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Geographic variation of floral traits in *Nicotiana glauca*: Relationships with biotic and abiotic factors

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ABSTRACT

Geographic pattern of phenotypic variation can appear in a clinal or a mosaic fashion and can evidence adaptive or non-adaptive variation. To shed light on the mechanisms underlying this variation, we studied the relationships between geographic variation of floral traits and both biotic and abiotic factors of the hummingbird-pollinated plant, *Nicotiana glauca*, across its natural range. We obtained floral measures of 38 populations from an area about 1600 km long and 1050 km wide and an altitude range from 7 to over 3400 m. We used a MANOVA to detect between-population differentiations in flower traits and a DFA to determine the traits that best discriminate between populations. To test for associations between floral traits and climatic variables we used correlation analysis. We explored any possible distance-based pattern of variation (either geographic or altitudinal) in floral traits or bill length of pollinators using Mantel tests. Finally, we used a multiple regression to analyze simultaneously the effects and relative importance of abiotic predictor variables and bill length on corolla length. We found a high variation in flower traits among populations. Morphometric traits were the ones that best discriminated across populations. There was a clinal pattern of floral phenotypic variation explained by climatic factors. Differences in floral phenotypic distances were structured by altitudinal distances but not by geographic distances. Bill length of the hummingbird pollinators was structured both by altitudinal and geographic distances. Differences in bill length of hummingbird pollinators explained differences in corolla length across populations. Our findings support the assumption of flower evolution at a broad geographic scale. Floral traits seem to be structured not only by altitude but also by climatic factors.

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1. Introduction

Studies on geographic variation in phenotype provide insights into the mechanisms of evolution and have long contributed to a better understanding of diversification within and among species (Gould and Johnston, 1972; Thompson, 1994; Futuyma, 1998; Thompson and Cunningham, 2002). When geographic patterns of phenotypic variation are detected, they can appear in a clinal or a mosaic fashion and can evidence adaptive or non-adaptive variation (e.g., Endler, 1977; Anderson and Johnson, 2008; Toju, 2008; Pérez-Barrales et al., 2009). Differentiation among populations of a species may result, among other factors, from divergent natural selection (Herrera et al., 2006), or may be

facilitated by isolation by distance (Gould and Johnston, 1972; Futuyma, 1998). Flower phenotype has traditionally been associated with the relevant traits of the most frequent pollinators (Stebbins, 1970) or functional groups of pollinators (Fenster et al., 2004). Thus, local differences in these functional traits may be promoting differentiation among populations, i.e., the differentiation of pollination ecotypes adjusted to these assemblages (e.g., Miller, 1981; Armbruster, 1985; Robertson and Wyatt, 1990; Johnson and Steiner, 1997; Hansen et al., 2000; Barrett et al., 2004; Schueller, 2004). However, morphological variation between populations may be subtle and differences may not be evident within a small geographic range, across which gene flow via pollen or seeds may occur. Geographic variation across mosaics of environmental conditions, which may impose selection on phenotypic traits, should be studied in species with wide distribution ranges, where pollinator assemblages greatly vary and where gene flow between distant populations is limited (Thompson, 1994). Herrera et al. (2006) proposed a five-step approach to determine the causes of geographic variation in

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floral traits. The five steps include: 1- Documenting geographic variation in pollinators. 2- Testing whether geographically variable floral traits are targets of selection by pollinators. 3- Examining whether the selection gradient on floral traits is related to geographic variation in the pollinator fauna. 4- Quantifying spatial correlation between selection gradients and phenotypic values. 5- Determining whether population differences in floral traits have a genetic basis.

Evidences of congruent variation between plant traits and pollinators were found for other plant-pollinator systems (e.g., Johnson and Steiner, 1997; Gómez and Zamora, 1999, 2000; Fausto et al., 2001; Totland, 2001; Blionis and Vokou, 2002; Elle and Carney, 2003; Valiente-Banuet et al., 2004; Anderson and Johnson, 2008). Some works have studied the relationships between variation in floral phenotypes and biotic and abiotic factors at a large geographic scale (Thompson, 1997; Boyd, 2002; Thompson and Cunningham, 2002; Silva-Montellano and Eguiarte, 2003; Medel et al., 2007; Anderson and Johnson, 2008). In addition, congruent variation across populations between flower and pollinator traits that are relevant in reciprocal mechanical fit has been demonstrated in other studies (e.g., Steiner and Whitehead, 1990; Johnson and Steiner, 1997; Boyd, 2002; Sánchez-LaFuente, 2002; Anderson and Johnson, 2008).

In general, population dynamics and natural selection in plant-animal systems can be attributed to different biotic and abiotic factors at different spatial scales (e.g., McGeoch and Price, 2005; Herrera et al., 2006; Laine, 2005; Anderson and Johnson, 2008; Toju, 2008). However, how those processes at multiple spatial scales are incorporated into ecological and evolutionary interactions at the species level is less understood. Elucidating floral trait variations at multiple spatial scales is not only challenging but also important for understanding the ecological and evolutionary processes of plant–pollinator interactions. We studied the geographic patterns of phenotypic variation in floral traits of *Nicotiana glauca* Graham, a hummingbird-pollinated plant native to South America. This species occupies an extensive ecological and geographic range where marked variation in flower phenotype is evident. We hypothesized that floral phenotype was associated with both the biotic and abiotic environmental contexts. Relative geographic position (altitude and geographic distances) and/or climatic variables (temperature and rainfall) may accompany clinal variation of flower traits (e.g., altitude: Anderson and Johnson, 2008; Toju, 2008; latitude: Boyd, 2002; Herrera et al., 2006; longitude, temperature and rainfall: Mascó et al., 2004). The available evidences suggest that hummingbirds play an important role in the variation of *N. glauca* floral traits. Positive correlations between flower length and bill length of the most frequent pollinator have been found in five populations, suggesting the occurrence of pollination ecotypes (Nattero and Cocucci, 2007). In addition, phenotypic selection on corolla length has been found to be locally acting in favour of flower-pollinator adjustment in six populations (Nattero et al., 2010). Hummingbird bill length is mainly related to three flower traits: the combination of corolla tube length and width, which may limit access to nectar rewards (Temeles et al., 2002; Temeles and Kress, 2003), and style length, because this trait is involved in a mechanism of adjustment to receive pollen in the stigma from the same place where it was deposited on the hummingbird bill. In addition, nectar concentration may be a trait overriding corolla length, which may affect hummingbird preferences (Baker, 1975). Based on the previous knowledge on *N. glauca* pollinator system, we postulated that this pattern of association between bill length and flower length would also occur at a larger geographic scale, and that such association would explain an important part of the broad geographic variation in floral traits.

