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The Role of Animal Pollination in Plant Speciation: Integrating Ecology, Geography, and Genetics

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Key Words

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Abstract

Although animal pollination is often proposed as a major driver of floral divergence, questions remain about its importance in plant speciation. One issue is whether pollinator specialization, traditionally thought necessary for floral isolation, is prevalent enough to have played a major role in speciation. Furthermore, the ecological and geographic scenarios under which pollinator transitions occur are poorly understood, and the underlying genetic factors are just beginning to be uncovered for a few systems. Nevertheless, macroevolutionary studies consistently show that transitions to animal pollination are accompanied by an increase in diversification rate. Here we consider several models and diverse empirical data on how pollinators could influence speciation. We conclude that floral isolation is rarely, if ever, sufficient to cause speciation on its own, but that it acts synergistically with other isolating mechanisms. A more comprehensive approach is the key to an improved understanding of the role of pollinators in angiosperm speciation.

1. INTRODUCTION

Assortative mating: a pattern in which individuals tend to mate with other individuals exhibiting a similar phenotype

Floral isolation: reduced pollen transfer from anthers to stigma between species compared to within species, caused by differences in floral traits

The diversification of the angiosperms has long fascinated biologists. Many hypotheses have been put forth to explain the rapid radiation and overall species richness of flowering plants. One popular idea is that the evolutionary innovation of flowers has enabled specialized associations with animals for pollination, and that these associations drive divergence. Because a plant's reproductive success is tied to the attraction and manipulation of its floral visitors, pollinators can exert selection on such floral traits as reward, timing, shape, color, scent, and display. Geographic differences in pollinator assemblage can therefore causedivergent selection on floral traits (Grant 1949, Grant & Grant 1965), and as animal pollinators themselves evolve, the pollinator assemblages provide shifting phenotypic optima for the plants they pollinate (Schemske 2009, Thompson 2005).

Floral trait evolution in response to the local environment can result in assortative mating upon sympatric contact through floral isolation. We define floral isolation to mean a reduction in interspecific pollen transfer relative to intraspecific pollen transfer that is caused by floral traits. It may involve differences in the identity, timing, or behavior of the pollinators, as well as mechanical isolation through differences in pollen placement. The evolution of strong assortative mating is synonymous with speciation under the biological species concept. In this way, biotic pollination may drive diversification by causing reproductive isolation and directly increasing speciation rates.

Biotic pollination may contribute to diversification in other ways. Sympatric lineages that directly compete for pollinators or produce unfit hybrids may diverge in floral phenotype through character displacement or reinforcement (reviewed in Armbruster & Muchhala 2008). In this scenario, floral isolation is favored by natural selection after plant lineages already have diverged in other traits that contribute to reproductive isolation. Biotic pollination may also reduce the extinction rate for rare plants by assuring pollen transport among isolated individuals. Conversely, specialized biotic pollination may leave plant lineages vulnerable to extinction because of spatial or temporal variation in pollinator visitation (Waser et al. 1996).

Although floral isolation has been a focus of plant speciation studies for more than a half century (Grant 1949), it remains a controversial topic. How does it evolve, and how important is it as a cause of plant biodiversity? Here we review these issues and discuss key areas for future work.

2. SPECIALIZATION IN POLLINATION SYSTEMS AND THE FLORAL ISOLATION HYPOTHESIS

Results from empirical studies of a variety of plant-pollinator communities show that most flower-visiting animals visit multiple plant species and most biotically pollinated plants are visited by multiple species of animals (Bawa 1990, Olesen & Jordano 2002, Schemske et al. 1978). Many pollinators are opportunists with labile preferences, and at least under some conditions, spatial and temporal variation in the pollinator assemblage should favor generalization in pollinator attraction by the plant (Waser et al. 1996). Some therefore have proposed that pollination is too generalized to contribute to reproductive isolation and that the role of plant-pollinator interactions in speciation has been overemphasized (Waser 2001).

What Is Meant by Specialization?

At present, there is little agreement about what constitutes specialized relationships. Plants may adapt to functional groups of multiple pollinator species that exert consistent selection pressures rather than to a single pollinator species (Fenster et al. 2004). Pollinator species also vary greatly

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in their contribution to a plant's reproductive success because of their relative visitation frequency and efficiency at pollen transport. For both reasons, counting individual species of pollinators in an attempt to quantify the degree of specialization can be misleading. These issues are exemplified by Waser et al. (1996) and Fenster et al.'s (2004) analyses of the same dataset of plant-pollinator interactions; counting the number of functional groups standardized by their visitation frequency rather than the number of species of likely pollinators per plant gave a strikingly different picture of the level of specialization. Ideally, pollinator importance would be estimated for each floral visitor before inferring the level of specialization of a pollination system (Reynolds & Fenster 2008), although this painstaking work is rarely done (but see Schemske & Horvitz 1988). Rather, the vast majority of studies equate visitation with pollination, so that much of our empirical understanding of plant-pollinator relationships is based on a proxy measure for pollination.

Pollinator importance: the product between visitation rate and per visit effectiveness at depositing pollen or causing seed set

What Is the Role of Pollinator Specialization in Speciation?

Many community-level studies of plant-pollinator interactions assume that a lack of evidence for widespread specialization of plant-pollinator interactions undermines the importance of floral isolation in speciation. However, floral isolation requires differences in pollination among closely related plant species, not within the plant community in general. For example, plants pollinated by different functional groups of pollinators, e.g., hummingbirds versus bees, are reproductively isolated regardless of the level of pollinator specialization. Similarly, assortative mating can result from plants sharing pollinators that differ in the relative frequency and effectiveness of visits. Counts of the number of species of pollinators per plant are not the relevant metrics to understand the importance of floral isolation; instead, we need to estimate the overlap in pollinator assemblage for closely related plant species, incorporating measures of pollinator importance.

Grant (1949) summarized variation in the importance of floral isolation across a large sample of plant genera by assessing the proportion of species-level diagnostic characters that are floral versus vegetative. He assumed that groups for which floral isolation is important should have diagnostic floral characters. To date, however, most evidence for a role of floral specialization in speciation comes from case studies of co-occurring, interfertile pairs of closely related species (e.g., Grant 1949, 1992; Kay & Schemske 2003; Ramsey et al. 2003; Smith et al. 2008c). Yet these species pairs may have been chosen for study because of their obvious floral differences. Studies of a broader range of plant taxa are necessary before any general inferences about the importance of floral isolation are possible.

