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Pollination genetics: Using molecular genetic underlying floral traits to study pollination ecology in an evolutionary context

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ABSTRACT

Pollination ecology and plant–pollinator relationships are major topics in studies of floral evolution and plant speciation. The genetic basis of floral traits is a key factor in determining the evolutionary response to pollinator-mediated selection. Despite the important role of genetic background in evolutionary studies, it has rarely been incorporated into pollination studies. Extensive literature on the reciprocal effect of pollinators and floral traits on each other exists, yet only a few studies tested the effect of molecular genetic variation of pollination-relevant floral traits on pollinator behavior. Here, I review these studies and propose a framework to study pollinator-mediated selection in an evolutionary genetic context.

Two lines of research are used to study the genetic basis of pollination. The first approach connects candidate genes for floral traits with pollinator behavior. These genes can be modified, and the consequent reaction of the pollinators' behavior is then observed. The second approach uses indirect estimation of the genetic effect by constructing the genetic architecture that underlies floral characteristics, and quantifying its indirect effect on pollinator behavior and consequent measured fitness. This connection between pollinator behavior and genetics of floral traits, accompanied by the effect of pollinators on plant fitness, can be combined into a cascade of effects in a hierarchical statistical model that gives pollination ecology studies an evolutionary insight.

Keywords: floral evolution, flower color, gene expression, insect behavior, path analysis, pollination ecology, pollinator-mediated selection, quantitative trait loci

THE EVOLUTIONARY SIGNIFICANCE OF POLLINATION SYSTEMS

Floral characters have been shown in numerous studies to be selected by pollinators (e.g., Campbell, 1989; Galen, 1989; Medel et al., 2003; Lau and Galloway, 2004; Armbruster et al., 2005; Irwin and Strauss, 2005; Buide, 2006). Pollinator-mediated selection is the consequence of the pollinators' choice of behavior, and has been proposed to play a major role in floral evolution, sometimes creating divergence among populations that can lead to pre-zygotic isolation and speciation (Grant, 1949, 1994; but see Armbruster and Muchhala, 2008).

Pollinators are attracted to the flowers by a suite of cues. Visual cues are important for detection of flowers by animal pollinators. For example, there is evidence that larger displays are seen by pollinators from further distances and attract more visitors (e.g., Galen, 1989; e.g., Campbell et al., 1991; Conner and Rush, 1996; Ne'eman and Kevan, 2001; Cariveau et al., 2004; Fenster et al., 2006). Floral fragrance is another attractive

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signal (Kunze and Gumbert, 2001; Gegear, 2005; Huber et al., 2005). Other cues, such as heat and echo fingerprints, also exist in flowers to attract pollinators from a distance (Raguso, 2004; Terry et al., 2004). All these floral cues are used as a signal for the reward, which is the main attraction for the pollinators, and the extent of advertisement is associated with the amount of the reward (Cohen and Shmida, 1993). The strong association between floral cues and pollinator behavior suggests that these traits are under natural selection exerted by the pollinators.

Evolutionary response to natural selection on floral traits requires underlying genetic variation (Endler, 1986). The phenotypic variation in the above floral characteristics, as well as many other floral traits, generally has a genetic component (Bradshaw et al., 1998; Juenger et al., 2000; Fishman et al., 2002; Hodges et al., 2002; Stuurman et al., 2004; Goodwillie et al., 2006; Bouck et al., 2007; Baack et al., 2008).

Floral traits that are tightly linked genetically or are pleiotropically interacted are expected to vary in concert (Hodges et al., 2002; Bouck et al., 2007). Even without genetic physical linkage, floral traits can co-vary under similar selection regimes to create “pollination syndromes” (Fenster et al., 2004). Genetic constraints, such as negative genetic correlations, can create contrasting evolutionary trends in floral traits (Ashman and Majetic, 2006; Caruso, 2006). Similarly, selection on the same trait by an agent other than pollinators (Strauss and Whittall, 2006) can mask the effect of pollinator-mediated selection on floral traits. Overall, massive literature exists for the quantitative genetic basis underlying floral traits, as well as for trade-offs in the evolution of floral traits (for review, see Conner, 2006). This literature, dealing mostly with heritability, correlations, and floral integration, is beyond the scope of the present review, and will be discussed elsewhere.

