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MOLECULAR SIGNATURES OF SELECTION ON REPRODUCTIVE CHARACTER DISPLACEMENT OF FLOWER COLOR IN *PHLOX DRUMMONDII*

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Character displacement, which arises when species diverge in sympatry to decrease competition for resources or reproductive interference, has been observed in a wide variety of plants and animals. A classic example of reproductive character displacement, presumed to be caused by reinforcing selection, is flower-color variation in the native Texas wildflower *Phlox drummondii.* Here, we use population genetic analyses to investigate molecular signatures of selection on flower-color variation in this species. First, we quantify patterns of neutral genetic variation across the range of *P. drummondii* to demonstrate that restricted gene flow and genetic drift cannot explain the pattern of flower-color divergence in this species. There is evidence of extensive gene flow across populations with different flower colors, suggesting selection caused flower-color divergence. Second, analysis of sequence variation in the genes underlying this divergence reveals a signature of a selective sweep in one of the two genes, further indicating selection is responsible for divergence in sympatry. The lack of a signature of selection at the second locus does not necessarily indicate a lack of selection on this locus but instead brings attention to the uncertainty in depending on molecular signatures to identify selection.

KEY WORDS: **Anthocyanin biosynthesis, population structure, reinforcement, selective sweep.**

Character displacement occurs when two species have different phenotypes in an area of range overlap but similar phenotypes in areas where their ranges do not overlap. Character displacement driven by resource competition in sympatry is termed ecological character displacement (ECD) (Brown and Wilson 1956; Losos 2000), whereas character displacement reflecting reduced mating between species in sympatry is termed reproductive character displacement (RCD) (Grant 1972; Howard 1993; Pfennig and Pfennig 2009). Reinforcing selection, which is selection that favors increased prezygotic isolation in sympatry between populations that are partially isolated postzygotically, often causes RCD (Butlin 1987; Servedio and Noor 2003; Ortiz-Barrientos et al. 2009).

Investigations of character displacement have clarified our understanding of several basic evolutionary patterns and processes (Pfennig and Pfennig 2009). For example, it is now recognized that character displacement is an alternative to competitive exclusion when two ecologically similar species exist in the same area (Slatkin 1980; Schluter and McPhail 1992; Losos 2000; Pfennig and Murphy 2002; Dayan and Simberloff 2005; Grant and Grant 2006). Additionally, RCD can be involved in either completing the process of speciation (reinforcement) or in reducing gamete wastage after speciation has been completed (Butlin 1987; Liou and Price 1994; Noor 1995; Higgie et al. 2000; Nosil et al. 2003; Servedio and Noor 2003; Smadja and Ganem 2005; Jang and Gerhardt 2006; Kay and Schemske 2008; Ortiz-Barrientos

et al. 2009). The study of character displacement has also aided in understanding broader evolutionary processes such as correlated evolution (Pfennig and Pfennig 2005), phenotypic plasticity (Pfennig and Murphy 2000), and sexual selection (Higgie and Blows 2007).

Most discussions of character displacement assume that intraspecific divergence arises primarily via natural selection (Howard 1993; Pfennig and Pfennig 2009). Numerous authors have described criteria to identify ECD and RCD and to distinguish them from other patterns of local adaptation (Waage 1979; Howard 1993; Servedio and Noor 2003). However, it has seldom been recognized that neutral forces, such as restricted gene flow and drift, can also cause patterns of divergence similar to character displacement (but see Schluter and McPhail 1992). For example, a neutral mutation may arise in a population in the region of sympatry with another species. The frequency of this allele may drift to high frequency in the original population as well as neighboring populations connected by gene flow. However, such a neutral mutation could be absent in allopatric populations for at least two reasons: (1) by chance, it may not have spread to populations in the zone of allopatry; or (2) the same environmental factor that determines the second species range boundary (and hence the border between sympatry and allopatry for the first species) may also separate populations of the first species, either due to physical barriers to gene flow or local adaptations causing restricted gene flow between sympatric and allopatric populations. Despite this possibility, rarely has the alternative hypothesis that drift leads to divergence been explicitly addressed in studies of character displacement (Marko 2005; Lehtonen et al. 2009).

The classic method for assessing if selection is responsible for phenotypic divergence within a species is to quantify selection on the divergent trait(s) in different environments (Kawecki and Ebert 2004; Hoeksema and Forde 2008; Hereford 2009). This approach has greatly increased our understanding patterns of diversity and local adaptation by documenting competition, divergent selection, and variation in hybridization (see the following reviews: Losos 2000; Servedio and Noor 2003; Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009).

Nevertheless, this approach can have limitations. For example, because there may be substantial temporal and spatial variation in selective environments across species' ranges, it can be difficult to extrapolate results from short-term, spatially restricted experiments to an understanding of the forces shaping patterns of divergence across many populations over many generations. Moreover, it is often difficult to measure selection in natural environments and to evaluate both the costs and the benefits of hybridization and competition across the range of an organism (but see Pfennig and Pfennig 2005; Grant and Grant 2006). Instead, most mate-choice tests and cost of hybridization assays are in artificial settings, and lifetime fitness often cannot be quantified (Noor 1995; Saetre et al. 1997; Pfennig and Murphy 2000; Cooley et al. 2001; Pfennig and Simovich 2002; Hobel and Gerhardt 2003).

A complementary approach that can circumvent some of these problems is to compare the magnitude of heritable trait divergence with that exhibited by neutral genetic variation. Although this approach has become common in demonstrating divergent selection (McKay and Latta 2002; Le Corre 2005; Edelist et al. 2006; Raeymaekers et al. 2007), it has seldom been applied to cases of apparent character displacement. If phenotypic differentiation in the heritable character is significantly greater than that for neutral genetic variation, one may conclude that processes other than drift and restricted gene flow, such as selection, contribute to phenotypic divergence (Gockel et al. 2001; Crispo et al. 2006; Keller et al. 2009; Antoniazza et al. 2010); however, the inverse is not necessarily true: neutral divergence as great as that exhibited by the character does not imply the absence of selection. Additionally, if the genes responsible for phenotypic variation are known, analysis of patterns of genetic variation in these genes may reveal signatures of selection (Charlesworth et al. 1997, 2003; Durrett and Schweinsberg 2004; Nielsen 2005). One advantage of this approach is that it integrates the effects of both neutral and adaptive divergence over substantially longer time periods and greater spatial scales than is possible with the classical approach of directly measuring selection. In this way, molecular analysis can complement direct studies of natural selection by demonstrating that experimentally derived patterns of selection have been characteristic over longer periods and thus likely explain patterns of divergence.

Here, we use this type of molecular approach to investigate if neutral genetic drift, coupled with restricted gene flow, has given rise to a pattern similar to RCD in the native Texas wildflower *Phlox drummondii*. Throughout most of its range, *P. drummondii* has the light-blue flower-color typical of the *Phlox* clade; but on the eastern edge of the range, in a region that is sympatric with the closely related, light-blue flowered *P. cuspidata*, *P. drummondii* has dark-red flowers (Levin 1985; Turner et al. 2003). *Phlox drummondii* thus exhibits the characteristic pattern of character displacement in that the two species have the same flower color in allopatry but different flower colors in sympatry with *P. cuspidata*. It has been suggested that this pattern is caused by reinforcement (Levin 1985). Hybrids between the two species are largely, though not completely, sterile (Levin 1967; Ruane and Donohue 2008), thus allowing for some introgression between the species (Levin 1975). Moreover, there is experimental evidence indicating that dark-red-flowered *P. drummondii* experience less interspecific hybridization than light-blue-flowered individuals (Levin 1985). However, it has yet to be determined if these hybridization differences result in selection favoring the red morph in sympatry or if selection favors maintaining the blue

morph in allopatry. Consequently, it remains to be determined whether selection, as opposed to restricted gene flow and drift, are responsible for flower-color divergence in *P. drummondii*. In this report, we distinguish between these hypotheses by comparing patterns of variation in multiple codominant microsatellite loci to the pattern of differentiation found in flower color between eastern (sympatric) and western (allopatric) populations of *P. drummondii*.

In addition, because we have previously identified the genes involved in the flower-color change in sympatric populations of *P. drummondii* (Hopkins and Rausher 2011), we investigate if these genes exhibit a signature of a selective sweep. In particular, we asked whether these genes exhibited less genetic variation in sympatric populations than in allopatric populations. Because the sympatric flower color is derived—the dark-red color of sympatric populations is the only known occurrence of red in the genus *Phlox*—a selective sweep favoring alleles producing dark-red flowers may have reduced variation in the two flower-color genes in sympatry (Charlesworth et al. 1997, 2003; Nielsen 2005).

Finally, even if a signature of a selective sweep has decayed, it may be possible to detect continuing selection by assessing the degree of divergence in genes involved in flower-color change. Both theory and empirical studies demonstrate that population subdivision and local selection is expected to result in the accumulation of neutral divergence near loci subject to divergent selection (Charlesworth et al. 1997; Storz and Kelly 2008). Although gene flow and recombination tend to inhibit the accumulation of divergence in most of the genome, differences may accumulate near the selected loci due to reduced effective migration at that region of the genome. Such divergence would be expected between populations that differed in the allele favored at the selected loci but would not be expected between populations in which the same allele was favored. We therefore attempted to determine whether in genomic regions surrounding the flower-color loci there is greater divergence between dark-red and light-blue populations than between populations with the same flower color. As with attempts to detect a selective sweep, absence of this type of divergence cannot be taken as evidence for absence of selection, because there may not have been sufficient time to accumulate the expected divergence.

Methods

STUDY ORGANISM

Phlox drummondii is an annual herb native to East and Central Texas, commonly found along roadsides and in pastures and agricultural fields. Germination occurs typically in November and plants overwinter as rosettes and flower from March through July. The closely related *P. cuspidata* is also an annual herb found in sympatry with eastern populations of *P. drummondii* (Levin 1985; Turner et al. 2003). This species produces light-blue flowers that are similar in appearance to, though smaller than, those of *P. drummondii*. The two species overlap substantially in flowering time, although *P. cuspidata* begins and ends flowering somewhat earlier than *P. drummondii* (Levin 1967, 1975). Both species are pollinated by the same species of butterflies (Levin 1985), predominantly the pipevine swallowtail (*Battus philenor*) (R. Hopkins, pers. obs.).

