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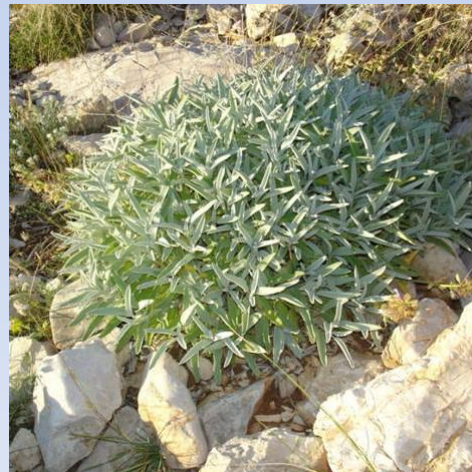
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CHLOROPLAST DNA DIVERSITY AND PHYLOGEOGRAPHY OF *Salvia officinalis* L. AND *Salvia lavandulifolia* Vahl



Salvia officinalis L. - common, garden or Dalmatian sage

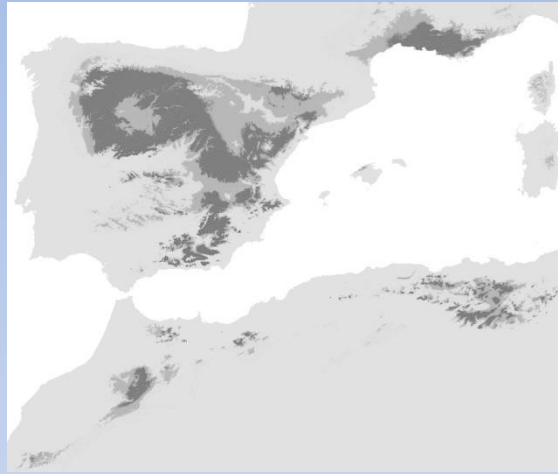
- type species of the genus *Salvia*



- thujones (12.5-62.8 %) are present in the highest percentage among almost hundred of compounds detected in the essential oil of Common sage (Jug-Dujaković et al. 2012)
- *Salvia officinalis* L. - with other 11 European species is a part of the section *Salvia* (*S. lavandulifolia*, *S. grandiflora*, *S. eichlerana*, *S. triloba*, *S. candelabrum*, *S x hegelmaieri*, *S. blancoana*, *S. brachyodon*, *S. ringens*, *S. pinnata*, *S. scabiosifolia* /Hedge 1972/)

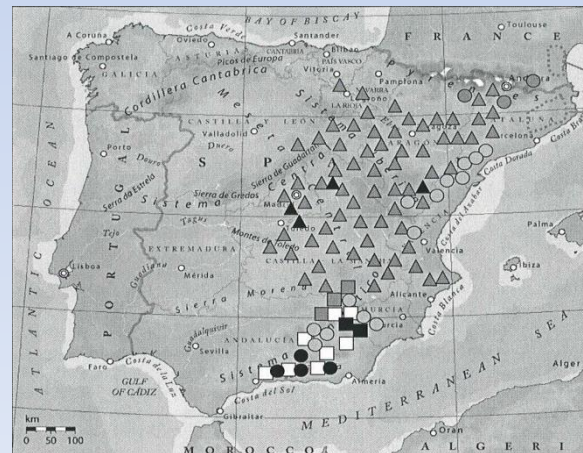
Salvia lavandulifolia Vahl - Spanish or Lavender-leaved sage

- subspecies of the *S. officinalis* ? (Reales 2004)



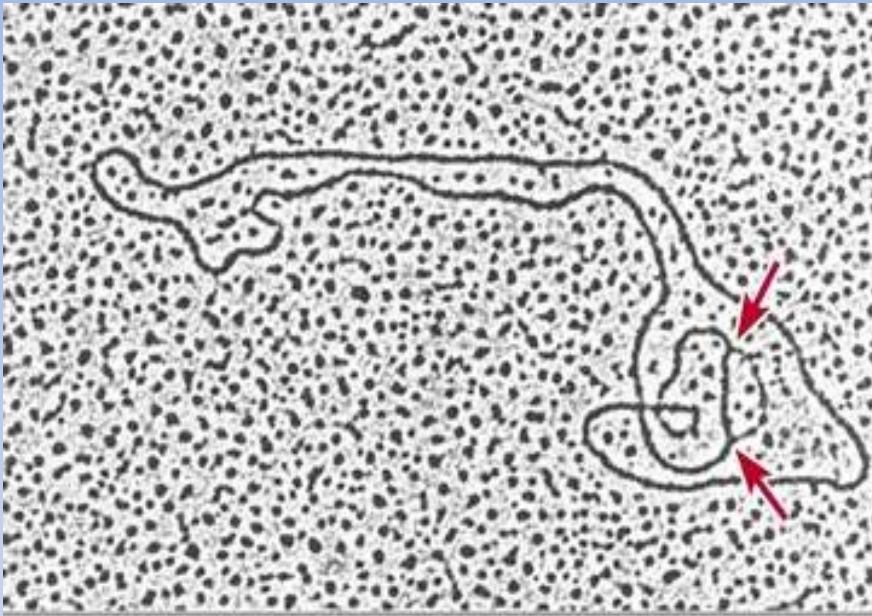
- 1,8-cineole (15.5 - 55.1 %) is present in the highest percentage in the essential oil of Spanish sage (Herraiz-Peñalver et al. 2015)

subsp. *lavandulifolia* ▲
 subsp. *oxyodon* ◻
 subsp. *blancoana* ◼
 subsp. *vellerea* ●
 subsp. *mariolensis* ▲
 (Flora Iberica / Saez 2010/)



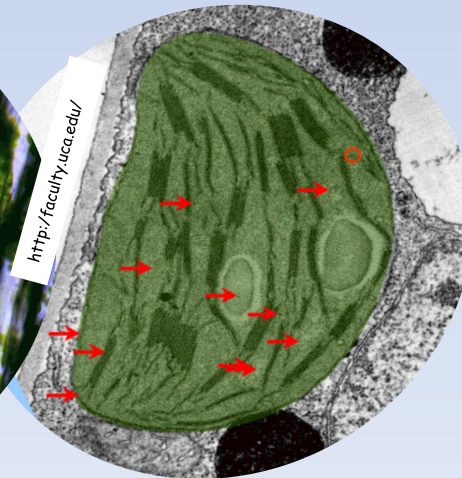
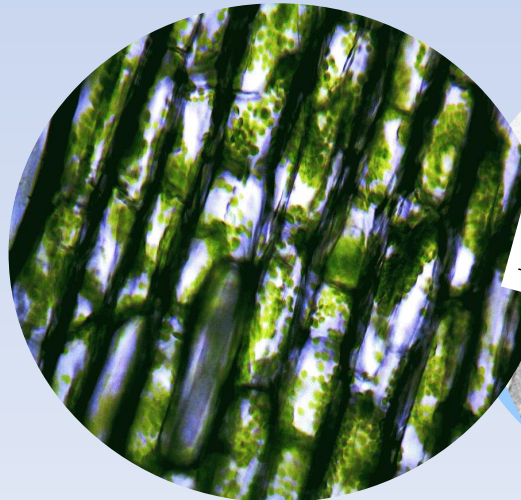
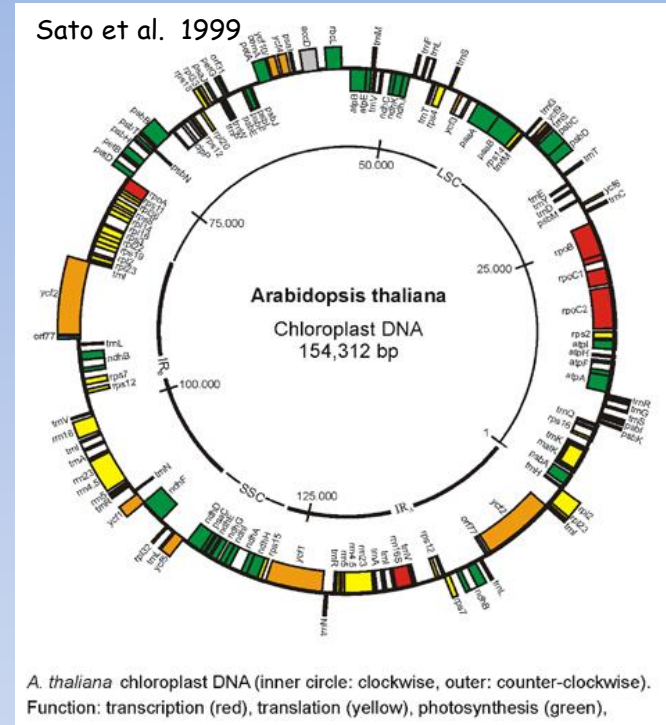
- ◼ Intermediate forms between subsp. *blancoana* and subsp. *oxyodon*
- ▲ Intermediate forms between subsp. *vellerea* and subsp. *lavandulifolia*
- Intermediate forms between subsp. *vellerea* and subsp. *oxyodon*

