Morphological Analysis of Short-Tooth Sage (*Salvia brachyodon* Vandas) Populations

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Summary

Short-tooth sage (*Salvia brachyodon* Vandas) is an endemic and one of the rarest plant species of the Dinaric karst. Although older literature indicates its presence in more localities, only two have been confirmed at the present time, Mt. Orjen (border of Bosnia and Herzegovina and Montenegro) and Pelješac peninsula (Croatia). Due to its very narrow distribution and economically very interesting essential oil composition short-tooth sage might become more endangered in the future. The aim of this research was to determine and compare inter-and intra- population morphological diversity of the only two noted natural populations of short-tooth sage by analyzing 16 quantitative morphological traits. Pelješac population of short-tooth sage showed considerably higher values for traits related to habitus and plant height, while the Mt. Orjen population showed higher values for traits related to the calyx and higher occurrence of sessile glandular hairs on the flower pedicels. When interpreting the obtained results, we should consider the fact that the habitats on Pelješac peninsula and Mt. Orjen greatly differ. In order to get better insight into the diversity and differentiation of short-tooth sage populations and to define efficient protection measures an additional analysis of populations using molecular markers are recommended.

Key words

endemic species, morphological diversity, *Salvia brachyodon,* short-tooth sage

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Introduction

Short-tooth sage (*Salvia brachyodon* Vandas), together with 11 European *Salvia* species, belongs to the section *Salvia* of the genus *Salvia* (Hedge, 1972). Currently only two localities are confirmed (Fig. 1): Mt. Orjen, on the border between Bosnia and Herzegovina and Montenegro (*locus classicus,* Vandas, 1899) and Pelješac peninsula (Croatia). Another two localities (Konavle and Mt. Mosor), known from The Ivo and Marija Horvat Herbarium (ZAHO) collection and from literature (Girometta, 1930) are recorded only once and therefore need additional verification. On the basis of presently known localities, short-tooth sage is one of the rarest plant species of Dinaric karst. Therefore, short-tooth sage is classified as near threatened (NT) and has the endemic status in Croatia (Nikolić, 2014) while in Montenegro, it is classified as an endangered species (EN) (Petrović et al., 2008). Short-tooth sage together with *Erica manipuliflora* Salisb., grows on the highest peak of Pelješac peninsula (St. Ilija) on the shallow soil on dolomitic limestone within Dalmatian black pine (*Pinus nigra* Arnold) forest (Trinajstić, 1986) or along the forest edges, together with *Genista sylvestris* Scop. subsp *dalmatica* (Bartl.) and *Erica manipuliflora* Salisb., assemble Mediterranean garrigues (Horvatić, 1958). On Mt. Orjen short-tooth sage grows also on the shallow soils on dolomitic limestone, but contrary to the Pelješac peninsula, on the edges and clearings of thermophyllous pubescent oak (*Quercus pubescens* Willd.) forest extending to the belt of littoral beech (*Fagus sylvatica* L.) forest. On this location, short-tooth sage can also be an element of rocky pastures and dry grasslands often accompanied by a number of additional endemic and rare species such as *Genista sylvestris* Scop. subsp. *dalmatica* (Bartl.) H.Lindb., *Centaurea glaberrima* Tausch, *Euphrasia dinarica* (Beck) Murb. and *Edraianthus tenuifolius* (Waldst. et Kit.) A.DC. (Abadžić and Šilić, 1982).

Short-tooth sage is a small shrub with relatively large leaves on a long petiole, densely arranged on the base of stem. Leaf blade is simple or often pinnately compound with 3-5 leaflets. Young leaves are densely covered by hairs, developed leaves are mostly glabrous on upper side with white hairs on the lower side, while older leaves are coriaceous and yellowish with prominent veins and less hairy. Pale-lilac-blue flowers covered with glandular hairs form loosely racemes. Flowers are situated on pedicel of different length with numerous glandular hairs. Bracts are of various shapes and sizes, early caducous. The tubular bell-shaped calyx with visible longitudinal veins is approx. 8-10 mm long, covered by glandular hairs. Calyx teeth have a wide triangular shape, tapered at the top. Petals are 3-4 times longer than the calyx. The flowers contain two fertile stamens with short filaments. Both stamens are connected and form a characteristic pollination mechanism for the genus *Salvia*. The pistil consists of two carpels and two stigmas that, together with the stamens, protrude out of corolla tube. One ovary produces four triangular shiny and smooth nutlets (mericarps), more or less dark in colour (Barbalić, 1956). *S. brachyodon* is a diploid species with $2n = 14$ chromosomes (Maksimović et al., 2007). Flowering period is from July to August.

