Original article

Erratic pollination, high selfing levels and their correlates and consequences in an altitudinally widespread above-tree-line species in the high Andes of Chile

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ABSTRACT

Unfavorable temperatures and weather conditions for biotic pollination in above-tree-line alpine habitats predict self-compatibility, high levels of autogamy and small flower size ("autogamy reproductive assurance hypothesis"), or alternatively, compensatory measures such as greater flower longevity and larger display size so as to capture scarce visits and maintain outcrossing ("increased pollination probability hypothesis"). We assess these possibilities in a fine-tuned study of *Chaetanthera euphrasioides* (Asteraceae) populations located above-tree-line in the Andes of central Chile, where prior, independently obtained information on community flower visitation rates is available. Visitation by flies and Andrenid bees was highly erratic in all populations and among years, and the rates well below the community averages. We found evidence for high levels of self-compatibility, equally high autogamous potential, low genetic diversity and high and similar F_{IS} in all populations studied, associated with no clear trends in floral morphology. Strong decoupling of *C. euphrasioides* reproductive biology with community-level pollinator availability in the alpine fails to support either of the above-mentioned hypotheses and suggests early acquisition of autogamy with present-day pollinator conditions being adequate to maintain low visitation rates at all elevations. Our study provides the only instance where alternative hypotheses on alpine breeding systems have been tested with prior access to independently quantified community-level flower visitation rates.

1. Introduction

A number of studies have revealed that pollinator abundance and diversity suffer progressive reductions above the tree-line (Arroyo et al., 1982, 1985; Arroyo and Squeo, 1990; Totland, 1994; Bergman et al., 1996; Totland, 1997). In the high Andes of central Chile, where extensive quantitative studies have been performed, community-level flower visitation rates (based on a survey of 134 plant species) are generally low and decline by over 50% from the lower to the upper alpine over a corresponding altitudinal range of 1400 m (Arroyo et al., 1985). This altitudinal reduction in pollinator availability is paralleled by an altitudinal turnover in the major pollinator groups whereby high energy-demanding endothermic hymenopterans tend to be replaced by passively thermoregulating lepidopterans and less energy-demanding dipterans (Arroyo et al., 1982). Altitudinal turnover in pollinator assemblages has also been documented

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on other high mountains (Medan et al., 2002), including, interestingly, at the level of visitors to particular plant species (Galen, 1996; Zoller et al., 2002). Both the altitudinal decline in flower visitation rates and the eventual replacement of high energy requiring pollinators by less energy-demanding groups have been interpreted as the result of lower temperatures and increased cloudiness at the higher elevations as they affect the ability of insects to become active and maintain a positive energy balance (Arroyo et al., 1982).

A critical question in alpine ecology concerns how above-tree-line, altitudinally widespread plant species that reach the upper alpine and subnival zone deal with the progressively deteriorating conditions for biotic pollination just described. This same question of course, can be posed at different spatial scales, such as above and below the tree-line or even in pollinator-rich environments, where reduced pollinator service to some species accrues because of intensive competition by other plant species for the resource (Motten, 1986). However, conditions for pollinators become most critical toward the upper vegetation limit. Therefore, if anywhere, it should be here that clear expressions of adaptive compromises in breeding system and floral morphology on the part of plants are found.

The traditional "autogamous reproductive assurance hypothesis", which sees the acquisition of predominant self-pollination as circumventing the need for abundant pollination, has been proposed as a possible evolutionary response for alpine and arctic species facing impoverished pollinator conditions (Mosquin, 1966; Billings, 1974). In accordance with this idea, pollen limitation has been demonstrated in a number of alpine species (Galen, 1985; Stenström and Molau, 1992; Miller et al., 1994; Totland and Eide, 1999; Totland and Sottocornola, 2001) including in the low alpine belt in central Chile where pollinator availability is still relatively high in relation to the upper alpine belt (Muñoz and Arroyo, 2004). Under this hypothesis, shifts toward autogamy and high selfing rates could be expected above the tree-line and especially toward the upper vegetation limit, with concomitant reductions in flower size and population heterozygosity, and increased inter-population differentiation. However, other adaptive responses are possible for coping with decreased pollinator availability in the alpine. What will here be called the "increased pollination probability hypothesis", deriving initially from the earlier naturalists in terms of flower showiness (Müller, 1881) and from Arroyo et al. (1985) in terms of compensation through flower longevity (see also Kudo and Molau, 1999; Utelli and Roy, 2000; Fabbro and Körner, 2004), seeds increases in flower longevity or display size compensating for the scarce pollinator visits at the higher and colder elevations. Other variants of this general idea see some high elevation pollinators (bumblebees) as being more efficient than lowland pollinators (Bingham and Orthner, 1998; Bingham, 1999) or the abandonment of insect pollination for wind pollination (Arroyo and Squeo, 1987; Gomez and Zamora, 1996; Totland and Sottocornola, 2001). Increased flower longevity in the alpine has now been reported in several geographical areas of the world (Arroyo et al., 1985; Primack, 1985; Kudo and Molau, 1999; Utelli and Roy, 2000; Blionis and Vokou, 2001; Fabbro and Körner, 2004). Indeed, Rathcke (2003) recently provided experimental evidence for the effectiveness of increased floral longevity in increasing female reproductive success in a non-alpine environment. Plants growing in the alpine have also been shown to allocate proportionately more of their resources to flowers than lowland plants (Fabbro and Körner, 2004). With respect to display, in a seminal paper, Galen (1996) provided evidence that changes in pollinators in the alpine can influence flower size, indicating that fine floral evolutionary adjustments are indeed possible. Under these last various scenarios, relatively high outcrossing rates and considerable genetic variation could be maintained.