Chloroplast DNA

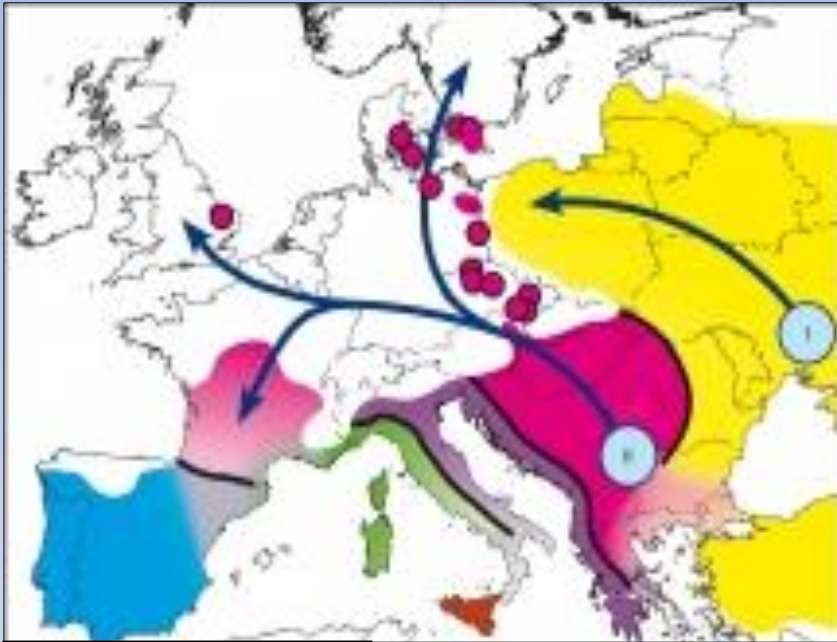


<https://www.slideshare.net>

0.25 μm

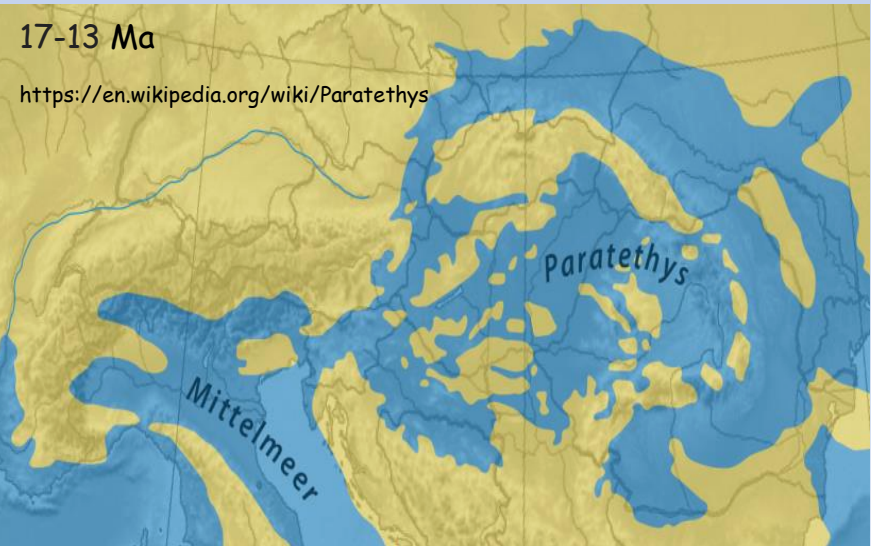
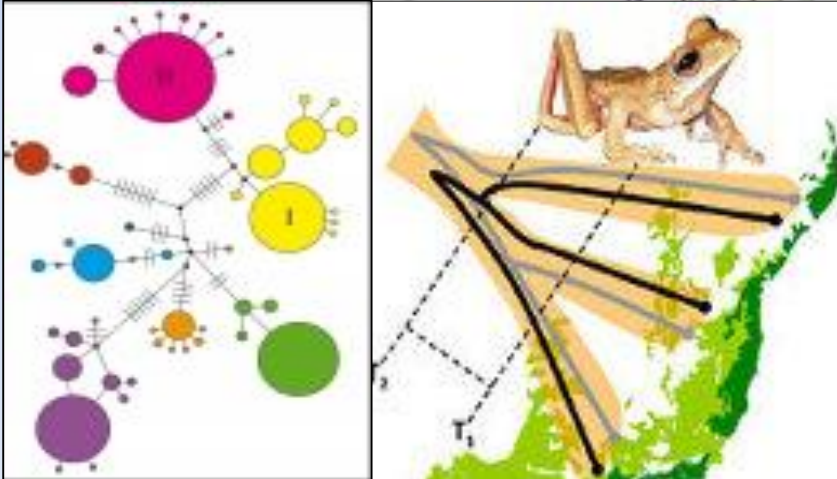


Phylogeography



The oldest findings of pollen of the genus *Salvia* originate from the lower Miocene in Mexico (Graham, 1999) This indicates an age of the genus of about 25 Ma.

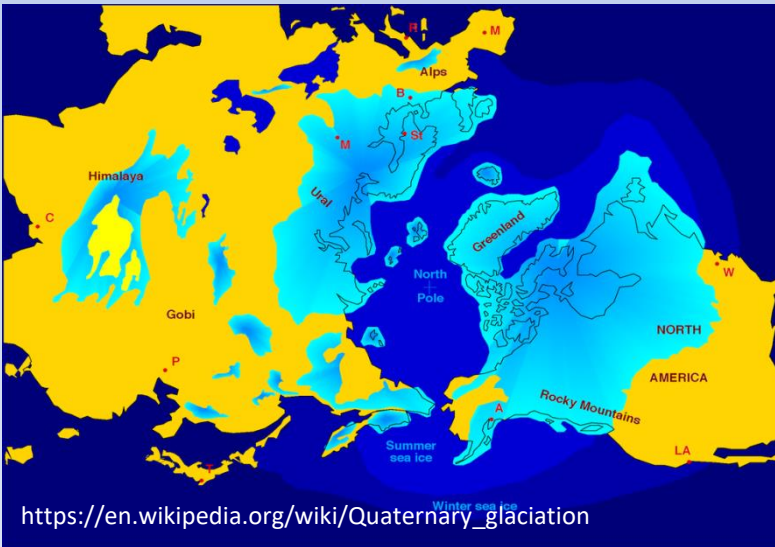
During the Miocene and Pliocene the genus spread extensively from 23 to 2.5 Ma (Claßen-Bockhoff et al. 2002.).





