Genetic divergence causes parallel evolution of flower color in Chilean *Mimulus*

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Summary

- Deciphering the genetic architecture of phenotypic change provides a framework for understanding how evolution proceeds at a genetic level, and paves the way for work at the molecular level.
- A series of intra- and interspecific crosses were used to investigate the genetic control of recently evolved floral pigmentation phenotypes in a group of closely related *Mimulus* species from central Chile.
- An intraspecific polymorphism was found to be controlled by a single Mendelian locus. Differences between species, by contrast, were composed of multiple independent patterning elements, including both Mendelian and polygenic traits. The most striking phenotypic novelty in this group, anthocyanin pigmentation in the petal lobes, has evolved three times independently.
- The results illustrate how genetically simple modular elements can interact with polygenic or quantitative traits to create complex new phenotypes. The repeated evolution of petal lobe anthocyanins suggests that natural selection may have played a role in the evolution of red coloration in the Chilean *Mimulus*, and shows that red coloration has been achieved via different genetic pathways in these closely related species.

Introduction

The question of whether traits evolve via many loci of small effect or a few loci of major effect has a long and controversial history, beginning with the opinions of Darwin (1859) and Fisher (1930) that phenotypic change is composed of numerous minor mutations. The opposite view, of discontinuous changes caused by single genes, was given by Darwin's cousin Francis Galton (1894) and later by Mendelian advocates such as Bateson (1913). A more extreme version of discontinuous evolution 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 (Orr, 1998). Although theoretical arguments can be made for all sides, additional empirical data on the genetic basis of phenotypic change are needed to help 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 these 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 of phenotypes is of great interest to researchers. Not only is it considered to be evidence of natural selection when found in taxa inhabiting similar environments (Endler, 1986; Schluter, 2000), but the determination of its genetic basis can reveal the degree of evolutionary constraint experienced by underlying biochemical pathways. Repeated use of the same gene to achieve a particular phenotype would suggest that very few genes are available to respond to selection, as a result of either a scarcity of genes or an abundance of pleiotropic constraints. The opposite finding, that changes at a variety of loci can create a similar phenotypic result, would suggest that there is a great deal of flexibility (and little predictability) in evolution.

A classic system for testing the predictions of evolutionary theory is floral pigmentation (Mol *et al.*, 1998; Hirschberg,

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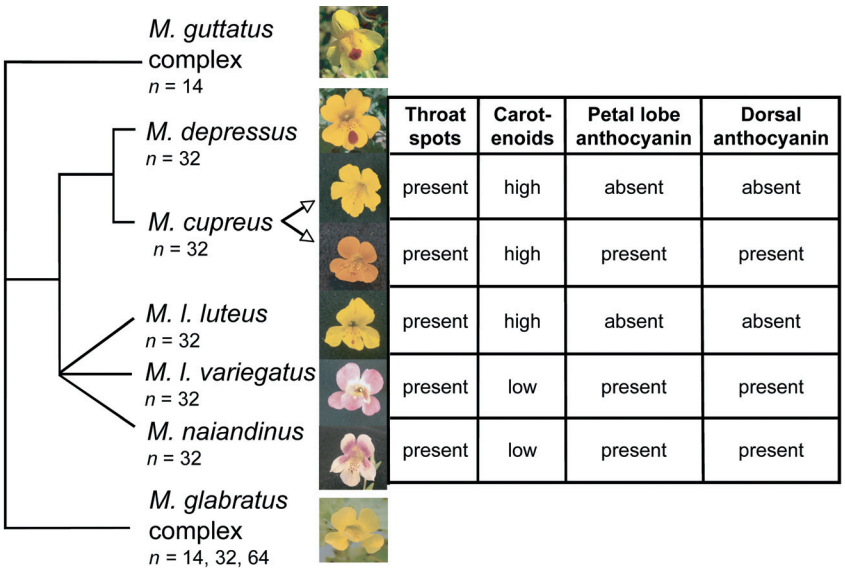

Throat spots	Carot-enoids	Petal lobe anthocyanin	Dorsal anthocyanin
present	high	absent	absent
present	high	present	present
present	high	absent	absent
present	low	present	present
present	low	present	present

Fig. 1 Floral traits and chromosome counts in Chilean *Mimulus*. Phylogenetic relationships are based on Beardsley *et al*. (2004), sequence data from A. Cooley (unpublished) and Grant (1924). *Mimulus depressus* photograph is courtesy of Station Alpine Joseph Fourier.

2001; Durbin *et al*., 2003). Pigment biosynthesis pathways are reasonably simple and well understood, and generate clear phenotypes that tend to be evolutionarily labile. In plants, the two major classes of pigment are the anthocyanins, which produce red, purple and blue colors, and the carotenoids, which are typically yellow or orange. The anthocyanin pathway has been a particular focus of evolutionary biologists for both practical – it is especially compact and well characterized – and historical reasons. Early work on maize anthocyanin phenotypes made major contributions to several fields of research, including the regulation of gene networks and biosynthetic pathways (for example, Emerson & Anderson, 1932; McClintock, 1950, 1968; Chen & Coe, 1977), and laid the groundwork for continued evolutionary research.

