Evolution of Floral Color Patterning in Chilean *Mimulus*

by

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Greg Wray

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Evolution can be studied at many levels, from phenotypic to molecular, and from a variety of disciplines. An integrative approach can help provide a more complete understanding of the complexities of evolutionary change. This dissertation examines the ecology, genetics, and molecular mechanisms of the evolution of floral anthocyanin pigmentation in four species of *Mimulus* native to central Chile. Anthocyanins, which are responsible for the red and purple colors in many plants, are a valuable model for studying evolutionary processes. They are ecologically important and highly variable both within and between species, and the underlying biosynthetic pathway is well characterized. The focus of this dissertation is dramatic diversification in anthocyanin coloration, in four taxa that are closely related to the genomic model system *M. guttatus*.

I posed three primary questions: (1) Is floral pigment diversification associated with pollinator divergence? (2) What is the genetic basis of this diversification? (3) What is the molecular mechanism of the increased production of anthocyanin pigment? The first question was addressed by evaluating patterns of pollinator visitation in natural populations of all four study taxa. Flower color showed little effect of flower color on pollinator behavior, implying that pollinator preference probably did not drive pigment evolution in this group. However, the segregation analyses on intra- and interspecific crosses, which were performed to answer question (2), revealed that petal
anthocyanin pigmentation has evolved three times independently in the study taxa, suggesting an adaptive origin. In addition to pollinator attraction, anthocyanins and their biochemical precursors protect against a variety of environmental stressors, and selection may have acted on these additional functions.

One of the three examples of increased petal anthocyanin pigmentation was further dissected at the molecular level, using candidate gene testing and quantitative gene expression analysis. This single-locus trait maps to a transcription factor, McAn1, which is differentially expressed in high- versus low-pigmented flowers. Expression of the anthocyanin structural genes is tightly correlated with McAn1 expression. The results suggest that the molecular mechanism for increased petal pigmentation is a mutation cis to McAn1 that alters the activity level of the anthocyanin biosynthetic pathway.
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And finally, I would like to thank my brother, sisters, and parents, none of whom are scientists and all of whom have nevertheless learned enough science to understand what it is that I do, and why, and who have cheered me on at every step.
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1. Is floral diversification associated with pollinator divergence?

1.1 Introduction

The phenotypic diversity of flowers is both visually striking and evolutionarily intriguing. Since Kölreuter (1761) and Sprengel (1793; 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 coloration may result from pleiotropic effects of pigmentation biosynthetic pathways (Armbruster 1993; Schemske and Bierzychudek 2007; Smith et al. 1
In press). 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 coloration (Grant 1924). Although *Mimulus* is increasingly a focus of ecological, evolutionary and genomic research (Wu et al. 2007), 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 color and pigment patterning (Fig. 1). *Mimulus luteus* var. *luteus* has the classic “yellow monkeyflower” color 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. 
*naianinus* are vegetatively quite similar, with long stems, internodes, and pedicels, and few flowers per plant. They are primarily distinguished by flower color. *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).
Figure 1: Study sites, species ranges, and typical flowers of the study taxa. SCL: Santiago, Chile. The distribution of Mimulus l. luteus within Chile is shown in yellow. Chilean distributions of M. l. variegatus and M. cupreus are indicated by the blue dotted and red dashed line, respectively. 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. 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: Volcán San José (SJ); Río Pangal (RP); Río Tinguiririca (RT); Laguna del Maule (LM); Termas de Chillan (TC); Laguna del Laja (LL). Below each photo is the taxon name and the study sites at which it occurs.
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; vonBohlen 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) raises 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; vonBohlen 1995) but never quantitatively assessed. Our purpose is to determine the extent to which this system shows potential for the maintenance of floral variation by pollinator preference. To that end, we address two basic questions:

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?

1.2 Materials and Methods

1.2.1 Study taxa and sites

*Mimulus luteus* var. *luteus*, *M. l. var. 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).

1.2.2 Measurement of floral trait variation

We collected maternal families consisting of up to four ripe fruits per plant from 10 – 20 haphazardly selected plants per species per location, spaced to approximately sample the entire population. We germinated seeds in a common garden in the Duke University greenhouses, with 18 h days with supplemental lighting from high-pressure sodium lights. We randomly selected ten maternal families per population per species, for a total of 120 families, and planted seeds from each family in two 5-cm pots filled with Fafard 4-P potting soil. After germination, we transplanted four plants per family into individual pots. Germination rates were low in some families, so family sizes range from one to four.

We measured floral traits on a single, randomly chosen flower from each plant, 2 – 8 h after the flower opened (Fig. 2). Nectar volume was calculated using calibrated 5 μL glass capillary tubes (Drummond Scientific, Broomall, PA, U.S.A.). All other size measurements were made to the nearest 0.1 mm using digital calipers (Mitutoyo America Corporation, Aurora, IL, U.S.A.).

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), 10 plants from four
families (*luteus* x *naiandinus* hybrid swarm), and eight plants from four families (*M. cupreus*), using a temperature-calibrated handheld Brix refractometer (QA Supplies, Norfolk, Virginia, U.S.A.). Three flowers per plant were dissected and the drop of nectar at the base of the corolla was collected using a 5 µl glass capillary tube and placed on the refractometer plate. Nectar was diluted two- or three-fold with water if it exceeded refractometer’s detection limit of 32% dissolved solids.

**Figure 2: Landmarks for morphological measurements.** Diagrams show front and side views of a *M. l. luteus* flower. Lettering indicates (a) maximum throat width, (b) corolla length, (c) maximum tube height, (d) stigma-anther separation, and (e) stigma height.

To determine whether the study taxa differ in UV patterns, we measured their spectral reflectance in the 200 – 380 nm range with a fiber optic probe (R400-7 reflection

To identify the biochemical basis of the red pigmentation in the study taxa, we extracted anthocyanins from corollas of a single individual of *M. l. luteus*, *M. l. variegatus*, and *M. cupreus* by Anthocyanidin pigments (unglycosylated precursors to the anthocyanins) were extracted by soaking 0.5 g 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$_2$O = 30:3:10). Pigments were identified by comparing spot color and *R_f* values to reported values of all naturally occurring anthocyanin compounds (Harborne 1967).

**1.2.3 Analyses of floral trait variation**

We performed an analysis of variance on the full dataset in order to identify differences among the four taxa. We used a MANOVA of all traits except sugar content 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.
We used a nested ANOVA 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. We calculated $F$ ratios for each level using the appropriate Mean Square denominators of population and family, respectively (Sokal and Rohlf 1981; Ramsey and Schafer 2002).

We conducted a canonical variate analysis (CVA) on the full dataset 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, North Carolina, U.S.A., 2002). The CVA was performed in JMP (JMP IN 5.1, SAS Institute, Cary, North Carolina, U.S.A., 2003).

### 1.2.4 Pollinator visitation

In order to compare pollinator assemblages across the study taxa, we examined patterns of pollinator visitation in January and February 2005 at four locations: RP, RT, LM, and TC. Observation periods were 30 minutes each, ranged from pre-dawn (6:00...
am) to dark (9:00 pm), 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 haphazardly selected location for each observation period. At each site we conducted 40 to 70 observation periods over three to five days, for a total of 120 h of observations.

We counted the number of open flowers per quadrat; densities ranged from 3 – 261 flowers·m$^{-2}$ (50.8 ± 3.80 flowers·m$^{-2}$; mean ± SE). We identified each pollinator entering the quadrat and recorded visits until the pollinator flew out of range or visited a flower outside the quadrat. In the *luteus* x *naiandinus* hybrid swarm, we also recorded the color phenotype of each flower visited. 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. We calculated the visitation rate at each quadrat (flowers visited·quadrat flower number$^{-1}$·0.5 h$^{-1}$), and then calculated a mean visitation rate per quadrat for each population. We tested for variation in visitation rate with a univariate ANOVA, with taxon as a fixed effect.

### 1.2.5 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), we also measured stigma closure 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.

1.2.6 Pollinator behavior in a hybrid swarm

The Rio 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 m x 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 Jan. 2005, we scored each open flower 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. 6 for
photos of representative phenotypes. Nineteen foraging bouts of individual *B.
dahlbomii* were recorded, thirteen on 20-22 Jan. and six on 27-28 Jan., lasting a total of
356 minutes. 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. We also conducted a G-test 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 $\chi^2$ test.

1.3 Results

1.3.1 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’ $\lambda = 0.0852$, $F$ approximation = 36.92, $P < 0.0001$). 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 but 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. 3). 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 (Table 2). 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-colored portions of *M. l. luteus*, *M. l. variegatus*, and *M. cupreus* corollas all contained a single type of anthocyanin pigment. Spot color, Rf values, and comparison to known standards indicate that this pigment is cyanidin (Fig. 4).
Table 1: Means ± SE of floral characters in Chilean *Mimulus*. Nectar volume is in µl; nectar sugar is in % dissolved solids; the three ‘standardized’ variables are unitless; other variables are in mm. Sample sizes for nectar sugar are in the text; sample sizes for other traits are in the column headings. SA/SH is stigma-anther separation divided by stigma height. A significant effect of species (ANOVA) is denoted by asterisks: *P* < 0.05 (*); **P** < 0.001 (**). Significance groupings in each row are indicated by the superscript letters a, b, and c (*P* < 0.05, Tukey’s Studentized range test).

<table>
<thead>
<tr>
<th></th>
<th>cupreus (n = 101)</th>
<th>luteus (n = 142)</th>
<th>variegatus (n = 32)</th>
<th>naiandinus (n = 27)</th>
<th>luteus x naiandinus (n = 34)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corolla length***</td>
<td>19.3 ± 0.17a</td>
<td>26.9 ± 0.26b</td>
<td>27.4 ± 0.53bc</td>
<td>26.1 ± 0.53b</td>
<td>29.0 ± 0.54c</td>
<td>0.648</td>
</tr>
<tr>
<td>Corolla width***</td>
<td>10.2 ± 0.19a</td>
<td>15.3 ± 0.20b</td>
<td>15.3 ± 0.34b</td>
<td>14.5 ± 0.43b</td>
<td>16.0 ± 0.44b</td>
<td>0.543</td>
</tr>
<tr>
<td>Corolla height***</td>
<td>5.9 ± 0.12a</td>
<td>9.7 ± 0.19b</td>
<td>10.2 ± 0.20b</td>
<td>9.9 ± 0.27b</td>
<td>9.7 ± 0.18b</td>
<td>0.660</td>
</tr>
<tr>
<td>Nectar volume***</td>
<td>0.11 ± 0.032a</td>
<td>1.7 ± 0.13b</td>
<td>1.7 ± 0.23b</td>
<td>1.3 ± 0.19b</td>
<td>1.6 ± 0.15b</td>
<td>0.260</td>
</tr>
<tr>
<td>Stigma-anther***</td>
<td>0.98 ± 0.183a</td>
<td>2.5 ± 0.17b</td>
<td>2.5 ± 0.22b</td>
<td>2.1 ± 0.36b</td>
<td>3.4 ± 0.21b</td>
<td>0.163</td>
</tr>
<tr>
<td>Stigma height***</td>
<td>12.9 ± 0.18a</td>
<td>26.1 ± 0.27b</td>
<td>28.2 ± 0.50c</td>
<td>26.8 ± 0.42bc</td>
<td>27.5 ± 0.47bc</td>
<td>0.851</td>
</tr>
<tr>
<td>SA / SH*</td>
<td>0.06 ± 0.014a</td>
<td>0.09 ± 0.006ab</td>
<td>0.09 ± 0.007ab</td>
<td>0.08 ± 0.013ab</td>
<td>0.12 ± 0.007b</td>
<td>0.038</td>
</tr>
<tr>
<td>Width / length***</td>
<td>0.53 ± 0.008a</td>
<td>0.57 ± 0.006b</td>
<td>0.56 ± 0.007ab</td>
<td>0.56 ± 0.017ab</td>
<td>0.55 ± 0.012ab</td>
<td>0.055</td>
</tr>
<tr>
<td>Height / length***</td>
<td>0.31 ± 0.006a</td>
<td>0.36 ± 0.004b</td>
<td>0.37 ± 0.005b</td>
<td>0.38 ± 0.011b</td>
<td>0.34 ± 0.006c</td>
<td>0.238</td>
</tr>
<tr>
<td>Nectar sugar</td>
<td>9.0 ± 2.80</td>
<td>47.3 ± 1.90</td>
<td>35.5 ± 1.97</td>
<td>n/a</td>
<td>50.2 ± 2.40</td>
<td>0.846</td>
</tr>
</tbody>
</table>
Table 2: Nested analyses of six morphological characters in *Mimulus luteus* var. *luteus* (*n* = 138) and *M. cupreus* (*n* = 97). Population was nested within species and family within population. Significant effects of family, population and species are denoted by asterisks: *P* < 0.05 (*); *P* < 0.01 (**); *P* < 0.001 (**). VC, Variance Component.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Corolla length (mm)</th>
<th>Corolla width (mm)</th>
<th>Corolla height (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS</td>
<td>F</td>
<td>VC (%)</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>3322</td>
<td>81.33***</td>
<td>79.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population</td>
<td>6</td>
<td>40.85</td>
<td>3.19**</td>
<td>2.74</td>
</tr>
<tr>
<td>Family</td>
<td>57</td>
<td>12.82</td>
<td>3.30***</td>
<td>6.87</td>
</tr>
<tr>
<td>Error</td>
<td>170</td>
<td>3.89</td>
<td>10.73</td>
<td>2.69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Nectar volume (µl)</th>
<th>Stigma-anther distance (mm)</th>
<th>Stigma height (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MS</td>
<td>F</td>
<td>VC (%)</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>146.4</td>
<td>5.07**</td>
<td>34.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population</td>
<td>6</td>
<td>28.86</td>
<td>25.54***</td>
<td>34.78</td>
</tr>
<tr>
<td>Family</td>
<td>57</td>
<td>1.13</td>
<td>1.51*</td>
<td>3.68</td>
</tr>
<tr>
<td>Error</td>
<td>170</td>
<td>0.75</td>
<td>26.66</td>
<td>1.63</td>
</tr>
</tbody>
</table>

Note: *VC* stands for Variance Component.
Figure 3: Canonical variate analysis on six floral characters of Chilean *Mimulus*.
Symbols indicate *Mimulus cupreus* (open circles; n = 100), *M. l. luteus* (triangles; n = 142), *M. l. variegatus* (closed circles; n = 32), *M. naiandinus* (squares; n = 27), *M. l. luteus* x *M. naiandinus* (crosses; 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).
Figure 4: Thin Layer Chromatography identification of floral petal pigments. Lanes (A) and (B) provide known cyanidin and pelargonidin standards for visual comparison, extracted from *Ipomoea purpurea* and *I. quamoclit* respectively. Subsequent lanes correspond to *M. l. variegatus* (C); *M. l. luteus* yellow floral tissue (D); *M. l. luteus* red-spotted floral tissue (E); and orange-flowered *M. cupreus* (F). The yellow tissue of *M. l. luteus* contains no anthocyanidin pigment, as indicated by the absence of pink color in that lane. Lanes C, E, and F all have Rf values corresponding to cyanidin.

