PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

rstb.royalsocietypublishing.org

Research

Cite this article: Wessinger CA, Hileman LC, Rausher MD. 2014 Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Phil. Trans. R. Soc. B* **369**: 20130349. http://dx.doi.org/10.1098/rstb.2013.0349

One contribution of 14 to a Theme Issue 'Contemporary and future studies in plant speciation, morphological/floral evolution and polyploidy: honouring the scientific contributions of Leslie D. Gottlieb to plant evolutionary biology'.

Subject Areas:
evolution, genetics, genomics

Keywords:
Penstemon, pollination syndrome, QTL, genetics of adaptation, phenotypic correlation

Author for correspondence:
Mark D. Rausher
e-mail: mrausher@duke.edu

†These authors contributed equally to this study.

Electronic supplementary material is available at http://dx.doi.org/10.1098/rstb.2013.0349 or via http://rstb.royalsocietypublishing.org.

Royal Society Publishing

Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*

Carolyn A. Wessinger[1], Lena C. Hileman[1,†] and Mark D. Rausher[2,†]

[1]Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA
[2]Department of Biology, Duke University, Durham, NC 27708, USA

Distinct floral pollination syndromes have emerged multiple times during the diversification of flowering plants. For example, in western North America, a hummingbird pollination syndrome has evolved more than 100 times, generally from within insect-pollinated lineages. The hummingbird syndrome is characterized by a suite of floral traits that attracts and facilitates pollen movement by hummingbirds, while at the same time discourages bee visitation. These floral traits generally include large nectar volume, red flower colour, elongated and narrow corolla tubes and reproductive organs that are exerted from the corolla. A handful of studies have examined the genetic architecture of hummingbird pollination syndrome evolution. These studies find that mutations of relatively large effect often explain increased nectar volume and transition to red flower colour. In addition, they suggest that adaptive suites of floral traits may often exhibit a high degree of genetic linkage, which could facilitate their fixation during pollination syndrome evolution. Here, we explore these emerging generalities by investigating the genetic basis of floral pollination syndrome divergence between two related *Penstemon* species with different pollination syndromes—bee-pollinated *P. neomexicanus* and closely related hummingbird-pollinated *P. barbatus*. In an F_2 mapping population derived from a cross between these two species, we characterized the effect size of genetic loci underlying floral trait divergence associated with the transition to bird pollination, as well as correlation structure of floral trait variation. We find the effect sizes of quantitative trait loci for adaptive floral traits are in line with patterns observed in previous studies, and find strong evidence that suites of floral traits are genetically linked. This linkage may be due to genetic proximity or pleiotropic effects of single causative loci. Interestingly, our data suggest that the evolution of floral traits critical for hummingbird pollination was not constrained by negative pleiotropy at loci that show co-localization for multiple traits.

1. Introduction

Flowering plants rely on pollen vectors for their reproductive success, which has led to evolutionary diversification in floral phenotypes. This diversification includes the repeated emergence of distinct floral pollination syndromes—stereotypical combinations of floral traits that attract and facilitate pollination by a particular functional group of pollinators [1–3]. These floral traits include floral morphology (flower shape and the morphology or orientation of reproductive structures), nectar characteristics, scent and colour.

In western North America, the hummingbird pollination syndrome has evolved at least 129 times in a variety of angiosperm lineages, generally from an ancestral bee pollination syndrome [4]. Many of these hummingbird-adapted species have close relatives or even sister species that are bee-pollinated. Thus, shifts from bee to hummingbird pollination syndrome occur relatively frequently and rapidly, and, interestingly, seem to be unidirectional in most taxa. Bee-to-hummingbird pollinator shifts involve stereotypical changes in a variety of floral traits, some of which are adaptations to attract hummingbird pollinators

and facilitate pollination by them, others of which are adaptations to exclude bee pollinators [5]. The most dramatic case of repeated adaptation from bee to hummingbird pollination in North America comes from the genus *Penstemon* [4]. There are approximately 284 species in this group, the majority of which display the ancestral bee pollination syndrome. However, there have been an estimated 10–21 independent evolutionary transitions to the hummingbird pollination syndrome, with no cases of reversals [6,7]. These transitions are highly convergent and involve a shift from blue, purple or pink flowers to red flowers, an increase in nectar volume, a decrease in nectar sugar concentration, loss of a 'landing platform' formed by the lower petals, lengthening and narrowing of the corolla tube, and lengthening of the stamens and style yielding exsertion of the reproductive organs [8].

While parallel evolutionary transitions between bee and hummingbird pollination syndromes are common, little is known about the molecular identity of the underlying genetic changes. Yet, some empirical evidence from quantitative trait locus (QTL) studies on pollination syndrome divergence suggests that there may be common patterns at the genetic level. For example, previous QTL studies have suggested that traits such as flower colour and nectar volume often involve few substitutions of medium to large effect [9–13], while changes in morphological characters tend to involve more loci with smaller effects [12,14,15]. In addition, these studies have revealed that QTLs for different traits sometimes co-localize (e.g. [9,14–18], reviewed by Hermann & Kuhlemeier [19]), suggesting either that some adaptive substitutions have pleiotropic effects on more than one trait or that multiple adaptive substitutions, each affecting a single trait, are tightly linked. Exceptions to these general patterns exist [15,19], and because the genetic architecture of floral syndrome transitions has been examined in only a few species it is unclear to what degree these patterns are representative.

Developmental constraints on the evolution of complex phenotypes such as pollination syndromes arise when pleiotropic adaptive substitutions generate mutational covariance among multiple component traits. When this pattern of covariance fortuitously causes adaptive change in each trait, adaptation towards the optimal multi-trait phenotype will be accelerated. However, a potential constraint arises when the pattern of mutational covariance causes adaptive change in one trait, but maladaptive change in a second trait. This type of antagonistic pleiotropy will reduce the net selective coefficient for the adaptive substitution, slowing the rate of evolution. Moreover, evolution to the optimum multi-trait phenotype will require additional substitutions that 'correct' the effects of fixing mutations with antagonistic pleiotropy. In this situation, a QTL analysis would be likely to identify, for some pairs of traits, co-localizing QTLs that exhibit antagonistic pleiotropy, unless for some *a priori* reason developmental constraints largely prevent the fixation of such mutations. For example, adaptation to hummingbird pollination can include increased floral tube length and decreased floral tube width compared with bee-pollinated flowers. Antagonistic pleiotropy would be evident if QTLs for these traits co-localize to the same genomic region and the alleles derived from the hummingbird-pollinated lineage have the effect of increasing both tube length and tube width. In the absence of pleiotropy, adaptive substitutions will affect only one trait and each character can evolve independently towards the optimal phenotype. In this situation, one should not see substitutions that are counter to the direction of selection, i.e. substitutions that decrease a character when it is advantageous to increase it.

In this study, we begin to address these issues by conducting a preliminary QTL analysis of differences in floral traits between two closely related species of *Penstemon* with contrasting pollination syndromes. One species, *P. neomexicanus*, is pollinated primarily by bees and displays floral characteristics typical of the bee pollination syndrome (see below). The other species, *P. barbatus*, is pollinated primarily by hummingbirds and displays characters typical of the hummingbird pollination syndrome. Using this system, we characterized QTLs for trait differences between the two species and asked two questions: (i) whether the effect sizes of QTLs conform to the patterns seen in previous studies of other systems and (ii) whether there is evidence of antagonistic pleiotropy in co-localizing QTLs. This comprises a first step towards the molecular dissection and characterization of major QTLs underlying trait divergence in this study system.