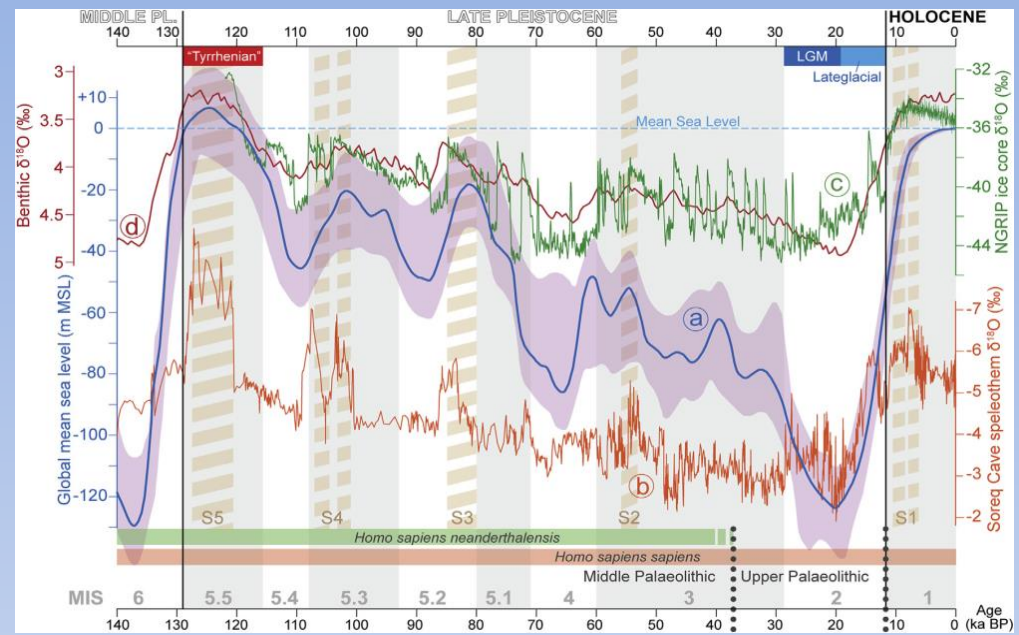
https://en.wikipedia.org/wiki/Messinian_salinity_crisis

Messinian Salinity Crisis (5.77-5.33 Ma)



https://en.wikipedia.org/wiki/Quaternary_glaciation

Several glaciation periods in the Pleistocene (2,5 Ma to 12000 years)



Reconstruction of global sea level in the past 140 ka modeled from palaeoclimatic, palaeoenvironmental and archaeological data (Benjamin et al. 2017)



Adriatic Sea during the Last Glacial Maximum (LGM) (Sikora et al. 2014.)

Environmental Niche Modelling

- process of using computer algorithms to predict present, the distribution of species in geographic space on the basis of incomplete information of occurrence and climate data (other variables such as soil type, water depth, and land cover can also be used)
- climate variables (WorldClim, Hijmans 2005):

BIO1 = Annual Mean Temperature	BIO10 = Mean Temperature of Warmest Quarter
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))	BIO11 = Mean Temperature of Coldest Quarter
BIO3 = Isothermality (BIO2/BIO7) (* 100)	BIO12 = Annual Precipitation
BIO4 = Temperature Seasonality (standard deviation *100)	BIO13 = Precipitation of Wettest Month
BIO5 = Max Temperature of Warmest Month	BIO14 = Precipitation of Driest Month
BIO6 = Min Temperature of Coldest Month	BIO15 = Precipitation Seasonality
BIO7 = Temperature Annual Range (BIO5-BIO6)	BIO16 = Precipitation of Wettest Quarter
BIO8 = Mean Temperature of Wettest Quarter	BIO17 = Precipitation of Driest Quarter
BIO9 = Mean Temperature of Driest Quarter	BIO18 = Precipitation of Warmest Quarter
	BIO19 = Precipitation of Coldest Quarter
- models are post-processed and visualised in computer programs for creating and using maps (e.g. ArcGIS Esri, Redlands, CA, USA).
- application in conservation biology, ecology, evolution etc.

Aims of the research

- 1) Sequence two non-coding chloroplast DNA regions of *S. officinalis* and *S. lavandulifolia* individuals from 88 natural populations from Iberian, Balkan and the Apennine Peninsulas
- 2) Determine the number and geographical distribution of chloroplast haplotypes
- 3) Match the results of chloroplast DNA analysis with the results of Ecological Niche Modeling
- 4) Compare genetic diversity between two closely related species as well as among Iberian, Apennine and Balkan Peninsulas
- 5) Find out centers of genetic diversity and centers of uniqueness (important for the future conservation, breeding programs and cultivation of these species!)
- 6) Identify possible refugia during unsuitable periods (e.g. glaciations) as well as migration routes during expansion periods

MATERIAL AND METHODS



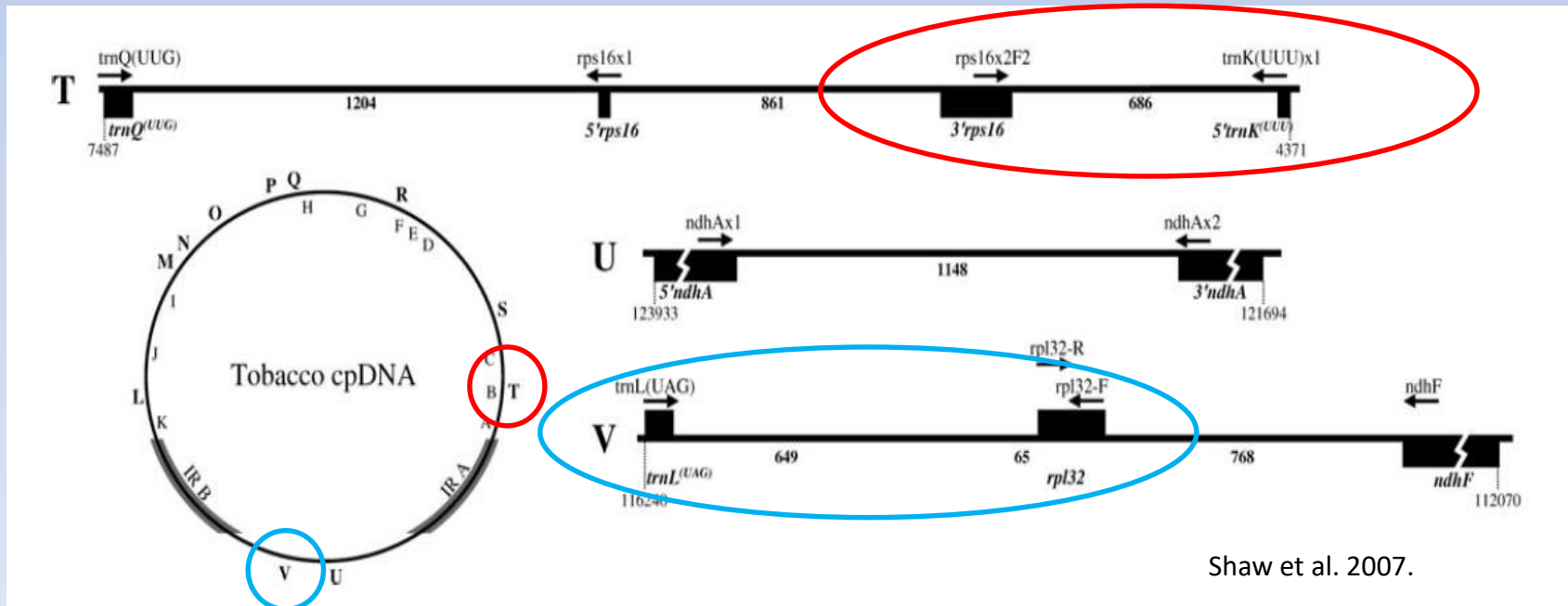
318 individuals from 61 populations of *S. officinalis*



130 individuals from 27 populations of *S. lavandulifolia*

DNA isolation, PCR amplification and purification of PCR products

- GenElute™ Plant Genomic DNA Kit (Sigma®)
- Nanophotometer Implen®
- PCR amplification



Shaw et al. 2007.