Short-tooth sage leaves contain 1.6% of essential oil. The main compounds are sesquiterpens (67.8%) (Maksimović et al., 2007). In the short-tooth sage population from Mt. Orjen, a high percentage of 1.8-cineole was recorded (36.9%). This compound has an allelopathic effect on other plant species and protects short-tooth sage plants from herbivores. In the Pelješac population, that percentage of 1.8-cineole is considerably lower (1.6%), attributed to different growth conditions. Interestingly, short-tooth sage shows no presence of thujones or only traces of it (Tzakou et al., 2003). That fact bears special significance if we

Figure 1. Short-toth sage at two confirmed localities: Mt. Orjen, on the border between Bosnia and Herzegovina and Montenegro (left) and Pelješac peninusla, Croatia (right).

know for thujons negative side effects in a prolonged medicinal usage of Dalmatian sage (*S. officinalis* L). Knowing the economic value of the essential oil of common sage, short-tooth sage might become very interesting in the future for both gatherers and producers. It is intriguing that during an organized buying up of common sage, the gatherers were selling the short-tooth sage as well, pointing out its more beneficial healing properties than in common sage (Barbalić, 1956).

The preliminary research of the genetic diversity of short-tooth sage from the Pelješac peninsula indicated low genetic diversity of this species as well as the existence of genetic bottleneck in its recent past (Pruša, 2011). The obtained results showed that it is urgent to revise the present threat category for short-tooth sage.

The aim of this research was to determine inter- and intra-population morphological diversity of the only two known natural populations of short-tooth sage.

Material and methods

Plant material was collected in full bloom. The Pelješac samples were collected near the highest peak Sv. Ilija (843 m.a.s.l.; 42° 59' 45'' N, 17° 09' 29'' E) in late August, while the ones from Mt. Orjen on the locality Vrbanj (1024 m.a.s.l.; 42° 34' 12'' N, 18° 28' 29'' E) in mid July. In total 30 individuals were collected per locality. Herbarium voucher specimens are deposited in Herbarium ZAGR (http://herbarium.agr.hr) at the University of Zagreb, Faculty of Agriculture, Zagreb, Croatia (Voucher nos. 33441 and 33444).

A total of 60 herbarium specimens were analyzed using 16 quantitative morphological traits (Table 1). During the determination and measuring of the most of traits Olympus Pro-lux Microscope and Dino-Lite pro Digital Microscope were used. Olympus Pro-lux Microscope was used for determination of the type of hairs on flower pedicels and calices while Dino-Lite pro Digital Microscope was used for measuring of the length of calyx, calyx lobes, calyx tips, and the type of calyx venation (Fig. 2). Traits like length of flower, number of internodes on the inflorescence axis and number of primary branches did not required optical devices (Fig. 3).

The univariate analysis of variance using PROC GLM in SAS (SAS Institute, 2004) was conducted in order to test mean differences between two short-tooth sage populations in 16 quantitative morphological traits.

Principal component analysis (PCA) based on 16 traits was performed using PROC PRINCOMP in SAS. The biplot was constructed by two principal components showing analysed individuals and traits.

A discriminant analysis using PROC STEPDISC, PROC DISCRIM and PROC CANDISC in SAS was performed to evaluate the utility and importance of 16 quantitative morphological traits by determining which were most useful in maximally discriminating populations. A stepwise discriminant analysis (PROC STEPDISC) was performed in order to select a subset of traits for use in discriminating between populations. Chosen subset of morphological traits was evaluated for the performance as discriminant criterions (PROC DISCRIM) for correct classification of plants into their respective species by estimating the

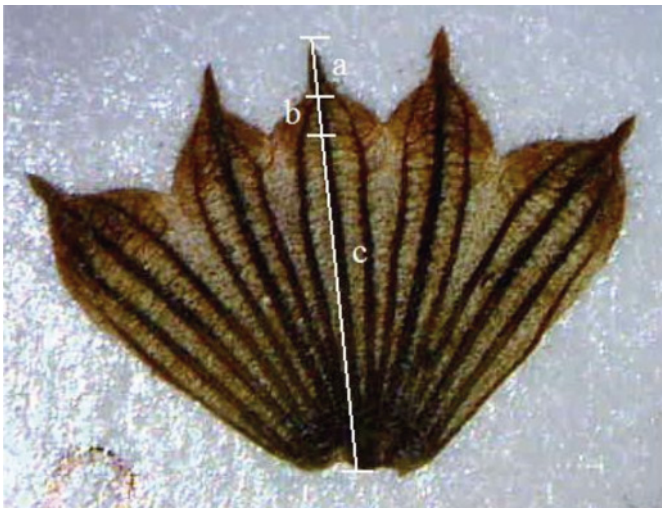

Figure 2. Measured morphological traits of short-toth sage calyx: a) length of calyx tips, b) length of calyx lobes, c) length of calyx.

