

The Color Genes of Speciation in Plants

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The genes underlying speciation remain largely undiscovered. An article published in this issue of *GENETICS* presents results related to “Genetic Dissection of a Major Anthocyanin QTL Contributing to Pollinator-Mediated Reproductive Isolation Between Sister Species of *Mimulus*.” Yuan *et al.* (2013) provide compelling evidence that the R3 *MYB* gene causes differences in anthocyanin concentration in the flowers of *Mimulus lewisii* and *M. cardinalis*, and is in fact likely responsible for pollinator-mediated reproductive isolation in areas where the two species co-occur. This commentary discusses general implications from the results of Yuan *et al.* (2013), and frames them in terms of both the genetic basis of color and species evolution.

COLORS in nature bring partners together (Darwin 1871), signal distaste to predators (Bates 1862), and create interactions between radically different organisms (*e.g.*, Bawa 1990). Colors also signal reward (Hamilton and Zuk 1982), or possibly atavistic preferences (Ryan 1998), and drive fundamental evolutionary processes such as sexual selection in animals (Andersson 1994) and pollination preferences in plants (Grant 1949). These processes have major implications for the origin of traits and species both in the animal and plant kingdoms. Although we have made remarkable progress in identifying the genes responsible for color in nature (Wessinger and Rausher 2012), we still know very little about the color genes of speciation (Rieseberg and Blackman 2010). In this issue, Yuan *et al.* (2013) are helping to fill this gap by identifying some of the genes responsible for flower color variation in a clear case of pollinator-mediated reproductive isolation (RI) in monkeyflowers (Bradshaw *et al.* 1995; Schemske and Bradshaw 1999; Ramsey and Schemske 2002). Their findings fundamentally advance our knowledge of speciation in plants and further open the doors for understanding the evolution of color and its consequences in nature.

Pollinator isolation, a common prezygotic isolating barrier in flowering plants, reduces gene flow between populations because different pollinators (*e.g.*, birds vs. bees) or the same pollinator (pollinator constancy) transfer pollen predominantly between conspecific and not heterospecific flowers (Bradshaw and Schemske 2003; Hopkins and Rausher 2012). In recent years, a number of studies have demonstrated that shifts in flower color are common in a variety of plant lineages (Rausher 2008) and that some of them cause changes in pollinator behavior leading to pollinator isolation (Hopkins and Rausher 2012). However, very few studies have found the genes responsible for flower-color variation associated with demonstrated cases of plant speciation (Rieseberg and Blackman 2010). For instance, Hopkins and Rausher (2011) identified genetic changes in two genes involved in the anthocyanin biosynthetic pathway (ABP) that cause changes in flower pigment intensity (from violet to dark red) between co-occurring populations (sympatry) of the plants *Phlox drummondii* and *P. cuspidata*. Notably, pollinators display visitation fidelity to flowers sharing the color of the first flower they visited, even though the pollinator is able to visit the flowers of both species. Because color shifts do not occur outside areas of sympatry, the evolution of sympatric flower color differences in *Phlox* likely evolved in response to partial hybrid sterility between the two species and thus reinforced prezygotic reproductive isolation in the system (reviewed in Hopkins 2013).

The discovery of speciation genes requires systems of study with detailed knowledge of the reproductive barriers that separate two species and the ability to perform classical and molecular genetic experiments with them (*e.g.*, QTL mapping and genetic transformation). Traditionally, this has been the domain of model systems, such as *Drosophila* and *Arabidopsis*. However, Yuan *et al.* (2013) demonstrate that *Mimulus* is joining their ranks, not only by providing these tools of discovery, but also by enabling future genetic experiments in ecological settings. In a tour de force, Yuan and colleagues discover a gene that causes pollinator isolation between *Mimulus lewisii* and *M. cardinalis* by using a series of QTL

fine-mapping experiments coupled with stable genetic transformation of candidate genes, and RNA interference-induced knockouts. Further, the authors use clever genetic and functional approaches to disentangle the contributions of *cis*-regulatory vs. coding-sequence variation on the evolution of traits important for adaptation and speciation. Although the evolution of flower-color differences is probably not important in many species (e.g., sunflowers and many *Asteraceae*), these results expand our understanding of how speciation occurred between these two species and suggest how cases of pollinator isolation may work in other systems.