Flower-color divergence between allopatric and sympatric populations of *P. drummondii* is due to *cis*-regulatory changes in two genes in the anthocyanin biosynthetic pathway (Hopkins and Rausher 2011). One change, which influences floral hue (red vs. blue), markedly reduces expression of the gene coding for the branching enzyme flavanone-3′5′-hydroxylase (F3′5′h). This down regulation prevents the production of blue pigments and results in the production of only red floral pigments. The other *cis*-regulatory change increases pigment intensity by increasing the expression of an *R2R3-Myb* transcription factor, which regulates the anthocyanin structural genes and thereby causes greater pigment production. Although we have cloned and identified the coding sequences of both of these genes, we have not yet identified the *cis*-regulatory changes that are responsible for the expression changes. Consequently, all subsequent analyses to detect signatures of selection are restricted to examining patterns in the coding sequences.

POPULATION SURVEY

Fourteen populations were surveyed in 2007, 12 populations in 2009, and 13 populations were visited in the spring of 2010 for a total of 39 populations (Table 1, Fig. 1). Population sampling was designed to include approximately equal numbers of dark-red and light-blue populations. Five populations were called "mixed" because they contained at least 35% of both alleles at the flower-color loci. We chose sites that were at least 5 km apart from each other (with the exception of populations 1 and 2) and attempted to represent as much of the documented range of *P. drummondii* as possible, with concentrated sampling in the center of the range on either side of the flower-color divergence boundary. For each population, we surveyed flower-color proportions, collected leaf tissue for DNA extraction, and recorded GPS location.

Flower-color surveys were performed by tallying flower-color counts along three to five transects through the length of each population. Every individual that touched a line transect was scored for color. Within each population, we counted 100–500 individuals depending on the size of the population. For populations surveyed in 2007, we recorded both floral hue (red vs. blue) and intensity (light vs. dark) phenotypes. We assumed genotypes were in Hardy–Weinberg equilibrium and estimated the recessive allele frequency, q, as the square root of the frequency of the corresponding phenotypic frequency and then estimated p as

Table 1. Characteristics of the 39 populations sampled, with the year sampled and the GPS coordinates indicated. Color indicates whether the population consisted predominantly of light-blue individuals (LB), dark-red individuals (DR), or contained a mixture of both color phenotypes (MIX). The counts for the two hue colors and the two intensity colors are indicated as well as the estimated allele frequencies at both loci.

			GPS			Color counts							
Number	Name	Year	North	West	Color	Blue	Red	Light	Dark	q (red)	p (blue)	q (light)	p (dark)
1	77N1	2010	30.5556	−97.0027	LB	100	0			0	1		
2	36N1	2007	30.4159	−96.6037	LB	219	6	217	8	0.16	0.84	0.98	0.02
3	RRanch	2010	30.3624	−98.1254	LB	100	0			0	1		
4	21W1	2010	30.3457	−96.9001	LB	100	0			0	1		
5	696N1	2007	30.3236	−97.2902	LB	200	0	200	0	0	1	1	0
6	DOG95	2007	30.2927	−97.3423	LB	150	0	142	8	0	1	0.97	0.03
7	969E1	2009	30.1773	−97.4176	LB	385	10			0.16	0.84		
8	80N3	2009	29.7648	−97.7605	LB	100	0			0	1		
9	466E7	2007	29.5050	−97.7292	LB	192	8	167	33	0.20	0.80	0.91	0.09
10	181E1	2010	29.2697	−98.3112	LB	100	0			0	1		
11	108S3	2007	29.2653	−97.6382	LB	188	0	166	12	0	1	0.97	0.03
12	119S1	2007	29.0180	−97.5371	LB	200	0	200	0	0	1	1	0
13	181E2	2010	29.0161	−98.0494	LB	100	0			0	1		
14	72W2	2007	28.9034	−97.6817	LB	247	17	187	13	0.25	0.75	0.97	0.03
15	792N1	2007	28.8422	−97.8456	LB	99	0	92	7	0	1	0.96	0.04
16	239E1	2010	28.7398	−97.6305	LB	100	0			0	1		
17	239E2	2010	28.6156	−97.3272	LB	100	0			0	1		
18	239E3	2010	28.5162	−97.1143	LB	100	0			0	1		
19	202W1	2010	28.3566	−97.3488	LB	100	0			0	1		
20	281N1	2010	28.2895	−98.1138	LB	100	0			0	1		
21	969E2	2009	30.1344	−97.3711	MIX	428	95			0.43	0.57		
22	535W2	2009	29.9294	−97.2980	MIX	357	100			0.47	0.53		
23	466E11	2009	29.4844	−97.6784	MIX	162	41			0.45	0.55		
24	108S5	2009	29.1488	−97.6023	MIX	384	64			0.38	0.62		
25	80N1	2007	29.0150	−97.8179	MIX	140	37	104	61	0.46	0.54	0.79	0.21
26	POC2104	2007	30.0814	−97.0872	DR	0	200	0	200	1	0	0	1
27	2571E1	2007	30.0260	−97.2967	DR	18	182	0	200	0.95	0.05	0	1
28	153E1	2007	30.0042	−96.9856	DR	0	200	0	200	1	0	0	1
29	535W1	2009	29.9760	−97.1927	DR	5	384			0.99	0.01		
30	20S1	2009	29.9030	−97.5678	DR	0	100			1	0		
31	713E1	2009	29.8352	−97.4106	DR	0	100			1	0		
32	442W1	2009	29.7708	−97.4229	DR	0	100			1	0		
33	304S1	2009	29.6866	−97.4177	DR	0	100			1	0		
34	80S1	2007	29.6404	−97.6613	DR	0	200	0	200	1	0	0	1
35	71FS2	2010	29.5288	−96.4412	DR	0	100			1	0		
36	466E12	2007	29.4812	−97.6557	DR	0	200	0	200	1	0	0	1
37	466E10	2009	29.4676	−97.6015	DR	0	100			1	0		
38	90AFS1	2010	29.4540	−96.8770	DR	0	100			1	0		
39	71FS1	2010	29.3647	−96.3705	DR	0	100			1	0		

$1 - q$ (Table 1). After evaluating the counts and discovering that the light-allele and the blue-allele frequencies are almost perfectly correlated ($r_{(n=14)} = 0.98$, $P < 0.0001$), we restricted our scoring in subsequent years to recording flower hue (red vs. blue) and estimating allele frequency at the hue locus. Because flower-color variation in this species is not a quantitative trait, but instead controlled by two Mendellian-like loci, we did not use Q_{ST} to measure phenotypic population differentiation; instead, we used the allele frequency estimates at the hue locus to calculate single-locus F_{ST} across all populations.

We collected leaf tissue for genotyping from 15 to 16 individuals haphazardly chosen from each of the 39 locations, which

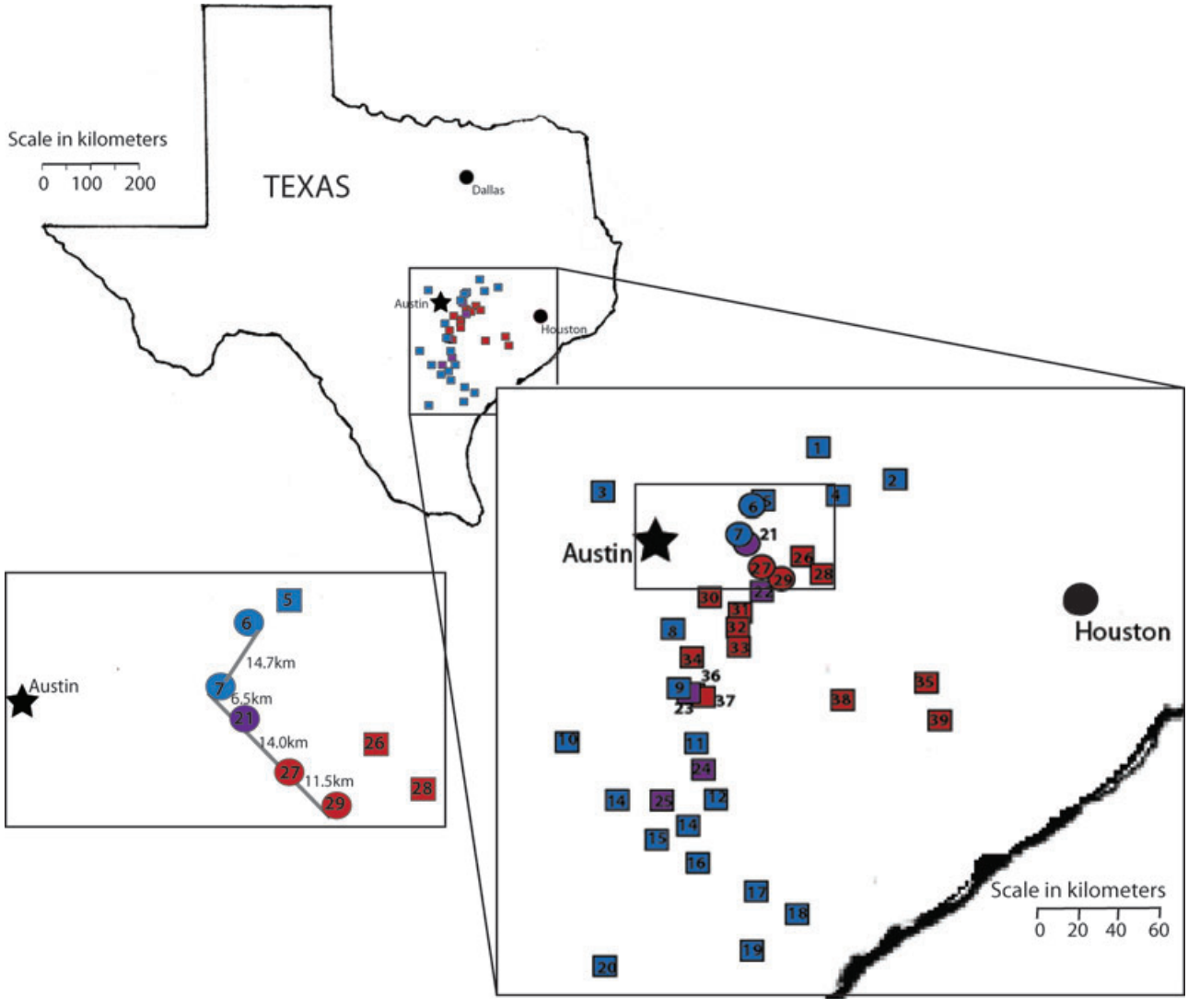

Figure 1. **Map of Texas showing locations of 39 sampled populations. Blue squares indicate populations with light-blue flower color, red squares indicate populations with dark-red flower color, and purple squares indicate mix flower-color populations. The first inset shows all populations numbered as indicated in Table 1. The second inset shows the five populations (in circles) used to assess variation at the flower-color loci and the distance between neighboring populations in kilometers.**

yielded a total of 605 samples from throughout the range of *P. drummondii*. Effort was made to distribute the sampling throughout the population and no neighboring plants were collected. From mixed populations, half the individuals we sampled were dark-red and half were light-blue.