2. Material and methods

(a) Study system

Penstemon barbatus (figure 1*b*) occurs throughout the American Southwest at high elevations and displays a hummingbird pollination syndrome: bright red flowers with a long narrow tube, sharply reflexed lower petals, exserted style and stamens, and large amounts of dilute nectar. *Penstemon neomexicanus* (figure 1*a*) is a closely related species [6] that occurs in the Sacramento mountains of New Mexico. This species retains the ancestral bee pollination syndrome: blue-purple flowers that are shorter and wider, having lower petals that form a landing platform for bees, and producing small amounts of concentrated nectar. *Penstemon* species have four fertile stamens: one pair that are longer and one pair that are shorter. In *P. barbatus*, both sets of stamens are relatively long compared with those in *P. neomexicanus* flowers, as are the styles (figure 1*c*).

Seeds from each species, a generous gift from Prof. Paul Wilson (California State University, Northridge), were germinated after two months of cold stratification. We generated an F_2 mapping population of approximately 180 individuals by crossing these two species and intercrossing three F_1 individuals. The F_2 plants, along with the parental and F_1 individuals, were grown in the Duke University greenhouses under controlled conditions and supplemental light (18 h daylight).

(b) Phenotypic measurements

We measured floral traits on five *P. neomexicanus* individuals, three *P. barbatus* individuals, three F_1 hybrids and 157 F_2 individuals. We measured six morphological traits (figure 1*c*) on three flowers per individual on the day the flower opened. First, we measured style length using digital callipers, which allowed us to let the entire gynoecium remain attached to the plant for potential seed production. We carefully removed the rest of the flower (petals and stamens) and digitally photographed the exterior (side and head-on views) and cross section of each flower using a small stage. We included rulers at the same focal distance as the flowers for calibration. We used ImageJ [20] to determine the following five morphological measurements: floral tube length, floral tube width, the angle formed by the lower petal and the lengths of the long and short stamens.

We measured two nectar traits, volume and sugar concentration, for three to five flowers per individual. Nectar was collected by contacting nectaries with 2 µl glass capillary tubes (Drummond Scientific, Broomall, PA, USA). Nectar volume was

Figure 1. Floral phenotypes of (*a*) *P. neomexicanus* and (*b*) *P. barbatus*. (*c*) Floral morphological traits measured.

measured by counting the number of completely filled capillary tubes and multiplying by 2 µl; partially filled tubes were measured with digital callipers and this length was converted into microlitres. Nectar concentration was measured using a Master-53M refractometer (Atago, Bellevue, WA, USA).

Flower colour segregated as a binary trait in this F_2 population, with individuals having either blue-purple or red flowers. Previously, we characterized the genetic basis of the flower colour difference between *P. neomexicanus* and *P. barbatus* [13]. We determined that the shift to red flowers in the lineage leading to *P. barbatus* is due to redundant loss-of-function mutations in the enzyme *flavonoid 3′,5′-hydroxylase* (*F3′5′h*). Genetic substitutions to this locus in *P. barbatus* are sufficient for producing a shift from blue-purple to red flowers. However, a second locus that acts epistatically to *F3′5′h* determines flower colour in individuals that are heterozygous at *F3′5′h*. For our analyses here, flower colour was coded as a binary trait, with '0' denoting red flowers and '1' denoting blue-purple flowers.

(c) Statistical analyses

Trait values were averaged for each individual. We assessed whether parental trait values differed significantly using *t*-tests implemented in JMP Pro 11 (SAS Institute, Cary, NC, USA). We calculated Spearman's correlations between all pairwise combinations of traits in the F_2 population using R v. 2.13.0, and assessed significance using a Bonferroni correction.

(d) Multiplexed shotgun genotyping

To generate genotyping markers for QTL mapping, we performed a modified form of multiplexed shotgun genotyping (MSG) [21] on parental DNA and DNA from 96 F_2 individuals. We extracted genomic DNA from frozen leaf tissue using a CTAB–chloroform extraction protocol [22] and quantified DNA concentration using a Qubit BR kit (Invitrogen, Carlsbad, CA, USA). We digested 50 ng of DNA from each individual in separate reactions using AseI (New England Biolabs, Ipswich, MA, USA), ligated unique barcoded adapters to each sample and pooled samples, yielding two pools each containing 96 samples. The first pool consisted of the 96 F_2 individuals. The second pool contained 12 replicate samples of each of the two parental DNA samples, along with the first 72 F_2 individual samples. We purified the pooled samples using AMPure beads (Beckman Coulter, Danvers, MA, USA) at 1.5× volume and size selected DNA fragments of 250–300 bp from an agarose gel using a Pippen Prep (Sage Science, Beverly, MA, USA). DNA from this gel fragment was extracted using the Qiaquick kit (Invitrogen). For each DNA sample, we amplified eight replicate reactions containing 2 ng library DNA for 14 cycles each using Illumina sequencing

primers, then each set of eight replicate PCR reactions was pooled. Each library was sequenced on an Illumina Hi-Seq 2500 lane (100 base-pair single-end reads; Illumina, San Diego, CA, USA) at the University of Kansas Genome Sequencing Core using standard Illumina protocols.

(e) Construction of linkage map

We processed the raw sequencing reads using STACKS [23]. First, we de-multiplexed reads from each lane, removed low-quality reads and removed reads with ambiguous barcodes. We then concatenated duplicate samples (i.e. for parental samples and those F_2 samples that were represented in both sequencing lanes). We used the STACKS pipeline denovo_map.pl to identify unique loci within each individual, produce a catalog of homologous loci found in the two parental samples, and assign hard genotypes for each individual at each locus present in the catalogue. We enforced a minimum of three reads to create a 'stack' (genetic locus) in each parent, a minimum of eight reads per stack for a genotype to be called in any individual and a maximum distance of four nucleotides between alleles of a particular stack. Highly repetitive stacks (greater than 2 s.d. from the mean) were removed. We then extracted markers that met two critiera: (i) they had fixed differences between the two parental samples (i.e. AA in *P. neomexicanus* and BB in *P. barbatus*) and (ii) they were genotyped in at least 88 of the 96 F_2 individuals. Details of the sequencing output are reported in the electronic supplementary material, table S1.

We used R/QTL [24] to construct a linkage map. We identified markers with identical genotypes across all individuals and removed the duplicates. We used a χ^2-test implemented in R/QTL to identify markers with distorted segregation patterns and removed markers with $p < 0.000001$. This filtering step removed markers with true severe segregation distortion, but also removed markers that had been incorrectly genotyped. Markers were then separated into linkage groups (LGs) as suggested by Broman [25] using a maximum recombination frequency threshold of 0.35 and a minimum logarithm of odds (LOD) threshold of 6.0. Markers were ordered along each LG using the ripple function in R/QTL. We added CAPS marker data at *F3′5′h* to our marker dataset to determine where this locus resides in the linkage map.

(f) QTL mapping

We performed QTL analysis of floral traits using the Haley–Knott method in R/QTL. Flower colour was treated as a binary trait in all analyses. We identified QTL positions as locations where the log-ratio (LR) statistic was greatest comparing a model of the presence of a QTL at that position to a model of no QTL present using the scanone function and assessed significance using LOD scores. For each trait, we calculated a genome-wide significance LOD threshold corresponding to a false discovery rate of 0.05 by performing 1000 permutations of the data. We tested for the presence of multiple QTL peaks on a single LG and for interactions between identified QTL using the scantwo function. For each significant QTL, we obtained 1.5-LOD confidence intervals (CIs), as well as estimates of additive allelic effects and dominance deviations. QTL effect sizes were reported as the proportion of variation explained (PVE) and relative homozygous effect (the homozygous additive effect divided by the mean phenotypic difference between *P. neomexicanus* and *P. barbatus*). We fit a model containing all main QTL effects and, for each trait, obtained an estimate of the total phenotypic variation explained by the model. The degree of dominance for each QTL was calculated as |dominance deviation|/|additive effect|.

