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NATURAL HYBRIDIZATION BETWEEN *SALVIA OFFICINALIS* L. AND *SALVIA FRUTICOSA* MILL. (LAMIACEAE) ON THE ISLAND OF VIS (CROATIA): EVIDENCE FROM MORPHOLOGICAL AND MOLECULAR DATA

Epigenetic vs. genetic diversity in natural plant populations: A case study of Croatian endemic *Salvia* species

Croatian Science Foundation Project

Common (Dalmatian) sage (*Salvia officinalis* L.)

Salvia officinalis L.
Image processed by Thomas Schoepke
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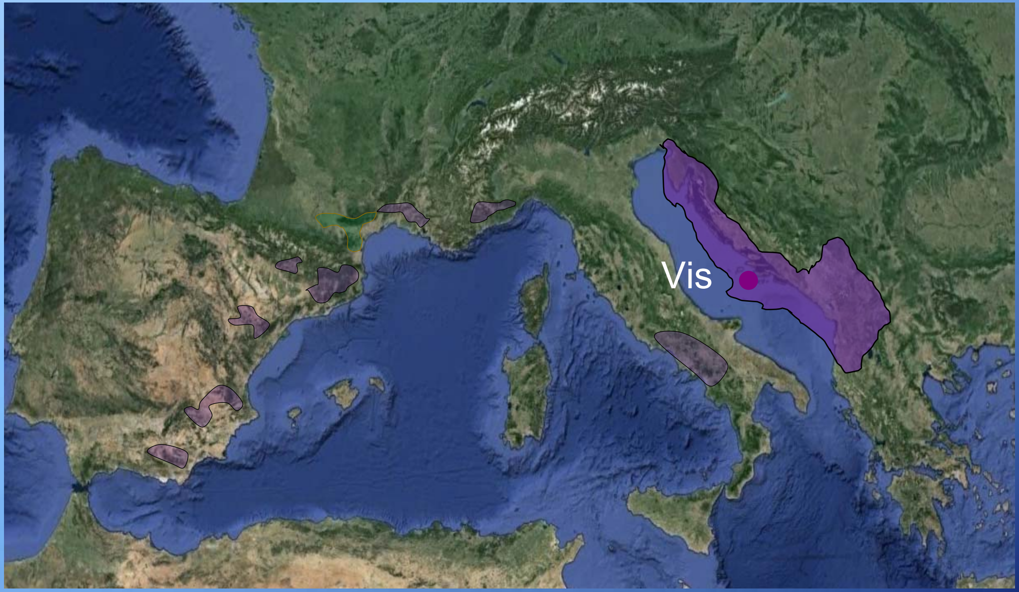
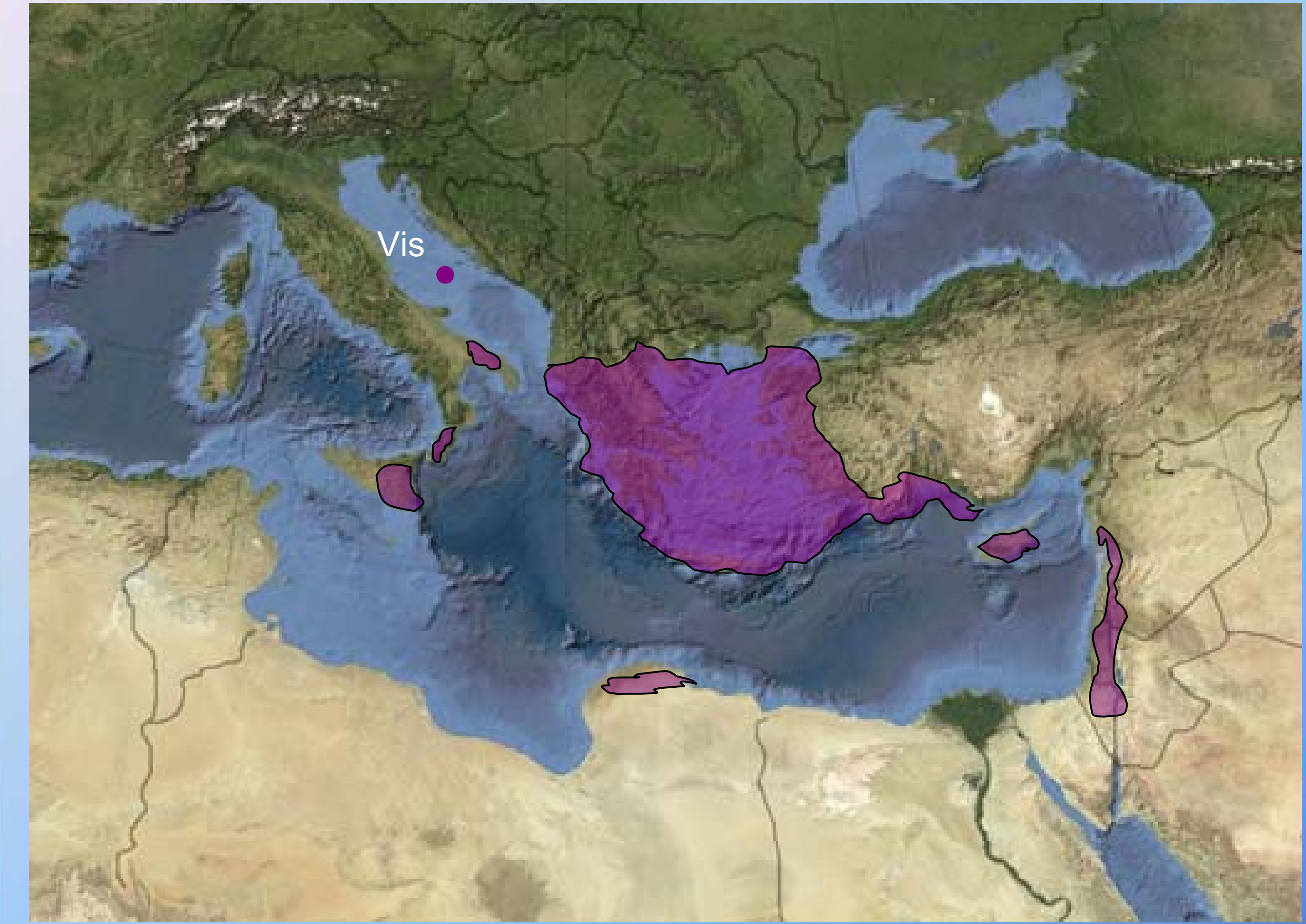
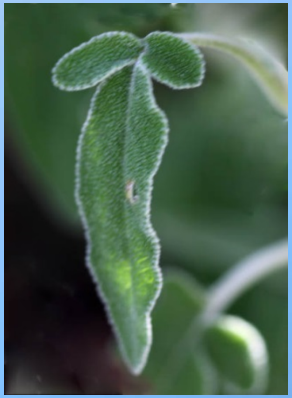