1.3.2 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 (1,230 of 1,233 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 flower⁻¹h⁻¹ (Table 3).
*Mimulus cupreus* received far fewer visitors than members of the *luteus*-like group at all locations where it occurred (0 – 0.02 visits flower\(^{-1}\)h\(^{-1}\)). Visitation rate varied significantly by species \((F = 8.49, P < 0.001)\), but a Tukey’s Studentized range test confirms that this is a result of significant differences only between *M. cupreus* and the other three taxa (Table 2). The lack of *M. cupreus* pollinators was not due to low floral density: *M. cupreus* had an intermediate floral density of 34.5 ± 5.8 flowers m\(^{-2}\) (mean ± SE) versus 45.5 ± 3.7 flowers m\(^{-2}\) (M. *l. luteus*), 28.0 ± 3.3 flowers m\(^{-2}\) (M. *l. variegatus*), and 23.7 ± 3.6 flowers m\(^{-2}\) (*M. naiandinus* x M. *l. luteus* hybrid zone).

**Table 3: 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 minutes of observation); “Hours” is the numbers of hours of observation for that dataset. The visitation rate for each quadrat was calculated as visits flower\(^{-1}\)h\(^{-1}\); the mean across quadrats is shown in the last column. *Mimulus cupreus* differed significantly from the other taxa in visitation rate, as indicated by the superscript letters a and b \((P < 0.001, \text{Tukey’s Studentized range test})\).

<table>
<thead>
<tr>
<th>Species</th>
<th>Population</th>
<th>Visits</th>
<th>Flowers</th>
<th>Hours</th>
<th>Rate per flower</th>
<th>Mean rate per quadrat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>luteus</em>(^a)</td>
<td>LM</td>
<td>316</td>
<td>1975</td>
<td>21</td>
<td>0.32</td>
<td>0.29 ± 0.109</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>213</td>
<td>757</td>
<td>12.5</td>
<td>0.56</td>
<td>0.45 ± 0.113</td>
</tr>
<tr>
<td><em>luteus</em> x <em>naiandinus</em>(^a)</td>
<td>RT</td>
<td>280</td>
<td>1207</td>
<td>28</td>
<td>0.46</td>
<td>0.48 ± 0.073</td>
</tr>
<tr>
<td><em>variegatus</em>(^a)</td>
<td>RP</td>
<td>425</td>
<td>1650</td>
<td>29.5</td>
<td>0.32</td>
<td>0.35 ± 0.070</td>
</tr>
<tr>
<td><em>cupreus</em>(^b)</td>
<td>RT</td>
<td>5</td>
<td>425</td>
<td>2</td>
<td>0.024</td>
<td>0.025 ± 0.020</td>
</tr>
<tr>
<td></td>
<td>LM</td>
<td>4</td>
<td>5130</td>
<td>19.5</td>
<td>0.002</td>
<td>0.003 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>0</td>
<td>231</td>
<td>7</td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
</tbody>
</table>


1.3.3 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 ($R^2 = 0.933, P < 0.001$). In *M. l. luteus*, *M. l. variegatus*, and *M. naiandinus*, stigma closure was usually highest at mid-day (71.9 ± 7.26%; mean ± SE), and decreased by dusk to a mean of 70.8 ± 6.19% (Fig. 5). About a quarter of these closed stigmas reopened overnight, suggesting ineffective pollinator visitation in those cases. The stigmas of bagged control flowers generally did not close (% closure = 9.8 ± 2.53%). 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 12.1 ± 5.16% at mid-day, and 10.9 ± 4.46% 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).
Figure 5: Patterns of stigma closure in Chilean Mimulus over a 24-h period at four locations. Location names are given in bold, 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.

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).

1.3.4 Pollinator behavior in a hybrid swarm

The nineteen foraging bouts that we observed at RT comprised 1464 flower
visits. Bouts ranged from 16 – 316 visits (77.1 ± 15.72; mean ± SE). There was no effect of
day on the mean number of visits per bout ($F = 1.21$, df = 18, $P = 0.286$) or on the mean of
the phenotypes visited with respect to either yellow ($F = 0.05$, df = 18, $P = 0.822$) or red ($F$
$= 0.02$, df = 18, $P = 0.997$) pigmentation. A total of 554 and 714 flowers, respectively, were
open on the Jan. 21 and 27 censuses. All 16 possible phenotypes were observed,
although the *luteus*-like coloration was by far the most common, comprising 27.5% of the
population on average (Fig. 6). 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 ($G_{\text{H}} = 1.9$, df = 3, $P =$
ns). Frequencies of yellow phenotypes did show significant heterogeneity ($G_{\text{H}} = 1651,$
df = 3, $P < 0.001$), due mainly to a reduction in the “1” class (23% versus 14% on Jan. 21
and Jan. 27, respectively) and an increase in the “4” class (29% versus 37%). We
therefore compared the behavior of each bee to floral frequencies from the
 corresponding census, rather than to the mean of the two censuses.
Figure 6: Comparison of population frequency and visitation frequency of floral phenotypes in a *Mimulus l. luteus* x *M. naiandinus* hybrid swarm. Flowers are assigned to one of 16 categories, based on intensity of yellow pigmentation (Y-axis) and red pigmentation (X-axis). Grey shaded squares depict the frequency of each phenotype in the entire population, averaged over two censuses. Census sample sizes were $n = 554$ open flowers (Jan. 21) and $n = 714$ open flowers (Jan. 27, 2005). Orange circles indicate the floral-phenotype mean $\pm$ SE of 19 individual foraging bouts by *B. dahlbomii*. Sample sizes for the foraging bouts range from 16 – 316 flowers visited, mean $\pm$ SE = 77.1 $\pm$ 15.72.

Heterogeneity across individual foraging bouts was highly significant, whether the data were divided into 16 categories ($G^2 = 599.5$, df = 15, $P < 0.0001$) or four red categories ($G^2 = 351.3$, df = 3, $P < 0.0001$) and then four yellow categories ($G^2 = 713.1$, df = 3, $P < 0.0001$). As shown in Fig. 6, 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 overvisited *luteus*-like flowers ($\chi^2 > 3.84$, df = 1, $P < 0.05$).

Transitions between phenotypes were non-random, with a significant excess of like-to-like transitions for both red ($\chi^2 = 267.6$, df = 1, $P < 0.0001$) and yellow ($\chi^2 = 620.9$, df = 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 1,416 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).

### 1.4 Discussion

Our 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*. Our
work indicates that flower color 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 color patterning diversity. Future studies should evaluate elements such as parasite interactions, water availability, and soil composition.

1.4.1 Limited effect of flower color on pollinator preference

This study reveals 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 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. in press). 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 x 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 nonrandom 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 behavior of *B. dahlbomii*, even in the complete absence of floral color 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 color differences in the Chilean *Mimulus* presently have little influence on pollinator behavior. There are several alternative hypotheses that could explain the existence of species- or subspecies-specific flower color. This study spans only six 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 habitat. Another hypothesis is that floral variation is selectively unimportant and due instead to genetic drift. Given the multigenic basis of flower-color differences in the study taxa (A. Cooley, unpubl. data), this seems unlikely.

Finally, floral anthocyanin variation could be due to non-pollinator sources of selection. Whittall and Strauss (2006) review several examples of floral color 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).

1.4.2 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. data), 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 to that 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 are 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, 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 color. 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 3,630 yellow *M. cupreus* flowers was observed to be visited, which is even less than the three visits out of 1,500 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 color.

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 colors (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, G. Carvallo, pers. obs.). There are several examples of highly selfing showy-flowered plants, including *Mimulus platycalyx* (Dole 1992; Lin and Ritland 1997a), *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, *Mimulus cupreus* autogamously selfs much more readily in the greenhouse than members of the *luteus*-like group (A. Cooley, unpubl. obs.). Its low nectar content, relatively small flower size, and reduced stigma-anther separation are also consistent with a highly selfing mating system.
1.4.3 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 color.

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.
2. The genetic architecture of novel traits: Convergent evolution of floral color patterning in Chilean *Mimulus*

2.1 Introduction

Relating phenotypic differences to their causal genetic and molecular changes is a central goal of evolutionary biology. An important first step towards this objective is to determine the genetic architecture of trait divergence, using any of a variety of mapping approaches. The data that are generated can identify genomic regions that are good candidates for further study, and can also help address a number of long-standing evolutionary questions.

One such question is whether traits evolve via many loci of small effect or a few loci of major effect. The issue has a long and controversial history, beginning with the opinions of Darwin (1859) and Fisher (1930) that phenotypic change is extremely gradual and composed of numerous minor mutations. The opposite view, of discontinuous changes due to single genes, was given by Darwin’s cousin Francis Galton (1894) and later by Mendelian advocates such as Bateson (1913). A more extreme version, that evolution often occurs in large jumps, was argued by De Vries (transl. 1910), Goldschmidt (1940) and Gould (1980). The discussion has continued in recent times with the proposal of an exponential distribution of effect sizes, including a few fairly large changes early in the adaptive process, and many small changes (Orr 1998).

While theoretical arguments can be made for all sides, additional empirical data on the genetic basis of phenotypic change are needed to truly resolve this debate.
An interesting implication of the gradualist viewpoint is that it should be very difficult to exactly repeat an evolutionary trajectory. Two organisms that independently evolve the same trait are likely to do so via distinct genetic routes, simply because there are so many possible genes that could contribute. A caveat is that the number of loci “available” to contribute to evolutionary change is determined, not only by the number of genes in the genome that affect a given trait, but also by how pleiotropically constrained those genes are. Antagonistic pleiotropy, in which a genetic change has opposite effects on fitness through two or more different functions performed by the same gene (Williams 1957), can limit that gene’s responsiveness to natural selection, thereby reducing the total number of “available” loci.

Repeated evolution is often described as either parallel or convergent. While uses of the terms have varied (Arendt and Reznick 2008), parallel evolution at the genetic level generally refers to achieving the same outcome (phenotype) from changes in the same starting condition (at the level of an amino acid, gene, or biochemical pathway). Genetic convergence refers to repeated evolution of a phenotype via changes in different amino acids, genes, or biochemical pathways (Zhang and Kumar 1997; Arendt and Reznick 2008). Both parallel and convergent evolution, when found in groups of organisms inhabiting similar environments, are considered evidence of natural selection (Endler 1986; Schluter 2000).

Parallel and convergent evolution have been observed frequently in both wild and domesticated organisms (e.g. Copeland and Edwin 1946; Zhang 2003; Colosimo et al. 2005; Hovav et al. 2008). Repeated evolution has piqued researchers’ interest because
of its frequency and its putative association with adaptation, but also because of its implications for understanding the mechanisms of evolution. A prevalence of parallel evolution in nature would suggest that very few genes are available to respond to selection, due either to a scarcity of genes or an abundance of pleiotropic constraint. Prevalence of convergence would suggest that there is great flexibility (and little predictability) in how a phenotype is achieved.

A classic system for testing the predictions of evolutionary theory is floral pigmentation. Pigment biosynthesis pathways are reasonably simple and well characterized, and generate clear phenotypes that tend to be evolutionarily labile. In plants, the two major classes of pigments are the anthocyanins, which produce red, purple, and blue colors, and the carotenoids, which are typically yellow or orange. Studies of both pigments have yielded insights into connections between genotype and phenotype (Mol et al. 1998; Hirschberg 2001; Durbin et al. 2003). The anthocyanin pathway has been a particular focus of evolutionary biologists, for both practical reasons – the pathway is especially compact and well characterized – and historical ones. Early work on maize anthocyanin phenotypes made major contributions to several fields of research, including the regulation of gene networks and biosynthetic pathways (e.g. Emerson and Anderson 1932; McClintock 1950, 1968; Chen and Coe 1977), and laid the groundwork for continued evolutionary research on anthocyanin biosynthesis.

