doi:10.1111/j.1558-5646.2010.01165.x

POPULATION GENETICS, PLEIOTROPY, AND THE PREFERENTIAL FIXATION OF MUTATIONS DURING ADAPTIVE EVOLUTION

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Received November 13, 2009
Accepted September 29, 2010

Ongoing debate centers on whether certain types of mutations are fixed preferentially during adaptive evolution. Although there has been much discussion, no quantitative framework currently exists to test for these biases. Here, we describe a method for distinguishing between the two processes that likely account for biased rates of substitution: variation in mutation rates and variation in the probability that a mutation becomes fixed once it arises. We then use this approach to examine the type and magnitude of these biases during evolutionary transitions across multiple scales: those involving repeated origins of individual traits (flower color change), and transitions involving broad suites of traits (morphological and physiological trait evolution in plants and animals). We show that fixation biases can be strong at both levels of comparison, likely due to differences in the magnitude of deleterious pleiotropy associated with alternative mutation categories. However, we also show that the scale at which these comparisons are made greatly influences the results, as broad comparisons that simultaneously analyze mutliple traits obscure heterogeneity in the direction and magnitude of these biases. We conclude that preferential fixation of mutations likely is common in nature, but should be studied on a trait-by-trait basis.

KEY WORDS: **Adaptation, flower color, pleiotropy, population genetics.**

There has been renewed interest in developing a general theory of adaptation (Orr 2005). Recent work in this area has focused primarily on characterizing the expected distribution of mutational effect sizes for traits evolving due to natural selection (Orr 1998, 2000). By contrast, a relatively neglected aspect of the theory of adaptation is to understand whether natural selection preferentially targets certain genes, or certain types of genetic change, over others. This question has been of particular interest in the "evo–devo" literature, in which there has been much discussion (Stern 2000; Carroll 2005a, b, 2008) but little attempt to develop a formal analysis of whether selection targets specific genes.

In particular, this question has been stated and argued in many forms over the last several years (Stern 2000; Carroll 2005a, b; Prud'Homme et al. 2007; Wray 2007; Hoekstra and Coyne 2007; Lynch and Wagner 2008; Stern and Orgogozo 2008, 2009; Wagner and Lynch 2008). The common theme to these discussions has been an attempt to ask whether certain kinds of genetic change contribute more frequently than other types to phenotypic change. In the context of this question, "genetic change" usually refers to "substitutions," which we define as fixed genetic differences at specific loci between populations or species (see Table 1 for definitions of terms used).

As has been stated previously (Wray et al. 2003; Hoekstra and Coyne 2007; Stern and Orgogozo 2008), it is important to recognize that two distinct processes can affect whether particular types of mutations become fixed preferentially between species

Table 1. Definition of terms used in this study.

Term	Definition
Substitution	A fixed genetic difference between populations or species
Substitution spectrum	A vector of the probabilities of substitutions in each mutation category
Substitution bias	Deviation from an equal contribution of mutation categories to substitutions
Mutation spectrum	A vector of the probabilities of spontaneous mutations in each mutation category
Mutation bias	Deviation from equal probabilities of mutation for different mutation categories
Fixation spectrum	A vector of the probabilities of fixation in each mutation category
Fixation bias	Deviation from equal fixation probabilities for different mutation categories
Pathway gene	A gene that encodes an enzyme responsible for the biosynthesis of anthocyanin pigments
Transcription factor	A gene coding for a protein that activates or represses expression of another gene
Coding mutation	A mutation leading to a change in function that results from complete deletion, rearrangement, or alteration of the amino acid sequence or splicing patterns of a protein or RNA molecule
cis-regulatory mutation	A mutation in a gene's promoter, enhancer, introns, or untranslated regions that directly reduces or enhances the expression levels of that gene

(the "substitution bias") (Table 1). First, some mutations for a particular novel trait may arise more frequently than others. This process is termed "mutation bias." Recent arguments by Carroll (2008) invoke this process to argue that *cis*-regulatory mutations should predominate in morphological evolution. In his view, morphological evolution is primarily about making and breaking connections in gene regulatory networks, which in most cases can be achieved only through *cis*-regulatory mutations. Second, mutations may have different probabilities of being fixed once they arise. This process is termed "fixation bias." The usual explanation for biased fixation is that different mutation types experience different magnitudes of deleterious pleiotropy (Stern 2000; Wray et al. 2003; Carroll 2005a, 2008; Stern and Orgogozo 2008, 2009). Typically, it is argued that *cis*-regulatory mutations incur less pleiotropy than coding mutations (as defined in Table 1) because coding mutations alter the gene products in all tissues where that gene is expressed, whereas *cis*-regulatory mutations are believed to alter expression of that gene in a smaller subset of tissues. For example, Stern and Orgogozo (2008, 2009) argue that biased fixation contributes substantially to the observation that *cis*-regulatory mutations are more common in morphological divergence between species.

An additional point that frequently is overlooked in these studies is the possibility that the scale with which these comparisons are made might obscure heterogeneity in the direction or magnitude of biases. For example, because the genetic and regulatory networks that control individual traits are different, and are thus subject to different mutational and selective forces, individual traits and taxa could vary in the direction or magnitude of these biases. If true, this would make it dangerous to combine data across traits or across taxa into a single analysis, and it would raise the question of whether generalizations can be made about substitution biases for broad categories of traits or taxa.

For these reasons, it would be desirable to use an approach that explicitly differentiates between biases associated with rates of mutation and probabilities of fixation. In this article, we begin by describing such an approach. This method has its roots in the classical literature on the genetics of adaptation (Kimura 1983; Orr 1998) and thus is a general extension of the work in that area. A second objective of this report is to determine the utility of this method for interpreting the causes of substitution bias. To do so, we compare two datasets that examine trait evolution at different scales: independent, evolutionary transitions of the same trait (flower color transitions), and transitions involving broad suites of traits and taxa (Stern and Orgogozo 2008). We demonstrate that our method is capable of detecting biases in the predicted directions for analyses involving single traits. However, interpretation becomes obscured at larger scales of comparison, likely because of extensive heterogeneity in the direction and magnitude of these biases. We conclude by discussing the likely causes for biases and present potential alternatives to this problem of scale.

