

# How variable is delayed selfing in a fluctuating pollinator environment? A comparison between a delayed selfing and a pollinator-dependent *Schizanthus* species of the high Andes

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**Abstract** Delayed selfing has been considered the best-of-both-worlds response to pollinator unpredictability because it can provide reproductive assurance without decreasing outcrossing potential. According to this hypothesis, selfing rates in delayed selfing species should be highly variable in fluctuating pollinator environments. To test this prediction, as well to explore the consequences of delayed selfing on genetic patterns, we compared two sister species that grow in the high Andes of Chile: *Schizanthus grahamii* that exhibits delayed selfing and *Schizanthus hookeri*, which is self-compatible but requires pollinators for seed set. We estimated genetic diversity within and among five populations of each species using six shared microsatellites. Our results indicated that selfing rates in *S. grahamii* (range 0.07–0.81) were significantly more variable than in *S. hookeri* (range 0–0.26). The highest levels of selfing were found in the populations of *S. grahamii* located at highest altitudes ( $r = 0.78$ ) and at northern margin range, where pollinators are probably more scarce. These populations also showed the lowest allelic richness and heterozygosity values. Southern populations of *S. grahamii* had mixed mating, and showed heterozygosity and diversity values close to those detected for *S. hookeri* along all the sampled range. Selfing in this species results from geitonogamy, and did not covary with altitude. *Schizanthus grahamii* showed greater population differentiation than *S. hookeri*. Overall, our results indicated that selfing rates were widely variable in *S. grahamii*, with some populations predominantly selfing and others showing mixed mating. This pattern may be associated with the strong fluctuations in pollinator service that typically occur in the high Andes of Chile.

**Keywords** Delayed selfing · High Andes · Mixed mating system · Self-compatible · Pollinator failure

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## Introduction

Understanding the causes and consequences of mixed mating system evolution, in which both selfing and outcrossing occur, presents a major challenge for evolutionary biologists (Goodwillie et al. 2005). Early models of plant mating system evolution argued that predominant selfing and predominant outcrossing were alternative evolutionary optima (Lloyd 1979; Lande and Schemske 1985), predicting that intermediate selfing rates will be infrequent in natural populations. However, today it is widely recognized that mixed mating systems are common in natural populations of animal pollinated species (Aide 1986; Vogler and Kalisz 2001; Barrett 2003; Goodwillie et al. 2005; Iqic and Kohn 2006). This pattern has been attributed in part to the inability of self-compatible plants with large floral displays to avoid vector-mediated self-pollination (geitonogamy), however several mechanisms have been proposed that could yield evolutionarily stable mixed-mating systems (Holsinger 1988; Uyenoyama et al. 1993; Sakai 1995; Porcher and Lande 2005; Goodwillie et al. 2005).

Delayed selfing is one of the strategies that can favor the evolution of mixed mating (Iwasa 1990; Sakai 1995). In this mode of autonomous selfing (i.e., within-flower fertilization without the intervention of an external agent), self-pollination is delayed until after the opportunity for outcrossing has passed. Unlike other modes of selfing that occur before (prior selfing) or at the same time (competing selfing) as the opportunities for outcrossing, delayed selfing incurs no pollen or seed discounting (Schoen and Brown 1991; Lloyd 1992; Schoen and Lloyd 1992; but see Vaughton and Ramsey 2010). This selfing mode has been considered the best-of-both-worlds response to pollinator unpredictability because it provides reproductive assurance without decreasing potential for outcrossing (Kalisz and Vogler 2003). According to this hypothesis, selfing rates in delayed selfing species should be highly variable in fluctuating pollinator environments. When pollinators fail and flowers are pollinated only by autonomous-selfing, selfing rates should be as high as in prior-selfing species, whereas when pollinators are abundant and flowers receive cross- or geitonogamous pollen, selfing rates should be as low as in the case of non-autonomous self-compatible species. Although the best-of-both-worlds hypothesis is widely accepted in the literature, few studies have estimated selfing rates in natural populations of delayed selfing species subjected to high pollinator fluctuations (but see Kalisz et al. 2004), and no comparative studies between species with different modes of selfing have been conducted.

