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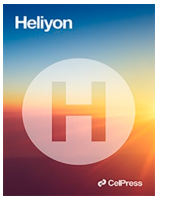

Research article

Genetic structure of populations of *Salvia ceratophylloides* endemic to southern Calabria (southern Italy)

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A B S T R A C T

Assessing the degree of genetic diversity and differentiation of rare or endangered endemic species is essential to evaluate the conservation status of populations and successively implement appropriate conservation strategies. We investigated the population structure of *Salvia ceratophylloides* Ard., a scapose hemicryptophyte endemic to Calabria (southern Italy), both to answer questions about its genetic structure and to determine whether the actual population size has undergone significant demographic changes in the near past. The data obtained from the census showed that the populations are characterised by a greater number of adult individuals than juveniles and are on declining. The genetic analysis carried out on 99 individuals from four populations of the species under study, shows a mean expected heterozygosity value of 0.50 and an overall differentiation value of 0.083. The population structure shows that the four studied populations are distinct genetic units, genetically linked to four different ancestral gene pools. Bayesian analysis based on ABC models indicates that the present populations underwent a significant reduction in size in the past. This corresponds to the demographic decline at the end of the 19th century, which according to the literature, was due to the strong anthropic pressure (agriculture, grazing, fire and plantations) of Reggio Calabria suburbs. We can therefore conclude that populations are not affected by inbreeding and low genetic diversity and that there is no immediate danger of genetic erosion, and that the problems associated with population decline, past and present, are exclusively due to anthropogenic causes.

1. Introduction

One of the most critical challenges to global biodiversity loss is the protection and conservation of threatened species, which requires an understanding of their biology and all the factors involved in maintaining their populations in the wild [1–3]. Although conservation strategies must consider biological, ecological and even political factors, the importance of considerations on the genetic structure of the species should not be underestimated [4]. Genetic diversity is not usually considered in biodiversity monitoring, and is considered separately from other conservation issues with the consequence that more than 10 % of genetic diversity might already be

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lost for many threatened and non-threatened species [5]. This is particularly true for plants which are endemic to the Mediterranean region, which is one of the 35 biodiversity hotspots, i.e. regions with a high species richness and endemism concentration where biodiversity is highly threatened [6]. Indeed, these areas are prone to disturbances from various sources that can have significant and often irreversible impacts [1]. Species from these areas have evolved by adapting to the unique climatic and geological conditions of the region and can now be considered as representatives of local plant biodiversity, also for their role for conservation of rare genotypes [7,8].

Plant species' evolution is closely influenced by genetic variability and differentiation [9]; the latter is strongly affected by both exogenous and endogenous variables, including distribution area, reproduction mode (closely linked to pollination type), seed dispersal processes, and life traits [10]. Gene flow through pollination and seed dispersal is more important for establishing the genetic diversification and structure of the population in fragmented environments than in continuous ones [11]. Degradation, fragmentation and reduced habitat availability affect the distribution of species, population size and, in particular, the genetic variability and population structure [12–16]. High levels of genetic variability conserve the population structure [17], which is fundamental for endemic and/or endangered species. Both aforementioned categories can be characterised by small populations growing in highly fragmented habitats and thus, more susceptible to environmental and biological stresses [18,19], as well as reduced genetic diversity [20]. In this way, the analysis of genetic variability and population structure is one of the main priorities of conservation biology. The importance of genetic variability and inbreeding depression for the species' ability to adapt to changing climate is an issue that has also been widely debated in the past [21]. To this end, though more modern techniques are now also being employed, the use of simple sequence repeat (SSR) molecular markers is still widely adopted in population genetic surveys of several species [22–25] including different species of Lamiaceae [26–28] as well such as those from the genus *Salvia* [29–31]. Molecular genetic markers are essential for understanding the speciation processes, species adaptability, their evolutionary patterns and mechanisms. All of the above can be used to apply concrete conservation strategies and protection measures for endangered species to aid with population restoration and ensure long-term survival [9,32–35].

The degree of genetic variability and differentiation of four relict populations of *Salvia ceratophylloides* Ard. (Lamiaceae), endemic to suburban Reggio Calabria (Southern Italy), were analysed, with the aim of answering both the question of their genetic structure and to estimate whether the actual population size has undergone significant demographic changes in the recent past. Together with several other studies [36–38], this study will be the basis for the development of conservation and management strategies for this very valuable endangered species.

Fig. 1. *Salvia ceratophylloides* Ard.

2. Materials and methods

2.1. Species description

Salvia ceratophylloides Ard. is an endemic species of the Lamiaceae family with a highly restricted distribution range to Calabria region (southern Italy) belonging to the subgenus *Sclarea* (Moench) Benth. (section *Plethiosphace* Benth.) and to the *Salvia pratensis* L. group [39,40].