NEUTRAL GENETIC VARIATION

The 605 individuals from 39 populations were used to evaluate population genetic structure within *P. drummondii* to determine if neutral genetic differentiation corresponded to patterns of flower-color differentiation. We extracted DNA from leaf tissue using a modified cetyltrimethyl ammonium bromide (CTAB) extraction (Hopkins and Rausher 2011). Each individual was genotyped at nine microsatellite loci (Table 2). All loci were developed using primers from Fehlberg et al. (2008) and Wu (2006). Microsatellites were amplified using the Qiagen multiplex kit (Qiagen Inc., Valencia, CA), analyzed using capillary electrophoresis and fragment analysis on an ABI 3730 × 1 DNA Analyzer, and were scored by eye using the program GENEMARKER (SoftGenetics, 2005, State College, PA). We determined whether the loci were in Hardy–Weinberg equilibrium within a population to detect loci that are likely to have high rates of allelic dropout (Morin et al. 2009) using Arlequin version 3.1 (Excoffier et al. 2005).

Genetic variation in these presumably neutral makers was analyzed in a number of ways. First, we used a hierarchical model, analysis of molecular variance (AMOVA), to explicitly test for significant genetic variation differentiating populations according to flower color. We partitioned the total variation into three

Table 2. Microsatellite loci information. Shown are the average number of alleles (Na), the observed heterozygosity (Ho), and the expected heterozygosity (He) at each locus averaged across all 39 populations. $^{+}$Loci discovered by amplifying, cloning, and sequencing marker Ipo121 (Wu 2006). $^{\#}$Loci discovered by amplifying, cloning, and sequencing marker Phl33 (Fehlberg et al. 2008).*Loci discovered by Fehlberg et al. (2008).

Name	Primers (5′–3′)	Size (bp)	Na (SD)	Ho (SD)	He (SD)
RH_PD2^{+}	CTACTGTAAGAGATGTGGCAACAACC	237–241	3.44 (0.68)	0.513 (0.19)	0.474 (0.13)
	GTTGAAAAATCCTCCCCCATTGTGG				
RH_PD3^{+}	CTACTGTAAGAGATGTGGCAACAACC	269–281	3.97 (0.81)	0.633 (0.19)	0.52 (0.10)
	GTTGAAAAATCCTCCCCCATTGTGG				
RH_PD4^{+}	CTACTGTAAGAGATGTGGCAACAACC	171–183	5.54 (1.21)	0.471 (0.17)	0.744 (0.085)
	GTTGAAAAATCCTCCCCCATTGTGG				
RH_PD7$^{\#}$	GTTCAGCCGGGAAGGGAATATAGCG	107–117	3.97 (1.27)	0.472 (0.21)	0.606 (0.15)
	CCCTTCACTCTTAACCCGATCCG				
RH_PD8$^{\#}$	GTTCAGCCGGGAAGGGAATATAGCG	118–136	4.54 (1.02)	0.402 (0.16)	0.583 (0.16)
	CCCTTCACTCTTAACCCGATCCG				
Phl68*	ACGCAACAACCAAACTCCAT	241–259	6.23 (1.61)	0.400 (0.12)	0.744 (0.08)
	GATGCAGCCACACGAGTTTA				
Phl84*	TCAGACTAGGGGAGGCAGTG	171–201	7.54 (1.52)	0.728 (0.13)	0.806 (0.074)
	TTCTTTACCGCTGGCTGAAT				
Phl113*	TGTCCACATGGGCTTGACTA	472–500	5.59 (1.79)	0.614 (0.03)	0.653 (0.02)
	ACGTACACGCCCAACTAAGG				
Phl137*	TCGGGCACCAGATTTTATTC	204–220	3.82 (0.88)	0.325 (0.17)	0.675 (0.12)
	TTCGACCCCCAGATAGTCAG				

components: (1) between color morph, (2) among populations within color, and (3) within population (Arlequin version 3.1). For this analysis, we excluded the five mixed populations and only used populations that were predominantly light-blue or dark-red. We present the result for using F_{ST}-like estimates to compute the distance matrix, but using R_{ST}-like estimates (Slatkin 1995) show the same results. Including the mixed populations as a third color category or as part of either of the fixed color categories produced similar results.

In many systems with large ranges and potentially restricted gene flow or short migration distances, there is a pattern of isolation by distance. If this pattern exists in *P. drummondii*, it may interfere with detecting an additional pattern of differentiation associated with flower color. Furthermore, lack of isolation by distance would indicate there is extensive gene flow across the range of the species. We tested for isolation by distance using a Mantel test with 9999 permutations in GenAlEx (Peakall and Smouse 2006). We used a pairwise geographic distance matrix created from the GPS coordinates using the web program *Geographic Distance Matrix Generator* (Ersts 2010) and a pairwise F_{ST} genetic distance matrix created by Arlequin version 3.1.

Finally, we used the Bayesian clustering program STRUCTURE (Pritchard et al. 2000) to probabilistically assign all 605 individuals to a varying number of clusters (k). We used an admixture model with independent allele frequencies and no population identity prior. We ran 10 replicates for each of $k = 1$–16 with a burn-in of 10,000 Markov Chain Monte Carlo (MCMC) steps followed by 50,000 iterations. We used Structure Harvester version 0.56.4 (Evanno et al. 2005) to determine that k best fit the data by calculating Δk based on L(k). Δk peaked at $k = 3$ clusters, and based on our a priori hypothesis of population subdivision according to flower colors, we further analyzed the data for $k = 2$ as well as $k = 3$. The replicates of $k = 2$ and $k = 3$ were consolidated for graphical representation using CLUMP (Jakobsson and Rosenberg 2007) and DISTRUCT (Rosenberg 2004). We examined the cluster assignments for a pattern indicating grouping by flower color. For direct comparison, we calculated the mean probability of assignment to a particular cluster by flower color for both $k = 2$ and $k = 3$. The likelihood of an individual being assigned to a given cluster was determined by combining the likelihoods from the 10 replicates using the program CLUMP (Jakobsson and Rosenberg 2007).

GENETIC VARIATION AT FLOWER-COLOR LOCI

In examining genetic variation at the flower-color loci, we focused on five populations along a 38 km transect spanning the flower-color transition (Fig. 1, inset). Along this transect, we sampled two populations with light-blue flower color, one mixed population, and two populations with dark-red flower color. We extracted DNA from leaf tissue of 10 individuals from each population using the CTAB procedure described above. For each individual, we amplified 532 base pairs of the third exon in the *R2R3-Myb*

transcription factor (intensity locus), and 666 base pairs of the first exon of *F3′5′h* (hue locus). For each individual, we amplified each gene fragment, direct sequenced both fragments in both directions, cloned each fragment, and sequenced four colonies. We amplified the products using Qiagen Taq DNA Polymerase (Qiagen Inc.) as per manufacture instructions. We ligated polymerase chain reaction products into pGEM®-T Easy Vector (Promega Co, Madison, WI) and transformed into chemically competent cells. Chromatograms from each individual were visually validated and then manually assembled into multiple sequence alignments. Heterozygous sites were confirmed by a double peak in the direct sequence chromatograms and both alleles appearing in sequences derived from direct picks of individual clones. Additional clones were sequenced if there were missing alleles as indicated by double peaks in the direct sequences and to clarify questionable base calls. Haplotypes were determined by cloned sequences. For each gene, both alleles from every individual were aligned using Sequencher version 4.8 (Gene Codes Co, Ann Arbor, MI) and analyzed using DnaSP (Librado and Rozas 2009).

SIGNATURE OF A SELECTIVE SWEEP

To determine if the derived alleles show evidence of a selective sweep, we used a variety of diversity statistics to evaluate genetic diversity at both loci within each population. All statistical calculations were performed in DnaSP (Librado and Rozas 2009). For each of the five populations, we calculated the number of segregating sites (S), the number of haplotypes (H), the average pairwise difference between sequences (K), and the Shannon–Wiener diversity index for haplotypes (I). We also evaluated the average total nucleotide diversity (π) with a Jukes–Cantor correction (Jukes and Cantor 1969) and the nucleotide diversity for synonomous (π_S) and nonsynonomous sites (π_A). Finally, we calculated Tajima's D and Fu and Li's F-statistic to evaluate if the sequence evolution was significantly different from the expectation under neutral processes. Tajima's D can detect positive selection through deviations in the estimates of θ by the number of segregating sites and average number of nucleotide differences (Tajima 1989). Confidence limits were calculated assuming the statistic follows a beta distribution (Tajima 1989). The F-test detects positive selection through examining variation in number of external-branch versus internal-branch mutations (Fu and Li 1993). In addition to calculating each of these summary statistics within each of the populations, we also grouped the two dark-red populations and the two light-blue populations to determine if patterns were consistent and that one anomalous population did not skew the interpretation of our results.

We used two different tests to compare the levels of diversity at each locus within populations with different flower colors and looked for a pattern of lower diversity in the dark-red populations. First, we focused on two measures of diversity: π, the mean pairwise difference between sequences, estimated with the Jukes–Cantor correction, and I, the Shannon–Wiener diversity index for haplotypes. The Shannon–Wiener diversity index is traditionally used to evaluate species diversity in and between populations (Shannon 1948; Hutcheson 1970) but is also useful for evaluating genetic diversity in and between populations (Lewontin 1972). The value of this index is that it reflects both the amount of genetic variation and the evenness of the variation. We used the Jackknife procedure (Cohen 1969; Gray and Schuncany 1972) to test the hypothesis that the parameter average for the two red populations differed from the average for the two blue populations. Because means calculated by the Jackknife procedure are asymptotically normal, the ratio of the mean to the standard deviation is approximately t-distributed and can be used to test the hypothesis that the parameter (or difference in parameter averages) is equal to zero (Gray and Schuncany 1972). The Jackknife procedure also generally produces less-biased estimates of parameter values (Gray and Schuncany 1972).

