

Pollinator-mediated selection in a specialized pollination system: matches and mismatches across populations

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Abstract

Most studies on pollinator-mediated selection have been performed in generalized rather than specialized pollination systems. This situation has impeded evaluation of the extent to which selection acts on attraction or specialized key floral traits involved in the plant-pollinator phenotypic interphase. We studied pollinator-mediated selection in four populations of *Nierembergia linariifolia*, a self-incompatible and oil-secreting plant pollinated exclusively by oil-collecting bees. We evaluated whether floral traits experience variable selection among populations and whether attraction and fit traits are heterogeneously selected across populations. Populations differed in every flower trait and selection was consistently observed for corolla size and flower shape, two traits involved in the first steps of the pollination process. However, we found no selection acting on mechanical-fit traits. The observation that selection occurred upon attraction rather than mechanical-fit traits, suggests that plants are not currently evolving fine-tuned morphological adaptations to local pollinators and that phenotypic matching is not necessarily an expected outcome in this specialized pollination system.

Introduction

Pollinator-mediated selection is considered a major factor in shaping the evolution of the flower phenotype (e.g. Stebbins, 1970; Faegri & van der Pijl, 1979; Waser, 1983; Fenster *et al.*, 2004). The preference of pollinator species for some flowers variants within populations often creates a within-population covariance between some components of the flower phenotype and plant fitness that influences the subsequent evolution of flower characters provided that some genetic assumptions are fulfilled (Lande & Arnold, 1983). A multitude of studies on pollinator-mediated selection have been carried out in the last 25 years (reviewed in Kingsolver *et al.*, 2001; Herrera *et al.*, 2006), confirming that pollinators are a

driving force in the evolution of flower traits, such as corolla size (e.g. Campbell, 1989; Galen, 1989; Conner *et al.*, 1996; Medel *et al.*, 2003), corolla shape (e.g. Herrera, 1993; Nagy, 1997; Gómez *et al.*, 2006), corolla colour (e.g. Campbell *et al.*, 1997; Nagy, 1997), stigma exertion (e.g. Conner *et al.*, 1996), nectar guides (e.g. Medel *et al.*, 2003) and flowering date (e.g. Campbell, 1989; Johnston, 1991). Implicit in most of these studies is the idea that the morphology of flowers reflects the action of long-term natural selection imposed by particular guilds of pollinators (Waser, 1983; Waser *et al.*, 1996). Pollinator-mediated selection is by definition a local scale phenomenon. However, interactions between plants and pollinators have different local evolutionary outcomes depending on the community context of each population. Unfortunately, most evidence of pollinator-mediated selection has come from single rather than replicated populations (see review in Herrera *et al.*, 2006), which complicates our ability to make useful generalizations about the evolution of plant-pollinator relationships in broader spatial and temporal contexts. For example, several studies have shown variable selection as populations often face variation in the species

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composition of pollinator assemblages across localities (e.g. Campbell *et al.*, 1997; Totland, 2001; Caruso *et al.*, 2003; Anderson & Johnson, 2008), and/or variation in local abiotic conditions (e.g. Caruso *et al.*, 2003; Maad & Alexandersson, 2004). Because plants may adapt to different pollinator species across populations, identification of the relevant floral traits involved in local adaptation represents an important step in understanding the mechanisms that determine the formation of ecotypes and incipient pollinator-mediated speciation (see reviews in Waser & Campbell (2004) and Herrera *et al.* (2006).

The phenotypic pollinator-plant interface, defined as the set of traits that mediate the interaction, is a useful context for understanding potential variation in reciprocal selection across the landscape (Thompson, 2005). Despite some variation in method and interpretation (e.g. Gomulkiewicz *et al.*, 2007; Hanifin *et al.*, 2008), the extent of phenotypic matching and mismatching may roughly represent the potential for reciprocal selection. High levels of phenotype matching imply a potential for strong reciprocal selection because any change in the phenotypic mean of one species is expected to have an important fitness impact on the other. On the contrary, low levels of matching are indicative of low potential for reciprocal selection because of the different metric scale of the involved phenotypes. Recent advances in evolutionary theory, however, suggest that phenotype matches and mismatches across sites can be a common outcome in systems subject to coevolutionary dynamics (e.g. Brodie & Ridenhour, 2003; Whittall & Hodges, 2007; Hanifin *et al.*, 2008; Anderson & Johnson, 2009). Furthermore, because only one partner may evolve adaptations to the interaction in some localities but not others, there is a strong potential for the occurrence of complex coevolutionary mosaics at the geographical level (Thompson, 2005; Anderson & Johnson, 2009).

Flower traits under pollinator-mediated selection can be classified in two categories. First, attraction traits involved in pollinator attraction are important during the first step of the pollination process but do not necessarily participate in the process of pollen removal from anthers and pollen deposition on stigmas. Because pollinators often discriminate between flowers in response to flower size, shape, colour and number (e.g. Waser & Price, 1983; Geber, 1985; Campbell, 1989; Galen & Stanton, 1989; Mitchell, 1994; Vaughton & Ramsey, 1998), it is expected that floral characters engaged in pollinator attraction evolve in situations of pollen limitation and pollinator discrimination among floral variants (e.g. Johnston, 1991; Totland, 2001). Second, traits involved in the mechanical fit between flower and pollinator structures are involved in the fine-tuned phenotypic matching of the pollination process and permit quantification of the match or mismatch in the phenotypic interphase between flowers and pollinators. Mechanical-fit traits are thus most relevant to the second step of the pollination process, after pollinators have already been

attracted to flowers (see Fenster *et al.*, 2004). Matched phenotypes in plant-pollination relationships require precision (*sensu* Armbruster *et al.*, 2004), that is, a consistent morphology that ensures that some floral parts and not others contact the same part of the pollinator body in repeated visits and/or influence the movement of the pollinator whereas natural selection will always work on accuracy (*sensu* Armbruster *et al.*, 2004), that is, the closeness of the individual plants to an optimum. Even though attraction and mechanical-fit traits are necessary for successful pollination, most studies on pollinator-mediated selection have focused on the first.