Salvia ceratophylloides (Fig. 1) is a perennial herbaceous plant (scapose hemicryptophyte), between 30 and 90 cm tall, with erect or ascending stems, normally lignified and branched at the base, densely pubescent with both glandular and simple hairs. The leaves are pinnate-parted with toothed lobes. The basal and stem leaves are petiolate, those of the inflorescence are sessile. The inflorescences are very showy, measuring 20–30 cm long, and can be branched at the base, and consist of 5–8 whorls counting 4–6 flowers each. The corolla is bilabiate, purple, pubescent on the outside, with the upper lip folded into a hood over the stamens and measure 15–25 mm long. The fruit is a peculiar schizocarp: a microbasarium consisting of four dark brown, spherical to ovoid mericarps. The main flowering period occurs in spring, from April to June, and a second flowering period in autumn, from October to November [36]. Pollination is entomophilous, mediated mainly by Hymenoptera, particularly of the genera *Eucera* sp., *Bombus* sp., *Apis* sp. (personal observation). Fruiting occurs a few weeks after flowering. Seed dispersal is mainly carried out by ants (myrmecochory) [36,41],

Salvia ceratophylloides is a strongly aromatic plant, rich in essential oils [42], the composition of the volatiles produced by the glandular hairs suggests that they are involved in chemical defence against insects, which are mainly responsible, together with anthropogenic impact, to the decline in *S. ceratophylloides* populations [36,37,43]. Physiological studies conducted on this species have also shown that it possesses a high adaptive capacity to climate change and develops resilient forms of defence [44,45].

2.2. Study area and habitat

The distribution area of *Salvia ceratophylloides* lies entirely within the municipality of Reggio Calabria in southern Italy [38]. *S. ceratophylloides* is assessed as an endangered species and classified as "Critically Endangered" (CR) according to the IUCN [46] criteria and categories: indeed, it covers an 'extent of occurrence' (EOO) of 4.2 km^2 and an 'area of occupancy' (AOO) of 7 km^2 [36, 47]. Until 2008, this species was considered extinct [48–50] as it was no longer found in the stations cited in the literature [51–53]. However, over a decade ago its presence was once again reported at the stations of Puzzi and Mosorrofa, two villages close to the city of Reggio Calabria [54] the other two stations at Aretina and San Todaro were found as part of research undertaken into the conservation biology of this species [36–38]. To date, *S. ceratophylloides* populations do not benefit from any form of protection: it is not included in protected areas or Special Protection Area (SPA) and/or Special Areas of Conservation (SAC), nor is it included in the lists established by the Calabria Region for the protection of wild flora (Regional Law No. 47 of December 7, 2009 as amended).

The species grows in hills and valleys, at altitudes between 250 and 450 m a.s.l., characterised by layers of loose sands alternating with banks of Pliocene calcarenites [55]. The mean annual temperature is 18 °C while the mean annual rainfall is 600 mm, concentrated in November and December, and a dry summer period of about 5 months [36]. The studied populations fall within the Mediterranean pluviseasonal oceanic bioclimatic zone, with an upper thermo-Mediterranean thermotype and a lower sub-humid ombrotype [56].

Salvia ceratophylloides grows wild in the EEC Directive 92/43 habitat: "5330 thermo Mediterranean and predesert scrub", subtype "32.23 Diss dominated garrigues", Mediterranean steppe grasslands with *Ampelodesmos mauritanicus* (Poir.) Dur. & Schinz. *S. ceratophylloides* also grows in sandy steppe grasslands with *Artemisia campestris* subsp. *variabilis* (Ten.) Greuter and in garrigues characterised by *Cistus creticus* L. subsp. *creticus* and *Thymbra capitata* (L.) Cav [36,47].

2.3. Population census

The census of individuals was carried out throughout the area occupied by the examined stations (Table 1), using the methodology proposed by Hegland et al. [57] for the populations of *Salvia pratensis*, a species allied to *S. ceratophylloides*.

All individuals at each site were counted and sorted into five 'life stages': S = seedlings with cotyledons and one or two main leaves; category J/I that groups = (J) young seedlings with two/three pairs of leaves and (I) immature plants with three/four pairs of leaves; because in *S. ceratophylloides* the two stages are difficult to distinguish. V = non-reproductive vegetative adults with at least five pairs of leaves; G = generative adults, where one to five floriferous stems up to 50 cm high with large basal rosettes, are present. In category

Table 1
Populations of *Salvia ceratophylloides* Ard. used in this study and characteristics of the studied sites.

Location	Mosorrofa	San Todaro	Puzzi	Aretina
Altitude (m a.s.l.)	380	340	370	300
Exposure	NO	NO	NE	NO
Aspect (°)	20	0	5	20
Habitat	Grasslands with *Ampelodesmos mauritanicus* (Poiret) Dur. et Sch.	Garrigues with *Cistus creticus* L.	Grasslands with *Ampelodesmos mauritanicus* (Poiret) Dur. et Sch.	Garrigues with *Cistus creticus* L.
Distribution area (m^2)	22	1.210	521	130

G, two sub-classes of size were distinguished: Gs = small generative adults with one or two floriferous stems; Gl = large generative adults with more than two flower stems, usually the largest individuals are also the oldest (3–8 years). Each station was divided into sub-areas homogeneous in terms of habitat and population density to census the entire population. The area and number of individuals per "life stage" were then recorded for each sub-area.