Studies of populations of altitudinally wide-ranging above-tree-line species where there is prior *independent* knowledge of tendencies in pollinator availability at the community-level provide an ideal means for distinguishing between the alternative hypotheses proposed. Inclusion of populations in the upper subnival belt should be particularly informative, as here conditions for biotic pollination reach their extreme. Yet in the few studies on this theme published to date, the key information on general trends in community pollinator availability has not been available, such that in rigor, it is unclear as to whether the prerequisites for testing hypotheses about breeding system tendencies are really given. That is, low pollinator availability shown on some high mountains might not necessarily characterize all alpine areas, at least to the same degree. Variation in wind speed, cloudiness, and summer rainfall can be expected to influence the altitude above which significant declines in pollinator availability become manifest on different mountain systems, and even within particular mountains systems between areas that differ in exposition.

Here, we undertake a comprehensive study of representative populations of alpine *Chaetanthera euphrasioides* (Asteraceae), found in the same area of the central Chilean Andes where precipitous declines in community-level flower visitation rates have been previously documented in above-tree-line habitats (Arroyo et al., 1985). Specifically we ask whether the high seed set observed throughout the range results from strong autogamy in all populations, or from some kind of compensation for differences in community-level flower visitation rates in the lower and upper alpine. We investigate breeding system, floral morphology, floral longevity, and pollinator visitation rates and provide indirect estimates of selfing rate (F_{IS}) for several populations of the focal species drawn from above the tree-line. Our study also allows consideration of the more general question of the predictive value of community-level trends in flower visitation rate when it comes to individual species.

2. Materials and methods

2.1. *Study species and sites*

C. euphrasioides is one of many annuals in the alpine flora of the generally arid northern and central Chilean Andes. Annuals are uncommon in wetter northern hemisphere alpine areas, but this is not the case in the drier Andes, where up to 15% of the high alpine flora can be annual species (Arroyo et al., 1998). *C. euphrasioides* is distributed from 2200 to 3500 m and thus spans the entire altitudinal range of the alpine belt (lower alpine: 2200–2700 (2600) m; upper alpine: 2800 (2700)–3100 m; subnival zone: 3200–3600 m). It forms dense continuous populations that are quite showy when flowering. Plants have an open spreading habit (to ca. 10 cm wide), with the capitula borne terminally on the branches. Total number of flower heads per plant varies from 1 to 21. The rays are white (to occasionally yellow). Arroyo et al. (1982) reported dipterans and a small bee visiting the heads of *C. euphrasioides* in a valley to the north of the present study site. The achenes of *C. euphrasioides*, which fall close to the mother plant and drop their pappus upon making contact with the soil, can be found in the persistent seed bank (Arroyo et al., 1999).

Research was carried out in the Valle Nevado area, Chile (33°19′S 70°14′W to 33°21′S 70°16′W) over two main periods: from early austral summer of 2002 to early autumn of 2003 (November–March) (hereafter Yr-1); late spring of 2003 to early autumn of 2004 (October–March) (Yr-2), with determination of breeding system in one population carried out in February 2002. Mean annual air temperature is 9.0 °C at 2200 m and 1.0 °C at 3500 m (Cavieres et al., 2000). Large interannual fluctuations in winter snow depth, a long, sunny, snow-free summer period interrupted occasionally above 2700 m by light afternoon convective precipitation in the form of rain, non-persistent snow or hail, and afternoon summer cloudiness above 3100 m, are characteristic (Arroyo et al., 1981). Work was carried out in populations located at 2460, 2810, 3050, and 3315 m. Populations around 2200–2300 m were discarded on account of extensive grazing disturbance; occasional outlying populations at 3500 m were too small for the study design. The three lowermost populations studied occur in the same valley on S- to SW-facing slopes while the uppermost occurs on a SE-facing slope at the head of the aforementioned valley. Mean air temperatures at peak flowering for the altitudinal extremes in this study (interpolated from Cavieres and Arroyo, 1999) are 11.8 °C (2460 m) and 4.6 °C (3315 m). Yr-1 was an unusually heavy snow year with a late summer, while Yr-2 was a light snow year with an earlier summer. Mean community-level flower visitation rates for the same general alpine area vary from 0.7×10^{-2} visits per min in the lower alpine belt to around 0.3×10^{-2} visits per min in the upper alpine belt, the differences being significant (Arroyo et al., 1985).

