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Is Floral Diversification Associated with Pollinator Divergence? Flower Shape, Flower Colour and Pollinator Preference in Chilean *Mimulus*

A. M. COOLEY[1,*], G. CARVALLO[2] and J. H. WILLIS[1]

[1]*Department of Biology, Duke University, Durham, North Carolina 27708, USA and* [2]*Departamento de Ciencias Ecológicas, Universidad de Chile, Casilla 653, Santiago, Chile*

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• *Background and Aims* Adaptation to different pollinators is thought to drive divergence in flower colour and morphology, and may lead to interspecific reproductive isolation. Floral diversity was tested for association with divergent pollinator preferences in a group of four closely related wildflower species: the yellow-flowered *Mimulus luteus* var. *luteus* and the red-pigmented *M. l. variegatus*, *M. naiandinus* and *M. cupreus*.
• *Methods* Patterns of pollinator visitation were evaluated in natural plant populations in central Chile, including both single-species and mixed-species sites. Floral anthocyanin pigments were identified, and floral morphology and nectar variation were quantified in a common garden experiment using seeds collected from the study sites.
• *Key Results Mimulus l. luteus, M. l. variegatus* and *M. naiandinus* are morphologically similar and share a single generalist bumblebee pollinator, *Bombus dahlbomii. Mimulus cupreus* differs significantly from the first three taxa in corolla shape as well as nectar characteristics, and had far fewer pollinator visits.
• *Conclusions* This system shows limited potential for pollinator-mediated restriction of gene flow as a function of flower colour, and no evidence of transition to a novel pollinator. *Mimulus cupreus* may experience reduced interspecific gene flow due to a lack of bumblebee visitation, but not because of its red pigmentation: rare yellow morphs are equally undervisited by pollinators. Overall, the results suggest that factors other than pollinator shifts may contribute to the maintenance of floral diversity in these Chilean *Mimulus* species.

Key words: *Mimulus*, Chile, pollinator preference, floral morphology, flower colour, pigment patterning.

INTRODUCTION

The phenotypic diversity of flowers is both visually striking and evolutionarily intriguing. Since Kölreuter (1761) and Sprengel (1793; see translation in Lloyd and Barrett, 1996) first proposed that the function of flowers is to attract insects, plant–pollinator relationships have been the focus of a large body of research (reviewed in Fenster *et al.*, 2004). Subsequent studies have shown that insect pollinators often have strong preferences for particular floral characters (Muller, 1883; Knuth, 1906; Baker, 1963; Grant and Grant, 1965; Ollerton, 1996; Waser, 1998) and that this can lead to reproductive isolation between divergent floral morphologies (Hodges and Arnold, 1994; Bradshaw *et al.*, 1998; Bradshaw and Schemske, 2003; Ippolito *et al.*, 2004). However, the degree to which such traits generally predict pollinator type is debated (Waser *et al.*, 1996; Ollerton, 1998). Evolutionary diversification of floral traits can occur for many other reasons, potentially uncoupling the evolution of floral traits from pollinator-mediated selection (Whittall and Strauss, 2006). For example, divergence in floral display size may be related to shifts in breeding systems (Totland and Schulte-Herbruggen, 2003; French *et al.*, 2005; Raguso *et al.*, 2007), and the evolution of alternative floral colouration may result from pleiotropic effects of pigmentation biosynthetic pathways (Armbruster, 1993; Schemske and Bierzychudek, 2007; Smith *et al.*, 2008). A major challenge is to evaluate the relative importance of pollinators in floral evolution.

The wildflower genus *Mimulus* is an excellent system for studying plant–pollinator relationships because of its tremendous diversity in floral morphology and colouration (Grant, 1924). Although *Mimulus* is increasingly a focus of ecological, evolutionary and genomic research (Wu *et al.*, 2008), the relationship between pollinator preference and floral evolution is unknown for most species in the genus (but see Schemske and Bradshaw, 1999; Streisfeld and Kohn, 2007). Here, we examine the pollination biology of a group of four closely related *Mimulus* species from central Chile. These species are thought to be recent tetraploid derivatives of the genomic model *M. guttatus* (Vickery *et al.*, 1968; Vickery, 1995) and belong to the section Simiolus, a large monophyletic group that is normally characterized by yellow corollas with red spots along the throat (Beardsley and Olmstead, 2002).

In contrast to the presumably ancestral 'yellow monkeyflower' phenotype, the study taxa vary greatly in flower colour and pigment patterning (see Fig. 1). *Mimulus luteus* var. *luteus* has the classic 'yellow monkeyflower' colour pattern, while *M. l. variegatus* has a white or pale-yellow corolla with purplish anthocyanin pigment covering all five petals. *Mimulus naiandinus* has a similarly pale corolla with pink pigment on the upper two petals and parts of the lower three petals. *Mimulus l. luteus*, *M. l. variegatus* and *M. naiandinus* are vegetatively quite similar, with long stems, internodes and pedicels, and few

* For correspondence. E-mail amc34@duke.edu

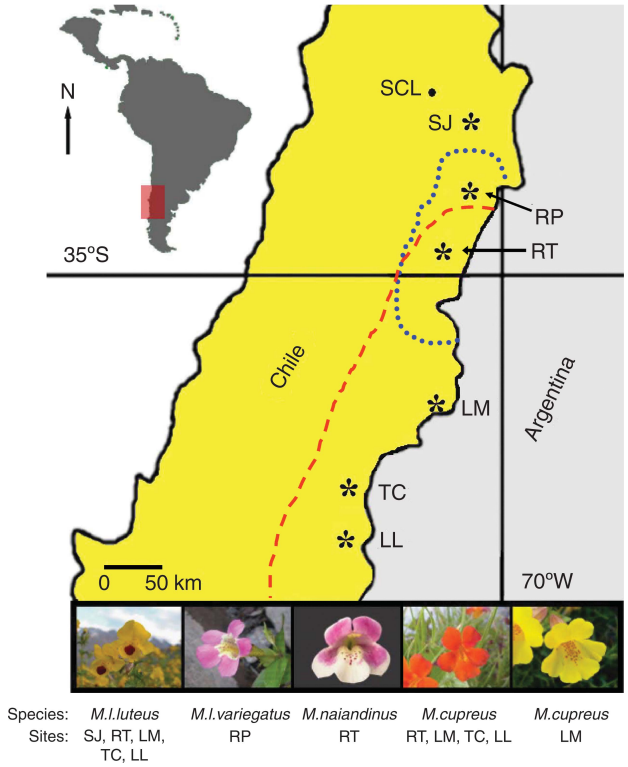

FIG. 1. Study sites, species ranges, and typical flowers of the taxa studied. The distribution of *Mimulus l. luteus* within Chile is shown in yellow. The Chilean distributions of *M. l. variegatus* and *M. cupreus* are indicated by the blue dotted and red dashed line, respectively. A rare yellow morph of *M. cupreus* is known only from LM. Only two populations of *M. naiandinus* have been found (one of which is the RT study site), both within the range of *M. l. variegatus*. The primary range of *M. l. luteus* is in the eastern, montane region of Chile, but there are some reports of populations in the central and coastal regions as well (von Bohlen, 1995). Ranges are redrawn from von Bohlen (1995); the study taxa have occasionally been found in Argentina but their distributions there are unknown. Study sites are denoted by asterisks, and are abbreviated as: SJ, Volcán San José; RP, Río Pangal; RT, Río Tinguiririca; LM, Laguna del Maule; TC, Termas de Chillan; LL, Laguna del Laja. SCL denotes the location of Santiago, Chile. Below each photograph is the name of the taxon and a list of the study sites at which it occurs.

flowers per plant. They are primarily distinguished by flower colour. *Mimulus cupreus*, in contrast, has a compact, bushy habit, short pedicels and numerous flowers per plant. These architectural differences are observed under greenhouse conditions as well as in the field (A. Cooley, pers. obs.). The corolla of *M. cupreus* is dark orange throughout, although a yellow morph with *luteus*-like pigmentation is found in at least one population (LM; Fig. 1).