Selfing has important consequences on the amount and pattern of genetic variation within and among populations (Charlesworth and Wright, 2001; Ingvarsson 2002; Glémin et al. 2006). Selfing reduces heterozygosity, effective population size (Schoen and Brown 1991; Charlesworth et al. 1993), and genetic diversity (Hamrick and Godt 1996; Charlesworth and Wright 2001; Glémin et al. 2006). Selfing can also decrease gene flow through pollen, leading to strong population differentiation (Wright 1969; Hamrick and Godt 1996; Nybom 2004). The relationship between selfing and genetic diversity has been examined by numerous comparative studies of closely related taxa; most of which support the expected reduction in genetic diversity as well greater population differentiation of selfers with respect to their outcrossing relatives (Fenster and Ritland 1992; Charlesworth and Charlesworth 1995; Hamrick and Godt 1996; Awadalla and Ritland 1997; Dvorak et al. 1998; Liu et al. 1998; Williams et al. 2001; Sweigart et al. 2007; Mable and Adam 2007; Yan et al. 2009). However, none of these comparative studies included delayed selfing species, and the question of whether genetic diversity patterns in delayed selfing populations are similar to those reported for other modes of selfing remains relatively unexplored.

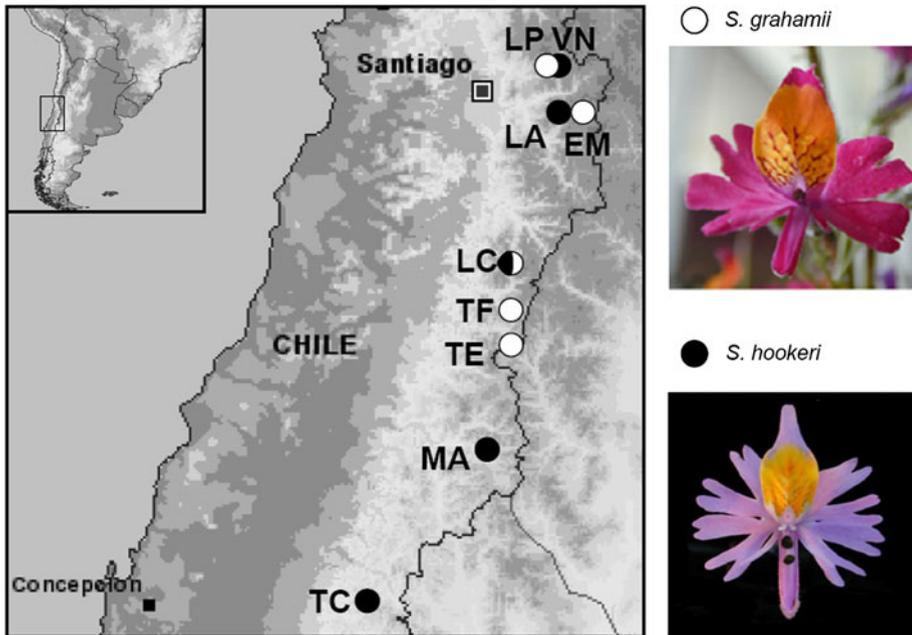
*Schizanthus grahamii* Gillies ex Hook. and *S. hookeri* Gillies ex Grham. are two herbaceous sister species that grow principally in the high Andes of central Chile, where community level flower visitation rates are low (Arroyo et al. 1985), and pollinator visitation tends to be erratic (Arroyo et al. 2006). Both species are self-compatible, but differ in their capacity for autonomous selfing (Pérez et al. 2009). In *S. grahamii*, the style elongates continuously throughout anthesis until the stigma eventually makes contact with the anthers at the end of anthesis, allowing delayed selfing. The flowers of *S. hookeri*, in contrast, are strongly herkogamous and dichogamous, and require pollinators for seed set. Like *S. grahamii*, this species has a large floral display, and therefore can be self-pollinated by geitonogamy. Ancestral reconstruction of pollination and mating systems of the genus were previously undertaken using molecular data (Pérez et al. 2006, 2009). This data showed that the clade containing *S. hookeri* and *S. grahamii* arose from an ancestor dependent on pollinators for seed set (Pérez et al. 2006, 2009). Besides their differences in capacity for autonomous selfing, *S. hookeri* and *S. grahamii* differ in the identity of major pollinators. The flowers of *S. hookeri* are visited by bees, hummingbirds and dipterans, whereas the flowers of *S. grahamii* are visited principally by hummingbirds. Some occasional visits of bees have also been recorded in southern populations, which have large and reddish flowers. Previous supplemental-hand pollination experiments with emasculated flowers showed that pollinator service is highly variable in natural populations of *S. grahamii*, and frequently pollen deposited by vectors is not sufficient for full fruit set (a phenomenon termed pollinator failure). In such cases, autonomous selfing provides reproductive assurance. Pollinator failure is less frequent and less variable in populations of *S. hookeri* than in populations of *S. grahamii* (Pérez et al. 2009). Given that pollinator service is low and unpredictable for *S. grahamii*, increases in autonomous selfing and occasional outcrossing could be expected. On the other hand, given that pollinator service is often adequate in *S. hookeri*, outcrossing and some geitonogamy could be common. Therefore, selfing rates should be greater and more variable in *S. grahamii* than in *S. hookeri*.

