EVOLUTIONARY TRANSITIONS IN FLORAL COLOR

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Abstract

The tremendous diversity in flower color among angiosperms implies that there have been numerous evolutionary transitions in this character. The common wisdom is that a large proportion of these transitions reflect adaptation to novel pollinator regimes. By contrast, it has been recently suggested that many of these transitions may instead have been driven by selection imposed by other agents on pleiotropic effects of flower-color genes. I evaluate the evidence for these alternative hypotheses and find that while there is circumstantial evidence consistent with each hypothesis, there are not definitive examples of flower-color evolution conforming to either hypothesis. I also document three macroevolutionary trends in flower-color evolution and discuss how these trends may be related to each other.

Introduction

Angiosperms exhibit a tremendous diversity of flower colors, with sister species often differing in the intensity, hue, or patterning of the corolla. This diversity implies that there have been numerous evolutionary transitions in flower color. The observation that floral color is at least loosely correlated with other floral traits, resulting in the common recognition of "pollinator syndromes" (Fenster et al 2004), suggests that many of these transitions have been adaptive. Moreover, the apparent importance of showy flowers in attracting pollinators has led to the common interpretation that pollinators are the primary selective agents influencing flower color and that transitions to different colors represent adaptation to different suites of pollinators. This has led to the common belief that floral-color diversity is a largely a product of coevolution between plants and their pollinators, a proposition I call the "conventional wisdom".

In the first part of this essay, I inquire into the causes of evolutionary transitions in flower color. I first evaluate the evidence supporting the conventional wisdom. I then consider evidence supporting alternate interpretations, including the possibilities that many flower-color transitions are non-adaptive or that many reflect natural selection on pleiotropic effects of genetic variants that affect flower color.

The astounding variety of floral hues, intensity, and patterns of pigmentation makes it appear as if there are few constraints on the evolution of flower color. However, constraints become apparent when one examines macroevolutionary trends in floral-color

transitions. In the second part of this essay, I describe these trends and discuss how properties of flower-color genes and the biochemical pathways they encode may contribute to establishing these trends.

Pollinators as Selective Agents on Flower Color

The idea that evolutionary change in flower color reflects adaptation to novel pollinators can be traced back at least as far as Darwin (see Fenster et al. 2004 for historical review). The primary evidence supporting this contention is the existence of "pollinator syndromes", groups of floral traits that typically occur together in plants pollinated by a particular agent. Examples include (1) bird-pollinated flowers, which typically are red or orange and have elongated floral tubes, reduced floral limbs, exserted stigmas, copious dilute nectar, and articulated anthers; (2) bee-pollinated flowers, which typically are blue or purple, have short, wide tubes, wide limbs, inserted stigmas, small amounts of concentrated nectar and non-articulated anthers; and (3) moth-pollinated flowers, which are typically white, fragrant, and open at night.

Although the generality of floral syndromes have been questioned (Robertson 1928; Waser et al. 1996; Ollerton 1996, 1998), and clearly not all flowers exhibit standard syndromes, there is substantial evidence to indicate that many species conforming to a particular syndrome are in fact pollinated primarily by the agent associated with that syndrome (Fenster et al 2004). This evidence indicates that we

should take seriously the hypothesis that interactions with pollinators have driven the evolution of flower color in many, if not all, species.

While the existence of pollinator syndromes is consistent with this hypothesis, it is also consistent with others. For example, like any evolutionary change, a particular flower-color transition may have occurred by genetic drift. Alternatively, it may have been driven by selection on pleiotropic effects of flower-color alleles (see below), even if the change is deleterious with respect to pollinator attraction. In either case, if the flower color change subsequently attracts novel pollinators, selection imposed by these pollinators may mold other floral characteristics to produce a standard floral-color syndrome. It is therefore possible for transitions between pollinator syndromes with different flower colors to occur without direct selection on flower color by pollinators. Consequently, no matter how common pollinator syndromes may be, their existence cannot be taken as definitive evidence that pollinator-mediated selection drives the evolution of floral color.

What type of evidence would constitute a definitive demonstration that a floral-color change reflects adaptation to novel pollinators? Ideally, it must be shown that (1) the change was caused by selection, and (2) the agent causing that selection was pollinators. As will be seen below, there are currently no species for which both of these conditions have been demonstrated. This lack of compelling evidence does not necessarily indicate, however, that pollinators are unimportant effectors of flower-color evolution. Rather, it just as likely reflects the difficulty of assessing both of these criteria

simultaneously for any given plant species. Because of this, it is worthwhile considering

how much evidence supports the generality of each condition separately. If, for example,

investigations seldom detect selection on flower-color variants, we may be led to think

that genetic drift is more commonly responsible for flower-color evolution than currently

believed.

For information about selection on, and pollinator responses to, flower-color

variation I conducted a literature search on Web of Science. Studies were included only

if they attempted to determine either whether flower-color variants differed in some

component of fitness or whether pollinators respond differently to different color variants

(Table 1). Information was obtained on 24 different species that exhibited variation in

floral color. For two species (*Antirrhinum majus* and *Ipomoea purpurea*), information

was available for two different color polymorphisms, and these are listed separately in

Table 1. Seven of the examples involve color divergence between populations or

interspecific hybrid zones in which selection was examined on potentially introgressing

color phenotypes (Table 1A). The remainder involve within-population color

polymorphisms (Table 1B).   Although this search cannot claim to be exhaustive, the

investigations it turned up are likely to be representative.

Selection on Floral-Color Variation

If the results from these investigations are taken at face value, selection on flower-

color variants is ubiquitous. Of 21 species that have been examined, 18 exhibit evidence

of selection on flower color phenotype (Table 1). However, a number of biases and

limitations associated with many of these studies restrict the degree of confidence that

can be placed on this conclusion. The first is possible reporting bias: it is likely that

evidence for selection is more likely to be reported than lack of evidence for selection.

This type of bias would mean that selection on flower-color morphs is less common that

the sample indicates.

