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A Hopeful Monster in *Aquilegia*: Uncovering the genetic basis and selective advantage of a naturally occurring floral homeotic mutant of *Aquilegia coerulea*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Ecology, Evolution, and Marine Biology

by

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- "just one drink" at Lama Dog
- Casual drinks
- …it'll be fiiiiiinneeeee
- "geltopia 2016 brah"
- Bahn Mi
- Sashimi grade
- Adiooooooooossss
- Floating
- Bagel Wednesday
- Bromance 1 and Bromance 2
- Reality blockers
- ……EGG

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ABSTRACT

A Hopeful Monster in *Aquilegia*: Uncovering the genetic basis and selective advantage of a naturally occurring floral homeotic mutant of *Aquilegia coerulea*

by

Zachary Alix Cabin

Broadly, the field of evolutionary biology aims to understand the spectrum of evolutionary mechanisms that generate the diversity of life. At one end of the spectrum is Darwinian gradualism – slow, gradual change driven by small effect mutations that likely accounts for an overwhelming majority of evolution over the past 4 billion years. At the other end sits saltational evolution – a mechanism that invokes large effect macromutations and evolutionary "leaps". The most extreme version of saltational evolution is Richard Goldschmidt's "hopeful monster": a single, large effect macromutation (likely affecting early development) that drastically alters the body plan (e.g. homeotic mutants). This radical change would almost always be deleterious ("hope*less* monster"), but on the rare occasion that this change occurred in the right ecological context, an entirely new lineage could arise. Unfortunately, natural evidence for hopeful monsters is almost nonexistent. For the first chapter of my dissertation, I begin by reviewing the current literature and report on a handful of studies that have identified large effect mutations that underly adaptive traits in nature and discuss why these examples do (or do not) fit into Goldschmidt's criteria for a hopeful monster. I then review some oft-cited examples of hopeful monsters and how they fit into Goldschmidt's criteria. Lastly, I introduce a case study for hopeful monsters. This is expanded upon in Chapter 2.

For my second chapter, I present my research on a naturally occurring homeotic mutant of the columbine *Aquilegia coerulea, A. coerulea* var. *daileyae*, in which the nectar-spurred petal is replaced with a second set of sepals, which do not produce nectar. Despite the expected negative effects of losing a pollinator reward, I find that floral herbivores, not pollinators, are driving strong, positive selection (s = 0.17-0.3) for the mutant morphology. Then, using population sequencing, haplotype analysis and SNP genotyping, I was able to identify the underlying locus (*APETALA3-3*) and multiple independently-derived causal loss-of-function mutations indicating an on-going soft-sweep. Elevated linkage disequilibrium around the two most common causal alleles indicates that positive selection has been ongoing for many generations. Furthermore, genotypic frequencies at *AqAP3-3* indicate a degree of positive assortative mating by morphology, indicating that this morphological shift could lead to a new lineage. Lastly, I frame this homeotic shift in a macroevolutionary lens, showing not only that petal loss is a viable and stable macroevolutionary transition, but also that the genetic mechanism in this study population (loss of function at *AP3*-3) mimics the genetic mechanism at the macroevolutionary level. By identifying a large effect macromutation affecting early development that is under selection in the proper ecological setting and plays a role in mating patterns, I present some of the strongest evidence to date of a hopeful monster.

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Part 1: Introduction

Broadly, the field of evolutionary biology aims to explain the patterns of natural variation around us. In 1859, Darwin's theory of evolution by natural selection provided a process that we can use to test and inevitably explain the adaptive variation around us. In short, Darwin proposed that evolution was inherently gradual, that small improvements would be favored from generation to generation, eventually resulting in unique species. With the rediscovery of Mendel's work and an increased understanding of genetics there was a shift to focusing on the evolution of the *population*, rather than the organism. Then, with the application of mathematic and statistical models, this idea of gradualism naturally was specifically applied to genetic change and it was assumed that the small phenotypic changes were controlled by small effect genetic changes (*1*, *2*). In the mid 1900's, all of these ideas came together (with the assistance of Fisher, Haldane, Dobzhansky, and Wright) to form what is referred to as the "Modern Synthesis" (MS) (*3*). The overarching doctrine, for the purposes of our discussion here forth, is that all evolution occurs gradually, through small effect mutations to many genes resulting in small phenotypic differences upon which selection can act. Although this dogma dominated in the late 20th century, as DNA sequencing capabilities have advanced and the genetic basis of ecologically important traits have been identified, the strict adherence to a gradual mechanism of evolution has been re-examined (*4*).

Complete adherence to a strictly gradual, Darwinian mode of evolution has been debated since Darwin introduced the idea in 1859 (*5*–*8*). Most notable was Richard Goldschmidt (RG) in his book *The Material Basis of Evolution*. The principal argument of this text is that while the slow and gradual process of microevolution might account for differences *within* species, these mechanisms do not account for the differences *between* species. True species, he argued, are separated by "bridgeless gaps" and thus would require evolutionary "jumps" (*6*, *8*, *9*). In 1940, he coined the term "hopeful monster" to better explain the patterns of macroevolution that he felt could not be explained by the gradual, Darwinian view of evolution. Instead of small effect micromutations, RG hypothesized a

single, large effect macromutation (likely affecting early development) would result in a radically different body plan. This radical difference would almost always be deleterious (aka "hope*less* monster"), but on the rare occasion that this radical change was beneficial and occurred in the right ecological context, an entirely new lineage could arise (*6*).

RG was not the first to propose a saltational ("jumping") view of evolution as an alternative to the gradual mode that dominated the field. Galton, Bateson, and De Vries all had some variation of these ideas (*10*). Even Fisher and Haldane, architects of the MS, both agreed with Goldschmidt that these evolutionary jumps are possible and could explain such phenomena as mimicry (*3*). A commonality in all of these views of saltational evolution is that it most likely occurs via regulatory genes like transcription factors (dubbed "rate genes" or "switch genes" by Goldschmidt) that alter the developmental fate of tissues. The best examples at the time were homeotic mutants (where one organ grows in the place of another) of *Drosophila melanogaster* such as *Antennapedia*. RG in fact worked with homeotic mutants as he saw them as sort of "proof of concept" for hopeful monsters (*9*). While homeosis may have been a proof of concept, the natural evidence in support of hopeful monsters was – and in many ways, still is – lacking.

Population genetics, mathematical models, discoveries of intermediate forms in the fossil record and experimental evidence were all amassed in the latter half of the 20th century that overwhelmingly supported a gradual, Darwinian mode of evolution (*11*, *12*). As evidence for gradualism mounted, evidence for RG's hopeful monster was still lacking and as such, was losing validity. In the framework of population genetics, the probability of a new mutation fixing in a population is low, even for an advantageous mutation (*11*). Furthermore, as the argument goes, once a hopeful monster appears, there would be no conspecific with which the monster could mate, thus eradicating any hope (pun very much intended) of passing the causal mutation on to the next (*12*, *13*).

Even still, many argued that outright dismissal of RG's ideas was incorrect, but instead it was rather a question of relative frequency of occurrence. While gradualism is undoubtedly the most common mode of evolution, there are still some major transitions between higher taxa in both plants and animals that do not appear to have arisen in a gradual way (*14–17*). Even with numerous "missing links" identified, many argue that there is still an impressive *lack* of intermediate forms in the fossil record (*14*, *18*).

By the 1980's to the early 2000's as the field of developmental biology and DNA sequencing was advancing, it was clear that large effect loci were more common than originally thought(*1*, *19–21*). Quantitative Trait Locus (QTL) studies were starting to identify regions of the genome (regions, not specific genes or mutations) responsible for large effect changes in ecologically important traits (*22–25*) and developmental studies were showing that small changes to timing (heterochrony) and location (heterotopy) of gene expression could lead to drastic changes in phenotype (*19*, *21*). Developmental studies were identifying genes that controlled organ identity in plants (*21*) and animals (*26*) and when these loci were mutated, the resultant phenotypes would phenocopy highly diverged lineages (*16*, *27*, *28*). For example, in the crustacean *Parhyale hawaiensis*, knockdown of the homeotic patterning gene *Ultrabithorax* (*Ubx*), there is a homeotic shift whereby walking appendages are transformed into an additional set of feeding appendages (maxipilleds). This phenotype (additional maxipilleds) is seen in many crustaceans, indicating that relatively simple genetic changes (location and intensity of *Ubx* expression) can have drastic, lineage-defining effects (*27*). Homeosis has been implicated in floral evolution many times (*29–31*) and it is possible that homeotic shifts may have been the mechanism allowing a shift from gymnosperms to angiosperms – an answer to what Darwin called an "abominable mystery" (*32*). Additionally, evolutionary theory has also started to suggest saltational events are more probable that originally expected (*33*, *34*).

All in all, whether evolution proceeds by strict Darwinian gradualism or via saltation can be viewed as a continuum as well as the underlying effect sizes of beneficial mutations (Figure 1). At the

right-most end are small-effect beneficial mutations. These mutations are easily fixed in large populations but can be lost by drift in small populations (*35*). They will most likely be involved in gradual changes though if evolution is concentrated in relatively short time periods followed by stasis, can appear saltational over long time-spans (*36*, *37*). On the left-most end of the spectrum are large effect beneficial mutations. These mutations will be rarer but will necessarily cause saltational changes, even when viewed on short time-scales. The most extreme version of these mutations are RG's hopeful monsters. Unfortunately, as has been mentioned previously, evidence for hopeful monsters is lacking. There are a few reasons for this. One approach to identifying a true hopeful monster would be to show that a difference between two established taxa is the result of a single genetic change, radically altering the morphology. Any analysis of this type is fully comparative in nature (*16*, *17*, *27*) and it cannot be shown that the mutation arose before or after the split and the fitness implications are unknown. Another approach would be to study a natural population in which there was a drastic morphological mutant and attempt to uncover the genetic basis and fully connect genotype, phenotype, and fitness (*38–40*). While this solves part of the issue in a comparative analysis (lack of natural data), there is no way of knowing if the mutation will lead to a stable, cladogenetic split – a requirement of a hopeful monster. Even still, at its core RGs idea of a hopeful monster is that a single, large effect macromutation (genotype) effecting early development and drastically altering the morphology of the organism (phenotype) would be beneficial in the right ecological context and lead to a new lineage (fitness). Thus, being able to fully connect these three tenets of biology is our best bet to identify and possible hopeful monsters.

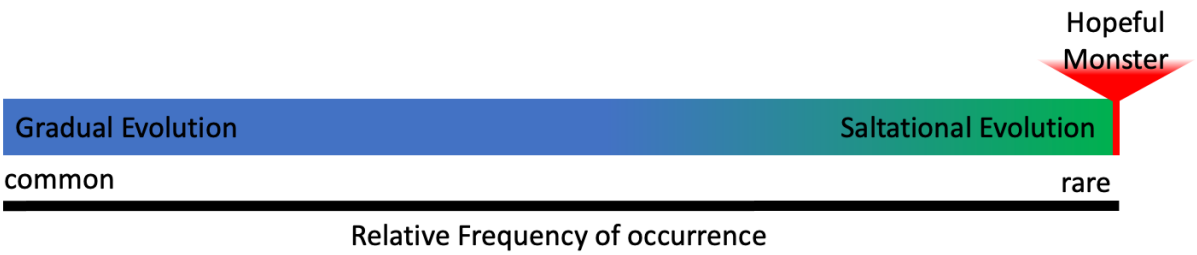

Figure 1. The spectrum of beneficial mutations. While most beneficial mutations are likely small and contribute primarily to gradual evolution (blue), there is growing evidence that medium-large effect mutations have been involved in adaptive change (green). At the most extreme end of the spectrum sits RG's hopeful monster (red), which necessarily results in saltational evolution.

In this review/document/chapter, I will review a subset of the small number of studies that have been able to fully connect G-P-F[1] (or at least come close), discuss how they do (or do not) fit into RGs framework, what we have learned and any questions that remain. Following that, I will review the few hypothesized examples of hopeful monsters (both in plants and animals) to update our knowledge on the occurrence of this phenomenon in nature. Lastly, I will introduce a naturally occurring homeotic mutant, *Aquilegia coerulea* var. *daileyae*, as the best example to date of a hopeful monster being selected for in the field.

Part 2: Measuring evolution in nature: Connecting genotype, phenotype, and fitness

There is a limited number of studies that have been able to fully connect G-P-F for adaptive traits. There are a cornucopia of studies that have uncovered a strong connection between variable phenotypes and fitness (*41–45*). There have also been a number of studies that have identified the genetic basis of variable traits in nature (*46*). There are other groups of studies that have uncovered genetic differences between defining characteristics of well-established taxa, but these studies are

[1] For the remainder of this document, I will refer to "connecting genotype to phenotype to fitness" as "connecting G-P-F".

comparative in nature and thus we cannot be sure if the mutations that have been identified were causal for cladogenesis or not as it is unclear if the genetic changes occurred before or after lineage splitting (*16*, *17*, *47*, *48*). Below, I will begin by reviewing a subset of the studies that have been able to partially connect genotype, phenotype, and fitness.

Close, but no cigar

Two of the oft-mentioned systems that have mapped this continuum (G-P-F) are threespine sticklebacks (*Gasterosteus aculeatus*) and monkeyflower (*Mimulus spp.*). Each of these examples have mapped a 'large effect QTL' for a phenotype of interest, but these QTL may be a single gene with a single mutation or a cluster of genes, each harboring their own function. One thing that remains unclear in each of these scenarios are the actual mutation(s) driving the phenotypic difference. As one of the requirements of a hopeful monster is that a single mutation (and the nature of said mutation) caused the large-effect phenotypic change, these examples do not fit into the framework (although, neither of these studies attempt to prove otherwise).

Stickleback

Marine threespine sticklebacks (*Gasterosteus aculeatus*) have recently (~20,000 years ago) colonized freshwater lakes and in almost all of these colonization events, freshwater sticklebacks evolve in several ways but most notably show a reduction in lateral bony armor from ~35 plates on each side ("complete" morph) to anywhere from 0-9 plates on either side ('low" morph) (*49*). QTL mapping identified a single large effect QTL that explains 75% of the variation in plate number and patterning (and 4 minor "modifier" loci, (*23*)). Additional genetic analyses narrowed the large effect QTL from a 539kb to a 16kb region that was highly associated with plate phenotype. The strongest candidate gene within this region is the *Ectodysplasin* gene (*Eda*), a signaling molecule with known function in ectodermal structures including bone, teeth, and fish scales (*49*). Haplotype reconstruction and phylogenetic analyses suggests almost all freshwater populations share a similar 'low' *Eda* haplotype that is present in marine populations at low frequencies (0.2-3.8%), indicating repeated

evolution from standing genetic variation. Consistent, rapid, repeatable shifts in morphology signals strong selection for this phenotype in this environment and experimental evidence and long-term monitoring projects show similar selection coefficients favoring the low *Eda* allele in freshwater populations ($s_{complete}$ = 0.49-0.52) (*49–51*), but there is also evidence that shows a similar selection coefficient (s_{low} = 0.50) at an earlier life stage that favors the "complete" allele (*50*). The running hypothesis is that armor plates are costly to produce in freshwater lakes and not producing them allows for an increased growth rate (and body size is positively correlated with reproductive fitness) but a recent study showed that although body size and genotype at *Eda* are both correlated with fitness (number of offspring produced), the effect of *Eda* genotype on body size does *not* account for the fitness benefit for the "low" allele (*51*). What remains clear is that there are environmental factors (*52*), additional modifier loci (*23*), and clear pleiotropic effects of *Eda* alleles (*53*). Additionally, though much work has been done with this system, the exact mutation(s) that drive the difference in phenotype is still unknown (*54*).

Mimulus

Mimulus lewisii is a, light pink flower with a wide landing platform and corolla that produces minimal nectar and is primarily bee pollinated. The closely related *M. cardinalis* is a narrow, red, nectar-rich flower that is primarily pollinated by hummingbirds. These species grow in sympatry in some parts of their ranges. Schemske and colleagues (*55*) set out to determine (a) which traits were most important for species recognition and (b) the genetic basis of these traits. A QTL mapping study uncovered at least one "major effect" QTL for every trait measured, specifically pigment (carotenoid) production and deposition and nectar volume (*55*). Plants from an F2 population from a cross between the species were transplanted in an area where these plants grow in sympatry and both main pollinators were present and researchers compared visitation rates with phenotypic measurements. Plants characterized as having less carotenoid and anthocyanins (more pink) and larger size (most

similar to *M. lewisii*) were much more likely to get visited by bees whereas plants with more anthocyanins (more red) and high nectar volumes (most similar to *M. cardinalis*) were more likely to get visited by hummingbirds. These findings suggest that pigment and nectar production are ecologically important traits that might be under selection and play a role in reproductive isolation.

