addresses

1 University of Zagreb, Faculty of Science,
Marulićev trg 9, HR- 10000 Zagreb, CROATIA; *E-mail: ivanrad@biol.pmf.hr
2 University of Zagreb, Faculty of Agriculture,
Svetošimunska 25, HR-10000 Zagreb, CROATIA
3 University of Montenegro, Faculty of Natural Sciences and Mathematics,
Džordža Vašingtona bb, 81000 Podgorica, MONTENEGRO

# Morphometric and genetic diversity in short-toothed sage (*Salvia brachyodon* Vandas)

Ivan RADOSAVLJEVIĆ[1*], Sandro BOGDANOVIĆ[2], Monika PRUŠA[1], Zlatko SATOVIC[2], Danijela STEŠEVIĆ[3], Zlatko LIBER[1]

summary

Short-toothed sage (*Salvia brachyodon* Vandas) is Illyrian-Adriatic endemic species. It is reliably known to grow only in two localities: at the summit Sv. Ilija on Pelješac peninsula in Croatia, and near the village Vrbanj on Mt. Orjen at the border between Bosnia and Herzegovina, and Montenegro. In order to study population diversity of short-toothed sage, the samples from both localities have been analysed using 16 morphological traits and eight microsatellite primers previously developed for Dalmatian sage (*Salvia officinalis* L.) and successfully cross-amplified in short-toothed sage. The morphological analysis revealed that two populations could be discriminated using a minimal set of two morphological traits: length of calyx tips and the length of inflorescence. The short-toothed sage tends to form lax cushions that can cover up to few dozen square meters, thus suggesting clonal reproduction via stolones. The molecular analysis confirmed that the clonal propagation is frequent in short-toothed sage given that out of 180 individual plant samples, only 98 were of unique multilocus genotype.

key words

*Salvia brachyodon* Vandas,
microsatellites,
morphometry,
diversity,
clonality

*Fig. 1. Short-tooth sage (*Salvia brachyodon *Vandas) growing sympatrically with Dalmatian sage (*Salvia officinalis *L.) at the summit Sv. Ilija on Pelješac peninsula, Croatia*

## introduction

Short-tooth sage (*Salvia brachyodon* Vandas; Fig. 1), together with 11 European *Salvia* species, belongs to the section *Salvia* of the genus *Salvia* (Hedge, 1972). Currently, only two localities where this species grows have been confirmed: Mt. Orjen, on the border between Bosnia and Herzegovina, and Montenegro (*locus classicus*, Vandas, 1899) and the Pelješac peninsula (Croatia). Another two localities (Konavle and Mt. Mosor), known from collection of the Herbarium ZAHO and from literature (Girometta, 1930), are recorded only once and therefore need additional verification. 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).

The aim of this research was to assess population diversity and differentiation of short-toothed sage from two known localities by morphological and molecular analyses.

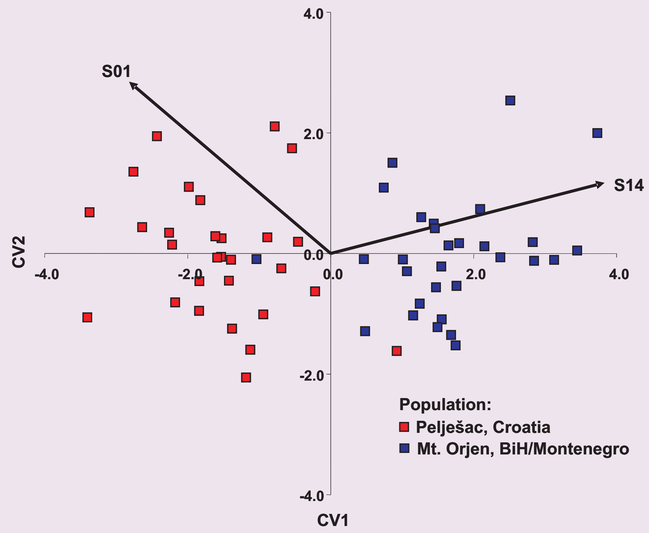


*Fig. 2. Biplot of the canonical discriminant analysis based on the length of calyx tips (S14) and the length of inflorescence (S01), two quantitative morphological traits that best discriminate between short-tooth sage (*Salvia brachyodon *Vandas) populations from Pelješac, Croatia and Mt. Orjen, BiH/Montenegro*

## materials 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. A total of 60 herbarium specimens were analyzed using 16 quantitative morphological characters: S01 Length of inflorescence (cm), S02 No. of internodes, S03 No. of primary branches, Flower pedicel trichome types present/absent (S04 Patent eglandular, S05 Short glandular, S06 Long capitulate glandular, S07 Sessile glandular, S08 Stalked glandular), Calyx trichome types present/absent (S09 Patent eglandular, S10 Long capitulate glandular, S11 Stalked glandular), S12 Length of calyx (mm), S13 Length of calyx lobes (mm), S14 Length of calyx tips (mm), Calyx venation (S15 Parallel with slight anastomosing, S16 Parallel with very prominent anastomosing). 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. 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.