2.2. Pollinator visitation

We quantified pollinator activity on the four populations of *C. euphrasioides* in Yr-1 and Yr-2 in accordance with Utelli and Roy (2000), whereby independent observers simultaneously monitored insect visitation at three points of the population for periods of 25 min, alternating with a 5 min rest, over a total of three mostly sunny days per population (3 days × 4 populations × 2 years × 3 points × number of 25 min observation periods). The number of 25 min observation periods varied from 24 to 30, there being fewer than 30 in the two uppermost populations on one (3050 m) or both (3315 m) years on account of cold, cloudy, late afternoon conditions at these higher elevations. Capitula of *C. euphrasioides* open at mid-morning and close early in the afternoon, with the period of capitulum opening being somewhat shorter at higher elevations. Observations were made on all plants of *C. euphrasioides* contained within 1 m × 1 m quadrats, from 10:30 to 11:30 h in the morning to 03:30–04:55 h in the afternoon, the exact initial and final time of observation depending on the elevation and the particular day. For each 25 min period, the total number of open capitula was recorded, along with ambient temperature (taken at ca. 5 cm above ground level) (Arroyo et al., 1982). Data for all simultaneous observations in a population were pooled to obtain one estimate of capitula visitation rate per each 25 min observation period (as per Utelli and Roy, 2000). Number of capitula observed per 25 min period in the total sampling area of 3 m^2 varied from a yearly mean of 402.6 (2450 m) to 82.6 (3310 m) with an average of 278 considering all populations, reflecting different local plant densities. Wind pollination was apriorily discarded on the basis of the very low pollen production and the sticky nature of the pollen.

2.3. Breeding system and open-pollination seed set

Degree of self-compatibility and capacity for unassisted spontaneous self-pollination were assessed in the field over the period February 2002–2004. The following three tests were performed: a) hand self-pollination; b) pollen augmentation by hand cross-pollination; c) unassisted spontaneous self-pollination, all with prior bagging in the bud stage, and with subsequent re-bagging to ease capture of the mature achenes. All treatments on a particular population were performed over the same summer season. Because few open heads at the same stage of development can be found on an individual plant, we were forced to conduct the three tests on different plants (maximum of one head employed per plant). Each disc floret was pollinated on the day the stigmas became bifurcate and receptive and again on the following day (as they showed signs of age) to ensure successful pollination. All disc florets in a capitulum open within a period of 2–3 days, even though the capitula themselves can remain open for much longer periods, and some unusually small capitula can remain open for only 1 day. Seed set (% achene set) was assessed 2.5–3 weeks following pollination. The final number of plants included in the analysis varied between treatments and populations as a result of loss of material on account of wind and animal damage and occasional seed predation resulting from oviposition in the early bud stage prior to isolating the plants from pollinators (2460 m: 22–26 plants per treatment; 2810 m: 37–54; 3050 m: 9–20; 3315 m; 6–12). Because the number of disc florets varies according to the size of the capitulum among plants of the same population, % achene set provides a better comparative measure of fecundity than absolute achene set. Open seed set was assessed in Yr-1 for all populations and compared with additional independent sets of unassisted self-pollination data taken the same year as the open-pollination trials in each population. For open-pollination, a single capitulum per plant was tagged on haphazardly selected plants in the field and the near-mature achenes collected before they reached the dispersal stage. Sample sizes for open-pollination varied from *N* = 29 to 54. The unassisted pollination sample sizes varied from $N = 24$ to 49. Although apomixis is invariably associated with the perennial habit (Bicknell and Koltunow, 2004), in order to assure that apomixis was not a confounding factor in our results, in the summer of 2005–2006, one head per each of several plants at 2810 m ($N = 13$), 3050 m ($N = 17$) and 3315 m ($N = 15$) was carefully excised with a razor blade below the level of the stigma, taking care not to damage the ovules. The heads (containing a sum total of 133–173 disc florets per population) were bagged and later checked for seed set.

2.4. *Capitulum characteristics*

We sought to determine whether the capitula are more attractive and remain open for longer periods of time where the community-level pollination rates are known to be lower, thus compensating for the low visitation rates. For capitulum display we considered open capitulum diameter and the number of showy, but sterile ray florets per capitulum in relation to total floret number. Analyses showed that the two measures of capitulum display were uncorrelated in three cases and negatively correlated in the remaining cases. Since the expectation is that *both* head size and proportion of rays increase (increased pollination probability hypothesis), or both *decrease* (autogamous reproductive assurance hypothesis), and significant correlations were lacking in a number of cases, these two characteristics may be considered separately. Floral characteristics were assessed on 100 plants per population, repeatedly over Yrs-1 and -2 (4 populations × 2 years × 100 plants = 800 plants; Set A plants). Full capitulum diameter was measured in the field with digital calipers (Mitutoyo) around mid-day on sunny days for one capitulum on each plant. Each measured capitulum was harvested and conserved in 70% alcohol so as to count the number of ray and disc florets later in the laboratory. For each plant, we also recorded total capitulum number. The proportion of ray florets was obtained by dividing ray floret number by total floret number (the sum of the ray and disc florets).

The Asteraceous capitulum reflects extreme consolidation of several, simple inflorescence flowers into a tight head that is functionally equivalent to a simple flower. The size of the Asteraceous capitulum is known to be plastic and affected by plant size (Battjes and Bachmann, 1994). Because adult plants of annual *C. euphrasioides* are susceptible to size variation reflecting interannual differences in snowfall and local population density, some environmentally-determined variation in capitulum size could be expected. Accordingly, additional sets of 100 plants per year and population (Set B plants) (including the root system) were harvested well into the flowering season (but before achene dispersal had begun) with total capitulum number recorded for each plant. The Set B plants were dried for 72 h at 70 °C in a laboratory incubator, and weighed individually on an Ohau Analytical Plus Precision Balance (0.0001 g precision). Dry weight for Set B plants was regressed on capitulum number for each of the four populations and 2 years, with highly significant regressions obtained in each case (R^2 range: 0.843–0.922), thus allowing us to use capitulum number as surrogate for plant size. The two step process described here was unavoidable: full dry weight could not be obtained for the Set A plants, on account of fixing one capitulum per plant in alcohol for morphological measurements. Finally, at peak flowering in each population, unopened capitula (one per plant) were monitored from capitulum opening until closure in order to determine capitulum longevity. The final data set for head longevity considered 30 (2810 m) to 38 (2460 m) plants.