2.4. Plant material collection

The plant material was collected from four different populations located in Aretina, San Todaro, Mosorrofa and Puzzi (Table 1). The populations are found from a minimum of 0.80 Km (between Aretina and San Todaro) to a maximum of 4.14 Km apart (between Mosorrofa and San Todaro) (Fig. 2). The sampled populations are the most relevant of the species, with the largest number of individuals, distributed in different habitats populated by *S. ceratophylloides* [38]. Between these populations we recorded the presence of small, scattered clusters of plants, which were not sampled in this study.

For each population, the intact young basal leaves of 30 plants at the life stage of small generative adults (Gs) were sampled. Sampling was carried out all at the same time in late spring, the optimal period for species phenology.

2.5. DNA extraction

Total genomic DNA was extracted from roughly 100 mg of fresh tissue using the Qiagen© Plant DNeasy kit, according to the manufacturer's instructions with the addition of a liquid nitrogen and steel beads during the sample preparation step. The average yield was around 30 ng/μl as estimated by NanoDrop (Thermo Fisher Scientific, Waltham, USA). Genotyping of all plants was done by six heterologous microsatellite markers (SSRs) from *Salvia officinalis* [29], chosen among those that yielded a simple pattern of amplification and whose sequences are reported in Table 2. All polymerase chain reactions (PCRs) with the DNA extracted were performed on a Mastercycler Gradient (Eppendorf AG, Hamburg, Germany) in 15 μl volume containing 1 μl of DNA (~1 ng/μl) and 14 μl mix. The six primer pairs were labelled with FAM, HEX and TAMRA fluorescent dyes for multiplexed genotyping. Cycling conditions were optimised for all loci, which differed for the annealing temperature (T_a). Initially, DNA was denatured for 5 min at 94 °C followed by 40 cycles of 94 °C for 45 s, annealing temperature (T_a) for 30 s, and 72 °C for 90 s. A final 8 min extension at 72 °C was included.

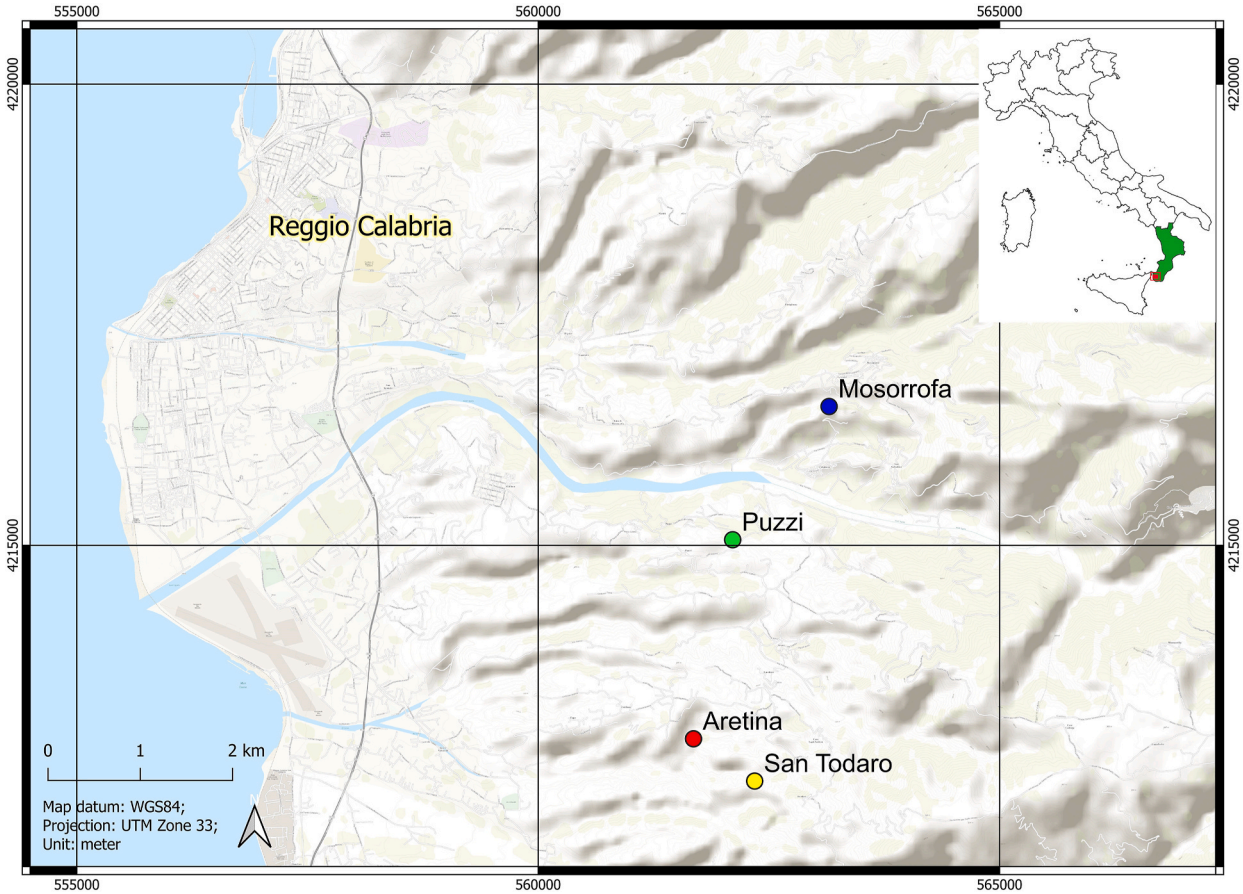

Fig. 2. Distribution map of the *Salvia ceratophylloides* Ard. studied populations.