Ranges of the study taxa overlap geographically. The most abundant species, *M. l. luteus*, often co-occurs with one of the other taxa at a given location. Although we were unable to find a sympatric population of *M. l. luteus* and *M. l. variegatus*, the *M. naiandinus* and *M. cupreus* study sites all contained *M. l. luteus* with either partly overlapping (RT, TC, LL) or fully intermingled (LM) distributions.

The distinctive phenotypes of the Chilean *Mimulus* have been consistently maintained at least since European botanists began working in South America in the 18th and 19th centuries, with the possible exception of *M. naiandinus* (Grant, 1924; von Bohlen, 1995). Despite such long-standing and dramatic floral variation, the Chilean *Mimulus* remain virtually unstudied.

Here, we examine whether the unique and geographically restricted floral diversification in the Chilean *Mimulus* is associated with variation in pollinators. One study of a single *M. l. luteus* population (Medel *et al.*, 2003) has raised this possibility: bees preferred flowers with small and arrow-shaped red spots, while hummingbirds chose flowers with larger and more heart-shaped spots. Such results suggest that pollinators could potentially drive phenotypic divergence in the Chilean *Mimulus*, particularly considering that much greater pigment variation exists between species than within a single population. Some interspecific floral shape variation has also been noted (Grant, 1924; von Bohlen, 1995) but never quantitatively assessed. The purpose of the current study was to determine the extent to which this system shows potential for the maintenance of floral variation by pollinator preference. To that end, two basic questions were addressed. (1) What is the extent of floral differentiation in traits potentially relevant to pollinator discrimination? (2) Does pollinator discrimination by floral phenotype exist in natural populations?

MATERIALS AND METHODS

Study taxa and sites

Mimulus luteus var. *luteus*, *M. l. variegatus*, *M. naiandinus* and *M. cupreus* are native to central Chile and have overlapping distributions, as described in Grant (1924) and von Bohlen (1995). They readily produce hybrids in the greenhouse and sometimes also in nature, but little is known about their genetic distinctness. *Mimulus l. luteus* is the most widely distributed (29 – 45°S, sea level to 3650 m a.s.l), while the others have more limited ranges (Fig.1). All are found predominantly along streams or seeps in premontane habitat. They flower between November and March, with the peak of flowering typically in January and February (G. Carvallo, pers. obs.). To make comparisons across similar habitats we focused on the overlapping region of the *M. l. luteus* and *M. cupreus* distributions, and identified five study locations that contained one or more taxa (Fig. 1).

Measurement of floral trait variation

Maternal families were collected, consisting of up to four ripe fruits per plant from 10–20 randomly selected plants per species per location, spaced so as to approximately sample the entire population. Seeds were germinated in a common garden in the Duke University greenhouses, with 18 h days using supplemental lighting from high-pressure sodium lights. Ten maternal families per population per species were randomly selected, for a total of 120 families, and seeds from each family were planted in two 5-cm pots filled with Fafard 4-P potting soil (Fafard, Agawam, MA,

USA). After germination, four plants per family were transplanted into individual pots. Germination rates were low in some families, so family sizes ranged from one to four.

Floral traits were measured on a single, randomly chosen flower from each plant, 2–8 h after the flower opened (Supplementary Information, Fig. S1; available online). Nectar volume was calculated using calibrated 5-mL glass capillary tubes (Drummond Scientific, Broomall, PA, USA). All other size measurements were made to the nearest 0·1 mm using digital calipers (Mitutoyo America Corporation, Aurora, IL, USA).

Nectar sugar content was measured at a later date, on 22 plants from eight families (*M. l. luteus*), 13 plants from five families (*M. l. variegatus*), ten plants from four families (*luteus* × *naiandinus* hybrid swarm), and eight plants from four families (*M. cupreus*), using a temperature-calibrated handheld Brix refractometer (QA Supplies, Norfolk, VA, USA). Three flowers per plant were dissected and the drop of nectar at the base of the corolla was collected using a 5-mL glass capillary tube and placed on the refractometer plate. Nectar was diluted two- or three-fold with water if it exceeded the refractometer's detection limit of 32% dissolved solids.

To determine whether the study taxa differ in UV patterns, their spectral reflectance was measured in the 200–380 nm range with a fibre optic probe (R400–7 reflection probe, Ocean Optics Inc., Dunedin, FA, USA), coupled with an ultraviolet light source and a multichannel spectrometer (USB2000, Ocean Optics).

In order to identify the biochemical basis of the red pigmentation in the study taxa, anthocyanins were extracted from corollas of a single individual of *M. l. luteus*, *M. l. variegatus* and *M. cupreus*. Anthocyanidin pigments (unglycosylated precursors to the anthocyanins) were extracted by soaking 0·5 g of petal tissue for 1 h in 20–30 mL of 2N HCl, followed by boiling the solution to less than 1·5 mL, adding a few drops of isoamyl alcohol, and resuspending in MeOH with 1% HCl. Anthocyanidins were applied to cellulose-coated glass thin-layer chromatography plates, and were developed for 6–8 h in forestal solvent (acetic acid : HCl : $H_2O = 30 : 3 : 10$). Pigments were identified by comparing spot colour and R_f values to reported values of all naturally occurring anthocyanin compounds (Harborne, 1967)

Analyses of floral trait variation

An analysis of variance was performed on the full dataset in order to identify differences among the four taxa. A MANOVA of all traits except sugar content was used followed by univariate ANOVAs on each trait separately, with taxon as a fixed effect. Nectar sugar content was separately evaluated using a fully nested ANOVA. Taxon was considered a main effect, family was nested within taxon, and individual within family; all three effects were considered random.

A nested ANOVA was used to evaluate population- and family-level variation relative to interspecific variation in *M. l. luteus* and *M. cupreus*. Only families with two or more progeny were included in this analysis. Population was considered a main effect and family was nested within population. Both effects were considered random. *F*-ratios were calculated for each level using the appropriate mean-square denominators of population and family, respectively (Sokal and Rohlf, 1981; Ramsey and Schafer, 2002).

A canonical variate analysis (CVA) was conducted on the full dataset in order to illustrate the extent to which floral morphology successfully classifies the study taxa, relative to our identification based on floral pigmentation and vegetative morphology. A CVA (Fisher, 1936; Campbell and Atchley, 1981) is more appropriate for the data than a principal components analysis (PCA), as PCA assumes that the data belong to a single group or sample with no known substructure (Sokal and Rohlf, 1981; Ramsey and Schafer, 2002). All ANOVAs and MANOVAs were performed in SAS (SAS Institute, Cary, NC, USA, 2002). The CVA was performed in JMP (JMP IN 5·1, SAS Institute, Cary, NC, USA, 2003).