PCR primers:

rpl32_trnL	trnL(UAG): CTG CTT CCT AAG AGC AGC GT rpl32-F: CAG TTC CAA AA A AAC GTA CTT C
rps16_trnK	rpS16x2F2: AAA GTG GGT TTT TAT GAT CC trnK(UUU)x1: TTA AAA GCC GAG TAC TCT ACC

PCR solution (total 25 µl):

- 8.35 µl Nuclease-Free Water (Qiagen®).
- 2.50 µl 10xPCR Buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂; TaKaRa®)
- 2.00 µl dNTP (dATP, dCTP, dGTP, dTTP) mix (2.5 mM; TaKaRa®)
- 1.00 µl 5 µM PCR primer (PCR solution 1: *rps16x2F2*; PCR solution 2: *rpl32F*)
- 1.00 µl 5 µM PCR primer (PCR solution 1: *trnK^{UUU}*; PCR solution 2: *trnL^{UAG}*)
- 0.15 µl Taq HS DNA polymerase (5 U/ µl; TaKaRa)
- 10.00 µl DNA (c=0.5 ng/µl)

PCR program (GeneAmp® PCR System 9700 /Applied Biosystems®/):

- 94 °C 5 min,
- 35 cycles:
 - 94 °C 45s (DNA denaturation)
 - 53 °C 1 min (primers' binding)
 - 72 °C 1 min (DNA synthesis)
- 72 °C 10 min

Purification of PCR products:

- 5.0 μl PCR products
- 0.5 μl (10 U) exonuclease I
- 1.0 μl (1 U) FastAP™ alkaline phosphatase
- incubation at 37 °C 15 min
- stop the reaction by heating the mixture at 85 °C for 15 min

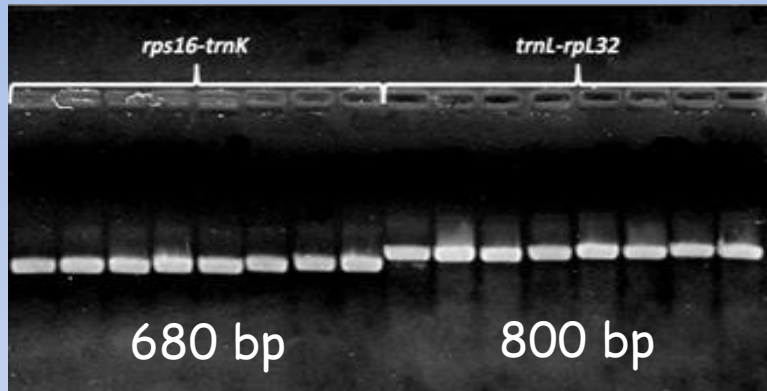
DNA sequencing:

- automated Sanger dideoxy sequencing approach using BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems®) and capillary electrophoresis on ABI 3730xl DNA Analyzer (Applied Biosystems®)

Data analysis

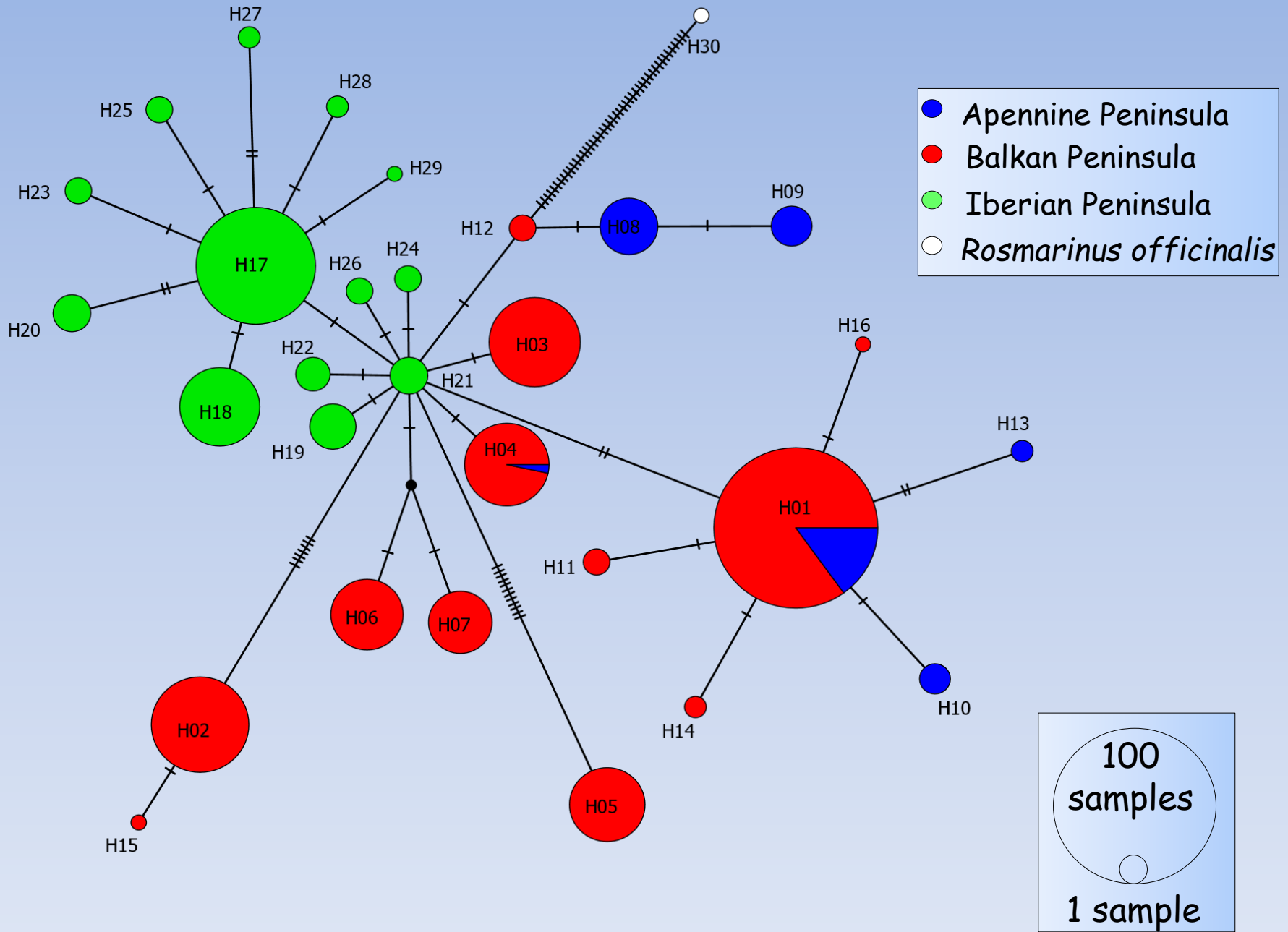
- Chloroplast DNA sequences were assembled, concatenate and aligned using Geneious 6.1.8. computer program (Kearse et al., 2012)
- Levels of within-population haplotype diversity were quantified by calculating the number of haplotypes (h), number of haplotypes per number of individuals and unbiased haplotype diversity (Hd; Nei, 1987) using Arlequin ver. 3.5 (Excoffier et al., 2010).
- Statistical parsimony networks were constructed from chloroplast sequence data (*rpl32_trnL* + *rps16_trnK*) using TCS network option (Clement et al., 2002) in PopART computer programme (<http://popart.otago.ac.nz>). Chloroplast DNA sequence of *Rosmarinus officinalis* L. was used as outgroup. Indels longer than 1 bp were considered as single base-pair and treated as fifth character state.
- Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) statistics were calculated using Arlequin to test for evidence of range expansion. The significance of both test statistics was tested using 10,000 bootstrap replicates.
- Ecological Niche Modelling for potential present, past and future distributions of *Salvia officinalis* and *S. lavandulifolia* were done by MAXENT ver. 3.3.3k using an algorithm for identifying species' suitable environmental space from incomplete information of occurrence (Phillips et al 2006). Models were based on WorldClim bioclimatic variables (Hijmans 2005). Models were processed and visualized in ArcGIS ver. 10.1. (Esri, Redlands, CA, USA)

