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Morphological and chemical evidence of *Teucrium* × *rohlenae* K. Malý (Lamiaceae), a new hybrid in Croatia

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Abstract – Several natural hybrids of *Teucrium montanum* L. and *T. polium* L. have been described in certain parts of the area where these two species are sympatric. A new population with intermediate individuals that share morphological characteristics of both *T. montanum* and *T. polium* has been found in Croatia (Bisko near Trilj), and most likely represents a hybridogenous taxon. The aim of this study is to compare the morpho-anatomical and phytochemical characteristics of the two parental species and their putative hybrid, as well as to determine the most important distinguishing characters between these three groups. In order to describe the variability and significance of morpho-anatomical and phytochemical differentiation of the analysed groups several multivariate statistical analysis were conducted (PCA, CDA, DFA, UPGMA-clustering). All analyses confirm the separation of two species, *T. polium* and *T. montanum*, and reveal the intermediate position of the putative hybrid. In this way, data on morphological and anatomical analysis together with data on the composition of the essential oils support the assumption of the hybridogenous origin of the intermediate individuals. Based on descriptions and original indication of parental species of previously described hybrids *T.* × *castrense* Verg., *T.* × *bogoutdinovae* Melinkov and *T.* × *rohlenae* K.Malý, we found that the intermediate specimens collected in Croatia perfectly correspond to *T.* × *rohlenae*, a hybridogenous taxon discovered by Rohlena in the vicinity of Kotor in Montenegro, and thus prove the presence of a new natural hybrid in the flora of Croatia.

Keywords: Adriatic flora, anatomy, hybridization, morphology, phytochemistry, *Teucrium*

Introduction

The genus *Teucrium* L. is represented by approximately 250 species whose centre of the diversity is in the Mediterranean region (Harley et al. 2004). The genus includes shrubs, subshrubs, and herbaceous perennials, rarely annual or biennial plants, mostly growing on open exposed rocky grounds, rock crevices and screes. Based on the characteristics of the calyx and life forms the genus *Teucrium* is separated into seven sections. The section *Polium* (Mill.) Schreb. comprises 19 species, where *T. montanum* L. and *T. polium* L. belong to the same phylogenetic lineage (Tutin and Wood 1972, Salmaki et al. 2016). As a result of the wide distribution of these two species, chromosome numbers vary greatly within this area. *T. montanum* is diploid or tetraploid with chromosome numbers 2n = 13, 22, 26, 26+0–

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2B, 26+0–7B, 30, 60. Chromosome numbers for *T. polium* are 2n = 26, 26+3–4B, 52, 78, and it can be found as diploid, tetraploid and hexaploid (Ranjbar et al. 2018).

These two taxa are semi-woody, evergreen small shrubs. *T. polium* is erect up to 45 cm high, whereas *T. montanum* is usually prostrate, growing up to 30 cm. The most prominent morphological differences of this species are observed in the indumentum and leaf shape. *Teucrium polium* is distinguished by a very dense indumentum composed of glandular and non-glandular multicellular, uniseriate, branched hairs, whereas *T. montanum* has a less dense indumentum composed of glandular and non-glandular multicellular, uniseriate and unbranched hairs (Jurišić Grubešić et al. 2007, Lakušić et al. 2010). *Teucrium polium* is characterised by a dense, hairy calyx, in contrast to the calyx of *T. montanum* whose calyx has fewer hairs. The calyx teeth of *T. polium* are moderately obtuse, while *T. montanum* has acute often setaceous teeth (Maurer 1967, Diklić 1974).

These two species are found in sympatry in certain parts of their distribution, with several hybridogenous taxa being described as: *T.* × *castrense* Verg., *T.* × *bogoutdinovae* Melinkov and *T.* × *rohlenae* K.Malý. The hybridogenous taxon between *T. montanum* and *T. polium* first described was collected in July 1907 and June 1908 near the town of Castres in south France by Verguin (Verguin 1908). Verguin named that plant *T.* × *castrense*, with *T. polium* ssp. *polium* Briquet and *T. montanum* L. as supposed parental species. Fifty years later, Maurer found individuals with morphological characteristics intermediate between the species *T. montanum* and *T. polium* on dunes near Lignano, North Italy (Maurer 1967). This was the first finding of *T.* × *castrense* in Italy (Maurer 1967). Another hybridogenous taxon has been described by Rohlena in the area near Kotor in Montenegro (Rohlena 1922). However, according to the Shenzhen Code and Art. H.2.1. (Turland et al. 2018) it was invalidly described as *T. montanum* × *polium* (and Malý validly renamed it as *T.* × *rohlenae* (Malý 1950). Subsequently, *T.* × *bogoutdinovae* from Moldova was described (Melnikov 2014). The species *T. reuticum* Bogoutdinova and *T. polium* were listed as parents for *T.* × *bogoutdinove* which was described on a rocky terrace of the River Reut (Bogoutdinova 1991). Given that *T. reuticum* is considered a heterotypic synonym of *T. montanum* (Eur+Med 2006), *T.* × *bogoutdinove* can also be treated as a hybrid between *T. montanum* and *T. polium*.

