EVOLUTION OF COMPLEX TRAITS: THE CASE OF *ERYSIMUM* COROLLA SHAPE

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The evolution of flower shape has attracted the attention of biologists for at least two hundred years. Although much information is accumulating on the genetic architecture of flower shape, information on its adaptive significance is much scarcer. Using geometric morphometrics, we have explored the microevolution of corolla shape in *Erysimum mediohispanicum* during the past decade. We have found that, by contrast with conventional wisdom, corolla shape shows great variation even between co-occurring individuals. This variation can have strong fitness consequences, with reproductive success being associated with specific corolla shapes. Corolla shape seems to act in *E. mediohispanicum* as an honest signal, since it is associated with reward (nectar and pollen) and determines the preference pattern of important pollinators. Finally, since pollinator fauna varies geographically in this generalist plant species, we have detected a geographic mosaic of selection on *E. mediohispanicum* corolla shape in southeastern Spain that has resulted in a pattern of local adaptation. We hope that this review will encourage other evolutionary biologists to explore corolla shape microevolution, helping to unravel Darwin's "abominable mystery."

Keywords: *Erysimum mediohispanicum*, corolla shape, pollinator-mediated selection, geographic mosaic of selection, local adaptation.

Introduction

The evolution of flower shape has attracted the attention of biologists working in disparate disciplines, including evolutionary developmental biology, evolutionary ecology, pollination biology, paleobiology, and phylogeny (Coen et al. 1995; Donoghue et al. 1998; Cubas et al. 1999; Endress 1999; Ree and Donoghue 1999; Galen and Cuba 2001; Busch and Zachgo 2009; Hileman and Cubas 2009). Paleontological and phylogenetic studies have shown that the ancestral angiosperm flowers were actinomorphic (radially symmetric). Floral zygomorphy (bilateral symmetry or monosymmetry) has evolved several times in different angiosperm lineages from actinomorphic ancestors (Stebbins 1974; Reeves and Olmstead 1998; Ree and Donoghue 1999; Dilcher 2000; Olson 2003; Rudall and Bateman 2003, 2004; Knapp 2010). Floral zygomorphy is considered a key innovation promoting speciation and diversification in angiosperms, since it is associated with the largest plant families (Sargent 2004; but see Kay et al. 2005).

The genetic control of flower shape and symmetry is well known, mostly because of comprehensive research on several model organisms (Cronk et al. 2002). Monosymmetry in the snapdragon *Antirrhinum major* (Plantaginaceae) is controlled by the activity of a network of interacting genes, including the much-studied *CYCLOIDEA* and *DICHOTOMA* (*CYC* and *DICH*, resepctively; Luo et al. 1996, 1999; Cubas et al. 2001; Hileman et al. 2003), two recently duplicated TCP genes de-

termining dorsal identity, and the MYB transcription factors *RADIALIS* and *DIVARICATA* (*RAD* and *DIV*, resepctively; Preston and Hileman 2009). In the snapdragon relative *Linaria vulgaris* (Plantaginaceae), Cubas et al. (1999) showed that floral symmetry is determined by an epigenetic mutation in *LCYC*. A similar role for *CYC*-like genes in flower symmetry has been proposed for other plant groups, such as Gesneriaceae (Zhou et al. 2008). It seems that *CYC* homologues are also involved in flower development and symmetry in many different plant families, such as Asteraceae (Broholm et al. 2008), Fabaceae (Citerne et al. 2006; Feng et al. 2006; Wang et al. 2008), and Brassicaceae (Busch and Zachgo 2007). Knowledge of the genetic basis of flower symmetry in distantly related species is accumulating very rapidly (Busch and Zachgo 2009; Preston and Hileman 2009).

Irrespective of its genetic architecture, zygomorphy is thought to have evolved as a consequence of strong selection exerted by specialized pollinators (Neal et al. 1998; Endress 2001), because it increases both flower attractiveness (Lehrer et al. 1995; Møller 1995; Rodríguez et al. 2004; Gong and Huang 2009) and pollen-transfer efficiency (Endress 1999). However, in contrast with the copious information on its genetics, empirical evidence on the adaptive significance of flower shape is scarce (Theißen 2000; Hileman et al. 2003). Several studies have found that some flower visitors have innate preferences for bilateral symmetry (Møller and Eriksson 1995; Møller and Sorci 1998; Rodríguez et al. 2004). For example, asymmetric *Epilobium angustifolium* (Onagraceae) flowers received fewer bumblebee visits than symmetric flowers (Møller 1995). However, available data are not conclusive, because Weeks and Frey (2007) showed no apparent preference for symmetrical *Hesperis matronalis* (Brassicaceae)

flowers, Frey et al. (2005) did not detect any increase in bumblebee visitation to *Impatiens pallida* (Balsaminaceae) symmetrical flowers, and asymmetry did not negatively affect pollinator visitation by bee flies and beetles to *Gortesia diffusa* (Asteraceae; Midgley and Johnson 1998). Most of these studies have been performed, however, under the framework of fluctuating asymmetry. In contrast, the pollinator preference patterns for polysymmetrical versus monosymmetrical flowers have seldom been studied (but see Rodríguez et al. 2004). Information about the reproductive consequences of different corolla shapes is also inconclusive. Corolla shape, acting in combination with other floral traits such as corolla color, seems to act as a barrier between the bee-pollinated *Mimulus lewisii* (Phrymaceae) and the hummingbird-pollinated *Mimulus cardinalis* (Bradshaw et al. 1998; Schemske and Bradshaw 1999). Similarly, Galen and Cuba (2001) showed that in *Polemonium viscosum* (Polemoniaceae), bumblebee pollinators select for funnel-shaped corollas, whereas nectar-thieving ants prefer flared, short corollas. In contrast with these outcomes, Herrera (1993) found only slight selection acting on the hawkmoth-pollinated *Viola cazorlensis* (Violaceae) corolla shape, whereas Weeks and Frey (2007) did not find any effect of flower symmetry in *H. matronalis* pollinator-mediated seed production, and Frey et al. (2005) did not find any effect of flower symmetry on *I. pallida* seed production.

A probable reason for this dearth of studies is the difficulty of quantifying intraspecific variation in flower shape and symmetry in plants. Selection on flower shape has hitherto been studied either by considering it qualitatively (i.e., radial vs. bilateral symmetry) or by describing it as a variable composed of linear measurements (but see Herrera 1993). However, flower shape is a complex trait that can hardly be described by linear approximations. To avoid this simplification, during the past decade we have used geometric morphometric tools (Bookstein 1991; Zelditch et al. 2004) to study *Erysimum mediohispanicum* (Brassicaceae) corolla shape microevolution. We review in this article the main methodological aspects of and the most striking results obtained from our research. In doing so, we aim to encourage a unified framework for study of the evolution of flower shape and symmetry.