The wildflower genus *Mimulus* displays numerous examples of variation in floral coloration, due to differences in both anthocyanins (Ferro et al. 1972; Wilbert 1997; Cooley et al. 2008) and carotenoids (Vickery and Olson 1956; Goodwin and Thomas
One dramatic example of a recent diversification in floral pigmentation is provided by several closely related *Mimulus* from Chile (Fig. 7). These taxa belong to a large monophyletic group within *Mimulus*, the “yellow monkeyflowers” (Beardsley and Olmstead 2002). Since virtually all species in the yellow monkeyflower group are characterized by highly similar floral coloration, consisting of a yellow corolla with red spots along the throat, the exceptions to this rule can confidently be considered to be derived. Within the Chilean *Mimulus* presented in Figure 7, three taxa (*Mimulus luteus* var. *variegatus*, *M. naiandinus* and *M. cupreus*) have novel floral pigmentation characteristics, while *M. l. luteus* and *M. depressus* exhibit the ancestral yellow monkeyflower phenotype.

The Chilean *Mimulus* are closely related to the genomic model system *M. guttatus*, which facilitates the development of markers and genomic techniques (Wu et al. 2008). They are all interfertile and easily maintained in the greenhouse, making genetic analysis straightforward. Although they are not distinct species according to the Biological Species Concept, due to their interfertility (Dobzhansky 1937; Mayr 1942, 1963), taxonomically recognized groups will sometimes be referred to here as species for clarity of writing.

We ultimately hope to understand the evolutionary and molecular mechanisms that have facilitated floral divergence in the study taxa. As a first step, we have taken a classical genetic approach to investigating the control of novel pigmentation phenotypes in the three red-pigmented taxa relative to *M. l. luteus*, with primary focus on *M. l. variegatus* and *M. cupreus*. *Mimulus l. luteus* was selected as a
representative of the ancestral yellow monkeyflower phenotype; *M. l. variegatus* is thought to be the closest relative of *M. l. luteus*; *M. cupreus* is more distantly related, and its position sister to another yellow-flowered species (*M. depressus*) suggested that its floral anthocyanin pigmentation might have arisen independently of *M. l. variegatus*. Here we perform a series of crosses within and between species, and analyze trait segregation patterns in order to genetically dissect the floral pigment diversification exhibited by the Chilean *Mimulus*.

Figure 7: Floral traits and chromosome counts in Chilean *Mimulus*. Phylogenetic relationships are based on Beardsley et al. (2004), sequence data from A. Cooley (unpubl. data), and Grant (1924). *Mimulus depressus* photo is courtesy of Station Alpine Joesph Fourier.
2.2 Materials and Methods

2.2.1 Study taxa

Members of the Chilean *Mimulus* floral radiation are recent tetraploids, with \( n = 30 - 32 \) chromosomes, compared to \( n = 14 \) in the closely related *M. guttatus* (Fig. 7; Vickery et al. 1968). *Mimulus depressus* and *M. luteus* var. *luteus* have the floral phenotype common to nearly all members of the yellow monkeyflower group, of a yellow corolla with red spots of anthocyanin restricted to the throat and lower central petal (Fig. 7). Derived floral pigmentation phenotypes are found in *Mimulus l. variegatus* (a purple corolla with a white or very pale yellow throat); *M. naiandinus* (a white corolla with pink color spreading out from the throat); and *M. cupreus*, which is usually a dark coppery orange. Yellow morphs of *M. cupreus* with the typical yellow monkeyflower patterning have been found in at least one population (Cooley et al. 2008). Unless otherwise specified, “*M. cupreus*” here refers to the more common orange morph. The available sequence data suggest that *M. depressus* and *M. cupreus* form a group sister to *M. l. luteus, M. l. variegatus* and *M. naiandinus* (Beardsley and Olmstead 2002; Beardsley et al. 2004, A. Cooley, unpubl. data).

We obtained seeds from natural populations of *M. luteus* var. *luteus, M. l. variegatus, M. naiandinus*, and both orange and yellow morphs of *M. cupreus* in the foothill region of central Chile (Table 4). In order to simplify genetic analyses, we created inbred lines of each taxon, including both color morphs of *M. cupreus*. Lines were created by five to eight generations of self-fertilization with single-seed descent.
Table 4: Seed collection sites. All seeds were collected from natural populations in central Chile, in the foothills of the Andes along the banks of streams or rivers. Seeds were collected in December or January, 2001-2005.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Collection Site</th>
<th>Location</th>
<th>Elevation (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. l. luteus</em></td>
<td>El Yeso / Cajón del Maipo</td>
<td>33.4°S, 70.0°W</td>
<td>2600</td>
</tr>
<tr>
<td><em>M. l. variegatus</em></td>
<td>Río Cipreses</td>
<td>34.2°S, 70.3°W</td>
<td>1200</td>
</tr>
<tr>
<td><em>M. cupreus</em></td>
<td>Laguna del Maule</td>
<td>36.0°S, 70.3°W</td>
<td>2300</td>
</tr>
<tr>
<td><em>M. naiandinus</em></td>
<td>Termas del Flaco</td>
<td>34.5°S, 70.4°W</td>
<td>1000</td>
</tr>
</tbody>
</table>

2.2.2 Greenhouse conditions

Seeds for inbred lines and crosses were sowed in 2-in pots using Fafard 4-P soil-free potting mix. Plants were maintained in the Duke University greenhouses under 18 h per day lighting and twice-daily watering. Peters Professional fertilizer was applied every 2 wk, alternating between general purpose (N:P:K = 20:10:20) and low-phosphorus (N:P:K = 15:0:15) formulas. Blossom Booster (N:P:K = 10:30:20) was applied weekly to enhance flowering.

2.2.3 Crossing design

In order to determine the genetic basis of interspecific differences, we first crossed inbred lines of two of the derived-phenotype species (*M. l. variegatus* and *M. cupreus*) to the ancestral phenotype represented by *M. l. luteus*. Hybrid F1 individuals were self-fertilized to yield segregating F2 populations, as depicted in Fig. 9a and 9b.

To further investigate the results of the two initial crosses, additional crosses were conducted in the same manner, generating the F2 populations depicted in Fig. 9c-e.
In some cases, backcrosses to *M. l. luteus* or the yellow morph of *M. cupreus* were performed to verify particular observations, as discussed in the Results. Each cross was conducted reciprocally, but since there was no evidence of parental effects for any of the traits examined, data from both directions of each cross were combined in all cases. Sample sizes differed for each cross and are presented in the Results section.

2.2.4 Assessment of segregating phenotypes

Flowers from the F$_2$ progeny of each cross were scored for the presence or absence of anthocyanin on the petal lobes (Fig. 8c), and the top of the corolla tube (“dorsal anthocyanins,” Fig. 8d-f). Note that “dorsal pigment” refers to a diffuse layer of anthocyanin on the back of the upper two petals, but it can sometimes be seen faintly on the front of those two petals as well (Fig. 8c).

In the *M. l. variegatus x M. l. luteus* cross, petal lobe anthocyanin pigmentation varied in spot size (but not intensity), while carotenoid pigmentation varied in intensity (but not in spatial patterning). To quantitatively measure anthocyanin spot size, we photographed one flower per plant of parental, F$_1$, and F$_2$ individuals from a head-on, “pollinator’s-view” perspective, and digitally removed the throat area in order to examine only the petals. We used ImageJ v1.31 ([http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)) to calculate the extent of red (anthocyanin) pigmentation, expressed as a percentage of total petal surface area, for the corolla as a whole as well as for each of the five petals individually.
Figure 8: Anthocyanin pigmentation phenotypes scored in *M. luteus* var. *luteus*, *M. l. variegatus*, and their F₁ and F₂ hybrids. (a) throat spots; (b) bottom spot; (c) petal lobe anthocyanin spot; (d) dorsal anthocyanins present; (e) dorsal anthocyanins absent; f) the faint front band of pigment associated with dorsal anthocyanins.

Next page: Figure 9. F₂ progeny of inter- and intraspecific crosses show segregating floral pigmentation patterns. The inbred parental lines used for each cross are outlined in green, with their F₁ hybrid in the middle. Representative photos of F₂ progeny are outlined in blue. F₂ photos illustrating inferred genotypes are outlined in yellow. Inferred genotypes of the parental lines are: aa bb (*M. l. luteus*), AA bb (*M. l. variegatus*), and aa BB (orange morph of *M. cupreus*). (a) *M. l. luteus* x *M. l. variegatus*; (b) *M. l. luteus* x orange *M. cupreus*; (c) orange *M. cupreus* x *M. l. variegatus*; (d) orange x yellow *M. cupreus*; (e) *M. naiandinus* x *M. l. variegatus*. White boxes in 2c denote “recombinant” bright red and light pink colors, as discussed in the Results.
2.3 Results

2.3.1 Hybrids exhibit complex phenotypes

Although the purple-flowered *M. l. variegatus* and orange-flowered *M. cupreus* have petal lobes that superficially appear solid colored (Fig. 7), crosses to the ancestral phenotype (as represented by the yellow-flowered *M. l. luteus*) revealed considerable complexity in the spatial distribution of red anthocyanin pigments. In *M. l. variegatus* x *M. l. luteus* F$_2$s, anthocyanins on the petal lobes were distributed in solid, rounded, relatively large patches (Fig. 9a). A cross between *M. cupreus* and *M. l. luteus*, in contrast, yielded F$_2$ progeny with a spray of very small specks of anthocyanin on the petal lobes (Fig. 9b). A cross between the two derived phenotypes showed a combination of both patterns, apparently segregating independently of one another (Fig.
9c): *M. l. variegatus* x *M. cupreus* F$_2$s show both the smooth rounded patches of *M. l. variegatus* and the irregular speckling of *M. cupreus*.

The purple and orange colors of *M. l. variegatus* and *M. cupreus*, respectively, are not caused by a change in anthocyanin pigment type: in both taxa, only the pigment cyanidin is present at detectable levels (Cooley et al. 2008). Rather, the F$_2$ populations show that overall flower color results from an interaction between the red anthocyanins and the yellow carotenoid pigments. Plants with light anthocyanin pigmentation on a dark yellow (high carotenoid) background are orange, similar to the *M. cupreus* parent, while plants with dark anthocyanin pigmentation on a white (low carotenoid) background are purple, similar to *M. l. variegatus*. Trait recombination in the *M. l. variegatus* x *M. cupreus* cross creates additional colors not seen in the parental lines: light pink (light anthocyanin pigmentation with low carotenoid levels; Fig. 9c, first white box) and bright red (dark anthocyanin pigmentation with high carotenoid levels; Fig. 9c, second white box).

### 2.3.2 Petal lobe anthocyanin differences between species are controlled by a single locus

While some aspects of hybrid floral pigmentation were spatially complex or appeared to vary in a quantitative manner, a number of F$_2$ individuals completely lacked anthocyanins in their petal lobes in both the *M. l. variegatus* x *M. l. luteus* and the *M. cupreus* x *M. l. luteus* crosses. We therefore tested whether the presence versus absence of petal lobe anthocyanins might have a simple genetic basis, by determining segregation ratios in F$_2$ and backcross populations. The simplest model, single-gene
control with complete dominance, predicts that 75% of $F_2$s and 50% of BC$_1$s (first-generation backcross to the recessive parent) should exhibit the dominant trait.

In crosses between the purple-flowered *M. l. variegatus* and the yellow-flowered *M. l. luteus*, presence versus absence of petal lobe anthocyanins did segregate as a single Mendelian locus. The derived state (presence of petal lobe spots, from *M. l. variegatus*) was dominant, yielding a 3:1 ratio in the $F_2$s (Table 5). A backcross to *M. l. luteus* showed the expected 1:1 ratio, confirming the single-locus control of petal lobe anthocyanins.

The orange-flowered *M. cupreus* showed a similar pattern, with presence versus absence of petal lobe anthocyanins segregating as a single locus in *M. cupreus* x *M. l. luteus* $F_2$ progeny (Table 5). The putatively derived (*M. cupreus*) state was again dominant to *M. l. luteus*.

* Mimulus naiandinus* was not subjected to extensive analysis. However, $F_1$ hybrids of *M. naiandinus* x *M. l. luteus* exhibited petal lobe anthocyanins (A. Cooley, unpubl. data), indicating that the gain of pigment is, once again, dominant to the ancestral phenotype.

### 2.3.3 Petal lobe anthocyanin polymorphism within species is controlled by a single locus

The presence versus absence of petal lobe anthocyanins in orange versus yellow morphs of *M. cupreus* is controlled by a single locus (Table 5). The $F_1$ is indistinguishable from the orange parent, indicating that the “orange” allele (anthocyanin presence) is completely dominant to the “yellow” allele (anthocyanin absence). Unlike the
interspecific cross of orange \( M. \text{cupreus} \) to the yellow-flowered \( M. \text{l. luteus} \), no pigment-intensity variation within the “pigment presence” class of \( F_2 \)s was observed (Fig. 9d).

Table 5: Segregation of dorsal and petal anthocyanins does not differ significantly from single-locus Mendelian ratios. For all samples, d.f. = 1 and a \( \chi^2 > 3.84 \) would show significant deviation from the hypothesized segregation ratio at \( P < 0.05 \). Abbreviations: \( L = M. \text{l. luteus} \); \( V = M. \text{l. variegatus} \); \( Co = \) orange morph of \( M. \text{cupreus} \); \( Cy = \) yellow morph of \( M. \text{cupreus} \). The null hypothesis for a single-locus trait is 3:1 in \( F_2 \) populations, and 1:1 in backcross (BC\(_1\)) populations. The “observed ratio” indicates the numbers of progeny exhibiting the dominant phenotype (presence of dorsal or petal lobe anthocyanins) versus the recessive phenotype (absence of dorsal or petal lobe anthocyanins).

