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CONTRASTING PATTERNS OF FLORAL AND MOLECULAR VARIATION ACROSS A CLINE IN *MIMULUS AURANTIACUS*

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Abstract.—Steep clines in ecologically important traits may be caused by divergent natural selection. However, processes that do not necessarily invoke ongoing selection, such as secondary contact or restricted gene flow, can also cause patterns of phenotypic differentiation over short spatial scales. Distinguishing among all possible scenarios is difficult, but an attainable goal is to establish whether scenarios that imply selection need to be invoked. We compared the extent of morphological and genetic differentiation between geographically structured red and yellow floral races of *Mimulus aurantiacus* (bush monkeyflower; Phrymaceae). Flower color was assessed in a common garden as well as in the field to determine whether variation was genetic and to quantify the extent of geographical differentiation. Population genetic differentiation at marker loci was measured for both chloroplast and nuclear genomes, and the degree of population structure within and among the floral races was evaluated. Flower color shows both a strong genetic basis and a sharp geographic transition, with pure red-flowered populations in western San Diego County and pure yellow-flowered populations to the east. In the zone of contact, both pure and intermediate phenotypes occur. Patterns of genetic differentiation at marker loci are far less pronounced, as little of the variation is partitioned according to the differences in flower color. Phenotypic differentiation (Q_{ST}) between populations with different flower colors is much greater than neutral genetic differentiation (F_{ST}). When comparisons are made between populations of the same flower color, the opposite trend is evident. Limited neutral genetic structure between the floral races, combined with sharp differentiation in flower color, is consistent with the hypothesis that current or recent natural selection maintains the cline in flower color.

Key words.—Cline, flower color, gene flow, isolation by distance, *Mimulus*, selection.

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Different historical scenarios can result in similar patterns of clinal variation (Haldane 1948; Slatkin 1973; Nagylaki 1975; Endler 1977). Where clines involve ecologically important traits, differentiation may have arisen in allopatry by either selection or drift followed by secondary contact. The time in allopatry and the time since secondary contact are important factors that may affect patterns of genetic differentiation across the cline. Clines may also arise in continuously distributed populations if divergent selection is strong enough to overcome the constraining effects of gene flow or if gene flow is sufficiently restricted to allow for neutral differentiation of phenotypic traits (Endler 1977). Because multiple scenarios can result in similar patterns of clinal phenotypic variation, rarely is one able to identify the particular historical scenario under which geographic patterns of phenotypic divergence arose (Endler 1977; Barton and Hewitt 1985; Harrison 1990; Durrett et al. 2000).

One approach for understanding the factors shaping clinal variation is to attempt to determine whether a given pattern implies ongoing natural selection acting on phenotypic traits. Recent work has focused on comparing levels of differentiation between morphological traits and neutral genetic markers across ecological gradients (Thorpe et al. 1996; Smith et al. 1997; Schneider et al. 1999; Storz 2002). Several outcomes and interpretations are possible, and they depend on the degree of concordance between differentiation in neutral genetic markers and phenotypic traits. Morphological traits and neutral markers may demonstrate strong and concordant clinal divergence. This would imply either past isolation followed by recent secondary contact or that both types of traits exhibit similar patterns of isolation by distance due to restricted gene flow. Given this pattern, no inference can be drawn regarding selection on phenotypic traits (Barton and Hewitt 1985).

On the other hand, lack of structure at neutral genetic loci, coupled with strong differentiation in heritable phenotypic traits, suggests a role for current or recent natural selection under one of the following scenarios: (1) the morphological cline arose due to strong selection within a contiguous population without previous geographic isolation; (2) gene flow following secondary contact has erased any structure at neutral loci, while current selection maintains the morphological cline; or (3) a short period of allopatry coupled with strong divergent selection caused morphological differentiation, but the time in allopatry was too short for neutral divergence to arise. Therefore, by comparing patterns of geographic variation in neutral and morphological characters, we can evaluate whether selection likely plays a role in maintaining clinal phenotypic divergence (e.g. Smith et al. 1997; Schneider et al. 1999; Storz 2002).

Here we revisit a previously described hybrid zone between red- and yellow-flowered forms of *Mimulus aurantiacus* (bush monkeyflower; Phrymaceae) in southern California. These perennial subshrubs of the section *Diplacus* show extensive geographic variation in floral traits (McMinn 1951; Beeks 1962; Beardsley et al. 2004). They have been described in detail for the variation that they exhibit not only in flower color, but also in floral shape, nectar volume, and ecology (McMinn 1951; Beeks 1962; Waayers 1996; Tulig 2000). In particular, there is a steep gradient in flower color between coastal and inland regions in San Diego County, California, with coastal populations containing red flowers, and inland populations containing yellow flowers. Where the forms

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come into contact, a narrow hybrid zone occurs in which both pure and intermediate floral types are found.

Grant (1981, 1993a,b) interpreted these differences to be the result of selection on floral traits in allopatry to maximize visitation and pollen transfer by alternative pollinators. He proposed that the red form was primarily hummingbird pollinated, while the yellow form was primarily hawkmoth pollinated. Grant (1993a) viewed the presence of hybrid forms as indicative of secondary contact because he felt that it was unlikely for such extensive divergence to have arisen without the aid of geographic barriers to gene flow. Under such a scenario, neutral markers would be as highly diverged as phenotypic traits provided that contact was recently re-established following a substantial period of isolation. If neutral markers are much less diverged than are the ecologically important traits, then current or recent selection is implicated in the maintenance of clinal variation.

In this study, we measure the amount of morphological and genetic differentiation between these southern California floral races of *Mimulus aurantiacus* to determine whether there is evidence of recent secondary contact following a long period of allopatry. In addition, we attempt to distinguish whether the maintenance of clinal variation could be attributed to purely neutral processes or whether scenarios involving ongoing (or at least recent) natural selection should be invoked. We quantify flower color in field and common-garden populations to determine the extent of the geographic differentiation and to evaluate the degree to which genes are responsible for differences in flower color. Next, we genotype plants at multiple chloroplast and nuclear loci to examine how genetic variation is structured within and among populations and floral races. We then compare the extent of population variation in flower color to that for marker loci and test the degree to which floral differentiation among populations is explained by geographic versus genetic distance. If flower color is strongly heritable and more diverged between the regions than neutral genetic variation, this provides evidence consistent with a role for current or recent selection in maintaining morphological differentiation (Smith et al. 1997; Orr and Smith 1998; Schneider et al. 1999).

