Quantitative Trait Loci Affecting Differences in Floral Morphology Between Two Species of Monkeyflower (Mimulus)

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ABSTRACT

Conspicuous differences in floral morphology are partly responsible for reproductive isolation between two sympatric species of monkeyflower because of their effect on visitation of the flowers by different pollinators. *Mimulus lewisii* flowers are visited primarily by bumblebees, whereas *M. cardinalis* flowers are visited mostly by hummingbirds. The genetic control of 12 morphological differences between the flowers of *M. lewisii* and *M. cardinalis* was explored in a large linkage mapping population of F_2 plants ($n = 465$) to provide an accurate estimate of the number and magnitude of effect of quantitative trait loci (QTLs) governing each character. Between one and six QTLs were identified for each trait. Most (9/12) traits appear to be controlled in part by at least one major QTL explaining ≥25% of the total phenotypic variance. This implies that either single genes of individually large effect or linked clusters of genes with a large cumulative effect can play a role in the evolution of reproductive isolation and speciation.

THE genetic basis of adaptation depends upon the number, magnitude of effect, and mode of action of quantitative trait loci (QTLs) controlling adaptive traits (Maynard Smith 1983; Barton and Charlesworth 1984; Coyne 1992), but the values of these parameters have been debated largely in the absence of empirical data (Orr and Coyne 1992). QTL mapping has been suggested as an experimental method to distinguish between Fisher's infinitesimal model, in which quantitative traits are controlled by a very large number of loci, each with a very small phenotypic effect (Fisher 1930), and the oligogenic model, which postulates that continuous phenotypic variation may be because of a few loci with very large effects (reviewed in Tanksley 1993).

We have searched for QTLs affecting floral traits likely to be involved in reproductive isolation between natural populations of two species of monkeyflower: *Mimulus lewisii* and *M. cardinalis* (Figure 1). The pale pink flowers of *M. lewisii* have broad, flat petals and yellow nectar guides and are pollinated primarily by bumblebees (Figure 1A). The red flowers of *M. cardinalis* have reflexed petals, forming a tubular corolla, and are pollinated by hummingbirds (Figure 1B). The relative position of the stigma and anthers differs greatly between the two Mimulus species; they are inserted within the corolla in *M. lewisii* (Figure 1C), requiring visiting bumblebees to crawl into the corolla tube to effect pollination, but they are exserted in *M. cardinalis* to make contact with the forehead of hummingbirds probing the corolla tube (Figure 1D). The two Mimulus species are largely allopatric throughout their ranges in western North America because of different elevational distribution, but in the zone of sympatry in the Sierra Nevada mountains of California, interbreeding is rare or nonexistent (Hiesey *et al.* 1971). However, when the two species are hybridized by artificial means, the F_1 plants are vigorous and fertile, suggesting that barriers to gene flow between *M. lewisii* and *M. cardinalis* in nature are partly because of differential rates of visitation or pollen transfer by bumblebees and hummingbirds, and that pollinators discriminate between the two Mimulus species based on flower morphology (Hiesey *et al.* 1971).

In this Mimulus system, we suggest that the genetics of reproductive isolation in sympatry can be understood as the genetics of differences in floral structure, and that the genetics of floral structure can be determined by mapping the QTLs that control characters such as flower color, corolla shape and size, nectar volume, and pollinator access to the plant reproductive organs.

In a previous study we used this approach to investigate the genetic basis of floral traits presumed to affect pollinator attraction, reward, and the efficiency of pollen removal and deposition in a *M. lewisii* × *M. cardinalis* F_2 population (Bradshaw *et al.* 1995). For each of the eight floral traits measured, we found at least one QTL explaining ≥25% of the total phenotypic variation and concluded that reproductive isolation in this system may

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be partly because of genes of large effect. Here we report the results of a much-expanded effort to characterize the genetic basis of floral traits in these species. We have increased the number of traits measured from eight to 12 and increased the size of the mapping from 93 to 465 F_2 progeny. The large F_2 sample in this study was intended to reduce bias in estimating QTL number and magnitude of effect (Beavis 1994, 1998).

MATERIALS AND METHODS

Mapping pedigree construction: Seeds of *M. lewisii* and *M. cardinalis* were collected in Yosemite National Park, California. The *M. lewisii* seed source population is from Tioga Road and the *M. cardinalis* from Wawona. Two *M. lewisii* parent plants, L1 and L2, and two *M. cardinalis*, C1 and C3, were grown in the University of Washington Botany greenhouse from field-collected seed. The following reciprocal intra- and interspecific crosses were made (female parent listed first): L1 × L2, C3 × C1, L1 × C1, C3 × L2. A single F_1 plant, LC1, was grown from seeds collected from the L1 × C1 cross, and another F_1 plant, CL2, was grown from C3 × L2 seeds. These two F_1 plants were mated reciprocally to produce F_2 plants with either a *M. lewisii* (LC1 × CL2) or a *M. cardinalis* (CL2 × LC1) cytoplasmic background. By outcrossing the F_1 plants to produce the F_2, we prevented segregation distortion due to homozygosity of deleterious recessive alleles.

Phenotypic trait measurements: Seeds of each of the two pure parental (L1 × L2 and C3 × C1), F_1 (L1 × C1 and C3 × L2), and F_2 crosses (LC1 × CL2 and CL2 × LC1) were sown in a peat/pumice soil mix. Lighting was a mixture of ambient sunlight and mercury vapor lamps with 17-hr days. Five weeks after the seeds were sown, seedlings were drawn at random from each cross type and transplanted to 4000-cm^3 pots. Thirty plants each of C3 × C1, L1 × L2, L1 × C1, and C3 × L2 and 240 plants each of LC1 × CL2 and CL2 × LC1 were placed in a completely randomized 24-row × 25-column matrix at 25-cm × 25-cm spacing in the greenhouse. Plants were flood-irrigated to provide continuous access to water and fertilized as necessary. Prior to flowering, 15 of the 480 F_2 plants died and were not available for analysis.

Twelve floral traits were measured in the parental, F_1, and F_2 plants. The mean of two randomly drawn flowers per plant was used as an estimate of each phenotypic trait value. Petal anthocyanin concentration (purple pigment) was estimated by punching 6-mm disks from the lateral petals, extracting the anthocyanins with 0.5-ml methanol/0.1% HCl, and determining the absorbance at 510 nm. Petal carotenoid concentration (yellow pigment) was estimated similarly, using methylene chloride for extraction and measuring absorbance at 450 nm. Petal width was measured on a flattened lateral petal. Corolla width and projected area of the corolla were determined by analysis (NIH Image; http://rsb.info.nih.gov/nih-image) of video images of whole flowers taken in a plane perpendicular to the long axis of the corolla tube, which approximates the viewing angle of approaching pollinators. The degree of petal reflexing was estimated separately for the upper pair and lateral pair of petals, by visually ranking the reflexing on a scale of one to five, with one being assigned to the unreflexed petals typical of *M. lewisii* and five given to petals fully reflexed against the corolla tube as they are in *M. cardinalis*. Nectar volume was measured with a graduated pipet tip. Stamen and pistil length were measured with digital calipers from the base of the calyx to the center of the anther on the longest stamen or to the cleft in the stigma. Corolla aperture width and height were measured from the video images.

Genotypic data collection: DNA was extracted from each F_2 plant using a FastPrep homogenizer and FastDNA extraction kits (BIO 101, Vista, CA). Random amplified polymorphic DNA (RAPD) reactions were done essentially as described by Williams *et al.* (1990), except that the final reaction volume was 10 μl with 1 ng of template DNA, and the thermocyling conditions were reduced to 35 cycles of 5 sec at 95°, 30 sec at 36°, and 60 sec at 72°. RAPD primers were obtained from Operon Technologies (Alameda, CA). Reaction products were visualized on ethidium-stained 1.4% agarose gels. Each gel was scored by two people working independently, and discrepancies were resolved by carrying out additional RAPD reactions if necessary. A single dominant flower color marker, *yup* (*y*ellow *up*per; Hiesey *et al.* 1971; Bradshaw *et al.* 1995), was also scored.