To determine if the QTL for these traits had a similar correlation with pollinator behavior (i.e., genotype effected pollinator behavior as much as phenotype), visitation was similarly compared with genotype at each QTL marker. Plants that were homozygous for the recessive *M. cardinalis* allele (*C*) at the pigmentation QTL (*YELLOW UPPER*) had deep orange and red flowers and received 80% less bee visitation than plants that were heterozygous for the '*lewisii*' allele (*L*). The QTL associated with nectar volume had similar effects on pollinator behavior. Plants homozygous for the *C* allele – which appears to be dominant and acts in an additive matter (heterozygotes have intermediate amounts of nectar) – received double the hummingbird visitations than *LL* individuals, and heterozygotes were visited an intermediate amount (*55*, *56*). Whereas flower color appears to be most important for bee recognition, hummingbirds exert more selection on nectar production. Together, these loci are implicated in phenotype, reproductive fitness, and reproductive isolation. Unfortunately, while other pigmentation QTL have been narrowed down to a specific gene, neither *YUP* nor the nectar volume QTL have been mapped to a specific gene (or genes), and no specific mutations have been identified (*57*).

Summary

While both of these examples link genetics to phenotype and fitness, neither were able to identify specific mutations, exemplifying how difficult it is to link the entire continuum. Whereas researchers were able to show consistent selection for the 'low' *Eda* allele and functional evidence implicating the locus in the body armor phenotype, the exact mutation driving the difference remains unknown. Additionally, there appear to be numerous pleiotropic functions of *Eda* blurring the exact relationship between phenotype and fitness. In *Mimulus*, on the other hand, the relationship between

phenotype and fitness is much more clear and there do not seem to be any pleiotropic effects of either QTL identified. Unfortunately, these large effect QTL represent regions of the genome, not specific loci or mutations. Even still, would these examples fit into RGs criteria for hopeful monsters? The hopeful monster is hypothesized to be a *monstrous* deviation from the bauplan of the organism, so changes in plate armor and pigmentation don't fall into that category (see much more on pigmentation and hopeful monsters in the next section). The presumed genetics also don't appear to fit into RGs ideas. *Eda* is a signaling molecule and in some ways may act as a "rate gene" affecting plate development, but it also appears to have a litany of pleiotropic effects that result in differential fitness effects. Additionally, it is unclear if any phenotype (body armor in *G. aculeatus* or flower color or nectar volume in *Mimulus*) is under monogenic or polygenic control.

Part 3: Three-peat

There are a few studies/systems that have been able to connect G-P-F. Each of the below examples measure strong selection on a variable trait in natural populations and identify specific mutations to specific genes underlying the variation. It is worth noting that all five of the following examples concern pigmentation and therefore worth discussing if color shifts should be considered as candidates for a hopeful monster.

Timema cristinae

Timema cristinae is a species of wingless stick insect that has adapted to be tightly associated with its host plant. There are three different color morphs of *Timema,* each with a specific host plant. These associations are mainly driven by predator avoidance, as each color morph is most cryptic on their respective host plants (*58*). There are two green morphs, one with a white dorsal stripe (GS) and one without (G), and a third brown melanistic morph (M). The GS morph is most cryptic on the leaves of *Adenostoma fasciculatum* whereas the G morph is most cryptic on *Ceonothus spinosus*. The M morph is conspicuous on the leaves of both hosts, but cryptic on the stems (it is also the least

common of the morphs) (*59*). Predator exclusion experiments in their natural habitat found that if predators were excluded, both morphs survived equally well on both host plants. If predators were not excluded, each morph only succeeded on their respective host plant (*58*, *59*). There is some work that suggests this selection is somewhat frequency dependent, that the cryptic morph is more strongly favored when it is the rare morph, and less so as it becomes more and more common, possibly allowing a maintenance of variation within a population. From a genetic point of view, there seems to be a relatively simply genetic basis. Green color is dominant to the melanistic, and unstriped is dominant to striped. The locus controlling color and stripe pattern ("*Mel*-stripe") has been narrowed to a 10.5 Mb haplotype of reduced recombination that can be grouped into 3 phases: *s* (green, striped), *u* (green), and *m* (melanistic). Recent work has identified a large (~1Mb) deletion at one end of the *Mel-stripe* locus that is highly associated with a shift from melanistic to green morphs. In closely related polymorphic species that do *not* carry this deletion, SNPs within the deletion region show strong phenotype associations. Interestingly, the species used in this analyses (*T. chumash*) greatly varies in pigmentation, including green, beige, pink, and dark brown morphs. These data indicate that color can be under multigenic control (as in *T. chumash*) or driven by large 'supermutations' that act as a developmental switch (as in *T. cristinae*) (*40*).

Peromyscus

In deer mice (*Permosycus maniculatus*), it has been shown that there is strong selection for coat color against particular soil backgrounds. In the dark-soil regions of Nebraska Sand Hills *P. maniculatus* live on dark-colored soils and are characterized by having darker coat color, a lower dorsal-ventral boundary (melanic dorsal pigmentation goes farther down the side of the body before switching to light ventral pigmentation), and a pronounced melanic tail stripe. In the (relatively) newly-formed (8-15,000 years ago) light-soil dunes of the Nebraska Sand Hills, *P. maniculatus* are characterized by lighter coat color, higher dorsal-ventral boundary (light ventral pigmentation comes further up the side of the body), and a less pronounced tail stripe (*39*). This variation in pigmentation

is seen as an adaptation to crypsis against avian predators and appears to be a mostly controlled by variation at one locus in particular, *Agouti*. A combination of regulatory and coding changes seems to be working together to alter each of these phenotypes, all together explaining up to 53% of variation seen in nature. One deletion in particular, a serine deletion (ΔSer), is associated with both a lighter tail stripe and ventral color, explaining 23.1% and 16% of the variation, respectively (*39*). Functional data shows that the ΔSer variant lacks binding capability and thus produces significantly less pheomelanin (yellow pigment) (*60*). Furthermore, molecular signatures and experimental evidence point to strong selection coefficients favoring this mutation, ranging from 0.126 to 0.32 (*39*, *60*).

Pepper Moth

Perhaps one of the best examples of rapid evolution by natural selection comes in the case of the pepper moth, *Biston betularia* (*61*). Prior to the Industrial Revolution, there was a single known color morph of *B. betularia* – the namesake, black and white "peppered" pattern. In 1848, the *carbonaria* form (all black) was first noted in Northern England and slowly spread south to London by the late 1800's. By the mid 1950's, the *carbonaria* form had reached estimates as high as 90% frequency throughout most of England before declining again in the mid 1970's. This rapid change has oft been cited as one of the most recognized examples of contemporary, rapid evolution and has been attributed to a combination of environmental pollution and avian predation (*62*).

Pollution from the industrialization of England during the mid 1800's left the normally pale trunks trees blackened from smoke and soot (*B. betularia* are commonly found on trunks of trees). Additionally, lichens and epiphytic plants which normally provide a cryptic stage for the wild type moths struggled to survive in this environment, removing another source of camouflage. As such, the once cryptic peppered moth was now conspicuous to avian predators and allowed the *carbonaria* form to rise rapidly in frequency. Many selection experiments have been conducted (see (*61*) for a comprehensive review) and, despite some methodological complaints (which are rather weak (*61*, *63*)) the reason for this rapid change is clear – reduced predation on the more cryptic morph. For

example, (*64*) showed that on lighter substrates, the wild type was at an advantage whereas on darker substrates, the *carbonaria* form had the upper hand (wing?). This study (along with many others of its time), used dead, frozen moths and as such, received criticism as to its perceived lack of realism (*61*). More recently (and in an effort to combat some of these criticisms), a 6-year study for which over 4,800 live moths were released showed strong selection against the *carbonaria* (*63*). In this study, since pollution is not at the levels it was in the late 1800's (or even mid 1900's, surprisingly), the trees were mostly light, and as such the wild type was more cryptic. Cook and colleagues calculated a selection coefficient (*s*) of ~0.1, which is sufficient to describe the recent rapid decline in *carbonaria*.

Molecular signatures indicated similarly strong selection favoring the *carbonaria* morph during the industrialization. The *carbonaria* has long been known to be dominant to wild type and controlled by a single locus. Genetic mapping narrowed the region of interest to a 1.4 Mb and researchers in 2011 further narrowed that region to a 200kb locus with strong signatures of selection (elevated linkage disequilibrium, $D' = 0.9$). Amazingly, they show that this "core region" is present in multiple populations across England, suggesting a single mutational origin that subsequently spread through the region (*65*). Further work from the same group identified a 20kb transposable element inserted within the first intron in the gene *cortex*. The effect of this insertion in *cortex* function is unclear, but there is heightened expression of a particular splice isoform carrying this insertion during wing morphogenesis (*66*). Although this gene has been shown to have a specific function in regulating meiosis in gametogenesis, it has been implicated in other Lepidopteran taxa as a target of selection for pigmentation (*67*).

Morning Glory

Shifts in flower color are not uncommon by any means (*68*) and are generally attributed to pollinator mediated selection. Genetically speaking, many of these shifts are driven by a few large effect loci (*56*, *68*) to different components of the pigment production pathways. In *Ipomoea purpurea*, the common morning glory, the shift from purple to white flowers appears to be no

exception. The locus controlling this shift is known as the *A* locus where the mostly dominant *A* allele produces purple flowers (*Aa* flowers are not as deep of a purple as *AA* plants) and the recessive *a* allele halts anthocyanin production, resulting in white flowers (*69*). The mutation in question is a transposable element insertion in the chalcone synthase gene, the first enzyme of the anthocyanin biosynthetic pathway (ABP), that renders the gene non-functional (*70*). Although this locus and phenotype have no effect on outcrossing rates (white and purple flowers have similar outcrossing rates), white flowers benefit from increased selfing resulting in a transmission advantage for the *a* allele. Despite this benefit, white flowers make up only ~1% of flowers across 22 populations (*71*). Controlled field experiments uncovered severely decreased survival for *aa* plants, bringing to light a deleterious pleiotropic effect of the *a* allele and providing an explanation for the maintained rarity of the mutant morphology. It is assumed that this pleiotropic effect is the result of the complete loss of the ABP, as anthocyanins are used for a cornucopia of processes throughout a plant (*69*).

Orchid

An interesting example of this continuum can also be found in the Alpine Orchid, *Gymnadenia rhellicani*. Kellenberger and colleagues (*38*) identified the genetic basis of an overdominant floral pigment phenotype and showed the selective agent maintaining the variation in the population. This is not only one of a subset of examples to connect the continuum, but additionally rare in showing a real-world example of overdominance with no detrimental effects of either homozygote class.

In a population in N. Italy, there is a population of *G. rhellicani* that is polymorphic for flower color with 62% black (wild type), 28% red, and 10% white. This dynamic was first described in 1906 but was more closely monitored starting in 1997 and since then, the black morph has been slowly decreasing while the red and white increase in frequency, likely indicative of some type of selective forces at work.

In fact, Kellenberger found that the intermediate red morph set significantly more fruit (reproductive fitness) than either of the other two morphs (which were not significantly different from one another). Pollinator observations indicated two main pollinators: bees, which prefer black flowers, and flies, which prefer the white flowers. While these pollinators showed preference for the extreme morphs, both were seen visiting the red morph at high levels, thus driving the increased fruit set. Thus, pollinator-mediated selection is maintaining the pigment variation in this population.

Comparative transcriptomics showed a marked decrease in expression of *anthocyanin synthase* (*GrANS1*), an enzyme integral to the last steps of the anthocyanin biosynthetic pathway (ABP), but there were no mutations that correlated with phenotype. This, as well as the maintenance of anthocyanidin at the base of the corolla of the white morph indicated a regulatory change. Further association studies showed a strong correlation with an *R2R3-MYB* (*GrMYB1*) transcription factor, a family of proteins heavily involved with anthocyanin production. They identified a SNP in the last exon with three states: C (wild type), G, and A. Both the C→G and C→A transversions turn a Tyrosine (TAC) into a premature stop codon (TAG, TAA), removing the last 43 amino acids of the protein. SNP genotyping showed all plants homozygous for C had high *ANS* expression and were darkly pigmented, whereas plants homozygous for either of the alternate alleles (or heterozygous G/A) showed little to no *ANS* expression or pigmentation. Intermediate (red) flowers carried one functional C allele and produced intermediate amounts of pigment. Functionally, RNAi of *GrMYB1* showed a reduction in color and expression of both *GrMYB1* and *GrANS*. Thus, nonsense mutations to *GrMYB1* control pigmentation in *G. rhellicani* and this polymorphism is maintained by pollinator-mediated selection on floral color (*38*).

Common Ground

Each of these studies demonstrate elegant experimental design coupled with extensive analyses to fully connect G-P-F. Common amongst the studies mentioned in this section is the general subject matter – pigmentation. Why might this be? Well, for starters, color is one of the best studied

characteristics in the field (*68*, *72*). It is relatively easy to phenotype and can be easily broken down as a both categorial (presence/absence) or continuous (hue/brightness/chroma) trait and the genetics are incredibly well understood. In both angiosperms and vertebrates, the pigmentation pathway has been well studied and is highly conserved (*72*, *73*), is easily quantifiable (*72*, *74*), has clear effects on fitness, and the trait itself is evolutionarily labile and there is a lot of variation both within and between species (*68*, *75*). This gives researchers strong candidate genes for a trait that harbors a lot of variation, thus increases the likelihood of finding the genetic cause.

Timema stands alone from the above examples from a genetic point of view. While no specific locus was identified, a specific mutation (a 1Mb deletion) was identified. This macromutation results in a binary phenotype in *T. cristinae*: green or melanistic. This pattern more closely fits into the idea of a hopeful monster. On the other hand, that same 1Mb region in another species (*T. chumash*) exhibited highly polygenic control of the same trait but resulted in a very continuous phenotype. This pattern fits perfectly in line with the ideas of the MS and small effect micromutations. In one species there is evidence of gradual change, and in the other there is evidence for saltational change. The fact that the same phenotype can be controlled by the same locus through such differing mechanisms is fascinating. Although color may not be the best candidate for hopeful monsters (see below), evidence for large effect macromutations succeeding in a stable species is an important finding.

If a hopeful monster is a radical phenotypic change with simple genetic basis that could create new lineages, would RG consider color shifts hopeful monsters? I would argue not. For starters, RG himself didn't consider them to be. In *The Material Basis of Evolution* he placed them in the category of *micromutation*, undoubtedly important for evolution within species and, if the situation was optimal, one color morph could supplant the ancestral (anagenesis), but of little importance to lineage splitting (cladogenesis) (*6*). Color did not divide *true* species, as he saw it, but more or less as a divider between subspecies, a type of local adaptation. In fact, much of the variation

heretofore discussed would likely fall in that category for RG. Now, how RG drew the line between micro- and macroevolution is up for debate, especially since we know that color shifts can be adaptive and drive reproductive isolation (see (*76*), but the essence of his argument remains, color shifts are not of the same ilk as monsters like homeotic mutants. Secondly, a large part of RGs idea was to explain the notable lack of intermediate forms in the fossil records. As color is not retained in fossilized specimens (with the few rare exceptions (*77*)), it is not a good fit for RGs hypothesis. Lastly, the very nature of a hopeful monster is a radical change resulting from slight perturbations in early development that most of the time should *not* survive. It is the rare few that are able to create entirely new lineages, thus being hope*ful*. In the next section, I will review some of the best-known examples of hopeful monsters in plants and animals.

Part 4: Hope Springs Eternal

So, the question remains…do hopeful monsters exist? Perhaps the better question is "*have* they ever existed?". The strongest defense of the existence of lineage-splitting monsters comes via macroevolutionary mechanisms and the fossil record. There are two core arguments: (1) the mechanism of microevolution (small, gradual steps) cannot create the "bridgeless gaps" between good, true species and higher-level taxa (see (*78*, *79*)), and (2) the notable *lack* of intermediate forms in the fossil record suggests that saltational jumps are not only possible, but likely plausible (*3*, *7*, *14*, *18*). It is important to note that I am not attempting to argue that saltational jumps and hopeful monsters are the *only* way in which taxa might diverge. Many (if not all) of the examples described and referenced above very well may lead to cladogenetic splits. I am, again, focusing on a singular end of the spectrum of evolutionary mechanisms (hopeful monsters) and placing current research in that framework.