In this paper, we evaluate the extent to which changes in the pollination environment influence the strength and form of selection acting upon flower traits involved in pollinator attraction and mechanical fit in a specialized pollination system. *Nierembergia linariifolia* Graham (Fig. 1a) is a plant species with oil-secreting flowers that receives the pollination service of solitary oil-collecting

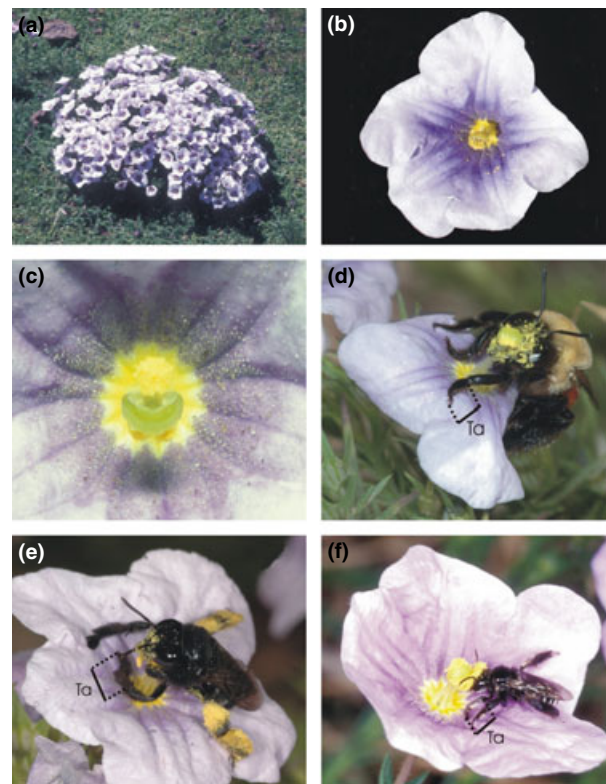


Fig. 1 Visual description of the study system. (a) Plant of *Nierembergia linariifolia* in the flowering peak. (b) Frontal view of the flower showing the labellum in the upper side. (c) Detail of the elaiophore area showing the stamen and stigma column surrounded by oil-secreting glands. (d) The large-sized *Centris tricolor* collecting oil with basitarsus and mediotarsus of forelegs. (e) *Tapinotaspis chalybaeae* collecting oil with the basitarsus and mediotarsus of the mid legs. (f) The tiny bee *Chalepogenus nigripes* collecting oil with basitarsus of the forelegs. Ta: tarsus.

bees. This pollination system has been previously described as a very specialized mutualistic relationship because the plant depends exclusively on a restricted group of pollinators for reproduction (Cocucci, 1991; Cosacov *et al.*, 2008) and pollinator bees have highly specialized morphological structures (Cocucci *et al.*, 2000). This system is especially suitable for this study for three reasons. First, previous studies indicate that pollinator bee species of *N. linariifolia* vary strongly in their oil-collecting behaviour and morphological structures (Cocucci, 1991; Cocucci *et al.*, 2000). Second, there is ample variation in the species composition of the oil-collecting bee assemblage across populations of *N. linariifolia* (Cosacov *et al.*, 2008). Third, *N. linariifolia* is pollen-limited by quantity (i.e. *via* pollinator visits and number of pollen grain deposited on stigmas) and quality (i.e. *via* compatibility of the pollen delivered) in different populations and depends on pollinators for reproduction (Cosacov *et al.*, 2008). Given that we have previous information about pollinator assemblages at the study area that would allow us to formulate predictions about the strength and targets of selection at different sites, the aim of this paper is to identify the floral traits (attraction or mechanical-fit traits) under selection in this specialized pollination system. We also evaluate whether the resulting selection pattern among populations reflects expectations from the geographical mosaic of evolving interactions. More specifically, we evaluate (i) whether floral traits experience variable selection among populations and (ii) whether populations differ in the types of traits under selection, that is, whether attraction and fit traits are heterogeneously selected across populations.

Materials and methods

Pollination natural history

Nierembergia linariifolia (Solanaceae) is a perennial shrub endemic to Central Argentina. Flowers are violet and salverform, i.e. with a thin nectarless tube and a well develop, conspicuous, somewhat horizontally spreading part of the corolla, the corolla limb (Figs 1a, b and 2a). One petal lobe of the corolla limb, which is called the labellum, is usually larger than the remaining lower ones (Fig. 1b). The very thin corolla tube serves to display the limb above the foliage. The fertile parts, consisting of five stamens and the stigmatic portion of the pistil are exerted, emerging from the centre of the almost flat limb (Cocucci, 1991). Flower reward is represented in this genus by fatty oils distributed in a restricted area of the corolla limb (Fig. 1c). Flowers are scentless and last about 3 days. Previous studies have reported that pollinating bees belong to three different genera: *Centris* (*C. tricolor*), *Tapinotaspis* (*T. chalybaea*) and *Chalepogenus* (*C. nigripes*, *C. brevipilli* and *C. parvus*) (Simpson & Neff, 1981; Cocucci, 1991; Cocucci *et al.*, 2000; Cosacov *et al.*, 2008). *Centris tricolor* (Fig. 1d) has oil-collecting structures

on the basitarsus and mediotarsus of the forelegs and mid legs. In *N. linariifolia*, *C. tricolor* uses only the forelegs to collect oil. When this bee lands on the flowers, it takes the stamen column in its mandible and touches the fertile part with its head while sweeping the elaiophore area (Fig. 2a). *Tapinotaspis chalybaea* (Fig. 1e) visits *N. linariifolia* flowers exclusively (Cocucci, 1991; Cocucci *et al.*, 2000), and their oil-collecting structures are located on the basitarsus and mediotarsus of the mid legs. When this bee visits the flowers, it lands on and embraces the central column with the forelegs. The hind legs often help to position the bee on the flower by grabbing the interlobular notches between the ventral and the lateral

