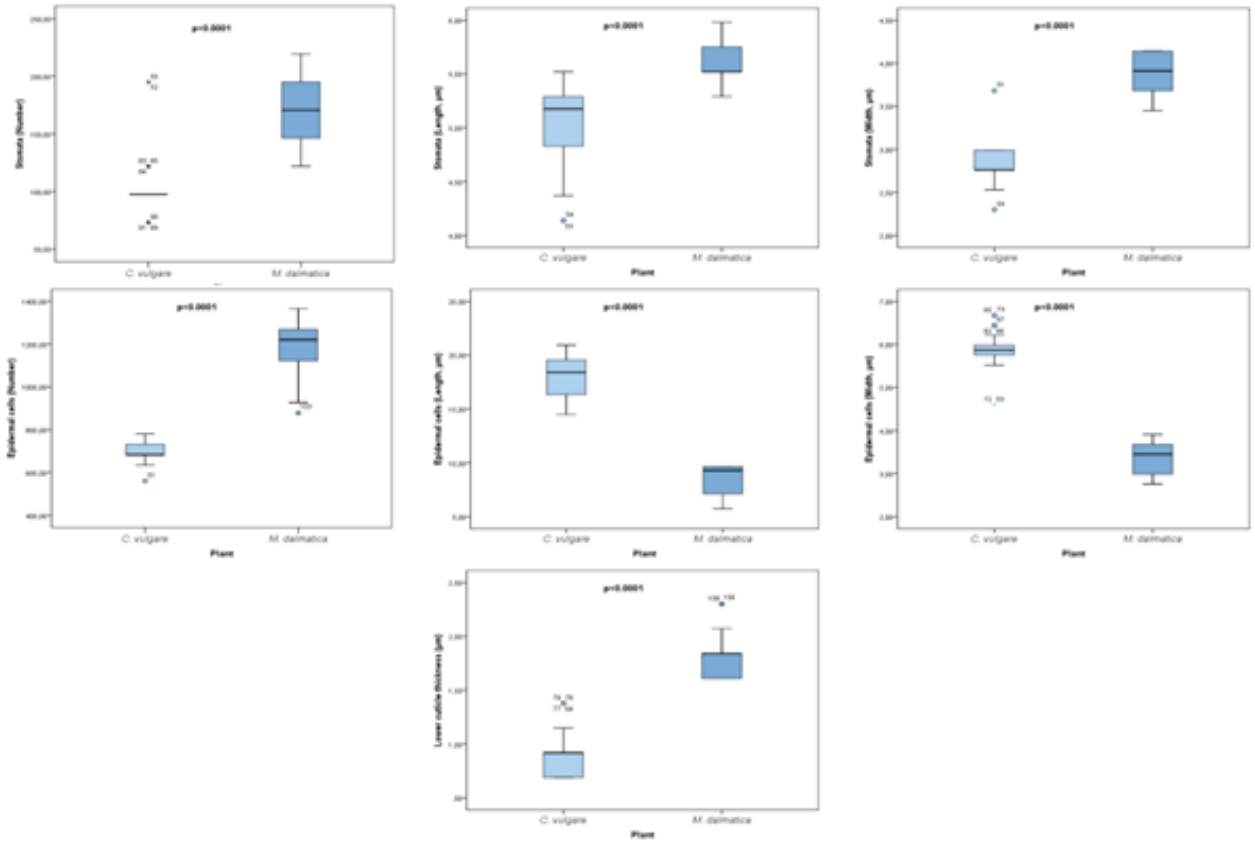

Fig. 9. Significant differences between the mean values of the leaf indicators for abaxial epidermis of *Micromeria dalmatica* and *Clinopodium vulgare*.

DNA barcoding is a molecular technique that employs a short, standardized region of genetic material to differentiate and identify species. This method enables rapid and accurate species identification, thereby facilitating biodiversity monitoring, ecological studies, and the detection of cryptic or invasive species (Wilson et al., 2019). For the molecular comparison of *Micromeria dalmatica* to the existing sequences of genus *Clinopodium*, two DNA regions were amplified: the ITS1 region encoded in plant cell chromosomes and the tRNALeu spacer region encoded in chloroplasts. The DNA sequences obtained were blasted against the NCBI database using the blastx algorithm, and a dataset of representative sequences was selected and downloaded for further local analysis. The sequences for *Micromeria dalmatica* ITS1 and tRNALeu regions obtained in this study were then aligned to the local dataset using the MAUVE algorithm embedded in MEGA 12 software using default software settings. The alignment data were subsequently used for phylogenetic analysis using the Maximum Parsimony method with a consistency index of 0.636175, the retention index of 0.812433, and the composite index of 0.516849 for all sites and parsimony-informative sites. The Maximum parsimony tree was constructed using the Subtree-Pruning-Regrafting algorithm. The evolutionary analyses were conducted using MEGA 12.

