Current Biology 24, 1995–1999, September 8, 2014 ©2014 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2014.07.027

Report

Strong Reinforcing Selection in a Texas Wildflower

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Summary

Reinforcement, the process of increased reproductive isolation due to selection against hybrids, is an important mechanism by which natural selection contributes to speciation [1]. Empirical studies suggest that reinforcement has generated reproductive isolation in many taxa (reviewed in [2–4]), and theoretical work shows it can act under broad selective conditions [5–11]. However, the strength of selection driving reinforcement has never been measured in nature. Here, we quantify the strength of reinforcing selection in the Texas wildflower *Phlox drummondii* using a strategy that weds a population genetic model with field data. Reinforcement in this system is caused by variation in two loci that affect flower color [12]. We quantify sharp clines in flower color where this species comes into contact with its congener, *Phlox cuspidata*. We develop a spatially explicit population genetic model for these clines based on the known genetics of flower color. We fit our model to the data using likelihood, and we searched parameter space using Markov chain Monte Carlo methods. We find that selection on flower color genes generated by reinforcement is exceptionally strong. Our findings demonstrate that natural selection can play a decisive role in the evolution of reproductive isolation through the process of reinforcement.

Results and Discussion

Evolutionary biologists have long tried to understand the role of natural selection during speciation [13–15]. Quantifying the strength of selection acting on reproductive isolating mechanisms could provide key insights into the process of speciation. Previous studies investigating reinforcement have shown that hybridization between taxa is costly and that adaptations found in sympatric populations decrease hybridization (e.g., [16–19]), but these studies fall short of quantifying the strength of reinforcing selection. That task is challenging because the strength of reinforcing selection depends on the rate of hybridization, which varies in space with the relative frequency of the hybridizing species.

We quantified the strength of selection in one of the best-studied examples of reinforcement, flower color variation in *Phlox drummondii*. We developed an innovative method that combined data collected from natural populations and a mathematical model. This approach allowed us to estimate the forces of selection acting in sympatry and allopatry, as well as gene flow between populations.

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Throughout most of their ranges, *P. drummondii* and *Phlox cuspidata* have flowers with a similar light-blue color. However, in regions where they occur in sympatry, *P. drummondii* has dark-red flower color. The evolution of flower color is due to reinforcement [19, 20]. Hybrids between the two *Phlox* species are nearly sterile, which creates strong selection against hybridization [21, 22]. Field experiments demonstrate that individuals with dark-red flowers hybridize 50% less frequently than those with light-blue flowers [19]. The color polymorphism is caused by variation at two unlinked loci that change the type and amount of anthocyanin pigments produced in the flower [12]. The hue locus controls expression variation of the gene *Flavonoid 3′5′-hydroxylase*. The derived allele (*h*) is recessive and causes red rather than blue flowers. The intensity locus is a *cis*-regulator of an *R2R3-Myb* transcription factor that controls expression of key genes in the anthocyanin biosynthesis pathway. The derived allele (*I*) is dominant and causes flowers to be dark rather than light. The ancestral light-blue flower color (*iiH_*) is near fixation in allopatry, whereas the derived dark-red flower color (*I_hh*) is near fixation in sympatry. The recombinant dark-blue (*I_H_*) and light-red (*iihh*) plants are found in areas where the two predominant colors come into contact.

Steep Clines in Flower Color

Geographically structured variation in phenotypes often results from spatially varying selection [23–27]. Clines in allele frequencies can be formed by the counteracting forces of homogenization from gene flow and differentiation from selection. The width of a cline reflects a balance between these two opposing forces [23].

We characterized how *P. drummondii* flower color varies across space by surveying natural populations throughout central Texas. We phenotyped 12,024 *P. drummondii* individuals for flower color from 32 populations across five transects (shown in Figure 1; data in Table S1) and found abrupt clines in *P. drummondii* flower color. To quantify the width of these clines, we used maximum likelihood to fit sigmoid curves to the frequencies of the recessive *ii* (light) and *hh* (red) genotypes across the five transects (as in [28, 29]). The results from combining all five transects are shown in Figure S1 available online, and results from each transect individually are in Table S3. The widths of the clines in light (*ii*) and red (*hh*) flower color are 4 km and 3.7 km, respectively.