RESULTS



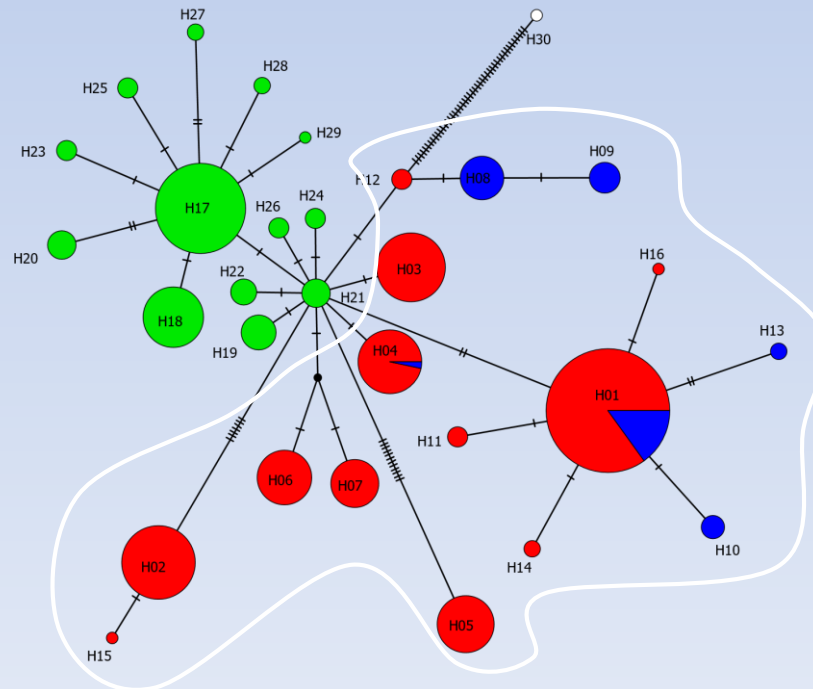
Haplotype	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12	H13	H14	H15	H16	H17	H18
S. off.	113	40	35	30	24	22	17	14	7	4	3	3	2	2	1	1	0	0
S. lav.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	27

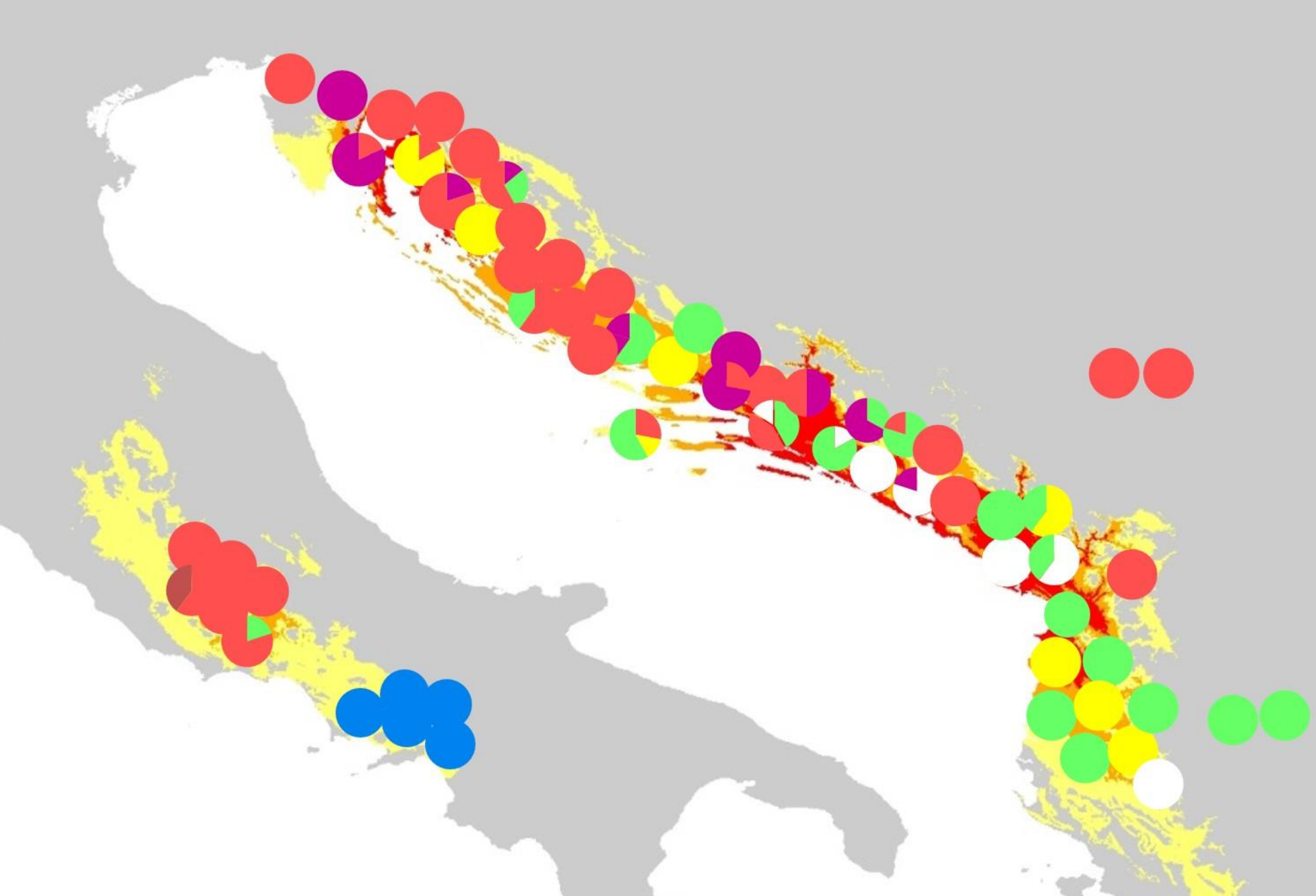
Haplotype	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	No individuals	No haplotype	Haplotype diversity (Nei, 1987)
S. off.	0	0	0	0	0	0	0	0	0	0	0	318	16	0.8233
S. lav.	9	6	6	5	3	3	3	3	2	2	1	130	13	0.7363



TCS Network (29 haplotypes + outgroup)

