

EVOLUTIONARY TRANSITIONS IN FLORAL COLOR

Mark D. Rausher¹

Department of Biology, Duke University, Durham, North Carolina 27708, U.S.A.

The tremendous diversity in flower color among angiosperms implies that there have been numerous evolutionary transitions in this character. The conventional wisdom is that a large proportion of these transitions reflect adaptation to novel pollinator regimes. By contrast, recent research suggests that many of these transitions may instead have been driven by selection imposed by nonpollinator agents of selection acting 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 no definitive examples of flower color evolution conforming to either hypothesis. I also document four macroevolutionary trends in flower color evolution: color transitions rates are often asymmetrical; biases favoring loss of pigmentation or favoring gain of pigmentation are both observed, but bias favoring transition from blue to red flowers seems more common than the reverse bias; transitions from blue to red often involve inactivation of branches of the anthocyanin pathway; and color transitions often involve loss-of-function mutations. Finally, I discuss how these trends may be related to one another.

Keywords: floral evolution, anthocyanin, natural selection, pollination biology, pollinator choice, pleiotropy.

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 often correlated with other floral traits, resulting in the common recognition of “pollination syndromes” (Faegri and van der Pijl 1966; 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, a proposition I call the “conventional wisdom” (Faegri and van der Pijl 1966; Grant 1993; Fenster et al. 2004).

In the first part of this article, 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 alternative interpretations, including the possibilities that many flower color transitions are nonadaptive 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 article, I 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 (for historical review, see Fenster et al. 2004). The primary evidence supporting this contention is the existence of “pollination syndromes,” groups of floral traits that occur together typically in plants pollinated by a particular agent. Examples include (1) bird-pollinated flowers, which are typically red or orange and have elongated floral tubes, reduced floral limbs, exerted stigmas, and copious dilute nectar; (2) bee-pollinated flowers, which are typically blue or purple and have short, wide tubes, wide limbs, inserted stigmas, and small amounts of concentrated nectar; and (3) moth-pollinated flowers, which are typically white and fragrant, have long floral tubes, and open at night.

Although the generality of floral syndromes has been questioned (Robertson 1928; Ollerton 1996, 1998; Waser et al. 1996) 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 most effectively 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 pollination 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, even if the change is deleterious with

¹ E-mail: mrausher@duke.edu.

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 pollination syndromes with different flower colors to occur without direct selection on flower color by pollinators. Consequently, no matter how common pollination 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 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 is currently believed.

For information about selection on and pollinator responses to flower color variation, I conducted a literature search on the Web of Science (keywords used were “flower color variation,” “flower color evolution,” “selection on flower color,” and “flower color polymorphism”). 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. Nine 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 involved within-population color polymorphisms (table 1B). Although this search cannot claim to be exhaustive, the investigations it turned up are likely to be representative of studies that have examined selection on and pollinator discrimination among flower color variants.

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.

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. In only two of the investigations listed in table 1 was any attempt made to randomize or otherwise account for the effects of the genetic background (the two different polymorphisms in *I. purpurea*), and even in these investigations, associations between the focal polymorphism and moderately linked loci were probably not disrupted. 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 experimentally, selection is measured for only 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. It should be noted that the four studies that infer fitness differences by comparing distributions of color morphs along a transect with distribution of neutral markers (“cline” in column 3 of table 1) do not suffer from this bias because the approach implicitly considers all components of fitness.

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 are 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 they also do 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 definitive, 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 that available evidence suggests that color transitions are often adaptive, there is little definitive evidence that pollinators are driving that adaptation. It is certainly true that in most cases that have been investigated (13 of 15 species in table 1),

Table 1

Investigations Examining either Selection on or Pollinator Discrimination among Floral Color Morphs in Nature

Species	Colors compared	Fitness differences	Divergent selection	Differential pollinator visitation	Pollinators impose selection?	Pleiotropy suggested	References
A. Cases involving population divergence or divergence between closely related species in flower color:							
<i>Antirrhinum majus</i>	Red/yellow	Cline	Yes	Whibley et al. 2006
<i>Antirrhinum majus</i>	Yellow/white	sp, ss	...	D ^a	Jones and Reithel 2001
<i>Aquilegia caerulea</i>	Blue/white	D	...	Miller 1981
<i>Aquilegia formosa</i> , <i>Aquilegia pubescens</i> ^b	Dark/pale red	Cline	Yes	Hodges and Arnold 1994
<i>Ipomopsis aggregata</i> , <i>Ipomopsis tenuituba</i> ^b	Red/white	fp, sp, ss	...	D	Yes	...	Campbell et al. 1997; Melendez-Ackerman et al. 1997; Melendez-Ackerman and Campbell 1998; Anderson and Paige 2003
<i>Iris fulva</i> , <i>Iris brevicaulis</i> ^b	Blue/red	D	Wesselingh and Arnold 2000
<i>I. fulva</i> , <i>Iris hexagona</i> ^b	Blue/red	D	Emms and Arnold 2000
<i>Linanthus parryae</i>	Blue/white	Cline, sp	Yes	No	...	Yes	Schemske and Bierzychudek 2001, forthcoming
<i>Mimulus aurantiacus</i> <i>Mimulus lewisii</i> ,	Red/yellow	Cline	Yes	D	Streisfeld and Kohn 2005
<i>Mimulus cardinalis</i> ^b	Pink/orange	D	Schemske and Bradshaw 1999; Bradshaw and Schemske 2003
B. Cases involving within-population flower color polymorphisms:							
<i>Phlox pilosa</i>	Pink/white	sp	...	I	Levin and Kerster 1967
<i>Phlox drummondii</i>	Red/pink	sp	...	I	Levin 1969, 1972, 1985
<i>Platystemon californicus</i>	Yellow/white	spf	...	D	Hannan 1981
<i>Clarkia gracilis</i>	Purple/white	sp, ss	Jones 1996
<i>Claytonia virginica</i>	Four discrete colors	None (fp)	Frey 2004
<i>Dactylorhiza sambucina</i>	Yellow/red	pr, sp, ss	Gigord et al. 2001; Pellegrina et al. 2005
<i>Delphinium nelsonii</i>	Blue/white	sp	...	D	Yes	...	Waser and Price 1981, 1993
<i>Hydrophyllum appendiculatum</i>	Blue/white	sp	...	No	Wolfe 1993
<i>Ipomoea purpurea</i>	Blue/white (<i>ww</i>)	ss, sm, fln	...	D	...	Yes	Rausher and Fry 1993; Rausher et al. 1993; Fry and Rausher 1997
<i>I. purpurea</i>	Blue/white (<i>aa</i>)	v, sp	Yes	Coberly 2003; Coberly and Rausher 2003
<i>Linaria canadensis</i>	Purple/blue	fls, frs	Wolfe and Sellers 1997
<i>Linum pubescens</i>	Purple/yellow	None (fn, sp, sm)	Wolfe 2001
<i>Lobelia maritima</i>	Purple/white	None (sp)	Gomez 2000
<i>Phlox drummondii</i>	Red/white	v, sp	Yes	Levin and Brack 1995
<i>Raphanus raphanistrum</i>	Yellow/white	ss	...	D	Kay 1976; Stanton et al. 1989
<i>Raphanus sativus</i>	Yellow/others	ss	...	I	Yes	...	Irwin and Strauss 2005