2.5. *Genetic diversity*

Measures of genetic diversity were obtained for all populations from allozyme variation. Early in the Yr-2 season, young actively growing plants were harvested over the interval from October (2460 m) to December 2003 (3315 m) and ground in an extraction buffer as per Mitton et al. (1979). Eight enzyme systems covering 11 putative loci were satisfactorily resolved with electrophoresis in 12% starch gels using two buffer systems, with staining procedures as per Murphy et al. (1996) (see Appendix A for details of loci and sample sizes). Conventional descriptors of genetic diversity (including multilocus H_T – total genetic diversity, H_S – mean genetic diversity within populations, G_{ST} – proportion of total genetic diversity among populations) were obtained with POPGENE version 1.31 (Yeh et al., 1997). *F*-statistics were calculated according to Weir and Cockerham (1984). A permutation test (GENETIX Software version 4.05, Belkhir et al., 2004) was used to test significant deviations of F_{IS} from HW equilibrium (single locus: 5000 permutations; overall F_{IS}: 10,000 permutations). Significantly positive F_{IS} is indicative of a significant substructure at the level of the populations observed (Wahlund effect). This substructure can be due to various causes such as limited gene flow or selfing. If selfing is the only cause of population structuration, the selfing rate (s) can be calculated from the multilocus F_{IS} as $s = 1 - (1 - F_{IS})/(1 + F_{IS})$, (Weir, 1996 as per Dudash and Fenster, 2001; Gaiotto et al., 2003). As s increases monotonously with F_{IS} (for $0 < FIS < 1$), F_{IS} can be used for comparing selfing rates across populations, everything else being equal. This estimate of selfing rate has the important advantage of reflecting a population's historical selfing tendencies (Dudash and Fenster, 2001) and thus is appropriate in environments where the rates could be expected to fluctuate widely between years, as in high alpine environments.

2.6. *Statistical analyses*

Measures of capitulum display were analyzed with two-way ANCOVA with Year and Population as fixed factors and capitulum number as a covariate. Year was considered as a fixed factor on the grounds that the second year was not selected randomly in relation to the first year of the study. The proportion of ray florets was transformed using the arcsine-square root transformation. Where criteria for using parametric methods (as per Zar, 1996) were not met, as in the breeding system tests, we employed the non-parametric Kruskal–Wallis test. The Mann–Whitney *U*-test with a Bonferroni sequential correction was used as an a posteriori test for the Kruskal–Wallis test.

3. Results

3.1. Pollinator visitation

The temperature at 5 cm above the ground was much higher than air temperature at 2.5 m (Fig. 1). However, in spite of the warmer conditions close to the ground, the capitula of *C. euphrasioides* received very few pollinator visits, mostly by Bombyllidae, miscellaneous other flies and a species (or perhaps two) of tiny high elevation Andrenid bee(s). In the over 93 h of field observations made in Yrs-1 and -2, the number of visits totaled 656 for a sum total of 62,187 capitula observed in all 25 min periods (224 in total). No visits were recorded for fully 77.7% of the observation periods. Visits were received by flies and Andrenid bees in similar proportions (flies: 49% of visits; Andrenid bees 51% of visits) in the lower alpine populations. All visits received at 3310 m pertained to the tiny bees. In *C. euphrasioides* the pollen is carried out of the corolla tube by the stigma as it grows upward. The shortly exerted disc floret stigmas become coated with sticky, pale yellow pollen such that any insect visiting the heads naturally comes into contact with the pollen and stigmas. However the efficiency of the visitors as pollinators probably varies. The Andrenid bees, which collect pollen, tend to work the capitula for longer periods than dipterans, and become completely covered with pollen.

The visitation pattern was highly erratic among the four populations and 2 years (Fig. 1). In Yr-1, there was a significant population effect (Kruskal–Wallis ANOVA: $H = 40.528$, $df = 3{,}114$, $P < 0.0001$), with visits being restricted to population 2460 m, and not surprisingly, significant differences between that population and the remaining three (Mann–Whitney *U*-test with Bonferroni sequential correction). A significant effect was again found in Yr-2 (Kruskal–Wallis ANOVA: $H = 43.569$, $df = 3{,}110$, $P < 0.0001$). The 2460 m population continued to receive visits, while none were recorded again at 2810 m. However, in contrast to the previous year, the 3050 m population received a minimal number of visits while the 3315 m population received an a significantly higher number of visits with respect to other populations (Mann–Whitney *U*-test with Bonferroni sequential correction). The lowermost population, in turn showed a significantly higher visitation rate than the two intermediate ones (Mann–Whitney *U*-test with Bonferroni sequential correction). Adjusting the 25 min visitation rates for total time available for pollination per day and assuming stigmas stay open for 1 day, leads to average overall visitation probabilities of 0.184 (2460 m), 0 (2810 m), 0.002 (3050 m) and 0.663 (3310 m) visits (without taking revisits into account). These values indicate significant pollination deficits in all populations in relation to the community means.

