ANCESTRAL RECONSTRUCTION OF FLOWER MORPHOLOGY AND POLLINATION SYSTEMS IN *SCHIZANTHUS* (SOLANACEAE)[1]

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Concerted changes in flower morphology and pollinators provide strong evidence on adaptive evolution. *Schizanthus* (Solanaceae) has zygomorphic flowers and consists of 12 species of annual or biennial herbs that are distributed mainly in Chile and characterized by bee-, hummingbird-, and moth-pollination syndromes. To infer whether flowers diversified in relation to pollinator shifts, we traced the evolutionary trajectory of flower traits and visitors onto a phylogeny based on sequence data from ITS, *waxy*, and *trnF*/*ndhJ* DNA. Maximum-likelihood ancestral reconstruction of floral traits suggests that ancestral *Schizanthus* had a bee-pollination syndrome. The hummingbird syndrome evolved in *S. grahamii*, a high elevation species in the Andes. The moth syndrome evolved in the ancestor of three species that inhabit the Atacama Desert. Results of mapping flower visitors onto the phylogeny show that the shift from bee to hummingbird pollination concurred with a shift in pollinators as predicted by the syndromes. However, the same pattern was not found for the moth syndrome. Visits by moths were observed only in one of the three moth-syndrome species, and at a very low rate. This mismatch suggests either anachronic floral characters or maintenance of rare, imperceptible moth pollination backed up by capacity for autonomous selfing. Overall, results suggest that diversification of flower traits in *Schizanthus* has occurred in relation to pollinator shifts.

Key words: bees; Chile; floral evolution; hummingbirds; molecular phylogeny; moths; pollination syndromes; Solanaceae.

One of the major features of flowering plants is that particular suites of floral characters are associated with specific groups of animal pollinators (Stebbins, 1974; Fægri and van der Pijl, 1979; Pellmyr, 2002). The idea that different pollinator syndromes (flower phenotypes specialized for different groups of pollinators) evolve from the direct influence of different pollinator species has become a central tenet in flowering plant evolution (e.g., Grant and Grant, 1965; Stebbins, 1974; Fægri and van der Pijl, 1979). Implicit in the syndrome concept is the assumption that pollinator-mediated selection is the main force driving floral diversification into highly specialized morphologies (e.g., Johnson et al., 1998; Goldblatt et al., 2001). However, several studies have suggested that historical and random processes unrelated to flower adaptation and developmental constraints can also influence the differential representation of flower morphologies across taxa (e.g., Armbruster, 2002; Herrera et al., 2002). These results altogether have stimulated a reevaluation of the validity of the syndrome concept to predict pollination systems (e.g., Waser et al., 1996; Johnson and Steiner, 2000). One way to examine whether well-defined floral trait combinations evolved in relation to particular pollination systems consists of evaluating their concomitant changes and joint evolutionary trajectories in a phylogenetic context (Fenster et al., 2004 and cited references). This approach allows the examination of historical effects in floral evolution and removal of the variation in floral traits introduced by evolutionary relatedness (Fenster et al., 2004). Despite these evident advantages, studies of this kind have been undertaken in a mere 12 groups of plants (Fenster et al., 2004). In this paper, we study the relationship between flower evolution and pollinator service in the solanaceous genus *Schizanthus* using a phylogenetic approach.

The genus *Schizanthus* Ruiz and Pavón, endemic to the southern South American Andean region, is distributed between 22–40° S (Fig. 1). Molecular (Olmstead and Palmer, 1992; Martins and Barkman, 2005) and morphological data (Grau and Grönbach, 1984) indicate that *Schizanthus* diverged early from the rest of the family Solanaceae, constituting the monogeneric tribe Schizanthoideae (Olmstead and Palmer, 1992). Extant *Schizanthus* comprise 12 species of annual to sometimes biennial herbs that grow to 1 m high. They occur in diverse habitats including the desert, coastal, high Andean, and mediterranean-climate regions of Chile, with two species reaching the Argentinean side of the Andes. Flowering in most species occurs during the austral spring-summer, although northern desert populations emerge only in high precipitation years concurring with El Niño events. The floral morphology in the genus *Schizanthus* is unusual among members of Solanaceae. The flowers are bilabiate and strongly zygomorphic, resembling the papilionaceous flower (Fig. 1) (Walters, 1969; Grau and Grönbach, 1984; Knapp, 2002). The corolla consists of five petals fused into a tube. The three uppermost petals constitute the upper lip, comprising a banner and two dissected lateral sections. The two lower petals are deeply dissected, and their inner portion is fused to form a keel that in some species retains the stamens prior to explosive pollen discharge (Cocucci, 1989). The outer portions of the lower petals constitute the wings. Moth- and bee-pollination syndromes have been previously described by Coccuci (1989). The moth-pollination syndrome consists of white corollas with long tubes; backward-reflexed and highly dissected, lateral sections; reduced lower lips; and

