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The outcome of natural hybridization in plants is difficult to predict. The symmetry of the hybridization depends on numerous elements, such as the viability and fertility of hybrid offspring, the initial sizes of the parental populations, and pollinator preferences. If hybridization is asymmetrical, it is expected that the inheritance will be oriented towards the same parental taxon on both molecular and morphological levels. Using molecular and morphological markers, we analysed the natural hybridization between *Salvia officinalis*, an indigenous species well adapted to the local environment, and *S. fruticosa*, a non-native species struggling with marginal environmental conditions. Results reveal that hybridization has an asymmetrical pattern and that gene flow is oriented towards *S. fruticosa*. However, the hybrid individuals resulting from back-crossing with *S. fruticosa* are more morphologically similar to *S. officinalis*. We believe that environmental pressure is responsible for such a hybridization outcome. To achieve the phenotypic optimum previously achieved by *S. officinalis*, hybrids use the genetic potential gained from the hybridization to become morphologically more like the native species.

Keywords: *Salvia*, hybridization, phenotype, genotype



Fig. 1. *Salvia officinalis* (A), hybrid (B) and *S. fruticosa*; locality near Komiža, island of Vis, Croatia where *S. officinalis* and *S. fruticosa* grow sympatrically

Contrasting patterns of phenotypic and genetic variation in hybrid offspring: Causes and consequences

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The *Salvia* section of the genus *Salvia* includes 13 species, with Dalmatian sage (*S. officinalis* L.; Fig. 1A) as its type species. The distribution range of *S. officinalis* includes the eastern Adriatic coast from the Gulf of Trieste in north to north-western Greece in the south (Pignatti 1982) as well as parts of the central and southern Apennines (Di Pietro 2011). Another easily recognizable member of this section is Greek sage (*S. fruticosa* Mill.; Fig. 1C) that extends from Cyrenaica, Sicily, and southern Italy through the southern part of the Balkan Peninsula to West Syria (Hedge 1982).

In the central Adriatic region, *S. fruticosa* can be found on the island of Vis, Croatia (Fig. 1D), where the plant grows sympatrically with *S. officinalis*. Because of the well-organized Greek colony called Issa that was founded on the island of Vis in the 4th century BC, it is likely to assume that this disjunct population is of sub-spontaneous origin rather than being a member of an indigenous flora.

The hybridization between these species has never been documented to occur spontaneously while artificial hybrids have been reported (Dudai et al. 1999). The most reliable morphological trait used in the field for identification is calyx shape. The *S. officinalis* calyx is larger and strictly bilabiate, whereas the *S. fruticosa* calyx is smaller, tubular and actinomorphic (Reales et al. 2004). Individuals characterized by a slightly bilabiate calyx are considered to be of a hybrid origin (Fig. 1B).

The aims of this study were (1) to analyse phenotypic diversity of *S. officinalis*, *S. fruticosa* and their putative hybrids using qualitative and quantitative morphological traits; (2) to analyse genetic diversity and structure, and (3) to determine the prevalence of hybridization and assess the nature and levels of inter-species gene flow.