TESTING FOR MUTATION BIAS AND FIXATION BIAS

The question of whether different categories of mutations vary in their contribution to evolutionary change is equivalent to asking whether, on average, substitution rates are the same. Kimura (1983) showed generally that the substitution rate, K, is equal to the product of the population mutation rate, $2N\mu$ where N is population size and μ is the per-copy mutation rate, times the probability, λ, that a mutation is fixed once it has arisen, that is, $K = 2N\mu\lambda$. Orr (1998) recognized that μ and λ could vary for different categories of mutations, and thus that substitution rates may vary for different types of mutations, so that the expected rate of substitution for category i mutations is $K_i = 2N\mu_i\,\lambda_i$. The category-specific mutation rate can be represented by $\mu_i = \mu\theta_i$,

where μ is the overall rate of mutations producing the novel phenotype and θ_i is the proportion of those mutations that are category i mutations, which yields

$$K_i = 2N\mu\ \theta_i\ \lambda_i.$$

From this expression, the expected number of category i mutations fixed in some time period T is

$K_i\ T$, and the proportion of fixed mutations that are of category i is

$$r_i = K_i T/(\Sigma_i K_i T) = \theta_i \lambda_i / \Sigma_i \theta_i \lambda_i. \quad (1)$$

When r_i is the same for all i, then the different categories of mutation are expected to contribute equally to evolutionary change in a trait. Generally, this will be true only if $\theta_i = \theta_j$ and $\lambda_i = \lambda_j$ for all i, j. By contrast, if $\theta_i \neq \theta_j$ or $\lambda_i \neq \lambda_{j,}$ or both, then $r_i \neq r_j$, that is, not all categories of mutation will contribute equally to evolutionary change in a trait. (We ignore as improbable the possibility that differences in μ and λ exactly compensate to make substitution probabilities equal.) This provides a formal explanation for previous assertions that either mutation bias or fixation bias can cause some categories of mutation to contribute more to adaptive substitution than others (Wray et al. 2003; Hoekstra and Coyne 2007; Stern and Orgogozo 2008).

This argument leads naturally to several definitions: we define the substitution spectrum as the vector $(r_1, r_2, \ldots, r_n)$, the mutation spectrum as the vector $(\theta_1, \theta_2, \ldots \theta_n)$, and the fixation spectrum as $(\lambda_1, \lambda_2, \ldots, \lambda_n)$, where the indices $1, \ldots, n$ represent the different categories of mutations. Using these definitions, specific hypotheses can be tested in a straightforward manner. For example, the null hypothesis that all categories of mutation contribute equally to adaptive change (i.e., there is no substitution bias) can be evaluated with a χ^2 test to determine whether $r_i = 1/n$ for all i. Similarly, the null hypothesis of no mutation bias can be evaluated with a χ^2 test to determine whether $\theta_i = 1/n$ for all i. Because estimates of λ_i typically are unavailable in the literature, a similar test cannot be performed for fixation bias. However, from equation (1) above, when the λ_i are all equal,

$$r_i = \theta_i / \Sigma_i\ \theta_i = \theta_i,$$

that is, the substitution spectrum should equal the mutation spectrum. Consequently, a fixation bias may be tested using a two-way contingency analysis where one factor is mutation category and the other is number of substitutions versus number of mutations.

This approach requires that mutational categories be defined a priori. Several categorization schemes have been employed in previous discussions. One commonly used scheme assigns mutations to one of two categories: *cis*-regulatory mutations versus coding mutations (Wray 2007; Hoekstra and Coyne 2007; Stern and Orgogozo 2008, 2009). By contrast, Wagner and Lynch (2008) and Lynch and Wagner (2008) argue that mutations in transcription factor (TF) coding sequences often will have reduced pleiotropic effects similar to *cis*-regulatory mutations, suggesting that there should perhaps be three categories recognized: *cis*-regulatory, coding mutations in TFs, and coding mutations in other genes (those that encode all other proteins or mature RNA molecules that are not TFs). Others have suggested the categories "regulatory" (including all mutations that alter gene expression) and "functional" (coding mutations in other genes) (discussed in Stern and Orgogozo 2008; also see Streisfeld and Rausher 2009a).

We believe that there is no categorization scheme for mutations that satisfies all cases. For example, there is no reason to expect that all *cis*-regulatory mutations are modular and thus have reduced pleiotropic effects, nor that all coding mutations are highly pleiotropic (Wray et al. 2003; Wagner and Lynch 2008; Lynch and Wagner 2008). For any particular study, investigators should employ the scheme that biologically is most relevant for their purposes. This means that there must be both a justifiable reason for classifying different mutations into particular categories and the classification scheme used must be applicable to all traits and genes examined in that study. In most cases, this will vary based on the trait or traits being compared and on what is known a priori about the magnitude of deleterious pleiotropy on different types of genetic change.

FLOWER COLOR AND THE ANTHOCYANIN PATHWAY

Our first application of the approach described above is intended to examine whether and why certain categories of mutation predominate during evolutionary transitions in flower color. Such transitions are numerous (Rausher 2008), and it often is thought that flower color differences between populations or species are adaptive because of their prominent role in the attraction of animal pollinators (Grant 1993; Fenster et al. 2004). Among angiosperms, anthocyanins are common floral pigments that give rise to blue, purple, and red colors, and the flavonoid biosynthetic pathway responsible for anthocyanin pigmentation is highly conserved (Holton and Cornish 1995; Winkel-Shirley 2001). Evolutionary transitions in flower color typically are of one of two types: (1) shifts in intensity of pigmentation, usually from pigmented flowers (red, blue, purple) to flowers lacking anthocyanin pigments (white or yellow flowers); or (2) changes in the type of anthocyanins found in flowers, which results in a change in hue (e.g., blue/purple to red) (Rausher 2006).

Changes in pigment intensity are caused by altering the amount of flux through the pathway; for shifts to unpigmented flowers, flux is completely blocked. Previous investigation has shown that functional deactivation or reduced expression of any of the pathway enzymes can result in a loss of pigmentation (Grotewold 2006). By contrast, transitions in floral hue frequently are achieved by altering the flux down different branches of the