Second, we used a permutation test to evaluate if point estimates of π with a Jukes–Cantor correction and I, the Shannon–Wiener diversity index, differed from a null expectation of a single panmictic population. We randomly chose 20 sequences from the pool of all 100 possible sequences (ignoring sampling location identity), and calculated both π with a Jukes–Cantor correction and I. We performed this random sampling 10,000 times and created a distribution of diversity values expected if our sequence sampling was taken from one panmictic population including all 100 sequenced alleles. We calculated two-tailed 95% confidence intervals to determine significant deviations from random and plotted the five population estimates of diversity on the distribution of diversity estimates.

SIGNATURE OF DIVERGENCE

To evaluate if populations with different flower colors show more divergence at *F3′5′h* and *Myb* than populations with the same color, we first calculated two statistics that estimate divergence between two populations: D_{xy} and D_a. The first of these is the average pairwise number of nucleotide differences per site between populations, and the latter corrects the former by subtracting the average genetic diversity within populations, that is, $D_a = D_{xy} - \pi$. We also calculated summary statistics that evaluate the amount of genetic variation between populations relative to the amount within populations. Specifically, we calculated F_{ST} based on average sequence diversity and G_{ST} based on average haplotype diversity. Higher F_{ST} and G_{ST} values between divergent populations than between populations with the same flower color would constitute evidence of accumulated divergence at the selected loci. One potential confounding factor in these analyses is that populations with different flower colors are more geographically distant from each other than populations with the same

flower color. To determine whether this difference confounds our results, we tested for a relationship between genetic differentiation (as measured by F_{ST} and G_{ST}) and geographic distance (in kilometers) using a Mantel test. F_{ST} and G_{ST} were calculated in DnaSP and the Mantel test was performed in GenAlEx.

Results

PHENOTYPIC VARIATION

Phenotypic surveys indicated that most populations were either fixed or nearly fixed for the light and blue alleles or fixed or nearly fixed for the dark and red alleles (Table 1). Using allele frequency estimates at the hue locus, we calculated F_{ST} for flower color as 0.80 indicating high levels of between-population differentiation and low levels of within-population variation. As reported by Levin (1985), dark-red populations generally occur east of light-blue populations (Fig. 1). Moreover, this transition occurs over a very short distance (8 km or less), as revealed, for example, by comparing populations 7, 21, and 27 and populations 9, 23, 36, and 37 (Fig. 1).

NEUTRAL GENETIC VARIATION

If selection is responsible for flower-color divergence in the face of gene flow, we would expect two patterns: (1) F_{ST} for flower color should be much greater than F_{ST} for neutral markers; and (2) populations with different flower color should exhibit little divergence at neutral markers. Our analysis of neutral variation confirms these expectations. Neutral genetic variation in *P. drummondii*, as estimated from 605 individuals across 39 sampling locations, shows high levels of admixture among populations and little differentiation corresponding to flower-color variation. The nine microsatellite loci were in Hardy–Weinberg equilibrium at most locations; therefore, we assumed low levels of allelic dropout. Microsatellite F_{ST} between populations averaged 0.115 (R_{ST} = 0.17), only 14% that of flower-color F_{ST}, confirming expectation (1). The elevated F_{ST} for flower color is consistent with the sharp differentiation in the frequency of the red allele at the hue (*F3′5′h*) locus across the sampled populations, which is not seen in the most frequent allele at each microsatellite locus (Fig. 2A).

Explicitly testing for differentiation at microsatellite molecular markers among populations of different flower color using an AMOVA reveals no variation explained by flower-color group, confirming expectation (2). Only 11% of the genetic variation is explained by among-population within-color group differences, and the remaining variation (89%) is explained by within-population variation (Table 3).

This pattern is confirmed by the STRUCTURE analysis, which shows low levels of microsatellite differentiation between populations and little differentiation between populations of different flower color (Fig. 3). The optimum number of clusters (k), using Evanno et al. (2005) method of calculating Δk, is 3, but we analyzed both $k = 3$ and $k = 2$ further. For $k = 2$, most

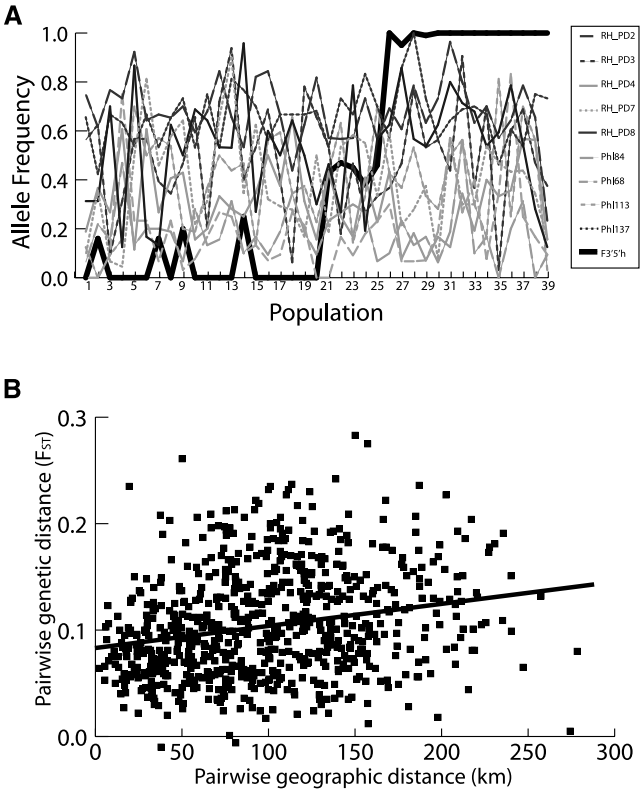

Figure 2. **(A) Variation in allele frequencies across the 39 populations. Red line shows estimated frequency of the red allele at the flower hue locus (*F3′5′h*) and is therefore indicative of population flower color. The gray lines show frequencies of the most common allele at each of the nine microsatellite loci. The sharp differentiation in flower color and red-allele frequency is not evident in the microsatellite alleles. (B) Population genetic differentiations show weak isolation by distance (R_{xy} = 0.23, P = 0.007). Pairwise genetic distance (as measured by F_{ST}) is regressed onto pairwise geographic distance.**

Table 3. **Analysis of molecular variation (AMOVA) results indicating no variation explained by between color differences.**

Source	DF	Sum of squares	Variance component	Percentage of variation
Between color	1	16.35	0.007	0.26
Among population within color	32	400.85	0.321	11.26
Within populations	1026	3004.62	2.522	88.48

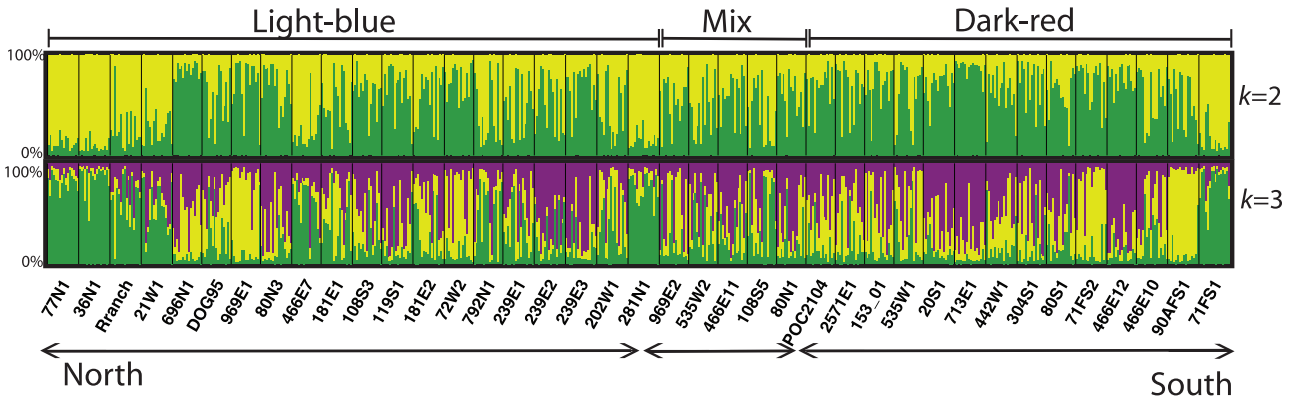

Figure 3. Structure results for $k = 2$ and $k = 3$ show no pattern of differentiation by flower color or by north-south geographic location. The small black lines separate populations, named at the bottom of the graph. Each individual is a small bar color coded according to probability of clustering in a particular group. Populations are ordered north to south within each flower-color category.

individuals from most locations are not assigned to a single cluster. Moreover, light-blue populations do not differ substantially from dark-red populations in average likelihood of individuals being assigned to the yellow cluster (0.49 ± 0.31 vs. 0.64 ± 0.26 for light-blue and dark-red populations, respectively). Consistent with this interpretation, in 28 of 34 nonmixed populations, individuals have a greater than 20% average likelihood of being in both clusters. Of the remaining six populations, four have an average likelihood of individuals being included in the yellow cluster of less than 20%. Three of these are light-blue populations and one is dark-red. The final two populations have an average likelihood greater than 80% of being in the yellow cluster. One of these is light-blue and one dark-red. These extreme populations are thus represented by both light-blue and dark-red populations and thus do not provide evidence of microsatellite differentiation between the two types of populations.

The analysis for $k = 3$ also exhibits little evidence of differentiation among-population color types (Fig. 3). On average an individual from a light-blue population has a nearly equal probability of being in any of the three clusters ($M_{\text{green}} = 0.38 \pm 0.31$, $M_{\text{yellow}} = 0.28 \pm 0.26$, $M_{\text{purple}} = 0.34 \pm 0.28$), and the same is true for individuals from dark-red populations ($M_{\text{green}} = 0.20 \pm 0.24$, $M_{\text{yellow}} = 0.39 \pm 0.29$, $M_{\text{purple}} = 0.40 \pm 0.30$).

These differences between patterns of variation in flower color and in microsatellites thus indicate that flower-color divergence is likely the result of divergent selection and not genetic drift in isolated portions of the species range. Additional evidence for gene flow between red and blue populations is that there is little isolation by distance in *P. drummondii*. Although a MANTEL test indicates a significant correlation between genetic distance and geographic distance ($R = 0.2$, $P = 0.007$), this correlation is weak and explains only 4% of the between-population differences in microsatellite allele frequencies (Fig. 2B and Table S1).

