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Pollinator-Mediated Selection on Flower Color Allele Drives Reinforcement

Robin Hopkins* and Mark D. Rausher

Reinforcement is the process by which reduced hybrid fitness generates selection favoring the evolution of stronger prezygotic reproductive barriers between emerging species. Using common-garden field experiments, we quantified the strength of reinforcing selection in nature by demonstrating strong selection favoring an allele conferring increased pigment intensity in the plant *Phlox drummondii* in areas of sympatry with the closely related species *Phlox cuspidata*. Incomplete hybrid sterility between the two species generates selection for traits that decrease interspecies hybridization. In contrast, selection on this locus is undetectable in the absence of *P. cuspidata*. We demonstrate that reinforcing selection is generated by nonrandom pollinator movement, in which pollinators move less frequently between intensely pigmented *P. drummondii* and *P. cuspidata* than between lightly pigmented *P. drummondii* and *P. cuspidata*.

Reinforcement is the evolution of increased prezygotic reproductive isolation due to selection favoring decreased hybridization between diverging groups of individuals or emerging species (1–4). A. R. Wallace first proposed that selection against hybrids might favor the evolution of novel prezygotic isolating barriers (subsequently termed the Wallace effect) in 1889 (5). Although this idea has been controversial, recent theoretical and empirical work suggests that reinforcement may often play an important role in increasing reproductive isolation in nature (1, 3, 4, 6, 7). However, the magnitude of reinforcing selection in nature is generally unknown, as are the genes upon which such selection acts. Theoretical models have demonstrated that direct environmental selection can be more effective in influencing trait evolution than reinforcing selection (6, 8–11), but previous investigations of reinforcement have rarely differentiated between these two types of selection [but see (12, 13)].

Flower color variation in *Phlox drummondii* has been hypothesized to be an example of reinforcement (14). The geographic range of this species partly overlaps with that of a congener, *P. cuspidata*, in eastern Texas. Both species have the light-blue (sometimes called violet or pink) flower color characteristic of most *Phlox* species in allopatric areas of their ranges, whereas *P. drummondii* has dark-red flowers in regions sympatric with *P. cuspidata* (15). In the region of sympatry, populations of the two species frequently grow in close proximity; produce hybrids in nature that have high, but not complete, ovule and pollen sterility; and exhibit some interspecific gene flow (16, 17). In *P. drummondii*, the difference between the ancestral light-blue flower color and the derived dark-red flower color is caused by mutations in the *cis*-regulatory regions of two genes (18). Down-regulation of the gene coding for the enzyme Flavonoid 3′5′-hydroxylase

(*F3′5′h*) alters the anthocyanin pigment composition of flowers and changes them from blue to red. At this hue locus, the ancestral “blue” allele (*H*) is dominant to the derived “red” allele (*h*). Up-regulation of an *R2R3-Myb* transcription factor increases the amount of pigments produced, resulting in increased color intensity. At this intensity locus, the derived “dark” allele (*I*) is dominant to the ancestral “light” allele (*i*). Western, allopatric *P. drummondii* populations are fixed for the *i* and *H* alleles, whereas eastern, sympatric populations are fixed or nearly fixed for the *I* and *h* alleles, and the two recombinant flower colors, light-red (*iihh*) and dark-blue (*I-I-H-*), occur only near the boundary between allopatric and sympatric populations (15).

Patterns of neutral genetic variation across the range of *P. drummondii* suggest extensive gene flow between allopatric and sympatric populations, indicating that natural selection and not genetic drift is likely responsible for the geographic pattern of flower color variation (15). To determine whether selection in sympatry is due primarily to environmental factors acting directly on flower color variation, rather than to effects of reinforcement, we performed a common-garden field experiment designed to detect selection in the absence of *P. cuspidata*. We measured average fitness of the four flower-color double-homozygote genotypes in their natural habitat. Three generations of crosses were performed to produce seeds of known flower-color genotype and to randomize the genetic background of loci unlinked to the two flower-color loci (19). For clarity, we will refer to the homozygous color genotypes by their corresponding flower color throughout the remainder of this paper. A total of 2720 seeds were planted in a randomized block design, with 170 individuals per genotype per block, at the University of Texas Stengl research station (Smithville, Texas). This station is located within the sympatric region of *P. drummondii* and *P. cuspidata* and contains natural populations of both species (19).