In pollination studies it is usually difficult to incorporate the effect of genetic divergence (or similarity) among plants on pollinator behavior into a comprehensive model. Indeed, there are currently only a handful of studies that manipulated plant genetics and measured pollinator behavior (e.g., Bradshaw et al., 1998; Bradshaw and Schemske, 2003; Hoballah et al., 2007). The genetic variation, either natural or manipulated, affects pollinator behavior, indirectly through floral characteristics, and pollinator behavior in turn affects fitness that forms the evolutionary response of the traits. In order to understand the evolutionary trajectories of response to pollinator-mediated selection, it is essential to test the connection between pollinator behavior and the genetic components of the floral traits, as well as the effect on plant fitness (Fig. 1).

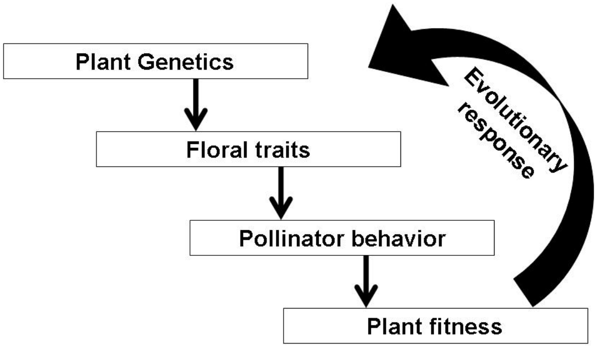

Fig. 1. Proposed framework for studying the evolutionary response of floral traits to pollinator-mediated selection. Arrows symbolize effects of each level on the other level; plant genetic composition or background affects floral trait phenotypes, and the response of pollinators to these traits affects the fitness of the plant. Evolutionary response of the trait, expressed by differential fitness, affects the genetic combination of the next generation of the plant.

Here, I review the approaches taken in pollination genetic studies, and propose potential directions of future research. I argue that the genetic background of the plant is important for understanding the evolutionary context of flower–pollinator interactions, and for understanding the evolutionary significance of pollinator-mediated selection. It is essential to test the effect of the genetic background of floral traits on pollinator behavior and preferences, alongside the effect of pollinator behavior on the evolutionary response of floral traits.

PLANT GENES AFFECTING POLLINATOR BEHAVIOR THROUGH FLORAL TRAITS

The complete causal genetic and developmental pathway from the molecular mechanism to a floral trait is understood in only a few cases (Clegg and Durbin, 2003). Floral traits that are involved in the attraction of pollinators can be controlled by either a single polymorphic gene controlling the trait, or by a few to many genes throughout the genome. In the latter case, the trait is usually quantitative and will be discussed later in this paper. For some floral traits it is possible to pinpoint the exact gene controlling it by associating allelic variation with floral polymorphism. This is especially true for floral color, which is usually controlled by a few genes, and it is possible to associate discrete floral genotypes with individual genes (Clegg and Durbin, 2000; Durbin et al., 2003; Schwinn et al., 2006). The biosynthetic pathways that determine floral pigmentation are well understood, and many of the genes involved have been isolated and their expression measured in various species (Durbin et al., 2003; Whittall et al., 2006). A few studies have shown that the natural variation in floral color can be due to differences both in the DNA sequence (and hence, in enzymatic activity) and in the expression of these genes (e.g., Schwinn et al., 2006; Whittall et al., 2006; e.g., Hoballah et al., 2007). Floral color is an important trait that is often correlated with other floral traits, resulting in pollination syndromes (Fægri and van der Pijl, 1979; Fenster et al., 2004). The conventional wisdom is that floral color is a trait adapted to attract the pollinators, and that different colors represent adaptations to a variety of pollinator visual systems (Fægri and van der Pijl, 1979; Grant, 1993). Thus, flower color is a useful trait for studying the genetics of floral adaptation to pollinators.