<table>
<thead>
<tr>
<th>Cross</th>
<th>Anthocyanin location</th>
<th>Observed ratio (dominant:recessive)</th>
<th>( H_0 )</th>
<th>( \chi^2 )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>L x V ( F_2 )</td>
<td>Dorsal</td>
<td>188 : 67</td>
<td>3 : 1</td>
<td>0.22</td>
<td>ns</td>
</tr>
<tr>
<td>L x V BC(_1)</td>
<td>Dorsal</td>
<td>152 : 145</td>
<td>1 : 1</td>
<td>0.16</td>
<td>ns</td>
</tr>
<tr>
<td>L x Co ( F_2 )</td>
<td>Dorsal</td>
<td>146 : 61</td>
<td>3 : 1</td>
<td>2.21</td>
<td>ns</td>
</tr>
<tr>
<td>L x V ( F_2 )</td>
<td>Petal lobes</td>
<td>187 : 68</td>
<td>3 : 1</td>
<td>0.38</td>
<td>ns</td>
</tr>
<tr>
<td>L x V BC(_1)</td>
<td>Petal lobes</td>
<td>149 : 148</td>
<td>1 : 1</td>
<td>0.0017</td>
<td>ns</td>
</tr>
<tr>
<td>L x Co ( F_2 )</td>
<td>Petal lobes</td>
<td>146 : 61</td>
<td>3 : 1</td>
<td>2.21</td>
<td>ns</td>
</tr>
<tr>
<td>Co x V ( F_2 )</td>
<td>Petal lobes</td>
<td>382 : 128</td>
<td>3 : 1</td>
<td>0.0059</td>
<td>ns</td>
</tr>
<tr>
<td>Co x Cy ( F_2 )</td>
<td>Petal lobes</td>
<td>204 : 70</td>
<td>3 : 1</td>
<td>0.044</td>
<td>ns</td>
</tr>
</tbody>
</table>

2.3.4 The same locus controls both intra- and interspecific variation

The rarity of the yellow morph of \( M. \text{cupreus} \) suggests that it could represent a secondary loss of function, in which case it is likely – given the number of potential causal genes – that different loci would control the derived yellow phenotype in \( M. \text{cupreus} \) and the ancestral yellow of \( M. \text{l. luteus} \). Alternatively, yellow-flowered \( M. \text{cupreus} \) could represent unsorted ancestral standing variation, or recent introgression due to hybridization between \( M. \text{l. luteus} \) and the orange morph of \( M. \text{cupreus} \). Under
the latter scenarios, the same gene should be responsible for the yellow phenotype in both species.

In order to determine whether the yellow morph of *M. cupreus* is due to secondary loss of anthocyanin function, or to an ancestral allele, we crossed it to *M. l. luteus*. Only yellow-flowered progeny appeared, in the F₁ and in 32 F₂ plants. This failure to complement indicates that the absence of pigment is controlled by the same locus in both *M. l. luteus* and the yellow morph of *M. cupreus*, and that the genetic bases of intra- and interspecific variation (with respect to the presence of petal lobe anthocyanins in *M. cupreus*) are identical.

### 2.3.5 Presence of petal lobe anthocyanin is convergent in *M. l. variegatus*, *M. cupreus* and *M. naiandinus*

We tested for independent evolution of the presence of petal lobe anthocyanins, in the purple-flowered *M. l. variegatus* compared to the orange-flowered *M. cupreus* and the pink-flowered *M. naiandinus*, by intercrossing them and looking for F₂ progeny with no petal lobe anthocyanins. Absence of petal lobe anthocyanins in the F₂ offspring of two fully-pigmented parents would indicate recombination between two loci, showing that a different gene controls petal pigmentation in each parent.

The presence of petal lobe anthocyanins is dominant in all three taxa, as can be seen by the fully-pigmented F₁s shown in Figure 9. If pigment presence is controlled by the same locus in a given species pair, then all of their F₁ and F₂ hybrid progeny should have petal lobe anthocyanins, although other aspects such as intensity and spatial distribution might vary. Under a two-locus model (independent origins of the trait via
different loci), a fraction of the $F_2$s should experience recombination or independent assortment between the two petal-pigment loci, and individuals receiving the recessive “absence” allele at both loci should be completely lacking in petal lobe anthocyanins. If the two loci are unlinked, $1/16$ of the $F_2$ progeny ($6.25\%$) will have unpigmented petal lobes.

In the $F_2$ progeny of *M. l. variegatus* x *M. cupreus* (Fig. 9c), 23 out of 509 plants ($4.52\%$) showed a complete lack of petal lobe anthocyanins. That number is not significantly different from the 31.8 individuals expected under a model of two unlinked loci ($df = 1, \chi^2 = 2.60, P > 0.05$) and indicates that petal pigmentation is controlled by different loci in *M. l. variegatus* and *M. cupreus*.

In a cross between *M. l. variegatus* and *M. naiandinus* (Fig. 9e), seven of 72 plants had pure white petal lobes, which is not significantly different from the 4.75 expected under a model of two unlinked loci ($\chi^2 = 1.14, df = 1, P > 0.05$). Two of the seven pure-white recombinants are shown in Fig. 9e. These data indicate that petal lobe anthocyanins are controlled by different loci in *M. l. variegatus* and *M. naiandinus*.

### 2.3.6 Dorsal anthocyanin pigmentation has a simple and shared genetic basis in *M. l. variegatus* and *M. cupreus*

We used a combination of interspecific crosses to analyze another derived patterning element, dorsal anthocyanin pigmentation. Both *M. l. variegatus* and *M. cupreus* have anthocyanin pigment on the dorsal (top) portion of the corolla limb, while *M. l. luteus* and most other yellow monkeyflower species do not.
Dorsal anthocyanin pigmentation segregated as a single Mendelian locus in \textit{M. l. luteus} x \textit{M. l. variegatus}. The derived state (presence of dorsal pigmentation, from \textit{M. l. variegatus}) was dominant, yielding a 3:1 ratio in the F_{2}s (Table 5). Its single-locus nature was confirmed in a backcross to \textit{M. l. luteus}, which showed the expected 1:1 ratio (Table 5). Likewise, dorsal pigmentation segregated as a single locus in \textit{M. l. luteus} x \textit{M. cupreus} F_{2} progeny (Table 5). Again, the putatively derived allele (presence of dorsal pigmentation, from \textit{M. cupreus}) was dominant.

We tested whether dorsal pigmentation in \textit{M. l. variegatus} and \textit{M. cupreus} share a single genetic basis by crossing the two taxa and examining the F_{1} and F_{2} progeny. In contrast to the data for petal lobe anthocyanins, all progeny appeared to exhibit dorsal pigmentation, suggesting that this trait is controlled by the same locus in both taxa. However, the intensity varied substantially, and in a few cases was so faint that it was difficult to distinguish from pigment absence.

\textbf{2.3.7 Petal and dorsal pigmentation are independently controlled in \textit{M. l. variegatus} but not in \textit{M. cupreus}}

Dorsal pigmentation segregated independently of petal pigmentation in \textit{M. l. luteus} x \textit{M. l. variegatus}, indicating that these traits are controlled by distinct loci in \textit{M. l. variegatus} (Table 6). In \textit{M. cupreus}, in contrast, the two traits were completely co-inherited, indicating that they are controlled by the same or very tightly linked loci (Table 6).
Table 6: Independent segregation of dorsal and petal anthocyanins in *M. l. variegatus*, but not in *M. cupreus*. Data were collected from BC\(_1\) populations (*M. l. variegatus* x *M. l. luteus* or *M. cupreus* x *M. l. luteus*, backcrossed to *M. l. luteus*).

<table>
<thead>
<tr>
<th></th>
<th>Dorsal anthocyanins:</th>
<th>Petal anthocyanins:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>variegatus*</td>
<td>Yes (Dd)</td>
<td>76</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No (dd)</td>
<td>69</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>cupreus</td>
<td>Yes (Dd)</td>
<td>146</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No (dd)</td>
<td>0</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

2.3.8 Presence and spatial patterning of pigment are separately controlled in *M. cupreus*

The availability of a yellow morph of *M. cupreus* permitted us to test whether the production of petal pigmentation and its punctate distribution are pleiotropic effects of a single genetic change. If so, then plants lacking one trait should also lack the other. If the two traits are separately controlled, then loss of petal lobe anthocyanin production does not necessarily imply loss of the “speckling” function.

We tested the single-locus model by crossing a yellow morph of *M. cupreus* to *M. l. variegatus*. The F\(_1\) hybrid had petal lobe anthocyanins, consistent with the dominance of the *M. l. variegatus* petal lobe anthocyanin allele. These pigments were distributed in a speckled fashion, rather than in rounded spots as seen in *M. l. variegatus* (Fig. 10). This shows that the yellow morph of *M. cupreus* has a functional allele(s) for “speckling,” despite lacking the petal lobe anthocyanin allele, which indicates that the two traits are separately controlled in *M. cupreus*. 
Figure 10: F₁ hybrids of *M. l. variegatus* and the yellow morph of *M. cupreus* show a punctate distribution of petal anthocyanin pigmentation. Three flowers from a single F₁ plant are shown.

2.3.9 A major locus contributes to spot size in *M. l. variegatus*

Although presence versus absence of petal lobe anthocyanins behaved as a discrete trait, anthocyanin spot size varied considerably when present. We quantified spot size in *M. l. luteus* × *M. l. variegatus* progeny in order to determine whether this trait has a quantitative genetic basis. Spot size in this study is expressed as the percentage of a petal’s surface area that is colored by anthocyanin pigment.

The parental taxa differed greatly in spot size (Fig. 11). Anthocyanin pigmentation ranged from 0.1-9.7% of the total petal surface area in *M. l. luteus* (*n* = 15, mean ± SE = 4.5 ± 0.6%), and was confined to a single spot on the bottom petal. *Mimulus l. variegatus* had anthocyanin on all five petal lobes, covering 75.6-85.1% of the total petal surface (*n* = 16, 81.6 ± 0.7%). The F₁ and F₂ hybrids were intermediate in extent of pigmentation, consistent with additivity of the parental alleles (F₁: *n* = 22, 36.9 ± 3.3%; F₂: *n* = 50, 38.5 ± 4.3%; Fig. 4).
The majority of F₂ progeny were similar to either the *M. l. luteus* parent or the *M. l. variegatus* parent: 30% of the F₂s fell within the *M. l. luteus* range; 26% were within or very slightly below the *M. l. variegatus* range (Fig. 11). The remaining 44% were evenly distributed across the intermediate values. A Castle-Wright estimator of the minimum number of factors, $\hat{n}_c$, was 1.06, consistent with a single gene of large effect contributing to spot size (Castle 1921). However, the degree of variation observed in the F₂s suggests that additional genes are probably also important.

### 2.3.9 Correlation of petal lobe anthocyanins among petals

Most species of *Mimulus* have anthocyanin pigment on the lower central petal, often in the form of a small red spot at the base of a double row of throat spots that are thought to act as nectar guides (Medel et al. 2003). This feature is referred to here as the “bottom spot”. *Mimus l. variegatus* also has anthocyanin pigmentation on the other four petals. These represent the *variegatus*-like petal spots discussed previously, and are referred to here as “PLA (Petal Lobe Anthocyanin) spots”. We compared patterns of variation between bottom and PLA spots in order to determine the extent to which these two patterning elements are independently controlled.

Hybrid F₁ and F₂ individuals of *M. l. luteus x M. l. variegatus* had a bottom spot of variable size. The PLA spots varied in number as well size: most plants displaying petal lobe anthocyanins had spots on all petal lobes, but some plants showed among-flower variation, with two to five anthocyanin-pigmented petals per flower. We compared phenotypic segregation patterns of individual petals, to evaluate the extent to which the
five petals are coordinately controlled. We found that spot size on the bottom petal is controlled partly, but not entirely, independently of the other petals.

In the F2 progeny, the size of the bottom spot was much larger in plants with PLA spots \( (n = 68, \text{mean } \pm \text{SE} = 74.9 \pm 2.4\%) \) compared to plants lacking PLA spots \( (n = 33, 19.7 \pm 3.2\%; \text{Fig. 12}) \). This difference was highly significant \( (t = 13.38; P < 0.0001) \).

Bottom-spot size did not differ significantly between plants with variable numbers of PLA spots versus plants that consistently had anthocyanin on each petal of every flower \( (n = 25, 70.8 \pm 4.7\%; \text{vs. } n = 43, 77.2 \pm 2.7\%; t = 1.28; P = 0.205) \).