Greek sage (*Salvia fruticosa Mill.*)





syn. *Salvia triloba* L.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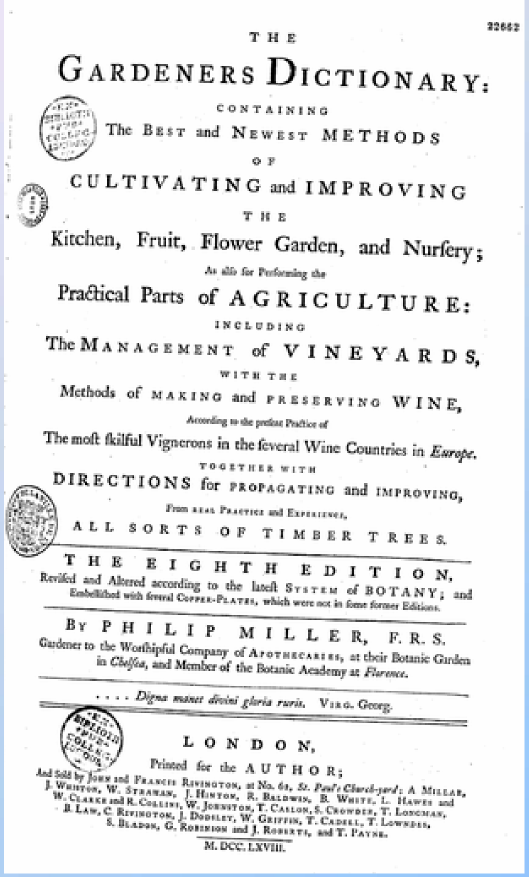
Hybrid sage (*Salvia* x *auriculata* Mill.)



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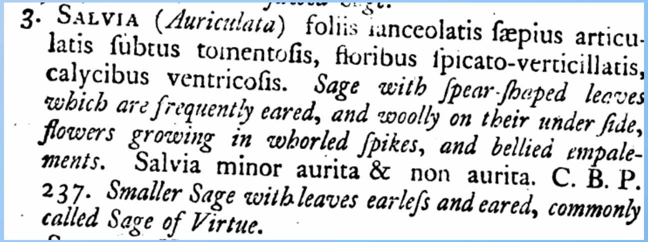
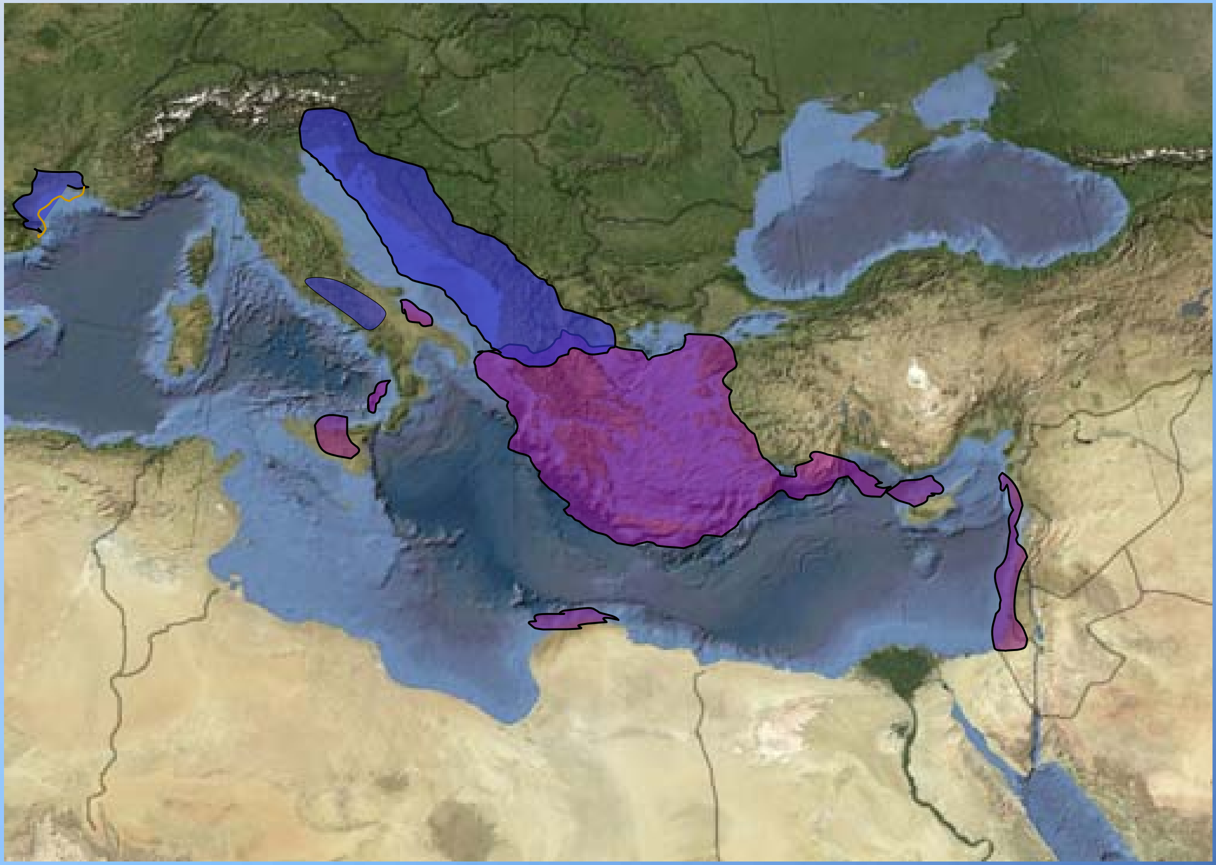
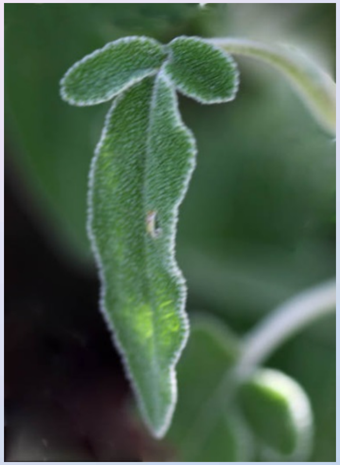