Linkage map construction: DNA was extracted from each of the two F_1 (LC1, CL2) and 465 F_2 plants. A pooled sample of five L1 × L2 plants was used to represent *M. lewisii* parental DNA, and five C3 × C1 offspring were pooled to represent the *M. cardinalis* parents. RAPD markers were chosen to give genome coverage as complete as possible with the minimum number of RAPD primers based upon the results of our previous mapping work (Bradshaw *et al.* 1995). A total of 66 RAPD markers (from 22 primers) were scored, of which 63 were inherited in a dominant manner (band-present allele dominant to the band-absent allele) and three were codominant. Because dominant markers linked in repulsion are not very informative for linkage analysis (Ott 1985; Knapp *et al.* 1995), the genotypic data were divided into two sets of coupling-phase markers. One set represents the *M. lewisii* genome (dominant markers homozygous recessive in the *M. lewisii* parent plants) and the other represents the *M. cardinalis* genome. Codominant marker genotypes were included in both data sets. MAPMAKER 3.0 (Lander *et al.* 1987) was used to infer linkage relationships and marker order, with thresholds for linkage of LOD ≥7 at a maximum distance of 40 cM, and for order at a LOD ≥2. Not all markers could be ordered unambiguously at LOD ≥2, so a framework set of markers was chosen with approximately equal spacing between markers, having as many markers as possible in common with our previous map (Bradshaw *et al.* 1995). The framework marker map order is supported at LOD ≥2.

Initially, separate linkage maps were made for the two F_2 subpopulations with different cytoplasmic backgrounds (reciprocal crosses LC1 × CL2 and CL2 × LC1). Because the most likely order of framework markers was found to be the same in the LCCL and CLLC subpopulations, a consensus map using recombination data from all 465 F_2 plants was constructed for QTL analysis to improve QTL detection power.

QTL mapping: QTL mapping was done first with MAPMAKER/QTL 1.1 (Lincoln *et al.* 1992) to permit a direct comparison with previously published QTL maps (Bradshaw *et al.* 1995). The consensus map was used with phenotypic and genotypic data from all 465 F_2 plants. A threshold for detection of LOD ≥2.7 was used, giving a genome-wide nominal significance level of $\sim P = 0.05$ (Lander and Botstein 1989; Bradshaw *et al.* 1995). Phenotypic data for traits with severely skewed distributions (anthocyanin concentration, carotenoid concentration, and nectar volume) were square-root-transformed to improve normality.

QTLs tentatively identified in MAPMAKER/QTL were examined with QTL Cartographer (Zeng 1994) using untransformed phenotypic data. QTL Cartographer was used in its interval mapping mode (Model 3), the same method employed by MAPMAKER/QTL, for all 12 traits and both coupling-phase linkage maps. The Model 3 likelihood ratio profile for each trait was examined for evidence of multiple QTLs

on a single linkage group, using the criterion of a likelihood ratio decline of ≥9 (approximately equivalent to a LOD score decline of 2) between adjacent peaks. If evidence for more than one QTL peak was found, the trait was analyzed by composite interval mapping with Model 6 (number of background parameters set to 5; window size set to 10). For all Model 3 and Model 6 analyses in QTL Cartographer, 100 permutations (Churchill and Doerge 1994) of the phenotypic data were done, and the threshold value of the test statistic chosen to give an experiment-wise Type I error rate of $P < 0.05$ for declaring the presence of a QTL. Churchill and Doerge (1994) recommend using 1000 permutations per trait; however, this proved to be too demanding computationally for the full data set. For the simplest model (Model 3) alone it took more than a month on a SPARCstation LX (Sun Microsystems, Palo Alto, CA) to perform 100 permutation tests for the 12 phenotypic traits and the two coupling-phase maps. There was very close agreement between the threshold values determined for each of the two coupling-phase maps.

The magnitude of QTL effect was estimated as the percent of F_2 phenotypic variance explained (PVE; Paterson *et al.* 1988; Stuber *et al.* 1992; Bradshaw *et al.* 1995; Paterson *et al.* 1995). An arbitrary criterion of ≥25% PVE was used to define a major QTL.

QTL mode of action was taken to be the most likely of three constrained models (dominant, recessive, additive) tested using the "try" command in MAPMAKER/QTL. If one of these models was more likely than the others by a LOD difference of >1, that was considered to be strong evidence in favor of the proposed mode of action.

Segregation distortion: Segregation ratios different from the 3:1 expected for dominant RAPD markers or the 1:2:1 expected from codominant RAPD markers were examined for significance ($P \leq 0.01$) using a χ^2 test with one or two degrees of freedom, respectively.

RESULTS

Phenotypic trait distributions: *M. lewisii* and *M. cardinalis* have strikingly different floral morphology, consistent with their differences in pollinators (Figure 1). We identified 12 floral traits that distinguish the two Mimulus species (Table 1). The traits were divided into three broad categories: pollinator attraction, pollinator reward, and pollen transfer efficiency (Bradshaw *et al.* 1995). Attraction traits included flower color, size, and shape characters, such as pigment concentration (anthocyanin and carotenoid), petal size, corolla width and projected area, and two petal-reflexing metrics. Reward was determined by nectar volume. Efficiency traits were stamen and pistil length and corolla aperture height and width, characters expected to play a role in pollen removal and deposition by animal pollinators. The mean values of these traits in the two parental species, their F_1 hybrids, and their distribution in the F_2 are shown in Figure 2. For eight of the 12 traits the mean phenotypic values for the parental species differ by more than seven units of standard deviation ($\Delta\sigma$; Table 1). For other traits, such as projected area of the corolla, there is relatively little difference between the parental species. All 12 floral characters segregate widely in the F_2 generation (Figure 2). There was no convincing pattern of bi- or trimodal distributions as might be expected if the traits were highly heritable and controlled by a single Mendelian locus, except in the case of carotenoid concentration where there is a large class of F_2 plants with values near zero. The presence of yellow carotenoid pigment throughout the flower petals depends upon a single locus called *yup* (*y*ellow *up*per; Hiesey *et al.* 1971; Bradshaw *et al.* 1995). Non-normal trait distributions in the F_2 were observed for many of the floral characters.

Linkage mapping: Of the 66 RAPD markers scored, 20 also appear on the published linkage maps developed in our previous *M. lewisii* × *M. cardinalis* F_2 pedigree (Bradshaw *et al.* 1995). This makes alignment and direct comparison of linkage and QTL maps possible between the two experiments (Figure 3). For the sake of convenience, we will refer to the previously published QTL mapping experiment as EXP1 (Bradshaw *et al.* 1995) and the current experiment as EXP2.

The consensus framework map for EXP2 has the same marker order as that for EXP1, providing additional evidence that the order is correct (Figure 3). There are eight linkage groups, A–H, found in each experiment. The haploid chromosome number of *M. lewisii* and *M. cardinalis* also is eight (Hiesey *et al.* 1971), but it is possible that linkage groups A–H do not represent all eight of these chromosomes. Only linkage groups A, B, and C have homologs from each parental species recognized by codominant markers. It is conceivable that some of the other five linkage groups are unrecognized homologs of each other.

QTL mapping: Using interval mapping with MAPMAKER/QTL and QTL Cartographer Model 3, between one and six QTLs were detected for each trait. A total of 47 QTLs for all 12 traits (counting only once each QTL on a pair of linkage groups known to be homologs) were found (Table 1; Figure 4). Five of the traits showed evidence of more than a single QTL (see materials and methods) on linkage group A when tested by simple interval mapping: carotenoid concentration (A_L), projected area (A_L), lateral petal reflexing (A_L and A_C), upper petal reflexing (A_C), and corolla aperture height (A_C). Composite interval mapping with QTL Cartographer Model 6 suggests that there really are two QTLs for projected area on linkage group A_L, and the results in Table 1 and Figure 4 reflect this revised total of 48 QTLs. The QTLs for petal reflexing on linkage group A appeared to be single peaks when analyzed with Model 6. The QTLs for carotenoid concentration and aperture height on linkage group A were not found at all when tested with Model 6, presumably because of reduced detection power in the multiple regression (Zeng 1994).