Within a flower, the sizes of the PLA spots were tightly and significantly correlated with one another \( (n = 98; \text{adjusted } R^2 = 0.885; F = 746.70; P < 0.0001) \). The correlation between bottom-spot size and PLA-spot size was also significant but smaller in magnitude, whether plants lacking PLA spots were included \( (n = 98; \text{adjusted } R^2 = 0.476; F = 89.21; P < 0.0001) \) or excluded \( (n = 69; \text{adjusted } R^2 = 0.334; F = 35.07; P < 0.0001) \).
Figure 11: Anthocyanin spot size in *M. l. luteus* x *M. l. variegatus* hybrids. The percentage of petal surface area colored by anthocyanin pigment is indicated for: (a) the parental taxa *M. l. luteus* (white bars) and *M. l. variegatus* (black bars), and F1 progeny (grey bars); (b) F2 progeny (striped bars). Data are taken from one flower per plant, with throat area removed as described in the Methods section. *n* = 15 (*M. l. luteus*); 16 (*M. l. variegatus*); 30 (F1s); 100 (F2s).
Figure 12: Size of the center-petal spot is small when no other petals are anthocyanin-pigmented, and large when additional petals are anthocyanin-pigmented. A and B denote significance groupings based on a t-test. The y-axis indicates the percentage of the bottom petal surface area that is red-pigmented.

2.4 Discussion

Finding the genetic basis of phenotypic change is a major challenge in evolutionary biology. We have used a classical genetic approach to evaluate a recent diversification in floral pigment patterning, in a group of South American wildflowers. Our primary goal was to determine the genetics of differences in floral coloration among four Mimulus taxa: Mimulus luteus var. luteus, M. l. variegatus, M. cupreus and M. naiandinus. We found that differences between species were comprised of multiple independent patterning elements, including both Mendelian and polygenic traits. The most striking phenotypic novelty, the appearance of petal lobe anthocyanin
pigmentation, has evolved three times independently, which suggests that extensive red coloration may confer some adaptive benefit.

2.4.1 Major- and minor-effect patterning elements combine to create novel phenotypes

At least six distinct patterning elements were identified using intra- and interspecific crosses. Two pattern elements acted in Mendelian fashion: (1) presence of petal lobe anthocyanin in the purple-flowered *M. l. variegatus*, and (2) presence of petal lobe anthocyanin in the orange-flowered *M. cupreus*. Subsequent genetic mapping has confirmed the independence of these two elements (A. Cooley, unpubl. data). The single Mendelian locus underlying trait (2) is responsible for both the intraspecific orange-yellow polymorphism within *M. cupreus* and the interspecific difference between *M. cupreus* and the yellow-flowered *M. l. luteus*. The locus also appears to control dorsal pigmentation in both *M. l. variegatus* and *M. cupreus*.

Three poly- or multigenic pattern elements were found: (3) anthocyanin spot size in *M. l. variegatus*; (4) carotenoid intensity in *M. l. variegatus*; and (5) anthocyanin intensity in *M. cupreus*. Trait (6), the finely speckled spatial distribution of anthocyanin in *M. cupreus*, acts independently of the petal lobe anthocyanin locus as demonstrated by its continued activity in a yellow morph of *M. cupreus*.

For trait (3), and probably (4) and (5) based on visual assessment, a high rate of recovery of parental phenotypes in the F$_2$s implicates a single major locus combined with modifier loci of small effect. Traits (3) and (4) appear to segregate independently of each other in a hybrid F$_2$ population. For trait (6), no estimate of the number of genes
involved has been obtained due to the difficulty of finding a measure of “speckliness” with which to evaluate hybrid $F_2$ progeny.

We conclude that a minimum of five major genetic changes contribute to color pattern diversification in this system, four of them associated with the distribution of red anthocyanin pigments. However, the continuous variation detected for traits (3), (4), and (5) is consistent with the presence of multiple additional loci of individually small effect. The observed pattern, of several major changes and multiple minor changes, is intermediate between the extreme models of gradual (Darwin 1859; Fisher 1930) and saltational (De Vries 1910; Gould 1980) evolution, and is most consistent with the exponential distribution of effect sizes proposed by Orr (1998).

2.4.2 The same locus controls both intra- and interspecific variation

Comparing trait variation within and between species can illuminate the evolutionary history of that trait (e.g. Lexer et al. 2005). We examined a rare yellow-flowered morph of $M. cupreus$ that occasionally co-occurs with orange-flowered $M. cupreus$. Historically, the yellow morph might have arisen by introgression from the yellow-flowered $M. l. luteus$ or $M. depressus$; it might represent unsorted ancestral standing variation; or it might represent a secondary loss of the petal lobe anthocyanins that characterize the more common orange morph. Our data suggest that the hypothesis of secondary loss is unlikely. Rather, the observed control of both intra- and interspecific variation by a single locus suggests a role for either recent introgression or
unsorted standing variation, and highlights the contribution of ancestral alleles to extant, intraspecific diversity.

2.4.3 Convergent evolution of petal lobe anthocyanins

Anthocyanin pigment covers the entire petal lobe of *M. l. variegatus* and *M. cupreus*, and most of the petal lobe of *M. naiandinus* (Fig. 7). Such extensive pigmentation is highly unusual within the yellow monkeyflower group, and the three taxa are very close relatives, raising the question of whether petal lobe anthocyanins might have arisen a single time in their common ancestor. If so, pigment presence should be controlled by the same genetic locus in all three. Independent evolution of the trait, in contrast, could have occurred multiple times at the same locus (parallel evolution), or via different loci (convergent evolution).

The recovery of F$_2$ hybrid progeny with no petal lobe anthocyanin pigmentation, in a cross between the purple-flowered *M. l. variegatus* and the orange-flowered *M. cupreus*, reflects recombination between two distinct loci. This can be described by a simple genetic model in which petal lobe anthocyanins are controlled by a dominant allele at Locus “A” in *M. l. variegatus*, and by a dominant allele at Locus “B” in *M. cupreus*. A cross between the two taxa yields a doubly heterozygous F$_1$ (Aa Bb) with anthocyanin-pigmented petal lobes. Up to 1/16 of the F$_2$ progeny will receive the “aa bb” genotype and show no petal lobe anthocyanin pigmentation, as shown in Fig. 9c.

Similarly, a cross between *M. l. variegatus* and *M. naiandinus* revealed progeny with pure white petals, which shows that petal lobe anthocyanins are
controlled by different loci in the two taxa. Since *M. naiandinus* is more closely related to
*M. l. variegatus* than to *M. cupreus* (Fig. 7), the most parsimonious explanation is that
petal lobe anthocyanins evolved independently in all three red-pigmented taxa.

Petal pigmentation is convergent between *M. l. variegatus* and both *M. cupreus*
and *M. naiandinus*, indicating that there are multiple genetic routes to achieving red
petals. Although pleiotropic constraints are thought to be prevalent in the anthocyanin
biosynthetic pathway (Rausher 2006), there is clearly some degree of evolutionary
flexibility, in that at least two different loci can independently create very similar
outcomes. Further tests will be required to determine whether the presence of petal lobe
anthocyanins in *M. cupreus* and *M. naiandinus* is parallel or convergent.

While the altered coloration does not appear to affect pollinator preference
(Cooley et al. 2008), the convergent evolution of a similar trait in three closely related
taxa inhabiting similar environments does suggest that the trait may be adaptive (Endler
1986; Schluter 2000). Flavonoid compounds, including anthocyanins as well as products
derived from side branches of the anthocyanin biosynthesis pathway, offer protection
against stressors such as insect herbivory, heat, and ultraviolet radiation (Bernays *et al.*
Coberly and Rausher 2003). Increased activity of the anthocyanin biosynthesis pathway
and its multiple side branches could be beneficial during floral development for reasons
not directly related to flower color.
2.4.1 Does diversity originate from multiple genes or multiple alleles?

In parallel and convergent evolution, the *same* trait is achieved in several species via one or many loci. The same distinction can be made for trait *diversification* – do multiple loci contribute, or is the same locus involved repeatedly? Trait variation through high allelic diversity at a single locus may seem less likely, but it has been documented in the vertebrate immune response system (Potts and Wakeland 1990; Rogers and Kaufman 2008) and, albeit less dramatically, in the regulation of anthocyanin pigment in maize (Emerson and Anderson 1932; Radicella *et al.* 1992).

As with parallel versus convergent evolution, diversity originating from a single locus is most likely if very few genes affect a trait, or if most of the relevant genes have strong pleiotropic constraints. The more genes that are “available” to natural selection, the more likely it is that novel traits will evolve using a variety of different loci.

The anthocyanin biosynthesis pathway involves a fairly small number of genes, several of which are thought to be under strong evolutionary constraint due to pleiotropic effects (Rausher 2008). The Chilean floral diversification involves at least five major anthocyanin-related genetic changes, as well as multiple changes of smaller phenotypic effect. Have all of these variants accumulated in the few anthocyanin genes that are relatively non-pleiotropic? Do the pleiotropic genes in fact contribute to the observed pigment variation?

In at least one case examined here, phenotypic diversity arises from multiple alleles at a single locus: dorsal pigmentation in *M. l. variegatus*, presence of petal and dorsal pigment in *M. cupreus*, and absence of petal pigment in *M. l. luteus* are all
determined by the same genetic region, locus (2) in the list presented at the beginning of the Discussion. However, several other pattern elements were shown to segregate independently of one another, indicating that multiple different loci are likely to be involved, despite the small gene number and high pleiotropic constraint of the anthocyanin biosynthesis pathway.

Interestingly, all of the study taxa are tetraploid relative to other members of the yellow monkeyflower group (Fig. 7). Polyploidization creates a duplicate copy of every gene, which, if maintained, is potentially freer to evolve. By expanding the available evolutionary material and potentially reducing pleiotropic constraints on some gene copies, gene duplication is thought to facilitate phenotypic diversification (Force et al. 1999; Lynch and Force 2000). Polyploidization could contribute to diversification in the Chilean *Mimulus* by allowing one copy of the anthocyanin biosynthesis pathway to specialize on petal lobes, while the other retains the “yellow monkeyflower” pattern function. Similarly, additional copies of a regulatory sequence (either cis or trans to the structural genes) could promote color pattern variation by subfunctionalizing. In order to discover whether gene or genome duplication played a role in the Chilean *Mimulus* diversification, it will be necessary to identify the individual causal genes.
3. Changes in gene regulation underlie flower color evolution in *Mimulus cupreus*

3.1 Introduction

An enormous variety of genomic changes can impact evolution. Phenotypic differences both within and between species have been linked to causes ranging from whole genome duplications and large-scale chromosomal rearrangements to single nucleotide substitutions (Soltis and Soltis 1999; Rieseberg 2001; Deutschbauer and Davis 2005; Masly et al. 2006). The extensive amino acid similarity between chimpanzees and humans first led to the suggestion that regulatory changes, which can be defined as mutations that affect gene expression levels, predominate in defining species differences (King 1975). Subsequent work in the field of evolutionary development highlighted the importance of regulatory changes, particularly of *cis*-regulatory changes, which are typically in noncoding regions and affect the expression of an adjacent gene. Proponents of a major role for *cis*-regulatory mutations argue, among other things, that *cis* regions face fewer constraints than coding regions and may therefore evolve more rapidly (Stern and Orgogozo 2008).

The data currently available do not show a definitive prevalence of either *cis* or coding mutations associated with phenotypic diversity (Hoekstra and Coyne 2007). However, there is great interest in further exploring the issue, with the hope that a more complete understanding of the relationship between molecular and phenotypic evolution will allow us to make concrete predictions about the type of molecular variant most likely to affect a particular trait. A predictive framework would accelerate the
process of finding the genetic basis for adaptive evolutionary innovations, and for harmful variation including human diseases. It is also possible that no such framework can be developed, and that evolution is in fact a highly contingent process dictated largely by chance historical events (Coyne and Hoekstra 2007). More empirical data are needed to expand our understanding of the genetic and molecular mechanisms that control different types of traits.

The molecular mechanisms of evolution are best studied in a system for which the underlying biosynthetic pathways and regulatory networks are well understood. Pigment biosynthesis has long been a model of choice for this type of work (Mol et al. 1998; Bennett and Lamoreux 2003; Hoekstra 2006). Pigmentation is a natural "reporter system", as pigment presence versus absence is easily visible and is a reliable indicator of whether the biosynthetic pathway is fully functional and expressed in a particular tissue or cell type. Pigmentation pathways are often relatively compact, and pigments and their biochemical precursors contribute to a host of fitness-related traits (Hoekstra 2006; Whittall and Strauss 2006; Rausher 2008).

The anthocyanin pathway, which produces red and purple hues in plants, is a prime example of the utility of pigment biosynthesis to evolutionary biologists. Vegetative and floral anthocyanin coloration are highly labile across the angiosperms, creating a wealth of opportunities for the study of variation among interfertile taxa. The pathway comprises only a handful of enzyme-encoding genes, which simplifies candidate gene approaches to finding the genetic basis of pigment variation. Three types of transcription factors (bHLH, R2R3 MYB, and WD40 proteins) are known to
physically interact to regulate expression of the structural genes (Koes et al. 2005).

Research on the anthocyanin pathway has already yielded insights into the evolution of
genes and gene networks, and into the connections between genetic variation and
phenotypic effects (Durbin et al. 2003; Rausher 2006).

Previously, we analyzed the biochemical and genetic basis of floral anthocyanin
variation in five *Mimulus* species from central Chile. These taxa, referred to here
collectively as the *luteus* group, are monophyletic and belong to a larger assemblage
known as the “yellow monkeyflowers”. The yellow monkeyflowers, which form a large
monophyletic group and include the model species *M. guttatus*, exhibit strongly
conserved floral pigmentation consisting of a yellow corolla with anthocyanin
production restricted to small spots on the throat and lower petal (Grant 1924). Floral
pigmentation in the *luteus* group, in contrast, is remarkably diverse. These species
produce the same types of pigment as the other yellow monkeyflowers are thought to
have: yellow carotenoids (Goodwin and Thomas 1964; Nitsche et al. 1969) and one type
of red anthocyanin pigment, cyanidin (Cooley et al. 2008). However, in contrast to most
of the yellow monkeyflowers, the spatial distribution of pigment varies greatly across
members of the *luteus* group.

