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Do they really hybridize? A field study in artificially established mixed populations of *Euphrasia minima* and *E. salisburgensis* (Orobanchaceae) in the Swiss Alps

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Abstract Hybridization and introgression in the European species of Euphrasia depend on the relationships between the species, on flower size and habitat. Hybridization between *Euphrasia minima* and *Euphrasia salisburgensis* was investigated in their natural habitat using artificial sympatric populations of both species in the Swiss Alps. The insect behavior in the populations suggests, that cross-pollination is likely to occur. A number of putative hybrids were detected by morphological characteristics, and their hybrid origin was verified using RAPD analysis. The predominance of RAPD bands in one of the species and the occurrence of these bands in some plants of the second species point to earlier introgression events. The number of hybrids found in the artificial populations together with results of earlier studies indicate that insect visits and cross-pollination in small-flowered *Euphrasia* species in lower alpine regions may be more common than has been suggested in the past.

Keywords *Euphrasia minima* ·
Euphrasia salisburgensis · Field experiment ·
Insect behavior · Hybridization · Introgression ·
RAPD · Discriminant analyses

Introduction

The *Euphrasia* species of the Northern Hemisphere (section *Euphrasia*) are hemiparasitic herbs of either subsection *Ciliatae* or subsection *Angustifoliae*. Within both

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subsections taxonomy is difficult due to large intraspecific variability, to small interspecific differences and to the occurrence of hybrids (Yeo 1968). The close relatedness of some of the species within the same subsection is reflected by successful interspecific cross-pollination (Liebst and Schneller 2005; Yeo 1966, 1976). Artificial crosses of taxa of different subsections may result either in low seed set or in a seed set similar to that resulting from intraspecific crossing or selfing. The F1 hybrids of such crosses are either sterile or bear only a few seeds (Liebst 2006; Yeo 1966). In a few cases, hybridization between diploid and tetraploid *Euphrasia* species has been observed (Liebst and Schneller 2005; Pugsley 1930; Yeo 1956).

Artificial pollination may illustrate the interfertility of species, but cannot be used to estimate the probability or the frequency of interspecific cross-pollination in nature. Preconditions for hybridization between insect-pollinated species include the occurrence of at least two species in the immediate neighborhood and of overlapping flowering periods. Mixed populations of *Euphrasia* species have frequently been found in Europe (von Wettstein 1893; Yeo 1966). The probability of interspecific cross-pollination in these populations depends on the availability of pollinators and on the breeding system of the taxa, which is strongly associated with the size of the corolla (von Wettstein 1896; Yeo 1966, 1978a; French et al. 2005). Cross-pollination is common in large-flowered species, whereas the small-flowered species are predominantly selfing. In alpine populations of the small-flowered *E. minima* and *E. willkommii* few or no flower visitors have been detected (Kreisch 1996; Gomez 2002). However, a few hybrids of small flowered alpine *Euphrasia* taxa have been found in the last two centuries suggesting that cross-pollination at least occasionally occurs (see references in von Wettstein 1896; Vitek 1986).

Both the small and the large flowers of *Euphrasia* are adapted to the same type of pollinators (Yeo 1968) and are mainly visited and pollinated by flies, hover flies (Diptera, Syrphidae) and bees (Hymenoptera, Apidae s.l.). According to Schultz (see Knuth 1909) the nectary is well developed in the larger-flowered species and less well developed or absent in the smaller-flowered ones. Pollen seems to be at least as much an attraction as the nectar, particularly for Syrphidae (Yeo 1968). The mechanisms of pollination in *Euphrasia* and the flower biology have been described in detail by von Wettstein (1896) and Yeo (1968).

The tetraploid, small-flowered species *E. minima* (subsection *Ciliatae*) and *E. salisburgensis* (subsection *Angustifoliae*) are among the most common *Euphrasia* species in the Swiss Alps. Despite their different ecological preferences, sympatric and parapatric populations occur. Both species are successfully selfing (Liebst 2006). It is unlikely that their flowers may attract many insects, however, Yeo (1966) argued that even between small-flowered *Euphrasia* species crosses may be common. Because *E. minima* and *E. salisburgensis* belong to different subsections, it is expected that hybrids are highly sterile (Yeo 1968). Nevertheless, an artificial F2 generation was raised in a garden experiment from seed resulting from artificial selfing and crossing of F1 hybrids of *E. minima* and *E. salisburgensis*, and from artificial back-crossing of the F1 hybrids with the parental species (Liebst 2006).

So far, inter- and intraspecific crossing and selfing in *Euphrasia* have been investigated exclusively by artificial pollination (Liebst 2006; Yeo 1976). In the present study, for the first time pollination by insects and hybridization in the natural habitat of the species were investigated. Artificially established, mixed populations of *E. minima* and *E. salisburgensis* were used to answer the following questions: (1) Do the flowering periods of *E. minima* and *E. salisburgensis* overlap sufficiently to allow interspecific pollination? (2) Are the flowers of *E. minima* and *E. salisburgensis* visited by insects and does the insect's behavior potentially allow pollen transfer between the species? (3) Can hybrids establish in a natural habitat?

Materials and methods

Species

Euphrasia minima Jacq. ex DC., subsection *Ciliatae*, and *E. salisburgensis*, subsection *Angustifoliae* (Wettst.) Joerg. are annual, hemiparasitic herbs. *E. minima* is a facultative hemiparasite but grows much more vigorously when it is attached to a suitable host plant (Heinricher 1924; Matthies 1998). Although many species are suitable hosts for

Euphrasia, there are strong differences in their quality as host plants (Yeo 1964; Matthies 1998).