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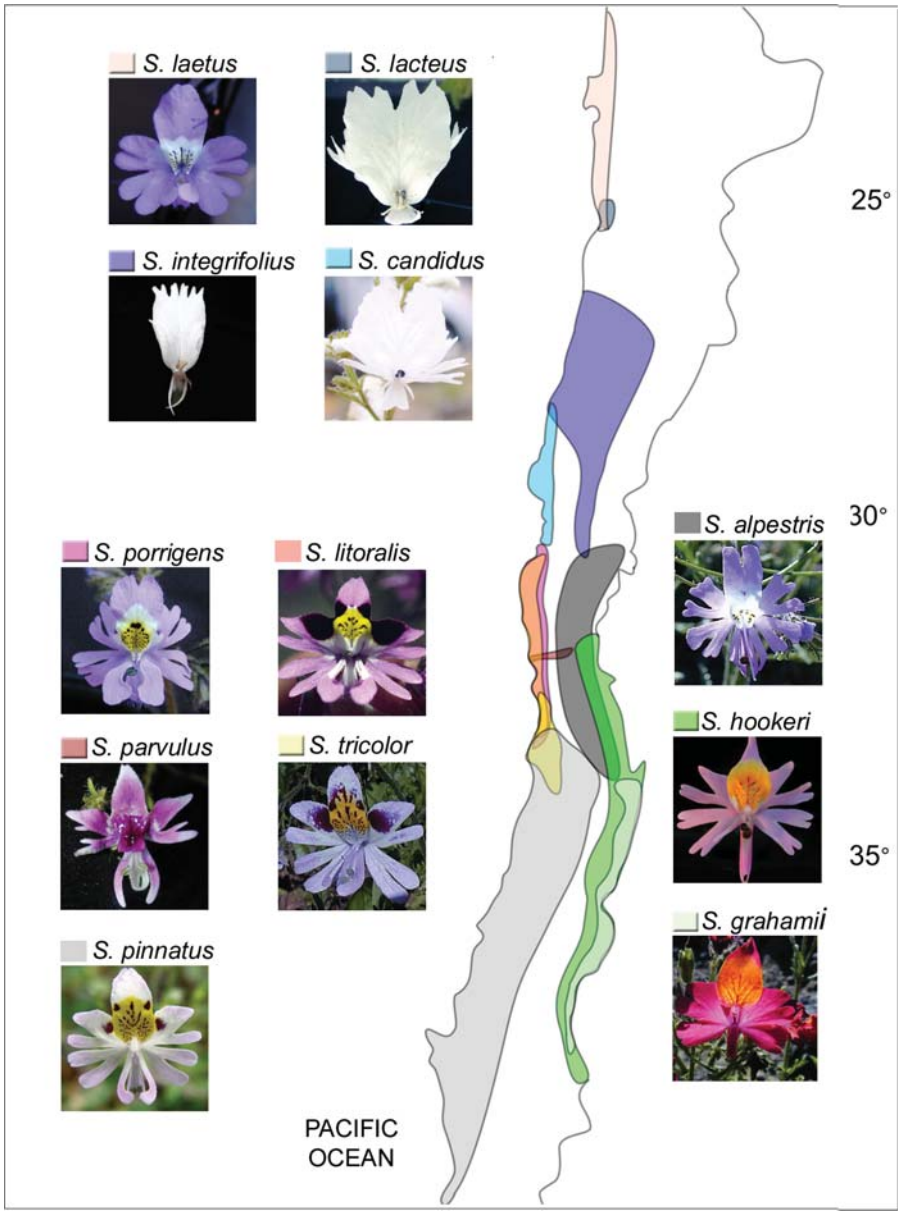

Fig. 1. Distribution of *Schizanthus* species in Chile.

the absence of explosive pollen discharge. The bee-pollination syndrome consists of pink-purple corollas, nectar guides, nonreflexed lateral sections, lower lips extended as landing platforms, and the presence of explosive pollen discharge. In addition, a hummingbird-pollination syndrome was detected during this study, in which the flowers (with the exception of the banner) are red and the tubes long. Such flowers lack a landing platform and have non-explosive pollen discharge.

To examine transitions between pollination syndromes, we first reconstructed the phylogeny of the genus *Schizanthus* using the nrDNA internal transcribed spacer region (ITS), nrDNA granule-bound starch synthase gene (GBSSI, or *waxy*), and cpDNA *trnF*/*ndhJ* intergenic spacer region. We measured floral traits for all *Schizanthus* species and observed flower visitors in nine of 12 species. Both floral morphology and flower visitors were subsequently mapped onto the resulting phylogeny and ancestral states reconstructed. This procedure allowed us to address the following questions: (1) Do changes in flower morphology and pollinator service occur in a concerted way? (2) Do transitions from one pollination syndrome to another take place without intermediate stages? (3) Does flower morphology evolve independently of phylogenetic effects? The answers to these questions relate to the more general one, (4) does flower diversification occur in relation to pollinator shifts in *Schizanthus*?

MATERIALS AND METHODS

Phylogenetic reconstruction—Leaves and floral buds of all 12 species of *Schizanthus* were collected in the field in Chile between 25–37° S and dried

TABLE 1. List of *Schizanthus* species with data on location in Chile, research performed, and GenBank accession number: *trnF-ndhJ*, ITS, *waxy*. Data on location include study site, elevation, GPS coordinates when available, and date. Research abbreviations: d, collection of plant material for DNA studies; m, collection of flowers for morphological analyses; n, nectar measurement; o, observation of flower visitors.

Taxon	Location	Research	Accession number
S. alpestris Poepp. ex Benth.	Cuesta la Viñita, 820 m, 29°51′ S 70°49′ W, Nov. 2002	dmno	DQ299443, DQ299455, DQ299431
S. candidus Lindl.	Playa Arrayán, 40 m, 28°15′ S 71°09′ W, Nov. 2002	dmno	DQ299437, DQ299449, DQ299425
S. grahamii Gill. ex Hook.	Termas del Flaco, 1750 m, Jan. 2001	dm	DQ299434, DQ299446, DQ299422
	La Parva, 2350 m, Jan. 2003	no	
	Valle Nevado, 2480 m, 33°21′ S 70°16′ W, Jan. 2001	o	
S. hookeri Gill. ex Graham	Valle Nevado, 2450 m, 33°21′ S 70°16′ W, Jan. 2001	dmno	DQ299435, DQ299447, DQ299423
	Portillo, 2850 m, Feb. 2001	o	
	Termas de Chillán, 36°50′ S 71°05′ W, Feb. 2002	o	
S. integrifolius Phil.	Alto del Carmen, 710 m, 28°45′ S 70°29′ W, Sep. 2002	dmno	DQ299436, DQ299448, DQ299424
S. lacteus Phil.	R.N. Paposo, 144 m, 25°06′ S 70°27′ W, Sep. 2000	dmo	DQ299438, DQ299450, DQ299426
S. laetus Phil.	R.N. Paposo, 455 m, 25°00′ S 70°26′ W, Sep. 2000	dm	DQ299439, DQ299451, DQ299427
S. litoralis Phil.	Parque Nacional Fray Jorge, 30°38′ S 71°40′ W, Oct. 2002	dm	DQ299445, DQ299457, DQ299433
S. parvulus Sudzuki	R.N. Las Chinchillas, 580 m, 31°29′ S 71°05′ W, Oct. 2002	dm	DQ299442, DQ299454, DQ299430
S. pinnatus Ruiz et Pav.	R.N. Río Clarillo, 800 m, 33°43′ S 70°56′ W, Nov. 2001	dmno	DQ299441, DQ299453, DQ299429
S. porrigens Graham	Puente Juan Soldado, 150 m, 29°38′ S 71°17′ W, Sep. 2002	dmno	DQ299444, DQ299456, DQ299432
S. tricolor Grau et Gronb.	Papudo, 130 m, 32°31′ S 71°28′ W, Oct. 2002	dmno	DQ299440, DQ299452, DQ299428