Materials and Methods

Although Thompson (1993) most recently described both of these floral races as part of the species complex, *Mimulus aurantiacus,* the ''red'' floral race has also been recognized as *Mimulus puniceus*, and the ''yellow'' floral race has been named *Mimulus aurantiacus* ssp. *australis* (Munz 1973). These forms are distributed throughout San Diego, Orange, and Riverside Counties in extreme southern California, but according to Beeks (1962), there is greater discontinuity in floral traits and more extreme geographical structure found among San Diego County populations than elsewhere. Therefore, we focused our sampling on populations from the San Diego series (sensu Beeks 1962), but we also sampled five populations from the more northern regions of southern California (i.e., Orange and Riverside Counties) and three other nearby subspecies from the section *Diplacus* for comparison with the San Diego group (see Table S1 available online only at http://dx.doi.org/10.1554/05-514.1.s1). Further taxonomic difficulties surround the entire section *Diplacus*, and to avoid confusion over the nomenclature of these forms, we simply refer to the San Diego series of populations as the red and yellow floral races, but refer to the other subspecies as defined by Munz (1973).

Floral Measurements

We quantified flower color in 23 natural populations across San Diego County between 2001 and 2004 using an Ocean Optics USB2000 spectrophotometer (Dunedin, FL). This device measures the proportion of light that is reflected off of an object at each wavelength between 300 and 700 nm, standardized against fully reflecting (white) and fully absorbing (black) reference standards. Using these spectra, we calculated typical measures of color, including chroma, brightness, and hue according to Endler's (1990) segmental classification method. Briefly, this method segments the spectrum into four bins of equal wavelength (A = 300–400 nm; B = 400–500 nm; C = 500–600 nm; D = 600–700 nm), and the proportion of total brightness (total proportion of light reflected between 300 and 700nm) in each of these bins is calculated. The bins used in this study are a slight modification from those used by Endler (1990), so that we could take into account the reflectance patterns in the ultraviolet portion of the spectrum (300–400 nm). The relative brightness values were used to calculate chroma = $[(D - C)^2 - (B - A)^2]^{1/2}$ and hue = $\sin^{-1}[(C - A)/\text{chroma}]$. Chroma is a measure of the purity or intensity of a color, while hue represents the typical meaning of color (i.e. red, blue, yellow, green, etc.). Two flowers per plant from 10–25 plants per population ($N = 362$ plants; mean/population = 15.7) were collected and their reflectance spectra measured. To standardize measurements across flowers and because flower color fades somewhat over the lifetime of a flower, care was taken to only measure first-day flowers. Since flowers are slightly protogynous, with the anthers dehiscing on the second day that the flower is open (Fetscher and Kohn 1999), we measured only open flowers with indehiscent anthers. In addition, we more extensively sampled four populations in the hybrid zone. We collected one flower per plant from every plant within 1 m on either side of three 75 m transects and measured standardized reflectance spectra from these flowers.

To determine whether flower color and morphology have a strong genetic basis, we quantified the degree of morphological variation between coastal and inland populations grown in a common environment and compared these measurements to data collected in the field. We created 18 full-sib families from crosses conducted in the field in the spring of 2001 within two populations (one coastal and one inland). The fruits were collected when mature, and seed was germinated in the University of California San Diego greenhouses. Seedlings were transplanted to three-gallon pots, grown outdoors in a partial shade environment, and watered every two to three days. Two first-day flowers per plant from 168 plants were collected for floral measurements. Corolla length was measured as the length from the base of the calyx to the end of the corolla tube. Corolla tube width was measured as the width across the corolla tube at the notches below the two lateral petals. These measurements were taken using

a dial caliper, to the nearest 0.1 mm. Nectar volume was measured using 5-μl capillary tubes. Flower color of common-garden plants was measured using a spectrophotometer, as described above.

We were also interested in the biochemical basis of the pigments responsible for the differences in flower color. The two most common floral pigments are the anthocyanins, which are responsible for the red, blue, and purple colors (Harborne 1976), and the carotenoids, which often produce yellow and orange colors (Goodwin and Britton 1988). We investigated whether anthocyanins were the only pigments found in red flowers or whether a combination of anthocyanins and carotenoids was present. In addition, we examined whether production of red pigment was reduced or absent in yellow flowers. To measure the concentration of the red, putative anthocyanin pigment found in lateral petal lobes, hole punches were taken from the lateral petals of flowers. Anthocyanins were extracted in methanol/0.1% HCl, and absorbance was recorded at 510 nm (Wilken 1982; Schemske and Bradshaw 1999). We were unable to develop a method for extracting yellow (putatively carotenoid) pigments, but we examined the presence of both red and yellow floral pigments in different cellular regions of the petal lobes using light microscopy for both types of flowers.

Population Genetic Structure

Tissue collection and DNA isolation

Fresh leaf tissue was collected from 10–19 individuals per population from 31 populations across southern California ($N = 527$ individuals; see Table S1 available online). Leaves were either stored on ice until they could be frozen in liquid nitrogen and stored at −80°C or were immediately desiccated in silica and stored at room temperature. Total genomic DNA was isolated using either the Qiagen (Valencia, CA) DNeasy kit or a modified version of the rapid extraction protocol described by Edwards et al. (1991). The modifications involved grinding the leaf tissue under liquid nitrogen, and instead of an isopropanol precipitation, we used 0.3M sodium acetate and 2 volumes of 100% ethanol, followed by a wash with 70% ethanol. Stock DNA was diluted 1:10 before polymerase chain reaction (PCR) analysis.

Chloroplast DNA PCR-RFLP

Initially, we PCR amplified 11 noncoding introns and intergenic spacers using universal primers from a subset of 10 individuals from the most spatially distant populations and digested the products using 13 restriction endonucleases to detect restriction-site mutations (Taberlet et al. 1991; Demesure et al. 1995; Dumolin-Lapegue et al. 1997). All PCR was carried out in 10-μl reactions using 10mM Tris pH 8.3, 50mM KCl, 0.01% gelatin, 0.01% Triton X-100, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer, 1 unit of Taq DNA polymerase, and approximately 25 ng of genomic DNA. Restriction digests were carried out in 15-μl reactions using 1 unit of enzyme, 1.5 μl of the appropriate restriction enzyme buffer, and 100 μg/ml BSA, and incubated at either 37°C or 65°C (enzyme dependent) for 1.5 hours.

From this initial screening, three primer/enzyme combinations showed restriction-site variation (see Table S2 available online only at http://dx.doi.org/10.1554/05-514.1.s1). These regions were PCR amplified from all individuals, digested using the appropriate restriction enzyme, and separated on 2% agarose gels stained with ethidium bromide. During the analysis of one of these regions (trnH-trnK spacer; Demesure et al. 1995), several length variants in addition to a restriction-site mutation were detected. We sequenced a set of these individuals and discovered a 175-bp region at the 5′ end of this spacer consisting of numerous insertion/deletion mutations (indels). We next designed internal primers that spanned this region, labeled one of the primers with the fluorescent dye HEX, separated PCR products by denaturing polyacrylamide gel electrophoresis, and visualized them using the Typhoon 9100 scanner (Amersham Biosciences, Piscataway, NJ). Each new length variant was sequenced in at least one individual. We then scored the frequency of different length variants in each of the different populations. A fourth polymorphic region was developed using sequence provided by P. Beardsley (Idaho State University, Pocatello, ID) from the chloroplast trnL intron that showed the presence of a 5-bp indel. We scored the presence versus absence of this polymorphism by designing internal PCR primers that amplify a 200-bp fragment containing the indel, and separating it based on size using denaturing polyacrylamide gel electrophoresis as before.