No significant differences in survival or reproductive success among the flower-color genotypes were observed (table S2). We noted that

survival was slightly lower for two derived genotypes (dark-blue and dark-red), compared with the ancestral genotype (light-blue), whereas it was slightly higher for the derived genotype light-red (Fig. 1A). The number of fruits produced was slightly higher for all three derived genotypes compared with light-blue (Fig. 1B), but these differences were also not statistically significant (table S3). There were no detectable differences among genotypes for number of seeds per fruit (table S4). Female fitness, the product of survival and fruit production, was also slightly higher for the derived genotypes, compared with light-blue genotype (Fig. 1C), but again none of these differences were statistically significant (19). Overall, we did not detect environmental effects acting directly on flower color favoring the derived allele at either the hue or the intensity locus in the area of sympatry.

To examine whether reinforcing selection generated by hybridization with *P. cuspidata* favors the derived allele at either flower-color locus, we established blocks consisting of 30 plants of one of the double-homozygous genotypes (“focal plants”), as well as 105 light-blue plants of a stock line. We followed 30 of the light-blue stock individuals as “reference plants” to control for environmental variation among blocks. In addition, we planted 115 *P. cuspidata* plants in each block. We collected fruits from reference and focal plants and randomly chose 100 to 150 seeds from each

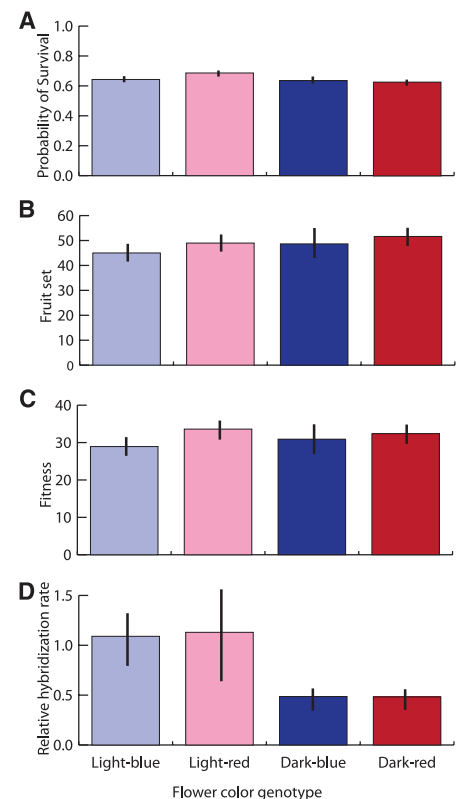


Fig. 1. Fitness components for each flower color genotype. (A) Survival probability ($n = 1909$). (B) Fruit production ($n = 1240$). (C) Fitness ($n = 1909$). (D) Relative hybridization rate ($n = 931$). Bars indicate 1 SE.

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focal and reference genotype in each block to genotype and determine whether the paternal parent was *P. drummondii* or *P. cuspidata* (19). Using this paternity test, we calculated the hybridization rate for focal and reference genotypes within each block.

Across the four blocks, the hybridization rate (proportion of seeds sired by *P. cuspidata*) varied between 28 and 44% for the light-blue reference plants, which indicated substantial overall interspecific hybridization. The hybridization rates of the light-blue and light-red focal plants were similar to those of their respective reference plants (Fig. 1D and table S5, a and b). In contrast, the hybridization rates of dark-blue and dark-red focal plants were more than 50% lower than the reference plants (Fig. 1D and table S5, a and b). Thus, we conclude that the dark allele (*I*) at the intensity locus significantly decreases hybridization between *P. drummondii* and *P. cuspidata*. Given conservative empirical estimates of hybrid sterility of ~90% (17) and the average light-blue reference plant hybridization rate of 0.43, the reduction in hybridization translates into a selection coefficient of 0.32 favoring the dark allele (19). The two species of *Phlox* are commonly found in intermixed populations with spatial proximity similar to that in our experiments. In these populations, the strong reinforcing selection documented here would increase the frequency of the dark allele rapidly to fixation. Extensive gene flow between *P. drummondii* populations (15), including those without nearby *P. cuspidata*, has

likely resulted in the subsequent spread of the dark allele throughout the sympatric region. To our surprise, there appeared to be no effect of the red allele at the hue locus on hybridization, even though this allele is fixed in sympatric populations. Taken together with the lack of difference in fitness among genotypes in the first experiment, the difference in hybridization rates between dark and light plants supports the hypothesis that reinforcing selection is responsible for the fixation of the dark allele in sympatric populations.