At this point, we can walk through a thought experiment. If we wanted to find a hopeful monster, where can we look to increase our odds of discovery? Hopeful monsters are, by definition, a saltational phenomenon. For this reason, categorical traits might prove more fruitful than continuous

traits. Furthermore, RGs idea of the hopeful monster focused on developmental changes stemming from small changes in "rate" genes (e.g., transcription factors). Is there a trait that meets these criteria (categorical traits plus developmental anomalies) and has been heavily studied and identifiable (like pigmentation)? Together, this leads us to organ identity (and, eventually, homeotic mutants, just as RG proposed). Finally, we can ask if there is any group of organisms somewhat predisposed to these types of changes. Many have argued that plants, given their repetitive, meristematic, relatively "open" body plan would be more capable of surviving developmental macromutations than animals which go through development once during a highly coordinated embryogenesis (*30*, *31*, *80*). In fact, Darwin's "abominable mystery" – the remarkably sudden and prolific origin of flowering plants – could have been a hopeful monster resulting from a homeotic change and every flowering plant today a descendant of that mutant (*31*, *32*, *81*). Most recently, the strongest examples of hopeful monsters in nature come from floral organ transitions (homeotic mutants), all controlled by a suite of transcription factors in the MADS-box gene family (*31*, *82*). This, though, does not exclude the existence (or evidence of) these mutants in animals. The most analogous argument in animals would be body segmentation in insects which is controlled by a suite of transcription factors known as *Hox* genes. Here I will give two of the better examples of possible monsters (one for both animals and plants). These are not necessarily the *only* examples, but were chosen for either the clear connection between one taxa and a sister lineage (*17*) or for being a homeotic change with possible effect on reproductive fitness (*83*).

Cirripedia

Cirripedia is a type of crustacean with many characteristics that are intriguing to say the least. The have a calcareous shell (akin to bivalves) as well as "biramous" appendages (a crustacea trait). Most intriguing for our discussion is the notable *lack* of abdomen in the group (*17*). In 2006, Géant and colleagues showed that in all three orders of Cirripedia, the *Hox* gene *Abd-A* was apparently missing. *Abd-A* determines abdominal organ identity, as when it is non-functional abdominal

segments resemble thoracic segments, not only in appendages but also nervous and digestive system formation. In other words, *Abd-A* is required for proper development of the abdomen (*26*). Researchers were unable to detect expression of any protein or transcript and sequencing efforts revealed a high prevalence of repetitive regions in the presumed genetic location of *Abd-A*. *Hox* gene clusters are impressively conserved across taxa, not only in function but in synteny as well (*26*)). All other *Hox* homologs were identified in expression analysis and *in situ*. In the sister taxon, Ascothoracida, *Abd-A* is present and has conserved functionality. It is also worth noting that there are no repetitive elements throughout the rest of the *Hox* cluster in Cirripedia or *Hox* clusters in general, signaling an apparently unique molecular characteristic of an otherwise highly conserved region (*17*).

Are modern day Cirripedia hopeful monsters? Probably not, as they are extant species that have no doubt gradually changed and adapted to their environments. Are they the descendants of a hopeful monster? Maybe! Unfortunately, these analyses are necessarily comparative and it is unclear if these genetic changes were the cause of the change or a result. Additionally, there is no obvious fitness benefit to not having a fully developed abdomen. Herein lies the problem of describing hopeful monsters at the macro-evolutionary level. These are *comparative* analyses. Assuming all members of one taxon shared the same homeotic shift (and conserved genetics) compared to their sister taxon, it is almost impossible to know whether or not this homeotic change caused the split or occurred after the split. Although the genetic evidence is strong in Cirripedia and work with segmentation and *Hox* genes in other crustaceans show simple homeotic expression changes phenocopy sister taxa (*16*, *27*, *28*), the natural evidence is inherently lacking. To determine if these changes might have caused a lineage split, there would need to be a current example of a naturally occurring homeotic shift in a polymorphic population in which reproductive isolation and relative fitness could be measured. Research of these types of mutants in nature is, not surprisingly, lacking (*83*, *84*). There are very few examples of a naturally occurring homeotic mutant in nature, and these tend to be in plants.

Capsella bursa-pastoris

Homeotic mutants in plants are not uncommon (*21*, *29*, *30*), but these are often of the horticultural or lab variety. While these are integral to understanding developmental genetics (especially floral mutants), their effect on fitness in nature is either unknown or obviously negative. In a macroevolutionary sense, evidence suggests that homeotic shifts may underlie the "abominable mystery" that is the origin of flowering plants as well as the shift from actinomorphic to zygomorphic flowers (*8*, *85–87*). In natural populations, homeotic mutants have been observed somewhat regularly, but often only as a single flower on an inflorescence, or a single plant that fails to reproduce (what Goldschmidt would call a "hope*less* monster") (*84*, *86*). Some commonly cited examples for naturally occurring homeotic mutants (hopeful monsters) that have managed to persist are; (1) *Vinca minor flore pleno* (stamen to petal transition), (2) *Linaria vulgaris* var. *Peloria* (peloric mutant (zygomorphic to actinomorphic flower)), and (3) *Clarkia concinna* var. *bicalyx* (petal to sepal transition) (*88–90*). While each of these are examples of stable homeotic mutants in nature, they persist mainly because they propogate vegetatively (1-2), are primarily self-pollinated (3), or are seed sterile (2) and thus the mutants likely have little to no impact on overall fitness and subsequently are unlikely to spur new lineages. *Linaria vulgaris* var. *Peloria* is perhaps the best candidate, as the shift from zygomorphic to actinomorphic flower has occurred many times in angiosperms and represents major shifts between lineages, as RG hypothesized (*90*). The genetic basis of this shift has been identified as an epigenetic change (methylation) to the gene *CYCLOIDEA*, a transcription factor heavily implicated in determining floral symmetry during early floral development. Unfortunately, this mutant is seed sterile and can only propagate vegetatively (*91*) and is therefore is unlikely to result in a new, successful lineage.

The most appealing (and oft-cited front runner) candidate as a hopeful monster is *Capsella bursa-pastoris* and the naturally occurring petal to stamen mutant, *C. bursa-pastoris* var. *Spe* (*83*, *92*). The mutant variety has established itself in many wild type habitats, grows in sympatry with the

wild type, and has been described for over 200 years. For this reason, it is assumed to have similar, if not occasionally higher fitness, than the wild type. Although each morph produces similar numbers of seeds per fruit, the wild type produces more flowers (and fruits) and therefore overall seed production is much higher for the wild type morph. Alternatively, seeds from the var. *Spe* mutant have higher germination rates, another important fitness factor. While there seems to be some compensatory selection for floral architecture vs germination, the petal to stamen transition likely has little effect on overall fitness of the mutant as this plant is mainly self-pollinated (*83*, *92*). Although a specific gene (or genes) or mutation have not been identified, genetic mapping indicates a single region of the genome that is syntenic with the location of the C-class floral identity gene *AGAMOUS* in *Arabidopsis* (*31*). *AGAMOUS* is implicated in the ABC model of floral development (*21*) and is integral to stamen and carpel formation. Ectopic *AGAMOUS* expression in the 3rd floral whorl (normally consisting of petals) results in a replacement of petals with stamen in other taxa (*93*).

Does the var. *Spe* mutant fit into RGs framework of a hopeful monster? It is a homeotic shift likely controlled by a transcription factor that alters early development. Furthermore, as it occurs alongside the wild-type in multiple populations fitness differences can be measured and although there are some aspects for which the mutant has higher fitness, the wild-type is favored through other mechanisms, making the mutant phenotype fitness neutral. Since *Capsella* doesn't heavily rely on pollinators for reproduction, alterations to floral structure are unlikely to result in reproductive isolation and therefore the var. *Spe* mutant is unlikely to result in a new lineage. While the mutations may drastically alter floral shape, none of the taxa (except *Linaria*) rely on pollinators for reproduction, so there is no *a priori* reason to assume that these changes would affect reproductive fitness and therefore, unfortunately, still don't fulfill RGs ideas of a hopeful monster.

Where are we now?

Unfortunately, the data regarding survival of homeotic mutants in nature is scarce (*84*). All of the examples mentioned above either have no natural fitness data (Cirripedia) or, at best, have a

neutral effect on fitness in nature (*Vinca, Clarkia, Linaria,* and *Capsella*). Cirripedia may have been a hopeful monster when it first arose, but it is hard to say whether it thrived *because* of the loss of abdomen. As this group is a fully established taxon, the analysis as it currently stands is entirely comparative and is therefore difficult to determine if abdomen loss caused a split or was a result of a split.

It is interesting that both the Cirripedia and *Capsella* examples above are *losses*, not gains. It is easy at first to assume that a hopeful monster must be a gain, but there is no reason for this. Most of the examples RG gives in his description (limb reduction in Dachsunds, tail reduction in Archaeopteryx, wing reduction in cormorants) are losses or reductions as opposed to gains (*6*, *17*). Another hypothesized hopeful monster is the paedomorphic salamander, the Mexican axolotl (*Ambystoma mexicanus*). The retention of the paedomorphic state is classified as a "failed metamorphosis" and is derived characteristic and may in fact be also be a loss of function phenotype (*94*). This is not to say that a gain-of-character mutation cannot fit into RGs framework, rather that losses might be more plausible from both a genetic and developmental perspective (*95*). Cooption and heterotopic expression of regulatory networks could underly many major evolutionary transitions and key innovations ((*96*, *97*).

The last (often overlooked) aspect of a hopeful monster is that it *must* occur in the right ecological context. The developmental shift from typical ray-finned fish to flatfish, for example, would be monstrous if the proto-flatfish lived in the water column like the average fish. But if they already spent a lot of time living on the ocean floor, then the laterally compressed body plan and asymmetric brain case and most importantly asymmetric eye placement, all of a sudden might be beneficial (*6*, *98*).

The way I see it is as such: there are two types of hopeful monsters – the micromonster and macromonster. The macromonster could be something like a Cirripedia ancestor. A radical change that has since spawned a separate evolutionary branch. Micromonsters would be represented by a

radical change within a population that might spawn a new species and *could* go on to spur a new diversified lineage. Arguments against macromonsters are similar to the Cirripedia argument made above: they are comparative and we cannot be sure that the change was causal of the split. Arguments against micromonsters are that they are just hyper-successful varieties. But it has to be recognized that any macromonster must begin as a micromonster – a radical change to a single individual in a population of, for lack of a better term, the ancestral state. And until the success of a micromonster is shown, it will be difficult to accept the possibility of a macromonster. Ideally, a research program would combine these two ideas. Using a micromonster, specific genes and mutations could be identified along with fitness differences and evidence of reproductive isolation that could lead to a new species. This could then be corroborated with a macroevolutionary comparison showing that the phenotypic shift represented by the micromonster is stable at a macroevolutionary level. Next, I will introduce a homeotic mutant of *Aquilegia coerulea*, *A. coerulea* var. *daileyae*, in which the nectar producing petals are replaced with a second set of sepals. We show that this 'micromonster' is under strong selection in the field and have identified the gene and multiple, independently occurring loss-of-function mutations causing the phenotype. We show asymmetric pollen movement that could lead to reproductive isolation and, subsequently, a new species. This morphological shift – the loss of nectariferous petals – has been shown many times within the Ranunculaceae, indicating that the loss is a stable macroevolutionary shift. Thus, *A. coerulea* var. *daileyae* represents the best-known example of RGs hopeful monster.

"Hope is a good thing" – *A. coerulea* var. *daileyae*

The genus *Aquilegia* provides a fantastic opportunity to study the evolution and genetics of complex traits. It is a relatively young (~7 million years old) but widespread (entire northern hemisphere) and speciose (70 species) genus that has undergone a recent, rapid radiation (*99–101*). The structure that is believed to have led to this recent, rapid adaptive radiation is a modified petal with a nectar spur (*36*, *102*). These modified petals are thought to be intimately associated with a

specific pollinator (*100*). It has been shown that there is high inbreeding depression in *Aquilegia* and it is hypothesized that these nectar spurs, by being associated with specific pollinators, promote outcrossing and are therefore integral to the reproductive success (fitness) of an individual (*103*, *104*).

A. coerulea (wild type, WT) follows the general *bauplan* of the genus (five petaloid sepals alternating with five nectariferous petals) and occurs in the southern and central Rocky Mountains in rocky outcrops in montane and subalpine habitats (2,100-3,700m), receiving full sun and summer snowmelt (*105*, *106*) (Figure 2). In contrast, *A. coerulea* var. *daileyae* (mutant, var. *daileyae*, D) is a naturally occurring homeotic mutant in which the petals have been transformed into a second set of sepals (Figure 1) (*106*). The loss of petals removes the nectar reward and would therefore presumably be detrimental for pollinator visitation by decreasing outcrossing and therefore fitness. Despite this likely fitness cost, we currently know of three populations where the mutant is prevalent; Reynolds Park (~25%), Hermit Park (100%) and Smookler (100%). The repeated occurrence and apparent success of this mutant indicates that it is at least selectively neutral and may have some selective advantage over the wild type. These populations are atypical of most *A. coerulea* populations as they occur at the geographic edge (eastern foothills of the Rocky Mountains) and lower elevational limit (2,200-2,500m) of the species range. Additionally, as opposed to most populations that occur in open meadows and rocky outcrops with full sun and plenty of summer snowmelt, these populations all occur in mixed conifer forests with partial sun and no summer snowmelt and may therefore experience unusual selection pressures (i.e., increased competition/predation, decreased resources, increased stress) (*107*). Three years of observation of a polymorphic population (Reynolds Park) show consistent, strong positive selection for the D morph. Each year, the D morph sets significantly more fruit (no difference in flowers/plant, fruit size, or seed number) and this difference in fruit set is driven by reduced floral herbivory (s = 0.17-0.3). This is the first example of strong selection

favoring a homeotic mutant alongside the WT morph, satisfying the important ecological aspect of a proposed hopeful monster.

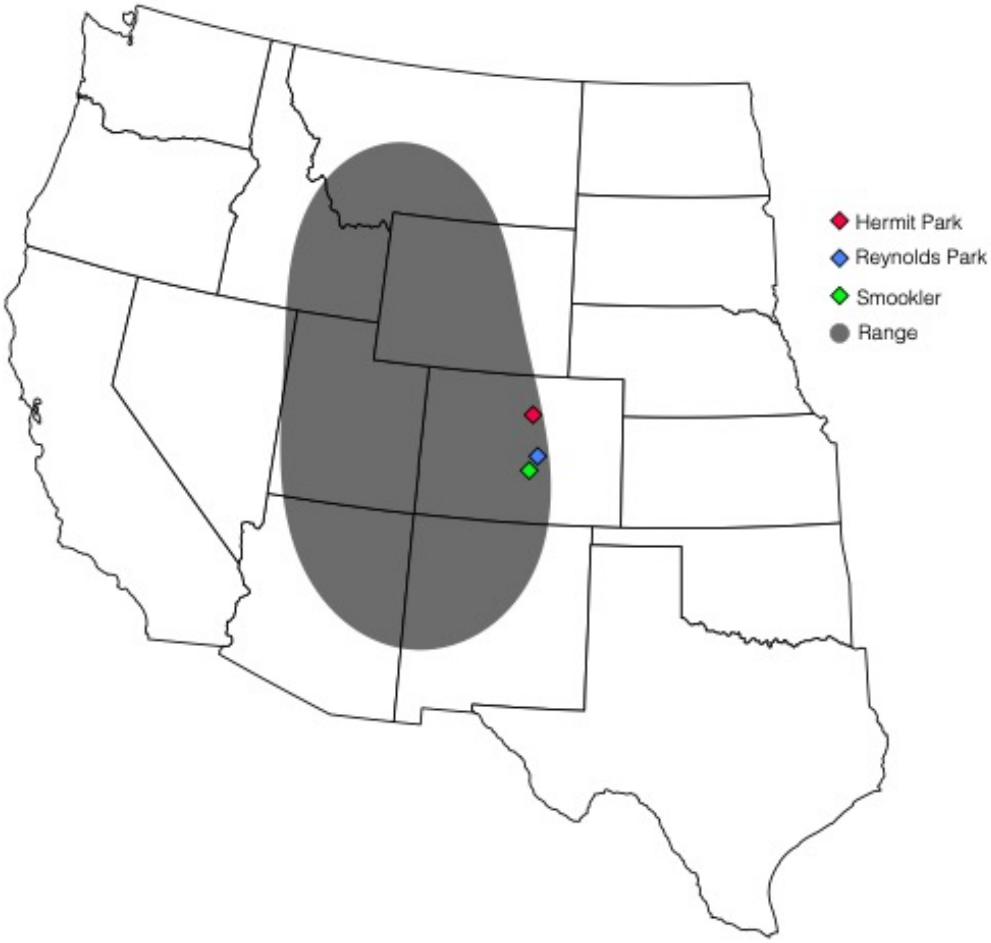

Figure 1. Top: Range map for *A. coerulea* with known *A. coerulea* var. *daileyae* populations marked. **Bottom**: From left to right – photos of *A. coerulea* (WT, side, front) and *A. coerulea* var. *daileyae* (D, side, front).

