### Geographical variation in floral traits of the tree tobacco in relation to its hummingbird pollinator fauna

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*Nicotiana glauca*, a hummingbird pollinated plant, exhibits geographical variation in several floral traits. We examined whether geographical differentiation occurred for different flower characters and if this differentiation could be explained, at least in part, by the existence or abundance of different hummingbird species in the respective pollinator assemblages. The comparison between five populations showed significant variation in six floral traits and two female fitness measures. The traits that better discriminated between populations were corolla length and corolla width. There were metric correlations between corolla length and style length in all populations studied and, in four of the five populations, both corolla length and width were also correlated. Among plants in each population, seed weight was correlated positively and significantly with style exertion, suggesting that fruit quality is dependent on the degree of cross pollination. Assemblages of hummingbirds differed between populations in species composition, visitation frequencies, and bill length. Linear regression involving bill length of the more frequent hummingbird pollinators and corolla length yielded positive and significant relationships. Thus, there appears to be an adjustment between pollinators and flowers traits that have high incidence in the among population variation. © 2007 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2007, **90**, 657–667.

ADDITIONAL KEYWORDS: corolla length – hummingbird assemblages – hummingbird bill length – *Nicotiana glauca* – pollination.

Flowers are among the most complex and diversified objects in the plant kingdom; they vary in colour, scent, size, morphology, and in the type and amount of reward they offer to their animal pollinators (Proctor, Leo & Lack, 1996). This diversity is believed to be the result of varied pollination strategies.

Agreements between flower and pollinator traits have traditionally been used to recognize adaptations of flowers to pollinators. Specific combinations of such traits supposedly adjusted to specific animal pollinator types have formed the basis of the long time paradigmatic syndrome concept (Faegri & van der Pijl, 1979). However, little empirical evidence has been provided to show that such traits actually are or were selected by pollinators. Such evidence may be provided by studying within a population the effect of trait variation on fitness through phenotypic selection (Nilsson,

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1988; Galen, 1989). Because this approach assesses present selective conditions, it does not necessarily explain the evolution of flower characters over time to the present. The current state of characters is the result of selection over many years, and phenotypic selection has been shown to vary in intensity and direction from year to year (Maad, 2000). A possibility to enable study of selection on characters from a more distant time to the present is to correlate across populations the variation of flower and pollinator traits.

We have adopted the latter approach based of the following rationale. Mutualistic relationships (e.g. plant-pollinator interactions), show dynamic patterns of specializations, which are moulded in different ways both locally and geographically by natural selection through the community context in which the interaction occurs over long periods of time (Thompson, 1994). Intraspecific geographical variation could be present due to differences in pollinator assemblages between sites, and in this way flowers adjust to local pollinators (Miller, 1981; Robertson & Wyatt, 1990; Gilbert *et al.*, 1996; Johnson & Steiner, 1997; Temeles & Kress, 2003).

When availability of pollinators is geographically patchy in discontinuously distributed populations or heterogeneous in continuously distributed plant populations, shifts in flower traits should be expected depending on the effectiveness and abundance of local pollinators. Because flowers will be moulded in each population by the most effective pollinator (Stebbins, 1970; Johnson, 1997), such patchiness or heterogeneity should lead to the formation of pollinator ecotypes.

Studies on geographical variation in flower traits of one species in relation to pollination are not very numerous and were made using different approaches. Most work is based on one or a few focal traits (typically spur length) that are analysed with a univariate approach. Some studies have a multivariate approach and include traits not directly involved in a pollination mechanism (e.g. calyx measures) (Herrera et al., 2002). The inclusion of other traits that may be indirectly correlated to the focal ones is necessary because the pattern of variation of one trait may be actually explained as the effect of selection on other morphologically integrated traits (Lande & Arnold, 1983; Armbruster et al., 1999). Regarding flower length not only the mean is an interesting trait to associate to pollinations specificity, but also, as shown by Fenster (1991), a low variance may be an indicator of longtermed directional and stabilizing selection in specialized plant-pollinator systems.

Studies seeking the match between flower and pollinator traits are based either on the comparison of data from two populations (Robertson & Wyatt, 1990; Johnson, 1997) or on the correlation analysis of several populations (Herrera *et al.*, 2002; Johnson & Steiner, 1997). However, the results obtained in these studies show that between-population differences not always reflect selective action of current pollinators.