Table 2
Primer sequences (F = forward; R = reverse), annealing temperatures (T_a), observed allele size in base pairs and PIC (Polymorphism Information Content) for the six SSRs used in this study. M = primers with the same M value were amplified in the same multiplexed PCR. The chromophore used with each primer is indicated.

Locus	M		Primer Sequence (5′–3′)	T_a	size	PIC
SoUZ004-FAM	1	F:	GCGATTGCAGGACTATCTATC	53 °C	152–162	0.11
		R:	CACGCTCATATTTATTCTTCTG			
SoUZ001-FAM	2	F:	GCAAACGCACTTTCCCACAA	53 °C	103–137	0.64
		R:	TTGTGCCCAAAACCTCCTCCTGATG			
SoUZ006 -TMR	1	F:	TGCAGAATGAGGCACCTACAG	53 °C	234–260	0.52
		R:	ATCACGGGCCTTTTCTTCCT			
SoUZ009-HEX	2	F:	GATGGGCTTGTGGTTGCAGA	54 °C	190–250	0.91
		R:	TCCACACACTCCACCATTGC			
SoUZ013-FAM	3	F:	ACCATGCCCAAAGACCATAA	54 °C	162–192	0.32
		R:	GGCTTCTCCCCTCGAATAAC			
SoUZ026-TMR	3	F:	TTCATCTTTGACCGGAAAAC	54 °C	146–182	0.48
		R:	CATGTGGTGATGCGAGATTC			

Amplification products were then sent to Macrogen (Seoul, South Korea) for fragment analysis. Allele sizing was performed on the Thermo Fisher Connect™ Cloud using the MSA (Microsatellite Analysis) app.

2.6. Data analysis

Allele frequencies, observed and expected heterozygosity were used to estimate genetic variation. Weir and Cockerham's [58] estimators of *F*-statistics were applied to analyse genetic diversity both within (F_{IS}) and amongst (F_{ST}) populations. Principal coordinate analysis (PCoA) was performed based on pairwise genetic distances between the individuals, under an infinite-allele-model. For the above analyses we used Genetix 4.05.2 [59] and the scatterplot3d package for R [60]. Polymorphism information content (PIC), was calculated with Cervus 3.0.7 [61]. To obtain the number of private alleles for each population we used the GenAlEx 6.5 software package [62]. Then, the presence of *null* alleles was estimated using Genepop version 4.7.5 [63] with the expectation maximisation (EM) algorithm [64].

To detect genetic characteristics of recent bottleneck events we used the software BOTTLENECK 1.02.2 [65]. We conducted four tests, with 1000 simulation iterations, under the TPM (Two-Phase Model) mutational model, which allows multiple step-mutation, as suggested by Piry [66] for SSRs. The Wilcoxon's test was used, being the most robust for less than 20 analysed loci.

Then, we used the Bayesian clustering analyses implemented in the program Structure 2.3.1 [67] to estimate the most likely number of ancestral gene clusters, *K*, via the Evanno Δ*K* method [68], using Harvester [69]. Ten independent runs were performed for each *K* between one and ten, with both the "admixture" and "no admixture" models using prior population information with LOCPRIOR [70], 50,000 MCMC iterations with a 10,000 iteration burn-in period. Based upon the value found for *K*, we then investigated the presence of genetic structuring in the studied populations.

We used the DIYABC version 2.1.0 algorithm [71] to investigate the demographic history of the ancestral genetic clusters identified by Structure by a coalescent-based approximate Bayesian computation (ABC). First, we allowed both present and past effective population sizes to vary freely, as to ensure that every possible demographic event could occur. To confirm that a scenario with population size change was supported, we tested the same scenario against the opposite one (*i.e.* contraction vs. expansion) via logistic regression, as in previous works [72–74].

Posterior parameter distributions were estimated under the best scenario using a linear regression on the 1 % closest simulations and applying a logit transformation to parameter values. Posterior distributions were obtained for three composite parameters: population diversity parameters N0μ0 (present), N1μ1 (past) and ratio r0 = N0μ0/N1μ1, plus all single parameters. We focused interspecific comparisons on the composite parameter r0 because it correctly represents ratios of present-to-past effective population sizes and on the single parameter T, the number of generations since the detected demographic event.

Table 3
Population census. Number of individuals occurring in the four studied populations, subdivided by "life stage" and population density (n. individuals/m^2).

Life stage	Mosorrofa	San Todaro	Puzzi	Aretina	Total
S	2	4	3	1	10
JI	24	29	21	9	83
V	20	133	155	8	316
Gs	14	166	103	115	398
Gl	3	92	133	2	230
Total	63	424	415	135	1037
Density	2.86	0.35	0.80	1.04	0.55

3. Results

3.1. Population census

The studied populations of *S. ceratophylloides* comprise a total of 1037 individuals (628 breeding individuals GS + GL). They are divided into the four populations as follows: 63 in the Mosorrofa population, 135 in Aretina, 415 in Puzzi and 424 in San Todaro, the latter being also the largest in terms of occupied area. The population density ranges from 2.86 individuals/m^2 in Mosorrofa, that occupies the smallest area, to 0.35 individuals/m^2 in the largest population of San Todaro, with an average value of 0.55 individuals/m^2 (Table 3).