Floral development has been widely studied in the core eudicots and is generally attributed to the interaction of a certain class of transcription factors known as MADS-box genes (Type II MIKC, specifically), otherwise known as the ABC model of floral development. In short, A-class genes expressed alone code for sepals (1st, outermost whorl), A- and B-class genes code for petals (2nd whorl), B- and C-class genes code for stamen (3rd whorl), and C-class genes code for carpels (4th, innermost whorl) (*21*). These transcription factors form tetramers with other A, B, and C-class genes and determine organ identity early in floral development. Previous work has shown that prior to the diversification of the Ranunculales, there were multiple duplications in the *paleoAP3* lineage (the ancestral B-class gene) yielding three paralogs; *AP3-1, AP3-2, and AP3-3* (*108*). Expression work has shown that *AP3-3* has been sub-functionalized to the petal and has therefore been implicated in the wide diversity of petal morphology seen within the group. Furthermore, within the family there have been nine independent losses of petals (*109*) and these losses are highly correlated with a loss of function or severely lowered expression of AP3-3 paralogs (although outward expression of the C-class gene AGAMOUS1 cannot be ruled out (*47*, *48*)). In *Aquilegia*, knock-down experiments have shown that *AP3-3* functionality is necessary for production of the petal and when silenced the flower reverts to 1st whorl sepals (*110*), identical to the var. *daileyae* phenotype. This provided us with a strong candidate gene for the mutant phenotype. DNA sequencing and SNP genotyping identified 4 loss of function (LOF) mutations to *AP3-3* that are almost perfectly associated with the var. *daileyae* phenotype. Haplotype construction and analyses indicate that each of these mutations represent independent alleles arising from previously functional WT haplotypes. Furthermore, molecular signatures of selection indicated extended strong, ongoing positive selection on mutant *ap3-3* alleles, corroborating the selection measured in the field. This is in line with Goldschmidt's view of saltational evolution, which he proposed could occur through "developmental macromutations" in

"rate" or "controlling" genes (i.e., transcription factors) that by altering development could have drastic impacts on the phenotype of an organism (*14*).

Particular to plants, pollinator abundance and diversity may change drastically at the edge of the range which may drive a shift in mating systems (*111*). Unlike the previously mentioned examples (*88–90*, *92*), *Aquilegia*, though not an obligate outcrosser (*105*, *112*), depends heavily on pollinators for reproduction. There is high inbreeding depression in *A. coerulea* (*103*) and it has been shown that hawkmoth abundance is associated with higher outcrossing rates, and therefore higher fitness (*112*). The lack of spurs on the mutant phenotype likely prevents the predominant pollinator (hawkmoth) from efficiently transferring pollen between flowers (*113*, *114*) which we would expect to lead to higher inbreeding among D morphs. SNP genotyping of four neutral, unlinked loci indicate exactly this pattern, suggesting restricted pollen dispersal for D plants that is likely driven by pollinator behavior. Additionally, SNP genotyping at *AP3-3* revealed that the population boasts a significant lack of *AP3-3*/*ap3-3* heterozygotes, a result that is also likely driven by asymmetric pollen movement. These patterns of assortative mating could lead to a cladogenetic split between *A. coerulea* and *A. coerulea* var. *daileyae*, satisfying another tenet of RGs framework for a hopeful monster.

Floral homeotic mutants are capable of surviving in the wild through autogamous fertilization or clonal reproduction, but, until now, little to no data has been collected on floral homeotic mutants that drastically impact reproductive fitness in nature. From a microevolutionary lens, the data presented in the following chapter provides a complete example of natural selection in a polymorphic population. By identifying the source of selection (floral herbivory) and the concomitant impact on reproductive fitness (fruit set) and the specific, causal mutations, we can fully connect genotype, phenotype and fitness. Additional genomic signatures around the causal alleles bolsters the evidence of selection measured in the field. From a macroevolutionary lens, we are showing concrete evidence

of a homeotic mutant being favored over a wild-type in a natural population. Loss of nectariferous petals has happened many times in the Ranunculaceae and is the defining characteristic of many taxa (*48*). This repeated loss signifies that it is an evolutionarily stable transition, capable of spurring new lineages (no pun intended). These data are instrumental in the debate between saltational and gradual evolution, for a homeotic mutant that is both under positive selection and effects floral design and possibly pollinator behavior might alter gene flow and lead to a new species.

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Chapter 2: A Hopeful Monster in *Aquilegia*

Uncovering the genetic basis and selective advantage of a naturally occurring floral homeotic mutant of *Aquilegia coerulea*

Summary

In 1933, Goldschmidt coined the term “hopeful monster” to symbolize his view of how new lineages of organisms may arise and his ideas have been debated ever since. He envisioned simple genetic changes that result in radical phenotypic abnormalities, usually unfit but occasionally surviving and establishing a new species. Here we describe a naturally occurring floral homeotic mutant of the columbine *Aquilegia coerulea* that is lacking the characteristic nectar spurs of the genus. While it would be expected that this loss of pollinator reward would be disadvantageous to the mutant morphology, we find that the mutant is under relatively strong, positive selection (s = 0.17-0.3) due to reduced floral herbivory. We identify the underlying locus (*APETALA3-3*) and multiple independently-derived causal loss-of-function mutations indicating an on-going soft-sweep. Elevated linkage disequilibrium around the two most common causal alleles indicates that positive selection has been ongoing for many generations. Lastly, genotypic frequencies at *AqAP3-3* indicate a degree of positive assortative mating by morphology. Together, these data not only provide a compelling example of the initial stage of the establishment of a hopeful monster, but also provide a particularly clear example of linking genotype to phenotype to fitness.

Keywords: positive selection, homeotic mutant, hopeful monster, soft sweep, assortative mating, herbivory, selection coefficient

Introduction

Darwin famously argued that evolution proceeds gradually through many changes of small effect, slowly improving one form over another (*1*). However, Richard Goldschmidt, while recognizing gradual microevolution within species, viewed the origin of new species and lineages as arising through single saltational macromutations that caused large changes in body plan resulting in "bridgeless gaps" between taxa (*2*). He referred to an individual with such a macromutation as a "hopeful monster" because most often they would be manifestly unfit, but occasionally, if they occurred in the proper ecological setting, the "monster" could be favored by natural selection[2]. Supporting Goldschmidt's ideas are comparative analyses showing that changes in the number or expression patterns of genes involved in organ development (e.g., homeotic patterning genes (*3*–*7*) or organ symmetry genes (*8*, *9*)) are correlated with major morphological differences between taxa, though whether these differences evolved through single macromutations or the accumulation of many smaller changes is often unknown. In addition, it is unclear whether these changes occurred at the time of speciation (resulting in cladogenesis) or subsequently. While theory has predicted that large effect mutations should be fixed during adaptation and the identification of large-effect QTL supports this prediction (*10*–*12*), these examples do not fundamentally affect organismal form as Goldschmidt envisioned and it is often unclear whether single mutations underlie their effects. Also supporting Goldschmidt's view are single homeotic mutations which can drastically alter body plans, though these are usually clearly less fit (*13*). In plant populations, a few examples of naturally occurring floral homeotic mutants have been documented but they either represent very rare individuals in a population (*14*) where a selective advantage could not be determined or occur in predominantly selfing or clonal

species where they are unlikely to promote reproductive isolation (*15*, *16*). As a result, whether hopeful monsters could account for some patterns of evolution has remained controversial as no clear example of a known single 8macromutation (with a monstrous effect) being favored in a natural population has been observed (*17–19*). To our knowledge, we provide the first strong example of a hopeful monster in a natural population.

The columbine genus, *Aquilegia*, is noted for having petals with long, tubular nectar spurs. These nectar spurs have been considered as a 'key innovation' (*20*) driving diversification in the genus as the length, shape and color of these nectar spurs are correlated with the major pollinators of each species (*21*). *A. coerulea* ("WT", Fig. 1A) follows the basic *bauplan* of the genus having five flat, petaloid sepals in the outermost whorl and five petals in the second whorl, each with a tubular outgrowth (nectar spur). Pollination has been extensively studied in this species (*22*, *23*) and there are two major pollinators: hawkmoths, which probe the long spurs for nectar, and bumblebees, which collect pollen. The abundance of hawkmoths (but not bumblebees) in a population is significantly associated with higher outcrossing rates (*23*). *A. coerulea* suffers from severe inbreeding depression (*24*) and thus traits promoting outcrossing (e.g. hawkmoth visitation) should be favored.

In 1897, a homeotic mutant where the petals are converted into a second set of sepals (10 sepals, 0 petals), was described as *A. coerulea* var. *daileyae* ("D", Fig. 1B) (*25*). Apart from the replacement of nectariferous petals with non-nectar bearing sepals, there appear to be no differences in floral structure between the two morphs (Fig. 1) (*25*). Despite this radical change in 2nd whorl organ identity, it was noted at the time that the mutant form appeared to

be increasing in frequency (*25*), and 100 years later, the high abundance of the D morph was noted approximately 24 km south of the original locality in central Colorado (Reynolds Park Open Space) (*26*). The high abundance of the D morph is surprising given that the loss of petals and any nectar reward would likely discourage hawkmoth visitation (outcrossing). Even if hawkmoths attempted to visit D plants, their long tongues would prevent them from coming into close contact with the reproductive organs of the flower (Fig. 2E).

Alas, both pollinators are present in this population and appear to visit both morphs (Supplemental Video S1), indicating that the loss of nectar spurs may not be as detrimental as expected and the D mutant might at worst be selectively neutral, or there may be some other factor favoring the mutant morphology. This population is atypical of most *A. coerulea* populations as it occurs at the geographic edge (eastern foothills of the Rocky Mountains) and lower elevational limit (2,400m) of the species range (2,100-3,700m) (*25*, *27*). Additionally, as opposed to most populations that occur in open meadows and rocky outcrops with full sun and plenty of summer snowmelt, this population occurs in mixed conifer forests with partial sun and no summer snowmelt and may therefore experience unusual selection pressures (i.e., increased competition/predation, decreased resources, increased stress) (*28*). Our first field season (2014) suggested floral herbivory may drive a difference in reproductive success between the two morphologies, so this was chosen as the main selective pressure to measure over the next two seasons (2015-2016). Floral herbivory can exert strong selection on floral morphology (*29*, *30*), spotlighting the importance of studying non-pollinator agents of selection on floral shape (*31*). This population (Reynolds Park, Fig. 1C) provided us with a unique opportunity to study the apparent success of this homeotic mutant

in a naturally occurring common garden.

Here, we use a combination of field observation and molecular techniques to show relatively strong, consistent, and ongoing positive selection for a radical floral mutant. We first establish floral herbivory, not pollinators, as the source of selective pressure driving differential reproductive output (fruit set) between the morphs. Then, using a combination of sequencing and mutation genotyping we identified the locus (*APETALA3-3*) and multiple, independently-derived, recessive loss-of-function mutations that underlie the phenotypic shift, thus fully connecting genotype, phenotype, and fitness. This information allowed us to examine molecular signatures of selection around the locus and show heightened patterns of linkage disequilibrium (LD) around the independently derived mutant alleles resembling a multiple-origin soft sweep over many generations (*32*). Lastly, the drastic difference in flower morphology between morphs suggests that pollinator behavior or pollen dispersal dynamics may be affected as genotypic frequencies at the causal locus indicate a significant degree of positive assortative mating by morph. Taken together, these findings show that radical homeotic mutants can indeed succeed in natural populations and potentially lead to a new lineage.

Results

Floral herbivory favors homeotic mutant

In each of three flowering seasons (2014-2016), we mapped the position of every flowering plant in one portion of Reynolds Park (Fig. 1C) and recorded the number of flowers produced

and whether fruits matured. The vast majority of plants had flowers of either WT or D morphology though rare individuals with intermediate morphology (or variable morphology within and/or between flowers) were observed (Fig. S2A-B, Table S7) which we included as mutants in analyses. We found that across all three flowering seasons D flowers were significantly more likely to produce fruits than WT flowers (s_{WT} = 0.17 – 0.3, Table S5, Fig. 3A) even though there were large differences among seasons in the overall frequency of fruit-set. Environmental factors such as water availability and temperature likely affect fruit-set as, for example, 2016 was particularly hot and dry during the growth season (Table S2) and fruit set was substantially lower. Similarly, 2017 was an even drier year and we found few plants producing mature flowers (resulting in a cancelled field season). However, environmental factors are unlikely to explain the differences in fruit-set between the morphs within any season since the two morphs did not differ in their spatial distribution in the population and grew as close as a centimeter apart (Fig. 1C, S1). Furthermore, the number of flowers produced per plant, which could be an indication of resource availability, did not differ between the morphs in any year (Fig. 2A). Thus, abiotic factors (e.g., soil chemistry, sunlight etc.) are unlikely to be responsible for the differences between the morphs in fruit production in any season.

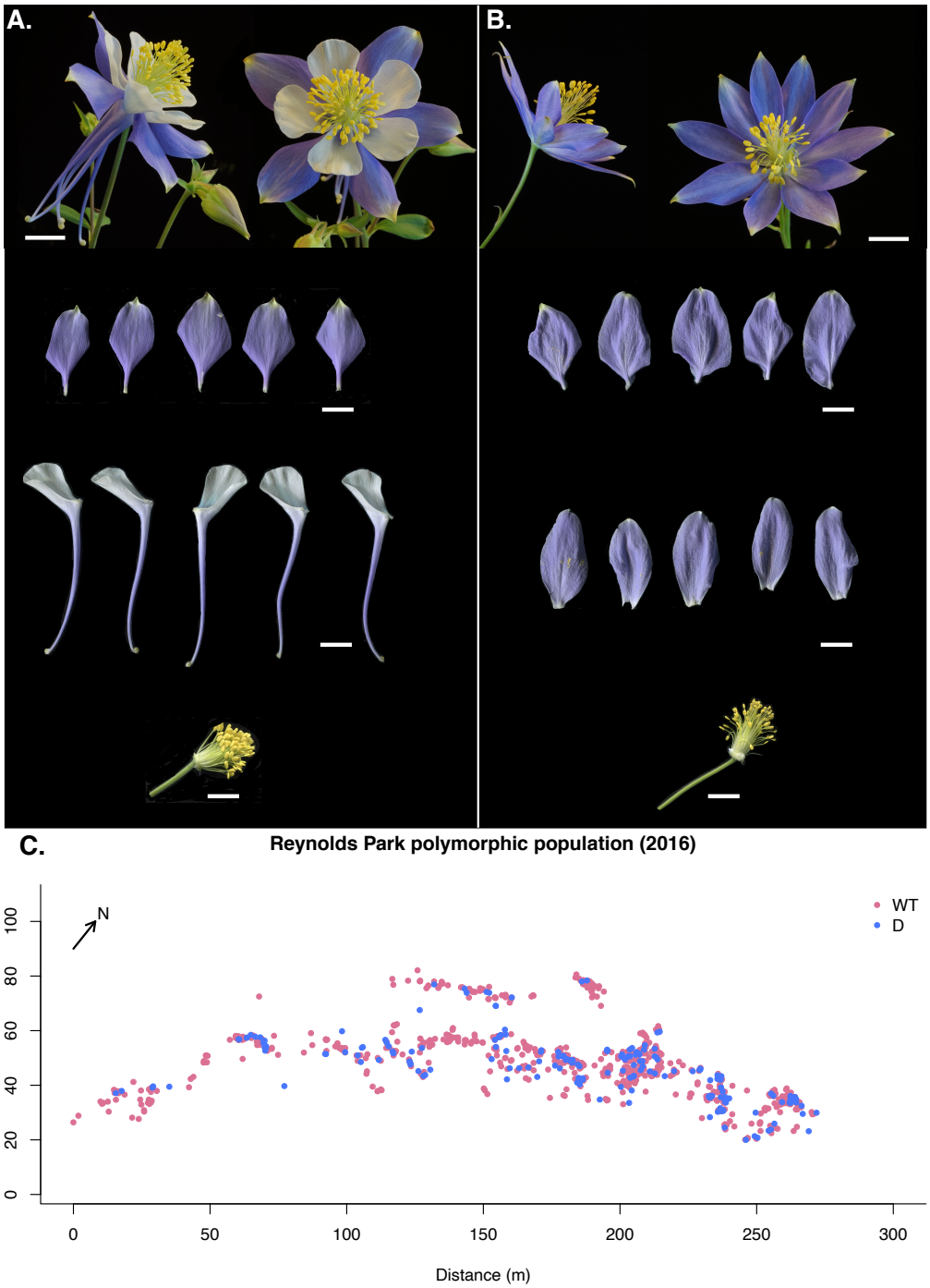


Figure 1. **Panel A.** *Aquilegia coerulea* (WT). **Panel B.** *A. coerulea* var. *daileyae* (D, mutant). **Top Row:** whole flower side view, front view. Rows 2-4: example of flower dissection for each morph. **Row 2** shows the outermost whorl (sepals). **Row 3** shows second whorl with characteristic nectar spurs for the WT and mutated second whorl sepals for D. **Row 4 shows** inner whorls (stamen, stamenodia, carpels). Scale bar = 1 cm. **C.** Map of study site in Reynolds Park Open Space, CO in 2016.