Fig. 1. Known occurrences of *Teucrium* × *rohlenae* K.Malý based on literature data (A) and recently discovered populations (B, C). A – Kotor in Montenegro, B – Matokit, and C – Trilj, both in Croatia.

We conducted several field trips, during June 2018 in Bisko near Trilj in Croatia (Fig. 1), where *T. montanum* and *T. polium* grow sympatrically. There we found individuals with morphological characteristics intermediate between these two species, which a represent hybridogenous taxon.

The aim of our study is to compare morpho-anatomical and phytochemical characteristics of parents and their putative hybrid and to determine the most important characters of differentiation.

Materials and methods

Because of the small population sizes of *T. montanum* and the putative hybrid in their narrow hybridization zone (Bisko near Trilj, Croatia), and their presumed endangered status, destructive sampling was limited to a minimum, which resulted in a relatively limited number of analyzed individuals. A total of 29 specimens were selected and scored for analyses. Those include 10 specimens of *T. polium*, 13 specimens of *T. montanum* and six specimens of their putative hybrid. Specimens were deposited in the Herbarium of the Institute of Botany and Botanical Garden of the Faculty of Biology, University of Belgrade (BEOU – herbarium code follows Thiers 2019).

Analysed samples are as follows:

T. montanum

- CROATIA, Trilj, Bisko (43.579400° N, 16.695995° E), pseudomacchia, leg.: D. Lakušić, B. Lakušić, M. Zbiljić, 30.06.2018, (BEOU – 54028)

T. polium ssp. *capitatum*

- CROATIA, Trilj, Bisko (43.579400° N, 16.695995° E), pseudomacchia, leg.: D. Lakušić, B. Lakušić, M. Zbiljić, 30.06.2018, (BEOU – 54027)

Putative hybrid "*T. montanum* × *polium*"

- CROATIA, Trilj, Bisko (43.579400° N, 16.695995° E), pseudomacchia, leg.: D. Lakušić, B. Lakušić, M. Zbiljić, 30.06.2018, (*Teucrium* × *rohlenae* K. Malý, BEOU – 54029)

– CROATIA, Dalmatinska Zagora, Vrgorac, Mt. Matokit, Zavojane, Zekulić-Roč (43.244743° N, 17.264857° E), rocky ground pasture, leg.: M. Vukojević, 13.06.2015, (sub. *T. montanum* L., ZA-GR – 40074).

For morphological analysis samples of stem with leaves (without terminal inflorescence), leaves, bracts, calyx and flowers from terminal inflorescences were stored in solution of glycerol and ethanol (1:1). Anatomical sections of leaves were preserved on permanent slides, prepared by the standard method for light microscopy. Cross-sections of leaves were cut on a Reichert sliding microtome (up to 15 µm thick). The sections were stained with safranin (1%, w/v, in 50% ethanol) and alcian blue (1%, w/v, aqueous). All slides were mounted in Canada balsam after dehydration (Lakušić et al. 2010). Morphometric analysis included 21 morphological and 23 anatomical characters.

Plant material (3–4 g) was extracted with *n*-hexane (1:10) for 24 hours at room temperature. The extraction was repeated, and the extracts were united and concentrated under the reduced pressure.