Codominant nuclear markers

We used PCR primers provided by J. Willis (Duke University, Durham, NC) to amplify several short (i.e., 200–600 bp) single-copy nuclear introns. We PCR amplified and sequenced 11 of these introns from 11 individuals from spatially distant regions. From these sequences, three single nucleotide polymorphisms (SNPs) were detected from three different introns. We scored these SNPs using a PCR-RFLP approach known as dCAPS (derived cleaved amplified polymorphic sequence; Neff et al. 2002). This method allowed us to genotype the SNPs using restriction digestion, even though the SNP did not introduce a restriction site. A single approximately 30-bp PCR primer was designed that contained a nucleotide mismatch to the template DNA. This mismatch occurred 1–3 nucleotides upstream of the target SNP, so the presence of the mismatch and the SNP together introduced a restriction site. The primer was cleaved from the allele that contained the restriction site and the difference in allele size was separated using a high-resolution agarose gel stained with ethidium bromide. The program dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html) was used for primer design and restriction enzyme selection. We followed this procedure for all of the SNPs from the same 31 populations used for the chloroplast markers.

We also sequenced the second intron of the floral development gene, *Leafy* (*Lfy*; Weigel et al. 1992). Degenerate PCR primers were designed from a homologous sequence of *Mimulus guttatus*. Genomic DNA was PCR amplified, and the PCR product was cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). We sequenced 10 of the clones and aligned the sequences to the *M. guttatus* cDNA and genomic DNA sequences to assess homology and determine the

position of the intron. Two different putative copies were detected, as the introns showed no sequence similarity between the copies. Internal PCR primers were designed that were intron (and therefore copy) specific. We sequenced both strands of one of these copies in 33 individuals. A single-nucleotide indel was detected in some of the sequences. Due to difficulty with PCR amplification, allele variation in this marker was scored using two rounds of PCR. We first amplified the entire intron using two external primers that spanned the intron. This PCR product was then used as a template for a second PCR that used one new internal primer that flanked the indel and contained a single nucleotide mismatch in order to introduce a restriction site when combined with the insertion. Product from the second PCR was digested with the restriction enzyme HpyCH4V that cleaved only one of the two alleles.

Amplified fragment length polymorphism

We developed amplified fragment length polymorphisms (AFLPs) following the method of Vos et al. (1995) from a subset of five red-flowered and five yellow-flowered populations from San Diego County (total $N = 150$; see Table S1 available online). We first digested genomic DNA using both EcoRI and MseI restriction enzymes, followed by immediate ligation of the linkers. The double digest was aided by the addition of 100 μg/ml BSA and 0.3 M spermidine. A preselective PCR was performed using primers that contained one additional nucleotide 3′ of the linkers, followed by a selective PCR using primers with three additional nucleotides. Two sets of selective PCR primers were used, with one primer in each case fluorescently labeled with the dye HEX. PCR products were subjected to capillary electrophoresis using an ABI 3100 sequencer, and scored for peak sizes using the GeneMapper version 3.5 software (Applied Biosystems, Foster City, CA).

Data Analysis

We employed several statistical methods for detecting population structure. For the cpDNA data, haplotype frequencies were scored within and among populations. For the SNPs, we scored the electrophoretic variants as separate alleles and determined the allele frequencies in each population. For the AFLP, we counted each well-defined peak size as a separate dominant Mendelian locus and scored the presence (dominant phenotype) versus absence (recessive) of each peak in the different individuals. We calculated population pairwise F_{ST} values using the Arlequin version 2.0 (Schneider et al. 2000) software package for the SNP data. We also calculated neutral genetic differentiation using Nei's genetic distance based on gene frequencies from AFLP markers.

For the San Diego County red, yellow, and hybrid populations, we estimated gene diversity for each type of molecular marker. For the cpDNA markers, gene diversity was calculated as the probability that two haplotypes were different; for the SNPs, it was measured as the expected heterozygosity. To avoid having to make the assumption that populations were in Hardy-Weinberg equilibrium, we used a Bayesian approach to estimate expected heterozygosity for the dominant AFLP dataset (Holsinger et al. 2002).

We then performed both individual-locus and multilocus analyses of molecular variance (AMOVA) using Arlequin (Schneider et al. 2000). These analyses hierarchically partitioned molecular variation into within- and among-population components to estimate genetic structure in predefined groups of populations. Permutation tests were used to determine statistical significance (Excoffier et al. 1992). The individual-locus AMOVAs allowed us to determine which loci were contributing most to any underlying genetic structure, and the multilocus test was simply the weighted average of the individual locus AMOVAs. For the first analysis, we performed separate AMOVAs that individually tested each of the three nearby *Diplacus* subspecies against a group that contained the red, yellow, and hybrid populations to determine whether the San Diego populations were more highly differentiated from these other subspecies than they were from each other. We also tested these same San Diego populations against a group of red, yellow, and hybrid populations from north of San Diego County to examine Beeks' (1962) assertion that these regions reflected distinct population series. These analyses were performed using only the cpDNA and SNP data sets, as AFLP data were collected only from red- and yellow-flowered populations within San Diego County. The final analysis focused on the extent of variation within and among the San Diego County red, yellow, and hybrid populations. We tested the red-flowered populations against the yellow-flowered populations, and we separated the red-flowered, yellow-flowered, and hybrid populations into three groups.

Q_{ST} versus F_{ST}

To determine whether the observed cline in flower color can be attributed to the effects of current or recent selection, we compared the extent of divergence in a quantitative morphological character (flower color) to divergence in neutral DNA markers. We used the quantitative trait analogue of Wright's (1951) measure of F_{ST}, termed Q_{ST} (Lande 1992; Spitze 1993; Merilä and Crnokrak 2001; McKay and Latta 2002), to assess levels of between-population variation in flower color. Q_{ST} measures the proportion of variance (phenotypic variance, in this case) in a quantitative trait that is attributable to differences between populations. Our common-garden experiment provides convincing evidence that the variation in flower color is due primarily to variation in genes as opposed to environmental effects (see Results).

We compared measures of Q_{ST} with F_{ST} (calculated from codominant SNP markers) for pairwise combinations of populations within San Diego County. Q_{ST} was calculated using one-way ANOVA to partition the phenotypic variance from spectrophotometer measurements of hue into within- and between-population components (Lynch and Walsh 1998). If the trait in question is completely neutral and has evolved due to genetic drift, then the values of Q_{ST} and F_{ST} should be similar. On the other hand, a value of Q_{ST} that exceeds F_{ST} indicates divergent selection between the populations. A third outcome, that F_{ST} exceeds Q_{ST}, indicates stabilizing selection for the same phenotype (reviewed in Merilä and Crnokrak 2001; McKay and Latta 2002). Specifically, we made six comparisons. We evaluated measures of Q_{ST} and

F_{ST} between populations within each floral race, between populations of the different floral races, between populations within the hybrid zone, and between populations of either floral race and hybrid populations. If current selection is contributing to the observed clinal variation in flower color, then comparisons between populations with different flower colors will yield Q_{ST} values in excess of F_{ST}. We considered there to be a statistically significant difference between Q_{ST} and F_{ST} for groups of comparisons whenever the 95% confidence intervals did not overlap.