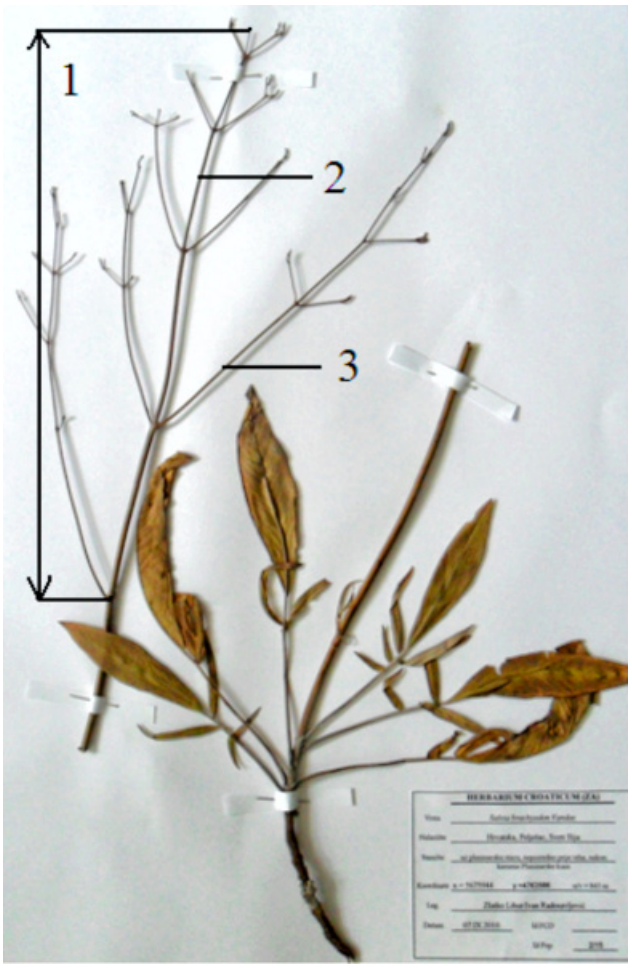

Figure 3. Measured morphological traits of short-tooth sage inflorescence: 1 - length of inflorescence, 2 - number of internodes, 3 – number of primary branches.

probabilities of misclassification with cross validation. In order to establish a minimal set of traits needed for the correct classification, the traits were re-evaluated for the performance as discriminant criterions in order from most important to least important. A canonical discriminant analysis (PROC CANDISC) was performed based on the minimal set of traits that differentiated best between populations and the first two canonical variables were plotted.

Results and discussion

The analysis of 16 morphological traits revealed differences between plants that could be related to their population affiliation. Eight out of 16 quantitative morphological traits were found significant between populations at $P < 0.05$ (Table 1). Pelješac population exhibited significantly higher values than Orjen population in traits related to inflorescence (S01 Length of inflorescence; S02 Number of internodes; S03 Number of primary branches) while the Orjen population has significantly higher values in traits related to the presence of some types of trichomes on flower pedicel (S06 Trichome type: Long capitulate glandular; S07 Trichome type: Sessile glandular) and calyx (S11 Trichome type: Stalked glandular) as well as in traits related to calyx (S12 Length of calyx; S14 Length of calyx tips).

Table 1. Mean values and ranges of 16 quantitative morphological traits in two short-tooth sage populations (Pelješac, Orjen)