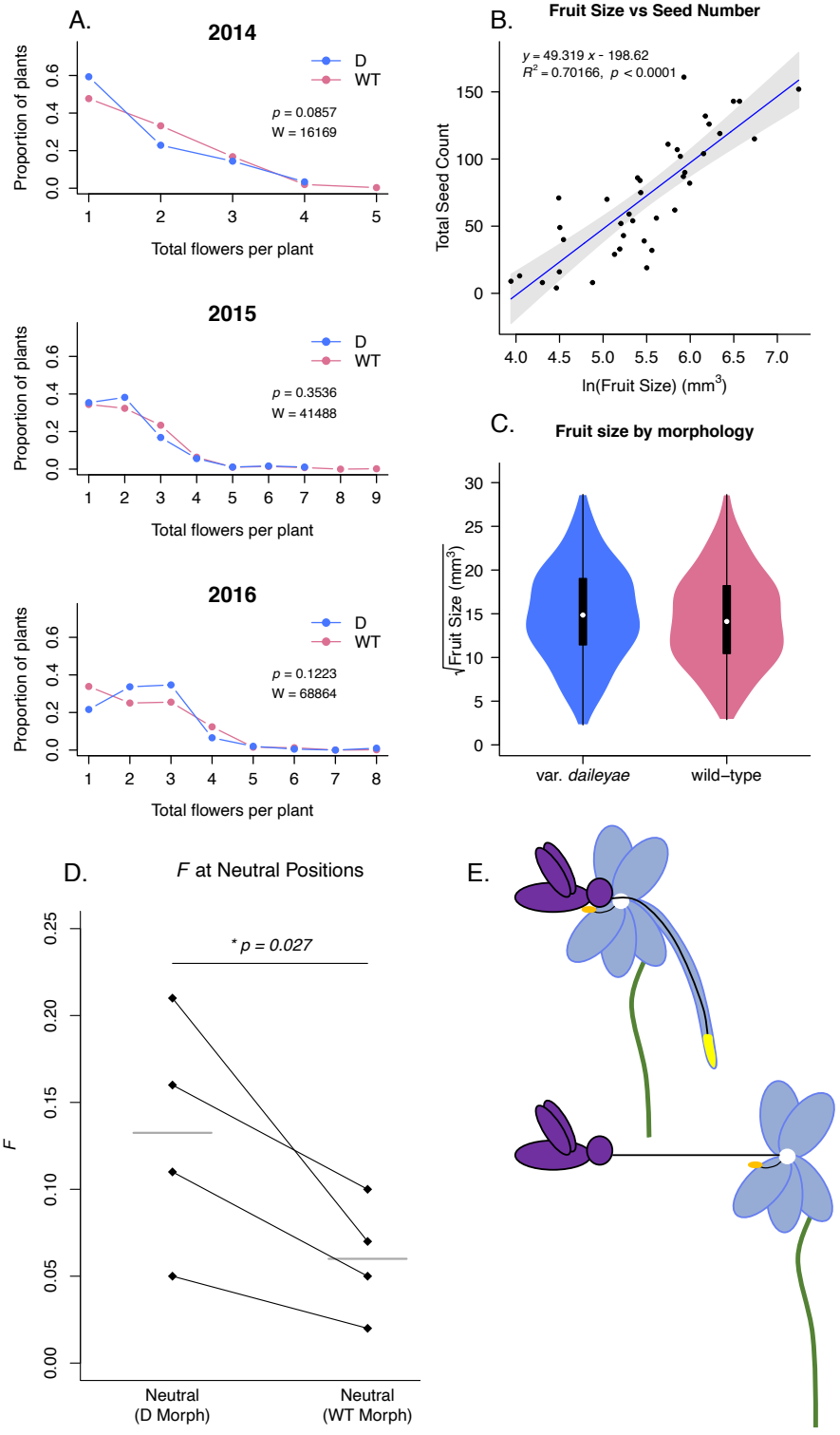


Figure 2. A. Flowers per plant. Flowers per plant did not differ between the two morphs for any of the flowering seasons. Statistics generated with a Wilcoxon Rank Sum test. **B.** Fruit size correlation with seed production. **C.** Fruit size by morphology. Fruit size did not differ between morphs ($t = 1.08$, $p = 0.28$). Color scheme from A carried through to C. **D**. Inbreeding coefficient (F) at neutral loci in the genome by morphology. One-tailed t-test ($t = 3.07$, d.f. =3). Gray lines indicate mean F. **E.** Cartoon schematic indicating the mechanical separation resulting from a hawkmoth attempting to feed on a spurless D plant.

Differences in pollinator visitation between the morphs could also result in differences in fruit and/or seed production. For instance, the differences in visual or olfactory cues could affect pollinator attraction. We observed both major pollinators (hawkmoths and bumblebees) visiting *A. coerulea* at RP in all three field seasons. In 2014 we used GoPro video cameras to record visitation to flowers of both morphs growing close together and observed hawkmoths and bumblebees visiting both morphs and that individual pollinators often transitioned between the morphs (Supplemental Video S1, Table S4). Hawkmoths did attempt to visit the nectarless D morph but they are unlikely to be efficient pollinators since their long tongues prevent their bodies from contacting the reproductive organs as they do when feeding on nectar in the long spurs of WT flowers (Fig. 2E, Video S1). Thus WT flowers are likely pollinated by both hawkmoths and bumblebees while D flowers are likely predominantly pollinated by just bumblebees.

Flowers of *A. coerulea* are self-compatible and can set fruit without pollination, though when pollinators are excluded from visiting flowers the resulting fruits have significantly fewer seeds (*22*, *33*). Thus if differences in visitation between the morphs are generally more pronounced than we observed, this could result in differences in fruit set or fruit size. For open-pollinated flowers, the first flower to open on an inflorescence is significantly more likely to set a fruit and to be larger than subsequent fruits (*33*). Therefore, for each season we compared fruit-set for plants producing the same number of flowers (1, 2 or 3) and lacking any signs of floral herbivory (see below). We found only one out of nine comparisons to be marginally significantly different between the morphs (Table S4) indicating overall similar levels of pollination. In 2016 we also measured the size of fruits produced, which strongly

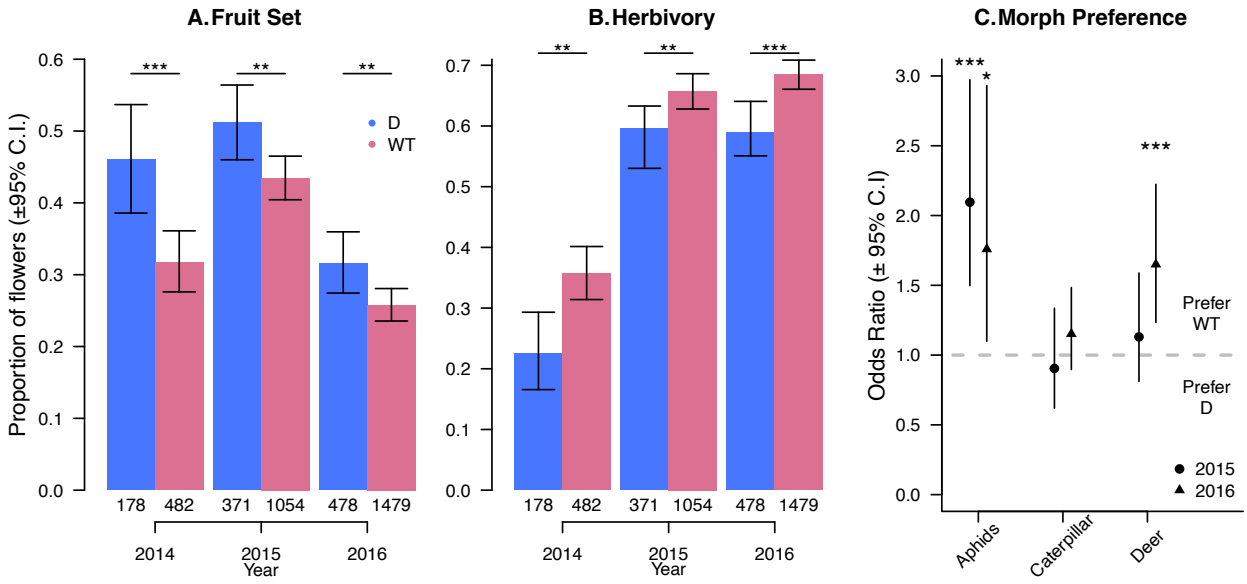
correlates with the number of seeds produced (Fig. 2B-C). Again, for plants without signs of floral herbivory, the morphs did not differ in fruit size for the first flower produced, though fruit size was larger for plants producing larger numbers of flowers (ANOVA, $p = 2.36 \times 10^{-5}$, Table S1). Thus, we find little evidence that pollinators differentially affect the number or size of fruits produced by the two morphs.

While pollination does not appear to affect fruit and seed production, the morphs could differ in the quality of seeds produced. Since hawkmoths are associated with higher outcrossing in *A. coerulea* while bumblebees are not (*23*), the predominance of bumble bee pollination for D plants could result in their being more highly inbred. We determined the inbreeding coefficient (*F*) at four unlinked, neutral (4-fold degenerate) SNP positions for the two morphs and found significantly higher *F* among D compared to WT plants ($t = 3.07$, $p = 0.027$, Fig. 2D). Because the morphs do not differ in their spatial structure nor seed dispersal, reduced pollen dispersal for D plants likely explains this result. Taken together, we found no evidence that differences in pollination would cause natural selection favoring the D morph and instead found evidence for a potential negative effect (higher inbreeding) which might be detrimental to D plants via inbreeding depression which has been documented in *A. coerulea* (*24*).

In contrast to the results for pollinators, across all three seasons we found significantly greater floral herbivory on WT flowers (Fig. 3B). We observed three floral herbivores – aphids, caterpillars, and deer. Aphids feed on the pedicels of flowers (and sometimes the flowers themselves), often causing wilted and stunted floral development (Fig. S2C-D).

Caterpillars (primarily *Platypolia anceps,* Fig. S3) preferentially grazed on reproductive organs (as well as sepals and petals, Fig. S2E), often resulting in complete loss of fruit production. Mule deer (*Odocoileus hemionus*) were observed to eat entire flowers (Fig. S2F, Video S2). In 2015 and 2016 we recorded damage due to each specific herbivore and, using logistic regression, we found that caterpillars showed no morph-preference in either year, aphids were significantly associated with WT flowers in both years and Mule Deer showed a preference for WT flowers, though this was significant only in 2016 (Fig. 3C, Table S6). Thus the greater floral herbivory on WT flowers is very likely to be the cause of their significantly lower fruit set (Fig. 3A-B). While the year-to-year agent of selection may vary (aphids or aphids and deer), the result was the same – selection favoring the D flowers due to reduced floral herbivory.

Figure 3. Fruit set and herbivory by floral morph in RP. A-B. Proportion of flowers that successfully set fruit (**A**) or suffered any type of herbivory (**B**) across three years (2014-2016). Color scheme for **A** carried through for **B**. The numbers below columns represent sample size (# flowers). Statistics generated from a χ^2 analysis. **C.** Odds ratios for logistic regression of herbivore type on morphology. *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$.



Multiple independent loss of function mutations cause the floral mutant phenotype

An obvious candidate gene underlying the D phenotype is the B-class floral identity gene *APETALA3*-3 (*AqAP3-3*), as previous work has shown knockdown of expression of this transcription factor in *A. coerulea* results in a homeotic shift from petals to sepals, while no other organs are affected (*34*). Thus, we sought to determine if loss-of-function (LOF) mutations at *AqAP3-3* are associated with the D morphology. Initial PCR amplification and Sanger sequencing indicated multiple indels making interpretation difficult. Therefore, we assessed sequence variation using Illumina sequencing of an approximately 3.6 kb PCR product around the locus from 101 plants (81 with morph data, Data S4). We identified 55 variable positions, four of which were deletions likely to create LOF mutations: two single bp deletions (d^{1}, d^{1b}) that would each cause frameshifts and a major truncation of the protein, a nine bp deletion (d^{9}) that causes the loss of highly conserved amino acids in the DNA-binding domain (*35*), and a 682 bp deletion (d^{682}) immediately upstream of the gene, eliminating a known *cis*-regulatory element (Fig. 4A) (*36*). We collectively refer to these as *d* mutations. Genotypes at these loci were highly correlated with flower morphology. All 32 D plants were either homozygous for one of these mutations or heterozygous for two of them. In contrast, 39 of the 49 WT plants either had none or were heterozygous for only one of these mutations. The remaining 10 WT plants appeared homozygous for one of the *d* mutations but were also homozygous at every other site suggesting that one allele might not have amplified (Fig. S4). We tested this possibility using PCR-based genotyping probes (*37*) for the three most common *d* mutations (d^{1}, d^{9}, d^{1b}) and found that all 10 plants were actually heterozygous for a single *d* mutation.

To further test that these LOF alleles underlie the D phenotype, we genotyped all 850 flowering plants (D = 198, WT = 652) from 2016 using the genotyping probes. No more than two *d* alleles were found in any individual (pairwise LD, $r^2 = 1$) confirming complete linkage disequilibrium among these LOF alleles, so we assigned an overall genotype to each individual as *d*/*d* (homozygous or heterozygous), *d*/*wt* or *wt*/*wt*. These genotypes were exceptionally strongly associated with floral phenotype ($p = 1.49 \times 10^{-153}$, Table 1, Table S7, Data S3). Fifteen D plants did not carry two of the assayed *d* alleles and therefore may carry one or two unassayed *d* alleles (e.g., d^{682}). The 13 WT plants genotyped as *d*/*d* could be the result of rare recombinants between *d* haplotypes, compensatory mutations, allelic dropout due to primer site variation, or mistakes in sample labeling or processing. Additionally, a second copy of *AqAP3-3* (*AqAP3-3b*) has been described as having very low expression in *A. coerulea* (*34*) and it is possible that epistatic variation at this locus accounts for not only these mismatches, but also for the few plants with intermediate morphology.

Table 1. Genotype-Phenotype associations at *AqAP3-3*.

Genotype	Phenotype		χ^2
	D	WT	
d/d	108	8	700.78 $p = 1.42 \times 10^{-151}$ d.f. = 3
d/d (het)	75	5	
d/wt	13	355	
wt/wt	2	284	

Lastly to determine whether the *d* mutations are likely the causal mutations and whether they evolved independently, we phased the sequence data and constructed a haplotype network (Fig. 4B) (*38*). We identified functional haplotypes as those that when heterozygous with a *d*

mutation produce WT morphology (Fig.4B). Haplotypes with different *d* mutations are all separated by functional haplotypes indicating independent origins. Mutant (*d*) haplotypes that differ from functional *wt* haplotypes by just the d^9 and d^{1b} mutations indicate that these mutations are in fact causal. Similarly, a haplotype carrying the d^1 mutation and a second SNP differentiates it from a functional haplotype (e.g. haplotypes 10 and 12, Fig. 4B). However, both states of the second SNP occur in other functional haplotypes indicating that the d^1 mutation is causal., These results strongly suggest that the D morph is caused by multiple recessive LOF mutations at *AqAP3-3* and that these mutations originated independently from different functional *wt* haplotypes (Fig. 4B).

Figure 4 (next page). Variation at *AqAP3-3*. A. Gene diagrams describing the *wt* and four *d* haplotypes. Numbers to the right of each diagram indicate the number of unique and total haplotypes containing inferred causal deletion mutations. The right-pointing arrow on the *wt* haplotype indicates the transcriptional start site. **B.** Minimum spanning haplotype network for *wt* and *d* haplotypes in the population. *d* haplotypes were identified when present in D plants. *wt* haplotypes were identified from WT plants heterozygous with a *d* haplotype. "Presumed *wt*" haplotypes did not carry an obvious LOF mutation and only occurred in WT plants but never with a known *d* haplotype, thus we cannot be certain of their effect on morphology. Specific positions that separate *wt* and *d* haplotypes are indicated (e.g. arrowhead, box, diamond, etc.) where they separate both *wt* and *d* haplotypes and multiple *wt* haplotypes. Colored hash marks indicate *d* mutations. Size of circle corresponds to frequency in the sample.

