doi:10.1111/j.1558-5646.2009.00880.x

EVOLUTION AND COEXISTENCE OF POLLINATION ECOTYPES IN AN AFRICAN *GLADIOLUS* (IRIDACEAE)

Bruce Anderson,[1,2,3] **Ronny Alexandersson,**[1,4] **and Steven D. Johnson**[1]

[1] *School of Biological and Conservation Sciences, University of KwaZulu-Natal, Private Bag X01 Scottsville, Pietermaritzburg, 3209, South Africa*

[2] *Department of Botany and Zoology, University of Stellenbosch, P. Bag X1, Matieland, 7602, South Africa*

[3] *E-mail: banderso.bruce@gmail.com*

Received June 15, 2009
Accepted September 23, 2009

Pollinator-mediated selection has been suggested as a key driver of speciation in plants. We examined the potential role of hawkmoth pollinators in driving allopatric divergence and maintaining sympatric coexistence of morphotypes in the African iris *Gladiolus longicollis*. Floral tube length in this species varies from 35 mm to 130 mm across its geographic range and reflects the prevailing tongue lengths of local hawkmoth assemblages. The distribution of floral tube lengths is bimodal with two relatively discrete categories—long (about 90 mm) or short (about 50 mm)—that match the bimodal distribution of hawkmoth tongue lengths in eastern South Africa. At a contact site between these two floral morphs, we found few individuals of intermediate length, suggesting limited gene flow between morphs despite their interfertility. A difference in flowering phenology appears to be the main isolating barrier between morphs at this site. Long- and short-tubed morphs differed markedly in the chemical composition of their floral fragrance, a trait that could be used as a cue for morph-specific foraging by hawkmoths. Positive directional selection on tube length was found to occur in both morphs.

KEY WORDS: **Assortative mating, floral scent, flowering phenology, geographic mosaic, hawkmoth, phenotypic selection, pollination ecotypes, speciation.**

Pollinator-mediated selection plays a key role in shaping flower morphology (Nilsson 1988; Campbell et al. 1996; Medel et al. 2003; Whittall and Hodges 2007; Anderson and Johnson 2008; Bloch and Erhardt 2008; Muchhala and Thomson 2009), and many believe that pollinators frequently drive the speciation process in plants (Grant and Grant 1965; Johnson 2006). For example, plants with wide geographic ranges may traverse complex pollinator mosaics (as outlined by Johnson 2006, also see Althoff et al. 2005; Whittall and Hodges 2007; Godsoe et al. 2008) varying in absolute or relative pollinator composition. As a result, the shapes and strengths of pollinator-mediated selection on flower form may vary between populations and this may lead to geographic variation in floral characters, such as flowering phenology (Olsson and Ågren 2002) and flower morphology (Schemske and Horvitz 1989; Johnson 1997; Ehlers et al. 2002; Rey et al. 2006; Perez-Barrales et al. 2007). In support of this, empirical studies have also demonstrated that geographical variation in flower morphology is often connected with geographical differences in pollinator assemblages (Johnson 1997; Boyd 2004; Muchhala 2007). Many studies linking floral morphology and pollinators have focused on allopatric pollination ecotypes, in which the same plant species has several different pollinators across its range (e.g., Robertson and Wyatt 1990; Johnson and Steiner 1997): few examine both allopatric populations and the contact zones in which those "incipient species" meet and potentially interbreed.

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[4] Current address: Biology Education Centre (IBG), Uppsala University, Norbyvägen 14, SE-752 36 Uppsala, Sweden.

In this study, we take a two-step approach to study the speciation process. First, we examine patterns of covariance for the adaptive trait corolla length and its proboscis length counterpart in allopatric populations of a morphologically variable South African iris, *Gladiolus longicollis*, and its hawkmoth pollinators. Second, we examine possible factors that allow these divergent forms to coexist in sympatry.

Gladiolus longicollis is pollinated by nocturnal hawkmoths (Goldblatt and Manning 1998; Alexandersson and Johnson 2002). It occurs in two major flowering forms: one with a short-tubed corolla and one with a long-tubed corolla. Populations in the northern part of the distribution appear to have long corollas (tubes up to 130 mm), whereas populations in the south appear to have short corollas (tubes as short as 35 mm; Fig. 1, Table S1). Alexandersson and Johnson (2002) documented strong positive directional selection for tube length in a population on Mount Gilboa in the central part of the distribution range. This showed that the length of the floral tube in relation to hawkmoth proboscis length is of great importance for reproductive success (e.g., the tube needs to be as long or longer than the proboscis for efficient transfer of pollen; Alexandersson and Johnson 2002), as also demonstrated in other plant species with deep flowers, (Nilsson 1988; Johnson and Steiner 1997; Maad 2000; Maad and Alexandersson 2004; Anderson and Johnson 2008; Anderson and Johnson 2009; Muchhala and Thomson 2009).

Alexandersson and Johnson (2002) found that the long-tongued hawkmoth *Agrius convolvuli* is the primary pollinator of the long-tubed morph of *G. longicollis* (See Fig. S1) at Mount Gilboa and that these moths carry large *G. longicollis* pollen loads. Shorter-tongued hawkmoths were seldom seen visiting the long-tubed plants and those captured had much smaller pollen loads (if any). In 2003, we visited the Gilboa population earlier than usual and found a form of *G. longicollis* with short floral tubes nearing the end of its flowering season but which was nevertheless coflowering with longer-tubed individuals, which were just beginning to flower. In 2005, further down the mountain, we found another contact zone where short- and long-tubed plants were in flower simultaneously. We hypothesized that one way in which these morphs may be maintained in sympatry, particularly at sites where flowering overlaps strongly, is if the short-tubed form has different, shorter-tongued pollinators than the long-tubed form. Long-tongued moths may have a preference for long-tubed *G. longicollis* flowers because these tend to have more nectar than short-tubed flowers (Fig. S2) whereas short-tongued hawkmoths which are unable to reach the nectar in the long-tubed flowers (Alexandersson and Johnson 2002, Fig. S2) should prefer to visit short-tubed flowers. One of the ways in which pollinator fidelity would be possible is if the two forms possessed different scent cues that would allow them to be discriminated.

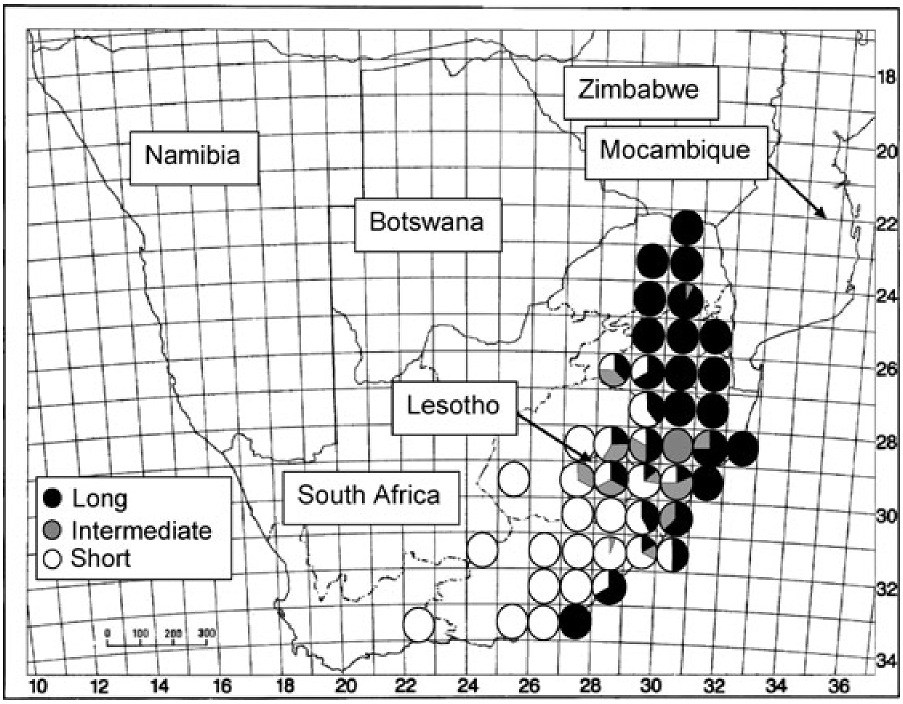

Figure 1. Geographical variation in corolla tube length of *Gladiolus longicollis* in South Africa, Lesotho, and Swaziland. Pie charts are based on measurements of 257 herbarium specimens. Each pie chart shows the proportion of long- (>80 mm), intermediate- (60–80 mm), and short-tubed (<60 mm) specimens collected within each square limited by the longitudes and latitudes (e.g., the square between 30°E–31°E and 22°S–23°S consists of only plants with tubes longer than 80 mm).