3. SALVIA (*Auriculata*) foliis lanceolatis ſæpius articulatis ſubtus tomentoſis, floribus ſpicato-verticillatis, calycibus ventricoſis. *Sage with ſpear-ſhaped leaves which are frequently eared, and woolly on their under ſide, flowers growing in whorled ſpikes, and bellied empalements.* Salvia minor aurita & non aurita. C. B. P. 237. *Smaller Sage with leaves earleſs and eared, commonly called Sage of Virtue.*



Salvia officinalis x *Salvia fruticosa* cv. *Newe Ya'ar* No.4 (Dudai et al 1999)



S. fruticosa

X

S. officinalis

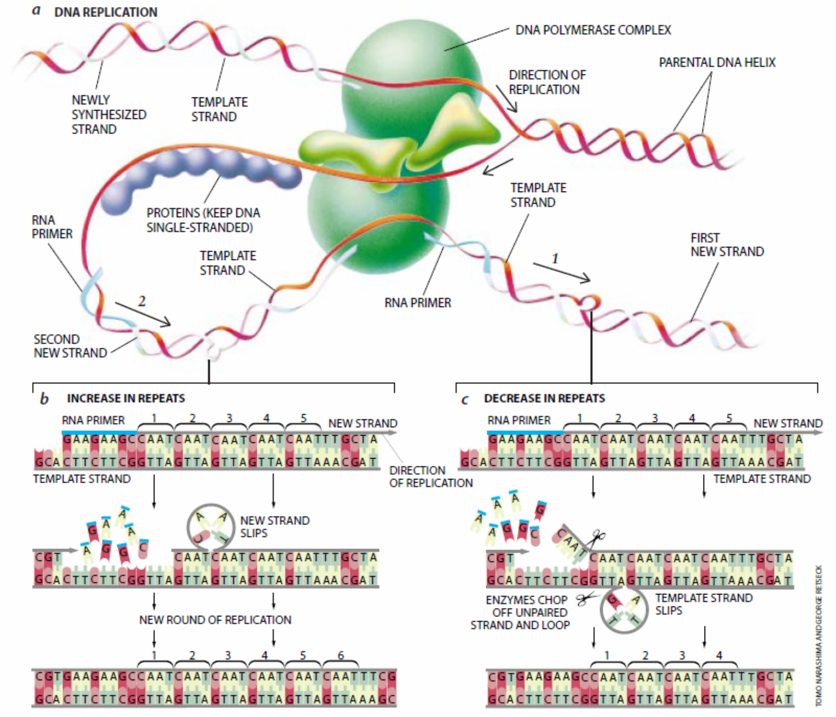
=

hybrid

Aim of the research:

- confirm or reject the hypothesis of the existence of natural hybridization on the island of Vis using up-to-date molecular, morphological and statistical methods

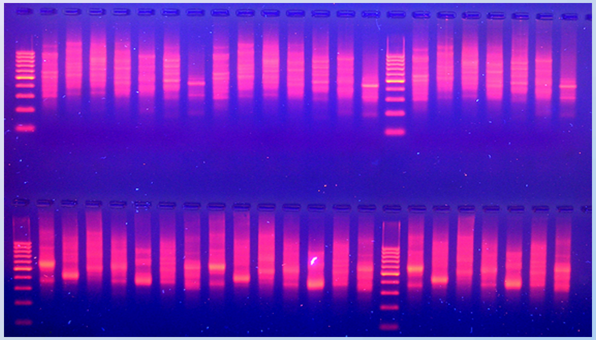
MICROSATELLITES
(Simple Sequence Repeats – SSR)