Both *E. minima* and *E. salisburgensis* are widespread in the Alps. In Switzerland, the altitudinal distribution of *E. salisburgensis* ranges from colline to alpine regions, while *E. minima* is usually restricted to subalpine and alpine regions (Hess et al. 1972). *E. salisburgensis* grows mainly on basic soils, *E. minima* prefers acidic substrates. The species can be morphologically separated by the two main characters used for the separation of the subsections: in subsection *Ciliatae* ciliate capsules and leaves with contiguous teeth; in subsection *Angustifoliae* glabrous capsules (or capsules with few small cilia) and leaves with at least some teeth distant (Yeo 1978). The color of the corolla is yellow or white in *E. minima* and white or lilac in *E. salisburgensis*. Like most *Euphrasia* flowers they also have violet longitudinal veins forming guide marks that converge to the throat (Yeo 1966) and yellow spots on the lower lip and throat.

Establishment of the artificial populations

Two to three ripe fruits from about 400 individuals of *E. minima* and *E. salisburgensis* were collected at three locations in the Swiss Alps in large sympatric or parapatric populations in autumn 2001: (1) Canton Tessin, Piora (PI); Alpe Tom (2,049 m, 46°32′51.69″N 8°41′19.65″E) to Cadagno di fuori, Cadagno di dentro and Alpe di Piora (2,013 m, 46°32′50.62″N 8°42′56.33″E); (2) Canton Uri, Andermatt (AM); Nätschen (rail stop Matterhorn–Gotthard Bahn, 1,890 m, 46°38′38.66″N 8°36′37.05″E) to Gütsch (Oberstafel, about 2,399 m, 46°39′26.20″N 8°37′14.77″E); (3) Canton Tessin, Valle Bedretto (VB); Alpe Cruina (2,050 m, 46°28′21.17″N 8°25′34.93″E). From here onward these populations are named "origin populations". To facilitate the discrimination of the species in the artificial populations, yellow flowered *E. minima* individuals were chosen as seed donors. Fruit collecting resulted in about 12,000 seeds per species and population, except for *E. salisburgensis* in VB, where only 7,000 seeds were collected (for details see Liebst 2006).

The experimental area was a 15 × 7 m plot in a pasture in the Pian Murinascia in Val Piora (Canton TI, about 1,980 m, 46°32′40.98″N 8°43′46.80″E). In this area single *E. minima* and *E. salisburgensis* plants and also some *E. alpina* (diploid, atypical forms) and *E. hirtella* plants (diploid) naturally occurred. Within the experimental area for each origin population four plots of 75 × 75 cm (without *Euphrasia* plants) were prepared for seeding by cutting away grass and herbs and then removing the plant litter. For a more even distribution of the seeds, each plot was divided in nine sub plots (25 × 25 cm). The seeds from each origin population were mixed. About 300 of

each *E. minima* and *E. salisburgensis* seeds were sown into each subplot of the populations PI and AM, and about 300 *E. minima* and 190 *E. salisburgensis* seeds were sown in each subplot of the population VB. After sowing, the seeds were covered with a fine layer of quartz sand.

Record of flowering plants

The number of *E. minima* and *E. salisburgensis* plants were counted at seven days during the flowering-period in 2002 and at six days in 2003. All plants with at least one open flower were counted. Based on these data, the day with the maximum number of flowering plants (peak of flowering) and the sowing success were determined (proportion of seeds that developed into a mature plant at the peak of flowering in 2002).

Observation of flower visitors

In the vegetation periods 2002 and 2003 a total of 102 10-min observation periods (between 08.00 and 18.00) were carried out in the artificial populations. During the observation periods the behavior of any insect that visited a flower of *E. minima* or *E. salisburgensis*, discriminating between Hymenoptera and Diptera and ants, butterflies or beetles, respectively, was described. Insect behavior was recorded until 10 min were over, until the insect visited the flower of a different genus or until the insect left the plot (whichever was shorter). The behavior of the insects as either: (1) visiting one *Euphrasia* flower, (2) visiting two or more flowers within the same inflorescence, (3) visiting two or more flowers of different individuals of the same *Euphrasia* species, (4) visiting two or more flowers of individuals of different *Euphrasia* species were described. For the latter *E. hirtella* and *E. alpina* that also occurred in some of the plots were included. Some beetles sitting in *Euphrasia* flowers were caught and determined to family level.

Additional to the 10-min observation periods any flower visitor that was present during the flowering plant counts was also recorded. A chi-square test was applied to test the dependence of insect visits on the species.

Morphological analyses

For the morphological analyses, plants in the artificial populations in 2003 and 2004 were collected. Altogether 88 *Euphrasia* plants from the population PI, 74 plants from the population AM, and 52 plants from VB were used for morphological and discriminant analyses.

All plants were determined according to Hess et al. (1972). If a plant showed characters both of *E. minima* and *E. salisburgensis* and/or if its corolla changed color from

yellow to white, it was considered as plant of hybrid origin and is referred to as such.

The flower color of each plant was recorded and then all plants were pressed and dried. A calyx and the largest of the bracts with the maximum number of teeth were mounted on a sheet of paper using transparent adhesive tape, and photographed with a digital camera. Quantitative characters of calyces and leaves were measured using the program tpsDig 2.02 (Rohlf 2004). Quantitative flower and fruit characters were measured under a stereomicroscope at magnifications of 6.4 and 16×, respectively, in one fully developed flower and in one fruit per plant after boiling. Four qualitative and 12 quantitative characters were scored and two ratios were calculated (Table 1; Fig. 1).