The analysis of the ITS1-based phylogenetic tree, illustrated in Fig. 10, shows a scattered distribution of *Micromeria* species in subclades along with *Clinopodium, Bystropogon, Monarda*, and *Mentha* species, for instance. In this case, *M. dalmatica* was clustered together with *C. vulgare* and *C. chynense*. On the tRNALeu-based phylogenetic tree, *M. dalmatica* is clustered together with members of the genus *Clinopodium*, while other members of the genus are in the same cluster with *Mentha* sp., *Monarda* sp., and *Glechon* sp. (Fig. 11). The higher consistency of the clustering on trnl-F based tree can be explained by the higher degree of sequence similarity of chloroplast genomes, but also may imply the evolutionary origin of *Micromeria* species. In this regard, its chloroplast

markers should be considered less reliable for species discrimination within the group.

As it is illustrated in Fig. 10, the ITS1-based phylogenetic tree provides better possibilities for M*icromeria* species determination. However, the sequence distances among the *Micromeria* genus are similar to the distance between *Micromeria* and *Clinopodium*. This finding is by the previous molecular studies of the *Micromeria* genus and supports classification revisions made earlier.

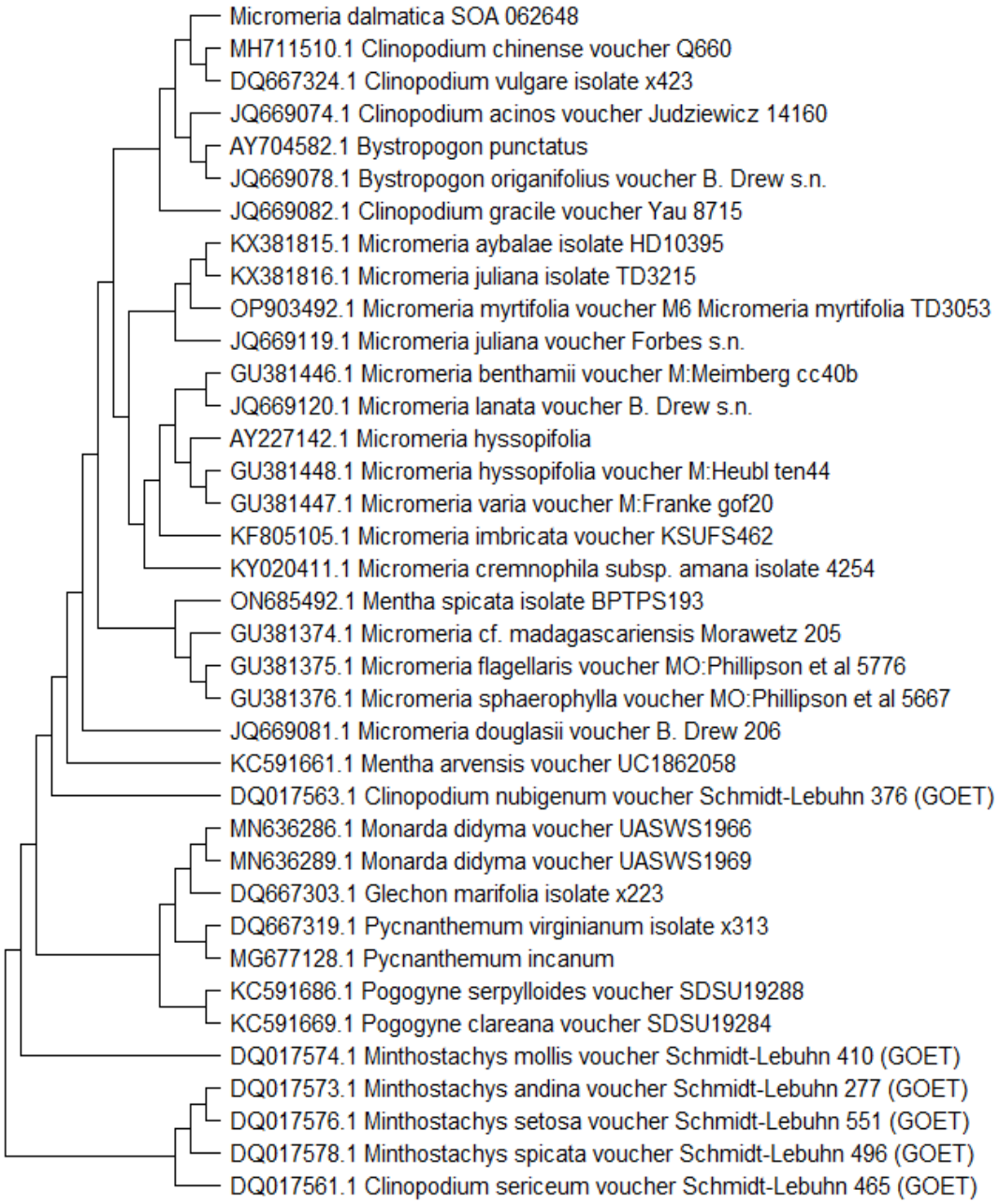

Fig. 10. Maximum parsimony tree of *Micromeria* and related genera, based on ITS1 data.