Pollinator visitation

In order to compare pollinator assemblages across the study taxa, patterns of pollinator visitation were examined in January and February 2005 at four locations: RP, RT, LM and TC (see Fig. 1). Observation periods were 30 min each, ranged from pre-dawn (0600 h) to dark (1900 h), and were spaced evenly throughout the day. With few exceptions, each hour of daylight was observed at least twice per site. The observed area was demarcated by a 1-m^2 quadrat, which was moved to a new randomly selected location for each observation period. At each site 40–70 observation periods were conducted over 3–5 d, for a total of 120 h of observations.

The number of open flowers per quadrat was counted; densities ranged from 3–261 flowers m^{-2} ($50{\cdot}8 \pm 3{\cdot}80$ flowers m^{-2}; mean ± s.e.). Each pollinator entering the quadrat was identified and visits were recorded until the pollinator visited a flower outside the quadrat. In the *luteus* × *naiandinus* hybrid swarm, the colour phenotype of each flower visited was also recorded. A visit was defined as entry far enough into the flower to contact the stigma. Wasps and smaller insects were not included, as they did not touch the stigma. The visitation rate at each quadrat was calculated (flowers visited per quadrat flower number per 0·5 h) and then a mean visitation rate per quadrat was calculated for each population. Variation in visitation rate was tested for with a univariate ANOVA, with taxon as a fixed effect.

Patterns of stigmatic closure

The stigmatic lobes of *M. l. luteus*, *M. l. variegatus*, *M. naiandinus* and *M. cupreus* are touch-sensitive and close 5–10 s after tactile stimulation. Experimental studies of other *Mimulus* species indicate that stigmas typically reopen within a few hours in the absence of pollen deposition, but remain closed if hand-pollinated with sufficiently high pollen loads (Dudash and Ritland, 1991; Fetscher and Kohn, 1999). Daily patterns of stigmatic

closure therefore are expected to reflect patterns of successful pollinator visits.

While observing pollination visits at each location (RP, RT, LM and TC), stigma closure was also measured over 24-h periods. In the evening, prior to each 24-h period of observation, unopened flower buds were marked with numbered masking tape and either covered with fine mesh to exclude pollinators (control group; $n = 232$) or left unmanipulated to allow pollination ($n = 429$). Buds that did not open overnight were excluded from the data. We then recorded whether or not stigmas were closed in experimental flowers at dawn, mid-day and dusk, as well as dawn of the following morning. Control flowers were unbagged and examined at dusk.

Pollinator behaviour in a hybrid swarm

The Río Tinguiririca site provided an opportunity to investigate the potential for variation in individual pollinator preferences. A patchy population of *M. naiandinus* extends for nearly a mile along the south bank of the river, and is gradually replaced by *M. l. luteus*, which extends upstream (eastward) for several more miles. The study site was located in the zone of overlap between the two species. At this site a variety of floral pigmentation phenotypes were intermingled along a small (35 × 6 m) riverside gravel bar, including the parental types *M. l. luteus* and *M. naiandinus*, as well as apparent hybrids that differed greatly in the quantity and distribution of red (anthocyanin) and yellow (carotenoid) pigmentation. We focused solely on the predominant pollinator, *Bombus dahlbomii*, which was responsible for >99% of the visits at this location.

On 21 and 27 January 2005, each open flower was scored for the extent of yellow and red pigmentation. Each pigment was scored using a 4-point scale (minimal pigment = 1, maximal = 4), yielding 16 possible phenotypes, with *M. l. luteus* ranking 1 for red and 4 for yellow, and *M. naiandinus* being the reverse (red = 4, yellow = 1; see Fig. 4 for photographs of representative phenotypes). Nineteen foraging bouts of individual *B. dahlbohmii* were recorded, thirteen on 20–22 January and six on 27–28 January, lasting a total of 356 min. Each bee was followed from the time that it first visited a flower to the time that it flew out of sight. Floral phenotypes were recorded in the order visited.

A *G*-test for goodness of fit (Sokal and Rohlf, 1981) was used to determine whether the frequency of each floral phenotype in the population was consistent with the proportion of visits it received. A *G*-test was also conducted for heterogeneity in floral-phenotype composition, across individual *B. dahlbomii* foraging bouts, to determine whether individual pollinators varied in floral preferences.

To evaluate the degree of pollinator constancy, each flower visited by a given *B. dahlbomii* was categorized by whether it had the same phenotype as the previously visited flower ('same') or not ('different'), based on the 16-category system described above. The frequency of flowers in 'same' versus 'different' categories was compared to the frequency expected under a null hypothesis of random phenotype choice, using a χ^2 test.

RESULTS

Measurement of floral trait variation

In a common garden environment, *M. l. luteus*, *M. l. variegatus* and *M. naiandinus* (hereafter referred to as the '*luteus*-like group') were morphologically similar but significantly different from *M. cupreus* (Table 1). *Mimulus cupreus* individuals were significantly smaller than plants in the *luteus*-like group with respect to stigma height, stigma–anther separation, and corolla length, width and height. These univariate results were confirmed by a highly significant MANOVA (Wilks' $\lambda = 0{\cdot}0852$, F approximation = 36·92, $P < 0{\cdot}0001$). Corolla shape differed as well, with a narrower tube relative to overall flower size (smaller width : length and height : length ratios) in *M. cupreus*. *Mimulus cupreus* differed significantly from the *luteus*-like group in nectar traits, with substantially lower nectar volume and sugar content. UV reflectance was not observed in any of the taxa and was excluded from further analysis.

Canonical variate analysis, using all traits except sugar content, was effective in distinguishing *M. cupreus* from the *luteus*-like group (>99%), but was unable to separate taxa within the *luteus*-like group (Fig. 2). The discrimination between *M. cupreus* and the *luteus*-like group was achieved almost entirely (97·7%) by the first linear discriminant function (CAN1).

Multiple populations were available for *M. l. luteus* and *M. cupreus*, allowing us to examine variation within species. Variation was significant at the level of populations and families within populations (Supplementary Information, Table; available online). All six traits showed a significant effect of family, consistent with a genetic basis for trait variation. All but corolla height varied significantly among populations within species. For most traits, population-level variation was small in magnitude and the interspecific differences accounted for over 70% of the total variance.

The red-coloured portions of *M. l. luteus*, *M. l. variegatus* and *M. cupreus* corollas all contained a single type of anthocyanin pigment. Spot colour, R_f values and comparison with known standards indicated that this pigment is cyanidin (Supplementary Information, Fig. S2; available online).

Pollinator visitation

Despite major differences in floral pigment patterning among *M. l. luteus*, *M. l. variegatus* and *M. naiandinus*, all three were visited almost exclusively (1230 of 1233 visits) by a single generalist bumblebee, *Bombus dahlbomii*. Three visits were by unidentified small bees. Per-flower visitation rates for each population ranged from 0·32–0·56 visits per flower h^{-1} (Table 2).