Note. Ellipsis in table indicates relevant data not reported. For fitness differences, 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. For divergent selection, yes = experimental confirmation that different morphs favored in different areas. For differential pollinator visitation, D = direct observation indicates differential pollinator visitation; I = indirect evidence indicates differential pollinator visitation. For whether pollinators impose selection, yes = experimental confirmation that pollinators impose selection on color. For pleiotropy suggested, yes = evidence provided that fitness effects not due to interactions with pollinators.

^a Experiments conducted in nonnative habitat.

^b Experiments conducted in or across hybrid zone.

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. For example, consider a situation in which individual pollinators specialize on one flower color morph or the other, so that there is little pollinator movement between morphs. Even though there may be an inherent tendency to specialize on one morph, so that more individual pollinators visit that morph, this differential visitation will not cause differences in either male or female fitness of the two morphs, as long as seed production is not pollen limited. Therefore, simply demonstrating that pollinators discriminate among morphs is insufficient for concluding that 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 that show evidence of pollinator discrimination, only three have been investigated in this way. Irwin and Strauss (2005) 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 haplotype 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 *Delphinium nelsonii*, compared to blue-flowered individuals, had reduced seed production when pollination was by natural pollinators. By contrast, when plants were hand pollinated, there was no difference in seed production. Moreover, the observation that pollinators visited white-flowered plants at a lower rate suggests that the difference in seed production is due to greater pollinator limitation in the whites, caused by pollinator discrimination. This study convincingly demonstrates that pollinator preferences cause differences in the female component of fitness between the floral color morphs.

Finally, Melendez-Ackerman and Campbell (1998) examined a hybrid zone between *Ipomopsis aggregata* and *Ipomopsis tenuituba*, in which red-flowered *I. aggregata* had higher seed production and outcross male success than did 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 did white flowers. In another experiment, when flowers of the two pure species and the hybrids were painted red, differences in both visitation rates and fitness were almost completely eliminated. It should be noted, though, that selection by pollinators has not been demonstrated outside the hybrid zone; it is therefore unclear that

the selection documented in this study is responsible for flower color divergence between the two species.

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 intrapopulation 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. Information from these species may be misleading regarding the validity of the conventional wisdom that flower color transitions reflect adaptation to novel pollinators 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 are presumed to generate evolutionary transitions in flower color. 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 on the basis of divergence in the frequencies of neutral genetic markers (e.g., Spitze 1993; Storz 2002). Evidence of this type has been reported for four species (*A. majus*, *Linanthus parryae*, *Mimulus aurantiacus*, and a hybrid zone of *Aquilegia formosa* and *Aquilegia 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 when measured in a single population or hybrid zone (table 1A). However, none of the studies reporting fitness differences conducted reciprocal transplants, so it is not known whether selection is divergent, as opposed to, say, purifying. Moreover, in only one species (*I. 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.

Color Transitions Not due to Selection by Pollinators

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, Wright (1943) argued that this variation is selectively neutral and that even extreme divergence in gene frequencies between populations was caused by genetic drift. By contrast, subsequent observations and experiments led Epling et al. (1960) to conclude that this variation was subject to selection, but Wright (1978) continued to disagree. Only recently has this debate apparently been resolved in Epling's favor by Schemske and Bierzychudek (2001, forthcoming), who have demonstrated that neutral markers do not exhibit the same clines as seen in flower color frequencies and in the higher fitness of the local morph in reciprocal transplant experiments.

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

Many of the enzymes involved in anthocyanin synthesis are also required for the synthesis of other flavonoid compounds (fig. 1). Noting that these compounds in turn influence ecological and physiological traits in addition to flower color 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 survival, flower production, seed production, or biomass for 10 species, with flower color polymorphisms and differences in damage by natural

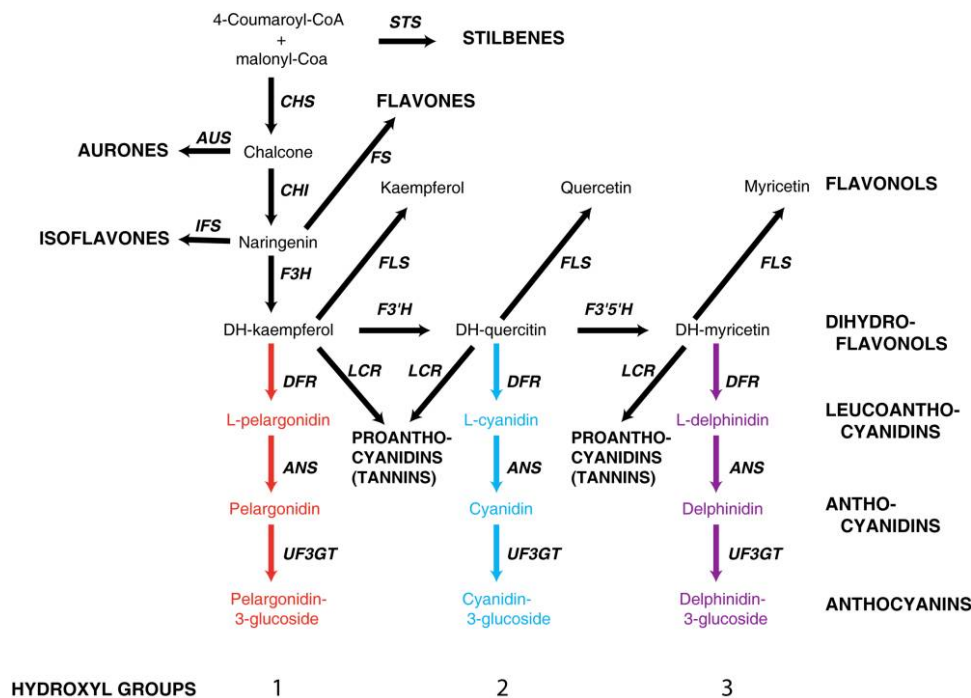


Fig. 1 Schematic diagram of anthocyanin pathway. Each of the three branches of the pathway leading to different classes of anthocyanins is colored differently. Branches leading to other types of flavonoids are not colored. Specific compounds are listed in lowercase. Classes of compounds are listed in bold uppercase. Enzymes are listed in uppercase. *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. Hydroxylation state is the number of hydroxyl groups on the beta ring of the anthocyanidin.