3.2. Breeding system and open-pollination seed set

The percentage of filled achenes was high in all three breeding system treatments in every population (Fig. 2). There were no treatment effects in any population (2460 m: Kruskal–Wallis test: $H = 0.140$, $df = 2{,}74$, $P = 0.932$; 2810 m: $H = 1.177$, $df = 2{,}102$, $P = 0.555$; 3050 m: $H = 1.132$, $df = 2{,}42$, $P = 0.568$; 3315 m: $H = 0.692$, $df = 2{,}27$, $P = 0.708$). No seed set resulted from the capitula from which the stigmas were excised in any population, eliminating the possibility that seed set obtained in the self-pollination treatments derives from apomixis. Open-pollination seed set (% filled achenes) was high in all populations (83.3–95.5%). There were no significant differences between open-pollination seed set and spontaneous pollination seed set except at 3050 m (Mann–Whitney *U*-test: $U = 287.0$, $P < 0.05$), where unassisted spontaneous self-pollination produces approximately 7% less achenes than open-pollination. Thus, the high levels of open-pollination seed set in *C. euphrasioides* could be

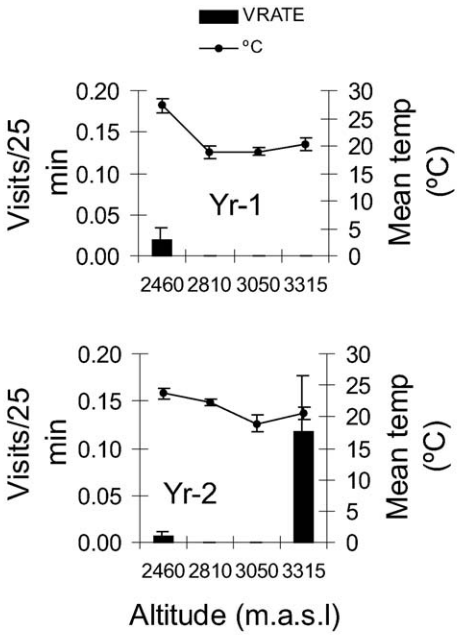

Fig. 1 – Mean head visitation rates (± 2S.E.) in populations of *C. euphrasioides* in Yr-1 (upper figure) and Yr-2 (lower figure). Mean temperature (± 2S.E.) taken 5 cm above ground level is also shown.

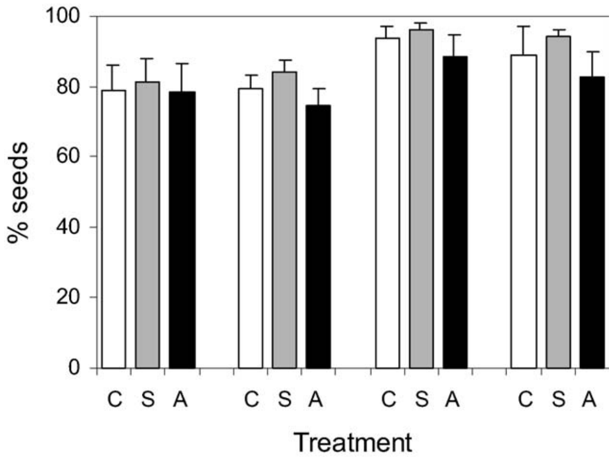

Fig. 2 – Comparison of mean (± 1S.E.) seed set (proportion of ovules maturing to achenes) in populations of *C. euphrasioides* under different breeding system treatments. C, hand cross-pollination; S, hand self-pollination; A, unassisted spontaneous self-pollination.

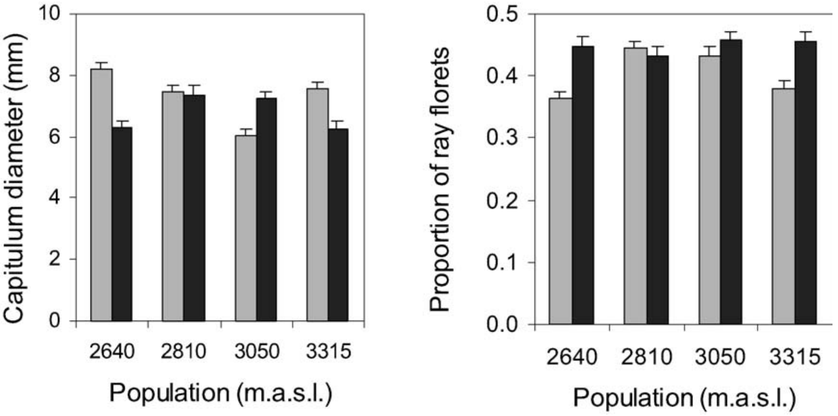

Fig. 3 – Capitulum diameter and proportion of ray florets (mean ± 2S.E.) for Yr-1 (gray bars) and Yr-2 (black bars) in populations of *C. euphrasioides*.

achieved either entirely (2460, 2810, 3315 m) or in great measure (3050 m) through unassisted self-pollination.