with silica gel immediately after collection. Information on location and study sites are shown in Table 1. Genomic DNA was extracted from each species using the DNEasy Plant mini kit (Quiagen, Hilden, Germany). The *trnF*/*ndhJ* regions of the cpDNA and the ITS region of nDNA were amplified by PCR in two stages following Hershkovitz and Zimmer (1997), i.e., a double-strand amplification followed by separate asymmetric amplification of each strand using primers internal to the first. In the case of ITS, the primer pair TTTCTTTTCCTCCGCTTA and GAAGGAGAAGTCGTAACAAG was used for the double-strand amplification and the internal primers ITS4 (White et al., 1990) or N18L18 (Hershkovitz and Zimmer, 1997) for the asymmetric amplifications. In the case of the *trnF*/*ndhJ* spacer region, the primer pair GYTGGTAGAGCAGAGGACTG and TGGATAGGATGGCCCTTAC was used for the double-strand amplification and the internal primers, either GGACTGAAAATCCTCGTGTC or TGGATAGGATGGCCCTTAC for the asymmetrical amplifications. A region of *waxy* spanning introns 4–8 of the corresponding sequence of *Solanum tuberosum* (van der Leij et al., 1991) was amplified in one stage using the primer pair CTAYAARMGAGGGGTT GATCG and GCRTTCATCCAGTTGATTTTC.

Because the *waxy* PCR fragment from *S. alpestris* could not be sequenced directly, we cloned it using the InsTA clone PCR product cloning kit (Fermentas, Hanover, MD, USA) and XL1-Blue Epicurian cells. *Waxy* fragments from 15 colonies were amplified and sequenced. Two different sequences were obtained; one of them was most variable. The two *waxy* clones of *S. alpestris* formed a sister pair in preliminary phylogenetic analyses. Only the most conserved clone was considered for final phylogenetic and reconstruction analyses for simplicity.

Purified products were sequenced using the ABI Prism BigDye Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, California, USA) on an ABI 3100 sequencer. In the case of ITS and *trnF*/*ndhJ*, the single strand PCR products of both directions were sequenced using the internal primers described. In the case of *waxy*, the double-strand PCR products were sequenced in both directions using the internal primers GGCACACTGCTC TACTTCC or GRTAKGCAATGTTGTGGATGC. Sequences from both strands of each PCR product were examined, compared, and corrected using the program Bioedit (Hall, 1999) from which a consensus sequence was generated. Sequence data were manually aligned in Bioedit.

Bayesian inference of phylogeny with Markov chain Monte Carlo sampling was conducted for the concatenated sequences of the three DNA regions using MrBayes 3.04 (Huelsenbeck and Ronquis, 2001). A general time-reversible model of DNA substitution and shape parameter of the gamma distribution (GTR + G) model was used with parameters partitioned across the genes. One cold chain and three heated chains were run simultaneously for one million generations, and one tree per 100 generations was sampled. The first 200 trees were discarded as burn-in, and Bayesian posterior probabilities were estimated on the 50% majority rule consensus of the remaining 9800 trees. Trees were also constructed by maximum parsimony in PAUP* version 4.0 (Swofford, 1999), and bootstrap support at nodes was computed for 1000 replicates of data (Felsenstein, 1985). The three DNA regions were analyzed separately and in combination. Equal-weighted parsimony analyses were carried out by heuristic search with tree-bisection-reconnection (TBR) branch swapping and random sequence addition replicates. To test for conflicts between the different DNA data sets, we conducted an incongruence length difference test (ILD, Farris et al., 1985) with 500 random partitions.

Because *Schizanthus* is a strongly isolated genus in the Solanaceae (Grau and Grönbach, 1984; Tétényl, 1987; Olmstead and Palmer, 1992; Martins and Barkman, 2005), and the risk of homoplasy is high when a highly divergent outgroup from the ingroup is used to root the trees (Swofford et al., 1996; Graham et al., 2002), we used the midpoint-rooting criterion. The midpoint procedure places the root between the most distant taxa under the assumption that molecular evolution between these taxa is clock-like. To test this assumption, likelihood ratio tests (Felsenstein, 1988) were performed for each DNA sequence to compare the likelihood scores of data with and without forcing the molecular clock. The likelihood ratio was assumed to be χ^2 distributed with N taxa minus two degrees of freedom. The molecular clock-like assumption was not rejected for any of the three data sequences, therefore justifying the use of the midpoint rooting.

Floral characters and visitors—One flower per each of 10 plants per population (one population per species) was collected in the field. The flowers were preserved in 70% alcohol (Table 1) and dissected later to separate the upper and lower lip so as to flatten the corolla. The flattened flowers were scanned, and the following morphological traits recorded using SigmaScan Pro 5.0 (SPSS, 1998): (1) corolla size, obtained as the sum of the lower and upper lip areas; (2) degree of corolla dissection, calculated as the ratio between the squared perimeter of the lips and corolla size; (3) relative size of the lower lip, calculated as the ratio between the lower lip and corolla size; and (4) relative length of corolla tube, calculated as the ratio between the lengths of the corolla tube and the major axis of the corolla. In addition, the following characteristics were measured directly in the field: (5) lateral section orientation of the upper lip, (backward-reflexed or non-reflexed orientation); (6) corolla color (white: without evident nectar guides when examined in daylight; pink-purple: light pink to purple lateral sections and/or keels, and nectar guides, often with dark-purple spots; or red: red lateral sections and keel); (7) type of pollen presentation (explosive or non-explosive); (8) nectar volume, obtained from 10 different female-phase bagged flowers at 1000, 1600, and 2300 using microcapillary tubes (1 μL and 5 μL). Nectar measurement times were selected so as to cover the range of daily times of nectar secretion found for different pollination syndromes.