M. lewisii and *M. cardinalis* are perennial herbs that occupy distinct habitats along altitudinal gradients in the mountains of western North America and display striking differences in flower color (Wu *et al.* 2008). Although other reproductive barriers prevent gene flow between the two species, previous studies revealed that pollinator isolation is the most important reproductive barrier in the system (Ramsey *et al.* 2003). Hummingbirds prefer the red flowers of *M. cardinalis* whereas bees more often visit the pink flowers of *M. lewisii*. Both pigment concentration and nectar load differ between the species, and each trait contributes a fraction to pollinator isolation. These reproductive barriers act early in the life cycle of the organism so they are expected to contribute disproportionately to reproductive isolation between *M. lewisii* and *M. cardinalis* even though other subsequent barriers are quite strong in absolute terms (e.g., conspecific pollen precedence leads to ~70% RI, and F1 hybrids have great reductions in pollen viability leading to ~66% RI; Ramsey *et al.* 2003).

The red flowers of *M. cardinalis* have petals rich in carotenoids (yellow pigment) and anthocyanins (pink pigment) and are full of nectar. In contrast, the pink flowers of *M. lewisii*, in addition to little nectar, have small amounts of both pigments in their petals. Genetic experiments under natural conditions have shown that swapping the QTL region that controls carotenoid deposition in petals between the two species (the *YUP* locus) is sufficient to generate an almost complete shift in relative pollinator preferences in the field (Bradshaw and Schemske 2003), although *YUP* seems to more strongly affect absolute visitation rate in bees than in hummingbirds (Schemske and Bradshaw 1999). Similar to carotenoid deposition, the concentration of anthocyanins in the petals of the two species also determines pollinator visitation by controlling red intensity in the two species (Bradshaw *et al.* 1995; Schemske and Bradshaw 1999). Yuan *et al.* (2013) show that R3 MYB (*ROSE INTENSITY1*, or *ROI1*) is the gene underlying the QTL with the greatest effect (41% of the variation observed in the mapping population) on anthocyanin concentration, and thus pinkness in this system. These results are consistent with previous genetic mapping experiments in which QTL controlling the concentration of anthocyanin largely determined visitation of hummingbirds to red plants (Schemske and Bradshaw 1999). It is notable that should the authors have followed only a candidate gene approach, they would have not iden-

tified *ROI1* (i.e., R3 MYB was not previously implicated in anthocyanin production) as a gene responsible for pollinator isolation in this system, thus demonstrating that traditional QTL-mapping approaches remain fundamental for understanding the genetic basis of traits important in evolution.

Anthocyanins are hydrophilic compounds that belong to the general class of secondary metabolites in plants known as flavonoids (Koes *et al.* 2005). The very well-described ABP is highly branched and includes a variety of precursors that may lead to either red or blue pigment. A number of regulatory proteins, including members of the R2R3 MYB family, regulate the activity of the ABP (Wessinger and Rausher 2012). These transcriptional regulators occur in a variety of tissues and developmental stages and are known to be involved in many biological processes ranging from stomatal aperture (Liang *et al.* 2005) and freezing tolerance (Agarwal *et al.* 2006) to defense against herbivores (Kaur *et al.* 2010) and fruit (Allan *et al.* 2008) and flower-color differences (Rausher 2008) across a variety of flowering plants. Complementing previous studies (Quattrocchio *et al.* 1999; Schwinn *et al.* 2006; Hopkins and Rausher 2011) where MYB-related transcription factors play an important role in determining flower color, Yuan *et al.* (2013) found that *ROI1* is homologous to the R3 MYB gene in *Arabidopsis* and thus a member of the MYB family of transcriptional regulators. Unlike R2R3 MYB genes, which have been shown to upregulate the ABP, R3 MYB genes seem to downregulate the expression of genes in the ABP, thus leading to small amounts of red pigment in the flower petals (Koes *et al.* 2005). Thus, as predicted, the *ROI1* gene reduces anthocyanin concentration in the pink flowers of *M. lewisii* but increases it in the red flowers of *M. cardinalis*.