3.3. *Capitulum characteristics*

The heads of *C. euphrasioides* varied from a mean diameter of 6.05 mm (3050 m; Yr-1) to 8.20 mm (2540 m; Yr-1). The proportion of ray florets ranged from 36.5 (2640 m, Yr-1) to 45.8 (3050 m, Yr-2) (Fig. 3). Capitulum number proved to be an excellent surrogate for plant size in all populations (see Section 2). Positive correlations between capitulum number and capitulum size were found in seven out of eight cases, thus justifying the decision to use capitulum number as a covariate in the analysis of variance. Year, population and their interaction had significant effects on total capitulum size and proportion of ray florets per capitulum (two-way ANCOVA, Table 1). However, no evidence emerged for a consistent pattern of excessively small or excessively large capitula in the populations corresponding to the upper alpine (3315, 3050 m) where overall community-level pollination rates are lowest, as would is predicted by the two contrasting hypotheses under consideration, respectively (Fig. 3). Notably, the interannual variation in the capitulum measurements in a population can be higher than the variation among populations in a given year indicating significant plasticity in the capitulum and strong local environmental effects (Fig. 3).

Individual capitula remained open for one to 11 days with the mean fluctuating between 3.84 (3050 m) and 7.83 days (2810 m) (Table 2). There was a significant population effect on capitulum longevity (Kruskal–Wallis test: $H = 44.62$, $df = 3{,}132$, $P < 0.001$), but only the 2810 m population stood out, being significantly different from the remaining three populations (Mann–Whitney test with Bonferroni correction: $P = 0.008$). We believe that the longer lived capitula at 2810 m are an artifact of unseasonably cold weather brought on by an intense summer snow storm leaving the plants at that elevation covered in snow and without pollination for 2 days. Thus there is no evidence of head longevity compensation for the predicted lower numbers of visits in *C. euphrasioides* in the upper alpine.

Table 1 – Two-way ANCOVA (with capitulum number—Cap—as covariate) of the effect of year and population on two capitulum characteristics reflecting attractivity in *C. euphrasioides*

ANCOVA	*Df*	SS	*F*	*P*
Capitulum diameter				
Cap	1	107.264	95.14	< 0.0001
Year	1	46.336	41.10	< 0.0001
Population	3	52.813	15.61	< 0.0001
Year × population	3	249.415	73.74	< 0.0001
Error	791	891.783		
Total	799	1414.973		
Proportion of ray florets				
Cap	1	0.829	159.33	< 0.0001
Year	1	0.321	61.76	< 0.0001
Population	3	0.140	8.94	< 0.0001
Year × population	3	0.247	15.82	< 0.0001
Error	791	4.113		
Total	799	6.025		

3.4. *Genetic diversity*

The 2460 m population showed four polymorphic loci, while the remaining populations had 2 (Table 3; see also Appendix A for details). Total unique genotypes recorded in the isozyme profiles were 15, with no one genotype being dominant. Although the mean number of alleles was highest at the lowest elevation (Table 3, there was no significant population effect (Kruskal–Wallis test, $H = 1.835$, $df = 3{,}44$, $P = 0.61$). Likewise, although highest expected and observed heterozygosity was found at 2460 m (Table 3), no significant population effect was revealed (H_e: $H = 1.98$, $df = 4{,}44$, $P = 0.58$: H_o:

Table 2 – Head longevity in *C. euphrasioides*. Different letters indicate significant differences (Mann–Whitney *U*-test, with Bonferroni correction). *N* = number of plants. In all cases one head per plant was studied

Population	Mean (days)	N	2S.E.
2460 m	4.26 ^{a}	38	0.77
2810 m	7.88 ^{b}	30	0.92
3050 m	3.84 ^{a}	32	0.64
3315 m	3.28 ^{a}	32	0.27

Table 3 – Measures of genetic variation (mean ± 2S.E.) and F_{IS} in populations of *C. euphrasioides*. N: number of polymorphic loci, P: percentage of polymorphic loci, A: mean number of alleles per locus, H_e: mean expected heterozygosity, H_o: mean observed heterozygosity across all populations. F_{IS}: multilocus F_{IS}. *: significant deviation from HW equilibrium. See Appendix 1 for further details

Population	N	P	A	H_e	H_o	F_{IS}
2460 m	4	36.4	1.72 ± 0.66	0.120 ± 0.10	0.042 ± 0.05	+0.633*
2810 m	2	18.2	1.45 ± 0.62	0.052 ± 0.08	0.028 ± 0.04	+0.470*
3050 m	2	18.2	1.18 ± 0.24	0.043 ± 0.07	0.014 ± 0.02	+0.672*
3315 m	2	18.2	1.27 ± 0.39	0.043 ± 0.07	0.018 ± 0.02	+0.589*
Mean	2.5 ± 1.0	22.7 ± 9.1	1.40 ± 0.13	0.065 ± 0.04	0.026 ± 0.02	+0.591 ± 0.087

$H = 1.36$, $df = 3{,}44$, $P = 0.72$). Single locus F_{IS} was positive and differed significantly from 0% to 80% of the cases (Appendix A), indicating strong heterozygote deficiency in all four populations. All polymorphic loci in the two lowermost populations deviated significantly and positively from HW equilibrium, while 50% did so in the two uppermost populations (Appendix A). Multilocus F_{IS} was positive and differed significantly from 0 in all populations (Table 3).