The census shows that all examined population have a few seedlings (S), below five individuals, and a low number of juveniles/immature (J-I), except for the Mosorrofa station, where J-I is the most represented life stage (Fig. 3A). The vegetative (V) life stage has a high number of individuals in the larger stations with a greater number of plants, i.e. Puzzi and San Todaro (Fig. 3B–C). The small generative (Gs) is represented in all populations with many individuals: at the Aretina station it is the most represented with 115 plants (Fig. 3D). The life stage Gl is not very well represented except at the Puzzi station where 133 individuals occur (Table 3, Fig. 3E).

3.2. Genetic variability

Of the sampled *S. ceratophylloides* plants, 99 were successfully genotyped for six SSRs loci. Genetic diversity was estimated by means of the standard indices H_e, H_o and the average number of alleles per locus; the results are reported in Table 4. On average, the four populations show heterozygosity values ranging between 0.46 and 0.57. The number of alleles detected is again very close in all populations and an excess of heterozygous genotypes is indicated by the F_{IS} values, which are all below 0.022. As far as null alleles calculation is concerned we did not obtain a regular pattern, all populations had *null* alleles at one or two loci; loci *SoUZ01* and *SoUZ06* in Aretina; locus *SoUZ04* in Mosorrofa; loci *SoUZ4* and *SoUZ6* in Puzzi; loci *SoUZ01* and *SoUZ09* in San Todaro.

Analysis of population bottlenecks revealed no significant deviation from the expected heterozygosity for any locus (data not shown). This finding was also supported by the distribution of allelic frequencies, which in our case remain in a normal L-shaped distribution indicating that our populations are at mutation-drift equilibrium and did not undergo any genetic bottleneck event.

The overall genetic differentiation value estimated by F_{ST} was 0.083 with a confidence interval (95 %) ranging from 0.014 to 0.167. Values between population pairs are in the range from 0.067 (Mosorrofa – Puzzi) to 0.115 (Aretina - Mosorrofa) as reported in Table 5.

Principal Coordinate Analysis (PCoA) was then used to visualise genetic distances between plants, with the results depicted in Fig. 4. These results are generally in agreement with the differentiation estimates. The clusters of the San Todaro (yellow dots) and Aretina (red dots) populations being the most apart and the two populations show the second highest differentiation value (F_{ST} = 0.101). On the other hand, the clusters of the individuals of the Mosorrofa (blue dots) and Puzzi (green dots) populations are close to each other, and the two populations display the lowest differentiation value (F_{ST} = 0.067).

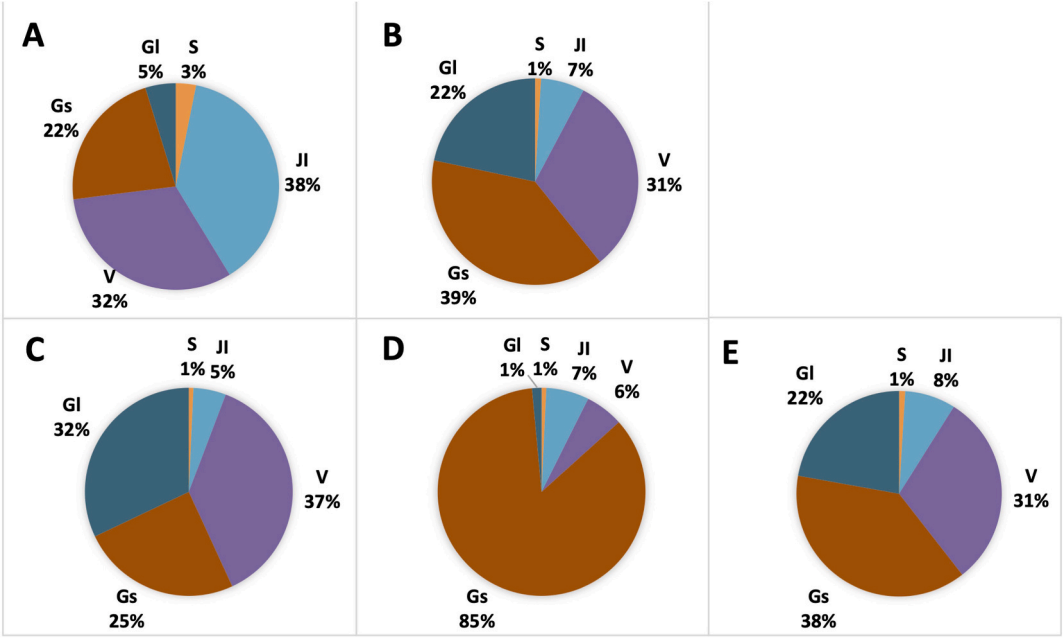

Fig. 3. Distribution of the "life stages" in the examined populations (A- Mosorrofa; B- San Todaro; C- Puzzi; D- Aretina) and in all examined populations (E).