Exploring the Corolla Shape of *Erysimum mediohispanicum*

Some Notions on Geometric Morphometrics

Geometric morphometrics (GM) is a sophisticated technique allowing the collection, exploration, and quantitative study of the shape of objects (Bookstein 1991; Lele and Richtsmeier 2001; Marcus et al. 1993; Zelditch et al. 2004). During the past two decades, this technique has experienced a revolution (Adams et al. 2004), and it is now frequently used to solve questions regarding evolution of complex phenotypes in very diverse organisms (Lawing and Polly 2009; Mitteroecker and Gunz 2009; Schaefer and Bookstein 2009). GM differs from traditional morphometrics (based on distances, distance ratios, angles, etc.) in that GM uses the overall geometry of an object throughout the entire analysis and permits accurate statistical analysis of shapes (Mitteroecker and Gunz 2009).

Although there are many ways of representing, describing, and analyzing the shape of objects, the most common the GM technique uses landmark points aligned with one another (Bookstein 1991). To perform a landmark-based analysis of shape, the first requirement is to identify the landmarks on the surface of the target trait. Landmarks are precise locations on biological forms that hold some developmental, functional, structural, or evolutionary significance. Therefore, they should provide adequate coverage of morphology and be found repeatedly and reliably in many different individuals, and their topological position cannot vary relative to the other landmarks (Zelditch et al. 2004). If we are interested in describing the two-dimensional shape of objects, landmarks must be coplanar. Landmarks are digitized most frequently from a digital photo of the target object, and for this any landmark is characterized by its Cartesian coordinates.

In GM, "shape" denotes the geometric properties of any object other than the object's overall size, position, and orientation (the "form" of an object comprises both its shape and size). For this reason, once landmarks are digitized, it is necessary to convert their coordinates into real shape variables. The most commonly used method is called Procrustes superimposition, a least squares–oriented approach involving three steps (Rohlf and Slice 1990): (1) translation of the landmark configuration of all objects so that they share the same centroid; (2) scaling of the landmark configuration so that they have the same centroid size; and (3) rotation of the landmark configurations to minimize squared Euclidean distances between homologous landmarks. When more than two landmark configurations are rotated, the algorithm is called generalized Procrustes analysis (GPA; Rohlf and Slice 1990; Slice 2001). The coordinates resulting from GPA are called Procrustes shape coordinates (or just shape coordinates). This procedure eliminates nonshape variation in configurations of landmarks by superimposing landmark configurations using least squares estimates for translation and rotation parameters. Now the only remaining difference between objects is their shape. A main advantage of this method is that it offers a way to analyze shape that permits a wide range of multivariate methods for answering biological questions and provides a straightforward way to visualize the corresponding shape changes (Klingenberg 2010).

The next step is to visualize shape differences between individuals. The most prominent approach is modeling shape change using deformation grids, a technique dating back to D'Arcy Thompson (1917). A deformation is a smooth interpolation function that maps points in one form to corresponding points in another form (Zelditch et al. 2004). The function most often used to interpolate deformation is the thin-plate spline (TPS; Mitteroecker and Gunz 2009). The TPS is applied to decompose shape deformations into a range of geometrically independent components called partial warps, which describe a pattern of relative landmark displacements based on the spacing and location of landmarks in the reference form (Bookstein 1991; Zelditch et al. 2004). So the combination of all components completely describes any shape change.

After GPA, it is possible to compute the relative warps (RWs), which are principal components of the covariance matrix of the partial-warp scores and uniform components (Walker 2000; Adams et al. 2004). This is equivalent to a

principal-components analysis of the Procrustes shape coordinates (Mitteroecker and Gunz 2009). In two-dimensional GM analyses, it generates $2p - 4$ orthogonal RWs (where p is the number of landmarks). Each RW explains a given variation in shape among specimens. Thus, RWs summarize shape differences among specimens (Adams et al. 2004), and their scores can also be saved for use as a data matrix to perform standard statistical analyses (Zelditch et al. 2004).

Erysimum mediohispanicum *Natural History*

Erysimum mediohispanicum is a biennial to perennial monocarpic herb found in many montane regions of southeastern Spain at elevations of 1100–2000 m, inhabiting forests and subalpine scrublands (fig. 1). Plants usually grow for 2–3 yr as vegetative rosettes and then die after producing one to eight reproductive stalks, which can display between a few and several hundred hermaphroditic, slightly protandrous, bright-yellow flowers (Gómez et al. 2006). During anthesis, most flowers are oriented in a vertical or quasi-vertical plane with respect to the flowering stalk (fig. 1*A*). This species belong to a species complex distributed all along the Iberian Peninsula and formed by six species or semispecies (fig. 1*B*). *Erysimum mediohispanicum* is the most widely distributed species, occu-pying two large areas: one in the southeast and the other in the north-northeast of Spain (fig. 1*B*).

In the Sierra Nevada of southeastern Spain, *E. mediohispanicum* is patchily distributed from 1600 to 2300 m asl, forming small populations comprising tens to several hundreds of individuals. Most of our research has been performed in eight populations spanning the complete altitudinal range of the species (fig. 1*C*). In spite of their relative proximity, populations are clearly differentiated from each other, and genetic divergence among populations is high ($G_{st} = 0.22 \pm 0.007$, based on 164 RAPD markers; $F_{st} = 0.32$, based on trnL-trnF choloroplast DNA).