Mimulus cupreus received far fewer visitors than members of the *luteus*-like group at all locations where it occurred (0–0·02 visits per flower h^{-1}). Visitation rate varied significantly by species ($F = 8{\cdot}49$, $P < 0{\cdot}001$), but a Tukey's Studentized range test confirmed that this is a result of significant differences only between *M. cupreus* and the other three taxa (Table 2). The lack of

TABLE 1. *Means ± s.e. of floral characters in four Chilean* Mimulus *species and in* M. l. luteus × M. naiandinus *hybrids. Nectar volume is expressed in μL; nectar sugar is the percentage of dissolved solids; the three 'standardized' variables are unitless; all other variables are expressed in mm. Sample sizes for nectar sugar content are indicated in the text; sample sizes for all other traits are shown in the heading of each column. SA/SH represents stigma–anther separation divided by stigma height*

	M. cupreus ($n = 101$)	*M. l. luteus* ($n = 142$)	*M. l. variegatus* ($n = 32$)	*M. naiandinus* ($n = 27$)	*M. l. luteus* × *M. naiandinus* ($n = 34$)	R^2
Corolla length***	$19{\cdot}3 \pm 0{\cdot}17^{a}$	$26{\cdot}9 \pm 0{\cdot}26^{b}$	$27{\cdot}4 \pm 0{\cdot}53^{bc}$	$26{\cdot}1 \pm 0{\cdot}53^{b}$	$29{\cdot}0 \pm 0{\cdot}54^{c}$	0·648
Corolla width***	$10{\cdot}2 \pm 0{\cdot}19^{a}$	$15{\cdot}3 \pm 0{\cdot}20^{b}$	$15{\cdot}3 \pm 0{\cdot}34^{b}$	$14{\cdot}5 \pm 0{\cdot}43^{b}$	$16{\cdot}0 \pm 0{\cdot}44^{b}$	0·543
Corolla height***	$5{\cdot}9 \pm 0{\cdot}12^{a}$	$9{\cdot}7 \pm 0{\cdot}19^{b}$	$10{\cdot}2 \pm 0{\cdot}20^{b}$	$9{\cdot}9 \pm 0{\cdot}27^{b}$	$9{\cdot}7 \pm 0{\cdot}18^{b}$	0·660
Nectar volume***	$0{\cdot}11 \pm 0{\cdot}032^{a}$	$1{\cdot}7 \pm 0{\cdot}13^{b}$	$1{\cdot}7 \pm 0{\cdot}23^{b}$	$1{\cdot}3 \pm 0{\cdot}19^{b}$	$1{\cdot}6 \pm 0{\cdot}15^{b}$	0·260
Stigma-anther***	$0{\cdot}98 \pm 0{\cdot}183^{a}$	$2{\cdot}5 \pm 0{\cdot}17^{b}$	$2{\cdot}5 \pm 0{\cdot}22^{b}$	$2{\cdot}1 \pm 0{\cdot}36^{b}$	$3{\cdot}4 \pm 0{\cdot}21^{b}$	0·163
Stigma height***	$12{\cdot}9 \pm 0{\cdot}18^{a}$	$26{\cdot}1 \pm 0{\cdot}27^{b}$	$28{\cdot}2 \pm 0{\cdot}50^{c}$	$26{\cdot}8 \pm 0{\cdot}42^{bc}$	$27{\cdot}5 \pm 0{\cdot}47^{bc}$	0·851
SA/SH*	$0{\cdot}06 \pm 0{\cdot}014^{a}$	$0{\cdot}09 \pm 0{\cdot}006^{ab}$	$0{\cdot}09 \pm 0{\cdot}007^{ab}$	$0{\cdot}08 \pm 0{\cdot}013^{ab}$	$0{\cdot}12 \pm 0{\cdot}007^{b}$	0·038
Width/length***	$0{\cdot}53 \pm 0{\cdot}008^{a}$	$0{\cdot}57 \pm 0{\cdot}006^{b}$	$0{\cdot}56 \pm 0{\cdot}007^{ab}$	$0{\cdot}56 \pm 0{\cdot}017^{ab}$	$0{\cdot}55 \pm 0{\cdot}012^{ab}$	0·055
Height/length***	$0{\cdot}31 \pm 0{\cdot}006^{a}$	$0{\cdot}36 \pm 0{\cdot}004^{b}$	$0{\cdot}37 \pm 0{\cdot}005^{b}$	$0{\cdot}38 \pm 0{\cdot}011^{b}$	$0{\cdot}34 \pm 0{\cdot}006^{c}$	0·238
Nectar sugar	$9{\cdot}0 \pm 2{\cdot}80$	$47{\cdot}3 \pm 1{\cdot}90$	$35{\cdot}5 \pm 1{\cdot}97$	n/a	$50{\cdot}2 \pm 2{\cdot}40$	0·846

A significant effect of species, in a one-way ANOVA, is denoted by asterisks following each trait: * $P < 0{\cdot}05$; *** $P < 0{\cdot}001$. Significance groupings in each row are indicated by the superscript letters a, b and c ($P < 0{\cdot}05$, Tukey's Studentized range test).

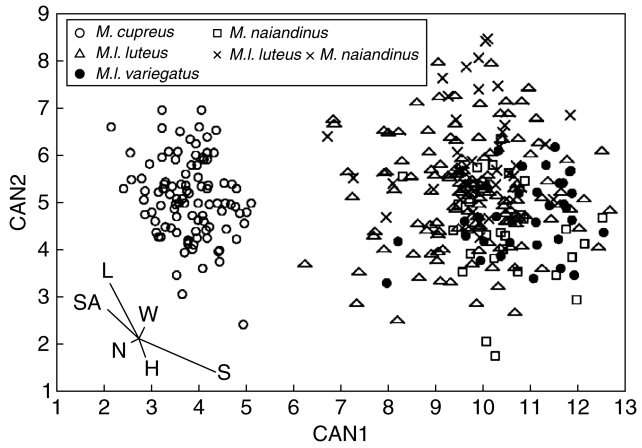

FIG. 2. Canonical variate analysis on six floral characters of Chilean *Mimulus*. Symbols indicate *Mimulus cupreus* ($n = 100$), *M. l. luteus* ($n = 142$), *M. l. variegatus* ($n = 32$), *M. naiandinus* ($n = 27$), *M. l. luteus* × *M. naiandinus* ($n = 34$). Loadings for the six variables are shown in the lower left corner, using the following abbreviations: L, corolla tube length; W, corolla width; H, maximum corolla height; S, stigma height; SA, stigma–anther separation; and N, nectar volume.

M. cupreus pollinators was not due to low floral density: *M. cupreus* had an intermediate floral density of $34{\cdot}5 \pm 5{\cdot}8$ flowers m^{-2} (mean ± s.e.) versus $45{\cdot}5 \pm 3{\cdot}7$ flowers m^{-2} (*M. l. luteus*), $28{\cdot}0 \pm 3{\cdot}3$ flowers m^{-2} (*M. l. variegatus*) and $23{\cdot}7 \pm 3{\cdot}6$ flowers m^{-2} (*M. naiandinus* × *M. l. luteus* hybrid zone).