Flower visitors were observed over three sunny days for nine species (one population per species). In the case of moth-syndrome species, observations were extended up to 6 d because of the low visitation rates. For two populations of *S. hookeri* and three of *S. grahamii* were observed. In the remaining species, only one population could be studied because most of these species only appear abundantly during El Niño years (in our case, the spring season of 2000 and 2002). Temporal considerations prevented direct observations of pollinators of *S. parvulus*, *S. laetus*, and *S. litoralis*. Flower visitors were observed directly or

by using binoculars in 2-m^2 patches for one 30-min period per hour between 0900 and 2400 hours. For the dark hours, observations were performed under a red light source. Total flowers observed per species per 30 min ranged from 207 to 617. Flower visitors were trapped for identification, grouped as bees, dipterans, and lepidopterans, and identified to the maximum resolution possible. The effectiveness of flower visitors for bees and dipterans was judged by observing the presence or absence of *Schizanthus* pollen on the insect body. We did not trap hummingbirds. However, the flower-handling pattern of the white-sided hillstar, *Oreotrochilus leucopleurus*, the only hummingbird observed, was consistent with effective pollination. Because of their extremely low visitation rates, we were unable to trap moths. The effectiveness of moths as pollinators on *Schizanthus* is less certain.

Ancestral reconstruction of floral traits and flower visitors—Maximum-likelihood ancestral reconstructions of floral characters and flower visitors were undertaken using the majority rule consensus tree recovered from the Bayesian analysis of the combined nuclear and chloroplast dataset. The only polytomy detected in the consensus tree was resolved by assigning a branch length of 0.00001. Ancestral states of qualitative floral traits were reconstructed using Mesquite (Maddison and Maddison, 2004) based on a one-parameter model. Ancestral states of continuous floral characters were estimated in ANCML (Schluter et al., 1997), which assumes a Brownian model of evolution. Ancestral reconstructions of the presence/absence of hummingbird and bees visitors were conducted separately using maximum parsimony in Mesquite (Maddison and Maddison, 2004). Moths were not considered for ancestral reconstructions because of their unknown effectiveness and low visitation rates. The influence of phylogeny on the evolution of continuous floral traits was evaluated by fitting Pagel's (2002) λ phylogeny-scaling parameter included in Continuous (Pagel, 2000). When $\lambda = 1.0$, trait evolution is influenced strongly by phylogeny; when $\lambda = 0.0$, trait evolution is independent of phylogeny. Lambda parameters were fitted by maximum likelihood. Nested likelihood ratio tests were undertaken to determine whether the observed values differed significantly from 0.

RESULTS

Phylogenetic reconstruction—The alignment of the chloroplast and nuclear regions for the 12 species considered a total of 2223 positions. ITS varied the most with 53 of 644 variable sites (8.2%), of which 36 were parsimony-informative. This was followed by *waxy* with 64 of 856 variable sites (7.5%), of which 39 were informative. The chloroplast region was the most conserved with 34 of 723 variable sites (4.7%), with 14 sites informative. Figure 2A shows the majority rule consensus tree recovered from the Bayesian analysis of the combined nuclear and chloroplast data set. Three major clades within the genus *Schizanthus* were recovered. Clade A consisted of *S. alpestris* positioned sister to a second unresolved clade containing *S. candidus*, *S. integrifolius*, and *S. lacteus*. Clade B contained *S. hookeri* and *S. grahamii*. Clade C placed *S. laetus* with two sister subclades, one containing *S. litoralis* and *S. porrigens*, and the other *S. tricolor*, *S. pinnatus*, and *S. parvulus*. Clades A and B were strongly supported by the three DNA regions when analyzed separately, but clade C was only supported by the *waxy* data (Fig. 2B–D). Other differences among the data sets relate to the root position and the clustering of *S. litoralis* with *S. porrigens*. This subclade was strongly supported by the ITS data, but not by the more informative *waxy* data set. Despite these differences, the ILD test did not provide evidence of conflicts between the three data sets.

Floral morphology and flower visitors—Floral measurements for the 12 species of *Schizanthus* are shown in Table 2. We assigned each *Schizanthus* species to a pollination syndrome on the basis of their floral traits and without consideration of their visitors. *Schizanthus grahamii* was assigned to the hummingbird-pollination syndrome; *S. candidus*, *S. integrifolius*, and *S. lacteus* to the moth-pollination syndrome; and the remaining species to the bee-pollination syndrome. Reflecting the diverse floral morphology in the genus, a wide range of flower visitors was observed, including hymenopterans, dipterans, lepidopterans, and hummingbirds (Table 3, Appendix). Bee visits were frequent in six species, *S. porrigens*, *S. tricolor*, *S. alpestris*, *S. pinnatus*, *S. integrifolius*, and *S. hookeri*. Bee visits were accompanied by long-tongued fly visits in *S. pinnatus*, by moths and butterflies in *S. integrifolius,* and by hummingbirds and flies in *S. hookeri*. *Schizanthus grahamii* was visited by hummingbirds only. In