Many previous analyses of clines have estimated the strength of selection using the width of the cline and independent dispersal estimates [30–33]. The models on which those analyses are based assume that there is no epistasis between loci, that alleles are under weak selection ($s < 0.1$), and that alleles are not dominant. Those assumptions are violated in many systems, including *Phlox*. To avoid that problem, we exploited our knowledge of this system's natural history and the genetic basis of flower color to create a population genetic model tailored to the life cycle of *Phlox*.

A Population Genetic Model to Estimate Evolutionary Forces

We estimated the strength of selection acting on *P. drummondii* flower color by fitting a spatially explicit population genetic

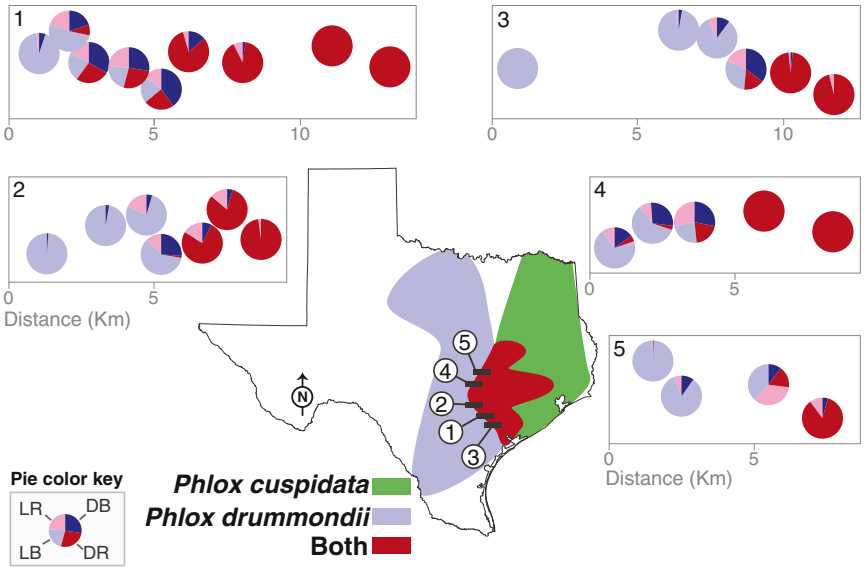

Figure 1. Flower Color Frequency in *Phlox drummondii* Populations across Five Transects

The transects are shown as insets, with each of the 32 populations represented by pie diagrams. Each pie diagram shows the frequency of flower color phenotype within a population, with wedge color corresponding to light-blue (LB), dark-blue (DB), light-red (LR), and dark-red (DR) flower color.

model to our data from the field (e.g., [34]). Our model comprised a set of recurrence equations describing how selection, migration, mating, and reproduction act in demes along transects. By simulating these equations to equilibrium, we obtained predicted genotype frequencies for a given set of parameters. We fit the model by using the likelihood of the observed color frequencies given the frequencies predicted by the model. We found the maximum likelihood estimates by heuristic search using a Markov chain Monte Carlo method. Below is a brief conceptual description of the model; full details of the model and how it was fit are in the Supplemental Experimental Procedures.