We hypothesized that floral tube length variation in *G. longicollis* has resulted from adaptation to local hawkmoth pollinator assemblages and has led to the establishment of isolating barriers. Our aims were to establish (1) whether there is a positive relationship between floral tube length and the prevalence of long-tongued hawkmoths across sites, (2) whether floral tube length has a bimodal distribution and whether this reflects the distribution of hawkmoth tongue lengths, (3) whether differences in floral tube length among allopatric populations are maintained in "contact zones," (4) whether there is an association between floral tube length and scent cues that could be used by hawkmoth pollinators, (5) whether there is an association between floral tube length and flowering phenology, (6) whether there is an association between tongue length and phenology of hawkmoths, (7) whether there are incompatibilities between morphs, and (8) whether patterns of selection can explain the maintenance of long- and short-tubed morphs.

Materials and Methods

STUDY SPECIES

Gladiolus longicollis (Iridaceae) is a self-incompatible, perennial herb, with creamy yellow or white flowers possessing a narrow floral tube 35–130 mm in length that contains nectar at the base (Goldblatt and Manning 1998; Alexandersson and Johnson 2002). It grows in low open grassland, and fire in the preceding winter is crucial for the stimulation of flowering. It is widespread in southern Africa, and exists from the southern Cape up to the Northern Province and Swaziland (Goldblatt and Manning 1998). In the past, the species has been divided into two subspecies: short-tubed subsp. *longicollis* and long-tubed subsp. *platypetalus*, but current taxonomic opinion is that the floral variation is clinal and not easily partitioned into intraspecific categories (Goldblatt and Manning 1998).

Flowering in *G. longicollis* occurs in spring to early summer, and plants typically produce a single flower, although up to seven flowers can occur on an inflorescence (R. Alexandersson pers. obs.). The flower is closed during daytime and opens gradually from about 1700 h in the afternoon on sunny days, or earlier during cloudy conditions. The flower produces a strong sweet fragrance during the evening and fruit maturation occurs 3–4 weeks after flower senescence.

GEOGRAPHICAL VARIATION IN FLORAL TRAITS AND HAWKMOTH ASSEMBLAGES

Proboscis–corolla covariation in herbarium and museum specimens

To document the distribution pattern of flower morphology in *G. longicollis,* specimens were obtained from the Bolus Herbarium (University of Cape Town), KwaZulu-Natal Herbarium (Durban), Compton Herbarium (Kirstenbosch), Bews Herbarium (University of KwaZulu-Natal), and CE Moss Herbarium (University of Witwatersrand). Herbarium specimens date as far back as 1888 and span the months of July to February. For each specimen ($n = 255$), corolla tube length from the base of the tube to the point where the tube starts to flare was measured to the nearest 0.1 mm using digital callipers. Based on the natural dichotomy of *G. longicollis* tube length across its entire range (see results section), we divided the data into plants with "tubes shorter than 60 mm," "between 60 mm and 80 mm," and "longer than 80 mm." The proportions of specimens corresponding to these limits were calculated for grid squares delimited by degrees of latitude and longitude (e.g., 22°S to 23°S and 30°E to 31°E; Fig. 1).

To establish the geographical mosaic of potential hawkmoth pollinators, we obtained data from the Transvaal and Iziko South African museums on the distributions of hawkmoth species with tongue lengths in excess of 30 mm ($n = 832$ specimens). Localities were recorded at the same full degree grid scale as the plant specimens. Because we were not able to measure their rolled up proboscides, we divided moth species into short (30 mm $<$ proboscis $<$ 45 mm) and long (proboscis $>$ 60 mm) categories based on our records of tongue lengths from specimens of the same species collected by ourselves over the last eight years. These categories correspond to a natural dichotomy in hawkmoth tongue lengths (see results section). We used univariate regression to establish whether the average corolla length of *G. longicollis* has a positive relationship with the percentage of long-tongued moths across grid squares where data on both hawkmoths and *G. longicollis* were available ($n = 30$ grid squares). All regression analyses included in this study were based on data that met the assumptions for this method.

Distribution patterns of herbarium and moth specimens collected over the geographic range of the interaction, including a site (Mount Gilboa) with unusually high levels of corolla length variation, were analyzed for bimodality using the equations outlined by Der and Everitt (2002). Skewness (M_3), kurtosis (M_4), and sample size (n) are used to calculate a coefficient of bimodality (b), where $b = (M_3^2 + 1)/(M_4 + 3(n - 1)2/(n - 2)(n - 3))$. If b is greater than 0.55, frequency distributions are taken to be bimodal (Der and Everitt 2002).

Proboscis–corolla covariation in field collected specimens

To determine whether corolla length reflects the prevailing assemblages of short- and long-tongued hawkmoths at finer geographic scales, four long-tubed and seven short-tubed *Gladiolus* populations were chosen (Table S1). The sites were chosen to cover much of the *G. longicollis* distribution range. The study populations were distributed from Buffelskloof in the north (25°S: 30°E) to Mkambati in the south (31°S: 29°E; Table S1).

The length of the floral tube was measured for 4–151 (median = 50) plants at each site (Table S1). Floral traits were measured during peak flowering in the populations, except for the Mkambati-site where flowering was finished at the time of our visit. The floral measurements from the Mkambati-site were thus obtained from herbarium specimens.

To quantify the pollinator fauna in each population, hawkmoths were caught using a light trap equipped with a 250 W mercury vapor lamp. Moths were captured on one to four evenings during peak flowering of *G. longicollis* at each site (except at Mkambati where plants had finished flowering) from about 1830 h to 2130 h. The tongues of captured hawkmoths were unrolled and measured to the nearest 0.1 mm using a digital caliper. The relationship between the proportion of moths with long tongues (>60 mm) and tube length was explored using univariate regression.

Observations of pollinators in unimodal populations

To determine if flower morphology was matched by the tongue length morphology of visiting moths, we spent 45 nights observing flowers in unimodal short- and long-tubed populations. Moths visiting flowers of *G. longicollis* were identified and, if possible, captured. The tube length of the flower that they were captured on was measured and the tongue of the moth was unraveled and then measured with callipers. Later, the moths were swabbed with glycerol gel containing fuschin stain and the number of *G. longicollis* pollen grains counted. Captured moths were classified as belonging to either the long- or short-tongued guild and their fidelity to plants of matching tube morphology (i.e., either short or long) was analyzed using *G*-tests.

COEXISTENCE OF SHORT- AND LONG-TUBED MORPHS

Pollinator observations

We also spent 29 nights observing moths at the contact zone on the summit of Mount Gilboa where both morphotypes co-occur. Although we had hoped to explore assortative mating via pollinator preferences at this site, flowering phenology of these morphotypes were separated in time during both years of observation (unlike in 2003 when flowering of both forms overlapped). We nevertheless captured moths on both *G. longicollis* morphs to determine if there are associations between the tongue length of visitors and flower depth.