No.	Plant organ	Trait (unit)	*P*(*F*)	Pelješac		Orjen	
				Mean	Range	Mean	Range
S01	Inflorescence	Length of inflorescence (cm)	***	60.52	19.4-94.0	36.77	14.8-66.9
S02	Inflorescence axis	Number of internodes	***	10.17	2.0-17.0	7.30	4.0-11.0
S03	Inflorescence axis	Number of primary branches	***	20.33	4.0-32.0	14.50	8.0-22.0
S04	Flower pedicel	Trichome type: Patent eglandular (0/1)	ns	0.98	0.7-1.0	0.99	0.8-1.0
S05	Flower pedicel	Trichome type: Short glandular (0/1)	ns	0.98	0.5-1.0	0.95	0.4-1.0
S06	Flower pedicel	Trichome type: Long capitulate glandular (0/1)	*	0.28	0.0-1.0	0.52	0.0-1.0
S07	Flower pedicel	Trichome type: Sessile glandular (0/1)	***	0.66	0.0-1.0	0.96	0.6-1.0
S08	Flower pedicel	Trichome type: Stalked glandular (0/1)	ns	0.02	0.0-0.3	0.06	0.0-0.4
S09	Calyx	Trichome type: Patent eglandular (0/1)	ns	0.99	0.8-1.0	1.00	1.0-1.0
S10	Calyx	Trichome type: Long capitulate glandular (0/1)	ns	0.97	0.8-1.0	0.99	0.8-1.0
S11	Calyx	Trichome type: Stalked glandular (0/1)	*	0.79	0.0-1.0	0.92	0.4-1.0
S12	Calyx	Length of calyx (mm)	**	8.44	7.2-10.5	9.05	7.5-10.3
S13	Calyx	Length of calyx lobes (mm)	ns	0.84	0.3-1.7	0.95	0.7-1.3
S14	Calyx	Length of calyx tips (mm)	***	0.48	0.2-0.8	0.91	0.6-1.3
S15	Calyx	Calyx venation: parallel with slight anastomosing (0/1)	ns	0.48	0.0-1.0	0.53	0.0-1.0
S16	Calyx	Calyx venation: parallel with very prominent anastomosing (0/1)	ns	0.52	0.0-1.0	0.47	0.0-1.0

P(*F*), significance of ANOVA's *F*-test: *** significant at $P < 0.001$, ** significant at $0.001 < P < 0.01$, * significant at $0.01 < P < 0.05$,.
ns depicts non-significant values ($P > 0.05$)

Table 2. Pearson's correlation coefficients between 16 quantitative morphological traits and scores of the first three principal components. Trait numbers are indicated in Table 1

No. trait	Principal components					
	PC1		PC2		PC3	
S01	0.847	***	0.183	ns	0.054	ns
S02	0.887	***	0.149	ns	0.150	ns
S03	0.894	***	0.153	ns	0.144	ns
S04	-0.127	ns	-0.184	ns	-0.623	***
S05	-0.060	ns	-0.205	ns	0.477	***
S06	-0.497	***	-0.194	ns	0.503	***
S07	-0.438	***	-0.248	ns	-0.070	ns
S08	-0.177	ns	0.006	ns	0.698	***
S09	-0.372	**	0.013	ns	0.068	ns
S10	0.128	ns	-0.298	*	0.271	*
S11	-0.352	**	-0.047	ns	0.042	ns
S12	-0.536	***	0.551	***	0.271	*
S13	-0.335	**	0.579	***	-0.147	ns
S14	-0.609	***	-0.060	ns	-0.143	ns
S15	0.101	ns	-0.912	***	-0.008	ns
S16	-0.101	ns	0.912	***	0.008	ns
Eigen value	3.860		2.650		1.601	
% of variance	24.12		16.57		10.01	

*** significant at $P < 0.001$, ** significant at $0.001 < P < 0.01$,
* significant at $0.01 < P < 0.05$, ns depicts non-significant values ($P > 0.05$)

Principal component analysis (PCA) based on 16 quantitative morphological traits in two short-tooth sage populations revealed that first three principal components had an eigenvalue > 1 and accounted for 50.69% of the total variation (Table 2). The strong positive correlations ($r > 0.70$; $P < 0.001$) were observed between the first principal component (PC1) and the length of inflorescence (S01), the number of internodes (S02) and the number of primary branches (S03). The second principal component (PC2) was strongly positively correlated ($r = 0.912$; $P < 0.001$) with the calyx venation (parallel with slight anastomosing; S15) while the strong negative correlation ($r = -0.912$; $P < 0.001$) was observed between the second principal component and the calyx venation (parallel with very prominent anastomosing; S16). The biplot constructed by the first two principal components is presented in Fig. 4. PC1 explaining 24.12% of total variation tended to separate the individuals according to their population origin.