Here we compared levels of genetic variation and selfing rates of the delayed selfer *S. grahamii* and its non-autonomous self-compatible congeneric *S. hookeri*. Five populations of each species located in the high Andes of central Chile were sampled, including one site where the species occur sympatrically. We used a combination of six microsatellite loci to estimate genetic diversity, inbreeding coefficient, selfing rate and the proportion of outcrossing of each population. Population structure was also estimated for each species. In this way, we attempt to answer the following questions: (1) How variable are selfing rates in a delayed selfing species growing in a highly fluctuating pollinator environment such as the high Andes? (2) Does *S. grahamii* exhibit higher and more variable selfing rates than its non-autonomous self-compatible congeneric *S. hookeri*? (3) Does *S. grahamii* exhibit less genetic variation than *S. hookeri* within populations? (4) Is there more differentiation among populations in *S. grahamii* than in *S. hookeri*?

## Materials and methods

### Study species

*Schizanthus hookeri* and *S. grahamii* are annual- and occasionally biennial herbs endemic to Chile and Argentina (Grau and Grönbach 1984). The distributional ranges of these species overlap considerably: *S. grahamii* grows at high elevations in the Andes between 33°S and 39°S; *S. hookeri* grows at mid and high elevations between 29°S and 38°S



**Fig. 1** Map of central Chile showing the ten sampling locations of *Schizanthus hookeri* (black circles) and *S. grahamii* (white circles). One location (LC) harboring both species growing sympatrically is marked by a half white/solid circle. The list of localities and population abbreviations are given in the “[Appendix](#)”

(Grau and Grönbach 1984). Both species produce a large floral display of zygomorphic and bilabiate papilionaceous flowers (Fig. 1). The two species differ in floral morphology and pollinator visitors. *Schizanthus hookeri* has a typical bee-pollination associated with pink–purple corollas, nectar guides, lower lips extended as landing platforms, and explosive pollen discharge. Accordingly, flowers of *S. hookeri* are visited by bees, but also by dipterans and hummingbirds (Pérez et al. 2006). *Schizanthus grahamii* has a hummingbird pollination syndrome with flowers that lack a landing platform and have non exerted and non-explosive pollen discharge stamens. Southern populations of *S. grahamii* have red flowers (reddish morph), while northern populations have pink–purple flowers (pink morph) or flowers with reduced lateral sections, partially yellow (yellowish morph) (Pérez 2011). Flowers of *S. grahamii* are principally visited by hummingbirds (Pérez et al. 2006, 2009). The fruit in both species is a dehiscent capsule up to 15 mm long and 10 mm wide. Each capsule contains from 20 to 100 rough-surfaced small seeds up to 2 mm long. Apparently seeds are not wind or animal dispersed, and usually fall to the ground near the mother plant.