Scent analysis

To determine if scent cues differ between the forms in a contact zone, we analyzed the scent of 12 *G. longicollis* individuals with short corolla tubes (41–52 mm) and 11 individuals with long corolla tubes (92–103 mm) on the slopes of Mount Gilboa. Freshly opened flowers were enclosed in a polyacetate bag soon after dusk (1800–1900 h) and the air from the bags was pumped continuously for 10–30 min at 200 mL/min through a filter containing 1 mg of tenax (Tenax, Baltimore, MD) and 1 mg of carbotrap (Sigma-Aldrich, St. Louis, MO) activated charcoal held between plugs of silanized glass wool. Ambient control samples were obtained using the same procedure with an empty bag. These filters were thermally desorbed in the 1079 injector of a Varian CP-3800 gas chromatograph by means of a Chromatoprobe device (Amirav and Dagan 1997). The GC was coupled to a Varian 1200 quadrupole mass spectrometer in electron impact ionization mode (Varian, Palo Alto, CA) and equipped with a 30 m × 0.25 mm (film thickness 0.25 μm) Alltech EC-WAX column. The carrier gas was helium with a flow rate of 1 mL/min. The 1079 injector was held at 40°C for 2 min with a 1:20 split to flush any trapped air, then increased to 200°C at 200°C/min in splitless mode, held for 10 min. After a 3 min hold at 40°C, the GC oven was ramped up to 240°C at 10°C/min, held for 12 min. Mass spectra were tentatively identified the using the NIST/EPA/NIH mass spectral library (NIST05) and the identities of the most abundant compounds were verified by comparing their mass spectra and retention times with those of authentic standards. Compounds present in similar quantities in the ambient controls were ignored. Peak areas in the total ion chromatograms were integrated using Varian Workstation software and divided by total peak area (excluding known contaminants) to obtain relative proportions for compounds. Differences between morphs in the relative percentages of abundant compounds in the total scent emitted were analyzed using *t*-tests that do not assume equal variance. Probability values from these tests were adjusted using the sequential Bonferroni method to limit the chance of type 1 errors (Rice 1989). The overall relationship between morphs and scent composition was explored by multidimensional scaling (MDS) of a Bray–Curtis matrix of similarity for the 23 scent samples using the software package PRIMER 6 (Clarke and Gorley 2006).

Nectar analysis

We measured nectar volumes, sugar concentrations, and the distance to the nectar in flowers to ascertain whether pollinators could reach the nectar and whether tube length was related to reward quality. The 20 randomly selected short-tubed flowers had corolla lengths ranging from 42 mm to 51 mm and the 20 long-tubed flowers had corollas ranging from 82 mm to 106 mm. The flowers were backlit so that the nectar level could be seen within the flower. The distance from the corolla mouth to the nectar was measured using callipers. The nectar was then extracted with a capillary tube and the volume was measured before the concentration was determined using a Bellingham and Stanley refractometer. Total sugar was calculated according to Bolten et al. (1979) and the differences in the distance to nectar as well as the total sugar content per flower was analyzed using *t*-tests.

Phenology

To determine the flowering patterns of long- and short-tubed *G. longicollis* morphs and whether these are synchronized with the availability of long- and short-tongued moths, respectively, surveys were conducted at the Mount Gilboa summit site on a weekly basis for 13 weeks. Each week we recorded the number of *G. longicollis* plants flowering in an area measuring approximately 40,000 m^2. Moths were captured in a permanent light trap set up on the top of the mountain. Pyrethroids were placed in the bottom of the trap to kill moth visitors.

Interfertility of morphs

To establish the cross-compatibility of morphs, flower buds of long-, intermediate-, and short-corolla morphs were bagged on the lower slopes of Mount Gilboa because here they are sympatric and have strongly overlapping flowering (there is less overlap in flowering on the summit). Once the stigmas unfurled, the corolla tubes were measured and the stigmas were hand pollinated using pollen from plants with either short-, intermediate-, or long-tubed flowers. These were then rebagged and harvested once seeds had formed. We used simple linear regression to test whether the slopes of the relationships between corolla length and seed set differed from zero for the various crossing treatments.

Phenotypic selection

The relationship between corolla tube length and fecundity was investigated by measuring the corolla tube lengths (to the nearest mm) of 463 single flowered *G. longicollis* plants on the summit of Mount Gilboa, and then determining fruit and seed set of these plants. Height of these plants was also measured. The measured plants spanned the entire flowering time of the species. After each plant was measured, it was given a unique tag so that at the end of the season we were able to harvest the fruits, count the seeds, and relate these measures of fecundity to both tube length and plant height using regression analysis. We also hand cross-pollinated 35 short-tubed and 35 long-tubed individuals at the Gilboa site to establish if there is a relationship between tube length and seed set in flowers whose stigmas are saturated with pollen.

The relationships between tube length, inflorescence height, and measures of fecundity were analyzed for a signal of phenotypic selection. First, we tested whether hand pollination had a different effect on seed set to natural pollination using analysis of covariance (ANCOVA). We conducted separate analyses for early- and late-flowering plants (corresponding to short- and long-tubed, morphs, respectively) as we could not be certain that they belong to the same gene pool for selection. Then, we used multiple regression to detect directional selection on seed set with corolla length and plant height as predictive variables using the methods set out by Lande and Arnold (1983) and Alexandersson and Johnson (2002). First, we calculated relative fitness by dividing an individual's absolute fitness by the population average. Trait values were then standardized by subtracting the population mean value from the individual trait value and dividing by the standard deviation. This was then repeated using univariate regression analysis. These analyses were run separately for fruit set (when zero counts were included) as well as for seed set (when zero counts were excluded from the analysis). We also used univariate and multivariate quadratic regression to test for stabilizing selection on the above traits.

Results

GEOGRAPHICAL VARIATION IN FLORAL TRAITS AND HAWKMOTH ASSEMBLAGES

The measurement of herbarium specimens confirmed our initial impression that northern and eastern populations generally consist of long-tubed plants and the southwestern populations of short-tubed plants (Fig. 1). The overall frequency distribution for corolla length (Fig. 2A) is bimodal, both when analyzed from herbarium

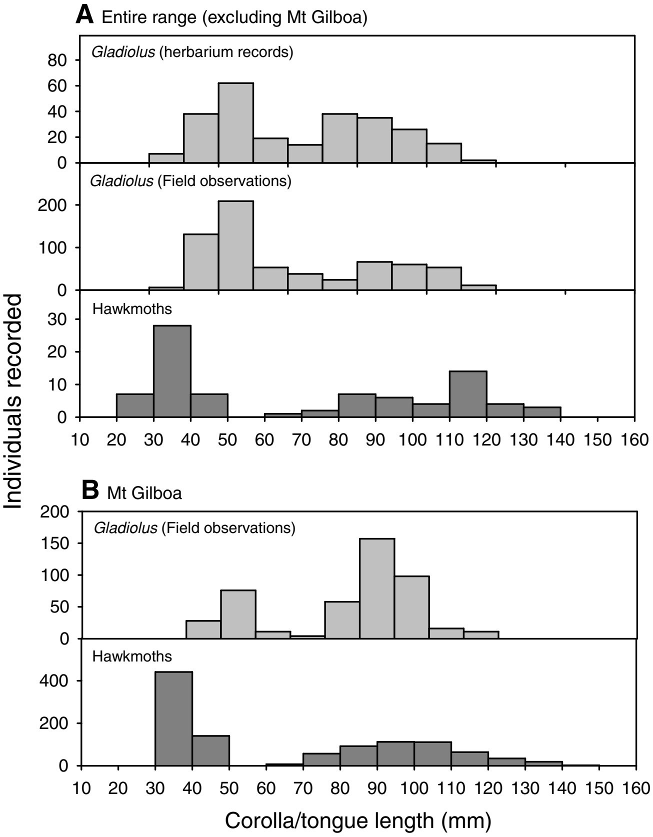

Figure 2. Frequency distributions of *G. longicollis* corolla tube lengths and moth tongue lengths. (A) Over the entire range of *G. longicollis* (excluding Mount Gilboa) using herbarium specimens, as well as focused collections of both plants and hawkmoths at specific populations. (B) At a contact zone on the top of Mount Gilboa only, using data collected by us in the field.

records or field observations ($b = 0.60$ or 0.71 respectively). Both of these histograms are very similar to the bimodal ($b = 0.71$) histogram of proboscides from moths captured over the range of *G. longicollis* (Fig. 2A).