Mean pairwise genetic divergence (F_{ST}) for contiguous populations was: 2460 vs. 2810 m: 0.413; 2810 vs. 3050 m: 0.809; 3050 vs. 3315 m: –0.017 (in this case, not significantly from 0 at $P < 0.05$). In *C. euphrasioides* most of the genetic variation, as indicated by the large G_{ST} value, is distributed among populations: $H_T = 0.42$; $H_S = 0.18$; and $G_{ST} = 0.58$.

4. Discussion

Visitation rates in *C. euphrasioides* were low, erratic and highly variable between years. Based on the community rates given in Arroyo et al. (1985) *C. euphrasioides*' flower visitation rate (expressed on a comparable per min basis) at 2460 m (0.6×10^{-3} visits per min) is fully one order of magnitude lower than the community average. For 3050 m (0.9×10^{-4}), the rate is two orders of magnitude lower. At 3315 m (0.2×10^{-2}) the rate is about one-third lower. These last comparisons, along with the total lack of visits registered at 2810 m over the 2 years, show that *C. euphrasioides* not only attracts far fewer visits than an average species in these alpine communities, but also is more disadvantaged in a relative sense in the lower alpine belt, where there are numerous very showy-flowered species.

C. euphrasioides turned out to be highly genetically self-compatible and capable of unassisted self-pollination. Seed set in plants tested for unassisted selfing was equal or similar to open-pollination seed set. The F_{IS} values obtained in *C. euphrasioides* (0.47–0.67) indicate high selfing rates (around 70% if selfing were the only cause of population substructure), placing *C. euphrasioides* within or close to Vogler and Kalisz's (2001) category for predominant selfing (> = 80%). Thus while our species is capable of undergoing fairly high levels of selfing, it is by no means an obligate selfer in practice, as might have been concluded from the field measures of its breeding system. In fact, the indirect method possibly overestimates the true level of selfing. The achenes of *C. euphrasioides* lose their pappus shortly after dispersal and only undergo primary dispersal. That is the high F_{IS} could be partially a reflection of limited dispersal. In any case, results from *C. euphrasioides* stress the importance of providing measures of selfing rates in addition to breeding system determinations in fine-tuned ecological studies such as that carried out here. Breeding system per se addresses a species' physiological and structural potentials for undergoing selfing, whereas selfing rates reflect realized amounts of selfing and will depend strongly on local variation in pollinator activity. To date, estimates of selfing rates for alpine species are limited to a single population of *Epilobium anagallidifolium* in the Colorado Rockies (Kelley and Latta, 1998), which was reported to be strongly selfing. No previous study addressing plant breeding system in the alpine has attempted to provide this critical information for a series of populations drawn throughout the entire alpine belt as has been done here.

As in the case of breeding system and F_{IS}, head diameter, the proportion of ray florets per head and head longevity bore no relationship to the drop-off in overall community-level pollinator availability, neither in the direction expected under the "autogamy fertility assurance hypothesis" nor in that expected under the "increased pollination probability hypothesis". The resulting seed set is however very stable across populations due to the similar levels of genetic self-compatibility and spontaneous selfing capacity among all four populations. Considering breeding system, selfing rates and floral characteristics, it can be seen that *C. euphrasioides* is strongly decoupled from the principal abiotic gradients that affect pollinator availability. Indeed, our results taken together are in lines with the conclusion that there are no consistent differences between populations or altitudinal tendencies in breeding system, selfing rates, floral morphology and floral longevity in the alpine in *C. euphrasioides*. The conclusion regarding altitude per se is tentative as we were unable to replicate the system. Indeed, this difficult to do in north–south trending ranges such as the Andes, in a meaningful way.

Lack of differences between the populations that are spread over 1400 m elevation naturally brings up the question as to when and where autogamy originally arose in *C. euphrasioides*, and if indeed, pollinator availability in alpine has been relevant at all in molding its breeding system. In view of the fact that many alpine species in Central Chile are likely to have arisen from lineages found at lower elevations in the Mediterranean-type climate woodlands where autogamous annuals abound (Arroyo et al., 1983; Arroyo and Uslar, 1993), *C. euphrasioides* ancestor might already have acquired autogamy by the time it reached the alpine life-zone. In this proposed ancestral habitat, late spring drought restricts water availability for shallowly rooted annuals, such that autogamy could be a response to selection for rapid reproductive completion, as per Aarssen's

(2000) "time limitation hypothesis". In this case, success of *C. euphrasioides* throughout the alpine today could be seen as a result of possession of a preadapted breeding system. Here it is worthwhile recalling that the three uppermost populations of *C. euphrasioides* exhibit fewer polymorphic loci than the lowermost population, and that the two uppermost populations show non-significant interpopulation divergence. This suggests an upward colonization mode of founder populations carrying a reduced sample of the total genetic variation found at lower elevations. Resolution of these kinds of problems in alpine ecology clearly requires integrating ecological and phylogenetic approaches.