Specifically, the aims of this work were to evaluate: 1) whether there was a large-scale geographic structure of variation in floral traits of *N. glauca* across its natural distribution; 2) whether any

particular abiotic factor best accounted for this variation and if there was any distance-based pattern in this structure; 3) whether there was any geographic structure of variation among populations in hummingbird traits functionally relevant to flower-pollinator fit; and 4) whether floral trait variation among populations was explained by hummingbird functional traits (bill length).

2. Materials and methods

2.1. Plant life history

N. glauca is a native perennial shrub, widely distributed in subtropical zones of western South America (northern Argentina and Southern Bolivia; Goodspeed, 1954). Populations occur on riverbanks, roadsides and disturbed areas. Plants are distributed in isolated patches, with usually fewer than 15 plants and exceptionally more than 70 in each population. Hummingbirds are the only pollinators recorded for native (Giacomelli, 1905; Parker and O’Niell, 1980; Loayza et al., 1999; Nattero and Cocucci, 2007; Nattero et al., 2010) and introduced populations of *N. glauca* in central and North America (Hernández, 1981; Schueller, 2004, 2007). The flowering season extends from early September to April; flowers are tubular and have a reduced corolla limb. Plants are self-compatible and although some spontaneous autogamy may occur, visitation by hummingbirds significantly enhances seed and fruit set (Schueller, 2004; Nattero and Cocucci, 2007). Fruits are upright capsules with terminal slits through which seeds are shed when branches are shaken (Daumann, 1928).

2.2. Study sites and flower traits

We sampled 38 populations of *N. glauca*, 30 in northern Argentina and eight in southern Bolivia from September 2001 to April 2008 (Fig. 1, Appendix A). We measured corolla length, style length, corolla width, and nectar concentration in 15 haphazardly chosen flowers from each of the 10 to 86 plants sampled in each population. Plant size varies greatly within each population, with plants being from about 1 m to 4 m tall; however, we did not note differences in flower size from basal branches to the top of the shrub. We measured morphometric characters with a digital calliper to the nearest 0.01 mm, and nectar concentration (percent sucrose, g/g) with a hand refractometer (Atago). In this species, at the base of the corolla tube, there is a short nectar chamber that may prevent nectar evaporation. Since nectar volume and total sugar production increase as a function of flower age (Galletto and Bernardello, 1993), we collected first-day flowers to standardize measurement of nectar concentration. Plant mean values for each of the floral variables were obtained and used in the analyses.

2.3. Hummingbird assemblages

We recorded the pollinators that visit *N. glauca* flowers in 18 populations from Argentina and southern Bolivia. Observations in each population were made in 6–33 periods of thirty–min, from 8:00 to 18:00 h during 1–15 sunny days. In populations where more than one species was seen visiting the flowers, only the most frequent one was incorporated in the analyses; the most frequent pollinator made at least 60% of all the visits in all cases (see below).

We measured bill lengths of the hummingbird species that were recorded visiting *N. glauca* from specimens deposited at “Museo Argentino de Ciencias Naturales” (MACN) which had been captured in the respective geographic provinces of the study sites. This collection represents the whole distribution range of each species in Argentina and Bolivia. We measured exposed bill culmen lengths (hereafter bill length) with a calliper to the nearest 0.01 mm.

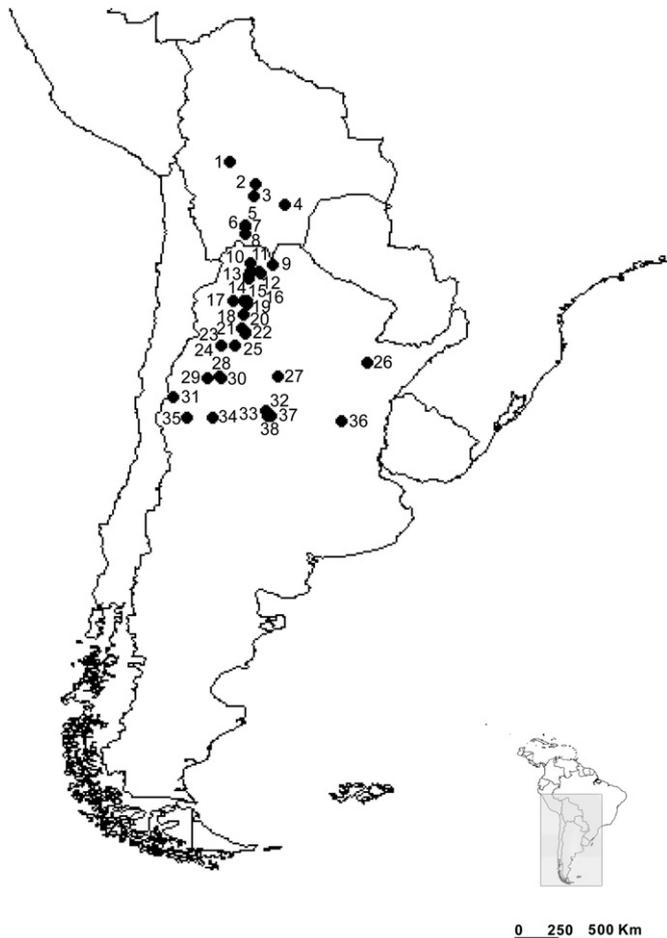


Fig. 1. Location of the 38 *Nicotiana glauca* populations studied in northern Argentina and southern Bolivia. Numbers beside each population correspond to population numbers from Appendix A.

