



# Genetic diversity and reproductive ecology of the sage-leaved rockrose, *Cistus salviifolius* L., in the Swiss Alps

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**Abstract** Range marginal populations are often perceived to have lower conservation value compared to those in the core area. The allocation of resources to maintain peripheral populations is therefore often questioned. The sage-leaved rockrose (*Cistus salviifolius* L.) is a self-incompatible and obligate seeder widely distributed in the Mediterranean area but rare and patchily distributed in Switzerland at its range margin on the southern slopes of the Alps. Here, we combined analysis of genetic diversity with pollinator surveys and field studies of reproductive ecology to compare peripheral *Cistus* populations in the Alps with range central populations in the Mediterranean. Our results showed no differences in genetic diversity

between peripheral and central populations and between fragmented and connected ones at its range margin in the Alps. Although the fragmented populations were visited by more abundant and species richer pollinators (bees and wasps), they showed lower number of seeds and higher self-compatibility compared to the connected ones, which excludes the pollination limitation hypothesis. Overall, our study highlights that peripheral populations of *C. salviifolius* in the Alps are likely to contribute to maintain genetic diversity, while showing variation in reproductive ecology, and are therefore important for the conservation of this species.

**Keywords** AFLP · Conservation · Marginal populations · Genetic variation · Pollination

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## Introduction

When considering genetic variation of populations across species distribution range and their potential to undergo evolutionary changes, two main contrasting hypotheses have been reported in the literature: the center–periphery *versus* the core–leading-edge hypotheses (Pironon et al. 2017). According to the center–periphery hypothesis, the abundance, density, and genetic diversity of populations are likely to decrease towards the range margin (Eckstein et al.

2006). This is because central populations are thought/are likely to experience favorable habitat and climatic conditions compared to peripheral ones, where habitat tends to be less suitable and more stressful (Hoffmann and Blows 1994; Holt and Keitt 2000; van Heerwaarden et al. 2009; Van Rossum et al. 2003). High level of random genetic drift and inbreeding as well as low level of gene flow present in peripheral populations are expected to lead to a reduction in genetic diversity within populations (Hoffmann and Blows 1994; Lammi et al. 1999; Young et al. 1996), and increased genetic differentiation among populations (Vakkari et al. 2006). In addition, population size and isolation might affect the flower-pollinators interaction networks (Moeller 2006), potential leading to potential pollination limitation and reduced plant reproductive success (Ågren 1996; Jennersten 1988). However, this hypothesis is not consistently supported by empirical studies, as marginal populations often show similar demographic performances and levels of genetic diversity as population located at the center of a species range (see Pironon et al. 2017 for a synthesis). In this regard, the core-leading-edge hypothesis provides a more dynamic explanation for patterns otherwise attributed to a static contemporary center-periphery (Dai and Fu 2011; Eckert et al. 2008; Pironon et al. 2017). According to the ‘leading-edge’ model of colonization, range expansions are likely to lead to lower levels of genetic diversity at the colonization front due to a combination of rare long-distance dispersal events followed by exponential population growth, as it is observed in postglacial colonization bottlenecks (Hewitt 2000). In addition, mating system and reproductive ecology could show differences in leading-edge populations, reflecting the lower abundance of pollinators and/or lower population density (Pannell et al. 2015). For example, loss of self-incompatibility has been repeatedly described in small and highly isolated populations (Glémin et al. 2005; Silva et al. 2016). In fact, because of the potential adaptation to stressful environments, leading-edge populations are likely to harbor rare alleles and gene combinations which are important for adaptation to extreme environmental conditions and thus play a major role for preserving genetic variation (Eckstein et al. 2006; Hampe and Petit 2005; Lesica and Allendorf 1995). In the light of global change created by climate and human impact on landscapes, leading-edge populations may thus be important for

the evolution and conservation of species (Razgour et al. 2013).

The sageleaf rockrose *Cistus salvifolius* L. is an Eumediterranean shrub widespread across the entire Mediterranean area in Europe and occurring in a wide range of habitats, from sea level to the mountain regions in the Swiss Alps and Spain. *Cistus salvifolius* is considered to be a pyrophytic shrub, with seed germination being stimulated by the heat of wildfires, as well as by competition-free post-fire conditions (Thanos et al. 1992). Thus, fire and fire regime seem to be one of the most important limiting factors for the distribution of the species. The *C. salvifolius* is a diploid insect pollinated shrub. The inflorescence holds one or more round flower from April to May in the Mediterranean (one month later in the Alps) and the large white flowers are mainly visited by bees and wasps, and to a lesser by overflies and beetles. The *C. salvifolius* is outcrossing and highly self-incompatible (Bosch 1992). Self-incompatibility in large populations helps to prevent mating among individuals harboring the same self-incompatibility alleles. However, in small and fragmented populations, this mechanism may further reduce the number of potential mating partners and can thus be detrimental.

In Switzerland, *C. salvifolius* occurs only on the southern slope of the Alps, a fire prone region, characterized by an irregular winter fire regime (Pezzatti et al. 2010), and here, *C. salvifolius* populations largely overlap the distribution of wild fires (Moretti et al. 2006). The origin of its presence in the Alps is so far unknown: paleoecological investigations in the region have never reported the presence of pollen of *C. salvifolius*. First reports of the species on the southern slope of the Alps date back the eighteenth century (Haller 1768) and the nineteenth century (Franzoni 1890; Gaudin 1833), see Ceschi (1995) for a synthesis.

More recently, species distribution modeling (Moretti et al. 2006) have highlighted that change of fire regime in the past 20 years (Pezzatti et al. 2010) is threatening *C. salvifolius*, whose population survive at fragmented vegetation-free sites, such as emerging bedrock, ridge locations or steep slopes temporarily compensating for such fire-free periods. For this reason, in Switzerland, *C. salvifolius* is red listed as a vulnerable species due to the highly fragmented populations and their negative demographic trends over the past 10 years (Bornand et al. 2016).