Patterns of stigmatic closure

Patterns of stigma closure among locations closely reflected observed pollinator visitation rates. The fraction of flowers with closed stigmas at mid-day was highly correlated with overall visitation rate at each site ($R^2 = 0{\cdot}933$, $P < 0{\cdot}001$). In *M. l. luteus*, *M. l. variegatus* and *M. naiandinus*, stigma closure was usually highest at mid-day ($71{\cdot}9 \pm 7{\cdot}26\%$; mean ± s.e.), and decreased by dusk to a mean of $70{\cdot}8 \pm 6{\cdot}19\%$ (Fig. 3). About a quarter of these closed stigmas reopened overnight, suggesting ineffective pollinator visitation. The stigmas of bagged control flowers generally did not close (% closure = $9{\cdot}8 \pm 2{\cdot}53\%$). Consistent with its low visitation rates, *Mimulus cupreus* showed notably lower levels of stigma closure than members of the *luteus*-like group, never exceeding 30% (mean stigma closure was $12{\cdot}1 \pm 5{\cdot}16\%$ at mid-day and $10{\cdot}9 \pm 4{\cdot}46\%$ at dusk). At all sites except LM, 100% of closed *M. cupreus* stigmas reopened overnight, suggesting exceedingly low pollination success. There was little evidence of nocturnal pollination for any taxa, since only 3·2% of the stigmas that were open at dusk were closed the following morning (and rare visits by *B. dahlbomii* were observed just after the evening stigma check and immediately before the morning check, which could easily account for these rare exceptions).

Stigma closure in the bagged controls was probably due to afternoon 'bud pollination' just prior to bagging. *Bombus dahlbomii* strongly prefer open flowers, but sometimes force their way into unopened flower buds, presumably as the nectar stores of open flowers are depleted. Data collected on a single day showed that 0 of 56 visits were 'bud pollinations' in the morning, versus 11 of 60 visits at mid-day and 18 of 60 visits in the late afternoon (data not shown).

Pollinator behaviour in a hybrid swarm

The 19 foraging bouts that we observed at the RT site comprised 1464 flower visits. Bouts ranged from 16–316 visits ($77{\cdot}1 \pm 15{\cdot}72$; mean ± s.e.). There was no effect of day on the mean number of visits per bout ($F = 1{\cdot}21$, d.f. = 18, $P = 0{\cdot}286$) or on the mean of the phenotypes visited with respect to either yellow ($F = 0{\cdot}05$, d.f. = 18, $P = 0{\cdot}822$) or red ($F = 0{\cdot}02$, d.f. = 18, $P = 0{\cdot}997$) pigmentation. A total of 554 and 714 flowers, respectively, were

TABLE 2. *Pollinator visitation rates across species and populations. Population codes are as in Figure 1. 'Visits' is the total number of flowers visited for a given plant taxon at a given location; 'Flowers' is the total number of open flowers observed for that dataset (each flower received 30 min of observation); 'Hours' is the number of hours of observation for that dataset. The visitation rate for each quadrat was calculated as visits per flower h^{-1}; the mean across quadrats is shown in the last column*

Species	Population	Visits	Flowers	Hours	Rate per flower	Mean rate per quadrat
M. l. luteus[a]	LM	316	1975	21	0·32	0·29 ± 0·109
	TC	213	757	12·5	0·56	0·45 ± 0·113
M. l. luteus × *M. naiandinus*[a]	RT	280	1207	28	0·46	0·48 ± 0·073
M. l. variegatus[a]	RP	425	1650	29·5	0·32	0·35 ± 0·070
M. cupreus[b]	RT	5	425	2	0·024	0·025 ± 0·020
	LM	4	5130	19·5	0·002	0·003 ± 0·001
	TC	0	231	7	0	0

Mimulus cupreus differed significantly from the other taxa in visitation rate, as indicated by the superscript letters a and b ($P < 0·001$, Tukey's Studentized range test).

open on the 21 and 27 January censuses. All 16 possible phenotypes were observed, although the *luteus*-like colouration was by far the most common, comprising 27·5% of the population on average (Fig. 4). Because several phenotypic classes were very rare (<1% of the population), we also conducted analyses using only four categories. We examined variation in red pigment alone, and then in yellow pigment alone. With this method, no class contained fewer than 13 individuals. Frequencies of the four red phenotypes did not differ significantly between the two censuses ($G_H = 1·9$, d.f. = 3, P = ns). Frequencies of yellow phenotypes did show significant heterogeneity ($G_H = 1651$, d.f. = 3, $P < 0·001$), due mainly to a reduction in the '1' class (23% versus 14% on 21 and 27 January, respectively) and an increase in the '4' class (29% versus 37%). We therefore compared the behaviour of each bee with floral frequencies from the corresponding census, rather than to the mean of the two censuses.

Heterogeneity across individual foraging bouts was highly significant, whether the data were divided into 16 categories ($G_H = 599·5$, d.f. = 15, $P < 0·0001$) or four red categories ($G_H = 351·3$, d.f. = 3, $P < 0·0001$) and then four yellow categories ($G_H = 713·1$, d.f. = 3, $P < 0·0001$). As shown in Fig. 4, individuals had mean preferences ranging from highly *luteus*-like (little red, much yellow) to moderately *naiandinus*-like (much red, little yellow). An overall preference for *luteus*-like flowers (red = 1 or 2; yellow = 3 or 4) was observed: compared to a null hypothesis that floral phenotypes should be visited in proportion to their frequency in the population, 14 of 19 bees significantly over-visited *luteus*-like flowers ($\chi^2 > 3·84$, d.f. = 1, $P < 0·05$).

Transitions between phenotypes were non-random, with a significant excess of like-to-like transitions for both red ($\chi^2 = 267·6$, d.f. = 1, $P < 0·0001$) and yellow ($\chi^2 = 620·9$, d.f. = 1, $P < 0·0001$) pigments. Transitions between the most *luteus*-like (red, yellow = 1, 4) and the most *naiandinus*-like (4, 1) phenotypes did not account for any of the 1416 observed transitions. However, 30 transitions did occur between the most *luteus*-like flowers (1, 4) and moderately *naiandinus*-like flowers (0, 2; 1, 2; and 1, 3). Nine transitions occurred between the most *naiandinus*-like flowers (4, 1) and moderately *luteus*-like flowers (2, 0; 2, 1; and 3, 1).

DISCUSSION

The main goal in this study was to evaluate whether floral diversity in the wildflower species *Mimulus luteus* var. *luteus*, *M. l. variegatus*, *M. naiandinus* and *M. cupreus* is associated with variation in pollinators. We were motivated by earlier findings (Medel *et al.*, 2003) on the importance of floral anthocyanins to bee versus hummingbird pollinators, and by the extreme differences in floral pigmentation amongst the study taxa. In this study, a single bumblebee species was responsible for the vast majority of all floral visits, suggesting little opportunity for pollinator discrimination among taxa. Visitation rates were high in all taxa except *M. cupreus*. The results indicate that flower colour differences are not associated with distinct pollinator assemblages, and that the only potential source of pollinator-mediated reproductive isolation is individual variation within a single generalist pollinator. Despite the overall lack of pollinator differentiation, species-specific floral phenotypes in the Chilean *Mimulus* are long-standing. Other, perhaps abiotic, factors may instead contribute to the maintenance of this colour-patterning diversity. Future studies should evaluate elements such as parasite interactions, water availability and soil composition.

Limited effect of flower colour on pollinator preference

This study revealed two distinct patterns of pollinator-mediated reproductive isolation: (1) amongst the morphologically similar members of the *luteus*-like group (*M. l. luteus*, *M. l. variegatus* and *M. naiandinus*), assortative mating may exist but is unlikely to pose a strong barrier to gene flow; and (2) *M. cupreus* appears to be largely selfing and thus reproductively isolated from the *luteus*-like group by its low rate of pollinator visitation.