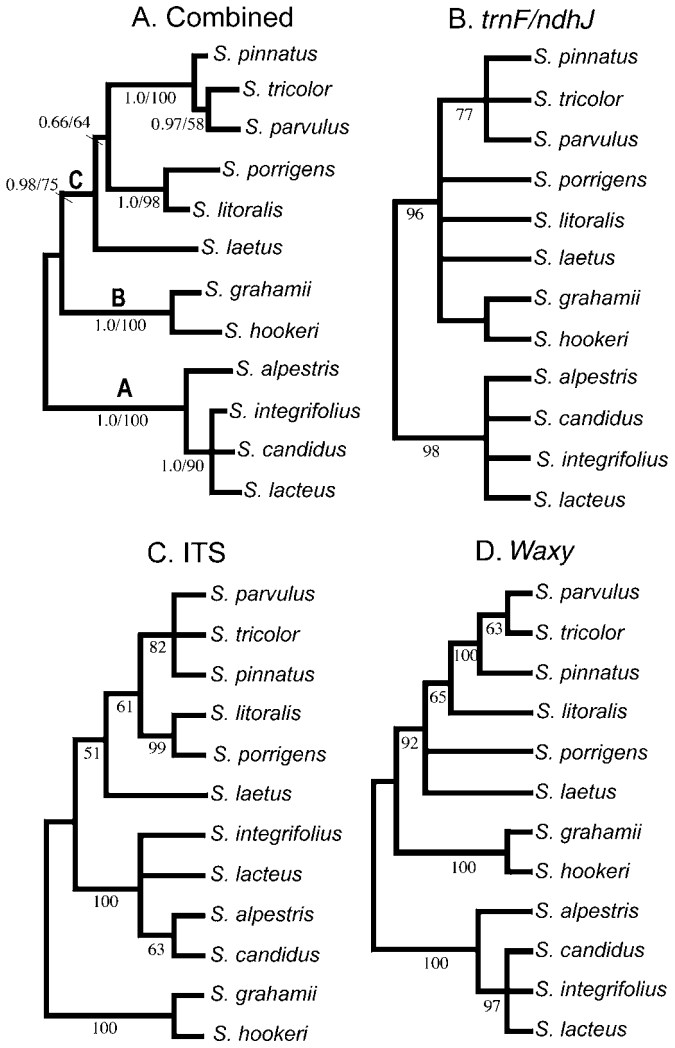

Fig. 2. Analysis of relationships within *Schizanthus*. (A) Phylogram of the midpoint-rooted majority rule consensus tree based on Bayesian analysis of the combined three DNA sequences. Numbers below each branch are the BI a posteriori probabilities/most parsimonious (MP) bootstrap of each clade > 50%. (B) The strict consensus tree from the 14 most parsimonious *trnF/ndhJ* trees of 37 steps (consistency index, CI = 0.92; retention index, RI = 0.91). (C) The strict consensus tree from the three most parsimonious ITS trees of 70 steps (CI = 0.86, RI = 0.88). (D) The most parsimonious *waxy* tree of 70 steps (CI = 0.96; RI = 0.97). Numbers below each branch are the MP bootstrap support of each clade >50%.

TABLE 2. Floral traits measured in *Schizanthus* species; corolla size; degree of corolla dissection; relative size of the lower lip; relative length of the corolla tube; lateral section of the upper lip reflexed (R) or not reflexed (NR); white (W), light pink-purple (P) or red (R) corolla; presence (P) or absence (A) of explosive pollen discharge; nectar volume (μl) measured at three times. The pollination syndrome is indicated. Sample size was one flower per each of 10 plants.

Species	Corolla size (Mean, cm^2)	Corolla dissection (Mean)	Lower lip (Mean)	Tube length (Mean)	Corolla color	Petal reflection	Pollen discharge	Nectar volume (Mean ± SE) 1000	1600	2200	Syndrome
S. alpestris	1.06	129.7	0.24	0.38	P	NR	P	0.28 ± 0.03	0.18 ± 0.01	0.14 ± 0.02	Bee
S. candidus	3.82	176.9	0.07	0.44	W	R	A	1.41 ± 0.04	1.29 ± 0.16	1.67 ± 0.27	Moth
S. grahamii	4.26	74.4	0.14	0.30	R	NR	A	2.28 ± 0.13	2.24 ± 0.09	1.78 ± 0.24	Hummingbird
S. hookeri	2.82	116.6	0.25	0.48	P	NR	P	2.34 ± 0.27	1.70 ± 0.19	1.13 ± 0.23	Bee
S. integrifolius	2.30	236.0	0.11	0.60	W	R	A	2.79 ± 0.20	3.23 ± 0.23	3.37 ± 0.41	Moth
S. lacteus	1.44	121.2	0.10	0.38	W	R	A	—	—	—	Moth
S. laetus	2.18	56.1	0.21	0.14	P	NR	P	—	—	—	Bee
S. litoralis	3.62	60.4	0.28	0.16	P	NR	P	—	—	—	Bee
S. parvulus	0.71	78.3	0.34	0.13	P	NR	P	—	—	—	Bee
S. pinnatus	2.65	94.2	0.25	0.16	P	NR	P	0.15 ± 0.02	0.19 ± 0.04	0.06 ± 0.02	Bee
S. porrigens	3.27	92.4	0.31	0.13	P	NR	P	0.06 ± 0.02	0.08 ± 0.03	0.03 ± 0.01	Bee
S. tricolor	2.75	96.2	0.23	0.15	P	NR	P	0.07 ± 0.03	0.06 ± 0.01	0.04 ± 0.02	Bee

spite of the higher sampling effort in *S. lacteus* and *S. candidus*, visitors were not recorded.

Ancestral reconstruction of flower traits and flower visitors—Ancestral reconstruction of floral traits indicated that the ancestral flower in the genus *Schizanthus* was medium sized (2.8 cm^2), little dissected (106), with a medium-sized lower lip (0.20), and was long-tubed (0.31) with non-reflexed lateral petals, pink to purple corollas, and explosive pollen discharge (Fig. 3). The combination of characters suggests the putative ancestral species had a corolla similar to the extant *S. alpestris,* albeit larger than this species. The analyses also indicated that floral traits related to moth pollination, such as dissected corollas, small lips, reflexed lateral petals, presence of a white corolla, and lack of explosive pollen discharge, evolved in the clade containing the desert species *S. candidus*, *S. integrifolius*, and *S. lacteus* (Fig. 3). Reconstruction also indicated that traits associated with hummingbird pollination such as red corollas, a smaller lower lip, and loss of explosive pollen discharge evolved only in *S. grahamii*. Finally, all six species in clade C (*S. laetus*, *S. porrigens*, *S. litoralis*, *S. tricolor*, *S. parvulus*, *S. pinnatus*) maintain some ancestral bee-syndrome traits (such as explosive pollen discharge, light pink to purple corolla color, and non-reflexed petals), but at the same time evolved an increased size of the lower lip and a short corolla tube in comparison to the ancestral situation. Reconstruction of flower visitors indicate that ancestral flowers were visited by bees (Fig. 4) and that hummingbird pollination was acquired only in the clade containing the Andean species *S. hookeri* and *S. grahamii*. Bee pollination was lost in *S. grahamii* and in the desert species *S. candidus* and *S. lacteus*. The phylogenetic scaling parameter (λ) did not differ from 0.0 for corolla size ($\lambda = 0$, $P = 1.0$), relative lower lip size ($\lambda = 0.36$, $P = 0.07$), corolla dissection ($\lambda = 0.47$, $P = 0.11$), and relative corolla tube length ($\lambda = 0.76$, $P = 0.17$), indicating that the different components of the flower have evolved independently of any phylogenetic effects in this genus.

DISCUSSION

Molecular phylogeny—The combined results for ITS, *waxy*, and *trnF*/*ndhJ* produced a well-resolved phylogeny. Our molecular phylogenetic hypothesis was partially congruent with the morphological classification of Grau and Grönbach (1984), who recognized four groups: (1) *S. candidus*, *S.*

TABLE 3. Percentage of visits by hymenopterans, dipterans, lepidopterans, and hummingbirds to species of *Schizanthus*. Visitor taxa are detailed in Appendix. PS, Pollination syndrome, B, Bee; H, Hummingbird; M, Moth; N, total number of visits. FO, mean number of flowers observed on a daily basis.