To explore how well our recovered QTLs explained the observed correlations among traits in the F_2 individuals, we calculated expected correlations based on the effect sizes and dominance deviations of each QTL and compared them with the observed phenotypic correlations. We first calculated the genotypic value for each genotype at a QTL in the following way: if the three genotypes at the QTL are *BB*, *NB* and *NN*, corresponding to the homozygote for the *P. barbatus* allele, heterozygote and homozygote for the *P. neomexicanus* allele, then the respective genotypic values were $-a$, d and a, where a is the estimated additive effect of the QTL and d is the estimated dominance deviation. When a QTL did not significantly influence a trait, all three values were set to 0. Next, for a pair of traits, the set of QTLs affecting one or both traits was chosen. QTLs that co-localized for the two traits were considered to be the same QTL, with the criterion for co-localization being overlap of the 1.5-LOD CIs. However, we assumed the QTLs for tube width and style length on LG 3 were not co-localized because of their broad CIs and because their most likely positions were at opposite ends of the LG. From this set of QTLs, the genotypic values for all multi-locus genotypes were calculated by summing the estimated genotypic values over loci.

We then calculated the covariance of the expected genotypic values for the two traits across multi-locus genotypes, weighted by their expected frequencies in the F_2 individuals, as well as the variances of the expected genotypic values. We assumed the phenotypic covariance was equal to the expected genetic covariance, i.e. that environmental deviations for the two characters were uncorrelated. However, we did not assume the environmental variances were zero. We therefore calculated the expected phenotypic variance of a trait by dividing the expected genetic variance by the summed per cent variance explained for all QTLs affecting the trait. Finally, the expected phenotypic correlation was calculated by dividing the expected covariance by the square root of the product of the expected phenotypic variances. This procedure assumes that there are no epistatic effects between QTLs and therefore that genotypic values are additive across loci. This assumption was required because epistatic interactions are difficult to estimate using a small population of F_2 individuals because many multi-locus genotypes have little replication. As long as epistatic effects are small compared with additive and dominance effects, however, our procedure should provide a good approximation of the correlations expected from the identified QTLs.

3. Results

(a) Phenotypic distributions

Penstemon neomexicanus and *P. barbatus* differed significantly for all measured phenotypic traits (table 1), despite high levels of variance in nectar traits. Flower colour segregated as a binary trait with 123 individuals having blue-purple flowers and 39 individuals having red flowers. The phenotypic distributions for the remaining traits were continuous and unimodal (figure 2), suggesting a polygenic basis. For all traits except colour, the F_1 phenotype was intermediate between the two parental phenotypes.

(b) Phenotypic correlations

We identified several phenotypic correlations among traits in the F_2 population (figure 3). Interestingly, the direction of all significant correlations was consistent with the floral trait combinations found in the parent species. The most strongly correlated traits were tube length, and short and long stamen lengths, with an average pairwise correlation of 0.79. These strong correlations suggest that pleiotropic loci may affect all three of these traits in the same direction and that QTLs for these traits should co-localize. We designate this set of traits 'TLS'. The set of TLS traits is more weakly correlated with style length (mean correlation = 0.35), suggesting some overlap in loci influencing

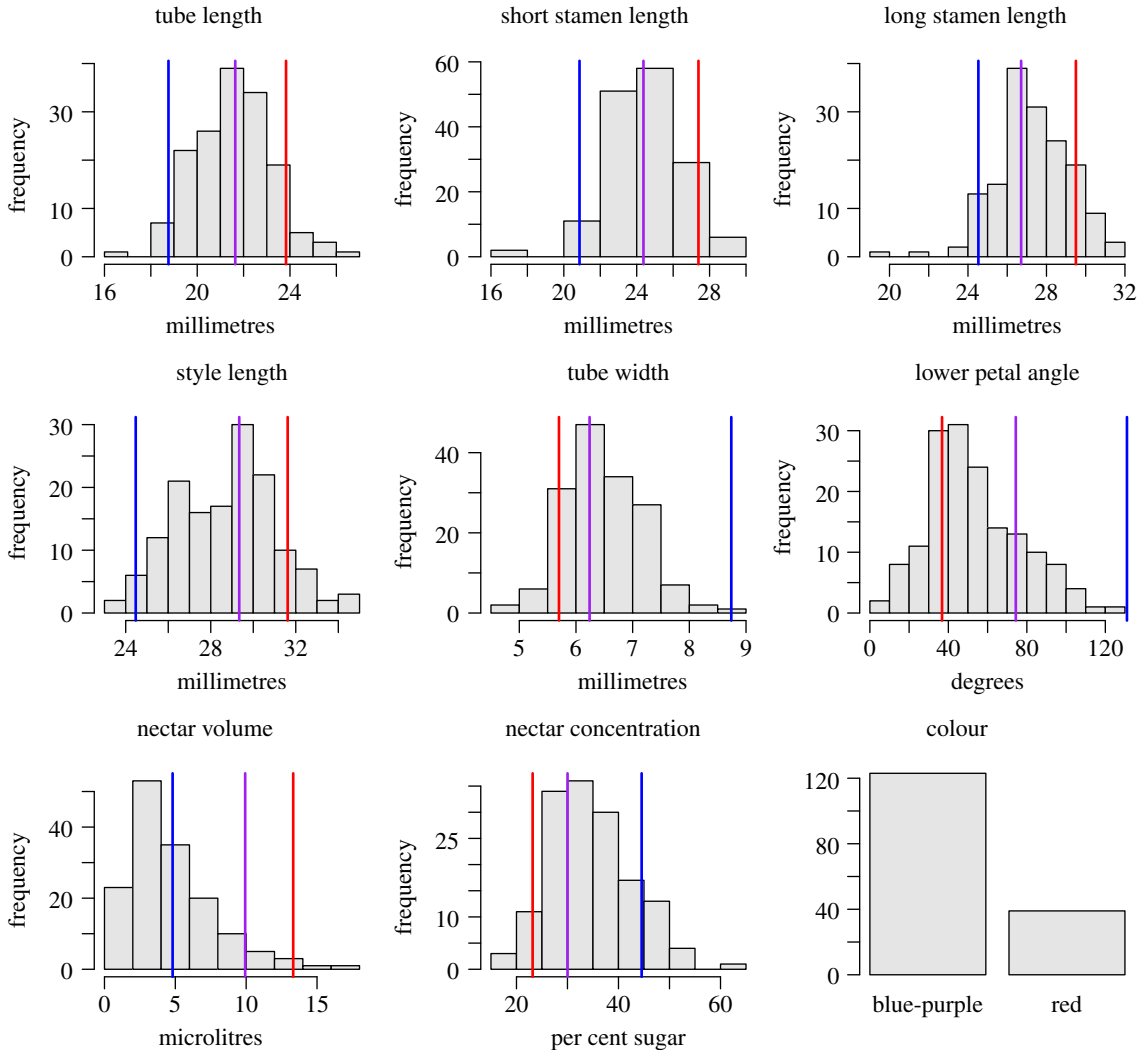

Figure 2. Histograms of floral traits in the F_2 population. Phenotypic means for *P. neomexicanus P. barbatus*, and F_1 hybrids are marked with blue, red and purple lines, respectively.

Table 1. Means and standard deviations for floral traits in *P. neomexicanus*, *P. barbatus* and F_1 hybrids. Reported are the results from *t*-tests comparing the mean phenotypic values for *P. neomexicanus* versus *P. barbatus*. Asterisks denote statistical significance at the level of $p < 0.05$.