Regardless of methodological issues in quantifying specialization, it is unclear how strong floral isolation must be to be a driver of plant speciation. One contentious issue is whether floral divergence can be initiated and proceed in sympatry (Armbruster & Muchhala 2008, Waser 2001). The argument against this is the same as that against sympatric speciation driven by disruptive sexual selection (reviewed in Coyne & Orr 2004, pp. 127–30). For floral isolation to evolve in sympatry, divergent floral traits would need to confer strong floral isolation through differential pollinator attraction nearly instantaneously, and the plant lineages would need to quickly evolve ecological differences that allow for their stable coexistence. Sympatric speciation through floral isolation is improbable, and there are no well-supported empirical examples. We focus instead on the classic hypothesis of floral isolation involving geographic isolation during the initial stages of floral divergence, with different pollinator assemblages across a landscape applying divergent selection pressures (Grant 1949). In this scenario, strong floral preference by pollinators at the initial stages of floral divergence is not required. When divergent floral phenotypes experience secondary sympatry, other ecological traits or intrinsic genetic incompatibilities acquired in

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allopatry also will contribute to reproductive isolation, so that pollination by itself need not provide complete assortative mating. In this scenario, the necessary strength of assortative mating required by floral isolation depends critically on the strength of isolation provided by other mechanisms. We treat this issue in greater detail below.

Pollination syndrome: a suite of floral characters associated with the preferences, behavior, and morphology of a specific functional group of pollinators

Sexual deception: a pollination system in which flowers mimic chemical and/or visual female insect mating signals to attract male insect pollinators

What Is the Evidence that Pollinator Specialization Contributes to Floral Isolation?

We have attempted to compile a comprehensive list of documented or putative cases of floral isolation in **Supplemental Table 1**. (Follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org.**) In many plant lineages, pollination syndromes, or suites of floral traits associated with pollinator functional groups, are evolutionarily labile (e.g., Armbruster & Baldwin 1998, Beardsley et al. 2003, Fulton & Hodges 1999, Ippolito et al. 2004, Kay & Schemske 2003, Kay et al. 2005, Martin et al. 2008, Perret et al. 2003, Ramsey et al. 2003, Smith et al. 2008b, Thomson & Wilson 2008, Tripp & Manos 2008, Whittall & Hodges 2007). For several of these systems, field studies have confirmed the contribution of pollinator shifts to reproductive isolation. For example, hummingbird pollination has evolved repeatedly from bee pollination in the neotropical genus *Costus*, and sympatric species pairs differing in syndrome have almost no overlap in pollinator use (Kay & Schemske 2003).

Some plant groups may not exhibit divergent syndromes, but rather diverge in the identity or relative importance of pollinators within a functional group. For example, the Iochrominae is a group of predominantly hummingbird-pollinated shrubs in the Andes, where Smith et al. (2008c) found that sympatric species pairs sharing a hummingbird syndrome exhibit a mean proportional similarity in pollinator assemblage of 0.57 (on a scale from 0 to 1). This indicates substantial, but incomplete, floral isolation caused by differences in the identity and relative importance of hummingbird species for pollination. The insect-pollinated genus *Calochortus* shows a similar pattern, in which sympatric species pairs have overlapping pollinator assemblages, but many individual pollinator species show a significant preference for one *Calochortus* species over the other (Dilley et al. 2000). Another class of examples involves systems with evidence of coevolution between plants and pollinators at the species level, in which small changes in floral traits or pollinator preferences are thought to drive speciation. These interactions, in which both the plant and animal species are highly specialized, are rare and often involve pollinating seed consumers or sexual deception. The most striking examples include pollinating seed consumer mutualisms of the figs, yuccas, senita cacti, and Phyllantheae, and the sexually deceptive orchids (Cozzolino & Widmer 2005, Fleming & Holland 1998, Kawakita & Kato 2009, Pellmyr et al. 1996, Ronsted et al. 2005).

Some plant lineages exhibit specialization in other features, e.g., divergence in flowering time (Stiles 1975) or the site of pollen placement on the pollinator (Armbruster et al. 1994, Grant 1994, Kay 2006, Kress 1983). Individual pollinators of the same species may also show floral constancy by preferentially visiting a floral type (Chittka et al. 1999). Although empirical studies suggest that strong floral constancy is uncommon (Chittka et al. 1997, Heinrich 1976) and unlikely to be a primary mechanisms of reproductive isolation, it may complement other isolating mechanisms. We hypothesize that this latter type of specialization—divergence in the relationship with the same pollinator species—may often be caused by character displacement or reinforcement, whereas other types of specialization may be more likely the result of differences in the abundance and effectiveness of pollinators over space and time. Without a cost to using the same pollinator species the same way, closely related plants should be similarly selected for the floral phenotype that most efficiently uses the pollinator.

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3. EVOLUTIONARY DYNAMICS OF POLLINATOR SHIFTS

One of the unresolved mysteries of pollinator-mediated speciation is the process by which shifts between pollinators occur. Research in this area has focused on cases where floral traits appear fine-tuned to the main pollinator. It is difficult to determine how plants adapted to one functional group of pollinators could shift to a different one (that is, an adaptive peak shift). Yet, we know that shifts among syndromes occur, in some lineages repeatedly (Kay et al. 2005, Perret et al. 2003, Thomson & Wilson 2008, Whittall et al. 2006). For the most part, we are unable to explain the process of pollinator shifts under complex ecological circumstances.

Adaptive peak: a particular combination of allele frequencies or traits that maximizes the mean fitness of a population

Most effective pollinator principle (MEPP): floral evolution is driven by the most frequent and effective pollinator(s) in the local environment

When Do We Expect Shifts to Arise?