For the molecular analyses each population was represented by 90 individuals. Eight microsatellite primers developed for Dalmatian sage (*S. officinalis* L.) were used for the analysis: SoUZ001, SoUZ002, SoUZ004, SoUZ005, SoUZ006, SoUZ007, SoUZ011 and SoUZ014 (Molecular Ecology Resources Primer Development Consortium, 2010; Radosavljević et al., 2011; 2012). To quantify the ability of markers to resolve between two individuals, the unbiased probability of identity (*PI*; Waits et al., 2001) was estimated for each locus in each population using the GIMLET software (Valiere, 2002). Clonal diversity was assessed by calculating the number of clonal lineages i.e. distinct genotypes (*G*), maximum clonal size (max $n_g$), genotypic richness (*R*; Dorken and Eckert, 2001) and Simpson's evenness index (*V*; Hurlbert 1971) in GenClone 2.0 (Arnaud-Haond and Belkhir, 2007). Genetic distances between all pairs of short-tooth sage samples were calculated using the proportion-of-shared-alleles distances ($D_{PSAM}$; Bowcock et al., 1994) as implemented in MICROSAT (Minch et al., 1997) and unrooted phylogenetic tree was created using Neighbor-joining algorithm as implemented in NEIGHBOR programme of the PHYLIP ver. 3.6b software package (Felsenstein, 2004). The reliability of the tree topology was assessed via bootstrapping (Felsenstein, 1985) over 1,000 replicates.

## 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 $P < 0.05$. Pelješac population exhibited significantly higher values than Mt. Orjen population in traits related to inflorescence (S01, S02, S03) while the Mt. Orjen population has significantly higher values in traits related to the presence of some types of trichomes on flower pedicel (S06, S07) and calyx (S11) as well as in traits related to calyx (S12, S14).

Six out of 16 quantitative morphological traits were chosen by stepwise discriminant analysis (SDA) as the best differentiating factors between the populations. 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 traits chosen by SDA 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 of most important to least important. Overall classification success of the discriminant function based exclusively on the first trait (S14) was only slightly lower (93.33%), while using the first two traits (S14 and S01) the same level of classification success was obtained (96.67%) as by using the whole subset of traits chosen by SDA. The canonical discriminant analysis based on two traits revealed the first canonical discriminant variate (CV1) explained 100% of the variation between populations. Thus, the ordination diagram revealed a clear differentiation between populations along the CV1 (Fig. 2).

*Table 1. Genetic diversity of short-tooth sage populations from Pelješac and Mt. Orjen as assessed by eight microsatellite markers*

| Population | *N* | *G* | max $n_g$ | *R* | *V* |
|---|---|---|---|---|---|
| Pelješac | 90 | 73 | 7 | 0.809 | 0.800 |
| Mt. Orjen | 90 | 25 | 18 | 0.270 | 0.914 |

*N - sample size, G - number of clonal lineages i.e. distinct genotypes, max $n_g$ - maximum clonal size, R - clonal richness, V - Simpson's evenness index*

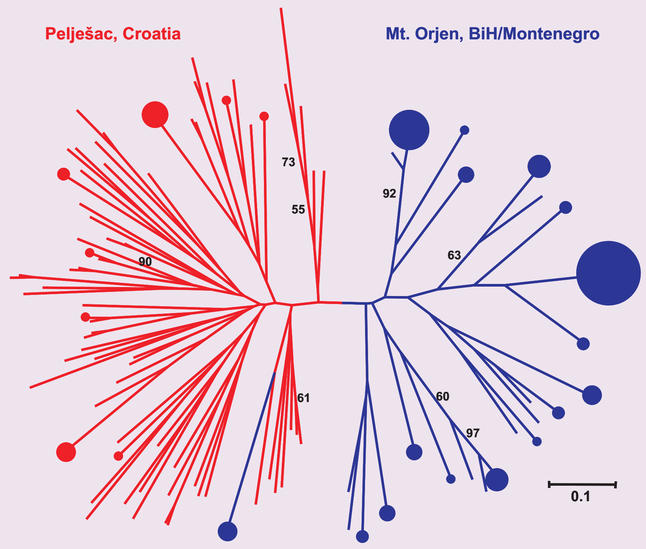


*Fig. 3. Unrooted Neighbour Joining tree of short-tooth sage (Salvia brachyodon Vandas) samples from Pelješac, Croatia and Mt. Orjen, BiH/Montenegro. Numbers above branches indicate bootstrap support percentage over 50% in 1,000 pseudoreplicates. The size of the circles at the branch tips is proportional to size (no. of individual plant samples) of the clonal lineage*

Eight microsatellite loci yielded a total of 74 alleles. The unbiased probability of identity (PI) values indicated that eight microsatellite loci were sufficiently polymorphic to allow identification among more than $4 \times 10^8$ individuals ($PI = 2.34 \times 10^{-9}$). Only 98 out of 180 samples had unique multilocus genotypes. While in Pelješac population 73 unique genotypes were detected, in Mt. Orjen population only 25 genotypes were unique. Genotypic richness (*R*) was considerably higher in Pelješac than in Mt. Orjen population. On the other side, maximal clonal size as well as clonal equitability measured by Simpson's evenness index (*V*) was higher in Orjen than in Pelješac population (Table 1). In the Neighbor-joining tree it can be seen that the great majority of the individuals grouped together according to population affiliation, and none of the multilocus genotypes were shared between populations (Fig. 3).

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