Studies in only a few species have shown the full path from a gene underlying pollination-associated floral traits to the behavior of the pollinators, and most dealt with floral coloration traits (Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003; Dyer et al., 2007; Hoballah et al., 2007; Kessler et al., 2008). The first study using this approach was work in *Mimulus* (Bradshaw et al., 1998; Schemske and Bradshaw, 1999; Bradshaw and Schemske, 2003). *Mimulus cardinalis* flowers are red due to high concentrations of anthocyanin and carotenoid pigments, and are pollinated by hummingbirds, while the flowers of *M. lewisii* are pink, low in anthocyanin pigment, and are pollinated by bumblebees (Schemske and Bradshaw, 1999). Observations on F_2 hybrids produced by crossing the two species, combined with genotypic data, revealed that bee visitation rate was significantly higher in plants that contained the *M. lewisii* allele in the YUP locus (Schemske and Bradshaw, 1999). A consequent experiment used near-isogenic lines, in which the YUP locus contained the allele of one of the species on the genetic background of the other species. In this experiment, *M. cardinalis* with the *M. lewisii* YUP allele had bee-associated color and received many more visits by bees than by hummingbirds, and vice versa (Bradshaw and Schemske, 2003).

The genus *Petunia* comprises species with distinct pollination syndromes, and a genetic study of these syndromes has shown that they are controlled by a few large-effect quantitative trait loci (QTL) (Stuurman et al., 2004). *Petunia axillaris* has white flowers and a hawkmoth pollination syndrome, while *P. integrifolia* has a violet-reddish flower and is visited primarily by solitary bees (Ando et al., 2001). Anthocyanin biosynthesis of floral color in these species is controlled by the transcription factor AN2. Loss of its function results in absence of anthocyanin pigmentation in *P. axillaris* (Quattrocchio et al., 1999). The effect of variation of the AN2 gene on pollinator preferences was tested by introgressing the AN2 chromosomal segment of either *P. axillaris* (AN2 inactive) or *P. integrifolia* (AN2 active) into a third species, *P. hybrida* genetic background. In addition, an active AN2 gene was transformed from *P. integrifolia* into *P. axillaris*. These plants were presented to pollinators and, indeed, bees preferred flowers with an expressed AN2, i.e., flowers with anthocyanin pigmentation, while hawkmoths preferred white flowers of which AN2 gene was non-functional, as in *P. axillaris* (Hoballah et al., 2007). This study showed that variation in one gene with a large phenotypic effect directly influenced pollinator behavior, and thus, in the *Petunia* case, can play a major role in reproductive isolation.

Following a similar rationale, experiments in snapdragon (*Antirrhinum majus*) flowers used plants with a single mutation that affected floral coloration to test the effect of these mutations on pollinator behavior (Dyer et al., 2007). The MIXTA locus controls petal epidermal cell shape, and its mutant changes the light reflectance from the pigment-containing cells. Although there were differences in the color and structure of the mutant and the wild type, bumblebees in a Y-maze experiment did not prefer the pink flowers of the mutant over the red and shiny flowers of the wild type. Moreover, naïve bees did not develop any preference for either. Another mutant, NIVEA, is non-pigmented and has much higher UV reflectance than the wild-type flowers. Despite the much stronger signal, bumblebees preferred the wild type over the mutant (Dyer et al., 2007). These results, although counter to expectations, are also pioneering as they measured the direct effect of the genetic polymorphism in one gene of the plants on pollinator behavior. In this case, the research failed to connect genetic polymorphism with pollinator behavior, but its importance is in the attempt to do so.

While visual cue is detectable to the human senses, there might be an invisible floral trait that manipulates the behavior of pollinators. Floral scent and secondary compounds in the nectar are factors that attract or repel, respectively, visitors to the flowers (Kessler and Baldwin, 2007; Raguso, 2008b). Flowers of the tobacco *Nicotiana attenuata* produce both fragrant benzyl acetone that attracts visitors, and nicotine that is laced to the nectar and repels visitors. Kessler et al. (2008) manipulated these two compounds by silencing the genes encoding them, using RNAi. They showed that the combination of both the attractant and the repellent is necessary to maximize seed-set and seed siring (female and male fitness, respectively), and increase out-cross rate. The presence of nicotine in the nectar had a dual effect on visitors to the flowers. First, it shortened the time of visit in the flowers, which increased chances for out-crossing. Second, it repelled two other visitors that harmed the attractive flowers, namely, nectar robbers and herbivorous insects (Kessler et al., 2008). The importance of this study is the pinpointing of the molecular genetic basis of a "push–pull" manipulation of the flower on its pollinators. This study also used molecular techniques that separated the effect of two secondary floral chemicals on pollinators' behavior, which is almost impossible in regular pollination ecology studies (Raguso, 2008a).