Species	PS	Hymenoptera	Diptera	Lepidoptera	Hummingbirds	*N*	FO
S. alpestris	B	100	0	0	0	342	340
S. candidus	M	—	—	—	—	0	617
S. grahamii[2]	H	0	0	0	100	81	447
S. grahamii[3]	H	—	—	—	—	0	354
S. hookeri[1]	B	95.2	0	0	4.8	1580	350
S. hookeri[2]	B	87.4	0	0	12.6	1416	562
S. hookeri[3]	B	79.5	5.9	0	14.6	390	207
S. integrifolius	M	81.2	0	18.8	0	32	523
S. lacteus	M	—	—	—	—	0	312
S. pinnatus	B	82.9	17.1	0	0	1751	314
S. porrigens	B	100	0	0	0	503	339
S. tricolor	B	100	0	0	0	1759	424

Notes: Locations for multiple specimens are *Schizanthus grahamii*[2], La Parva; *S. grahamii*[3], Valle Nevado; *S. hookeri*[1], Valle Nevado; *S. hookeri*[2], Portillo; *S. hookeri*[3], Termas de Chillán.

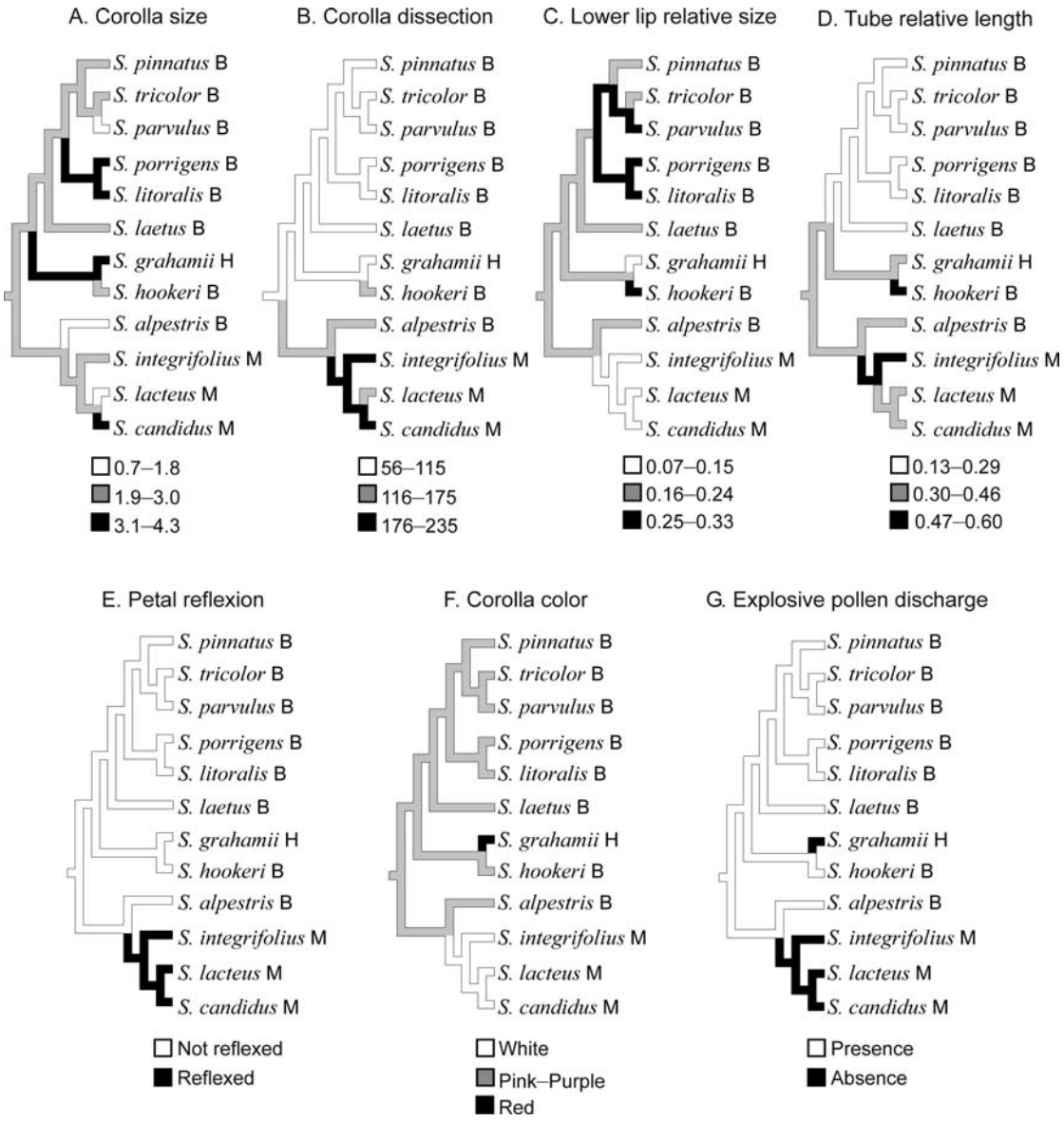

Fig. 3. Maximum-likelihood reconstructions of ancestral states for continuous (A–D) and qualitative (E–G) floral traits associated with pollination syndromes. Ancestral reconstructions for continuous characters were performed in the natural continuous scale but are represented as three categories for simplicity. Pollination syndromes are indicated after each species name: B, bee; H, hummingbird; M, moth.

integrifolius, and *S. lacteus*; (2) *S. hookeri* and *S. grahamii*; (3) *S. tricolor* and *S. pinnatus*; (4) *S. litoralis* and *S. porrigens*. They suggested that *S. laetus* is most closely related to the fourth group and that *S. alpestris* and *S. parvulus* are isolated taxa with no clear affinities. Our molecular data support the recognition of the four groups as well as the suggested relationship of *S. laetus*. *Schizanthus alpestris* is also included in the first group, and *S. parvulus* forms part of the clade comprising the third group. Interestingly, multiple *waxy* clones were obtained from a single individual of *S. alpestris*. Within-individual variation of *waxy* has been observed in other Solanaceae (Peralta and Spooner, 2001). This variation could be attributable to diverse causes, for example, allelic variation, presence of more than one *waxy* locus (Evans et al., 2000), or polyploidy. Irrespective of the causes involved, the two *waxy* clones of *S. alpestris* belong to the same clade of the phylogeny.