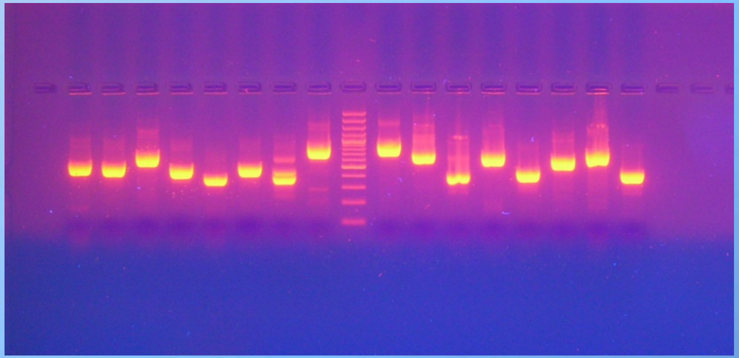


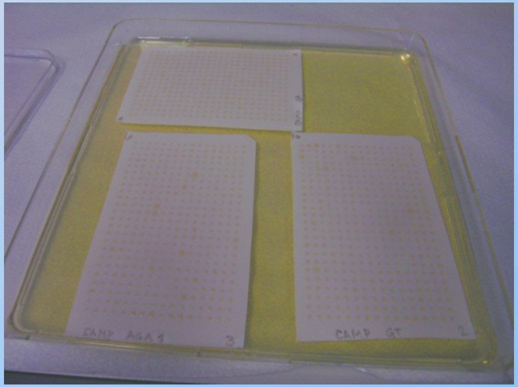
TOMO NARASHIMA AND GEORGE RETSECK

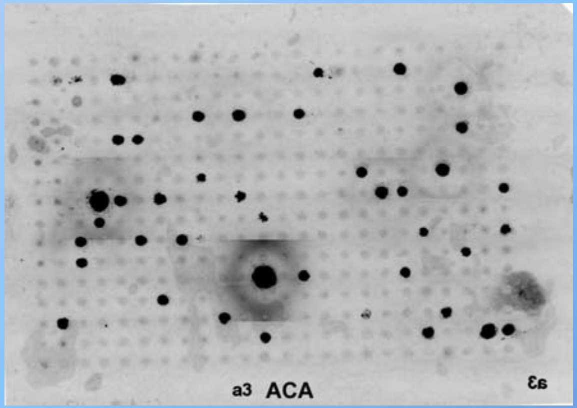
Moxon and Wills 1998

Isolation and characterization of polymorphic microsatellites markers from common sage (*Salvia officinalis L.*)









Testing of new SSR markers in 25 individuals from a natural population of the common sage

(Molecular Ecology Resources Primer Development Consortium 2010; Radosavljević et al 2011; Radosavljević et al 2012)

Locus	GenBank	Repeat motif	T_a (°C)	Size range (bp)	N_a	H_O	H_E	PIC
SoUZ001	GQ376512	$(AG)_{15}$	55	159-195	15	0.833	0.905	0.897
SoUZ002	GQ376513	$(TG)_{11}$	55	199-222	11	0.600	0.772	0.744
SoUZ003	GQ376514	$(GT)_{13}$	55	193-210	9	0.640	0.666	0.641
SoUZ004	GQ376515	$(CA)_{19}$	55	195-227	12	0.625	0.824	0.804
SoUZ005	GQ376516	$(AC)_{20}$	55	131-160	9	0.300	0.808	0.787
SoUZ006	GQ376517	$(CT)_{31}$	55	204-244	14	0.833	0.882	0.872
SoUZ007	GQ376518	$(GT)_{11}$	55	214-227	8	0.583	0.722	0.691
SoUZ008	GQ376519	$(TC)_{15}$	55	190-210	10	0.708	0.800	0.773
SoUZ009	GQ376520	$(TG)_{15}$	55	209-250	9	0.609	0.738	0.699
SoUZ010	GQ376521	$(GA)_{22}$	55	137-222	26	0.800	0.934	0.931
SoUZ011	GQ376522	$(GA)_{25}$	55	175-227	19	0.760	0.930	0.926
SoUZ012	GQ376523	$(CTT)_{26}$	55	179-281	25	0.880	0.921	0.916
SoUZ013	GQ376524	$(AAC)_{8}$	55	202-229	8	0.739	0.740	0.702
SoUZ014	GQ376525	$(AGA)_{10}$	55	201-237	12	0.870	0.878	0.866
SoUZ015	GQ376526	$(GTT)_{12}$	55	136-188	14	0.720	0.853	0.839
SoUZ016	GQ376527	$(TTC)_{20}$	55	128-191	21	0.880	0.927	0.923
SoUZ017	HQ401010	$(CAA)_{13}$	55	198-231	7	0.375	0.624	0.585
SoUZ018	HQ401011	$(GAA)_{20}$	55	170-260	21	0.417	0.931	0.927
SoUZ019	HQ401012	$(AGA)_{16}$	55	153-183	9	0.667	0.701	0.675
SoUZ020	HQ401013	$(GAA)_{16}$	55	198-246	10	0.500	0.769	0.736

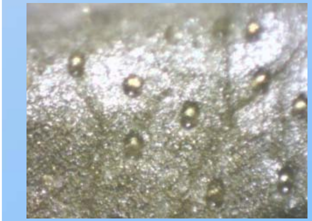
Results of PCR amplification in closely related species

SSR lokus	*S.brachyodon*	*S. fruticosa*	*S. pratensis*	*S. sclarea*	*S. verticilata*	*R. officinalis*
SoUZ001	+					
SoUZ002	+					+
SoUZ003		+		+		
SoUZ004	+					
SoUZ005	+	+	+			
SoUZ006	+	+	+	+		
SoUZ007	+	+				+
SoUZ008	+		+		+	+
SoUZ009	+	+	+	+	+	+
SoUZ010						
SoUZ011	+					
SoUZ012						
SoUZ013	+	+				
SoUZ014	+	+	+	+	+	
SoUZ015						
SoUZ016		+				
SoUZ017						
SoUZ018						
SoUZ019						
SoUZ020	+	+			+	

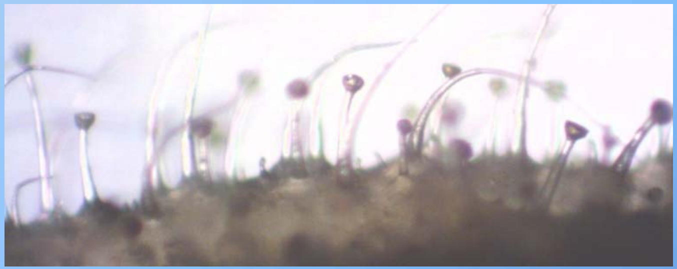
23 qualitative (binary) morphological traits