Nicotiana glauca is a cosmopolitan shrub, native to Northern Argentina and Southern Bolivia but, naturalized in four continents (Goodspeed, 1954). The pollinators of N. glauca are always birds, namely Trochillidae in America, Nectarinidae in Africa and Meliphagidae in Australia (Hernández, 1981; Schueller, 2004). In the region where N. glauca grows naturally, there are diverse hummingbird assemblages that interact with the plant. We examined whether there is allopatric differentiation of flower characters and if it can be explained, at least in part, as an adjustment to pollinators. The latter implies that contemporary pollinators have been exerting selective pressures on flower phenotype. Hummingbirds have been found to feed from and pollinate flowers from much shorter to much longer than their bills (Snow & Snow, 1972; Feinsinger, 1976; Arizmendi & Ornelas, 1990; Cotton, 1998), apparently contradicting the view

that flowers evolved in concert with their pollinating hummingbirds. Through experiments with artificial flowers (Temeles, 1996) and a comparative study (Temeles *et al.*, 2002), it has been shown that flower width in relation to flower length is actually a better measure of adjustment between flowers and hummingbirds. Underlying this view is the simple reason that long flowers will exclude short billed hummingbirds if they are also relatively narrow.

Several criteria must be met to support the hypothesis that a plant is represented by pollination ecotypes in portions of its range. As summarized by Robertson & Wyatt (1990), these criteria are that both plants and pollinators must have evolved under the conditions where they are studied, and primary pollinators must differ between different portions of the plant's range. It must also be demonstrated that plant morphology differs between these areas, and specific mechanisms by which local pollinators could influence natural selection on plant characters must be identified. Finally, variation in the characters hypothesized to be adapted to different pollinators must be genetically based.

In the present study, we examined the relationship between geographical variation in floral traits and female reproductive success in natural populations of *N. glauca* along with the species composition and visitation frequencies of the local pollinator assemblages. These assemblages vary considerably in composition and bill size across large distance scales, so we expected selection on flower traits to differ strongly. We specifically asked: (1) were there geographical differences in floral morphology and fitness measures; (2) were there phenotypic correlations between floral traits within different populations; and (3) did pollinator abundance and bill length explain differences in floral morphology?

#### MATERIAL AND METHODS

#### STUDY SITES

We studied five populations of *N. glauca*, four in Argentina (Los Cardones at  $26^{\circ}40'17.7''S$ ;  $65^{\circ}49'4.1''W$ , 1978 m, Cuesta de Miranda at  $29^{\circ}20'14.4''S$ ;  $67^{\circ}41'31.5''W$ , 1559 m, Costa Azul at  $31^{\circ}23'48.4''S$ ;  $64^{\circ}26'34''W$ , 148 m and La Falda at  $31^{\circ}05'49.5''S$ ;  $64^{\circ}30'34.4''W$ , 992 m) and one in southern Bolivia (between Tupiza and Villazón on route 14 at km 71,  $21^{\circ}32'30.1''S$   $65^{\circ}38'20.5''W$ , 2910 m) (Fig. 1).

In general, populations were located on dry river beds or in disturbed sites, such as at roadsides where soil had been recently exposed. Plants were distributed in loose patches, frequently being less than 15 in number and exceptionally more than 70.

Nicotiana glauca individuals are tall shrubs, 1–4 m in height, with dense inflorescences distributed all over the plants, each one having dozens of flowers



Figure 1. Distribution map of the five Nicotiana glauca populations.

opening at the same time. Plants are self-fertile, but do not always self-pollinate spontaneously because anthers may be at the same or at a lower level than the stigma. The flowering season extends from early September to April. Flowers last for approximately 3 days, have diurnal anthesis, and are scentless; corollas are tubular and, in Tupiza, Los Cardones, Costa Azul, and La Falda, flowers are yellowish green in colour whereas the Cuesta de Miranda population has colour polymorphism including dark red, reddish yellow, and yellow morphs.

Plant density was calculated as the number of plants over an area ranging between 500  $m^2$  and 1200  $m^2$ .

In one previous study (Galetto & Bernardello, 1993), the total nectar volume produced by a flower was found to be  $20.0 \pm 8.07 \,\mu\text{L}$  in excluded flowers, and nectar extraction was found not to stimulate secretion. Nectar volume and total sugar production increased as a function of flower age.

#### VARIABLES MEASURED

We performed random sampling on plants from each population, from five to 20 flowers per plant, to measure sugar concentration and five morphometric flower traits (Fig. 2): corolla length, style length, corolla width, calyx length, and stamen length. We took morphometric measurements with a digital calliper (accuracy of 0.01 mm). Corolla length was the distance from the base of the calyx to the tip of one randomly chosen lobe. Style length was the distance from the base of the calyx to the stigma. Style exertion was calculated as the difference between corolla length and style length. Corolla width was the maximum external width of the flower tube at the throat, its most expanded portion (Fig. 2). The throat is a characteristic portion of the tube in many Nicotiana species and is where anthers are located (Goodspeed, 1954); thus, we are confident that this measure is homologous across species and individuals of one species. Calyx length was the distance from the base of the calvx to the tip of one randomly chosen sepal. Stamen length was the distance from the base of the calyx to the tip of the longest stamen. Sugar concentration (percent sucrose,  $g g^{-1}$ ) was measured with a hand refractometer (Atago). Calyx length and stamen length were only measured in three of the five populations.