QTL magnitudes ranged from as high as 84.3% PVE to as low as 3.3% PVE (Table 1). The distribution of QTL effects for all traits on both coupling-phase maps is shown in Figure 5. The distribution is strongly skewed, suggesting that for many traits there are one or a few

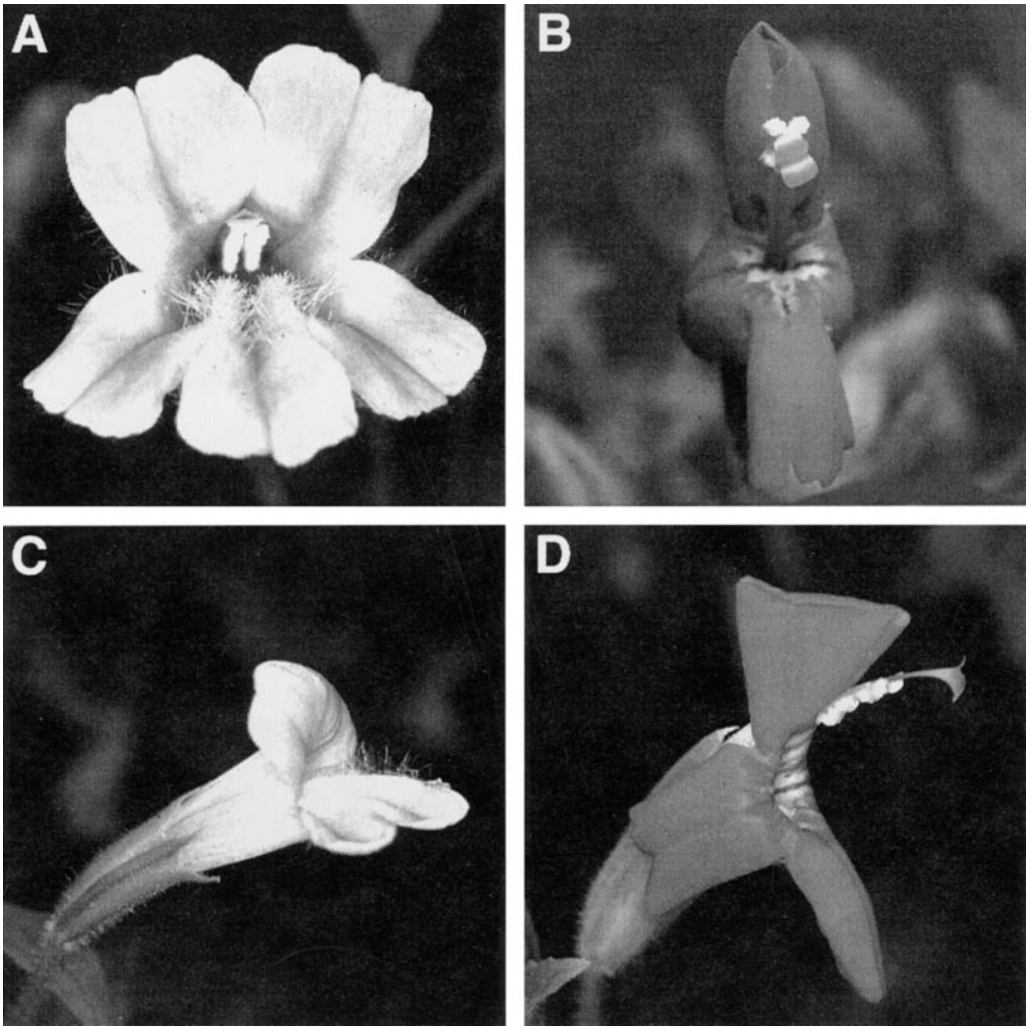

Figure 1.—*Mimulus lewisii* (A, C) and *M. cardinalis* (B, D) flowers. Flowers are shown from the front (A, B) as an approaching pollinator views them. In side views (C, D), the relative positions of the stigma and anthers are shown.

QTLs of large effect and several other QTLs with smaller effects. If a threshold of 25% PVE is employed to declare a major QTL (Bradshaw *et al.* 1995), most (9/12) of the floral traits are controlled by at least one major QTL. Only anthocyanin pigment concentration and the two corolla aperture characters have no major QTLs detected (Table 1; Figure 4).

The direction of QTL effect for most traits can be predicted from the phenotypes of the two parental species. Alleles from *M. cardinalis* always increase anthocyanin and carotenoid concentration, petal reflexing, nectar volume, stamen and pistil length, and corolla aperture height (Table 1). For most of these characters, the distribution of phenotypes in the F_2 is bounded by the values measured in the parental species (Figure 2). For other traits, especially size-related characters such as corolla width, petal width, and projected area, the effect of the *M. cardinalis* allele is less predictable, and transgressive segregation in the F_2 is common (Figure 2). This may be because corolla width and projected area traits are composite characters affected by other characters such as petal width and reflexing. The tendency of *M. cardinalis* alleles to have larger petals with larger projected area is countered by the tendency to produce more reflexed petals, making the projected area smaller.

There is a clear asymmetry in the distribution of dominance between *M. lewisii* and *M. cardinalis* alleles. Of 26 unique QTLs (*i.e.*, a QTL on homologous linkage groups is counted only once) whose mode of action could be determined with a confidence of LOD $\geq$1, 18 appear to be inherited in a dominant/recessive manner, and at 15 of these loci the *M. cardinalis* allele is recessive (Table 1).

Interaction among QTLs: Although the division of the linkage data into two separate coupling-phase genetic maps prevents an exhaustive search for epistatic interactions among loci, we see evidence of interaction between a classical Mendelian locus and a QTL. There is a single locus on linkage group D_C, *yup*, which determines whether yellow carotenoid pigments are deposited in the upper epidermis of the petals (Hiesey *et al.* 1971; Bradshaw *et al.* 1995). *M. cardinalis* is homozygous for the recessive allele of *yup*, allowing carotenoids to accumulate in chromoplasts. The carotenoid pigments together with the purple anthocyanins give *M. cardinalis*

TABLE 1

Floral trait QTLs mapped in a *Mimulus lewisii* × *M. cardinalis* F_2

	Trait	Linkage group	PVE (%)	Mode of action	*M. lewisii* parental phenotype (mean ± σ)	*M. cardinalis* parental phenotype (mean ± σ)	Δσ	LL homozygote mean at QTL	CC homozygote mean at QTL	Homozygote sample size
Attraction	Anthocyanin concentration (A_{510})	D_C	20.6	R	0.09 ± 0.04	0.87 ± 0.20	18	0.15	0.34	137
	Carotenoid concentration (A_{450})	A_L	84.3	R[a]	0.13 ± 0.29	1.39 ± 0.32	8	0.08	0.76	12
		C_L	75.7	R[a]				0.15	0.98	76
		D_C	83.0	R				0.23	0.93	137
	Lateral petal width (mm)	A_L	31.5	A	9.5 ± 0.8	16.1 ± 1.4	7	10.9	14.5	12
		B_L	17.9	d				11.5	14.1	158
		B_C	19.4	a				11.5	14.3	62
		C_L	17.1	d				14.0	13.9	76
		C_C	14.4	r				12.6	11.8	169
		D_C	5.4	r				12.7	13.7	137
		E_L	42.1	r[a]				11.3	15.3	85
		H_C	13.5	R				12.2	14.0	169
	Corolla width (mm)	A_L	31.5	R[a]	27.8 ± 3.7	22.6 ± 4.1	1	25.3	24.0	35
		A_C	9.1	R				29.7	26.2	243
		B_L	3.3	d				26.3	29.2	158
		B_C	3.3	d[a]				26.3	29.2	62
		C_L	11.3	a				30.8	24.9	76
		C_C	14.9	d[a]				32.4	26.6	169
		D_C	5.6	r				29.4	25.7	137
		G_L	9.4	r[a]				26.6	31.6	220
	Corolla projected area (mm^2)	$A_{L(1)}$	16.5	D	583 ± 177	559 ± 134	1	481	513	48
		$A_{L(2)}$	10.6	R				608	511	35
		B_L	10.4	d				501	649	158
		B_C	12.2	a				494	664	66
		C_L	9.7	d				696	550	76
		C_C	40.5	D[a]				802	499	150
		E_L	14.7	R[a]				492	511	85

(*continued*)