The 'yellow monkeyflower' group of the genus *Mimulus* is characterized by a conserved pattern of floral pigmentation, consisting of a yellow corolla with small red spots along the throat, in contrast with the red and pink corollas found elsewhere in the genus. This strongly supported monophyletic group contains *c*. 35 species [including clades J and K in Beardsley & Olmstead (2002) and Beardsley *et al*. (2004)], 30 of which display characteristic yellow corollas. The phylogenies of Beardsley & Olmstead (2002) and Beardsley *et al*. (2004) suggest that yellow corollas are ancestral to the group. This hypothesis permits a maximum of five independent gains of red flower color (in *M. exiguus*, *M. latidens* and the taxa discussed below), whereas the alternative hypothesis of a red-flowered ancestral state requires at least seven independent losses.

Three exceptions to the 'rule' of yellow corollas are contained within a small monophyletic group native to central Chile, referred to here as the *luteus* group. All three taxa (*Mimulus luteus* var. *variegatus*, *M. naiandinus* and *M. cupreus*) show substantial increases in floral anthocyanin pigmentation relative to the ancestral state, whereas their close relatives *M. l. luteus* and *M. depressus* exhibit the classic yellow monkeyflower phenotype (Fig. 1). With the goal of obtaining a better understanding of the evolution of floral pigmentation in the *luteus* group, we used a series of intra- and interspecific crosses to determine the genetic basis of color patterning in the three red-pigmented taxa relative to *M. l. luteus*. Our data permitted us to address the following specific questions.

- Is the genetic basis of the derived floral phenotypes simple or complex?
- Do the same loci contribute to both intra- and interspecific variation?
- Do the same loci contribute to similar phenotypes in different species?

Materials and Methods

Study taxa

Members of the *luteus* group are recent tetraploids, with $n =$ 30–32 chromosomes, compared with $n = 14$ in the closely related *M. guttatus* (Vickery *et al*., 1968). Their ranges are partially overlapping (vonBohlen, 1995) and they are pollinated by hummingbirds, insects and self-pollination (Medel *et al*., 2003; Cooley *et al*., 2008). *Mimulus depressus* and *M. luteus* var. *luteus* have a yellow corolla with red spots of anthocyanin restricted to the throat and lower central petal (Fig. 1). Derived floral pigmentation phenotypes are found in *M. 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 coppery orange. Yellow morphs of *M. cupreus* have been found in one of four surveyed populations (Cooley *et al*., 2008). Unless otherwise specified, '*M. cupreus*' refers here to the more common orange morph. Sequence data suggest that *M. depressus* and

Table 1 Seed collection sites

Taxon	Collection site	Location	Elevation (m)
M. l. luteus	El Yeso/Cajón del Maipo	33.4 S, 70.0 W	2600
M. l. variegatus	Río Cipreses	34.2 S, 70.3 W	1200
M. cupreus	Laguna del Maule	36.0 S, 70.3 W	2300
M. naiandinus	Termas del Flaco	34.5 S, 70.4 W	1000

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 2001–05.

M. cupreus form a group sister to *M. l. luteus*, *M. l. variegatus* and *M. naiandinus* (Beardsley *et al.*, 2004).

Mimulus luteus var. *luteus* and *M. l. variegatus* have historically been considered as varieties of the same species, whereas the other taxa have been accorded species status based on vegetative and floral morphology (Grant, 1924). All of the study taxa are interfertile, sometimes producing hybrids in nature in areas of sympatry (Cooley *et al.*, 2008), and thus are not distinct species according to the 'Biological Species Concept' (Dobzhansky, 1937; Mayr, 1942). However, the term 'species' is used here for clarity of writing.

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 1). In order to simplify the 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.

Glasshouse conditions

All seeds were sown in 2-in pots using Fafard 4-P soil-free potting mix. Plants were maintained in Duke University glasshouses with 18 h d^{-1} 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.

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 F_1 individuals were self-fertilized to yield segregating F_2 populations, as depicted in Fig. 2a,b.

To further investigate the results of the initial crosses, additional crosses were conducted in the same manner, generating the F_2 populations shown in Fig. 2c and Fig. S1 (see Supporting Information). In some cases, backcrosses to *M. l. luteus* or the yellow morph of *M. cupreus* were performed to verify particular observations, as discussed in Results. Each cross was conducted reciprocally but, as 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 Results.

Assessment of segregating phenotypes

Flowers from the progeny of each cross were visually scored for the presence or absence of anthocyanin on the petal lobes (Fig. 3c) and the top of the corolla tube ('dorsal anthocyanins'; Fig. 3d,e). It should be noted that 'dorsal pigment' refers to a diffuse layer of anthocyanin on the back of the upper two petals, but can sometimes also be seen faintly on the front of these two petals (Fig. 3f).

In the *M. l. variegatus* × *M. l. luteus* cross, carotenoid pigmentation varied in intensity (but not in spatial patterning), whereas petal lobe anthocyanin pigmentation varied in spot size (but not intensity). Carotenoid intensity was quantified for three flowers per plant in the parentals, the F_1 and a small sample of F_2s ($N = 20$) using a spectrometer. For each flower, a flat portion of petal lobe tissue that was not pigmented by anthocyanin was selected for analysis. Spectral reflectance in the visible range was measured in 0.34 nm segments with a fiber optic probe (R400-7 reflection probe, Ocean Optics Inc., Dunedin, FL, USA) coupled with a visible light source and a multichannel spectrometer (USB2000, Ocean Optics Inc.). Two regions of the visible spectrum were of particular interest: 430–470 nm (blue–violet) and 575–600 nm (yellow). Estimates for each flower were obtained by averaging across data points in the 430–470 nm and 575–600 nm ranges. Species differences were tested for significance with one-way ANOVAs (JMP 7.0, SAS Institute, Cary, NC, USA, 2007).