2.4. Climatic and geographic variables

We obtained the January and July mean air temperatures (°C) and yearly mean rainfall (mm) for the 30 Argentine populations, as derivations of agro-climatic potentials, which cover a time span of about 30 years (De Fina, 1992). We included mean air temperature of the warmest summer month (January) and coldest winter month (July). We obtained geographic position and altitude data with a GPS (Garmin, Etrex).

2.5. Statistical analysis

We performed a multivariate analysis of variance (MANOVA) on flower trait measurements to detect between-population differences in all traits. Flower traits were not transformed because they were normally distributed.

To determine which traits best discriminated between populations we performed a discriminant function analysis (DFA) using four floral variables as predictors (corolla length, corolla width, style length, and nectar concentration). We excluded from this analysis three populations whose flowers were lacking nectar. This analysis gives the correlation coefficients between traits and the DFA axes. Thus, the highest correlations indicate which traits best contribute to the discrimination between populations. We calculated Wilk's lambda F statistic. This statistic is useful for assessing the statistical significance of among-group differences in centroid location in the multivariate space (Di Rienzo et al., 2000).

To test the association between phenotypic traits and climatic variables, we performed Pearson product-moment correlations between climatic variables and the first DFA axes of floral phenotype.

We used Mantel tests to determine a possible distance-based pattern of variation, in terms of proximity between populations. We compared geographic and altitudinal distances and phenotypic distances (floral traits) between populations pairs (e.g., Anderson and Johnson, 2008; Pérez-Barrales et al., 2009). The Mantel test is a technique used to estimate the association between two independent matrices describing the same populations and to determine whether the association is stronger than expected by chance (Sokal and Rohlf, 1995). The normalized Mantel coefficient, calculated as the correlation between the pairwise elements of both matrices, was used to test significance. Significance was tested through a randomization test by randomly permuting the order of the elements within one matrix (rows and columns are permuted in tandem). We performed all possible Mantel tests between pairs of distance and dissimilarity matrices. For geographic distance we constructed two matrices, i.e., altitudinal and geographic distances, and for phenotypic dissimilarities, other two matrices, i.e., overall phenotypic distance and distance in mean corolla length only. Altitudinal distances were calculated as the altitudinal differences between populations, and geographic distance, as the flat distance between them. Matrices with geographic and altitudinal distances between all population pairs were built from the geographic positions using the GPS Track Maker program (Ferreira Júnior, 2004).

We also used Mantel tests to determine a possible association between geographic and altitudinal distances matrices with functional differences (bill length) of the hummingbird pollinators. These Mantel tests were performed by building a primary matrix with Euclidean distances in bill length of hummingbird pollinators for populations where these had been recorded and the two secondary matrices either of geographic or altitudinal geographic distances. We also analyzed the association between flower phenotype and bill length dissimilarities with Mantel tests. Finally, we used a correlation to assess the relationship between corolla length and bill length of the most frequent hummingbird pollinator across the 18 populations. We used multiple regressions to analyze simultaneously the effects and relative importance of geographic or climatic predictor variables, together with bill length on corolla length. The geographic variables considered were latitude, longitude, and altitude and the climatic variables considered were January and July mean air temperatures and yearly mean rainfall.

3. Results

3.1. Geographic structure of floral trait variation in *N. glauca*

MANOVA for all flower traits showed significantly different mean values between populations (Wilk's lambda = 0.05; $p < 0.0001$). In the population with the largest flowers, mean flower measures were nearly twice as long as in the populations with the smallest flowers (Table 1). Mean corolla length ranged between 30.42 and 57.57 mm in Cuesta del Obispo II and Potosí, respectively; mean style length ranged between 28.62 and 56.22 mm for the same populations; mean corolla width ranged between 6.02 mm and 11.03 mm recorded in Embarcación and Potosí populations, respectively and mean nectar concentration range between 14.80 g/g and 40.60 g/g in Camiri and Valle Fertil, respectively (Table 1).

The DFA showed significant differences in flower phenotype between the 35 populations (Wilk's lambda 0.02; $p < 0.0001$). The first DFA axis, explaining 75.04% of the total variance, was

Table 1
Mean \pm SD for four floral traits in 38 *N. glauca* populations. Numbers beside each population name correspond to labels from Fig. 1.

