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Geographic variation in floral scent of *Echinopsis ancistrophora* (Cactaceae); evidence for constraints on hawkmoth attraction

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Variation in floral phenotype (color, depth, nectar) suggests incipient specialization for bee or hawkmoth pollination across the geographic distribution of *Echinopsis ancistrophora*, with flower depth ranging from 4.5 to 24 cm. We used chemical and behavioral analyses to test whether fragrance has evolved in concert with morphology in these Andean cacti. Floral scent (145 total compounds) was collected using dynamic headspace methods and analyzed with gas chromatography–mass spectrometry, revealing subspecies-specific odors dominated by sesquiterpenes in *E.* ssp. *ancistrophora* and *arachnacantha* and fatty acid derivatives or aromatics in *E.* ssp. *cardenasiana* and *pojoensis*. Compounds indicative of sphingophily were not consistently found in moth-pollinated plants, and total scent emissions were significantly lower in populations with nocturnal anthesis. In wind tunnel assays, *Manduca sexta* moths were attracted to scent of ssp. *ancistrophora* from both bee and hawkmoth-pollinated populations, but not to scent of ssp. *cardenasiana*. However, hawkmoths were most attracted to the methyl benzoate-dominated scent of a distant relative, *Echinopsis mirabilis*. Thus, hawkmoth-pollinated descendants of the *E. ancistrophora* lineage may be phylogenetically constrained to emit weak, sesquiterpene-dominated fragrances that are not optimally attractive to hawkmoths, or floral scent may be under stronger selection by destructive flower visitors.

The evolution of floral phenotype is thought to be driven by complex interactions with mutualists (including pollinators), plant enemies (nectar robbers, herbivores, florivores), pathogens and abiotic stresses (Galen and Cuba 2001, Frey 2004). Speciation in radiating angiosperm groups is often accompanied by diversification of floral traits (Hodges 1997, Manning and Goldblatt 2005). The last decade has witnessed an increase in experimental tests of the 'adaptedness' of variation in floral color and shape to different pollinators (Wilson 1995, Schemske and Bierzychudeck 2001, Wilson et al. 2004) and the degree (if any) to which such variation influences reproductive isolation (Waser 1998, Schemske and Bradshaw 1999). Floral scent is also expected to contribute to pollinator specificity in some lineages (reviewed by Raguso 2004, Schiestl 2005), but its role in evolutionary pollinator shifts and adaptive radiation has rarely been studied.

Here we introduce the Andean cactus genus *Echinopsis* as a novel system in which to study the integration of floral phenotypes. A pervasive problem among adaptive studies of floral phenotype is that color, shape and scent occur in non-random combinations in many plant lineages and, as correlated traits, cannot easily be 'deconstructed' in fitness-related experiments (Herrera 2001). In contrast, flowers of the *Echinopsis ancistrophora* species complex show unexpected combinations of traits, such as pink flowers with medium-depth floral tubes and nocturnal anthesis (*E. ancistrophora* ssp. *cardenasiana*) and white flowers with short-to-intermediate (up to 15 cm) depth floral tubes and diurnal anthesis (*E. ancistrophora* ssp. *ancistrophora*). These populations may represent early stages of local pollinator adaptation or, alternatively, patterns of genetic drift or introgression from other extant or extinct *Echinopsis* populations. The unusual de-coupling of floral traits that define traditional pollination syndromes in the *Echinopsis ancistrophora* group provides an opportunity to study the selective pressures responsible for constructing floral phenotypes without artificial hybrids, transgenic plants or floral manipulation.

Geographic variation in the floral characteristics of *Echinopsis ancistrophora* has led to the description of four recognized taxonomic entities (Hunt 1999):

1. *E. ancistrophora* ssp. *ancistrophora* is characterized by white flowers of varying tube length (4.5–24 cm) with scattered populations in northwestern Argentina.
2. *E. ancistrophora* ssp. *cardenasiana* has pink, medium to long tubed (ca 10 cm) flowers and is found in southern Bolivia.

3. *E. ancistrophora* ssp. *pojoensis* has pink to red, medium to long tubed (ca 9 cm) flowers and is distributed in central Bolivia.
4. *E. ancistrophora* ssp. *arachnacantha* has yellow, orange or red, short tubed (ca 6 cm) flowers and grows in central Bolivia as well (see Table 1 and Fig. 1 for details and distribution maps).

Floral traits and limited field observations suggest bee pollination for subspecies *cardenasiana, pojoensis* and *arachnacantha* across isolated populations in Bolivia. In this paper, we focus primarily on populations of *E. ancistrophora* ssp. *ancistrophora,* in which dramatic variation in floral depth, nectar production and anthesis time among isolated populations suggest local pollinator adaptation, to bees in short to medium depth flowers that open in the morning or late at night, and to hawkmoths in long-tubed flowers with vespertine anthesis. Field observations confirm that hawkmoths pollinate the four populations with flower tubes longer than 15 cm (which produce large standing crops of nectar), and that matinal bees pollinate all other populations (having little or no nectar) (Table 1, Schlumpberger unpubl.). Bee pollination remains possible in the four long-tubed, hawkmoth-pollinated populations on the morning after anthesis, but the extent to which this occurs in nature will require additional field observations.

In this paper, we explore the composition of floral phenotypes in these highly variable cacti, with a particular emphasis on the fit between scent chemistry and floral morphology among bee- and moth-pollinated populations of *E. ancistrophora* ssp. *ancistrophora*. We sought to determine whether scent chemistry and emission rates are non-randomly associated with flower color, depth or anthesis time in ways predicted by traditional pollination syndromes. We were especially interested in potential evolutionary shifts to or from hawkmoth pollination, which we have studied in the Nyctaginaceae (Levin et al. 2001), Solanaceae (Raguso et al. 2003, 2006) and Onagraceae (Raguso et al. 2007), so we analyzed floral scent from 13 different populations of white-flowered *E. ancistrophora* ssp. *ancistrophora,* which vary in floral depth, nectar production and time of anthesis, and are pollinated by bees and/or hawkmoths (Table 1).

We also wished to test the effects of floral scent variation on hawkmoth attraction. We used a laboratory colony of *Manduca sexta*, which has been thoroughly characterized for its innate responses to floral traits in laboratory bioassays (Raguso and Willis 2002, Raguso et al. 2005). Behavioral assays were performed in a laminar flow wind tunnel, the arena of choice for testing odor-guided moth attraction (Willis and Arbas 1991, Fraser et al. 2003).

Table 1. Studied taxa and populations of *Echinopsis ancistrophora*. Mean flower lengths based on n ≥10, except for [1] n =1, [2] n =2, [3] n =4, and [4] n =5.

Taxa (Hunt 1999)	Flower color	Flower length	Country, province/dept.	Location	GPS and elevation
E. ancistrophora ssp. *arachnacantha*	orange, red	5.5 cm	Bolivia, Santa Cruz	Samaipata Torrecillas plus cultivated plants	18°09′S, 063°55′W; 1530 m 17°51′S, 064°37′W; 2730 m
E. ancistrophora ssp. *pojoensis*	red	8.4 cm[4]	Bolivia, Cochabamba	cultivated plants	
E. ancistrophora ssp. *cardenasiana*	pink	9.2 cm[3]	Bolivia, Tarija	cultivated plants	
E. ancistrophora ssp. *ancistrophora*	white	6.4 cm	Argentina, Salta	Quebrada de Toro, Puente del Toro	24°52′S, 065°41′W; 1800 m
E. ancistrophora ssp. *ancistrophora*	white	7.6 cm	Argentina, Salta	Quebrada de Toro, south Puente Toro	24°53′S, 065°42′W; 1760 m
E. ancistrophora ssp. *ancistrophora*	white	9.5 cm	Argentina, Salta	Quebrada de Toro, El Mollar	24°51′S, 065°42′W; 1800 m
E. ancistrophora ssp. *ancistrophora*	white	10.5 cm[1]	Argentina, Salta	Quebrada de Toro, Rio Blanco	24°54′S, 065°40′W; 1620 m
E. ancistrophora ssp. *ancistrophora*	white	10.5 cm	Argentina, Salta	Cuesta del Cebilar	25°40′S, 065°29′W; 1800 m
E. ancistrophora ssp. *ancistrophora*	white	11.4 cm	Argentina, Salta	La Caldera, Campo Alegre	24°34′S, 065°21′W; 1550 m
E. ancistrophora ssp. *ancistrophora*	white	11.4 cm	Argentina, Salta	Quebrada de Escoipe, Escoipe	25°11′S, 065°46′W; 2250 m
E. ancistrophora ssp. *ancistrophora*	white	13.5 cm[1]	Argentina, Salta	Quebrada de Escoipe, Huayra Huasi	25°09′S, 065°43′W; 1850 m
E. ancistrophora ssp. *ancistrophora*	white	13.9 cm	Argentina, Jujuy	Quebrada de Humahuaca, Volcán	23°55′S, 065°28′W; 2130 m
E. ancistrophora ssp. *ancistrophora**	white	16.4 cm	Argentina, Jujuy	Abra Santa Laura	24°29′S, 065°18′W; 1800 m
E. ancistrophora ssp. *ancistrophora**	white	18.8 cm[2]	Argentina, Salta	Calderilla	24°40′S, 065°22′W; 1400 m
E. ancistrophora ssp. *ancistrophora**	white	19.9 cm	Argentina, Jujuy	Termas de Reyes	24°10′S, 065°28′W; 1740 m
E. ancistrophora ssp. *ancistrophora**	white	20.8 cm	Argentina, Jujuy	El Fuerte	24°15′S, 064°25′W; 1450 m

*Populations with flowers opening in the evening and visited by hawkmoths (unpubl.).