**Figure 2.** Flower structure of *Nicotiana glauca*. A, corolla length; B, stamen length; C, corolla width; D, calyx length; E, style length; A–E, style exertion.

#### FITNESS MEASURES

We estimated two measures of female fitness for each individual: fruit set and mean seed weight/fruit. Fruit set assesses pollination intensity as a proportion of pollinated flowers whereas seed weight/fruit assesses the quality of mating. We were able to measure the proportion of fruit set/plant because flowers not turning into fruits fall leaving a scar on the inflorescence axis. This allowed us to be able to count the number of fruiting and nonfruiting flowers (scars). To calculate seeds weight/fruit, we collected and stored separately 30 capsules shortly before opening. After opening, the seeds were allowed to dry at 60 °C for 48 h and then weighed on with an Ohaus Explorer Balance (accuracy of 0.1 mg).

In one population (La Falda), seed weight/fruit was compared between sets of open pollinated and bagged flowers (approximately ten flowers per treatment and for each of ten plants) to determine whether open pollination resulted in better mating.

#### POLLINATORS

Pollinators were considered to be those visitors entering the flower tube and making contact with the stigmas and anthers.

At each site during a measured time interval, and with a fixed number of flowers, we recorded pollinators as well as their number of visits per flower; thus, the visitation rates per population for each pollinator species could be calculated as flowers min<sup>-1</sup>. These records were obtained over two successive flowering periods for each population. Pollinators were always hummingbirds. For the species of hummingbirds observed, we measured bill lengths (as exposed culmen taken from the nostril to the end of the bill) on specimens deposited in the 'Museo Argentino de Ciencias Naturales' that had been captured in the respective political provinces of the study sites. We are confident that the mean measures of these museum specimens are a good estimates of the local mean because bill lengths did not differ between provinces within the range covered by our study (P > 0.05). Analysis of variance (ANOVA) showed significant differences between geographical regions, only for *Chlorostilbon aureoventris* (F = 4.13; d.f. = 72; P < 0.0001) which was acting as pollinator in two nearby sites.

#### STATISTICAL ANALYSIS

We performed one-way ANOVA to determine whether fitness measures or floral traits vary between populations. We tested the assumptions of normality for all traits using the Shapiro–Wilks and Lilliefors tests in Statistica (StatSoft Inc., 1995). As an appropriate technique to determine which variables help to discriminate between populations, we used the discriminant function analysis (DFA) in InfoStat (Di Rienzo *et al.*, 2000). Four traits measured in all populations (corolla length, corolla width, style length and nectar concentration) were used for this analysis.

As stated above, the study of corolla length variance is particularly important. To compare corolla length variation independently of the mean, raw data were In-transformed before standard deviation and variance for each population were calculated. Equality of variances between all pair of sites was tested with the F-statistics (Sokal & Rohlf, 1995).

To establish whether there was any association between phenotypical variables, the Pearson product moment correlation coefficients were calculated for each population. Probability values of these correlations were corrected for type I error by Bonferroni sequential test (Sokal & Rohlf, 1995).

For assemblages of two or more pollinators, we used the homogeneity G-test to test if any pollinator species was a more frequent visitors than others. A simultaneous procedure of the unplanned homogeneity test was used to compare overall frequencies between sites (Sokal & Rohlf, 1995).

To test whether variation of floral traits was explained by pollinator traits, correlation analysis was performed between floral morphometric traits and bill length of the most frequent local pollinator. Two of the five sites do not differ in bill length of the most frequent pollinator; thus, we expected that similar mean values of floral traits in these two sites would support the hypothesis of adjustment to local pollinator.

#### RESULTS

# WERE THERE ANY GEOGRAPHICAL DIFFERENCES IN FLORAL MORPHOLOGY AND FITNESS MEASURES?

There were large and significant differences between populations for all morphological traits, sugar concentration and for both fitness measures (Table 1). Differences were particularly big for fruit set. Flower width in relation to flower length was homogeneous across most sites, being significantly higher only in Costa Azul.