Population	Number of plants sampled	Corolla length (mm) \times \pm SD	Corolla width (mm) \times \pm SD	Style length (mm) \times \pm SD	Nectar concentration (g/g) \times \pm SD
1. Cochabamba	12	35.17 \pm 1.48	6.77 \pm 0.55	33.77 \pm 1.16	16.70 \pm 0.64
2. Sucre	15	38.23 \pm 2.41	7.61 \pm 0.49	34.69 \pm 3.13	20.63 \pm 1.91
3. Potosí	10	57.57 \pm 2.49	11.03 \pm 0.75	56.22 \pm 2.37	19.26 \pm 1.18
4. Camiri	10	36.33 \pm 1.05	7.03 \pm 0.26	34.28 \pm 1.89	14.80 \pm 0.21
5. Río Blanco	18	51.20 \pm 2.78	10.52 \pm 0.97	49.52 \pm 2.98	20.76 \pm 2.16
6. Tupiza	69	41.89 \pm 4.94	8.43 \pm 0.88	39.35 \pm 4.50	27.82 \pm 4.80
7. Yuruma	10	48.16 \pm 2.41	9.69 \pm 0.63	45.20 \pm 2.38	18.36 \pm 1.38
8. Ramadas	10	54.93 \pm 3.44	10.44 \pm 0.78	53.41 \pm 4.31	22.48 \pm 0.87
9. Embarcación	23	33.14 \pm 1.17	6.02 \pm 0.45	30.76 \pm 2.01	30.17 \pm 1.93
10. Chorrillos	15	42.09 \pm 1.56	8.34 \pm 0.54	39.57 \pm 1.55	22.60 \pm 1.53
11. San Francisco	10	32.87 \pm 1.00	8.36 \pm 0.40	30.24 \pm 0.98	21.45 \pm 0.85
12. Santa Clara	10	33.37 \pm 1.22	6.36 \pm 0.28	31.02 \pm 1.16	–
13. Tilcara	14	37.98 \pm 1.61	8.35 \pm 0.44	35.98 \pm 1.64	23.95 \pm 3.35
14. El Colorado	12	39.55 \pm 1.61	7.61 \pm 0.21	36.83 \pm 1.54	–
15. Purmamarca	10	37.82 \pm 2.02	8.95 \pm 0.63	34.19 \pm 1.94	–
16. Obispo I	10	35.62 \pm 0.43	7.54 \pm 0.40	33.69 \pm 0.50	24.62 \pm 1.83
17. Obispo II	12	30.42 \pm 1.88	6.90 \pm 0.48	28.62 \pm 1.70	26.27 \pm 3.55
18. Obispo III	10	36.98 \pm 1.01	7.90 \pm 0.34	34.86 \pm 0.46	23.25 \pm 1.21
19. Ampascachi	11	37.89 \pm 3.80	7.01 \pm 0.46	35.44 \pm 3.44	27.90 \pm 2.43
20. Cafayate	12	35.85 \pm 0.95	6.98 \pm 0.28	33.58 \pm 0.88	23.08 \pm 1.10
21. Los Cardones	18	41.09 \pm 2.91	8.33 \pm 0.52	38.54 \pm 2.37	24.95 \pm 6.25
22. Qda de Belén	10	46.34 \pm 4.29	8.50 \pm 0.71	44.85 \pm 4.40	21.14 \pm 1.97
23. La Angostura	15	42.99 \pm 2.66	7.80 \pm 0.69	40.01 \pm 2.67	20.69 \pm 2.99
24. Tafi del Valle	15	42.98 \pm 2.10	7.80 \pm 0.55	38.99 \pm 3.68	21.09 \pm 1.30
25. Cuesta de la Chilca	19	42.10 \pm 3.46	8.46 \pm 0.40	39.42 \pm 3.57	18.16 \pm 1.22
26. Bella Vista	18	37.21 \pm 0.66	6.41 \pm 0.23	35.44 \pm 0.73	23.25 \pm 9.65
27. Loreto	10	36.13 \pm 0.26	6.74 \pm 0.14	33.97 \pm 0.40	36.13 \pm 0.78
28. Sanagasta	22	38.17 \pm 5.48	7.14 \pm 1.19	37.06 \pm 5.45	21.36 \pm 4.82
29. Río Miranda	12	44.30 \pm 2.17	8.32 \pm 0.55	42.76 \pm 2.23	19.16 \pm 1.04
30. Dique Los Sauces	21	43.11 \pm 2.96	7.49 \pm 0.65	41.01 \pm 2.86	31.37 \pm 5.76
31. Cuesta de Miranda	71	39.75 \pm 2.79	8.07 \pm 0.63	37.73 \pm 2.66	25.26 \pm 2.92
32. La Falda	30	34.34 \pm 1.65	6.62 \pm 0.22	32.63 \pm 1.54	22.00 \pm 1.79
33. La Calera	35	40.34 \pm 1.31	6.37 \pm 0.28	38.66 \pm 1.26	20.42 \pm 3.05
34. El Zonda	10	40.80 \pm 0.40	7.17 \pm 0.18	38.69 \pm 0.36	23.53 \pm 1.37
35. Valle Fértil	12	36.82 \pm 0.22	7.93 \pm 0.53	34.78 \pm 0.45	40.60 \pm 8.76
36. Paraná	10	37.00 \pm 2.23	7.22 \pm 0.43	35.91 \pm 2.23	25.60 \pm 3.17
37. Costa Azul	86	38.04 \pm 2.44	7.20 \pm 0.46	36.34 \pm 2.14	22.56 \pm 2.01
38. Córdoba	32	31.98 \pm 2.20	6.97 \pm 0.72	31.23 \pm 2.14	23.56 \pm 1.19

influenced mostly by the three morphometric characters, as shown by their high standardized discriminant coefficients, and weakly by nectar concentration (Table 2). Since nectar concentration was very weakly correlated with this axis and only accounted for a small portion of the interpopulation variation, we performed subsequent analyses only with morphometric traits. The second axis only explained 8.61% (accumulated 83.65%) of the total variance and was moderately correlated with corolla width and style length (Table 2).

3.2. Relationship between floral traits and abiotic factors and distance-based pattern in *N. glauca*

Correlations between DFA axes of flower phenotypic variation and climatic variables showed strong associations of the first two

phenotypic axes with two climatic variables (January and July mean temperature). In addition, the first phenotypic axis was associated with rainfall (Table 3).