A neutral cline in phenotypic traits may form if migration occurs primarily between nearby populations (Kimura and Weiss 1964). The expected outcome of such a scenario is isolation by distance, whereby neutral and phenotypic divergence increase in parallel with geographic distance (Wright 1943; Slatkin 1993; Hutchison and Templeton 1999). We used matrix correspondence tests (MCTs) implemented in Permute version 3.4 (Legendre et al. 1994) to assist in disentangling the effects of ongoing natural selection from neutral demographic processes. MCTs were used to measure the degree of association between two distance matrices using a regression framework, with statistical significance tested by means of randomization (Legendre et al. 1994; Manly 1997). We first examined whether our genetic marker data showed evidence for isolation by distance by comparing pairwise population estimates of F_{ST} with measures of pairwise geographic distance (Slatkin 1993; Hutchison and Templeton 1999; Storz 2002). The presence of significant isolation by distance can indicate that populations have reached an equilibrium between migration and genetic drift (Hutchison and Templeton 1999).

We then used simple, pairwise MCTs to determine the predictive power of geographic and genetic distances on between-population flower color differences (Q_{ST}). Finally, we used partial MCTs, which provide standardized partial regression coefficients, to determine the effect of each predictor matrix (genetic or geographic distance) on the response variable (phenotypic distance) while controlling for the effect of the other predictor variable. This way, the association between flower color and geography could be assessed after accounting for the effect of neutral genetic differentiation and vice versa. If a significant association persists between Q_{ST} and geographic distance after we control for the effects of neutral genetic divergence, we can reject the hypothesis that neutral processes are sufficient to explain the presence of the morphological cline (Malhotra and Thorpe 2000; Storz 2002).

Results

Floral Traits

Typical reflectance spectra of a red and yellow flower are presented in Figure 1. Red flowers have a peak reflectance at about 700 nm and yellow flowers have a peak at about 580 nm. Yellow flowers also have a second reflectance peak at approximately 380 nm, in the ultraviolet portion of the spectrum, which is completely absent from red flowers. Strong geographic structuring of flower color is evident in San Diego County, with coastal populations consisting only of red flowers and inland populations containing only yellow flowers (Fig. 2; Table 1). The transition in flower color is restricted to a region approximately 20 km wide. Where the two forms come together, hybridization occurs and an increased amount of variation in flower color is apparent. Although the variation in the hybrid zone is due predominantly to the presence of both red and yellow floral types, intermediate flower colors also occur here and are not found elsewhere (Fig. 2).

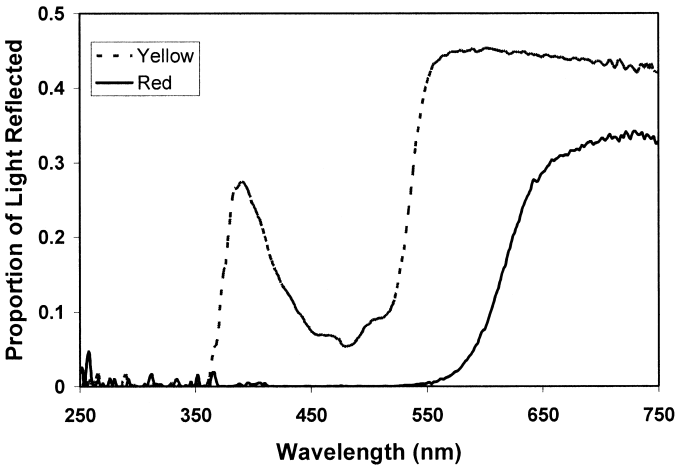

FIG. 1. Typical standardized reflectance spectra from one red flower and one yellow flower grown in a common garden. The wavelength at which the reflectance curve peaks denotes the color that is directly visible (approx. 580 nm for yellow and 700 nm for red). For the yellow flower, the smaller peak at approximately 380 nm reflects in the ultraviolet.

When seeds from field crosses are grown in a common environment, there are significant differences between coastal and inland populations for all measured floral traits. Measures of flower color from common-garden plants do not differ from field-collected flowers (Table 1). This indicates a strong genetic basis for flower color. Although we did not directly measure floral shape and nectar volume from field-collected flowers, differences reported here in common-garden plants are in agreement with measures from field studies (Waayers 1996; Tulig 2000). Floral traits are highly differentiated among the races and we recover similar phenotypes when plants are grown under uniform environmental conditions (Table 1).

Plants from yellow-flowered populations contain longer and wider corolla tubes, and significantly less nectar than red-flowered populations. Flower color is more divergent between the populations than are the other traits, as the distributions of flower color are completely nonoverlapping and separated by seven to 15 standard deviations (Table 1). In addition, absorbance by red, putative anthocyanin pigments extracted from flower petals is much higher in red flowers than in yellow flowers, where it is virtually absent (Table 1). Under the microscope, a second pigment, likely a carotenoid, is present in both floral races in roughly similar quantities and appears restricted to the chromoplasts. In contrast, the putative anthocyanin pigment is seen only in red flowers and is restricted to the vacuoles.

TABLE 1. (A) Floral trait summary statistics from red- and yellow-flowered populations grown in a common environment. (B) Flower color statistics calculated from all 16 natural red- and yellow-flowered populations. Anthocyanin abs. refers to the absorbance of extracted anthocyanin pigments at 510 nm. ΔSD refers to the average number of standard deviations differentiating the populations at each floral trait. Statistical significance between the morphs for each comparison is indicated. All flower color comparisons between common-garden and field-grown plants show no significant differences ($P > 0.15$).