Data from all populations were pooled for statistical analyses. Mean and standard error were calculated for species and hybrids. Because most of the data did not show normality even after transformation, differences between

Table 1 List of characters

Qualitative characters		
Flowers	Color[a]	1 White
		2 Yellow
		3 Yellow to white
		4 Violet
Bracts	Hairs on the underside[a]	0 Absent
		1 Present
Capsules	Cilia on the retuse apex[a]	1 Absent
		2 < 0.2 mm
		3 ≥ 0.2 mm
	Cilia on the surface[a]	0 Absent
		1 Present
Quantitative characters		
Leaves	Total length (a)[a]	mm
	Largest width between two teeth (b)	mm
	Lateral length of the top tooth (c)[a]	mm
	Width of the upper lateral tooth (d)[a]	mm
	Angle of the top tooth (e)[a]	
	Number of leaf teeth pairs	
Flowers	Length of the upper lip (f)	mm
Calyces	Toothlength (g)[a]	mm
	Toothlengt (h)[a]	mm
	Width of the calyx (i)[a]	mm
Stems	Number of lateral shoots[a]	
	Nodium of the first flower	
Indices		
Leaves	Ratio total length (a)/width (b)[a]	
	Ratio total length (a)/tooth length (c)[a]	

Small types in parenthesis refer to Fig. 1

[a] Characters that were used in the discriminant analyses in at least one artificial population
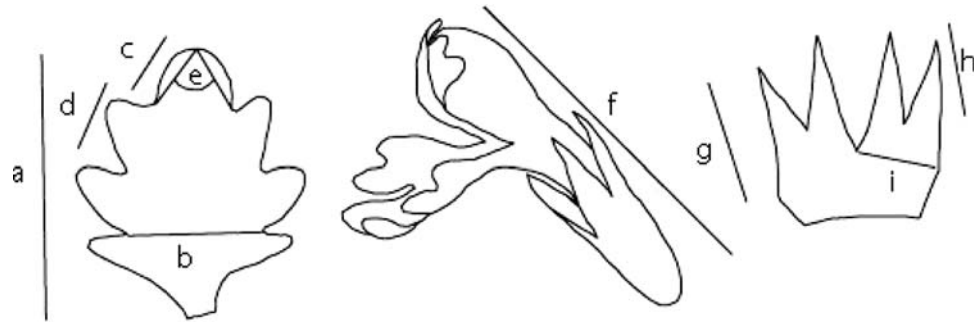
species and between species and hybrids were analyzed by nonparametric Kruskal–Wallis tests, followed by pairwise comparisons (Mann–Whitney tests).

The dependence of qualitative morphological characters on species and hybrids was tested by chi-square tests.

Discriminant function analysis

Discriminant analysis was used to test whether *E. minima*, *E. salisburgensis* and putative hybrids could be separated by morphological characters. Characters showing non-normality were transformed according to the equation $x'_{ij} = \ln x_{ij}$ (x_{ij} = measured value of character i in plant j), except for the character "number of lateral shoots" which includes zeros and was transformed according to the equation $x'_j = \ln(100 x_j + 1)$ (Sokal and Rohlf 1981). The numerators and denominators of the ratio characters were ln transformed prior to division. Finally, vector transformation was applied to all characters according to the equation $x'_{ij} = x_{ij} - x_{imin}/x_{imax} - x_{imin}$ (Gower 1971). This transforms all data to scores between 0 and 1, allowing the combination of quantitative and qualitative characters (Brochmann 1987).

In the first step, all independent variables were entered in the discriminant analysis. Characters with the smallest Wilks's lambda values (test of equality of group means) were chosen for the main analysis. The maximum number of characters used for the analysis depended on the number of individuals in the smallest group (maximal number of characters = number of individuals in the smallest group − 1). Statistical analyses were performed using SPSS 12.1.

RAPD analysis

Species and hybrids were identified by RAPD analyses. Plants collected in 2004 were used for the analyses. Total DNA was isolated from dried plant material using the Qiagen RNeasy MiniKit and the manufacturer's protocol, but with the incubation time extended to 20 min. In most cases the whole plant excluding roots, flowers and fruits was used to get 6–15 mg of dried material. The isolated DNA was stored at −20°C until amplification.

The DNA concentration was determined by visualizing the samples on 0.8% (w/v) agarose gel electrophoresis in 1× TAE buffer and by comparing the band intensity with DNA standards. The DNA concentration of the samples varied between <3 and 6 ng/μl.

Primer screening was performed using four individuals of each species and 55 of altogether 80 primers (Operon Technologies, kit A-D). Twenty-seven primers yielded in amplification products, six of them produced 40 reproducible bands. The reproducibility of the patterns was tested by repeated amplifications and by variation of the reaction mixtures (modifying the MgCl$_2$ and/or DNA concentration). The PCR reactions were performed according to Liebst (2006).

The plants from PI, VB and AM were analyzed separately. The amplification products for the different samples were compared with each other and screened for the presence or absence of specific bands. A similarity coefficient $S = M_{ab}/N_t$ was calculated for each pair of individuals (De Greef and Triest 1999) and each primer, where M_{ab} is for the number of all matches in the two individuals tested and N_t stands for the total number of different bands identified for the tested primer (being constant for each primer). The resulting similarity matrix was the basis for a cluster analysis (UPGMA clustering, Ntsyspc2.02i, Exeter software, 1986–1998).

Results

Sowing success and flowering phenology

The sowing success varied between about 3.3 and 4.2% in *E. minima* and between about 4.4 and 5.0% in *E. salisburgensis*. The flowering period of both species overlapped in all populations (Fig. 2).