Table 4
Genetic variability. Expected (H_e) and observed (H_o) heterozygosity, average number of alleles/locus (N_a), mean number of private alleles (Npa) and inbreeding coefficient (F_{IS}) are reported for each population.

Population	n° plants	H_e	H_o	N_a	Npa	F_{IS}
Aretina	29	0.48	0.47	4.2	1.17	0.022
Mosorrofa	26	0.46	0.61	3.7	0.33	−0.332
Puzzi	15	0.57	0.70	4.7	0.83	−0.215
San Todaro	29	0.47	0.60	3.7	0.83	−0.266
Mean		**0.50**	**0.59**	**4.1**		

Table 5
Genetic differentiation. F_{ST} values between population pairs. All values are significant at the 0.05 level.

	Mosorrofa	Puzzi	San Todaro
Aretina	0.115	0.099	0.101
Mosorrofa	–	0.067	0.091
Puzzi	–	–	0.075

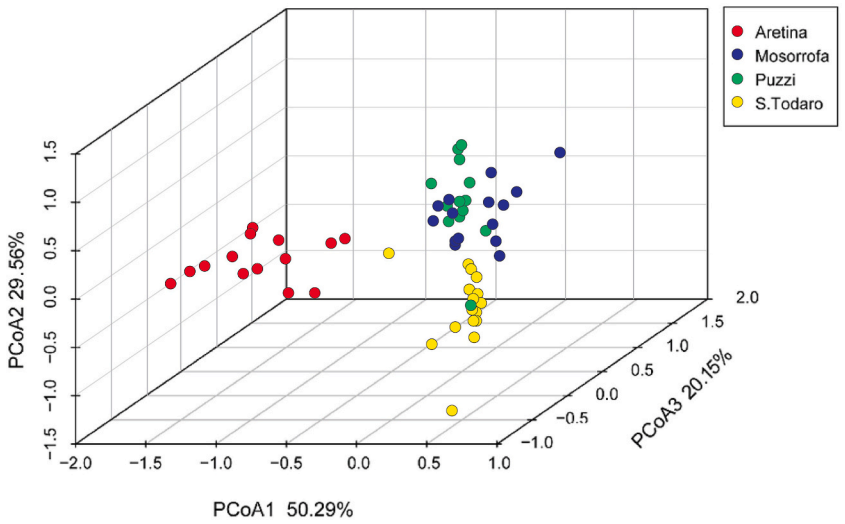

Fig. 4. Principal Coordinate Analysis. Each individual is plotted in the 3-D Euclidean space defined by the first three principal coordinates. Red dots = Aretina; Green dots = Puzzi; Blue dots = Mosorrofa; Yellow dots = San Todaro. Percentage of the total variation explained by each PCoA is indicated.

3.3. Population structure

We used STRUCTURE to determine most probable number of ancestral gene pools and to analyse the genetic composition of each population. By using both the admix and no admix models, the most probable number for *K* is four **[Supplementary Fig. 1]** as estimated by the Evanno method. Based upon this estimate, we used CLUMPAK to produce the bar plot of Fig. 5. The provenance from a single genetic cluster is clear for each of the studied populations, with the partial exception of Puzzi (the "green" cluster), that shows in the admixture model some introgression from the Mosorrofa (blue) gene pool. In the non-admixture model only a few individuals, two in the Puzzi population, one in San Todaro, shows evidence of mixed origin **[Supplementary Fig. 2]**.

3.4. Demographic analysis

Salvia ceratophylloides underwent some extreme event in the last few decades as indicated by its surmised extinction until its rediscovery in the 2000's [48,49], therefore, we deemed appropriate to detect possible demographic events occurred for the four *S. ceratophylloides* populations. To do this, each of the four homogeneous gene pools identified by STRUCTURE was studied by a coalescent-based Approximate Bayesian Computing (ABC) approach as implemented by DIYABC [71].

In ABC modelling, under the reasonable assumption that the mutation rate has not varied, $r_0 < 1$ indicates a demographic contraction event, $r_0 > 1$ an expansion event. The results of the analysis are reported in Table 6 as median values. For all populations of *S. ceratophylloides* we found $r_0 < 1$, signal of a demographic reduction at a given moment in the past.

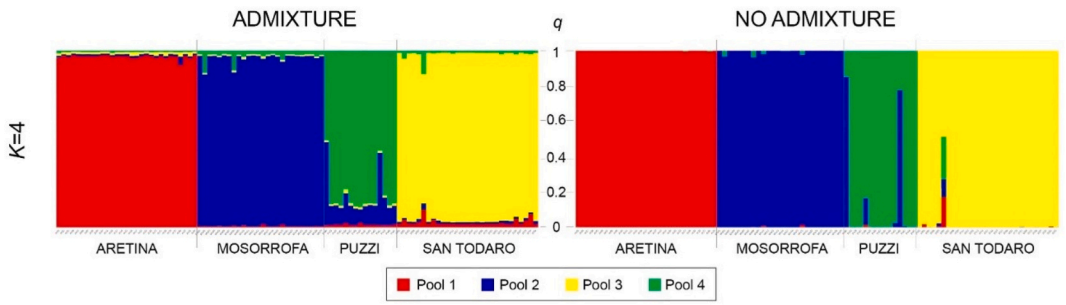

Fig. 5. Population structure. Bar plot for the genetic structure of the four populations of *Salvia ceratophylloides* Ard. The bar plots for $K = 4$ are shown for both the admixture model (left column) and the non-admixture one (right). Each vertical bar represents the genetic contribution to a single plant genome of the ancestral gene pool of a given colour.