C. euphrasioides shows similarities, yet important differences with other alpine species studied to date. As in *C. euphrasioides*, Gugerli (1998) found no difference in open seed set among alpine populations of *Saxifraga oppositifolia* in the Swiss Alps. However, in direct contrast to *C. euphrasioides*, *S. oppositifolia* is a strongly outcrossing species and would seem to be an ideal candidate for the "increased pollination probability hypothesis". Nevertheless, in the absence of visitation rate and flower longevity data in the last study, it cannot be ruled out that pollinator availability is not limiting in this system. Akhalkatsi and Wagner (1996) found no difference in percent seed set between alpine and subnival populations of *Gentianella caucasea*; however again, in the absence of breeding system and visitation rate data, it is difficult to interpret the significance of this trend. In *Espletia schultzii*, a strongly outcrossing insect-pollinated species in the tropical Venezuelan Andes, breeding system remains fairly constant among populations (Sobrevila, 1989). Wind pollination appears at higher elevations (Berry and Calvo, 1989), suggesting a case of increased pollination probability via a shift from insect pollination. *C. euphrasioides* bears some similarity to self-compatible *Cerastium alpinum*, recently studied by Totland and Schulte-Herbrüggen (2003) in the Norwegian alpine, except that pollinator visitation is much lower and more sporadic in our species. Clearly, the number of detailed comparative studies that include populations from the highest elevations is far too limited for evaluating the real effect of any decreased pollination availability on plant breeding system on high mountains at this stage.

Finally, the low and highly erratic visitation patterns in *C. euphrasioides* seem to reflect local competition for pollinators with other plant species. For example the almost total lack of visits over the 2 years at 3050 m occurs in the same area where *C. planiseta*, a second, but much more showy annual species of *Chaetanthera*, is known to be visited by similar insect ensembles visiting *C. euphrasioides* (C. Torres, Universidad de Concepción, personal communication). Such strong competition for pollinators among local plant assemblages in pollinator-scarce alpine environments would tend to provoke high variance among the flower visitation rates of alpine species. Even at the upper limit of the vegetation, some species can be expected to successfully draw pollinators away from others (and thus exhibit quite high pollination rates), while others are likely to be severely underserviced (as in the case of *C. euphrasioides*). This scenario, rather than favoring a single autogamy assurance pathway for alpine plant breeding system evolution, as has so often been espoused, would tend to select for a diversity of breeding systems ranging from strongly selfing species (such as *C. euphrasioides*), perhaps many species with mixed mating systems, to highly outcrossed species. This last prediction finds good support from the wide range of levels of genetic diversity in alpine plants (Galen et al., 1991; Abbott et al., 1995; Odasz and Savolainen, 1996; Stanton et al., 1997; Kelley and Latta, 1998; Gugerli et al., 1999; Bingham and Ranker, 2000; Stenström et al., 2001; Quiroga et al., 2002; Till-Bottraud and Gaudeul, 2002; *C. euphrasioides*, this study). Testing this idea in several alpine areas around the world would add much to our knowledge of the reproductive biology of alpine plants.

5. Conclusions

C. euphrasioides is strongly adapted for autogamy and shows high selfing rates throughout the entire alpine belt in central Chile. Flower visitation rates are low and erratic and strongly decoupled from the community-level visitation rates, to the extent that the community flower visitation rates have low predictive value for this particular species. This species fails to conform to the "autogamous reproductive assurance hypothesis" and to the "increased pollination probability hypothesis" at the level of the alpine life zone. It may have acquired autogamy at an earlier stage in the subtending lowland Mediterranean scrublands on account of selection for rapid reproductive maturation, thus becoming preadapted to the low visitation rates at the highest elevations in the central Chilean Andes.

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Appendix A

Allele frequencies for 11 isozyme loci for populations of *C. euphrasioides* in the significant F_{IS} deviation from HW equilibrium

Locus	2460 m	2810 m	3050 m	3315 m
PGI 1				
(N)	46	46	45	46
1	0.0556	0.0652	0	0
2	0.8556	0.7283	0.256	0.2391
3	0.0889	0.2065	0.744	0.7609
F_{IS}	+0.575*	+0.545*	+0.828*	+0.766*
PGI 2				
(N)	39	36	33	33
1	0	0	0	0
2	1	1	1	1
PGM 1				
(N)	45	44	45	46
1	0.0667	0.0227	0	0.0109
2	0.7556	0.9205	0.9556	0.9457
3	0.0889	0.0227	0.0444	0.0435
4	0.0889	0.0341	0	0
F_{IS}	+0.412	+0.256*	-0.035	-0.037
PGM 2				
(N)	27	35	38	38
1	0	0	0	0
2	1	1	1	1
6PGD 1				
(N)	45	46	45	46
1	0	0	0	0
2	1	1	1	1
6PGD 2				
(N)	16	24	22	26
1	0	0	0	0
2	1	1	1	1
IDH 1				
(N)	24	27	45	46
1	0.1667	0	0	0
2	0.7083	0	1	1
3	0.1250	1	0	0
F_{IS}	+0.824			
ME				
(N)	45	46	46	46
1	0	0	0	0
2	1	1	1	1
MDH 1				
(N)	46	46	45	46
1	0	0	0	0
2	1	1	1	1
SKDH 2				
(N)	43	34	34	46
1	0.9070	1	0	0
2	0.0930	0	1	1
F_{IS}	+0.730			
ADH 1				
(N)	45	40	45	46
1	0	0	0	0
2	1	1	1	1

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