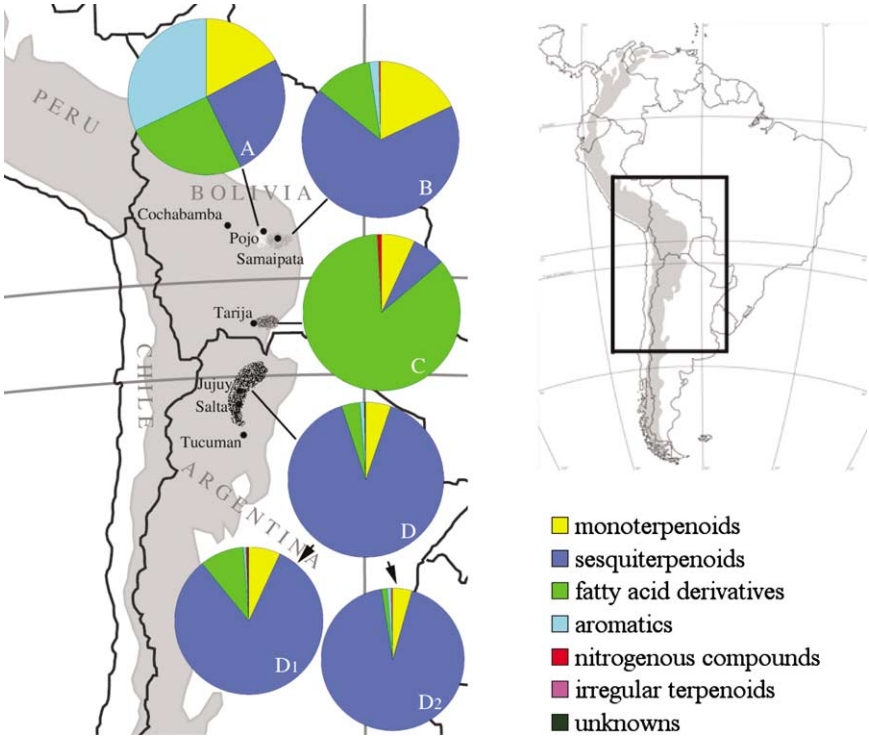

Fig. 1. Geographic variation of floral scent composition among four subspecies of *Echinopsis ancistrophora* in Bolivia and Argentina. Shaded areas indicate the Andean cordillera. Pie charts depict mean scent composition (% of total GC peak area) per subspecies. (A) ssp. *pojoensis*; (B) ssp. *arachnacantha*; (C) ssp. *cardenasiana*; (D) ssp. *ancistrophora*; D1. subset of D: populations with flowers primarily pollinated by hawkmoths; D2. subset of D: populations with bee pollination.

Our data were used to test the following adaptive hypotheses:

1. Fragrance composition and emission rates are correlated with flower color, depth, nectar production and anthesis time as predicted by classical pollination syndromes (Vogel 1954, Faegri and van der Pijl 1971):
   1a. Hawkmoth pollinated populations should be white with longer floral tubes, greater nectar volumes, nocturnal anthesis and the strongest scent emissions, with greater aromatic ester or nitrogenous volatile content.
   1b. Bee pollinated populations should have variable colors and shorter floral tubes, lower nectar volumes, diurnal or variable anthesis, and weaker, more variable scents.
2. The scent of hawkmoth pollinated flowers should be more attractive to a representative nocturnal hawkmoth, *Manduca sexta*, than the scent of bee pollinated taxa, on a per-flower basis.

Material and methods

Details concerning plant materials, hawkmoths, wind tunnel dimensions and protocols for scent collection/analysis are provided in Supplementary material Appendix 1.

Variation of scent emission

A complete list of studied subspecies and populations, their origins and floral trait characteristics is given in Table 1. For each of the four subspecies of *E. ancistrophora*, and for 13 populations of ssp. *ancistrophora* we calculated the mean percentages for all odor compounds and compound classes, and the standard error (except for three populations in which only a single specimen was sampled). Visualization of differences in floral scent composition for each individual was accomplished at the level of compound classes via multidimensional scaling, using Euclidean distances (SPSS 13). Before analysis, compound class proportions were transformed to Z-scores (unit variance). Differences of scent composition at the compound level were estimated by ANOSIM (Clarke and Gorley 2001) with 10 000 permutations, after calculating Bray–Curtis similarity index, in order to avoid grouping entities as similar due to shared absence of specific compounds. Square root and fourth root transformations of data were performed to test whether results would differ if the impact of the few dominant GC peaks (e.g. (E)-nerolidol) were reduced. In order to investigate individual differences in odor emission within a population, we compared floral scent composition and emission rates of five different plants within three populations (Escoipe, El Mollar and Calderilla) of ssp. *ancistrophora*. Besides comparing relative fractions of compound classes, we also compared relative emissions of the main volatiles, (E)-nerolidol and germacrene D.

Correlation of floral scent emission with floral syndrome and geography

Flowers of four populations of *E. ancistrophora* ssp. *ancistrophora* (Abra Santa Laura, Calderilla, El Fuerte, Termas de Reyes) are visited by hawkmoths in nature (Schlumpberger unpubl.). Several studies show that hawkmoth pollinated Neotropical cacti and other sphingophilous plants emit floral scents rich in aromatic alcohols and esters (Knudsen and Tollsten 1993, Kaiser 1993, Kaiser and Tollsten 1995, Hoballah et al. 2005). We compared the scent of flowers from these four populations with floral scent of bee-pollinated populations of the same subspecies. For each compound class we compared the mean proportions in % of both syndromes using the Mann–Whitney U-test (1-tailed). The same test was used to compare differences of the numbers of compounds emitted by flowers representing both syndromes, and for relative scent emission rates (GC peak areas).

Furthermore, we performed simple and partial Mantel tests to ask whether floral scent composition is correlated with the geographic distance between populations or, alternatively, with variation in floral depth as one measure for the observed differences in floral biology (using the zt software, Bonnet and Van de Peer 2002). The floral scent distance matrix was comprised of the same Euclidean distances used for multi-dimensional scaling (above), whereas a separate distance matrix was calculated from differences in floral depth. Geographic distances were calculated with a great circle calculator (spherical earth model), using our own GPS data in all but one accession (ssp. *pojoensis* was not found in its natural habitat during the field trip, therefore approximate coordinates of a known population were taken from a map). Mantel tests (with 99 999 random iterations) were performed for the entire data set and for ssp. *ancistrophora* only.

Scent emission day versus night

Hawkmoth pollinated flowers that last only one night (e.g. *Datura wrightii*, Raguso et al. 2003) typically lose turgor the following morning and may emit less floral scent as they senesce. Because long-tubed flowers of *Echinopsis ancistrophora* ssp. *ancistrophora* open in the evening but remain open during part of the following day, we compared scent composition patterns and emission rates during night and day (within the first half of the night, and during the following morning). Only those long-flowered populations (Calderilla, Termas de Reyes, El Fuerte) in which both diurnal and nocturnal samples could be collected were included. We sampled four flowers at night and five flowers during the day, including two flowers that were sampled both night and day. We used the Mann–Whitney U-test (1-tailed) to test the null hypotheses that diurnal samples were not less strongly scented than nocturnal ones and that relative amounts of compound classes emitted did not differ significantly.

Behavioral assays

We used *Manduca sexta*, a nectar-feeding hawkmoth distributed throughout the Americas (Raguso and Willis 2005), as representative of the guild of potential pollinators of the plants studied here. These moths pollinate night-blooming cacti and other plants throughout northern Argentina (Ando et al. 2001, Nattero et al. 2003, Moré et al. 2006). We used moths of both sexes in wind tunnel experiments, with a balanced sex ratio whenever possible. Because flowering in *E. ancistrophora* plants is brief and unpredictable, behavioral assays could not follow a randomized block design. Thus, sex ratios often were unavoidably biased by the chance availability of moths on the days when flowers bloomed. However, our previous studies indicated no significant sex biases in attraction or feeding behaviors in response to *Magnolia grandiflora* (Magnoliaceae) floral scent and Bergamot oil odors (Raguso et al. 2005). We evaluated the results for both sexes separately and found no significant differences (data not shown), then combined data for subsequent analyses.

We tested the attractiveness of scent from single flowers from plants of different populations of white-flowered *E. ancistrophora* ssp. *ancistrophora* (three populations with short–medium length flowers and three with long flowers), and from pink-flowering *E. ancistrophora* ssp. *cardenasiana* (from three different accessions, i.e. different collection numbers). We also tested *M. sexta* responses to the distantly related *Echinopsis mirabilis*, which is characterized by a simple, benzenoid-dominated floral scent. Methyl benzoate makes up 60–90% of scent emission, and is accompanied by small amounts of long chain hydrocarbon aldehydes and esters, without sesquiterpenoids. Odor emission rates of *E. mirabilis* flowers were even weaker than those of medium to weakly scented flowers of *E. ancistrophora* ssp. *ancistrophora*. For a positive control, we used scent from single flowers of *Magnolia grandiflora*, which were available throughout our study and were previously observed to attract naïve *M. sexta* (Raguso et al. 2005). Single flowers were tested in all experiments; all cactus flowers were fully open for only one day and thus were used only once. For each test stimulus, the bagged cactus flower remained intact and attached to the plant, whereas *Magnolia* branches with single flowers were cut and placed into water, with only the flower contained within the plastic bag during the experiment. For a negative control, we used compressed air only, purified as described.

Evaluation of hawkmoth behavior

We used wind tunnel assays to test relative attractiveness of different odors to naïve *M. sexta* moths. A positive response was indicated by stereotypic upwind, zig-zag flight towards the odor source. Proportions of moths responding to different odors were tested using a G-test as described by Goyret et al. (2007). Differences in the distances flown in the wind tunnel were tested with a one-way ANOVA, followed by a one-tailed Mann–Whitney U test for differences between short to medium and long-flowered *E. ancistrophora* ssp. *ancistrophora*. Finally, proportions of

moths showing the proboscis extension reflex (PER) to each odor were compared using G-tests as described above.