The pollination system of *E. mediohispanicum* is highly generalized. During five years of study in eight focal populations, we have recorded more than 150 species of flower visitors belonging to more than 25 families and six insect orders (Gómez et al. 2008*b*, 2009*b*). Most of these species belong to Hymenoptera (more than 60 species) and Coleoptera (more than 40 species). They exhibit a wide range in body size, mouthpart length, and foraging behavior. Body size ranged from 0.3 mg in *Melighetes minutus* (Nitidulidae) to 130 mg in *Anthophora aestivalis* (Anthophoridae). Mouthpart length ranged from <1 mm in several beetles to several centimeters in some butterflies. Most pollinators had mouthparts shorter than *E. mediohispanicum*'s corolla tube depth (10 mm); only
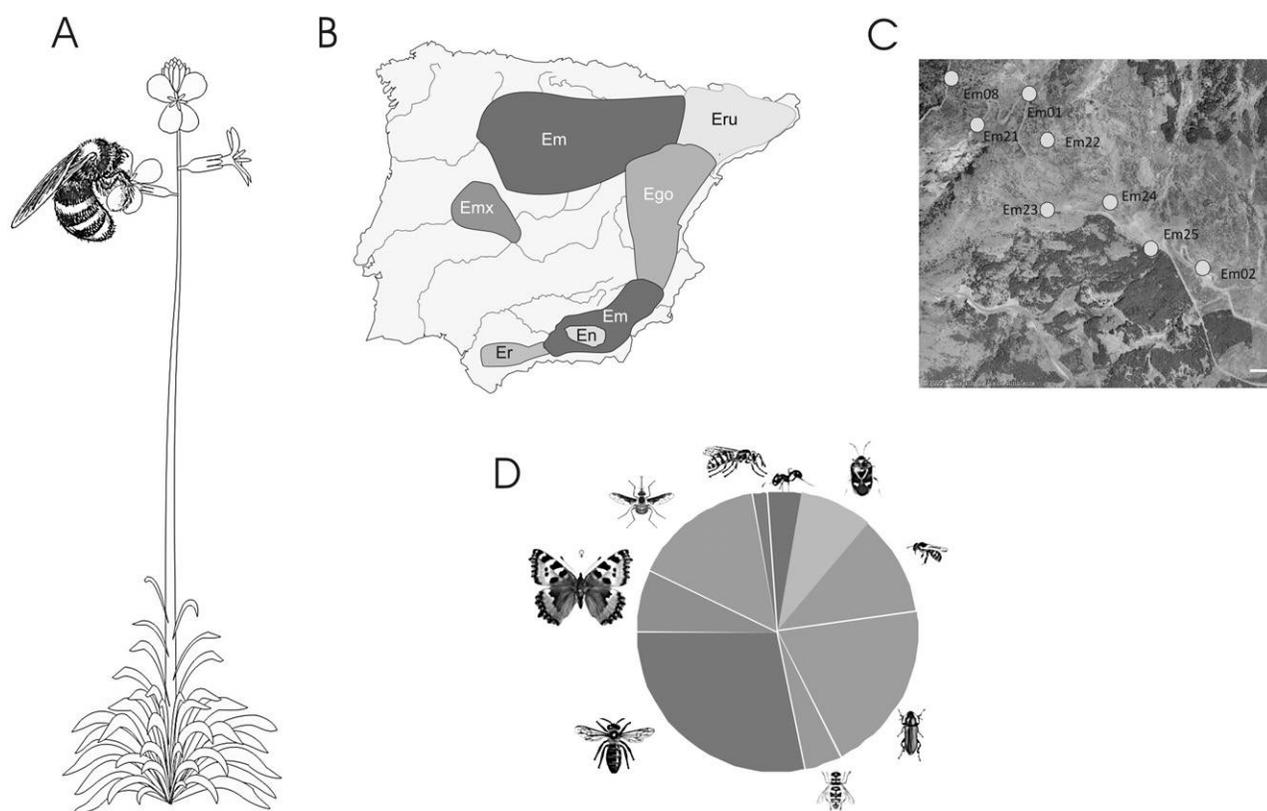
Fig. 1 *Erysimum mediohispanicum* natural history. *A*, Habit of an ideal plant, showing the position of the flowers. *B*, Geographic distribution of the *nevadense* complex in the Iberian Peninsula: *Em* = *E. mediohispanicum*, *En* = *E. nevadense*, *Er* = *E. rondae*, *Eru* = *E. ruscinonense*, *Emx* = *E. mexmuellieri*, *Ego* = *E. gomezcampoi*. *C*, Geographical location of the eight focal populations of *E. mediohispanicum* in the Sierra Nevada (southeastern Spain); scale bar = 200 m. *D*, Relative abundance of the flower visitor functional groups; all populations (8) and years (8) pooled.

a few species (large bees, bee flies, and butterflies) have mouthparts as long as or longer than that. Some pollinators visited *E. mediohispanicum* flowers mostly for nectar (bee flies, butterflies, wasps, male bees), while others collected large amounts of pollen (beetles, female bees). Only a few species, such as the solitary bees *Osmia brevicornis* and *Andrena agilissima*, are crucifer specialists (Gómez et al. 2007).

The pollinators of *E. mediohispanicum* can be grouped into nine main functional groups according to their size, foraging behavior, diet, and so forth: ants, bee flies, hoverflies, large bees, small bees, beetles, butterflies, wasps, and others (mostly muscoid flies and bugs). Large bees, small bees, bee flies, and beetles are the most abundant and widely distributed flower visitors (fig. 1*D*).

Determining Corolla Shape in *E. mediohispanicum*

To determine corolla shape, we digitally photograph *E. mediohispanicum* flowers, using a standardized procedure. Flowers are photographed at anthesis, to avoid ontogenetic effects, and always in front view and planar position. The up-down position of the flower is determined by its relative position with respect to the vertical flowering stalk (fig. 1*A*). We have defined 32 coplanar landmarks along the outline of the flowers and the aperture of corolla tube (fig. 2*A*); the number of landmarks was chosen to provide comprehensive coverage of the flower shape (Roth 1993; Zelditch et al. 2004). Thus, we use two-dimensional GM to describe a tridimensional