The largest corollas and styles were from Tupiza and the smallest from La Falda. Tupiza also had the largest standard deviation of the ln-transformed corolla length measures. Length differences between corolla and style were smaller in La Falda and Costa Azul than in the other three populations. These differ-

Table 1.	$Mean \pm SD$	for six	floral	traits	and t	wo	fitness	measures	of.	Nicotiana	glauca	in f	ive	popu	lation	$\mathbf{s}$
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Traits	Tupiza, mean $\pm$ SE (N)	Los Cardones, mean ± SE (N)	Cuesta de Miranda, mean ± SE (N)	Costa Azul, mean ± SE (N)	La Falda, mean $\pm$ SE (N)	ANOVA between populations
Corolla length (mm)	$42.59 \pm 5.09^{d}$	$41.09 \pm 2.91^{cd}$	$39.75 \pm 2.79^{\mathrm{bc}}$	$37.86 \pm 2.79$ b (77)	$35.17 \pm 1.60^{a}$	$F_{4,255} = 16.58$ P < 0.001
Ln [Corolla length (mm)]*	(13) $3.74 \pm 0.12^{b}$ (79)	(10) $3.71 \pm 0.07^{a}$ (18)	(10) $3.68 \pm 0.07^{a}$ (76)	(11) $3.67 \pm 0.06^{a}$ (76)	(10) $3.56 \pm 0.05^{a}$ (10)	1 < 0.001
Style length (mm)	$40.10 \pm 4.72^{d}$ (79)	$38.54 \pm 2.37^{\circ}$ (18)	$37.73 \pm 2.66^{\circ}$ (76)	$36.02 \pm 1.66^{\mathrm{b}}$ (77)	$33.65 \pm 1.96^{a}$ (10)	$F_{4,255} = 49.80$ P < 0.001
Corolla width (mm)	$8.55 \pm 0.91^{\circ}$ (79)	$8.33 \pm 0.52^{ m bc}$ (18)	$8.07 \pm 0.63^{b}$ (76)	$7.13 \pm 0.51^{a}$ (77)	$\begin{array}{c} 6.93 \pm 0.36^{\rm a} \\ (10) \end{array}$	$F_{4,255} = 48.44$ P < 0.001
Calyx length	$\begin{array}{c} 15.84 \pm 1.84^{\rm b} \\ (69) \end{array}$	-	$\begin{array}{c} 15.80 \pm 1.76^{\rm b} \\ (76) \end{array}$	$\begin{array}{c} 12.50 \pm 0.76^{\rm a} \\ (67) \end{array}$	_	$F_{2,219} = 105.82$ P < 0.001
Corolla length/ corolla width	$5.00 \pm 0.46^{a}$ (79)	$\begin{array}{c} 4.94 \pm 0.28^{\rm a} \\ (18) \end{array}$	$\begin{array}{c} 4.95 \pm 0.43^{\rm a} \\ (76) \end{array}$	$5.51 \pm 0.29^{b}$ (77)	$\begin{array}{c} 5.09 \pm 0.30^{a} \\ (10) \end{array}$	$F_{4,255} = 25.70 P < 0.001$
Stamen length	$37.86 \pm 4.28^{b}$ (69)	-	$36.54 \pm 2.89^{b}$ (76)	$\begin{array}{c} 34.86 \pm 1.50^{a} \\ (67) \end{array}$	_	$F_{2,209} = 27.62$ P < 0.001
Style exertion	$2.55 \pm 0.62^{ m b}$ (79)	$3.13 \pm 1.34^{\circ}$ (18)	$2.50 \pm 1.21^{ m b}$ (76)	$2.01 \pm 0.71^{a}$ (77)	$1.53 \pm 0.50^{a}$ (10)	$F_{4,259} = 12.49$ P < 0.001
$\begin{array}{c} {\rm Sugar \ concentration} \\ {\rm (g \ g^{-1})} \end{array}$	$\begin{array}{c} 26.32 \pm 4.58^{\rm b} \\ (58) \end{array}$	$20.90 \pm 4.09^{a}$ (14)	$\begin{array}{c} 22.98 \pm 4.09^{\rm a} \\ (35) \end{array}$	$\begin{array}{c} 26.78 \pm 1.52^{\rm b} \\ (49) \end{array}$	$21.81 \pm 0.99^{a}$ (9)	$F_{4,121} = 8.97$ P < 0.001
Seeds weight/fruit (mg)	$4.64 \pm 0.18^{\circ}$ (72)	$3.03 \pm 0.70^{ab}$ (10)	$3.45 \pm 0.99^{b}$ (75)	$3.19 \pm 0.13^{ m b}$ (73)	$\begin{array}{c} 2.43 \pm 0.57^{a} \\ (10) \end{array}$	$F_{4,235} = 14.80$ P < 0.001
Prop. fruits/plant	$\begin{array}{c} 0.36 \pm 0.25^{a} \\ (79) \end{array}$	$\begin{array}{c} 0.18 \pm 0.22 \ ^{\rm b} \\ (18) \end{array}$	$0.39 \pm 0.16^{a}$ (76)	$\begin{array}{c} 0.41 \pm 0.18^{a} \\ (77) \end{array}$	$0.88 \pm 0.03$ <sup>c</sup> (10)	$F_{4,255} = 551.18$ P < 0.001

The analysis of variance (ANOVA) column shows results of comparisons between populations. Different superscript letters indicate significant differences between populations in a Duncan a posteriori test at the P < 0.05 level. *N*, number of plants.