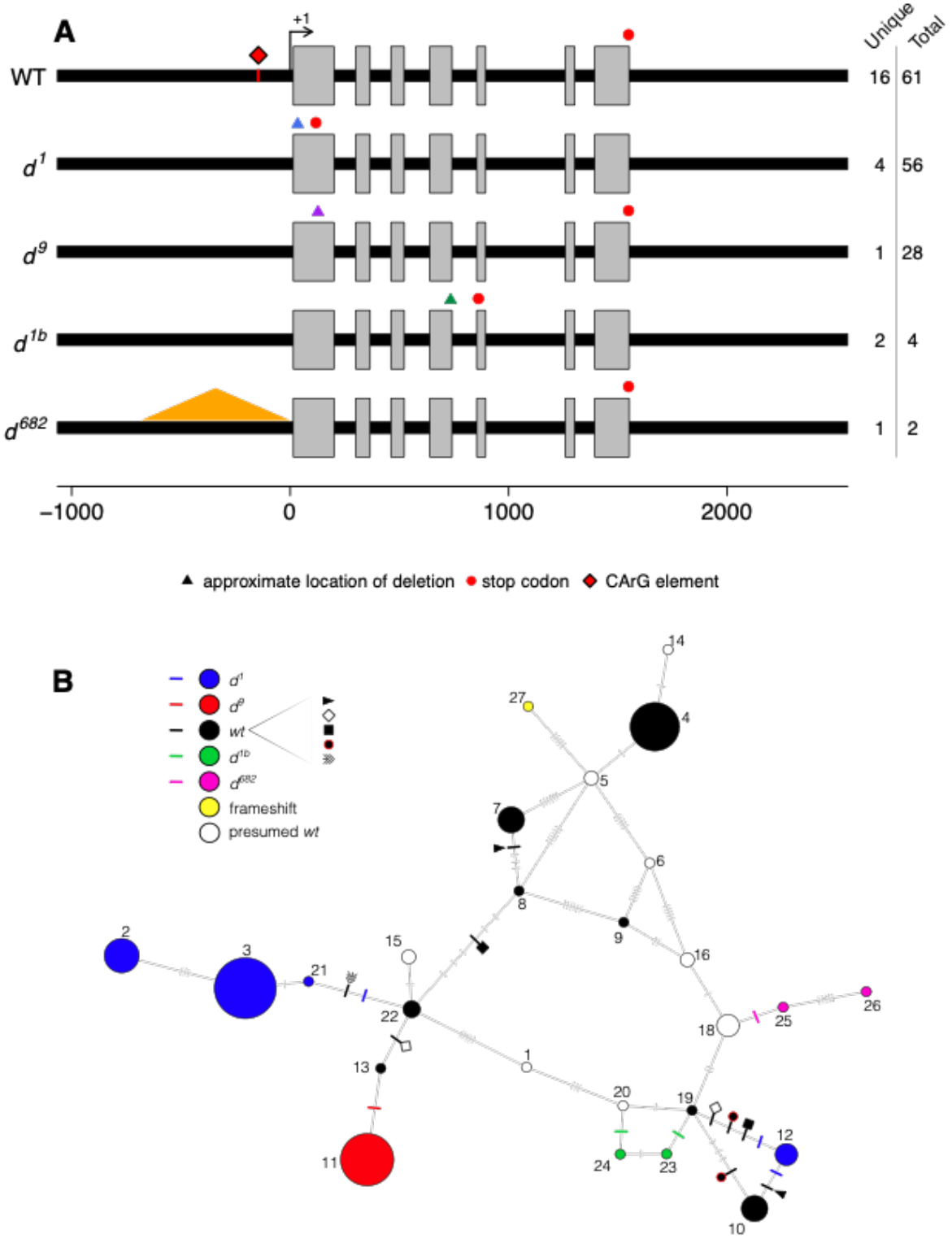


Figure 4. Variation at *AqAP3-3.*

Molecular signatures of selection

While we found consistent selection favoring D plants across three consecutive seasons, variable selection in the past or at other life stages acting directly on *AqAP3-3* or closely linked genes could enhance, eliminate, or reverse the effect of selection due to floral herbivory (*39*). If, however, directional selection has consistently favored *d* alleles for many generations, then we would expect to see heightened linkage disequilibrium (LD) surrounding *d* alleles compared to ancestral *wt* alleles (*40*). We therefore calculated Extended Haplotype Homozygosity (EHH) using the haplotypes identified for the two most common *d* alleles, d^1 and d^9, compared to the *wt* haplotypes (Fig. 5) (*40*, *41*). EHH for *wt* haplotypes rapidly declines within 1 kb, similar to that found for other loci in *Aquilegia* (*42*). In contrast, the two *d* alleles have much higher levels of EHH extending beyond 1.1 kb and 2.5 kb up- and downstream of the mutations, respectively (Fig. 5). These results indicate that *d* alleles quickly rose to high frequencies due to positive selection over many generations.

Morph based assortative mating

Given the large difference in morphology it seems likely that mating patterns will be influenced through either changes in pollinator behavior or asymmetric pollen movement (or some combination therein). Pollinator observations noted above revealed that both hawkmoths and bumblebees visit both morphs at least to some degree but differential pollen placement could still be driving some amount of assortative mating (Fig. 2E). Assortative mating between morphs would specifically cause a deficit of *d*/*wt* heterozygotes above that observed for *d*/*d* heterozygotes (e.g. d^1/d^9). The observed genotypes strongly departed from Hardy-Weinberg expectations ($\chi^2 = 23.97$, $p = 2.5 \times 10^{-5}$, d.f. = 3, Table 2) and standardized

residuals indicated that this was largely the result of a lack of *d*/*wt* heterozygotes (but not *d*/*d* heterozygotes), along with an excess of *d*/*d* homozygotes (Table 2).

The excess of *d/d* homozygotes agrees with the elevated *F* at neutral loci mentioned previously and is likely a result of higher inbreeding among D plants driven predominantly by bumblebee pollination. Since *A. coerulea* is known to suffer from inbreeding depression, this elevated inbreeding is unlikely to be beneficial for D plants. While this may signify some amount of assortative mating between the morphs in this population, that would only serve to speed up the rate at which D and WT might diverge but would not alone result in a fitness difference between the morphs. In other words, pollinators are unlikely to be the driving force of selection favoring the D plants, but rather an accompanying, supplementary factor.

Table 2. Observed and expected (from Hardy-Weinberg) genotype counts at *AqAP3-3*. Standardized residuals in bold indicate significant contribution to the χ^2 statistic.

Genotype	Observed	Expected	Standardized Residuals	χ^2
d/d	116	81.96	3.76	23.97 $p = 2.50 \times 10^{-5}$ d.f. = 3
d/d (het)	80	87.92	-0.84	
d/wt	368	420.24	-2.55	
wt/wt	286	259.88	1.62	

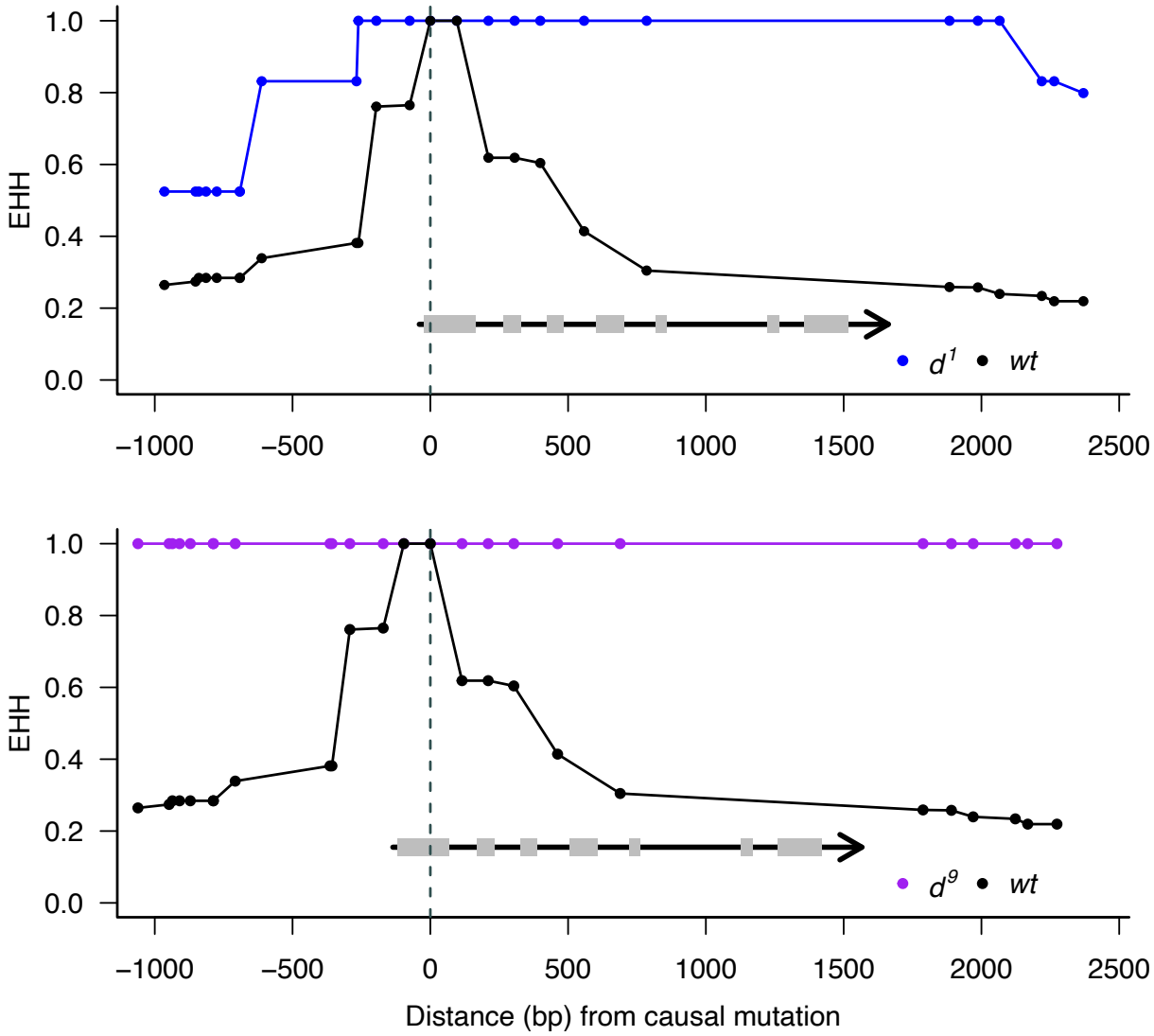


Figure 5. Extended Haplotype Homozygosity. Extended Haplotype Homozygosity (EHH) for d^1 (top) and d^9 haplotypes (bottom) relative to *wt*. Each point represents a variable position. Dashed lines correspond to location of the causal mutation. Gene diagram for *AqAP3-3* indicated in each figure. EHH calculated using selscan.

Discussion

Here we have identified a homeotic mutant with a strong fitness advantage in a natural population as well as the ecological drivers of selection, the underlying locus and multiple causative mutations. Our measurement of selection against the WT morph (s = 0.17-0.3) is similar to or greater than measures of strong selection in notable, classic examples of evolution such as the pepper moth (*Biston betularia*) and pocket mice (*Chaetodipus intermedius*) (*43*, *44*). Furthermore, knowledge of the genetic underpinnings of the mutant allowed us to identify molecular signatures suggesting that selection has been positively acting on multiple independent alleles over many generations and changes in mating patterns, such as assortative mating by morphology.

Although pollinators are often considered to be the main driver of shifts in floral morphology, there are multiple lines of evidence that suggest herbivores are driving the fitness patterns measured in this population, not pollinators. First, video evidence (and personal observation across three years in the same population) shows both pollinators visiting both morphs (Video S1, Table S4), although the long tongue of a hawkmoth likely prevents any efficient pollen transfer for D plants (Fig. 2E). As a result, a majority of the pollen moved by hawkmoths will be *wt* pollen. Bumblebees visit both morphs, but may not be as efficient of pollinators (since they groom) as hawkmoths (which do not groom), as hawkmoths are associated with higher outcrossing rates in *A. coerulea* (*45*). This observation is supported by inbreeding levels measured at neutral positions in the genome which suggest higher inbreeding among D plants. As *A. coerulea* is known to suffer from inbreeding

depression (*24*), this interaction is at best selectively neutral and might have some negative consequences for D plants. Second, if there were a difference in visitation, it does not appear to be affecting seed number or fruit size, which are the same between the two morphs. Third, plants that show no sign of herbivory are equally likely to successfully set fruit, regardless of morphology. Overall, while pollinators may play a role in reproductive isolation, they do not appear to be driving selection for the D morph.

Once D morphs were present in the population, natural selection may or may not immediately favor them. Aphids and deer were found to prefer WT flowers and this could occur when D plants are at low frequencies if, for example, petals provide an innate visual or olfactory cue. Loss of petals results in not only a clear visual change (large protruding spur to flat sepal), but also a color shift (white petal "blades" to purple sepals), loss of nectar production and quite possibly a change in floral scent (petals are often the organ producing floral scent (*46*)). In the case of aphids, loss of nectar production may result in lower phloem flow and decreased aphid population growth rate resulting in less damage to D flowers (regardless of the D frequency in the population). Also, aphids identify their host plant through a cocktail of chemical cues and will leave an incorrect host for an alternate one and, in some cases, refuse to feed (*47*, *48*). Together, these changes could make D plants less attractive to aphids and give early, rare D plants an almost immediate selective advantage over WT plants. Alternatively, D plants may need to reach a high enough frequency so that herbivores can learn to prefer one over the other. For example, deer may learn to prefer WT flowers that have sweet nectar in their spurs. It has been shown that large mammal herbivory can be inconsistent across generations and still elicit a strong evolutionary response, even

cancelling out and reversing selection driven by pollinators (*29*, *30*). Regardless of the specific cause, the overall effect of loss of nectariferous petals is likely causing the difference in herbivory.

An alternative hypothesis would be that a locus that is tightly linked to *AqAP3-3* is driving the difference in herbivory or has some other fitness advantage. This is unlikely for a number of reasons. First, the genomic architecture surrounding *AqAP3-3* is surprisingly gene-poor. Within 10kb up or down-stream of *AqAP3-3* there are only three annotated genes and only one of these has been functionally annotated (Aqcoe5G180700, a xylulose kinase-related protein, which has no obvious involvement with herbivory). No other genes occur within 20kb of *AqAP3-3*. The likelihood of tight linkage between *d* alleles and any alternative allele on these loci is low, especially since LD begins to break down within ~2.5kb of the d^1 mutation (Fig. 5). While we cannot exclude the possibility that a regulatory element or genes that have no functional annotation in the region are responsible, we find this to be unlikely. In other words, we don't find any obvious candidate gene closely linked to *AqAP3-3* that could be driving the selection measured.

Second, this hypothesis would require either that the d^1 and d^9 mutations both arose on an alternative haplotype carrying a separate favorable mutation or that other favorable mutations arose separately only on haplotypes carrying the d^1 and d^9 mutations. For the first scenario, selection would favor the alternative haplotype(s) before the *d* mutations occurred so we would expect to see at least one closely related high frequency *wt* haplotype, but this is not the case (Fig. 4B). The second scenario would imply that favorable alleles linked to *AqAP3-3*

only arose on *d* haplotypes which, presumably, would be very rare. Thus the simplest explanation of our data is that the loss of *AqAP3-3* functionality and the corresponding homeotic conversion of petals to sepals also results in differential herbivory which causes selection for the D morphology.

All in all, this study is a clear example of a multiple origin soft sweep. This occurs when multiple beneficial alleles arise before any single allele is able to reach fixation (*32*). These types of sweeps are far more likely when there is a large mutational target (like functionally equivalent LOF mutations) or the selected allele is recessive as it takes time for the allele(s) to rise to high enough frequency to occur as a homozygote and selection can act (*32*, *49*). For the D morph, both of these conditions are met as many mutations can cause LOF alleles at *AqAP3-3* and the phenotypic effect appears to be completely recessive (i.e., we find no evidence of a fitness advantage of WT *d/wt* plants over *wt*/*wt*, Table S8). Furthermore, the time between the original mutation and selection favoring D plants may be relatively long and allow for additional *d* alleles to accumulate before selection can bring them to high frequency.

The *d* alleles appear to have relatively recent independent origins as they occur on separate haplotypes that show extended regions of LD relative to *wt* haplotypes. Our data suggest that the d^1 allele is likely the oldest because it has the highest allele frequency (28%) among *d* alleles and relatively high degree of haplotype variation compared to the second most common allele, d^9, (13%, Fig. 4-5). Other *d* alleles were too rare to assess haplotype variation but also appear to have originated from separate *wt* haplotypes (Fig. 4B).