GENETIC VARIATION AT FLOWER COLOR LOCI—TESTS FOR SELECTIVE SWEEPS

We focused our analysis of genetic diversity at the flower color loci on five populations forming a transect across the cline in flower color (Fig. 1, smallest inset). Genetic diversity at both flower-color loci within all five populations along the transect is moderately high (Table 4). In the first exon of the hue locus, *F3′5′h*, each population contains 24–31 segregating sites and has nucleotide diversity (π) values ranging from 0.00764 to 0.0158. A signature of a selective sweep would correspond to reduced levels of variation at the derived red allele compared to the ancestral blue allele. The two dark-red populations do have less genetic diversity than the two light-blue populations. The pattern is exhibited by all measures of diversity including number of segregating sites (K), number of haplotypes (h), haplotype diversity (I), and π.

This hypothesis was further examined by applying the Jackknife procedure to the difference in average parameter values for the two types of populations. Specifically, we formed the composite parameter (average of parameter for the two red populations) − (average of parameter for the two blue populations) and tested whether this difference equaled zero for π and I. For both parameters, average diversity was significantly lower in the dark-red populations ($t_{19} = 4.39$, $P < 0.001$ for π; and $t_{19} = 4.02$, $P < 0.001$ for I).

We also used a permutation test to determine if estimates of π and I in the red populations were significantly lower than would be expected by chance from sampling across all populations. The test indicated that for both measures of diversity, dark-red populations had significantly lower diversity than expected by chance in a panmictic population (Fig. 4A,C). This pattern of lowered diversity is consistent with a past selective sweep at this locus.

A pattern of lower genetic diversity is also consistent with a population bottleneck or founder event. If these two red

Table 4. Genetic diversity at flower-color loci *F3′5′h* (A) and *R2-R3 Myb* (B), for each of the five populations in the transect. No value of Tajima's D was significantly different from neutral as assessed by a two-tailed test assuming a beta distribution (Tajima 1989). No value of Fu and Li's *F*-statistic was significantly different from neutral as compared to the critical values obtained by Fu and Li (1993). *N* = sample size; *S* = number of segregating sites; *H* = number of haplotypes; *I* = Shannon–Wiener index of haplotype diversity; *K* = average number of nucleotide differences; *Pi* = pairwise nucleotide diversity with Jukes–Cantor correction; *Pi*(S) = pairwise nucleotide diversity of synonymous sites; *Pi*(A) = pairwise nucleotide diversity of nonsynonymous sites.

(A)

Population	*N*	*S*	*H*	*I*	*K*	*Pi* JC (SD)	*Pi*(S)	*Pi*(A)	Tajima's D	Fu and Li *F*-statistic
Red (29)	20	24	10	2.08	7.42	0.01126 ± 0.0011	0.03254	0.00466	0.37725	0.5246
Red (27)	20	26	13	2.39	5.05	0.0076 ± 0.0014	0.01973	0.00386	−1.40391	−1.5693
Mix (21)	20	35	13	2.29	10.43	0.0158 ± 0.0014	0.04453	0.00703	−0.10173	−0.36923
Blue (7)	20	31	19	2.93	9.07	0.01376 ± 0.0011	0.04039	0.00554	−0.08729	0.21646
Blue (6)	20	31	16	2.59	9.98	0.01519 ± 0.0015	0.03713	0.00835	0.42307	0.36103
Average red	20	25	11.5	2.24	7.03	0.01057 ± 0.0009	0.03068	0.00445	−0.8843	−0.71622
Average Blue	20	31	17.5	2.76	10.51	0.01598 ± 0.0007	0.04221	0.00788	−0.2218	−0.1642

(B)

Population	*N*	*S*	*H*	*I*	*K*	*Pi* JC (SD)	*Pi*(S)	*Pi*(A)	Tajima's D	Fu and Li *F*-statistic
Red (29)	20	10	5	1.05	2.75	0.00525 ± 0.00117	0.01576	0.0025	−0.07543	−0.43038
Red (27)	20	19	17	2.79	5.91	0.01127 ± 0.00089	0.02433	0.00785	0.30192	0.73747
Mix (21)	20	20	13	2.44	4.89	0.00933 ± 0.00131	0.01933	0.00672	−0.50057	−0.62325
Blue (7)	20	18	14	2.62	4.53	0.00864 ± 0.00097	0.01961	0.00575	−0.39805	0.1861
Blue (6)	20	10	8	1.87	2.64	0.00502 ± 0.00069	0.01332	0.00281	−0.21908	−0.87054
Average red	20	14.5	11	1.92	4.82	0.00826 ± 0.00058	0.02304	0.00555	−0.22642	0.23702
Average blue	20	14	11	2.25	4.06	0.00683 ± 0.00034	0.01687	0.00531	−0.045048	0.04493

populations were recently colonized, we would expect genetic diversity at all loci to be lower, similar to that found at *F3′5′h*. To evaluate this possibility, we investigated the levels of within-population genetic diversity at the nine microsatellite loci described above. We calculated the Fixation index (F_{IS}) and the Shannon–Wiener diversity index (*I*) (Table S2) within each of the 39 populations described above and then asked whether the two dark-red populations used in the transect (population 27 and 29) had lower estimates of genetic diversity at the microsatellite loci than the other populations. We found all five focal populations fell within one standard deviation of the mean for both F_{IS} ($M = 0.12 \pm 0.10$) and *I* ($M = 1.17 \pm 0.13$). Furthermore, we found no evidence of reduced diversity in the two dark-red populations ($M_{FIS} = 0.15 \pm 0.003$, $M_I = 1.23 \pm 0.10$), compared to the light-blue populations ($M_{FIS} = 0.11 \pm 0.03$, $M_I = 1.20 \pm 0.12$). These results are inconsistent with the hypothesis that these two dark-red populations underwent a recent founder event or bottleneck, but are consistent with genetic variation around the hue locus (*F3′5′h)* undergoing a selective sweep.

Despite the lower overall genetic diversity at *F3′5′h* in red populations, tests of neutrality do not show significant signatures of positive selection in these populations. Tajima's D and Fu and Li *F*-statistics both indicate no significant deviation from neutral evolution across all populations (Table 4A). These tests are known to be conservative and may be more sensitive than previously discussed tests to recombination degrading signatures of selective sweeps and small sample sizes (Simonsen et al. 1995).

Unlike the hue locus, the third exon of the intensity locus, the *R2R3-Myb* transcription factor, shows no signature of a selective sweep (Table 4B, Fig. 4B,D). This region contains 10–19 segregating sites per population with average nucleotide diversity within population (π) ranging from 0.00502 to 0.01127. Although there is variation among populations in the amount of diversity at the *R2R3-Myb* locus, we do not find that the dark-red populations are less diverse than the light-blue populations. In fact, one dark-red population (2571E1) is the most diverse and the other is the least diverse (535W1). The Jackknife tests for differences between light-blue and dark-red populations were not significant for π or *I* ($t_{19} = -0.39$, $t_{14} = -0.53$, $P > 0.5, 0.1$, respectively). The permutation test for values of π and *I* show one light-blue and one dark-red population with lower diversity than expected by chance in a panmictic population and one dark-red population with higher diversity than expected by chance. As with *F3′5′h*, Tajima's D and Fu and Li's *F*-statistics do not indicate positive selection at this locus.

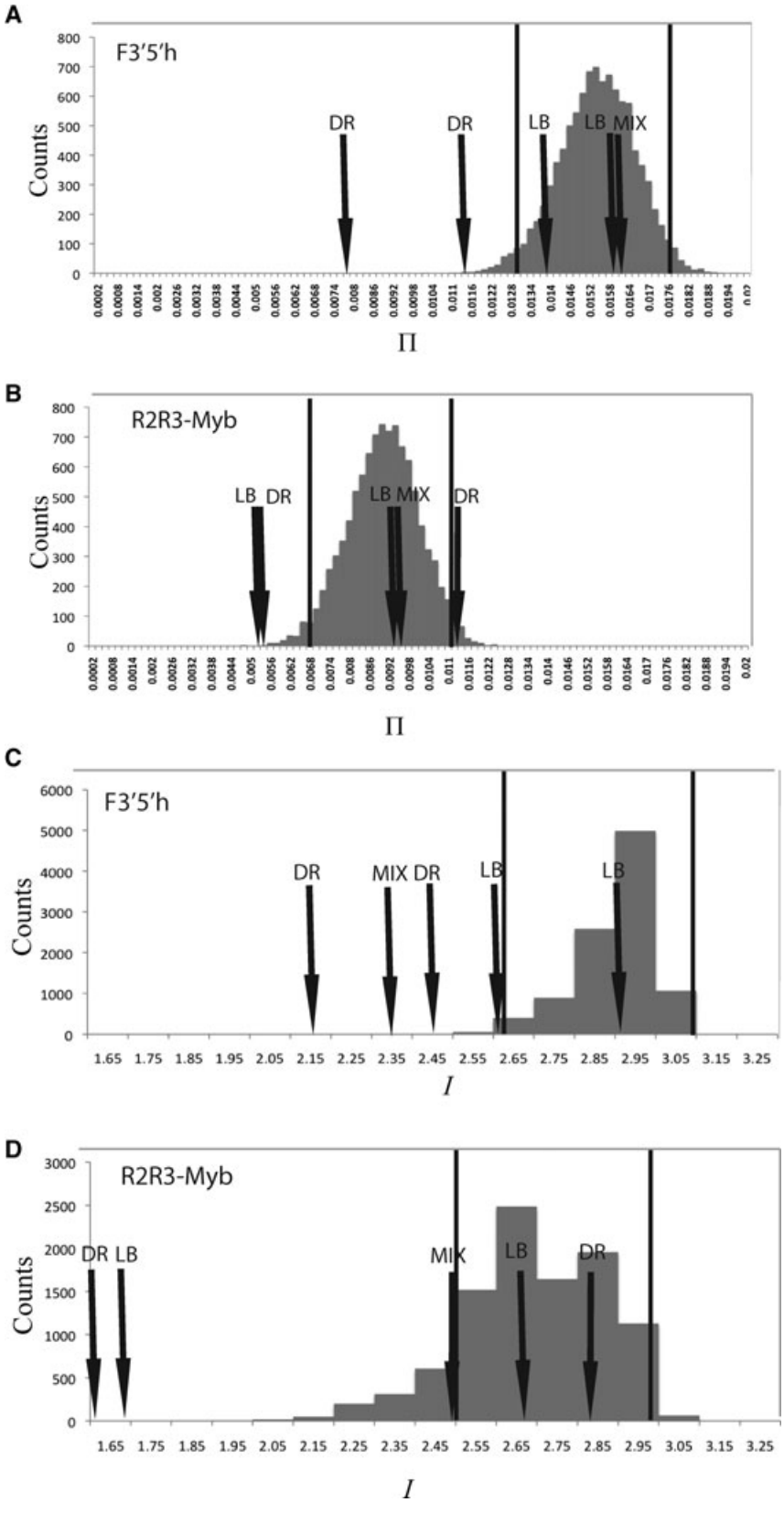

Figure 4. Permutation test for average pairwise distance (π) within each population for the hue locus, F3′5′h (A) and the intensity locus, R2R3-Myb (B). Permutation test for average haplotype diversity (*I*) within each population for *F3′5′h* (C) and *R2-R3 Myb* (D). In gray is the distribution of values for 20 alleles randomly sampled without replacement from the all 100 sequenced alleles. The arrows indicate the actual values for each of the sampled populations, with the flower color indicated above. The area within the black bars represents 95% of the permuted values.