\*Equality of variances between all pair of sites was tested using *F*-statistics.

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ences also indicate the shortest anther-stigma distance (see below). Sugar concentration was highest in Costa Azul (Table 1). The Duncan a posteriori test shows that the differences in corolla length between populations were gradual, with no site completely overlapping with others. Other morphometric traits showed overlap between populations (Table 1). The Ftest for homogeneity of corolla length variance showed that Tupiza had a variance significantly higher than any other site and that sites with a lower variance did not differ significantly between each other (Table 1).

In the La Falda population, seed weight in self pollinated flowers was significantly lower than in open pollinated flowers ( $F_{1,38} = 57.20$ ; P < 0.0001). Seed weight/fruit was highest in Tupiza, whereas plants of Los Cardones, Cuesta de Miranda, and Costa Azul had, on average, practically the same seed weight/ fruit. In La Falda, individuals showed, on average, the lowest seed weight, but the highest fruit set/plant, among the five sites.

For the Cuesta de Miranda population, where N. glauca exhibits flower colour polymorphism, none of floral traits measured exhibited significant differences among the three colour morphs (corolla length  $F_{3,75} = 1.61$ , P = 0.195; style length  $F_{3,75} = 1.74$ , P = 0.166; corolla width  $F_{3,75} = 2.53$ , P = 0.089; calyx length  $F_{3,75} = 1.74$ , P = 0.166; stamen length  $F_{3,75} = 1.41$ , P = 0.247; sugar concentration  $F_{3,31} = 1.61$ , P = 0.195).

DFA performed for the five populations was highly significant (Wilks' lambda = 0.3914; F = 8.11; P < 0.0001). DFA discriminated one major component accounting for 72.85% of the variation among the populations studied (Table 2). This factor was strongly correlated with the three flower measures: corolla length, corolla width, and style length (Table 2). Factor two accounted for 25.27% of the total variance and was best correlated with corolla width (Table 2). The DFA indicated that 63% of the plants were correctly assigned to their source population. The population

**Table 2.** Canonical discriminant function for flowers traits in five populations of *Nicotiana glauca* of a discriminant function analysis

Trait	Axis 1	Axis 2
Corolla length	0.58*	-0.25
Style length	-0.51*	0.39
Corolla width	$0.73^{*}$	$-0.74^{*}$
Sugar concentration	0.12	$0.22^{*}$
Eigenvalue Percentage variance explained	$0.90 \\ 72.85$	$0.31 \\ 25.27$

Traits strongly correlated (P < 0.001) with the component are indicated by an asterisk.

with the highest number of plants correctly assign was La Falda (90%) and that with the highest number of misassigned plants was Cuesta de Miranda (37%).

#### WERE THERE PHENOTYPIC CORRELATIONS BETWEEN FLORAL TRAITS WITHIN AND AMONG DIFFERENT POPULATIONS?

Corolla length and style length showed the most consistent correlation, being significant across populations (Table 3). Only in La Falda were corolla length and corolla width not correlated. In Tupiza and in La Falda populations, sugar concentration was correlated with corolla length and style length. In the populations where stamen length was measured, it was correlated with all other four morphometric measures (Table 3).

Within populations, none of the flower traits showed significant correlations with seed weight/fruit. In Tupiza and in Costa Azul populations, corolla length, style length, and corolla width were correlated with fruit set (Table 3). Additionally, in Tupiza, calyx length and sugar concentration were significantly correlated with fruit set.

## DID POLLINATORS EXPLAIN DIFFERENCES IN FLORAL MORPHOLOGY?

Five hummingbird species accounted for all visits in the five populations (Table 4). Populations differed in the composition of the hummingbird assemblages, and in the bill length of the hummingbird species that constituted these assemblages (Table 5). All humming-bird species differed significantly in bill length ( $F_{4.58} = 318.28$ ; P < 0.0001).

Population density varies from 3 plants m<sup>-2</sup> in La Falda population to 12.50 plants m<sup>-2</sup> in the Costa Azul population. Plant density did not affect the visitation rates observed because the correlation between plant density and visitation rate was not significant (r = 0.26; P = 0.673; N = 5). In Tupiza, Patagona gigas was the only hummingbird pollinating N. glauca and it was the one with the longest bill among all the hummingbirds observed. In Los Cardones, two hummingbird species were observed as pollinators: Oreotrochillus leucopleurus and Sappho sparganura. These species have medium and short bills, respectively, with, O. leucopleurus being by far the more frequent pollinator. Overall visitation frequency was not significantly different from Tupiza (Table 4). In Cuesta de Miranda, Heliomaster furcifer was the more frequent pollinator with P. gigas acting as an occasional pollinator. Overall visitation frequency was intermediate. An additional species present in this population, C. aureoventris, was recorded visiting N. glauca flowers but as a secondary nectar robber.