Notably, the magnitude of the event is very similar for all populations, roughly indicating a 300-fold reduction in size, compatible with the alleged "extinction" of the species. The median age of demographic transitions of all populations varied between 224 and 394 generations and showed a remarkably consistent lower limit between 16 and 19 generations. The contraction episode was then compared with the alternative expansion event: the contraction shows a relative probability higher than 0.95, allowing us to safely assume that the contraction event was the most likely. To lend further strength to our findings, the posterior probability distribution of $\log_{10} r_0$ was plotted against the expected distribution in the case of constant effective population size **[Supplementary Fig. 3]**. The graphs show a sharply defined episode of decline in size for all populations, suggesting that even a relatively low number of markers was able to capture this event.

4. Discussion

In this work, the demographic and the genetic structure of the relict populations of *Salvia ceratophylloides* was studied so to better understand the chance of survival and persistence of this endangered species in its natural habitat. Out of the narrow endemic Mediterranean plants, which can represent up to about 30 % of the species total [75], only a limited fraction has been described also by genetic analyses. The study on this species is an ideal case for rare endemic characterized by fragmented distribution over a small area and facing extinction risk.

Salvia ceratophylloides is a very rare and localised endemic species that survives with a few scattered populations in the suburbs of Reggio Calabria city. According to IUCN criteria [46], is assessed as critically endangered (CR) in relation to the "Area of Occupancy" (AOO <10 Km^2) [36,47,54]. The census of the four populations shows that they are in severe decline, as all the populations observed, except Mosorrofa, consist mostly by adult breeding individuals (628; GS + GL) and very few young ones (S, JI and V). Conversely, Mosorrofa population is made up of more juvenile individuals (JI and V) and is the smallest in terms of surface area and number of total individuals and higher density probably due to the particular conformation of the territory which is often subject to landslides and fires [37,76]. The population of Mosorrofa is therefore the one most potentially at risk of extinction. San Todaro, Puzzi and Aretina populations, on the other hand, have more adults than juveniles, highlighting a regressive aptitude; this is particularly evident in the Aretina population, where the number of juveniles (S, JI, V) is about 13 % of the total number of individuals. What we have highlighted is in line with the analyses carried out by several authors on the population structure of Mediterranean endemic plants, which are dominated by adults rather than juveniles, since these endemics often live in difficult environments where there is less competition from more widespread species, showing an ecological strategy based more on persistence than on recruitment [77,78].

The amount of genetic variability found was medium to high, as indicated by the H_e values, ranging from 0.46 (Mosorrofa) to 0.57 (Puzzi). Fragmented populations are expected to display low genetic variabilities, but the rule is not a general one [79,80]. In our case, the high heterozygosity values are probably due to the fact that, in the past, populations consisted of a larger number of individuals, which allowed for greater gene flow. However, even in the congeneric species *Salvia brachyodon* Vandas, endemic to the Pelješac Peninsula (Croatia), H_e estimates using SSR markers in 15 natural populations gave values in the range 0.33–0.84 [29], showing that even in other small endemic populations, H_e values can be in the range of those found in our study. The inbreeding coefficient (F_{IS}) value shows that there is an excess of heterozygosity in the populations compared to the expected value according to Hardy-Weinberg

Table 6
Parameter estimation by ABC modelling. θ_0, θ_1: median of past and present diversity index ($\theta_n = N_n\mu_n$, with N_n the effective population size and μ_n the mutation rate at time n); r_0: median of the ratio θ_0/θ_1. T: Median of the time (in generations) of the demographic event. The credible intervals at 95 % are indicated.

	θ_0	θ_1	r_0	T
Aretina	0.13 [0.007–0.90]	35.5 [4.8–88.6]	0.0036	394 [19.0–4960]
Mosorrofa	0.05 [0.004–0.35]	22.3 [3.6–77.0]	0.0022	224 [16.8–2920]
Puzzi	0.09 [0.005–0.70]	29.4 [4.6–83.3]	0.0030	290 [15.8–4560]
San Todaro	0.09 [0.006–0.60]	30.5 [5.3–84.3]	0.0029	310 [16.6–4480]

equilibrium indicating that even given the small distribution range, three out of four populations have a healthy level of genetic diversity and are not undergoing inbreeding events.