A second limitation is that in most of the investigations listed in Table 1, it is

impossible to differentiate between selection on flower color itself and selection on

genetically linked traits. This problem is especially acute in studies that attempt to detect

selection by measuring the fitnesses of different color variants. In only two of the

investigations of species in which fitnesses were directly measured was any attempt made

to randomize the genetic background (the two different polymorphisms in *Ipomoea*

purpurea), and even in these investigations associations between the focal polymorphism

and moderately linked loci were probably not disrupted. This limitation is probably less

of a problem for studies that detected selection by comparing the spatial distribution of

flower-color morphs with the spatial distribution of neutral markers ("cline" in Table 1),

since in these species recombination has probably had sufficient time to break up

associations between even closely linked genes. Nevertheless, because studies taking this

approach constitute only a small fraction of the studies that attempted to detect selection,

the inability to identify unambiguously the target of selection may mean that direct

selection on flower-color variation is much less common than the sample seems to

indicate.

A third possible bias in many of these studies arises from the fact that in most investigations that attempt to quantify fitness, selection is measured only for some components of fitness. In cases in which fitness differences are found in one or more fitness components, it is unlikely that fitness differences in unmeasured components will just compensate the measured components to produce net neutrality. By contrast, in cases in which no fitness differences were detected, it is very possible that differences would be exhibited in other fitness components, and hence in net fitness. This type of bias will lead to underestimating the prevalence of selection. Because lack of selection was reported for only three of the species examined, however, the extent of this bias will be minor in the sample reported here.

Given these limitations, what is to be concluded about the prevalence of selection on floral-color variation? The most legitimate conclusion is that the evidence suggests that color variants are usually not selectively neutral, but that this has been shown definitively for only a handful of species (those exhibiting flower-color clines). Thus, the current data is *consistent* with the hypothesis that selection on floral-color variation is ubiquitous and therefore likely responsible for many, if not most, evolutionary transitions in flower color, but it also does not exclude alternative hypotheses. This issue will likely be settled only by future investigations that distinguish between direct selection on floral phenotypes and selection on linked traits. This will be most easily achieved by introgressing different flower-color alleles into the same genetic background (e.g. Bradshaw and Schemske 2003) and measuring fitness on the resulting isogenic lines. To

be convincing, however, this approach will require genetic documentation that the lines are truly isogenic, i.e. that they differ at only the flower-color locus.

Selection Driven by Pollinators

As described above, the conventional explanation for floral-color transitions is that they represent adaptations to exploiting different types of pollinators. While I argued in the previous section that available evidence suggests that color transitions are often adaptive, there is little convincing evidence that pollinators are driving that adaptation. It is certainly true that in most cases that have been investigated (14 of 15 species in Table 1), pollinators discriminate among color phenotypes and usually visit some morphs more frequently than others. However, while such discrimination *could* cause fitness differences among color morphs, one can imagine situations in which no fitness differences result from discrimination.   Therefore, this type of evidence is insufficient for determining whether pollinators impose selection on flower color.

Instead, some sort of experimental demonstration that pollinators cause fitness differences is required, either by manipulating pollinator access or by manipulating floral characters. Of the 15 species in Table 1 showing evidence of pollinator discrimination, only three have been investigated in this way. Irwin and Strauss (2003) examined a two-locus color polymorphism in a naturalized population of *Raphanus sativus*. Using progeny analysis, they estimated the proportions of the four paternal haploid haplotypes in each of two treatments: one in which pollination occurred naturally, and one in which maternal plants were pollinated with a mixture of pollen in which the haplotypes

frequencies were equal to those produced by flowers in the population. Proportions were different between the two treatments, indicating that some aspect of pollinator behavior generated differences in the male component of fitness among the flower-color genotypes.

Using a similar approach, Waser and Price (1981) found that rare white-flowered individuals of *Delphinia nelsonii* had reduced seed production, compared to blue-flowered individuals, when pollination was by natural pollinators. By contrast, when plants were hand-pollinated, there was no difference in seed production. Coupled with observations that pollinators visited white-flowered plants at a lower rate, this study convincingly demonstrates that pollinator preferences cause fitness differences between the floral color morphs.

Finally, Melendez-Ackerman and Campbell (1998) examined a hybrid zone between *Ipomopsis aggregata* and *I. tenuituba*, in which red-flowered *I. aggregata* had higher seed production and outcross male success than hybrid genotypes or the white-flowered *I. tenuituba*. In addition, pollinators (primarily hummingbirds) preferentially visited *I. aggregata*. The causal link between differential visitation and fitness differences was established by experimentally manipulating floral color to disassociate effects of flower color from other floral characteristics. When *I. aggregata* flowers were painted either red or white, red flowers received more visits and sired more seeds than white flowers. In another experiment, when flowers of both pure species and of hybrids were

painted red, differences in both visitation rates and fitness were almost completely

eliminated.

Because evidence for pollinator-mediated selection has been found when sought,

it is tempting to infer that, in general, differential pollinator visitation among color

morphs will tend to cause fitness differences. With only three species examined,

however, one cannot at this point place too much confidence in this inference.

Selection for Divergence

More than half of the species listed in Table 1 (i.e. those in part B of the table)

report on intra-population color polymorphisms, rather than color divergence between

populations. Moreover, for most of these species, it is believed (admittedly often without

much evidence) that some sort of balancing selection maintains the polymorphism.

Because it is not clear that the selective agents responsible for balancing selection are

necessarily similar to those responsible for the divergent selection between populations or

species that is presumed to generate evolutionary transitions in flower color, information

from these species may be misleading regarding the validity conventional wisdom that

flower-color transitions reflect adaptation to novel pollinators. Instead, it may be most

meaningful to focus only on species that exhibit geographic divergence in flower color

(Table 1A).

For such cases, we would like to know (1) whether the divergence is adaptive, and

(2) whether pollinators are the selective agents driving divergence. One way of

demonstrating that divergence is adaptive is to show that spatial divergence in color is much greater than expected based on divergence in the frequencies of neutral genetic markers (e.g. Spitze 1993, Storz 2002). Evidence of this type has been reported for four species (*Antirrhinum majus*, *Linanthus parryae, Mimulus aurantiacus*, and a hybrid zone of *Aquilegia formosa* and *A. pubescens*; see references in Table 1a). Unfortunately, only for *M. aurantiacus* is there evidence of pollinator discrimination between morphs, and in this investigation there is no experimental evidence showing that pollinators cause fitness differences.