Anthocyanin spot size was quantified in the same cross. Photographs of one flower per plant from parental, F_1 and F_2 populations were taken from a head-on, 'pollinator's view' perspective. The throat area was digitally removed in order to examine the petals only. ImageJ v1.31 (http://rsb.info.nih.gov/ij/) was used to calculate the extent of red (anthocyanin) pigmentation as a percentage of total petal surface area.

The intensity of dorsal pigmentation was not quantified. Although dorsal pigment was clearly either present or absent in the *M. l. variegatus* × *M. l. luteus* cross, it is likely that the pigment varied in intensity when present. However, its uneven variegated distribution made it difficult to quantify via the

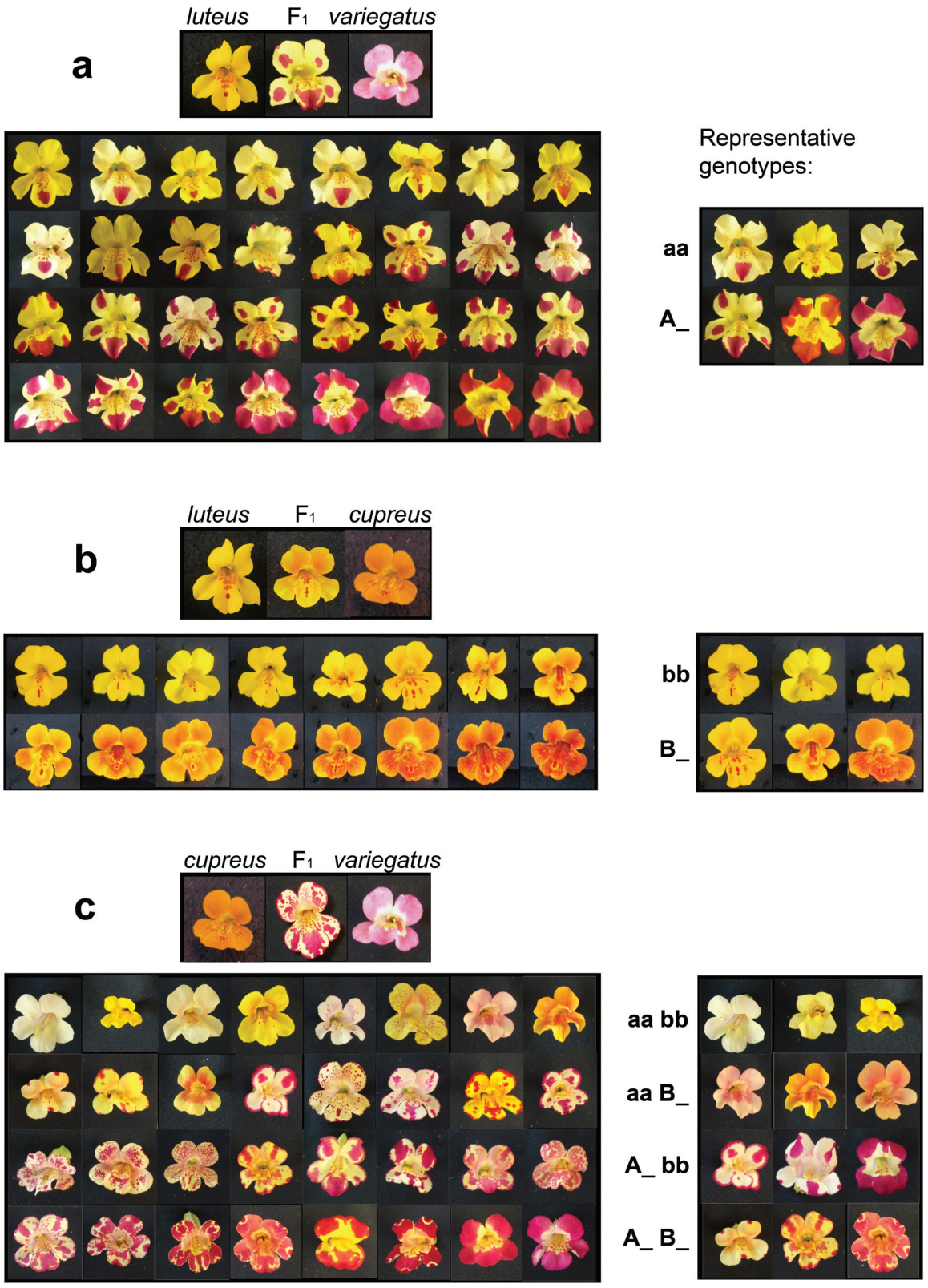

Fig. 2 F_2 progeny of interspecific crosses show segregating floral pigmentation patterns. The inbred parental lines used for each cross are labeled at the top of each figure section, with their F_1 hybrid in the middle and F_2 progeny below. F_2 photographs illustrating inferred genotypes are shown on the right-hand side of the figure. Inferred genotypes of the parental lines are: aa bb (*Mimulus luteus* var. *luteus*), AA bb (*M. l. variegatus*) and aa BB (orange morph of *M. cupreus*). (a) *Mimulus luteus* var. *luteus* × *M. l. variegatus*; (b) *M. l. luteus* × orange *M. cupreus*; (c) orange *M. cupreus* × *M. l. variegatus*. White boxes in (c) denote 'recombinant' bright red and light pink colors, as discussed in Results.