Manual pollen transfers indicate that there is no difference between light and dark flowered plants in fertilization success of *P. cuspidata* pollen (14). These data suggest that dark-flowered individuals are not less compatible with *P. cuspidata* pollen than light-flowered individuals. It is therefore likely that nonrandom patterns of pollinator visitation between *Phlox* species with different flower colors explain our observed variation in hybridization. We examined patterns of pollinator visitation in experimental arrays to test this hypothesis. We constructed three arrays containing light-blue plants, *P. cuspidata* plants, and either light-red, dark-red, or dark-blue focal plants (19). We observed a total of 181 pollinators making a total of 2301 transitions between plants.

The primary visitors to both *Phlox* species in these arrays, as in natural populations, were *Battus philenor* butterflies (108 individuals observed) and various species of skippers (Lepidoptera, family Hesperidae) (73 individuals observed) (table S6a). Both types of pollinators displayed

similar movement patterns and visited both *Phlox* species extensively (table S6b). In arrays with light-red plants, there is no evidence of pollinator constancy, as measured by the Bateman's Constancy Index (19, 20) (table S6c). Pollinators were equally likely to visit light-blue and light-red plants after visiting *P. cuspidata* (Table 1A) (19). This pattern is consistent with finding no difference in hybridization rates between these two genotypes. In addition, pollinators were equally likely to visit a *P. cuspidata* plant after visiting a light-blue or a light-red plant, which suggested that pollen wastage through interspecific fertilization does not differ between these two genotypes (Table 1A) (19).

In contrast, pollinators in arrays containing either dark-blue or dark-red plants exhibited a significantly higher species-level Bateman Constancy Index for dark-flowered genotypes compared with light-flowered genotypes (table S6c). In particular, pollinators were half as likely to visit dark plants as light-blue plants after visiting a *P. cuspidata*—a pattern that explains the reduced hybridization observed in plants with dark pigmentation (Table 1, B and C) (19). Pollinators were also substantially less likely to visit a *P. cuspidata* after visiting a dark-blue or dark-red plant than after visiting a light-blue plant (Table 1, B and C) (19), which suggested that darkly pigmented plants waste less pollen on interspecific pollination. Although we did not directly measure male fitness in our field experiments, this observation indicates that the dark allele may significantly increase male fitness, in addition to female fitness, relative to the light allele. It is possible that pollinators could be responding to pleiotropic effects of the intense allele (e.g., nectar volume or concentration), but this seems unlikely given the visual orientation of the primary pollinator, *B. philenor* (21).

Our investigations provide no explanation for why sympatric populations of *P. drummondii* have evolved red flowers. Although previously we used patterns of genetic variation at this locus and at neutral markers to show that natural selection drove the fixation of the red (*h*) allele in the region of sympatry (15), in the current study, we detected neither fitness differences nor differences in levels of interspecific hybridization between genotypes at the hue locus. One possible explanation for these contrasting results is that selection due to environmental factors favors the red allele in sympatry but that the magnitude of this selection is too small to detect given the power of our analysis (as may be evidenced by Fig. 1C). A second possibility is that environmental selection operates only intermittently on this locus and was absent during our experiments. Finally, a third possibility is that a selective agent, such as another type of pollinator, generated selection in the past on the hue locus but is no longer present. Although hitchhiking by the red allele in a selective sweep involving a closely linked gene is a formal possibility, this seems unlikely because it would have required that the favored mutation arose on a rare haplotype carrying the red allele.

Table 1. The percentage of transitions by pollinators across flower types. (A) Pollinator movement between colors and species within arrays containing light-red *P. drummondii*. (B) Pollinator movement between colors and species within arrays containing dark-blue *P. drummondii*. (C) Pollinator movement between colors and species within arrays containing dark-red *P. drummondii*. The percentages of transitions between *P. cuspidata* and *P. drummondii* plants are in bold.