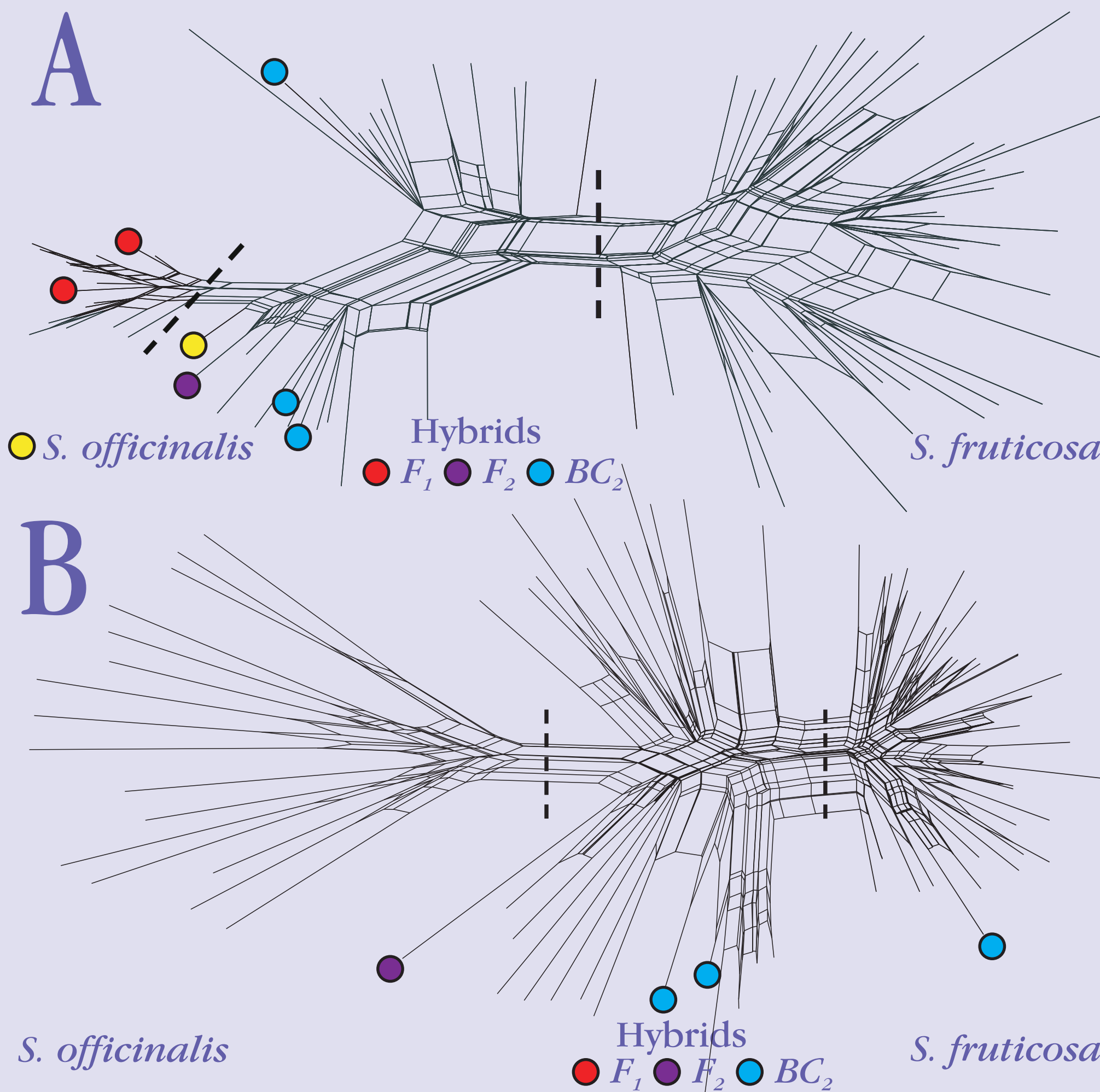


Fig. 2. Neighbor-net diagrams based on (A) 23 qualitative and 19 quantitative morphological traits, and (B) microsatellite marker data of *S. officinalis*, *S. fruticosa* and their hybrids

Plant material: Leaf tissue and branches with inflorescences were collected from 79 *Salvia* individuals in the locality near Komiža, island of Vis, Croatia. In total, 16 samples were identified as *S. officinalis*, 38 as *S. fruticosa* and 25 as their putative hybrids.

Morphometric analyses: 23 qualitative and 19 quantitative traits were selected and measured based on a previous taxonomic revision of the section *Salvia* (Reales et al. 2004). Shannon's information index as a measure of qualitative trait diversity was calculated for each trait in each taxon and the results were compared by repeated measures analysis of variance. To test for differences in mean values between taxa in 19 quantitative traits univariate analysis of variance was performed. To combine the information from the qualitative and quantitative traits into a single distance measure, Gower's distance (Gower 1971) was calculated between all pairs of individuals. A neighbour-net diagram, which is well suited to depicting reticulate relationships (Bryant & Moulton, 2004), was constructed from Gower's distances using SplitsTree 4 (Huson & Bryant 2006).

Microsatellite data analysis: Seven microsatellite loci that were previously characterized in *S. officinalis* and successfully cross-amplified in *S. fruticosa* (MERPDC et al. 2010; Radosavljević et al., 2011; 2012) were used. GENEPOP 4.0 (Raymond & Rousset 1995) was used to estimate population genetic parameters. As a measure of the number of alleles per locus independent of sample size, allelic richness (N_{ar}) was calculated by FSTAT v.2.9.3.2 package (Goudet 1995). Genetic distances between pairs of samples were calculated using the proportion-of-shared-alleles distances (Bowcock et al. 1994) and a NeighbourNet diagram was produced using SplitsTree4. A model-based clustering method was applied to infer the genetic structure and define the number of clusters using STRUCTURE ver. 2.3.3 (Pritchard et al. 2000). The Bayesian method implemented by NewHybrids 1.1 (Anderson & Thompson 2002) was used to assign individuals to one of six classes: two pure and four putative hybrids (F_1 , F_2 and backcrosses with the parental populations).