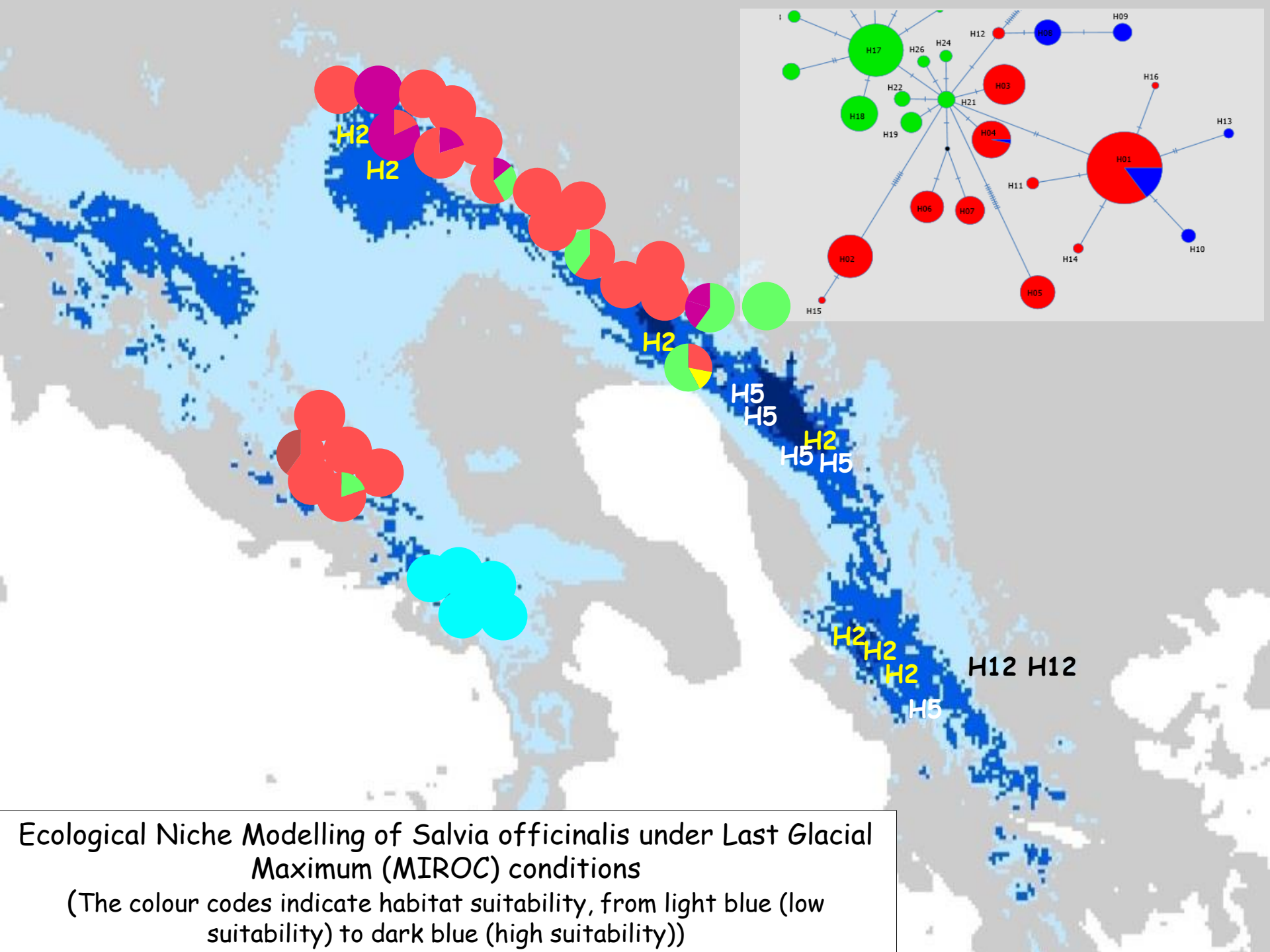
So	H01	H02	H03	H03	H04	H05	H05	H06	H06	H01	H01	H03	H07	H01	H02	H01
So	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12	H13	H14	H15	H16
H01																
H02	9															
H03	3	8														
H04	3	8	2													
H05	14	19	13	13												
H06	4	9	3	3	14											
H07	4	9	3	3	14	2										
H08	4	9	3	3	14	4	4									
H09	5	10	4	4	15	5	5	1								
H10	1	10	4	4	15	5	5	5	6							
H11	1	10	4	4	15	5	5	5	6	2						
H12	3	8	2	2	13	3	3	1	2	4	4					
H13	2	11	5	5	16	6	6	6	7	3	3	5				
H14	1	10	4	4	15	5	5	5	6	2	2	4	3			
H15	10	1	9	9	20	10	10	10	11	11	11	9	12	11		
H16	1	10	4	4	15	3	5	5	6	2	2	4	3	2	11	





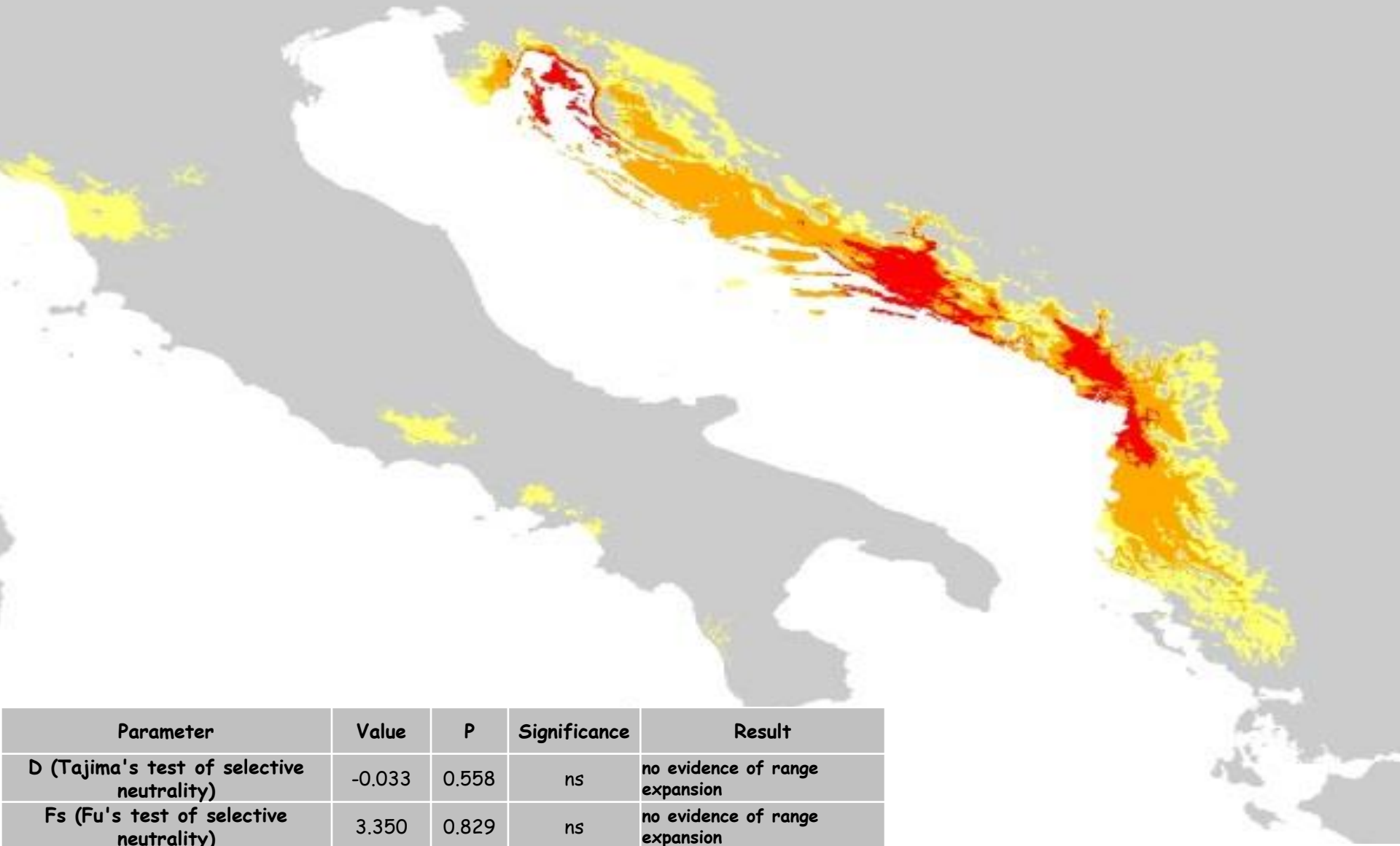
Ecological Niche Modelling of *Salvia officinalis* /present/

(The colour codes indicate habitat suitability, from yellow (low suitability) to red (high suitability))