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 do 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. At least two 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 nonexistent. Second, it cannot be ruled out that fitness differences were not caused by pollinators in many of the cases reported by Strauss and Whittall.

The most convincing example of selection on pleiotropic effects from Strauss and Whittall (2006) is represented by Levin and Brack (1995), who showed that color morphs of *Phlox drummondii* 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. Also, in *L. parryae*, extensive pollinator observations (Schemske and Bierzychudek 2001) indicated no pollinator discrimination among morphs, which makes it difficult to believe that the selection on flower color documented by Schemske and Bierzychudek (2001, forthcoming) was imposed by pollinators, although pollinator-imposed selection was not directly examined.

In the remaining studies listed by Strauss and Whittall, fitness traits for which differences among morphs were reported were 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 drought 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 that pollinators did not impose the fitness differences observed in Warren and Mackenzie's investigation, albeit on a possible pleiotropic effect of flower color variation.

Finally, the three intriguing studies demonstrating differential susceptibility to natural enemies reported by Strauss and Whittall (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 because 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. The issue is not whether loci affecting flower color also have pleiotropic effects; in a variety of species, flower color variants also influence characters such as trichome density, root hair formation, cell morphology, and vacuolar size (Walker et al. 1999; Spelt et al. 2002; Morita et al. 2006), any of which could, in turn, affect fitness. Rather, the issue is that there is little evidence indicating that these or other pleiotropic effects are subject to selection. 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

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 derived characters (see Ackermann and Weigend 2006; also see Thomson and Wilson 2008). By contrast, in these taxa, 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 four general macroevolutionary trends in flower color evolution that the literature suggests are relatively common: (1) floral color transition rates are often unequal; (2) in general, transitions from blue to red flowers, and from pigmented to white flowers, are more common than the reverse transitions, although some taxa exhibit the reverse pattern; (3) 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; and (4) transitions from blue to red flowers, and from pigmented to white flowers, typically involve loss-of-function (LOF) mutations.

I then describe how these four 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). Because diversification and extinction differences for taxa with different character states can confound estimates of transition rates (Nosil and Mooers 2005; Maddison 2006), such analyses should ideally jointly estimate both diversification/extinction rates and transition rates. Techniques for doing this are still in their infancy, with the first method being published only now (Maddison et al., forthcoming). In the absence of this approach, character state reconstruction can be used to assess the reasonableness of patterns deduced from analysis of only transition rates (Nosil and Mooers 2005).

Despite potential pitfalls of transition state analysis, one study has used this approach successfully. 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 strongly suggest more frequent flower color transitions in one direction but fail either to estimate actual transition rates or to sample multiple trees. Using a single tree, Whittall 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*, P. Wilson, A. D. Wolfe, W. S. Armbruster, and J. D. Thomson (unpublished manuscript) 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 21) blue/purple to red transitions and no convincing reverse transitions (see also Thomson and Wilson 2008).

When we use less rigorous criteria, several other taxa appear to exhibit biased transition rates. 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, *Ipomoea horsfalliae*). By contrast, no reverse transitions of either type were inferred. In the Sinningieae, white hawkmoth- or bat-pollinated flowers have evolved independently five times, with no instances of regain of pigmentation (Perret et al. 2003). And in *Iochroma*, floral anthocyanins have been lost five times, with only one regain (Smith and Baum, forthcoming). Moreover, an analysis using 100 Markov chain Monte Carlo-generated trees shows that transition rates to white are substantially higher than the reverse transition rate (S. D. Smith and M. D. Rausher, unpublished data).

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 tend to arise from pigmented clades and have short branch lengths. 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), in which red-flowered, hummingbird-pollinated lineages tend to be confined to the tips, and in the phylogenies of *Sinningia* reported by Perret et al. (2003), in which white-flowered, hawkmoth- and hummingbird-pollinated lineages appear to be confined to the tips. Unfortunately, although this pattern may have implications for evolutionary potential, it has not been verified statistically by any analysis, largely because the appropriate methodology is only now appearing (e.g., Maddison et al., forthcoming).

Directions of Asymmetric Transition

I have documented several taxa in which color transitions between pigmented and white were asymmetric. Although the sample size is small, four taxa (*Ipomoea*, *Sinningia*, *Iochroma*, and *Aquilegia*) appear to show greater rates of transition from pigmented to white flowers, while only one taxon (*Costus*) exhibits the reverse pattern. In addition, in both *Dalechampia* and *Acer*, there have been three independent gains of pigmentation and no losses (Armbruster 2002), although transition rates have not been compared statistically for these species. It thus appears that in different taxa, the favored direction of transition between pigmented and unpigmented may differ.

While I am unaware of any taxa in which the transition rate from red to blue/purple is greater than the reverse transition rate, a number of taxa exhibit the opposite pattern. As described above, in *Penstemon* and *Ipomoea*, there are numerous transitions from blue to red but no reversals. The same appears to be true in the Antirrhineae (three transitions from blue to red and no reversals; Ghebrehiwet et al. 2000). By contrast, in *Sinningia*, transitions from red to blue appear to be as common as the reverse transitions. Again, although the sample size is small, there appears to be a trend for the transition from blue to red to be favored, although reverse transitions do occur.