For many of the remaining species exhibiting flower-color divergence, there is evidence for both differential pollinator visitation and differential fitness between morphs (Table 1B). However, none of the studies reporting fitness differences conducted reciprocal transplants, so that it is not known if selection is *divergent*, as opposed to, say, purifying. Moreover, in only one species (*Ipomopsis aggregata* and *I. tenuituba* hybrid zone), is there convincing evidence that pollinators impose selection on color morphs, and in this case there is no evidence that flower-color divergence is adaptive.

Remarkably, then, we lack any example in which flower-color divergence can be reliably attributed to adaptation to different types of pollinators.   This of course does not mean that the conventional wisdom is incorrect: arguably, for no species have investigators conducted the entire set of experiments required to verify or falsify this hypothesis. Nevertheless, the absence of firm support for this hypothesis indicates that alternative explanations for flower-color transitions should be seriously entertained.

Non-pollinator-driven transitions

Genetic Drift

One alternative to the conventional wisdom is that color divergence is caused by genetic drift. The only case for which the neutrality hypothesis has been seriously argued is the blue-white polymorphism in *Linanthus parryae*, which is controlled by variation at a single Mendelian locus. Using data on spatial distribution of color morphs, Sewell Wright (1943) argued that this variation is selectively neutral, and that even extreme divergence in gene frequencies between populations was caused by genetic drift. Subsequent observations and experiments led Epling et al. (1960), by contrast, to conclude that this variation was subject to selection, but Wright continued to disagree (1978). Only recently has this debate apparently been resolved in Epling's favor (Schemske and Bierzychudek 2001, in press).

One reason that neutrality of color variation has seldom been championed is undoubtedly the apparent ubiquity of selection on flower-color variants. Nevertheless, it is premature to conclude that neutrality is rare. As discussed above, for only a few species can fitness differences among morphs be unambiguously ascribed to variation in floral-color genes, rather than to variation at linked genes. It is thus possible that much of the flower-color variation we see in nature is actually selectively neutral.

Selection on Pleiotropic Effects

Noting that genes that influence floral color also often influence other ecological and physiological traits in plants (Shirley 1996), a number of authors have suggested that flower-color evolution may often be influenced by selection on these pleiotropic effects rather than, or in addition to, selection imposed by pollinators (Rausher and Fry 1993, Simms and Bucher 1996, Fineblum and Rausher 1997, Armbruster 2002, Irwin et al. 2003). Indirect selection on flower color through genetic correlations thus provides an alternative hypothesis for explaining floral color transitions.

Because evidence for pleiotropic fitness effects of color variants has been reviewed recently by Strauss and Whittall (2006), I will simply summarize and comment on their conclusions. These authors (see their Table 7.2) report evidence of fitness differences among morphs in either survival, flower production, seed production or biomass for ten species with flower-color polymorphisms, and differences in damage by natural enemies for three additional species. Unfortunately, because they do not report investigations in which no fitness differences were reported (e.g. Table 1), their data does not allow even a crude assessment of how frequently such pleiotropic effects occur. Nevertheless, it is clear from their compilation that pleiotropic fitness effects of color variants may be common.

A number of caveats must be attached to this conclusion, however. First, as described above, evidence that these effects are due to true pleiotropy of flower-color genes rather than to effects of linked genes is non-existent. Second, it is not clear that in

all cases reported by Strauss and Whittall, the fitness differences were *not* caused by

pollinator behavior. In one case, *Phlox drummondii* (Levin and Brack 1995), color

morphs differed in viability. Because it is difficult to imagine pollinators affecting

survival before flowering begins, the fitness differences in this species can be ascribed to

an agent other than pollinators.

In the remaining cases, fitness traits for which differences among morphs were

reported were either flower number, seed number (or fruit number), or biomass. Biomass

by itself is not a fitness component, but rather a trait that contributes to fitness

components such as viability, seed production, and siring success. While biomass is

often highly correlated with true fitness components (e.g. seed production) in plants, this

correlation needs to be demonstrated in each investigation in order for biomass by itself

to be used as evidence of differential fitness. One of the studies reported by Strauss and

Whittall (on *Echium plantagineum*; Burdon et al. 1983) should be discounted for this

reason.

For six of the remaining species, the primary evidence for the importance of

pleiotropic effects is differential seed production among morphs. While differential seed

production may be due to pleiotropic effects, it may also be caused by differential

pollinator visitation (e.g. Waser and Price 1981), and it is not clear that for any of these

six species differences in visitation can be ruled out as causing the observed differences

in morph fitness. Five of the species were examined by Warren and Mackenzie (2001),

who allowed open pollination by natural pollinators in their experiment. Although in

their experiment the color morph producing the most seeds switched between draught and watered treatments, the authors do not rule out the possibility that pollinator behavior also switches in these treatments (e.g. morphs may switch rankings in nectar production, causing a change in pollinator preference). Because pollinator effects were not decoupled experimentally from other sources of selection, we cannot be certain pollinators did not impose the fitness differences observed in Warren and Mackenzie's investigation or in the other study that has documented differential seed production among morphs (Schemske and Bierzychudek 2001, in press).

Finally, the three intriguing studies demonstrating differential susceptibility to natural enemies reported by Strauss and Whitall (2006) demonstrate the potential for these kinds of organisms to act as agents of selection on flower color. However, differential damage or infection by itself does not imply differential fitness, since many plants are tolerant of damage and infection (Kniskern and Rausher 2006, Weis and Franks 2006). In this context, while Frey (2004) reported both differential herbivory and differential fitness among color morphs in *Claytonia virginica*, selection on flower color was not significant when examined in a multivariate context that included floral size and leaf area, so it is not clear that either pollinators or natural enemies caused fitness differences among flower color genotypes.