The aim of this study was to provide a contribution in the understanding on the importance and role of these peripheral and fragmented populations where climatic and environmental conditions differ substantially from those at the range center (Ceschi 1995; Moretti et al. 2006, 2008). In particular, our study aimed to (1) investigate genetic diversity within and between populations of *C. salviifolius* at its range margin in Southern Switzerland (i.e. peripheral populations) and core population in the Mediterranean core area (i.e. central populations), and (2) assess genetic variability in fragmented and connected peripheral populations. Pollination experiments and pollinator surveys were applied to test for pollination limitation and for evidence of reduced self-incompatibility in the fragmented compared to the connected populations in the Northern range margin in Southern Switzerland.

## Materials and methods

### Study area

The study area is located on the southern slope of the Swiss Alps in central Europe (Canton Ticino, 8°70′–8°85′ E, 46°10′–46°20′ N; Fig. 1) at an elevation ranging between 298 and 795 m a.s.l. and it is part of the insubric bioclimate region (Berger and Walther 2006). The climate is warm and temperate with a mean annual precipitation of 1600–1700 mm and a temperature of about 12 °C (mean monthly temperatures of 1 °C in January and 22 °C in July). In contrast to the more southerly regions with a Mediterranean climate, the rainfall is higher in summer (June–September, ca. 800 mm) than in winter (November–February, ca. 400 mm). The soil is siliceous and vegetation is dominated by former coppice stands of the European chestnut *Castanea sativa*, followed by oak (*Quercus* sp.) and beech (*Fagus sylvatica*). These forests were traditionally managed as coppice and woody pastures, and are now undergoing an abandonment processes (Conedera et al. 2001) leading to an expansion of the forested area into clearings, gaps and other open habitats often occupied by warm loving vegetation (Moretti et al. 2008; Staehli 2005). The southern slope of the Alps is the Swiss region with the highest number of wildfires occurring mainly in winter in contrast to the Mediterranean summer fire regime. During the last

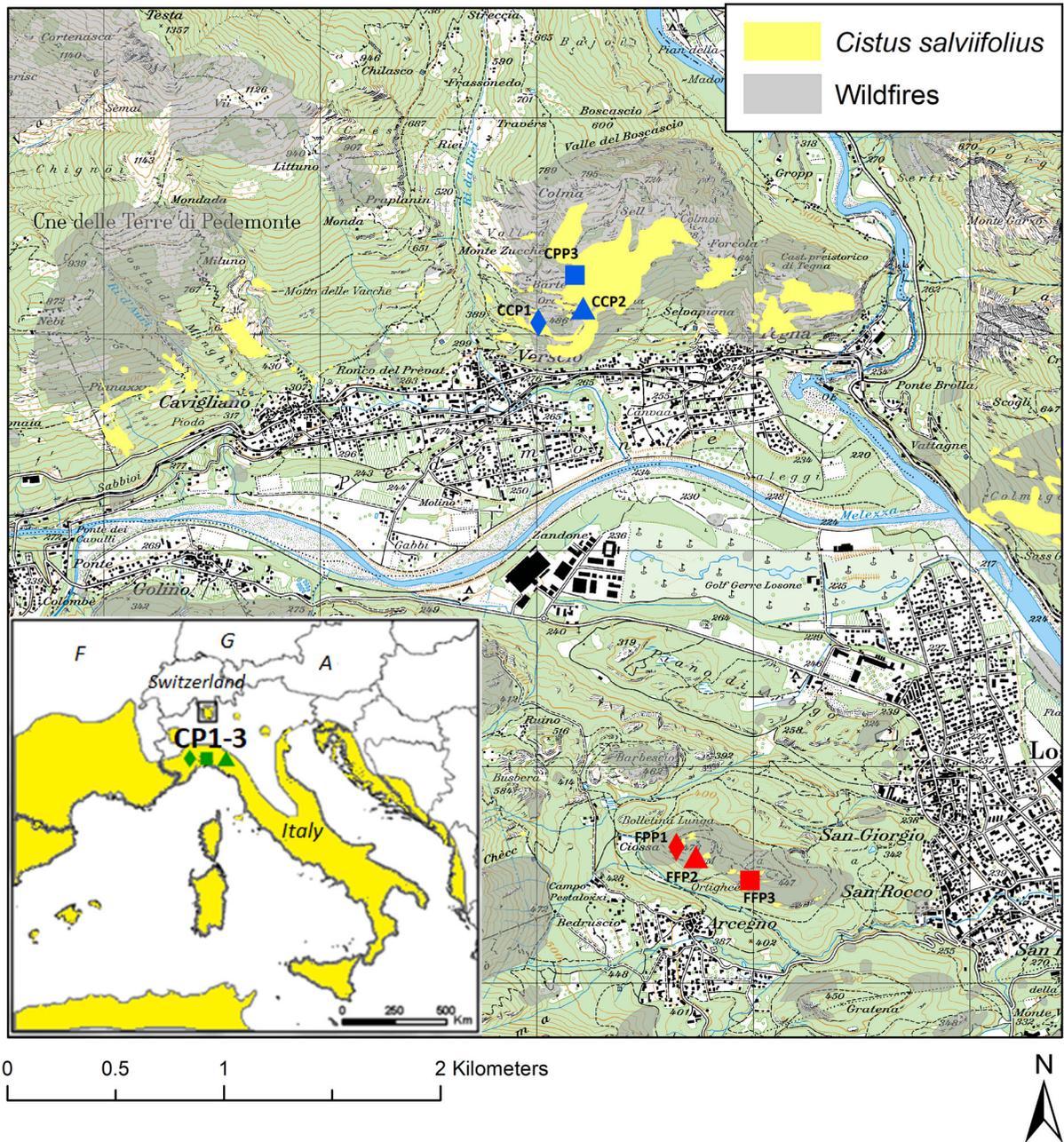
50 years, 90% of the area affected by wild fires in Switzerland was located in this region. Since 1990, the fire frequency decreased dramatically from 91 fires and 910 ha per year in the period 1968–1990 to 57 fires and 357 ha in the period 1991–2002 due to the improvement of the fire-fighting techniques and fire prevention measures (Pezzatti et al. 2010). During the same period, systematic surveys have revealed the steady decrease of *C. salviifolius* in Switzerland such that nowadays the species is restricted to rocky outcrops where competition from the steadily expanding surrounding wood lands is lower (Moretti et al. 2006). This has led to a patchy distribution of *C. salviifolius* at the northern range margin.