### Sampling

Plant material of *S. hookeri* was collected from five sites located between 33°S and 36°S in the high Andes of Central Chile at elevations ranging between 1750 and 2450 m (Fig. 1; “[Appendix](#)”). Plant material of *S. grahamii* was also collected from five sites located between 33°S and 35°S at elevations ranging between 1960 and 2420 m. These sites included one locality (LC, Laguna Los Cristales) where species occur sympatrically. A random sample of 20 individuals was collected from each population.

## DNA extraction and amplification

Leaves and floral buds were collected and immediately dried in silica gel. Genomic DNA was extracted from each sample using the DNEasy Plant mini kit (Quiagen, Hilden, Germany). A total of 200 individuals were genotyped for six microsatellite loci shared by the two species, using the primers and protocol described by Pérez et al. (2011). PCRs were carried out in 10  $\mu$ L reaction volumes containing 5 ng of template DNA, 1.6 pmol of the reverse primer, 0.8 pmol of M13-tailed forward primer (M13 forward sequence and microsatellite forward primer), 1.6 pmol of fluorescently labeled (6-FAM,1-vic or NED) M13 universal primer, Taq DNA polymerase (GoTaq, Promega), 5  $\mu$ L of 2X GoTaq Master Mix (supplied with the enzyme). Cycling conditions consisted of an initial denaturing step of 5 min at 95  $^{\circ}$ C, followed by 30 cycles of 30 s at 95  $^{\circ}$ C, 45 s at 52  $^{\circ}$ C, 45 s at 72  $^{\circ}$ C, and a final elongation step at 72  $^{\circ}$ C for 10 min. For genotyping, 1  $\mu$ L of the PCR product was added to 22  $\mu$ L formamide and 0.5  $\mu$ L LIZ-400 size standards. The mixture was run on the ABI PRISM 310 (Applied Biosystems), and analyzed using Peak Scanner<sup>TM</sup> Software version 1.0 (Applied Biosystems).

## Genetic diversity

The mean number of alleles per locus ( $N_a$ ) and Shannon Information Index ( $I$ ) were estimated for each population using GenALex version 6 (Peakall and Smouse 2006). Observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_E$ ), multi-locus fixation index  $F_{IS}$  and linkage disequilibrium coefficient for each pair of loci were estimated using Genetix 4.02 (Belkhir et al. 2001). Statistical significance of  $F_{IS}$  and linkage disequilibrium were calculated constructing null distributions with 100,000 random permutations in Genetix. Selfing rates ( $s$ ) were estimated by two approaches: (1) from  $F_{IS}$  using the relation  $s = 2F_{IS}/(1 + F_{IS})$  and (2) from the two-locus heterozygosity disequilibrium values ( $g_2$ ), using the software RMES. Unlike those derived from  $F_{IS}$ , selfing rates estimated from  $g_2$  are not sensitive to null alleles and scoring errors (David et al. 2007). To test whether selfing rates were significantly greater and more variable in *S. grahamii* than in *S. hookeri*, we performed a one-tailed, unequal variance  $t$  test and an  $F$  variance ratio test, respectively (Sokal and Rohlf 1995).

## Genetic differentiation

Population divergence was quantified using  $F_{ST}$  (Weir and Cockerham 1984) in Genetix 4.02 (Belkhir et al. 2001). Genetic differentiation within and among populations was further measured by analysis of molecular variance (Excoffier et al. 1992) using GenALex version 6 (Peakall and Smouse 2006).