Altitude ranged from 7 to 3327 m, whereas geographic distances varied from 9 to 1678 km, with Paraná and Cochabamba as the most distant populations. The Mantel tests showed significant associations between the altitudinal distance matrix and the two phenotypic distance matrices (i.e., overall phenotypic distance and distance in mean corolla length only) meaning that differences in phenotypic distances were structured by altitudinal distances (Table 4, Fig. 2). The geographic distance matrix did not show association with phenotypic matrices (Table 4), indicating that differences in phenotypic distances were not significantly structured by geographic distances.

Table 2
Standardized discriminant coefficients and percentage of explained variance of the first two axes of a Discriminant Function Analysis (DFA) for four flower traits (corolla length, style length, corolla width, and nectar concentration) in 35 populations of *N. glauca* from northern Argentina and southern Bolivia.

	Canonical axis 1	Canonical axis 2
Corolla length	–0.873	–0.344
Style length	–0.866	–0.496
Corolla width	–0.758	0.534
Nectar concentration (g/g)	–0.180	–0.408
Variance explained (%)	75.04	8.6

Table 3
Pearson product–moment correlation coefficients (*r*) between climatic variables (January and July mean air temperature and mean annual rainfall) and the first two axes of a DFA for four flower traits (corolla length, style length, corolla width, and nectar concentration) in 30 populations of *N. glauca* from northern Argentina and southern Bolivia.

	Canonical axis 1	Canonical axis 2
January temperature	–0.341**	–0.455***
July temperature	–0.310**	–0.251**
Rainfall	–0.364**	–0.071

p* < 0.01; *p* < 0.001.

Table 4

Mantel pairwise comparisons between distance matrices constructed with hummingbird bill length, floral traits (corolla length, width, style length and nectar concentration), and geographic distances between *N. glauca* populations. Z is the Mantel statistic of association and r is the moment–product correlation between the matrices.

	Matrices	Geographic distance	Altitudinal distance	Hummingbird bill length $((y_i - y_j)^2)^{1/2}$	Phenotypic distance (Euclidean)	Corolla length $((y_i - y_j)^2)^{1/2}$
Geographic distance (38 populations)		Z	0.457	0.195	0.760	0.589
		r	0.245*	0.209**	0.190	0.052
Altitudinal distance (38 populations)		Z		0.814	0.231	0.124
		r		0.294**	0.287**	0.256**
Hummingbird bill length (18 populations)	$((y_i - y_j)^2)^{1/2}$	Z			0.207	0.401
		r			0.224*	0.349**
Flower traits (38 populations)	Phenotypic distance (Euclidean)	Z				0.167
		r				0.690**
	Corolla length $((y_i - y_j)^2)^{1/2}$	Z				
		r				

* $p < 0.05$; ** $p < 0.005$.

3.3. Hummingbird assemblage among *N. glauca* populations and geographic structure of functional trait variation

Six hummingbird species were the most frequent pollinators in the 18 populations where pollinators were recorded (Table 5). We recorded more than one pollinator species (Table 5) in only three populations. Bill length of these hummingbirds ranged from 16.64 mm in *Chlorostilbon lucidus* to 49.20 mm in *Patagona gigas* (Table 5).

The Mantel test showed a significant association between bill length of pollinating hummingbirds and both geographic and altitudinal distance matrices (Table 4). Thus, distant populations had the greatest differences in bill length among the most frequent pollinators.

3.4. Relationships between corolla length and bill length among *N. glauca* populations

Population mean values for corolla length of *N. glauca* and bill length of the most frequent hummingbird pollinator were significantly correlated $r = 0.69$; $p < 0.01$ (Fig. 3). The Mantel tests showed

significant associations of both flower phenotype and corolla length with the bill length matrix (Table 4). For the multiple regressions that include bill length with geographic or climatic predictor variables, bill length remained a significant predictor of corolla length (Tables 6 and 7).

4. Discussion

Previous studies conducted on a low number of populations revealed a pattern of phenotypic variation (Nattero and Cocucci, 2007) and of phenotypic selection in corolla length (Nattero et al., 2010). The major goal of the present study was to determine if such pattern could be extrapolated to the scale of the species' natural range, i.e., central Argentina and southern Bolivia, and to which extent environmental factors are potential drivers of local flower phenotype in this species.

4.1. Spatial pattern of floral trait variation and hummingbird pollinators

We confirmed previous reports on variation of *N. glauca* floral traits (Nattero and Cocucci, 2007), using a larger population data set ($n = 38$) that covers the natural range of this species. The DFA showed that populations can be discriminated by three morphometric traits (corolla length, corolla width and style length). The highest percentage of variation of the mean flower phenotype was due to differences among populations and between-population variation was better explained by morphometric characters than by nectar concentration. Consequently, morphometric floral traits would have more potential for geographic differentiation of populations than nectar concentration.

Altitudinal distance is apparently a more decisive factor in phenotypic differentiation, as shown by the stronger and significant association between floral phenotype and altitudinal distance. There are at least two hypotheses that should be explored to explain the departure from expectations of isolation by distance: i) because of the mode of seed dispersal (gravity) and pollen dispersal (hummingbirds), gene exchange could be more difficult across vertical than across horizontal distances; ii) phenotypic differences could be better explained by phenotypic plasticity (genotypic by environment effect) across altitudinal ranges. Correlation analysis revealed that this species was significantly correlated with climatic variables (January and July mean air temperature and mean annual rainfall). This suggests the existence of a geographic cline, which could be an indirect expression of a climatic cline, because

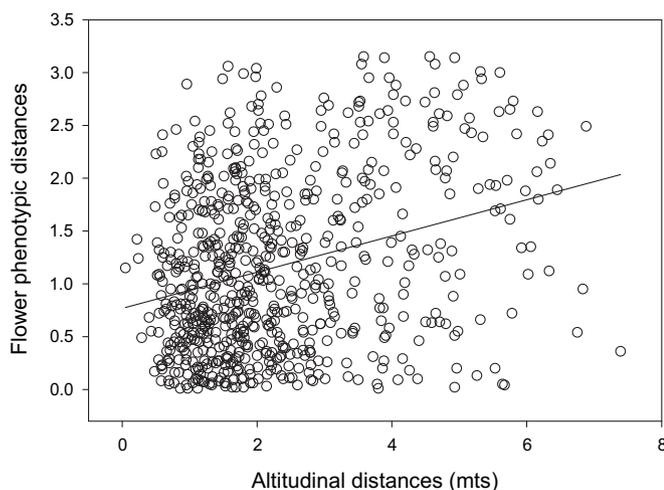


Fig. 2. Scatterplot of pairwise values of flower phenotypic distances (Euclidean distances) against altitudinal distances for 38 populations of *Nicotiana glauca*. Some data points are obscured owing to overlap.