The qualitative and quantitative analysis of hexane extracts was done using the GC and the GC/MS method. The GC analysis was performed on an Agilent 6890N GC system with a 5975 MSD and FID detectors. The column was HP-5 MS (30 m × 0.25 mm, 0.25 µm film thickness). Two µL were injected and the injector temperature was 200 °C with a 10:1 split ratio. Helium was used as a carrier gas (1.0 mL min^{-1}, constant flow mode). The column temperature was linearly programmed (60–280 °C, rate 3 °C min^{-1}, 280 °C for 5 min). The transfer line was heated at 250 °C and the FID detector at 300 °C. EI mass spectra (70 eV) were obtained in the m/z range of 35–550. The retention indices (Kovat's retention index, RI) of essential oil components were experimentally determined relative to two series of *n*-alkanes (C_8-C_{20} and C_{21}-C_{40}). Their spectra were obtained under the same chromatographic conditions. Identification of compounds was based on comparison of their retention indices (RI) and mass spectra with those from authentic samples and/or the NIST AMDIS software, Wiley, the Adams database and available literature (Adams 2007). Relative percentages of the identified compounds were computed on the basis of the peak areas obtained by FID detector.

The chemical analysis was performed on all compounds (28) of hexane extracts or only on terpene (17) compounds.

In order to describe the variability and significance of morphoanatomical and phytochemical differentiation of analysed groups Principal component analyses (PCA) and Canonical discriminant analysis (CDA) were used, as well as an UPGMA (unweighted pair group method with arithmetic mean) clustering analysis based on Mahalanobis distances for mopho-anatomical data and Pearson's distances for chemical data in the UPGMA clustering method. Discriminant function analysis (DFA) was used to estimate the contribution of individual characters to the overall discrimination. Statistical analyses were performed using Statistica v.8.0 (StatSoft 2007).

Results

Multivariate analysis of the morpho-anatomical characters

Principal component analysis (PCA) and Canonical discriminant analysis (CDA) performed on morpho-anatomical characters revealed clear separation of *T. polium* and *T. montanum,* as well as clear intermediate position of the putative hybrid (Fig. 2A,B). Both analyses revealed a clear separation of three groups along the x-axis, whereas CDA revealed clear separation of putative hybrid along both discriminant axes.

Cluster analysis performed on all morpho-anatomical characters classified *T. polium* and the putative hybrid in the first cluster, while *T. montanum* belongs to the second cluster (Fig. 3A). On the other hand, the cluster analysis based on leaf anatomy revealed the hybrid individuals are closer to *T. montanum* (Fig. 3B).

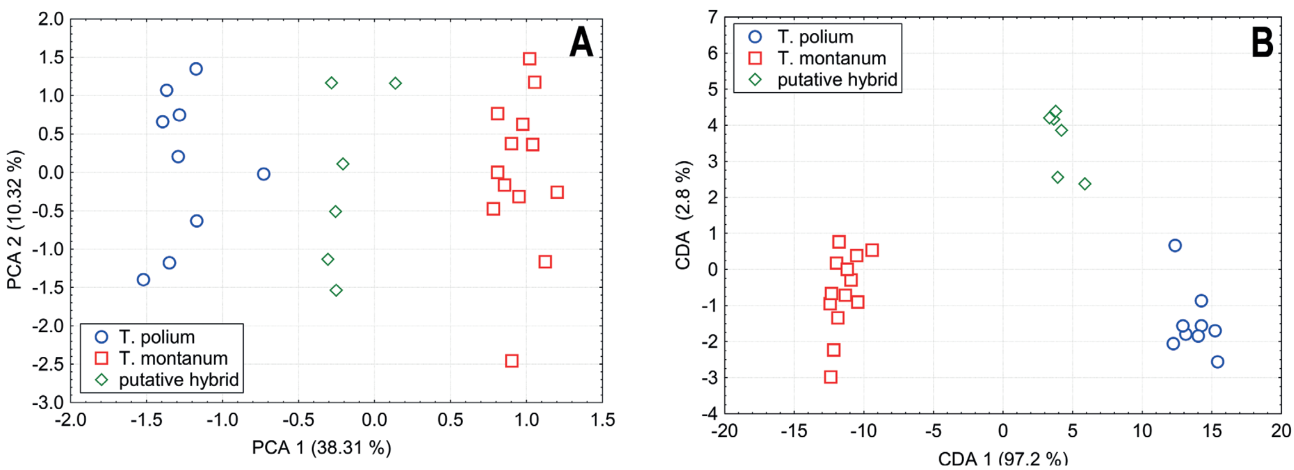

Fig. 2. Multivariate analysis of the morpho-anatomical data for the *Teucrium montanum*, *T. polium* and the putative hybrid in their narrow hybridization zone in Bisko near Trilj (Croatia). A – Principal component analysis (PCA), B – Canonical discriminant analysis (CDA).