## Results

### Within population genetic diversity

Except for the population of *S. grahamii* denoted LP, all populations were polymorphic for the six microsatellite loci. The lowest allelic richness ( $n_a$ ), Shannon information index ( $I$ ) and heterozygosity values ( $H_o$  and  $H_E$ ) were found in the northern populations of *S. grahamii*, LP ( $n_a = 2.2$ ;  $I = 0.44$ ;  $H_o = 0.07$ ;  $H_E = 0.25$ ) and EM ( $n_a = 2.2$ ;  $I = 0.49$ ;

**Table 1** Genetic variation and selfing rates at 6 microsatellite loci for five populations of the delayed autonomous selfing *Schizanthus grahamii* and five populations of its non-autonomous self compatible congeneric *S. hookeri*

	na	I	H <sub>O</sub>	H <sub>E</sub>	F <sub>IS</sub>	s(F <sub>IS</sub> )	s(g <sub>2</sub> )	N° loci
<i>S. hookeri</i>								
LA	5.3	1.32	0.52	0.65	0.17***	0.29	0.17	6
VN	4.3	1.19	0.62	0.72	0.13**	0.23	0	6
LC-H	6.5	1.50	0.55	0.71	0.24***	0.38	0	6
TC	4.5	1.07	0.52	0.56	0.12*	0.21	0.06	6
MA	4.0	1.08	0.43	0.58	0.22***	0.36	0.26	6
<i>S. grahamii</i>								
LP	2.2	0.44	0.07	0.25	0.75***	0.85	0.61	3
EM	2.2	0.49	0.04	0.32	0.87***	0.93	0.81	6
LC-G	5.0	1.15	0.42	0.60	0.27***	0.43	0.53	6
TE	5.0	1.28	0.44	0.67	0.37***	0.54	0.29	6
TF	4.7	1.03	0.48	0.55	0.15**	0.26	0.07	6

Shown for each population are: mean number of alleles per locus (na), Shannon's information index (I), observed (H<sub>O</sub>) and expected (H<sub>E</sub>) heterozygosity, inbreeding coefficient (F<sub>IS</sub>), and selfing rates estimated from F<sub>IS</sub> (s) and from g<sub>2</sub>. The number of loci useful is also shown

H<sub>O</sub> = 0.04; H<sub>E</sub> = 0.32) (Table 1). The southern populations of *S. grahamii* (TE, LC, TF) had genetic diversity measures and heterozygosity values two to ten times greater than northern populations (range na = 4.7–5.0; I = 1.03–1.28; H<sub>O</sub> = 0.42–0.48; H<sub>E</sub> = 0.55–0.67). These values were close to those detected in the pollinator-dependent *S. hookeri* along all the sampled range (na = 4.0–6.5; I = 1.07–1.50; H<sub>O</sub> = 0.43–0.62; H<sub>E</sub> = 0.58–0.72). When all populations of *S. grahamii* and *S. hookeri* were compared, marginal differences in I (*t* test; *p* = 0.057; Table 2) and significant differences in H<sub>O</sub> (*t* test; *p* = 0.03, Table 2) were detected among species.

#### Linkage disequilibrium, inbreeding coefficients and selfing rates

Significant linkage disequilibrium between loci was detected only in two populations, EM (with four of 15 locus pairs) and TF (with two of 15 locus pairs). Fixation indices F<sub>IS</sub> across six loci were positive in all populations, indicating substantial heterozygote deficit in both species (Table 1). F<sub>IS</sub> indices and F<sub>IS</sub>-based selfing rates were significantly greater (*t* test, Table 2) in *S. grahamii* than in *S. hookeri*. F<sub>IS</sub>-based selfing rates varied widely among populations of *S. grahamii* from a low of 0.26 in TF to ~0.93 in EM, and tend to be higher at higher altitudes (*r* = 0.77, *p* = 0.09). Variation in s (F<sub>IS</sub>) was significantly less among populations of *S. hookeri*, ranging from 0.21 to 0.38 (*F* test: *p* = 0.01). The same tendency was detected when selfing rates were estimated with the g<sub>2</sub>-method (*F* test: *p* = 0.05; Table 2). In general, this method gave lower estimates of selfing rates than the F<sub>IS</sub>-based method. For example, although the F<sub>IS</sub> for VN and LC-H were very significant and yielded substantial selfing rates of 0.23 and 0.38, the g<sub>2</sub> method yielded s = 0. Selfing rates estimated with this method varied between 0.70 and 0.81 in *S. grahamii*, and were strongly correlated with altitude (*r* = 0.91, *p* = 0.03). No significant correlation between selfing rates and altitude were detected for *S. hookeri*.