structure. Although this simplification may be problematic in some cases, we consider it an appropriate way to describe the pattern of corolla shape variation that is important for understanding the interaction between *E. mediohispanicum* and its pollinators. Landmarks are defined by reference to the midrib (landmarks 1, 9, 17, and 25), the primary veins (landmarks 2, 8, 10, 16, 18, 24, 26, and 32), and the secondary veins (landmarks 3, 4, 6, 7, 11, 12, 14, 15, 19, 20, 22, 23, 27, 28, 30, and 31) of each petal, as well as the connection between petals (landmarks 5, 13, 21, and 29; see fig. 2*A*). The whole set of 32 landmarks was always present in all populations and years studied during our research. We consider all of these to be type I landmarks; however, landmarks 5, 13, 21, and 29 are supported as much by geometric as by histological evidence and may be considered type II landmarks (see Zelditch et al. 2004 for landmark definitions). We capture the landmarks with the software tpsDig, version 1.4 (available at the Stony Brook Morphometrics Web site, http://life.bio.sunysb.edu/morph/index.html). Then, the two-dimensional coordinates of these landmarks are determined for each plant, and the generalized orthogonal least squares Procrustes average configuration of landmarks is computed via the GPA superimposition method (Rohlf and Slice 1990; Slice 2001). GPA is performed with the software tpsRelw, version 1.11 (available at http://life.bio.sunysb.edu/morph/index.html). In these analyses, we always consider the flowers as nonarticulated structures, because the relative position of the petals does not change during their functional life (see Adams 1999 for a discussion on articulated structures). After GPA, the RWs are computed with
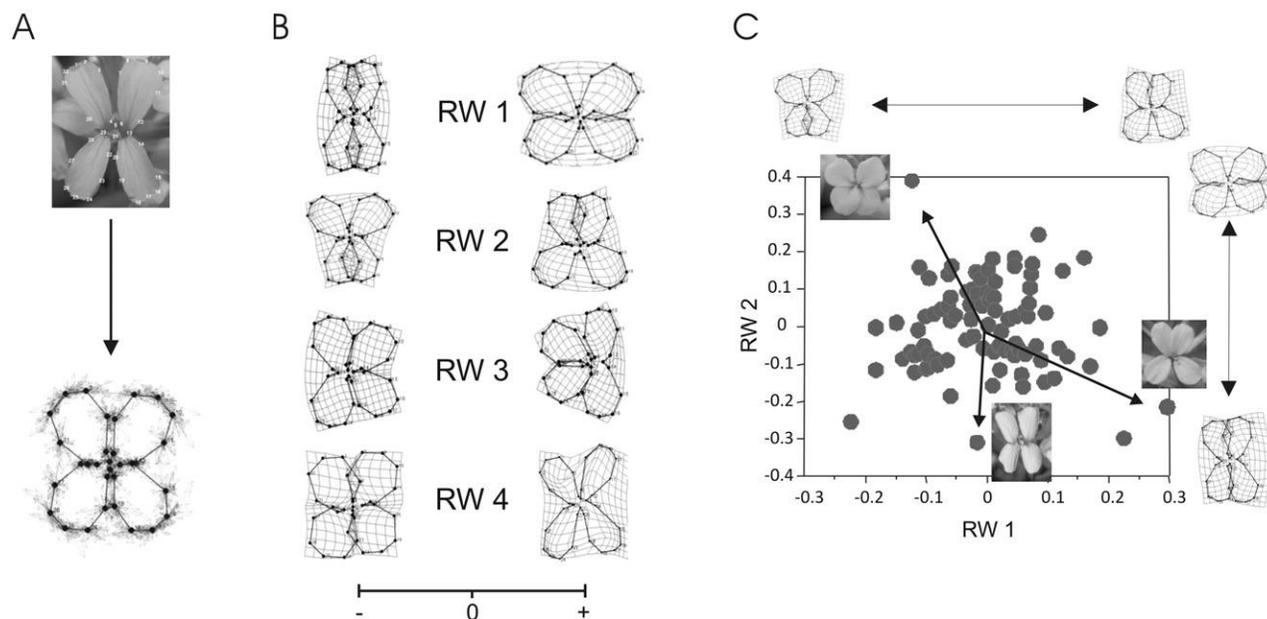
Fig. 2 Determination of *Erysimum mediohispanicum* corolla shape by means of geometric morphometric (GM) tools. *A*, *Erysimum mediohispanicum* corolla, showing the location of the 32 landmarks used in the GM analysis (top) and the consensus corolla shape after the GM analysis (bottom). *B*, Variation in corolla shape produced by the relative warps (RWs) explaining more than 5% of the overall variation in shape. *C*, Between-individual within-population variation in corolla shape (plant population Em21 for 2005 is shown as an example). The panel represents the location of each co-occurring individual plant with respect to the first two RWs and the real shapes of three individuals having contrasting values of these RWs.

the methodology explained above. This procedure generates a consensus configuration, the central trend of an observed sample of landmarks, which is similar to a multidimensional average (fig. 2*A*).

We have quantified *E. mediohispanicum* corolla shape in all years since 2003 in 4–56 populations per year. In all cases, we used one flower per plant, to maximize interindividual variation. Nevertheless, we were aware that some intraindividual variation may occur. For this reason, we have explored intraindividual variation in some years (2002, 2005, and 2010). For example, in 2010 we determined corolla shape in 5–15 flowers per plant in 15 plants belonging to two Sierra Nevada populations (Em17 and Em25). After performing a discriminant analysis on RWs, we found that no single flower was misclassified as belonging to a different individual, whereas misclassification occurred in only 3% of the 205 pairwise comparisons when the landmark configurations were compared with Procrustes discriminant analysis (A. J. Muñoz-Pajares, M. Abdelaziz, J. Bosch, B. Herrador, F. Perfectti, and J. M. Gómez, unpublished manuscript). More remarkably, we have always found the same pattern of interindividual variation in corolla shape (fig. 2*B*). Four RWs each consistently account for more than 5% of the variance in shape. Each RW is associated with a deformation pattern in corolla shape. Thus, RW1 is associated with a change in the parallelism of the petals, RW2 with a change in the size of abaxial (lower) and adaxial (upper) petals, RW3 with a change in vertical symmetry, and RW4 with a change in corolla roundness (fig. 2*B*). Most important, individual plants within a population varied significantly in the values obtained for each of these four shape components (fig. 2*C*). Plants displaying corollas with very different shapes are frequently in the same population (fig. 2*C*). That is, although corolla shape has been referred to as a very fixed trait in plants, there are some species where this trait is very variable, allowing for the functioning of natural selection.

Microevolution of Corolla Shape: Quantifying Natural Selection on Corolla Shape

Ever since the seminal Lande and Arnold (1983) paper introducing a quantitative method to explore natural selection in the wild (the so-called selection gradients) and Endler's (1986) cornerstone book developing an algorithmic definition of natural selection and urging deeper attention to the functioning of selection in the real world, there has been a dramatic increase in the number of studies devoted to exploring the phenotypic selection of diverse traits in disparate organisms. Plant sciences and floral biology have not escaped this trend, and studies on floral evolutionary ecology are currently abundant (see, e.g., compilations in Lloyd and Barrett 1986 and Harder and Barrett 2006). Our knowledge about the selective scenarios, selective agents, and fitness consequences has been much improved for many floral traits. Unfortunately, these quantitative methods have so far been applied mostly to traits varying continuously along one or a few dimensions. These are traditionally traits associated with size (flower size, flower diameter, spur length, plant height) or quantity (nectar production, volatile production, secondary metabolite production). In contrast, multidimensional traits describing complex

patterns of variation of floral phenotype cannot be studied with the same approach. Corolla shape, a crucial trait driving the evolution of many plant groups, is an unequivocal example of this kind of trait. Alternative approaches are thereby necessary to explore the evolutionary ecology and quantitative genetics of floral shape.