No.	Organ	Trait	Abbr.
1	Inflorescence	Occurance of bracts in the first lower verticillaster	BLV
2	Inflorescence	Occurance of bracts in the second and upper verticillasters	BUV
3	Inflorescence	Occurance of leaves in the zone of inflorescence	LI
4	Inflorescence axis	Trichome type: 1 Eglandular 1.1 Patent	IEP
5	Inflorescence axis	Trichome type: 1 Eglandular 1.2 Subadpressed	IEA
6	Inflorescence axis	Trichome type: 2 Glandular 2.1 Short glandular	IGS
7	Inflorescence axis	Trichome type: 2 Glandular 2.2 Long capitulate	IGL
8	Inflorescence axis	Trichome type: 2 Glandular 2.3 Sessile	IGSS
9	Inflorescence axis	Trichome type: 2 Glandular 2.4 Stalked	IGST
10	Inflorescence branches	Occurance of secondary branches	OSB
11	Floral bract	Trichome type: 1 Eglandular 1.1 Patent	BEP
12	Floral bract	Trichome type: 1 Eglandular 1.2 Subadpressed	BEA
13	Floral bract	Trichome type: 2 Glandular 2.1 Short glandular	BGS
14	Floral bract	Trichome type: 2 Glandular 2.2 Long capitulate	BGL
15	Floral bract	Trichome type: 2 Glandular 2.3 Sessile	BGSS
16	Floral bract	Trichome type: 2 Glandular 2.4 Stalked	BGST
17	Flower pedicel	Trichome type: 1 Eglandular 1.1 Patent	FEP
18	Flower pedicel	Trichome type: 1 Eglandular 1.2 Subadpressed	FEA
19	Flower pedicel	Trichome type: 2 Glandular 2.1 Short glandular	FGS
20	Flower pedicel	Trichome type: 2 Glandular 2.2 Long capitulate	FGL
21	Flower pedicel	Trichome type: 2 Glandular 2.3 Sessile	FGSS
22	Flower pedicel	Trichome type: 2 Glandular 2.4 Stalked	FGST
23	Calyx	Calyx appendices	CAP

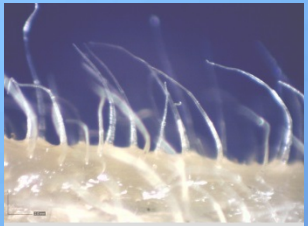
IGST



FGSS



BGS i IGL



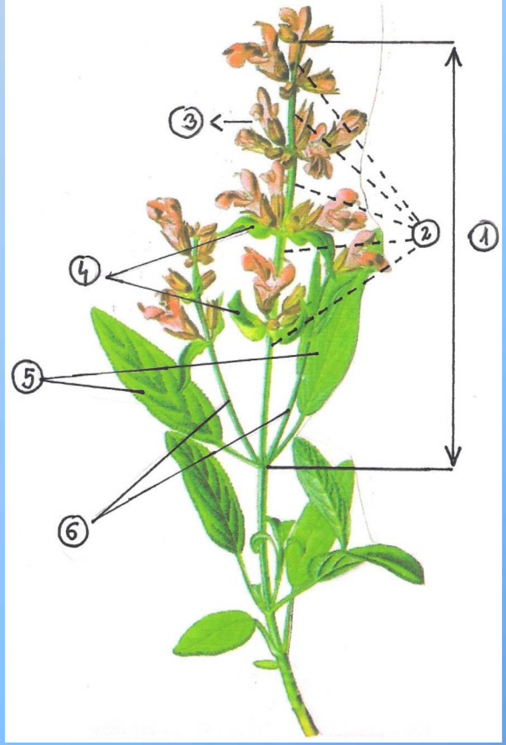
FEP

BEA

Calyx with or without apendices

19 quantitative morphological traits

No.	Organ	Trait	Abbr.
1	Inflorescence	Inflorescence length	IL
2	Inflorescence	Number of internodes on inflorescence	NII
3	Inflorescence	Number of flowers in lower verticillaster	NFV
4	Inflorescence branches	Number of primary branches	NPB
5	Floral bract	Bract length	BL
6	Floral bract	Bract width	BW
7	Calyx	Trichome type: 1 Eglandular 1.1 Patent	CEP
8	Calyx	Trichome type: 1 Eglandular 1.2 Subadpressed	CEA
9	Calyx	Trichome type: 2 Glandular 2.1 Short glandular	CGS
10	Calyx	Trichome type: 2 Glandular 2.2 Long capitulate	CGL
11	Calyx	Trichome type: 2 Glandular 2.3 Sessile	CGSS
12	Calyx	Trichome type: 2 Glandular 2.4 Stalked	CGST
13	Calyx	Calyx type: actinomorphic (radial symmetry)	CA
14	Calyx	Calyx type: almost actinomorphic	CAA
15	Calyx	Calyx type: zigomorphic (bilabiate)	CZ
16	Calyx	Calyx length	CL
17	Calyx	Calyx lobes length (min)	CLMI
18	Calyx	Calyx lobes length (max)	CLMX
19	Calyx	Calyx venation: reticulate	CNR



1 - IL, 2 - NII, 3 - NFV, 4 – BLV (Occurance of bracts in the first lower verticillaster) and BUV (occurance of bracts in the second and upper verticillasters), 5 – LI (occurance of leaves in the zone of inflorescence), 6 – NPB ili OSB (occurance of secondary branches)

CA

CL, CLMI and CLMX

CZ

MATERIAL AND METHODS

Molecular analysis

Plant material: silica-gel dried leaf tissue of 79 plants

DNA isolation: GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich®)

PCR program: touchdown PCR (94° C for 5 min; five cycles of 45 s at 94° C, 30 s at 60° C, which was lowered by 1° C in each cycle, and 90 s at 72° C; 25 cycles of 45 s at 94° C, 30 s at 55° C, and 90 s at 72° C; and an 8-min extension step at 72° C)

Detection of alleles at seven SSR loci: capillary electrophoresis on an ABI 3730XL analyzer (Applied Biosystems®). The results were analyzed using GeneMapper 4.0 software.