**Table 2** Comparison of genetic diversity between populations of *S.s grahamii* and *S. hookeri*

	<i>S. hookeri</i> mean ( $\sigma$ )	<i>S. grahamii</i> mean ( $\sigma$ )	<i>P</i> value means	<i>P</i> value variances
I	1.23 (0.18)	0.88 (0.39)	0.06	0.08
Ho	0.53 (0.07)	0.29 (0.22)	0.03	0.02
F <sub>IS</sub>	0.18 (0.05)	0.48 (0.31)	0.05	0.00
s(F <sub>IS</sub> )	0.29 (0.08)	0.60 (0.28)	0.03	0.01
s(g <sub>2</sub> )	0.10 (0.11)	0.46 (0.29)	0.02	0.05

**Table 3** Pairwise population F<sub>ST</sub> values for *Schizanthus hookeri* and *S. grahamii*

<i>S. hookeri</i>	LA	LC_H	MA	CHI
VN	0.13	0.08	0.23	0.23
LA		0.19	0.24	0.25
LC_HOO			0.18	0.24
MA				0.24
<i>S. grahamii</i>	EM	LC-G	TF	TE
LP	0.62	0.53	0.54	0.45
EM		0.46	0.52	0.44
LC_GRA			0.32	0.17
TF				0.11

### Genetic differentiation among populations

AMOVA detected significant differentiation among populations for both species. In the case of the delayed autonomous selfing *S. grahamii*, differences among populations accounted for 51 % of the total genetic variation. By contrast, only 33 % of variation was partitioned among populations of the non-autonomous self compatible *S. hookeri*. Bootstrap-derived confidence intervals for F<sub>ST</sub> values indicated that the degree of population differentiation was significantly greater in *S. grahamii* (CI<sub>95 %</sub> = 0.35–0.52) than in *S. hookeri* (CI<sub>95 %</sub> = 0.16–0.30). Northern populations of *S. grahamii* exhibited the highest levels of differentiation with respect to their conspecific populations, with a mean pairwise F<sub>ST</sub> of 0.53 for LP, and 0.51 for EM (Table 3). There was less divergence among southern populations of *S. grahamii*, with pairwise F<sub>ST</sub> values that ranged from 0.11 to 0.33. Populations of *S. hookeri* also showed low divergence with pairwise F<sub>ST</sub> values that ranged from 0.08 to 0.25 (Table 3).

### Discussion

In agreement with the “best-of-both-worlds hypothesis”, we found that selfing rates in delayed selfing *S. grahamii* were more variable than in the pollinator-dependent *S. hookeri*, even though the geographical range sampled for the former species was smaller. This tendency was observed either when selfing rates were estimated using the fixation index F<sub>IS</sub> or disequilibrium g<sub>2</sub> values, although often the former method gives estimations higher

than the latter. The great variability in selfing rates found in *S. grahamii* may be a result of strong fluctuations in pollinator service. We do not have enough data to show that selfing rate variation in *S. grahamii* is correlated with variation in pollinator failure rate as has been described for other species with delayed selfing (Kalisz et al. 2004), but several lines of evidence support this hypothesis. First, *S. grahamii* occurs in the high Andes of central Chile, where community-level flower visitation rates are low and decrease with elevation (Arroyo et al. 1985) and visitation tends to be erratic (Arroyo et al. 2006). Second, this species has a specialized pollination system (being visited principally by hummingbirds) that makes it vulnerable to spatial and temporal pollinator fluctuations (Pérez et al. 2009). Third, pollinator observations have been conducted in several populations and often flower visitation rates tends to be low (Pérez et al. 2006; and unpublished data). Four, supplemental hand-pollination experiments with emasculated flowers showed that pollinator service is highly variable in natural populations of *S. grahamii*, and that frequently pollen deposited by vectors is not sufficient for full fruit set. The same set of data showed that delayed selfing provides reproductive assurance when pollinators fail. Pollinator failure in natural populations of *S. hookeri* is lower and less variable than in *S. grahamii* (Pérez et al. 2009). The generalized pollination system of *S. hookeri* (which is visited by bees, Diptera, flies and hummingbirds) probably allows it to make better use of the low and erratic pollinator visitation rates recorded in the high Andes of central Chile.