Klingenberg and Leamy (2001) and Klingenberg and Monteiro (2005) have proposed that, as for any other phenotypic trait, selection for shape can be calculated by means of selection differentials *s* and selection gradients *β*. The former describes the total effect of selection on shape without distinguishing between direct and indirect selection, whereas the latter allows inquiry into the causal basis of selection and represents the direct effect of each shape variable separately (Klingenberg and Monteiro 2005). In both types of analyses, the shape variables considered can be either the partial warps or the RWs. We have always used RWs as shape variables during our research because they can be interpreted easily as shape deformation patterns (fig 2*B*, 2*C*). Nevertheless, this interpretation should be considered carefully, since selection gradients are not shape changes per se (Klingenberg et al. 2010).

Selection differential on corolla shape can be quantified as the vector of covariances between fitness and the complete set of RWs, deriving the vector from a two-block partial least squares analysis (PLS) between shape and fitness (Rohlf and Corti 2000) and determining the covariance between fitness and shape predicted by this parsimonious model. The multivariate selection gradients can be estimated by multiple regressions of fitness on shape variables (Klingenberg and Monteiro 2005), using Lande and Arnold's (1983) standardized selection gradients and introducing as independent variables the GPA-generated RWs. Klingenberg and Monteiro (2005) recommend visualizing the expected shape of individuals with different fitnesses directly as changes in landmark positions (Rohlf et al. 1996; Adams and Rosenberg 1998). Finally, we have used structural equation modeling (SEM) with latent constructs (Shipley 2000; Pugesek 2003) as an additional methodology to estimate the relationship between the corolla shape and fitness. This method allows us to consider flower shape as a single, inclusive, and multidimensional character (Adams and Rosenberg 1998). For this, we consider corolla shape as a latent construct defined by the set of shape variables (RWs). This approach is interesting because it allows us to consider corolla shape as a complex trait at the same time as it allows us to identify which shape variable is most affected by natural selection. In addition, this approach allows for an explicit consideration of the selective pressures (i.e., pollinators) mediating selection on each plant trait.

We have quantified selection on *Erysimum mediohispanicum* corolla shape from 2002 to 2009 in one to eight populations per year. These analyses have repeatedly proved that corolla shape is significantly associated with fitness in most studied populations of *E. mediohispanicum* in the Sierra Nevada (Gómez 2008; Gómez et al. 2006, 2008*b*, 2009*b*). So, for example, figure 3*A* shows the selection differential obtained in 2003 by means of PLS in a population pollinated mostly by the nitidulid *Meligethes maurus*. This analysis indicates that corolla shape has a significant effect on plant fitness through both seed production and juvenile production (Gómez et al. 2006). Figure 3*B* shows the selection regime acting
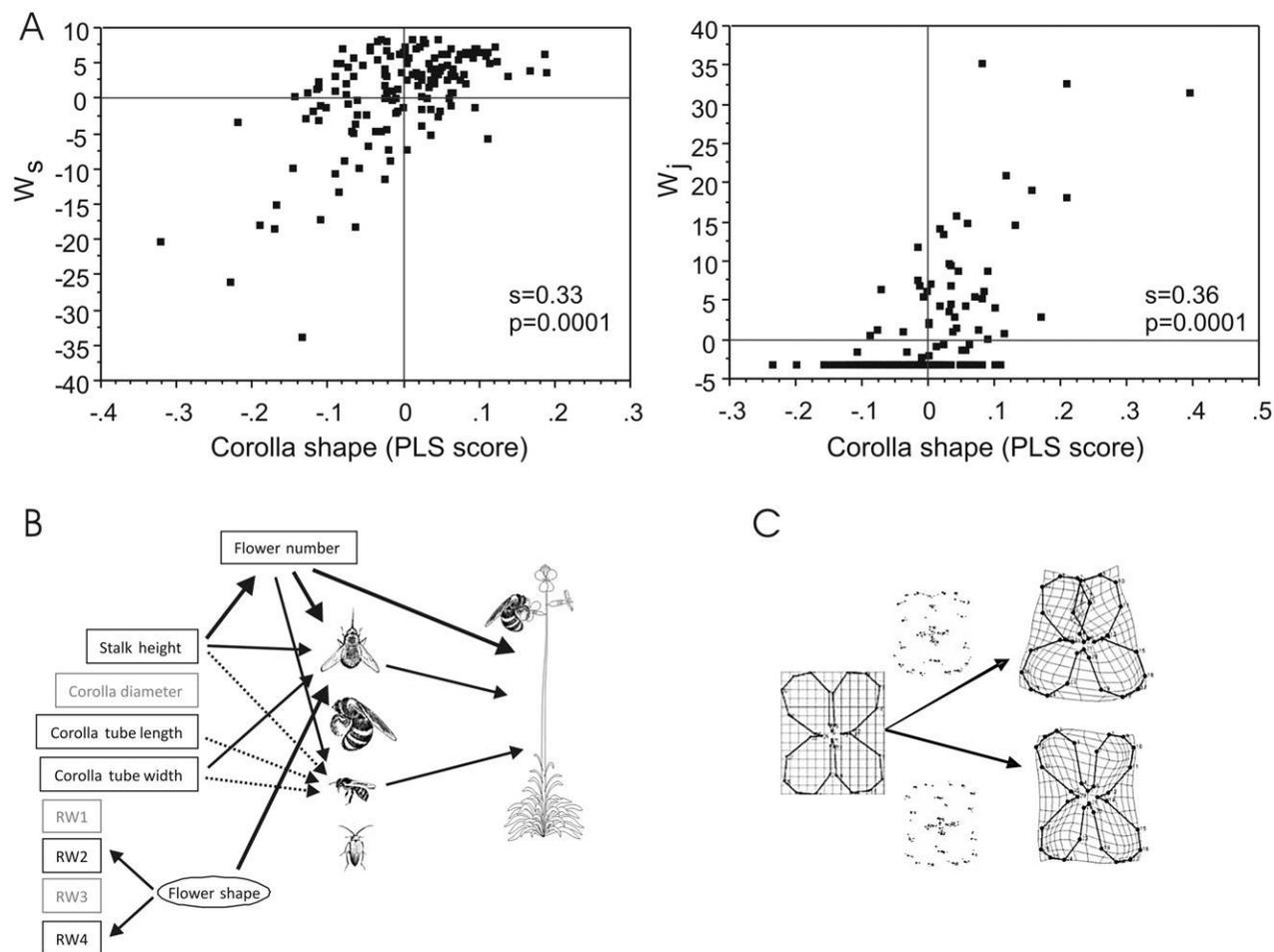
Fig. 3 Quantification of selection on *Erysimum mediohispanicum* corolla shape. *A*, Selection differential through two estimates of fitness (W_s = number of seeds, W_j = number of juveniles) by means of partial least squares analysis. *B*, Structural equation modeling showing the pollinator-mediated selection acting on corolla shape (and other plant traits). *C*, Selected corolla shapes according to selective regime described in *B*.