	Red morph Mean (SD, range)	Yellow morph Mean (SD, range)	ΔSD	*P*
A. Common-garden				
Flower color:				
Chroma	0.93 (0.02, 0.85–0.98)	0.46 (0.04, 0.38–0.56)	14.9	<0.001
Hue	0.04 (0.02, 0.00–0.10)	0.64 (0.06, 0.50–0.81)	14.2	<0.001
Brightness	0.08 (0.01, 0.05–0.11)	0.21 (0.02, 0.16–0.26)	7.0	<0.001
Anthocyanin abs.	0.33 (0.08, 0.20–0.52)	0.04 (0.01, 0.01–0.05)	6.3	<0.001
Flower shape:				
Corolla length (mm)	33.9 (1.8, 29.4–37.6)	37.2 (2.7, 30.2–43.5)	1.4	<0.001
Corolla width (mm)	11.2 (1.0, 5.70–13.7)	15.4 (1.8, 12.4–20.4)	2.9	<0.001
Nectar volume (μL)	1.5 (1.4, 0.00–6.51)	0.2 (0.4, 0.0–2.3)	1.4	<0.001
B. Field				
Flower color:				
Chroma	0.96 (0.03, 0.87–0.99)	0.53 (0.07, 0.40–0.73)	8.6	<0.001
Hue	0.04 (0.03, 0.00–0.13)	0.59 (0.06, 0.40–0.74)	11.2	<0.001
Brightness	0.08 (0.02, 0.03–0.13)	0.21 (0.03, 0.12–0.32)	5.2	<0.001

Genetic Variation

The cpDNA PCR-RFLP analysis resolved 23 different haplotypes from the four loci. Most of the polymorphism was in the form of length variation due to insertion/deletion mutations. Haplotype A is very widespread and occurs in 22 of the 31 populations (see Table S3 available online only at http://dx.doi.org/10.1554/05-514.1.s1). It is found at a frequency greater than 0.5 in 16 of the 31 populations. Novel haplotypes (i.e., absent from the San Diego populations) are found in high frequency among the other *Diplacus* subspecies. There are also high frequency haplotypes found in populations north of San Diego County that are not found in the San Diego County red, yellow, or hybrid populations.

Gene diversity estimates from the San Diego red, yellow, and hybrid populations are presented in Table 2. Red- and yellow-flowered populations show roughly similar levels of diversity for each type of marker. The cpDNA markers show the lowest levels of diversity, and the SNPs show the highest. Hybrid populations have a higher overall diversity in the cpDNA, but intermediate levels in the SNPs. SNPs have about three times more genetic diversity than the AFLPs. Expected heterozygosity is rather high in the SNPs, ranging from 0.37 to 0.84.

Population Structure

Partitioning of haplotype and allele frequencies depends on the marker type and the type of analysis. AMOVA consistently demonstrates significant population structure that separates the San Diego red, yellow, and hybrid populations from both *M. clevelandii* and *M. aridus* (Table 3A). The *M. longiflorus* populations are marginally differentiated from the San Diego populations at the cpDNA markers ($P < 0.1$), but not at the SNPs. Therefore, with the possible exception of *M. longiflorus*, the red and yellow floral races in San Diego County appear to be more closely related to each other than to other southern California *Diplacus* subspecies. There is significant differentiation between northern and southern red- and yellow-flowered populations at two of the cpDNA markers, but this pattern is absent from the SNP data.

Chloroplast DNA haplotypes show no evidence of differentiation by flower color in San Diego County (Fig. 3B; Table 3B), despite significant among-population haplotype frequency variation at three of the four loci. SNP and AFLP loci exhibit a slightly different pattern. For these markers, AMOVA finds significant differentiation ($P < 0.05$) between red- and yellow-flowered populations. For the SNPs and AFLPs, 7.2% and 8.1% of the variation is partitioned among red- and yellow-flowered forms, respectively (Table 3B). The structure among red- and yellow-flowered populations can be attributed to only a small number of loci. In only one of the four SNP loci (*dLFY*) is a significant amount of the variation partitioned among the flower color groups (Fig. 3C). Based on the individual-locus AMOVA, 23.2% of the variation can be attributed to the among-group level at this locus. For the AFLP, 14 of the 100 polymorphic loci show significant amounts of variation between red- and yellow-flowered forms (Table 3B). For these loci, the range of variation explained at the among-group level is 7.0%–39.4%. For all three datasets, a significantly larger portion of the variation is partitioned among populations of the same flower color, and more of the loci show significant variation at this level than between flower colors (Table 3B). Further partitioning of the data by including the hybrid populations as a third group (in addition to the red and yellow groups) does not affect the results for the cpDNA and removes the significant among groups effect in the SNP dataset.

Q_{ST} versus F_{ST}

Measures of Q_{ST} between populations of the different floral races are greater than F_{ST} (Fig. 4). By contrast, estimates of Q_{ST} between populations within each floral race are similar to or less than F_{ST}. Matrix correspondence tests indicate the

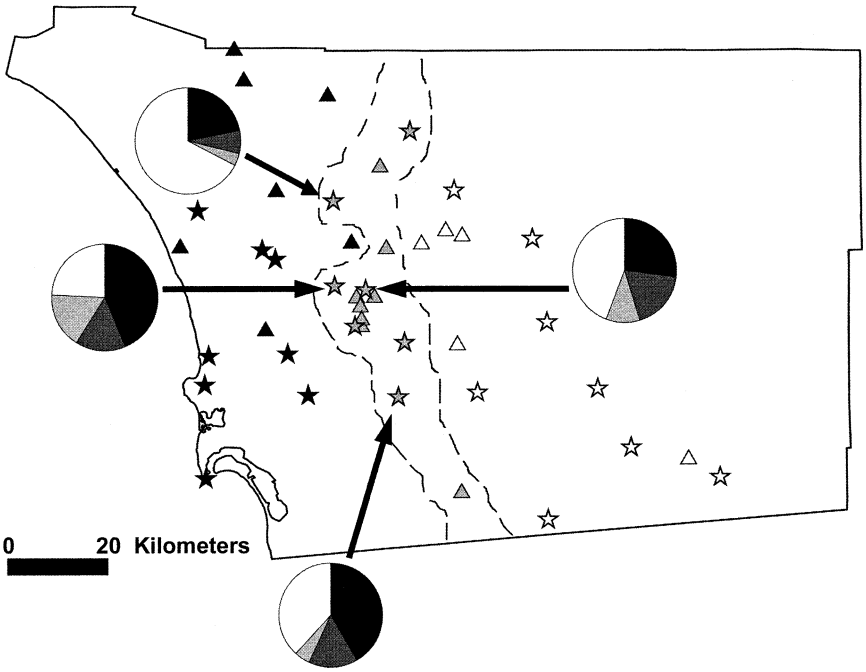

FIG. 2. Map of *Mimulus aurantiacus* populations in San Diego County, California. Stars represent those populations for which we measured flower color with the spectrophotometer. Triangles denote additional populations that were visually inspected for flower color. Pie charts show the range of variation in flower color from four hybrid populations. The bins correspond to measurements of hue taken from spectrophotometer data in pure populations and denote flower color as follows: pure red (black; range = 0.00–0.13), pure yellow (white; range = 0.40–0.80), intermediate colors in hybrid populations (gray; range = 0.13–0.27; light gray; range = 0.27–0.40). Shading of the symbols is the same as that for the pie charts. The dashed line shows the approximate location of the transition zone between pure red- and pure yellow-flowered populations.

TABLE 2. Gene diversity estimates (±1 SD) for three types of molecular markers within San Diego populations of *Mimulus aurantiacus*. For the cpDNA, diversity is estimated as the probability that two haplotypes are different. For the single nucleotide polymorphism (SNP), gene diversity is calculated as expected heterozygosity, and for the amplified fragment length polymorphism (AFLP), we estimate expected heterozygosity according to Holsinger et al. (2002). AFLPs were measured from a subset of five red-flowered (R) and five yellow-flowered (Y) populations. H, hybrid populations. Population codes defined in Table S1, available online.