Six out of 16 quantitative morphological traits were chosen by stepwise discriminant analysis as the best differentiating factors between the populations (Table 3). The results indicated that the length of calyx tips (S14) was the most important factor (partial $R^2 = 0.675$) contributing to the differentiation of populations, followed by the length of the inflorescence (S01; partial $R^2 = 0.195$). The discriminant function based on six quantitative

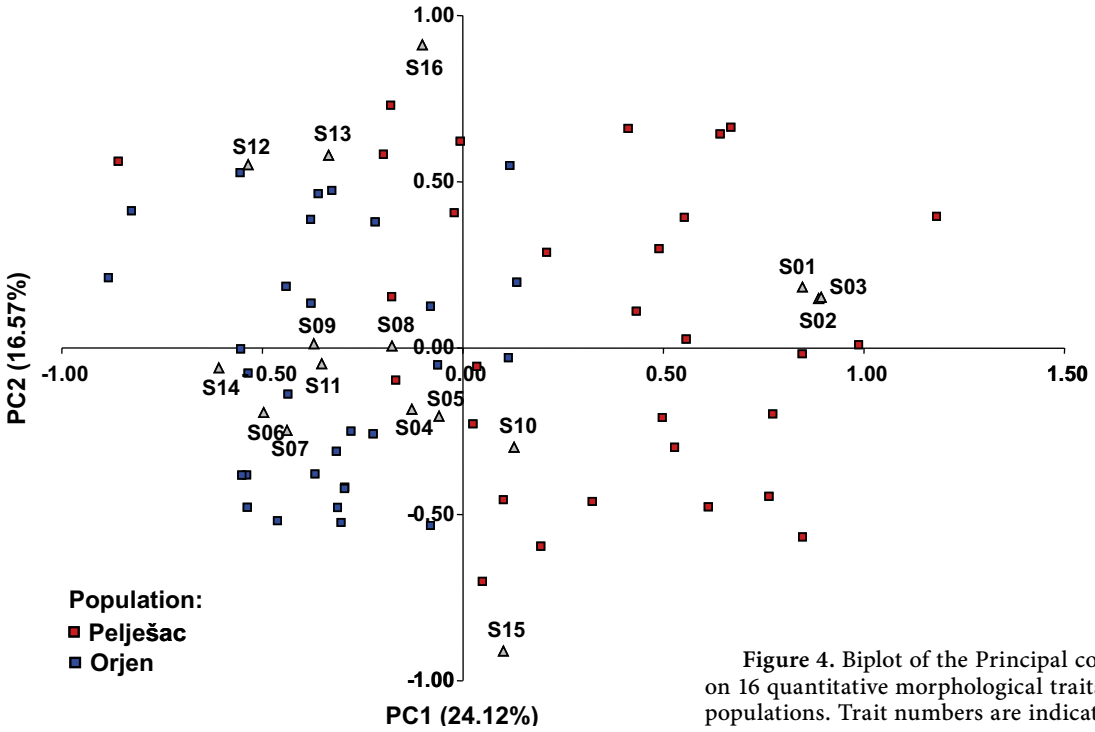

Figure 4. Biplot of the Principal components analysis based on 16 quantitative morphological traits in two short-tooth sage populations. Trait numbers are indicated in Table 1

Table 3. Stepwise discriminant analyses selection summary for significant traits discriminating between short-tooth sage populations and the percentage of classification success of the discriminant function based on an increasing number of traits using cross-validation.

No.	Trait	Partial R^2	Wilks' λ	$P(\lambda)$	% of success
S14	Length of calyx tips	0.675	0.325	***	93.33
S01	Length of inflorescence	0.195	0.261	***	96.67
S07	Flower pedicel / Trichome type: Sessile glandular	0.132	0.227	***	96.67
S10	Calyx / Trichome type: Long capitulate glandular	0.108	0.202	***	96.67
S12	Length of calyx	0.074	0.187	***	96.67
S11	Calyx / Trichome type: Stalked glandular	0.047	0.179	***	96.67

$P(\lambda)$, significance of Wilks' λ: *** significant at $P < 0.001$, ** significant at $0.001 < P < 0.01$, * significant at $0.01 < P < 0.05$,.
ns depicts non-significant values ($P > 0.05$)

traits chosen by stepwise discriminant analysis displayed 96.67% classification success after cross-validation indicating its usefulness in population discrimination. The traits were then re-evaluated for the performance as discriminant criterion in order from most important to least important. Overall classification success of the discriminant function based exclusively on the first trait (length of calyx tips; S14) was only slightly lower (93.33%; four out of 60 individuals were misclassified), while using the first two traits (the length of inflorescence; S01) the same level of classification success was obtained (96.67%; two out of 60 individuals were misclassified) as by using the whole subset of traits chosen by stepwise discriminant analysis. The canonical discriminant analysis based on two traits revealed that the first canonical discriminant variate (CV1) explained 100% of the variation between populations and it was highly correlated with both traits, positively with the length of calyx tips (S14) and negatively with the length of inflorescence (S01). Thus, the ordination diagram revealed a clear differentiation between populations along the CV1 (Fig. 5).