Mutations in the coding region of the *ROI1* gene are not responsible for the observed phenotypic difference between the two populations of *M. cardinalis* and *M. lewisii*. Although Yuan *et al.* found amino acid changes that could have suggested a role for failed DNA-protein interactions driving high production of anthocyanin, through expression analyses and complementation tests, the authors were able to conclude that regulatory regions of *ROI1*, and not another protein, were responsible for differences in *ROI1* expression in the petals of the two *Mimulus* species. This result is important, as it suggests that *cis*-regulatory evolution could be responsible for morphological differences between species, and more so for variation in traits that directly cause reproductive isolation (Hoekstra and Coyne 2007; Stern and Orgogozo 2008). However, this postulate has been evaluated in great detail when considering flower-color evolution as a whole. For instance, Wessinger and Rausher (2012) found that transitions between flower colors might involve functional or regulatory changes depending on the species and the aspect of the pigmentation pathway being considered. Although the origin of *ROI1* mutations controlling flower-color variation could be idiosyncratic to these *Mimulus* species, they contribute to the ongoing debate on the role of *cis*-regulatory elements to the evolution of traits and physiologies.

A description of natural variation in candidate speciation genes leads to greater understanding of their role in the evolution of reproductive isolation. A missing piece of the puzzle in this study is whether the mutations responsible for changes in anthocyanin concentration between *M. cardinalis* and *M. lewisii* are fixed between them or polymorphic in the two species. These patterns need to be revealed to better understand the role of natural selection in driving speciation. However, patterns of nucleotide variability in speciation genes can be complex and not necessarily aligned to phenotypic patterns of variation. Consider a scenario in which the nucleotide changes in *RO11* separating the two species are loss-of-function mutations. Such a possibility is not unlikely given that in most cases, flower-color transitions result from such kind of mutations (Wessinger and Rausher 2012). Because *RO11* is a repressor, mutations that stop its transcription will lead to the accumulation of anthocyanin and therefore to the emergence of greater red intensity in the flower (although other pigments would be needed for recapitulation of original phenotypes). Since there are presumably more ways to destroy than to gain a function, populations of *M. cardinalis* could each carry a different nonfunctional mutation affecting the repressor activity of *RO11* and as a consequence could be highly polymorphic across the entire species range. In contrast, the *cis*-regulatory region in *M. lewisii* would be expected to carry a functional allele and be monomorphic. Therefore, lack of fixed differences between species at the DNA sequence level does not equate to lack of fixed differences at the functional or phenotypic level, thus suggesting that a species-wide analysis of variability in speciation genes is a fruitful avenue to understanding the genetic basis of phenotypic divergence.

Difficulties in identifying causal mutations for flower-color variation in this system will not stop the authors from performing field experiments in the wild. These experiments are laborious, but not rare in the *Mimulus* system (e.g., Lowry and Willis 2010) or new to the authors, who have set the standards in the community (Bradshaw *et al.* 1995; Schemske and Bradshaw 1999). For instance, *M. lewisii* lines carrying a *M. cardinalis* *RO11* region, and appropriate control lines, could be exposed to field conditions under which both bees and pollinators are exposed to these flowers. These kinds of experiments may test the effect of shifting pollinators through changes in regulation, such as those created by mutations in *RO11*. Further, as the authors suggest, promoter-swapping experiments could investigate the mutational target size in *cis*-regulatory vs. coding regions for a loss-of-function variant. It may not be long before we know the answer to this question as the authors have in hand such precious genetic resources and the unique opportunity to study the interaction between these important loci and variation in their natural environment. It would be interesting to perform these experiments in genetic backgrounds where the information contained in the *YUP* locus is also manipulated (Bradshaw and Schemske 2003). The evolution of pigment combinations may ultimately be the main cause of complex and abrupt transitions like the one observed in this system.

Speciation between *M. cardinalis* and *M. lewisii* is a textbook example of how geography and pollinators play a major role in keeping two species apart. This, and data from other studies, has led many students of speciation to consider that reproductive barriers acting during the early stages of the life cycle of an organism (i.e., prezygotic barriers) perhaps play a more important role in speciation than those barriers acting further down development (e.g., hybrid sterility). This is because the net number of individuals available for heterospecific crosses is sequentially and progressively reduced every time a reproductive barrier manifests (i.e., by the time the production of hybrids could occur, the number of available flowers with heterospecific pollen is already very small, even if such hybrids would all be rather sterile). Further work on speciation genes should move beyond asking whether natural selection leaves a signature on their sequences and explore the relationships between reproductive barriers at different stages of development. R3 MYB proteins, for instance, are known to control plant trichome and root-hair development in certain plants (e.g., Tominaga-Wada *et al.* 2013), perhaps suggesting that cases of adaptation to local conditions could perhaps lead to the incidental evolution of changes in other traits such as flower color.

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