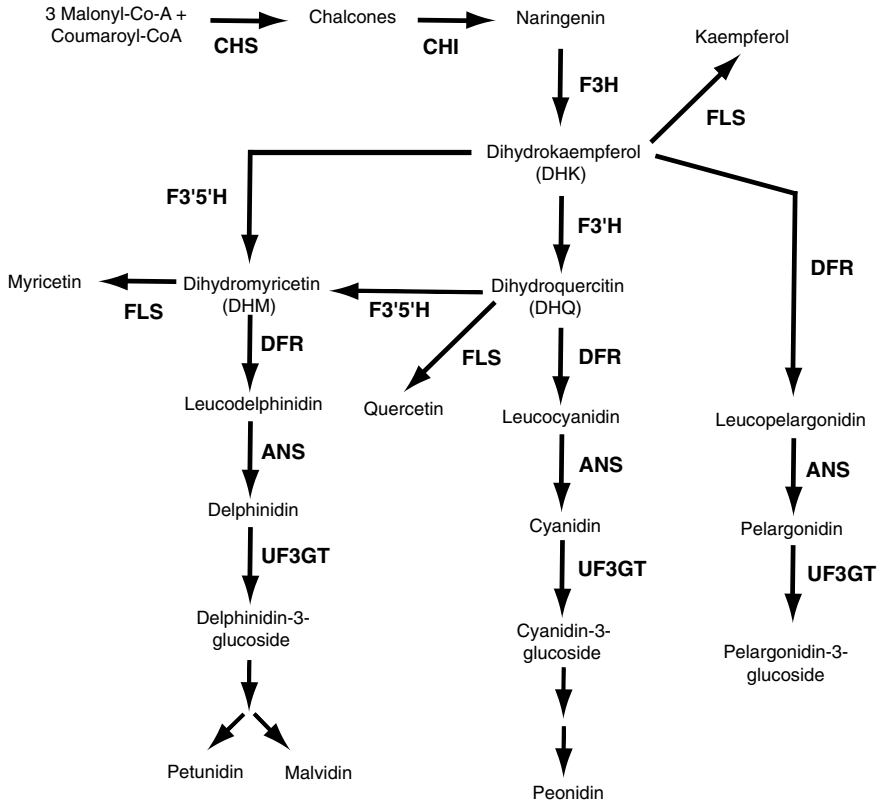

Figure 1. **A schematic of the three major branches of the anthocyanin biosynthetic pathway. Each enzyme is listed as an abbreviation beside the arrows, whereas the products of each enzymatic reaction are listed below the arrows. The FLS enzyme (flavonol synthase) is not required for pigment production, but is necessary for the synthesis of the three flavonols: kaempferol, quercetin, and myricetin. Modified forms of the anthocyanidins are shown below the cyanidin and delphinidin branches. Abbreviations: CHS = chalcone synthase; CHI = chalcone isomerase; F3H = flavonoid-3-hydroxylase; F3′H = flavonoid-3′ hydroxylase; F3′5′H = flavonoid 3′5′ hydroxylase; FLS = flavonol synthase; DFR = dihydroflavonol-4-reductase; ANS = anthocyanidin synthase; and UF3GT = UDP-flavonoid-3-glucosyl-transferase.**

pathway (Fig. 1), and they typically involve changes from derivatives of the cyanidin or delphinidin pigments to the red pigments derived from pelargonidin (e.g., Scogin and Freeman 1987; Zufall and Rausher 2004; Rausher 2008; Streisfeld and Rausher 2009a). Because these changes in floral color occur via distinct biochemical mechanisms, we define changes in pigment intensity and changes in floral hue as separate traits during our analyses of mutation and fixation biases.

The pathway topology, its transcriptional regulation, and our knowledge of the potential pleiotropic consequences of mutations in the genes encoding these pathway enzymes or TFs, allow us to make predictions about mutation and fixation biases for these types of flower color change. Moreover, our knowledge of the biochemical, developmental, and genetic bases of anthocyanin pigment production provide a priori justification for the classification of three categories of mutation, as defined in Table 1: (1) coding mutations in pathway genes (COD(*P*)); (2) *cis*-regulatory mutations in pathway genes (CIS(*P*)); and (3) either coding or *cis*-regulatory mutations in TFs. We note that the first two categories are analogous to those described by Stern and Orgogozo (2008) in their analysis of the types of mutations contributing to phenotypic evolution (see below). In particular, CIS(*P*) mutations include mutations in the promoter, enhancer, introns, and untranslated regions that directly affect expression of that gene, whereas COD(*P*) mutations include mutations in exons that alter amino acid composition, as well as mutations that give rise to gene deletions and altered splicing that affect protein function. Moreover, we add a third category that includes TF mutations that is based on our expectations of the magnitude of deleterious pleiotropy associated with these mutations. We combine coding and *cis*-regulatory mutations within TFs because data typically are not available to distinguish between them. Our predictions for mutation and fixation biases, along with the justifications for these mutational categories, are described below:

(1). For changes in pigment intensity, substitutions will preferentially involve either TF or CIS(P) mutations. Functional alteration or downregulation of any enzyme in the pathway can reduce or eliminate floral pigmentation (Grotewold 2006). However, because pathway enzymes also are used

to produce other flavonoids with important physiological and ecological functions (Koes et al. 1994; Shirley 1996, Winkel-Shirley 2002; Besseau et al. 2007), functional inactivation is likely to incur substantial deleterious pleiotropy (e.g., Coberly and Rausher 2008). By contrast, because both TF and CIS(*P*) mutations may be modular and tissue-specific, they have the potential to affect flavonoid production only in floral tissues (e.g., van Tunen et al. 1989, 1991; Quattrocchio et al. 1993; Rausher 2006; Streisfeld and Rausher 2009b). Thus, it is expected that these mutations will have fewer deleterious pleiotropic effects and a higher fixation probability than COD(*P*) mutations.

Based on what is known about TF's regulating anthocyanin production, we can also make more specific predictions about the particular class of TF that would be fixed preferentially. Three types of TF are involved in the coordinate, transcriptional control of the pathway genes: members of the R2R3-MYB (MYB), basic helix-loop-helix (bHLH), and WD40-repeat (WDR) families (Koes et al. 2005; Ramsay and Glover 2005). Due to their high copy number and often tissue-specific function, mutations in MYB proteins are expected to have fewer pleiotropic effects than mutations in bHLH or WDR proteins. For example, different MYB copies typically activate anthocyanins in separate tissues (Quattrocchio et al. 2006; Schwinn et al. 2006; Morita et al. 2006; Stracke et al. 2007; Gonzalez et al. 2008). Consequently, mutations affecting floral anthocyanin-regulating MYBs are unlikely to affect flavonoid production in other tissues, and thus are expected to have relatively few deleterious pleiotropic effects. By contrast, the same bHLH and WDR proteins involved in floral anthocyanin production also are known to be expressed more broadly and to regulate the development of additional characters besides flavonoids, such as trichomes, vacuolar pH, root hairs, seed coat mucilage, and seed proanthocyanidins (Ramsay and Glover 2005; Broun 2005; Koes et al. 2005). Because mutations in these genes likely affect flower color as well as these other characters, they are expected to have lower probabilities of fixation compared to mutations in MYB proteins that control only floral anthocyanins. Consequently, mutations in MYBs are predicted to demonstrate a fixation bias among all TF mutations.