Table 5. Pairwise genetic distance between each population in the transect at *F3′5′h* (A) and *R2-R3 Myb* (B). Above the diagonal are the average pairwise nucleotide differences between populations (D_{xy}) and below the diagonal are the net pairwise nucleotide differences between populations (D_a). Pairwise genetic divergence between populations in the transects at *F3′5′h* (C) and *R2-R3 Myb* (D). Above the diagonal are G_{ST} estimates calculated from haplotype diversity and below the diagonal are F_{ST} estimates calculated from nucleotide diversity. Bold accents distances between populations with different flower colors.

(A)					
F3′5′h	Red (29)	Red (27)	Mix (21)	Blue (7)	Blue (6)
Red (29)		0.03465	0.03542	**0.02288**	**0.04683**
Red (27)	0.19981		0.03121	**0.01877**	**0.04252**
Mix (21)	0.25093	0.30968		0.02564	0.03825
Blue (7)	**0.10204**	**0.1206**	0.07926		0.01469
Blue (6)	**0.24628**	**0.28784**	0.19184	0.16718	
(B)					
R2R3-Myb	Red (29)	Red (27)	Mix (21)	Blue (7)	Blue (6)
Red (29)		0.12927	0.14114	**0.13433**	**0.09907**
Red (27)	0.1799		0.0149	**0.01198**	**0.03197**
Mix (21)	0.09221	0.05217		0.019	0.048
Blue (7)	**0.18894**	**0.10903**	0.03224		0.04252
Blue (6)	**0.22857**	**0.17921**	0.20915	0.19868	
(C)					
F3′5′h	Red (29)	Red (27)	Mix (21)	Blue (7)	Blue (6)
Red (29)		0.01182	0.01802	**0.0138**	**0.01768**
Red (27)	0.00234		0.01652	**0.01206**	**0.01655**
Mix (21)	0.00438	0.0047		0.01718	0.01924
Blue (7)	**0.00141**	**0.00145**	0.00126		0.01171
Blue (6)	**0.00428**	**0.00507**	0.00364	0.00287	
(D)					
R2R3-Myb	Red (29)	Red (27)	Mix (21)	Blue (7)	Blue (6)
Red (29)		0.00999	0.00797	**0.00854**	**0.00662**
Red (27)	0.0018		0.01078	**0.01102**	**0.00985**
Mix (21)	0.00073	0.00056		0.00906	0.00901
Blue (7)	**0.00165**	**0.00115**	0.00015		0.00853
Blue (6)	**0.00151**	**0.00176**	0.00188	0.00174	

GENETIC VARIATION AT FLOWER-COLOR LOCI—TESTS FOR DIVERGENCE

When divergent selection maintains different alleles across populations, independent accumulation of neutral substitutions can lead to elevated divergence between different allele classes relative to between similar allele classes (Charlesworth et al. 1997; Storz and Kelly 2008; Feder and Nosil 2010). If this type of accumulation has occurred at the flower-color loci, we would expect to see greater divergence between populations with different flower colors than between populations with the same flower colors. Instead, we found that measures of divergence do not show greater differentiation between populations with different color alleles (Table 5). For *F3′5′h*, D_{xy} averaged 0.015 (±0.003) between populations with different flower colors and 0.012 (±0.0001) between populations with the same flower color (Table 5A). The average D_a value was 0.003 (±0.0004) for both comparisons. Similarly, neither F_{ST} nor G_{ST} was higher for the different-color comparison (F_{ST}: 0.189 ± 0.092 vs. 0.18300 ± 0.023 and G_{ST}: 0.033 ± 0.014 vs. 0.025 ± 0.014 for between populations with different and similar alleles, respectively) (Table 5B). Finally, there was no significant relationship between either F_{ST} or G_{ST} and geographic distance (Mantel test: $R_{xy} = 0.218$, $P = 0.265$, and $R_{xy} = 0.466$, $P = 0.072$, respectively).

We found similar patterns for the *R2R3-Myb* (Table 5C,D). D_{xy} and D_a show little difference between populations with different and similar flower colors (D_{xy}: 0.009 ± 0.002 vs.

0.009 ± 0.0001; D_a: 0.0017 ± 0.0001 vs. 0.0015 ± 0.0002 for populations with different vs. similar flower colors, respectively). Furthermore, F_{ST} and G_{ST} are actually lower for populations with different flower colors than for populations with the same flower color (F_{ST}: 0.176 ± 0.05 vs. 0.189 ± 0.013 and G_{ST} 0.069 ± 0.057 vs. 0.085 ± 0.061), although not significantly so. Again, there was no significant relationship between geographic distance and F_{ST} or G_{ST} ($R_{xy} = 0.549$, $P = 0.056$ and $R_{xy} = 0.40$, $P = 0.18$).

Discussion

Across the range of *P. drummondii*, populations show a sharp transition in flower color. Western populations contain solely, or almost solely, light-blue-flowered individuals. The boundary between these populations and those with solely dark-red-flowered individuals in the eastern portion of the species' range is separated by a cline spanning as little as 8 km (Fig. 1). Moreover, this boundary corresponds to the western limit of the distribution of the congener *P. cuspidata* (Levin 1985; Turner et al. 2003). This pattern of phenotypic differentiation in *P. drummondii* appears to reflect character displacement: sympatric populations of *P. drummondii* differ in flower color from *P. cuspidata*, whereas allopatric populations do not. As with most patterns of character displacement, divergence between sympatric and allopatric populations has been interpreted as being caused by selection. Specifically, it has been argued that this divergence is caused by reinforcing selection to reduce maladaptive hybridization between the two species (Levin 1985). However, direct measures of selection causing the divergence are lacking.

Here, we test the alternative hypothesis that restricted gene flow and genetic drift caused this pattern of flower-color divergence. We find that neutral genetic variation shows no pattern of differentiation corresponding to flower-color divergence. Instead, our data show a pattern consistent with selection maintaining locally adapted phenotypes in the face of gene flow between populations. Also consistent with this interpretation is the finding that one of the loci contributing to the evolution of dark-red flowers in sympatry shows reduced variation potentially caused by a recent selective sweep.

SELECTION FOR FLOWER-COLOR DIVERGENCE

The shift in flower color across the range of *P. drummondii* occurs over a very small geographic scale with populations fixed for different colors growing as close as 8 km apart. Analyses of variation at presumed neutral genetic markers within *P. drummondii* show low levels of differentiation between populations and no pattern of genetic differentiation between regions with different flower colors. Furthermore, despite the 300 km north-south span and 190 km east-west span of our sampling, we see a low correlation between genetic distance and geographic distance. This result indicates low isolation by distance and implies moderate gene flow between even geographically distant populations. Although we have not explicitly tested the relative effects of time versus gene flow on neutral genetic structure, the weak isolation by distance makes it unlikely we will find restricted gene flow between sympatric and allopatric populations. These results lead us to conclude that the pattern of neutral genetic differentiation is markedly different from the pattern of flower-color variation and thus support the hypothesis that selection was responsible for the transition to dark-red flowers in eastern populations of *P. drummondii*.

The steepness of the flower-color transition in *P. drummondii* is similar to other intraspecific phenotypic clines that have been maintained by selection despite high levels of gene flow. For example, there is a 10–20 km wide cline in flower color in the bush Mimulus (Streisfeld and Kohn 2005), and steep clines in coat color in both the rock pocket mouse (Hoekstra et al. 2004) and the oldfield mouse (Mullen and Hoekstra 2008). As with many similar clines that have been studied (McKay et al. 2001; Storz 2002; Storz and Dubach 2004; Hall et al. 2007; Antoniazza et al. 2010; Santure et al. 2010), these three clines have been hypothesized to be due to local adaptation to variation in biotic or abiotic environmental differences, specifically, to optimize pollination or predator crypsis, respectively. There has also been extensive research investigating clines resulting from hybridization between species (Barton and Hewitt 1985), but this work focuses on variation between species rather than within species (e.g., Marchant et al. 1988; Sperling and Spence 1991; Bert and Arnold 1995; Bridle et al. 2001; Ruegg 2008; Ouanes et al. 2011). Despite the abundance of examples of RCD and ECD in animals and plants, there have been only limited of investigations of population structure in these species (Marko 2005; Lehtonen et al. 2009; Rice and Pfennig 2010). Thus, this study represents one of the first comparisons of heritable phenotypic variation involved in character displacement and neutral population genetic variation.

IMPLICATIONS FOR REINFORCEMENT AND SPECIATION

Although the agents causing divergent selection on flower color in *P. drummondii* have yet to be definitively explicated, some experimental evidence suggests that selection favoring dark-red flowers in eastern populations reflects reinforcement to reduce interspecific hybridization with *P. cuspidata*. The process of reinforcement has been hypothesized not only to complete the process of speciation between two incipient species but also, in some cases, to initiate the process of speciation by causing the evolution of prezygotic isolation between populations of the species undergoing reinforcement (Howard 1993; Hoskin et al. 2005; Higgie and Blows 2007; Lemmon 2009; Rice and Pfennig 2010). When

reinforcement causes RCD, there is divergent selection within a species for different mating signals or mating preferences in different parts of the range (i.e., allopatry vs. sympatry). Although this divergence in mating traits can result in further isolation with sympatric heterospecifics, it can also decrease gene flow between the sympatric and allopatric populations of conspecifics (Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009). As has been documented in a number of systems, diverged mating traits can cause prezygotic reproductive isolation directly (through mate choice and assortative mating) and potentially postzygotic isolation (through hybrid disadvantage in mate or resource acquisition) between populations of the same species (Hoskin et al. 2005; Svensson et al. 2006; Higgie and Blows 2007; Pfennig and Ryan 2007; Lemmon 2009).