While the establishment of D individuals is likely to be slow initially, the subsequent increase would be enhanced by assortative mating. Our data at *AqAP3-3* indicates a degree of assortative mating by morph, which is likely caused by the mechanics of plant-pollinator interactions. In particular, hawkmoths likely facilitate matings among WT plants because when they probe the long nectar spurs with their long proboscis it brings their body into full contact with the flower's reproductive organs causing pollination (Fig. 2E, Video S1). When hawkmoths probe D plants their body is held far away from the reproductive organs, likely preventing efficient pollen transfer (*21*, *50*, *51*). Learned hawkmoth avoidance would only serve to enhance the assymetric pollen movement in the population. In contrast, bumblebees have access to anthers of both morphs, but D plants may be more attractive because of greater pollen availability via reduced removal by hawkmoths (*23*, *45*, *52*, *53*). Therefore, bumblebees may disproportionately move *d* pollen between D plants. Future studies documenting pollen transfer in this population would be especially useful in clarifying the degree of assortative mating.

To be a true paragon of Goldschmidt's hopeful monster, however, the D mutant would have to lead to a cladogenetic split from *A. coerulea,* which would require some combination of a reduction in gene flow between morphs and divergent selection. While this is unlikely to occur within RP where we find no evidence of divergent selection, speciation may occur if floral herbivory varies among populations. In populations without floral herbivores WT plants likely have an advantage over D plants because WT plants likely have higher outcrossing rates (Table 2) and *A. coerulea* is known to suffer from inbreeding depression

(*24*). This is likely the result of hawkmoth pollination, which is correlated with increased outcrossing rates in this species(*23*, *45*). Thus, divergent selection for the two floral morphs may well occur among populations and if populations become fixed for alternate morphs, pollinators (particularly hawkmoths) may strongly discriminate between them. We note that Reynolds Park is at the very edge of the species' range and may be experiencing unusual selective pressures, like floral herbivory, compared to other populations (*28*).

While we cannot know for sure whether the D mutant will ultimately result in speciation, comparative studies show multiple plant lineages are associated with the loss of nectariferous petals. Within the Ranunculaceae there are nine independently evolved apetalous lineages (*54*) suggesting that selection has favored these losses despite the presumed mutualistic advantage of producing a food reward for pollinators, perhaps because of interactions with herbivores. Similarly, the loss of the nectar spur, but not the entire petal, has occurred in a single species of *Aquilegia*, *A. ecalcarata* (*55*, *56*). While these losses could have occurred subsequent to lineage splitting, the loss of nectar rewards suggests that pollinators and pollination would have been affected, which could lead to reproductive isolation and thus promote cladogenesis. Furthermore, seven of the nine petal losses are association with loss of function at *AP3*-3. This all implies that not only is loss of nectariferous petals is an evolutionary stable and successful transition, but the genetic mechanism (loss of function at *AP3-3*) is stable as well.

In summary, the D mutant fits extraordinarily well into Goldschmidt's idea of a hopeful monster. A "monstrosity" (homeotic mutant) controlled by a "rate gene" (*AqAP3-3*), that

"alters the developmental fate of the tissue" (petal to sepal transition). It is 'hopeful' in that it has a clear fitness advantage (herbivory avoidance) and affects mating patterns such that it could lead to a new lineage. Consequently, *A. coerulea* var. *daileyae* is a clear example of a hopeful monster.

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Resource Availability

Resource Availability: All raw data (datas1-Data S4) and Supplemental Videos (S1-S2) are available on the Dryad Digital Repository: https://doi.org/10.25349/D9HC81. Amplicon sequencing data is deposited on the SRA under bioproject PRJNA660745. CO1 sequences are available in the genbank Database (Accessions MW302492-MW032499). All statistics were calculated in R v 4.0.3. All code is available upon request. Any additional information required to reanalyze the data reported in this chapter is available from the lead contact upon request.

Data and Movie Captions

Movie S1
Example pollinator videos from Reynolds Park, Colorado (2014).

Movie S2
Video evidence of Mule Deer feeding on A. Coerulea in Reynolds Park, Colorado (2015).

Data S1
Field Data and measurements (morphology, flower number, herbivory, fruit set, fruit size (2016 only)) for three flowering seasons (2014-2016).

Data S2
X-Y coordinates for RP population (2014-2016).

Data S3
SNP-genotyping results using genotyping probes at aqap3-3 and four neutral positions.

Data S4
Sequencing information. Full list of sequenced samples and morphologies, all variable positions, and any regions excluded from variant identification.

Materials and Methods

***Aquilegia* greenhouse and growth conditions.** Seeds collected in 2014 were first cold stratified at 4ºC on moistened filter paper in petri dishes for 4 months. Upon germination, individual seedlings were transplanted into a plug tray and grown under long-day conditions

(20ºC, 16/8) until the 2nd true leaf appeared. Plants were then transferred to ¼-gallon and finally ½-gallon pots in the greenhouses. To induce (and control) flowering, plants were put into vernalization (4ºC, short day, 8/16) for 8 weeks.

Data and Sample Collection. We surveyed the RP population in late June of each year (2014-2016) when the plants flowered. We numbered and tagged each plant with aluminum tags and noted flower morphology and in 2015 and 2016 evidence of specific herbivores. We also collected a small amount of tissue for DNA extraction and mapped the location of each plant (see below). We returned in early August to assess fruit production and to collect 2-3 seeds from fruits. During this time we again noted evidence for herbivore damage. Aphid evidence was simply presence/absence. Caterpillars were either identified on the flower, or through evidence such as partially eaten spurs, holes in floral tissue, or partial removal of anthers, staminodia, or carpels (Fig. S2E). During seed collection, caterpillar evidence could be identified through partially eaten carpels or, when no carpels remained, the floral receptacle was intact atop the inflorescence. Mule deer were observed to eat entire flowers leaving characteristic stumps on the flower pedicles or main inflorescence (Fig. S2F, Video S2).

Population Mapping. To create the spatial map, we first set up two reflective targets (T1, T2) a fixed distance apart (D_T). Then, using a laser distance measurer (Leica Disto D2 Laser Distance Measurer), we measured the distance from each plant to T1 (D_1) and T2 (D_2). It was impossible to measure the distance for all plants to the same targets, so multiple "target groups" were created. In every new target group, at least three plants from a neighboring

target group were included as “links”. These links are crucial not only for spatial accuracy, but to ensure neighboring target groups are oriented correctly to each other. For example, there were 16 target groups throughout the population in 2016. Simple algebra allowed us to convert measured distances (D_1, D_2) into unique *x*-*y* coordinates for every plant in the population (Fig. 1C, Data S2). This was done for 2014, 2015 and 2016, but only the map from 2016 is presented here. Coordinates for all years are available in Data S2. More detailed methods and equations available upon request.

Caterpillar Identification. Caterpillars (Fig. S2E) were collected in 100% EtOH. DNA was extracted following Gloor and Engles (*57*). Approximately 650 bp of the mitochondrial gene *cytochrome oxidase 1* was PCR amplified using the LepF and LepR primers from Hajibabaei (*58*) and an initial denaturation at 95ºC for 2 min followed by 35 cycles at 95ºC for 30 sec, then 57.6ºC for 30 sec, then up to 72ºC for 1 min. Sanger sequencing was conducted at the UC Berkeley Sequencing Facilities. Sequences were trimmed using Geneious version 9.1.6 and consensus sequences for each sample were generated (http://www.geneious.com) (*59*) and then blasted against the NCBI database (Fig. S3). Sequences are available on the GenBank Database (accession nos. MW302492-MW032499).

DNA Extraction. Field collected (“FC”) leaf tissue was collected every year (2014-2016) in RP for every flowering plant. Leaf tissue was stored in silica gel desiccant until processing at UCSB. Samples grown in the greenhouse (“GH”) were snap-frozen in LN_2 prior to DNA extraction. All DNA was extracted using the MagAttract 96 DNA Plant Core Kit (Qiagen) on

the BioSprint 96 (Qiagen) and quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

PCR Amplification and library preparation. We designed primers (F: 5' GAGAGACCTTGGTGGGGAGA 3', R: 5' AGCCAGCTTTACCGTACACC 3') around *AP3-3* (locus identifier: Aqcoe5G180800.1) to amplify the entire gene and ~1 kb up and downstream (total amplicon size = ~3.6 kb). We used Qiagen Multiplex PCR Kit (Qiagen). The PCR amplification protocol consisted of initial denaturation at 95ºC for 15 min followed by 36 cycles at 94ºC for 30 sec, then 61.9ºC for 1:30, then 72ºC for 4 min. PCR products were quantified using a Qubit 2.0 fluorometer (ThermoFisher Scientific). Dual-indexed libraries were prepared using ½ reactions of NEBNext Ultra II (New England Biolabs) for Illumina and 10ng of input DNA. Libraries were sequenced on the Illumina NextSeq 500 (UCSB CNSI BNL).

SNP Genotyping. In order to get accurate allele frequencies for *d* alleles, we employed real-time PCR allele specific SNP Genotyping (*37*). For the d^{1} and d^{9} alleles, we designed custom Taqman SNP Genotyping Assays (ThermoFisher). For the d^{1b} mutation and neutral SNPs (for measuring inbreeding), we designed a custom IDT rhAMP SNP Genotyping Assay (IDT). Taqman assays were run with iTaq Universal Probes Supermix (BIORAD), whereas the IDT assay was run with IDT rhAMP master mix. PCR protocols followed recommendations included with master mix. Plants with ambiguous morphs were excluded (n=7). These plants were either in early bud in the field (at which point nectar spurs are not visible, n=2) or all floral tissue had fallen off prior to sample collection in Reynolds Park

(n=5). All reactions were run on the BIORAD CFX-Connect Real-Time PCR Detection System. SNP Genotyping data can be found in Data S3.

Spatial Analyses. Spatial distribution of all plants in 2016 was modeled in R (*60*) following the methods of Kellenberger (53). Initially, the map was subset into 16 windows of the same size (17m x 30m, Fig. S1A). A Kolmogorov–Smirnov test was used to determine overall randomness of the spatial distribution of plants in the population. Differences in spatial distribution patterns between D and WT morphs were computed with a Studentised Permutation Test with 999 random permutations (Fig. S1B). All spatial analyses were implemented in R with the package spatstat v.1.64-1 (*62*).

Floral Measurements. We used a Wilcoxon rank sum test to assess whether either morph produced more flowers per plant for all three flowering seasons (Fig. 2A). In 2016, we collected entire, undehisced fruits lacking evidence of herbivores but having a range of sizes within 1 km of the study population and measured the height (h) and width in two directions (w_1, w_2) of each fruit (Data S1) and counted the total number of developed seeds. We modeled the volume of the fruit as an ellipsoid with the following equation:

$$V = \frac{4}{3}\pi\left(\frac{w_1}{2} \times \frac{w_2}{2} \times h\right)$$

We correlated fruit volume and seed number using a simple linear regression (Fig. 2B). In 2016 we similarly measured fruit volume for all fruits in the study population (Data S1).

After square root transformation to approximate normality, we tested whether floral morphs differed in fruit volume (Fig. 2C).

Variant Identification. Sequences were aligned to the *A. coerulea* 'Goldsmith' v3.1 reference genome (*63*) (https://phytozome.jgi.doe.gov) using the Burrows-Wheeler aligner (*64*) and then sorted and indexed with samtools 01.1.19 (*65*). Sequence data is available in the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA660745. We scanned sequences for large structural mutations using the IGV browser[44] and subsequently excluded those regions from the analysis. Small indels and SNPs were identified using GATK (HaplotypeCaller (-dontusesoftclippedbases), CombineGVCFs, GenotypeGVCFs) (*66*). For variants in the coding region, we determined if they altered amino acid positions and if any appeared only in D plants. See Data S4 for a full list of samples, variable positions, and excluded regions.

Sample and Variant Filtration for haplotype phasing. Average sequence coverage was 1278x (GATK DepthOfCoverage). Many samples showed clear amplification bias for one allele (Fig. S4). We thus excluded individuals with ≤75x (n = 22) from the haplotype phasing as their genotype calls were often incorrect. We excluded variant sites that fell within large structural mutations and filtered the remaining variants to include only biallelic sites with a MAF ≥ 5% and high mapping quality (MBQ > 30). This MAF cutoff resulted in the two rarest *d* mutations to be excluded (d^{1b} and d^{682}) though we flagged samples carrying

these alleles for downstream analyses. One variable position was added to represent a 280bp deletion (Data S4).

Haplotype Phasing and Analysis. Filtered VCFs were formatted with GATK and PLINK (*67*) and haplotypes were constructed using SHAPEIT2 (v2.r904, default settings, UCSB KNOT) (*68*). Since some GH plants were offspring of sequenced FC plants, for parent-offspring pairs that were both sequenced and phased, we removed haplotypes that were identical by descent. To be conservative, if both the parent and offspring were heterozygous for the same haplotypes, both of the offspring haplotypes were removed from the analysis. Lastly, 11 haplotypes were removed as they were the clear result of allelic drop out.

Allelic drop-out (ADO) occurs when one chromosome (allele) fails to amplify during the initial PCR reaction and therefore does not get sequenced. We could identify sequence data that resulted from ADO by finding individuals that, according to the sequence data, were homozygous for a single *d* allele and at every position across the entire amplicon, but WT morph. To be conservative, all of these individuals were kept in the analysis but only contributed a single haplotype to the EHH calculations. We note that we were only able to identify ADO in D individuals, raising the possibility that it occurred in WT plants and went unnoticed, possibly inflating homozygosity measures for *wt* haplotypes. This implies that our estimation of *wt* haplotype decay might be rather conservative. If a sample suffered from ADO and the remaining haplotype was IBD, then that haplotype was removed as well (effectively removing that individual from the analysis). Lastly, we created a minimum spanning haplotype network (http://popart.otago.ac.nz) (*38*). We identified specific positions

that separated *d* haplotypes and *wt* haplotypes (Fig 4B). Each of these, with the exception of one position, also separated functional *wt* haplotypes. The position in question (10,481,304, marked as the "triple arrowhead" in Fig 4B) only appears on haplotypes 2, 3, and 21 (all d^1 haplotypes). This mutation is a C/T transition and occurs upstream of the gene. While it only occurs on those three haplotypes, it is *not* present on haplotype 12, the other d^1 haplotype suggesting it does not have a substantial effect on function.

Genotypic LD. To further confirm the apparent complete LD between *d* alleles, we used the SNP genotyping data to calculate pairwise genotypic LD between the three most common *d* alleles (d^1, d^9, d^{1b}). The --r2 tool in PLINK (v1.07) calculates r^2 between multiple sites with unphased genotypic data (*67*).

Supplementary Tables

Table S1.
ANOVA for comparing fruit size amongst first flowers between morphs, grouped by number of flowers per plant. FPP = flowers per plant. Superscripts indicate significant differences for fruit sizes for differing number of FPP (1, 2, 3) according to a Tukey HSD test. All fruit size measurements square-root transformed.

	DF	SS	MS	*F*	*p*	FPP mean fruit sizes		
						1	2	3
Morph	2	103	102.6	2.683	0.104			
FPP	1	878	438.9	11.482	2.36×10^{-5}	16.6^{a}	20.8^{b}	22.5^{b}
Morph*FPP	2	102	51	1.334	0.267			

Table S2.
Temperature and Precipitation in Evergreen, CO (elev: 6,985ft), 24km away from Reynolds Park (elev: 7,870 ft) during the reproductive season from 2014-2017. Notice how 2016 is notably hotter and drier.

	Temperature (ºF)				Precipitation (in)			
	May	Jun	Jul	Aug	May	Jun	Jul	Aug
Average	65	75.6	81.5	79.4	2.59	2	2.33	2.28
2014	62.7	75.1	79.4	76.1	3.27	1.15	4.68	2.84
2015	57.1	76.1	77.6	80.5	6.9	3.18	2.85	1.62
2016	60.5	80.2	82.9	77.6	1.06	1.21	1.88	1.66
2017	61.7	77.2	80.8	76.1	4.07	0.4	1.9	2.01

Table S3.
Fruit set for plants with the same number of flowers (1, 2, or 3) that also had no herbivory. FPP = flowers per plant. *P-values* were generated with a Fisher's Exact test and then adjusted with a Benjamini-Hochberg correction.

		D		WT		
FPP	Year	Fruit	No Fruit	Fruit	No Fruit	Adjusted *p*
1	2014	32	21	40	43	0.98
2	2014	19	17	45	53	0.84
3	2014	22	8	26	37	0.04*
1	2015	18	4	36	14	1
2	2015	26	8	33	13	0.8
3	2015	21	6	39	21	0.95
1	2016	14	3	56	10	0.81
2	2016	16	12	33	33	0.84
3	2016	29	22	29	31	1

Table S4.
Pollinator data gathered from GoPro videos in 2014. We quantified both total visits and number of transitions within and between morphs (transition matrix) for both pollinators.