We showed that extensive anthocyanin pigmentation in the flower petals has
evolved three times independently in the *luteus* group, each time via a single-locus
dominant allele (Cooley and Willis in prep). Here we explore the molecular basis of one
of these evolutionary changes, the gain of petal anthocyanins in *Mimulus cupreus*. *M.
cupreus* has a dark coppery orange corolla, which is caused by a punctate layer of
anthocyanin pigmentation that extends throughout the petals. Crosses to the closely related, yellow-flowered *M. luteus* var. *luteus* show that the presence versus absence of petal anthocyanins is controlled by a single nuclear locus (Cooley and Willis in prep), with the “presence” allele showing complete dominance.

A rare yellow-flowered morph of *M. cupreus* is known to co-occur with the orange morph in at least one population (Cooley et al. 2008). A single locus controls presence versus absence of petal anthocyanins in an intraspecific cross between the two morphs. The yellow morph of *M. cupreus* fails to complement when crossed to *M. l. luteus*: all F$_1$ and F$_2$ progeny show the recessive yellow phenotype, indicating that the same locus is involved for both the intra- and interspecific flower color differences (Cooley and Willis in prep). We use a combination of candidate gene and expression studies to find the locus and identify the mechanism responsible for the orange-yellow polymorphism within *M. cupreus*.

### 3.2 Materials and Methods

#### 3.2.1 Plant materials and crosses

We obtained seeds from Laguna del Maule (36.0 S, 70.3 W, 2300 m.a.s.l.), a site in the precordillera region of central Chile containing both the common orange morph of *Mimulus cupreus* and a rare, but locally abundant, yellow morph. Seeds were planted in the Duke University greenhouse under the conditions described in Cooley and Willis (in prep).
Previous work (Cooley and Willis in prep) showed that the petal color polymorphism of *M. cupreus* is controlled by a single nuclear locus, with the "orange" allele (presence of petal lobe anthocyanins) dominant to the "yellow" allele (absence of petal lobe anthocyanins). With the goal of identifying the gene underlying the causal locus, we created segregating F$_2$ populations to use for both the testing of candidate anthocyanin-related genes and quantitative gene expression studies. The orange- and yellow-flowered parents consisted of inbred lines created by five to eight generations of self-fertilization with single-seed descent. One orange and one yellow parent were crossed to obtain F$_1$ individuals. Self-fertilization of a single F$_1$ was used to generate segregating F$_2$ progeny.

### 3.2.2 Identifying candidate genes in *M. cupreus*

We considered all structural and regulatory genes involved in cyanidin production to be candidates for the *M. cupreus* polymorphism (Fig. 13). Our general strategy was to clone each candidate in *M. cupreus* based on sequence similarity to the closely-related model system *M. guttatus*, and then to find sequence- or length-based polymorphism between the orange- and yellow-flowered parental inbred lines. These polymorphisms were used to screen each locus for an association between genotype and flower color, in a segregating F$_2$ population.
Figure 13: The cyanidin biosynthetic pathway. Cyanidin, one of several types of anthocyanin pigment, is the product of the precursor compounds listed in regular font and the six enzymes listed in bold capital letters.

3.2.3 PCR, sequencing, and genotyping

Degenerate primers were designed to genes for the anthocyanin enzymes shown in Fig. 13, based on alignments between *M. guttatus* genomic DNA (http://www.mimulusevolution.org) and published sequences from other angiosperm species (obtained from GenBank). Portions of each gene were cloned and sequenced from floral cDNA, using inbred lines of both orange and yellow *M. cupreus*.

Genomic DNA for sequencing was extracted from young floral bud tissue using a modified hexadecyl trimethyl-ammonium bromide (CTAB) chloroform extraction
protocol (Lin and Ritland 1997b; Kelly and Willis 1998). Standard PCR conditions were used to amplify anthocyanin-related genes: 3 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 55°C, and 45-60 s at 72°C, with a final 5 min incubation at 72°C.

PCR products were gel-purified using the QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA) and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Vector inserts from single colonies were PCR amplified with universal M13F and M13R primers (Invitrogen), and sequenced using the Big Dye standard protocol (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 DNA Analyzer (Applied Biosystems).

Many loci showed intron-based length differences between the “orange” and “yellow” alleles. These loci were turned into more easily-scored genotyping markers, by fluorescently labeling one primer of each primer pair (using a 5’ incorporated dye). Each length-polymorphic locus was amplified using the PCR conditions described above, and was run on an ABI 3730 DNA Analyzer. Genotypes of orange and yellow parental and F_2 individuals were examined by eye using the program GeneMarker (SoftGenetics, State College, PA, USA).

3.2.4 Treatment of gene duplicates

Any time that a degenerate primer pair amplified two or more genes in _M. cupreus_, specific primers were designed to differentiate them. In order to confirm that additional sequences represented distinct loci, rather than residual heterozygosity, silent-site divergence in exons was estimated between each putative gene pair, and
compared to silent-site divergence between the orange and yellow alleles of each locus. Since the *luteus* group is thought to be allopolyploid (Vickery *et al.* 1968), more variation is expected between paralogs (which originated from two divergent genomes) than between alleles of a single gene (which, in most cases, should share a relatively recent common ancestor).

To test whether one of the amplified copies showed evidence of degeneration, we compared exon sequence of each gene to *Antirrhinum majus* or to *Ipomoea purpurea*. If one gene copy is is maintained by purifying selection and the other is nonfunctional, the latter should show more nonsynonymous divergence from the outgroup.

### 3.2.5 Identifying anthocyanin-related R2R3 MYBs

The R2R3 MYB family is particularly large and diverse in plants, with 125 copies in *Arabidopsis thaliana* (Stracke *et al.* 2001). Only a small subset (one or a few loci, depending on the species) is involved in regulating the anthocyanin biosynthesis pathway. In order to design *M. cupreus* primers specific to the anthocyanin-regulating MYBs, we wished to find the correct gene(s) in a closely related species. We therefore conducted a phylogenetic analysis of the R2R3 MYBs in the model system *Mimulus guttatus*, with the goal of distinguishing between anthocyanin-related and –unrelated members of this large gene family.

As a first step, we reconstructed the MYB gene tree presented by Schwinn *et al.* (2006) and placed additional sequences on it. In addition to the six angiosperm species analyzed by these authors (*Antirrhinum majus, Petunia hybrida, Lycopersicon*
esculentum, Vitis vinifera, Arabidopsis thaliana, and Zea mays), we included candidate genes from M. guttatus (http://www.mimulusevolution.org), M. aurantiacus (Streisfeld and Rausher in press), and three R2R3 MYB genes from Ipomoea that are known to be involved in anthocyanin regulation (Morita et al. 2006).

Previously published cDNA sequences and amino acid translations were obtained from GenBank (http://www.ncbi.nlm.nih.gov). The M. aurantiacus sequence was kindly provided by M. Streisfeld. Genes from M. guttatus were identified using tBLASTx (Altschul et al. 1990) between the M. guttatus genome (http://www.mimulusevolution.org) and two anthocyanin-regulating R2R3 MYB genes, Rosea1 and Venosa, from A. majus. Sequences with BLAST scores higher than e^{-15} were virtually unalignable and lacked considerable portions of the exons, and were excluded from further analysis. Genes missing one or both of the functional domains (R2 and R3) were also excluded. A total of 54 M. guttatus sequences were retained and were included in the gene tree. Sequences that grouped with anthocyanin-regulating MYBs from other angiosperms were used to design degenerate primers for M. cupreus.

A key feature of the anthocyanin-regulating MYBs is that they must physically interact with a bHLH protein in order to activate transcription. In Arabidopsis thaliana, six amino acid positions show high conservation across MYBs that physically interact with bHLH transcription factors (Zimmerman et al. 2004). We examined sequences obtained from M. cupreus to determine whether they retained these bHLH interaction sites.
3.2.6 Phylogenetic analysis of R2R3 MYBs

Amino acid sequences of the R2R3 MYBs were aligned using Clustal X (Thompson et al. 1997) with default gap penalties within BioEdit 7.0.5 (Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008; http://www.mbio.ncsu.edu/BioEdit/bioedit.html), followed by manual optimization of the alignment. Consistency with the alignment of Schwinn et al. (2006) was maintained as closely as possible. As in Schwinn et al. (2006), the highly variable C-terminal domain (encompassing the majority of the third and final exon) was excluded, as it was found to be phylogenetically uninformative.

A neighbor-joining tree of the amino acid data was constructed using PAUP 4.0b10 (Swofford 2003), under a full heuristic search with the objective function set to minimum evolution and distances set to mean character differences. A minimum evolution tree was similarly constructed, with distances set to mean character differences, using a full heuristic search. Three distantly-related R2R3 MYBs (AtMYB0, AtMYB90, and AtMYBWER), previously isolated from Arabidopsis thaliana but not thought to be involved in the anthocyanin biosynthesis pathway (Kranz et al. 1998) comprised the outgroup. Support for all trees was estimated using a full heuristic bootstrap analysis with 10,000 replicates.

3.2.7 Gene prediction in M. guttatus

Association between floral phenotype and genotype at a candidate locus could be caused by a gene closely linked to the candidate. In order to test this possibility, we
used the gene prediction program GenScan ([http://genes.mit.edu/GENSCAN.html](http://genes.mit.edu/GENSCAN.html)) to analyze a 1-Mb region of the *M. guttatus* genome. This segment is estimated to encompass 10.6 cM, based on recombination rates between nearby markers (Y.-W. Lee, pers. comm.).

The GenScan output list of predicted genes was examined for anthocyanin-related enzyme-encoding and regulatory genes. Possible candidates were further explored by conducting a protein-protein BLAST search of Genbank ([http://www.ncbi.nih.gov](http://www.ncbi.nih.gov)) to determine whether the sequence's best matches were to anthocyanin-related genes in other angiosperms. Pilot mapping studies have shown good conservation of marker order and marker distance between *M. guttatus* and *M. cupreus* (A. Cooley, unpubl. data), suggesting that data from *M. guttatus* can be used to make reasonable conclusions about genomic patterns in *M. cupreus*.

### 3.2.8 Quantitative gene expression assays

Quantitative PCR (qPCR) was performed to test the hypothesis that downregulation of anthocyanin biosynthetic enzymes is responsible for the pigmentation difference between orange and yellow *M. cupreus*. Specific primers were designed for each enzyme-encoding gene in the pathway, as well as for the R2R3 MYB transcription factor *McAn1*, followed by cloning and sequencing of cDNA to confirm that only the correct locus was amplified. To rule out primer-mismatch as a potential cause of low levels of RT-PCR product, each gene pair was also amplified and sequenced from genomic DNA, using the conditions previously described.
Two copies of the reference gene, *actin1*, were initially found. One paralog of *actin1* was arbitrarily selected, and was amplified with copy-specific primers. Initial tests of the selected copy showed no significant difference in expression between orange and yellow inbred lines of *M. cupreus*. All primers were designed with annealing temperatures of 58.5 - 61°C, and amplified fragments 80 - 290 bp in length. All qPCR primer sequences are listed in Table 7.

Table 7: Primers used for quantitative PCR. “Length” indicates the length in base pairs of the amplified cDNA fragment. Primer sequences are shown 5’ to 3’.

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<thead>
<tr>
<th>Gene</th>
<th>Length</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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<tr>
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<td>CAGTATGAAAGCACACACGCG</td>
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<td>GGTGAGTTTCCCTCACAC</td>
</tr>
<tr>
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<td>288</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>ANS2</td>
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<td>TCATTGGCGTACTCCTCCTTT</td>
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<tr>
<td>Actin</td>
<td>121</td>
<td>CAGTCACACTGTTCGCGATT</td>
<td>CATGTACCTCTCTCGGTGA</td>
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</tbody>
</table>

Petal tissue for qPCR was obtained from the two parental lines, two orange F2 plants, and two yellow F2s, at three bud stages: early (bud fully concealed by a closed calyx); intermediate (calyx open, bud beginning to emerge); and late (bud fully emerged and nearing anthesis). Following initial surveys of temporal patterns of gene expression, tissue was collected only from the early bud stages of eight additional F2s
(four orange and four yellow) as well as the F1 hybrid. 40-50 mg of petal tissue were harvested from each plant, placed immediately on dry ice, and stored at -80°C until needed for RNA extraction.

RNA was extracted from frozen petal tissue using an RNeasy Plant Mini Kit (QIAGEN) with on-column DNAses to eliminate DNA contamination. Extracted RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and standardized to 100 ng/µL. cDNA was synthesized from total RNA, and diluted 1:2 to create a working stock. qPCR assays were performed using a 20 µL reaction with Finnzymes SYBR-Green PCR mix (Espoo, Finland), Rox Dye as a passive reference, and 1 µL of cDNA. On a subset of plates, dilution controls (full strength, 10%, and 1% dilutions of starting template) and no template controls were included. All controls performed as predicted. Reactions were run in 96-well plate format on an ABI 7000 (Applied Biosystems, Foster City, CA) for 40 cycles with an annealing temperature of 58°C, with three technical replicates per sample.

For each gene, the expression level of the inbred parental line of orange M. cupreus (early stage buds) was set to zero and the log-change of each individual was calculated relative to that sample.