Although quantitative genetic studies have identified some of the genetic mechanisms by which pollinator shifts can proceed (Bouck et al. 2007, Bradshaw et al. 1998), the ecological conditions under which shifts occur are not well understood. Intuitively, dramatic changes in ecological conditions such as the extirpation of a principal pollinator should result in strong selection for a pollinator transition (Campbell 2008). Similarly, an expansion of a species range that is not accompanied by a range expansion of the main pollinator(s) may initiate a pollinator transition (Ramsey et al. 2003). Declining pollinator populations may have an underlying abiotic cause, such as climate change (Memmott et al. 2007), and shifts in plant community composition could lead to changes in the available pollinator community.

Alternatively, the availability of a superior pollination system, through a range shift or floral mutation, could initiate a pollinator shift. For example, Maad & Nilsson (2004) show that eye-attachment of pollinia on moths is more efficient for pollen import in *Plantanthera* orchids than the ancestral trait of tongue-attachment, and they suggest that extreme pollen limitiation in portions of a species' range has driven evolutionary shifts in pollinia placement. These differences in pollinia placement then confer strong mechanical floral isolation upon secondary sympatry.

One of the earliest models of how plant-pollinator specialization might operate is Stebbins' (1974), Most Effective Pollinator Principle (MEPP). The MEPP postulates that pollinators vary in their effectiveness at transporting pollen and that selection favors traits that promote visitation by the most frequent and effective pollinator. Given that most plant species can potentially interact with multiple pollinator species, what conditions favor evolutionary specialization on the most effective pollinator versus generalization? Recent models predict that whether a plant species evolves to specialize or generalize depends on the fitness trade-offs involved in attracting multiple pollinators (Aigner 2001, Muchhala 2007, Sargent & Otto 2006; **Figure 1**). Specialization also has advantages beyond promoting visitation by the most effective pollinator. For example, specialization on a pollinator can ensure conspecific pollen transport in a diverse plant community (Sargent & Otto 2006).

Expanding on the MEPP, Thomson (2003) introduced the idea that a poor pollinator acts essentially as a parasite by wasting pollen that could have been transported by a superior pollinator. By this reasoning, when a more effective pollinator becomes available, plants should evolve to deter the inefficient pollinator and attract the efficient one (Castellanos et al. 2004). For example, hummingbirds are thought to be more efficient at transporting pollen than bumblebees, which may explain the prevalence of transitions in that direction (Thomson & Wilson 2008). Work on *Mimulus* supports the assertion that traits that attract more hummingbirds can also serve to deter bees, and vice-versa (Bradshaw et al. 1998, Schemske & Bradshaw 1999).

An alternative to the MEPP is that pollinator shifts evolve through reinforcement, although this possibility has rarely been explored. Under this scenario, incipient differences in a trait

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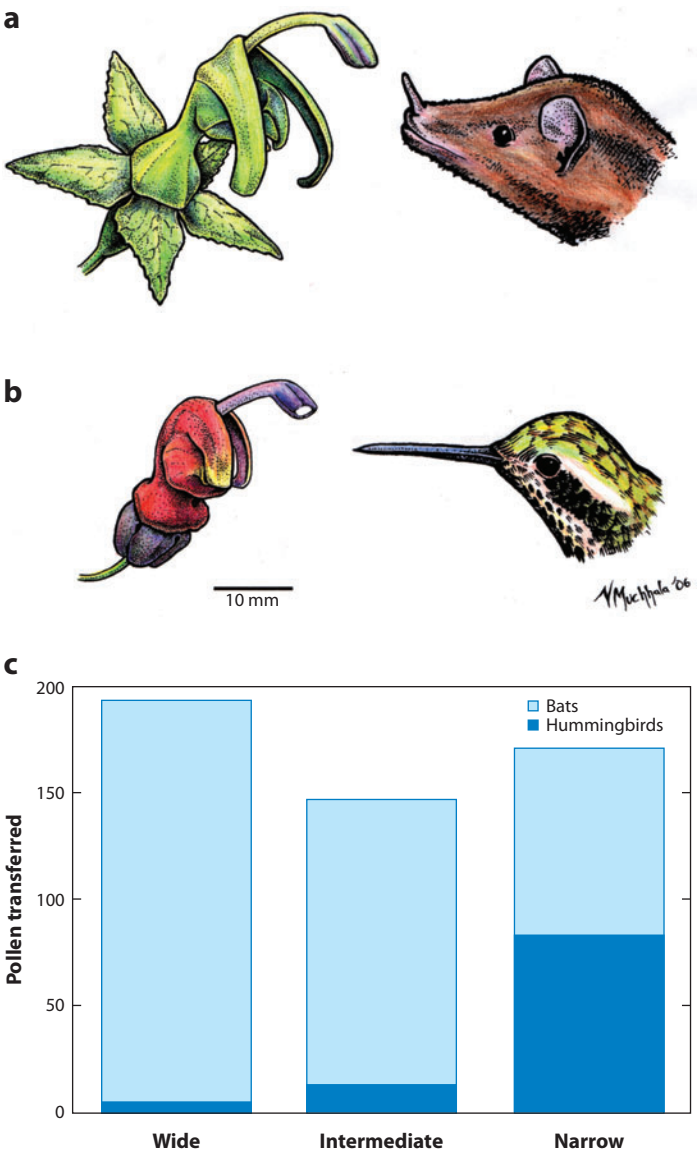


Figure 1

Differences among species and/or habitats in the shape of the fitness trade-offs associated with attracting different pollinators may play a key role in the evolution of floral isolation. In particular, concave trade-offs, in which intermediate forms are at a disadvantage, are likely to result in specialization rather than generalization (Sargent & Otto 2006). Muchhala (2007) published one of the first examples of data consistent with a concave trade-off between adaptations corresponding to pollination by bats (*a*) or hummingbirds (*b*) in the genus *Burmeistera*. Muchhala's data (reproduced in *c*) demonstrate that bats are more efficient at moving pollen between wide flowers, whereas hummingbirds excel at pollen transfer between narrow flowers (although in no case did hummingbirds transfer more pollen than bats). Intermediate flower widths transfer the least pollen. Muchhala speculates that narrow corolla width persists in areas where bat pollination is relatively rare. His data indicate that intermediate phenotypes are less fit, suggesting that the shape of the trade-off could underlie the evolution of specialization in this system. Illustration by N. Muchhala.

contributing to premating isolation, such as pollinator use, are increased by direct selection to reduce the frequency of maladaptive hybridization. Although the importance of reinforcement in plant speciation is debated (Kay & Schemske 2008), a recent study proposes it as an explanation for floral isolation among plant species in the Cape Flora. A comparative analysis found that pollination shifts are more commonly associated with edaphic shifts when a sister species pair are in contact than when they are allopatric, suggesting that pollinator shifts evolve upon secondary contact (van der Niet et al. 2006).