Data sampling was designed to compare peripheral *versus* central *Cistus* populations, and fragmented *versus* continuous populations. Two populations were selected on the southern slopes of the Swiss Alps (Canton Ticino) at the northern range margin of *C. salviifolius*, where two contrasting spatial distribution of the species occurred: fragmented, peripheral population (8°44′36″ E, 46°09′57″ N; 520–625 m a.s.l.; FPP hereafter), and connected, peripheral population (8°44′08″ E, 46°09′21″ N; 430–460 m a.s.l.; CPP hereafter). A third population was located in the Mediterranean, in the core distribution area of *C. salviifolius*, near Genoa, Italy (8°54′54″ E, 44°25′54″ N; 220–315 m a.s.l.; CP hereafter). Within each *C. salviifolius* population, we selected three sampling sites (FPP<sub>1-3</sub>, CPP<sub>1-3</sub>, CP<sub>1-3</sub> respectively) at a distance of about 200 m from each other.

### Genetic research

#### *Plant material and DNA extraction*

Young leaves were collected in spring 2005 from 25 individuals in each site of each population, dried in silica gel and stored at room temperature. Twenty mg of dry leaf material was ground in 2 ml Eppendorf tubes to extract genomic DNA by eluting it in 50 µl of double distilled water using the CTAB method (Doyle and Doyle 1987). From a subset of samples, the DNA concentration was quantified with a Fluorometer (Turner BioSystems, Sunnyvale, CA, USA). These quantified samples served as templates to estimate DNA concentrations of the remaining samples.



**Fig. 1** Distribution maps of *Cistus salvifolius* (yellow area) in Southern Europe and the location of the three investigated populations: connected peripheral population CPP and fragmented peripheral population FPP at the Northern range margin in the Alps (Canton of Ticino, Switzerland,) and in the range core population CP in the Mediterranean (Genoa, Liguria, Italy).

The colored symbols indicates the three distinct sites per populations investigated during the study, i.e., CPP<sub>1-3</sub> (blue color), FPP<sub>1-3</sub> (red color), CP<sub>1-3</sub> (green color), respectively. Within each sites data were sampled in sampling plots (not shown)

**Table 1** List of EcoRI–MseI primer pair combinations and indications of the three selective nucleotides on each of the primers used for the selective amplification

Primer combination code	Forward primer EcoRI + 3	Reverse primer MseI + 3
EM1	EcoRI + AAC	MseI + CCG
EM2	EcoRI + ACG	MseI + CAG
EM3	EcoRI + ACT	MseI + CCG
EM4	EcoRI + AGC	MseI + CCG
EM5	EcoRI + AGG	MseI + CCC
EM6	EcoRI + AGG	MseI + CCT

### AFLP analysis

The genetic diversity of the *Cistus salviifolius* was investigated using the AFLP (Amplified fragmented length polymorphism) technique. AFLP were widely used in the '90 s and particularly suitable for genetic study of populations where specific primers are unknown (Schmidt and Jensen 2000; Travis et al. 1996). This technique has still been used to investigate central *versus* peripheral populations in several plant species until very recently (e.g., Duffy et al. 2009; Kropf 2012; Plenk et al. 2017).

We applied AFLP as described by Vos et al. (1995) with minor modifications described by Bratteler et al. (2006). We used the restriction enzyme combination EcoRI/MseI for DNA digestion, while various combinations of EcoRI (E) primers with 3 selective nucleotides and MseI (M) primers with 3 selective nucleotides were used for selective amplifications (Table 1).

The best twelve out of the 25 sampled individuals were chosen according to the quality of the peaks produced by ABI PRISM 3100-*Avant* Genetic Analyzer (Applied Biosystems) in the six primer combinations. Runs were then analyzed with GeneMapper 3.7 (Applied Biosystems). The labeling of the peaks was manually controlled by their visual inspection. The size of fragments used for analyses ranged from 50 to 350 bp. The results of band identification were then used to create a qualitative matrix of presence (1) or absence (0) data for each individual and population. The number of polymorphic AFLP loci and Nei's gene diversity  $h$  for the six different primer pairs in the nine populations were calculated from the binary matrix

using PopGen version 1.31. Nei's gene diversity  $h$  is defined as

$$h = 1 - \sum x_i^2$$

where  $x_i$  is the population frequency of each allele (1 or 0) at locus  $i$ . Nei's index produces values from 0 to 0.5. Differences in gene diversity between sites were then tested with ANOVA Post Hoc Tests LSD using SPSS 11.0.2. To estimate the partitioning of AFLP genetic variation between and within populations, analysis of molecular variance (AMOVA) was carried out, using Arlequin software, version 2 (Excoffier et al. 2005). Pairwise genetic differences between the three populations (pairwise  $F_{ST}$ ) were calculated by employing the software package GenALEX Version 6 (Smouse and Peakall 1999). Then, a principal coordinate analysis (PCO) was performed based on these genetic pairwise differences.