Genetic distance and Neighbor-Net diagram: proportion-of-shared-alleles distances (D_{psa}) was calculated using MICROSAT computer programe (Minch et al 1997). Based on distance matrix reticulate evolutionary eventes were visualized by SPLITS TREE (Huson and Bryant 2006)

Population genetic structure: a model-based clustering method was applied using STRUCTURE software (Pritchard et al 2000)

Determination of hybrid status: Bayesian method implemented by NEW HYBRIDS software (Anderson and Thompson 2002) was used to assign individuals into six classes

Morphological analysis

Plant material: 79 herbarium specimens

Morphological characteristics: 23 qualitative and 19 quantitative traits

Gower distance: in order to combine information form qualitative (binary) and quantitative morphological traits Gower's distance (Gower, 1971) was calculated between all pairs of individuals using PAST (Hammer et al. 2001)

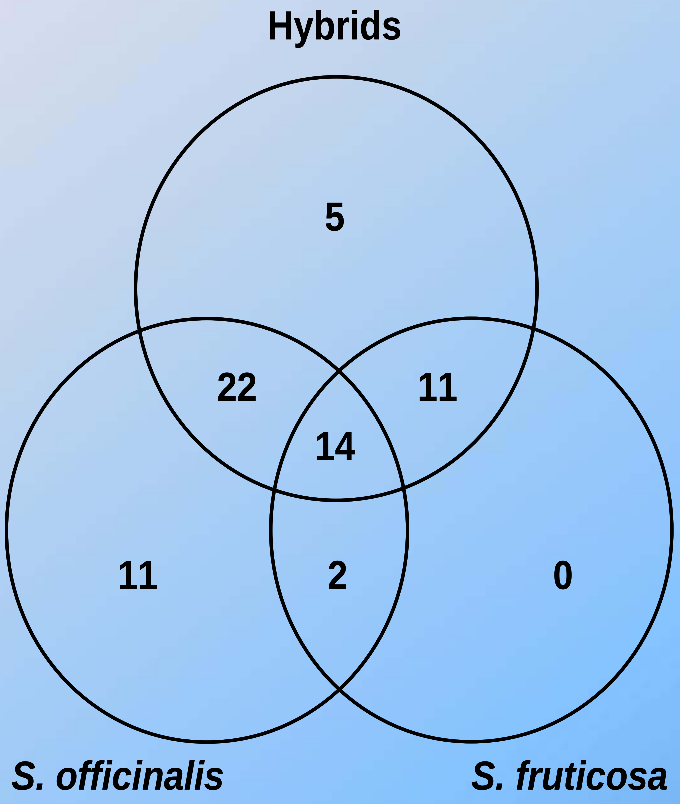
Neighbor-Net diagram: to depict reticulate relationships from Gower’s distances SPLITS TREE ver. 4,0 was used (Huson and Bryant 2006)

RESULTS

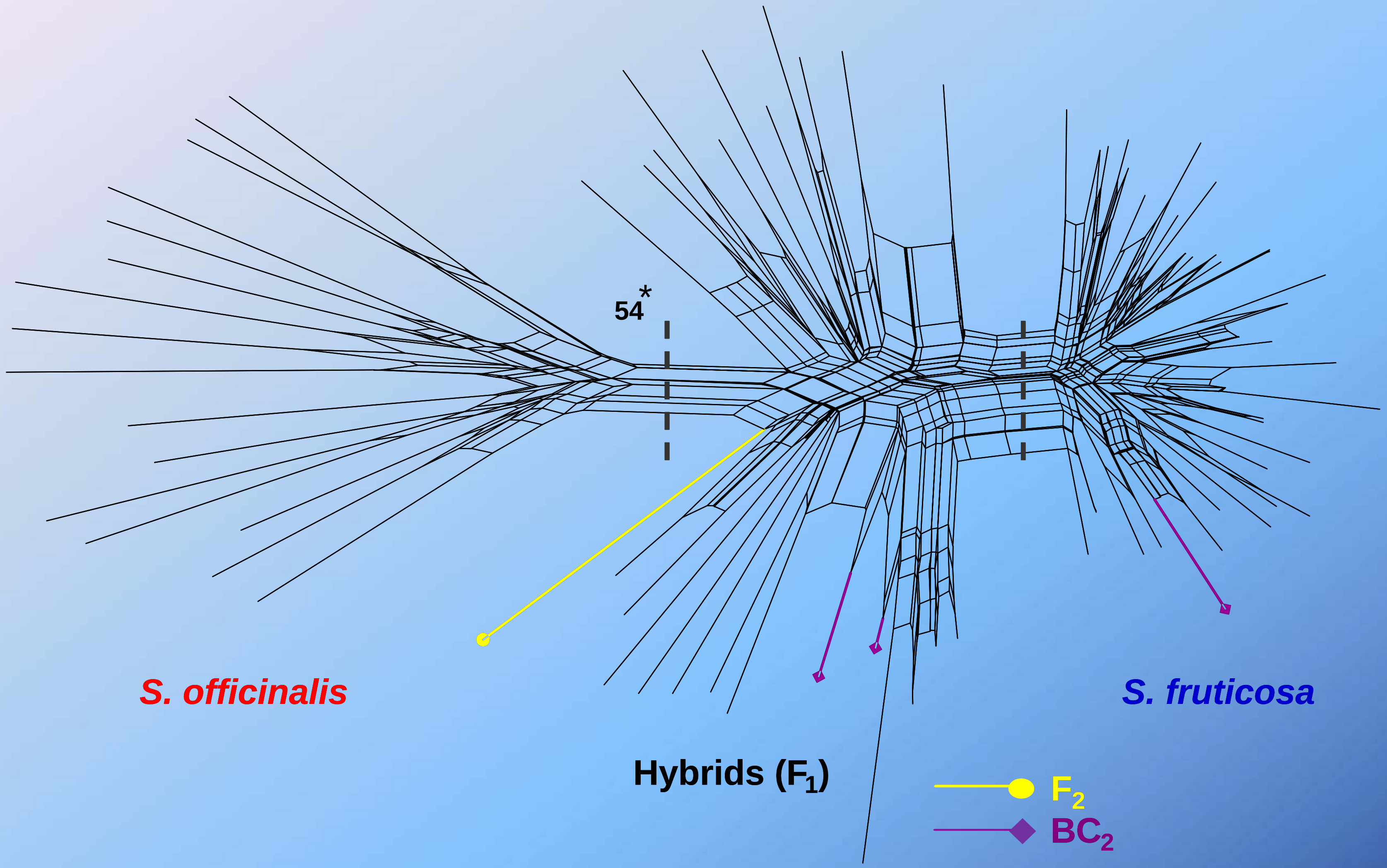
Allelic diversity of seven microsatellite loci scored in 79 Salvia plants from the island of Vis, Croatia.