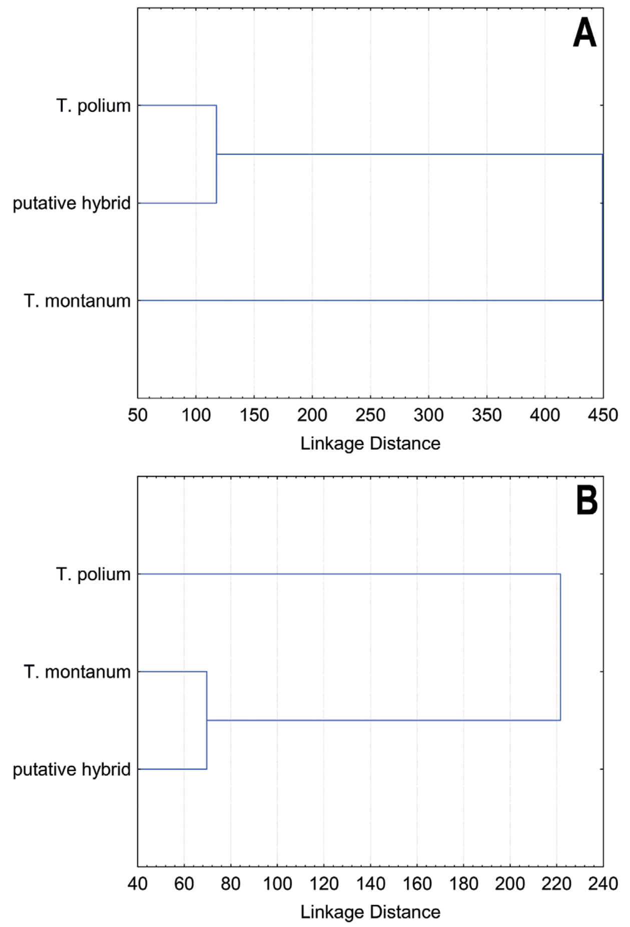

Fig. 3. Cluster (UPGMA) analysis of the morpho-anatomical data for the *Teucrium montanum*, *T. polium* and putative hybrid in their narrow hybridization zone in Bisko near Trilj (Croatia). A – for all morpho-anatomical characters, B – only for the anatomical characters.

Characters that predominantly contribute to the distinction among the groups are: coverage of adaxial indumentum, thickness of cuticle, number of teeth on the edge of a leaf, bract length, stem length, frequency of lateral tuft on stem nodes, distance between calyx base and tooth base, length of the narrow part of the calyx teeth and number of terminal inflorescences (Tab. 1).

Chemical analysis of volatile compounds

The chemical analysis of the aerial parts' volatile compounds revealed that total hydrocarbons (saturated and unsaturated) were the main compounds in all investigated samples (82.9–90.7%). Sesquiterpene hydrocarbons and oxygenated sesquiterpenes were present in amounts that did not exceed 10.2% and monoterpene hydrocarbons only about 1% (Tab. 2).

The chemical compositions of the extracts of the studied groups were quite similar. Saturated and unsaturated hydrocarbons represented the main compounds (33.2–75.0% and 14.1–49.6%). Saturated hydrocarbons containing 25, 29 and 31 C atoms, pentacosane (33.6% and 46.5%), nonacosane (9.2–17.5), untriacontane (0.2–12.3%) as well as unsaturated untriacontene (11.4–48.4%) were dominant.

The cluster analysis of all compounds of hexane extracts (Fig. 4A) revealed a similarity in the composition of the volatiles of the aerial parts of *T. polium* and the putative hybrids. The extracts of *T. polium* and the putative hybrid were characterised by a pentacosane (46.5 and 33.6%), compound that was not present in the *T. montanum* volatiles. Unsaturated untriacontene (48.4%) was the main compound in the *T. montanum* extract.

Tab. 1. Discriminant functional analysis summary of the 18 most important morpho-anatomical characters for 29 specimens of *Teucrium montanum*, *T. polium* and the putative hybrid in their narrow hybridization zone in Bisko near Trilj (Croatia). Invariable and highly correlated characters were excluded from analysis.