The genetic differentiation values (F_{ST}) between pairs of population range from 0.067 to 0.115 values in the same range than species with similar size and distribution and analysed by the same molecular techniques [81,82]. In particular, the Aretina and San Todaro populations, despite their proximity are the most differentiated in absolute terms with the first having the highest F_{ST} values. This can be explained by the geographical isolation of the population, which compared to the others is embedded in a valley floor at 340 m a.s.l. surrounded by hills between 425 and 464 m a.s.l. that can be represent barriers for many species of pollinating insects. In favour of this interpretation, the lowest F_{ST} value is observed in the Mosorrofa and Puzzi populations, which are not separated by geographical barriers. It should also be noted that the population of San Todaro is characterised by a high number of breeding individuals (Gl and Gs), which favours cross-pollination within the population. Despite the small geographic distances, the high differentiation between the four populations indicates that they all deserve consideration from a conservation point of view, in order to preserve the species' adaptive potential in response to environmental changes, such as climate change or further habitat fragmentation. These results are confirmed by the genetic analysis of the population structure, showing that the populations were already ancestrally different, thus representing the contribution of four different gene clusters. This can also indicate that isolation-by-distance did not play a role in shaping the genetic differentiation.

Coalescent-based Bayesian analyses show that the studied population underwent a dramatic demographic decline; the estimated confidence interval for this event falls for the four populations at approximately the same time. Despite the indirect estimate, this finding is supported by the population dynamics analysis by means of the life stages. In fact, the four populations show very few non-breeding individuals and many breeding individuals, a clear sign of regression and demographic decline. The estimated time is also in agreement with historical data, setting the time for the demographic event between the end of 19th and the beginning of 20th century: from the literature data, this species was present in some areas where it is now extinct [51–53]. As reported by Lacaita [52,53], the first report for Reggio Calabria is attributed to Gussone, who found the species near Pietrastorta (Reggio Calabria) in July 1827. About 50 years later, Macchiati [51] claims to have found the species near Gallico, Cataforio and Gallina, localities near Reggio Calabria; to confirm this, Lacaita [53] cites an herbarium specimen in Turin collected by Macchiati under the village of Gallico (Reggio Calabria). In all the localities mentioned, the species is now extinct due to heavy urbanisation [36,37]. However, it should be remembered that the species was already rare and extinct in some places in the early 1900s, as reported by Lacaita [53], who wrote that he no longer found *S. ceratophylloides* in the locality previously collected by Gussone, but observed a few individuals some kilometres further away, between Terreti and Straorino (Reggio Calabria). Lacaita [53] also stated that the population of Pietrastorta was destroyed by cultivation, confirming that the probable cause of the population decline is due to the agricultural and urban expansion of the city of Reggio Calabria.

Since *S. ceratophylloides* is a perennial herbaceous plant (scapose hemicryptophyte) and that according to personal field observations it has an average age of about 7/8 years (survival time of a plant), the time period roughly spanning 1880–1920 falls within the confidence credible interval for the inferred population decline. Our results are a direct, although inferential, evidence of the effects on *S. ceratophylloides* due by human-induced changes habitat. The use of land for agriculture and grazing, the increasing demand for road infrastructure and the request for new housing probably caused the loss of *S. ceratophylloides* populations reported in the literature. In fact, the currently known localities in the Reggio Calabria area do not correspond to any of the historically reported populations but are about 15 Km away from them. In the recent past, in addition to agriculture and urbanisation, conifer plantations, often associated with frequent and repeated fires, have also been threats and pressure factors [36,83].

5. Conclusions

Endemic taxa with a restricted distribution, consisting of small and isolated populations, can persist in specific ecological niches and avoid extinction by means of ecological specialisation and habitat-specific adaptations essential for survival. However, habitat fragmentation and degradation, with the addition of more modern problems, such as the rising temperatures in the Mediterranean area under the global climate changes, can affect population structure and genetic diversity.

Albeit the loss of genetic diversity has been a hot topic for several decades, very few efforts have been made to detect climate change effects on genetic diversity with international biodiversity agreements ignoring the issue altogether.

The present study of *Salvia ceratophylloides*, a rare endemic species of the Italian flora, shows that the populations are not undergoing inbreeding, given the small geographic area and harbour a healthy level of genetic diversity, this suggests that there is no immediate risk of genetic erosion. The four populations under study are clearly distinct genetic entities that, whether assuming gene flow or not, are genetically pertain to four distinct ancestral gene pools. On the other hand, the census shows ageing populations with a low number of young individuals. In particular, the coalescent-based analysis of the genetic structure highlighted a past and current demographic decline of the population, which can be attributed mainly to anthropic causes, such as urbanisation, the increase in areas devoted to agriculture and grazing, often associated with frequent and repeated fires and conifer plantations, all activities recognized to increase the probability of species extinction, range reduction and habitat degradation.

These threats will probably stay in the near future, given the semi-urban localization of the *S. ceratophylloides* populations, so attention is required to protect this once considered extinct species. In particular, our findings will also be useful to establish *ex-situ* collections in botanical gardens, both for immediate conservation of the species and for the purpose of maintaining plant material for reintroduction.

Data availability statement

The data associated with this study have not been deposited in a public archive, because are included in the article/supporting material/referenced in the article.

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CRediT authorship contribution statement

Valentina Lucia Astrid Laface: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marta Cavallini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonino Di Iorio:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Gianluca Lombardo:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Giorgio Binelli:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Agostino Sorgonà:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Carmelo Maria Musarella:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation, Conceptualization. **Giovanni Spampinato:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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