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Phenotypic diversity: The Shannon's diversity index based on 23 qualitative traits was significantly different between taxa with means of 0.165, 0.418 and 0.710 for *S. officinalis*, hybrids and *S. fruticosa*, respectively. The number of monomorphic traits was the highest in *S. officinalis* (17) and lowest in *S. fruticosa* (one). Fifteen out of 23 quantitative traits were the most variable in *S. fruticosa*. ANOVA indicated statistically significant mean differences in all 19 quantitative traits in the analysed taxa. A neighbour-net diagram unambiguously separated the parental taxa from each other and the hybrids (Fig. 2A). The highest levels of within-taxon Gower's distances were observed in *S. fruticosa*, followed by hybrids and then by *S. officinalis*.

Analyses of morphological variation resulted in clear delimitation of taxa and show contrasting patterns of the extent of phenotypic variation between the taxa. *S. officinalis* was characterized by almost complete morphological uniformity. In *S. fruticosa*, significantly higher levels of phenotypic variation were found. Although such contrasting results are a bit unexpected, they can be explained by the fact that the population of *S. officinalis* is a true "core" population, owing its morphological stability to its long persistence in the study area. In contrast, the population of *S. fruticosa* is of anthropogenic origin and can be treated as an archaeophyte and "edge" population. The studied population is in the northernmost area of the species distribution, and it presumably faces marginal environmental conditions resulting in high levels of phenotypic plasticity. When a population is faced with changing environmental conditions, a full spectrum of phenotypes is produced (Pigliucci 2001), and natural selection is responsible for selecting the phenotype with high fitness and optimum traits under the new conditions (Wund 2012).

Table 1. Microsatellite diversity of *S. officinalis*, *S. fruticosa* and their hybrids

Species	<i>n</i>	<i>N_{ar}</i>	<i>H_o</i>	<i>H_e</i>	<i>F_{is}</i>
<i>S. officinalis</i>	16	7.00	0.634	0.751	0.156 ^{***}
Hybrids	25	6.66	0.786	0.680	-0.156 ^{ns}
<i>S. fruticosa</i>	38	3.17	0.304	0.334	0.090 [*]

n - sample size, *N_{ar}* - allelic richness, *H_o* - observed heterozygosity, *H_e* - expected heterozygosity, *F_{is}* - inbreeding coefficient (significance: ****P* < 0.001, ***P* < 0.01, **P* < 0.5, ns - non-significant)