TABLE 1

Continued

	Trait	Linkage group	PVE (%)	Mode of action	*M. lewisii* parental phenotype (mean ± σ)	*M. cardinalis* parental phenotype (mean ± σ)	Δσ	LL homozygote mean at QTL	CC homozygote mean at QTL	Homozygote sample size
	Upper petal reflexing (1–5)	A_L	51.4	a	1.72 ± 0.29	4.67 ± 0.36	9	2.17	3.99	35
		A_C	37.7	R				2.34	3.80	269
		D_C	7.1	r				3.40	3.50	137
		E_L	4.7	r[a]				2.79	3.35	85
		H_C	5.0	a				2.94	3.48	169
	Lateral petal reflexing (1–5)	A_L	68.8	R[a]	1.05 ± 0.16	4.13 ± 0.59	8	1.80	4.06	35
		A_C	34.4	R				2.16	3.60	269
		D_C	16.2	R				2.75	3.67	137
		E_L	64.5	R[a]				2.12	4.22	85
Reward	Nectar volume (μl)	B_L	33.2	A	8.1 ± 2.8	119.5 ± 29.2	15	23.2	68.8	158
		B_C	33.1	A				22.8	68.8	62
		E_L	30.2	r[a]				27.1	64.8	85
Efficiency	Stamen length (mm)	A_L	46.6	a	25.1 ± 0.6	43.1 ± 1.3	22	30.4	37.8	12
		A_C	23.3	a				31.3	36.4	269
		B_L	10.2	A				33.1	37.0	158
		B_C	12.5	A				32.9	37.2	66
		D_C	17.6	R				34.0	37.1	137
		E_L	14.6	A				31.3	35.3	85
		H_C	24.0	R				33.0	37.1	169
	Pistil length (mm)	A_L	40.5	A	28.7 ± 0.8	49.1 ± 1.0	15	33.9	42.5	12
		A_C	18.9	a				35.2	41.0	269
		B_L	4.8	A				38.1	41.3	158
		B_C	5.7	A				37.8	41.2	66
		D_C	33.1	R				39.6	43.0	137
		E_L	49.6	A				34.4	44.0	76
		H_C	14.1	r				37.8	41.6	169

(*continued*)

TABLE 1

Continued

Trait	Linkage group	PVE (%)	Mode of action	*M. lewisii* parental phenotype (mean ± σ)	*M. cardinalis* parental phenotype (mean ± σ)	Δσ	LL homozygote mean at QTL	CC homozygote mean at QTL	Homozygote sample size
Corolla aperture width (mm)	A_L	9.6	a	7.1 ± 0.9	3.9 ± 0.6	3	6.17	4.81	21
	A_C	16.3	d[a]				6.37	4.79	269
	B_L	12.0	D				4.54	5.36	158
	B_C	11.9	D[a]				4.51	5.33	66
	D_C	15.1	a				4.69	4.61	137
	F_C	17.7	d[a]				5.73	4.65	68
	G_L	4.8	d				5.33	5.13	137
	H_C	6.5	a				5.49	4.75	169
Corolla aperture height (mm)	A_L	22.6	A	2.6 ± 1.0	4.5 ± 1.1	3	3.60	5.64	12
	A_C	5.1	a				4.38	5.24	243
	C_C	19.4	r				4.40	4.48	169
	H_C	7.2	r				4.54	5.42	158

"Linkage group" refers to the map in Figure 3. PVE is the percent of F_2 phenotypic variance explained, as calculated by interval mapping in MAPMAKER/QTL. The most likely mode of action of each QTL is shown with reference to the *M. cardinalis* allele. If the *M. cardinalis* allele is recessive to the *M. lewisii* allele, an "R" is found in the "Mode of action" column. "D" and "A" stand for "dominant" and "additive," respectively. Uppercase letters are used when the statistical support for the most likely model is LOD >1 higher than any of the alternative modes.

[a] The QTL is in repulsion linkage phase with the RAPD markers (see text). The phenotypic mean and standard deviation are shown for the parental *M. lewisii* and *M. cardinalis*. The Δσ column shows the standardized difference (in units of standard deviation) between parental mean phenotypes. The phenotypic means of F_2 plants homozygous for the *M. lewisii* (LL) or *M. cardinalis* (CC) allele at the QTL are given. The number of F_2 plants homozygous for the RAPD marker nearest the QTL is shown to indicate the severity of segregation distortion near the QTL. Approximately 116 homozygotes are expected if the locus is segregating in the Mendelian 3:1 ratio.

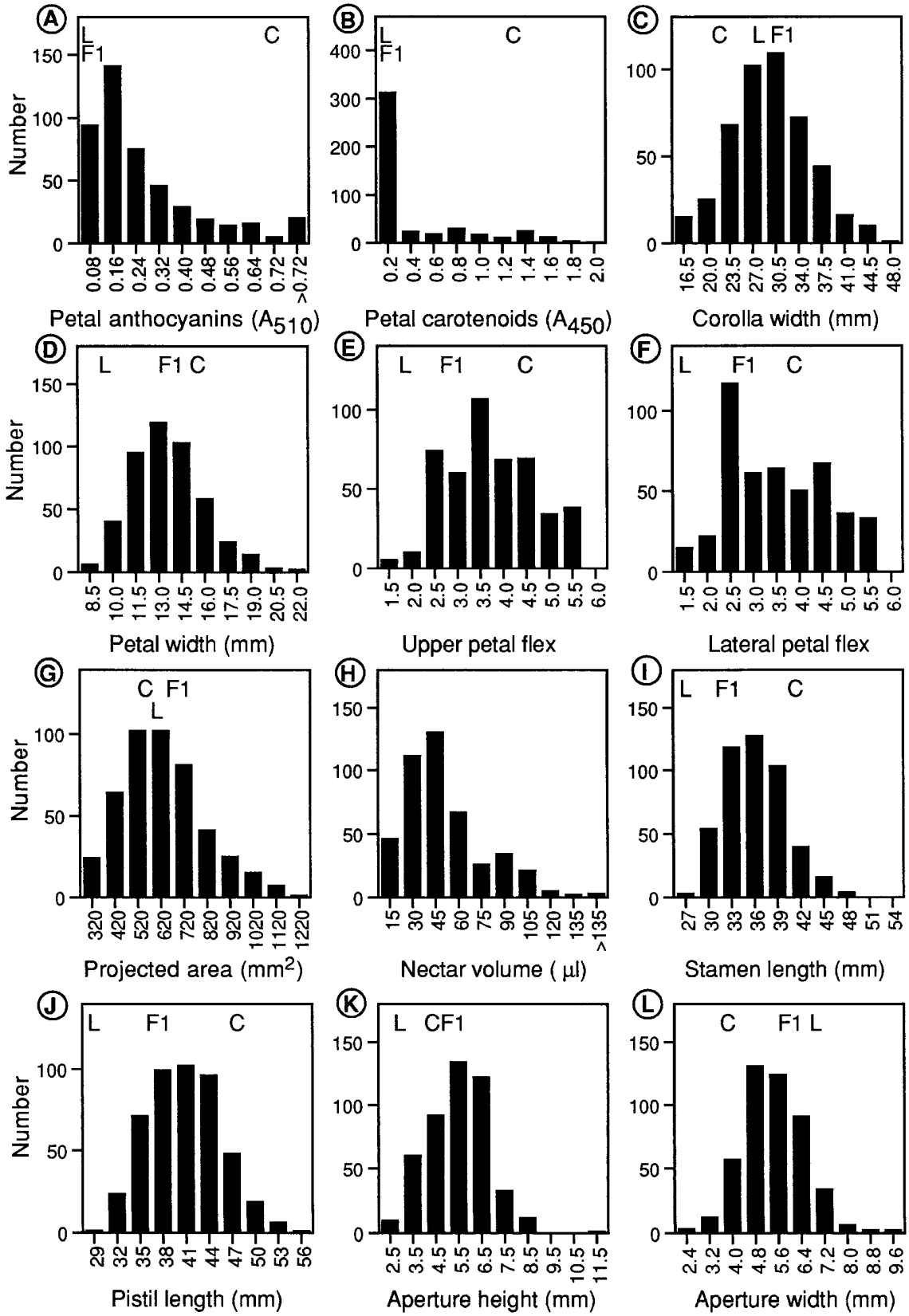

Figure 2.—Mean phenotypic trait values of *M. lewisii* (L), *M. cardinalis* (C), their F_1 hybrid, and the distribution of trait values for 465 F_2 offspring.