When obtained results were analysed great differences between habitat conditions on Pelješac peninsula and Mt. Orjen had to be considered. The greater incidence of glandular hair and longer calyx tip length in the plants from Mt. Orjen might be interpreted as additional protection from UV irradiation and herbivores.

Conclusions

Having conducted morphological analysis on the populations of short-tooth sage from Pelješac peninsula and Mt. Orjen, several differences between them can be emphasised:

1. The univariate analysis of variance showed a significant value differences in eight of the total of 16 morphological traits. The Pelješac population showed considerably higher values

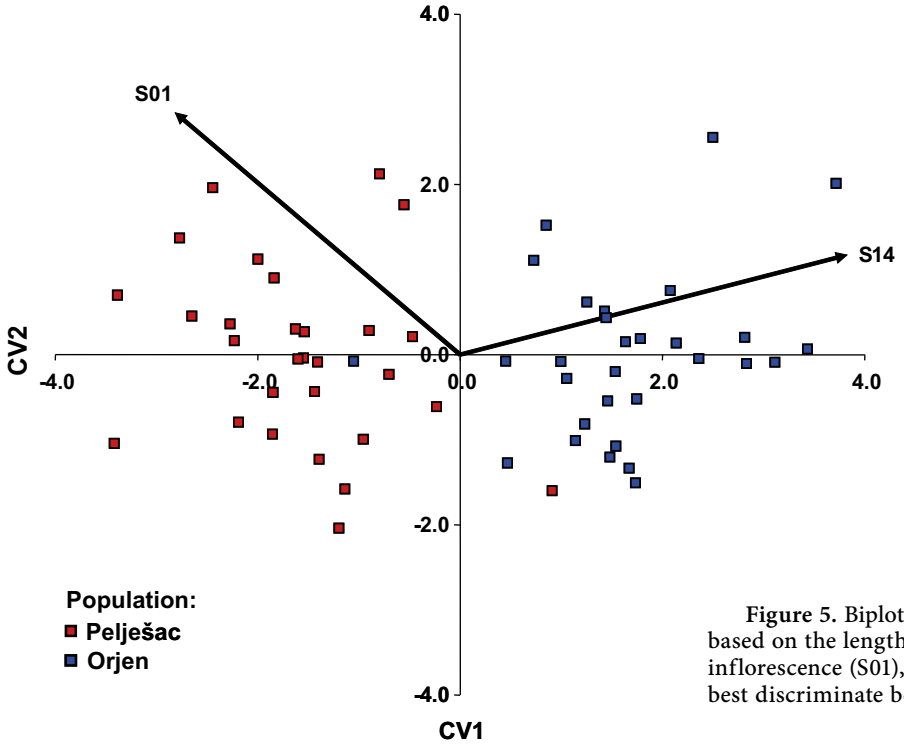

Figure 5. Biplot of the canonical discriminant analysis based on the length of calyx tips (S14) and the length of inflorescence (S01), two quantitative morphologcial traits that best discriminate between two short-tooth sage populations.

for traits related to habitus and plant height, while the Mt. Orjen population showed higher values for traits related to the calyx and higher incidence of sessile glandular hair on the flower pedicels.

2. Six out of 16 quantitative morphological traits were chosen by stepwise discriminant analysis as the best differentiating factors between two populations (length of calyx tips, length of inflorescence, flower pedicel with sessile glandular trichome type, length of calyx, calyx with long capitulate glandular trichome type and calyx with stalked glandular trichome type) with the success rate of 96.67%. The minimal set of traits yielding the same level of classification success comprised only two traits: length of calyx tips and length of inflorescence.
3. When interpreting the obtained results, we should consider the fact that the habitats on Pelješac peninsula and Mt. Orjen greatly differ and that the morphological distinctions may represent the adaptation of the plants to specific habitat conditions.
4. The morphological population analysis will provide a better insight into the current fitness of the short-tooth sage populations, help define efficient protection measures and represent an important step towards possible future cultivation and selection as well as help in elution of complex phylogenetic and taxonomic relations within the section *Salvia*.

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