(2). Mutations causing a change in floral hue are more likely to be COD(P) or CIS(P) than TFs. Most changes in floral hue involve the redirection of pathway flux from branches producing more hydroxylated anthocyanidins to less hydroxylated anthocyanidins (dephinidin is the most hydroxylated, pelargonidin is the least hydroxylated). This can be accomplished by downregulating or inactivating the branching enzymes F3′H and F3′5′H (Fig. 1). Current evidence suggests that the anthocyanin TFs regulating *F3′h* and *F3′5′h* also coordinately control the expression of other pathway genes (Mol et al. 1998; Koes et al. 2005). Thus, it is unlikely that mutations involving TFs would be capable of producing red flowers—any downregulation of the genes encoding the two branching enzymes due to a mutation in a TF also would downregulate other enzymes and reduce or abolish pigment production. This would result in white or yellow flowers, rather than red flowers. Therefore, we predict that the mutation spectrum for changes in floral hue will show that COD(*P*) or CIS(*P*) mutations in the branching enzymes are common, but mutations involving TF are rare.

(3). Evolutionary transitions from blue to red flowers are more likely to involve CIS(P) than COD(P) mutations. Given the potential for a strong mutation bias against TF mutations causing changes in floral hue (see above), we predict that evolutionary transitions in floral hue preferentially will fix CIS(*P*) mutations instead of COD(*P*) mutations. This is because CIS(*P*) mutations have the potential to affect anthocyanin production only in flowers, and thus to incur less deleterious pleiotropy (Streisfeld and Rausher 2009a; Des Marais and Rausher (2010).

Materials and Methods

FLOWER COLOR

Mutation spectrum

We obtained estimates of the mutation spectrum for changes in floral pigment intensity and floral hue primarily from reports of spontaneous mutations described in the horticultural literature. We used a literature search of ISI Web of Science with a number of keyword combinations: "flower anthocyanin," "floral anthocyanin," "flower color mutant," and "anthocyanin mutant." This survey produced a list of investigations that characterized the molecular changes responsible for spontaneous mutations affecting pigment intensity and floral hue in horticultural varieties of different plant species. It also included a small number of cases involving color polymorphisms segregating at low frequency within natural populations. This list was augmented with examples of the molecular characterization of flower color mutations from two other sources: (1) from a mutagenesis screen among 2300 families and 200,000 *Petunia hybrida* plants (van Houwelingen et al. 1998); and (2) examples included in Table 1 of Chopra et al. (2006) that identified the molecular basis of flower color mutations in *P. hybrida, Antirrhinum majus,* and *Ipomoea* spp.

One caveat to this approach is that these data may not represent the true mutation spectrum. Ideally, one would collect a random sample of spontaneous mutations producing the phenotypic change of interest and determine how many mutations fall into each category. However, to our knowledge, such a sample of mutations is not available for any phenotypic change. Nevertheless,

we believe that any biases in our data likely are small. In particular, horticulturalists typically preserve all phenotypically identifiable mutations that they observe, usually without a priori knowledge of their genetic basis. This is certainly the case for all of the studies included in our survey and represents mutations of both large and small effect (e.g., see Coen et al 1986; Almeida et al. 1989). Thus, even though this set of mutations does not represent an exhaustive list of all known flower color mutations, there is little reason to believe that the proportion of mutations identified in each category is biased.

Although some of the mutations included in our survey were due to frameshift, nonsense, and point mutations (see Supporting information), a large proportion was caused by transposon insertions, which also seem to contribute substantially to segregating natural variation and fixed differences between species (Quattrocchio et al. 1999; Clegg and Durbin 2000; Zufall and Rausher 2003; Schwinn et al. 2006). Some of these mutations were unstable, resulting in restored streaks of pigmentation in flowers due to excision by the transposon in somatic cells. We include these unstable mutants for two reasons. First, they often produce substantial changes in flower color. For example, most mutants causing loss of pigmentation produce flowers that are primarily white with a few colored streaks. Similarly, in those causing change in floral hue, most of the background color is altered, whereas excision results in streaks of the original color. Second, evidence suggests that in plants, excision of transposons often is imprecise. Imprecise excision usually yields stable alleles with the mutant color phenotype, and such stable mutants are common in natural populations (e.g., Habu et al. 1998; Quattrocchio et al. 1999; Zufall and Rausher 2003). In addition, variation in the transposon sequence itself or in the background genotype can greatly reduce excision frequency (e.g., Habu et al. 1998), resulting in a quasi-stable phenotype. Assuming the probability of imprecise excision does not differ among categories of mutation, unstable insertions should reflect the mutation spectrum of resulting stable mutations.

For each mutation in the dataset, several features were tabulated, including the change in phenotype (i.e., altered pigment intensity or change in floral hue), the gene that is affected, the mutation category (COD(*P*), CIS(*P*), or TF), the molecular nature of the mutation (i.e., frameshift, transposon, etc.), and whether the phenotypic effects of the mutation are manifested in just flowers or in other tissues (see Table S1).

Substitution spectrum

To estimate the substitution spectrum, we searched the literature exhaustively for all examples involving genetically characterized fixed differences in flower color between populations or between species. For each example, we tabulated the same features as for the mutation spectrum.

STERN AND ORGOGOZO DATASET

As a second application of our method, we reanalyzed the complete dataset of Stern and Orgogozo (2008). Here, the goal is to compare the results from the flower color analyses described above with a dataset that examines transitions involving broad suites of traits (morphological and physiological trait evolution in plants and animals) to determine whether the different scales of comparison affect our interpretation of substitution biases. The Stern and Orgogozo (2008) dataset presents an exhaustive literature survey of characters in which the genetic basis of phenotypic traits in plants and animals was characterized. In their analysis, the frequency of *cis*-regulatory (CIS) versus coding (COD) mutations involved in either morphological or physiological evolution was contrasted at different taxonomic levels (i.e., domestication, within-species, between-species, and between genera).

We tabulated genetic changes using four categories: (1) taxon (plants or animals); (2) trait (morphology or physiology); (3) mutation category (COD or CIS); and (4) divergence (within-species or between-species). In all cases, assignment to different category states was based on categorization by the original authors. This tabulation is presented in Table S2. For category (4), we combined the authors' categories "Domestic" and "Intraspecific" to form our "Within Species" category, and their "Interspecific" and "Intergeneric" categories to form our "Between Species" category. We believe this is appropriate because the "Within Species" category essentially represents the spectrum of spontaneous mutations that appeared under domestication or that segregate in wild populations. This is analogous to our characterization of the mutation spectra in the flower color datasets above, but here a larger fraction of mutations are segregating, and thus potentially subject to selection. Although little information is present regarding selection on the wild variants, it seems likely that many represent nearly neutral or slightly deleterious variants that have not fixed in populations, although there are clearly some that have been subject to selection. We thus believe that this categorization scheme represents at least a crude approximation of the mutation spectrum. However, we recognize that if a truly random collection of mutations were available, the true mutation spectrum might differ from the collection of mutations we estimated, and this might affect our conclusions. By contrast, even if what we are calling the mutation spectrum is influenced by an element of selection, our "Between Species" category represents mutations that have fixed between species, and thus represents the set of mutations that have likely passed through an additional selective filter. Hence, a difference between the "within species" and "between species" spectra still indicates a fixation bias. Finally, even though this dataset has its limitations, it is the only comprehensive dataset currently available with which to compare the flower color data.