What Role does Geographic Variation Play in Pollinator Shifts?

It may be appropriate to envision plant species as adapting to a geographically shifting pollinator climate, rather than a single type of pollinator or functional group (Grant & Grant 1965). The Grants described clinal patterns of selection for different pollinators throughout a species range, resulting in a compromise: The degree to which the plant species as a whole is adapted to a particular pollinator depends on the relative frequency of effective visits. Additionally, where population density declines across a species' range from the center to the range boundary, theoretically species will more closely track the optimal phenotype at the range center, while gene flow restricts adaptation near the boundary (Kirkpatrick & Barton 1997). We expect that the degree to which floral traits are adapted to the local pollinator environment depends on geographic variation in the optimal floral phenotype, differences in population density across a species' range, and the extent of gene flow among populations. Furthermore, because the floral phenotype may be mismatched to the pollinator assemblage in peripheral populations, divergence can occur rapidly in the event that gene flow is disrupted (**Figure 2**). Although few studies have done the meticulous work necessary to demonstrate variation in pollinator-mediated selection across a species range (but see Anderson & Johnson 2008), it could be an important clue in determining the capacity for future pollinator-mediated divergence. We expect that geographic variation in pollinator-mediated selection is driven not just by variation in the pollinator assemblage, but also by variation in the community of coflowering plants that may compete for pollinators or facilitate pollination through sharing (Moeller 2004, Sargent & Ackerly 2008).

4. THE GENETIC BASIS OF FLORAL ISOLATION

To understand how speciation proceeds, it is crucial to identify the nature and distribution of genetic changes causing reproductive isolation. Specifically, we need to know the number and location of loci involved; and for each allele, we must know the magnitude of its phenotypic effect, whether it is dominant or recessive, and whether it is structural or regulatory. Ideally, it would be possible to understand the timing and order of the appearance and fixation of underlying mutations. Is speciation caused by a series of genes of small effect? Could speciation be initiated by large-effect mutations that allow a lineage to make a leap toward a new adaptive peak? Can reproductive isolation evolve from changes in the frequencies of alleles that are already present in a population or does it depend on new mutations? We are at the beginning of an era in which it is becoming possible to answer these types of questions.

Floral isolation is an attractive subject for genetic studies of speciation. Traits such as flower color, shape, and reward are relatively easy to measure, and their impact on prezygotic isolation through pollinator specificity can be experimentally tested. Closely related plant species isolated primarily by pollinators may be compatible in artificial crosses, facilitating the genetic dissection of key traits. Aside from the case of simple Mendelian color polymorphisms, however, genetic studies of floral traits until recently were restricted to the statistical estimation of genetic variances

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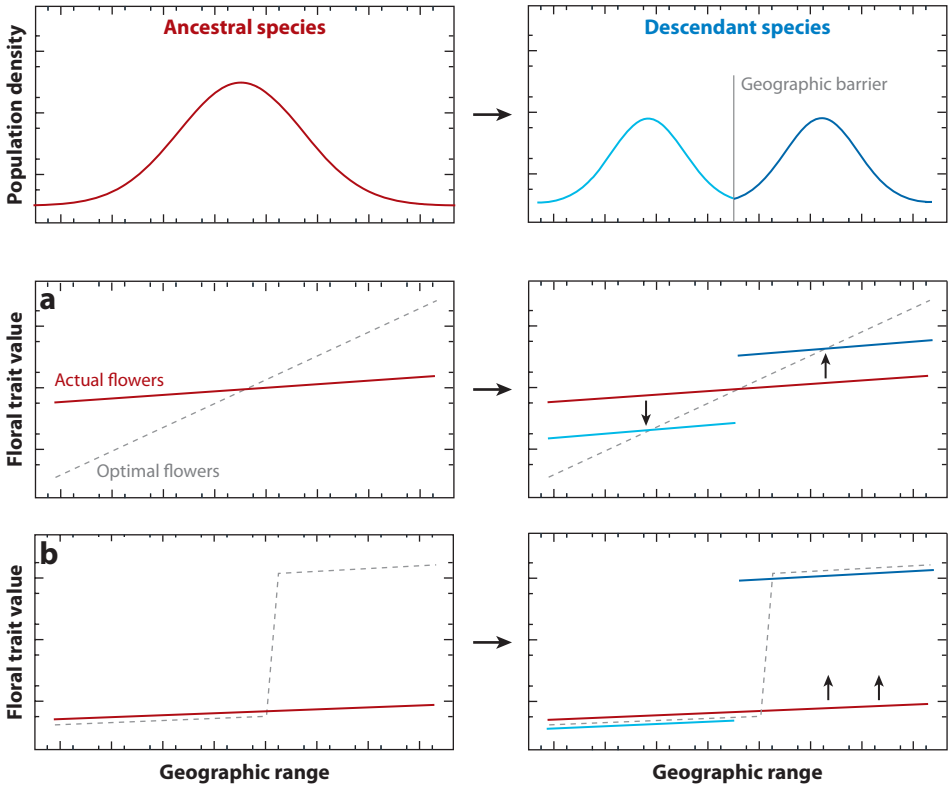


Figure 2

(*a*) Floral evolution across a geographic range with pollinators driving a linear environmental gradient. Kirkpatrick & Barton (1997) presented a model demonstrating how gene flow from the high-density center of a species geographic range could prevent peripheral populations from tracking the local optimum of a trait. Applying their model to the floral phenotype could help explain a lack of perfect correspondence between floral traits and the local most effective pollinator (*left*). We extend their idea to incorporate a vicariance event (*right*) that allows isolated populations to quickly evolve toward the optimal phenotype at the new centers of highest population density. Upon secondary contact the descendant species retain the divergent floral phenotypes favored at their range centers, and may even undergo character displacement in sympatry. (*b*) Floral evolution across a geographic range with discrete floral optima driven by concave trade-offs in pollinator attraction, e.g., between pollination syndromes. The optimal floral phenotype across the range takes the shape of a step function (*left*), with the actual floral phenotype matching the optimum at the range center. Assuming sufficient genetic variance for a large phenotypic shift, geographic isolation would promote a quick shift to a new optimum (*right*), and the new floral phenotypes would coexist upon secondary sympatry.