Taxa in the *luteus*-like group are distinct in floral pigmentation but are morphologically similar. All three were pollinated primarily by *Bombus dahlbomii* in this study, in contrast to observations by Medel *et al.* (2003) that

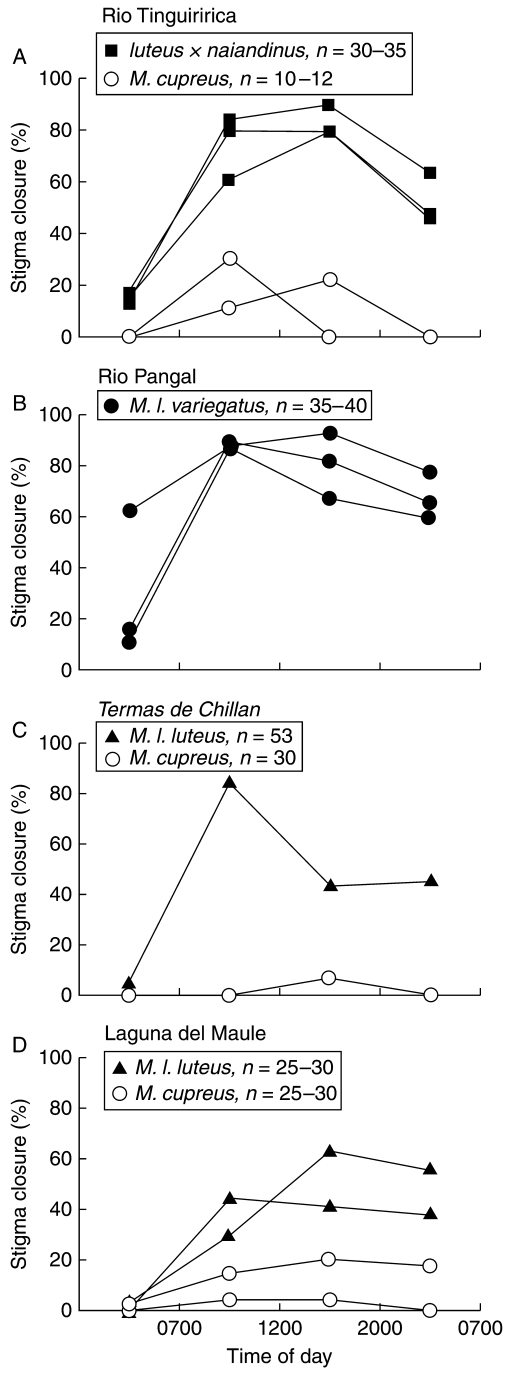

FIG. 3. Patterns of stigma closure in Chilean *Mimulus* over a 24-h period at four locations. Location names are followed by the species present at each location. Sample size in the experimental group is indicated by *n*. Controls (not shown) have sample sizes of approximately 0·5*n*. Each line represents data collected in one 24-h period; up to three replicates (on different days) were performed per species per site. Time-points are shown along the *x*-axis; the *y*-axis shows the percentage of flowers in the experimental group with closed stigmas.

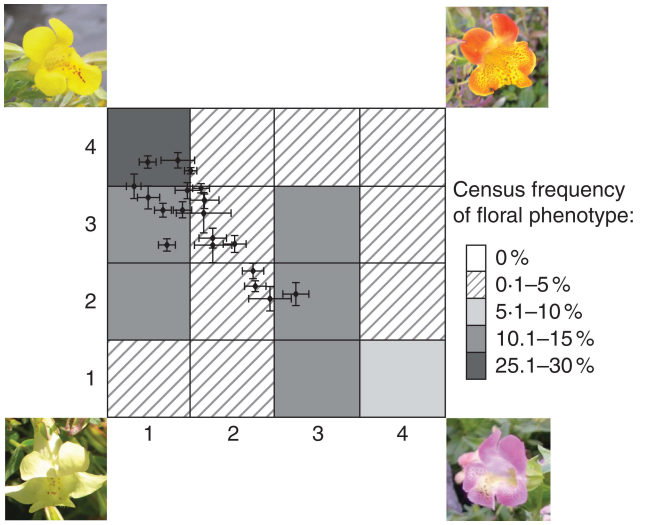

FIG. 4. Individual variation in *Bombus dahlbomii* visits to 16 floral phenotypes in a *Mimulus l. luteus* × *M. naiandinus* hybrid swarm. Each square in the grid represents one of 16 possible phenotypes, based on the extent of red pigmentation (*x*-axis; 1 = minimal, 4 = maximal) and yellow pigmentation (*y*-axis; 1 = minimal, 4 = maximal). The photographs illustrate the following phenotypes, clockwise from top left: (red, yellow) = (1,4); (4,4); (4,1); (1,1). Squares are shaded according to the frequency of the corresponding phenotype, averaged over two censuses. Census sample sizes were $n = 554$ open flowers (21 January 2005) and $n = 714$ open flowers (27 January 2005). The symbols indicate the floral-phenotype mean ± s.e. of 19 individual foraging bouts by *B. dahlbomii*. Sample sizes for the foraging bouts range from 16–316 flowers visited (mean ± s.e. = 77·1 ± 15·72).

hummingbird visits were relatively common in a single, more northerly population of *M. l. luteus*. Pollinator assemblages vary with latitude, and hummingbird visitation is more common at the northern edge of the range of *M. l. luteus* than in the sympatric regions further south (Medel *et al.*, 2007). Hummingbird and bumblebee pollinators could potentially diverge in their preferences in the northern part of the *M. l. luteus* range, but this would have little impact on interspecific gene flow, as the other study taxa do not occur in that region.

Despite the generalist nature of *B. dahlbomii*, the *M. l. luteus* × *M. naiandinus* hybrid swarm at Río Tinguiririca (RT) does show significant variation in the classes of floral phenotypes visited by different *B. dahlbomii* individuals. Since the data were collected from a natural population with non-random distributions of floral phenotypes, it is not clear whether visitation patterns arise from individual preference for particular pigment types or from spatial clustering of flowers. A randomized array would be required to distinguish between the two alternatives.

At RT, clustering occurred for two reasons: (1) each plant has multiple open flowers at any given time, all of which have near-identical pigmentation; (2) the population includes several clusters of plants with similar floral pigmentation, including a large patch of mostly *luteus*-like plants towards the downstream end of the plot and a small patch of mostly *naiandinus*-like plants towards the upstream end. Although these clusters are separated by only about 10 m, such patchiness is likely to affect the floral composition of individual bees' foraging bouts. Bee flight patterns typically consisted of multiple visits within a single small patch, separated by longer flights to another patch.

Regardless of its cause, the variation across foraging bouts will to some extent reduce gene flow between *luteus*-like and *naiandinus*-like individuals at RT. Other regions of *Mimulus* sympatry in Chile tend to be even more spatially structured than the RT site, with partially, but not completely, overlapping populations of two taxa. Interspecific gene flow would then be somewhat limited by the localized foraging behaviour of *B. dahlbomii*, even in the complete absence of floral colour preferences.

Gene flow between phenotypes at RT, while not random, is probably still substantial, and presumably much greater than gene flow between disjunct populations of the same species. Even if no pollinator ever travels directly between the most *luteus*-like and the most *naiandinus*-like plants, indirect transmission will still occur via the intermediate phenotypes (Goulson and Jerrim, 1997; Leebens-Mack and Milligan, 1998; Broyles, 2002).