3.2.9 Statistical analyses

The effects of flower color, bud stage, and gene copy (paralog 1 versus 2) on gene expression were analyzed using one- and two-way ANOVAs. All statistical analyses were performed in JMP 7.0.1 (SAS Institute, Cary, NC).
3.3 Results

3.3.1 The anthocyanin biosynthetic pathway is duplicated in *M. cupreus*

PCR amplification of the enzyme-encoding genes yielded exactly two copies of most of the structural genes in the anthocyanin biosynthetic pathway. The exception was *Chs*, for which a highly divergent third copy (*Chs-3*) was found (56.6% synonymous and 15.5% nonsynonymous divergence from *Chs-1*; Table 8). Silent-site divergence was higher between putative gene duplicates (Ks = 0.078 - 0.214) than between orange versus yellow alleles of a single gene copy (Ks = 0 - 0.038), for all six steps of the pathway (Table 8).

For each step in the pathway, duplicate genes were compared to an outgroup species, *A. majus* or *I. purpurea*, to see whether one copy might be rapidly degenerating. *Chs-3* showed unusually high nonsynonymous divergence from *A. majus* (Ka = 0.167, compared to 0.055 for *Chs-1* and 0.053 for *Chs-2*). All other genes were closely matched to their putative paralog, in both synonymous and nonsynonymous divergence from the outgroup species (Table 8).
Table 8: Divergence in structural genes of the anthocyanin biosynthetic pathway.

“Allele divergence” compares alleles of orange-flowered and yellow-flowered inbred lines of *M. cupreus*. “Paralog divergence” compares Copy 1 and Copy 2 for each step in the anthocyanin pathway. *Chs-3* was compared to *Chs-1* to obtain a measure of paralog divergence. “Interspecific divergence” compares the orange-flowered *M. cupreus* allele to *Antirrhinum majus* (*Chs*, *Chi*, *F3’h*, *Dfr*) or *Ipomoea purpurea* (*F3h*, *Ans*). The number of synonymous or nonsynonymous sites in each analyzed sequence is shown in parentheses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele divergence</th>
<th>Paralog divergence</th>
<th>Interspecific divergence</th>
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<tr>
<td></td>
<td>Ks</td>
<td>Ka</td>
<td>Ks</td>
</tr>
<tr>
<td></td>
<td>(35.67)</td>
<td>(111.33)</td>
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<td>Chs-1</td>
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<td>0.2145</td>
</tr>
<tr>
<td></td>
<td>(164.75)</td>
<td>(495.25)</td>
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</tr>
<tr>
<td></td>
<td>(44.17)</td>
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</tr>
<tr>
<td></td>
<td>not measured</td>
<td>not measured</td>
<td>0.6928</td>
</tr>
<tr>
<td>Chi-1</td>
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</tr>
<tr>
<td></td>
<td>(71.08)</td>
<td>(237.92)</td>
<td>0.6050</td>
</tr>
<tr>
<td>Chi-2</td>
<td>0</td>
<td>0</td>
<td>0.0867</td>
</tr>
<tr>
<td></td>
<td>(71.08)</td>
<td>(237.92)</td>
<td>0.6050</td>
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<td>(55.5)</td>
<td>(193.5)</td>
<td>(65.17)</td>
</tr>
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</table>

3.3.2 Flower color cosegregates with an R2R3 MYB anthocyanin-related transcription factor

A phylogenetic tree of R2R3 MYBs revealed two “best candidates” for anthocyanin-related genes MYBs in *M. guttatus*, *MgAn1* and *MgAn2* (Fig. 14). Primers to *MgAn1* amplified many (at least five) different loci in *M. cupreus*. One of these, *McAn1*,
cosegregated with floral phenotype in an initial screen of eight F2 progeny. A subsequent screen of 283 F2 individuals (212 orange-flowered and 71 yellow-flowered plants) revealed a single recombination with the McAn1 locus: all orange-flowered plants had at least one copy of the "orange" allele, 70 yellow-flowered plants were homozygous for the "yellow" allele, and one yellow-flowered plant was heterozygous at McAn1.

It is possible that some of the orange-flowered F2s are heterozygous at the amplified locus, but homozygous dominant at the causal site, or vice versa; progeny testing would be required to identify this type of recombinant. A conservative conclusion, excluding all of the orange-flowered F2s, is that there was one recombination event in the 142 meioses represented by the 71 yellow F2s, placing the amplified marker approximately 0.70 cM away from the causal site.

When added to the MYB gene tree, McAn1 grouped with MgAn1, consistent with a role for McAn1 in anthocyanin regulation (Fig. 14). Of the six sites thought to be important for bHLH interaction in A. thaliana, five are identical at the amino acid level between M. cupreus and A. thaliana. The sixth site has undergone a conservative change, from leucine to cysteine, in both orange and yellow M. cupreus. Leucine and cysteine are both nonpolar, and are very similar in size and hydropathy (Biro 2006).
Figure 14: Minimum Evolution Tree for R2R3 MYBs from *M. guttatus*, *M. cupreus*, and other angiosperms. (a) 54 R2R3 MYBs from *M. guttatus* as well as MYBs from other angiosperm species, as described in the Methods. Bold horizontal lines indicate >80% bootstrap support. The dashed box encompasses all of the R2R3 MYBs known to regulate anthocyanin biosynthesis in dicots, and is shown in an expanded view in (b). (b) Anthocyanin-related R2R3 MYBs. Bootstrap support values >50% are shown. Species are indicated by the following two-letter abbreviations: Mg, *Mimulus guttatus*; Mc, *Mimulus cupreus*; Am, *Antirrhinum majus*; Vv, *Vitis vinifera*; Ma, *Mimulus aurantiacus*; In, *Ipomoea nil*; Ip, *Ipomoea purpurea*; At, *Arabidopsis thaliana*; Ph, *Petunia hybrida*; Le, *Lycopersicon esculentum*. Abbreviated gene names, as listed in GenBank, are given following each species designation.
3.3.3 Gene prediction in *M. guttatus*

In *M. guttatus*, the 1 Mb region surrounding *MgAn1* corresponds to approximately 10.6 cM and contains 180 predicted genes. A genomic survey showed that *MgAn1* (and its ortholog in *M. cupreus*, *McAn1*) are the most likely anthocyanin-related candidate genes in this region.

None of the 180 predicted genes corresponded to any of the enzymes in the anthocyanin pathway. Two truncated R2R3 MYB genes were found within 100 kb (approximately 1 cM) of *MgAn1*, but both are predicted to be nonfunctional: one was missing the exons 1 and 2, and the other, 30 kb downstream of the first, was missing exon 3. The lack of exons was confirmed by retrieving, manually translating, and visually examining the nucleotide sequence in that region. One R2R3 MYB was found, 25 kb away from *MgAn1* and in the opposite orientation, that could potentially be a functional anthocyanin regulator. Exons 1 and 3 were present and were similar to the corresponding exons in *MgAn1*. Exon 2 was not found; however, the 3 kb region separating exons 1 and 3 including a large amount of unreadable sequence (coded as "N" in the genome). While unreadable sequence is usually highly repetitive and noncoding, it is possible that exon 2 was in this region and that the gene is in fact an operational transcription factor.

One WD40 gene and two bHLH genes were found, at distances of 80-200 kb away from *MgAn1*. A search of GenBank showed that all three were very divergent from the WD40 and bHLH genes that regulate the anthocyanin pathway in other angiosperms.
3.3.4 Anthocyanin structural genes are expressed early in bud development

At Chs-1, which encodes the first committed step in the anthocyanin biosynthesis pathway, both orange- and yellow-flowered individuals showed highest expression in the early bud stage, intermediate expression in the intermediate bud stage, and lowest expression in the late bud stage (Fig. 15). A two-way ANOVA showed a significant effect of both flower color (d.f. = 1; $F = 10.68; P = 0.0038$) and bud stage (d.f. = 2; $F = 28.50; P < 0.0001$) on Chs-1 expression. The interaction term was not significant.

For Ans-1, which catalyzes the final step in the production of cyanidin pigment, orange- and yellow-flowered plants differed in their gene expression patterns. Orange-flowered plants again showed continuing downregulation over time, as seen for Chs-1. Yellow-flowered plants, however, were strongly and consistently downregulated at all three bud stages (Fig. 15). A two-way ANOVA revealed significant effects of color (d.f. = 1, $F = 16.78, P = 0.0005$), stage (d.f. = 2, $F = 8.71, P = 0.0018$) and a color x stage interaction (d.f. = 2, $F = 16.85, P < 0.0001$). A one-way ANOVA of only orange-flowered plants showed a significant effect of bud stage (d.f. = 2, $F = 35.11, P < 0.0001$); a one-way ANOVA of only yellow-flowered plants did not (d.f. = 2, $F = 2.22, P = 0.1589$). Significance groupings for both genes, based on a Tukey’s HSD test performed following a 2-way ANOVA, are shown in Fig. 15.
Figure 15: Expression of anthocyanin structural genes decreases with bud development. Gene expression for *Chis-1* (top) and *Ans-1* (bottom) is shown on a log scale relative to expression in the early bud stage of the orange-flowered *M. cupreus* parent for early, intermediate, and late bud stages. Black bars indicate the mean of orange-flowered F$_1$ and F$_2$ progeny; white bars indicate the mean of the yellow-flowered parent and F$_2$ progeny; error bars show standard error. Sample sizes for each color class were $N = 7$ (early bud stage) and $N = 3$ (intermediate and late bud stages). The letter below each bar shows significance groupings obtained from a Tukey’s HSD post hoc test.
3.3.5 “Late” anthocyanin enzymes are coordinately downregulated in plants with a recessive genotype at McAn1

Since the anthocyanin pathway appears to be most active in the early bud stage, we focused on this stage for analysis of the remaining anthocyanin genes. At each step in the pathway, flower color had a highly significant effect ($P < 0.005$ for each), with yellow-flowered plants showing lower expression than orange-flowered plants (Fig. 16). The difference was more pronounced for the four "late" genes ($F3h$, $F3'h$, $Dfr$, and $Ans$).

Expression patterns between the duplicate copies of all gene pairs were very similar. A significant difference between paralogous copies was detected only for $Ans$: $Ans$-1 was expressed at considerably lower levels than $Ans$-2 (d.f. = 1, $F = 20.41$, $P = 0.00017$). Orange-flowered plants had average gene expression levels (relative to the reference individual) of 53% ($Ans$-1) versus 202% ($Ans$-2). Yellow-flowered plants had average gene expression levels of 1.2% ($Ans$-1) and 9.1% ($Ans$-2). Since the two paralogs were not comparable, the effect of flower color was analyzed separately for each, and was found to be highly significant ($Ans$-1: $F = 41.77$, $P < 0.0001$; $Ans$-2: $F = 45.19$, $P < 0.0001$).

Locus x color interaction terms were not significant for any of the six steps in the anthocyanin pathway ($P > 0.20$ for all comparisons).
Figure 16: Flower color correlates with expression of anthocyanin enzyme-encoding genes. Expression is higher in orange-flowered plants (McAn1 genotype AA or Aa) than in yellow-flowered plants (genotype aa) for all twelve assayed genes. The difference is much greater in the two copies of F3h, F3′h, Dfr, and Ans than in the “early” genes, Chi and Chs. Black bars indicate the mean of orange-flowered F₁ and F₂ progeny; white bars indicate the mean of the yellow-flowered parent and F₂ progeny; error bars show standard error. Sample sizes for each gene were $N = 6$ (orange-flowered F₂s) and $N = 7$ (yellow-flowered F₂s plus the yellow-flowered parent).
Figure 17: Individual variation in McAn1 expression correlates with individual variation in enzyme-encoding genes of the anthocyanin biosynthesis pathway. The expression difference between each individual and the inbred orange-flowered *M. cupreus* parent is measured on a log scale. The X-axis shows expression at McAn1; the Y-axis shows expression of the six steps of the cyanidin pathway. White symbols represent yellow-flowered parental and F2 plants; filled symbols represent orange-flowered F1 and F2 plants; circles indicate one paralog; squares indicate the other paralog. Adjusted $r^2$ values are shown in the upper left corner of each graph. $P < 0.005$ for all steps of the pathway.
3.3.6 Expression at \textit{McAn1} correlates with expression of the anthocyanin structural genes

In the yellow-flowered \textit{M. cupreus} parent and F$_2$ progeny, expression of \textit{McAn1} was an average of 11.4\% relative to the orange-flowered parent. The effect of flower color was highly significant (d.f. = 1, $F = 29.43$, $P < 0.0001$). Within each color class, individual variation in \textit{McAn1} expression was strongly, significantly, and positively correlated with individual expression variation at all of the anthocyanin structural genes (Fig 17).

3.4 Discussion

We used a candidate gene approach to identify an R2R3 MYB transcription factor, \textit{McAn1}, which cosegregates with a floral anthocyanin polymorphism in a population of 283 \textit{Mimulus cupreus} F$_2$s. To test the causality of \textit{McAn1}, we measured expression levels of the anthocyanin biosynthetic enzymes that \textit{McAn1} putatively regulates, in the two parents, the F$_1$, six orange F$_2$s and six yellow F$_2$s. We found a highly significant correlation between expression of all six enzyme-encoding genes and flower color, \textit{McAn1} genotype, and \textit{McAn1} expression, with strikingly larger differences between color morphs for the four later genes in the pathway (\textit{F3h}, \textit{F3'h}, \textit{Dfr}, and \textit{Ans}) than for the first two genes (\textit{Chs} and \textit{Chi}). Although transformation would provide definitive proof of the mechanism underlying the orange-yellow floral polymorphism in \textit{M. cupreus}, the most straightforward interpretation of our data is that the change is in
the cis-regulatory region of the transcription factor McAn1. Below we present and evaluate three alternative hypotheses.