At the zone of secondary contact on Mount Gilboa, two distinct, nonoverlapping size classes of moth were also captured (Fig. 2B): Moths either had short proboscides between 20 mm and 50 mm (seven species) or long proboscides between 60 mm and 150 mm (two species). The bimodal distribution of moth tongue lengths ($b = 0.73$) at Gilboa was also matched by a bimodal distribution in the corolla lengths of *G. longicollis* ($b = 0.67$). Here, short corollas were between 20 mm and 60 mm. There were a few individuals with corollas of intermediate length (60–80 mm) and another size class peak between 80 mm and 120 mm.

Proboscis–corolla covariation

Using only herbarium and museum specimens we found that there was a positive relationship between the relative abundance of long-tongued hawkmoths and the tube length of *G. longicollis* ($P = 0.01$, $N = 29$, Fig. 3A). The greater the relative abundance of long-tongued hawkmoths in any locality, the longer the *G. longicollis* tube lengths in that locality.

A similar relationship was found when we trapped moths at specific localities spanning most of the tube length range in *G. longicollis* ($P = 0.04$, $N = 10$, Fig. 3B). Here, a total of 83 hawkmoths were captured (Table S1) at all of the sites except Highmoor, where no hawkmoths were captured despite four nights of trapping (Table S1). Long-tongued hawkmoths were always captured at long-tubed populations, but these moths were absent from three of the short-tubed sites (Highmoor not included; Table S1, Fig. 3B).

Observations of pollinators in unimodal populations

A total of seven short-tongued moths were seen visiting flowers in pure short-tubed populations, whereas no long-tongued moths were observed to visit (Table S2). In contrast, nine long-tongued and no short-tongued moths were seen visiting plants in pure long-tubed populations ($G_1 = 19.9$, $P < 0.00001$).

COEXISTENCE OF SHORT- AND LONG-TUBED MORPHS

Pollinator observations

As was observed for allopatric populations, there was an association between tube length and the tongue length of hawkmoth visitors in the contact zone on Mount Gilboa. Short-tubed plants at this site were observed to be visited by nine short-tongued moths and a single long-tongued moth, whereas sympatric long-tubed plants were visited by ten long-tongued moths and a single short-tongued moth (Table S2, $G_1 = 14.5$, $P < 0.0005$).

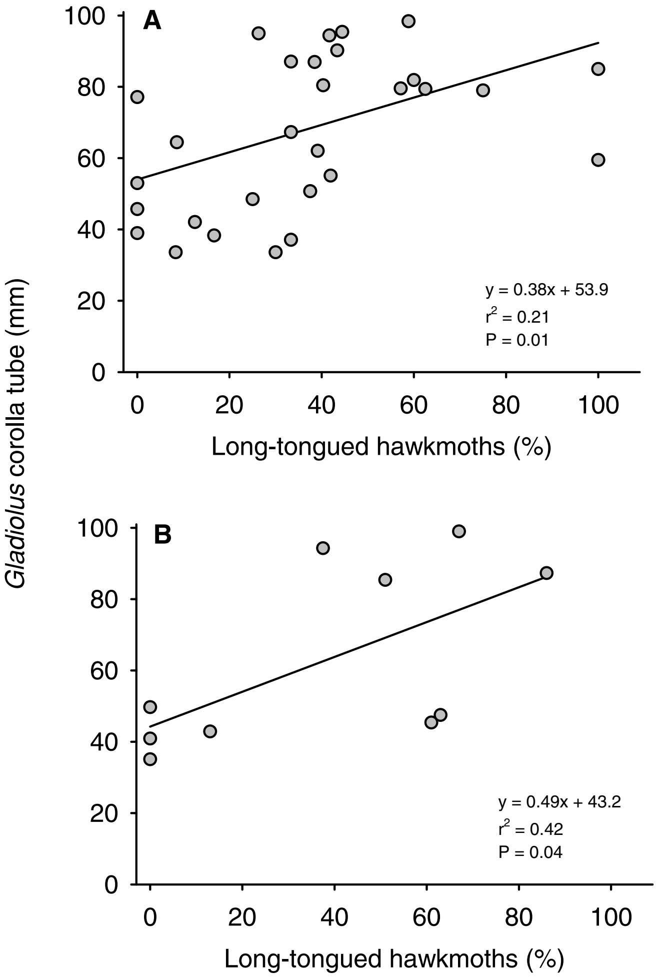

Figure 3. The relationship between the mean corolla tube length of *G. longicollis* and the fraction of hawkmoths with proboscides longer than 80 mm. (A) Data from herbarium and museum specimens, plotted at the scale of one degree (as in Fig. 1). (B) Data collected during sampling of selected populations covering a range of tube lengths.

Scent analysis

We found major differences in the chemical composition of scent emitted by flowers of the two morphs. In an ordination based on multidimensional scaling of scent data, the two morphs formed very distinct clusters (Fig. 4). The monoterpene, ocimene, and benzenoid compounds benzyl acetate, phenylacetaldehyde, benzyl alcohol, and phenylethyl acetate dominated the scent emitted from short-tubed flowers, whereas these compounds were absent or present in trace amounts in the scent emitted from long-tubed flowers (Fig. S3). On the other hand, scent of long-tubed flowers was strongly dominated by the monoterpene, alcohol, linalool, and benzenoid methyl benzoate (Fig. S3). The differences between morphs for all of these compounds, except methyl benzoate, remained significant ($P < 0.05$) after sequential Bonferroni correction. Other compounds, such as benzaldehyde,

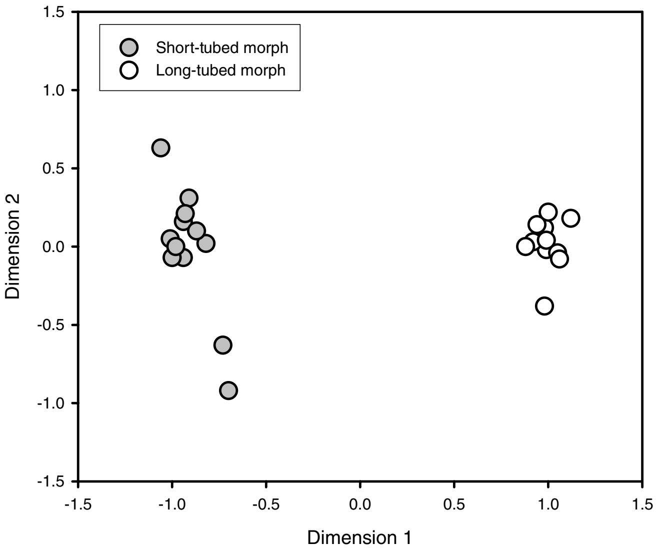

Figure 4. **Multidimensional scaling of scent profiles collected from 23 plants of *G. longicollis* from Mount Gilboa. Analysis of differences between morphs for separate compounds is given in Fig. S3.**

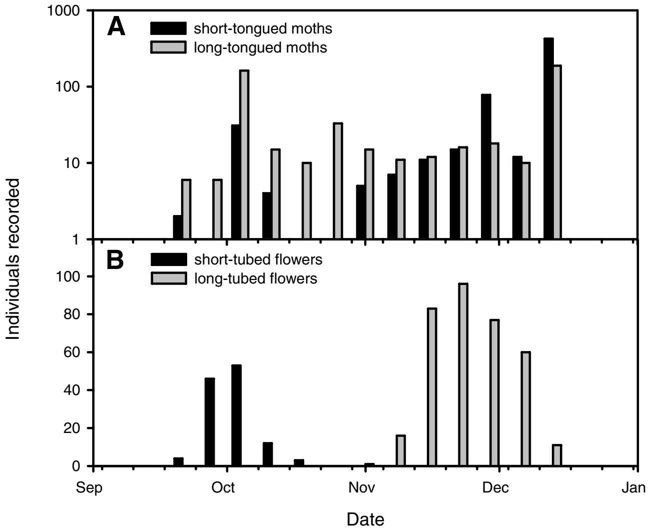

Figure 5. **Phenology of hawkmoths and *G. longicollis* flowers in a contact zone on Mount Gilboa recorded over a 12-week period. (A) Short- (<50 mm) and long- (>70 mm) tongued hawkmoths. (B) Short- (<60 mm) and long-tubed plants of *G. longicollus*.**

were present in similar proportions in the scent of both morphs (Fig. S3).