Molecular and morphological data indicate that *Schizanthus* diverged early from the rest of the Solanaceae (Grau and Grönbach, 1984; Olmstead and Palmer, 1992; Martins and Barkman, 2005), probably in the late Cretaceous to early Tertiary, as suggested by data on the origin and diversification of the Solanales (Magallón et al., 1999) and the earliest fossil record for the Solanaceae (Eocene; Collinson et al., 1993). At this time, tropical and subtropical conditions dominated the southern South American region (Hinojosa and Villagrán, 1997). Despite the ancient origin of *Schizanthus*, the molecular data suggest that the three major clades of the genus diverged recently. Considering the evolutionary rates for ITS in other herbaceous plants (Richardson et al., 2001) and the molecular clock, the age of the root would lie at about 5 million years

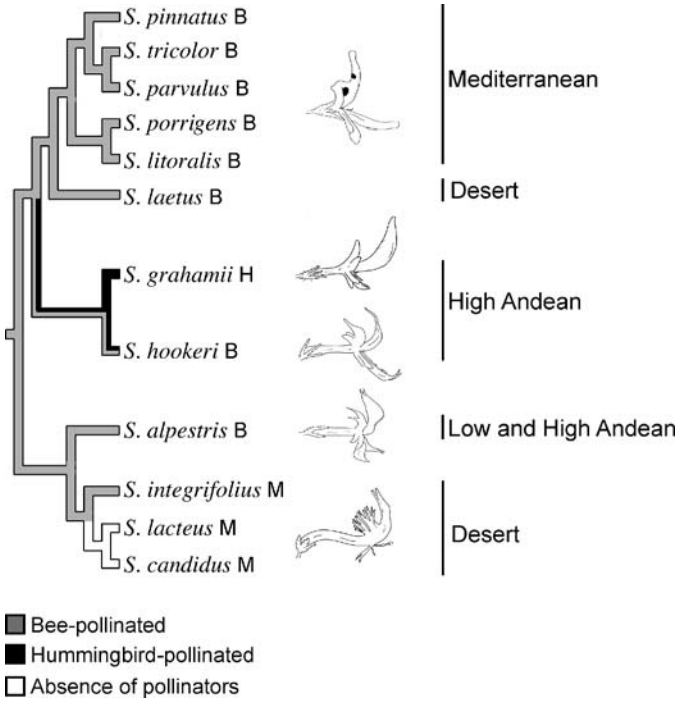

Fig. 4. Maximum-parsimony reconstruction of ancestral states for bee and hummingbird pollination in *Schizanthus*. In the absence of pollinator data for *S. parvulus, S. litoralis,* and *S. laetus*, character states were treated as ambiguous for ancestral reconstruction. In descendent order, drawings represent the lateral view of flowers of *S. tricolor, S. grahamii, S. hookeri, S. alpestris,* and *S. integrifolius*. The major ecosystem types for species are also indicated. Pollination syndromes are indicated after species names; B, bee; H, hummingbird; M, moth.

(my). The major clades of *Schizanthus* thus diverged when the current desert and semidesert regions were already arid and the Andean mountains had reached considerable height (Hinojosa and Villagrán, 1997).

Evolution of floral traits in association with flower visitors—Ancestral reconstruction of floral visitors suggests that the original flower of *Schizanthus* was bee-pollinated (Fig. 4). This is consistent with the floral trait reconstruction that gave ancestral flowers similar to those of extant bee-pollinated *S. alpestris* (Fig. 3, Table 3). Bee pollination (Fig. 4) and bee-syndrome traits (Fig. 3) are still conserved in clade C.

The only species showing a hummingbird-pollination syndrome was *S. grahamii* (Table 2). Its sister species, *S. hookeri,* lacks the suite of characters related to the hummingbird-syndrome, but has high nectar production (Table 2) and attracts some hummingbird attention (Table 3). The common ancestor of the two species presumably acquired hummingbird pollination (Fig. 4), and later *S. grahamii* evolved away from bees to specialize exclusively on birds (Figs. 3, 4). These two species are alpine species of the central Chilean Andes. Conditions for biotic pollination here tend to deteriorate with elevation (Arroyo et al., 1982, 1985). It is known that transitions to hummingbird pollination occur in environments with low insect activity (Cruden, 1972), thus the appearance of hummingbird pollination is not unexpected at high elevations, as indeed is seen in the northern tropical Andes (Kay et al., 2005).

Traits associated with moth pollination are found in *S. candidus*, *S. lacteus*, and *S. integrifolius*, which inhabit coastal and inland areas of the Atacama Desert. However, moth visitation was observed only in *S. integrifolius* and at a very low rate (only two visits in 30 h of observation during six consecutive nights) compared with bee visitation. Other plant species with a moth-pollination syndrome are visited by both diurnal and nocturnal visitors (e.g., Young 2002). In some cases, the effectiveness of nocturnal pollinators is greater even when diurnal visits are more abundant (Groman and Pellmyr, 1999). We do not have information on the effectiveness of bees and moths in *S. integrifolius* and thus cannot accept or reject the hypothesis that the moth syndrome evolved from the direct influence of the most effective pollinator. That the stigmas are receptive and pollen and nectar are available during the daylight hours suggests that effective diurnal pollination is not impossible (F. Pérez, unpublished data). The complete absence of flower visitors in *S. lacteus* and *S. candidus* is intriguing. In a pollinator exclusion experiment we found that 90–100% of the flowers in these species form fruits, indicating a high autonomous selfing capacity (F. Pérez, unpublished data). Darwin (1862) was perplexed by the presence of adaptations for selfing in plants with specialized floral morphology. Zhang et al. (2005) likewise recognized this paradox, suggesting that the retention of specialized pollination syndromes in plants with high selfing levels could represent an anachronism. Although we cannot rule out insufficient sampling, the persistence of the moth syndrome in *S. lacteus* and *S. candidus* could well be anachronic. Paleoecological studies in the Atacama Desert show that aridity has increased significantly over the last 3000 years (Latorre et al., 2002, 2003), which suggests that current conditions for moth pollination are perhaps less amenable than they were in the past, leading to a mismatch between floral morphology and current visitors. Chilean ecosystems are depauperate in terms of hawkmoth species richness (Ureta and Donoso, 1956). Whether this pattern is replicated in other genera inhabiting the Atacama Desert is unknown at present and needs to be assessed in future studies. On other hand, the conservative nature of the moth syndrome seems counterintuitive, given that floral evolution in *Schizanthus* is not strongly influenced by phylogeny. Possibly El-Niño-related climatic fluctuations impinging on pollinator abundance in species with high specialized morphology have promoted the acquisition of a backup breeding system that allows seed set in years when pollinators are infrequent.