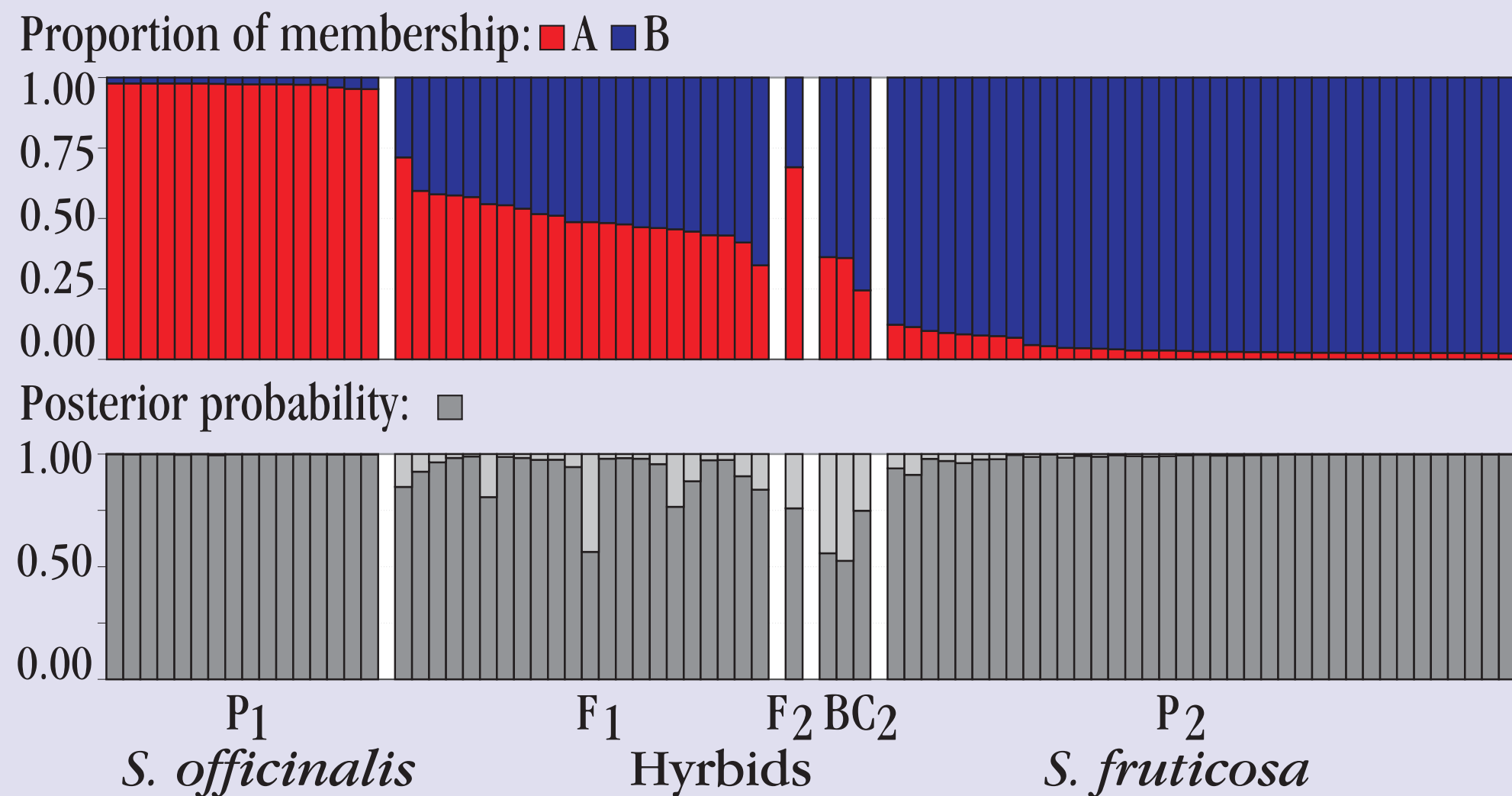


Fig. 3. Proportions of membership of each individual in each of the two clusters as estimated by the program Structure and the assignment of individuals into classes (parental, F_1 , F_2 and BC_2) as estimated by the program NewHybrids.

Genetic diversity and structure: A total of 65 alleles were detected using eight microsatellite markers, out of which 49 were present in *S. officinalis*, 27 were in *S. fruticosa* and 54 were in hybrids. Allelic richness of *S. officinalis* was comparable to that of the hybrids while *S. fruticosa* exhibited considerable lower values. The levels of H_o and H_e were significantly higher in both *S. officinalis* and in the hybrids than in *S. fruticosa* (Tab. 1). The neighbour-net analysis (Fig. 2B) clearly separated the parental taxa from the hybrids. The highest levels of within-taxon genetic distances were observed in *S. officinalis*, followed by hybrids and then by *S. fruticosa*.

The results of STRUCTURE strongly supported the presence of two clusters representing genetically well-defined populations belonging to parental taxa while the hybrid individuals were characterized by wide range of admixture proportions (Fig. 3). Using NewHybrids, the majority of the hybrid individuals (22 out of 25) were classified as F_1 . A single hybrid individual was classified as an F_2 , and two individuals were classified as back-crosses to *S. fruticosa*. All *S. fruticosa* samples were classified as pure parental except one that was classified as a back-cross to *S. fruticosa*.

As a result of specific evolutionary histories of the species, the population the autochthonous *S. officinalis* is characterized by increased levels of genetic diversity compared to with the allochthonous *S. fruticosa*. Consequently, *S. officinalis* participates in the hybridization with more alleles than *S. fruticosa*, resulting in high similarity of allelic richness levels between hybrids and *S. officinalis*.

As most of the hybrid individuals belong to F_1 generation it seems that they are predominantly sterile. The lack of back-crosses to *S. officinalis* suggests that the hybridization is introgressive to *S. fruticosa*. However, the BC_2 individuals are morphologically more similar to *S. officinalis* indicating that the environment plays a crucial role in shaping the process of hybridization.

results and discussion

materials and methods