Mutation and substitution biases were assessed statistically using χ^2 tests of equal representation of the mutation categories. These analyses were supplemented by two- and three-way G-tests, performed using an APL program written by MDR, following Bishop et al. (1975), to test for differences in bias between subsets of the data.

Results

FLOWER COLOR TRANSITIONS

Substitution bias

There are seven independent examples of studies that report genetic changes associated with altered floral pigment intensity (*Petunia*: Quattrocchio et al. 1999; three changes among *Antirrhinum* spp: Schwinn et al. 2006; *Aquilegia*: Whittall et al. 2006; *Mimulus aurantiacus*: Streisfeld and Rausher 2009b; and *Mimulus cupreus*: Cooley 2008). In *Antirrhinum*, genetic crosses identify two distinct alleles of the same MYB gene that have become fixed among species (Schwinn et al. 2006). Phylogenetic evidence also suggests that one of these alleles has arisen independently at least twice within the genus (Vargas et al. 2009). This implies that there have been at least three independent mutations that have become fixed among species of *Antirrhinum*.

In all seven cases, TF mutations are involved, which represents a statistically significant substitution bias ($P < 0.05$, Fisher's exact test of equal involvement of TF versus COD(P) + CIS(P) mutations; Table 3). In addition to the above examples, two additional *Antirrhinum* species with reduced pigment intensity are fixed for MYB TF mutations (Schwinn et al. 2006). However, because the phylogenetic relationships among these species are not resolved, it is unclear whether these represent independent mutations. Finally, Hoballah et al. (2007) identified four additional, independent loss-of-function mutations in a MYB TF during the transition to white flowers between *Petunia integrifolia* and *P. axillaris*. However, because only single accessions from each population were sampled, it is unclear whether these mutations were fixed between populations. To the extent that these additional cases represent independent substitutions, the substitution spectrum may include up to six additional examples that all involve MYB proteins. This would indicate a striking bias favoring substitutions in TFs in general, and MYB proteins in particular ($P < 0.001$, Fisher's exact test for both comparisons).

By contrast, for evolutionary transitions in floral hue, there is only one example in which the molecular basis of the change has been characterized (Des Marais and Rausher 2010). In this study, the evolutionary transition involves a CIS(P) mutation (see below). However, no conclusive statement of substitution bias can be derived until additional transitions involving fixed differences in hue are characterized between populations or species.

Table 2. Number of spontaneous mutations identified in the literature surveys for (A) different flower color transitions; and (B) among transcription factor families. Transcription factor mutations occur only for those phenotypic transitions involving altered pigment intensity. Mutation categories are defined in the text.

(A) Type of transition			
	Mutation category		
	COD(P)	CIS(P)	TF
Pigment intensity	29	8	32
Floral hue	9	0	0
(B) Type of transcription factor			
	MYB	bHLH	WDR
	5	20	7

Mutation bias

As described above, the predominant use of one type of genetic change in evolutionary transitions can be caused simply by high mutation rates associated with that type of change. In this section, we examine properties of the mutation spectrum for flower color transitions to determine whether mutation bias exists (Table 2). Several inferences can be drawn from these data.

(1) For mutations that alter floral hue, all of the documented cases involve COD(P); none involve CIS(P) or TF mutations (Table 2). Although there is only one example of an evolutionary transition altering floral hue, adequate numbers of spontaneous mutations have been characterized to allow conclusions about mutation bias to be drawn. There appears to be a significant mutation bias against both *CIS(P)* and TF for this type of flower color change (Table 3). The lack of TF mutations in our survey is not surprising, given that TF mutations are unlikely to alter floral hue (see above).

(2) The proportion of pigment intensity mutations that are CIS(P) is very low. Out of 69 documented mutations causing changes in pigment intensity (all greatly reduce or abolish anthocyanin pigments), only eight involved CIS(P) mutations (Table 2), which represents a significant deviation from equality of mutations of the three categories (Table 3). Thus, for both types of flower color change, CIS(P) mutations appear to occur at a very low frequency. By contrast, there is no bias in the proportion of intensity mutations that are either COD(P) or TF (29 vs. 32 mutations identified, respectively; $\chi^2 = 0.15$, df $= 1$, ns). However, the involvement of TF mutations affecting pigment intensity is significantly greater than in mutations causing changes in floral hue ($P < 0.006$, Fisher exact test). This result

Table 3. For each of the transitions examined, the mutation category that is overrepresented for substitution, mutation, and fixation biases are indicated. Mutation categories are abbreviated as follows: COD=coding mutation; CIS=*cis*-regulatory mutation; TF=transcription factor mutation; COD(*P*) and CIS(*P*)=coding mutation and *cis*-regulatory mutation in anthocyanin pathway gene, respectively. For the transitions involving floral color changes, three mutation categories were compared: COD(*P*), CIS(*P*), and TF. For the transitions involving plant and animal morphology and physiology, two mutational categories were compared: COD and CIS. (+)=Mutation category direct influences substitution bias, (−)=effect opposes substitution bias. NA: insufficient data.

Transition	Substitution bias	Mutation bias	Effect on substitution bias	Fixation bias	Effect on substitution bias
Pigment intensity	TF*	COD(*P*)/TF**	−	TF*	+
Floral hue	NA	COD(*P*)*	NA	NA	NA
Morphology (animal)	CIS***	COD	−	CIS***	+
Morphology (plant)	COD*	COD***	+	COD	+
Physiology (animal)	COD***	COD***	+	COD*	+
Physiology (plant)	CIS	COD***	−	CIS**	+

***$P<0.05$; **$P<0.01$; ***$P<0.001$.**

indicates that for different types of phenotypic change, the mutation spectrum can vary substantially.

(3) Among mutations altering pigment intensity that involve TFs, mutations in genes encoding bHLH and WDR proteins are involved more frequently than genes encoding MYB proteins. Thus, there appears to be a significant bias against mutations in MYB proteins ($\chi^2 = 12.43$, df $= 2$, $P < 0.01$).

Fixation bias

Comparison of the substitution and mutation spectra has yielded a number of patterns indicating differential probabilities of fixation across mutational categories. Several of these patterns are tentative because the sample size of fixed differences between species is small. Nevertheless, the patterns are all in the direction predicted a priori (see above).