Fig. 3 Anthocyanin pigmentation phenotypes scored in *Mimulus luteus* var. *luteus*, *M. l. variegatus* and their F_1 and F_2 hybrids. (a) Throat spots; (b) bottom spot; (c) petal lobe anthocyanin spot; (d) dorsal anthocyanins present; (e) dorsal anthocyanins absent; (f) faint front band of pigment associated with dorsal anthocyanins.

spectrometer, and its faintness and three-dimensionality made it difficult to quantify using ImageJ.

Results

Hybrids exhibit complex phenotypes

The purple-flowered *M. l. variegatus* and orange-flowered *M. cupreus* superficially appear to be solid colored (Fig. 1), but crosses to *M. l. luteus* revealed considerable complexity in the spatial distribution of red anthocyanin pigments. In *M. l. variegatus* × *M. l. luteus* F_2 progeny, anthocyanins on the petal lobes were distributed in solid, rounded, relatively large patches (Fig. 2a). A cross between *M. cupreus* and *M. l. luteus*, by contrast, yielded F_2 progeny with a fine spray of petal anthocyanins (Fig. 2b). A cross between the two derived phenotypes showed a combination of both patterns, apparently segregating independently of one another (Fig. 2c): *M. l. variegatus* × *M. cupreus* F_2 populations 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* are not caused by a change in anthocyanin type: in both taxa, only the pigment cyanidin is detectable (Cooley *et al.*, 2008). Rather, the F_2 populations show that flower color results from interaction of the red anthocyanins and the yellow carotenoids. Plants with light anthocyanin pigmentation on a dark yellow (high-carotenoid) background are orange, similar to *M. cupreus*, whereas plants with dark anthocyanin pigmentation on a white (low-carotenoid) background are purple, similar to *M. l. variegatus*. Independent assortment creates colors not seen in the parentals: light pink (light anthocyanin pigment with low carotenoid levels; Fig. 2c, first white box) and bright red (dark anthocyanin pigment with high carotenoid levels; Fig. 2c, second white box).

Differences in carotenoid intensity, but not patterning, were observed. *Mimulus luteus* var. *luteus* and yellow *M. cupreus* differed marginally in reflectance at 430–470 nm ($F = 7.81$, $P = 0.049$), but did not differ significantly at 575–600 nm ($F = 3.81$, $P = 0.123$). *Mimulus luteus* var. *variegatus* showed significantly greater reflectance than the two yellow-flowered

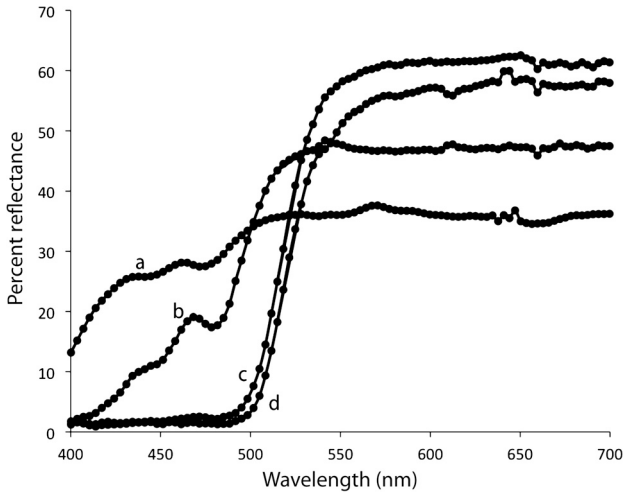

Fig. 4 Reflectance of visible light in floral petal tissue. The percentage of light reflected between 400 and 700 nm is shown for one representative flower of: (a) *Mimulus luteus* var. *variegatus*; (b) *M. l. variegatus* × *M. l. luteus* F_1 hybrid; (c) *M. l. luteus*; (d) yellow-flowered *M. cupreus*. For clarity, every 10th reading is shown here. Readings were taken on corolla tissue that was free of visible anthocyanin pigmentation in order to evaluate variation in carotenoids alone.

taxa in the 430–470 nm (blue–violet) range, indicating a lower degree of saturation or greater 'whiteness' ($F = 105.6$, $P < 0.001$; Fig. 4). In the 575–600 nm (yellow) range, *M. l. luteus* and yellow *M. cupreus* showed significantly greater reflectance than *M. l. variegatus* ($F = 84.27$, $P < 0.001$; Fig. 4). The *M. l. luteus* × *M. l. variegatus* F_1 progeny were intermediate between the two parental lines at both 430–470 nm and 575–600 nm (Fig. 4). The F_2 progeny showed a wide range of variation at both 430–470 nm and 575–600 nm, with few individuals approaching parental values (Fig. S2, see Supporting Information).