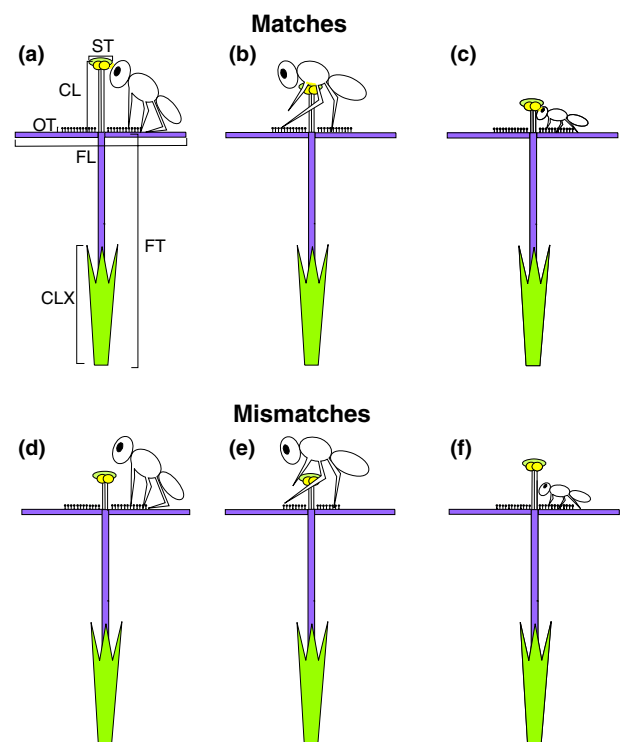


Fig. 2 Pollination mechanisms and matches and mismatches among floral mechanical-fit-related traits of *Nierembergia linariifolia* and pollinating bees. Matches: (a) Pollination mechanism of *Centris tricolor*, it only uses the forelegs to collect oil and touches the fertile parts of the flower with its head. (b) Pollination mechanism of *Tapinotaspis chalybaea*, it only uses mid legs to collect oil and contacted with the ventral side the fertile part of the flower. (c) Pollination mechanism of *Chalepogenus nigripes*, it uses the forelegs to collect oil and contacted with its head the fertile part of the flower. (d–f) Mismatches: (d) Small column prevent *C. tricolor* to contact fertile structures located at the top of the column. (e) Small columns do not reach the ventral side of *T. chalybaea*. (f) Large columns prevent the tinea bee *C. nigripes* from contacting the stamens and stigma. Large elaiophore areas may allow bees to collect oil on the periphery of the elaiophore without touching the fertile parts of the flowers. Flower traits in (a): ST, stigma; CL, column length; OT, oil trichome; FL, flower limb; FT, flower tube; CLX, calyx.

lobes of the flower. While sweeping the elaiophore area with semicircular movements of the extremely long mid legs, the fertile organs of the flower are contacted with the ventral side of the insect (Fig. 2b) (Cocucci *et al.*, 2000). *Chalepogenus* bee species are smaller than the other described bees; they also differ in having the oil collectors on the basitarsus of the forelegs only (Fig. 1f).

Study sites and sampling procedure

This study was conducted during the austral spring and summer of 2006–2007 in four populations: Capilla del Monte (CM hereafter, 30°52'31.0"S, 64°32'12.1"W, 980 masl), La Toma (LT hereafter, 30°49'56.5"S, 64°29'30.1"W, 1390 masl), Pampa de Achala (PA hereafter, 31°36'44.8"S, 64°48'44.2"W, 2194 masl) and Villa Ani-mí (VA hereafter, 31°00'54.7"S, 64°17'07.1"W, 674 masl). Visitation rate per flower was recorded on 50 randomly tagged plants per population. Observations were performed during several days on each population to cover the whole flowering period (2 months). For each observation day we recorded, the number of open flowers per plant, the number of times each plant was visited and the number of flowers probed per plant visit; we observed all study plants simultaneously for pollinator visits. In addition, at each population, pollinator bees were captured and their oil-collecting structures (for *C. tricolor* basitarsus and meditarisus of forelegs, for *T. chalybaea* basitarsus and meditarisus of the mid legs, for *C. nigripes* basitarsus of forelegs) (Fig. 1d–f) measured under a stereomicroscope using a digital caliper. In 101–175 plants per population, flower characters were measured from digital photographs in three flowers per plant, both from frontal and lateral perspectives. On the base of these measurements, we distinguished two categories of characters depending on their role in the pollination process: (a) Attraction-related traits, which include corolla size (area), total floral display and flower shape; and (b) Mechanical-fit-related traits, which include fertile column length (measured from the base of the limb to the top of the anthers) and elaiophore size (area). In this study, plant

flower shape is not acting in a way that it mechanically guides visitors to make contact with fertile organs. It may instead be important in short-distance orientation before landing. The fertile column length is a morphological trait that influences pollen transfer through adjustments with bee body size and behaviour (Fig. 2a–f). While large columns prevent small bees from contacting the fertile structures located at the top of columns (e.g. *C. nigripes*, Fig. 2f), small columns do not reach the ventral side of large bees (e.g. *T. chalybaea*, Fig. 2e). Elaiophore area, in turn, is a critical trait involved in effective pollination. It determines whether a bee moves close enough to the central column to touch it with its head while collecting oil. This trait allows (or excludes) bees from collecting oil near the fertile column depending on the reciprocal size adjustment (Fig. 2a–f). For example, large elaiophore areas may allow bees to collect oil on the periphery of the elaiophore without touching the fertile column of flowers (Fig. 2d–f). Even though the elaiophore area of *N. linariifolia* reflects UV wavelength and hence may signal reward to pollinators, visual guides are considered to act mainly as cues for short-distance orientation and landing on flowers (e.g. Lehrer *et al.*, 1995; Dafni & Kevan, 1997; Johnson & Dafni, 1998). We estimated a mechanical-fit index (MFI) between pollinators and flowers at each population from $MFI = 1 - (\text{mean oil-collecting structure length} / \text{mean elaiophore radius})$. According to this index, $MFI = 0$ indicates exact matching, $MFI > 0$ indicates that elaiophores are larger than insect oil-collecting structures, and $MFI < 0$ indicates that elaiophores are smaller than insect structures. MFI standard deviation was calculated by resampling the MFI with a jackknife procedure. Number of bees and flowers used to calculate the MFI at each population is given in Table 1. Insect morphology was measured with a digital caliper under a stereomicroscope. Flower measurements were made in *UTHSCSA* Image Tool (version 3.0 for Windows, San Antonio, TX, USA). Because shape is an important descriptor of flower morphology (e.g. Herrera, 1993; Medel *et al.*, 2003; Gómez *et al.*, 2006; Benitez-Vieyra *et al.*, 2009), we recorded flower shape descriptors using

Table 1 Descriptive statistics of the pollination process in four populations of *Nierembergia linariifolia*.