Pigment Changes in Blue-Red Transitions

The most common and widely distributed 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 (fig. 1). 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 having 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 suggest 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 exist, the pigments shift from entirely cyanidin to entirely pelargonidin anthocyanins (Zufall 2003). In a fifth species (*Ipomoea 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 exerted anthers and stig-

mas; 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 demonstrate that 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 (1987) conclusion. The *trnC-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 appendix). This pruned tree is presented as figure A1 in the appendix. Wilson et al. (2004; P. Wilson, A. D. Wolfe, W. S. Armbruster, J. D. Thomson, unpublished manuscript) 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 of sister groups were performed (table 2; for details, see appendix). 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 seven 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 two other cases, whether there was a reduction depended on which neighbor species was used as a comparison.

For *Penstemon kunthii*, if *Penstemon incertus*, *Penstemon caespitosus*, or *Penstemon confusus* was used as the paired blue-flowered species, then there was also a reduction in hydroxylation state. Only if *Penstemon clevelandii* was used as

Table 2

Correlation between Transition from Hummingbird-Pollinated Red and Bee-Pollinated Blue *Penstemon* Lineages and Corresponding Transition in Anthocyanidins Produced

Bee-pollinated species	Hummingbird-pollinated species	Anthocyanidin transition
<i>Antirrhinum majus</i>	<i>Keckiella ternata</i>	1 → 3, 2 → 3, or 3 → 3
<i>P. barrettiae</i>	<i>P. newberryi</i>	1 → 3
<i>P. incertus</i> group	<i>P. rostriflorus</i>	1 → 4, 2 → 4, or 3 → 3
<i>P. incertus</i> group	<i>P. lanceolatus</i>	1 → 5, 2 → 5, or 3 → 3
<i>P. linarioides</i> and <i>P. incertus</i> groups	<i>P. hartwegii</i> / <i>P. isophyllus</i>	1 → 5, 2 → 5, or 3 → 5
<i>P. incertus</i> group	<i>P. kunthii</i>	1 → 3, 2 → 3, or 3 → 3
<i>P. speciosus</i>	<i>P. labrosus</i>	2 → 5
<i>P. thompsonii</i>	<i>P. centranthifolius</i>	1 → 5
<i>P. perpulcher</i> group	<i>P. barbatus</i>	1 → 5

Note. All transitions are from bee to hummingbird pollination. See appendix for additional information. For anthocyanidin transition, 1 = delphinidin; 2 = delphinidin + cyanidin; 3 = cyanidin; 4 = cyanidin + pelargonidin; 5 = pelargonidin.

the paired blue-flowered species was there no change in hydroxylation level. For *Keckiella ternata*, comparison to any of eight blue-flowered species in the *Penstemon albertinus* group indicated a reduction in hydroxylation state; only when compared to the ninth species, *Penstemon caesius*, was there no change in hydroxylation state. Effectively, these results mean that in eight or nine of the nine comparisons, there was a decrease in hydroxylation state, and 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*, the delphinidin-producing branch of the pathway was inactivated early in the diversification of the genus, so that most blue species 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 (Hoshino et al. 2003; Zufall and Rausher 2004). A similar situation obtains in *Antirrhinum majus* (Stickland and Harrison 1974). These observations suggest that, in general, nonutilized pathway branches are potentially functional and all that is needed to produce redder flowers is inactivating the branch that 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 downregulated, 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. The correlation between

red flowers and the reduced hydroxylation state described above for *Penstemon* also indicates that branches of the pathway have repeatedly been inactivated in the transition to red flowers, although the genes involved have not been described.

Transitions Often Involve LOF Mutations

Available evidence indicates that many, if not most, flower color transitions result from LOF mutations in pigment pathway genes. LOF mutations can be of two types: mutations in coding regions that abolish enzyme function or mutations in regulatory regions (including coding regions of transcription factors) 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 eight taxa (table 3; note that for *Ipomoea 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, except *Mimulus aurantiacus*, one or sometimes two LOF mutations have been identified as causing the flower color change. (In *M. aurantiacus*, the direction of the color change has not been determined, so it is not known whether the change was a gain or a loss of function.) 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 taxa (*I. quamoclit*, *Ipomoea alba*, *Ipomoea 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 because they would not have been fixed if the pathway had not been previously inactivated.

LOF mutations cause floral color change either by 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 because it would require multiple simultaneous mutations restoring function (Rausher 2006).

This type of degeneration has been reported for the blue-red transition in *I. quamoclit* (Zufall and Rausher 2004). Another possible example may be the transition to white flowers

Table 3

Species for Which Genes Responsible for Naturally Occurring Color Morph Differences Have Been Identified

Species	Locus	Gene	Phenotype	LOF mutation	References
A. Within-population polymorphisms:					
<i>Antirrhinum</i>	<i>Rosea</i>	<i>myb</i> (tf)	Yellow ^a	?	Whibley et al. 2006
<i>Ipomoea purpurea</i>	<i>W</i>	<i>Ipmyb1</i> (tf)	White	Coding region deletions	Chang et al. 2005
<i>I. purpurea</i>	<i>A</i>	<i>CHS</i> (e)	White	Coding region insertion	Habu et al. 1998
<i>I. purpurea</i>	<i>P</i>	<i>F3'H</i> (e)	Pink	Coding region deletion	Zufall and Rausher 2004
B. Fixed change within a species:					
<i>Antirrhinum</i> (five species)	<i>Rosea</i>	<i>myb</i> (tf)	White	?	Schwinn et al. 2006
<i>Aquilegia</i> (<i>chrysantha</i> clade)	...	<i>DFR</i> (e)	Yellow	Downregulation	Whittall et al. 2006
<i>Ipomoea alba</i>	...	<i>CHS</i> (e), <i>DFR</i> (e)	White	Downregulation	Durbin et al. 2003
<i>Ipomoea ochracea</i>	...	<i>DFR</i> (e), <i>CHI</i> (e)	White	Downregulation	Durbin et al. 2003
<i>Ipomoea quamoclit</i>	...	<i>F3'H</i> (e), <i>DFR</i> (e)	Red	Downregulation	Zufall and Rausher 2004
<i>Mimulus aurantiacus</i>	...	<i>DFR</i> (e), <i>myb</i> (tf)	Yellow ^a	Downregulation	M. A. Streisfeld and M. D. Rausher, unpublished manuscript
<i>Petunia axillaris</i>	<i>An2</i>	<i>myb</i> (tf)	White	Coding region frameshift	Quattrocchio et al. 1999

Note. Ellipsis in table indicates relevant data not reported. LOF = loss of function. For gene, e = codes for enzyme; tf = codes for transcription factor.