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

Although some aspects of hybrid floral pigmentation varied continuously, a number of F_2 individuals completely lacked

Table 2 Segregation of dorsal and petal anthocyanins does not differ significantly from single-locus Mendelian ratios

Cross	Anthocyanin location	Observed ratio (dominant : recessive)	H_o	χ^2	P
L × V F_2	Dorsal	188 : 67	3 : 1	0.22	ns
L × V BC_1	Dorsal	152 : 145	1 : 1	0.16	ns
L × Co F_2	Dorsal	146 : 61	3 : 1	2.21	ns
L × V F_2	Petal lobes	187 : 68	3 : 1	0.38	ns
L × V BC_1	Petal lobes	149 : 148	1 : 1	0.0017	ns
L × Co F_2	Petal lobes	146 : 61	3 : 1	2.21	ns
Co × V F_2	Petal lobes	382 : 128	3 : 1	0.0059	ns
Co × Cy F_2	Petal lobes	204 : 70	3 : 1	0.044	ns

For all samples, d.f. = 1 and $\chi^2 > 3.84$ would show significant deviation from the hypothesized segregation ratio at $P < 0.05$. Abbreviations: Co, orange morph of *Mimulus cupreus*; Cy, yellow morph of *M. cupreus*; L, *M. l. luteus*; ns, not significant; V, *M. l. variegatus*. 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) vs the recessive phenotype (absence of dorsal or petal lobe anthocyanins).

anthocyanin in their petal lobes in both the *M. l. variegatus* × *M. l. luteus* and *M. cupreus* × *M. l. luteus* crosses. We therefore tested whether the presence vs absence of petal lobe anthocyanin 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 individuals and 50% of BC_1 individuals (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*, the presence vs absence of petal lobe anthocyanin pigment segregated as a single Mendelian locus. The derived state (anthocyanin presence, from *M. l. variegatus*) was dominant, yielding a 3 : 1 ratio in F_2 individuals (Table 2). A backcross to *M. l. luteus* showed the expected 1 : 1 ratio, confirming single-locus control. The orange-flowered *M. cupreus* showed a similar pattern, with the presence vs absence of petal lobe anthocyanin segregating as a single locus in *M. cupreus* × *M. l. luteus* F_2 progeny (Table 2). The putatively derived (*M. cupreus*) state was dominant.

Mimulus naiandinus was not subjected to extensive analysis. However, F_1 hybrids of *M. naiandinus* × *M. l. luteus* exhibited petal lobe anthocyanin (A. M. Cooley, unpublished), indicating that pigment gain is, again, dominant to the ancestral phenotype.

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

Petal lobe anthocyanin pigmentation in orange vs yellow morphs of *M. cupreus* is controlled by a single locus (Table 2). F_1 is indistinguishable from the orange parent, indicating that the 'orange' allele (anthocyanin presence) is completely dominant. Unlike the interspecific cross of orange *M. cupreus* to the yellow-flowered *M. l. luteus*, no pigment intensity variation was observed (Fig. S1).

The same locus controls both intra- and interspecific variation

The rarity of the yellow morph of *M. cupreus* suggests that it could be 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. cupreus* and the ancestral yellow of *M. l. luteus*. Alternatively, yellow *M. cupreus* could represent unsorted ancestral variation or recent introgression from *M. l. luteus*. Under the last two scenarios, the same gene should be responsible for the recessive yellow phenotype in both species.

To determine whether the yellow morph of *M. cupreus* is a result of the secondary loss of anthocyanin function, or of a previously existing allele, we crossed it to *M. l. luteus*. Only yellow-flowered progeny appeared in F_1 and 32 F_2 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 petal lobe anthocyanins) are identical.

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 dorsal anthocyanin pigment, whereas *M. l. luteus* and other yellow monkeyflowers do not.

Dorsal anthocyanin pigmentation segregated as a single Mendelian locus in the *M. l. luteus* × *M. l. variegatus* F_2 progeny, as well as in a backcross to *M. l. luteus* (Table 2). It also segregated as a single locus in *M. l. luteus* × *M. cupreus* F_2 individuals (Table 2). In both cases, the putatively derived allele (presence of dorsal pigmentation) was dominant.

Table 3 Independent segregation of dorsal and petal anthocyanins in *Mimulus luteus* var. *variegatus*, but not in *M. cupreus*

		Petal anthocyanins	
	Dorsal anthocyanins	Yes (Pp)	No (pp)
variegatus[1]	Yes (Dd)	76	72
	No (dd)	69	80
cupreus[2]	Yes (Dd)	146	0
	No (dd)	0	61

Data were collected from a backcross (BC_1) population of *M. l. variegatus* × *M. l. luteus* to *M. l. luteus* and an F_2 population of *M. cupreus* × *M. l. luteus*.
[1]H_o = 1 : 1 : 1 : 1, d.f. = 1, χ^2 = 0.93, not significant.
[2]H_o = 9 : 3 : 3 : 1, d.f. = 1, χ^2 = 263.78, $P << 0.001$.

We tested whether dorsal pigmentation in *M. l. variegatus* and *M. cupreus* shares a single genetic basis by crossing the two taxa and examining the F_1 and F_2 progeny. In contrast with 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.