Results

Scent variation between subspecies

We found a total of 145 volatile compounds in the floral scent of the four subspecies of *Echinopsis ancistrophora* (Table 2). Between three and 46 compounds were detected per flower, with a mean of 20.6 (ssp. *ancistrophora* 23.3, ssp. *cardenasiana* 18.7, ssp. *pojoensis* 14.2, ssp. *arachnacantha* 8.0). However, on average only eight compounds reached levels of 1% or more of the total odor emitted by individuals (minimum 1, maximum 15 compounds). Thus, the floral scent profiles of *E. ancistrophora* cacti were dominated by a few very abundant compounds, with many other, related compounds contributing trivial amounts to overall scent production.

The four subspecies of *E. ancistrophora* can be distinguished clearly by their mean patterns of scent composition at the level of compound class, with the exception of ssp. *arachnacantha* that shows similarities to ssp. *ancistrophora* (Fig. 1, Supplementary material Appendix 2). The odor bouquets of ssp. *ancistrophora* were dominated by sesquiterpenoids, making up roughly 86% of relative odor emission (Fig. 1 D, Supplementary material Appendix 2). In contrast, the scent of pink-flowered ssp. *cardenasiana* was dominated (85%) by compounds related to palmitic, linoleic, linolenic and other fatty acids (Fig. 1C). The floral scents of red/yellow flowered plants from ssp. *arachnacantha* also had high sesquiterpenoid content on average (68%, Fig. 1B), whereas the scent of red-flowered ssp. *pojoensis* was dominated by benzenoid (33%) and fatty acid derived compounds (25%) (Fig. 1A). On the level of individual compounds, the chromatograms of ssp. *ancistrophora* scent usually consisted of a large peak of (E)-nerolidol, germacrene D, spathulenol, or (E,E)-α-farnesene. In ssp. *arachnacantha*, (E)-nerolidol or 1,8-cineole typically were the most abundant scent compounds. An isomer of 9-octadecenal was the most prominent scent component of ssp. *cardenasiana*, while the highly variable scent of ssp. *pojoensis* was dominated either by spathulenol, nonanal or methyl benzoate (Table 2).

Variation within subspecies was especially high in ssp. *arachnacantha* and ssp. *pojoensis*, in which the scent of different individuals was dominated by either fatty acid derivatives, mono- or sesquiterpenes (ssp. *arachnacantha*), or by fatty acid derivatives, aromatics or sesqui- and monoterpenes in equal amounts (ssp. *pojoensis*). Comparatively little variation at the level of biosynthetic class was found in ssp. *ancistrophora* and *cardenasiana*, in which sesquiterpenoids and fatty acid derivatives, respectively, were the dominant scent components in all studied plants. In ssp. *ancistrophora*, (E)-nerolidol was the dominant scent component in 81% of all studied individuals, whereas germacrene D was the dominant component in 5%, with equal amounts of these compounds emitted by 3%. In the remaining 9% of studied plants, floral scent was dominated by varying amounts of the sesquiterpenoids spathulenol and (E,E)-α-farnesene.

Scent variation among and within populations of ssp. *ancistrophora*

We found no significant differences in the mean ranks of relative abundance of individual compound classes when populations of ssp. *ancistrophora* were grouped into putative syndromes of hawkmoth pollination (four populations) vs bee pollination (nine populations) using the Mann–Whitney U-test (monoterpenoids: $p = 0.44$, sesquiterpenoids: $p = 0.24$, irregular terpenoids: $p = 0.64$, fatty acid derivatives: $p = 0.99$, aromatics: $p = 0.32$, nitrogenous compounds: $p = 0.94$, unknowns: $p = 0.71$). Sesquiterpenoids dominated all individual plants from bee-pollinated populations (48.8–99.7% of all scent emitted, mean $92 \pm 11\%$ SD), and most (9 of 11) moth-pollinated individuals (0.8–99.7%, mean $71.8 \pm 31\%$ SD). The lower mean of the latter group reflects two individuals whose floral scent was dominated by monoterpenoids (56.5% of total scent). Flowers of moth-pollinated populations also emitted higher (and more variable) proportions of fatty acid derivatives (0–42%, mean $9.7 \pm 15\%$ SD) than did those of bee-pollinated individuals (mean $1.5 \pm 1.5\%$ SD). The mean number of compounds found in bee-pollinated flowers (25.6) was higher than in hawkmoth-pollinated flowers (17.9), but this difference was not significant ($p = 0.15$).

Comparing the relative amounts of the two most dominant individual compounds – (E)-nerolidol and germacrene D – in ssp. *ancistrophora*, we found that hawkmoth flowers emitted significantly less (E)-nerolidol than did bee flowers ($p < 0.001$), whereas no significant differences were found for germacrene D ($p = 0.26$). The results were similar when comparing absolute amounts (peak areas), with no significant differences in emission of germacrene D between bee and moth pollinated populations ($p = 0.64$); in contrast, the moth-pollinated populations emitted significantly smaller absolute amounts of (E)-nerolidol than the bee-pollinated ones ($p < 0.001$, Mann–Whitney U-test). (E)-nerolidol was the dominant compound in all but two of the 26 individuals studied from bee-pollinated populations, but was dominant in only six of 11 individuals from moth-pollinated populations, and never above 70% of total emissions per flower. Instead, higher amounts of spathulenol or (E,E)-α-farnesene were emitted from the remaining five individuals from hawkmoth pollinated populations.

Variation in scent composition among individuals from the same population was studied in three populations of ssp. *ancistrophora*. Variation on the level of compound classes was low in Escoipe, with sesquiterpenoids comprising between 94.8% and 99.7% of total scent and monoterpenoids accounting for most of the remaining (3.8%) scent emissions (Table 3). In contrast, scent composition was slightly more variable in flowers from El Mollar, with 67.5–96% sesquiterpenoid composition and larger amounts of monoterpenoids (up to 16.3%), fatty acid derivatives (up to 11.8%) and irregular terpenoids (up to 7.7%; Table 3). In the Calderilla population, scent was dominated by sesquiterpenoids (70.8–99.7%), and the contribution of monoterpenoids to scent composition was highly variable (0–25.6%; Table 3).

With respect to individual compounds, variation in the two most abundant sesquiterpenoids, (E)-nerolidol and

Table 2. Floral scent compounds (%) of 13 populations of *E. ancistrophora* ssp. *ancistrophora* (from left to right in order of increasing flower length), and the three subspecies with colorful flowers. Asterisks indicate compounds confirmed by injection of reference samples, n refers to individual flowers from individual plants. Retention times (RT) are for EC-wax column.

	RT	P. del Toro n = 4	s. P. Toro n = 2	El Mollar n = 5	Rio Blanco n = 2	Cebilar n = 3	Escoipe n = 5	H. Huasi n = 1	C. Alegre n = 3	Volcan n = 1	Abra Sta. L. n = 1	Calderilla n = 5	Termas de Reyes n = 3	El Fuerte n = 2	*E. arachnacantha* n = 4	*E. pojoensis* n = 3	*E. cardenasiana* n = 4
Monoterpenoids																	
Limonene*	7,86	0.04	0.16	0.01	0.06	0.08	0.17	0.51	0.14	0.11	0.43	0.02	0.64		0.25	4.92	
1,8-cineole*	7,99				0.07		0.14	5.02	1.27	1.40		0.06	0.40		15.41		
trans-β-ocimene	8,71																2.14
6-Methyl-5-heptene-2-one*	10,02	1.71		3.28	0.37	0.12	0.09		4.80			0.05	0.83	8.55	1.60	12.23	
(E,E)-2,6-dimethyl-1,3,5,7-octatetraene	11,72											0.74					0.22
Linalool	12,93								0.01		0.07						
Dodecatriene	13,94						0.25										
Geranial*	15,41											0.06	0.11				
(E,Z)-2,6-dimethyl-3,5,7-octatriene-2-ol	16,17											0.35					2.49
Octadecatetraene	16,32																0.13
(E,E)-2,6-dimethyl-3,5,7-octatriene-2-ol	16,39										2.33	3.13	0.38				
Geraniol	16,64											0.61					0.74
Geranyl acetate*	16,72	1.11		1.92	0.10	0.07	0.22	0.51	2.33			0.07	3.77		1.06		1.23
Unknown monoterpenoids		1.16	0.43	0.06	0.00	0.12	0.18	0.02	0.39	0.00	0.00	0.10	0.25	0.00	0.00	0.00	0.00
All monoterpenoids		4.02	0.59	5.27	0.60	0.39	1.04	6.06	8.95	1.51	2.82	5.18	6.37	8.55	18.33	17.16	6.94
Sesquiterpenoids																	
α-cubebene*	11,87	0.35			0.04		0.17			0.28							
α-copaene*	12,38	0.81	0.04	0.17	2.84	1.12	0.95		0.35	3.28		1.37	1.30				
β-bourbonene	12,76	0.32	0.09		0.27	0.48	0.63			8.03			0.15		0.11		
β-copaene*	12,98	0.19	0.04	0.06	0.89	0.16	0.53	0.10	0.07	2.18	0.11	0.62	0.05				
β-elemene	13,66			0.01	0.05	0.04	0.08			0.17		0.09					
(E)-caryophyllene*	13,78	0.21	0.03	0.03	0.29	0.13	0.62			1.65	0.66	0.57	0.08		2.00		
α-santalene	14,27						0.06	0.38	0.10								
β-santalene	14,44						0.08	0.27	0.07								
Humulene	14,68	0.02			0.20	0.03	0.20			0.29							
Germacrene D*	15,16	2.80	0.14	1.35	19.13	5.52	13.12	0.73	2.32	50.75	13.14	32.58	5.12			7.93	
Bicyclogermacrene	15,24	0.16		0.02	0.56	0.06	0.26			1.44	0.28	0.07	0.09				
(Z,E)-α-farnesene	15,32	0.11		0.04	0.18	0.07	0.61	0.61	0.17	2.48	0.22	0.31	1.05		0.12		
(E,E)-α-farnesene*	15,51	0.24		0.11	0.17		0.08	0.16	0.09				22.43	10.23			4.21
δ-cadinene	15,67	0.06		0.01	0.22		0.78	0.02	0.01	0.91		0.06					
β-sesquiphellandrene	15,83						0.04	0.38	0.10						0.03		
Dehydrogeosmin*	16,34	0.15			0.20		0.28	0.17	1.37	0.67							0.13
(Z)-nerolidol*	18,23	1.31		0.90		0.83	1.82	0.09	0.75						0.04		
(E)-nerolidol*	18,59	82.43	94.28	81.22	68.53	87.52	71.18	83.75	73.64	2.16	71.30	47.64	11.79	35.60	53.38	3.50	1.82
α-cadinol	20,58				0.03		0.25			0.72							
(E,E)-farnesyl acetate	20,94							0.28	0.07			3.33					
Spathulenol	21,1	0.24	0.27	3.25									13.86	35.90	0.04	9.44	
(E,E)-farnesol*	21,64	0.56	0.49	0.24	0.42		0.08	0.56	2.18			1.79					
Unknown sesquiterpenoids		4.95	2.45	2.92	4.47	1.84	6.60	4.19	3.62	21.58	3.25	4.93	3.37	7.97	8.30	4.88	0.77
All sesquiterpenoids		94.90	97.82	90.34	98.50	97.80	98.42	91.69	84.89	96.59	88.96	93.35	59.28	89.70	64.02	25.76	6.92
Fatty acid derivatives																	
Nonanal	10,91	0.41	0.44	1.10	0.27	0.07	0.02	0.49	0.88	0.40	3.49	0.08	2.25	0.33	4.49	17.17	3.94
Hexadecane	13,84	0.17		0.03	0.05				0.93		1.38		0.38				
Methyl hexadecanoate	20,48					1.06											
9-octadecenal isomer	21,92															4.71	49.00
Unknown fatty acid derivatives		0.22	0.48	2.32	0.00	0.68	0.12	0.00	0.40	0.00	0.48	0.03	30.33	0.75	10.36	3.10	32.32
All fatty acid derivatives		0.81	0.92	3.45	0.32	1.82	0.14	0.49	2.22	0.40	5.35	0.11	32.96	1.08	14.85	24.97	85.26