No.	Marker	Repeat Motif	Range	N_a	PIC
1	SoUZ003	$(GT)_{13}$	190-212	9	0.739
2	SoUZ006	$(CT)_{31}$	209-238	14	0.829
3	SoUZ007	$(GT)_{11}$	204-229	7	0.571
4	SoUZ009	$(TG)_{15}$	212-229	5	0.314
5	SoUZ013	$(AAC)_{8}$	178-229	10	0.746
6	SoUZ014	$(AGA)_{10}$	194-248	14	0.573
7	SoUZ020	$(GAA)_{16}$	201-225	6	0.454
	Total			65	
	Average			9.29	0.604

Na - number of alleles; PIC - Polymorphic Information Content

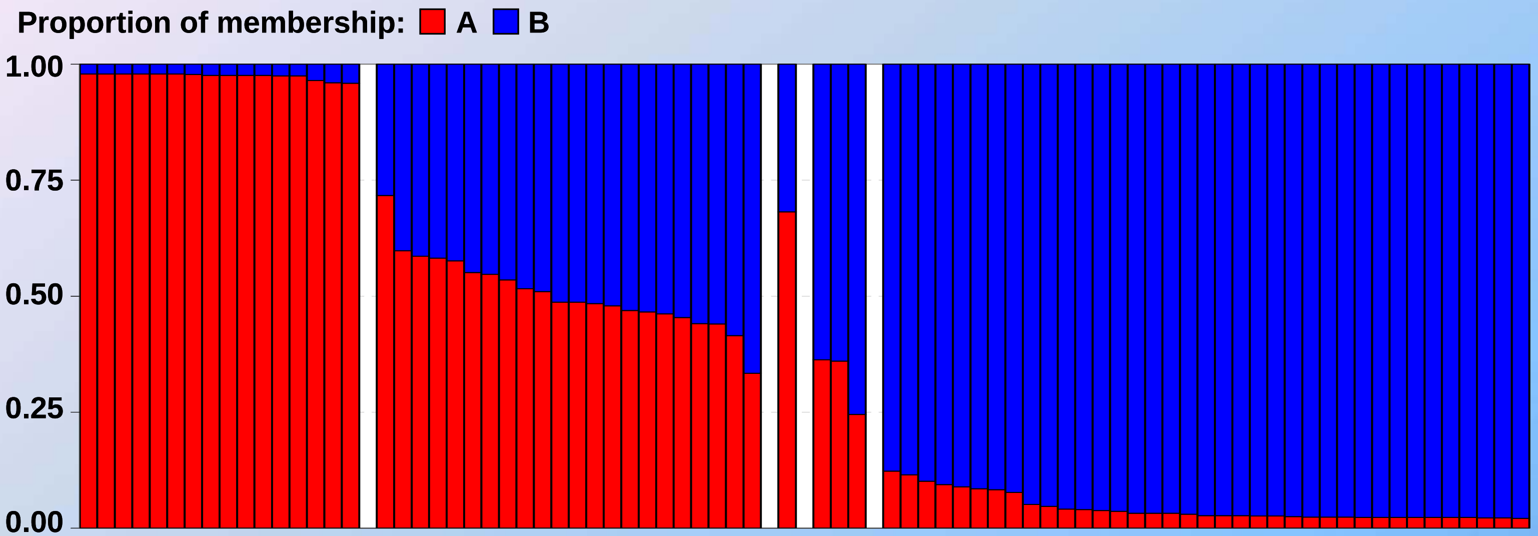


Venn diagram showing the share of 65 microsatellite alleles among two Salvia species and their hybrids