One sign of the development of this type of reproductive isolation between conspecific populations is divergence between populations at neutral loci (Rice and Pfennig 2010). However, we found no detectable divergence at presumed neutral loci between *P. drummondii* populations with different flower colors. Thus, at this point, there is no evidence that reinforcement of isolation between *P. drummondii* and *P. cuspidata* has contributed to the development of isolation between eastern and western populations of *P. drummondii*, although further investigation could indicate otherwise.

SELECTIVE SWEEPS

Selective sweeps generate a pattern of reduced variability at sites closely linked to the selected locus (Charlesworth et al. 1997, 2003; Nielsen 2005). This pattern is expected to decay over time as mutations accumulate and restore variability to presweep levels (Przeworski 2002). Although a signature of a selective sweep may indicate selection acting near a particular gene, these tests cannot rule out the possibility that a genetically linked locus is influencing the allelic distribution at the flower-color loci. Nevertheless, we detected a signature of a selective sweep at one of the two loci we examined. Specifically, at the hue locus, the derived, recessive, "red" allele of *F3′5′h* from eastern populations shows significantly less diversity than the ancestral, dominant "blue" allele from western populations. One alternative explanation for this pattern is that the eastern populations went through a recent bottleneck. If this explanation were true, we would expect other loci to exhibit a similar reduction in variability. Because we detected no evidence for lower diversity at other loci (microsatellites), we can rule out this alternative explanation.

By contrast, we found no evidence of decreased variation in the derived allele at the intensity locus (*R2R3-Myb)*. One explanation for this result is that there was a selective sweep, but that its signal has been degraded over time by recombination between the functional changes in the *cis*-regulatory region and the coding region. If the genetic distance between the functional changes and the coding region is similar for the two genes, or smaller in *Myb*, then the absence of a signature of a sweep in the *Myb* gene would suggest that its sweep occurred before that of *F3′5′h*. Alternatively, if this distance is greater in the *Myb* gene, it may have occurred later. Future identification of the site of the functional changes in these genes may allow us to infer whether recombination rates differ and thus, which of these explanations is more likely.

The second explanation for the absence of a signature of a selective sweep in the *Myb* gene is that fixation of the dark allele involved a "soft-sweep" (Hermisson and Pennings 2005; Pritchard et al. 2010). In this scenario, the causal mutation(s) existed in *P. drummondii* prior to the two species coming into secondary contact but was maintained at low frequencies due to slightly deleterious fitness consequences. By the time, the two *Phlox* ranges became sympatric—causing dark-red flower color to be advantageous—the adaptive mutation may have recombined onto many different genetic backgrounds, thereby forming multiple haplotypes associated with the causal adaptive mutations. Fixation would then drag with it most of the variation initially present around the *R2R3-Myb*, resulting in little reduction of variation.

LACK OF DIVERGENCE AT CAUSAL LOCI

Spatially varying selection can produce a peak of nucleotide diversity at a selected locus due to high linkage disequilibrium among neutral polymorphisms accumulated near selected mutations (Charlesworth et al. 1997; Storz and Kelly 2008; Feder and Nosil 2010). The magnitude of this effect will be proportional to how long divergent selection has operated and to how strong selection is, but inversely proportional to the rate of recombination between the selected site and the scored sites. An expected signature of this effect is greater divergence between alleles of different types than between alleles of the same type. We found no evidence for this effect at either *F3′5′h* or *Myb*. One explanation for this lack of differentiation is that there has been insufficient time because the evolution of red flowers for divergence to accumulate. Another possibility is that there has been substantial recombination between the coding regions of the genes, which we examined here and the *cis*-regulatory elements in which the causal changes have occurred. At this point, we have no evidence that would allow us to differentiate between these two possibilities.

Conclusion

Flower-color variation in *P. drummondii* has long been hypothesized to be an example of RCD caused by reinforcing selection on species boundaries. Here, we provide the first evidence for selection causing this pattern of phenotypic variation and rule out the alternative hypothesis that variation in flower color is caused

by neutral drift and restricted gene flow. This conclusion is supported by the much greater differentiation of flower-color alleles than is exhibited by neutral markers. It is also supported by evidence consistent with a selective sweep of the "red" allele at one of the causal loci. However, examination of various potential signatures of selection failed to reveal evidence for a sweep at the other causal locus, even though a comparison with neutral loci indicates selection did occur. This difference illustrates a general problem with relying on "signatures of selection" to detect selection on a specific gene: a signature is evidence for selection but a lack of signature is not evidence for lack of selection. In the absence of a signature of selection, other approaches, such as direct measurement of selection under field conditions, are warranted.

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LITERATURE CITED

Antoniazza, S., R. Burri, L. Fumagalli, J. Goudet, and A. Roulin. 2010. Local adaptation maintains clinal variation in melanin-based coloration of European barn owls (*Tyto alba*). Evolution 64:1944–1954.

Barton, N. H., and G. M. Hewitt. 1985. Analysis of hybrid zones. Annu. Rev. Ecol. Syst. 16:113–148.

Bert, T. M., and W. S. Arnold. 1995. An emperical test of predictions of 2 competing models for the maintenance and fate of hybrid zones: both models are supported in a hard-clam hybrid zone. Evolution 49:276–289.

Bridle, J. R., S. J. E. Baird, and R. K. Butlin. 2001. Spatial structure and habitat variation in a grasshopper hybrid zone. Evolution 55:1832–1843.

Brown, W. L., and E. O. Wilson. 1956. Character displacement. Syst. Zool. 5:49–64.

Butlin, R. 1987. Speciation by reinforcement. Trends Ecol. Evol. 2:8–13.

Charlesworth, B., M. Nordborg, and D. Charlesworth. 1997. The effects of local selection, balanced polymorphism and background selection on equilibrium patterns of genetic diversity in subdivided populations. Genet. Res. 70:155–174.

Charlesworth, B., D. Charlesworth, and N. H. Barton. 2003. The effects of genetic and geographic structure on neutral variation. Annu. Rev. Ecol. Evol. Syst. 34:99–125.

Cohen, J. E. 1969. Natural primate troops and a stochastic population model. Am. Nat. 103:455–477.

Cooley, J. R., C. Simon, D. C. Marshall, K. Slon, and C. Ehrhardt. 2001. Allochronic speciation, secondary contact, and reproductive character displacement in periodical cicadas (Hemiptera: Magicicada spp.): genetic, morphological, and behavioural evidence. Mol. Ecol. 10:661–671.

Crispo, E., P. Bentzen, D. N. Reznick, M. T. Kinnison, and A. P. Hendry. 2006. The relative influence of natural selection and geography on gene flow in guppies. Mol. Ecol. 15:49–62.

Dayan, T., and D. Simberloff. 2005. Ecological and community-wide character displacement: the next generation. Ecol. Lett. 8:875–894.

Durrett, R., and J. Schweinsberg. 2004. Approximating selective sweeps. Theor. Popul. Biol. 66:129–138.

Edelist, C., C. Lexer, C. Dillmann, D. Sicard, and L. H. Rieseberg. 2006. Microsatellite signature of ecological selection for salt tolerance in a wild sunflower hybrid species, *Helianthus paradoxus*. Mol. Ecol. 15:4623–4634.

Ersts, P. J. 2010. Geographic distance matrix generator V 1.2.3 American museum of natural history. Center for Biodiversity and Conservation. Available at: http://biodiversityinformatics.amnh.org/open_source/gdmg/. Accessed September 12, 2010.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611–2620.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol. Bioinform. 1:47–50.

Feder, J. L., and P. Nosil. 2010. The efficacy of divergence hithiking in generating genomic islands during ecological speciation. Evolution 64:1729–1747.

Fehlberg, S. D., K. A. Ford, M. C. Ungerer, and C. J. Ferguson. 2008. Development, characterization and trasferability of microsatellite markers for the plant genus *Phlox* (Polemoniaceae). Mol. Ecol. Resources 8:116–118.

Fu, Y. X., and W. H. Li. 1993. Statistical tests of neutrality of mutations. Genetics 133:693–709.

Gockel, J., W. J. Kennington, A. Hoffmann, D. B. Goldstein, and L. Partridge. 2001. Nonclinality of molecular variation implicates selection in maintaining a morphological cline of *Drosophila melanogaster*. Genetics 158:319–323.

Grant, P. R. 1972. Convergent and divergent character displaceent. Biol. J. Linn. Soc. 4:39–68.

Grant, P. R., and B. R. Grant. 2006. Evolution of character displacement in Darwin's finches. Science 313:224–226.

Gray, H. L., and W. R. Schuncany. 1972. The generalized jackknife statistic. Marcel Dekker, Inc., New York.

Hall, D., V. Luquez, V. M. Garcia, K. R. St Onge, S. Jansson, and P. K. Ingvarsson. 2007. Adaptive population differentiation in phenology across a latitudinal gradient in European Aspen (*Populus tremula*, L.): a comparison of neutral markers, candidate genes and phenotypic traits. Evolution 61:2849–2860.

Hereford, J. 2009. A quantitative survey of local adaptation and fitness tradeoffs. Am. Nat. 173:579–588.

Hermisson, J., and P. S. Pennings. 2005. Soft sweeps: molecular population genetics of adaptation from standing genetic variation. Genetics 169:2335–2352.

Higgie, M., and M. W. Blows. 2007. Are traits that experience reinforcement also under sexual selection? Am. Nat. 170:409–420.

Higgie, M., S. Chenoweth, and M. W. Blows. 2000. Natural selection and the reinforcement of mate recognition. Science 290:519–521.

Hobel, G., and H. C. Gerhardt. 2003. Reproductive character displacement in the acoustic communication system of green tree frogs (*Hyla cinerea*). Evolution 57:894–904.

Hoeksema, J. D., and S. E. Forde. 2008. A meta-analysis of factors affecting local adaptation between interacting species. Am. Nat. 171:275–290.