Table 5
Hummingbird pollinator species, percentage of visits, number of flowers visited, mean bill length (regional mean) and mean corolla length for 18 *N. glauca* populations from northern Argentina and southern Bolivia (modified from Nattero and Cocucci, 2007; Nattero et al., 2010).

Population	Hummingbird species	Percentage of visits at each site	Number of flowers visited (number of 30-min. observation periods)	Mean bill length $\bar{x} \pm \text{SD}$ (n)	Mean corolla length
Bella Vista	<i>Chlorostilbon lucidus</i>	100	29 (6)	20.49 \pm 1.06 (8)	37.21 \pm 0.66
Cafayate	<i>C. lucidus</i>	100	41 (8)	19.37 \pm 0.72 (6)	35.85 \pm 0.95
Cuesta de Miranda	<i>Helimaster furcifer</i>	75	137 (31)	33.93 \pm 0.69 ^{*2} (15)	40.99 \pm 1.91
	<i>Patagona gigas</i>	25	46 (31)	44.30 \pm 1.02 (4)	
Córdoba	<i>C. lucidus</i>	100	32 (12)	16.64 \pm 1.17 ^{*1} (6)	35.94 \pm 1.57
Costa Azul	<i>C. lucidus</i>	100	94 (33)	16.64 \pm 1.17 ^{*1} (6)	38.35 \pm 2.35
La Angostura	<i>Sappho sparganura</i>	100	34 (8)	21.53 \pm 2.09 (7)	39.89 \pm 1.51
Dique Los Sauces	<i>H. furcifer</i>	100	38 (8)	33.93 \pm 0.69 ^{*2} (15)	43.11 \pm 2.96
Embarcación	<i>S. sparganura</i>	100	54 (8)	21.16 \pm 1.86 (5)	33.14 \pm 1.17
La Calera	<i>C. lucidus</i>	100	45 (8)	16.64 \pm 1.17 ^{*1} (6)	39.62 \pm 1.18
La Falda	<i>C. lucidus</i>	60	10 (20)	16.64 \pm 1.17 ^{*1} (6)	37.10 \pm 2.62
	<i>S. sparganura</i>	20	10 (20)	21.92 \pm 1.02 (6)	
	<i>H. furcifer</i>	20	48 (20)	31.04 \pm 1.26 (9)	
Los Cardones	<i>Oreotrochilus leucopleurus</i>	90	90 (8)	19.46 \pm 1.08 (3)	42.18 \pm 3.11
	<i>S. sparganura</i>	10	8 (8)	20.04 \pm 0.69 (6)	
Obispo II	<i>C. lucidus</i>	100	11 (6)	19.37 \pm 0.72 (8)	30.42 \pm 1.88
Obispo III	<i>P. gigas</i>	100	16 (6)	49.20 \pm 1.37 (4)	36.98 \pm 1.01
Quebrada de Belén	<i>P. gigas</i>	100	21 (6)	43.42 \pm 1.09 (5)	46.34 \pm 4.29
Río Blanco	<i>P. gigas</i>	100	15 (6)	43.89 \pm 0.86 ^{*3} (8)	51.19 \pm 2.78
San Francisco	<i>Leucippus chionogaster</i>	100	36 (12)	26.36 \pm 1.19 (5)	32.87 \pm 1.01
Tilcara	<i>H. furcifer</i>	100	32 (8)	33.48 \pm 0.56 (4)	37.98 \pm 1.61
Tupiza	<i>P. gigas</i>	100	136 (13)	43.89 \pm 0.86 ^{*3} (8)	47.78 \pm 3.12

*1, *2, *3 correspond to bill length measures of the same hummingbird species from the same geographic region.

correlations between altitude, the most influential geographic variable, and all climatic variables were also strong and significant (altitude–January temperature $r: -0.84, p < 0.001$; altitude–July temperature $r: -0.39, p < 0.001$; altitude–Rainfall $r: -0.58, p < 0.001$). Accordingly, some climatic factors would be contributing to the geographic differentiation of floral traits. For example, the effects of prevailing seasonal winds, which probably determine seasonal changes in temperature and precipitation, would differ among the locations sampled. As demonstrated in several species (e.g., Endler, 1977; Jonas and Geber, 1999), flower and plant morphology changes in response to very simple abiotic factors, such as temperature, photoperiod, altitude, etc., which are unrelated to effects of local pollinators. Under this scenario, pollinators may select *N. glauca* populations and even plants within populations according to flower morphology.