The populations of *S. grahamii* located at highest altitudes on the northern range margin (LP and EM) showed the highest selfing rates.  $F_{IS}$ -based selfing was 0.93 for EM and 0.85 for LP. The  $g_2$ -based method gave lower estimates; 0.85 for EM and only 0.61 for LP. Similar bias has been detected in other studies, where the larger  $F_{IS}$ -based estimates have been considered as artifacts, because unlike  $g_2$ -based estimates, they are sensitive to null alleles and scoring errors (David et al. 2007). Biparental inbreeding, mating between relatives, can also cause increased homozygosities and inflated  $F_{IS}$ -based selfing values (Ritland 1984; Ellstrand and Elam 1993). Although the importance of this bias is apparently little in natural populations (Jarne and Auld 2006), it can increase in small and dense populations (Ellstrand and Elam 1993; Hodgins and Barrett 2006). Biparental inbreeding would be important in LP, which is notably smaller than the other populations. No obvious differences in size between the remaining populations of *S. grahamii* and *S. hookeri* were observed. It is also important to highlight that  $F_{IS}$  and  $g_2$ - methods produce estimates that integrate the inbreeding history over several generations. In addition both methods are sensitive to bias introduced by differential mortality between inbred and outcrossed progeny, and therefore selfing rates could in both cases be underestimated (Jarne and David 2008). Unfortunately, we could not test this hypothesis because the germination of *S. grahamii* seeds is very low.

Several authors have proposed that selfing rates should increase in habitats where pollinator service is limited (Bliss 1962; Arroyo 1973; Lloyd 1979). Pollinator abundance and activity have been shown to become limiting factors for successful pollination at high elevations (Arroyo et al. 1985; Bingham and Orthner 1998), and accordingly, it has been frequently hypothesized that selfing rates should increase with altitude. However, the few studies that have examined selfing rates along altitudinal gradients does not support this hypothesis (Bingham and Ranker 2000; Arroyo et al. 2006; Rahel et al. 2010). Here, we found a strong correlation between selfing rates and elevation, but this relationship can be confounded by latitude. Indeed, LP and EM are located at the highest elevations, but also in the northern margin range. A tendency toward increased selfing rates in geographical range margins has been detected for several species and it is also attributed to reduced pollinator service or plant density (Busch 2005; Mimura and Aitken 2007). No evidence

that pollinator service is lower in LP and EM than in the remaining populations is available, but supplemental pollination experiments with emasculated flowers performed in previous years indicates that it is highly fluctuating. For example, variation in pollinator failure rates from 5 to 85 % in two consecutive years was detected in LP (Pérez et al. 2009). Northern and southern populations of *S. grahamii* also differ in flower phenotype (Pérez 2011) and probably in the role of bees in pollination. Flowers visitors were recorded in LP, and two southern populations (TF and LC), and in all the cases hummingbirds were the most abundant visitors. However, bees visiting flowers were also recorded in TF and LC, accounting for 7 and 13 % of visits (Pérez et al. 2006, and unpublished data). It is not known how variable is pollinator failure in southern populations of *S. grahamii*, but it is possible that fluctuations in hummingbird abundance will be buffered by bees, producing lower selfing rates and higher genetic diversity. Indeed,  $F_{IS}$ -based selfing rates and genetic diversity of *S. grahamii* in the sympatric site LC were very similar to *S. hookeri*, suggesting non- autonomous selfing occur when this species become little more generalized.