Anatomy	Wilks' Lambda	Partial Lambda	F-remove (2.8)	P-level
Radius of the central nerve	0.001401	0.990041	0.040235	0.96076
Coverage of adaxial indumentum	0.001605	0.864346	0.627779	0.55815
Number of capitate hairs C	0.001599	0.867574	0.610556	0.56654
Thickness of adaxial epidermal cells	0.001537	0.90247	0.432283	0.66333
Thickness of cuticula	0.001675	0.828184	0.829843	0.47044
Morphology				
Leaf surface	0.002156	0.643504	2.215968	0.17148
Leaf base width	0.001557	0.890808	0.490304	0.62971
Number of teeth on edge of leaf	0.00197	0.704347	1.679023	0.24612
Bract length	0.001626	0.853201	0.688230	0.52991
Average width of bract	0.001747	0.794168	1.036716	0.39779
Stem length	0.001829	0.758577	1.273031	0.33113
Frequency of lateral tuft on stem's nodes	0.001786	0.776835	1.149100	0.36418
Average length of first three internodes	0.002502	0.554452	3.214335	0.09451
Distance between calyx base and tooth base	0.001631	0.850866	0.701094	0.52414
Length of narrow part of tooth	0.001512	0.917384	0.360227	0.70828
Width of tooth	0.001532	0.905726	0.416345	0.67296
Number of flowers in terminal inflorescence	0.001944	0.71378	1.603966	0.25957
Number of terminal inflorescences	0.001763	0.787023	1.082446	0.38366

Tab. 2. The chemical composition (%) of the volatiles of *Teucrium montanum*, *T. polium* and the putative hybrid in their narrow hybridization zone in Bisko near Trilj (Croatia). RI – retention index, t – trace (< 0.1%).

Compund	RI	*T. montanum*	putative hybrid	*T. polium*
alpha-Thujene	922	0.39	t	t
alpha-Pinene	929	0.18	0.29	t
Sabinene	968	0.40	0.50	t
beta-Pinene	972	0.13	0.16	0.18
beta-Bourbonene	1381	0.49	0.25	t
(E)-Caryophyllene	1416	t	0.97	2.32
(Z)-beta-Farnesene	1439	0.90	0.00	0.00
alpha-Humulene	1450	0.00	t	0.18
(E)-beta-Farnesene	1452	1.01	0.00	t
Germacrene D	1478	3.74	2.46	1.42
Bicyclogermacrene	1493	0.36	0.30	0.20
alfa-Bisabolene	1498	0.30	t	0.00
beta-Bisabolene	1505	t	0.00	0.27
cis-Sesquisabinene hydrate	1540	1.81	0.00	0.00
trans-Sesquisabinene hydrate	1576	0.48	0.00	0.00
epi-alpha-Cadinol	1637	0.51	0.00	0.00
Hexahydrofarnesyl acetone	1838	0.62	t	0.30
Tetracosane	2397	t	t	0.10
Pentacosene	2489	1.22	2.93	2.74
Pentacosane	2509	0.00	33.56	46.53
Hexacosane	2589	0.31	0.26	0.34
Heptacosane	2689	3.93	2.88	2.61
Octacosane	2789	0.93	0.57	0.70
Nonacosane	2890	17.45	13.43	9.20
Triacontane	2990	3.12	1.47	1.09
Untriacontene	3092	48.43	20.61	11.40
Untriacontane	3125	0.16	11.94	12.32
Dotriacontane	3187	7.31	3.09	2.13
Percentage of identified compounds		94.18	95.67	94.12
Monoterpene hydrocarbons		1.10	0.95	0.18
Sesquiterpene hydrocarbons		6.80	3.98	4.48
Oxygenated sesquiterpenes		3.42	0.00	0.30
Saturated hydrocarbons		33.21	67.20	75.02
Unsaturated hydrocarbons		49.65	23.54	14.14

On the other hand different results were obtained when only terpene compounds were used for cluster analysis (Fig. 4B). There was a certain similarity between the composition of monoterpenes and sesquiterpenes in extracts of *T. montanum* and the putative hybrid, mainly represented by higher germacrene D content (3.7% and 2.5%).