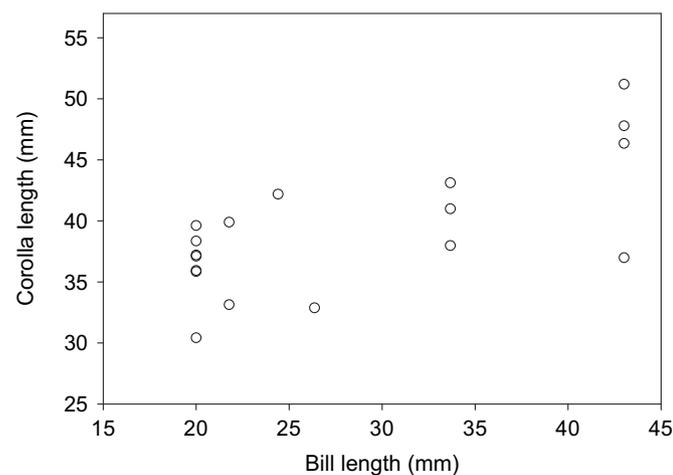


Fig. 3. Relationship between hummingbird bill length and corolla length of *Nicotiana glauca* flowers in 18 populations. Each symbol represents the mean trait value per population.

Differentiations in floral traits were structured by altitudinal distances. However, we did not measure vegetative traits or genetic markers to contrast with flower trait variation among populations and studies including those parameters remain to be done. Floral and vegetative traits belong to different plant modules, which are subjected to different environmental factors and selective agents, and variation of these two kinds of traits can be independent (e.g., Berg, 1960; Armbruster et al., 1999; Pérez-Barrales et al., 2009). Floral trait clines are related to latitudinal variation or geographic distances either in morphometric traits (Boyd, 2002; Silva-Montellano and Eguiarte, 2003; Herrera et al., 2006; Pérez-Barrales et al., 2009) or relative frequencies of flower morphs (Barrett et al., 2004). Blionis and Vokou (2002) detected altitudinal variation in flower phenotype (flowering time) in a *Campanula spatulata* subspecies. Anderson and Johnson (2008) found an altitudinal cline variation in proboscis length of the flies that pollinated *Zaluzianskya microsiphon* but not in the corolla length of the flowers. Lack of geographic cline in the results of other studies is attributable to a small geographic scale, the low number of populations studied or to very strong dispersal barriers. For example, Domínguez et al. (1998) found that most floral variation of *Rhizophora mangle* was evident between populations of Atlantic and Pacific coasts and not attributable to latitude.

Hummingbird bill length of the most frequent pollinator at each population was structured both by geographic and altitudinal distances. The altitudinal structure mainly seems to support the prediction that habitat is an important determinant of the evolutionary dynamics of flower–pollinator interaction across the *N. glauca* natural range distribution. However, studies examining environmental gradients of vegetative trait variation and genetic variation (neutral markers) are necessary to contrast these results.

4.2. Evolution of flower phenotype in *N. glauca*

The pattern of variation in flower phenotype was also significantly explained by a morphological trait of the most frequent pollinator: bill length of the hummingbird in each population.

Table 6Multiple regression model that tests the effect of bill length, together with geographic predictors variables, on corolla tube length of *N. glauca*.

Response variable	Bill length	Corolla length	Latitude	Longitude	Altitude	Model R^2	F	p
Corolla length	0.45**	–	0.63	–0.43	–0.00	0.55	3.93	0.0265

** $p < 0.001$.**Table 7**Multiple regression model that tests the effect of bill length, together with climatic predictors variables, on corolla tube length of *N. glauca*.

Response variable	Bill length	Corolla length	January temperature	July temperature	Rainfall	Model R^2	F	p
Corolla length	0.28*	–	0.20	–0.08	–0.00	0.31	1.22	0.356

* $p < 0.01$.

This pattern is in turn associated with the congruent variation between altitude and bill length across populations, as shown by the Mantel Test. A significant correlation between mean corolla length and mean bill length of the most frequent hummingbird pollinator in this system reported in a previous study supported the hypothesis of differentiation in local ecotypes (Nattero and Cocucci, 2007). The present study provides further evidence to support that hypothesis; indeed, at least in 18 populations evaluated, this correlation is maintained across a large scale and showed that populations of *N. glauca* with shorter or longer flowers had short or long-billed hummingbirds as the most frequent pollinators, respectively.

Most studies addressing the association between geographic variation in flower phenotype and pollinator variation have focused on species composition and visitation frequencies of the pollinator assemblages; however, very few take into account morphological traits of the pollinators that are relevant in the flower-pollinator fit (see review in Herrera et al., 2006 and also Medel et al., 2007, Anderson and Johnson, 2008). Nevertheless, in other plants variation in flower phenotype has been reported to be related to morphological variations of pollinators. Robertson and Wyatt (1990) found that differences in spur length of mountain and lowland populations of *Platanthera ciliaris* could be attributed to differences in proboscis length of butterflies in both regions. Other authors found that the evolution of spur length in the *Disa draconis* complex (Johnson and Steiner, 1997) and of *Satyrrium hallackii* (Johnson, 1997) has a similar basis, with flowers adjusted to different local pollinator species. In a study of the specialized mutualism between *Z. microsiphon* and the long-tongued fly *Prosoeca ganglbaueri*, Anderson and Johnson (2008) found that a strong correlation between the length of both the fly's proboscis and the flower's corolla tube. Similar results were obtained by Boyd (2002) from a multivariate study of *Macromeria viridiflora*, in which plants with short corollas occurring in northern populations were visited by smaller hummingbirds, whereas plants with long corollas present in southern populations were visited by larger ones. However, this conclusion is based on a categorical distinction of hummingbird body size.

The role of pollinators in the genus *Nicotiana* is one of the keys to understanding floral evolution and adaptive radiation in the Solanaceae family (Knapp, 2010). Floral evolution in this genus presents a great adaptive radiation owing to different pollinators (Knapp, 2010). According to Knapp (2010) results, adaptive evolution in *Nicotiana* operates at the micro and macroevolution scale. Beside selection, gene flow can take place at the same time in populations and still evolutionary processes can be detected. However, as we have not measured gene flow, we cannot rule out its importance. Following the five-step approach proposed by Herrera et al. (2006) to determine the causes of geographic variation in floral traits, and based on the present results, we

confirmed that two steps are met: (1) There were differences between populations in functional traits of pollinators (bill length) of the most frequent pollinator species at the local scale (see also Nattero and Cocucci, 2007) and these differences had a geographic structure. (4) Flower size varied gradually with altitude across populations and this variation was accompanied by bill length variation.