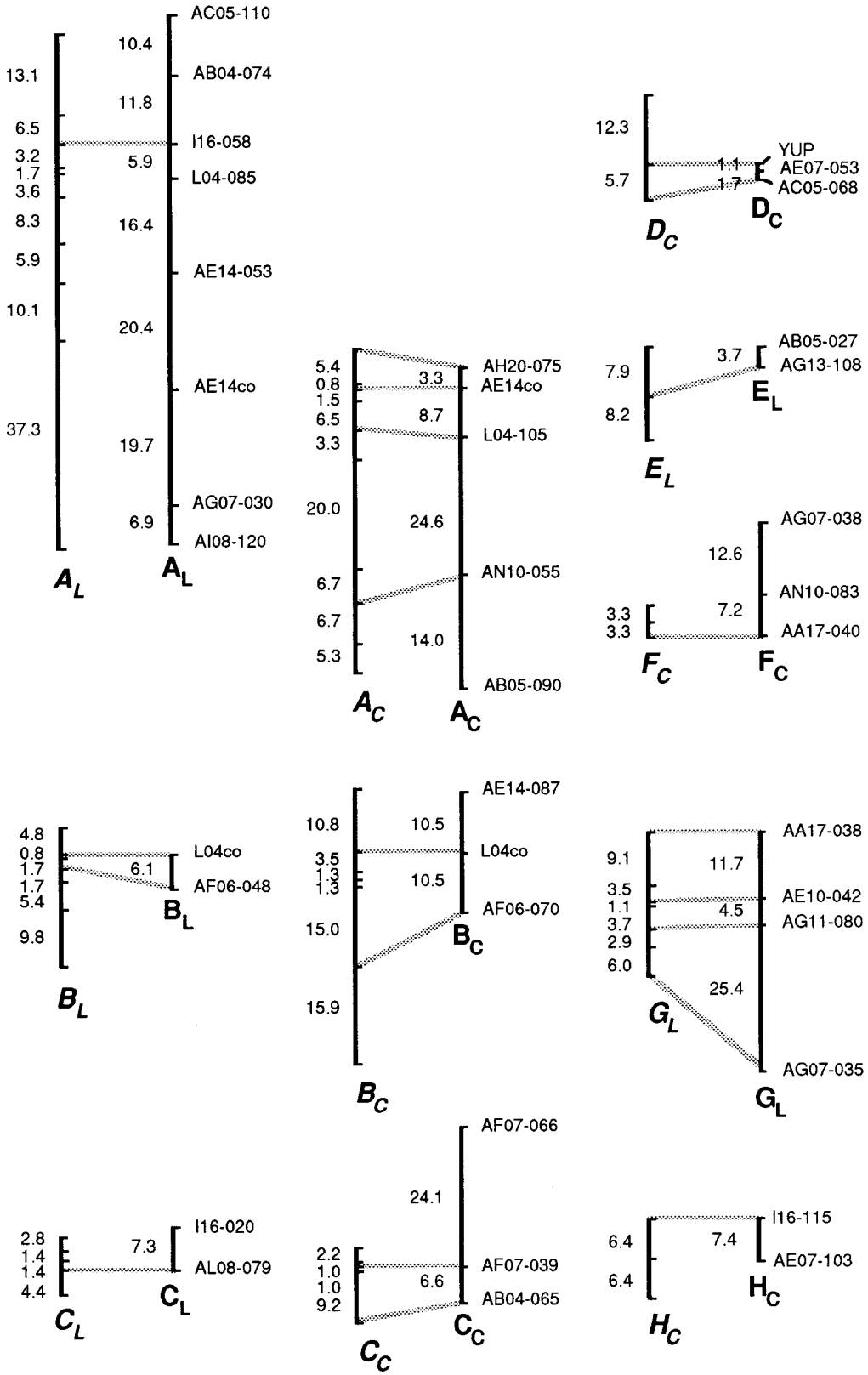

Figure 3.—Comparison of RAPD linkage maps made from independent populations of *M. lewisii* × *M. cardinalis* F_2 offspring. The linkage maps derived in EXP1 (Bradshaw *et al.* 1995) are shown to the left of the corresponding maps made in EXP2. RAPD marker names are shown only on the EXP2 maps and follow the convention of giving the Operon primer name followed by the approximate size of the segregating band (*e.g.*, AE14-053 indicates a band of about 530 bp produced from primer AE14). The "co" designation is used to denote a codominant marker (*e.g.*, L04co). Horizontal lines connecting the pairs of linkage groups indicate markers mapped in both EXP1 and EXP2. The eight linkage groups are designated with the letters A–H and are subscripted with L or C to indicate from which set of coupling phase markers they are derived. For example, linkage group A_L is the *M. lewisii* homolog of A_C from *M. cardinalis*, and linkage group E_L is known only from recombination within the *M. lewisii* genome and cannot be matched with a *M. cardinalis* homolog due to the lack of codominant markers. Estimated Haldane map distances (cM) between adjacent markers are shown to the left of the vertical line representing each linkage group.

its distinctive crimson petals. *M. lewisii* is homozygous for the dominant *yup* allele, which suppresses carotenoid deposition. When QTLs governing carotenoid pigment concentration are mapped in the F_2, the *yup* locus appears as a major QTL (Bradshaw *et al.* 1995; Table 1 and Figure 4 of this article). In addition to the essentially qualitative effect of the *yup* locus, two further putative major QTLs were found (in repulsion phase) on linkage groups A_L and C_L in EXP2 (Table 1; Figure 4). These QTLs are expected to be modifiers of carotenoid pig-

ment concentrations, epistatically controlled by *yup* and only expressed in *yup*/*yup* recessive homozygotes. The fact that *yup* is located on linkage group D_C on one coupling-phase map and the putative modifier QTLs are found on the other coupling-phase map on linkage groups A_L and C_L precludes a simultaneous estimate of QTL magnitude using a multiple-QTL statistical model. However, it is possible to perform a separate QTL analysis on the 137 F_2 plants homozygous for the recessive *yup* allele to measure the effect of the modifier QTLs on carotenoid concentration. When this was done using MAPMAKER/QTL, the modifier QTL on linkage group A_L was detected (peak LOD = 10.3; PVE = 43%), but the putative QTL on C_L was below the detection threshold (LOD = 2.2; PVE = 8%). Composite interval mapping with QTL Cartographer (Model 6) and permutation analysis confirm that the QTL on A_L is significant, but that the putative QTL on C_L is not. Note that the QTL magnitude estimates in this case reflect the variance explained only among recessive *yup*/*yup* homozygous F_2 plants, and that these values are much lower than the estimates of QTL magnitude made for the entire F_2 population (Table 1). This discrepancy and the fact that the sum of estimated magnitudes for carotenoid concentration QTLs exceeds 100% PVE (Table 1) probably are because of a violation of the assumption that QTLs act independently of each other. This problem could be corrected if there were a single unified linkage map with which to estimate QTL magnitudes using all codominant markers simultaneously.

Segregation distortion: Severe ($P < 0.01$) segregation distortion from the expected 3:1 Mendelian ratios for dominant markers was observed for at least one marker on every linkage group. In some cases the *M. lewisii* homozygotes are underrepresented and *M. cardinalis* homozygotes overrepresented (linkage group A), and in others the reverse is true (linkage group B). The number of homozygotes at the RAPD locus nearest each QTL is shown in Table 1 to give a sense of the amount of segregation distortion present near each QTL and to indicate the number of genotypes used to calculate the mean phenotype in the homozygous class of offspring. The number of null (band-absent) homozygotes is given because this is the only genotypic class that can be identified unambiguously with dominant RAPD markers.

In general there is no effect of cytoplasmic background on segregation distortion at the level of entire linkage groups (data not shown). The sole exception is linkage group C, which shows no segregation distortion in the CL2 × LC1 subpopulation (marker AL08-079 on C_L; marker AF07-066 on C_C; $P > 0.6$), but a twofold deficit of *M. lewisii* homozygotes and a corresponding surplus of *M. cardinalis* homozygotes in the LC1 × CL2 progeny with the *M. lewisii* cytoplasm (all markers; $P < 0.0001$).

DISCUSSION

Many of the traits of greatest interest to biologists exhibit continuous phenotypic variation and are inherited quantitatively. The resolution of quantitative traits into discrete Mendelian loci has made substantial progress since the development of genetic linkage maps based on molecular markers and computer software implementing improved statistical treatments of combined genotypic and phenotypic data. Most of this progress has been made by plant and animal breeders engaged in mapping QTLs controlling economically important traits such as crop yield or livestock quality (*e.g.*, Paterson *et al.* 1988; Stuber *et al.* 1992; Andersson *et al.* 1994; Georges *et al.* 1995).