(1) Alteration of floral pigment intensity involves preferential fixation of TF mutations. In all seven of the published studies referenced above, the change has involved modification of a TF, which in turn causes a marked downregulation of anthocyanin pathway genes. When compared to the spectrum of pigment intensity mutations, TF mutations are fixed disproportionately more frequently than COD(*P*) and CIS(*P*) mutations ($P = 0.025$, Fisher's exact test; Table 3). This statistic becomes even more highly significant if the additional, unconfirmed examples of TF substitution described above are included.

(2) Mutations in Myb TFs are preferentially fixed over mutations in bHLH and WDR. Five of the seven investigations of evolutionary reductions in pigmentation identified the specific TF involved, and in all cases the mutation occurred in a gene encoding an R2R3 MYB protein (Quattrocchio et al. 1999; Schwinn et al. 2006; Cooley 2008). When compared with the mutation spectrum of TF mutations (Table 2B), fixation of MYB mutations is overrepresented ($P = 0.0006$, Fisher's exact test). In addition, the six potential examples described above also all directly implicate substitutions in MYB genes, which may demonstrate a striking fixation bias favoring this class of TF.

(3) Evolutionary changes in floral hue may preferentially involve CIS(P) mutations. Des Marais and Rausher (2010) found that the transition from blue/purple to red flowers in the *Mina* clade of *Ipomoea* was due in large part to a CIS(*P*) mutation in the gene encoding *F3′h*. Although no statistical statement about fixation bias can be made from this one example, we note that it supports our prediction about the direction of fixation bias for this type of flower color change. In addition, Streisfeld and Rausher (2009a) found evidence suggesting that two other, independent transitions to red flowers among *Ipomoea* species also involved floral-specific downregulation of the gene encoding F3′H. In these cases, however, additional experiments would be required to demonstrate that downregulation is caused by CIS(*P*) mutations. To the extent that these transitions to red flowers also are caused by CIS(*P*) mutations, they would further support our prediction that CIS(*P*) mutations are fixed preferentially during evolutionary shifts in floral hue.

Relative contribution of mutation and fixation biases to substitution bias

For changes in pigment intensity, mutation bias contributes partially to the substitution bias, in that it favors both COD(*P*) mutations and TF mutations over CIS(*P*) mutations. The fixation bias strongly favors TF mutations over COD(*P*) mutations, producing the overall substitution bias favoring TF mutations (Table 3). By contrast, for floral hue transitions, the mutation spectrum lacks

evidence for TF mutations, which, as discussed above, is probably a result of the biochemical and developmental architecture of this transition. The resulting mutation bias significantly favors COD(*P*) mutations that alter hue. No inference can be made about substitution or fixation biases for transitions involving hue.

STERN AND ORGOGOZO DATASET

Our analysis of this dataset confirms a number of conclusions drawn by Stern and Orgogozo (2008). We do not reproduce the results leading to those conclusions here. Rather, we concentrate on results that either differ or were not discussed in their article. In these analyses, there are only two categories of mutations: coding mutations (COD) and *cis*-regulatory mutations (CIS).

Substitution biases

Plants and animals differ in both the magnitude and the direction of their substitution biases. For animals, changes in physiological traits preferentially involve COD mutations, whereas for morphological traits, CIS mutations are favored. By contrast, for plants, morphological changes preferentially involve COD mutations, whereas physiological changes trend toward CIS mutations (Table 3). This difference between plants and animals is highly significant, as reflected in the three-way interaction term in a three-way G-test involving factors taxon (plants vs. animals), trait (physiology vs. morphology), and mutation category (COD vs. CIS) (Table S3, Part A). Moreover, in both taxa, the differences in substitution bias between morphological traits and physiological traits are significant (two-way Fisher's exact tests: for plants, $P < 0.05$; for animals, $P < 0.0001$). Thus, it does not appear possible to make a general statement applicable to all taxa or traits describing the preferential use of COD or CIS mutations during evolutionary transitions involving morphological or physiological traits. Rather, the mutation category preferentially favored depends on the taxa and traits that are compared.

Mutation biases

For all four taxon × trait category combinations, there is a substantial mutation bias in which COD mutations are much more common than CIS mutations, and this bias is highly significant for all but animal morphology (Table 3). However, the magnitude of this bias varies for the different taxon × trait combinations. In a three-way analysis, neither the three-way interaction (mutational category × trait × taxon) nor any of the two-way interactions involving taxon were significant (Table S3. part B). We thus collapsed the table over the factor taxon and analyzed the resulting two-way table with factors mutation category and trait. This analysis showed that the relative proportion of CIS mutations differed for morphological and physiological traits (0.27 vs. 0.16, respectively; $G = 3.71$, $P = 0.055$), but this difference was similar for animals and plants. Largely consistent with the data on the flower color mutation spectra, this analysis suggests that mutation spectra have the potential to vary depending on the trait categories that are compared.

Fixation bias

For physiological traits, animals show a fixation bias favoring COD mutations, whereas plants exhibit a bias favoring CIS mutations. The three-way interaction term in a three-way G-test examines whether these biases between the taxa are significantly different from each other. This interaction term is highly significant (Table S3, part F). Moreover, for both taxa, the biases are individually significant when tested with a two-way G-test (Table 3). For morphological traits, animals exhibit a fixation bias favoring CIS mutations, although the bias in plants is toward COD mutations. As with physiology, the three-way term is highly significant (Table S3, part E), indicating that fixation biases differ for the two taxa. Separate analyses for each taxon indicate the bias is significant for animals, but not for plants. The most conservative interpretation of this result is that for animal morphology, there is a bias favoring CIS mutations, whereas for plants there is no bias. More to the point, this result again appears to indicate that a general statement covering all taxa and traits is not possible: for example, across all taxa, it is not possible to conclude that CIS mutations are preferentially fixed during morphological evolution but COD mutations are fixed preferentially during physiological evolution.

Relative contribution of mutation and fixation biases to substitution bias

For all four combinations of taxa and traits, fixation bias is in the same direction as the substitution bias. For plant morphology and animal physiology, the mutation bias is also in the same direction as the substitution bias. For these two categories, both mutation and fixation bias contribute directly to the substitution bias that favors COD mutations (Table 3). By contrast, for animal morphology and plant physiology, mutation bias is antagonistic to fixation bias: there is a mutation bias favoring COD mutations, whereas there is a fixation bias favoring CIS mutations. In these two cases, fixation bias predominates, giving rise to a net substitution bias favoring CIS mutations (Table 3).