Nectar analysis

The mean distance (± SD) from the flower entrance to the nectar in long-tubed plants (77.7 ± 10 mm) was significantly greater ($t_{36} = 16.7$, $P < 0.00001$, also see Fig. S2) than for the short-tubed plants (37.5 ± 4). In addition, the total amount of sugar found in the long-tubed plants was more than twice that found in the short-tubed plants (5.5 ± 3 mg vs. 2.1 ± 1 mg, $t_{36} = 4.6$, $P < 0.0001$).

Phenology

On Mount Gilboa, short- and long-tongued moths were generally present and abundant throughout the study period although their numbers were very variable (Fig. 5A). Availability of short- and long-tongued moths did not correspond with the flowering peaks of short- and long-tubed morphs of *G. longicollis*, respectively (Fig. 5B). In the year of study, flowering of the two morphs did not overlap on the summit, although in other years we have found overlap in flowering and thus potential for gene flow between the two morphs. On the lower slopes of Mount Gilboa, short and long morphs flowered simultaneously across a 4-year period (2005–2008, B. Anderson, pers. obs.).

Interfertility of morphs

Bagged buds set no seed ($n = 15$ plants) meaning that experimentally crossed plants were very unlikely to have been contaminated with other pollen after hand pollinations had been performed. Separate linear regressions for the three types of parental crosses revealed that, for short-tubed sires, seed set declined significantly ($F_{1,33} = 5.72$, $r^2 = 0.15$, $P = 0.02$) with increasing floral tube length of the recipient flower, whereas no relationship was found for the other paternal cross types (Fig. 6). On an average, short-tubed pollen donors sired 60.69 ± 5.6 (SE) seeds, whereas intermediate- and long-tubed donors sired 63.18 ± 8.1 and 74.05 ± 6.4, respectively ($F_{2,98} = 1.19$, $P = 0.31$).

Phenotypic selection

Of the 464 plants originally tagged, only 143 plants were available for harvest near the end of the season as grasshoppers decimated most of the *G. longicollis* plants before they had time to set seed.

Natural seed set showed a significant positive relationship with tube length for both short- (early-flowering) and long-tubed (late-flowering) morphs in univariate regression analyses (Fig. 7B, Table 1). In addition, stabilizing selection was found in early-flowering plants using univariate regression (Fig. 7B, Table 1). However, the significance of this quadratic curve was dependent on a single outlying data point. After this point was excluded there was significant directional selection on corolla lengths of early-flowers for both univariate and multivariate regression, irrespective of whether fruit set or seed set was considered as the measure of fecundity (Fig. 7B, Table 1). For hand-pollinated plants (saturated stigmas), the relationship between tube length and seed set was positive for the early-flowering morph and negative for the late-flowering morph (Fig. 7A). Hand pollination significantly increased seed set for the early-flowering morph ($F_1 = 21.5$, $P < 0.001$) with hand-pollinated plants setting on average 84.79 ± 27.91 (SD) seeds and naturally pollinated

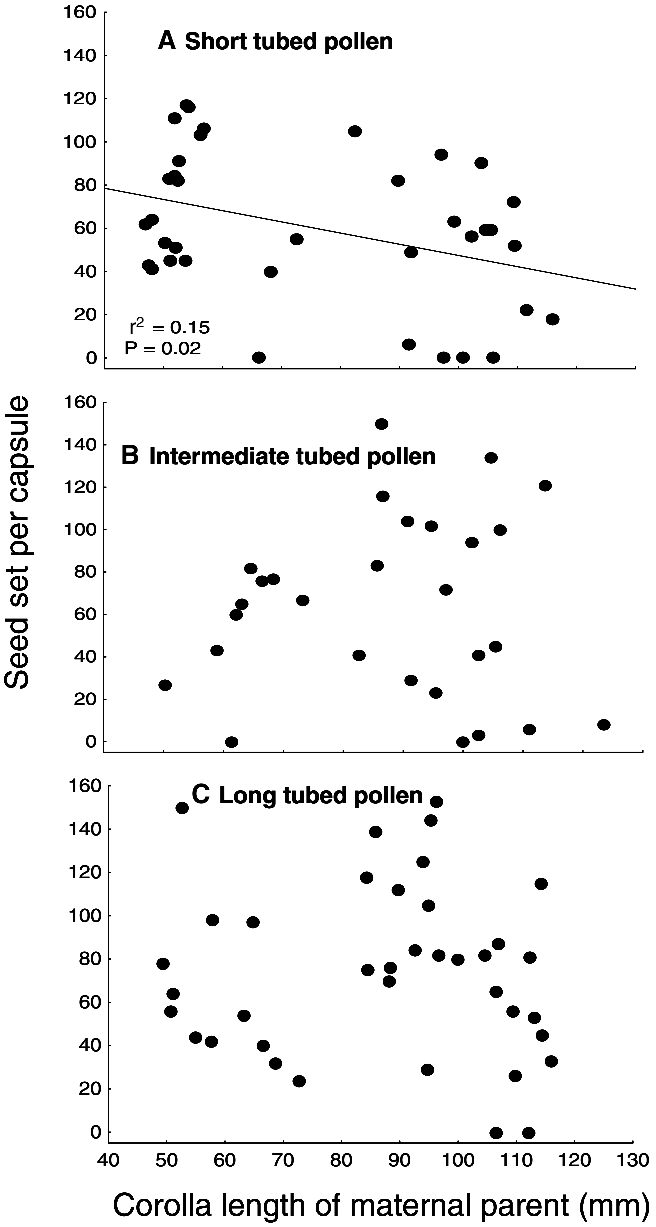

Figure 6. Seed set across a range of corolla tube lengths after each flower was hand crossed using pollen from (A) a short-tubed flower, (B) an intermediate-tubed flower, (C) a long-tubed flower.

plants setting 64.5 ± 42.20 (SD) seeds. There was also a significant interaction effect between hand pollination and tube length on seed set ($F_1 = 19.82$, $P < 0.001$) (Table 2). For the early-flowering morph, there was no significant interaction between hand pollination and tube length on seed set (Table 2). Multiple regression analyses showed positive directional selection on tube length for both short- and long-tubed morphs. Plant height also emerged as a significant predictor of fruit and seed set in these analyses (Table 1).

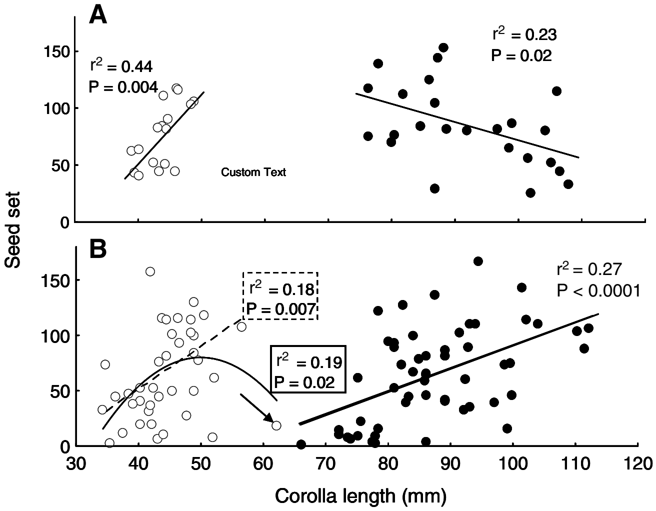

Figure 7. The relationships between corolla tube length and seed set for different *G. longicollis* phenotypes at two contact zones on Mount Gilboa. (A) Hand-pollinated plants (univariate linear regression). (B) Naturally pollinated plants (univariate quadratic and linear regression). If an outlier (indicated with an arrow) is excluded from the data for early-flowering plants, the relationship with the best fit was linear. Clear circles represent early-flowering or short-tubed individuals, whereas dark circles represent late-flowering or long-tubed individuals.