^a Anthocyanins absent.

in *I. alba*. In this species, both *CHS* and *DFR* are markedly downregulated, while *CHI* is not (Durbin et al. 2003). Because at least *CHS* and *CHI* are coordinately regulated by transcription factors (Mol et al. 1998) in all species that have been examined, it seems unlikely that a single mutation in a transcription factor is responsible for downregulation 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 downregulation of these two genes.

Causal Relationship among Trends

It is very likely that the four macroevolutionary trends identified above are causally interrelated. In particular, the asymmetry of transitions from blue to red and from pigmented to white seen in many taxa 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. First, they often involve coding region insertions or deletions of more than one base pair that cause frame shifts or premature termination, often as a result of imprecise transposon excision. Simple point mutations will be unable to reverse such changes. Moreover, once these knockout mutations have arisen, subsequent mutations that would be deleterious in a functional gene product are free to accumulate by genetic drift, rendering reversal even less likely. Second, inactivation of one gene in the pathway facilitates pathway degeneration, which again would presumably make reversal virtually impossible because it would require multiple simultaneous mutations. Examples of the former type include the *I. purpurea* mutations at all three loci and the *Petunia 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.

Although loss of floral pigments seems to occur at rates higher than those for regain of color in many taxa, it is clear that reversals occur, even frequently in some taxa (e.g., *Cos-*

tus, *Dalechampia*). Although no cases of the regain of pigmentation have been examined at the molecular level, these reversals most likely occur when the original LOF mutation is easily reversible. Cis-regulatory mutations that greatly reduce the expression of an anthocyanin structural gene or transcription factor are the most probable candidates for reversal because lost promoter binding sites can be recovered by a variety of mutational processes (Wray et al. 2003). Moreover, it seems probable that gene and pathway degeneration are less likely for LOF cis-regulatory mutants than for coding sequence mutants. Anthocyanin genes often exhibit differential expression patterns in different tissues, implying that expression in different tissues is controlled by different cis-regulatory elements. LOF mutations that abolish floral pigments will thus frequently not abolish anthocyanin production in vegetative tissues. Because anthocyanins and other flavonoids in vegetative tissues serve many ecologically and physiologically important functions (Shirley 1996), selection will tend to prevent degeneration of anthocyanin structural genes. This argument thus suggests that although there are no documented cases of cis-regulatory mutants causing changes in floral pigment color, we may have been looking in the wrong taxa: instead of taxa showing transition rates that are biased in favor of LOF, we should perhaps be examining taxa in which floral pigments have been regained multiple times.

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, whereby 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 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 nonancestral flower color have a shorter time to extinction or lower speciation rate (Maddison 2006), perhaps because inactivation of major parts of the

anthocyanin pathway allows less evolutionary flexibility. For example, shifts from blue to red associated with shifts to hummingbird pollination, as well as shifts from pigmented to white associated with moth or bat pollination, may involve a transition to increased pollinator specialization, which could render the lineage more susceptible to extinction. Alternatively, pleiotropic effects, such as increased susceptibility to herbivory, that have been ascribed to floral pigment change could also increase the probability of extinction of white-flowered lineages.

Differential rates of speciation and extinction among lineages with different characteristics have also been suggested for the concentration of self-compatible plant lineages at the tips of phylogenies (Igic et al. 2003; see Igic et al. 2008). 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.

Acknowledgments

I thank three anonymous reviewers for constructive comments on the manuscript. This article was written while I was a Triangle Sabbatical Fellow at the National Evolutionary Synthesis Center. Support was provided by National Science Foundation grant DEB-0448889.

Appendix

Correlation between Flower Color Change and Pigment Change in *Penstemon*

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* consensus tree for *Penstemon* and *Keckiella* (with *Antirrhinum majus* as outgroup) reported by Wolfe et al. (2006). This tree is the consensus of 2000 equally parsimonious trees. Floral color states were taken from work by 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 work by 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 by Wilson et al. (2004; P. Wilson, A. D. Wolfe, W. S. Armbruster, and J. D. Thomson, unpublished manuscript). The red-flowered species involved in this transition was then paired with a blue-flowered sister species or species group, and the anthocyanidin contents of the two species were compared. In some cases (e.g., *Penstemon kunthii*), there were multiple blue-flowered sister species that could have been used for the comparison because of lack of phylogenetic reso-

lution. In these cases, all possible comparisons were examined. In all cases of this type, the multiple comparisons gave consistent results. *Penstemon kunthii*, *Penstemon rostriflorus*, and *Penstemon lanceolatus* were compared to the same blue species (the four species in the clade with *Penstemon anguineus*). Although technically this makes these three comparisons non-independent, they most likely represent three independent transitions to red (P. Wilson, A. D. Wolfe, W. S. Armbruster, and J. D. Thomson, unpublished manuscript), as evidenced by the presence of the blue-flowered *Penstemon lentus* separating these two red-flowered species (broken blue line in fig. A1).

Anthocyanidin 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.