Our data suggest that flower colour differences in the Chilean *Mimulus* presently have little influence on pollinator behaviour. There are several alternative hypotheses that could explain the existence of species- or subspecies-specific flower colour. This study spans only 6 weeks within a single year, so we cannot evaluate annual variability in pollinator abundance. Other pollinators might be more important in other years, or at the very beginning or end of the flowering season. Floral divergence might have been driven by a pollinator that is now extinct or rare; increasing human activity in the Andean foothills has resulted in the destruction of potential *Mimulus* and pollinator habitats. Another hypothesis is that floral variation is selectively unimportant and due instead to genetic drift. Given the multigenic basis of flower-colour differences in the study taxa (A. Cooley, unpubl. res.), this seems unlikely.

Finally, floral anthocyanin variation could be due to non-pollinator sources of selection. Whittall and Strauss (2006) reviewed several examples of floral colour polymorphisms in which the more anthocyanic form exhibits higher tolerance to one or more forms of environmental stress. The probable explanation for this phenomenon is that flavonoids, the biochemical precursors of the red anthocyanin pigments (Harborne, 1967), are important in buffering plants against extremes of light and heat (Holton and Cornish, 1995; Chalker-Scott, 1999; Hoch *et al.*, 2001; Coberly and Rausher, 2003). An upregulation in floral anthocyanins may be associated with an overall increase in flavonoids, either in the flower alone or in the entire plant. In the desert annual *Linanthus parryae*, for example, two morphs that differ in floral anthocyanin quantity and distribution are maintained by strong and fluctuating abiotic selection. Patterns of selection are associated with annual variability in rainfall, possibly as a result of differential adaptation to soil chemistry between the two morphs (Schemske and Bierzychudek, 2001; Turelli *et al.*, 2001; Schemske and Bierzychudek, 2007).

Pollinator preference associated with flower shape?

In our common garden, *Mimulus cupreus* differed from members of the *luteus*-like group in multiple aspects of floral morphology as well as in its reproductive ecology. One possible concern is that morphology might differ between greenhouse and field conditions. However, a separate sample of field-collected versus greenhouse-raised plants from the same two populations did not differ significantly in corolla length (G. Carvallo, unpubl. res.), indicating that our results are likely to be consistent with patterns in natural populations.

While the *luteus*-like group showed high rates of pollinator visitation, comparable with those observed for other outcrossing species of *Mimulus* (Schemske and Bradshaw, 1999; Mitchell *et al.*, 2004), all three populations of *M. cupreus* had markedly low visitation rates. Low visitation rates were not due to a lack of bumblebee activity: at all three locations, *M. cupreus* co-occurred with another *Mimulus* species that received frequent and effective pollinator visits. Despite its lack of pollinator visitation, *Mimulus cupreus* has a high seed set both in the field and in the greenhouse (A. Cooley and G. Carvallo, pers. obs.), suggesting that it may frequently self-fertilize and thus may have little opportunity for genetic exchange with the other study taxa.

Discrimination against *M. cupreus* does not appear to be associated with flower colour. At Laguna del Maule, a yellow morph of *M. cupreus* occurs together with the characteristic orange morph. Both are intermingled with the yellow-flowered *M. l. luteus. Mimulus l. luteus* and yellow *M. cupreus* do not differ in ultraviolet reflectance or in the types of anthocyanin pigment that they contain, and have highly similar patterns of corolla pigmentation. Although *M. l. luteus* was very frequently visited at LM, only one out of 3630 yellow *M. cupreus* flowers was observed to be visited, which is even less than the three visits out of 1500 observed flowers received by orange-flowered *M. cupreus*, and opposite to the pattern expected if the pollinator avoidance of *M. cupreus* were due to its characteristic orange flower colour.

It is possible that *M. cupreus* is associated with a spatially or temporally variable pollinator that was not observed in this study. Long-tongued insects such as butterflies or bombyliids, for example, could easily reach into the relatively narrow throat of *M. cupreus*. As mentioned in the Results, a small number of bombyliids visited *M. cupreus* at Laguna del Maule. Bombyliids visit nectar-bearing flowers of many shapes and sizes, with a preference for blue and lavender colours (Kastinger and Weber, 2001). Adult populations of bombyliids are typically present for just a few weeks or months per year (Kastinger and Weber, 2001). Since all data were collected during one month at the height of the *M. cupreus* flowering season, bombyliids could potentially play a more important role at the beginning or end of the season. However, morphological data show a nearly complete lack of nectar in all populations of *M. cupreus*, suggesting that nectar-seeking insects are unlikely to be a common contributor to this plant's mating system.

Alternatively, despite its large and showy flower, *M. cupreus* may be a predominantly self-fertilizing species. Despite the absence of *M. cupreus* pollinators throughout the peak month of flowering, nearly every fruit that we examined was filled with seed (A. Cooley

and G. Carvallo, pers. obs.). There are several examples of highly selfing showy-flowered plants, including *Mimulus platycalyx* (Dole, 1992; Lin and Ritland, 1997), *Datura stramonium* (Motten and Antonovics, 1992) and the orchids *Ophrys apifera* and *Disa grandiflora* (Darwin, 1877). Additional genetic data are needed to confirm differences in outcrossing rate between *M. cupreus* and the other Chilean *Mimulus*. However, *M. cupreus* autogamously selfs much more readily in the greenhouse than members of the *luteus*-like group (A. Cooley, pers. obs.). Its low nectar content, relatively small flower size, and reduced stigma–anther separation are also consistent with a highly selfing mating system.

Conclusions

We have shown that the evolutionarily recent appearance of red-pigmented flowers in the 'yellow monkeyflower' section of *Mimulus* is not associated with a transition to 'red-flower' pollinators such as hummingbirds, or indeed to any new type of pollinator at all. The only major transition is one of mating system, with an apparent shift towards a more highly selfing strategy in *M. cupreus*. Selfing in *M. cupreus* may be associated with changes in flower shape, but does not appear to be a function of flower colour.

Classic 'pollinator syndromes' have indeed been found in other parts of the genus (Schemske and Bradshaw, 1999; Streisfeld and Kohn, 2007). This study illustrates the diversity of mechanisms of floral evolution within a single genus, and highlights the importance of an increased understanding of non-pollinator contributions to floral diversity.

SUPPLEMENTARY INFORMATION

Supplementary information is available online at http://aob.oxfordjournals.org/ and consists of two figures and a table as follows. Figure S1: landmarks for morphological measurements, with diagrams showing front and side views of a *M. l. luteus* flower. Figure S2: thin-layer chromatography identification of floral petal pigments. Table: nested analyses of six morphological characters in *Mimulus luteus* var. *luteus* and *M. cupreus*.

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LITERATURE CITED

Armbruster WS. 1993. Evolution of plant pollination systems – hypotheses and tests with the neotropical vine *Dalechampia*. *Evolution* **47**: 1480–1505.

Baker HG. 1963. Evolutionary mechanisms in pollination biology. *Science* **139**: 877–883.

Beardsley PM, Olmstead RG. 2002. Redefining Phrymaceae: the placement of *Mimulus*, tribe Mimuleae and *Phryma*. *American Journal of Botany* **89**: 1093–1102.

von Bohlen C. 1995. El género *Mimulus* L. (Scrophulariaceae) en Chile. *Gayana Botanica* **52**: 7–28.