on corolla shape, according to an SEM analysis, during 2005 in a population pollinated mostly by small bees and bee flies. In particular, in this population the selected corolla shapes are those associated with positive values of RW2 and RW4 (fig. 3C). According to our selection analysis (fig. 3B), it is clear that these two shape components are selected through bee fly preference. Putting together the results obtained during different years, we can conclude that corolla shape is under pollinator-mediated selection in *E. mediohispanicum*.

Corolla Shape Function

Pollinator Preference for Corolla Shape

It is traditionally assumed that the function of corolla shape is to attract effective pollinators (Lehrer et al. 1995; Møller 1995; Rodríguez et al. 2004; Gong and Huang 2009). We have explored the attractiveness of *Erysimum mediohispanicum* corolla shapes by experimentally quantifying the preference pattern displayed by its pollinators. For this, we built artificial flowers, using yellow construction paper to match (from a human perspective) the color of *E. mediohispanicum*

flowers. We built artificial flowers of nine different shapes. Eight of these shapes corresponded to the two extremes (positive and negative) of the four RWs that define *E. mediohispanicum*'s corolla shape, according to GM analyses (fig. 2B). The ninth flower shape corresponded to the consensus shape obtained in the same analyses (Gómez et al. 2006). Artificial flowers were of the same size as natural flowers and were individually arranged on 20-cm-tall wire stalks. To avoid any side effect of reward on pollinator behavior, we did not add any reward to the artificial flowers. We set up experimental arenas (80 cm × 120 cm) with 12 randomly distributed flowers of each of the eight extreme shapes and 48 flowers of the consensus shape (144 artificial flowers per arena). All insects approaching the artificial flowers were noted. We considered an approach to be successful if the insect landed on a flower or contacted it. We noted only those approaches made by those insects visiting natural *E. mediohispanicum* flowers in the three populations. The artificial flowers were visited by 1300 pollinators belonging to 66 species and the main *E. mediohispanicum* functional groups: large bees (144 visits), small bees (156 visits), bee flies (405 visits), hoverflies (427 visits), and beetles (168 visits).

Our experiment showed that *E. mediohispanicum*'s pollinators are able to discriminate between flowers differing exclusively in corolla shape. Most important, different pollinator functional groups exhibited different preference patterns (Likelihood ratio test = 87.08, df = 48, $P < 0.0001$; nominal logistic model). Beetles did not show any clear preference for any flower type (goodness of fit: $\chi^2 = 5.80$, df = 8, $n = 168$, not significant), suggesting that these pollinators visited the artificial flowers at random. Large bees mostly visited flowers with positive RW4 (fig. 4*A*). Small bees preferentially visited flowers with positive RW4 and positive RW1 (fig. 4*A*). Bee flies mostly visited flowers with positive RW1 and negative RW4 (fig. 4*A*). Finally, hoverflies visited flowers with positive RW1 and positive RW4 (fig. 4*A*).

Corolla Shape as Honest Signal

It is important to uncover the factors underlying the observed pollinator preference patterns. One prominent hypothesis states that pollinator preferences result from a functional link between floral traits and reward production (Ashman and Stanton 1991; Campbell et al. 1991; Cohen and Shmida 1993; Møller 1995; Blarer et al. 2002; Armbruster et al. 2005; Fenster et al. 2006). We actually found a link between corolla shape and both pollen and nectar production in *E. mediohis-*

panicum (fig. 4*B*). Specifically, we found that nectar production was highest in flowers with positive RW4 and RW2, while pollen production was highest in flowers with positive RW4 (Gómez et al. 2008*a*, 2008*b*).

Our study shows that the most rewarding flowers matched the corolla shape of artificial flowers preferentially visited by large and small bees. It is remarkable that both rewards were associated with the same corolla shape and that the two pollinator functional groups preferentially attracted to this corolla shape collected both rewards. These results support the idea that bees can use corolla shape as a signal for reward production in *E. mediohispanicum*. Since we used nonrewarding artificial flowers visited by wild, experienced pollinators, these presumably had learned the association between shape and reward on natural flowers (Smithson and Macnair 1997; Neal et al. 1998; Boisvert et al. 2007; Makino and Sakai 2007).