Flower visitors

In 30 of the overall 102 observation periods (17 h) at least one insect visited one or more flowers of *E. minima* or *E. salisburgensis*. During the counting of flowering
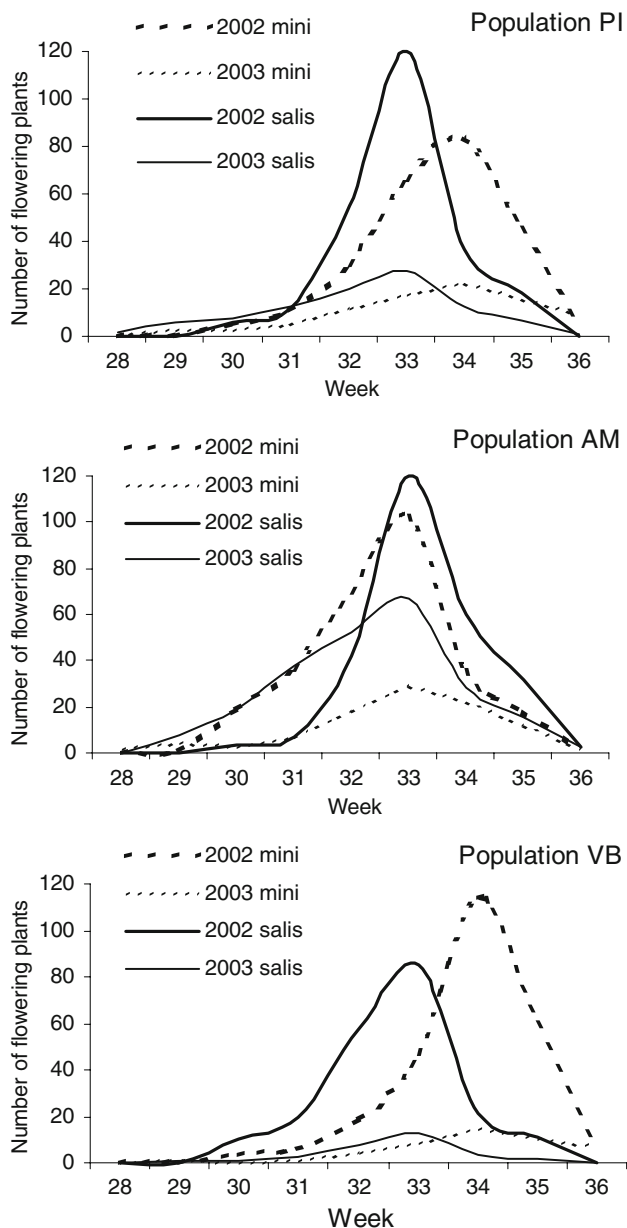
Fig. 2 Mean number of flowering plants of *E. minima* and *E. salisburgensis* in the plots of the artificial populations from Piora (PI), Andermatt (AM) and Valle Bedretto (VB) in 2002 and 2003
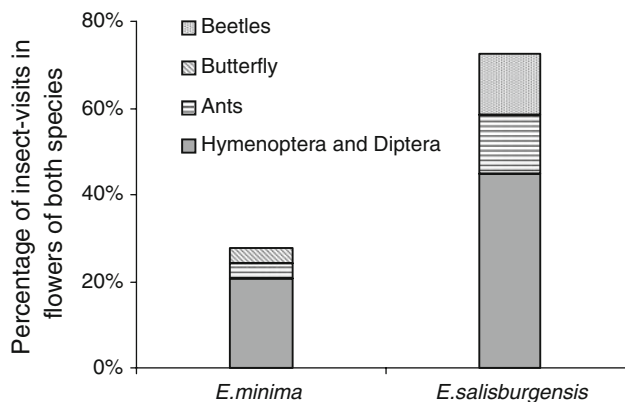

Fig. 3 Percentage of insects visiting one single *Euphrasia* flower (behavior 1)

The flower-visiting beetles were very small (about 2 mm) and were detected by chance when they crawled out or into a flower. In most cases they stayed in a flower during the whole census, probably feeding pollen. The ants behaved similar to the beetles. Ants and beetles either visited only one flower (behavior 1, Fig. 3) or 2–3 flowers of the same inflorescence (behavior 2). Overall, *E. salisburgensis* was more frequently visited by insects than *E. minima*, but the proportion of Hymenoptera and Diptera to ants and beetles was similar in both species (Fig. 3; Table 2).

Morphological analyses

Based on morphological characters, 109 *E. minima*, 80 *E. salisburgensis* and 25 putative hybrids were determined in 2003 and 2004. In 10 of the 14 characters Kruskall–Wallis tests revealed significant differences between species and/or between species and hybrids (Table 3). Pairwise comparisons found no significant differences in the corolla length of *E. minima* and *E. salisburgensis*. Furthermore, *E. minima* and the hybrids were similar in three characters and *E. salisburgensis* and the hybrids in one character (Table 3). The values of quantitative

Euphrasia plants (28 h), 15 flower visitors were detected. The *Euphrasia* plants were visited by Hymenoptera and Diptera, ants, small beetles (Nitidulidae and Curculionidae) and one single butterfly. Hymenoptera and Diptera (about 5–10 mm long) were the most frequent flower visitors and often visited more than one *Euphrasia* plant. Multiple visits of *E. salisburgensis* (2–9 plants, mean 3.5) occurred 10 times but only once for *E. minima* (nine plants). Twelve Hymenoptera and Diptera visited at least two different *Euphrasia* species during a census (behavior 4). Before or after visiting *E. salisburgensis*, Hymenoptera and Diptera often visited the large-flowered *E. alpina*.

Table 2 Observed and expected number of visitors in flowers of *E. minima* and *E. salisburgensis* (behavior 1)

	E. minima		*E. salisburgensis*	
	Observed	Expected	Observed	Expected
Hymenoptera and Diptera	5	4.5	13	13.5
Beetles, ants, butterfly	2	2.5	8	7.5

Chi-square 0.21, *df* 1, not sig.