Table 2 (*Continued*)

	RT	P. del Toro n=4	s. P. Toro n=2	El Mollar n=5	Rio Blanco n=2	Cebilar n=3	Escoipe n=5	H. Huasi n=1	C. Alegre n=3	Volcan n=1	Abra Sta. L. n=1	Calderilla n=5	Termas de Reyes n=3	El Fuerte n=2	*E. arachnacantha* n=4	*E. pojoensis* n=3	*E. cardenasiana* n=4
Aromatics																	
Benzaldehyde	12,72	0.03		0.03	0.08			0.13	1.14		0.13		0.70	0.18	0.60		
Methyl benzoate*	13,95		0.24	0.01	0.06		0.01	0.14	0.04			0.40	0.12	0.49	1.90	30.70	0.04
Methyl salicylate	15,66		0.10									0.07					
Benzyl alcohol*	16,85	0.24		0.05	0.17		0.07	1.42	1.13	0.29			0.44			1.41	
Phenethyl alcohol	17,26	0.01	0.33				0.01	0.07	0.59			0.08					
Unknown aromatics		0.00	0.00	0.02	0.01	0.00	0.00	0.00	1.04	0.00	0.24	0.00	0.00	0.00	0.10	0.00	0.00
All aromatics		0.28	0.68	0.11	0.32	0.00	0.09	1.76	3.94	0.29	0.37	0.55	1.27	0.67	2.60	32.11	0.04
Nitrogenous compounds																	
Methyl-anthranilate	20,57			0.01							0.12				0.20		0.01
Indole	22,39			0.80							2.37				0.20		0.84
All nitrogenous compounds				0.81							2.49				0.20		0.85
Irregular terpenoids																	
Unknown irregular terpenoids		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.12	0.00	0.00	0.00	0.00
All irregular terpenoids		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.12	0.00	0.00	0.00	0.00
Unknowns		0.04			0.26		0.30			1.22		0.39					

germacrene D, was high in the Escoipe population, with (E)-nerolidol ranging between 10.2% and 94.8% of all emitted compounds, and germacrene D ranging from 0 to 46.2% (Table 3). In contrast, all sampled individuals from El Mollar were dominated by (E)-nerolidol (61.1–91.1%), with only small amounts of germacrene D (0–3.6%; Table 3). In Calderilla, (E)-nerolidol was usually the dominant compound (28.6–67.8%), but germacrene D often reached similarly high levels (26–43.3%; Table 3).

Scent composition with relation to geography and flower depth

Multidimensional scaling by odor compound class grouped all but one of the short-tubed, bee-pollinated individuals of ssp. *ancistrophora*, together with the two moth-pollinated populations with shorter-tubed flowers (Abra Sta. Laura and Calderilla) (Fig. 2; stress 0.14, $R^2 = 0.96$). The two outlier populations of ssp. *ancistrophora* (Termas de Reyes and El Fuerte) are noteworthy for having the longest flower tubes of the species. These moth-pollinated populations differ from the others by relatively lower amounts of sesquiterpenoids, higher emission of monoterpenoids, and, in plants from Termas de Reyes, greater amounts of fatty acid derivatives (Table 2). To a great extent, these differences consist of spathulenol (in both populations) and pentadecane (in Termas de Reyes), along with other, less prominent hydrocarbons (Table 2). Among the three subspecies of *E. ancistrophora* with colored flowers, only individuals of ssp. *cardenasiana* form a tight cluster in odor space, reflecting the domination of fatty acid derivatives in their floral scent. (Fig. 2). Due to their high variation in scent composition, individuals of ssp. *arachnacantha* and ssp. *pojoensis* are scattered throughout the odor space in Fig. 2. When the populations with the smallest sample sizes ($n < 3$) are omitted from the analysis, the pattern described above does not change (stress 0.09, $R^2 = 0.98$). Similarly, removing ssp. *arachnacantha* (cultivated plants originating from several populations) from the analysis, or removing all samples taken from cultivated plants has no effect on the pattern (stress 0.07, $R^2 = 0.99$, or stress 0.06, $R^2 = 0.99$).

Odor space plots derived from the use of individual compounds in similarity matrices (ANOSIM), odor space plots are comparable to those shown in Fig. 2. In the interest of space, these graphs are not shown. Differences of floral scent profiles were most pronounced between bee-pollinated ssp. *ancistrophora* on the one hand and ssp. *pojoensis* and *cardenasiana* on the other hand ($R = 0.91$ and 0.97, $p = 0.05$ and 0.01). Transformation with square-root and fourth-root had no effect, indicating that results were not skewed by the overwhelming dominance of single compounds. Differences between bee and moth-pollinated populations of ssp. *ancistrophora* were modest but significant ($R = 0.5$, $p = 0.01$) but decreased with square root transformation. As above, we tested for the influence of populations with small sample sizes, and again we found no major changes when calculating the ANOSIM without those samples (bee-pollinated ssp. *ancistrophora* vs ssp. *pojoensis* $R = 0.95$, $p = 0.06$, bee-pollinated ssp. *ancistrophora* vs

Table 3. Variation of relative proportions (%) of compound classes and (E)-nerolidol and germacrene D within three populations of *E. ancistrophora* ssp. *ancistrophora*. Examples for strong within–population variation in (E)-nerolidol and germacrene D are printed in bold letters.

	monoterpenoids	sesquiterpenoids	fatty acid derivatives	aromatics	nitrogenous compounds	irregular terpenoids	unknowns	(E)-nerolidol	germacrene D
El Mollar	1.24	95.76	0.43	0.26	2.32	0.00	0.00	91.12	2.14
El Mollar	1.58	96.99	1.08	0.35	0.00	0.00	0.00	88.48	3.56
El Mollar	5.98	91.97	2.04	0.00	0.00	0.00	0.00	87.79	0.51
El Mollar	16.76	79.65	1.85	0.00	1.74	0.00	0.00	77.62	0.55
El Mollar	4.41	83.74	11.85	0.00	0.00	0.00	0.00	61.09	0.00
Escoipe	0.99	98.66	0.00	0.21	0.00	0.00	0.14	76.50	11.69
Escoipe	1.35	98.36	0.09	0.20	0.00	0.00	0.00	80.93	7.68
Escoipe	3.83	94.78	0.00	0.03	0.00	0.00	1.35	**10.22**	**46.24**
Escoipe	0.44	99.02	0.54	0.00	0.00	0.00	0.00	**94.83**	**0.00**
Escoipe	0.23	99.70	0.07	0.00	0.00	0.00	0.00	**93.43**	**0.00**
Calderilla	25.62	70.83	0.13	2.38	0.00	0.03	1.01	28.61	26.16
Calderilla	0.21	99.75	0.00	0.03	0.00	0.00	0.00	67.80	25.96
Calderilla	0.27	99.27	0.00	0.07	0.00	0.00	0.39	47.15	43.34
Calderilla	0.00	99.74	0.00	0.26	0.00	0.00	0.00	50.26	34.51
Calderilla	0.75	98.27	0.41	0.00	0.00	0.00	0.56	44.36	32.90

ssp. *cardenasiana* R = 0.99, p = 0.02, bee vs moth-pollinated ssp. *ancistrophora* R = 0.62, p = 0.02).