### Pollination experiments

Pollination experiments were performed to investigate pollination limitation and self-incompatibility in FPP and CPP in Southern Switzerland. Twelve individuals out of the 25 previously used in the genetic investigation were selected randomly and at a minimal distance of 5 m between individuals in each site of each population. The pollinator limitation experiment was carried out on 4 flowers per individual: two flowers were used as controls (Control), while the other two were hand pollinated with pollen grains of two other individuals flowering in the same site of each population (cross-pollination, CrossP). The self-incompatibility experiment was performed on the same individuals. Before anthesis, flowers were covered with nylon bags to prevent cross-pollination. After 1–2 days, bags were opened and the flowers pollinated with pollen from 2 flowers of the same plant individual (self-pollination, SelfP). Bags were removed after petals had withered.

The three treatments (Control, CrossP, SelfP) were repeated three times on the same individuals at weakly intervals between May 19 and June 5, 2006. A preliminary pollination experiment in 2005 ended with a high rate of flower loss due to the fragility of a particular breaking-point in the flower stem. In 2006, flower stems were fixed with adhesive tape to avoid flower and aborted fruits to drop before recording.

Fruit capsules were sampled between June 22 and July 11, 2006 and the numbers of healthy and aborted seeds per fruit counted by hand.

The mean number of healthy seeds (square root transformed) per individual and treatment was first analyzed using hierarchical linear models with population (CPP and FPP), site, treatments, and the population-by-treatment interaction as explanatory variables. The dataset was then split by population and analyzed again by hierarchical linear models as before. Treatment effects on the number of filled seeds were subsequently compared within study sites using Tukey's HSD test in R 2.3.1 (R Core Team 2005).

### Pollinator survey

During the preliminary pollination experiment in 2005, bees were collected in FPP and CPP to assess pollinator activity in the different *C. salviifolius* populations. Three yellow pans (Ø 30 cm; 40 cm high) were placed within each site of each population giving a total of 9 pans per population and 18 pans in the two populations. The pans were placed on the ground at the same high as *C. salviifolius* plants, and filled with water and detergent to avoid water tension on the surface. The minimum distance between pans within each site was ca. 10 m. The traps were emptied weekly from May 11 until June 6, 2005 during the main flowering period of *C. salviifolius* in the study

areas. Yellow pans are widely used to study pollinators in forests and agroecosystems as reliable methods to compare different habitats and landscapes (see Moretti and Legg 2009 for an application in burnt areas; see Westphal et al. 2008 for a synthesis). Complementary techniques, such as caching by using a swiping net, could not be used at the steep rocky slopes at the study sites.

Bees and wasps were then sorted out and identified to species level. Comparisons between the number of individuals and species in the different populations were tested with ANOVA Post Hoc Tukey's Test HSD using SPSS 11.0.2.

## Results

### Genetic research

The AFLP analysis of the 9 populations of *C. salviifolius* was performed using six primer pairs resulting in a total of 341 markers (Table 1). The percentage of polymorphic loci detected for each single primer pair combination in each site ranged from 19.2% (in FPP<sub>3</sub>) to 73.0% (in FPP<sub>1</sub>); (Table 2).

Overall, no significant differences were found in genetic diversity between the three populations (Table 3a), but there was a significant interaction 'population \* primer pair'. The primer pair EM2, for

**Table 2** Statistics for the number of polymorphic loci of six AFLP primer combinations (see Table 1) in 9 sites within 3 populations (FPP, CPP, CP) of *Cistus salviifolius* with a sample size of twelve individuals for each population ( $n$  = total

number of loci; % = percentage of polymorphic loci); FPP fragmented, peripheral population; CPP continuous, peripheral populations; CP core population

Population	Sites	Number of polymorphic loci											
		EM1 ( $n = 64$ )	%	EM2 ( $n = 52$ )	%	EM3 ( $n = 48$ )	%	EM4 ( $n = 74$ )	%	EM5 ( $n = 44$ )	%	EM6 ( $n = 59$ )	%
FPP	FPP <sub>1</sub>	40	62.5	11	21.5	7	14.6	54	73.0	16	36.36	32	54.2
	FPP <sub>2</sub>	45	70.3	34	65.4	23	47.9	44	59.5	21	47.7	45	76.3
	FPP <sub>3</sub>	13	20.3	10	19.2	11	22.9	30	40.5	13	29.6	24	40.7
CPP	CPP <sub>1</sub>	43	67.2	28	53.9	24	50.0	40	54.1	16	36.4	32	54.2
	CPP <sub>2</sub>	32	50.0	27	51.9	22	45.8	43	58.1	15	34.1	31	52.5
	CPP <sub>3</sub>	33	51.6	26	50.0	27	56.3	42	56.8	15	34.1	31	52.5
CP	CP <sub>1</sub>	42	65.6	31	59.6	23	47.9	52	70.3	20	45.5	30	50.9
	CP <sub>2</sub>	42	65.2	27	51.9	25	52.1	46	62.2	22	50.0	26	44.1
	CP <sub>y</sub>	42	65.2	31	59.6	24	50.0	41	55.4	30	68.2	29	49.2

**Table 3** a, b Mean values of Nei's gene diversity *h* and standard deviation (SD) over loci in the 3 populations of *Cistus salvifolius* (a) and 9 sites (i.e. 3 per population) (b) for the six primer pairs together (341 markers), and for each primer pair individually