Quantitative trait locus (QTL): a region of the genome associated with phenotypic variation in a continuous trait like nectar volume or flower color

Candidate gene: a gene suspected to cause or contribute to a particular phenotype, typically identified in model genetic organisms

and covariances, selection gradients, and responses to selection (Conner 2006). Two important advances have led to a better understanding of the genetics of floral isolation and have opened up entirely new fields of research. First, quantitative trait loci (QTL) mapping techniques have extended Mendelian analysis of simple genetic traits to the analysis of quantitative traits controlled by multiple genes, using molecular markers and arrays of segregating progeny. Additionally, the discovery and functional analysis of genes in model genetic systems have provided candidate genes for floral traits in wild plants. These approaches answer different, but complementary, questions about the genetic basis of trait differences.

Quantitative Trait Loci Mapping of Floral Traits Involved in Prezygotic Isolation

QTL mapping studies can identify the number of loci contributing to trait differences, the magnitude of their effects and mode of action, and their distribution throughout the genome. Following the groundbreaking studies of hummingbird-pollinated *Mimulus cardinalis* and its bee-pollinated sister species *M. lewisii* (Bradshaw et al. 1998), QTL studies of floral isolation have now been performed with several wild plants, such as *Iris fulva* and *I. brevicaulis* (Bouck et al. 2007), *Aquilegia formosa* and *A. pubescens* (Hodges et al. 2002), and *Petunia integrifolia* and *P. axillaris* (Galliot et al. 2006, Stuurman et al. 2004).

Reverse genetics: studying effects of specific genes on the phenotype with techniques like site-directed mutagenesis, RNA interference, virus-induced gene silencing and transformation

These studies have found major QTL controlling a large proportion of the phenotypic variance in traits important to pollinator preference. In *Mimulus*, Bradshaw et al. (1998) mapped QTL controlling more than 20% of the difference between species for flower shape, nectar volume, and flower color patterning, including a large QTL controlling the presence of carotenoids in the upper petals—as had been identified in earlier biosystematic studies (Hiesey et al. 1971). Major QTL also have been mapped for flower color, orientation, and spur length in *Aquilegia*; sepal length, color brightness, and nectar guide area in *Iris*; and nectar volume and floral elongation in *Petunia*. The importance of some of these large-effect QTL has been confirmed by further field studies. For example, Bradshaw & Schemske (2003) found that near isogenic lines introgressed with a single flower color locus increased bee visits 74-fold in the hummingbird-pollinated genetic background and increased hummingbird visits 68-fold in the bee-pollinated background. These studies show that shifts in pollination syndromes may involve major mutations, a result consistent with a model of adaptation involving the fixation of a few mutations of large effect when the population is far from the phenotypic optimum and many mutations of progressively smaller effect as the population nears the optimum (Fisher 1930, Orr 1998). Large beneficial mutations could be rapidly fixed by selection and allow a population quickly to bypass low points on an adaptive landscape between alternate pollination syndromes (**Figure 2**).

Although QTL studies have identified regions of the genome involved in floral isolation, they have not led to the identification, cloning, and functional analysis of actual genes. A QTL may comprise many linked genes, and positional cloning and sequencing of the genes of interest is difficult without other substantial genomic information. The advent of affordable expressed sequence tag (EST) sequencing (Bouck & Vision 2007), custom microarrays (Wayne & McIntyre 2002), and especially next generation sequencing techniques (Shendure & Ji 2008) may vastly increase the availability of markers for QTL studies and gene discovery.

Candidate-Gene Approaches to Floral Isolation

In contrast to QTL studies, candidate gene and reverse genetics approaches start with a gene of interest, typically identified in studies on model organisms, and attempt to determine its importance for the trait(s) under study. Floral symmetry genes provide an interesting example. Changes of expression patterns of *CYCLOIDEA*-like genes have been found to cause a recent reversal in zygomorphy in the bird-pollinated legume genus *Cadia* (Citerne et al. 2006) and are hypothesized to be involved in other shifts to bird pollination in wild relatives of the model legume *Lotus* (Cronk & Ojeda 2008). A study of *Antirrhinum majus* and its close relative *Mohavea confertiflora* found that differences in expression of *CYCLOIDEA* and *DICHOTOMA* underlie a shift to superficial radial symmetry that was hypothesized to be integral to the change from nectar-foraging to pollen-collecting bee pollinators (Hileman et al. 2003). To date, however, no study has linked changes in symmetry directly to reproductive isolation.

The genetic pathways controlling the synthesis of floral pigments may be more promising for identifying candidate floral isolation genes. The structural genes in the anthocyanin biosynthetic

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pathway are relatively conserved across angiosperms, but regulation of the expression of these genes differs among species (Durbin et al. 2003). It has been possible to examine changes in gene expression in the pathway that cause flower color differences involved in pollinator attraction and prezygotic isolation in wild plant systems. For example, a shift in flower color in *Ipomoea* from a purple-red bee-pollinated flower to an orange-red hummingbird-pollinated flower is caused by both a dramatic reduction in expression and the functional inactivation of a key enzyme leading to red-purple cyanidin production (Zufall & Rausher 2004). The result is production of orange-red pelargonidin. Similarly, a shift from blue bee-pollinated to white moth-pollinated flowers in *Petunia* has been found to involve loss-of-function mutations at a transcription factor that acts on structural genes late in the pathway, preventing anthocyanin production (Hoballah et al. 2007, Quattrocchio et al. 1999). In both *Ipomoea* and *Petunia*, a single gene mediates a change in flower color that influences pollinator attraction.