Bradshaw HD, Schemske DW. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**: 176–178.

Bradshaw HD, Otto KG, Frewen BE, McKay JK, Schemske DW. 1998. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics* **149**: 367–382.

Broyles SB. 2002. Hybrid bridges to gene flow: a case study in milkweeds (*Asclepias*). *Evolution* **56**: 1943–1953.

Campbell NA, Atchley WR. 1981. The geometry of canonical variate analysis. *Systematic Zoology* **30**: 268–280.

Chalker-Scott L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and photobiology* **70**: 1–9.

Coberly LC, Rausher MD. 2003. Analysis of a chalcone synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Molecular Ecology* **12**: 1113–1124.

Darwin CR. 1877. *On the various contrivances by which British and foreign orchids are fertilized*. New York: D. Appleton.

Dole JA. 1992. Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). *American Journal of Botany* **79**: 650–659.

Dudash MR, Ritland K. 1991. Multiple paternity and self-fertilization in relation to floral age in *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany* **78**: 1746–1753.

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* **35**: 375–403.

Fetscher AE, Kohn JR. 1999. Stigma behavior in *Mimulus aurantiacus* (Scrophulariaceae). *American Journal of Botany* **86**: 1130–1135.

Fisher RA. 1936. The use of multiple measurements in taxonomic problems. *Annals of Eugenics* **7**: 179–188.

French GC, Ennos RA, Silverside AJ, Hollingsworth PM. 2005. The relationship between flower size, inbreeding coefficient and inferred selfing rate in British *Euphrasia* species. *Heredity* **94**: 44–51.

Goulson D, Jerrim K. 1997. Maintenance of the species boundary between *Silene dioica* and *S. latifolia* (red and white campion). *Oikos* **79**: 115–126.

Grant AL. 1924. A monograph of the genus *Mimulus*. *Annals of the Missouri Botanical Garden* **11**: 99–389.

Grant V, Grant KA. 1965. *Flower pollination in the phlox family*. New York: Columbia University Press.

Harborne JB. 1967. *Comparative biochemistry of the flavonoids*. London and New York: Academic Press.

Hoch WA, Zeldin EL, McCown BH. 2001. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology* **21**: 1–8.

Hodges SA, Arnold ML. 1994. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proceedings for the National Academy of Sciences* 91: 2493–2496.

Holton TA, Cornish EC. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* **7**: 1071–1083.

Ippolito A, Fernandes GW, Holtsford TP. 2004. Pollinator preferences for *Nicotiana alata*, *N. forgetiana*, and their F_1 hybrids. *Evolution* **58**: 2634–2644.

Kastinger C, Weber A. 2001. Bee-flies (*Bombylius* spp., Bombyliidae, Diptera) and the pollination of flowers *Flora* **196**: 3–25.

Knuth P. 1906. *Handbook of flower pollination*. Oxford: Clarendon Press.

Kölreuter JG. 1761. *Vorläufige Nachrichten von einigen das Geschlecht der Pflanzen betreffenden Versuchen und Beobachtungen*. Leipzig: Gleditschischen Handlung.

Leebens-Mack J, Milligan B. 1998. Pollination biology in hybridizing *Baptisia* (Fabaceae) populations. *American Journal of Botany* **85**: 500–507.

Lin JZ, Ritland K. 1997. Quantitative trait loci differentiating the outbreeding *Mimulus guttatus* from the inbreeding *M. platycalyx*. *Genetics* **146**: 1115–1121.

Lloyd DG, Barrett SCH. Eds**. 1996.** *Floral biology: studies on floral evolution in animal-pollinated plants*. Chapter 1: Sprengel CK. Discovery of the secret of nature in the structure and fertilization of flowers. New York: Chapman and Hall, 1–43.

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

Medel R, Valiente A, Botto-Mahan C, Carvallo G, Pérez F, Pohl N, Navarro L. 2007. The influence of insects and hummingbirds on the geographical variation of the flower phenotype in *Mimulus luteus*. *Ecography* **30**: 812–818.

Mitchell RJ, Karron JD, Holmquist KG, Bell JM. 2004. The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. *Functional Ecology* **18**: 116–124.

Motten AF, Antonovics J. 1992. Determinants of outcrossing rate in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* **79**: 419–427.

Muller NH. 1883. *The fertilization of flowers*. London: Macmillan.

Ollerton J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant–pollinator systems. *Journal of Ecology* **84**: 767–769.

Ollerton J. 1998. Sunbird surprise for syndromes. *Nature* **394**: 726–727.

Raguso RA, Kelber A, Pfaff M, Levin RA, McDade LA. 2007. Floral biology of North American *Oenothera* sect. *Lavauxia* (Onagraceae): advertisements, rewards, and extreme variation in floral depth. *Annals of the Missouri Botanical Garden* **94**: 236–257.

Ramsey FL, Schafer DW. 2002. *The statistical sleuth: a course in methods of data analysis*. Pacific Grove, CA: Wadsworth Group.

Schemske DW, Bierzychudek P. 2001. Evolution of flower colour in the desert annual *Linanthus parryae*: Wright revisited. *Evolution* **55**: 1269–1282.

Schemske DW, Bierzychudek P. 2007. Spatial differentiation for flower colour in the desert annual *Linanthus parryae*: was Wright right? *Evolution* **61**: 2528–2543.

Schemske DW, Bradshaw HD. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the USA* **96**: 11910–11915.

Smith S, Ane C, Baum D. 2008. The role of pollinator shifts in the floral diversification of *Iochroma* (Solanaceae). *Evolution*. In press. doi: 10·1111/j.1558-5646·2008·00327.x

Sokal RR, Rohlf FJ. 1981. *Biometry*. New York: W.H. Freeman and Co.

Sprengel CK. 1793. *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*. Berlin: Vieweg.

Streisfeld MA, Kohn JR. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* **20**: 122–132.

Totland O, Schulte-Herbruggen B. 2003. Breeding system, insect flower visitation, and floral traits of two alpine *Cerastium* species in Norway. *Arctic, Antarctic and Alpine Research* **35**: 242–247.

Turelli M, Schemske DW, Bierzychudek P. 2001. Stable two-allele polymorphisms maintained by fluctuating fitnesses and seed banks: protecting the blues in *Linanthus parryae*. *Evolution* **55**: 1283–1298.

Vickery RK. 1995. Speciation by aneuploidy and polyploidy in *Mimulus* (Scrophulariaceae). *Great Basin Naturalist* **55**: 174–176.

Vickery RK, Crook KW, Lindsay DW, Mia MM, Tai W. 1968. Chromosome counts in the section *Simiolus* of the genus *Mimulus* (Scrophulariaceae). VII. New numbers for *M. guttatus, M. cupreus*, and *M. tilingii*. *Madroño* **19**: 211–218.

Waser NM. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. *Oikos* **81**: 198–201.

Waser NM, Chittka L, Price MV, Williams NM, Ollerton J. 1996. Generalization in pollination systems, and why it matters. *Ecology* **77**: 1043–1060.

Whittall JB, Strauss SY. 2006. Non-pollinator agents of selection on floral traits. In: Harder LD, Barrett SCH, eds. *Ecology and evolution of flowers*. Oxford: Oxford University Press, 120–138.

Wu CA, Lowry DB, Cooley AM, Wright KM, Lee Y-W, Willis JH. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. *Heredity* **100**: 220–230.