Discussion

DIVERGENCE IN ALLOPATRY

The results of this study suggest that divergence in corolla tube length in *G. longicollis* has been a result of adaptation to two different hawkmoth guilds that differ in mean tongue length. This is reflected in the matching bimodal distributions of tube length of *G. longicollis* and tongue length in the hawkmoths (Fig. 2A). Allopatric divergence seems to reflect a geographical mosaic in the distribution of these hawkmoth guilds. A positive relationship was found between the floral tube length of *G. longicollis* and the relative abundance of long-tubed hawkmoths across sites (Fig. 3). The short-tubed morph is found mainly in the southwest where the short-tongued moth guild predominates, whereas the long-tubed morph is found mainly in the northeast where the long-tongued moth guild is predominant (Figs. 1 and 3).

Gladiolus longicollis is nested within a clade of moth-pollinated species with short tubes varying from 8 mm to 63 mm in length, suggesting that the very long-tubes found in this species are derived (Goldblatt and Manning 1998). This may have happened in one or more populations where long-tongued moths are common, through Darwin's mechanism of directional pollinator-mediated selection on tube length, as demonstrated by Alexandersson and Johnson (2002), and according to the "most effective pollinator principle" (Stebbins 1970). The evolution and spread of long-tubes in populations in the northern and eastern

Table 1. **Multiple regression results examining directional selection and stabilizing selection on tube length and plant height in early- (top) and late- (bottom) flowering plants. Results were analyzed using univariate and multivariate regression as well as both seed set and fruit set as measures of fitness. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001; ****, *P*<0.09. Data from early-flowering plants were reanalyzed after the exclusion of a single outlying data point and the selection gradients presented for corolla length. We do not report the reanalyzed results for inflorescence height as their overall results stay the same.**

		Selection differentials (univariate)				Selection gradients (multivariate)			
Trait	Form of selection	Fruit set		Seed set		Fruit set		Seed set	
		β or γ	t	β or γ	t	β or γ	t	β or γ	t
Tube length	Directional	0.34**	2.91	0.28****	1.78	0.33*	2.38	0.27	1.39
Tube length (no outlier)	Directional	0.39**	3.42	0.43**	2.87	0.37**	2.80	0.40*	2.26
Tube length	Stabilizing	−0.09	−0.75	−0.39*	−2.25	−0.06	−0.49	−0.39****	−1.85
Tube length (no outlier)	Stabilizing	0.07	0.53	−0.09	−0.57	−0.06	−0.49	−0.07	−0.37
Inflorescence height	Directional	0.14	1.02	0.07	0.40	0.01	0.11	−0.02	−0.10
Inflorescence height	Stabilizing	0.08	0.62	0.04	0.20	0.03	0.22	−0.04	−0.24
Tube length	Directional	0.42***	4.00	0.52***	4.40	0.28*	2.38	0.35*	2.44
Tube length	Stabilizing	0.04	0.42	−1.97	−1.67	0.09	0.73	−0.10	−0.71
Inflorescence height	Directional	0.29*	2.39	0.46**	3.26	0.18	0.42	0.33*	2.31
Inflorescence height	Stabilizing	0.22****	1.82	0.06	0.43	0.23	1.94	0.08	0.57

regions was most likely facilitated by the predominance of long-tongued moths in these areas. Once a certain threshold corolla length is reached, short-tongued moths are completely precluded from accessing nectar from the flowers (See Alexandersson and Johnson 2003 and Fig. S2) and it is thus likely that they would quickly learn to avoid long-tubed plants. Long-tubed moths are likely to rob nectar from short-tubed plants in the southwest unless they have a suitable alternative nectar source. But because these moths are not common in the southwest, plants there are unlikely to adapt to these long-tongued moths. Geographical isolation would also facilitate the divergence of other pollinator-linked traits such as scent and flowering time, as well as adaptations to the abiotic environment.

Table 2. **ANOVA results for early- and late-flowering plants, examining the effect of treatment (hand-pollinated or naturally pollinated) and corolla length on seed set.**

Source	SS	df	*F*	*P*	R^2
Seed set early-flowering plants					
Model	12273.61	3	3.25	0.029	0.16
Intercept	3134.273	1	2.49	0.12	
Natural/hand pollination	1936.57	1	1.54	0.22	
Corolla length	8275.56	1	6.58	0.013	
Natural/hand pollination × corolla length	2287.78	1	1.82	0.183	
Seed set late-flowering plants					
Model	39153.71	3	10.56	0.0001	0.3
Intercept	2781.81	1	2.25	0.138	
Natural/hand pollination	26548.74	1	21.48	0.0001	
Corolla length	413.82	1	0.34	0.57	
Natural/hand pollination × corolla length	24498.91	1	19.82	0.0001	

ECOTYPE MAINTENANCE IN CONTACT ZONES

In most regions all plants are either completely long-tubed or completely short-tubed, suggesting that the greatest barrier to gene flow is the geographic isolation of ecotypes (see Ramsey et al. 2003). However, there are sites, such as Mount Gilboa, where differences in tube length are maintained in sympatry as evidenced by bimodal tube length distributions (Fig. 2B). This indicates that there are other gene flow barriers in addition to geographic ones. On the top of Mount Gilboa, for example, we found a strong association between floral tube length and flowering phenology (Fig. 5). Although this would serve as a strong isolating barrier preventing hybridization of the two ecotypes at this site, it is still apparently leaky, as in some years there is some overlap in flowering of the ecotypes (S. D. Johnson, pers. obs.) and on the lower slopes of Mount Gilboa both ecotypes flower simultaneously each year. Although the role of temporal isolation in plant speciation has been considered by some to be relatively unimportant (e.g., Rathcke and Lacey 1985), models predict that temporal isolation may be commonplace and very important in the speciation process (e.g., Stam 1983; Kondrashov and Kondrashov 1999; Doebeli and Diekmann 2003; Devaux and Lande 2008). Part of the reason for the proposed frequency of divergent flowering phenology is that flowering phenology can diverge through nonselective assortative mating that does not even require disruptive selection for flowering time divergence (e.g., Stam 1983; Devaux and Lande 2008). However, even weak disruptive selection should enhance phenotypic divergence (Devaux and Lande 2008) and when flowering time divergence is linked to adaptive traits, the two should work in concert to facilitate further divergence in both morphology and flowering phenology (Stam 1983, also see Weis 2005). Devaux and Lande (2008) suggest that traits such as short individual flowering time and long flowering season facilitate nonselective phenological divergence. In the *G. longicollis* contact zone, plants usually have a single flower that remains open for just a few days. In contrast, the flowering season of the Gilboa population is long (about 13 weeks) possibly making it very susceptible to nonselective divergence of phenology. Outside of theoretical models, several other studies also propose an important role for phenological divergence in the process of plant speciation and the maintenance of species boundaries in sympatry (e.g., Ellis et al. 2006; Marques et al. 2007).

Interestingly, there was no association between flowering time of *G. longicollis* morphs and the phenology of short- and long-tongued hawkmoths, as evidenced by light trapping (Fig. 5). This suggests that the observed association between the morphology of moth visitors and floral tube lengths reflects the foraging preferences of the different kinds of hawkmoths, and not simply their temporal availability. In contact zones, short-tongued moths may be expected to avoid long-tubed flowers because they are not able to reach the nectar (see Fig. S2) whereas long-tongued moths are expected to forage preferentially on long-tubed plants because of their higher reward quality. Indeed, we found that in the Gilboa contact zone, there was a significant degree of fidelity to short- and long-tubed *G. longicollis* plants by short- and long-tongued hawkmoths, respectively (Table S2).

We found an association between floral tube length and scent cues (Fig. 4) that may allow individual moths to distinguish between the two morphs and forage preferentially on the one which is most rewarding to them. Several studies suggest that scent differences between plants can play an important role in assortative mating (Plepys et al. 2002; Dötterl et al. 2005a; Huber et al. 2005; Waelti et al. 2008), and this may be especially so with moth-pollinated plants where scent is usually the primary cue for attracting pollinators (Waelti et al. 2008). Some of the scent compounds (e.g., benzyl acetate), which differ significantly between the flowers of *G. longicollis* ecotypes are known to be detectable by hawkmoth antennae and are used in food seeking behavior (e.g., Raguso et al. 1996; Raguso and Light 1998). The synthesis of phenylactetaldehyde, a compound found to differ between *G. longicollis* ecotypes, is probably dependent on the expression of a single gene (Kaminaga et al. 2006) and manipulation of this compound induced assortative mating in moth-pollinated species of *Silene* (Waelti et al. 2008). Unfortunately, we were not able to make pollinator observations in the contact zones where the ecotypes overlap strongly in flowering time, but our results do suggest that pollinators may have a "motive" in terms of rewards to discriminate between morphs, that there are scent differences which could be used as morph-specific cues, and that there is an association between morphs and the tongue length of moth visitors. Thus, assortative mating due to pollinator fidelity to morphs in contact zones with strong flowering time overlap (e.g., Hodges and Arnold 1994; Schemske and Bradshaw 1999; Ramsey et al. 2003; Aldridge and Campbell 2007) is a distinct possibility and will be tested in future studies.