The preference of bee flies for rounded flowers cannot be explained by a functional link between corolla shape and reward. We propose a potential mechanism related to the use of the corolla as a landing platform by pollinators (Neal et al. 1998). Upon landing on a flower, hoverflies, bees, and beetles frequently walk between flowers within the same plant. Bee flies, however, always fly between consecutively visited flowers, even within the same individual plant. Sometimes bee flies hover while collecting nectar, but at other times they land
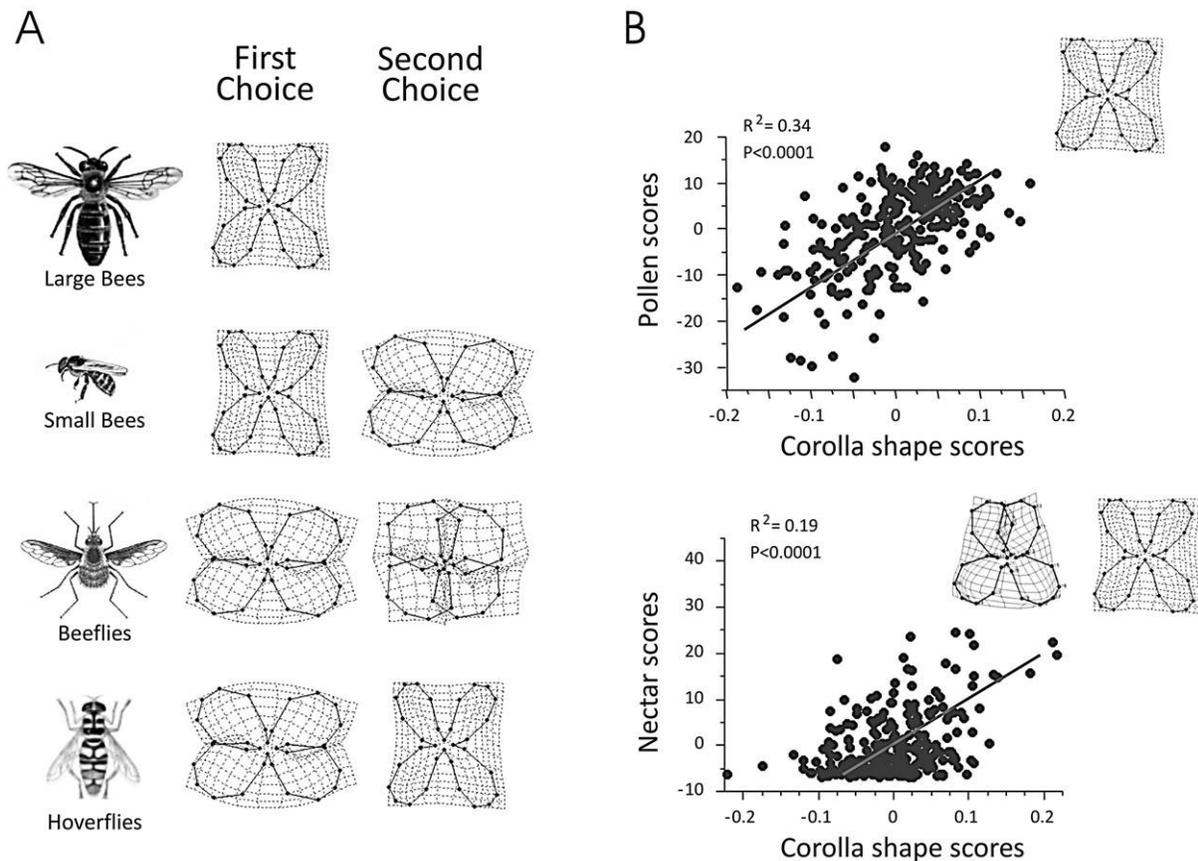
Fig. 4 Function of corolla shape. *A*, Pattern of preference for corolla shape by different flower-visitor functional groups. *B*, Relationship between corolla shape and pollen and nectar production, according to partial least squares analyses. The most-rewarded corolla shapes are shown.

on the flowers and collect nectar while standing on their second and third pairs of legs. Because hovering is very costly in terms of energy expenditure (Heinrich 1993), we believe that bee flies may choose to feed while standing on flowers that offer an appropriate landing platform (flowers with large, rounded petals). Although this hypothesis remains to be tested, it offers a plausible explanation for the observed bee fly preference pattern. Hoverflies showed a preference for both flowers with rounded petals and flowers with nonoverlapping petals. Interestingly, large, beelike hoverflies visiting *E. mediohispanicum* behave similarly to bees, frequently walking from flower to flower, while smaller, wasplike hoverflies behave like bee flies.

In brief, our study suggests that *E. mediohispanicum* corolla shape acts as reward signal, promoting an increase in pollinator visitation rate. This effect on pollinator attraction could be a mechanism explaining the observed phenotypic selection on this trait.

Spatial Structure in Selection Pattern on Corolla Shape

Geographic Mosaic of Selection on Corolla Shape

An outstanding characteristic of the pollinator fauna of *Erysimum mediohispanicum* is its spatial variation in abundance, diversity, and specific composition. Thus, although altogether we have recorded more than 150 species of flower visitors, the average number of pollinators per plant population ranges between 20 and 50 species (Gómez et al. 2007, 2008b, 2009a). Similarly, between-population overlap in pollinator assemblage is very low. For example, in the Sierra Nevada populations, two randomly selected plant populations shared, on average, only 43% of the flower visitors (Gómez et al. 2008b, 2009b). In addition, we noted above that different *E. mediohispanicum* pollinators exhibit different preference patterns for corolla shape. Thus, different *E. mediohispanicum* flower visitors display different preference patterns and differ in abundance across populations. Consequently, because of this geographic variation in pollinator fauna, the selection regimes occurring on corolla shape should vary spatially.

We explored the geographic mosaic of selection on corolla shape in 2005 in eight populations in the Sierra Nevada (southeastern Spain; fig. 1C). Corolla shape varies significantly among these populations (tables 1, 2). We found that selection strength on *E. mediohispanicum* corolla shape varies spatially, with some populations undergoing strong selection

Table 1

Procrustes ANOVA Comparing Differences in Corolla Shape between *Erysimum mediohispanicum* Populations in the Sierra Nevada (Spain)

Effect	Population	Residual
df	420	42, 420
SS	1.277	27.469
MS	.003	.001
Goodall's F	4.65	
Pillai's trace	1.67	
P	.0001	

Table 2

Pairwise Procrustes Distances

	Em01	Em02	Em08	Em21	Em22	Em23	Em24
Em02	.056						
Em08	.044	.050					
Em21	.054	.073	.063				
Em22	.027	.059	.048	.044			
Em23	.074	.054	.057	.056	.065		
Em24	.083	.074	.067	.063	.081	.056	
Em25	.051	.044	.065	.084	.055	.082	.106

Note. Underscoring indicates nonsignificant distances, according to canonical variate analysis.

(selective hotspots) and other populations undergoing weak or even null selection (selective coldspots; fig. 5A). In addition, we found that the selected corolla shape (that associated with highest relative fitness) varied among populations (fig. 5A). Whereas in some populations there was selection for flowers with narrow petals, in other populations there was selection for rounded or zygomorphic flowers (fig. 5A). We even found divergent selection in corolla shape, since some shape components, such as RW4, were selected in opposite directions in different populations (fig. 5A).

The locally selected corolla shape agreed highly with the preference pattern of the local pollinator fauna (Gómez et al. 2008b, 2009b). Thus, in populations dominated by large bees the fittest flowers were those having narrow petals, whereas in populations dominated by small bees the fittest flowers were those having a round outline, and in those dominated by bee flies the fittest flowers were zygomorphic (fig. 5A). The observed between-pollinator differences in preference patterns suggests the existence of a pollinator-mediated trade-off, since any modification in plant phenotype to attract a given pollinator will produce a decrease in attractiveness for other pollinators (Castellanos et al. 2004; Muchhala 2007). This means that different pollinators may exert opposite selective pressures in this plant species. When pollinator-mediated trade-offs are involved, any among-population variation in the abundance of pollinator functional groups may result in divergent selection (Aigner 2006; Sargent and Otto 2006). The outcome is a geographic mosaic of selection on different corolla shapes. Since corolla shape may vary along multiple dimensions, the resulting geographic mosaic for this trait will presumably be more complex than that appearing in other, simpler traits, such as flower size or corolla tube length.