Population	Species	Proportion of visits	Total number of flower visits	Visitation rate (h)	Oil-collecting structure (mm) $\bar{x} \pm SE$ (N)	Elaiophore radius (mm) $\bar{x} \pm SE$ (N)	MFI $\pm SE$
CM	<i>Centris tricolor</i>	0.99	3147	0.102 (56)	6.87 \pm 0.56 (6)	6.24 \pm 1.86 (175)	-0.10 \pm 0.02
CM	<i>Tapinotaspis chalybaea</i>	0.01	24	0.001 (56)	3.01 (1)*	6.24 \pm 1.86 (175)	0.52
LT	<i>C. tricolor</i>	1	1041	0.571 (22)	6.46 \pm 0.40 (10)	5.48 \pm 0.78 (150)	-0.18 \pm 0.01
PA	<i>Chalepogenus nigripes</i>	1	203	0.157 (17)	1.84 \pm 0.18 (9)	2.56 \pm 1.14 (165)	0.28 \pm 0.01
VA	<i>C. tricolor</i>	0.11	18	0.090 (16)	6.61 \pm 0.58 (16)†	2.02 \pm 1.14 (101)	-2.27 \pm 0.02
VA	<i>T. chalybaea</i>	0.50	541	0.399 (16)	3.01 (1)	2.02 \pm 1.14 (101)	-0.49
VA	<i>Chalepogenus parvus</i>	0.39	178	0.340 (16)	1.09 \pm 0.10 (7)	2.02 \pm 1.14 (101)	0.46 \pm 0.01

CM, Capilla del Monte; LT, La Toma; PA, Pampa de Achala; VA, Villa Ani-mí. Visitation rate was calculated from visits per flower h^{-1} .

A mechanical-fit index (MFI) was calculated as $1 - (\text{mean oil-collecting structure} / \text{mean elaiophore radius})$.

*Value replicated from the observed length recorded at VA.

†Average value from the observed length recorded in CM and LT.

a landmark-based methodology in the context of geometric morphometrics that permits quantification of shape regardless of size, rotation and scale of the geometric object (Rohlf & Marcus, 1993; Zelditch *et al.*, 2004). Landmarks should represent homologous anatomical loci that provide adequate coverage of the overall morphology which can be found repeatedly and reliably (Zelditch *et al.*, 2004). We defined 12 coplanar landmarks located along the outline of flowers (Fig. 4a). Landmarks were defined by reference to petal overlap, labellum width and petal length. Landmarks 3, 5, 7, 9 and 11 refer to petal overlap, landmarks 2 and 12 refer to labellum width, and landmarks 1, 4, 6, 8 and 10 refer to petal length. All geometric morphometric analyses were performed using the tps package (available in <http://life.bio.sunysb.edu/morph/index.html>). To obtain a consensus flower shape per plant, we performed a general Procrustes analysis that removed nonshape variation (i.e. translation, scaling and rotation) in the landmark coordinates. The Procrustes average configuration and relative warps (RW hereafter, which represent the principal components of the covariance matrix of the partial warp scores) were obtained separately for each population and their scores were used as a data matrix to perform standard statistical analyses. To visualize the displacement of landmarks relative to a theoretical consensus for each population, we used the thin-plate spline procedure that smooth configurations by minimizing the 'bending energy' of deformations (see Zelditch *et al.*, 2004). To test for flower shape differences among populations, we performed a canonical variates analysis (CVA) on the partial warp scores. Pairwise multiple comparisons based on the generalized Mahalanobis distance (D2) from CVA were performed to determine the populations that differed statistically in flower shape. This analysis was performed in PAST (Hammer *et al.*, 2001).

Statistical analyses

To estimate the direct force of selection acting on the phenotypic characters, excluding indirect selection through correlated traits, we applied the multivariate model of Lande & Arnold (1983). We performed phenotypic selection analysis on flower display (measured as the

number of open flowers), floral area, elaiophore area, fertile column length and the first two RW describing floral shape. The number of plants per population used for these analyses is given in Table 2. We estimated the multivariate gradients for directional selection and stabilizing/disruptive selection by using relative female fitness as the dependent variable. In addition to selection acting upon individual characters, we estimated the gradients of correlational selection for all pairwise combination of characters. Individual fitness (W) was recorded as the mean seed production from five randomly collected fruits per plant. Seeds were counted in the laboratory under a binocular microscope. With this value, we estimated the relative female fitness (w) as $W/\text{mean}(W)$, where $\text{mean}(W)$ is the mean individual fitness in the population. Quadratic regression coefficients for stabilizing/disruptive selection gradients were doubled to avoid underestimation as suggested by Stinchcombe *et al.* (2008). The significance of the selection coefficients was determined with multiple regressions. All traits were standardized to zero mean and unit variance. This transformation is equivalent to expressing the original individual trait in units of standard deviation to permit legitimate comparison of the strength of selection among populations (Lande & Arnold, 1983; Brodie *et al.*, 1995). To test whether the pattern and strength of pollinator-mediated selection differed across populations, we examined the statistical significance of population \times trait interactions in ANCOVA (Heisler & Damuth, 1987; Maad, 2000). The form of the selection surface acting upon the characters was examined by using the univariate cubic spline routine provided by Schluter (1988). The cubic spline procedure is a nonparametric fitting function that provides a quantitative prediction of fitness across a range of trait values. The standard error for the predicted regression surface was estimated by bootstrapping the dataset 5000 times.

Results

Pollinator and flower variation among populations

A total of four bee species belonging to three genera accounted for the total visitation rate in the four

Table 2 Coefficients of phenotypic selection on floral traits in four populations of *Nierembergia linariifolia*.