	Total ( <i>h</i> ± SD)	EM1 ( <i>h</i> ± SD)	EM2 ( <i>h</i> ± SD)	EM3 ( <i>h</i> ± SD)	EM4 ( <i>h</i> ± SD)	EM5 ( <i>h</i> ± SD)	EM6 ( <i>h</i> ± SD)
<b>(a) Populations</b>							
FPP	0.1906 ± 0.0935 <sup>a</sup>	0.1912 ± 0.1488	0.1018 ± 0.1422	0.1159 ± 0.1496	0.2347 ± 0.1734	0.1458 ± 0.1855	0.2403 ± 0.1820
CPP	0.1928 ± 0.0946 <sup>a</sup>	0.1771 ± 0.1723	0.1917 ± 0.1861	0.1783 ± 0.1832	0.1999 ± 0.1918	0.1219 ± 0.1813	0.2219 ± 0.1939
CP	0.1998 ± 0.0980 <sup>a</sup>	0.2173 ± 0.1806	0.1971 ± 0.1955	0.1803 ± 0.1803	0.2153 ± 0.1818	0.1904 ± 0.1837	0.1980 ± 0.1845
<b>(b) Sites</b>							
FPP <sub>1</sub>	0.1615 ± 0.0850 <sup>a</sup>	0.1798 ± 0.1760	0.0521 ± 0.1220	0.0489 ± 0.1360	0.2313 ± 0.1972	0.1291 ± 0.1896	0.1834 ± 0.2082
FPP <sub>2</sub>	0.2212 ± 0.1156 <sup>bfi</sup>	0.2376 ± 0.1988	0.1424 ± 0.1647	0.1556 ± 0.1940	0.1918 ± 0.2007	0.1472 ± 0.1872	0.2505 ± 0.1910
FPP <sub>3</sub>	0.0997 ± 0.0527 <sup>c</sup>	0.0424 ± 0.1082	0.0483 ± 0.1238	0.0703 ± 0.1391	0.1436 ± 0.2010	0.0923 ± 0.1729	0.1594 ± 0.2130
CPP <sub>1</sub>	0.1805 ± 0.0945 <sup>a</sup>	0.1767 ± 0.1689	0.1681 ± 0.1901	0.1422 ± 0.1841	0.1658 ± 0.1916	0.0958 ± 0.1613	0.1859 ± 0.2044
CPP <sub>2</sub>	0.1731 ± 0.0906 <sup>ai</sup>	0.1425 ± 0.1791	0.1646 ± 0.1925	0.1495 ± 0.1949	0.1747 ± 0.1915	0.1144 ± 0.1879	0.2049 ± 0.2161
CPP <sub>3</sub>	0.1896 ± 0.0992 <sup>abhi</sup>	0.1520 ± 0.1833	0.1847 ± 0.2002	0.1805 ± 0.1991	0.1836 ± 0.2011	0.1246 ± 0.1912	0.1738 ± 0.2022
CP <sub>1</sub>	0.2106 ± 0.1100 <sup>dfigh</sup>	0.2302 ± 0.2033	0.1910 ± 0.1973	0.1428 ± 0.1786	0.2368 ± 0.1990	0.1521 ± 0.2009	0.1870 ± 0.2044
CP <sub>2</sub>	0.1769 ± 0.0926 <sup>egi</sup>	0.1597 ± 0.1846	0.1867 ± 0.2073	0.1816 ± 0.1989	0.1761 ± 0.1844	0.1542 ± 0.2003	0.1606 ± 0.2055
CP <sub>3</sub>	0.1853 ± 0.0970 <sup>efgi</sup>	0.1842 ± 0.1844	0.1845 ± 0.1886	0.1590 ± 0.1963	0.1781 ± 0.2031	0.1874 ± 0.1863	0.1827 ± 0.2120

Cells within columns with different letters are significantly different ( $P < 0.05$ ); EM<sub>1-6</sub> = Primer combination code (see Table 1)  
 FPP fragmented, peripheral populations, CPP continuous, peripheral populations, CP core populations

**Table 4** a, b Hierarchical partitioning of genetic variation (AMOVA); (a) leading-edge (FPP and CPP) versus central (CP) populations; (b) fragmented (FPP) versus connected (CPP) populations

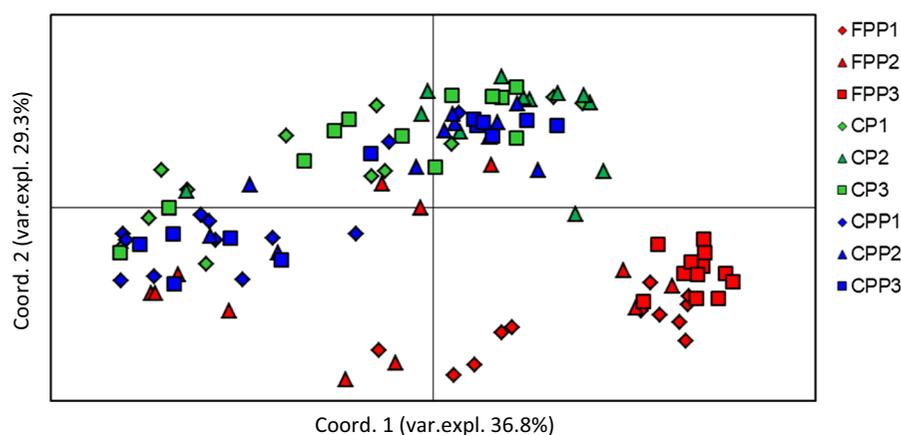
Source of variation	d.f	Sum of squares	Variance components	% total	F-Statistic	P value
(a) Leading-edge <i>versus</i> central						
Within sites	99	2997.833	30.2811	68.67		
Among sites within populations	6	481.944	4.1702	9.46	$F_{SC} = 0.12105$	< 0.001
Among populations	2	855.130	9.6456	21.87	$F_{CT} = 0.21874$	0.0019
Total	107	4334.907	44.0970	100	$F_{ST} = 0.31331$	< 0.001
(b) Fragmented <i>versus</i> connected						
Within sites	66	1923.500	29.1439	65.82		
Among sites within populations	4	363.944	5.1535	11.64	$F_{SC} = 0.15026$	< 0.001
Among populations	1	450.306	9.9811	22.54	$F_{CT} = 0.22542$	0.0968
Total	71	2737.750	44.2786	100	$F_{ST} = 0.34180$	< 0.001

The levels of significance are based on 10,100 permutations

example, revealed low levels of genetic diversity in FPP and significant differences between FPP and CP (ANOVA Post Hoc LSD Test  $P = 0.007$ ), and between FPP and CPP ( $P = 0.01$ ) (Table 3a), while primer pairs EM4 and EM6 found the highest genetic diversity in FPP. The remaining four primer pairs, EM1, EM2, EM3 and EM5, revealed the highest genetic diversity in CP. Substantial differences in gene diversity were also observed among the 9 sites (Table 3b). Across all six primer pair combinations, FPP<sub>3</sub> site in FPP clearly revealed the lowest genetic diversity. Substantial variation was, however, observed between primer pairs. For EM3, for example,

genetic diversity in FPP<sub>1</sub> was well below that observed in FPP<sub>3</sub>, while for EM2, genetic diversity in the two sites was similar.