GENETIC ARCHITECTURE OF POLLINATION SYSTEMS

Another approach connects the genetic architecture of floral traits with pollinator behavior through the latter's reaction to variation in floral phenotypes. The genetic analysis here reveals the genetic architecture underlying the effect of floral traits on the variation or differences of pollinator behavior. This approach does not require prior knowledge of the function of the genetic marker. For example, differences among plants exhibiting divergent pollination systems can be associated with genetic differences of neutral markers, such as microsatellites. Association of variation in genetic markers with quantitative phenotypic variation in floral traits enables mapping the loci that control these traits (quantitative trait loci, QTL) (Erickson et al., 2004). QTL studies provide insights into the genetic architecture of floral traits, and variation of this architecture can explain differences in floral traits between species that are pollinated by different pollinators (Bradshaw et al., 1998; Hodges et al., 2002; Stuurman et al., 2004). Large effects of a few QTL can be an indicator that selection, as opposed to a drift, has shaped the trait (Karrenberg and Widmer, 2008).

Studying the genetic basis of pollination systems cannot be based solely on identification of QTL underlying the floral traits, but also requires evidence that the variation of these loci is indeed responsible for variation in pollinator behavior. Two methods to use QTL variation to understand variation in pollinator behavior have been suggested. To the best of my knowledge, these are the only studies that used quantitatively floral QTL to explain pollinator behavior. Thus, I give here a broader description of these two studies.

The first used the behavior of the pollinators on individual plants as an extended phenotype of the plant, which can be mapped as a QTL. Such an approach was taken for Louisiana irises (Martin et al., 2008). Two species of Louisiana irises, *Iris fulva* and *I. brevicaulis*, are reproductively isolated through their pollination system; *I. fulva* is adapted to hummingbird pollination, while *I. brevicaulis* is bee-pollinated (Wesselingh and Arnold, 2000). Natural and artificial hybrids are intermediate for floral traits, such as color, shape, and nectar guides, and are visited by a mixture of hummingbirds, bees, and butterflies (Martin et al., 2008). These floral traits were genetically mapped throughout the *Iris* genome in a back-cross population (Bouck et al., 2007). The back-cross plants were planted in the natural habitat and were observed for animal visitors. Preferences of these visitors to the different genotypic classes were recorded, and the variation in visitors' preferences among the plants was used as a plant phenotypic trait to be mapped as QTL (Martin et al., 2008). These pollinator behavior QTL were then compared to a floral trait QTL map to find regions of co-localization of pollinator behavior with floral traits. A few QTL were found in the plant genome that affect pollinator behavior, including some QTL that differed among pollinator taxa, and some QTL that co-localized with floral trait QTL. The latter may hint at the mechanism of which selection is acting on floral traits through pollinator preferences and behavior. Also, these QTL could serve as domains for searching for candidate genes that affect pollination systems and ecological divergence among species (Arnold, 1994; Grant, 1994; Bouck et al., 2005). A note of caution: although the approach is intriguing, it should be noted that the QTL found are of the plant and not of the pollinators. This approach is indirect and aims to explore plant evolution, while the genetic basis of pollinator behavior and its cognitive source remain a "black-box" in that context. However, the method used by Martin et al. (2008) is important for further exploring the plant genomic regions associated with pollinator-relevant phenotypes.

A slightly different method used the allelic composition of floral trait QTL to test how this content affects floral phenotypes, and how these phenotypes, in turn, affect pollinator visitation rates (Sapir, 2009). Recombinant inbred lines of crop × wild sunflowers (*Helianthus annuus* L.) were genotyped by simple sequence repeats (SSRs; microsatellites), and a genetic map was constructed using these molecular markers (Baack et al., 2008). Floral traits were measured and mapped as QTL throughout the genome. The question was whether the variation in these QTL, representing the genetic architecture of floral traits, can explain the variation in pollinator behavior, mediated through floral phenotypes. Statistically, this connection was made using path analysis, which is a hierarchical set of regression analyses, solved together by structural equation modeling techniques (Mitchell, 1992). Results of these analyses showed that the full path from plant genetics to floral phenotype to pollinator behavior and to fitness is statistically valid for a subset of the pollinators, suggesting that there might be differential selection exerted on floral traits via differential behavior of pollinator functional groups. The method is promising because it uses a framework that can be relatively easily solved statistically, and gives clear interpretations of the mechanism underlying pollinator-mediated selection. Path analysis has been shown to be useful in quantifying selection in a hierarchical system of indirect effects (Kingsolver and Schemske, 1991; Mitchell, 1994; Scheiner et al., 2000; Cariveau et al., 2004; Irwin, 2006). It is a practical statistical method to incorporate the genetic basis of traits undergoing pollinator-mediated selection with pollinator behavior in order to get an evolutionary prediction for the pollination systems.