Our model describes five transects that cross two zones of selection corresponding to allopatry and sympatry. The locations of the borders between the two zones are assumed to depend on unobserved environmental factors, so the borders are free variables in the model (for effects of border position on model, see Table S4). Each transect consists of 36 demes. In each generation, the first event to occur is selection acting on flower color. The relative fitness of the four color phenotypes (light blue, light red, dark blue, and dark red) differs between the allopatry and sympatry zones but are assumed to be constant within each zone. After selection, pollen disperses between demes according to a symmetrical geometric dispersal function. Finally, the random union of pollen and ovules within each deme gives rise to the zygotes of the next generation. The assumption of random mating between flower colors is consistent with our field observations of pollinators [35]; we return to this point below. Random mating also assumes no self-fertilization, which is consistent with observations of self-incompatibility in *P. drummondii* and with little or no evidence for inbreeding seen in molecular data [36, 37]. The model depends on 12 parameters: three relative fitnesses in sympatry, three relative fitnesses in allopatry, the variance of the dispersal function, and the locations of the boundaries between the allopatric and sympatric zones in each of the five transects. We implemented a number of variations of this standard model in order to demonstrate the robustness of our results; see below for descriptions and results from these model variants. More complex (and realistic) models are conceivable but are likely not feasible to implement because of the larger number of parameters they would require.

For a given combination of parameter values, we simulated our model to determine the equilibrium genotype frequencies in each deme. These frequencies are used to calculate expected phenotypic frequencies as well as the frequencies of offspring phenotypes from each maternal flower color. We used likelihood to fit our model to two types of data collected from the five transects. The first data set consisted of flower color phenotypes from 12,024 individuals. The second data set contained 2,581 offspring flower color phenotypes from 382 maternal plants (data in Table S2). We compiled the second data set by phenotyping the flower color of maternal plants in the field, collecting their seeds, and growing these offspring to flowering in the University of Texas at Austin greenhouses. We gained power by fitting the model to both data sets simultaneously because doing so captured information about the clines, pollen movement between demes, and potential nonrandom mating.

We obtained maximum likelihood estimates and probability distributions for our parameters of interest by using a Metropolis-Hastings Markov chain Monte Carlo method. The results from our standard model are shown in Figure 2. The model predicted that within 5 km, the light-blue flowers would be replaced by dark-red flowers (Figure 2a shows the expected flower color phenotypic frequencies predicted by the model in solid lines compared to the observed frequencies from our data). Consistent with our field data, there was a peak of the recombinant phenotypes (dark blue and light red) in the center of the cline. Our model also predicted the flower color frequencies for offspring produced by maternal plants of each flower color within each population. These expected results are compared to the offspring phenotypes observed in our data set in Figure 2b.

Flower Color Fitness

We found strong selection acting on *P. drummondii* flower color in populations that are sympatric with *P. cuspidata* (Figure 3). The estimated relative fitness of the ancestral light-blue color flower compared to the derived dark-red color was 0.17 (that is, an 83% decline in fitness). The other flower color phenotypes were also significantly less fit than dark red (fitness: dark blue = 0.53, light red = 0.86).

Field experiments have shown that reinforcing selection is likely the cause of these large fitness differences in sympatry [19]. The results from our model are consistent with previous studies, demonstrating that the change in flower color from light blue to dark red substantially decreases hybridization and therefore increases the viable fruit production. Unlike the

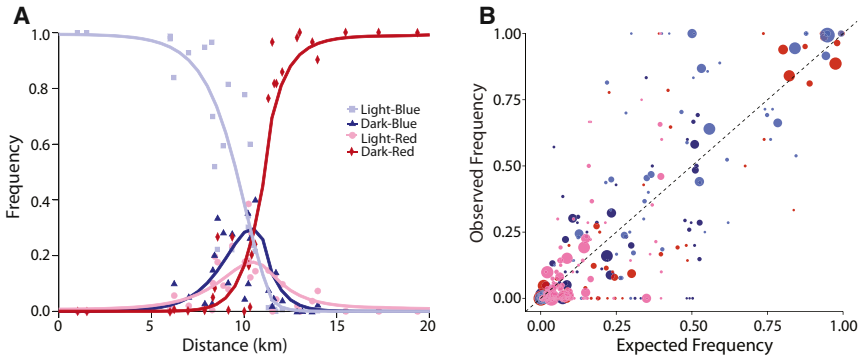

Figure 2. Comparison of Observed Frequency of *P. drummondii* Flower Color and Expected Frequency from Our Standard Model

(a) Phenotype frequencies observed from surveyed transects are shown as points, and expected frequencies are shown as lines. Data from all five transects are graphed together by aligning the border between allopatry and sympatry for each transect. Observed transect phenotype data are in Table S1.