Flower trait	CM (175)	LT (150)	PA (165)	VA (101)
Total flower display	0.03 \pm 0.03	-0.02 \pm 0.03	0.05 \pm 0.03	0.07 \pm 0.03*
Corolla size	0.18 \pm 0.04***	0.12 \pm 0.04**	0.11 \pm 0.03***	0.10 \pm 0.04**
RW1	0.11 \pm 0.03***	0.10 \pm 0.03**	-0.03 \pm 0.84	0.01 \pm 0.03
RW2	-0.01 \pm 0.03	-0.03 \pm 0.03	-0.10 \pm 0.03**	-0.02 \pm 0.03
Elaiophore area	0.00 \pm 0.03	-0.05 \pm 0.04	-0.05 \pm 0.03	-0.04 \pm 0.04
Stamen column length	-0.06 \pm 0.05	-0.02 \pm 0.02	0.03 \pm 0.03	-0.04 \pm 0.03

Population abbreviations as in Table 1. Parentheses indicate the number of plants sampled. Standardized gradients of directional selection ($\beta'_i \pm \text{SE}$) are shown. Only one significant gradient for stabilizing/disruptive selection was detected, and no correlational selection coefficient was significant (see Results section). Therefore, only gradients for directional selection are presented. Boldface values retained statistical significance after Bonferroni adjustment. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

populations. Similarly, the identity of the locally most important oil-collecting species changed across sites (Table 1). For example, while *C. tricolor* and *T. chalybaea* accounted for a high proportion of visits and had a high visitation rate in CM and VA, respectively, *C. nigripes* and *C. tricolor* were the only species found in PA and LT, respectively (Table 1). Even though *C. nigripes* visited *N. linariifolia* in CM, this tiny bee did not contact the flowers' fertile organs and behaved as an oil thief. The overall visitation rate per population was highest in LT by *C. tricolor* and lowest in CM by *T. chalybaea* (Table 1).

Flower traits involved in attraction and mechanical fit showed a wide variation among populations (Fig. 3). Stamen column length and total floral display showed respectively, the lowest and highest coefficient of variation in all populations (Fig. 3). Similarly, CVA analysis revealed overall and pairwise differences in flower shape among populations (Wilk's λ : 0.1391, $P < 0.001$; Mahalanobis distance, $P < 0.001$ for all contrasts, Fig. 4b). Likewise, there was an ample variation in the mechanical fit between pollinators and flowers as revealed by the positive and negative deviations observed among species and populations around the exact matching value (=0). Interestingly, extreme MFI-values were observed in *C. tricolor*, and such variation could not be attributed to variation in the insect oil-collecting structure but to variation in elaiophore radius among populations. For example, MFI-values were near to 0 in CM and LT (-0.1 and -0.18, respectively), where *C. tricolor* was the most frequent, if not the only pollinator species. In VA, the matching value decreased to -2.27, where *C. tricolor* accounts for a low proportion of visits to *N. linariifolia* (Table 1). This observation suggests that *N. linariifolia* seems to adjust elaiophore size to the most important pollinators in local assemblages.

Table 3 ANCOVA of character impact on female fitness (seed production) in *Nierembergia linariifolia*.

Source of variation	d.f.	MS	F
Population	3	3231.0	10.94***
Total floral display	1	236.2	0.80
Corolla size	1	4016.8	13.60**
RW1	1	1246.2	4.22*
RW2	1	1204.4	4.08*
Elaiophore area	1	527.6	1.79
Stamen column length	1	1.9	0.01
Population \times total floral display	3	186.3	0.63
Population \times floral area	3	1316.7	4.46**
Population \times RW1	3	745.8	2.53†
Population \times RW2	3	764.1	2.59†
Population \times elaiophore area	3	190.1	0.64
Population \times stamen column length	3	464.8	1.57
Error	558	295.4	

Degrees of freedom (d.f.), mean squares (MS) and *F*-values are listed. Boldface *F*-values retained statistical significance after Bonferroni adjustment. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, † $P < 0.10$.

Pollinator-mediated selection

Selection for floral traits involved in pollinator attraction was strong and relatively similar among populations. There was consistent directional selection to increase corolla size in the four populations (Table 2, Fig. 5). Likewise, flower shape, as revealed by RW1 and RW2, was selected in CM, LT and PA (Table 2). Visual inspection of flower shape through thin-plate splines revealed that significant coefficients in CM and LT were associated with compression of the lower petals and concomitant expansion of the labellum (Fig. 4c). In this way, plants with expanded labella may experience a higher pollination service from this pollinator species. Shape deformation in PA followed a different pattern. Flowers with a compression in the labellum and concomitant expansion of the lower petals had a higher relative fitness (Fig. 4c). The only species responsible for pollination in this population is the small bee *C. nigripes*, which suggests that selection on floral shape is entirely attributable to this pollinator species (Table 1). Floral display was under slight directional selection in VA, but this coefficient lost statistical significance after Bonferroni adjustment (Table 2). No flower trait involved in pollinator attraction was under stabilizing/disruptive and correlational selection with the exception of corolla size in CM, which experienced a significant stabilizing selection that retained significance after Bonferroni adjustment ($\gamma_{ii} = -1.284 \pm 0.209$, $P = 0.0025$).

Regarding traits involved in mechanical fit (elaiophore area and stamen column length), we found no evidence of directional selection in any population. Like attraction traits, no fit trait was under stabilizing, disruptive, or correlational selection. When the entire dataset was examined across populations, the strength of directional selection differed among populations as revealed by a significant overall model (ANCOVA, $F_{27, 531} = 4.11$, $P < 0.001$). Only the interaction term population \times corolla size was significant ($F_{3, 531} = 4.46$; $P = 0.0042$, Table 3). The remaining attraction and mechanical-fit traits did not interact significantly with population (Table 3), and therefore they were irrelevant in accounting for variation in pollinator-mediated selection among populations.