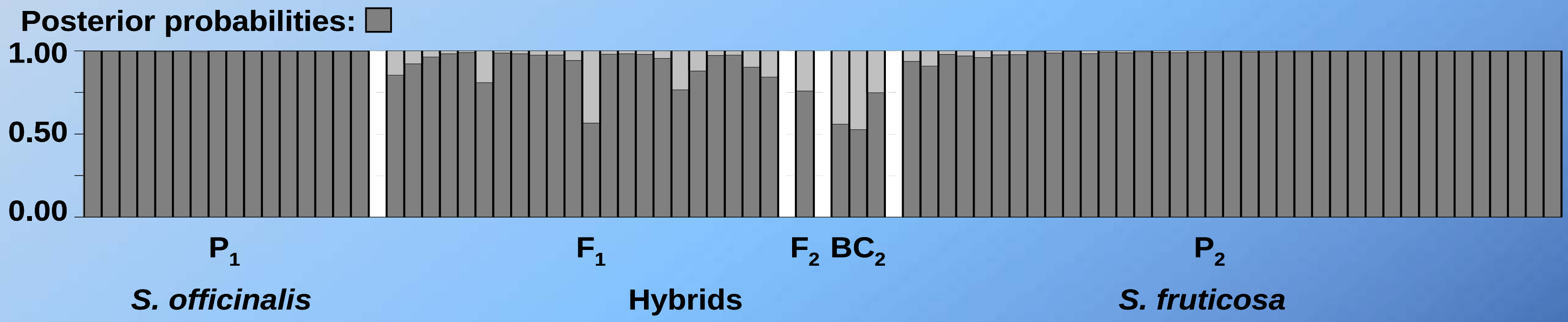


Neighbor-net diagram based on proportion-of-shared-alleles distance matrix among individuals belonging to S. officinalis, S. fruticosa and their hybrids

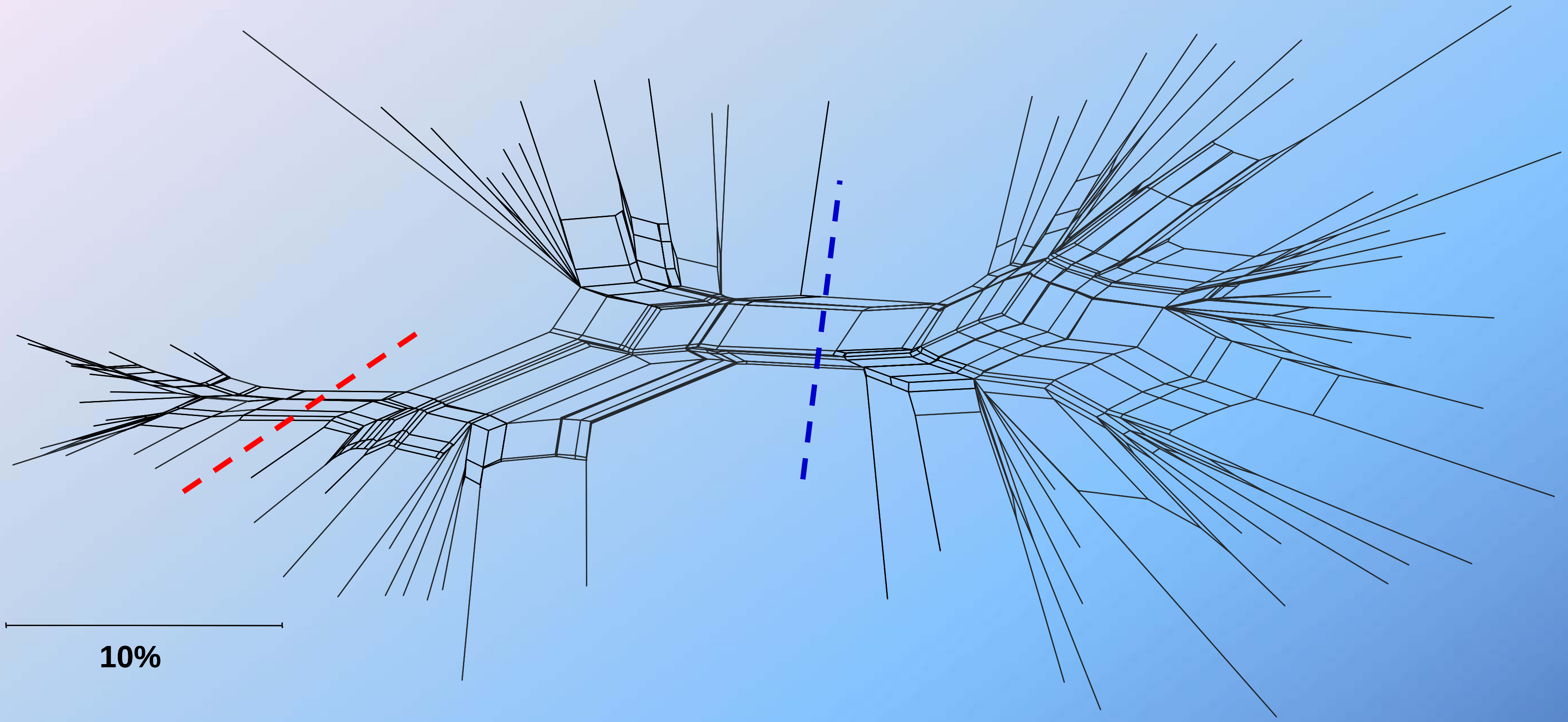
* *Boostrap support value was derived from Neighbor Joining analysis*



Proportions of membership of each individual in each of the two clusters as estimated by the program Structure (each individual plant is represented by a single vertical line)



Assignment of individuals into classes (parental S. officinalis and S. fruticosa, F_1, F_2, and BC_2) based on maximum posterior probabilities that each individual belongs to a particular class as estimated by the program NewHybrids



Salvia officinalis **Hybrids** ***Salvia fruticosa***

Neighbor-net diagram based on Gower distances among 23 qualitative and 19 quantitative morphological traits representing the relationships among 79 sage plants.

- - - *classification of individuals as it was in the SSR analysis*

CONCLUSIONS

- Molecular and morphological analyses confirmed the hypothesis about natural hybridization between *Salvia officinalis* and *Salvia fruticosa* on the island of Vis
- It is the first time that natural hybridization between these two sage species was confirmed
- Natural hybrids from the island of Vis represent a potential value for agronomy, pharmaceutical, food and cosmetic industries (e.g. they could unify good characteristics of both parental species or have unique combination of essential oil compounds)

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