The AMOVA comparing the central vs. the peripheral populations (Table 4a) showed that most variation was found within sites, but a substantial percentage of variation was explained by differences between the central and the two peripheral populations ( $F_{CT} = 0.2184$ ). The comparison between the FPP and CPP populations (Table 4b) also revealed that most of the variation was within sites, while among-population variation was large but not significant.

**Fig. 2** Principal coordinate plot based on individual genetic distances within and between fragmented (FPP<sub>1-3</sub>; red symbols), connected (CPP<sub>1-3</sub>; blue symbols) populations of *Cistus*

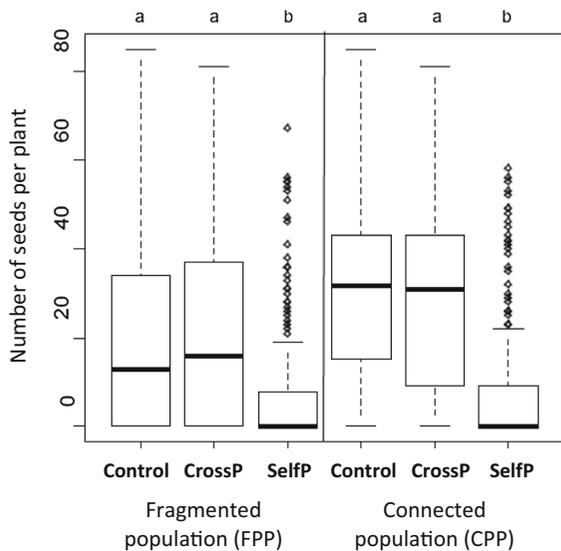
*salviifolius* in the Northern range margin in the Alps (Canton of Ticino Switzerland,) and in the range core population (CP<sub>1-3</sub>; green symbols) in the Mediterranean (Genoa, Liguria, Italy)

The geographic distance between the central population in Genoa and the Swiss leading-edge population, which is about 200 km, is much greater than the distance between the two Swiss leading-edge populations (FPP and CPP, approximately 2.5 km). Nevertheless, the three populations showed similar levels of pairwise genetic differentiation ( $F_{ST}$  CPP-CP = 0.2111,  $P < 0.001$ ;  $F_{ST}$  FPP-CP = 0.2701,  $P < 0.001$ ;  $F_{ST}$  FPP-CPP = 0.2620,  $P < 0.001$ ).

The Principal Coordinates (PCO) analysis based on 245 markers randomly chosen from the 341 markers (Fig. 2) separated all the connected sites in the core and margin range (CP<sub>1-3</sub> and CPP<sub>1-3</sub>) from most of the fragmented peripheral sites in FPP (mainly FPP<sub>3</sub> and few FPP<sub>2</sub> and FPP<sub>1</sub>) on the first axis, which explained 36.8% of the overall variance, and from the remaining FPP<sub>1</sub> and FPP<sub>2</sub> sites on the second axis, which explained 29.3% of the variance.

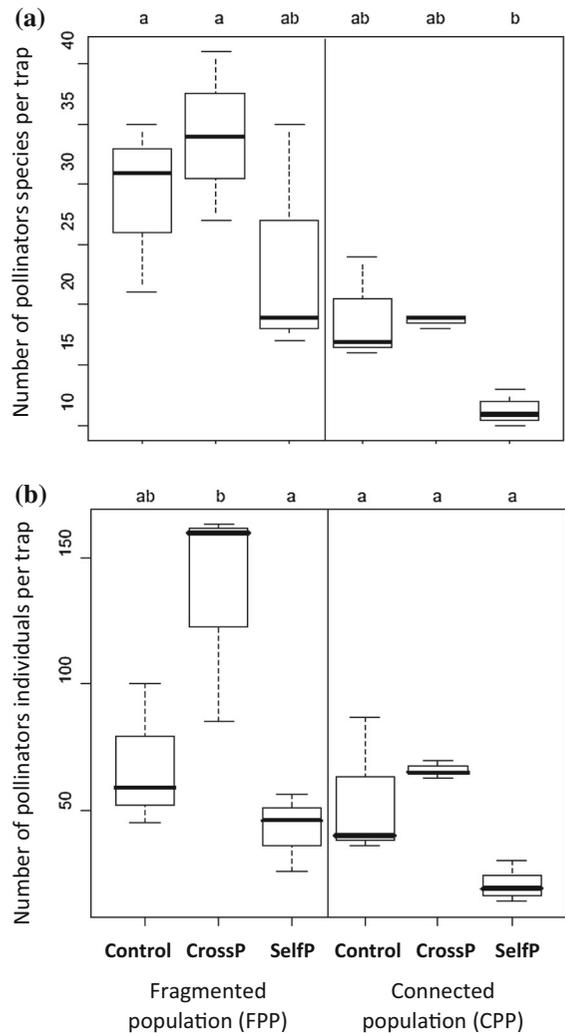
Pollination experiments

The response to pollination treatments (Control, cross-pollinated CrossP, self-pollinated SelfP) based on the number of seeds showed differences between the two leading-edge populations in Southern Switzerland (FPP and CPP) as indicated by a significant