Test-value from chi-square test

Table 3 Mean and standard error for quantitative morphological characters of *E. minima*, *E. salisburgensis* and their hybrids

Character	*E. minima* n = 109		*E. salisburgensis* n = 80		Hybrids n = 25		P	Not sign.
	Mean	Std. error	Mean	Std. error	Mean	Std. error		
Number of lateral shoots	0.08	0.03	0.16	0.04	0.08	0.05	*	s/h, m/h
Node of the first flower	6.22	0.19	6.69	0.23	6.04	0.40	–	–
Total leaf length, mm (a)	4.51	0.07	6.62	0.15	5.34	0.27	**	
Largest width between two teeth, mm (b)	2.58	0.06	2.55	0.08	2.62	0.15	–	–
Lateral length of the top tooth, mm (c)	1.37	0.03	2.00	0.04	1.57	0.09	**	
Width of the upper lateral tooth, mm (d)	1.22	0.02	1.80	0.04	1.43	0.09	**	
Angle of the top tooth (e)	53.36	0.86	36.95	0.81	49.64	2.08	**	
Number of leaf teeth pairs	2.95	0.05	2.78	0.06	2.88	0.10	–	–
Ratio total length (a) / width (b)	1.80	0.03	2.69	0.06	2.08	0.08	**	
Ratio total length (a) / length tooth (c)	3.36	0.05	3.32	0.05	3.46	0.09	–	–
Length of the upperlip, mm (f)	5.10	0.05	5.07	0.09	5.76	0.22	*	s/m
Length of the calyx tooth, mm (g)	2.72	0.05	3.73	0.08	3.20	0.16	**	
Length of the calyx tooth, mm (h)	2.02	0.05	2.64	0.06	2.22	0.12	**	m/h
Width of the calyx, mm (i)	2.06	0.03	2.18	0.03	2.05	0.07	*	m/h

Letters in parentheses refer to measurements explained in Fig. 1

Column *P*: levels of significance from Kruskal–Wallis tests for differences between the three groups (*$P < 0.05$; **$P < 10^{-12}$)

Column "not sign.": taxa that are not significantly different at the 0.05 level

m E. minima, *s E. salisburgensis*, *h* hybrid

characters of the putative hybrids in nearly all cases overlapped with values of one or both parental species. Mean character values of the hybrids were either intermediate or larger or smaller than those of the parental species. The upper lip of the corolla in all populations was significantly larger in the hybrids than in the putative parental species (Table 3).

With the exception of two white flowering plants, all *E. minima* plants had yellow corollas. *E. salisburgensis* plants from the artificial populations PI and AM flowered white, while three of the four *E. salisburgensis* in the artificial population VB had violet flowers. Most hybrids had white flowers (Fig. 4), but in two populations plants occurred whose flowers changed color from yellow to white during anthesis. This phenomenon was also observed in artificially produced hybrids of *E. minima* and *E. salisburgensis* and in (tetraploid) hybrids of *E. christii* and *E. hirtella* in previous garden experiments (Liebst and Schneller 2005; Liebst 2006). In plants from the populations PI and AM, cilia were mostly present on the surface of the capsules in *E. minima* and absent in the capsules of *E. salisburgensis*. In *E. minima* plants from the artificial population VB, capsule cilia and hairs on the leaves were present or absent in a similar number of plants. Hybrids either had glabrous or ciliate capsules and glabrous or hairy leaves (Fig. 4). *E. minima* and *E. salisburgensis* differed significantly in qualitative morphological characters (Table 4).

Discriminant analyses

Both qualitative and quantitative characters were used in the discriminant analysis (Table 5). Capsule and leaf indumentum characters were most powerful in discriminating species and hybrids (test of equality of group means, the two characters with the smallest Wilks's lambda values). The discriminant analysis resulted in statistically significant separation of species and hybrids in univariate and multivariate *F* tests ($P < 0.01$), with the first canonical function accounting for most of the spread (Fig. 5). A priori classification of *E. minima*, *E. salisburgensis* and hybrids was correct in 97.2, 94.7 and 64%, respectively.

RAPD analyses

In total 44 *E. minima*, 41 *E. salisburgensis* and seven plants determined as hybrids were used for RAPD analysis. A total of 40 repeatable polymorphic banding fragments were detected (540–3,000 base pairs, six primers), 24 of them occurring either with more than 80% in both species or with less than 80% in one or both of the species. The remaining 16 banding fragments occurred with ≥80% in one of the species, but with up to 25% in the second species too (Table 6). These bands were called "specific" for a species. UPGMA clustering did not separate the species and hybrids, when data of the populations were pooled and when all banding fragments were used in the analysis.

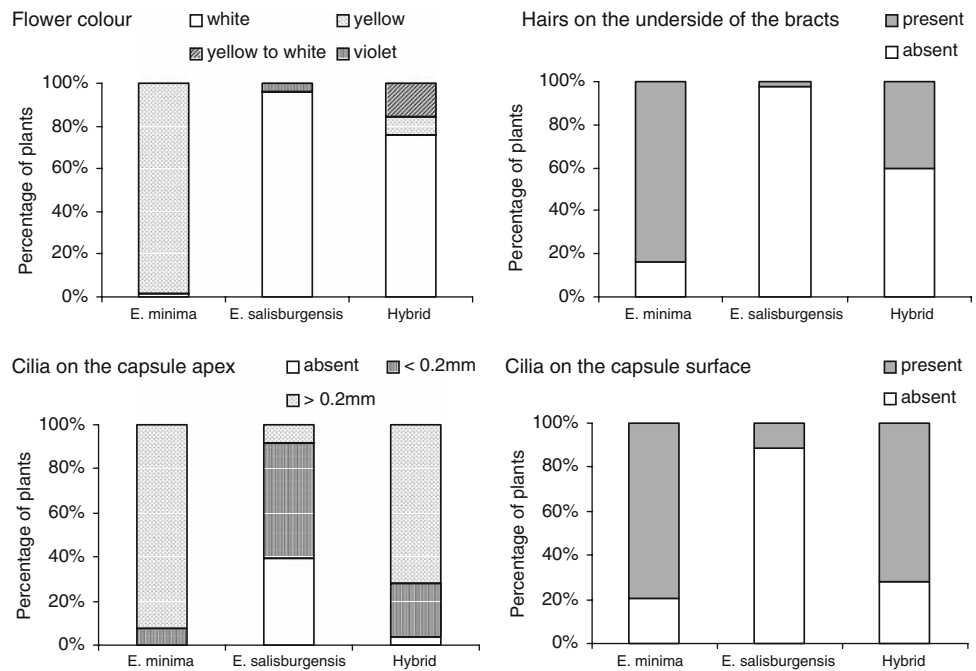**Fig. 4** Frequencies of qualitative morphological characters