What, then, can one conclude about the hypothesis that floral-color transitions are often driven by selective agents other than pollinators? About the same that was concluded for the conventional pollinator-driven hypothesis: that the evidence is

suggestive, but that there is not a single definitive example to support the hypothesis. What will be needed to evaluate this hypothesis conclusively for any particular situation? Again, as for the conventional hypothesis, two things will be required: (1) population differentiation in flower color must be shown to be due to natural selection on flower-color genes and not linked genes; and (2) the agent of selection must be shown unequivocally to be something other than pollinators, which will require some sort of experimental manipulation of purported selective agents. Until both of these criteria have been addressed for a number of species, we will be unable to say anything conclusive about whether pollinators or other agents are the primary cause of evolutionary transitions in flower color, or about the importance of genetic drift.

Macroevolutionary Trends

It is commonly believed that in many angiosperm taxa, bee pollination and blue-purple flowers are ancestral characters, while hummingbird-pollination and the typical red/orange flowers associated with bird pollination, are usually derived characters (see Ackermann and Weigand 2006). By contrast, transitions from bird to bee pollination, with an accompanying color transition, are generally perceived as rare. These observations imply an asymmetry in evolutionary transition rates between these two colors. In this section, I examine the evidence for this type of asymmetry and consider why it might exist. In doing so, I first identify three general macroevolutionary trends in flower-color evolution that the literature suggests are relatively common:

1.   Transitions from blue to red flowers, and from pigmented to white flowers, are more common than the reverse transitions.

2.   Transitions from blue to red flowers usually are caused by inactivating branches of the anthocyanin pathway, resulting in the production of different less-hydroxylated anthocyanins.

3.   Transitions from blue to red flowers, and from pigmented to white flowers, typically involve loss-of-function mutations.

I then describe how these three trends may be causally connected. I note here that this analysis is based primarily on patterns of anthocyanin pigment evolution. Information on macroevolutionary trends in flower-color changes involving carotenoids, the other major floral pigment, is largely lacking.

Asymmetry of floral-color transitions

Demonstrating asymmetry in color transitions requires a statistical assessment of whether transition rates in one direction of change differ from rates in the other direction. The ideal approach would take into account uncertainty due to both phylogeny and ancestral character state by averaging estimated transition rates over a large number of plausible phylogenies (Pagel and Lutzoni 2002). Unfortunately, this approach has been applied in only one study. Using 160,000 trees generated from a Bayesian analysis, Kay et al. (2005) found the rate of transitions from bee to bird pollination in *Costus* to be

significantly larger than the reverse rate, which was estimated to be zero. In this taxon,

the shift from bee to bird pollination typically involved a transition from white flowers

with pigmented stripes to fully-pigmented, red or yellow, corollas.

Two other investigations approach this ideal, but fail either to estimate actual

transition rates or to sample multiple trees. Using a single tree, Whitall et al. (2006)

demonstrated that in *Aquilegia*, the rate of transition from pigmented to unpigmented

(white) flowers was substantially and significantly greater than the rate of gain of

pigmentation. In fact, no reversals were inferred from ancestral state reconstruction.

And for *Penstemon*, Wilson et al. (unpublished) examined origins and reversals of

hummingbird pollination, which involved shifts between blue and red pigments, in a

sample of 1000 bootstrapped phylogenetic trees. Although they did not statistically

assess transition rates, they found numerous (minimum 10, maximum 25) blue/purple to

red transitions, and no convincing reverse transitions. Finally, in *Ipomoea* Rausher

(2006) identified, without any statistical analysis, but based on a phylogeny and ancestral

state reconstruction of Miller et al. (1999, 2004) and Zufall (2003), eight unambiguous,

evolutionarily independent transitions from pigmented to unpigmented flowers and four

unambiguous, evolutionarily independent transitions from blue to red flowers (we have

subsequently identified a fifth, *I. horsfalliae*). By contrast, no reverse transitions of either

type were observed.

While there are doubtless exceptions to this pattern of asymmetry in floral color

transitions, it seems clear that in many angiosperm taxa marked asymmetry is the rule.

One other pattern that may be associated with this asymmetry is "tipness". Rausher (2006) noted that in *Ipomoea*, white-flowered species strongly tend to occur at the tips of the phylogeny. Very few deep nodes are reconstructed as having white flowers. A similar pattern is evident in the phylogenies of *Penstemon* presented by Wolfe et al. (2006): red-flowered, hummingbird-pollinated species tend to occur on the tips. Unfortunately, although this pattern may have implications for evolutionary potential (see below), this pattern has not been verified statistically by any analysis.

Pigment changes in blue-red transitions

The most important type of floral pigment is the anthocyanins. Within angiosperms, there are three major classes of anthocyanin pigments: those based on the anthocyanidins pelargonidin, cyanidin, and delphinidin. These differ primarily in the number of hydroxyl groups on the beta ring of the molecule, with pelargonidins having the fewest, cyanidin having one more, and delphinidin yet one more. As hydroxyl groups are added, the peak of the absorbance spectrum shifts from the red end of the visible spectrum toward the blue end (Tanaka et al. 1998). Consequently, delphinidin-based anthocyanins tend to be purple, violet or dark blue, while cyanidin-based anthocyanins tend to be blue, magenta, or sometimes red, and pelargonidin-based anthocyanins are almost always red or orange.

Although floral color changes can occur without alteration of the pigment that is produced (e.g. most notably by altering vacuolar pH; Griesbach 1996), the general color differences among classes of anthocyanins suggests that most evolutionary transitions

from blue/purple to red/orange involve a switch from producing more-hydroxylated

anthocyanins to producing less-hydroxylated anthocyanins. Several examples support

this suggestion. In the genus *Ipomoea*, the red-flowered, hummingbird-pollinated species

in the *Mina* clade produce only pelargonidin-based anthocyanins. By contrast, the

ancestral state, represented by closely related species, consisted of blue-flowered, bee-

pollinated species that produce cyanidin-based anthocyanins (Zufall and Rausher 2004).

Moreover, in three out of four evolutionary transitions from blue to red flowers in

Ipomoea for which data exists, the pigments shift from entirely cyanidin to entirely

pelargonidin anthocyanins (Zufall 2003). In a fifth species (*I. purga*), the transition has

been from producing solely cyanidin-based anthocyanins to producing a mixture of

pelargonidin- and cyanidin-based compounds. The flowers of this species are

intermediate in color (magenta), but exhibit several traits characteristic of hummingbird

pollination (e.g. a reduced width:length ratio, increased nectar volume, reduced nectar

concentration, and exserted anthers and stigmas) (Zufall 2003). There are no known

cases of shifts from cyanidin to pelargonidin production without a change in flower color

from blue to red.