Petal and dorsal pigmentation are separately controlled in *M. l. variegatus*, but not in *M. cupreus*

Dorsal pigmentation segregated independently of petal pigmentation in *M. l. luteus* × *M. l. variegatus*, indicating that these traits are controlled by distinct loci in *M. l. variegatus* (Table 3). In *M. cupreus*, by contrast, the two traits were completely co-inherited, indicating that they are controlled by the same or very tightly linked loci (Table 3).

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 finely speckled distribution were 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, loss of petal lobe anthocyanin production would 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 individual 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. S3). Yellow *M. cupreus* must therefore have 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*.

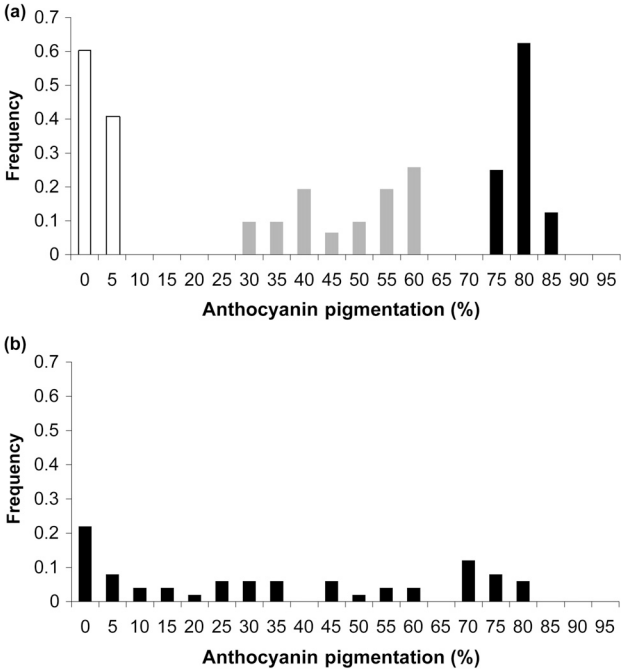

Fig. 5 Anthocyanin spot size in *Mimulus luteus* var. *luteus* × *M. l. variegatus* hybrids. The percentage of petal surface area colored by anthocyanin pigment is indicated for: (a) the parental taxa *M. l. luteus* (white bars) and *M. l. variegatus* (black bars), and F_1 progeny (grey bars); (b) F_2 progeny. Data were taken from one flower per plant, with the throat area removed as described in Materials and Methods. N = 15 (*M. l. luteus*); N = 16 (*M. l. variegatus*); N = 30 (F_1 individuals); N = 100 (F_2 individuals).

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

Although the presence vs absence of petal lobe anthocyanin behaved as a discrete trait, the anthocyanin spot size (in *M. l. luteus* × *M. l. variegatus* progeny) varied considerably when present. We measured the spot size quantitatively as a percentage of the petal surface area. Anthocyanin pigmentation ranged from 0.1 to 9.7% of the total petal surface area in our inbred lines of *M. l. luteus* (N = 15, mean ± SE = 4.5 ± 0.6%; Fig. 5), 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 F_1 and F_2 hybrids were intermediate in the extent of pigmentation, suggesting additive effects of the parental alleles (F_1: N = 22, 36.9 ± 3.3%; F_2: N = 50, 38.5 ± 4.3%; Fig. 5).

The majority of F_2 progeny were similar to either the *M. l. luteus* parent or the *M. l. variegatus* parent: 30% of the F_2 individuals fell within the *M. l. luteus* range; 26% were within or very slightly below the *M. l. variegatus* range (Fig. 5). The remaining 44% were evenly distributed across the intermediate values. A Castle–Wright estimator of the minimum number of factors, $\hat{n}_e$ (Castle, 1921), was 1.06, consistent with

a single gene of large effect contributing to spot size. However, the degree of variation observed in the F_2 progeny suggests that additional genes are probably also important.

Petal lobe anthocyanin evolved by genetic divergence in *M. l. variegatus*, *M. cupreus* and *M. naiandinus*

We tested for the independent evolution of the gain of petal lobe anthocyanin in the purple-flowered *M. l. variegatus* compared with the orange-flowered *M. cupreus* and the pink-flowered *M. naiandinus* by intercrossing them and evaluating the F_2 progeny. The absence of petal lobe anthocyanin in the F_2 offspring of two fully pigmented parents would indicate recombination between two loci, showing that a different gene controls petal pigmentation in each parent. If the two loci were unlinked, 1/16 of the F_2 progeny (6.25%) would be homozygous recessive at both loci and would display unpigmented petal lobes.

In the F_2 progeny of *M. l. variegatus* × *M. cupreus* (Fig. 2c), 23 of 509 plants (4.52%) showed a complete lack of petal lobe anthocyanin. This number is not significantly different from the 31.8 individuals expected under a model of two unlinked loci (d.f. = 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. S1), seven of 72 plants had pure white petal lobes. This number is consistent with the 4.75 expected under a model of two unlinked loci (d.f. = 1, $\chi^2 = 1.14$, $P > 0.05$), and indicates that petal lobe anthocyanins are controlled by different loci in the two parents.

Discussion

The determination of the genetic basis of phenotypic change is a major challenge in evolutionary biology. We used a classical genetic approach to evaluate floral diversification in pigment patterning in a group of South American wildflowers. Our primary goal was to determine the genetic basis of recently evolved floral phenotypes in *M. l.* var. *variegatus*, *M. cupreus* and *M. naiandinus*. We found that differences between species comprise 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. This observation further supports yellow as the ancestral flower color of yellow monkeyflowers, and suggests that extensive red coloration may confer some adaptive benefit.