Table 4 Observed and expected frequencies of qualitative characters and test-values from chi-square tests

	E. minima		E. salisburgensis		Hybrids	
	Observed	Expected	Observed	Expected	Observed	Expected
Hairs on the underside of the bracts						
Absent	18	56.8	79	42.2	15	13.0
Present	91	52.2	2	38.8	10	12.0
Chi-square 122.9, *df* 2, $P < 0.001$						
Cilia on the capsule apex						
No cilia or cilia <0.2 mm	8	45.1	74	33.5	7	10.3
Cilia >0.2 mm	101	63.9	7	47.5	18	14.7
Chi-square 137.3, *df* 2, $P < 0.001$						
Cilia on the capsule surface						
Absent	22	51.2	72	38.1	7	11.7
Present	87	57.8	9	42.9	18	13.3
Chi-square 92.2, *df* 2, $P < 0.001$						

When populations were analyzed separately and when only "specific" bands were used in the analysis, *E. minima* and *E. salisburgensis* were separated into two main clusters with the exception of seven plants (Fig. 6).

In most of the plants determined as hybrids a combination of bands predominantly occurring either in *E. minima* or in *E. salisburgensis* was found. Within the cluster *E. minima* of population PI, a cluster consisting of hybrids and one plant of *E. salisburgensis* (s1) and of *E. minima* (m2) was built. Each of the latter showed a combination of band patterns of both species, thus

revealing their hybrid origin (Fig. 6). Within the cluster *E. minima* of population AM, a cluster consisting of one hybrid and two plants of *E. minima* (m8, m11) and one *E. salisburgensis* plant (s10) was built. Banding patterns of the latter also revealed their hybrid origin (Fig. 6; Table 7).

Within the clusters of *E. salisburgensis* and *E. minima* in PI and AM, few plants of different species or hybrids were found. Banding patterns in most of these cases indicated that the plants were misidentified using morphological characters (Fig. 6; Table 7).

Table 5 Standardized canonical discriminant function coefficients

	Factor	
	1	2
Flower color[a]	0.252	0.597
Calyx tooth length (a)[b]	−0.205	−0.129
Calyx tooth length (b)[a]	0.074	0.165
Calyx width (c)[b]	−0.014	0.242
Capsule cilia surface	0.035	−0.407
Capsule cilia apex[a]	0.431	−0.507
Total leaf length (a)[b]	−0.096	−0.747
Top tooth length (c)[b]	−0.288	0.767
Lateral tooth width (d)[b]	−0.195	−0.005
Angle top tooth (e)[b]	0.423	−0.155
Hairs on the underside of the bracts[a]	0.424	0.501
Ratio leaf length (a)/width (b)[b]	−0.206	0.046
Ratio leaf length (a)/length tooth (c)[b]	−0.329	0.379
Number lateral shoots[b]	0.011	0.074
Variation explained (%)	97.1	2.9
Canonical correlation	0.920	0.379

Letters in parentheses refer to measurements explained in Fig. 1

Character transformation: [a] Vector transformation, [b] Element and vector transformation (Gower)
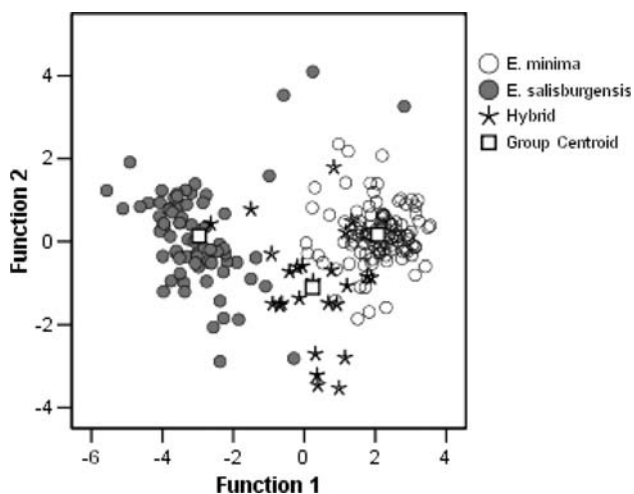

Fig. 5 Placement of plants of *E. minima*, *E. salisburgensis* and hybrids along the first and second discriminant function

Discussion

Preconditions for the production of hybrids

Overlapping flowering seasons in the artificial populations of *Euphrasia* enabled intra- and inter-specific cross-polli-nation of *E. minima*, *E. salisburgensis* and the indigenous large-flowered *E. alpina*, but neither the small- nor the large-flowered species attracted many insects. However, compared with studies of Gomez (2002) and Kreisch

Table 6 Polymorphic amplification products occurring in at least 80% of the individuals of one of the species and in not more than 25% of the individuals of the second species

Primer code	Primer sequence (5′ to 3′)	No. of polymorphic bands considered
OPA-19	CAAACGTCGG	1
OPC-13	AAGCCTCGTC	4
OPD-03	GTCGCCGTCA	4
OPD-19	CTGGGGACTT	5
OPD-20	ACCCGGTCAC	2

Primer code, primer sequence and the number of bands considered

(1996) (four or no visitors in small-flowered alpine *Eu-phrasia* species), the number of insect visits observed in the present study was much higher. The differences in the observed number of insects visiting *Euphrasia* flowers may be due to differences in the habitat, for instance the alti-tudinal zone (2,550–2,600 m in the studies of Kreisch (1996) and Gomez (2002), 1,960 m in the present study) or in the observation methods.