(b) Relationship between observed and expected flower color frequencies from the offspring data set. The x axis is the expected frequencies of offspring as predicted by our model, and the y axis is the observed frequency from our offspring data set. The dashed one-to-one line represents observed matching expected. Each point is the frequency of offspring flower color for a single maternal flower color within a population. Points are colored by maternal flower color. If multiple maternal plants from a single population have the same flower color, the frequencies are pooled. The size of each point is proportional to offspring sample size. Observed offspring data are in Table S2.

previous work, our current results go beyond estimating the hybridization rates of different flower colors by actually estimating the fitness of flower color phenotypes in nature.

In allopatry, *P. drummondii* with the light-blue flower color have a fitness advantage over the derived flower colors (Figure 3). Interestingly, we found that all three of the derived flower colors suffer from similar fitness loss (relative fitness: dark blue = 0.86, dark red = 0.90, light red = 0.89). These findings are consistent with field studies that demonstrate pollinator preference for light-blue *P. drummondii* flowers relative to other colors in allopatry, suggesting a paternal fitness advantage for individuals with the favored flower color [35]. Our results indicate that traits favored by reinforcement can harbor a cost and be disfavored in allopatry. This cost maintains the pattern, often used to recognize reinforcement, of greater divergence in allopatry relative to sympatry.

Selection on Flower Color Alleles

In order to better understand how selection acts on flower color variation, we calculated selection coefficients for alleles at the hue and intensity loci and the fitness epistasis between those loci. The relative fitness results described above are based on flower color phenotypes derived from allelic identity at both the hue and intensity loci. We can decompose the fitness of these phenotypes into selection acting on the hue locus and the intensity locus separately and determine whether fitness behaves additively or epistatically. For example, in sympatry we might expect selection to act against the light allele at the intensity locus and the blue allele at the hue locus. If fitness is additive, then selection against the light and blue alleles together in the light-blue individual is the sum of selection coefficients for the individual alleles. With epistasis, on the other hand, selection against the alleles in combination is either stronger (synergistic epistasis) or weaker (antagonistic epistasis) than in the additive case.

The results from our standard model support the hypothesis of local adaptation at the flower color loci and provide one of the few documented cases in which derived alleles are beneficial in their home environment but are maladapted to foreign environments. We also found fitness epistasis in both allopatry and sympatry.

In sympatry, we found that selection disfavored the ancestral blue allele (*H*) at the hue locus and the ancestral light allele (*i*) at the intensity locus (Table 1). Selection was strong at the hue locus: the blue allele was only half as fit as the red allele. These results are consistent with previous inferences of selection based on allele frequencies at color loci compared to neutral genetic markers [38]. Oddly, field experiments have not detected significant selection at the hue locus, but they do show strong selection acting on the intensity locus [19]. The inconsistency between the field studies and our model could be due to spatially or temporally varying selection that was not detected in the field experiment yet strongly affects the flower color allele frequencies across broad geographic space and evolutionary time.

Our model does not assume a particular source of selection and cannot differentiate between reinforcement and other sources of selection. Previous fieldwork found no evidence for ecological benefits of the derived flower colors, suggesting that ecological character displacement and adaption to abiotic or other biotic factors in the environment are unlikely. Field experiments have demonstrated direct evidence for reinforcing selection acting on flower color [19]. We therefore believe that selection is likely due to reinforcement.