	Corolla length	Style length	Corolla width	Calyx length	Stamen length	Sugar concentration	Fruit set	Seed weight
Tupiza Corolla length Style length Corolla width Calyx length Stamen length Sugar concentration Fruit set Seed weight	$0.973^{**}$ $0.648^{**}$ $0.899^{**}$ $0.954^{**}$ $-0.348^{*}$ $0.293^{*}$ 0.052	$0.716^{**}$ $0.884^{**}$ $0.984^{*}$ $-0.299^{*}$ $0.341^{*}$ 0.136	$0.568^{*}$ $0.787^{*}$ -0.229 $0.315^{*}$ 0.206	$0.863^{*}$ -0.363 $^{*}$ 0.343 $^{*}$ 0.039	$-0.179 \\ -0.002 \\ 0.106$	-0.320* -0.162	0.050	
Los Cardones Corolla length Style length Corolla width Sugar concentration Fruit set Seed weight	$0.994^{**}$ $0.636^{*}$ 0.209 $0.401^{*}$ 0.167	$0.601^{*}$ 0.219 0.366 0.162	$0.132 \\ 0.285 \\ 0.236$			$0.302 \\ 0.478$	-0.075	
Cuesta de Miranda Corolla length Style length Corolla width Calyx length Stamen length Sugar concentration Fruit set Sood weight	$0.967^{**}$ $0.636^{*}$ $0.443^{**}$ $0.977^{**}$ 0.004 0.011 0.195	0.212 0.380* 0.915** -0.060 0.033 0.212	$0.275^{*}$ $0.290^{**}$ 0.047 0.015 0.221	$0.450^{**}$ 0.050 0.169 0.096	0.020 -0.046 0.194	-0.015	0.017	
Costa Azul Corolla length Style length Corolla width Calyx length Stamen length Sugar concentration Fruit set Seed weight	0.785** 0.685** 0.613** 0.805** -0.098 -0.542** -0.051	0.212 0.444** 0.380* 0.925* -0.022 -0.416* 0.012	0.672** 0.473* 0.094 -0.320* -0.088	0.386* 0.094 -0.134 -0.134	-0.229 -0.172 -0.172	-0.077 0.297 0.373	0.229	
La Falda Corolla length Style length Corolla width Sugar concentration Fruit set Seed weight	0.981** 0.335 0.692* -0.423 0.143	0.375 0.679* -0.045 0.055	-0.124 0.311 -0.280			-0.910** 0.554	0.551	

**Table 3.** Pearson product moment correlation coefficient (r) among six floral traits and two fitness measures of *Nicotiana* glauca in five populations

Sample size as in Table 1.

\*P < 0.05; \*\*P < 0.001.

Three hummingbird species with medium and short bills were seen as pollinators in La Falda (Table 5). Visitation frequency was low and accounted for mainly by the short-billed hummingbird *C. aureoventris* and *S. sparganura* (Table 5). In the Costa Azul population, two hummingbird species were seen pollinating *N. glauca*. We were able to record visitation rate only for the more abundant *C. aureoventris*, with *S. sparganura* being seen occasionally in winter. The overall visitation frequencies in Costa Azul and La Falda were the lowest of the five sites and did not differ significantly between each other (Table 5).

Site	Plant density	Pollinator species	Percentage of visits in each site	Total number of flower visits for each hummingbird species	Overall pollinator visitation rate as flowers min <sup>-1</sup> (observation time in min)	Mean number of flower visits/ approach to a plant (plants 100 m <sup>-2</sup> )
Tupiza	8.75	Patagona gigas Viellot	100	136	0.348° (390)	7.27
Los Cardones*	10.00	Oreotrochillus leucopleurus Gould 1847	91.80	90	$0.375^{c}$ (240)	4.98
		Sappho sparganura Shaw	8.30	8		4
Cuesta de	6.66	Heliomaster furcifer Shaw	75	137	$0.146^{b} (940)$	7.04
Miranda*		Patagona gigas	25	46		6.69
Costa Azul	12.50	Chlorostilbon aureoventris Orbigny and Lafresnaye	100	94	$0.099^{a}\ (1005)$	9.17
La Falda*	3.00	Heliomaster furcifer	20.00	10	$0.113^{a}$ (600)	7.70
		Sappho sparganura	20.00	10		5.15
		$Chlorostilbon\ aureoventris$	60.00	48		10

Table 4. Pollinator hummingbird species, total number of flowers visits, visitation rates and number of flower visits/ hummingbird species in five populations of *Nicotiana glauca* 

Different superscript letters indicate significant differences between total number of visits in a homogeneity of replicates tested for goodness of fit at the P < 0.05 level.