The results from the Mantel tests allow us to reject the null hypothesis of no correlation between floral scent and geographic distance matrices. We found a marginally significant correlation of r = 0.38 (p = 0.049) for geographic distance between populations and odor composition independent of flower depth in the combined analysis of all subspecies and populations. When Mantel tests were limited to populations of ssp. *ancistrophora*, differences in floral scent and floral depth were significantly correlated (r = 0.34, p = 0.03), independent of geography.

Variation of scent emission rates

Scent emission rates differed between subspecies of *E. ancistrophora*, and both within and between populations of ssp. *ancistrophora*. Emissions per flower and per unit time were highest in ssp. *ancistrophora* (11843 ng = 11.8 µg), followed by ssp. *arachnacantha* (373 ng) and ssp. *cardenasiana* (729 ng), with minimal emission rates observed for ssp. *pojoensis* (17 ng) (Fig. 3A). On a per flower basis, mean rank hourly emission rates for ssp. *ancistrophora* were significantly greater than those for ssp. *arachnacantha* (p = 0.007), ssp. *pojoensis* (p = 0.004), and ssp. *cardenasiana*

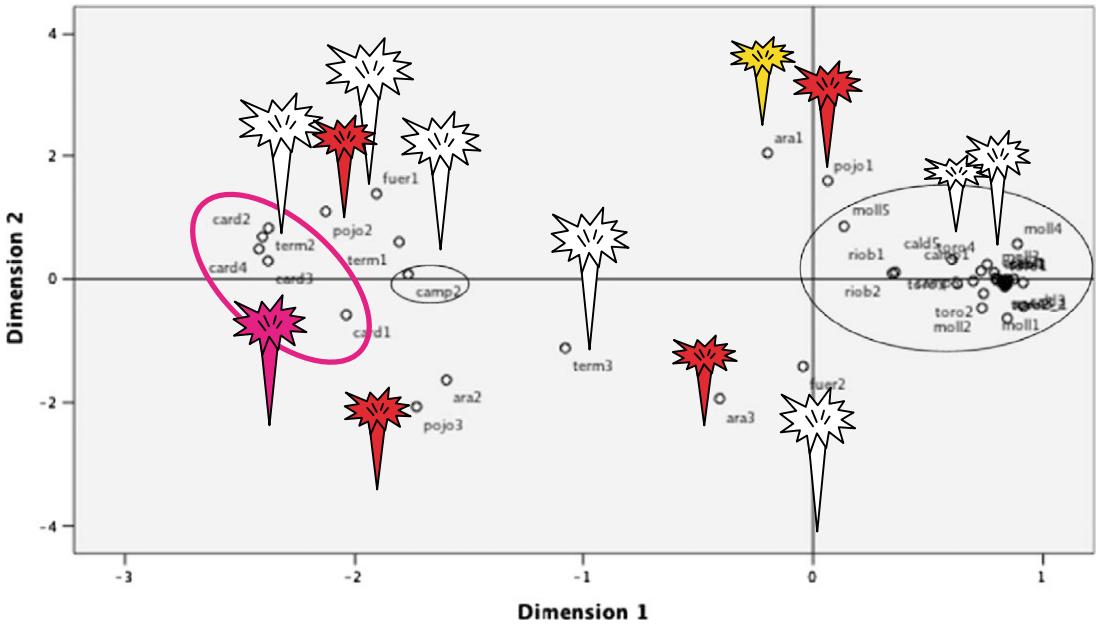

Fig. 2. Multidimensional scaling of compound class proportions of the three subspecies with colored flowers ssp. *arachnacantha* (arach), ssp. *cardenasiana* (card) and ssp. *pojoensis* (pojo) and the 13 populations of white-flowering ssp. *ancistrophora*. Circled in pink are all individuals of ssp. *cardenasiana*. Circled in black are all bee-pollinated and short-tubed, moth-pollinated populations of ssp. *ancistrophora*. The outlier populations (large white flowers) of ssp. *ancistrophora* from Termas de Reyes (term) and El Fuerte (fuer) are the longest-tubed moth pollinated taxa included in this study.

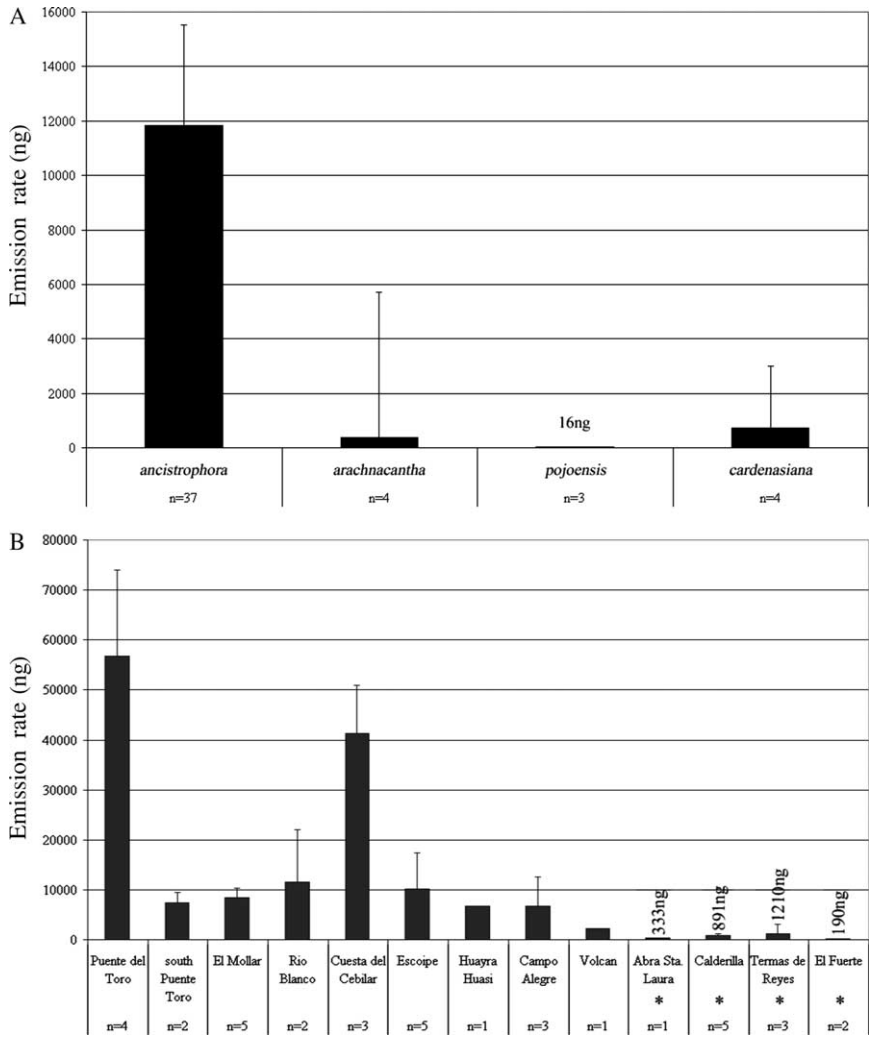

Fig. 3. Floral scent emission rates in ng scent per flower and hr ± SEM. (A) summary data for the four subspecies of *E. ancistrophora*; (B) data for 13 populations of ssp. *ancistrophora*, arranged in order of increasing floral depth. Asterisks indicate populations with long-tubed, moth-pollinated flowers.

($p = 0.047$) (Mann–Whitney U-test). Scent emission of ssp. *pojoensis* was significantly weaker than in the other subspecies with colorful flowers (*cardenasiana*: $p = 0.047$, *arachnacantha*: $p = 0.034$); the emission rates of ssp. *arachnacantha* and ssp. *cardenasiana* did not differ significantly ($p = 0.25$). When standardized for differences in floral mass, emission rates for ssp. *pojoensis* remained significantly lower than for ssp. *ancistrophora* ($p = 0.004$), ssp. *arachnacantha* ($p = 0.034$) and ssp. *cardenasiana* ($p = 0.034$).

Variation of scent emission rates also differed markedly among populations of ssp. *ancistrophora*. Unexpectedly, populations with short-tubed, bee-pollinated flowers generally emitted significantly greater amounts of scent per flower and hour (about 25-fold) than did populations of long-tubed, hawkmoth pollinated flowers ($p < 0.001$) (Fig. 3B).

Scent emission rates day versus night

The long-tubed, white flowers of ssp. *ancistrophora* open during the night and remain open during the early hours of the following day. We compared quantitative and qualitative odor emission during day vs night. The relative abundance of each compound class did not differ significantly between day and night. A slightly greater representation of aromatics ($p = 0.32$) and sesquiterpenoids ($p = 0.80$) at night and fatty acid derivatives ($p = 0.80$) during the day was not significant (Mann–Whitney U-test). Similarly, the total amounts of volatiles emitted did not vary significantly between night and day ($p = 0.62$), with slightly stronger emissions during the day, on average.