Analyses of floral anthocyanin production have also addressed questions about genetic constraints and convergence in floral evolution. In *Ipomoea*, in addition to inactivation of a key enzyme leading to cyanadin production, a downstream enzyme has lost the ability to metabolize substrates of the cyanidin branch, probably making the change from reddish-purple to red flowers irreversible (Zufall & Rausher 2004). An asymmetry of pollination syndrome shifts has been observed across many plant lineages; bee- to bird- pollination shifts are common, whereas bird-to-bee shifts are rare (but see Tripp & Manos 2008). This pattern is hypothesized to be driven by a consistent direction of selection toward hummingbird pollinators (Thomson & Wilson 2008), but a genetic constraint on color changes could also play a role. Whittall et al. (2006) examined the genetics of color change in *Aquilegia*, a genus with blue, red, and white flowers that are associated with bee, hummingbird, and moth pollination, respectively. They asked whether the direction of color changes has been conserved throughout the evolutionary history of the genus and whether convergent evolution of flower color has the same genetic basis. They found a consistent direction of color change and that most losses of color were due to down regulation of later steps in the pathway, although the mutations in regulatory genes causing this pattern may differ among losses. Their evidence suggests at least some losses are caused by a *trans* regulatory factor operating on two sequential steps late in the pathway. The losses of floral anthocyanin in *Aquilegia* also appear to be irreversible, and this constraint may contribute to the directional bias of shifts toward moth pollination.

5. FLORAL ISOLATION ACTS IN CONCERT WITH OTHER FACTORS TO PROMOTE SPECIATION

Plant-pollinator interactions have been the focal point of many studies of plant speciation, yet it is also apparent that floral isolation is rarely, if ever, sufficient to cause speciation alone. Indeed, the focus on floral isolation has been criticized in light of much empirical data showing ecological generalization (Waser 2001). Studies of closely related species that have quantified multiple forms of reproductive isolation (e.g., habitat isolation, phenological differences, pollinator specificity, pollen-pistil incompatibility, hybrid inviability, and infertility) invariably find that multiple isolating factors are important in maintaining species differences (Chari & Wilson 2001, Husband & Sabara 2004, Kay 2006, Martin & Willis 2007, Ramsey et al. 2003). Even figs and deceptive orchids, long thought to represent some of the best cases of pollinator-driven speciation and coevolution, experience some pollinator sharing among closely-related plant species, suggesting that other factors contribute to reproductive isolation (Cozzolino et al. 2005, Machado et al. 2005, Scopece et al. 2007).

Notably, even close relatives that exhibit striking differences in pollination syndrome are reproductively isolated by more than just pollinator specificity. For example, *Mimulus lewisii* and

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M. cardinalis exhibit strong floral isolation where they co-occur (Bradshaw et al. 1998, Bradshaw & Schemske 2003, Hiesey et al. 1971), but their ranges overlap over only a narrow band of elevation. Ramsey et al. (2003) suggested that ecogeographic isolation plays a primary role in their reproductive isolation, such that differential habitat adaptation prevents the species from experiencing extensive sympatric contact. In a series of reciprocal transplants, Angert & Schemske (2005) and Angert (2006) found that *M. lewisii* has a low survival rate in the high temperatures beyond its lower elevation range boundary, whereas *M. cardinalis* exhibits poor growth and reproduction owing to the shorter, cooler growing season above its high elevation range boundary. A similar pattern is observed in *Aquilegia formosa* and *A. pubescens*. These species show floral isolation in a narrow elevational zone of sympatry, are differently adapted to elevation, and show strong differences in edaphic affinity in sympatry (Grant 1952). In fact, the natural hybrid zones involving *A. formosa* and *A. pubescens* provide evidence that pollinator specificity alone is insufficient to cause complete reproductive isolation. Strong selection likely maintains the differences in floral traits and habitat affinity in the face of gene flow (Hodges & Arnold 1994). For both of these examples, geographic separation may have allowed adaptation to different pollinators to proceed in isolation, concurrently with adaptation to the local edaphic and climatic environment. The resulting floral differences may restrict hybridization upon secondary contact, but the differentiation in broad- or local-scale habitat affinity is not of secondary importance in causing speciation; if incipient species are ecologically equivalent, aside from floral differences, classical ecological theory would say they should not coexist.

Other relevant examples involve autopolyploids. Polyploidization confers strong postzygotic isolation, but until recently, prezygotic isolation was overlooked in these systems. Husband & Sabara (2004) studied the relative effects of postzygotic isolation, floral isolation, and habitat differentiation in the isolation of autotetraploid fireweeds from their diploid progenitors; they found that strong floral isolation was caused by pollinator fidelity. Thompson & Merg (2008) have similarly found that floral isolation plays a significant role in reproductive isolation between diploid and tetraploid *Heuchera grossulariifolia*. These studies demonstrate that floral isolation can play a contributing role in the establishment of polyploid lineages, although it is unclear whether the floral differences are a result of polyploidization, per se, or of genic adaptation in the isolated lineages.

Floral isolation is rarely a complete barrier (Chari & Wilson 2001, Kay 2006, Ramsey et al. 2003), but acts in concert with other isolating factors to reduce the homogenizing effects of gene flow, allowing divergent lineages to persist and further diverge. Without floral isolation, the same amount of allopatric divergence in ecological traits or other genetic factors may not be sufficient to cause speciation. For example, *Potentilla glandulosa* shows parallel adaptation and ecotypic differentiation across elevation along the same cross-section of the Sierra Nevada as the above *Mimulus* and *Aquilegia* examples, but lacks the floral isolation that has allowed the other groups to proceed on their own evolutionary trajectories without substantial gene flow (Clausen & Hiesey 1958, Lewis 1966).

Geographic patterns of plant diversity reinforce the idea that floral isolation can act synergistically with other ecological isolating factors. Many striking examples of floral isolation come from plant biodiversity hotspots in which high topographic and edaphic variation likely promotes divergent plant adaptation to both abiotic and biotic factors. Plant lineages differentiate along gradients of topography, microclimate, and soils, and areas with steep gradients in these factors are also likely to have high turnover in pollinator assemblages, because animals also respond to these factors. For example, the Cape Floristic Region in southern Africa has remarkably high plant species richness, rugged topography, high variability in microclimate and edaphic factors (Goldblatt & Manning 2002), and a stunning variety of pollination systems (Johnson & Steiner

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2003). In this system, pollination shifts appear to occur both through the classic model of divergence in isolation and through reinforcement (van der Niet et al. 2006). Other regions with high plant species richness and diversity of pollination systems include the California Floristic Province and the Andean-centered Neotropics, both of which exhibit extremes in topography, microclimatic variation and edaphic factors (Gentry 1982, Raven & Axelrod 1978).