Major and minor effect patterning elements combine to create unique phenotypes

Multiple color patterning elements were identified and genetically described using intra- and interspecific crosses. The abundant phenotypic variation observed in F_2 populations reveals an accumulation of distinct floral pigmentation traits in the parental taxa, many of them Mendelian and some quantitative.

A single locus simultaneously controls the presence of anthocyanin in both dorsal and petal regions of the *M. cupreus* corolla (Tables 2, 3), and is responsible for both intra- and interspecific variation in flower color (see Results). The same locus appears to confer dorsal pigmentation in *M. l. variegatus* (see Results), whereas a separate locus is responsible for petal lobe anthocyanin in *M. l. variegatus* (Table 3). Interestingly, the derived allele was dominant in all cases.

The presence vs absence of anthocyanin-pigmented regions always segregated in Mendelian fashion in both F_2 and backcross populations. By contrast, traits such as pigment intensity or extent suggested some degree of multigenic control. Anthocyanin spot size (Fig. 5) showed frequent recovery of parental phenotypes in the F_2 progeny, implicating a single major locus that is probably (given the extent of continuous variation in the F_2 progeny compared with the parents and the F_1 individuals) influenced by additional loci of small effect. Anthocyanin intensity in *M. cupreus* appeared to vary continuously in an interspecific cross (Fig. 2b), but not in an intraspecific cross (Fig. S1a), suggesting poly- or multigenic control of variation between but not within species. Carotenoid intensity varied continuously in *M. l. variegatus* × *M. l. luteus* F_2 individuals (Fig. S2), and the lack of progeny approaching parental values suggests a quantitative genetic basis for this trait.

The finely speckled spatial distribution of anthocyanin in *M. cupreus* was shown to act independently of the petal lobe anthocyanin locus (Fig. S3); however, its genetic basis was not further characterized because of the difficulty in quantifying speckliness.

The same locus controls both intra- and interspecific variation

Comparing a trait's variation within and between species can illuminate its evolutionary history (for example, Lexer *et al.*, 2005). We asked whether a rare yellow-flowered morph of *M. cupreus* might represent introgression from the yellow-flowered *M. l. luteus* or *M. depressus*, unsorted ancestral standing variation or secondary loss of petal lobe anthocyanins. 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 reveals a shared genetic basis for both intra- and interspecific variation.

Divergent 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. 1). 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. Parallel evolution of the trait, by contrast, could have occurred multiple times at the same locus or via different loci.

The recovery of F_2 progeny with no petal lobe anthocyanin pigmentation in a cross between purple-flowered *M. l. variegatus* and orange-flowered *M. cupreus* reflects recombination between two loci. This can be described by a simple genetic model with a dominant allele at locus 'A' in *M. l. variegatus* and a dominant allele at locus 'B' in *M. cupreus*. F_2 progeny receiving the 'aa bb' genotype lack petal lobe anthocyanin (Fig. 2c). *Mimulus luteus* var. *variegatus* × *M. naiandinus* yielded a similar result (Fig. S1b), showing that petal lobe anthocyanins are also controlled by different loci in these two taxa. As *M. naiandinus* is more closely related to *M. l. variegatus* than to *M. cupreus* (Fig. 1), the most parsimonious explanation is that petal lobe anthocyanins evolved independently in all three red-pigmented taxa using a variety of different loci. Incidentally, this result further confirms that yellow corollas are ancestral to the yellow monkeyflowers. If red were ancestral, it would have to have been lost and then independently regained at least once in the *luteus* group. The minimum number of changes to accommodate a red-flowered ancestor is therefore eight rather than seven, compared with a maximum of five changes given a yellow-flowered ancestor.

In the *luteus* group, there are clearly multiple genetic routes to achieving red petals. Although pleiotropic constraints are thought to be prevalent in anthocyanin biosynthesis (Rausher, 2006), there is clearly some evolutionary flexibility, in that at least two and perhaps three loci can independently create very similar outcomes. Further tests are needed to compare control of petal anthocyanins in *M. cupreus* and *M. naiandinus*.

Although flower color does not appear to influence pollinators (Cooley *et al.*, 2008), the independent evolution of red petals in three close relatives from similar environments suggests 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 pathway, protect against stressors, such as herbivory, heat and ultraviolet radiation (Bernays *et al.*, 1989; Holton & Cornish, 1995; Ayres *et al.*, 1997; Chalker-Scott, 1999; Hoch *et al.*, 2001; Coberly & Rausher, 2003). Increased activity of the pathway could be beneficial for reasons not directly related to flower color, for example by providing additional defensive compounds during floral development. It should be noted that all of the study taxa produce visible vegetative anthocyanins, particularly when stressed, and our line of *M. l. variegatus* produces constitutively higher levels of anthocyanin than the other taxa used here (A. Cooley, pers. obs.). However, visual assessment of *M. l. variegatus* × yellow *M. cupreus* F_2 plant photographs revealed no sign of covariation between floral and vegetative anthocyanin intensity (A. Cooley, unpublished).

Does diversity originate from multiple genes or multiple alleles?