Attractiveness of floral scent in behavioral assays

The attraction of *Manduca sexta* moths to the odor of single flowers differed significantly between species of *Echinopsis* ($G_h = 18.24$, $p = 0.0004$, $n = 102$). Eight out of 30 hawkmoths (27.9%) were attracted to floral scent of white-flowered *E. ancistrophora* ssp. *ancistrophora*. The scent from flowers with short to medium length (i.e. < 15 cm) nectar tubes (from three populations) attracted five out of 15 moths tested (30%), whereas scent from long-tubed (> 15 cm) flowers (from three populations) attracted three out of 15 moths (20%). In contrast, none of the 16 hawkmoths tested were attracted to the floral scent of

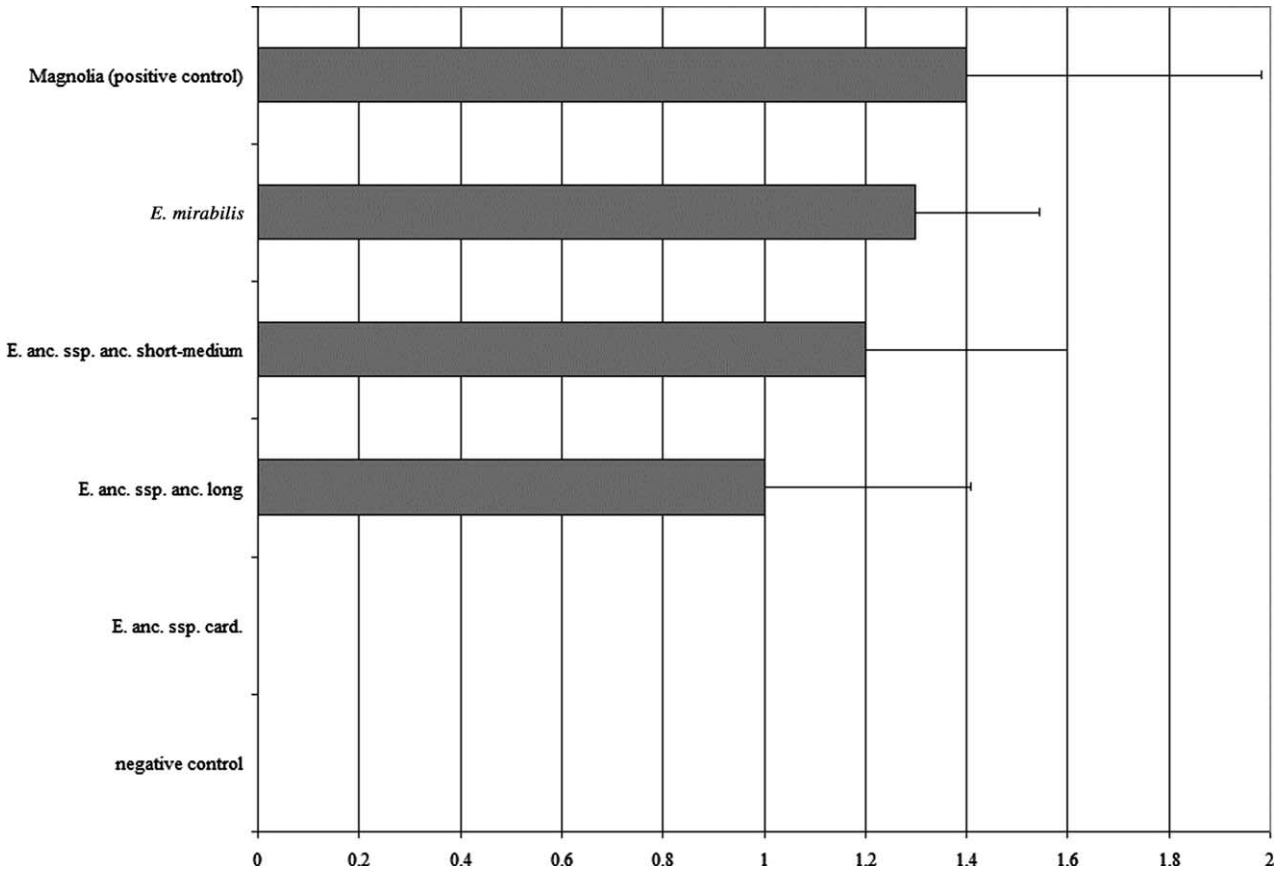

Fig. 4. Mean ± SD distance flown (m) by *Manduca sexta* moths in the wind tunnel towards the odor source.

pink-flowered, putatively bee-pollinated *E. ancistrophora* ssp. *cardenasiana*. Nine out of 16 moths (56%) were attracted to the odor blend of *E. mirabilis*, and 16 out of 30 hawkmoths (53%) were attracted to scent from the positive control (*Magnolia grandiflora*), whereas none of the ten moths exposed to the negative control (purified air) showed any behavioral response. Among the eight moths attracted to white cactus flowers, five reached the odor source (four in short to medium flowers vs one in long flowers), whereas 13 of 16 moths reached the odor source of the positive control.

On a finer spatial scale, the hawkmoths' zigzag flights extended furthest upwind in the presence of the odor of the positive control (*Magnolia grandiflora*), with a mean of 1.4 m (SD = 0.6, n = 15) followed by *E. mirabilis* (mean 1.3 m, SD = 0.6, n = 9), short to medium-tubed (< 15 cm) flowers of *E. ancistrophora* ssp. *ancistrophora* (mean 1.2 m, SD = 0.4, n = 5), and long-tubed (> 15 cm) flowers of ssp. *ancistrophora* (mean 1.0 m, SD = 0.4, n = 3), out of a maximum distance of 2 m (Fig. 4). Differences between these treatments were not significant (ANOVA, $p = 0.72$). Mann–Whitney U test showed no significant differences for upwind flight distances in short–medium white flowers vs long white flowers ($p = 0.79$).

Moths did not differ significantly in their proboscis extension reflexes (PER) to the odors of different treatments ($G_h = 6.33$, $p = 0.10$). At the release point in the wind tunnel, 10% of all moths flown to ssp. *ancistrophora* flowers spontaneously exhibited PER when placed within the odor plume, whereas 31% of all moths flown to *E. mirabilis* and 23% of all moths flown to the positive control (*Magnolia*) showed PER. Proboscis extension reflexes was never observed in response to the negative control (filtered air), nor to the odor of pink flowers of *E. ancistrophora* ssp. *cardenasiana*. Moths that exhibited PER upon being placed into the odor plume subsequently tracked the odor source in 67% of all cases.

Discussion

The results of our study were unexpected, in ways that suggest that floral scent in this lineage has not evolved in concert with floral color, tube length, and timing of anthesis, likely due to influences other than pollinator-mediated selection:

1. On the subspecies level, floral scent composition and emission rates appeared to be consistent with combinations of floral traits traditionally associated with pollination syndromes, due to the influence of ssp. *ancistrophora* (Fig. 3a). However, this result was an artifact of the association of hawkmoth pollination with only one of the four subspecies. When all populations of ssp. *ancistrophora* were examined, the actual relationship between scent emissions and floral depth was inverted, contrasting our first hypothesis, as short-tubed, bee-pollinated flowers consistently

emitted significantly stronger scents by all measures (Fig. 3b).

2. Similarly, differences in floral scent composition and emission rates between bee- and moth-pollinated populations of *E. ancistrophora* ssp. *ancistrophora* did not uphold predictions based on pollination syndromes (hypotheses 1a, 1b), despite extreme differences in floral tube length, time of anthesis and other floral traits.
3. Wind tunnel experiments showed that naïve *M. sexta* moths were attracted to floral scent from ssp. *ancistrophora*, but not to the fragrance of ssp. *cardenasiana*. Contrasting our second hypothesis, short and long-tubed populations of ssp. *ancistrophora* were equally attractive to the moths. However, none of these plants were as attractive as the more distantly related *Echinopsis mirabilis*, whose fragrance was emitted in smaller amounts but differed in its chemical composition. We discuss these results and their significance in greater detail below.

Chemical composition of floral scent

The volatile compounds and compound classes identified in our study, especially the dominant sesquiterpenes, are comparable to those of previously studied, diurnal cacti (Kaiser and Tollsten 1995, Schlumpberger et al. 2004). One noteworthy pattern was the statistical dominance of scent blends by relatively few compounds ((E)-nerolidol, germacrene D), accompanied by large numbers of biosynthetically related sesquiterpenoids in very low amounts ($<1\%$ of total emissions). Several other studies have identified odor blends dominated by one or a few compounds, particularly in highly specialized plants pollinated by male euglossine bees (Knudsen and Mori 1996) or by fig wasps (Grison-Pigé et al. 2002), but low abundance scent components often serve critical functions in these and other plant–pollinator relationships (Dodson et al. 1969, Schiestl 2005) and cannot be ignored. The large number of low-abundance sesquiterpenoids in our data set may represent biosynthetic by-products, as the enzymes responsible for (E)-nerolidol and germacrene-D biosynthesis in angiosperms produce several minor products, some potentially through spontaneous rearrangement (Schnee et al. 2002, Prosser et al. 2004). It also is possible that the low abundance compounds are rearrangement artifacts of the scent collection process. For example, (E,E)-2,6-dimethyl-3,5,7-octatrien-2-ol ((E)-ocimenol), (E,Z)-2,6-dimethyl-3,5,7-octatrien-2-ol ((Z)-ocimenol), and (E,E)-2,6-dimethyl-1,3,5,7-octatetraene result from oxidation and rearrangement when large amounts of (E)-β-ocimene are trapped on carbon filters (Kaiser 1993, Tholl et al. 2006).

Most subspecies of *E. ancistrophora* were distinguishable in terms of overall odor composition, on the level of compound class, with only ssp. *ancistrophora* and ssp. *arachnacantha* being similar (Fig. 1). Variation was observed within and among all three subspecies with colored flowers, especially in ssp. *arachnacantha* and ssp. *pojoensis* (Fig. 2). The plants of ssp. *arachnacantha* used for this study are known to descend from different natural populations, thus variation in scent chemistry in this group may reflect variation between populations. However, due to the large geographic distance between some subspecies, their remote habitats and brief, synchronized periods of flowering, we could not obtain all scent samples in situ. Because of our interest in the extreme morphological variation in ssp. *ancistrophora* we prioritized collecting scent samples from this subspecies only from plants we could study in their native habitats, and from as many populations as possible.