6. MACROEVOLUTIONARY TRENDS

Strong evidence exists that pollinators play a role in premating isolation in a few systems (e.g., *Mimulus*, *Aqueligia*, *Costus*); however, we lack detailed information for the majority of plants. This hampers our ability to evaluate the general importance of pollinator isolation on speciation. Comparative biologists have attempted to circumvent this problem by examining broad-scale associations between floral traits and diversification rates. A classic prediction is that transitions from abiotic to biotic pollination should be associated with a pattern of increased species richness. Biotic pollination has arisen multiple times in the history of flowering plants (Eriksson & Bremer 1992, Kay 2006, Sanderson & Donoghue 1994), providing independent replicates for comparative studies (**Table 1**). Although several studies support the prediction (e.g., Dodd et al. 1999, Eriksson & Bremer 1992, Kay et al. 2006), others do not. Those studies that failed to find an association were constrained by incomplete datasets (Bolmgren et al. 2003) or did not adequately control for phylogenetic relatedness among groups (Ricklefs & Renner 1994). Davies et al. (2004) generated a supertree of the angiosperms and examined the ten nodes with the highest contrasts in species richness. They did not find that these nodes consistently corresponded to changes in pollination mode, but this study did not directly test the hypothesis that biotic pollination is associated with higher species richness. Because the most comprehensive studies that use phylogenetic independent contrasts show a significant association (Dodd et al. 1999, Kay et al. 2006), we interpret this as cautious support for the hypothesis.

Does Pollinator Specialization Lead to Increased Diversification Rates?

To date, only a single study has performed a direct test for an association between clade species richness and specialized pollination across multiple lineages (Schiestl & Schluter 2009). Using phylogenetic independent contrasts across the orchids, they found a negative correlation between the mean number of pollinator species per plant species in a clade and species richness. Given

Table 1 Studies testing for an association between floral traits and diversification

Study	Trait	Number of independent contrasts	Proportion in expected direction	Reported significance	Relative species richness[a]
Dodd et al. (1999)	Pollination Mode (biotic versus abiotic)	22 (16)[b]	0.82 (0.69)	0.013 (0.033)	2.36 (5.73)
Sargent (2004)	Floral Symmetry	19 (22)	0.79 (0.73)	0.003 (0.035)	4.18 (2.8)
Hodges & Arnold (1995)	Nectar Spurs	6 (16)	0.83 (0.75)	NA (0.137)	3.25 (0.21)
Bolmgren et al. (2003)	Pollination Mode	16	0.56	0.3	1.43
Ree (2005)	Nectar Spurs	1	NA	0.0006	NA
Smith et al. (2008a)	Specialization	1	NA	0.20	NA

[a]Relative species richness is the ratio of species numbers in sister groups with alternative traits.
[b]Brackets indicate the results of a reanalysis of these contrasts using a more recent angiosperm phylogeny, as reported in Kay (2006).

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the lack of data for most plant lineages, a more common approach has been to examine whether increased diversification is associated with traits that are indirectly tied to specialized pollination. Along these lines, studies have demonstrated an association between species richness and nectar spurs (Hodges & Arnold 1995, Ree 2005) and bilateral corolla symmetry (Sargent 2004; **Table 1**). Nectar spurs restrict access to the reward and require a pollinator capable of manipulating the relatively complex flower and extracting the nectar, which should exclude the majority of potential pollinators in a community and increase the precision of pollen placement. Similarly, bilateral corolla symmetry is postulated to restrict the orientation of the pollinator on the flower, resulting in more precise pollen placement. Although support for the association between floral symmetry and clade diversity is diminished when updated phylogenetic information is accounted for, it remains significant (**Table 1**). Support for the association between nectar spurs and species richness is mixed; though there is clear evidence that *Aquilegia* diversified more rapidly with the advent of nectar spurs, the results of multiple comparison tests suggest that nectar spurs are an important determinant of species richness only under certain conditions (e.g., when there are no associated shifts in floral symmetry).

Limits of Tests for Diversification

The array of available approaches to test for key innovations or adaptive radiations is continually growing. Most methods either measure the association between a particular trait and increased clade size or use phylogenetic dating tools to test for an association between the diversification rate and the origin of a particular trait. Problematically, many methods cannot distinguish increased speciation from decreased extinction. Furthermore, current methods do not consider the fact that ancestral state reconstruction may be influenced by the effect of the trait on speciation and/or extinction rates (Goldberg & Igic 2008). A recent methodological advance appears to remedy both issues by incorporating character state change directly into a likelihood estimation of speciation and extinction rates (Maddison et al. 2007). Furthermore, this method can now take advantage of incompletely resolved phylogenies and datasets with missing taxa (FitzJohn et al. 2009). Finally, although comparative studies of biotic pollination and specific floral traits mostly support the hypothesis of pollinator isolation influencing diversification, the evidence is indirect. We would like to test for a direct association between the degree of plant-pollinator specialization and plant diversification rates, or ideally, between the incidence of pollinator-mediated floral isolation in a plant clade and its diversification rate. The key would be to reconstruct ancestral shifts between generalization and specialization and determine whether there was a significant diversification rate shift associated with the narrowing of the pollinator guild.

The Relative Importance of Floral Isolation

The importance of floral isolation to plant diversification varies widely across the angiosperms. Grant (1981) observed that perennial herbs are more likely to show pollination shifts than trees or annual herbs. Indeed, in our survey of documented or putative cases of floral isolation (**Supplemental Table 1**), we found that 58 out of 70 total cases involved herbaceous plant taxa, almost all of which were perennial. The cases were also not evenly distributed taxonomically. Most belonged to the asterid eudicot clade (28 cases), primarily the order Lamiales (14 cases); and the monocot clade (22 cases), primarily the order Asparagales (17 cases). As expected from macroevolutionary patterns of diversification rates, our survey was dominated by taxa exhibiting zygomorphic or irregular floral symmetry and fused floral parts, which are features that should increase pollinator specificity or restrict access to a reward. Specialized pollination may also be more important in

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some geographic regions than others. Verne Grant and many subsequent workers have studied floral isolation in temperate ecosystems, and approximately two-thirds of the cases in our survey involve temperate taxa (**Supplemental Table 1**). It is unclear how much of the focus on herbaceous and temperate taxa reflects convenience versus a true difference in the importance of floral isolation. Both plant and pollinator diversity are relatively low and wind-pollination is prominent in temperate ecosystems, which suggests we may be underestimating the significance of floral isolation in the tropics. Some tropical work supports Grant's hypothesis of the prevalence of floral isolation varying with life form, finding greater plant-pollinator specialization in herbaceous perennials, shrubs, vines and epiphytyes compared to long-lived trees (Bawa 1990, Gentry 1982); nevertheless, we understand very little about the importance of floral isolation in tropical systems.