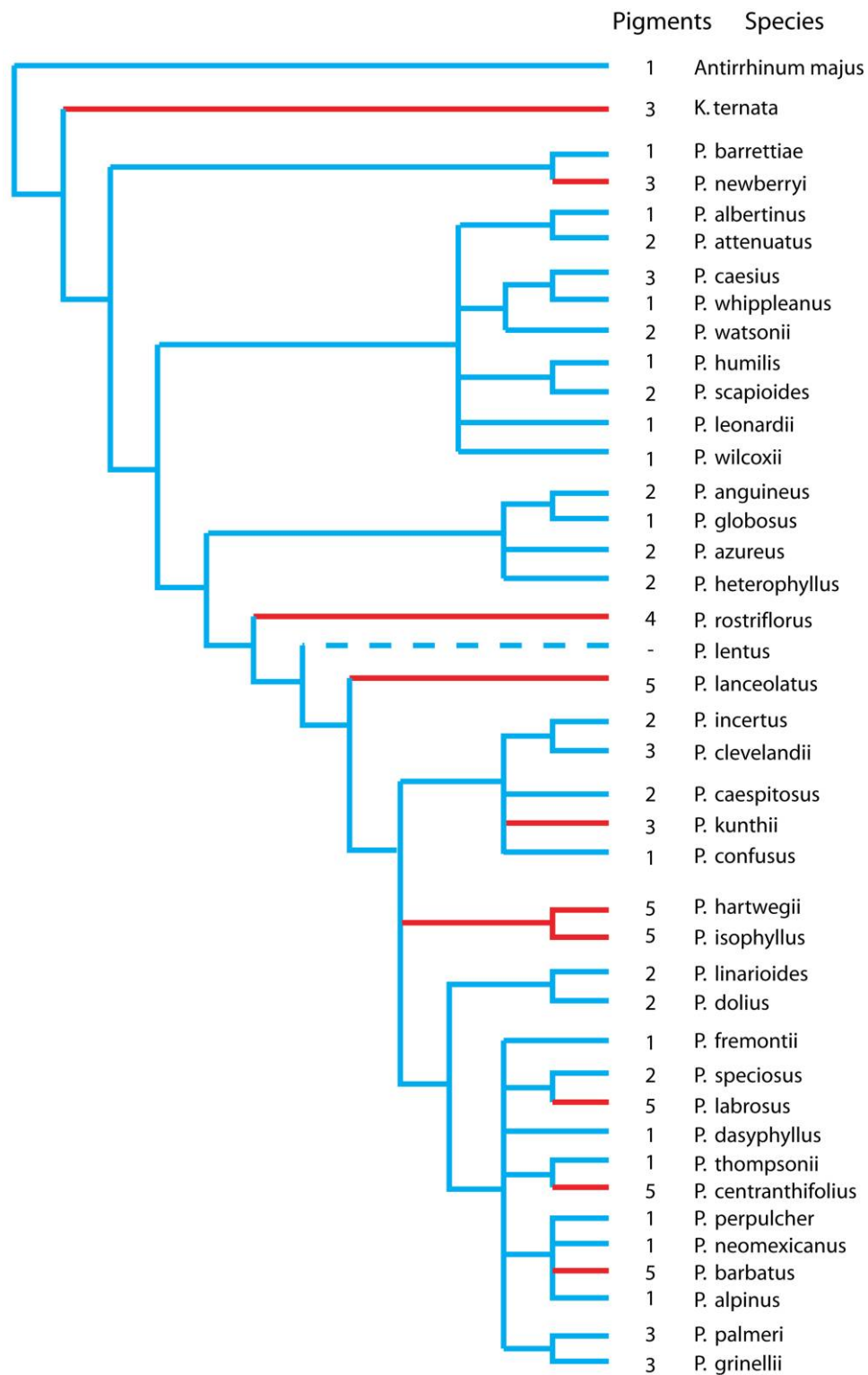


Fig. A1 The *trnC-D/T-L* consensus tree for *Penstemon* and *Keckiella* (with *Antirrhinum majus* as outgroup) from Wolfe et al. (2006), pruned to include only species (except *Penstemon lentus*) for which both floral color and floral anthocyanidins are known. Branch colors: blue = blue/purple flowers 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.