Scogin and Freeman (1987) noted a similar correlation between flower color and

anthocyanidin class in *Penstemon*. Because they did not have available a reliable

phylogeny of this genus, however, they were not able to convincingly evaluate whether

color transitions were caused by shifts in the class of anthocyanidin produced. With the

publication of a phylogeny of this group by Wolfe et al (2006), though, a

phylogenetically based analysis of this correlation is possible.

A crude analysis of the data now available for the genus *Penstemon* corroborates Scogin and Freeman's conclusion. The *trn* C-D/T-L tree of Wolfe et al. (2006) was pruned to include only those species for which information is known on both flower color and floral pigments (see supplemental material). This pruned tree is presented as Fig. 1 of the supplementary material. Wilson et al (2004, unpublished) demonstrate that each of the red-flowered lineages portrayed in this figure represents an independent transition from blue to red flowers. In order to determine whether these transitions are correlated with transition to a lower hydroxylation state, a series of independent contrasts (Felsenstein 1985) were performed (Table 2; see supplementary material for details). Each contrast included one red-flowered species (all hummingbird pollinated) and one or more blue-flowered species (all bee-pollinated). In some cases more than one blue-flowered species was used because of unresolved polytomies. However, in these cases, the results were similar, regardless of which blue-flowered species was compared to the red-flowered species.

In eight of nine contrasts, the shift from blue to red was coupled with a reduction in the hydroxylation state of the anthocyanins (Table 2). In the ninth case, involving *P. kunthii*, whether there was a reduction depended on which neighbor species was used as a comparison. If *P. incertus*, *P. caespitosus*, or *P. confusus* were used as the paired blue-flowered species, then there was also a reduction in hydroxylation state. Only if *P. clevelandii* is used as the paired blue-flowered species, was there no change in

hydroxylation level. In none of the nine comparisons was there a change to a higher

hydroxylation state. The probability of this correlation occurring by chance is < 0.01.

It thus seems that in at least two major angiosperm taxa, evolutionary transitions

from blue to red flowers, usually associated with transitions from bee to bird pollination,

are highly correlated with changes in the class of pigment produced, although for both

species more detailed analyses that incorporate uncertainty in tree topology are

warranted. Our understanding of the anthocyanin biosynthetic pathway in turn indicates

that this change in pigment type is most likely due to inactivation of one or more of the

major branches of that pathway.

The anthocyanin pathway has three major branches (Fig. 1), leading to

pelargonidin-, cyanidin-, and delphinidin-based anthocyanins. The branching enzymes

F3'H and F3'5'H are responsible for adding the additional hydroxyl groups characteristic

of the longer branches. Which class(es) of anthocyanins is(are) produced depends on the

amount of flux down each of these branches. The ancestral state of many angiosperm

groups, and perhaps of angiosperms as a whole, is the production of blue-purple flowers

that produce primarily or exclusively delphinidin-based anthocyanins (Rausher 2006).

Mutational studies of model organisms such as *Antirrhinum*, *Petunia*, and *Ipomoea*

demonstrate, however, that when branching enzymes are inactivated, flux is often

redirected along one of the other branches, indicating that the enzymes in these branches

are often fully functional. For example, wild-type *Petunia* produce deep purple flowers

with delphinidin anthocyanins. Mutational inactivation of F3'5'H, however, results in

the production of redder flowers that produce primarily cyanidin anthocyanins (Griesbach 1996). In *Ipomoea*, all but the most basal blue species lack a functional delphinidin-producing branch and produce only cyanidin (Zufall 2003). In three of these species, mutations in the branching enzyme F3'H inactivate the cyanidin branch, which directs flux down the pelargonidin branch to produce red/pink flowers (Zufall and Rausher 2004; Hoshino et al 2003). A similar situation obtains in *Antirrhinum majus* (Stickland and Harrison1974). These observations suggest that in general, non-utilized pathway branches are potentially functional, and all that is needed to produce redder flowers is to inactivate the branch down which is currently associated with maximum flux.

The genetics of blue-red flower-color transition has been examined in *Ipomoea quamoclit* and supports the hypothesis that this transition is commonly accomplished by inactivating a branch of the anthocyanin pathway. In this species, *F3'H* is almost completely down-regulated and the enzyme DFR has become a substrate specialist, unable to process the precursor to cyanidin (Zufall and Rausher 2004). These two changes completely block flux down the cyanidin branch and redirect it down the pelargonidin branch.

Transitions often involve loss-of-function mutations

Available evidence indicates that many, if not most, flower-color transitions result from loss-of-function (LOF) mutations in pigment pathway genes. LOF mutations can be of two types: mutations in coding regions that abolish protein function, or mutations in cis-regulatory regions that reduce or eliminate protein expression. Extensive work on

spontaneous mutations in model organisms such as *Petunia*, *Antirrhinum*, and *Ipomoea* has demonstrated that LOF mutations in both structural and regulatory genes of the anthocyanin pathway typically result in either loss of pigmentation or change in color of the corolla (Holton and Cornish 1995; Mol et al 1998).

The genetic changes associated with floral color change in nature have been documented for nine taxa (Table 3; note that for *I. purpurea* there are three different color change polymorphisms). Four of the studies involve natural flower-color polymorphisms, and seven involve fixed changes within a species. In every case, one, or sometimes two, LOF mutations have been identified as causing the flower-color change. These mutations are roughly equally divided among structural genes and transcription factors, and between coding-region and cis-regulatory region mutations. One caveat is that in four of the species (*I. quamoclit*, *I. alba*, *I. ochracea*, and *Aquilegia spp.*), the genetic changes identified have not been shown by crosses to be the only changes influencing floral color. Consequently, they may not be the original changes that produced the flower-color transition. Instead, they may reflect subsequent degeneration of one or more branches of the pathway after the original mutation abolished function (Zufall and Rausher 2004). Even if this is the case, however, the documented changes provide indirect evidence that LOF mutations were responsible for flower-color change, since they would not have been fixed if the pathway had not been previously inactivated.