A Pollinators in arrays with the light-red *P. drummondii* moved

From	To (%)		
	Light-red	Light-blue	<i>P. cuspidata</i>
Light-red (252)	32	44	24
Light-blue (219)	50	22	28
<i>P. cuspidata</i> (185)	34	31	35

B Pollinators in arrays with the dark-blue *P. drummondii* moved

From	To (%)		
	Dark-blue	Light-blue	<i>P. cuspidata</i>
Dark-blue (222)	51	38	11
Light-blue (198)	42	18	40
<i>P. cuspidata</i> (274)	7	32	61

C Pollinators in arrays with the dark-red *P. drummondii* moved

From	To (%)		
	Dark-red	Light-blue	<i>P. cuspidata</i>
Dark-red (255)	37	50	13
Light-blue (335)	37	25	38
<i>P. cuspidata</i> (378)	9	33	58

By measuring reinforcing selection acting on the dark flower–color allele in *P. drummondii* under natural sympatric conditions and by quantifying selection in the absence of *P. cuspidata*, we were able to compare the relative strengths of direct selection by other environmental factors and by reinforcing selection on a trait conferring increased pre-mating isolation in a region of sympatry. The absence of detectable fitness differences among flower color genotypes in the absence of *P. cuspidata* indicates that another agent of selection is unlikely to be involved in flower color divergence in *P. drummondii*. Although we cannot rule out small, statistically undetectable differences in survival or reproductive success favoring these genotypes, such differences would be of minor importance compared with the strong reinforcing selection acting on the intensity locus.

Many plants have evolved pre-mating reproductive isolation by switching pollinator types (e.g., from bees to hummingbirds) (22–24). Our work suggests that increased reproductive isolation can also be achieved by a single pollinator species through constancy of individual pollinators. In particular, if pollinators transition between flowers with similar phenotypes more frequently than between flowers with unlike phenotypes, this will decrease gene flow between unlike flowers. Constancy is commonly studied in bumble bees but rarely investigated in butterfly pollinators (20, 25). That the primary pollinator *Battus philenor* exhibits this type of constancy is not surprising, given that females of this species exhibit constancy for leaf shape when searching for oviposition sites (21).

Theoretical models indicate that the likelihood of successful reinforcement is greater when selection is strong, because this will counteract gene flow and recombination, which tend to reduce pre-mating isolation (26–28). Our results indicate that, at least in some cases, very strong reinforcing selection may act on a single allele and lead to increased reproductive isolation.

Theory also indicates that reinforcement is more easily achieved by a one-allele mechanism (4, 29), but empirical assessment of this prediction has been difficult because the genetic basis of reinforcement is understood in few systems (7). Our current demonstration of reinforcing selection acting on the dark allele indicates that reinforcement in *P. drummondii* involves a two-allele reinforcement mechanism. The intensity locus causes reproductive isolation only if the dark allele is present in *P. drummondii* and the light allele is present in *P. cuspidata*. Consistent with theory, we find that strong selection and high levels of hybrid sterility cause the spread of the dark allele through sympatric *P. drummondii* populations. We suspect all reinforcement mechanisms involving different floral phenotypes to which pollination vectors must respond will be two-allele assortative mating mechanisms, because pollinators must be able to discriminate between the novel phenotype in one species and the ancestral phenotype in both species.

Although reinforcement has been studied primarily in animals (3, 7), our work indicates that it may also be an important contributor to speciation in plants. If so, this phenomenon may provide a partial explanation for the tremendous diversity of floral color, floral morphology, and inflorescence structure that characterize flowering plants.

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Supporting Online Material

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Materials and Methods
Figs. S1 and S2
Tables S1 to S9
References

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Generation of Leaf Shape Through Early Patterns of Growth and Tissue Polarity

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A major challenge in biology is to understand how buds comprising a few cells can give rise to complex plant and animal appendages like leaves or limbs. We address this problem through a combination of time-lapse imaging, clonal analysis, and computational modeling. We arrive at a model that shows how leaf shape can arise through feedback between early patterns of oriented growth and tissue deformation. Experimental tests through partial leaf ablation support this model and allow reevaluation of previous experimental studies. Our model allows a range of observed leaf shapes to be generated and predicts observed clone patterns in different species. Thus, our experimentally validated model may underlie the development and evolution of diverse organ shapes.

The shapes of many plant and animal appendages are thought to develop under the influence of orthogonal organizing

systems (i.e., systems with axes that intersect at right angles) (1–4). However, it is unclear how these orthogonal systems lead to changes in tissue shape and how shape changes may themselves feed back to deform the organizing systems. Consider a square piece of tissue that deforms during growth (Fig. 1A). The tissue has an initial linear orthogonal system that organizes the pattern of morphogenesis (Fig. 1B, arrows). We might en-

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