Discussion

Trait variation among populations

The results of this study indicate that populations of *N. linariifolia* present ample and significant variation in floral traits regardless of their functional role in the pollination process (Fig. 3). Floral traits did not covary across populations but presented a relatively unique pattern of change, which suggests that *N. linariifolia* populations present different local combinations of trait states. Several studies have evaluated whether geograph-

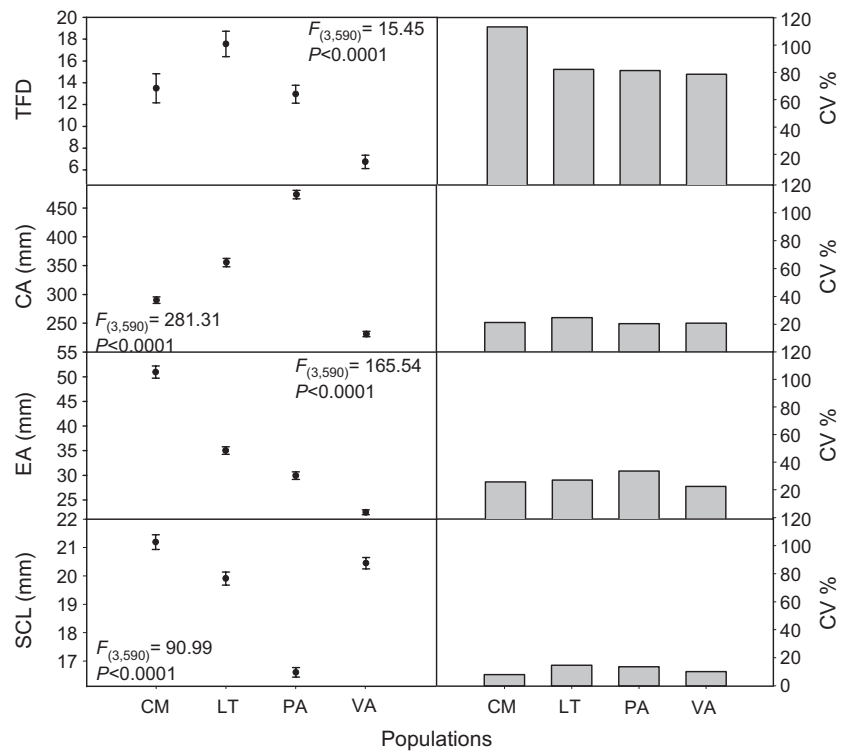


Fig. 3 Patterns of variation (mean \pm 1 SE) and coefficient of variation (CV) of plant and flower traits among populations of *Nierembergia linariifolia*. Plant and flower traits: SCL, stamen column length; EA, elaiophore area; CA, corolla area; TFD, total floral display. Populations: CM, Capilla del Monte; LT, La Toma; PA, Pampa de Achala; VA, Villa Ani-mí.

ical changes in pollinator assemblages associate with concomitant changes in flower morphology (e.g. Mitchell, 1994; Nattero & Cocucci, 2007; Anderson & Johnson, 2010), odour (e.g. Ackerman *et al.*, 1997), pollen and nectar availability (e.g. Stone *et al.*, 1998), phenology (e.g. Utelli & Roy, 2000) and colour (e.g. Miller, 1981; Medel *et al.*, 2007). Flowers are likely to adapt to bee morphology, but there is no selective pressure for bees to adapt to floral morphology. Thus, this system seems to be an example of a process of unilateral evolution (Anderson & Johnson, 2009). *Nierembergia linariifolia* flowers oil rewards are not concealed but located in a rather exposed fashion on the surface of the spreading corolla limb. Consequently, bees robbed the flowers of oil showing that they can still access the rewards despite the poor fit (e.g. *C. nigripes* in CM population, Fig 2f).

In this study, we found important variation in the phenotypic matching between bees and flowers of *N. linariifolia*. For instance, the MFI of pollinator species showed a high coefficient of variation (CV = 378%). Furthermore, the same pollinator species does not necessarily exhibit a comparable fit index between localities as revealed by *T. chalybaea* in CM (MFI = 0.52) and VA (MFI = -0.49). This result indicates that insect structures were smaller than their flower counterparts in some species and populations but not in others, rendering it difficult to extract useful generalizations on phenotypic matching. However, this MFI was lowest in populations with a single or one strongly predominant pollinator

species suggesting that in some populations, flowers and pollinators are adjusted.

Variation in floral traits across populations seems to relate both to the size of pollinator morphology and their foraging behaviour. For example, in the PA population, corolla size and stamen column length had respectively the highest and smallest values recorded in all populations. The only pollinator recorded in PA was the tiny *C. nigripes* (Table 1). Unlike the other bee species, *C. nigripes* needs a spacious limb area to move around and transfer the collected pollen to the hind legs. Stamen column length, in turn, must be small enough to allow pollen transfer between the anthers and the head of the small insect (Fig. 2c). On the contrary, in LT, where the large bee, *C. tricolor*, is the only pollinator, an inverse relationship between floral area and stamen column length was detected. The bee species performs in-flight oil-transfer from the front to hind legs. Flowers need to have a column length large enough to contact the relatively tall front head of this species to be successfully pollinated (see Fig. 1d, Fig. 2a). These extreme situations illustrate a wide variation in the fine-scale foraging behaviour, which leads to the expectation that changes in the composition of pollinator assemblages should be paralleled by a strong variation in the strength, sign and floral traits under selection in the four populations. However, contrary to expectations, there was a relatively homogeneous selection pattern among populations. Floral area was consistently under directional selection