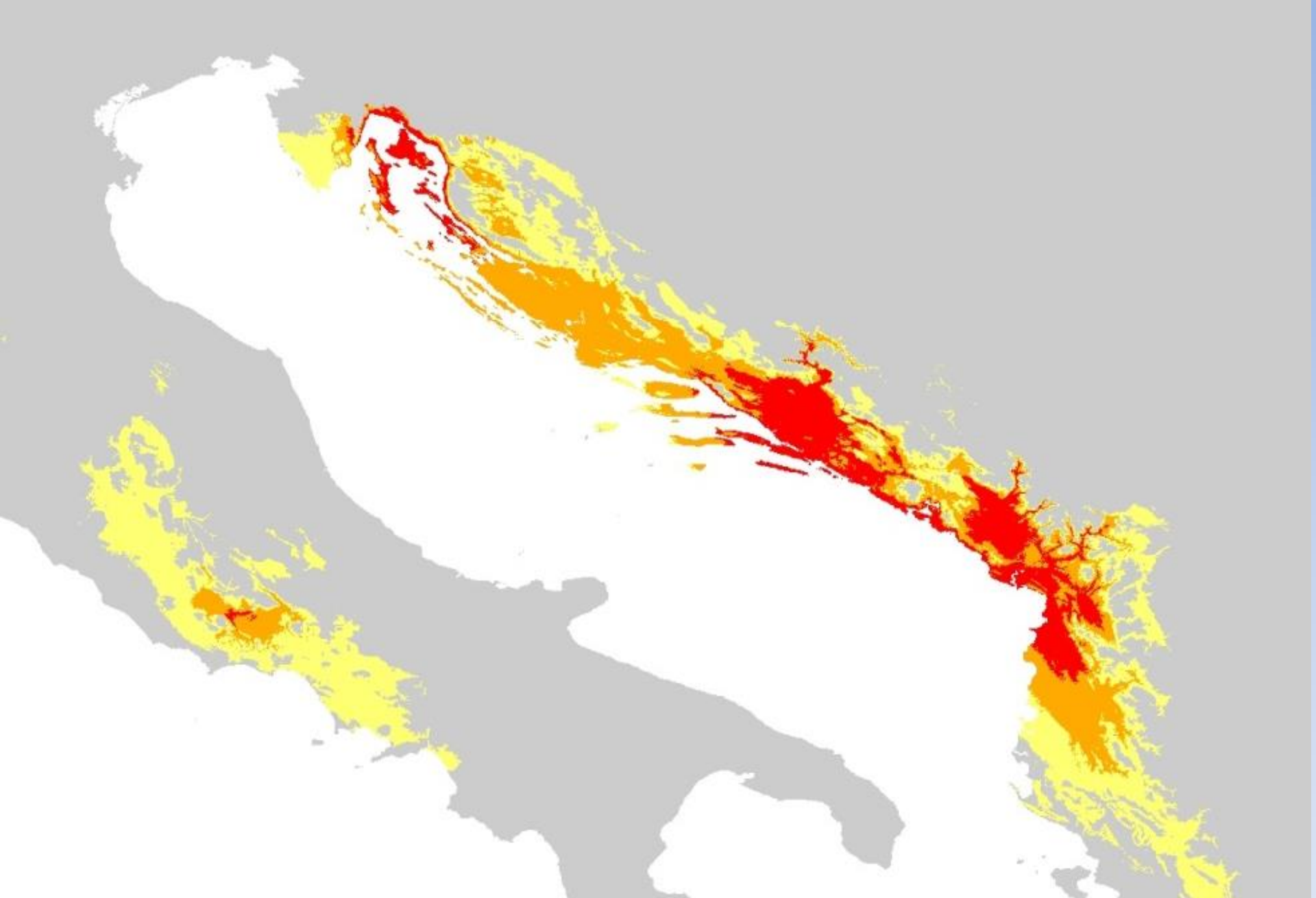
Ecological Niche Modelling of *Salvia officinalis* under Last Glacial Maximum (MIROC) conditions

(The colour codes indicate habitat suitability, from light blue (low suitability) to dark blue (high suitability))



Parameter	Value	P	Significance	Result
D (Tajima's test of selective neutrality)	-0.033	0.558	ns	no evidence of range expansion
Fs (Fu's test of selective neutrality)	3.350	0.829	ns	no evidence of range expansion

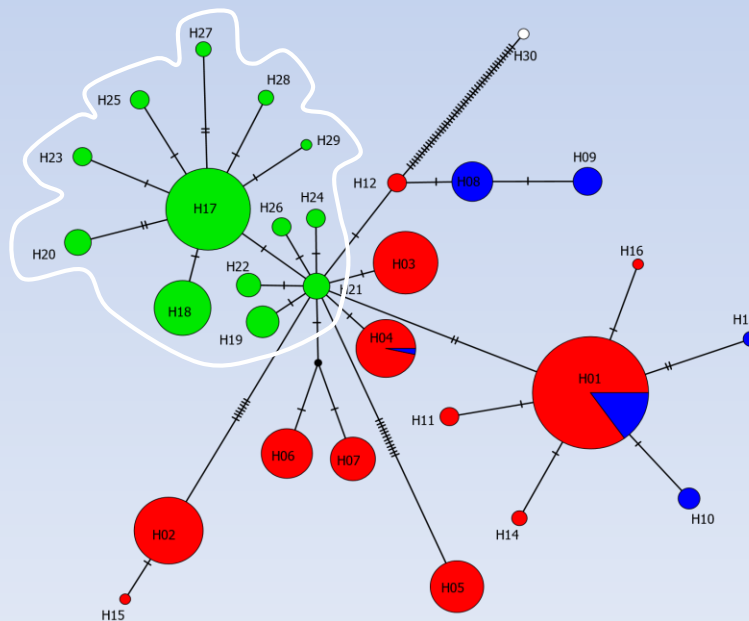
Ecological Niche Modelling of *Salvia officinalis* /future (2070)/
 (The colour codes indicate habitat suitability, from yellow (low suitability) to red (high suitability))



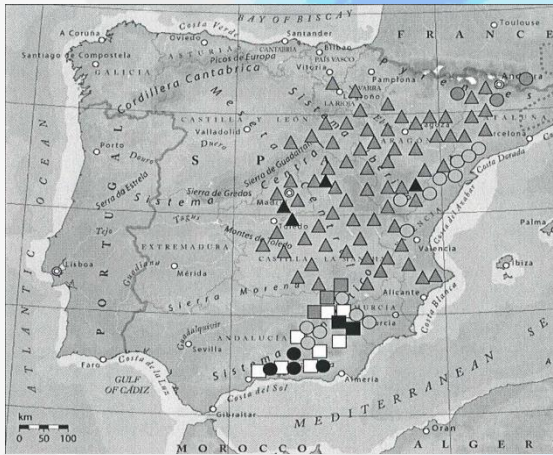
Ecological Niche Modelling of *Salvia officinalis* /present/

(The colour codes indicate habitat suitability, from yellow (low suitability) to red (high suitability))