That these same experimental methods could be used to study the genetic control of evolutionarily relevant complex traits such as reproductive isolation or adaptation in nature was recognized at the outset (Paterson *et al.* 1988), but very few QTL mapping experiments have been done in natural plant or animal populations (Bradshaw *et al.* 1995; Mitchell-Olds 1996; Lin and Ritland 1997). The reported results of essentially all QTL mapping experiments are consistent with the oligogenic model and not with the infinitesimal model (*e.g.*, Paterson *et al.* 1988; Stuber *et al.* 1992; Doebley and Stec 1993; Bradshaw *et al.* 1995). That is, much of the phenotypic variance in many traits appears to be under the control of one or a few major QTLs, perhaps modified by QTLs of minor effect. Often it is not clear whether this general outcome is a true reflection of the underlying genetics or a statistical artifact caused by sampling bias (Beavis 1994, 1998), incorrect specification of the mathematical model for QTL detection (Visscher and Haley 1996), or some other methodological problem.

Are major QTLs important in the evolution of natural populations? Most of the information regarding the magnitude of QTL effects in plants has come from studies of domesticated varieties. In reviews based largely on cultivated taxa, Hilu (1983) and Gottlieb (1984) concluded that many morphological characters in plants are controlled by genes of large effect. Similarly, recent mapping studies employing molecular markers have shown that major QTLs control differences in inflorescence architecture between maize and teosinte (Doebley and Stec 1993), differences in fruit size between cultivated tomato and a wild relative (Grandillo and Tanksley 1996), and differences in flowering time between cultivars of *Brassica oleracea* (Camargo and Osborn 1996). Because major genes in populations under cultivation can be fixed by strong artificial selection even when they possess negative pleiotropic effects (Coyne and Lande 1985), results derived from studies of domesticated varieties may not be relevant to the study of evolution in nature. Our finding of major QTLs for most characters contributing to the

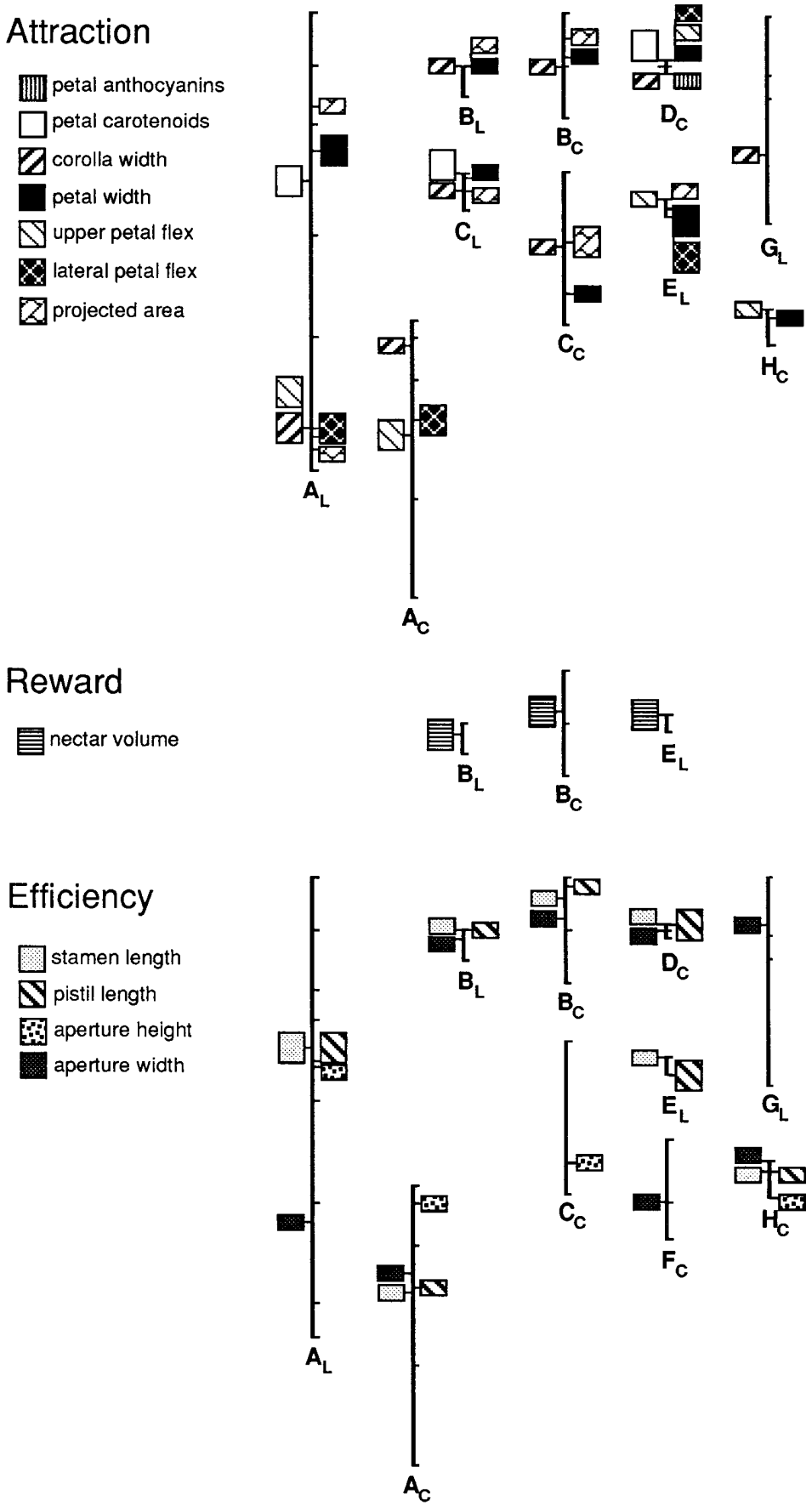

Figure 4.—QTL maps for 12 floral traits measured in EXP2. Boxes show the most likely position of the QTL, as determined by MAPMAKER/QTL and QTL Cartographer. The taller boxes indicate that a QTL magnitude is "major" (PVE $\geq$ 25%), and the shorter boxes denote "minor" QTLs.

floral divergence of *M. lewisii* and *M. cardinalis*, and results from other recent studies of natural populations (Mitchell-Olds 1996; Lin and Ritland 1997), provide support for the view that phenotypic differences between plants often may be controlled by QTLs of large effect. However, at present there are insufficient data to draw any generalizations about the magnitude of QTL effects in natural plant populations.

Do major QTLs contribute to the differences in floral morphology between *M. lewisii* and *M. cardinalis*? In the experiment described in this article, we find that the majority (9/12) of floral traits expected to be responsible for pollinator discrimination and reproductive isolation in nature are controlled by at least one major QTL (Table 1; Figure 4). Some single QTLs explain half or more of the phenotypic variance in the F_2 (Table 1). Thus, we conclude that major QTLs do contribute to the differences in floral morphology between *M. lewisii* and *M. cardinalis* (Figure 5) and that only a modest number of QTL allele substitutions might be required to reproductively isolate these two species.

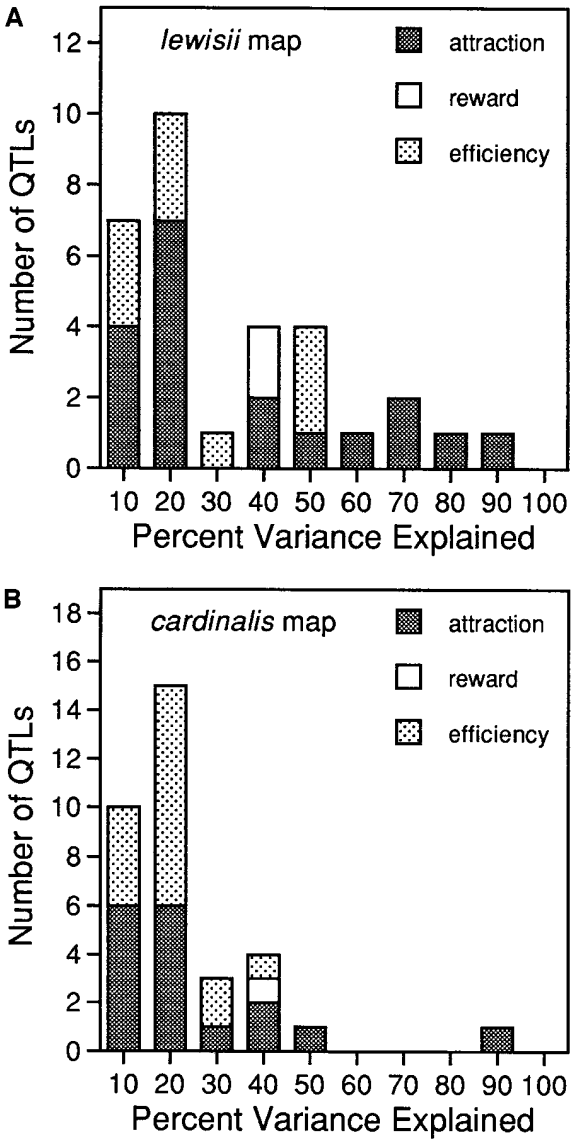

Figure 5.—Distribution of estimated QTL magnitudes from the *M. lewisii* (A) and *M. cardinalis* (B) coupling-phase maps. QTL magnitude data are taken from Table 1.