	P. neomexicanus ($n = 5$)	*P. barbatus* ($n = 3$)	F_1 ($n = 3$)	*t*-test (one-sided)
tube length (mm)	18.76 ± 0.38	23.83 ± 0.24	21.64 ± 0.23	$t = -11.249$ $p < 0.0001$*
tube height (mm)	7.65 ± 0.16	6.08 ± 0.16	7.33 ± 0.11	$t = 6.990$ $p = 0.0003$*
petal angle (degrees)	131.03 ± 6.37	36.78 ± 15.78	74.36 ± 9.88	$t = 5.538$ $p = 0.0079$*
tube width (mm)	8.74 ± 0.51	5.70 ± 0.35	6.24 ± 0.26	$t = 4.906$ $p = 0.0013$*
short stamen length (mm)	20.86 ± 0.85	27.39 ± 0.04	24.37 ± 0.48	$t = -7.678$ $p = 0.0008$*
long stamen length (mm)	24.53 ± 0.82	29.50 ± 0.53	26.71 ± 0.16	$t = -5.120$ $p = 0.0011$*
style length (mm)	24.47 ± 1.09	31.62 ± 0.25	29.34 ± 0.72	$t = -6.381$ $p = 0.0011$*
nectar volume (μl)	4.81 ± 1.73	13.32 ± 3.06	9.93 ± 2.32	$t = -2.425$ $p = 0.0428$*
nectar concentration (per cent sugar)	44.54 ± 2.84	23.17 ± 1.11	30.01 ± 7.29	$t = 7.018$ $p = 0.0004$*

these traits. Tube width, by contrast, is not significantly correlated with any of the length traits, though it is moderately correlated with nectar concentration. Finally, among the morphological traits, petal angle was moderately negatively correlated with the TLS traits but moderately positively correlated with tube width, suggesting some overlap in loci contributing to species differences in these traits.

Nectar concentration and nectar volume were weakly negatively correlated, as might be expected if increased nectar volume caused a dilution of the sugars present. Although we expected no correlations between nectar traits and morphological traits, nectar concentration was weakly to moderately positively correlated with tube width and petal angle, and negatively correlated with tube length. Finally, nectar volume

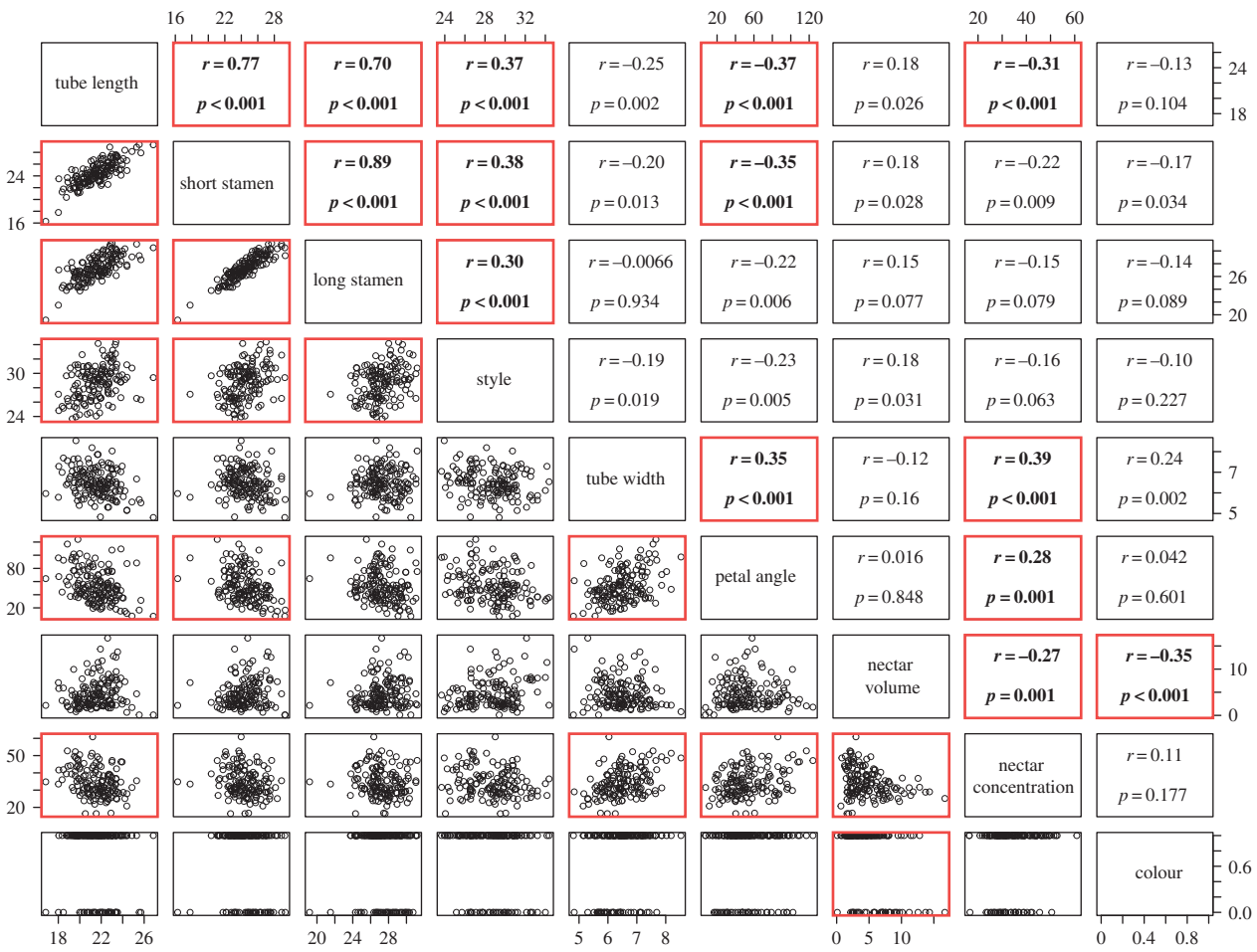

Figure 3. Floral trait correlations in the F_2 population. Scatterplots of all traits are given below the diagonal and Spearman's correlation coefficients and associated *p*-values are given above the diagonal. Statistically significant correlations after a Bonferroni correction ($p < 0.00139$) are in bold and outlined in red.

was correlated with flower colour, with greater nectar volumes associated with red flowers.

(c) Multiplexed shotgun genotyping and linkage map results

We obtained an average of 180 million high-quality reads per sequencing lane from our MSG library preparation. We chose 876 markers that had fixed differences between the two parental samples and that could be confidently genotyped (using a minimum of eight reads per locus) in at least 88 of the 96 F_2 individuals. One F_2 individual that had an unusually low proportion of typed markers was removed; it was likely that this individual had low representation in the set of pooled MSG libraries. After removing duplicate markers, we removed markers displaying significant segregation distortion at $p < 0.000001$. This filtering step removed markers displaying true severe segregation distortion, and also markers we suspect having genotyping error. Even with a minimum of eight reads to genotype a given locus, stochasticity in the sequencing data could cause a true heterozygote to be genotyped as a homozygote, and many of the markers discarded at this step were deficient in heterozygotes (electronic supplementary material, table S2). We constructed a linkage map using a final set of 642 MSG markers. We added the previously genotyped locus *F3′5′h* [13] to this dataset.

We obtained eight LGs (figure 4), the expected chromosome number in these species [26]. The mean number of markers per LG was 80 (minimum = 23, maximum = 138). The total map distance was 1372.2 cM, with a mean distance of 2.2 cM between markers (maximum 38.4 cM). We suspect the excessive length of certain LGs (i.e. 2, 3, 4 and 7) may reflect genotyping errors in our marker dataset not removed through our filtering process. Markers were not distributed as evenly as expected across the linkage map (figure 4). This was probably due to the small size of our mapping population, which contains limited recombination events. If such patterns persist in future studies with larger mapping populations, it could suggest regions of low recombination, perhaps due to genomic inversions between these two species. Nevertheless, given the small mapping population used to generate this linkage map, we consider it to be adequate for the preliminary QTL mapping described here.