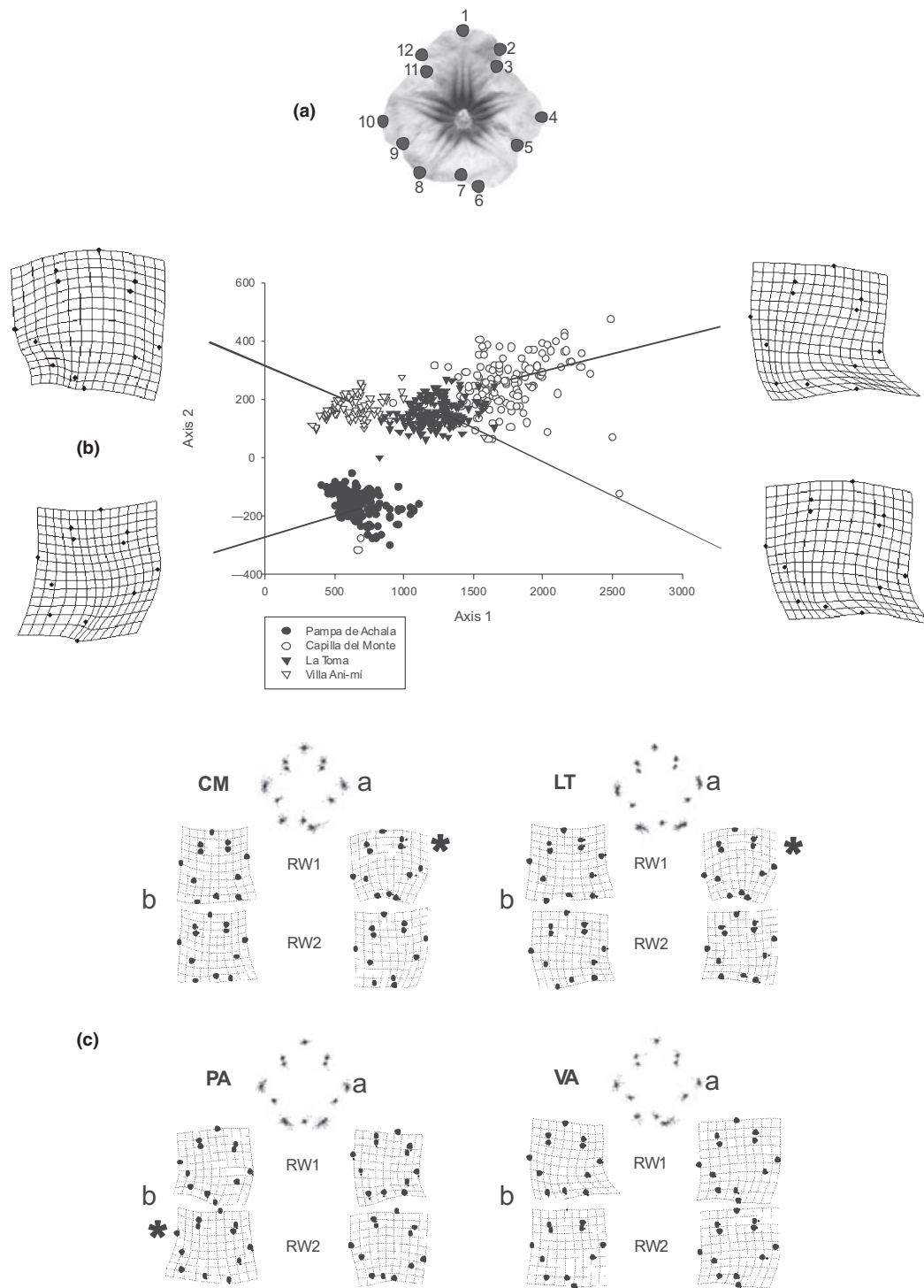


Fig. 4 Geometric morphometric analysis in four *Nierembergia linariifolia* populations. (a) Flower in frontal view showing the 12 landmarks located along the outline of the flower. (b) Ordination of the four populations along the first two axes in canonical variates analysis together with deformation grids showing the flower shape deformation for each population. (c) Visual representation of variation in flower shape. *a* Vectorial configurations for the 12 defined landmarks indicate the individual displacements from the consensus flower per population. *b* The extreme overall shape variation along the first two relative warps (RW1 and RW2) is depicted for each population. Asterisks above deformation grids indicate the shape configuration that is promoted by pollinator-mediated selection at each population. Populations named as in Fig. 3.

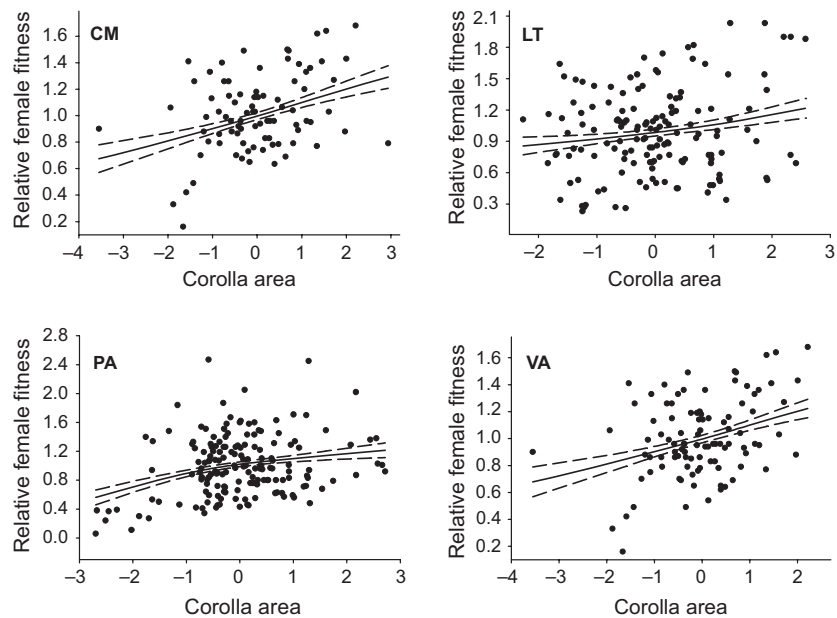


Fig. 5 Cubic spline estimates ($\lambda = 3$) for corolla area in the four populations of *Nierembergia linariifolia*. Populations named as in Fig. 3.

in every population that was analysed, and floral shape was selected in three of four populations. These results emphasize that in spite of the potentially wide variation in the selective scenarios, populations of *N. linariifolia* tend to be under similar rather than disparate selection forces.