The number of minor QTLs is less clear. The simplest interpretation of Figure 5 is that the modal QTL magnitude is in the range of 15–25% PVE. There seems to be a decline in QTL number at very low estimated magnitudes (0–15% PVE), but this is probably an artifact of the low power of QTL detection when QTL magnitudes are small (Beavis 1994, 1998; Lynch and Walsh 1998). It is likely that there are many minor QTLs affecting each of the 12 floral traits that have gone undetected even in this large F_2 mapping population and that the actual distribution of QTL magnitudes is skewed strongly toward smaller values.

These insights into the genetic architecture of adaptive traits in nature would have been very difficult to realize without genetic markers, linkage maps, and statistical methods for QTL mapping. For example, consider nectar volume in *M. lewisii* and *M. cardinalis*. Nothing about the phenotypic distribution of this character in the F_2 (Figure 2H) suggests anything other than a quantitative trait, and continuous distributions of phenotypes often are taken as evidence of polygenic inheritance. Yet, in two independent experiments (this article and Bradshaw *et al.* 1995), a QTL on linkage group B explains from one-third to two-thirds of the 14-fold difference in nectar volume between *M. lewisii* and *M. cardinalis* (Bradshaw *et al.* 1995; Table 1 of this article), leading to the conclusion that large changes in a complex trait like pollinator reward may be accomplished with a small number of loci having large effects on the phenotype.

QTL mode of action asymmetry between *M. lewisii* and *M. cardinalis*: Many of the QTLs identified in EXP2 possess a dominant/recessive mode of gene action, but the *M. cardinalis* allele is usually recessive and often increases the phenotypic value of a trait (Table 1). Examples include the QTL for anthocyanin concentration, each of the three QTLs for carotenoid concentration, and five of the 10 QTLs for petal reflexing. Such a high frequency of recessive alleles for floral traits is somewhat surprising, given that the fixation probability of advantageous recessive alleles is thought to be substantially lower than that for alleles expressed in heterozygotes, because of the low expected frequency of recessive homozygotes for new mutations in large, randomly mating populations (Haldane 1924, 1927). Partial self-fertilization has been shown to markedly increase the fixation probability of favorable recessive mutations, and this leads to the expectation of a higher frequency of adaptive recessive traits in derived, self-fertilizing species than in their outcrossing progenitors (Charlesworth 1992). Both *M. cardinalis* and *M. lewisii* are self-compatible (Hiesey *et al.* 1971), but their selfing rates are unknown. Because of the predominance of bee pollination in the

genus Mimulus, Charlesworth (1992) suggested that hummingbird pollination in *M. cardinalis* is the derived condition, a view consistent with patterns of the evolution of bird-pollinated plants in the western North American flora (Grant 1994). Furthermore, Charlesworth (1992) suggested that the high frequency of adaptive recessive traits found in this species by Hiesey *et al.* (1971) is consistent with the hypothesis that selfing increases the likelihood of adaptive evolution because of favorable recessive mutations. Mating system studies and a phylogenetic reconstruction of Mimulus section Erythranthe are now in progress to further examine how partial selfing may have contributed to the evolution of floral traits in *M. cardinalis* and *M. lewisii*.

Comparison with a previous mapping experiment in a *M. lewisii* × *M. cardinalis* F_2: In a previous QTL mapping experiment (EXP1; Bradshaw *et al.* 1995), eight floral traits were measured: anthocyanin concentration, carotenoid concentration, corolla width, petal width, nectar volume, nectar concentration, stamen length, and pistil length. At least one major QTL, explaining ≥25% of the phenotypic variance in the F_2, was mapped for each trait. However, the modest size of the F_2 mapping population (n = 93) and the non-normal distributions of several of the phenotypic traits may have limited our ability to identify QTLs and to estimate correctly the magnitude of their effects (reviewed in Lynch and Walsh 1998). First, the small F_2 population provided a relatively low QTL detection power (Lander and Botstein 1989; van Ooijen 1992; Beavis 1994, 1998). QTLs with effects as large as 10% of the phenotypic variance are expected to be detected only about one-third of the time in mapping populations of this size (van Ooijen 1992). Thus, the number of QTLs we identified was almost certainly an underestimate, and even some major QTLs likely were overlooked. Second, our EXP1 study employed a method of QTL analysis that assumes a normal distribution of phenotypes (Lander and Botstein 1989), yet the non-normal distribution of many phenotypic traits in the F_2 makes it problematic to set appropriate QTL detection test statistic thresholds (Churchill and Doerge 1994). Some trait data can neither be used in raw form nor be transformed to approach a normal distribution. Because most QTL mapping experiments are only capable of detecting QTLs of large effect, and because such major QTLs, by definition, must skew the distribution of F_2 phenotypes, it seems desirable to employ a distribution-free model for determining critical values of the QTL detection test statistic. Finally, there is also a problem of bias in estimating the magnitude of QTL effect in the same population used for QTL detection (Lande and Thompson 1990; Beavis 1994, 1998). Because QTL magnitudes are estimated only on those QTL likelihood peaks exceeding the detection threshold, sampling error in detection causes a systematic upward bias in the estimate of QTL effect. This bias is more pronounced the smaller the mapping population and the smaller the true QTL magnitude, and it can be up to threefold when F_2 progeny sizes of 100 are used and the true magnitude of the QTL is ~10% PVE. Thus, minor QTLs may become major simply because of sampling error, and a truly polygenic trait can appear to be oligogenic when analyzed by QTL mapping in a single small population (Beavis 1994, 1998).

The current experiment, EXP2, was designed to overcome some of the potential problems encountered in EXP1. First, the EXP2 mapping population was produced by outcrossing unrelated F_1 plants rather than by self-pollination to prevent segregation distortion due to homozygosity of deleterious recessive alleles, as may have happened in EXP1. The EXP2 pedigree was derived from an entirely different set of *M. lewisii* and *M. cardinalis* parents than was used in EXP1, so that any QTLs in common between the two experiments probably are a general feature of the two Mimulus species and not unique to a single pair of parents. Second, the F_2 sample size was increased from 93 to 465 plants, based on simulations showing that sample sizes of 400–500 are necessary to give high QTL detection power (van Ooijen 1992; Beavis 1994, 1998) and accurate estimation of QTL magnitude (Beavis 1994, 1998). Third, QTL detection in EXP2 was done by interval mapping to permit direct comparison with the results of EXP1, but QTL detection thresholds in EXP2 were set by permutation tests to eliminate false assignment of QTLs resulting from violation of the assumption that phenotypic trait data are normally distributed (Churchill and Doerge 1994).

In EXP2, QTLs were remapped for seven of the eight traits measured in EXP1 (nectar concentration was not measured in EXP2). Because the linkage maps in EXP1 and EXP2 were made with a common set of markers (Figure 3), it is possible to compare the QTLs detected for the seven traits measured in both experiments. A total of 12 QTLs (counting only once any QTL found on two linkage groups known to be homologs) for these seven traits were mapped in EXP1. Eleven of the 12 QTLs were found in similar map positions in EXP2 (Table 2). Only the QTL for petal anthocyanin concentration on linkage group B in EXP1 was not detected in EXP2, perhaps because of poor map coverage of this linkage group in EXP2.