The plausibility of reinforcement has been debated due to theoretical arguments regarding the "swamping" effects of gene flow. It has been postulated that the evolution of assortative mating in sympatric populations could be impeded by gene flow from allopatric populations into the zone of sympatry, making reinforcement difficult or impossible to evolve [39, 40]. The validity of this critique, and thus, the plausibility of reinforcement, largely depends on the previously unknown strengths of selection and migration. Here, we find that selection on flower color alleles is stronger than assumed by some theoretical models [6, 8] and more than adequate to prevent the potential swamping effect of gene flow.

There is also strong selection acting on both flower color loci in allopatric populations of *P. drummondi* (Table 1). We found that selection against the derived dark (*l*) and red (*h*) alleles maintains the ancestral light-blue flower color in areas without *P. cuspidata*. The strength of selection acting against the derived flower color alleles in allopatry is weaker than selection in sympatry, although selection in allopatry still represents a strong evolutionary force.

P. drummondii flower color loci showed strong epistasis for fitness in both sympatry and allopatry (Table 1). We quantified epistasis in allopatry as $\epsilon_a = 1 - w_{DB} + w_{DR} - w_{LR}$ and in sympatry as $\epsilon_s = 1 - w_{DB} + w_{LB} - w_{LR}$, where w_{DB}, w_{DR}, w_{LB}, w_{LR} are the relative fitness of the dark-blue, dark-red, light-blue, and light-red phenotypes, respectively. In sympatry, we found synergistic epistasis: the fitness cost of the light and the blue alleles together was greater than what was predicted by their

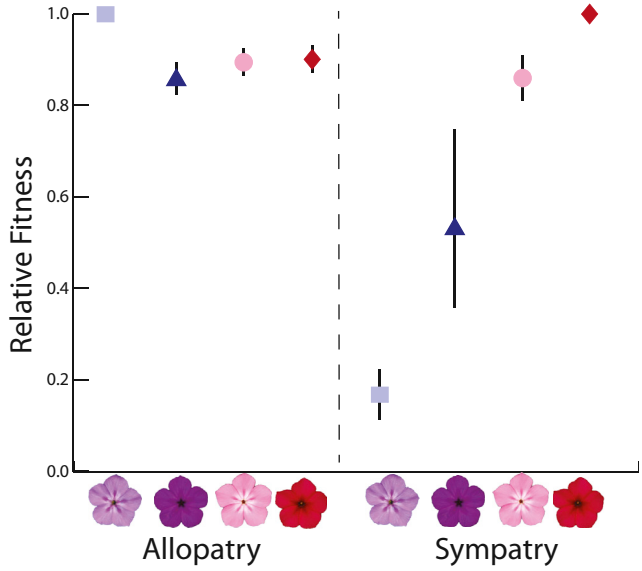

Figure 3. Estimated Relative Fitness of Flower Color Phenotypes in Allopatry and Sympatry

Points show mean estimates and are in order of light blue (squares), dark blue (triangles), light red (circles), and dark red (diamonds) within each zone. Error bars show 95% confidence regions.

individual additive effects. In allopatry, however, we found that epistasis was antagonistic: the fitness cost of red and dark alleles together was smaller than their additive effects.

The epistasis between the flower color loci was not apparent at the phenotypic or biochemical level. The dark allele (*I*) increased floral pigment production regardless of the hue of the flower, and the red allele (*h*) stopped the production of blue pigments, causing red flowers regardless of the genotype at the intensity locus [12]. Despite these seemingly additive effects on phenotype, the alleles showed epistatic effects on fitness. Interestingly, the direction of the epistasis was different in the two environments. The epistatic interactions between the flower color loci revealed the importance of understanding the genetic basis of complex traits in order to untangle how selection is acting on the trait as a whole and on the individual genetic components.

Significant Migration across Cline

Our estimate of migration suggests extensive gene flow between *P. drummondii* populations. The mean variance of the dispersal kernel was 0.83 km^2, which corresponded to an average parent-offspring dispersal distance of 0.91 km. In our model, migration occurred via pollen movement. These results indicate that about 50% of the pollination occurs within population, whereas the remaining pollen comes from at least a half kilometer away (Figure S2). Previous population genetic analyses of *P. drummondii* in central Texas found very little isolation by distance and low levels of population differentiation [38], which is consistent with the dispersal estimate from our model.