	Flower	Condition	Visits 6/28/14 17:30-21:00	Visits 6/29/14 13:54-17:00	Visits Total
BB	D1	male phase	11	3	14
	D2	just opening	2	2	4
	D3	female phase	1	0	1
	WT1	male phase	6	3	9
	WT2	male phase	11	4	15

TRANSITION MATRIX

		TO: D	TO: WT
FROM	D	2	8
FROM	WT	5	0

	Flower	Condition	Visits 6/26/14 19:40-20:01	Visits Total
HM	D1	male phase	2	2
	WT1	male phase	3	3
	WT2	male phase	3	3

TRANSITION MATRIX

		TO: D	TO: WT
FROM	D	NA	1
FROM	WT	2	1

Table S5.
Analysis of fruit set and herbivory by floral morph in each year. Selection coefficient against WT plants was measured as $s = 1 - W_{WT}$. In each year, W_D was set to 1, and W_{WT} was then measured relative to that value. All analyses were done on a per flower basis. * $p < 0.025$, ** $p < 0.005$, *** $p < 0.001$, d.f. = 1.

Year	Morph	No Fruit	Fruit	χ^2	s	No Herbivory	Herbivory	χ^2
2014	D	96	82	11.02***	0.3	138	40	9.81**
	WT	329	153			310	172	
2015	D	181	190	6.35*	0.17	155	216	6.42*
	WT	596	458			361	693	
2016	D	327	151	5.97*	0.19	192	286	11.56***
	WT	1094	379			467	1012	

Table S6.
Odds ratios (OR) from a logistic regression for specific herbivores regressed on morphology. OR greater than 1 indicate a preference for wild type, less than 1 for var. *daileyae*. Numbers in parentheses indicated 95% C.I. for the ORs. *$p < 0.05$, **$p < 0.025$, ***$p < 0.001$.

Herbivore	2015	2016
Aphids	2.10*** (1.50, 2.97)	1.73* (1.08, 2.90)
Caterpillars	0.90 (0.62, 1.34)	1.16 (0.90, 1.49)
Deer	1.13 (0.81, 1.59)	1.67*** (1.24, 2.25)
Aphid*Caterpillar	0.65 (0.28, 1.60)	1.16 (0.524, 2.67)
Aphid*Deer	3.95 (1.11, 25.28)	0.33** (0.14, 0.77)
Caterpillar*Deer	0.88 (0.34, 2.43)	0.59 (0.30, 1.17)
Aphid*Caterpillar*Deer	0.03** (0.0008, 0.446)	1.04 (0.14, 10.1)

Table S7.
Morph by genotype table expanded to include specific genotypes and prevalence of intermediate ("SS") phenotype in 2016. Bold values are used in Table 1 in the main text.

Genotype		Phenotype				
		var. *daileyae*			wt	
GT (adjusted)	GT (true)	D	SS	total	WT	total
d/d (homozygous)	d^1/d^1	74	4	**108**	7	**8**
	d^9/d^9	27	0		1	
	d^{1b}/d^{1b}	3	0		0	
d/d (heterozygous)	d^1/d^9	50	2	**75**	2	**5**
	d^1/d^{1b}	18	3		3	
	d^9/d^{1b}	2	0		0	
d/wt	d^1/wt	10	1	**13**	218	**355**
	d^9/wt	2	0		102	
	d^{1b}/wt	0	0		35	
wt/wt	*wt/wt*	2	0	**2**	284	**284**

Table S8.
Fruit set and herbivory data for 2016 parsed by genotype.

Genotype	No Fruit	Fruit	χ^2	No Herbivory	Herbivory	χ^2
d/*wt*	581	214	0.745	268	527	2.96
			$p = 0.39$			$p = 0.08$
wt/*wt*	480	158	d.f. = 1	187	451	d.f. = 1

Supplementary Figures

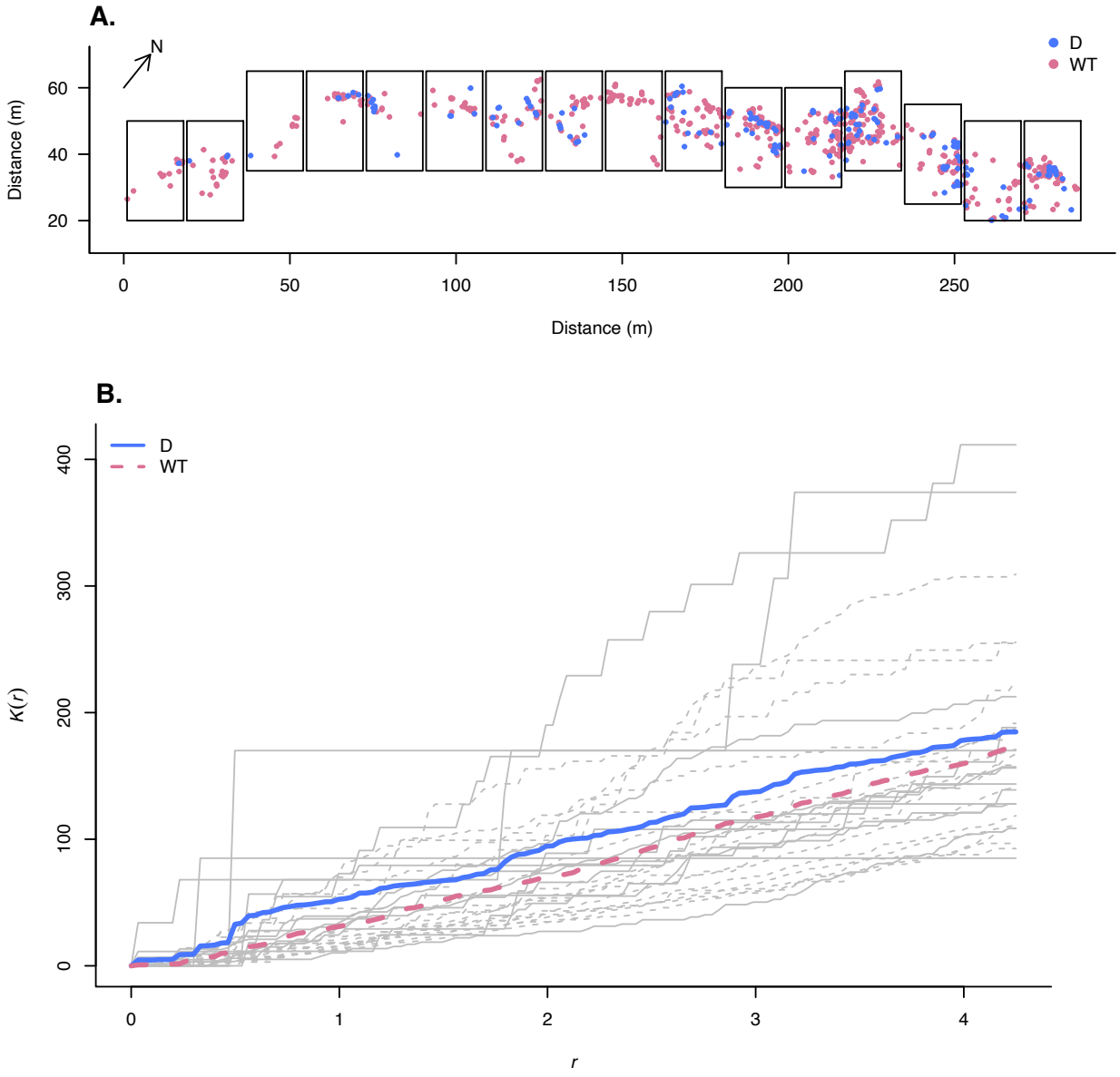

Fig. S1.
Spatial distribution analysis of D and WT plants within Reynolds Park. A. The population was subset into 16 disjunct, evenly sized quadrants (17m x 30m). Plants were not randomly distributed within the population as a whole (Kolmogorov-Smirnov Test, D = 0.274, $p < 0.00001$), with plants being more concentrated towards the NE portion. **B.** A Studentised permutation test found no significant difference in the spatial distributions of morphs ($T = 5.71$, $p = 0.28$). Gray lines show summary functions within each quadrat, means are plotted in color.

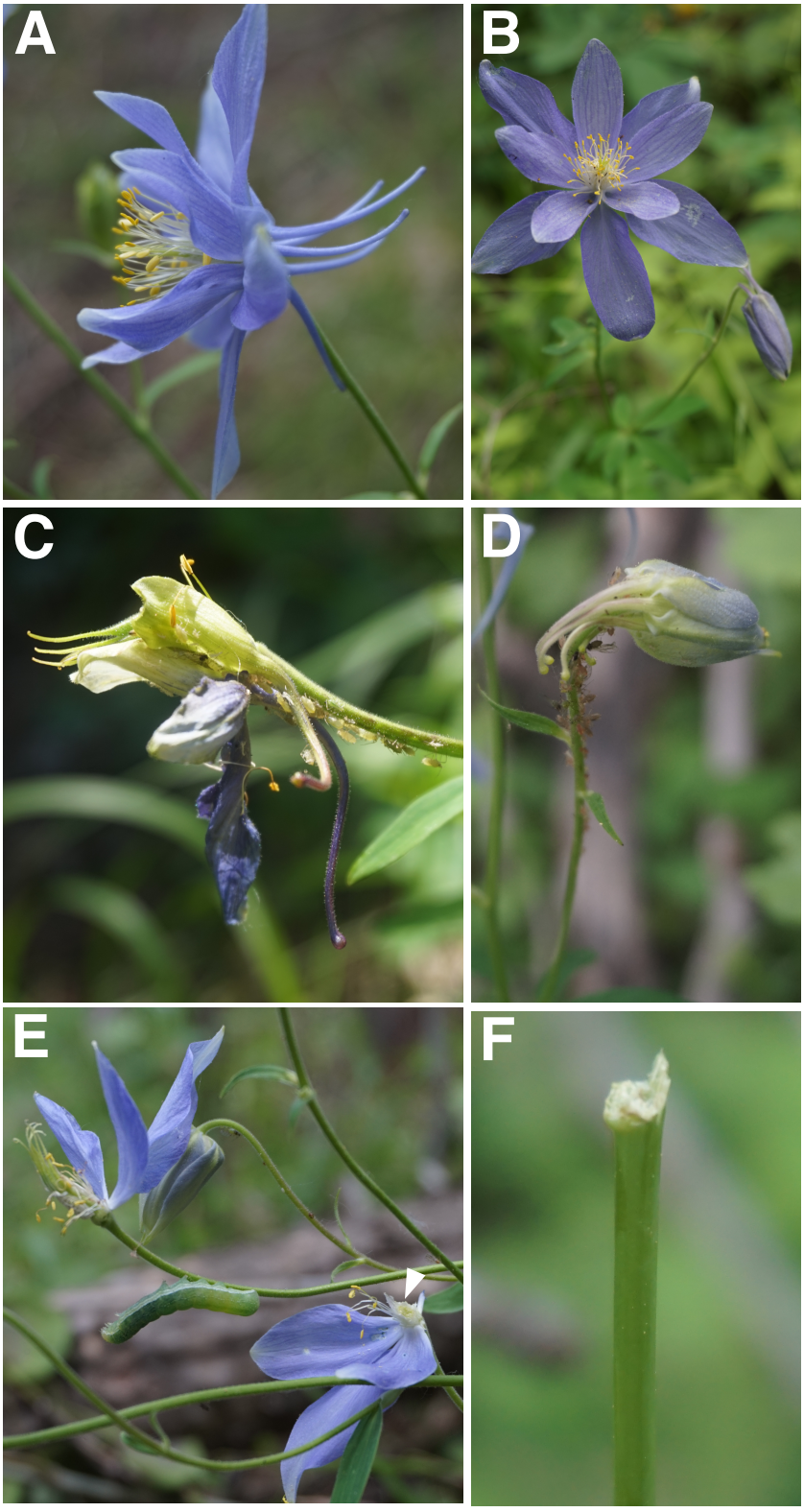

Fig. S2. Field photos. A-B. Rare intermediate 'short-spur' morphology side (**A**), front (**B**). This morph had long blue, sepaloid blades rather than the short white blades of WT petals and intermediate spur lengths with reduced nectaries. Plants with this morphology were classified as mutant in analyses. **C-F. Floral herbivores in Reynolds Park. C-D.** Aphid herbivory. **E.** Caterpillar herbivory. Arrowhead in **E** shows typical caterpillar damage. **F.** Deer herbivory leaves a stumped inflorescence.

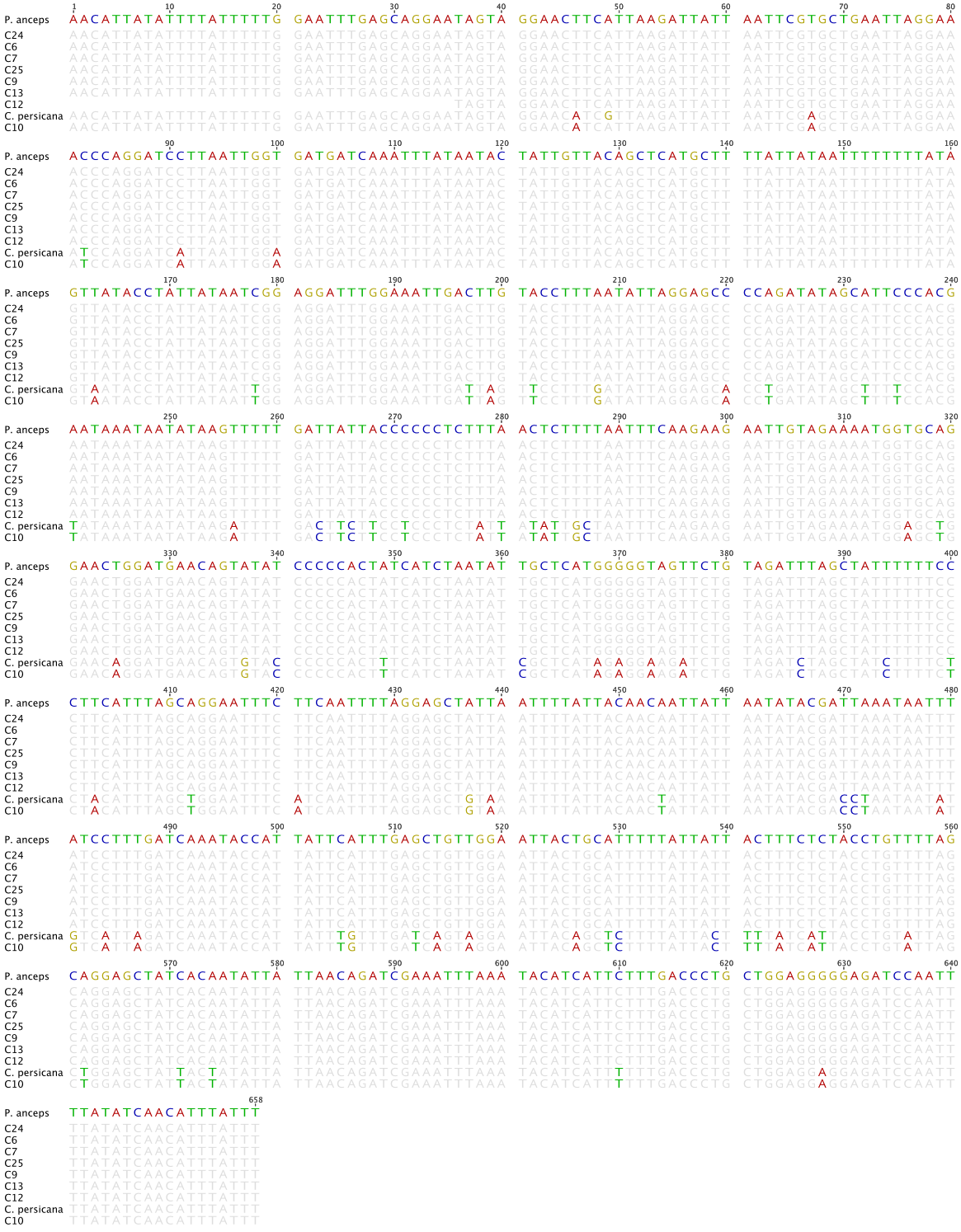

Fig. S3. *Platypolia anceps* is the predominant caterpillar herbivore.
CO1 sequences from eight collected caterpillars aligned to *Platypolia anceps* (GenBank: HM864392.1). All but one sample (C10) align perfectly to *P. anceps*. C10 aligned *to Clepsis persicana* (GenBank: JF703064), which is also shown here aligned to *P. anceps*.

Fig. S4.
Allele specific amplification bias. Each plot shows number of reads for the reference (ref) and alternate (alt) allele at three positions. Each point represents 1 individual that was called heterozygous at this position. Read counts were tested against a null hypothesis of equal number of reads and red points are significant using a chi-square test after a Bonferroni correction for multiple tests.