(d) QTL mapping results

We identified 23 total QTLs for the nine analysed traits (table 2). For nectar volume, there was one major QTL that explained greater than 51% of the trait variation and 67% of the difference between parental values. Similarly, there was one QTL for colour, which explained nearly two-thirds of the total flower colour variation in the F_2 population and 100% of the difference between parents. This QTL corresponds to *F3′5′h*, which resides directly under the peak LOD (figure 4). We have previously shown that this gene is responsible for the difference in colour between the two species [13]. For each of the remaining traits, there were three significant QTLs, explaining a total of 45–65% of the total proportion of trait variation. Assuming

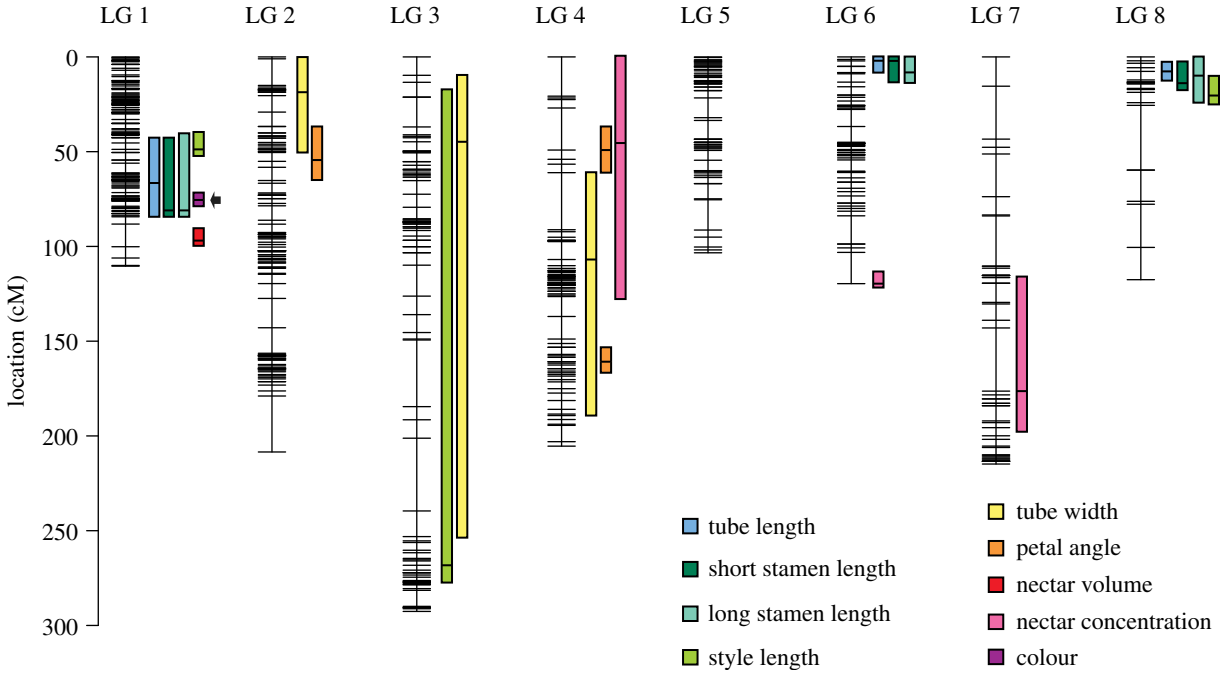

Figure 4. Linkage map and QTL positions marked with 1.5-LOD CIs. Small arrow indicates position of *F3′5′h* on LG 1 at 75.5 cM.

homozygous effects are additive across loci, the QTLs explain between 65 and 100% (mean = 80.7; colour excluded) of the differences between parental values. Given our small mapping population, these values may be somewhat inflated and we cannot rule out the existence of many additional QTLs with small effect.

All QTLs were in the direction of divergence between the two species, i.e. if the trait is larger in *P. barbatus*, the *P. barbatus* allele increases the value of the trait. Degree of dominance ranged from near absence of dominance to overdominance (table 2), though for none of the QTLs with nominal overdominance was the degree of dominance significantly greater than 1.0. Only tube width and style length exhibited directional dominance, with dominance deviation for all three QTLs having the same sign. This is consistent with the observation that for both traits the F_1 value was substantially shifted away from the parental mean towards the mean for *P. barbatus* (figure 2).

We found many areas of the genome where QTLs co-localize (figure 4). QTLs underlying length (TLS) traits were markedly coincident, with nearly identical QTLs for TLS traits on LG 1, 6 and 8. Significant QTLs for style length overlapped with these other length traits on LG 1 and 8. This is consistent with the highly significant pairwise correlations found among these length traits (figure 3). The large effect QTL for flower colour overlapped with the QTLs for length on LG 1, and, although it did not overlap, the large effect QTL for nectar volume was proximal to this cluster of QTLs as well. This clustering may explain the observed significant correlation between flower colour and nectar volume. Furthermore, floral tube width co-localized with lower petal angle on LG 2 and LG 4, along with nectar concentration, perhaps contributing to the observed correlations among these traits. Finally, the LOD CIs for style length and floral tube width overlapped on LG 3, however the peak LOD positions are on different ends of the chromosome.

The expected phenotypic correlations between traits, calculated from the estimated additive effects and dominance values of overlapping QTLs, are strongly correlated with the observed phenotypic correlations (figure 5). In calculating this correlation, we included the pairwise correlations among the TLS traits. However, since these appear to be highly co-localized and highly correlated, for other traits we used the average correlation with each TLS trait. The relationship between expected and observed trait correlation has a correlation coefficient of 0.92 and explains 85% of the variation in the observed correlations. Therefore, the identified QTLs capture the main features of segregating genetic variation among F_2 individuals. This suggests that we have not missed many QTLs with more than negligible effects, in agreement with the finding that the QTLs cumulatively explain most of the differences between parental values. However, the slope of the relationship is 1.88 (figure 5), whereas if the QTLs were a perfect predictor of observed phenotypes the slope would be 1.0. This difference is due largely to higher observed correlations among the TLS traits than expected from the QTL models. This suggests either that we may have failed to identify some important QTLs for these highly correlated traits, or that there was a positive environmental correlation that we were unable to account for (e.g. bigger flowers make values of all three TLS traits higher).

4. Discussion

(a) Genetic basis of pollination syndrome traits in *Penstemon*

Our study constitutes a preliminary examination of the genetic basis of floral trait divergence between *P. neomexicanus* and *P. barbatus*. Although our F_2 mapping population was of modest size, several patterns emerged. First, floral trait variation appears to be due to relatively few genetic loci of medium to large phenotypic effect. Two of the floral traits, flower colour and nectar volume, were each controlled by a single QTL of large effect, with the flower colour QTL completely explaining the between-species difference in floral colour and the nectar volume accounting for 67% of the difference

Table 2. Significant QTL positions, CIs and effect size estimates.