The very small beetles and ants observed in some of the *Euphrasia* flowers probably did not pollinate the flowers or pollinated flowers with pollen of the same inflorescence (geitonogamy). Conversely, the size and the behavior of most of the Hymenoptera and Diptera means that pollen could have been caught when creeping into a flower and deposited on the stigma of a second one.

Both *E. minima* and *E. salisburgensis* (and *E. hirtella* and *E. alpina*) were visited by Hymenoptera and Diptera, but *E. salisburgensis* was more frequently visited than *E. minima*, probably caused by a difference in the amount of nectar or pollen produced by each species. Both species possess small nectary discs at the bases of their ovaries (pers. obs.), but nectar could not be detected either in flowers of *E. minima* or in *E. salisburgensis* flowers. Large-flowered *Euphrasia* species are expected to produce more nectar than small-flowered species (Knuth 1909), but only one insect was observed successively visiting a large number of *E. alpina* flowers. There was no uniform pattern when insects visiting *E. salisburgensis* changed to the next plant. However, except in one case, insects visiting *E. minima* did not visit a second one, even if *E. minima* was as abundant as *E. salisburgensis* in the plot. These results indicate that insects probably discriminated between *E. minima* and *E. salisburgensis* by their flower colors.

Detection of hybrids

RAPD analyses

RAPD banding patterns confirmed the occurrence of hybrids between *E. minima* and *E. salisburgensis* in two of

Fig. 6 UPGMA clustering in the populations Piora and Andermatt. In population Valle Bedretto (not shown), no hybrids were detected and *E. minima* and *E. salisburgensis* were clustered in two groups similar to populations PI and AM. *m E. minima*, *s E. salisburgensis*, *h* hybrid
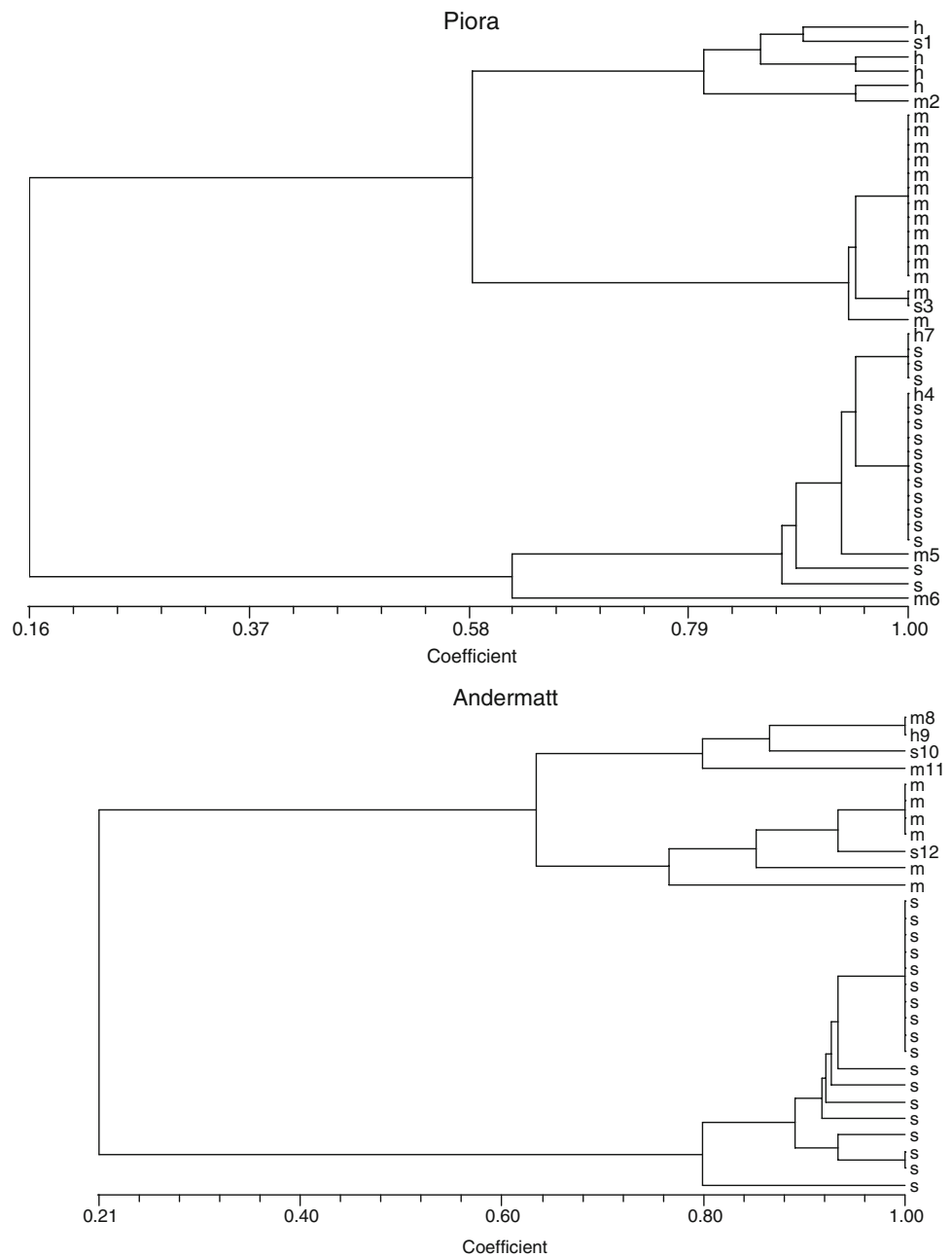

Table 7 RAPD bands of plants of the populations PI and AM

Plant	Piora							Andermatt				
	m2	m5	m6	s1	s3	h4	h7	m8	h9	s10	m11	s12
No. of bands specific for *E. minima*	7	0	6	7	8	0	1	12	8	8	8	8
No. of bands specific for *E. salisburgensis*	4	5	4	5	0	6	5	6	5	6	5	0