Population	Flower color	cpDNA	SNP	AFLP
CNM	R	0.0000 ± 0.0000	0.6810 ± 0.0388	0.2165 ± 0.0091
UCSD	R	0.4248 ± 0.0993	0.8063 ± 0.0371	0.2186 ± 0.0095
SMER	R	0.1176 ± 0.1012	0.8378 ± 0.0325	0.2055 ± 0.0104
LH	R	0.0000 ± 0.0000	0.8238 ± 0.0528	0.2335 ± 0.0089
ELT	R	0.3088 ± 0.1222	0.8217 ± 0.0451	0.2149 ± 0.0095
Mean		0.1702 ± 0.0645	0.7941 ± 0.0413	0.2178 ± 0.0095
LKW	H	0.7386 ± 0.0596	0.7270 ± 0.0614	
GDN	H	0.4248 ± 0.0993	0.7079 ± 0.0659	
MW	H	0.0000 ± 0.0000	0.7201 ± 0.0713	
DP	H	0.2092 ± 0.1163	0.7587 ± 0.0512	
SPT	H	0.0000 ± 0.0000	0.5587 ± 0.0830	
LCRD	H	0.3660 ± 0.1124	0.5587 ± 0.0825	
LKH	H	0.0000 ± 0.0000	0.6746 ± 0.0594	
DLZ	H	0.7516 ± 0.0754	0.7365 ± 0.0522	
Mean		0.3113 ± 0.0579	0.6801 ± 0.0659	
BC	Y	0.7843 ± 0.0849	0.7127 ± 0.0790	
ALP	Y	0.0000 ± 0.0000	0.7175 ± 0.0491	
BO	Y	0.0000 ± 0.0000	0.7270 ± 0.0549	
INJ	Y	0.0000 ± 0.0000	0.6185 ± 0.0795	0.2286 ± 0.0113
BCRD	Y	0.0000 ± 0.0000	0.5444 ± 0.0937	0.1941 ± 0.0106
POTR	Y	0.1176 ± 0.1012	0.8164 ± 0.0432	0.2362 ± 0.0092
PVT	Y	0.0000 ± 0.0000	0.4349 ± 0.1004	0.2443 ± 0.0090
LO	Y	0.0000 ± 0.0000	0.3708 ± 0.0785	0.2023 ± 0.0103
Mean		0.1127 ± 0.0230	0.6175 ± 0.0723	0.2211 ± 0.0101

TABLE 3. Results of analysis of molecular variance (AMOVA) for each class of molecular markers. (A) Comparisons of the San Diego County red (R), yellow (Y), and hybrid (H) populations with other members of *Diplacus*. North vs. South compares San Diego County populations (South) to populations of the same subspecies from Orange and Riverside Counties (North). (B) Comparisons among the San Diego County red, yellow, and hybrid populations. Statistical significance is based on 10,000 permutations.

	Within populations[2]		Among populations, within groups[3]		Among groups[4]	
Groups[1]	% variation explained	No. of significant loci	% variation explained	No. of significant loci	% variation explained	No. of significant loci
(A) San Diego (R, Y, H) vs. *Diplacus*						
cpDNA						
R, Y, H vs. *M. clevelandii*	12.27***	4	13.77***	4	73.96***	4
R, Y, H vs. *M. aridus*	12.00***	4	13.78***	4	74.22***	4
R, Y, H vs. *M. longiflorus*	46.23***	4	41.27***	4	12.51†	2
North vs. South (R, Y, H)	18.07***	4	21.64***	4	60.29***	2
SNP						
R, Y, H vs. *M. clevelandii*	50.53***	4	12.35***	4	37.12***	3
R, Y, H vs. *M. aridus*	26.64***	4	6.61***	4	66.75***	3
R, Y, H vs. *M. longiflorus*	75.84***	4	21.48***	4	2.68	0
North vs. South (R, Y, H)	79.26***	4	19.90***	4	0.84	0
(B) San Diego populations						
cpDNA						
Red vs. yellow	33.85***	3	62.74***	3	3.41	0
Red vs. yellow vs. hybrid	47.38***	4	50.02***	4	2.60	0
SNP						
Red vs. yellow	74.05***	4	18.75***	4	7.20*	1
Red vs. yellow vs. hybrid	79.80***	4	17.32***	4	2.88	1
AFLP						
Red vs. yellow	76.19***	68	15.67***	34	8.14*	14

[1] Group designations reflect differences in floral morphology among subspecies or geographic regions.
[2] Summarizes variation within populations without regard to flower morphology (i.e., group type).
[3] Summarizes variation among populations within floral morphology type.
[4] Summarizes variation among floral morphologies.
*$P < 0.05$; ***$P < 0.0001$; †$P < 0.1$.

presence of significant isolation by distance for neutral genetic markers, suggesting that populations may be in migration-drift equilibrium (Table 4). However, Q_{ST} shows a stronger relationship with geography than with either measure of genetic distance in pairwise MCTs. The effect of geographic distance remains strong even after accounting for the effect of genetic distance using partial MCTs. However, there is no effect of genetic distance calculated from SNPs and only a marginal effect of AFLP distance on flower color differentiation after we control for geographic distance (Table 4).

DISCUSSION

We have demonstrated that flower color in San Diego County *M. aurantiacus* has a strong genetic basis and shows a sharp geographic transition, with western, red-flowered populations steeply grading into eastern, yellow-flowered populations over a distance of approximately 20 km. However, patterns of genetic variation at neutral loci are far less pronounced, as little of the variation can be partitioned according to differences in flower color. Furthermore, a strong relationship between flower color and geography cannot be explained according to a neutral isolation by distance hypothesis. The most likely explanation for these results is that natural selection is responsible for the steep cline in flower color despite gene flow between floral races.

Floral Traits

All of the measured floral traits differ significantly between coastal and inland regions. Coastal plants contain red flowers with shorter and narrower corolla tubes, and more nectar as compared to inland, yellow-flowered plants. Flower color is more highly diverged between the races than are these other morphological traits. The similarity in measurements of flower color between common-garden plants and field-collected flowers indicates a strong genetic basis for flower color.

Spectrophotometry and microscopy suggest that differences in flower color are likely due to the presence of anthocyanins in red flowers and their absence in yellow flowers, as both floral types appear to have carotenoids in petal cells. This indicates that change in the anthocyanin biosynthetic pathway has eliminated red pigmentation in the yellow flowers, or red coloration is due to the gain of anthocyanin expression in red flower petals. Red pigmentation is present in stems and leaves of both races (M. Streisfeld, pers. obs.), implying that tissue-specific differences in gene regulation are likely responsible for differences in flower color (Mol et al. 1998).