An additional 16 QTLs for these seven traits were detected exclusively in EXP2 (Table 1; Figure 4). The increased QTL detection power is an expected consequence of the much larger F_2 progeny size used in EXP2 (Beavis 1994, 1998). In EXP1, the lowest estimated QTL magnitude was 18.7% PVE, but in EXP2 it was possible to detect QTLs with estimated magnitudes as small as 3.3% PVE (Table 1). Seven of the 16 QTLs unique to EXP2 have estimated magnitudes less than the smallest QTL in EXP1, implying that about half of the improvement in censusing QTLs in EXP2 is attributable to the increased power to find QTLs with small

TABLE 2

Floral trait QTLs mapped in two independent experimental F_2 populations derived from a *M. lewisii* × *M. cardinalis* cross

	Trait	Linkage group	EXP1 PVE (%)	EXP2 PVE (%)
Attraction	Anthocyanin concentration (A_{510})	B_L	33.5	ND
		B_C	21.5	ND
	Carotenoid concentration (A_{450})	D_C	88.3	83.0
	Corolla width (mm)	A_C	25.7	9.1
		C_L	68.7	11.3
	Lateral petal width (mm)	B_L	42.4	17.9
		B_C	41.2	19.4
		H_C	25.2	13.5
Reward	Nectar volume (μl)	B_L	48.9	33.2
		B_C	53.1	33.1
Efficiency	Stamen length (mm)	B_L	27.7	10.2
		B_C	27.5	12.5
		D_C	21.3	17.6
		E_L	18.7	14.6
	Pistil length (mm)	D_C	43.9	33.1
		E_L	51.9	49.6

The magnitudes of presumably homologous QTLs detected in two different mapping experiments are shown. EXP1 refers to QTLs mapped previously (Bradshaw *et al.* 1995). EXP2 is the current experiment, for which complete QTL data are given in Table 1. ND, not detected.

effects. The other half (9/16) of the improvement in counting QTLs in EXP2 is a result of finding QTLs of fairly large effect (>18.7% PVE). A few are of major magnitude, such as that for stamen length on linkage group A (Table 1; Figure 4). This reveals the relatively low detection power, even for major QTLs, in the EXP1 mapping population of 93 F_2 plants.

Every QTL ($n = 11$) detected in both EXP1 and EXP2 had a smaller estimated magnitude in EXP2, verifying an upward bias of magnitude estimates in small mapping populations as predicted by the simulation studies of Beavis (1994). The magnitude of QTL effects estimated in EXP2 ranged from 2.3 to 57.4% (mean = 16.1%) lower than those of presumably homologous QTLs in EXP1 (Table 2). The correlation coefficient between PVE estimates made for homologous QTLs in EXP1 and EXP2 is 0.77 ($n = 15$; $P = 0.0009$), showing that the relative QTL magnitudes are similar between the two experiments, in spite of the absolute differences. Of the nine major QTLs with estimated magnitude ≥25% PVE in EXP1 (not counting the QTL for anthocyanin concentration that was not detected in EXP2), only four—for carotenoid concentration, nectar volume, and two for pistil length—are also classified as major in EXP2 (Table 2; Figure 4).

In spite of the lower estimates of QTL magnitude in EXP2, it remains the case that major QTLs are found in EXP2 for most (6/7) floral traits measured in both EXP1 and EXP2 (Table 2) and for many (3/5) traits measured only in EXP2 (Table 1). None of the traits has QTLs of equal estimated magnitude distributed uniformly across the genome, as one might expect under Fisher's infinitesimal model of quantitative trait inheritance (Fisher 1930).

Limitations of this study: Despite the large size of the F_2 population used in EXP2, which exceeds that of most QTL mapping studies done in full sibships of crop, livestock, or model systems, this experiment has some notable limitations.

First, the lack of codominant markers makes it impossible to construct a single unified map for the F_2. The use of dominant RAPD markers in the F_2 results in the two coupling-phase dominant marker maps shown in Figures 3 and 4. Homologous linkage groups may be recognized by the sharing of a single codominant marker, but the maps cannot be merged based only on that one shared marker. In the absence of a unified map, it is impossible to make a complete count of the number of QTLs affecting each trait or to do a thorough search for epistatic interactions among loci. Perhaps QTLs whose mode of action indicates that they are linked in repulsion with the RAPD markers should be viewed with caution. Seventeen QTLs fitting this description (*i.e.*, recessive QTLs on *M. lewisii* linkage groups and dominant QTLs on *M. cardinalis* linkage groups) are indicated by a superior [a] in Table 1. Many (7/16) of these repulsion phase QTLs are also mapped in coupling on the homologous linkage group, giving us an opportunity to check for potential bias in estimating QTL magnitude based on linkage phase. Among the seven QTLs detected both in coupling and repulsion in EXP2, the estimate of QTL magnitude is smaller than the repulsion phase estimate in six cases, suggesting an upward bias in magnitude estimate in repulsion phase QTLs. The subject of QTL mapping with dominant markers in repulsion appears ripe for a theoretical and Monte Carlo simulation treatment. If we eliminate all repulsion phase QTLs from our analysis of EXP2, only corolla width and projected area would lose their major QTLs. QTL mapping experiments, especially those done in natural plant populations where marker development is almost nonexistent, will have to contend with all of these issues. The development of codominant markers such as microsatellites would be a desirable but expensive solution.

Second, segregation distortion in the F_2 could reduce the effective sample size by reducing the size of one genotypic class, which in turn might lower QTL detection power and affect estimates of QTL magnitude. On linkage group A_L as few as 12 F_2 plants were homozygous for the recessive RAPD marker, which might be considered marginal for estimation of phenotypic means, but

on other linkage groups the number of homozygotes seems adequate (Table 1). It is also possible for segregation distortion to increase the effective sample size of band-absent homozygotes, which should improve the precision of QTL parameter estimates. This topic deserves attention because segregation distortion is ubiquitous in linkage mapping experiments, but distorted data typically either are ignored (*e.g.*, Bradshaw *et al.* 1995; Liu *et al.* 1996) or discarded (*e.g.*, Lin and Ritland 1996), neither of which seems entirely satisfactory. Hiesey *et al.* (1971) have reported that there are no visible meiotic abnormalities in hybrids between the Sierra Nevada forms of *M. lewisii* and *M. cardinalis*, but we cannot rule out the presence of small differences in chromosome structure (cryptic structural differentiation; Stebbins 1950), nor can we infer that there are specific loci responsible for the segregation distortion.

Third, the relatively low resolution of the genetic linkage map makes it impossible to determine the fine structure of the QTLs. Does each QTL represent a single gene, many linked genes, or an entire linkage group? It is plausible that at least some of these major QTLs, such as the *yup* locus control of carotenoid pigmentation, could be due to allelic differences in a single gene (Hiesey *et al.* 1971; Bradshaw *et al.* 1995), but as yet there is no overwhelming evidence in support of this hypothesis. The small number of marker-delimited intervals in the Mimulus genetic map (Figure 3) makes it difficult to apply more powerful statistical methods for inferring the presence of multiple QTLs on a single linkage group, such as composite interval mapping (Zeng 1994). On the current Mimulus linkage map only the longest linkage group, A, showed any evidence of multiple QTLs for a single trait (Table 1; Figure 4).

All of these potential difficulties are being addressed in our Mimulus system by the development of near-isogenic lines (NILs) in which a major QTL interval from *M. lewisii* is introgressed into *M. cardinalis*, and vice versa, by marker-assisted backcrossing. NILs will allow a much better determination of QTL effect in a homogeneous genetic background. Once a defined chromosome segment has been introgressed, it can be broken up by further recombination to resolve the genetic structure of each QTL (Paterson 1998). NILs are now being made for two traits under major QTL control: carotenoid pigmentation (*yup*; linkage group D) and nectar volume (linkage group B).

Further experiments to elucidate the genetic basis of reproductive isolation in nature: There is substantial evidence from both EXP1 and EXP2 that three floral traits differentiating *M. lewisii* and *M. cardinalis* are under the control of one or more major QTLs. These major QTLs contribute to traits likely to affect pollinator attraction (carotenoid pigmentation), reward (nectar volume), and the efficiency of pollen deposition (pistil length). The most important remaining question is the extent to which each of these QTLs influences the frequency of bumblebee and hummingbird visitation and pollen transfer in nature. If alternate alleles of major floral QTLs are capable of causing marked shifts in pollinator preference or pollen transfer efficiency, then the evolution of reproductive isolation may not be a result only of the gradual accumulation of many mutations with small effects.

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