SYNTHESIS AND FUTURE DIRECTIONS

One important goal of studies in ecological genetics is to understand the molecular genetic mechanisms underlying ecological phenomena (Stinchcombe and Hoekstra, 2007). Above, I have categorized studies connecting genetics and pollination using either candidate genes controlling specific floral trait, or an indirect approach using the genetic architecture of floral traits. While the indirect approach can use non-model plants and is easier to perform than the candidate-gene approach, it provides only a suggestive causal pathway, without specifying the mechanism. On the other hand, three of the four studies that tested direct effects of a gene on pollinator behavior have been confined to only one trait, floral color (Bradshaw and Schemske, 2003; Stuurman et al., 2004; Hoballah et al., 2007). In numerous pollination ecology studies, traits other than color have been shown to be important for pollinator behavior, including floral odor, floral size and shape, and nectar traits (Campbell et al., 1991; Eckhart, 1991; Mitchell, 1994; Conner and Rush, 1996; Fenster et al., 2004; Raguso, 2004; Buide, 2006; Fenster et al., 2006; Martin et al., 2008; Smith et al., 2008). The study by Kessler et al. (2008) on *Nicotiana* that used molecular genetics of floral volatiles is an example of using molecular genetic tools for identifying important traits for pollination that otherwise are invisible to regular pollination ecological studies (Raguso, 2008a). This study emphasizes that more molecular knowledge is needed on the genetic basis of floral traits in the context of pollination.

While the elegant studies in *Mimulus*, *Petunia*, *Antirrhinum*, and *Nicotiana* are appealing, it should be noted that most floral traits are difficult to track down to the single-gene level, and usually there are diverse biochemical, developmental, and genetic mechanisms interacting with the gene–phenotype–pollinator pathway (Clegg and Durbin, 2003). In other words, most of the floral traits are quantitative and not inherited in a simple Mendelian way. Floral color is probably among the simplest floral traits that can be studied from gene to ecological interaction, but even this trait can be a complex of molecular and cellular interactions (Clegg and Durbin, 2003). Molecular evolutionary studies of floral development are another way to address pollination genetics. Homeotic genes or transcription factors can affect expression of floral traits and create quantitative differences that affect the pollination system (Krizek and Fletcher, 2005; Kramer, 2007; Theissen and Melzer, 2007). Genetic regulatory networks controlling floral development are a complication of the picture for pollination genetics (Espinosa-Soto et al., 2004; Chaos et al., 2006; Irish, 2008). While studying genetic networks in the context of evolutionary pollination ecology seems complicated, it could advance the understanding of the genetic basis of pollination interactions. High-throughput methods using, for example, microarrays or Expressed Sequence Tags (ESTs) from flowers can provide a broader view of the genes affecting pollination-relevant floral traits (Wellmer et al., 2004; Shiu and Borevitz, 2006; Shimamura et al., 2007). Once candidate genes controlling floral traits are identified, techniques such as gene silencing can be applied to test the effect on the pollination system (Hammond et al., 2001; Kessler et al., 2008). The advantage of such methods is that in a relatively short time, one can get a phenotypic change in a floral trait, as opposed to the laborious development of genetic lines (e.g., the genetic lines developed by Bradshaw and Schemske, 2003), that can be tested for the effect on pollinator behavior.

The studies in *Petunia* and *Mimulus* suggest that pollination systems can be shifted by a change in a single structural gene (Bradshaw and Schemske, 2003; Hoballah et al., 2007). On the other hand, studies in *Ipomoea* and *Aquilegia* suggest that regulation of the expression of genes controlling floral traits can create these shifts (Durbin et al., 2003; Whittall et al., 2006). Developing genetic libraries for expressed genes is another promising direction for identifying genes involved in the pollination system. This method is especially advantageous in genes that do not directly code for a floral trait, but create differences in floral traits through differential expression by regulatory function, controlling structural genes by epistasis (Tiffin et al., 1998). Using large-scale scanning of transcriptome or proteome and comparing it among different phenotypes may be useful to identify higher-level cellular interactions among genes and floral traits, and will provide an insightful view into the genetic basis of pollination-meaningful floral traits (Karr, 2007).