Local Adaptation in Corolla Shape

The synergistic effect of spatial variation in pollinator fauna and interspecific variation in preference implies that plants located at different populations were under contrasting selective pressures. Under these circumstances, it is expected that *E. mediohispanicum* corolla shape tends toward local differentiation across populations. Using translocation experiments, we demonstrated that plants are locally adapted to their pollinators and that this local adaptation is mediated, among other plant traits, by corolla shape (Gómez et al. 2009a). Thus,
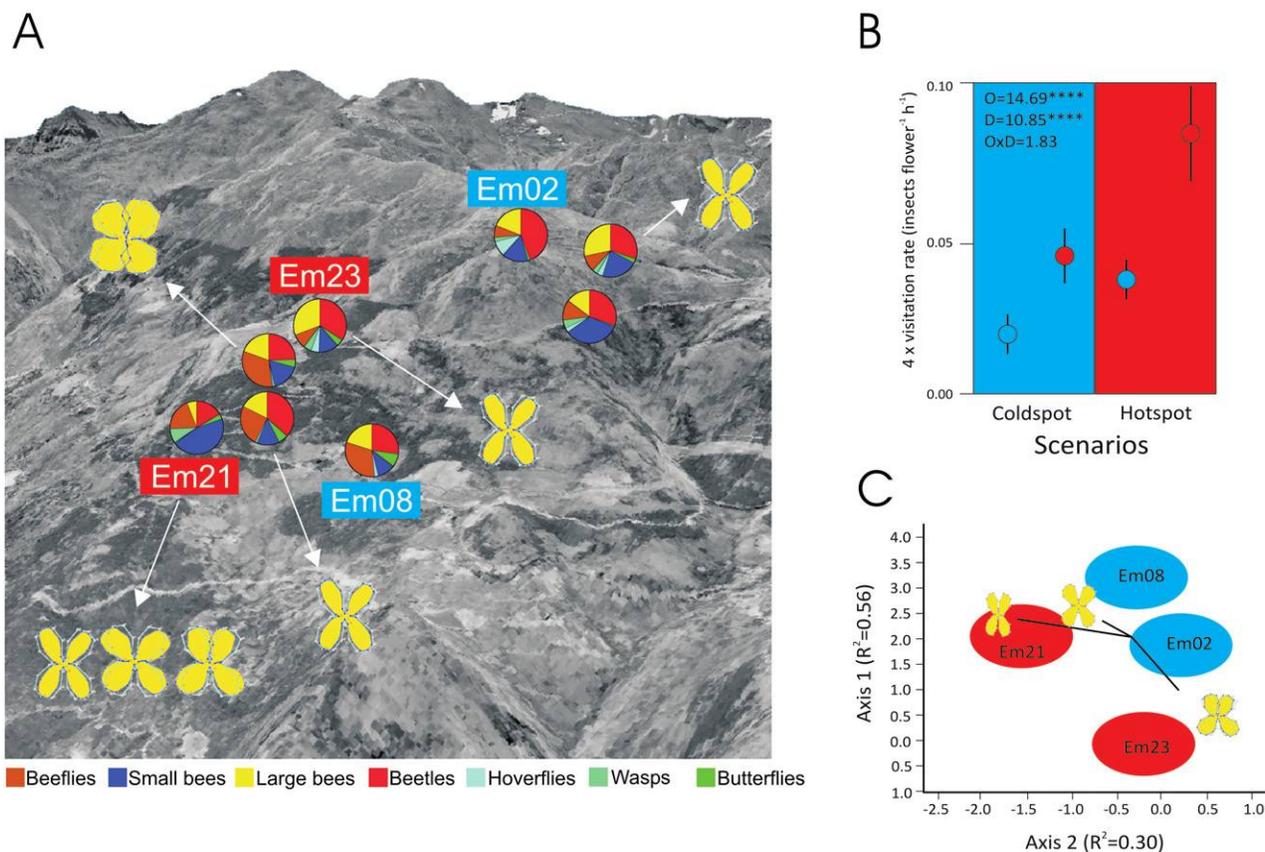
Fig. 5 Spatial structure of corolla shape selection. *A*, Geographic mosaic of selection in the eight focal populations from Sierra Nevada. Selected corolla shapes are shown in each population. *B*, Outcome of the translocation experiment. Plots show the attractiveness (estimated as mean ± 1 SD pollinator visitation rate) of plants from different origin (blue circles for coldspots, red circles for hotspots) in each of the two scenarios (blue background for coldspots, red background for hotspots). The populations used in the experiment are shown with their respective colors in *A*. *C*, Differences in corolla shape between populations used in the experiment on local adaptation.

plants originating from two 2005 hotspots (Em21 and Em23; fig. 5A) are visited more often by flower visitors than plants originating from selective coldspots (Em02 and Em08; fig. 5A), both in their own populations and in the coldspot populations (fig. 5B). Because pollinator visitation rate is significantly related to plant fitness, *E. mediohispanicum* seems to be pollen limited in the study area (Gómez et al. 2010), and pollinators are important selective agents in our plant species, this outcome strongly suggests local adaptation for hotspot plants and maladaptation for coldspot plants. A main difference between hotspot and coldspot plants is the shape of their corollas. Whereas plants originating from hotspots displayed corollas with positive RW1, RW2, and RW4, coldspot plants had corollas without any remarkable shape (fig. 5C). Observing figure 5A, we can note that those were the shapes under significant pollinator-mediated selection during previous selective episodes. All of this suggests a correspondence between the adaptation degree displayed by the experimental plants to pollinators and the overall selection strength experienced by their mother plants. Plants coming from more selective environments presumably produced attractive offspring; this attractiveness and local adaptation to pollinators are caused, at least partially, by the corolla shape displayed by the plants.

Conclusion

Our long-term research program shows that complex plant traits can be adequately investigated with specific methodologies. We have proposed in this article that geometric morphometrics can be successfully applied to explore the evolution of floral shape. By using this approach, we were able to find the pattern of variation in corolla shape, its heritability, the selection occurring on it, and the local adaptation associated with it. We hope that our article will persuade other plant evolutionary biologists to develop similar research with other plant species, since identifying the ecological factors driving corolla shape evolution may help to unravel Darwin 's "abominable mystery."

Acknowledgments

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