LOF mutations cause floral color change by either blocking branches of the pathway, forcing flux down other branches (e.g. blue-red transitions), or by blocking the

entire pathway so that no anthocyanins are produced (pigmented-nonpigmented

transitions). Once the branch or pathway has been inactivated, there is expected to be no

natural selection to maintain function in other elements of that branch or pathway.

Consequently, additional LOF mutations are expected to accumulate by genetic drift,

leading to "degeneration" of the branch or pathway. With the accumulation of more than

one LOF mutation, restoration of branch or pathway function is expected to be essentially

impossible, since it would require multiple simultaneous mutations restoring function

(Rausher 2006).

This type of degeneration has been reported for the blue-red transition in *Ipomoea*

quamoclit (Zufall and Rausher 2004; see above). Another possible example may be the

transition to white flowers in *Ipomoea alba*. In this species, both *CHS* and *DFR* are

markedly down-regulated, while *CHI* is not (Durbin et al. 1999). Because in all species

that have been examined, at least *CHS* and *CHI* are coordinately regulated by

transcription factors (Mol et al 1998), it seems unlikely that a single mutation in a

transcription factor is responsible for downregulaton of *CHS* and *DFR*. Instead, it seems

much more probable that either independent LOF mutations in the promoter region of

these two genes, or independent mutations in different transcription factors, have led to

the down-regulation of these two genes.

Causal relationship among trends

It is very likely that the three macroevolutionary trends identified above are

causally interrelated. In particular, the asymmetry of transitions from blue to red and

from pigmented to white can be explained by the observation that these transitions tend to involve LOF mutations. LOF changes are expected to be difficult to reverse for two reasons: (1) they often involve insertions or deletions of more than one base pair, and (2) they facilitate pathway degeneration (see above). Examples of the former include the *I. purpurea* mutations at all three loci and the *P. axillaris* mutant described in Table 3—all four of the cases for which we know the nucleotide changes responsible for LOF. Examples of pathway degeneration have been given previously.

While the predominance of LOF mutations can explain asymmetry in color transition rates, it cannot by itself explain a second apparent feature of floral color evolution: the tipness of transitions. Transitions tend to appear late in a phylogeny. There are at least two explanations for this pattern. The first is that ecological conditions favoring transitions became more common in more recent times. If this explanation is correct, then tipness reflects a temporal change in environments and selection regimes and a real change in the *rate* at which transitions occur. The second possible explanation is that lineages with non-ancestral flower color have a shorter time to extinction or lower speciation rate, perhaps because inactivation of major parts of the anthocyanin pathway allows less evolutionary flexibility. A similar explanation has been suggested for the concentration of self-compatible plant species at the tips of phylogenies (Igic et al 2003). If this explanation is correct, then the absence of deep nodes with a derived flower color results not from change in the rate of transitions, but from differential extinction of lineages in which transitions occur early. Although no information exists to decide

between these two explanations, both involve interesting macroevolutionary processes

that deserve further study.

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Table 1. Investigations examining either selection on, or pollinator discrimination among, floral color morphs in nature. Absence of entry in table indicates

relevant data not reported.

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Species	Colors compared	Fitness differences[1]	Divergent selection[2]	Differential pollinator visitation[3]	Pollinators impose selection?[4]	Pleiotropy suggested[5]	Refs

A.   Species exhibiting population-divergence in flower color

_____

Species	Colors compared	Fitness differences	Divergent selection	Differential pollinator visitation	Pollinators impose selection?	Pleiotropy suggested	Refs
Antirrhinum majus	red/yellow	cline	yes				Whibley et al. 2006
A. majus	yellow/white	sp; ss		D[6]			Jones and Reithel 2001
Aquilegia caerulia	blue/white			D			Miller 1981
A. formosa & A. pubescens[7]	dark/pale red	cline	yes				Hodges and Arnold 1994
Ipomopsis aggregata & I. tenuituba[7]	red/white	fp; sp; ss		D	yes		Anderson and Paige 2003 Melendez-Ackerman and Campbell 1998 Melendez-Ackerman et al 1997 Campbell et al 1997
Linanthus parryae	blue/white	cline; sp	yes	no		yes	Schemske and Bierzychudek 2001, unpublished
Mimulus aurantiacus	red/yellow	cline	yes	D			Streisfeld and Kohn 2005

Species	Color		Mode		Reference
Mimulus lewisii & M. cardinalis[7]	pink/orange		D		Bradsnaw and Schemske 2003 Schemske and Bradshaw 1999

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B. Species exhibiting within-population flower-color polymorphisms

_____

Species	Color		Mode		Reference
Phlox pilosa	pink/white	sp	I		Levin and Kerster 1967
P. drummondii	red/pink	sp	I		Levin 1969, 1972, 1985
Platystemon californicus	yellow/white	spf	D		Hannan 1982
Clarkia gracilis	purple/white	sp; ss			Jones 1996
Claytonia virginica	4 discrete colors	none (fp)			Frey 2004
Dactylorhyza sambucina	yellow/red	pr; sp; ss			Gigord et al. 2001 Pellegrino et al 2005
Delphinium nelsonii	blue/white	sp	D	yes	Waser and Price 1981, 1983
Hydrophyllum appendiculatum	blue/white	sp	no		Wolfe 1993
Iris fulva & I. breviaulis[7]	blue/red		D		Wesslingh and Arnold 2000
I. fulva & I. hexagona[7]	blue/red		D		Emms and Arnold 2000
Ipomoea purpurea	blue/white (*ww*)	ss; sm; fln	D	yes	Rausher et al. 1993 Rausher and Fry 1993

Fry and Rausher 1997

Species	Color morph	Selection	Pollinator visitation	Pollinator selection	Fitness non-pollinator	Reference
I.. purpurea	blue/white (*aa*)	v; sp			yes	Coberly 2003; Coberly and Rausher 2004
Linaria Canadensis	purple/blue	fls; frs				Wolfe and Sellers 1997
Linum pubescens	purple/yellow	none (fn; sp; sm)				Wolfe 2001
Lobelia maritime	purple/white	none (sp)				Gomez 2000
Phlox drummondii	red/white	v; sp			yes	Levin and Brack 1995
Raphanus raphinistrum	yellow/white	ss	D			Kay 1976; Stanton et al 1989
R. sativus	yellow/others	ss	I	yes		Irwin and Strauss 2005

[1] cline = selection deduced from comparison of color and neutral marker distributions; fln = flower number; fls = flower size; fn = fruit number; fp = fruit production; frs = fruit size; pr = pollen removal; sm = seed mass; sp = seed production; spf = seeds per fruit; ss = siring success; v = viability; none(x; y) = fitness components x and y examined but no differences detected between morphs.