Our results suggest that both morphs are naturally pollen limited, especially small individuals of each morph. Both morphs experience positive directional selection on tube length (Fig. 7). This by itself does not, however, explain the gap in tube length between short- and long-tubed morphs. Determining the precise shape of selection across the full range of tube lengths was not possible as intermediates were very rare in the contact zone (Fig. 2B) and the difference in flowering time of morphs at the Gilboa summit site meant that we could not pool all plants in a single selection analysis. In future studies, experimental arrays that include a full range of intermediate hybrids could be used to test the hypothesis that intermediate individuals are selected against because their nectar would be largely inaccessible to short-tongued hawkmoths and because they are effectively pollinated by long-tongued hawkmoths (Fig. 7). The fact that the hawkmoth selective agents are strongly dimorphic in size suggests that although selection gradients are positive for both *G. longicollis* ecotypes, there is still a dimorphism in the selective landscapes where large moths select for larger large flowers and small moths also select for larger small flowers.

That individuals of intermediate tube length could result from hybridization was confirmed by the fertility of crosses between short- and long-tubed morphs. Furthermore, plants with intermediate tube length were able to set seed and could thus contribute to the future break down of the two morphs through introgressive hybridization. However, our results suggest that a partial and asymmetric fertilization barrier between the two ecotypes may weaken the flow of genes between the ecotypes. Long-tubed plants pollinated by short-tubed donors produce fewer seeds than other plants (Fig. 6). This makes intuitive sense, because pollen from short-tubed plants may not have the resources to produce a pollen tube long enough to reach the ovules of long-tubed plants (see Plitmann and Levin 1983 and Roulston et al. 2000). However, most evidence suggests that pollen tube growth within the style uses resources from the female parent (see Cruden 2009). Pollen growth through the stigma on the other hand is autotrophic and because stigma depth may be correlated with style length, the amount of resources found within pollen grains may still play a role in determining pollen success (Cruden and Lyon 1985).

Conclusion

The association between pollinator and flower morphology together with the pollinator-mediated selection documented in this article as well as by Alexandersson and Johnson (2002) is compelling evidence for pollinators being the evolutionary force driving divergence of floral morphology in this system. Allopatric divergence of the morphs seems most likely on account of the geographical structure of corolla tube length variation and its correspondence to an apparent gradient in the abundance of long- and short-tongued hawkmoths (Figs. 1–3). The alternative scenario of sympatric divergence driven by strong disruptive selection cannot be completely excluded, but seems unlikely given that sympatric speciation in plants has not been convincingly demonstrated outside the realm of ploidy level changes (Coyne and Orr 2004; but see Savolainen et al. 2006).

Coexistence of the two ecotypes at Mount Gilboa, despite their ability to produce fertile hybrids (Fig. 7; P. Rymer, pers. comm.), indicates that there is substantial prezygotic reproductive isolation. The primary isolating barrier between morphs on the summit of this mountain seems to be a difference in flowering phenology (Fig. 5). However, overlap in flowering time of the morphs at other sites suggests that other isolating barriers are also important. One of these is likely to be assortative mating, based on the foraging preferences of long- and short-tongued hawkmoths and facilitated by differences in floral scent. To shed further light on this intriguing system, we are now collaborating with molecular biologists in a study using genetic markers to uncover the actual patterns of gene flow within and among ecotypes and to identify the gene regions linked to traits under selection.

ACKNOWLEDGMENTS

We thank S. A. Ros, B. Bullock, F. Field, P. Massinga, C. Peter, K. Thompson, J. Wissman, J. Roger, and P. Rymer for assistance during fieldwork and C. Potgieter for help with herbarium specimens. We are particularly grateful to J. Terblanche and M. van Kleunen who provided help with statistical analysis, as well as A. Ellis, and three anonymous reviewers for their insightful comments on the manuscript. We would like to especially thank J. Shykoff who handled this article with exceptional patience, and care as well as helping with the interpretation of our data. We also thank staff at Garden Castle NR, Highmoor NR, Kamberg NR, Umgeni Valley NR, Vernon Crookes NR, Mr. and Mrs. J. Burrows in Buffelskloof for giving permission to perform fieldwork within the nature reserve and on their private properties. This study was financially supported by grants from: The Royal Swedish Academy of Sciences for post-doctoral research (the program for scientific exchange between Sweden and South Africa) and STINT to RA, and from the South African National Research Foundation to SDJ+BA and the University of KwaZulu-Natal to BA.

LITERATURE CITED

Aldridge, G., and D. R. Campbell. 2007. Variation in pollinator preference between two *Ipomopsis* contact sites that differ in hybridization rate. Evolution 61:99–110.

Alexandersson, R., and S. D. Johnson. 2002. Pollinator-mediated selection on flower-tube length in a hawkmoth-pollinated *Gladiolus* (Iridaceae). Proc. R. Soc. Lond. B 269:631–636.

Althoff, D. M, K. A. Segraves, and O. Pellmyr. 2005. Community context of an obligate mutualism: pollinator and frugivore effects on *Yucca filamentosa*. Ecology 86:905–913.

Amirav, A., and S. Dagan. 1997. A direct sample introduction device for mass spectrometry studies and GC-MS analysis. Eur. J. Mass Spectrom., 3:105–111.

Anderson, B., and S. D. Johnson. 2008. The geographical mosaic of coevolution in a plant-pollinator mutualism. Evolution 62:220–225.

———. 2009. Geographical covariation and local convergence of flower depth in a guild of fly-pollinated plants. New Phytol. 182:553–540.

Bloch, D., and A. Erhardt. 2008. Selection toward shorter flowers by butterflies whose probosces are shorter than floral tubes. Ecology 89:2453–2460.

Bolten, A. B., P. Feinsinger, H. G. Baker, and I. Baker. 1979. Calculation of sugar concentration in flower nectar. Oecologia 41:301–304.

Boyd, A. E. 2004. Breeding system of *Macromeria viridiflora* (Boraginaceae) and geographic variation in pollinator assemblages. Am. J. Bot. 91:1809–1813.

Campbell, D. R., N. M. Waser, and M. V. Price. 1996. Mechanisms of hummingbird-mediated selection for flower width in *Ipomopsis aggregata*. Ecology 77:1463–1472.

Clarke, K. R, and R. N. Gorley. 2006. Primer v6: user manual. Primer-E, Plymouth, U.K.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer, Sunderland, MA.

Cruden, R. W. 2009. Pollen grain size, stigma depth, and style length: the relationships revisited. Plant Syst. Evol. 278:223–238.

Cruden, R. W, and D. L. Lyon. 1985. Correlations among stigma depth, style length, and pollen grain size: do they reflect function or phylogeny? Bot. Gaz. 146:143–149.

Der, G., and B. S. Everitt. 2002. A handbook of statistical analyses using SAS, 2nd ed. CRC Press LLC, Boca Raton, FL, pp. 117–130.

Devaux, C., and R. Lande. 2008. Incipient allochronic speciation due to non-selective assortative mating by flowering time, mutation and genetic drift. Proc. R. Soc. Lond. B 275:2723–2732.

Doebeli, M., and U. Dieckmann. 2003. Speciation along environmental gradients. Nature 421:259–264.

Dötterl, S., A. Jűrgens, K. Seifert, T. Laube, B. Weissbecker, and S. Schűtz. 2005. Nursery pollination by a moth in *Silene latifolia*: the role of odours in eliciting antennal and behavioural responses. New Phytol. 169:707–718.