The northern populations of *S. grahamii*, LP and EM, also showed the lowest allelic richness and heterozygosity values. The  $H_O$  values estimated for these populations are close to those estimated for predominantly selfing species (Nybom 2004). The remaining populations of *S. grahamii*, as well all sampled populations of *S. hookeri*, had heterozygosity values close to those reported for species with mixed mating systems (average  $H_O = 0.51$ ; Nybom 2004); moderate selfing rates were detected in these populations ( $s(F_{IS}) = 0.21–0.54$ ). In the case of *S. hookeri*, which is self-compatible and highly herkogamous, selfing probably results from geitonogamy. Indeed, the main pollinators of this species, *Bombus dalhombii* and bees of the genus *Megachile*, often visit several flowers within a plant. Variation in selfing rates among populations of *S. hookeri* might result from fluctuations in the abundance of these insects respect to other pollinators that move less within a plant. It is known, for example, that hummingbirds can respond to nectar asymmetry with increased single-flower visits (Carlson 2008), and therefore, an increase in hummingbird abundance would reduce geitonogamy. Other factors, including floral display, plant density, pollinator behavior, pollen load size might also explain variation in geitonogamy (Utelli and Roy 2008; Maad and Reinhammar 2004; Kropf and Renner 2008). Future studies are necessary to assess whether shifts in pollinator fauna cause variation in geitonogamy across populations of *S. hookeri*.

Our data also indicated that populations of *S. grahamii* were more differentiated than those of *S. hookeri*, even though the geographic range sampled for the former species was smaller. The northern populations of *S. grahamii* (LP, EM) which also had the highest selfing rates presented the highest levels of differentiation, compared to each other or to the southern conspecific populations. These results agree with a large set of studies showing that selfing increases differentiation among populations (Wright 1969; Hamrick and Godt 1996; Nybom 2004). High differentiation between northern and southern population of *S. grahamii* was reported previously using cpDNA data (Pérez 2011). This data, however, did not show differentiation between northern populations. Northern populations of *S. grahamii* also showed the most divergent floral morphology. The high level of selfing detected in these populations together with a lack of an effective mechanism of seed dispersal has probably produced a high genetic and morphological differentiation with respect to the remaining conspecific populations. Indeed, the fruit in the genus *Schizanthus* is a dehiscent capsule containing small, non-winged seeds that fall to the ground near the mother plant, where they germinate.

Overall, our results indicated that selfing rates were widely variable among populations of the delayed selfer *S. grahamii*, with some populations predominantly selfing and others

showing mixed mating. Further studies are needed to reveal which environmental conditions allow the maintenance of mixed mating in some populations.

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## Appendix

See Table 4.

**Table 4** Locations and voucher specimens of studied populations of *Schizanthus hookeri* and *S. grahamii*

<i>S. hookeri</i>		
LA	RM, Lagunillas, 2250 m, 33°33 S, 70°17 W	CONC 172479
LC-H	VI, Laguna de los Cristales, 2360 m 34°34 S, 70°32 W	CONC 171986
MA	VII, Laguna del Maule, 2050 m, 36°00 S, 70°33 W	CONC 172478
VN	RM, Valle Nevado, 2450 m, 33°21S, 70°17 W	CONC 172482
TC	VIII, Termas de Chillán, 1750 m, 36°54 S, 71°24 W	
<i>S. grahamii</i>		
LP	RM, La Parva, 2350 m, 33°20 S, 70°17 W	CONC 172483
EM	RM, Embalse del Yeso, 2420 m, 33°40 S, 70°05 W	CONC 172480
LC-G	VI, Laguna de los Cristales, 2360 m, 34°34 S, 70°32 W	CONC 171985
TE	VI, Laguna el Teno, 2170 m, 35°11 S, 70°30 W	CONC 172485
TF	VI, Termas del Flaco, 1960 m, 34°57 S, 70°25 W	HULS 6265

Location data include political region of Chile, study site, elevation (m a.s.l), latitude, and longitude

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