Comparative studies also show that plant lineages lacking obvious specialization in their pollination systems can exhibit high species diversity. For example, the wind-pollinated clade including the grasses and sedges is seven times more diverse than its animal-pollinated sister clade, the bromeliads (Kay et al. 2006). Similarly, it has been proposed that the rapid radiation of the Asteraceae, which contains nearly one-tenth of all angiosperm species, may be due to ancient gene duplication events that contributed to a vast capability for ecological speciation in this family (McKenzie & Barker 2008). Clearly, there are other factors besides pollination that drive plant diversification.

FUTURE DIRECTIONS

In spite of the many factors that may affect plant speciation and extinction, comparative studies generally support the hypothesis that animal pollination increases diversification rates; however, the lack of data on pollinators in most systems is a major obstacle to a better mechanistic understanding of this association. When evaluating plant-pollinator relationships, there is a trade-off between gathering the information necessary to directly show floral isolation between closely related plant species and gathering broad community-level data to evaluate the overall importance of floral isolation. Recent plant-pollinator network studies have begun to add quantitative measures of the frequency of interactions and even phylogenetic relationships among the interactors, and therefore show promise for a better understanding of the importance of floral isolation at a community scale (Rezende et al. 2007, Vazquez et al. 2005). Moreover, species-level phylogenetic studies can help to direct efforts toward collecting pollinator observations for radiations of plants that consist of partially sympatric close relatives. The biosystematic literature, especially for well-studied regions like the California Floristic Province, provides a treasure of information on plant groups that retain at least partial interfertility among species in artificial crosses, and these are the types of plants for which floral isolation may be especially important.

Pollinator transitions are key to the theory of pollinator-mediated speciation, but our understanding of the mechanisms by which they occur is rudimentary. In a few well-studied systems, we have learned that shifts from bee to hummingbird pollination involve the evolution of both attractants of hummingbirds and deterrents of bees and that the relatively higher efficiency of hummingbirds may drive these shifts (reviewed in Thomson & Wilson 2008). However, we need similar studies of other types of shifts in order to make general statements. Perhaps most importantly, we need more data on variation in pollinator-mediated selection gradients over a species range. Although a few key studies have documented geographic variation in floral traits that correspond to differences in pollination (e.g., Galen 1996, Pérez-Barrales et al. 2007), most studies relevant to floral isolation are restricted to a single location. We need to evaluate geographic divergence in floral traits in order to understand the dynamics of pollinator shifts and the potential role of gene flow in constraining floral adaptation.

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A better understanding of the genetic basis of floral isolation is also paramount. As knowledge of plant functional genetics accumulates, candidate gene approaches will become more important and be applied to a wider variety of floral traits. Quantitative polymerase chain reaction and reverse genetics techniques have become more affordable and accessible to those working on nonmodel plants, and these techniques will be important for testing candidate genes. As we learn more about plant genetics in general, we also will better understand pleiotropic constraints on floral evolution. For example, anthocyanins and their precursors are important for a wide variety of plant traits, such as protection against herbivory and UV light damage, nutrient uptake, and tolerance to abiotic stress, perhaps explaining why some flower color polymorphisms are not driven by pollinator preference (Whittall & Strauss 2006). Eventually, it should be possible to evaluate selection on floral traits in the context of the pleiotropic interactions of the underlying genes. There is a critical need to integrate candidate gene approaches with genetic mapping and field studies testing the importance of various floral characteristics to pollinator attraction. This is being done in a few systems, and we can expect to see exciting advances with the current whole genome sequencing of both *Mimulus* and *Aquilegia*, systems with a rich background of information on natural history and pollination.

In the past, a focus on floral isolation has tended to overlook a more comprehensive picture of speciation. We need to understand how pollinator specificity interacts with other isolating mechanisms, yet there are very few species pairs for which multiple isolating mechanisms have been quantified (reviewed in Lowry et al. 2008), and there are even fewer for which we understand the interactions among isolating mechanisms. To fully understand the role of plant-pollinator interactions in speciation, we need to know the sequence in which different isolating factors have evolved and their relative importance in maintaining species differences. This information will only come from many more detailed and comprehensive studies of isolating mechanisms both among divergent populations and between closely related species, as well as from comparative studies across many species pairs. Recent advances in methods that infer the evolution of species ranges on phylogenetic trees could help dissect the ecological and geographical aspects of lineage diversification (Ree & Smith 2008). Moreover, recognizing that neither floral isolation, local adaptation, nor postzygotic isolation is likely to be sufficient to cause speciation on its own, but that the synergy between these factors can rapidly promote divergence, may help to resolve the seeming paradox between incomplete floral isolation and the association between biotic pollination and the diversification of the angiosperms.

SUMMARY POINTS

1. Forward movement of the field is highly dependent on incorporating measures of pollinator importance into field pollination data.
2. New insights are likely to be gained if we relax the assumption that floral isolation must arise in sympatry in order to play a role in plant speciation.
3. To understand how transitions in pollination systems occur, we need to integrate ideas about trade-offs, geographic variation, and gene flow among populations.
4. Genetic studies should integrate quantitative genetic analysis with candidate gene approaches, and new genetic tools will expand the field to nonmodel plants.
5. Floral isolation must be studied in the context of other mechanisms of reproductive isolation to understand how it evolves and its relative importance.

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6. In spite of so much variation across plant lineages, we see macroevolutionary patterns of increased diversification with biotic pollination and specialized floral traits.

DISCLOSURE STATEMENT

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