Discussion

The last several years have seen an explosion of experimental studies seeking to identify the genetic and molecular bases of adaptive traits (e.g., Hoekstra et al. 2006; Rebeiz et al. 2009; Wittkopp et al. 2009; Chan et al. 2010). An important question that emerges from these studies is whether certain types of genes or mutations are preferentially and predictably selected to cause these phenotypic differences. However, no quantitatve framework

for addressing this question has been presented previously. One reason for this is that it is very difficult to determine the mutation spectrum for any particular trait. It has long been recognized that both mutational and selective forces can generate substitution bias (Wray et al. 2003; Hoekstra and Coyne 2007; Stern and Orgogozo 2008), making it necessary to have a reliable estimate of the mutation spectrum to distinguish between fixation and mutation bias. Thus, the standard approach in the literature for addressing this question has been to determine whether biases exist solely from substitution data (but see Stern and Orgogozo 2008, 2009).

In this article, we have developed a quantitative method that attempts to address whether mutation or fixation biases are responsible for biased substitution. In doing so, we use the distribution of spontaneous horticultural mutations affecting flower color change as an estimate of the mutation spectrum and compare this against data on evolutionary substitutions. Although we are not the first to use surveys of laboratory or spontaneous mutations to estimate the types of genes involved in generating particular phenotypes (Kingsley et al. 2009; Liao et al. 2010), these previous efforts did not explicitly compare the mutation and substitution data in a quantitative framework. Even though our estimates of the mutation spectra for floral hue and intensity are crude, they appear to be sufficient to reveal strong mutation biases, and in the case of pigment intensity, fixation biases as well. Moreover, our confidence in these estimates is strengthened by the fact that we detected fixation bias in the direction predicted by our knowledge of the structure and functioning of the anthocyanin biosynthetic pathway. We thus believe that this approach provides an improvement over earlier attempts to determine the causes of substitution bias.

A second goal of this article was to determine the utility of this method for interpreting the causes of substitution bias. We evaluated two datasets that examined trait evolution at different scales: independent, evolutionary transitions of the same trait, and transitions involving broad suites of traits and taxa. The former example (flower color transitions) probably provides the closest we have to an ideal dataset because its historical importance to both horticulturalists and molecular geneticists has led to a mutational distribution that likely resembles the true mutation spectrum. Moreover, as more examples of the molecular basis of evolutionary transitions in flower color become available in the near future, we will have more robust estimates of the substitution and fixation biases. Although there may be a limited number of traits for which such a comprehensive list of spontaneous mutations currently exists, as we begin to move away from single gene analyses toward whole-genome, network, and systems-level approaches in biology, it may be possible to generate these sorts of mutational data for a wide array of traits in the near future. Moreover, we note that the main objective of our comparison of the flower color results with the Stern and Orgogozo (2008) dataset was to highlight the difficulty in drawing strong conclusions about preferential fixation using such heterogeneous data. Thus, despite the obvious limitations of our approach, if multiple researchers employ this approach across a number of different traits, we should be able to determine not only whether particular traits exhibit a substitution or fixation bias, but also whether traits differ in the nature of these biases. In what follows, we focus our discussion on the extent of heterogeneity associated with these two datasets and the factors that might be responsible for generating it.

TRAIT AND TAXON HETEROGENEITY

Our analyses suggest that substantial heterogeneity exists among traits and taxa for the particular categories of mutations preferentially involved in evolutionary change. This heterogeneity is evident in differences in both the mutation and fixation spectra. Mutation spectra differed qualitatively for mutations affecting floral hue and pigment intensity: spontaneous mutations affecting hue were exclusively COD(*P*), whereas mutations affecting pigment intensity involved both COD(*P*) and TF at similar frequency. Differences among mutation spectra for the four taxon × trait combinations of the Stern and Orgogozo dataset were more quantitative: although COD mutations outnumbered CIS mutations in all four categories, the magnitude of the bias varied among combinations. In this case, however, there is the added caveat that these differences may in part be due to differences among the combinations in the extent to which selection has acted on within-species variation.

The limited data available on fixation bias suggest that it may differ between intensity and hue mutations: only TF mutations were involved in the evolution of altered pigment intensity, whereas in the one definitive example of the evolution of blue to red flowers, a CIS(*P*) mutation was involved. Similar heterogeneity was found among traits in the Stern and Orgogozo (2008) dataset: for animal morphological characters and plant physiological characters, fixation of CIS mutations was more likely than fixation of COD mutations, whereas for animal physiological characters and plant morphological characters, the opposite was true.

These patterns suggest that generalizations regarding biases in the types of mutations involved in phenotypic change are not appropriately applied to either all taxa or to broad trait categories. Why might these generalizations fail at these larger scales of comparison? One possibility is that the categorization of mutations is not appropriate at this scale of comparison. In particular, a *cis*-regulatory mutation is only expected to have fewer deleterious pleiotropic consequences compared to a coding mutation with phenotypically similar effects. Because selection is acting on phenotypes and not genotypes, there is no necessary reason to expect that a *cis*-regulatory mutation affecting a particular phenotypic

trait in plants is going to have fewer fitness consequences relative to a coding mutation that affects a completely different trait in animals. An additional (and not mutually exclusive) possibility is that broad comparisons that simultaneously examine large numbers of traits in a large number of organisms mask heterogeneity in the direction and magnitude of these biases. Although we do not know the scale at which this type of heterogeneity disappears from the data, we suspect it may be small. For example, our analysis of the Stern and Orgogozo dataset compared only two broad trait categories (morphological vs. physiological), but these categories are made up of multiple individual phenotypes, each of which is controlled by genes that are part of different genetic and regulatory networks. Thus, pooling these traits into such broad categories likely obscures additional heterogeneity that we have not accounted for. Our analysis of floral color evolution supports this contention: two similar types of phenotypic change that involve the same biochemical pathway actually have very different mutation spectra, resulting in biases favoring different types of mutations.

This heterogeneity may have important implications for how one should investigate whether particular mutations are fixed preferentially. The standard approach is to combine large numbers of traits examined in a large number of organisms and ask whether there is evidence of substitution bias. If, however, the traits combined are heterogeneous for either the magnitude or direction of bias, conclusions may be greatly influenced by the particular traits included in the analysis. Therefore, we suggest that it may not be possible to generate a uniform theory of preferential substitution. Instead, as shown here, the heterogeneity introduced by combining traits and organisms is so severe that it appears necessary to evaluate substitution biases on a trait-by-trait basis. As we demonstrate with the floral color analyses, examining multiple, independent origins of the same trait offers an alternative approach that greatly reduces this problem of heterogeneity (see also Kopp 2009). This approach has the added advantage that detailed knowledge of the biological characteristics of the trait and its underlying developmental and metabolic pathways allows the investigator to make informed a priori predictions about mutation biases and the magnitude of pleiotropy associated with different categories of genetic change. For example, our understanding of the topology of the anthocyanin pathway and its regulators led to several predictions about mutation and fixation biases that were supported by our analyses.