trait	QTL peak position	Peak LOD	1.5-LOD CI	additive effect (s.e.)	dominance deviation (s.e.)	PVE	relative homozygous effect	degree of dominance
tube length	1, 65.5	6.93	42.0–83.5	0.70 (0.15)	0.72 (0.22)	17.80	0.27	1.03
tube length	6, 2.1	4.44	0–8	0.59 (0.15)	−0.43 (0.23)	10.68	0.23	0.73
tube length	8, 5.7	8.97	3.36–10	0.90 (0.17)	−0.52 (0.24)	24.33	0.35	0.58
petal angle	2, 55.1	4.111	36.7–65.2	−9.92 (2.56)	−8.28 (3.98)	11.871	0.21	0.83
petal angle	4, 46	2.646	36–60	−9.75 (3.18)	2.97 (4.64)	7.361	0.20	0.30
petal angle	4, 160.8	4.443	152–166.7	−11.35 (2.91)	−12.88 (4.04)	12.94	0.24	1.13
tube width	2, 18.6	5.90	0–50.4	−0.30 (0.08)	−0.34 (0.11)	15.68	0.20	1.13
tube width	3, 44	4.93	10–265.4	−0.38 (0.08)	−0.10 (0.11)	12.76	0.25	0.26
tube width	4, 113.8	5.32	70–191.3	−0.38 (0.07)	−0.07 (0.10)	13.91	0.25	0.18
short stamen	1, 79.1	6.02	42.0–83.5	0.84 (0.18)	0.58 (0.26)	17.01	0.26	0.69
short stamen	6, 2.1	3.86	0–13.3	0.70 (0.18)	−0.26 (0.27)	10.31	0.21	0.37
short stamen	8, 12.2	5.94	3.4–16	0.79 (0.20)	−0.53 (0.29)	16.76	0.24	0.67
long stamen	1, 79.1	5.384	39.5–83.5	0.68 (0.19)	0.78 (0.27)	16.74	0.27	1.15
long stamen	6, 8	3.651	0–13.3	0.70 (0.18)	−0.36 (0.27)	10.86	0.28	0.51
long stamen	8, 6	4.974	0–22	0.89 (0.20)	−0.08 (0.29)	15.3	0.36	0.09
style length	1, 49.0	4.21	39.5–52	1.08 (0.24)	−0.20 (0.35)	11.88	0.30	0.19
style length	3, 270	4.47	20–280	1.07 (0.24)	−0.37 (0.34)	12.69	0.30	0.35
style length	8, 14.3	4.62	8–24	0.88 (0.27)	−0.71 (0.37)	13.17	0.25	0.81
nectar volume	1, 97.5	14.29	91.5–99.5	2.83 (0.34)	−2.24 (0.53)	51.47	0.67	0.79
nectar concentration	4, 38	4.25	0–126	−4.36 (1.03)	0.74 (1.49)	12.14	0.41	0.17
nectar concentration	6, 119.6	4.59	114–119.6	−4.35 (1.35)	−7.69 (1.65)	13.23	0.41	1.77
nectar concentration	7, 180.5	4.82	118–195.6	−3.34 (0.85)	−4.79 (1.53)	13.99	0.31	1.43
colour	1, 75.46	21.6	72.5–79.1			65.67		

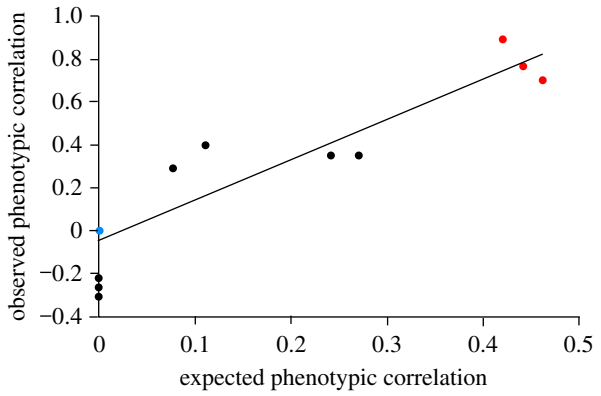

Figure 5. Relationship between observed phenotypic correlation and expected phenotypic correlation. Expected correlation is based on estimated additive effect and dominance deviation of QTLs. Each point represents a pair of traits (black and blue circles) or the average correlation between a trait and the three TLS traits (red circles). The blue circle represents eight points superimposed.

between the parental species. However, since the F_1 and F_2 mean nectar volumes do not fit the expected pattern under a single-locus model (figure 2), there may be additional QTLs for nectar volume that our analysis was too weak to identify or epistatic effects for which our analysis did not control. The remaining seven traits were each controlled by three QTLs of medium effect sizes. Assuming additivity of effect sizes, the QTLs for these traits account for between 65 and 91% (mean 78%) of the differences between parental means. Since the effect sizes of small effect QTLs can be overestimated in small mapping populations such as ours [27], we must interpret our results cautiously. Nevertheless, these results suggest that for all traits examined, evolutionary divergence was achieved largely by substitutions of medium to large effect. Presumably, there are additional loci of small effect involved, but these will be more difficult to detect. We also note that because a given QTL may harbour multiple loci affecting a trait, our estimate of the number of loci is a minimum. However, we believe this is unlikely for at least some of the traits (see below).

(b) QTLs of large effect size for colour and nectar volume

Nectar is a primary reward for pollinators and the amount produced varies with type of pollinator. As is true for *P. barbatus*,

hummingbird-pollinated flowers typically produce larger volumes of more dilute nectar compared with insect-pollinated plants [4]. This difference between *P. barbatus* and *P. neomexicanus* is due largely to a single QTL that explains about two-thirds of the difference between the parental species. Large effect QTLs have also been found to contribute to differences in nectar volume between bee- and hawkmoth-pollinated species of *Petunia* [11] and between bee- and hummingbird-pollinated species of *Mimulus* [9]. However, variation in nectar volume between insect- and hummingbird-pollinated species of *Ipomopsis* seems to be controlled by several small effect QTLs [15].

Nectar volume in particular may be an important first step in attracting a novel pollinator, particularly for hummingbirds [28]. The large effect QTL for nectar volume in *Mimulus* by itself influences pollinator attraction [29,30]. Moreover, addition of artificial nectar to *Penstemon spectabilis* (bee syndrome) flowers significantly increased hummingbird visitation [31]. Although this has not been experimentally tested, we expect that attraction of hummingbird pollinators requires a quantum jump in nectar volume and that incremental increases would not be likely to be detected by hummingbirds and hence would probably not affect hummingbird visitation or increase pollen export by bird pollinators. If true, this would explain the predominance of large effect QTLs involved in the evolution of increased nectar production in species pollinated by hummingbirds. This argument assumes that bird pollination is derived from insect pollination, as appears to be the case in *Penstemon* [6,7] and *Mimulus* [32]. However, bird pollination is considered derived in *Ipomopsis* [15], in which QTL effects on nectar volume are small. Experiments testing the effect of nectar volume on hummingbird visitation as well as additional information from other species are clearly needed to evaluate this explanation for large effect nectar volume QTLs.

Bradshaw *et al.* [9] found that the QTL with large effect on nectar volume also substantially decreased nectar sugar concentration. This pattern could be explained by a dilution effect if the causal locus simply determined how much water was secreted with a constant amount of sugar. In *P. barbatus*, however, we did not find this pattern. Although we detected three QTLs of moderately large effect influencing nectar concentration, none of them co-localized with the QTL for nectar volume. These two traits thus evolved independently. Although we cannot be sure of the order in which these two traits evolved, we believe the most reasonable expectation is that increased volume evolved first to attract and maintain visits by hummingbirds. It remains unclear whether decreased nectar sugar concentration is a positive adaptation for hummingbird pollination, functions to deter bee pollination or is an adaptation to favour energy savings for the plant, allowing reallocation to other fitness-enhancing functions [33,34]. Regardless, hummingbird pollinators continue to visit *P. barbatus* even though sugar concentration is relatively low. We suggest that once a large nectar volume had evolved, bird pollinators could be retained despite reductions in sugar concentration. Mutations that reduced allocation of sugars to nectar might then be favoured.

A single QTL of large effect was identified for the colour difference between *P. neomexicanus* and *P. barbatus*. This QTL corresponds to the gene *F3′5′h*. Wessinger & Rausher [13] demonstrated using co-segregation and functional analyses that this gene is redundantly non-functional in *P. barbatus* and completely explains the difference in flower colour between the two species. Similar large effect flower colour QTLs have been reported frequently for adaptation to different pollinators between pairs of species within *Aquilegia* [35], *Ipomopsis* [15], *Iris* [14,17], *Iochroma* [36], *Mimulus* [9,37] and *Petunia* [10,18]. One partial explanation for this pattern is that many flower colour transitions involve deactivation of parts or all of the anthocyanin pathway, which can be accomplished by inactivating mutations in one or a few genes [10,13,36–38]. In cases involving gain of function (e.g. *Ipomopsis*), a simple reversal of an inactivating substitution, particularly in a *cis*-regulatory region, might also be achieved by a single mutation and thus produce a QTL of large effect. Another, not mutually exclusive explanation for large effect flower colour QTLs is that small changes in colour may not substantially affect pollinator visitation. For example, in bird syndrome flowers, one primary function of red coloration is believed to be exclusion of bee pollinators because bees cannot easily distinguish red against a background of green vegetation [39]. Small shifts away from a blue, pink, purple or white ancestral state may not reduce contrast sufficiently to reduce bee visitation, whereas a major change to red is known to reduce visitation by bees and other insects [29,30,40,41].