Floral scent composition of bee and hawkmoth-pollinated populations of ssp. *ancistrophora* did not differ significantly, and lacked consistent emissions of monoterpene alcohols, nitrogenous and aromatic compounds often found in sphingophilous cacti (Kaiser and Tollsten 1995, Raguso et al. 2003), including other lineages of *Echinopsis* (Schlumpberger unpubl.). Variation on the level of compound classes was low among all but two populations of ssp. *ancistrophora*, and was not heavily affected by population of origin. Instead, population-level variation in ssp. *ancistrophora* was observed in the relative abundance of individual compounds (Table 3). For instance, individuals with low amounts of (E)-nerolidol often emitted high amounts of germacrene D, which likely represents allelic variation for sesquiterpene synthase enzymes that utilize the same substrate (farnesyl diphosphate) pools (above).

Floral scent emission rates

We predicted that *Echinopsis ancistrophora* ssp. *ancistrophora* flowers should produce more scent than the other subspecies for at least two reasons. First, all hawkmoth pollinated populations in our study belong to ssp. *ancistrophora*, and sphingophilous plants typically produce strongly scented flowers (Knudsen and Tollsten 1993, Raguso et al. 2003). Second, the relative isolation and limited periods of synchronized blooming that typify populations of ssp. *ancistrophora* suggested that strong scent would help attract pollinators to these ephemeral resource patches. Unlike many flower visiting bees, hawkmoths are not central place foragers, and may need to be attracted from a distance. Indeed, ssp. *ancistrophora* plants emitted several-fold more fragrance per flower or per unit floral mass than did other subspecies (Fig. 3a). Unexpectedly, this result was completely attributable to the short-tubed, day-blooming, bee-pollinated populations of ssp. *ancistrophora*, which emitted significantly higher amounts of total fragrance than did those from long-tubed, hawkmoth-pollinated populations (Fig. 3b), or any other taxa in this study. Although the long-tubed flowers from El Fuerte have six-fold greater fresh and dry mass than the short-tubed flowers of Puente del Toro, the latter emit at least ten-fold greater amounts of scent per flower or per dry floral mass (Fig. 3b). The inverse relationship between tube length and emission rate would appear anomalous in a single population, but was consistent over several populations across a broad geographical transect.

We have no immediate explanations for this pattern, which also was observed among South American species of *Nicotiana* (Raguso et al. 2006). Perhaps low emission rates in long-tubed populations reflect balancing selection by destructive flower visitors or nocturnal herbivores. The

weakly scented populations of ssp. *ancistrophora* with the longest floral tubes also have the largest nectar rewards and nocturnal pollen presentation, attract destructive nocturnal scarab beetles (Schlumpberger unpubl.), and may thus be more vulnerable to kairomonal 'eavesdropping' by florivores (Baldwin et al. 1997). This result highlights the perils of what Waser et al. (1996) refer to as 'syndrome thinking', in which non-pollinating floral visitors are disregarded, and guilds of taxonomically related pollinators (e.g. 'bees') are lumped into categories in which flower-visiting behavior is assumed to be somewhat stereotyped. Alternatively, weakly scented, long-tubed populations with low scent emissions per flower (or plant) could still produce attractive odor plumes if blooming is highly synchronous (e.g. *Peniocereus striatus*; Raguso et al. 2003) or populations are dense. Also, it is possible that increased CO_2 emissions associated with greater nectar secretion may compensate for low levels of floral scent in long-tubed populations, as floral CO_2 has been demonstrated to attract *M. sexta* in behavioral assays (Goyret et al. 2008). Additional experiments are required to evaluate these hypotheses.

Scent quantity, quality and hawkmoth attraction

In our wind tunnel experiments, the floral scent from several populations of *E. ancistrophora* ssp. *ancistrophora* was attractive to naïve *M. sexta*, whereas scent from *E. ancistrophora* ssp. *cardenasiana* was not. However, the fragrances of a distantly related night-blooming cactus (*E. mirabilis*) and the unrelated positive control (*Magnolia grandiflora*) were two to three times more attractive to *M. sexta* than floral scent from ssp. *ancistrophora*. This result was reflected in the lower number of attracted individuals, shorter distances flown, and lower PER frequencies in response to scent from individual flowers of ssp. *ancistrophora* (Fig. 4, Table 4). In natural populations, the synchronous flowering of conspecific cacti may result in a stronger olfactory signal from these plants, as suggested above. Certainly, an opportunistic hawkmoth might probe the pink flowers of ssp. *cardenasiana* if it encountered a large population of them (Haber and Frankie 1989). However, our assays show that the fragrance emitted from individual flowers of ssp. *cardenasiana* is insufficient to attract *M. sexta*, and the flowers offer little to no nectar (unpubl.). Increased attraction of *M. sexta* to the positive control may reflect the roughly 30-fold excess in total scent emission rates from *Magnolia grandiflora* flowers over those of the most strongly scented *Echinopsis*. It would be necessary to combine the odor of several *E. ancistrophora* ssp. *ancistrophora* flowers to test whether odor quality or quantity were responsible for their lower attractiveness to *M. sexta*. The small number of flowers produced per individual each year, combined with the brief life (one evening and morning) of each flower made this logistically unfeasible. On the other hand, fragrance emission rates from *E. mirabilis* were lower (65 ng) than those of *E. ancistrophora* ssp. *ancistrophora* and ssp. *cardenasiana*, suggesting that the marked differences in relative attractiveness between these taxa are due to the chemical composition, rather than the intensity, of floral scent.

Table 4. Attractiveness of floral odor from *E. ancistrophora* ssp. *ancistrophora* and controls to *Manduca sexta*.

	Total number of moths attracted (n tested)	Percentage of moths attracted (%)
All *ancistrophora* flowers	8 (30)	26.66
Medium/short flowers	5 (15)	33.33
Long flowers	3 (15)	20
ssp. *cardenasiana*	0 (16)	0
E. mirabilis	9 (16)	56.25
Positive control	16 (30)	53.30
Total	33 (92)	36.66

There is no obvious relationship between odor complexity and hawkmoth pollination in cacti, as other studies have identified from less than ten (Barthlott et al. 1997, Raguso et al. 2003) to more than 50 scent components (Kaiser and Tollsten 1995) per species. The two most dominant compounds in ssp. *ancistrophora*, (E)-nerolidol and germacrene D, are often (but not exclusively) found in sphingophilous flowers of other families (Kaiser 1993, Knudsen and Tollsten 1993). However, in several genera of moth-pollinated cacti studied so far, these two substances were mostly absent (Kaiser and Tollsten 1995, Barthlott et al. 1997, Raguso et al. 2003). Interestingly, we found comparable amounts of germacrene D in bee- and hawkmoth-pollinated populations, but (E)-nerolidol was emitted in significantly smaller amounts from hawkmoth pollinated populations. These results contrast with our expectations based on Knudsen and Tollsten's (1993) survey of fragrance in sphingophilous plants from Neotropical rainforests in Ecuador and Puerto Rico, which identified acyclic sesquiterpene alcohols (e.g. nerolidol and farnesol isomers) as characteristic scent components of hawkmoth-pollinated plants. Subsequent studies have identified nerolidol and farnesol isomers as major scent components ($>10\%$ of total) in some hawkmoth-pollinated Cactaceae (e.g. Selenicereus; Kaiser and Tollsten 1995) as well as Nyctaginaceae (Levin et al. 2001), Solanaceae (Raguso et al. 2003) and Apocynaceae (M. Moré et al. pers. comm.). However, nerolidol is the main scent compound in some bee-pollinated cacti from a different genus of the same tribe (Schlumpberger et al. 2004), and its dominance in ssp. *ancistrophora* may reflect a phylogenetic history involving bee pollination.

It is unclear why lower amounts of (E)-nerolidol were emitted in the long-tubed, hawkmoth pollinated populations than in the short-tubed, bee-pollinated populations of ssp. *ancistrophora*. One possibility is that nerolidol is ecologically costly in long-tubed *Echinopsis* flowers, such that it may attract floral visitors whose activities reduce reproductive output in these cacti. Trapping experiments with (E)-nerolidol as a lure should be conducted in the appropriate habitats to test this hypothesis. Another possibility is that nerolidol is relatively unattractive to hawkmoths, and may primarily attract bees in short- to medium-tubed, diurnal flowers. In *M. sexta*, (E)-nerolidol elicits antennal responses (coupled gas chromatography–electroantennographic detection = GC-EAD) only at high stimulus concentrations (Fraser et al. 2003) and may be a poor conditioning stimulus for PER (see Daly et al. 2008 for (Z)-nerolidol). Nevertheless, long and short tubed flowers of ssp. *ancistrophora* were equally attractive to *M.*

sexta in our wind tunnel assays, regardless of (E)-nerolidol content, and were significantly more attractive than those of ssp. *cardenasiana*, whose fragrance was dominated by fatty acid derivatives (Table 4, Fig. 4). Thus, the greater relative abundance of fatty acid derivatives in the fragrance of long-tubed populations (e.g. Termas de Reyes, Fig. 2) of ssp. *ancistrophora* simply represents an artifact of lower terpenoid content (Fig. 1, Table 2). On the other hand, all flowers of ssp. *ancistrophora* were significantly less attractive than flowers of *E. mirabilis*, whose chemically simple fragrance was dominated by methyl benzoate (Table 4, Fig. 4). Recent studies of *Petunia axillaris*, another hawkmoth pollinated plant from Argentina, demonstrate that methyl benzoate is a highly effective antennal stimulant for *M. sexta* (Hoballah et al. 2005).

Phylogeny, geography and floral evolution

If the innate odor preferences of *M. sexta* are representative of the guild of large, nocturnal American sphingids to which it belongs (Grant 1983, Haber and Frankie 1989, Moré et al. 2005), we might expect pollinator mediated selection to favor certain scent components over others, e.g. the dominance of methyl benzoate in hawkmoth pollinated flowers. Instead, methyl benzoate was sporadically detected, at comparably low levels (<1% of total scent), in three of four hawkmoth-pollinated and six of nine bee-pollinated populations of ssp. *ancistrophora*, and was dominant only in isolated individuals of ssp. *pojoensis* and the distantly related *Echinopsis mirabilis* (Table 2). Such paradoxical patterns might reflect several conflicting factors, including diffuse selection due to other pollinators, balancing selection via herbivores using floral scent as a kairomone (Baldwin et al. 1997) and phylogenetic constraint of the possible odors by virtue of limited biosynthetic capability in the ancestor of a given lineage (Raguso et al. 2006).