**Fig. 3** Mean number of seeds per plant for each treatment (Control; cross-pollinated, CrossP; self-pollinated, SelfP) in the three sites within the fragmented peripheral populations (FPP) and three sites within the connected peripheral populations (CPP) of *Cistus salvifolius* in the Alps

population-by-treatment interaction term ( $F_{2,242} = 4.55$ ,  $P = 0.012$ ) in the hierarchical model using the full dataset. In FPP, the mean number of healthy seeds was 31.6 (min: 0; max: 62.5) in cross-pollinated flowers (CrossP); 26.7 (min: 4; max: 67) in self-pollinated flowers (SelfP); and 27.2 (min: 0; max: 66.5) in control flowers (Control). In CPP, the number of healthy seeds was 33.6 (min: 0; max: 76) in CrossP, 24.1 (min: 3; max: 53) in S and 35.1 (min: 0; max: 77) in C. Because of the significant population-by-



**Fig. 4 a, b** Mean numbers of pollinator individuals (a), and pollinator species (b) per trap for each treatment (Control; cross-pollinated, CrossP; self-pollinated, SelfP) collected in the fragmented peripheral populations (FPP) and the connected peripheral populations (CPP) of *Cistus salvifolius* in the Alps

treatment interaction, differences between treatments were analyzed in each population separately.

When each population was analyzed separately, significant effects were found for the pollination treatments (FPP:  $F_{2,100} = 14.61$ ,  $P < 0.0001$ ; CPP:  $F_{2,101} = 48.01$ ,  $P < 0.0001$ ), but not for sites (FPP:  $F_{2,15} = 1.60$ ,  $P = 0.24$ ; CPP:  $F_{2,14} = 0.09$ ,  $P = 0.78$ ) and for the site-by-treatment interaction (FPP:  $F_{4,100} = 1.48$ ,  $P = 0.21$ ; CPP:  $F_{4,101} = 2.06$ ,  $P = 0.09$ ). In both populations, the pollen addition (CrossP) treatment did not lead to a significant change in seed production compared to the control (Fig. 3). Nevertheless, both Control and CrossP treatments produced more seeds in sites within CPP ( $28.63 \pm 19.29$ ) than in those within FPP ( $19.22 \pm 19.88$ ). Selfing strongly and significantly reduced the number of seeds in both populations (FPP:  $F_{2,100} = 21.09$ ,  $P < 0.0001$ ; CPP:  $F_{2,101} = 78.02$ ,  $P < 0.0001$ ; Fig. 3), but stronger in CPP (72.1%) than in the fragmented FPP (56.7%).

#### Pollinator surveys

A total of 103 pollinator species (41 bees; 62 wasps) (Fig. 4a) were identified from the 1164 individuals (439 bees; 725 wasps) (Fig. 4b) sampled; 39 species (37.9%) were sampled with five or more individuals involving 1057 individuals (90.8%). The number of pollinator species and individuals of both bees and wasps was significantly higher in FPP than in CPP (species  $F_{5, 12} = 5.358$ ,  $P = 0.008$ ; individuals  $F_{5, 12} = 7.037$ ;  $P = 0.003$ ). The highest number of species was sampled in two fragmented populations in FPP, i.e. FPP<sub>1</sub> ( $29 \pm 7.2$ ) and FPP<sub>2</sub> ( $34 \pm 7.0$ ). Differences were significant only between the fragmented FPP<sub>1</sub> and the connected CPP<sub>3</sub> ( $P = 0.035$ ), as well as between fragmented FPP<sub>2</sub> and connected CPP<sub>3</sub> ( $P = 0.006$ ) (Fig. 4b). High number of bee individuals were also sampled in fragmented FPP<sub>1</sub> ( $68 \pm 28.6$ ) and FPP<sub>2</sub> ( $136 \pm 44.2$ ), but only in the latter it was significantly higher than in fragmented FPP<sub>3</sub> ( $P = 0.007$ ) and in continuous CPP<sub>1</sub> ( $P = 0.019$ ) and CPP<sub>2</sub> ( $P = 0.049$ ).

## Discussion

### Genetic diversity

We found that genetic diversity in the peripheral populations of *Cistus salviifolius* in the Alps was similar to the range central population in the Mediterranean area. Despite the overall high genetic variation existing within the fragmented peripheral *Cistus* populations in the Alps, our study also revealed the occurrence of patches with lower genetic diversity and high genetic distance compared to those few hundred meters apart.

Our results are consistent with those reported by Farley and McNeilly (2000), who compared morphological and genetic diversity in *C. salviifolius* populations from a broad range of habitats across the Mediterranean. The occurrence of high levels of genetic variation was observed at all markers and was not driven by only a few loci. This discards the hypothesis of natural selection maintaining high genetic diversity by favoring selectively different alleles in peripheral populations growing in different environments (variation in fire regimes, microclimatic conditions, and topography) (Moretti et al. 2006).

The similarity in genetic variation between core vs. peripheral populations and the lack of decreasing in genetic diversity in the leading-edge *Cistus* populations observed in our study contrasts with the center vs. periphery hypothesis (e.g., Brown et al. 1996; Sagarin and Gaines 2002), which predicts that peripheral populations are more prone to extinction and genetically less diverse than those from the center as a consequence of bottlenecks, founder effects, inbreeding, or genetic drift (Durka 1999; Faugeron et al. 2004; Lammi et al. 1999; McCauley and Ballard 2002; Pironon et al. 2017). Records on the occurrence of *C. salviifolius* in the Alps date back to eighteenth century (see Ceschi 1995 for a synthesis). The species might have been dispersed by past transhumance activity from and to the Mediterranean regions, as suggested by Tramer et al. 1975/76, 1977/78) and maintained over time by regular fires in the region (Moretti et al. 2006, 2008), rather than by recent expansion driven by changes of climatic conditions and fire regime (Moretti et al. 2006). Dispersion from central populations to peripheral ones through animal-borne seeds might have led to founder effects and consequent reduction of genetic diversity and increase in genetic

differentiation in peripheral populations. However, the data analyzed in this study suggest that the continuous gene flow through seed dispersal was strong enough to replenish genetic diversity in peripheral sites. Alternatively, Swiss peripheral populations might be the remnants of a larger past distribution areas.