[2] yes = experimental confirmation that different morphs favored in different areas

[3] D = direct observation indicates differential pollinator visitation; I = indirect evidence indicates differential pollinator visitation.

[4] yes = experimental confirmation that pollinators impose selection on color

[5] yes = evidence provided that fitness effects not due to interactions with pollinators

[6] experiments conducted in non-native habitat

[7] experiments conducted in or across hybrid zone

Table 2. Independent transitions between pairs of red hummingbird- and blue bee-
pollinated *Penstemon* species and corresponding transition in anthocyanidins
produced. All transitions are from bee to hummingbird pollination. See
supplemental material for additional information.

Bee-pollinated Species	Hummingbird-pollinated species	anthocyanidin transition[1]
Antirrhinum majus	*K. tenata*	1 → 3
P. barrettiae	*P. newbarryi*	1 → 3
P. anguineus group	*P. rostrifolius*	1 → 4 or 2 → 4
P. anguineus group	*P. lanceolatus*	1 → 5 or 2 → 5
P. linearioides	*P. hartwegii/ P. isophyllus*	2 → 5
P. incertus group	*P. kunthii*	1 → 3 or 2 → 3 or 3 → 3
P. speciosus	*P. labrosus*	2 → 5
P. thompsonii	*P. centranthifolius*	1 → 5
P. perpulcher group	*P. barbadus*	1 → 5

[1] 1 = Delphinidin; 2 = Delphinidin + Cyanidin; 3 = Cyanidin; 4 = Cyanidin +
Pelargonidin; 5 = Pelargonidin

Table 3. Species for which genes responsible for naturally occurring color-morph

differences have been identified.

Species	Locus	Gene[1]	phenotype	LOF Mutation	Ref
A. Within-population polymorphisms					
Antirrhinum	*Rosea*	*myb* (tf)	yellow[2]	?	Whibley et al 2006
Ipomoea purpurea	*W*	*Ipmyb1* (tf)	white	coding region deletions	Chang and Rausher 2005
Ipomoea purpurea	*A*	*Chs* (e)	white	coding region insertion	Habu et al 1998
Ipomoea purpurea	*P*	*F3'H* (e)	pink	coding region deletion	Zufall and Rausher 2003
B. Fixed change within a species					
Antirrhinum (5 species)	*Rosea*	*myb* (tf)	white	?	Schwinn et al 2006
Aquilegia (*longissima* clade)		*Dfr* (e),	white	down-regulation	Whitall et al 2006
Ipomoea alba		*Chs* (e), *Dfr* (e)	white	down-regulation	Durbin et al 2003
Ipomoea ochracea		*Dfr* (e), *Chi* (e)	white	down-regulation	Durbin et al 2003
Ipomoea quamoclit		*F3'H* (e), *DFR* (e)	red	down-regulation	Zufall and Rausher 2004

Mimulus aurantiacus	*DFR* (e), *myb* (tf)	yellow[2]	down-regulation	Streisfeld and Rausher (unpublished)
Petunia axillaris	*An2* *myb* (tf)	white	coding region frameshift	Quattrocchio et al 1999

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[1] e = codes for enzyme; tf = codes for transcription factor

[2] anthocyanins absent

Figure 1. Schematic diagram of anthocyanin pathway. Each of the three branches of the

pathway leading to different classes of anthocyanins is colored differently.

Branches leading to other types of flavonoids are not colored. Specific

compounds are listed in lower case. Classes of compounds are listed in bold

upper case. Enzymes are listed in upper-case. *STS*: stilbene synthase. *CHS*:

chalcone synthase. *CHI*: chalcone-flavanone isomerase. *F3H*: flavanone-3-

hydroxylase. *DFR*: dihydroflavonol 4-reductase. *ANS*: anthocyanidin

synthase. *UF3GT*: UDP flavonoid glucosyltransferase. *F3'H*: flavonoid 3'