The geographic transition in flower color is abrupt, occurring over a distance of only about 20 km. In the region of overlap, intermediate flower colors segregate with the pure red and yellow forms. Although there are relatively few in-

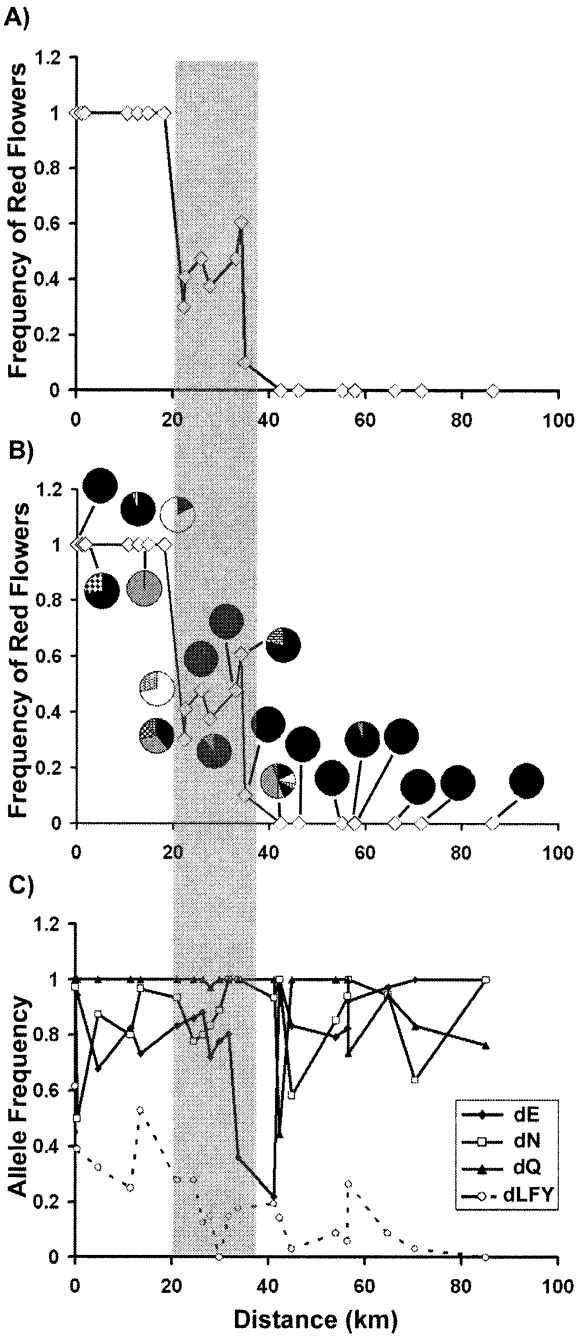

FIG. 3. Spatial variation in flower color and neutral genetic markers in red, yellow, and hybrid populations from San Diego County. (A) Cline in flower color, measured as the frequency of red flowers in each population. A flower is considered to be red if it has a range of hue between zero and 0.13. (B) cpDNA haplotype frequencies plotted as pie charts and mapped onto the cline in flower color in San Diego. Each haplotype is represented by a different shading pattern, with haplotype A in black. (C) Single nucleotide polymorphism allele frequencies from each of four loci. Because populations are situated in two dimensions, but the variation in flower color occurs primarily between eastern and western regions, we calculated geographic distance as the longitudinal distance from the most western population. The shaded area represents the approximate location of the hybrid zone in flower color.

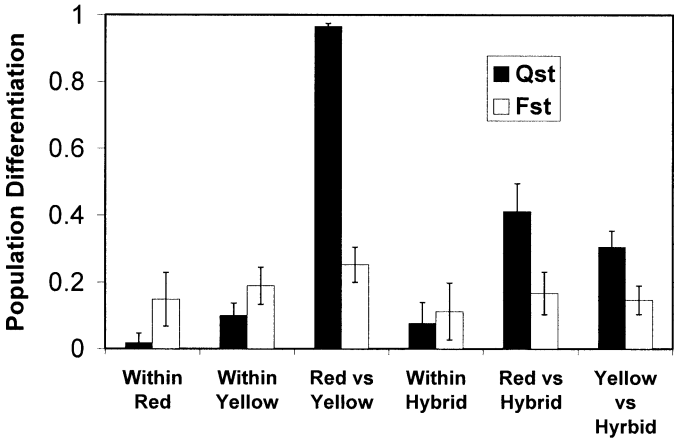

FIG. 4. Comparison of average Q_{ST} for flower color (black bars) and average F_{ST} (white bars) calculated from allele frequencies at four single nucleotide polymorphisms within and between red, yellow, and hybrid populations from San Diego *Mimulus aurantiacus*. Error bars represent the 95% confidence intervals.

termediate flower colors in the hybrid zone, these are the only locations where they are found. Other floral traits also change across the contact zone, but the clines in these traits are not as steep as for flower color (Waayers 1996). These traits are presumed to be important in pollinator attraction and effective pollen transfer (Grant 1993b), revealing a potentially strong ecological gradient that may result in different selective regimes on opposite ends of the cline. Additionally, because several of these floral traits appear to segregate independently in advanced generation hybrids (Streisfeld 2005), different unlinked genetic loci likely control trait variation. Taken together, these results suggest that it may be difficult to explain concordant clines at several ecologically important characters according to a purely neutral model of restricted gene flow and genetic drift.

Population Structure

The major finding from the analyses of genetic variation at neutral DNA markers is weak partitioning of molecular variation between the red and yellow floral races. Differences in flower color do not explain significant variation in the cpDNA markers, and although there is evidence for modest population structure between the floral races at the SNP and AFLP markers, only one of the four SNP loci and 14 of the 100 AFLP loci show significant differences between the races in allele frequency. Individual-locus analyses of molecular variance reveal that geographic differences in flower color cannot explain more than 39.4% of the variation at any of these loci. The presence of modest differentiation at only a handful of nuclear loci could indicate that introgression following secondary contact has erased some, but not all, neutral differentiation between floral races. Alternatively, gene flow between the races may be somewhat restricted, or differentiated marker loci may be linked to loci that are under divergent selection (Wilding et al. 2001).

Analyses of Q_{ST} and F_{ST} further support the results from AMOVA and show that differentiation in flower color between the floral races is far greater than differentiation in

TABLE 4. Pairwise and partial matrix correspondence tests (MCT) from red- and yellow-flowered populations for which both flower color (Q_{ST}) and either (A) codominant single nucleotide polymorphisms (SNPs) or (B) amplified fragment length polymorphisms (AFLPs) were scored. Genetic distance was calculated as pairwise F_{ST} from SNPs and as Nei's genetic distance based on gene frequencies for AFLP markers. Geographic distance is a matrix of the straight-line geographical distances between populations. For the pairwise MCTs, a single dependent variable (Y) was regressed against a single predictor matrix (X_1), with b representing the regression coefficient. When a second predictor matrix (X_2) is present, b represents the standardized partial regression coefficient of the effect of X_1 on Y, after accounting for the effect of X_2. Statistical significance is based on 10,000 randomized permutations (Legendre et al 1994; Manly 1997).