Ehlers, B. K., J. M. Olesen, and J. Ågren. 2002. Floral morphology and reproductive success in the orchid *Epipactis helleborine*: regional and local across-habitat variation. Plant Syst. Evol. 236:19–32.

Ellis, A. G., A. E. Weis, and B. S. Gaut. 2006. Evolutionary radiation of "stone plants" in the genus *Argyroderma* (Aizoaceae): unraveling the effects of landscape, habitat, and flowering. Evolution 60:39–55.

Godsoe, W., J. B. Yoder, C. I. Smith, and O. Pellmyr. 2008. Coevolution and divergence in the Joshua tree/yucca moth mutualism. Am. Nat. 171:816–823.

Goldblatt, P., and J. Manning. 1998. *Gladiolus* in Southern Africa. Fernwood Press, Vlaeberg, South Africa.

Grant, V., and K. A. Grant. 1965. Flower pollination in the Phlox family. Columbia Univ. Press, New York, NY.

Hodges, S. A., and M. L. Arnold. 1994. Floral and ecological isolation between Aquilegia Formosa and *Aquelegia pubescens*. Proc. Natl. Acad. Sci. USA 91:2493–2496.

Huber, F. K., R. Kaiser, W. Sauter, and F. P. Schiestl. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). Oecologia 142:564–575.

Johnson, S. D. 1997. Pollination ecotypes of *Satyrium hallackii* (Orchidaceae) in South Africa. Bot. J. Linn. Soc. 123:225–235.

———. 2006. Pollinator driven speciation in plants. Pp. 295–311 *in* L. D. Harder and S. C. H. Barrett, eds. Ecology and evolution of flowers. Oxford Univ. Press, Oxford, NY.

Johnson, S. D., and K. E. Steiner. 1997. Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* complex (Orchidaceae). Evolution 51:45–53.

Kaminaga, Y., J. Schnepp, G. Peel, C. M. Kish, G. Ben-Nissan, D. Weiss, I. Orlova, O. Lavie, D. Rhodes, K. Wood, et al. 2006. Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation. J. Biol. Chem. 281:23357–23366.

Kondrashov, A. S., and F. A. Kondrashov. 1999. Interactions among quantitative traits in the course of sympatric speciation. Nature 400:351–354.

Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. Evolution 37:1210–1226.

Maad, J. 2000. Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. Evolution 54:112–123.

Maad, J., and R. Alexandersson. 2004. Variable selection in *Platanthera bifolia* (Orchidaceae): phenotypic selection differed between sex functions in a drought year. J. Evol. Biol. 17:642–650.

Marques, I., A. Rossello-Graell, D. Draper, and J. M. Iriondo. 2007. Pollination patterns limit hybridization between two sympatric species of *Narcissus* (Amaryllidaceae). Am. J. Bot. 94:1352–1359.

Medel, R., C. Botto-Mahan, and M. Kalin-Arroyo. 2003. Pollinator-mediated selection on the nectar guide phenotype in the Andean Monkey flower, *Mimulus luteus*. Ecology 84:1721–1732.

Muchhala, N. 2007. Adaptive trade-off in floral morphology mediates specialization for flowers pollinated by bats and hummingbirds. Am. Nat. 169:494–504.

Muchhala, N., and J. D. Thompson. 2009. Going to great lengths: selection for long corolla tubes in an extremely specialized bat-flower mutualism. Proc. R. Soc. Lond. B. 276:2147–2152.

Nilsson, L. A. 1988. The evolution of flowers with deep corolla tubes. Nature 334:147–149.

Okamoto, T., A. Kawakita, and M. Kato. 2007. Interspecific variation of floral scent composition in *Glochidion* and ist association with host-specific pollinating seed parasite (Epicephala). J. Chem. Ecol. 33:1065–1081.

Olsson, K., and J. Ågren. 2002. Latitudinal population differentiation in phenology, life history and flower morphology in the perennial herb *Lythrum salicaria*. J. Evol. Biol. 15:983–996.

Perez-Barrales, R., J. Arroyo, and W. S. Armbruster. 2007. Differences in pollinator faunas may generate geographic differences in floral morphology and integration *in Narcissus papyraceus* (Alarcissiopapyraceris). Oikos 116:1904–1918.

Plepys, D., F. Ibarra, and C. Löfstedt. 2002. Volatiles from flowers of *Platanthera bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae). Oikos 99:69–74.

Plitmann, U., and D. A. Levin. 1983. Pollen–pistil relationships in the Polemoniaceae. Evolution 37:957–967.

Raguso, R. A., and D. M. Light. 1998. Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and ‘green-leaf volatiles’. Entomol. Exp. Appl. 86:287–293.

Raguso, R. A., D. M. Light, and E. Pickersky. 1996. Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. J. Chem. Ecol. 22:1735–1766.

Ramsey, J., H. D. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520–1534.

Rathcke, B., and E. P. Lacey. 1985. Phenological patterns of terrestrial plants. Annu. Rev. Ecol. Syst. 16:179–214.

Rey, P. J., C. M. Herrera, J. Guitian, X. Cerda, A. M. Sanchez-Lafuente, M. Medrano, and J. L. Garrido. 2006. The geographic mosaic in predispersal interactions and selection on Helleborus foetidus (Ranunculaceae). J. Evol. Biol. 19:21–34.

Rice, W. R. 1989. Analysing tables of statistical tests. Evolution 43:223–225.

Robertson, J. L., and R. Wyatt. 1990. Evidence for pollination ecotypes in the yellow-fringed orchid, *Platanthera ciliaris*. Evolution 44:121–133.

Roulston, T. H., J. H. Cane, and S. L. Buchmann. 2000. What governs protein content of pollen: pollinator preferences, pollen–pistil interactions or phylogeny? Ecol. Monogr. 70:617–643.

Savolainen, V., M. C. Anstett, C. Lexer, I. Hutton, J. J. Clarkson, M. V. Norup, M. P. Powell, D. Springate, N. Salamin, and W. J. Baker. 2006. Sympatric speciation in palms on an oceanic island. Nature 441:210–213.

Schemske, D. W., and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proc. Natl. Acad. Sci. USA 96:11910–11915.

Schemske, D. W., and C. C. Horvitz. 1989. Temporal variation in selection on a floral character. Evolution 43:461–465.

Stam, P. 1983. The evolution of reproductive isolation in closely adjacent plant-populations through differential flowering time. Heredity 50:105–118.

Stebbins, G. L. 1970. Adaptive radiation of reproductive characteristics in angiosperms. 1: pollination mechanisms. Annu. Rev. Ecol. Syst 1:307–326.

Waelti, M. O., J. K. Muhlemann, A. Widmer, and F. P. Schiestl. 2008. Floral odour and reproductive isolation in two species of *Silene*. J. Evol. Biol. 21:111–121.

Weis, A. E. 2005. Direct and indirect assortative mating: a multivariate approach to plant flowering schedules. J. Evol. Biol. 18:536–546.

Whittall, J. B., and S. A. Hodges. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. Nature 447:706–709.

Associate Editor: J. Shykoff

Supporting Information

The following supporting information is available for this article:

Figure S1. Long- and short-tubed ecotypes of *G. longicollis* found in a contact zone on Mount Gilboa.

Figure S2. The mean, maximum, and minimum recorded proboscis lengths for moths belonging to the long- and short-tongued guild, as well as the average, maximum, and minimum distance from the mouth of the corolla tube to the nectar in 20 long- and 20 short-tubed *G. longicollis* plants.

Figure S3. Comparison of the mean ± SE relative abundance of major compounds in the floral scent of long- ($n = 12$ plants) and short-tubed ($n = 11$ plants) ecotypes of *Gladiolus longicollis*.

Table S1. Geographical location (coordinates), mean corolla tube length, and hawkmoths captured in 11 populations of *Gladiolus longicollis* from South Africa (further information of geographical location, see Fig. 1).

Table S2. Hawkmoths captured (in light traps or with nets) or observed directly on flowers of *G. longicollis* at eight study sites and their proboscis dimensions and pollen loads.

Supporting Information may be found in the online version of this article.