\*Homogeneity rejected with G-test; P < 0.001

Table 5.	Assemblages of	of hummingbirds, mea	an bill length and	l corolla length in five	Nicotiana glauca populations
	0	<i>v</i>	0	0	

	La Falda	Costa Azul	Cuesta de Miranda	Los Cardones	Tupiza
Corolla length (mm), mean ± SD	$35.17 \pm 1.60$	$37.86 \pm 2.79$	$39.75\pm2.79$	$41.09\pm2.91$	$42.59 \pm 5.09$
Chlorostilbon aureoventris	$16.64 \pm 1.17 \ (4)^{*}$	$16.64 \pm 1.17 \ (4)^{*}$			
Sappho sparganura	$17.58 \pm 1.06$ (6)			$17.73 \pm 1.09 \; (21)$	
Oreotrochillus leucopleurus				$25.27 \pm 0.97$ (2)*	
Heliomaster furcifer Patagona gigas	$27.86 \pm 2.12$ (9)		$\begin{array}{c} 29.04 \pm 1.74 \; (15)^{*} \\ 38.81 \pm 1.36 \; (4) \end{array}$		$37.53 \pm 3.24 \ (2)^{*}$

Data in parenthesis indicate number of specimens measured. Asterisks indicate the most frequent pollinator in each population.

*Chlorostilbon aureoventris* was the hummingbird probing the highest number of flowers per approach to a plant, averaging 9.17 (Table 5). The hummingbird with the lowest number of flower visits per approach to a plant was *S. sparganura*, averaging 4.58 flowers per approach (Table 5).

Hummingbird assemblages did not vary between successive seasons except in Cuesta de Miranda, where *P. gigas* appeared only during the second season.

Because hummingbirds could not introduce their heads into the corolla tube because the corolla tube opening is too narrow, pollen placement on the pollinators was always on the bill independently of flower and bill length and there is no possibility of pollen deposition on the head of the hummingbirds.

Correlation involving bill length of the more frequent hummingbird pollinators for each population and corolla length were positive and significant (r = 0.88; P = 0.048; N = 5). Although the power of this correlation is low due to the small number of populations studied, there is an evident trend showing that longer flowers are matched with long-billed pollinators.

### DISCUSSION

#### FLOWER TRAITS

Studied floral traits differed significantly between populations. We expected these differences to be explained by selection of flower characters through distinct pollinator guilds. Corolla length and width gave for a better discrimination between populations than other traits and explained most of the among population variation. In *Nicotiana langsdorffii*, *Nicotiana alata* and *Nicotiana longiflora*, variation in corolla length is genetically based (East, 1916; Anderson, 1939) and we expected that genetic control of this trait would be consistent in the genus. However, definite conclusion still requires determining, for example through reciprocal transplants, how much of the observed variation in floral traits is due to the environment.

According to the view of Temeles and coworkers (Temeles, 1996; Temeles *et al.*, 2002), flowers pollinated by long-billed hummingbirds are expected to have narrower flowers in relation to length. However, we could not find that flowers were proportionately narrower in populations pollinated by long bill hummingbirds. In the present case, these two traits are highly correlated suggesting an allometric relationship. Thus, we suggest that corolla width did not evolve under selection by pollinators in *N. glauca*, but is rather overriding on corolla length.

Allometric relationships apparently also exist between other flower organs in N. glauca. Length of calyx, style, stamen, and corolla are positively and consistently correlated across populations suggesting strong flower integration.

Nectar concentration was negatively correlated with flower length in two populations and significantly so in only one. However, in three other populations, this correlation was both positive and mostly significant. A significant correlation between sugar concentration and fruit set again suggests that selection was acting in this traits but this was evident only in two populations.

Alternative hypotheses to explain flower trait variation are being considered. Data on the influences on floral traits of environmental factors, such as geographical and climatic ones, including a larger geographical scale and a larger number of populations, will be presented elsewhere (J. Nattero, A. N. Sérsic & A. A. Cocucci, unpubl. data).

#### FITNESS MEASURES

Fitness measures varied greatly between populations. Significant correlations between length of flower organs and fruit set in some populations suggest that flower length is subjected to selection. However, this was significant only in two populations, being positively correlated in one case, and negatively correlated in the other. Studies of phenotypic selection considering both direct and indirect effects should show how strong this selection is, and whether it acts differently in the various floral organs. A priori, it seems that calyx length could be driven by selection on the length of the corolla and fertile organs because it presumably is not subjected to selective pressures by pollinators.