Two other steps of this approach, namely: (2) phenotypic selection on corolla tube length via its influence on the maternal fitness component and (3) geographic differences in selection, were addressed elsewhere (Nattero et al., 2010). Corolla length has been identified as target of hummingbird-mediated phenotypic selection in six populations and the direction of selection is in agreement with the degree of morphological match between flower length and hummingbird bill length (Nattero et al., 2010).

The complete confirmation that population variation in floral traits is promoted by divergent natural selection awaits demonstration of the genetic basis of flower phenotypic variation in this species. However, the heritability of corolla tube length largely demonstrated in other species of *Nicotiana* (*N. langsdorffii*, *N. alata* and *N. longiflora*) (East, 1916; Anderson, 1939) strongly suggests the existence of genetic variation in this species. The studies on the floral evolution of this system we have conducted so far have contributed with strong evidences that suggest that pollinators are, at least in part, the cause of floral phenotypic differentiation in this plant species along its wide geographic range probably resulting from local adaptation of relevant floral traits.

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Appendix A

Site name, country, geographic position, altitude, January and July mean air temperature and mean annual rainfall of the 38 *N. glauca* Graham populations of northern Argentina and southern Bolivia. Numbers beside each population name correspond to labels from Fig. 1.

Population	Country	S Latitude	W Longitude	Altitude (m)	January mean air temperature (°C)	July mean air temperature (°C)	Mean rainfall (mm)
1. Cochabamba	Bolivia	17°40'02.0"	66°28'54.3"	3193			
2. Sucre	Bolivia	18°51'29.1"	65°08'31.8"	1868			
3. Potosí	Bolivia	19°28'58.0"	65°13'01.0"	2627			
4. Camiri	Bolivia	19°56'39.0"	63°31'26.4"	845			
5. Río Blanco	Bolivia	21°03'34.7"	65°38'23.4"	3401			
6. Tupiza	Bolivia	21°32'30.1"	65°38'20.5"	2910			
7. Yuruma	Bolivia	21°08'34.7"	65°38'23.4"	3401			
8. Ramadas	Bolivia	21°03'43.1"	65°38'15.2"	3250			
9. Embarcación	Argentina	23°14'49.2"	64°08'15.0"	287	28.1	15.3	421
10. Chorrillos	Argentina	23°05'16.2"	65°22'46.4"	3300	17.5	7.8	324
11. San Francisco	Argentina	23°36'22.1"	64°57'18.6"	1500	26.3	13.9	751
12. Santa Clara	Argentina	23°42'23.8"	64°51'26.4"	648	25.7	13.5	560
13. Tilcara	Argentina	23°35'35.0"	65°24'41.5"	2495	16.2	6.9	146
14. El Colorado	Argentina	23°45'02.1"	65°30'09.0"	2442	17.5	7.8	111
15. Purmamarca	Argentina	23°58'05.3"	65°27'01.3"	2020	17.5	7.8	111
16. Obispo I	Argentina	25°08'11.7"	65°36'23.6"	1207	21.9	10.9	575
17. Obispo II	Argentina	25°10'47.7"	65°46'35.4"	2248	16.3	7.2	115
18. Obispo III	Argentina	25°10'43.5"	65°48'59.9"	2670	17	7.7	146
19. Ampascachi	Argentina	25°20'55.8"	65°31'38.6"	1219	22.9	11.4	458
20. Cafayate	Argentina	25°55'42.2"	65°43'12.5"	1445	20.2	9.2	198
21. Los Cardones	Argentina	26°40'17.7"	65°49'04.1"	2734	19.8	8.6	240
22. Qda de Belén	Argentina	27°34'18.6"	67°00'03.0"	1300	24.9	9.2	411
23. La Angostura	Argentina	26°55'47.3"	65°40'36.5"	1930	18.6	18.1	398
24. Tafí del Valle	Argentina	26°53'28.6"	65°41'25.5"	1977	18.6	18.1	398
25. Cuesta de la Chilca	Argentina	27°38'10.1"	66°11'07.0"	1172	25.3	9.9	308
26. Bella Vista	Argentina	28°30'35.7"	59°03'02.5"	89	26.7	14.5	1045
27. Loreto	Argentina	29°16'28.4"	63°50'52.3"	369	25.8	11.5	597
28. Sanagasta	Argentina	29°15'49.5"	67°04'40.8"	930	25.2	9.7	103
29. Río Miranda	Argentina	29°20'14.4"	67°41'31.5"	1559	24.8	8.3	178
30. Dique Los Sauces	Argentina	29°23'25.7"	66°57'52.5"	517	26.8	11.2	327
31. Cuesta de Miranda	Argentina	30°23'32.7"	69°34'18.1"	1240	23.4	8.4	71
32. La Falda	Argentina	31°05'49.5"	64°30'34.4"	728	22.2	10.1	992
33. La Calera	Argentina	31°21'27.5"	64°19'08.1"	535	23.9	10	701
34. El Zonda	Argentina	31°30'45.4"	68°47'22.1"	622	25.9	7.8	95
35. Valle Fértil	Argentina	31°31'33.3"	67°23'52.0"	629	26.2	9.4	339
36. Paraná	Argentina	31°43'39.5"	60°29'07.3"	74	26.7	14.5	1045
37. Costa Azul	Argentina	31°23'48.4"	64°26'34.0"	148	23.6	9.9	709
38. Córdoba	Argentina	31°23'52.2"	64°13'58.6"	458	24.3	17.4	677

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