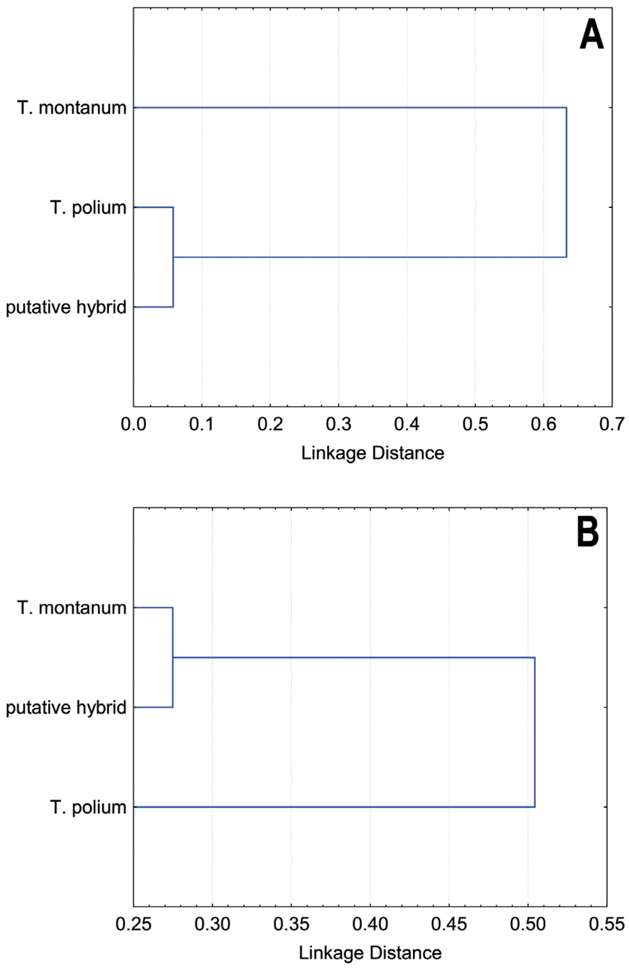

Fig. 4. Cluster (UPGMA) analysis of the chemical data of volatile compounds for the *Teucrium montanum*, *T. polium* and putative hybrid in their narrow hybridization zone in Bisko near Trilj (Croatia). A – for all compounds of hexane extracts (28), B – only for the terpene compounds (17).

Discussion

Intermediate position of putative hybrid

According to the leaf shape and the edge of a leaf with one or more teeth, majority of the hybrid individuals resemble *T. polium* (Fig. 5). Indumentum of the leaf of the hybrid is less dense than *T. polium* leaf indumentum, but more dense than indumentum of leaf *T. montanum*. In addition, non-glandular uniseriate branched hairs that dominate in *T. polium* and are absent in *T. montanum* are present in the putative hybrid. At the same time, the hybrid individuals contain many of the non-glandular uniseriate unbranched trichomes present in *T. montanum* but absent in *T. polium* (Jurišić Grubešić et al. 2007, Lakušić et al. 2010, Fig. 5).

Also, some hybrid individuals have short dense tufts of lateral shoots on the stem nodes, features typical for *T. polium*, but absent in *T. montanum* (Fig. 5).

Important indicators of the transition in morphological characters of the putative hybrid are the colour and number of flowers and the consistency of the inflorescences. *Teucrium polium* has a compact inflorescence with a large number

Fig. 5. Morphological characteristic of inflorescence, leaves and calyx and anatomical characteristic of leaf cross section of *Teucrium montanum* (m), putative hybrid (×) and *T. polium* (p) in their narrow hybridization zone in Bisko near Trilj (Croatia). 1 – inflorescence, 2 – leaves, 3 – abaxial leaf side, 4 – adaxial leaf side, 5 – calyx, 6 – leaf cross section: m6 – only non-glandular uniseriate unbranched trichomes in *T. montanum*, ×6 – both branched and uniseriate nonglandular trichomes in putative hybrid, p6 – only uniseriate branched nonglandular trichomes on both side of leaf in *T. polium*.

of small white flowers (between 13 and 51, on average 30); *T. montanum* has an a lax inflorescence with a small number of large pale yellow flowers (between 6 and 16, on average 11), and the hybrid individuals has compact inflorescence with a small number of white flowers (between 7 and 20, on average 13) (Fig. 5). Calyx morphology clearly shows intermediate forms. In terms of calyx dimensions (calyx tube length, calyx tooth length and width) the putative hybrids are between the parents: smaller than *T. montanum* and larger than *T. polium*. Additionally, the putative hybrid possesses setae on the top of the acutiform setaceous calyx teeth that are shorter than the setae of *T. montanum,* while setae are missing in *T. polium* (Fig. 5).