IMPORTANCE OF PLEIOTROPY

One commonly proposed hypothesis for fixation and substitution biases is differential pleiotropy (Stern 2000; Carroll 2005a). Based on our understanding of the biology of anthocyanin production in plants, the biases we observed in the evolution of floral color are consistent with, but do not prove, this hypothesis. Specifically,

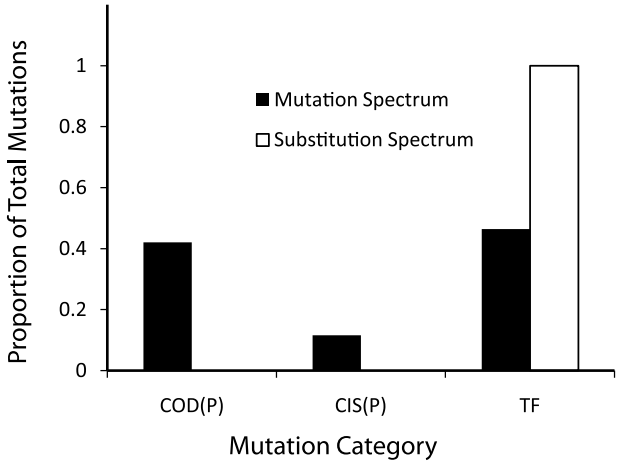

Figure 2. Mutation and substitution spectra for evolutionary transitions in floral pigment intensity. Mutation data are presented as the relative proportion of mutations in each mutational category, as defined in the text.

transitions involving alteration of pigment intensity, which most frequently involve loss of floral pigmentation, can be achieved through any of the three categories of mutation analyzed. Because in this case we are comparing different categories of mutation that all generate the same phenotype (change in pigment intensity), relative differences in the magnitude of deleterious pleiotropy likely account for the observed fixation biases among these mutation categories. As described above, COD(*P*) mutations are expected to incur substantial deleterious pleiotropy because these mutations affect gene products in all tissues in which the gene is expressed. By contrast, a CIS(*P*) mutation that causes tissue-specific changes in the expression of a pathway gene is expected to incur substantially less pleiotropy. Among TFs, inactivation of R2R3 MYB factors also is expected to incur relatively little deleterious pleiotropy, both because they are frequently floral-specific and because they appear to regulate only anthocyanin production. By contrast, inactivation of bHLH and WDR factors are expected to incur substantially more pleiotropy because they typically are expressed in a wider array of tissues and because they influence characters other than anthocyanin production.

Based on these considerations, our a priori expectation was that reduced levels of pleiotropy would lead to the disproportionate fixation of CIS(*P*) and TF mutations involving R2R3-MYB proteins. Although the sample size of substitutions affecting pigment loss is small, our results are consistent with this expectation: all transitions that have been sufficiently characterized involve MYB TFs. Although none involve CIS(*P*) mutations, this may simply reflect the low rate at which these mutations arise to generate this phenotype (Fig. 2). A limited amount of direct evidence also supports our expectation: data from field experiments in *Ipomoea purpurea* indicate there is substantial deleterious pleiotropy

associated with a coding mutation in an anthocyanin pathway gene that causes white flowers; by contrast little deleterious pleiotropy has been detected for a phenotypically similar mutation that inactivates the *IpMyb1* gene in this same species (Coberly and Rausher 2008). However, in this case, neither mutation has been fixed in populations, so it currently is unclear whether differential pleiotropy will lead to the preferential fixation of the *myb* mutant.

For floral hue transitions, our a priori expectation was that transitions from blue to red flowers disproportionately involve CIS(*P*) mutations. This prediction also is supported by our limited data but arguments about differential pleiotropy currently are based exclusively on indirect evidence. For example, in one survey of 110 angiosperm genera, Price and Sturgess (1938) found that all of the species examined produced either cyanidin- or delphinidin-derived pigments in leaves, but none produced pelargonidins. These results suggest that maintaining functioning cyanidin and delphinidin branches of the anthocyanin pathway in vegetative tissues are adaptive. This suggestion also is supported by transgenic experiments in *Petunia* and *Arabidopsis* in which it was found that an increase in quercetin in leaves (which requires a functional F3′H; Fig. 1) provides significantly higher tolerance to UV-B radiation relative to kaempferol (Ryan et al. 1998, 2001). These results thus suggest that it would be deleterious to inactivate the branching enzymes (F3′H and F3′5′H) throughout the plant. Instead, tissue-specific *cis*-regulatory mutations that reduce expression of the branching enzymes exclusively in flowers might limit these pleiotropic costs, which would allow these mutations to be fixed preferentially.

Our analysis of the Stern and Orgogozo (2008) dataset also suggests that differential pleiotropy contributes to substitution biases. However, in this case, the expected predictions for the direction of these biases are not clear. This likely reflects the difficulty associated with pooling data into such broad categories. For example, some have argued previously that physiological traits should evolve primarily due to coding mutations, whereas morphological traits should evolve predominantly through *cis*-regulatory mutations (Carroll 2005b). However, it is not always clear what constitutes a "morphological trait" or a "physiological trait" (see Hoekstra and Coyne 2007), and thus generalizations about the role of pleiotropy leading to fixation and substitution biases among these broad categories are difficult to formulate.

Although the patterns revealed by our analysis of the genetics of flower color transitions support our expectations, we realize that the sample sizes are small and that the patterns may change as more cases are examined. Moreover, more robust estimates of the mutation spectrum might result in different patterns of mutation and fixation biases. Finally, to confirm the hypothesized relationship between fixation bias and pleiotropy, empirical studies are necessary to quantify the fitness effects associated with different types of mutations under field conditions. Nevertheless, the consistency of our results with our a priori expectations about flower color evolution suggests that additional data of these types would contribute substantially to our understanding of the processes that cause substitution bias.

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Associate Editor: P. Phillips

Supporting Information

The following supporting information is available for this article:

Table S1. A list of spontaneous flower color mutations and substitutions fixed between populations or species obtained from the literature survey.

Table S2. Number of mutations reported by Stern and Orgogozo (2008), grouped by four categories: (1) taxa (plants or animals); (2) trait (morphology or physiology); (3) mutation category (coding mutation or *cis*-regulatory mutation); and (4) divergence (within-species or between-species).

Table S3. Analysis of substitution, mutation and fixation biases using data from Stern and Orgogozo (2008).

Supporting Information may be found in the online version of this article.

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