The large number of volatile compounds found in this study is a result of intensive odor collection over the entire range of *Echinopsis ancistrophora*. Although morphological flower characters did not vary strongly within populations of ssp. *ancistrophora* (unpubl.), floral scent composition varied considerably on the level of individual compounds within and among populations (Table 3). When all subspecies were included, the marginally significant correlation between geographic distance and floral scent composition (Mantel test) may be explained by the exclusively southern location of the subspecies with hawkmoth-pollinated populations. Interestingly, we found a significant correlation between floral scent and floral depth (Mantel test) within ssp. *ancistrophora*, independent of geographic distance. This correlation is not expressed by comparing the compound classes individually with the Mann–Whitney U-test (Results), nor by the results of our behavioral assays, and highlights the difficulty of interpreting variation in complex character states such as floral scent when the behaviorally active scent components (signal) cannot be distinguished from behaviorally neutral compounds (noise), which may function in plant defense or simply represent biosynthetic or phylogenetic artifacts (Raguso et al. 2007). In conclusion, the data presented here support Herrera's (1996) caveat that the characters from which floral phenotypes are constructed are not always optimally combined from the pollinators' perspective. Additional field studies and phylogenetic perspectives will be required to better interpret the intriguing – if counterintuitive – patterns of floral evolution in *Echinopsis ancistrophora*.

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References

Ando, T. et al. 2001. Reproductive isolation in a native population of *Petunia* sensu Jussieu (Solanaceae). – Ann. Bot. 88: 403–413.

Baldwin, I. T. et al. 1997. Patterns and consequences of benzyl acetone floral emissions from *Nicotiana attenuata* plants. – J. Chem. Ecol. 23: 2327–2343.

Barthlott, W. et al. 1997. *Selenicereus wittii* (Cactaceae): an epiphyte adapted to Amazonian Igapó inundation forests. – Plant Syst. Evol. 206: 175–185.

Bonnet, E. and Van de Peer, Y. 2002. Zt: a software tool for simple and partial Mantel tests. – J. Stat. Software 7: 1–12.

Clarke, K. R. and Gorley, R. N. 2001. Primer v6.1.6: user manual/tutorial. – Primer-E, Plymouth, UK.

Daly, K. C. et al. 2008. Characterizing psychophysical measures of discrimination thresholds and the effects of concentration on discrimination learning in the moth *Manduca sexta*. – Chem. Senses 33: 95–106.

Dodson, C. H. et al. 1969. Biologically active compounds in orchid fragrances. – Science 164: 1243–1249.

Faegri, K. and van der Pijl, L. 1971. The principles of pollination ecology (2nd ed.). – Pergamon Press.

Fraser, A. M. et al. 2003. Electroantennographic and behavioral responses of the sphinx moth *Manduca sexta* to host plant headspace volatiles. – J. Chem. Ecol. 29: 1813–1833.

Frey, F. M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). – Evolution 58: 2426–2437.

Galen, C. and Cuba, J. 2001. Down the tube: pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. – Evolution 55: 1963–1971.

Goyret, J. et al. 2007. The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. – J. Exp. Biol. 210: 1398–1405.

Goyret, J. et al. 2008. Context- and scale-dependent effects of CO_2 on nectar foraging by *Manduca sexta*. – Proc. Natl Acad. Sci. USA 105: 4565–4570.

Grant, V. 1983. The systematic and geographical distribution of hawkmoth flowers in the temperate North American flora. – Bot. Gaz. 144: 439–449.
Grison-Pigé, L. et al. 2002. Fig volatile compounds – a first comparative study. – Phytochemistry 61: 61–71.
Haber, W. A. and Frankie, G. W. 1989. A tropical hawkmoth community: Costa Rican dry forest Sphingidae. – Biotropica 21: 155–172.
Herrera, C. M. 1996. Floral traits and plant adaptation to insect pollinators: a devil's advocate approach. – In: Lloyd, D. G. and Barrett, S. C. H. (eds), Floral biology. Chapman and Hall, pp. 65–87.
Herrera, C. M. 2001. Deconstructing a floral phenotype: do pollinators select for corolla integration in *Lavandula latifolia*? – J. Evol. Biol. 14: 574–584.
Hoballah, M. E. et al. 2005. The composition and timing of flower odour emission by wild *Petunia axillaris* coincide with the antennal perception and nocturnal activity of the pollinator *Manduca sexta*. – Planta 222: 141–150.
Hodges, S. A. 1997. Floral nectar spurs and diversification. – Int. J. Plant Sci. 158: 81–88.
Hunt, D. 1999. CITES Cactaceae checklist (2nd ed.). – Kew Publishing.
Kaiser, R. 1993. The scent of orchids: olfactory and chemical investigations. – Elsevier.
Kaiser, R. and Tollsten, L. 1995. An introduction to the scent of cacti. – Flavour Fragrance J. 10: 153–164.
Knudsen, J. and Tollsten, L. 1993. Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth pollinated taxa. – Bot. J. Linn. Soc. 113: 263–284.
Knudsen, J. T. and Mori, S. A. 1996. Floral scents and pollination in Neotropical Lecythidaceae. – Biotropica 28: 42–60.
Levin, R. A. et al. 2001. Fragrance chemistry and pollinator affinities in Nyctaginaceae. – Phytochemistry 58: 429–440.
Manning, J. C. and Goldblatt, P. 2005. Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. – Int. J. Plant Sci. 166: 459–474.
Moré, M. et al. 2005. Sphingidae. Esfíngidos de Argentina. – L.O.L.A. (Literature of Latin America), Buenos Aires, Argentina.
Moré, M. et al. 2006. Specialized use of pollen vectors by *Caesalpinea gilliesii*, a legume species with brush-type flowers. – Biol. J. Linn. Soc. 88: 579–592.
Nattero, J. et al. 2003. Possible tobacco progenitors share long-tongued hawkmoths as pollen vectors. – Plant Syst. Evol. 241: 47–54.
Prosser, I. et al. 2004. Enantiospecific (+)- and (−)-germacrene D synthases, cloned from goldenrod, reveal a functionally active variant of the universal isoprenoid-biosynthesis aspartate-rich motif. – Arch. Biochem. Biophys. 432: 136–144.
Raguso, R. A. 2004. Why do flowers smell? The chemical ecology of fragrance-driven pollination. – In: Cardé, R. T. and Millar, J. G. (eds), Advances in insect chemical ecology. Cambridge Univ. Press, pp. 151–178.
Raguso, R. A. and Willis, M. A. 2002. Synergy between visual and olfactory cues in nectar feeding by naïve hawkmoths. – Anim. Behav. 63: 685–695.
Raguso, R. A. and Willis, M. A. 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. – Anim. Behav. 69: 407–418.
Raguso, R. A. et al. 2003. Trumpet flowers of the Sonoran Desert: floral biology of *Peniocereus* cacti and sacred *Datura*. – Int. J. Plant Sci. 164: 877–892.
Raguso, R. A. et al. 2005. Sensory flexibility in hawkmoth foraging behavior; lessons from *Manduca sexta* and other species. – Chem. Senses 30: 295–296, suppl. 1.
Raguso, R. A. et al. 2006. Phylogenetic fragrance patterns in *Nicotiana* sections Alatae and Suaveolentes. – Phytochemistry 67: 1931–1942.
Raguso, R. A. et al. 2007. Floral biology of North American *Oenothera* sect. *Lavauxia* (Onagraceae): advertisements, rewards and extreme variation in floral depth. – Ann. Miss. Bot. Gard. 94: 236–257.
Schemske, D. W. and Bradshaw, H. D. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). – Proc. Natl Acad. Sci. USA 96: 11910–11915.
Schemske, D. W. and Bierzychudeck, P. 2001. Evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. – Evolution 55: 1269–1282.
Schiestl, F. 2005. On the success of a swindle: pollination by deception in orchids. – Naturwissenschaften 92: 255–264.
Schlumpberger, B. O. et al. 2004. Musty-earthy scent in cactus flowers: characteristics of floral scent production in dehydrogeosmin-producing cacti. – Int. J. Plant Sci. 165: 1007–1015.
Schnee, C. et al. 2002. The maize gene terpene synthase 1 encodes a sesquiterpene synthase catalyzing the formation of (E)-beta-farnesene, (E)-nerolidol, and (E,E)-farnesol after herbivore damage. – Plant Physiol. 130: 2049–2060.
Tholl, D. et al. 2006. Practical approaches to plant volatile analysis. – Plant J. 45: 540–560.
Vogel, S. 1954. Blütenbiologische Typen als Elemente der Sippengliederung. – Fischer Verlag.
Waser, N. M. et al. 1996. Generalization in pollination systems, and why it matters. – Ecology 77: 1043–1060.
Waser, N. M. 1998. Pollination, angiosperm speciation and the nature of species boundaries. – Oikos 82: 198–201.
Willis, M. A. and Arbas, E. A. 1991. Odor modulated upwind flight of the sphinx moth, *Manduca sexta* L. – J. Comp. Physiol. A 169: 427–440.
Wilson, P. 1995. Selection for pollination success and the mechanical fit of *Impatiens* flowers around bumble bee bodies. – Biol. J. Linn. Soc. 55: 355–383.
Wilson, P. et al. 2004. A multivariate search for pollination syndromes among penstemons. – Oikos 104: 345–361.

Supplementary material available online as Appendix O16211 at www.oikos.ekol.lu.se/appendix. Appendix 1. Appendix 2.