Some significant correlations may be emphasized here to show the possible association between floral traits and self-pollination, either autogamous or geitonogamous. In La Falda, where style exertion is smallest, plants exhibited both the highest fruit set and the lowest seed weight/fruit. A shorter stigma exertion and narrower corolla indicate a greater longitudinal and radial proximity between tube rim (or anthers) and stigma, probably benefiting selfpollination. A high fruit set in this population can also be attributed to pollinator behaviour because here the more frequent visitor, C. aureoventris, visited the highest number of flowers per approach probably favouring bird-assisted geitonogamy. Thus, the La Falda population could have a high self-pollination intensity (autogamous and geitonogamous), but low quality matings (xenogamy). Also, differences in the direction of selection of corolla and style length as shown by the correlation between these traits and fruit set could suggest that mechanisms of pollen receipt differ between sites with short- and long-billed hummingbirds, flowers placing pollen, respectively, on the bill or on the face. However, pollen deposition was always on the bill independently from the corolla and bill length at each site. Our data, together with those of Schueller (2004), show that fruit set better reflects frequency of pollination flowers (including self-pollination) than quality of mating. Indeed, the population where we found fruit set to be negatively correlated with corolla length, visitation frequency was much lower than at other sites and mean stigma-anther distances was shortest, suggesting that a higher fruit set could be the result of a higher rate of self-pollination.

#### HUMMINGBIRD BILL LENGTHS AND CORRELATIONS WITH FLOWER TRAITS

The five hummingbird species observed pollinating N. glauca differed significantly in bill length. Guilds were composed alternatively of one or two long-billed species, a mixture of intermediate and short-billed species, or by one short-billed species. Patagona gigas is apparently an important and consistent pollinator of N. glauca in high mountain ranges of Bolivia and Argentina. In addition to the present data, there are old records from La Rioja in Argentina (Giacomelli, 1905) and several anecdotal ones from Salta

(Argentina) and other sites of Bolivia (D. Renison, pers. com.; A. A. Cocucci, unpubl. data).

Long corollas of Tupiza match with the long bills of the only pollinator, whereas the short corollas of Costa Azul and La Falda match with the short bills of the two species or more frequent pollinator species (*C. aureoventris* and *S. sparganura*). In the Los Cardones population, differences between corolla length and bill length are greater than in the other sites, so hummingbirds would have had to introduce their bills deeper into the flowers to have access to nectar. In the Cuesta de Miranda population, one hummingbird behaved as a secondary nectar robber, probably because flower length limits access for nectar to this hummingbird.

The positive and significant correlation between corolla length and bill length of the more frequent hummingbird pollinator, and the high incidence of corolla size in the among population variation are consistent with the hypothesis of flowers being moulded by local pollinators. The selection by pollinators could exert an important influence in the evolution of floral variation in this species. There are no evident local adaptations of the hummingbirds to corolla length because bill lengths of hummingbird species occurring at distant sites did not differ significantly. Thus, we assume that there is a unilateral adaptation of the flowers to the local pollinators rather than the opposite. Selection of corolla length, however, is not directly evident on a local scale, probably because the mode of selection, either univariate or correlational, could not be discerned here. Also, correlations between flower traits and fitness measures presented here must be interpreted cautiously because the effects on fitness of each hummingbird species at each site and the correlated effects of more than one trait were not analysed (Galen, 1989). However, we expect to show this in a subsequent study (J. Nattero, A. N. Sérsic & A. A. Cocucci, unpubl. data).

#### MECHANISM OF FLOWER LENGTH SELECTION.

Flowers of *N. glauca* spread pollen along the bill of its pollinators as it is introduced into the corolla tube. This mechanism does not fit in with any of the models available in the literature (Gregory, 1964; Miller, 1981; Nilsson, 1988; Robertson & Wyatt, 1990; Johnson, 1994; Johnson & Steiner, 1997) because plants may freely interchange pollen irrespective of their flower length (i.e. short flowers may pollinate long flowers and vice versa). For evolution of long flowers to take place, there must be a reproductive benefit to plants with longer flowers. We propose that this takes place by long flowers exporting and receiving larger amounts of pollen than short ones because they spread pollen over a larger extension of the pollinators' bill. Fenster (1991) argues that species with longer corolla tubes are associated with a low pollinator diversity and specificity of pollen placement, suggesting that the strength of stabilizing and/or directional selection on corolla tube length is greater for species with long corollas than those with short corollas. In the present study, corolla tube variance is greater in Tupiza where flowers are longer than in the other places, suggesting that phenotypic variation is not exhausted in this population.

We recognize that our conclusion must be taken cautiously. The evidence, although significant, shows a trend supporting predictions for the above mechanism (i.e. positive correlations between flower and bill length of the more frequent pollinators).

Similar mechanisms of pollen attachment over an extended area of the pollinator's mouth pieces should predictably have flower length adjusted to the pollinator bills and anthers placed near the mouth of the tube. Such is the case for all *Nicotiana* species, which have stamens included within the corolla tube, with some having very long flowers (Goodspeed, 1954).

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