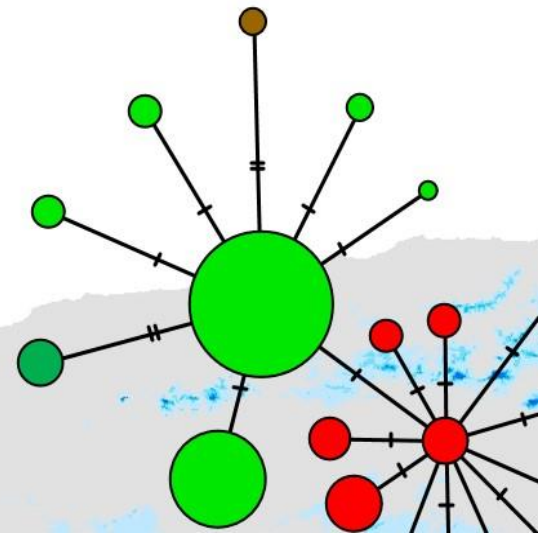
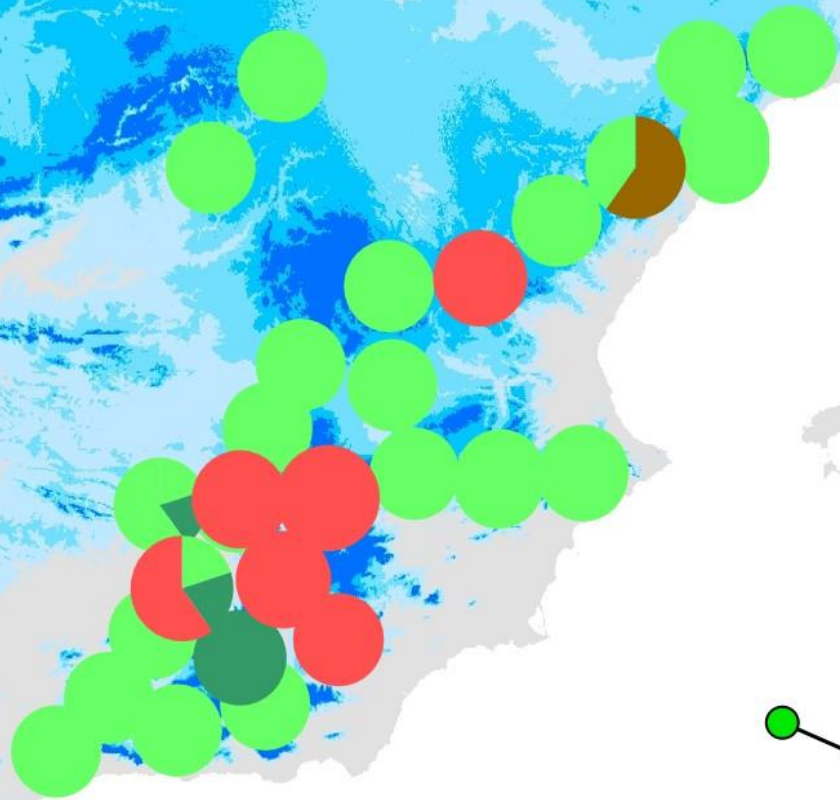
Slav	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29
Slav	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29
Slav	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27	H28	H29
H17													
H18	1												
H19	2	3											
H20	2	3	4										
H21	1	2	1	3									
H22	2	3	2	4	1								
H23	1	2	3	3	2	3							
H24	2	3	2	4	1	2	3						
H25	1	2	3	3	2	3	2	3					
H26	2	3	2	4	1	2	3	2	3				
H27	2	3	4	4	3	4	3	4	3	4			
H28	1	2	3	3	2	3	2	3	2	3	3		
H29	1	2	3	3	2	3	2	3	2	3	3	2	



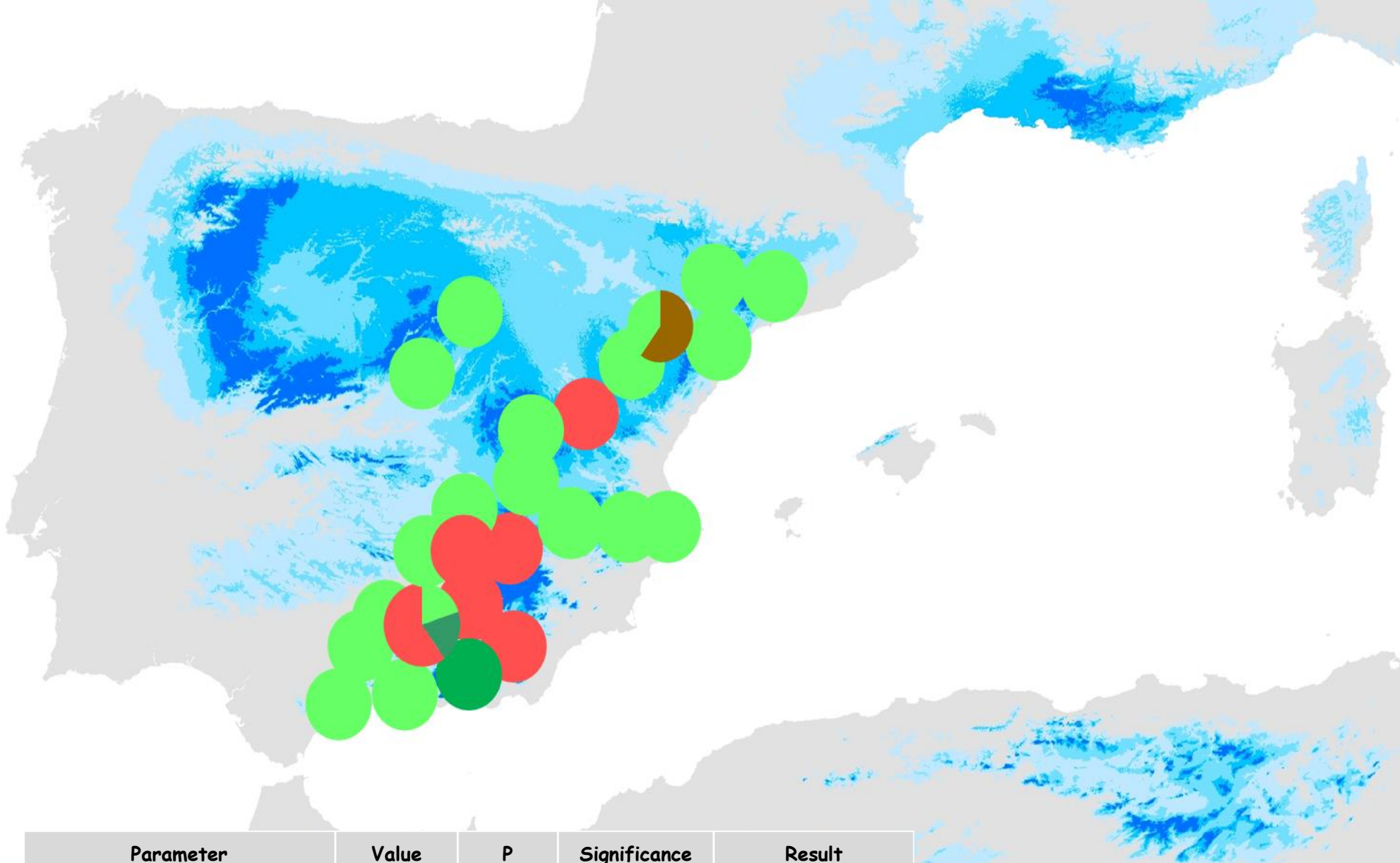
subsp. *lavandulifolia* ▲
 subsp. *oxyodon* □
 subsp. *blancoana* ■
 subsp. *vellerea* ○
 subsp. *mariolensis* ▲



■ Intermediate forms between subsp. *blancoana* and subsp. *oxyodon*
 ▲ Intermediate forms between subsp. *vellerea* and subsp. *lavandulifolia*
 ● Intermediate forms between subsp. *vellerea* and subsp. *oxyodon*



Ecological Niche Modelling of *Salvia lavandulifolia* /present/



Parameter	Value	P	Significance	Result
D (Tajima's test of selective neutrality)	-0.912	0.190	ns	no evidence of range expansion
Fs (Fu's test of selective neutrality)	-4.933	0.049	*	evidence of range expansion

Ecological Niche Modelling of *Salvia lavandulifolia* under present conditions

CONCLUSIONS

- 1) Chloroplast DNA diversity of *S. officinalis* and *S. lavandulifolia* was rather small (one mutation event differs most of the haplotypes).
- 2) *S. officinalis* and *S. lavandulifolia* don't share anyone haplotype (independent species!?).
- 3) The highest genetic diversity and uniqueness of haplotypes were found on the Balkan Peninsula.
- 4) Two independent methods (cpDNA diversity and ENM) confirmed several glacial refugia on the Balkan peninsula.
- 5) Several glacial refugia on Balkans have made demographic history of *S. officinalis* in this area stable (there is no evidence of range expansion)
- 6) *S. officinalis* on Apennine Peninsula showed clearly separated north and south genetic clusters
- 7) The northern Apennine populations contain chloroplast haplotypes characteristic of the eastern Adriatic coast (migration during glaciations!)
- 8) Southern Apennine populations showed the greatest similarity to several southern Balkan populations (old and relict populations!)
- 9) Star-like structure was a characteristic haplotype network's pattern of *S. lavandulifolia* suggesting sudden expansion during the recent history of the species (significant Fu's test of selective neutrality!)
- 10) Chloroplast DNA results didn't confirm morfological differences on which botanists had described a number of intraspecific taxa, intermediate forms and hybrids.

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