Finally, and in its general habitus, the putative hybrid has a transitional character. Namely, flowering stems of hybrid individuals are erect , as in *T. polium*, while lateral vegetative shoots are decumbent, as in *T. montanum*.

A previous work showed that *Teucrium* species from the Balkan Peninsula usually contain small quantities of essential oil (Kovačević et al. 2001). Sesquiterpenes were the main compounds in the essential oils of the aerial parts of *T. montanum* and *T. polium*. In the investigated samples from Montenegro germacrene D (15.0%), α-pinene (12.4) and β-eudesmol (10.1%) were the most abundant in *T. montanum,* while β-pinene (19.8%) and germacrene D (11.9%) in the oil of *T. polium* (Kovačević et al. 2001). The essential oil from the aerial parts of *T. montanum* from Croatia contained germacrene D (17.2%), β-pinene (12.3%) and β-caryophyllene (7.1%), while β-caryophyllene (52.0%) was dominant in the oil of *T. polium* (Bezić et al. 2011). By contrast, germacrene D (31%) was dominant in the essential oil of *T. polium* from Serbia, while δ-cadinene (8.1%) and β-caryophyllene (5.1%) were the main compounds in *T. montanum* (Radulović et al. 2012). Furthermore, the most recent study of the variability of essential oil of different populations of *T. montanum* from the central and south Balkan Peninsula (14 populations from Serbia, Greece and Albania), revealed extremely large differences in the chemical composition of the essential oils of the aerial parts of *T. montanum* (Marčetić et al. 2018). This study showed that the composition of essential oils was quite variable but the main compounds in almost all oils were germacrene D (trace-45.5%), sabinene (trace-23.1%), α-pinene (trace-20.7%), limonene (trace-20.4%), (*E*)-caryophyllene (2.9–14.5%), γ-cadinene (trace-13.8%) and δ-cadinene (trace-12.0%).

These results show that the composition of the essential oils of the two species is not clearly different, given that they contain similar compounds and similar pattern of the variability. It indicates that it is not possible to distinguish them on the basis of the chemical composition of essential oils alone, which is in accordance with the results of several recent studies that showed that variation in the composition of essential oils within a species appears to be the rule rather than the exception, and that the geographical distribution of different types of essential oils of plants is strongly corelated with the environmental conditions including climate, geological, pedological and phytosociological characteristics of the habitats (Kuštrak et al. 1984, Franz 1993, Jug-Dujaković et al. 2012, Lakušić et al. 2012).

However, together with data of morphological and anatomical analysis, the data of the composition of the analysed essential oils also support the assumption of the hybridogenous origin of intermediate individuals.

Taxonomical treatment and distribution

Brief descriptions of hybridogenous taxa *T.* × *castrense* and *T.* × *rohlenae* largely overlap with features of collected hybrid individuals (Verguin 1908, Rohlena 1922, Malý 1950, Maurer 1967). Considering that the subspecies *T. polium* ssp. *polium,* one parent of *T.* × *castrense,* is distributed only in the western parts of the Mediterranean (Tutin and Wood 1972, Euro+Med 2006) we have excluded the possibility that hybridogenic individuals from Croatia belong to these taxa.

At the same time, given that *T. polium* ssp. *capitatum* (L.) Archangeli and *T. polium* ssp. *vincentinum* (Rouy) D.Wood are distributed in Croatia (Nikolić 2019), and that the plant from Trilj belongs to the subspecies *T. polium* ssp. *capitatum* also distributed in Montenegro (Euro+Med 2006) we suggest the hybrid be included in taxon *T.* × *rohlenae*, originally found and described in area near Kotor in Montenegro (Rohlena 1922, Malý 1950).

We have analysed ZAGR herbarium specimens of *T. polium* and *T. montanum* collected in Croatia, and we found specimens that perfectly match the appearance of the hybrid *T.* × *rohlenae*. These specimens were determined as *T. montanum* and were collected in 2015 on Mt Matokit near Vrgorac in Dalmatinska zagora.

Even though *T.* × *rohlenae* has been registered only in three locations (Montenegro: Kotor, Croatia: Trilj and Matokit, Fig. 1), it is likely that its distribution is much wider, so we can expect hybrid individuals in areas where *T. montanum* and *T. polium* grow sympatrically.

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