(c) QTLs of small to medium effect for other characters

The remaining morphological characters examined each exhibited three QTLs of small to medium effect sizes (mean effect size as proportion of difference between species means $= 0.26 \pm 0.05$). This result is consistent with studies in other systems identifying multiple QTLs with small to medium effect for similar traits [11,14,15].

(d) Phenotypic correlations and QTL colocalization

We observed several interesting phenotypic correlations that generally corresponded to co-localization of QTLs. First, variation in TLS traits was highly correlated in the F_2 population, indicating the presence of shared or linked genetic control, and QTLs for these traits co-localized in a striking pattern. We identified three significant QTLs at nearly identical positions on LG 1, 6 and 8. For all three cases, the QTLs on LG 1 and 8 explained a larger proportion of the variation than the QTL on LG 6. Furthermore, the additive allelic effects and dominance deviations for each of these QTLs were similar across the different traits (table 2). Given these similarities, it is likely that each of these QTLs corresponds to a single locus affecting all three traits pleiotropically. It is theoretically possible that selection for strong character correlations could bring loci affecting different traits into tight linkage in genomic inversions [42], but we find it difficult to believe that this would occur in three different genomic locations. This inference is supported by the existence of loci affecting lengths of multiple floral characters identified in *Petunia* [18,43], *Solanum* [44] and other model systems [19].

There was greater variation for style length in the F_2 population and also weaker correlations between this trait and the other length traits. This may be the biological reality, or it may be due in part to measurement error. This trait was measured with digital callipers unlike the remaining traits that were measured in ImageJ from digital photographs. However, QTLs for this trait overlapped with the TLS QTLs on LGs 1 and 8, suggesting the presence of underlying loci that pleiotropically

affect both style length and TLS characters, which would give rise to the observed positive phenotypic correlations.

We identified several other correlations among floral traits and corresponding QTL co-localization. One interesting finding was the significant correlation between nectar volume and flower colour ($\rho = 0.35$, $p < 0.001$), with red flowers associating with higher nectar volumes. As discussed, both traits are due to major loci with large phenotypic effects. This correlation is probably due to modest genetic linkage of the two major QTLs underlying these traits. We suspect that several other character correlations are due to QTL linkage rather than pleiotropy, because the QTLs do not entirely overlap and different developmental systems are probably involved (e.g. petal angle and nectar concentration, tube width and nectar concentration). Overall, the high correlation between observed trait correlations and correlations expected from QTL co-localization suggests that the observed phenotypic correlations are largely explained by co-localization. Future fine-mapping experiments may elucidate whether the co-localization is due to pleiotropy or tight linkage.

(e) Lack of antagonistic pleiotropy

A striking pattern to emerge from our analysis is that all observed correlations are in the direction of adaptation. By this we mean that when two traits have higher values in *P. barbatus*, if those two traits are correlated in the F_2s, they have a positive correlation (e.g. positive correlations among TLS traits). By contrast, if one trait has a higher value in *P. barbatus* while a second has a lower value, then the correlation is negative (e.g. negative correlations between nectar volume and nectar concentration, and between tube length and tube width). Moreover, the same pattern is seen for co-localized QTLs: the sign of the additive effect of both traits is always in the direction of the sign of the change from *P. neomexicanus* to *P. barbatus*.

This pattern is easy to understand if co-localization of QTLs for two traits is due to tight linkage of two loci, each of which affects only one of the traits. In this situation, presumably adaptive mutations occurred and were fixed independently at the two loci. In a moderately sized F_2 population, the QTLs for these two loci would co-localize and the effects at both loci would be adaptive. By contrast, if co-localization is due largely to pleiotropic effects at individual loci, then the question arises as to why none of the QTLs exhibit antagonistic pleiotropy, i.e. exhibit effects in the direction of adaptation for one trait but opposite the direction of adaptation for another trait. Two answers to this question seem possible. One is that developmental constraint is such that mutational covariance is almost always in the direction of adaptation. We believe that this may be likely for the TLS traits because mutations that alter overall floral length may also affect the lengths of other floral organs in the same direction. The second explanation is that mutations exhibiting antagonistic pleiotropy are less favoured by natural selection. Such mutations have a lower net selective coefficient than mutations without antagonistic pleiotropy, because there is a deleterious effect on one of the traits. Since the probability of fixation of a mutation is proportional to the selection coefficient, pleiotropic mutations without antagonistic pleiotropy will therefore have a greater probability of fixation.

We suspect that both of these possibilities account for our failure to find evidence of antagonistic pleiotropy among QTLs that co-localize. On the one hand, for the reasons described above, we suspect that the mutation spectrum is highly constrained for the TLS traits, such that most mutations affecting one of these traits will affect the others in the same direction. On the other hand, for the correlations involving one morphological trait and one non-morphological trait (e.g. nectar volume and flower colour), we suspect that co-localizations arise from linkage of separate, trait-specific loci because we know of no obvious functional or developmental connections among the correlated traits. Finally, co-localization of loci affecting tube width and petal angle, or TLS traits and petal angle, are conceivably due to pleiotropy arising because each of these traits is determined by a common process of development. Clearly, additional work is needed to evaluate these hypotheses. Taken at face value, though, the absence of antagonistic pleiotropy suggests that most hummingbird syndrome traits evolve independently and are not constrained by pleiotropy.

Acknowledgements. We are grateful to Paul Wilson for seeds and Duke University Greenhouse for excellent plant care. We thank Nick McCool for assistance generating the MSG libraries and John K. Kelly for bioinformatic and QTL mapping advice.

Funding statement. This study was supported by research funds from the University of Kansas to L.C.H., including a GRF award, and NFS-IOS-1255808 and also by research funds from an NSF Grant DEB0841521 to M.D.R.

References

1. Grant V. 1949 Pollination systems as isolating mechanisms in angiosperms. *Evolution* **3**, 82–97. (doi:10.2307/2405454)
2. Baker HG. 1963 Evolutionary mechanisms in pollination biology. *Science* **139**, 877–883. (doi:10.1126/science.139.3558.877)
3. Stebbins GL. 1970 Adaptive radiation of reproductive characteristics in angiosperms, I: pollination mechanisms. *Annu. Rev. Ecol. Syst.* **1**, 307–326. (doi:10.1146/annurev.es.01.110170.001515)
4. Grant KA, Grant V. 1968 *Hummingbirds and their flowers*. New York, NY: Columbia University Press.
5. Castellanos MC, Wilson P, Thomson JD. 2004 'Anti-bee' and 'pro-bird' changes during the evolution of hummingbird pollination in *Penstemon* flowers. *J. Evol. Biol.* **17**, 876–885. (doi:10.1111/j.1420-9101.2004.00729.x)
6. Wolfe AD, Randle CP, Datwyler SL, Morawetz JJ, Arguedas N, Diaz J. 2006 Phylogeny, taxonomic affinities, and biogeography of *Penstemon* (Plantaginaceae) based on ITS and cpDNA sequence data. *Am. J. Bot.* **93**, 1699–1713. (doi:10.3732/ajb.93.11.1699)
7. Wilson P, Wolfe AD, Armbruster WS, Thomson JD. 2007 Constrained lability in floral evolution: counting convergent origins of hummingbird pollination in *Penstemon* and *Keckiella*. *New Phytol.* **176**, 883–890. (doi:10.1111/j.1469-8137.2007.02219.x)
8. Wilson P, Castellanos MC, Hogue JN, Thomson JD, Armbruster WS. 2004 A multivariate search for pollination syndromes among penstemons. *Oikos* **104**, 345–361. (doi:10.1111/j.0030-1299.2004.12819.x)
9. Bradshaw Jr HD, Wilbert SM, Otto KG, Schemske DW. 1995 Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). *Nature* **376**, 762–765. (doi:10.1038/376762a0)

10. Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J, Koes R. 1999 Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell* **11**, 1433–1444. (doi:10.1105/tpc.11.8.1433)
11. Stuurman J, Hobollah ME, Broger L, Moore J, Basten C, Kuhlemeier C. 2004 Dissection of floral pollination syndromes in petunia. *Genetics* **168**, 1585–1599. (doi:10.1534/genetics.104.031138)
12. Galliot C, Stuurman J, Kuhlemeier C. 2006 The genetic dissection of floral pollination syndromes. *Curr. Opin. Plant Biol.* **9**, 78–82. (doi:10.1016/j.pbi.2005.11.003)
13. Wessinger CA, Rausher MD. 2014 Predictability and irreversibility of genetic changes underlying flower color evolution in *Penstemon barbatus*. *Evolution* **68**, 1058–1070. (doi:10.1111/evo.12340)
14. Brothers AN, Barb JG, Ballerini ES, Drury DW, Knapp SJ, Arnold ML. 2013 Genetic architecture of floral traits in *Iris hexagona* and *Iris fulva*. *J. Hered.* **104**, 853–861. (doi:10.1093/jhered/est059)
15. Nakazato T, Rieseberg LH, Wood TE. 2013 The genetic basis of speciation in the *Giliopsis* lineage of *Ipomopsis* (Polemoniaceae). *Heredity* **111**, 227–237. (doi:10.1038/hdy.2013.41)
16. Bradshaw Jr HD, Otto KG, Frewen BE, McKay JK, Schemske DW. 1998 Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (Mimulus). *Genetics* **149**, 367–382.
17. Bouck A, Wessler SR, Arnold ML. 2007 QTL analysis of floral traits in Lousiana Iris hybrids. *Evolution* **61**, 2308–2319. (doi:10.1111/j.1558-5646.2007.00214.x)
18. Hermann K, Klahre U, Moser M, Sheehan H, Mandel T, Kuhlemeier C. 2013 Tight genetic linkage of prezygotic barrier loci creates a multifunctional speciation island in *Petunia*. *Curr. Biol.* **23**, 873–877. (doi:10.1016/j.cub.2013.03.069)
19. Hermann K, Kuhlemeier C. 2011 The genetic architecture of natural variation in flower morphology. *Curr. Opin. Plant Biol.* **14**, 60–65. (doi:10.1016/j.pbi.2010.09.012)
20. Abramoff MD, Magalhaes PJ, Ram SJ. 2004 Image processing with ImageJ. *Biophotonics Int.* **11**, 36–42.
21. Andolfatto P, Davison D, Erezyilmaz D, Hu TT, Mast J, Sunayama-Morita T, Stern DL. 2011 Multiplexed shotgun genotyping for rapid and efficient genetic mapping. *Genome Res.* **21**, 610–617. (doi:10.1101/gr.115402.110)
22. Doyle JJ, Doyle JL. 1987 A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **19**, 11–15.
23. Catchen JM, Amores A, Hohenlohe P, Cresko W, Postlethwait JH. 2011 *Stacks*: building and genotyping loci *de novo* from short-read sequences. *G3: Genes, Genomes Genet.* **1**, 171–182.
24. Broman KW, Wu H, Sen S, Churchill GA. 2003 R/qtl: QTL mapping in experimental crosses. *Bioinformatics* **19**, 889–890. (doi:10.1093/bioinformatics/btg112)
25. Broman K. 2010 Genetic map construction with R/qtl. Technical Report #214, University of Wisconsin-Madison, Department of Biostatistics and Medical Informatics.
26. Freeman CC. 1983 Chromosome numbers in Great Plains species of *Penstemon* (Scrophulariaceae). *Brittonia* **35**, 232–238. (doi:10.2307/2806022)
27. Beavis WD. 1994 The power and deceit of QTL experiments: lessons from comparative QTL studies. In *Proc. 49th Annual Corn and Sorghum Research Conference* (ed. DB Wilkinson), pp. 250–266. Washington, DC: American Seed Trade Organization.
28. Thomson JD, Wilson P. 2008 Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *Int. J. Plant Sci.* **169**, 23–38. (doi:10.1086/523361)
29. Schemske DW, Bradshaw Jr HD. 1999 Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proc. Natl Acad. Sci. USA* **96**, 11 910–11 915. (doi:10.1073/pnas.96.21.11910)
30. Bradshaw Jr HD, Schemske DW. 2003 Allele substitution at a flower color locus produces a pollinator shift in monkeyflowers. *Nature* **426**, 176–178. (doi:10.1038/nature02106)
31. Wilson P, Jordan EA. 2009 Hybrid intermediacy between pollination syndromes in *Penstemon*, and the role of nectar in affecting hummingbird visitation. *Botany* **87**, 272–282. (doi:10.1139/B08-140)
32. Beardsley PM, Payette S, Fortin MJ, Olmstead RG. 2003 AFLP phylogeny of *Mimulus* section Erythranthe and the evolution of hummingbird pollination. *Evolution* **57**, 1397–1410. (doi:10.1111/j.0014-3820.2003.tb00347.x)
33. Baker HG. 1975 Sugar concentrations in nectars from hummingbird flowers. *Biotropica* **7**, 37–41. (doi:10.2307/2989798)
34. Bolten AB, Feinsinger P. 1978 Why do hummingbird flowers secrete dilute nectar? *Biotropica* **10**, 307–309. (doi:10.2307/2387684)
35. Hodges SA, Whittall JB, Fulton M, Yang JY. 2002 Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Am. Nat.* **159**, S51–S60. (doi:10.1086/338372)
36. Smith SD, Rausher MD. 2011 Gene loss and parallel evolution contribute to species difference in flower color. *Mol. Biol. Evol.* **28**, 2799–2810. (doi:10.1093/molbev/msr109)
37. Streisfeld MA, Rausher MD. 2009 Altered *trans*-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. *Mol. Biol. Evol.* **26**, 433–444. (doi:10.1093/molbev/msn268)
38. Des Marais DL, Rausher MD. 2010 Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in *Ipomoea*. *Evolution* **64**, 2044–2054.
39. Spaethe J, Tautz J, Chittka L. 2001 Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proc. Natl Acad. USA* **98**, 3898–3903. (doi:10.1073/pnas.071053098)
40. Streisfeld MA, Kohn JR. 2007 Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *J. Evol. Biol.* **20**, 122–132. (doi:10.1111/j.1420-9101.2006.01216.x)
41. Streisfeld MA, Young WN, Sobel JM. 2013 Divergent selection drives genetic differentiation in an R2R3-MYB transcription factor that contributes to incipient speciation in *Mimulus aurantiacus*. *PLOS Genet.* **9**, e1003385. (doi:10.1371/journal.pgen.1003385)
42. Feldman MW. 1972 Selection for linkage modification: I. Random mating populations. *Theoret. Popul. Biol.* **3**, 324–346. (doi:10.1016/0040-5809(72)90007-X)
43. Venail J, Dell'Olivo A, Kuhlemeier C. 2010 Speciation genes in the genus *Petunia*. *Phil. Trans. R. Soc. B* **365**, 461–468. (doi:10.1098/rstb.2009.0242)
44. Chen KY, Cong B, Wing R, Vrebalov J, Tanksley SD. 2007 Changes in regulation of a transcription factor lead to autogamy in cultivated tomatoes. *Science* **318**, 643–645. (doi:10.1126/science.1148428)