Y	X_1	X_2	b	P	R^2
(A) Codominant SNPs					
Genetic distance (F_{ST})	geographic distance	—	0.456	0.001	0.208
Flower color (Q_{ST})	geographic distance	—	0.687	<0.001	0.472
Flower color (Q_{ST})	genetic distance (F_{ST})	—	0.231	0.039	0.053
Flower color (Q_{ST})	geographic distance	genetic distance (F_{ST})	0.735	<0.001	0.481
Flower color (Q_{ST})	genetic distance (F_{ST})	geographic distance	−0.104	0.185	
(B) AFLPs					
Genetic distance (Nei)	geographic distance	—	0.328	0.047	0.108
Flower color (Q_{ST})	geographic distance	—	0.770	0.003	0.593
Flower color (Q_{ST})	genetic distance (Nei)	—	0.459	0.023	0.211
Flower color (Q_{ST})	geographic distance	genetic distance (Nei)	0.694	0.005	0.641
Flower color (Q_{ST})	genetic distance (Nei)	geographic distance	0.231	0.045	

neutral markers. By contrast, comparisons between populations within each floral race show similar or lower amounts of neutral marker differentiation relative to phenotypic differentiation, indicating the possibility of stabilizing selection on flower color within the geographic regions. Additionally, even though F_{ST} values are roughly similar for comparisons made both within and between the floral races, Q_{ST} values increase almost 10-fold when we compare populations of the different floral races.

Matrix correspondence tests demonstrate a positive relationship between genetic and geographic distance, which suggests the presence of isolation by distance at these neutral markers. Geographic distance also predicts phenotypic differentiation in flower color, but this relationship is much stronger than the one obtained using genetic distance from either type of nuclear marker. In addition, associations between geographic and phenotypic distance remain strong after we control for the effects of genetic distance using partial MCTs. Conversely, genetic distance has only negligible power to predict phenotypic divergence after controlling for the effects of geographic distance. Therefore, phenotypic differences in flower color across the cline are much greater than a purely neutral model could account for, indicating that current or recent selection is responsible for maintaining floral variation (see also Thorpe et al. 1996; Malhotra and Thorpe 2000; Storz 2002). The weak evidence for neutral divergence among the races also argues against the possibility of recent secondary contact after a long period of allopatry.

Even though a significant amount of the molecular variation was attributable to differences in flower color in the AMOVA, this result was eliminated from the SNP dataset and remained only marginally significant ($P = 0.045$) for the AFLP data after we controlled for the effects of geographic distance in partial MCTs. The cause of the significant effect of flower color in the AMOVA can primarily be attributed to, on average, larger geographic distances between populations with different flower colors. We sampled populations of *M. aurantiacus* in San Diego County in two dimensions (north and south in addition to east and west), even though the major phenotypic transition occurs in one dimension (west to east). For the MCTs, we calculated geographic distance as the straight-line distance between populations, even though some long-distance comparisons represent distances between two populations of the same floral race (i.e., these represent north/south variation between populations within either red or yellow floral races). Therefore, our estimates of the association between Q_{ST} and geography may be conservative. Repeating the MCTs using the difference in longitude as an alternative measure of geographic distance strengthens the relationship between flower color and geography in pairwise and partial MCTs for both the SNP and AFLP datasets (data not shown). This new measure of geographic distance does not substantially affect the association between genetic and geographic distance, implying that levels of neutral differentiation (and gene flow) are as similar for comparisons between northern and southern populations as they are between eastern and western populations. In addition, the effect of Nei's genetic distance (from AFLP data) on flower color is eliminated when we control for east/west geographic distance between populations. This further suggests that neutral genetic divergence between the races is negligible.

These analyses rest on several assumptions. First, estimates of Q_{ST} based on phenotype must not be confounded by environmental and nonadditive effects. Phenotypic variance has been equated to additive genetic variance in many similar studies (e.g., Storz 2002; Saint-Laurent et al. 2003). Furthermore, our common-garden experiment, although not directly able to determine the magnitude of the different genetic components of phenotypic variation, clearly demonstrates that phenotypic differentiation in flower color is nearly entirely due to differences in genes as opposed to environmental effects. Nonadditive genetic components of variance (i.e., dominance and epistasis) appear to downwardly bias estimates of Q_{ST} (reviewed in Merilä and Crnokrak 2001), making our measure conservative.

Second, the randomization procedure used to evaluate partial MCTs has been criticized for providing inflated Type I error rates (false rejection of the null hypothesis of no effect

of a causal variable after accounting for the effect of a second causal variable; Raufaste and Rousset 2001; Rousset 2002). To the extent that these criticisms are valid (but see Legendre 2000; Castellano and Balletto 2002), caution should be used in interpreting the results of such tests. However, these criticisms do not apply to simple, pairwise MCTs (Raufaste and Rousset 2001). Thus, our findings of a strong relationship between Q_{ST} and geography, and only a weak relationship between Q_{ST} and genetic distance in simple pairwise tests are valid. Moreover, the statistical significance of the effect of Nei's genetic distance on Q_{ST} after controlling for geography in the partial MCT should be considered a maximum estimate. Therefore, the conclusions drawn here are robust to the criticisms raised against partial MCT tests and provide strong support for the hypothesis that current or recent selection is needed to account for the observed pattern of variation in flower color.

Conclusions

We find little evidence to support a hypothesis of recent secondary contact following a long period of allopatry. Grant's (1993b) contention that red and yellow floral races of *M. aurantiacus* diverged in allopatry may still be accurate, but either the time in allopatry was too short for much neutral divergence to arise, or secondary contact is old, and the cline in flower color has been maintained after secondary contact by selection. Alternatively, selection may have caused divergence in flower color in the absence of geographic barriers to gene flow. Differentiating among these alternatives is difficult and requires additional information such as direct estimates of current selection and migration. The fact that red-flowered plants are never seen growing on the eastern side of the cline and yellow-flowered plants are completely absent from the western region suggests that selection is strong enough to prevent the introgression of flower color despite gene flow at neutral loci.

This cline in flower color occurs even though both presumed pollinators (hummingbirds and hawkmoths) are found throughout the ranges of each floral form. Perhaps regional differences in relative abundance of each pollinator contribute to variation in the rate of successful pollination by each floral type (Beeks 1962; Grant 1993a; M. Streisfeld, pers. obs.). Similar studies of floral variation across ecological gradients have found comparable patterns of strong morphological differentiation, little or no neutral differentiation, and the presence of both alternative pollinators on each side of the contact zone (Hodges and Arnold 1994; Wolf et. al. 1997; Campbell et al. 1997; Fulton and Hodges 1999). In accord with the present study, these additional data suggest that selection frequently may be strong enough to maintain, or perhaps even create, geographic variation in floral traits in the absence of barriers to gene flow. Determining whether and to what extent floral divergence is mediated through selection for different pollinators is an important next step for elucidating the processes leading to adaptive population differentiation, and perhaps speciation.

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