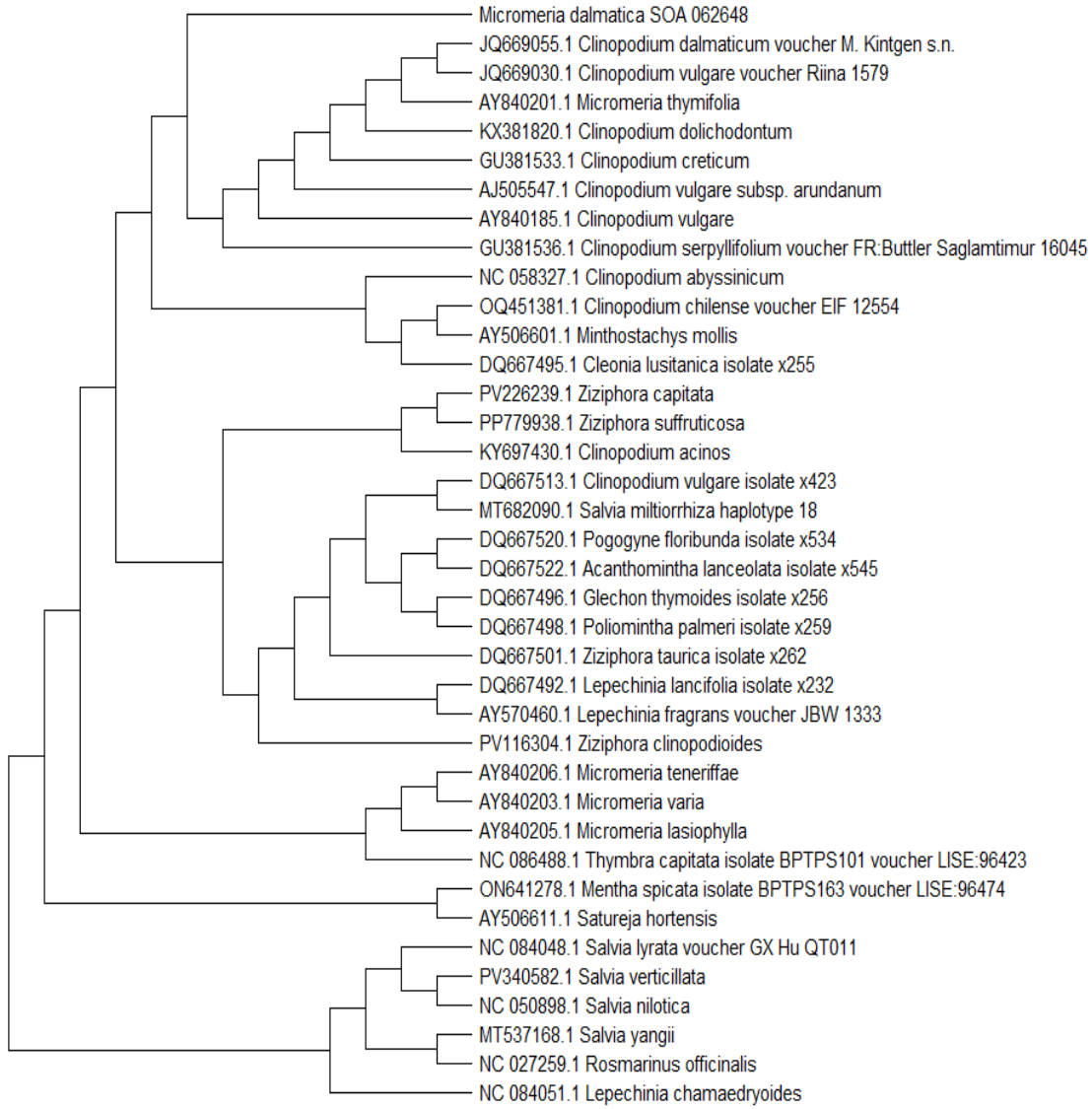

Fig. 11. Maximum parsimony tree of *Micromeria* and related genera, based on tRNALeu data.

Conclusions

The conducted study established a high level of identity between the sequences of *Micromeria dalmatica* and *Clinopodium vulgare*. The comparative anatomical analysis of the stomatal apparatus traits in *Micromeria dalmatica* and *Clinopodium vulgare* demonstrated that these traits have no taxonomic value for distinguishing the species. Both species are characterized by an identical type of stomatal apparatus and trichome type. The statistically significant differences found in the quantitative traits of the stomata on the upper and lower epidermis, the main epidermal cells on both epidermises, and the thickness of the upper and lower cuticle cannot serve as criteria for differentiating the two species. To clarify the phylogenetic relationships between *Micromeria dalmatica* and *Clinopodium vulgare* and to make well-founded taxonomic decisions, further karyological and morphological analyses are necessary.

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