All the above methods can be used to develop new directions in the field of pollinator-mediated floral evolution. One such promising direction is testing hypotheses on the relative effect of a single floral trait on pollinator behavior. Traditionally, this is done by crossing and artificially selecting for a trait value desired, but this can disrupt other floral traits due to genetic correlations (Gomez, 2000; Worley and Barrett, 2001; Hansen et al., 2003; Pérez-Barrales et al., 2007). By using molecular manipulations, only the trait of interest is modified, even when it controls by a genetic network. Another direction is identifying genes controlling pollinator behavior on a phylogenetic background. Ancestral reconstruction of pollination-relevant floral traits (e.g., Johnson et al., 1998; Kay et al., 2005; Pe'rez et al., 2006; Hu et al., 2008) is a useful source for understanding floral evolution. As for now, there are only a few studies that used direct observations of pollinators, in addition to inferring the pollination system from floral traits, to explore the role of pollinators in floral evolution, using phylogenetic trait reconstruction (Armbruster, 2002; Smith et al., 2008). Combining the candidate gene approach and phylogeny can pinpoint not only what modifications were made, but also the molecular evolution of the gene controlling the trait. This can bridge the gap between micro- and macro-evolution in pollination studies.

Overall, studies of the genetic basis of pollination systems are currently lacking enough power to make a strong statement on the role of pollinators in shaping the plant genome at the molecular level, as well as the role of the plant genome in affecting pollinator behavior. Despite the evidence for a strong effect of a single gene on pollinator behavior (Bradshaw and Schemske, 2003; Hoballah et al., 2007; Kessler et al., 2008), or the lack of such effect (Dyer et al., 2007), it is also clear that quantitative traits are important to shape the pollination system, and that their effect is more continuous (Martin et al., 2008). Moreover, connecting a specific change in a single (or a few) gene(s) to a shift in pollination system has been studied in only a few systems (Bradshaw and Schemske, 2003; Dyer et al., 2007; Hoballah et al., 2007; Kessler et al., 2008). While most of these systems (*Mimulus*, *Petunia*, and *Antirrhinum*) are becoming a model system for floral evolution (Hileman et al., 2003; Gerats and Vandenbussche, 2005; Wu et al., 2007), there is a need for similar studies in non-model systems.

Considering the framework suggested here (Fig. 1), the majority of the studies on the evolution of flower–pollinator interactions connect only two or three levels, but not the full path from gene to pollinator behavior (but see Galen, 1989, Campbell et al., 1991; and Conner, 2006). For example, there are many studies of the genes controlling floral development, the majority of them in the model plant *Arabidopsis thaliana* (Kramer, 2007; Irish, 2008), but also in other plants (e.g., Comba et al., 2000). In addition, there are many studies on the genetic architecture of floral traits, mainly at the form of QTL mapping (Juenger et al., 2000; Fishman et al., 2002; Hodges et al., 2002; Goodwillie et al., 2006; Bouck et al., 2007; Baack et al., 2008). The number of studies connecting variation in floral phenotypes with pollinator behavior is even higher, and only an exhaustive study would enable one to review them. Some of these studies also looked at the fitness effects of the pollinator behavior and estimated selection exerted by the pollinators on the measured floral traits (e.g., Campbell et al., 1991; Medel et al., 2003; Cariveau et al., 2004; Lau and Galloway, 2004). However, only in the examples given above there is a connection of the specific gene loci with both floral traits and pollinator behavior. The small number of such studies, and even fewer studies that connect all four levels of effects, from plant genetics to the evolutionary response through fitness, is striking. I propose the framework given in Fig. 1 as a guideline for studying the role of pollination in an evolutionary context. Studying all the steps, from the molecular genetic basis of floral traits, followed by the effect of its phenotypic expression on pollinator behavior, to the fitness outcome, is crucial for understanding floral evolution. There is a need for more studies that utilize this framework in order to generalize the evolutionary perspective on pollination systems.

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