hydroxylase. *F3'5'H*: flavonoid 3'5' hydroxylase. *LCR*: leucoanthocyanidins

reductase. *FS*: flavone synthase. *FLS*: flavonol synthase. *IFS*: isoflavone

synthase. *AUS*: aureusidin synthase. DH="dihydro". L="leuco".
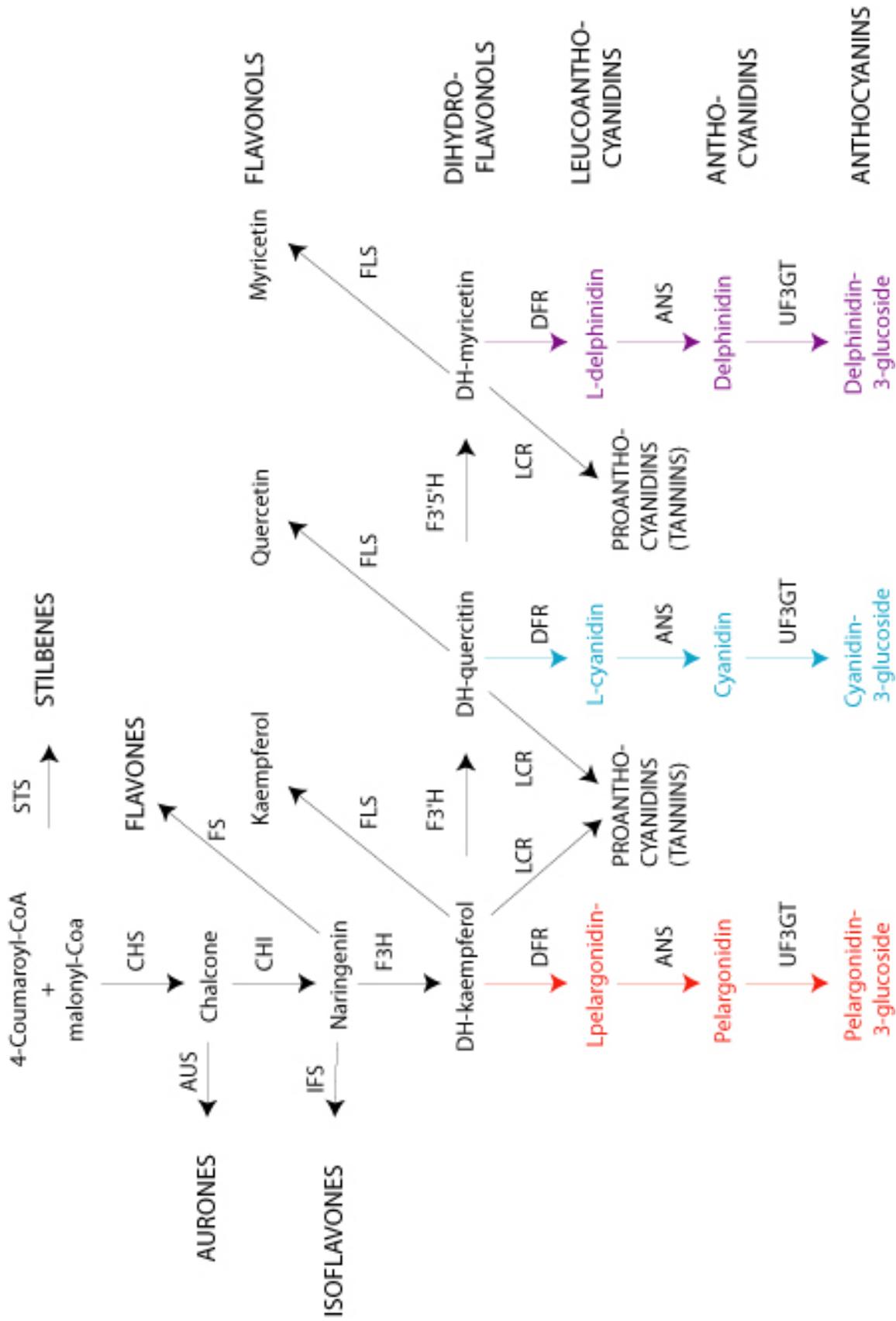
Supplemental Material

To examine the correlation between change in flower color and change in anthocyanidin pigment class in *Penstemon*, I began with the *trnC-D/T-L* concensus tree for *Penstemon* and *Keckiella* (with *Antirrhinum majus* as outgroup) reported by Wolfe et al (2006). This tree is the concensus of 2000 equally parsimonious trees. Floral color states were taken from Wilson et al (2004, 2006) and personal observation. Blue, violet, and purple flowers were considered "blue" in this analysis. All species of these colors are visited primarily by bees (Wilson et al 2004). Floral anthocyanidin content was taken from Scogin and Freeman (1987) and unpublished data from our laboratory.

Nine independent transitions from blue to red flowers were identified based on this phylogeny and the information presented in Wilson et al (2004 and unpublished ms). The red-flowered species involved in this transition was then paired with its most closely related blue-flowered sister species, and the anthocyanidin content of the two species were compared. In some cases (e.g. *P. kunthii*), there were multiple blue-flowered sister species that could have been used for the comparison because of lack of phylogenetic resolution. In these cases, all possible comparisons were examined. In all cases of this type, the multiple comparisons gave consistent results. *P. rostriflorus* and *P. lanceolatus* were compared to the same blue species (the four species in the clade with *P. anguineus*). Although technically this makes these two comparisons non-independent, the two transitions most likely represent two independent transitions to red (Wilson et al

unpublished), as evidenced by the presence of the blue-flowered *P. lentus* separating

these two red-flowered species (broken blue line in Supplemental Fig. 1).

Anthocyanidn content was coded 1 through 5, with increasing numbers

corresponding to decreasing average hydroxylation state. States 1, 3 and 5 correspond to

the production of one major anthocyanidin (delphinidin, cyanidin, and pelargonidin,

respectively), while states 2 and 4 correspond to two major anthocyanidin components

(delphinidin + cyanidin and cyanidin + pelargonidin, respectively). Transitions between

states for the paired red and blue species are reported in Table 2 as transitions from the

anthocyanidin state of the blue-flowered species to the anthocyanidin state of the red-

flowered species.

Supplemental Fig. 1.   *trnC-D/T-L* concensus tree for *Penstemon* and *Keckiella* (with

Antirrhinum majus as outgroup) from Wolfe et al (2006), pruned to include only species

(except *P. lentus*) for which both floral color and floral anthocyanidins are known.

Branch colors: Blue = blue/purple flowers that are bee pollinated; Red = red/orange

flowers that are hummingbird pollinated. Pigments: 1 = Delphinidin; 2 = Delphinidin +

Cyanidin; 3 = Cyanidin; 4 = Cyanidin + Pelargonidin; 5 = Pelargonidin. Broken line for

P. lentus indicates no anthocyanidin data available for this species.

Pigments	Species
1	Antirrhinum majus
3	K. ternata
1	P. borettiae
3	P. newberryi
1	P. albertinus
2	P. attenuatus
3	P. caesius
1	P. whippleanus
2	P. watsonii
1	P. humilis
2	P. scapioides
1	P. leonardii
1	P. wilcoxii
2	P. anguineus
1	P. globosus
2	P. azureus
2	P. heterophyllus
4	P. rostriflorus
-	P. lentus
5	P. lanceolatus
2	P. incertus
3	P. clevelandii
2	P. caespitosus
3	P. kunthii
1	P. confusus
5	P. hartwegii
5	P. isophyllus
2	P. linarioides
2	P. dolius
1	P. fremontii
2	P. speciosus
5	P. labrosus
1	P. dasyphyllus
1	P. thompsonii
5	P. centranthifolius
1	P. perpulcher
1	P. neomexicanus
5	P. barbatus
1	P. alpinus
3	P. palmeri
3	P. grinelli