Model Validation

We performed a number of checks to validate the power and robustness of our standard model. First, we tested the assumption of random mating with respect to flower color by adding an assortative mating parameter to our model. The assortative mating parameter estimate was not significantly different from zero (Table S5), which is consistent with our field observations that butterflies move randomly among *P. drummondii* with different flower colors [35]. Second, although previous studies have found that geometric and exponential functions adequately describe pollen dispersal [41, 42], we tested the robustness of our model to changes in the dispersal function by using a Gaussian migration kernel to describe migration. Our estimates of selection from the two models were statistically indistinguishable (Table S5). We also fit our model using each of the two data sets separately. We fit the phenotypic data collected from the five field transects without the offspring phenotype data set, and then we fit the offspring phenotype data set alone. Estimates based on the individual data sets were not significantly different from those based on using all the data together (Table S5).

Finally, we confirmed that our model accurately estimates the strength of selection and migration by simulating data sets with known parameter values. We simulated data using parameter values similar to those we estimated as well as stronger and weaker selection parameters. We found that the mean parameter estimates were within about 1 SD of the actual parameter values used to create the simulated data sets (Table S6), suggesting that our estimation procedure was performing correctly.

Table 1. Selection Coefficients and Epistasis for Flower Color Alleles in Allopatry and Sympatry

	Hue Locus		Intensity Locus		Epistasis	
Allopatry	s_h	−0.11 [−0.14, −0.08]	s_i	−0.14 [−0.17, −0.10]	ϵ_a	0.15 [0.11, 0.19]
Sympatry	s_H	−0.14 [−0.19, −0.09]	s_I	−0.47 [−0.69, −0.30]	ϵ_s	−0.22 [0.0, −0.37]

Parameter estimates from the standard model are shown with 95% confidence intervals in brackets. Negative selection coefficients indicate selection against an allele. Details on calculating selection coefficients and epistasis are in the Supplemental Experimental Procedures. Selection on the red allele (*h*) and blue allele (*H*) is indicated by s_h and s_H, respectively. Selection on the light allele (*i*) and the dark allele (*I*) is indicated by s_i and s_I. Epistasis in allopatry and sympatry is indicated by ϵ_a and ϵ_s.

Conclusions

Here, we report the strength of selection causing reinforcement in a natural system. We find very strong selection acting on two flower color loci in populations of *P. drummondii* sympatric with *P. cuspidata*. This selection is countered by weaker but significant selection favoring alternative alleles at both flower color loci in allopatric *P. drummondii* populations. Although directly measuring the strength of the selection in a natural field setting can provide valuable information about the process of evolution, these types of experiments are not feasible for some modes of selection, including reinforcement. Here, we use an innovative method of combining models with empirical observations to determine the strength of evolutionary forces causing a sharp cline.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, two figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.027.

Author Contributions

R.H., R.F.G., and M.K. conceived the method. R.H. and R.F.G. created the model. R.H., R.F.G., and M.D.R. collected the data. R.H., R.F.G., and M.K. wrote the manuscript.

Acknowledgments

We thank Sarah Otto, David L. Des Marais for assistance in field sampling and plant care, and Shane Merrell from the University of Texas at Austin greenhouses for assistance in plant husbandry. We thank Thomas Juenger for use of laboratory and greenhouse space and for advice throughout the project. The authors acknowledge the Texas Advanced Computing Center (TACC) at the University of Texas at Austin for providing high-performance computing (https://www.tacc.utexas.edu). This work was supported by a National Science Foundation (NSF) Postdoctoral Research Fellowship in Biology (DBI-1202719) to R.H. and by an NSF grant (DEB- 0819901) to M.K.

Received: May 28, 2014
Revised: July 9, 2014
Accepted: July 11, 2014
Published: August 21, 2014

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