3.4.1 Could the causal change be in an anthocyanin gene that is physically linked to McAn1?

Primers to McAn1 amplify a region that is tightly linked (~0.70 cM) to the causal site. Its ortholog in M. guttatus, MgAn1, resides on a 3 Mb scaffold (Scaffold 11, [http://www.mimulusevolution.org](http://www.mimulusevolution.org)) that is approximately 32 cM in length (Y.-W. Lee, pers. comm.), indicating a ratio of approximately 94 kb per cM in this region of the M. guttatus genome. Pilot mapping studies have shown excellent conservation of marker order and marker distance between M. guttatus and M. cupreus (A. Cooley, unpubl. data). Our best estimate, therefore, is that 0.70 cM in M. cupreus corresponds to approximately 66 kb. The causal locus could then be McAn1, or any gene up to 66 kb on either side of McAn1. However, the coordinate downregulation of multiple loci in yellow-flowered plants indicates that the causal change must be trans-regulatory relative to the enzyme-encoding genes, since the color phenotype is controlled by a single locus. This limits the list of obvious candidates to the MYB, bHLH and WDR transcription factors known to regulate anthocyanin enzyme production.

We used GenScan ([http://genes.mit.edu/GENSCAN.html](http://genes.mit.edu/GENSCAN.html)) to analyze the 500 kb upstream and downstream of MgAn1, the M. guttatus homolog of McAn1. Several fragments of MgAn1-like R2R3 MYBs were found in this 10 cM region, suggesting that localized gene duplication events are common in this regions of the genome. While they contained segments of high sequence similarity to MgAn1, most are predicted to be
pseudogenes as they lack substantial portions of the expected coding region. One gene, located 25 kb away from MgAn1, could potentially be a functional R2R3 MYB: it has an identifiable first and third exon which are similar in amino acid sequence to MgAn1, and a long (3 kb) intervening stretch of repetitive and unreadable sequence (coded as “N”). While the unreadable sequence is also likely to be highly repetitive, making this gene a probable pseudogene, it is possible that it masks a complete copy of Exon 2. Additional analysis would be required to definitively exclude this MgAn1-like R2R3 MYB.

bHLH and WD40 transcription factors were identified in the vicinity of McAn1, but they bore little similarity to anthocyanin-related bHLH and WD40 genes from other angiosperms. A search of the entire M. guttatus genome revealed three bHLH genes and four WD40 genes whose highest BLAST hits were to anthocyanin-related transcription factors in other angiosperms (A. Cooley, unpubl. data). None of these genes were located in or near the scanned region. MgAn1 is, then, the most plausible candidate gene in a 1 Mb region that corresponds to approximately 10 cM in M. guttatus.

3.4.2 Could the causal change be in the coding region of McAn1?

The expression of McAn1 in early buds is nearly 10-fold higher in orange-flowered petals than in yellow-flowered petals. However, it is conceivable that the original, causal change resided in the coding region of McAn1, and the change in expression arose later on and is only coincidentally associated with phenotype. A coding change might alter the affinity of the McAn1 protein for the promoter regions of its target genes, or might affect the stability of the mRNA product.
Two observations argue against, but do not disprove, this hypothesis. One is that crossing data show that the orange phenotype of *M. cupreus* is derived and the yellow phenotype is ancestral. If the common ancestor of *M. l. luteus* and the orange and yellow morphs of *M. cupreus* expressed *McAn1* at very low levels it is unlikely, although not impossible, that a nonsynonymous change in the few mRNA sequences produced could result in a strong upregulation of the target genes.

Secondly, *within* each of the two color classes, individual variation in *McAn1* expression correlates tightly with individual variation in expression of the target genes. These data strongly indicate that the primary determinant of anthocyanin enzyme production is the precise level of *McAn1* mRNA product, and exclude the possibility that the causal mechanism is altered affinity of the transcription factor for its targets. Regulation of mRNA level can occur post-transcriptionally via differential mRNA stability. However, transcript stability is thought to depend on noncoding sequences in the 3’ or 5’ untranslated region, rather than in the coding region (Mignone et al. 2002).

### 3.4.3 Could expression differences at *McAn1* be controlled in *trans* rather than in *cis*?

The gene or genes that regulate R2R3 MYB expression are unknown. It is possible that one such gene resides within 0.70 cM of *McAn1*, and a change in that gene is responsible for variation in *McAn1* expression. We cannot disprove this possibility, and we note the presence of one bZIP and two MADS-box transcription factors within 100 kb of *MgAn1* in *M. guttatus*, in addition to the bHLH and WD40 proteins discussed above. These transcription factors are all functionally uncharacterized in *Mimulus*, and
have not been linked to anthocyanin production in other angiosperms. We therefore consider them unlikely to regulate McAn1 expression.

3.4.4 Gene and genome duplication

Members of the luteus group, including *M. cupreus*, are recent tetraploids relative to *M. guttatus*. We had initially hypothesized that the additional genetic material created through polyploidization might contribute to floral diversification. In the case of *M. cupreus*, although we did find two duplicate copies of each enzyme-encoding gene, most paralogs showed no difference in gene expression. The causal locus was not, in fact, in an enzyme-encoding gene at all, but in (or near) a transcription factor that regulates their expression. This transcription factor, McAn1, has clearly had its share of gene duplication, as evidenced by the multiple closely related copies amplified even by most nondegenerate primer pairs that we designed based on the orthologous MgAn1.

The genomic region surrounding MgAn1 is littered with fragments of R2R3 MYBs that appear to be truncated and/or degenerate versions of MgAn1. Several transposon-related proteins are present as well (three, in the 200-kb portion of the scaffold most immediately surrounding MgAn1). The very large number of transposable elements found in the *M. guttatus* genome could be a primary driver of local gene duplication, potentially contributing to phenotypic diversification in *Mimulus*.

3.4.5 Why are both copies of the pathway maintained?

The genome duplication event in the luteus group (n = 32) must have occurred very recently, following divergence with *M. guttatus* (n = 14). This fact is strikingly
illustrated by the existence of two highly similar, identically regulated, and apparently fully functional copies of the anthocyanin biosynthesis pathway. Although it is possible that both copies of each enzyme-encoding gene are maintained by selection, we suspect that there has simply not yet been enough time for one copy to degenerate or to adopt a newer or more specialized function. The maintenance of both pathways suggests that the energetic cost of pathway duplication is not extraordinarily high. It would be interesting to know whether other tetraploid species in the luteus group also show activity of both copies of the pathway, and whether other biosynthetic pathways are retained in duplicated form as well.

3.4.6 Compartmental regulation of the anthocyanin biosynthetic pathway

The enzyme-encoding genes in the core anthocyanin pathway (Chs through Ans) fall into two distinct regulatory categories in M. cupreus. The “early” genes, Chs and Chi, show a significant but modest expression difference between orange- and yellow-flowered M. cupreus. The “late” genes, F3h, F3’h, Dfr, and Ans, are expressed more than 10-fold higher in orange-flowered plants. The anthocyanin pathway has been divided into early and late genes in other dicots as well, but membership in the two groups varies: Antirrhinum majus shows the same division as M. cupreus (Martin et al. 1991), whereas only Dfr and Ans act as “late” genes in the more distantly related Aquilegia (Whittall et al. 2006). Early versus late categorizations do not appear to apply to monocots. Zea mays shows coordinate regulation of the entire pathway (Goff et al. 1990). In Oncidium (orchid), downregulation of an R2R3 MYB correlated with the
downregulation of Chi and Dfr (Chiou and Yeh 2008). However, the first and last steps of the core pathway were unaffected. Clearly, the division of regulatory control in the anthocyanin pathway has evolved substantially over the course of angiosperm evolution.

The fact that McAn1 genotype covaries with the expression of all six steps of the pathway suggests that McAn1 physically interacts with all six. The magnitude of the orange-yellow expression difference in the “late” genes is roughly similar to the expression difference at McAn1, implying a direct relationship between McAn1 mRNA level and “late” gene expression. The expression difference in the “early” genes is, however, much more muted, which could be explained by the presence of additional factors mediating the interaction of McAn1 with its targets. The identity of the additional factor(s) is speculative at this point. One possibility is weaker binding of McAn1 to the “late” genes, causing those genes to be more sensitive to McAn1 transcript level. Binding affinity could be affected by changes in the coding region of McAn1 or in the cis-regulatory region of the enzyme-encoding genes. Alternatively, strong activation of Chs and Chi by other transcription factors (bHLH, WD40, or paralogs of McAn1) might override the effects of McAn1 expression differences.

Products of the CHS and CHI enzymatic reactions feed into pathways for the production of flavones and isoflavones, chemicals which have numerous functions including UV protection and plant signaling to root microorganisms (Dixon and Steele 1999). The participation of Chs and Chi in multiple pathways is thought to constrain their evolution (Rausher 2006). If the production of flavones or isoflavones in petal
tissue is beneficial, for example, then reduced expression of \textit{Chs} and \textit{Chi} in the petal may be selected against, while genes downstream of \textit{Chi} should experience no such selective pressure.

### 3.4.7 Pigmentation as a threshold trait

While \textit{McAn1} mRNA level varies dramatically by flower color, individual variation within each color class also correlates strongly with individual variation in the expression of its targets, indicating that the anthocyanin pathway is sensitive to very slight regulatory variation. Despite this sensitivity and continuous variability in expression level, only two discrete floral phenotypes are observed. Minor differences in enzyme production, therefore, do not generally translate to an altered phenotype.

This observation suggests a simple threshold model for pigment production: if \textit{McAn1} production in the early bud stage is at least 50\% of that observed in the orange parent, anthocyanin pigment is produced (Fig. 17). At lower levels, anthocyanin pigment is not produced: all yellow-flowered plants had \textit{McAn1} mRNA levels less than 32\% that of the orange parent. This model could be tested using overexpression vectors in yellow-flowered \textit{M. cupreus}, with \textit{McAn1} placed under the control of promoters of varying strength, in order to determine what levels of \textit{McAn1} mRNA production are associated with the activation of anthocyanin pigment.

The observed scatter in \textit{McAn1} expression could be due to chance or to environmental variation, or could be associated with genetic variation at other loci in the
genome. The mutation that determines flower color, which is in or very tightly linked
McAn1, appears to be sufficient to override this scatter.

3.4.8 Predicting the mechanisms of evolution

While “all types of molecular changes clearly contribute substantially to
molecular adaptation” (Carroll 2008), it may be possible to develop more specific
predictions within that framework. Researchers in the field of evolutionary
development have argued that morphological changes are largely cis-regulatory, while
physiological changes are not (Carroll 2005, 2008). It is not clear to which category
flower color belongs, and it may be that other, more useful ways to classify traits exist.

For example, traits may be categorized as gains or losses. Trait gains can arise
from the evolution of a new function in a structural gene (Zhang 2003), from a novel
deployment of that gene via a regulatory change (Gompel et al. 2005), or from increased
gene copy number (Perry et al. 2007). Trait loss has been associated with both loss of
function in enzyme-encoding genes and downregulation of their expression (Durbin et
al. 2003; Jeffery 2006). A reasonable general hypothesis is that changes in the coding
regions of structural genes are more likely to cause loss of function than gain of function,
since there are presumably more ways to disrupt a specialized enzyme than to increase
its activity.

A consistent pattern in anthocyanin pigmentation is that transitions are
unidirectional, with losses in the ability to produce pigment more common than gains
(Rausher 2008). The luteus group is a particularly rare phenomenon in that it exhibits
three independent gains of anthocyanin pigmentation (Cooley and Willis in prep). If it is true that enzymatic amino acid changes are more likely to cause trait loss than trait gain, then the best a priori candidates for pigment evolution in the luteus group might be regulatory mutations. These could be cis to the structural genes or in the cis or coding regions of a transcription factor. Molecular analysis of the other two gain-of-function traits, in the taxa M. l. variegatus and M. naiandinus (Cooley and Willis in prep), can provide a test of this hypothesis.

Genome-wide studies have found some patterns of evolutionary change associated with biological process. A survey of human and chimpanzee promoter regions by Haygood et al. (2007) found that genes involved in neural development and function, and in food uptake and metabolism, frequently showed accelerated rates of evolution in human cis-regulatory sequences. A study focused on coding regions (Clark et al. 2003) found that genes involved in olfaction and sensory perception showed the strongest evidence of accelerated amino acid evolution in humans. These results generate hypotheses that can be tested in other mammals. Similar surveys in plant species could also yield hypotheses about the types of mutations most frequently associated with various plant-specific biological processes.

3.4.8 Conclusions

We have shown that a single-locus gain-of-function trait, petal anthocyanin pigmentation in M. cupreus, cosegregates with the McAn1 transcription factor. McAn1 belongs to a family of transcription factors that have been found to control floral
anthocyanin patterning in a variety of other angiosperms. We conclude that the molecular mechanism underlying the color polymorphism is most likely to be a change in the \textit{cis}-regulatory region of \textit{McAn1}, because flower color cosegregates not only with genotype at \textit{McAn1} but also with expression at \textit{McAn1} and the twelve anthocyanin structural genes that are its regulatory targets. These data contribute to a growing body of literature on the molecular basis of phenotypic diversity, and provide support for the involvement of \textit{cis}-regulatory changes in the evolution of spatial patterning.
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