Transitions between pollination syndromes—Intermediate stages have been suggested to occur in the transition from one pollination system to another (Baker, 1963; Stebbins, 1974; Armbruster, 1993; Wilson et al., 2006). The combination of bee-syndrome traits and high nectar production in *S. hookeri* (reflecting a mixed syndrome with bees, long-tongued flies, and hummingbirds as pollinators) may represent a generalized intermediate stage in the bee-to-bird transition. This pattern agrees with Wilson et al. (2006) who suggested that "despecialized" stages might occur in the transition from bee- to hummingbird-pollination syndrome in *Penstemon.* Here bee-pollinated species experienced a "despecialization" transition through the acquisition of traits attractive to hummingbirds (such as high nectar production), but at the same time maintained traits that allowed bee pollination (pink color, landing platform). Subsequent "respecialization" occurred when pollination by hymenoptera was discouraged due to the appearance of traits that favor hummingbird pollination (e.g.,

reduction of the lower lip and acquisition of red). However, the situation in *S. hookeri* does not necessarily parallel that in *Penstemon*. This species of *Schizanthus* may represent a legitimate generalist species, rather than a transitional species. Generalized pollination systems would be adaptive at high elevations in the Andes where conditions for biotic pollination deteriorate with elevation. Reduction in pollinator resources driven by specialization in the stringent high elevation conditions may have promoted the acquisition of a backup breeding system as revealed by the observation that *Schizanthus grahamii*, a species that "respecialized" on hummingbirds, unlike *S. hookeri*, is capable of fruit formation in the absence of pollinators (F. Pérez, unpublished data).

Lability of flower trait—Our results indicate that λ estimates did not differ from 0 for all continuous floral traits, indicating that floral evolution was not strongly influenced by phylogeny. Repeated and independent changes of floral traits during phylogeny were observed (Fig. 3). For example, reduction of the lower lip and loss of explosive pollen discharge occurred independently in the hummingbird-pollinated *S. grahamii* and in the ancestor of the clade showing moth-syndrome traits (Fig. 3C, G). These results are consistent with other studies documenting that floral characters tend to be labile and often show independent evolution and reversals along phylogenies (Armbruster, 1993; Prather, 1999; Kimball and Crawford, 2004).

Conclusions—In *Schizanthus*, a number of floral traits have evolved in a concerted way with changes in floral visitors. While the maintenance of a suite of characters related to the bee syndrome in bee-pollinated species and the acquisition of characters related to the hummingbird syndrome in hummingbird-pollinated *S. grahamii* provide strong evidence for concerted evolution, the presence of traits related to a moth syndrome in the absence of moth pollinators fails to comply with this pattern. This mismatch suggests either anachronic floral characters possibly related to recent climate changes reducing the abundance of specialized pollinator taxa or maintenance of rare moth pollination backed up by capacity for autonomous selfing. The lack of close correspondence between floral morphology and pollinators in the moth-pollination syndrome reveals the importance of considering factors such as effectiveness of floral visitors and breeding system for a more complete understanding of patterns of floral evolution in *Schizanthus*. Overall, high floral diversification in the genus *Schizanthus* is a product of concerted changes associated with adaptation to different groups of pollinators in the mediterranean, high alpine, and desert ecosystems of Chile and adjacent Argentina.

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APPENDIX. Species visiting flowers of *Schizanthus* species and the number of visits. ALP, *S. alpestris*; CAN, *S. candidus*; GR2, *S. grahamii*, La Parva; GR3, *S. grahamii*, Valle Nevado; HO1, *S. hookeri,* Valle Nevado; HO2, *S. hookeri,* Portillo; HO3*, S. hookeri*, Termas de Chillán; INT, *S. integrifolius*; LAC, *S. lacteus*; PIN, *S. pinnatus*; POR, *S. porrigens*; TRI, *S. tricolor*.

Visitor taxon	Number of visits											
	ALP	CAN	GR2	GR3	HO1	HO2	HO3	INT	LAC	PIN	POR	TRI
Hymenopterans												
Apidae												
Alloscirtetica gazullai	0	0	0	0	0	0	0	0	0	855	0	0
A. gayi	0	0	0	0	0	0	0	0	0	0	0	1095
Anthophora paranaensis	0	0	0	0	0	0	10	0	0	0	0	0
Bombus dahlbomii	0	0	0	0	17	32	41	0	0	0	0	0
B. terrestris	0	0	0	0	0	0	8	0	0	0	0	0
Centris nigerrima	94	0	0	0	33	12	0	0	0	0	0	0
Svastrides melanura	0	0	0	0	53	347	0	20	0	0	0	0
Colletidae												
Leioproctus sp. 1	0	0	0	0	0	0	0	0	0	0	479	0
Leioproctus sp. 2	0	0	0	0	0	0	0	0	0	0	24	0
Leioproctus sp. 3	0	0	0	0	0	0	0	6	0	0	0	0
Leioproctus sp. 4	248	0	0	0	0	0	0	0	0	0	0	0
Halictidae sp.	0	0	0	0	0	0	0	0	0	0	0	9
Megachilidae												
Anthidium chilensis	0	0	0	0	0	0	0	0	0	597	0	655
Megachile semirufa	0	0	0	0	1093	847	251	0	0	0	0	0
Megachile sp.	0	0	0	0	308	0	0	0	0	0	0	0
Dipterans												
Tabanidae												
Mycteromyia conica	0	0	0	0	0	0	0	0	0	50	0	0
Promycteromyia sp.	0	0	0	0	0	0	0	0	0	92	0	0
Acroceridae												
Lasia corvine	0	0	0	0	0	0	0	0	0	157	0	0
Nemestrinidae												
Trichphthalma sp.	0	0	0	0	0	0	23	0	0	0	0	0
Lepidopterans												
Pieridae												
Tatochila sp.	0	0	0	0	0	0	0	4	0	0	0	0
Noctuidae sp.	0	0	0	0	0	0	0	2	0	0	0	0
Hummingbirds												
Oreotrochilus leucopleurus	0	0	81	0	76	178	57	0	0	0	0	0