Hoekstra, H. E., K. E. Drumm, and M. W. Nachman. 2004. Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. Evolution 58:1329–1341.

Hopkins, R., and M. D. Rausher. 2011. Identification of two genes causing reinforcement in the Texas wildflower *Phlox drummondii*. Nature 469:411–414.

Hoskin, C. J., M. Higgie, K. R. McDonald, and C. Moritz. 2005. Reinforcement drives rapid allopatric speciation. Nature 437:1353–1356.

Howard, D. J. 1993. Reinforcement: origin, dynamics and fate of an evolutionary hypothesis. Pp. 46–69 *in* R. G. Harrison, ed. Hybrid zones and the evolutionary process. Oxford Univ. Press, New York.

Hutcheson, K. 1970. A test for comparing diversities based on Shannon formula. J. Theor. Biol. 29:151–154.

Jakobsson, M., and N. A. Rosenberg. 2007. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23:1801–1806.

Jang, Y., and H. C. Gerhardt. 2006. Divergence in the calling songs between sympatric and allopatric populations of the southern wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae). J. Evol. Biol. 19:459–472.

Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules. Pp. 21–132 *in* H. N. Munro, ed. Mammalian protein metabolism, III. Academic Press, New York.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecol. Lett. 7:1225–1241.

Kay, K. M., and D. W. Schemske. 2008. Natural selection reinforces speciation in a radiation of neotropical rainforest plants. Evolution 62:2628–2642.

Keller, S. R., D. R. Sowell, M. Neiman, L. M. Wolfe, and D. R. Taylor. 2009. Adaptation and colonization history affect the evolution of clines in two introduced species. New Phytol. 183:678–690.

Le Corre, V. 2005. Variation at two flowering time genes within and among populations of *Arabidopsis thaliana*: comparison with markers and traits. Mol. Ecol. 14:4181–4192.

Lehtonen, P. K., T. Laaksonen, A. V. Artemyev, E. Belskii, C. Both, S. Bures, A. V. Bushuev, I. Krams, J. Moreno, M. Magi, et al. 2009. Geographic patterns of genetic differentiation and plumage colour variation are different in the pied flycatcher (*Ficedula hypoleuca*). Mol. Ecol. 18:4463–4476.

Lemmon, E. M. 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. Evolution 63:1155–1170.

Levin, D. A. 1967. Hybridization between annual species of *Phlox*—population structure. Am. J. Bot. 54:1122–1130.

———. 1975. Interspecific hybridization, heterozygosity and gene exchange in *Phlox*. Evolution 29:37–51.

———. 1985. Reproductive character displacement in *Phlox*. Evolution 39:1275–1281.

Lewontin, R. 1972. The apportionment of human diversity. Evol. Biol. 6:381–398.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Liou, L. W., and T. D. Price. 1994. Speciation by reinforcement of premating isolation. Evolution 48:1451–1459.

Losos, J. B. 2000. Ecological character displacement and the study of adaptation. Proc. Natl. Acad. Sci. USA 97:5693–5695.

Marchant, A. D., M. L. Arnold, and P. Wilkinson. 1988. Gene flow across a chomosomal tension zone. 1. Refelcts of ancient hybridization. Heredity 61:321–328.

Marko, P. B. 2005. An intraspecific comparative analysis of character divergence between sympatric species. Evolution 59:554–564.

McKay, J. K., and R. G. Latta. 2002. Adaptive population divergence: markers, QTL and traits. Trends Ecol. Evol. 17:285–291.

McKay, J. K., J. G. Bishop, J. Z. Lin, J. H. Richards, A. Sala, and T. Mitchell-Olds. 2001. Local adaptation across a climatic gradient despite small effective population size in the rare sapphire rockcress. Proc. R. Soc. Lond. B 268:1715–1721.

Morin, P. A., R. G. Leduc, F. I. Archer, K. K. Martien, R. Huebinger, J. W. Bickham, and B. L. Taylor. 2009. Significant deviations from Hardy-Weinberg equilibrium caused by low levels of microsatellite genotyping errors. Mol. Ecol. Resour. 9:498–504.

Mullen, L. M., and H. E. Hoekstra. 2008. Natural selection along an environmental gradient: a classic cline in mouse pigmentation. Evolution 62:1555–1569.

Nielsen, R. 2005. Molecular signatures of natural selection. Annu. Rev. Genet. 39:197–218.

Noor, M. A. 1995. Speciation driven by natural-selection in *Drosophila*. Nature 375:674–675.

Nosil, P., B. J. Crespi, and C. P. Sandoval. 2003. Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement. Proc. R. Soc. Lond. B 270:1911–1918.

Ortiz-Barrientos, D., A. Grealy, and P. Nosil. 2009. The genetics and ecology of reinforcement implications for the evolution of prezygotic isolation in sympatry and beyond. Year Evol. Biol. 1168:156–182.

Ouanes, K., L. Bahri-Sfar, O. K. Ben Hassine, and F. Bonhomme. 2011. Expanding hybrid zone between *Solea aegyptiaca* and *Solea senegalensis*: genetic evidence over two decades. Mol. Ecol. 20:1717–1728.

Peakall, R., and P. E. Smouse. 2006. GENALEX 6: genetic analysis in excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6:288–295.

Pfennig, D. W., and P. J. Murphy. 2000. Character displacement in polyphenic tadpoles. Evolution 54:1738–1749.

———. 2002. How fluctuating competition and phenotypic plasticity mediate species divergence. Evolution 56:1217–1228.

———. 2005. Character displacement as the "best of a bad situation": fitness trade-offs resulting from selection to minimize resource and mate competition. Evolution 59:2200–2208.

———. 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. Q. Rev. Biol. 84:253–276.

Pfennig, K. S., and M. J. Ryan. 2007. Character displacement and the evolution of mate choice: an artificial neural network approach. Philos. Trans. R. Soc. Lond. B 362:411–419.

Pfennig, K. S., and M. A. Simovich. 2002. Differential selection to avoid hybridization in two toad species. Evolution 56:1840–1848.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Pritchard, J. K., J. K. Pickrell, and G. Coop. 2010. The genetics of human adaptation: hard sweeps, soft sweeps, and polygenic adaptation. Curr. Biol. 20:R208–R215.

Przeworski, M. 2002. The signature of positive selection at randomly chosen loci. Genetics 160:1179–1189.

Raeymaekers, J. A. M., J. K. J. Van Houdt, M. H. D. Larmuseau, S. Geldof, and F. A. M. Volckaert. 2007. Divergent selection as revealed by P-ST and QTL-based F-ST in three-spined stickleback (*Gasterosteus aculeatus*) populations along a coastal-inland gradient. Mol. Ecol. 16:891–905.

Rice, A. M., and D. W. Pfennig. 2010. Does character displacement initiate speciation? Evidence of reduced gene flow between populations experiencing divergent selection. J. Evol. Biol. 23:854–865.

Rosenberg, N. A. 2004. DISTRUCT: a program for the graphical display of population structure. Mol. Ecol. Notes 4:137–138.

Ruane, L. G., and K. Donohue. 2008. Pollen competition and environmental effects on hybridization dynamics between *Phlox drummondii* and *Phlox cuspidata*. Evol. Ecol. 22:229–241.

Ruegg, K. 2008. Genetic, morphological, and ecological characterization of a hybrid zone that spans a migratory divide. Evolution 62:452–466.

Saetre, G. P., T. Moum, S. Bures, M. Kral, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature 387:589–592.

Santure, A. W., J. G. Ewen, D. Sicard, D. A. Roff, and A. P. Moller. 2010. Population structure in the barn swallow, *Hirundo rustica*: a comparison between neutral DNA markers and quantitative traits. Biol. J. Linn. Soc. 99:306–314.

Schluter, D., and J. D. McPhail. 1992. Ecological character displacement and speciation in sticklebacks. Am. Nat. 140:85–108.

Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: theory and data. Annu. Rev. Ecol. Evol. Syst. 34:339–364.

Shannon, C. E. 1948. A mathematical theory of communication. Bell Syst. Tech. J. 27:379–423.

Simonsen, K. L., G. A. Churchill, and C. F. Aquadro. 1995. Properties of statistical tests of neutrality for DNA polymorphism data. Genetics 141:413–429.

Slatkin, M. 1980. Ecological character displacement. Ecology 61:163–177.

———. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457–462.

Smadja, C., and G. Ganem. 2005. Asymmetrical reproductive character displacement in the house mouse. J. Evol. Biol. 18:1485–1493.

Sperling, F. A. H., and J. R. Spence. 1991. Structure of an asymmetric hybrid zone between 2 water strider species (Hemptera: Gerridae: Limnoporus). Evolution 45:1370–1383.

Storz, J. F. 2002. Contrasting patterns of divergence in quantitative traits and neutral DNA markers: analysis of clinal variation. Mol. Ecol. 11:2537–2551.

Storz, J. F., and J. M. Dubach. 2004. Natural selection drives altitudinal divergence at the albumin locus in deer mice, *Peromyscus maniculatus*. Evolution 58:1342–1352.

Storz, J. F., and J. K. Kelly. 2008. Effects of spatially varying selection on nucleotide diversity and linkage disequilibrium: insights from deer mouse globin genes. Genetics 180:367–379.

Streisfeld, M. A., and J. R. Kohn. 2005. Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. Evolution 59:2548–2559.

Svensson, E. I., F. Eroukhmanoff, and M. Friberg. 2006. Effects of natural and sexual selection on adaptive population divergence and premating isolation in a damselfly. Evolution 60:1242–1253.

Tajima, F. 1989. Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Turner, B. L., H. Nichols, G. Denny, and O. Doron. 2003. Atlas of the vascular plants of Texas. Botanical Research Institute of Texas, Fort Worth.

Waage, J. K. 1979. Reproductive character displacement in *Calopteryx* (Odonata, Calopterygidae). Evolution 33:104–116.

Wu, C. A. 2006. Characterization of microsatellite loci in *Ipomopsis* (Polemoniaceae) wildflowers. Mol. Ecol. Notes 6:921–923.

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Supporting Information

The following supporting information is available for this article:

Table S1. Below the diagonal is pairwise genetic distance (F_{ST}) and below the diagonal is pairwise geographic distance for each population pair. These values were used in the Mantel test to detect isolation by distance.

Table S2. Within-population diversity estimates based on microsatellite genotypes. Both Fixation index (F_{IS}) and the Shannon's Information Index (I) are listed.

Supporting Information may be found in the online version of this article.

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