Plant names refer to Fig. 6

the artificially established populations and detected plants of hybrid origin that had been identified either as *E. minima* or *E. salisburgensis*. In contrast to a previous study of Liebst (2006) in which specific RAPD bands occurred in each of the species, in the present study no such patterns have been found. The predominance of bands in one of the species and the occurrence of these bands in some plants of the second species point to introgression between *E. minima* and *E. salisburgensis* in the origin populations. Yeo (1978) argued that introgression occurs mainly when there

are strong barriers to interbreeding, for instance between diploids and tetraploids, and that it also takes place across the high sterility barrier between subsection *Angustifoliae* and *Ciliatae*. In these cases the F1 generation is expected to produce no or few offspring, but back-crossing the hybrids would more likely result in fertile offspring. Evidence for gene exchange between species is also given by a morphological characteristic. Some *E. salisburgensis* plants had ciliate instead of glabrous capsules, a characteristic important in the separation of subsections *Ciliatae* and *Angustifoliae*. The occurrence of these plants suggests introgression of genes from *E. minima*.

Morphological and discriminant analyses

The hybrids detected in the artificial populations may have resulted from interspecific cross-pollination (F1 hybrids) or from selfing or back-crossing of the hybrids (F2 hybrids); both types could have descended from the seedings. However, the RAPD banding patterns indicate that they may also be descendants from hybrids backcrossed with one of the species in their origin populations. Furthermore, they could be offspring from hybrids or from backcrossed hybrids present in the surroundings of the artificial populations. In contrast to F1 and F2 hybrids which express a combination of morphological characteristics of both species, offspring from hybrids backcrossed with one of the parental species often show the habitus of the species with which they were backcrossed and only few characteristic from the other (Liebst 2006). In the present study, the number of hybrids classified correctly was in accordance with the classification of F1 and F2 hybrids in the study mentioned above (Liebst 2006). Because the history of the plants identified as hybrids in the present study is unknown, no further interpretation of the classification in the discriminant analysis is possible. However it is assumed that due to the high sterility of F1 hybrids, few or no F2 hybrids occurred in the artificial populations.

Some of the plants identified as hybrids in the artificial populations differed from hybrids of *E. minima* and *E. salisburgensis* produced experimentally in a greenhouse experiment (Liebst 2006). In contrast to the latter that mostly had yellow flowers at least in the beginning of the anthesis and had hairs on the underside of the leaves, many of the hybrids in the artificial populations had white flowers and often had glabrous leaves. Both characters point to parental species with white flowers and glabrous leaves. Besides *E. salisburgensis* (4n) that has these characteristics, the white flowering, glabrous leaved *E. alpina* (2n) occurred in some of the plots and could be one hybrid parent. Insect-visits of both species indicate that interspecific cross-pollination is likely to occur. The different ploidy levels however should

suggest that viable hybrids are rare. Liebst and Schneller (2005) did not obtain offspring when crossing *E. minima* (4n) and *E. rostkoviana* (2n). In contrast, Pugsley (1930) and Yeo (1956) hypothesized that tetraploid and diploid *Euphrasia* species could produce triploid offspring, so hybridization between *E. alpina* and *E. salisburgensis* could not be excluded.

During the flowering period of *Euphrasia* only few insect-pollinated plant species flowered within and around the plots and consequently insects were seldom in the whole area. So, independent of the flower size of *Euphrasia*, the chance of cross-pollination was reduced. Yeo (1968, 1978a) argued that cross pollination of small-flowered *Euphrasia* species may be supported by an attractive plant species growing in the surrounding of *Euphrasia*, for instance by *Calluna vulgaris*, associated with *E. micrantha*. Yeo suggested that *E. micrantha* may depend on a sufficient frequency of mistaken visits by insects visiting the flowers of *Calluna*, to provide it with a moderate degree of outcrossing. Occasionally when surrounded by species with flowers different in color and size, mistaken visits of *Euphrasia* flowers may also occur thus increasing inter- and intraspecific crossing.

Between five and six putative hybrids were found in each of the artificial populations in 2003. Assuming that all of these plants had grown from seeds resulting from interspecific crossing in 2002 and assuming a germination and survival rate of 3.5%, an estimated 160 seeds were produced by interspecific pollination in each of the artificial populations in 2002. Further considering a mean seed set of 6.3 (result of artificial interspecific pollination of *E. minima* and *E. salisburgensis*, data not published), this corresponds to about 25 flowers that had to have been pollinated by insects. Taking into consideration that most flowers of *E. minima* and *E. salisburgensis* are selfing in an early stage of the anthesis, it may be expected that few seeds in a fruit resulted from cross-pollination. Thus, the number of flowers that had to be visited by an insect is probably much higher. This estimation demonstrates that even in small populations of *Euphrasia* with low abundance of insects a moderate number of flowers are probably cross-pollinated, thus both conserving the genetic variability within a population and allowing hybridization between species.

Conclusions

The small number of flowers and the small flower sizes did not prevent cross-pollination in the artificial populations of *E. minima* and *E. salisburgensis*. Considering that *Euphrasia* often naturally occurs in large populations and that vigorous plants may bear up to 25 open flowers at the same time (Liebst 1999), crossing probably is much more

frequent than observed in the present study and more common than has been expected so far.

They do hybridize in their natural habitat

Hybrids were detected in both years following the establishment of the artificial populations. Nearly all hybrids which were investigated by RAPD analyses revealed their descent from *E. minima* and *E. salisburgensis* by their banding patterns. RAPD banding patterns also revealed the hybrid origin of some plants that had been determined as *E. salisburgensis* or *E. minima*, respectively. The predominance of a band fragment in one of the species and the occurrence of this band in some plants of the second species point to introgression of genes from *E. minima* to *E. salisburgensis* and vice versa in their origin populations.

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