Literature Cited

- Ackermann M, M Weigend 2006 Nectar, floral morphology and pollination syndrome in Loasaceae subfam. Loasoideae (Cornales). *Ann Bot* 98:503–514.
- Anderson LL, KN Paige 2003 Multiple herbivores and coevolutionary interactions in an *Ipomopsis* hybrid swarm. *Evol Ecol* 17:139–156.
- Armbruster WS 2002 Can indirect selection and genetic context contribute to trait diversification? a transition-probability study of blossom-colour evolution in two genera. *J Evol Biol* 15:468–486.
- Bradshaw HD, DW Schemske 2003 Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* 426:176–178.
- Burdon JJ, DR Marshall, AHD Brown 1983 Demographic and genetic changes in populations of *Echium plantagineum*. *J Ecol* 71:667–679.
- Campbell DR, NM Waser, EJ Melendez-Ackerman 1997 Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *Am Nat* 149:295–315.
- Chang S-M, Y Lu, MD Rausher 2005 Neutral evolution of the nonbinding region of the anchocyanin regulatory gene *Ipmyb1* in *Ipomoea*. *Genetics* 170:1967–1978.
- Coberly LC 2003 The cost of white flowers: pleiotropy and the evolution of floral color. PhD diss. Duke University, Durham, NC.
- Coberly LC, MD Rausher 2003 Analysis of a chalcones synthase mutant in *Ipomoea purpurea* reveals a novel function for flavonoids: amelioration of heat stress. *Mol Ecol* 12:1113–1124.
- Durbin ML, KE Lundy, PL Morrell, CL Torres-Martinez, MT Clegg 2003 Genes that determine flower color: the role of regulatory changes in the evolution of phenotypic adaptations. *Mol Phylogenet Evol* 29:507–518.
- Emms SK, ML Arnold 2000 Site-to-site differences in pollinator visitation patterns in a Louisiana iris hybrid zone. *Oikos* 91:568–578.
- Epling C, H Lewis, FM Ball 1960 The breeding group and seed storage: a study in population dynamics. *Evolution* 14:238–255.
- Faegri K, L van der Pijl 1966 The principles of pollination ecology. Pergamon, Oxford. 248 pp.
- Fenster CB, WS Armbruster, P Wilson, MR Dudash, JD Thomson 2004 Pollination syndromes and floral specialization. *Annu Rev Ecol Evol Syst* 35:375–403.
- Fineblum WL, MD Rausher 1997 Do genes influencing floral pigmentation also influence resistance to herbivores and pathogens? the W locus in *Ipomoea purpurea*. *Ecology* 78:1646–1654.
- Frey FM 2004 Opposing natural selection from herbivores and pathogens may maintain floral-color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426–2437.
- Fry JD, MD Rausher 1997 Selection on a floral color polymorphism in the tall morning glory (*Ipomoea purpurea* L.): transmission success of the alleles through pollen. *Evolution* 51:66–78.
- Ghebrehiwet M, B Bremer, M Thulin 2000 Phylogeny of the tribe Antirrhinae (Scrophulariaceae) based on morphological and *ndhF* sequence data. *Plant Syst Evol* 220:223–239.
- Gigord LDB, MR Macnair, A Smithson 2001 Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylophiza sambucina* (L.) Soo. *Proc Natl Acad Sci USA* 98:6253–6255.
- Gomez JM 2000 Phenotypic selection and response to selection in *Lobelia maritima*: importance of direct and correlational components of natural selection. *J Evol Biol* 13:689–699.
- Grant V 1993 Origin of floral isolation between ornithophilous and sphingophilous plant species. *Proc Natl Acad Sci USA* 90:7729–7733.
- Griesbach RJ 1996 The inheritance of flower color in *Petunia hybrida* Vilm. *J Hered* 87:241–244.
- Habu Y, Y Hisatomi, S Iida 1998 Molecular characterization of the mutable *flaked* allele for flower variegation in the common morning glory. *Plant J* 16:371–376.
- Hannan GL 1981 Flower color polymorphism and pollination biology of *Platystemon californicus* Benth. (Papaveraceae). *Am J Bot* 68:233–243.
- Hodges SA, ML Arnold 1994 Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proc Natl Acad Sci USA* 91:2493–2496.
- Holton TA, EC Cornish 1995 Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7:1071–1083.
- Hoshino A, Y Morita, J-D Choi, N Saito, K Toki, Y Tanaka, S Iida 2003 Spontaneous mutations of the flavonoid 3'-hydroxylase gene conferring reddish flowers in three morning glory species. *Plant Cell Physiol* 44:990–1001.
- Igic B, R Lande, JR Kohn 2008 Loss of self-incompatibility and its evolutionary consequences. *Int J Plant Sci* 169:93–104.
- Igic G, L Bohs, JR Kohn 2003 Historical inferences from the self-incompatibility locus. *New Phytol* 161:97–105.
- Irwin RE, SY Strauss 2005 Flower color microevolution in wild radish: evolutionary response to pollinator-mediated selection. *Am Nat* 165:225–237.
- Irwin RE, SY Strauss, S Storz, A Emerson, G Gibert 2003 The role of herbivores in the maintenance of a flower color polymorphism in wild radish. *Ecology* 84:1733–1743.
- Jones KN 1996 Fertility selection on a discrete floral polymorphism in *Clarkia* (Onagraceae). *Evolution* 50:71–79.
- Jones KN, JS Reithel 2001 Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). *Am J Bot* 88:447–454.
- Kay KM, PA Reeves, RG Olmstead, DW Schemske 2005 Rapid speciation and the evolution of hummingbird pollination in Neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *Am J Bot* 92:1899–1910.
- Kay QON 1976 Preferential pollination of yellow-flowered morphs of *Raphanus raphanistrum* by *Pieris* and *Eristalis* spp. *Nature* 261:230–232.
- Kniskern JM, MD Rausher 2006 Environmental variation mediates the deleterious effects of *Coleosporium ipomoeae* on *Ipomoea purpurea*. *Ecology* 87:675–685.
- Levin DA 1969 The effect of corolla color and outline on interspecific pollen flow in *Phlox*. *Evolution* 23:444–455.
- 1972 The adaptedness of corolla-color variants in experimental and natural populations of *Phlox drummondii*. *Am Nat* 106:57–70.
- 1985 Reproductive character displacement in *Phlox*. *Evolution* 39:1275–1281.
- Levin DA, ET Brack 1995 Natural selection against white petals in *phlox*. *Evolution* 49:1017–1022.
- Levin DA, HW Kerster 1967 Natural selection for reproductive isolation in *Phlox*. *Evolution* 21:679–687.
- Maddison WP 2006 Confounding asymmetries in evolutionary diversification and character change. *Evolution* 60:1743–1746.
- Maddison WP, PE Midford, SP Otto 2007 Estimating a binary character's effect on speciation and extinction. *Evolution* (forthcoming).
- Melendez-Ackerman EJ, DR Campbell 1998 Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. *Evolution* 52:1293–1303.
- Melendez-Ackerman EJ, DR Campbell, NM Waser 1997 Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* 78:2532–2541.
- Miller RB 1981 Hawkmoths and the geographic patterns of floral variation in *Aquilegia caerulea*. *Evolution* 35:763–774.