In repeated evolution, we ask whether the *same* trait is achieved multiple times via one or many loci. The same question can be posed 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 & Wakeland, 1990; Rogers & Kaufman, 2008) and, albeit less dramatically, in the regulation of anthocyanin pigment in maize (Emerson & Anderson, 1932; Radicella *et al.*, 1992). As with repeated evolution, diversity originating from alleles at 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' for selection, the more likely traits are to evolve using multiple different loci.

The anthocyanin biosynthesis pathway involves relatively few genes, several of which are thought to be strongly constrained by pleiotropic effects (Rausher, 2008). The Chilean floral diversification involves several major anthocyanin-related genetic changes, as well as many changes of smaller phenotypic effect. Have all of these variants accumulated as alleles of the least-pleiotropic anthocyanin genes?

In several cases examined here, phenotypic diversity arises from different alleles at a single locus: the 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 locus. However, other pattern elements have been shown to segregate independently of one another, indicating the involvement of multiple other loci, despite the small size and high pleiotropic constraint of the anthocyanin pathway.

Interestingly, the *luteus* group is tetraploid relative to the other yellow monkeyflowers (Fig. 1). Polyploidization is an extreme example of gene duplication, which facilitates diversification by expanding the available evolutionary material and reducing pleiotropic constraints on some gene copies (Force *et al.*, 1999; Lynch & Force, 2000). Polyploidization could contribute to diversification in the *luteus* group by allowing one copy of the anthocyanin biosynthesis pathway to specialize on petal lobes (Fig. 3c), whereas the other retains the 'yellow monkeyflower' pattern function (Fig. 3a,b). Similarly, a duplicated regulatory sequence could promote color pattern variation through subfunctionalization. In order to discover whether genome duplication played a role in *luteus* group diversification, it will be necessary to identify the causal genes.

Gain-of-function vs loss-of-function traits

Losses of anthocyanin pigmentation are more common than gains in most of the angiosperm genera studied to date (Perret *et al.*, 2003; Rausher, 2006, 2008; Whittall *et al.*, 2006). Some

loss-of-function features have been traced to coding changes that disrupt structural genes (Coberly & Rausher, 2003; Zufall & Rausher, 2003). Others have been associated with the downregulation of structural genes (Whittall *et al.*, 2006), although we are unaware of any cases in which the regulatory change has been confirmed as either coding or noncoding.

An amino acid mutation in an enzyme may be more likely to reduce function than enhance it, because enzymes are structurally complex. The same is arguably true of amino acid changes in transcription factors. Accumulation of coding region mutations may help to explain the prevalence of pigment loss across the flowering plants.

However, gains of pigment function are known to occur, and indeed are moderately common, in at least two genera: *Dalechampia* and *Acer* (Armbruster, 2002). The genetic basis of floral pigment gain remains virtually unstudied, but we propose that *cis*-regulatory mutations are probable contributors. In contrast with coding sequence, *cis*-regions are not transcribed and thus should not be constrained by the need to maintain three-dimensional structure. In addition, transcription factor binding sites are quite short – typically 6–10 bp (Fairall & Schwabe, 2001) – which makes the acquisition of a new binding site by chance mutation relatively likely (Hahn *et al.*, 2003). The gain or loss of a transcription factor binding site, *cis* to a structural gene or a transcription factor, is a straightforward mechanism for activating or upregulating the anthocyanin biosynthetic pathway in a previously unpigmented part of a plant.

The *luteus* group is well suited for testing the *cis*-regulatory hypothesis. *Mimulus luteus* var. *variegatus*, *M. cupreus* and *M. naiandinus* represent three independent gains of function. If gains of function are more likely than losses of function to evolve via changes in *cis*, we might expect that the petal anthocyanin phenotypes in one or more of the three taxa should be associated with a *cis*-regulatory variant.

Conclusions

Many known color patterning changes behave as single-locus Mendelian traits. This study system illustrates how genetically simple, modular elements can interact with polygenic or quantitative traits (such as spot size in *M. l. variegatus* and pigment intensity in *M. cupreus*) to create complex new phenotypes. In the Chilean *Mimulus*, the repeated evolution of increased floral anthocyanin pigmentation suggests either a direct adaptive benefit or selection on pleiotropic effects of genes in the anthocyanin pathway.

Although trait segregation in mapping populations can provide information on the genetic architecture of evolution, a more specific molecular understanding requires the identification of the underlying genes. The petal lobe anthocyanin elements identified in this study are particularly amenable to further study because of their dramatic phenotype and simple genetic basis. We are now using a combination of mapping, candidate gene and functional studies to further explore the divergent evolution of petal lobe anthocyanins in *M. l. variegatus* and *M. cupreus*. By identifying the types of changes responsible for the creation of distinct pattern elements, we can begin to construct a model of how the underlying biosynthetic pathways have evolved to give rise to the floral diversity seen in this group of wildflowers.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Trait segregation in F_2 progeny of intra- and inter-specific crosses.

Fig. S2 Reflectance of light in the blue–violet (430–470 nm) and yellow (575–600 nm) ranges of the visible spectrum.

Fig. S3 F_1 hybrids of *Mimulus luteus* var. *variegatus* and the yellow morph of *M. cupreus* show a punctate distribution of petal anthocyanin pigmentation.

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