Species distribution modeling (Moretti et al. 2006) and systematic field surveys (Beffa 2006; Moretti et al. 2008) performed in the study region have shown that two main group of factors govern the current distribution of *C. salviifolius* in the Alps: rock cover, topography (slopes and aspects), and microclimate, on the one hand, and time elapsed since the last fire, on the other hand. While fire enable *C. salviifolius* to regenerate and temporarily expand, by triggering the seed germination including dormant seeds (Torrioni 2008), rock cover and topography provide habitat without competition-free from other plants (Moretti et al. 2008), although at possible risk of drought (personal observation). The same study demonstrated that during times without fires, isolated patches surrounded by forest tend to shrink and to disappear (up to 30% in case of 15 years return interval), leading to a high risk of extinction of entire patches.

Fragmentation may have failed to create immediate differentiation, because of two main reasons: on the one hand, the long life span of *C. salviifolius*, up to 15 years in the Alps (Beffa 2006; Roy and Sonie 1992), which can decrease the rate of genetic drift, and on the other hand, the long persistence of the seed bank of the species, more than 20 years (Cerabolini et al. 2003; Torrioni 2008) that is likely to contribute to buffer the population against the loss of genetic diversity (Nunney 2002; Vitalis et al. 2004).

The genetic consequences of physical fragmentation produced by advancing forest are not always found in genetic analyses because it takes many generations for genetic drift to result in significant effects on populations (Honnay et al. 2006). Hence, recent fragmentation associated with specific habitat and climatic conditions, local environmental heterogeneity, as well as fire history and fire regime are likely to super-impose their effects on genetic variation shaping the past and current distribution of the species, resulting in high genetic diversity and differentiation of the peripheral *Cistus* populations in the Alps.

## Pollinators and reproduction success

All the fragmented sites within the two peripheral populations in our study produced a lower average number of seeds and showed a slightly higher self-fertilization rate, despite the occurrence of a higher average number of pollinators (bees and wasps) and higher species richness compared to the connected ones. This suggests that, in the fragmented sites, pollinators were not as effective at ensuring cross-fertilization as in the connected sites, despite their higher abundance and richness. A possible reason is that, while yellow pans are suitable methods to describe the pollinator community at a given location (Westphal et al. 2008), they might not fully reflect the effective visit rate to *C. salviifolius* flowers and be affected by the visits to other plant species that flower at similar times in the same location. Fragmented *Cistus* populations are often surrounded and sometimes even suffocated by a dense vegetation dominated by heather, *Calluna vulgaris*, purple moor-grass, *Molinia caerulea*, and eagle fern, *Pteridium aquilinum*, as the typical intermediate successional stage after fire (Delarze et al. 1992; Moretti et al. 2008). Although we occasionally observed flower visits also by other generic pollinators than bees and wasp, such as overflies (Syrphidae), scarab beetles (Cetoniidae), longhorn beetles (Cerambycidae) and jewel beetles (Buprestidae), the rather small patches of *C. salviifolius* are likely to be overseen by pollinators, which might be attracted by other taller and more prominent plant species, such as the common broom, *Cytisus scoparius*, flowering at the same time as *C. salviifolius* (Moretti et al. 2006; Staehli 2005).

Systematic surveys and potential distribution scenarios of *C. salviifolius* in the Alps (Moretti et al. 2006) demonstrated that during times without fires, isolated patches surrounded by forest tend to shrink and to disappear, leading to a high risk of extinction of entire patches. Investigations by Beffa (2006) demonstrated that a lack of fire-induced regeneration, with fire-return interval longer than 15 years, leads to a lower density of *Cistus* plants and negative effects on vigor and fitness. Moreover, the present study showed that isolated sites and populations can harbor lower genetic diversity and produce less seeds than connected populations (Table 3b, Fig. 3). Taken together, these studies suggest that favorable management practices for the conservation of *C. salviifolius* in the

Alps include managing wild fire propagation and clearing the vegetation in late post-fire successional stages to reduce competition, ensure sufficient recruitment and avoid patch isolation. In this regard, agri-environmental measures can be implemented to counter the abandonment process and subsidize these favorable practices where they are not economically or socially advantageous (Andrello et al. 2018).

## Conclusion

We conclude that (1) the peripheral populations of *C. salviifolius* in the Alps conserve high genetic diversity and identity, (2) fragmented populations at range margins still retain genetic diversity, despite a potential for loss of genetic variation through inbreeding and self-fertilization in small and highly isolated *C. salviifolius* populations in the Alps, and (3) extended investigations that consider a higher number of populations especially in the peripheral areas are needed to better understand the mechanisms that allow leading-edge population to grow, reproduce and persist under a changing regime of wild fire and land use of the southern slopes of the Alps.

Meanwhile we recommend informing forest and conservation managers about the distribution and ecology of *C. salviifolius* south of the Alps and integrating the results out our study into forest management planning, fire regime regulation and fire-fighting.

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**Author contributions** MM and PC conceived and designed the study. PC collected the plant material in the field and ran the genetic analyses. AB and MM surveyed pollinators and ran the pollination experiment. PC, AB and MA analyzed data. All authors contributed to the writing of the manuscript.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** Since *Cistus salviifolius* a vulnerable species in Switzerland, the authors declare that, during the study, they treated the species and population with the greatest respect.

**Informed consent** The investigation at national park was conducted by obtaining due permission.

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