Selection on attraction traits

We detected significant selection in attraction traits (corolla size and floral shape) but not in mechanical-fit traits (elaiophore area and stamen column length). This result indicates that characters involved in the first step of the pollination process, that is, in the process of flower discrimination, have a higher chance of evolving under pollinator-mediated selection, provided that significant heritability exists (Ashman & Majetic, 2006). Corolla size was the only trait under consistent selection in all populations (Table 2, Fig. 5). Numerous studies have shown that pollinators prefer to visit large flowers over small ones (e.g. Galen, 1989; Campbell, 1991; Vaughton & Ramsey, 1998) because flower size is often associated with nectar and pollen reward (e.g. Galen, 2000; Fenster *et al.*, 2006). In this way, the association between flower size and reward may determine, in part, the choices made by pollinators and the subsequent variation in plant reproductive success.

Floral shape was under significant selection in three of four populations. Even though several studies have documented the importance of corolla shape in determining plant reproduction (e.g. Pellmyr, 1988; Herrera, 1993, 2001; Andersson, 1994; Johnson *et al.*, 1995; Galen & Cuba, 2001), relatively few studies have quantitatively evaluated whether flower shape evolves under pollina-

tor-mediated selection (e.g. Herrera, 1993; Medel *et al.*, 2003; Gómez *et al.*, 2006; Benitez-Vieyra *et al.*, 2009). In this study, shape variation was associated with changes in labellum shape, a trait that is directly involved in the landing behaviour. The bee *C. tricolor*, the most important pollinator in CM and the only pollinator in LT (Table 1), often used the labellum as a landing platform to collect oil from elaiophores (70% of landings). This observation suggests that shape may be an important floral attraction character that determines pollinator preference and adaptation to specialized pollinators.

It is likely that the intensity of selection acting on each population results from the combined action of breeding system and pollen limitation. It is known that selection on floral traits through female function may be particularly strong in pollen-limited populations (Totland, 2001; Ashman & Morgan, 2004), especially in self-incompatible species that depend entirely on exogamous pollen for successful reproduction (Burd, 1994). The breeding system of *N. linariifolia* has been extensively studied by Cosacov *et al.* (2008). In four of five studied populations, the species has a strictly self-incompatible system with high levels of pollen limitation both in quantity and quality components (Cosacov *et al.*, 2008). Our data on reproductive systems, comparing fruit set and seed set of bagged flowers to that of flowers exposed to pollinators, indicate that the only population showing some degree of spontaneous autogamy was VA (fruit set: 12%, seed set: 34%, when compared to open flowers). The remaining three populations did not produce seeds in the absence of pollinators. Selection for corolla size and complexity thus occur under conditions of competition for limited pollinators. Under such conditions plant and floral traits that increase visitation rate may be under

strong selection within populations as predicted by theory. Thus, selection on this trait is predictably sensitive to fluctuations in pollinator abundance.

Lack of selection upon mechanical-fit traits

Unlike traits related to the attraction of pollinators, mechanical-fit traits were not under significant selection in any population (Table 2). This result indicates that plants are not currently evolving fine-tuned morphological adaptations to local pollinator assemblages. There are four potential explanations that may account for this pattern. First, even though bee species differ in the way they collect oils from flowers, there is inherent variation in the foraging behaviour within species that may affect the precision of the pollination process. For example, *C. nigripes* showed different behaviours while visiting flowers. On the one hand, bees often land directly on the surface of the limb and collect oils by turning about and pointing towards the central column while contacting the flower fertile organs with the head (Fig. 2c). However, only sometimes bees of this species collect pollen by crawling to the top of the column. During this activity, the bee touches the fertile parts with its sternum, eventually pollinating the flowers (Cocucci, 1991). Consequently, any selective force associated with flower-pollinator mechanical matching can be hampered by behaviours that are only secondarily related to the pollination process. This phenomenon may constrain the 'precision' of this system *sensu* Armbruster *et al.* (2004), which may prevent the evolution towards optimal phenotypic values in this highly specialized system. Second, temporal variation in the composition of the pollinator assemblages may prevent fine-tuned matching between plants and pollinators because generalized rather than specialized flower phenotypes should be selected for. However, data in two of four populations indicate that consistent rather than variable composition of pollinator assemblages seems to characterize this system. For example, samplings during five nonconsecutive years revealed that *C. nigripes* was the only pollinator species present in PA (Lazarte *et al.*, unpublished data). Similarly, samplings during three nonconsecutive years in CM did not find additional oil-collecting bees to *C. tricolor* and *T. chalybaea* (Cosacov *et al.*, 2008). Consequently, even though we cannot reject the idea that temporal variation in the pollinator assemblages plays a role in determining the lack of mechanical-fit adjustments, its importance seems to be relatively minor judging by the lack of change in the composition of species assemblages among years. Third, fine-tuned flower adaptations may depend to a large extent on the identity and effectiveness of species belonging to the pollinator assemblage, as well as on the nature of the fitness trade-off associated with flower adjustments to different pollinator species (Aigner, 2001). The observation that more than one bee species

accounts for pollination service in some populations (e.g. CM and VA, see Table 1) suggests that incongruent and probably opposite selective forces determine net selection, eventually diluting the per species selective impact on flower adjustment (Fenster *et al.*, 2004). Attraction traits may be more strongly selected because all the bee species have relatively similar preferences. Unlike attraction traits, selection on mechanical-fit traits may be variable across populations, in part because of the size differences shown by pollinator species. Fourth, mechanical-fit traits, in contrast to attraction traits (such as total floral display or corolla size), are unlikely to be very strongly influenced by plant age or resource availability, contingent with habitat heterogeneity, showing reduced within-population variation. According to this, the low coefficient of variation showed by mechanical-fit traits (Fig. 3), may render detection of selection relatively difficult in comparison to traits influenced by resource availability, such as attraction traits. The low coefficients of variation shown by mechanical-fit traits, particularly on stamen column length, provide indirect support to this explanation (Fig. 3). Because low phenotypic variation may result from repeated stabilizing selection acting on a trait, the absence of selection on mechanical-fit traits is intriguing. Future studies on selection acting upon mechanical-fit traits probably need to consider methodologies that increase trait variation such as experimental manipulations, selected crosses between parents, or even the transplantation of plants from other populations to reveal the mechanisms underlying the, as yet in part, unpredictable selective outcomes.

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