- Miller RE, JA McDonald, PS Manos 2004 Systematics of *Ipomoea* subgenus *Quamoclit* (Convolvulaceae) based on ITS sequence data and a Bayesian phylogenetic analysis. *Am J Bot* 91:1208–1218.
- Miller RE, MD Rausher, PS Manos 1999 Phylogenetic systematics of *Ipomoea* (Convolvulaceae) based on ITS and *waxy* sequences. *Syst Bot* 24:209–227.
- Mol J, E Grotewold, R Koes 1998 How genes paint flowers and seeds. *Trends Plant Sci* 3:212–217.
- Morita Y, M Saitoh, A Hoshino, E Nitasaka, S Iida 2006 Isolation of cDNAs for R2R3-MYB, bHLH and WDR transcriptional regulators and identification of *c* and *ca* mutations conferring white flowers in the Japanese morning glory. *Plant Cell Physiol* 47:457–470.
- Nosil P, AO Mooers 2005 Testing hypotheses about ecological specialization using phylogenetic trees. *Evolution* 59:2256–2263.
- Ollerton J 1996 Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. *J Ecol* 84:767–769.
- 1998 Sunbird surprise for syndromes. *Nature* 394:726–727.
- Pagel M, F Lutzoni 2002 Accounting for phylogenetic uncertainty in comparative studies of evolution and adaptation. Pages 148–161 in M Lässig, A Valleriani, eds. *Biological evolution and statistical physics*. Springer, Berlin.
- Pellegrina G, F Bellusci, A Musacchio 2005 Evidence of post-pollination barriers among three colour morphs of the deceptive orchid *Dactylorhiza sambucina* (L.) Soo. *Sex Plant Reprod* 18:179–185.
- Perret M, A Chautems, R Spichiger, G Kite, V Savolainen 2003 Systematics and evolution of tribe Sinningieae (Gesneriaceae): evidence from phylogenetic analysis of six plastid DNA regions and nuclear ncgSP. *Am J Bot* 90:445–460.
- Quattrocchio F, J wing, K van der Woude, E Souer, N de Vetten, J Mol, R Koes 1999 Molecular analysis of the *anthocyanin2* gene of *Petunia* and its role in the evolution of flower color. *Plant Cell* 11:1433–1444.
- Rausher MD 2006 The evolution of flavonoids and their genes. Pages 175–211 in E Grotewold, ed. *The science of flavonoids*. Springer, Berlin.
- Rausher MD, D Augustine, A Vanderkooi 1993 Absence of pollen discounting in genotypes of *Ipomoea purpurea* exhibiting increased selfing. *Evolution* 47:1688–1695.
- Rausher MD, JD Fry 1993 Effects of a locus affecting floral pigmentation in *Ipomoea purpurea* on female fitness components. *Genetics* 134:1237–1247.
- Robertson C 1928 Flowers and insects: lists of visitors of four hundred and fifty-three flowers. Published by the author, Carlinville, IL.
- Schemske DW, P Bierzychudek 2001 Perspective: evolution of flower color in the desert annual *Linanthus parryae*: Wright revisited. *Evolution* 55:1269–1282.
- Forthcoming Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution*, doi: 10.1111/j.1558-5646.2007.00219.x.
- Schemske DW, HD Bradshaw 1999 Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc Natl Acad Sci USA* 96:11910–11915.
- Schwinn K, J Venail, Y Shang, S Mackay, V Alm, E Butelli, R Oyama, P Bailey, K Davies, C Martin 2006 A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* 18:831–851.
- Scogin R, CE Freeman 1987 Floral anthocyanins of the genus *Penstemon*: correlations with taxonomy and pollination. *Biochem Syst Ecol* 15:355–360.
- Shirley BW 1996 Flavonoid biosynthesis: “new” functions for an “old” pathway. *Trends Plant Sci* 1:377–382.
- Simms EL, MA Bucher 1996 Pleiotropic effects of flower-color intensity on herbivore performance on *Ipomoea purpurea*. *Evolution* 50:957–963.
- Smith SD, DA Baum Forthcoming Systematics of Iochrominae (Solanaceae): patterns in floral diversity and interspecific crossability. *Acta Horticult.*
- Spelt C, F Quattrocchio, J Mol, R Koes 2002 ANTHOCYANIN1 of petunia controls pigment synthesis, vacuolar pH, and seed coat development by genetically distinct mechanisms. *Plant Cell* 14:2121–2135.
- Spitze K 1993 Population structure in *Daphnia obtusa*: quantitative genetic and allozyme variation. *Genetics* 135:367–374.
- Stanton ML, AA Snow, SN Handel, J Berczky 1989 The impact of a flower-color polymorphism on mating patterns in experimental populations of wild radish (*Raphanus raphanistrum* L.). *Evolution* 43:335–346.
- Stickland G, BJ Harrison 1974 Precursors and genetic control of pigmentation. I. Induced biosynthesis of pelargonidin, cyanidin and delphinidin in *Antirrhinum majus*. *Heredity* 33:108–112.
- Storz JF 2002 Contrasting patterns of divergence in quantitative traits and neutral DNA markers: analysis of clinal variation. *Mol Ecol* 11:2537–2551.
- Strauss SY, JB Whittall 2006 Non-pollinator agents of selection on floral traits. Pages 120–138 in LD Harder, SCH Barrett, eds. *Ecology and evolution of flowers*. Oxford University Press, Oxford.
- Streisfeld MA, JR Kohn 2005 Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. *Evolution* 59:2548–2559.
- Tanaka Y, S Tsuda, T Kusumi 1998 Metabolic engineering to modify flower color. *Plant Cell Physiol* 39:1119–1126.
- Thomson JD, P Wilson 2008 Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *Int J Plant Sci* 169:23–38.
- Walker AR, PA Davison, AC Bolognesi-Winfield, CM James, N Srinivasan, TL Blundell, JJ Esch, MD Marks, JC Gray 1999 The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* 11:1337–1349.
- Warren J, S Mackenzie 2001 Why are all colour combinations not equally represented as flower-color polymorphisms? *New Phytol* 151:237–241.
- Waser NM, L Chittka, MV Price, NM Williams, J Ollerton 1996 Generalization in pollination systems, and why it matters. *Ecology* 77:1043–1060.
- Waser NM, MV Price 1981 Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* 35:376–390.
- 1993 Pollination behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* 302:422–424.
- Weis AE, SJ Franks 2006 Herbivory tolerance and coevolution: an alternative to the arms race? *New Phytol* 170:423–425.
- Wesselingh RA, ML Arnold 2000 Pollinator behaviour and the evolution of Louisiana iris hybrid zones. *J Evol Biol* 13:171–180.
- Whibley AC, NB Langlade, C Andalo, AI Hanna, A Bangham, C Thebaud, E Coen 2006 Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* 313:963–965.
- Whittall JB, C Voelckel, DJ Kliebenstein, SA Hodges 2006 Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in *Aquilegia*. *Mol Ecol* 15:4645–4657.
- Wilson P, MC Castellanos, JN Hogue, JD Thomson, WS Armbruster 2004 A multivariate search for pollination syndromes among penstemons. *Oikos* 104:345–361.
- Wilson P, MC Castellanos, A Wolfe, JD Thomson 2006 Shifts between bee and bird-pollination among penstemons. Pages 47–68 in NM Waser, J Ollerton, eds. *Plant-pollinator interactions: from specialization to generalization*. University of Chicago Press, Chicago.
- Wolfe AD, CP Randle, SL Datwyler, JJ Morawetz, N Arguedas, J Diaz 2006 Phylogeny, taxonomic affinities, and biogeography of *Penstemon* (Plantaginaceae) based on ITS and cpDNA sequence data. *Am J Bot* 93:1699–1713.

- Wolfe LM 1993 Reproductive consequences of a flower color polymorphism in *Hydrophyllum appendiculatum*. *Am Midl Nat* 129:405–408.
- 2001 Associations among multiple floral polymorphisms in *Linum pubescens* (Linaceae), a heterostylous plant. *Int J Plant Sci* 162:335–342.
- Wolfe LM, SE Sellers 1997 Polymorphic floral traits in *Linaria canadensis* (Scrophulariaceae). *Am Midl Nat* 138:134–139.
- Wray GA, MW Hahn, E Abouheif, JP Balhoff, M Pizer, MV Rockman, L Romano 2003 The evolution of transcriptional regulation in eukaryotes. *Mol Biol Evol* 20:1377–1419.
- Wright S 1943 An analysis of local variability of flower color in *Linanthus parryae*. *Genetics* 28:139–156.
- 1978 Evolution and the genetics of populations. IV. Variability within and among natural populations. University of Chicago Press, Chicago.
- Zufall RA 2003 Evolution of red flowers in *Ipomoea*. PhD diss. Duke University, Durham, NC.
- Zufall RA, MD Rausher 2004 Genetic changes associated with floral adaptation restrict future evolutionary potential. *Nature* 428:847–850.