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THE GENETIC ARCHITECTURE OF REPRODUCTIVE ISOLATION IN LOUISIANA IRISES: POLLINATION SYNDROMES AND POLLINATOR PREFERENCES

Noland H. Martin,[1,2,3] Yuval Sapir,[1,4] and Michael L. Arnold[1]

[1]*Department of Genetics, University of Georgia, Athens, Georgia 30602*

[2]*E-mail: nm14@txstate.edu*

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In animal-pollinated plants, pollinator preferences for divergent floral forms can lead to partial reproductive isolation. We describe regions of plant genomes that affect pollinator preferences for two species of Louisiana Irises, *Iris brevicaulis* and *Iris fulva*, and their artificial hybrids. *Iris brevicaulis* and *I. fulva* possess bee and bird-pollination syndromes, respectively. Hummingbirds preferred *I. fulva* and under-visited both *I. brevicaulis* and backcrosses toward this species. Lepidopterans preferred *I. fulva* and backcrosses toward *I. fulva*, but also under-visited *I. brevicaulis* and *I. brevicaulis* backcrosses. Bumblebees preferred *I. brevicaulis* and F_1 hybrids and rarely visited *I. fulva*. Although all three pollen vectors preferred one or the other species, these preferences did not prevent visitation to other hybrid/parental classes. Quantitative trait locus (QTL) mapping, in reciprocal BC_1 mapping populations, defined the genetic architecture of loci that affected pollinator behavior. We detected six and nine QTLs that affected pollinator visitation rates in the BCIb and BCIf mapping populations, respectively, with as many as three QTLs detected for each trait. Overall, this study reflects the possible role of quantitative genetic factors in determining (1) reproductive isolation, (2) the pattern of pollinator-mediated genetic exchange, and thus (3) hybrid zone evolution.

KEY WORDS: **Hybridization, pollination syndromes, pollinator behavior, prezygotic isolation, QTL analysis, speciation.**

The evolution of reproductive barriers is a key factor in the speciation process because it reduces genetic exchange in areas of overlap (Dobzhansky 1937; Mayr 1942; Coyne and Orr 2004). Although the genetic basis of postzygotic isolation has been studied extensively (e.g., Presgraves et al. 2003; Moyle et al. 2004), the genetic architecture of prezygotic isolation (i.e., How many genes underlie prezygotic barriers? Did these barriers evolve via few genes of large effect, or many genes of small effect?) is largely unknown (but see Ortiz-Barrientos and Noor 2005; Martin et al. 2007). For some plant clades, prezygotic isolation involves the added dimension of interactions between plants and pollinators. In such groups, differential visitation of pollinators (due to divergent floral morphologies) can lead to prezygotic isolation via assortative pollen flow, which may ultimately be a driving force in plant speciation (Grant 1994).

There are three main avenues by which plants may evolve pollinator isolation. First, selection resulting from competition for pollinators can facilitate the divergence of floral traits that attract different pollinators or enhance the efficiency of a specific pollinator. This will create prezygotic isolation between populations based on pollinator discrimination or efficiency (Grant

[3]Current address: Department of Biology, Texas State University, San Marcos, Texas 78666

[4]Current address: Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

1994; Aigner 2006; Sargent and Otto 2006). This mechanism is expected to affect sympatric, but not allopatric, populations of the interacting species. A second avenue for the development of prezygotic isolation involves selection against hybrid genotypes in zones of secondary contact; a process referred to as reinforcement (Dobzhansky 1937; Coyne and Orr 2004). The production of unfit hybrids may result in selection for individuals that make the "correct" choice to mate with conspecifics. The alleles underlying the prezygotic barrier (in this case pollinator isolation) must then spread into allopatric populations of the two taxa (Dobzhansky 1940; Hoskin et al. 2005). Finally, pollinator isolation may also evolve in allopatry, where different suites of pollinators exert divergent selection on floral morphologies, such that when the taxa become sympatric, pollinators discriminate between them (a distinct possibility for taxa in which there is currently substantial habitat isolation, i.e., Ramsey et al. 2003).

When reproductive isolation between plant species is not complete, interspecific pollination may lead to the formation of hybrid zones (see Arnold 1997 for review). Hybrid zones can function as a bridge for the introgression of alleles among the hybridizing taxa. The introgression of some alleles might be adaptive, as reflected by an increase in the fitness of the recipient taxon (see Arnold 2006 for examples). In regard to pollination systems, introgression might decrease the strength of selection on prezygotic reproductive isolation, eventually leading to an amalgamation of different floral forms.

Quantitative Trait Locus (QTL) analysis allows for the examination of the genetic architecture of adaptive differentiation between divergent lineages (reviewed in Erickson et al. 2004). QTL analyses enable a test of hypotheses regarding the contribution of individual QTLs to adaptive differentiation. This is accomplished by assessing the fitness effects of QTLs under different environmental conditions. For instance, a locus that contributes to the expression of a floral trait may have opposite effects in the presence of alternate pollinators (Bradshaw and Schemske 2003). Indeed, most floral traits are polygenic, with the alleles from different taxa demonstrating opposite phenotypic effects (e.g., Fishman et al. 2002; Hodges et al. 2002; Rieseberg et al. 2002; but see Bradshaw and Schemske 2003; Galliot et al. 2006). In cases in which the differentiating trait is controlled by a small number of loci with large effect, rapid evolution is considered more likely due to the fewer number of required steps (Bradshaw et al. 1995). Given that a major role for directional selection has been inferred in phenotypic divergence and speciation (Rieseberg et al. 2002), pollinator-mediated selection on floral traits, underlain by large-effect QTLs, could rapidly lead to reproductive isolation between different lineages.

A number of studies have defined floral trait QTLs among plant species (Bradshaw et al. 1995; Fishman et al. 2002; Hodges et al. 2002; Westerbergh and Doebley 2002; Stuurman et al. 2004; Goodwillie et al. 2006). However, most of the studies involving those plant taxa with pollen vectors did not include an analysis of the effects of the floral trait QTLs on pollinator behavior. One exception involved a study by Schemske and Bradshaw (1999) in which they detected a preference of bee visitors to *Mimulus* flowers as a function of the specific allele present at the *yup* locus—a locus controlling floral color. In addition, they also demonstrated an association between hummingbird visitation rates and a QTL that affected nectar.

In the present study, we examine the effect of divergent floral traits of the Louisiana Iris species, *Iris fulva* Ker-Gawler and *I. brevicaulis* Raf on pollinator behavior. These data allow a definition of regions (i.e., QTLs) in the *Iris* genome that impact the behavior of the pollinator species. *Iris fulva* and *I. brevicaulis* differ in many floral characters that are potential adaptations for attracting different categories of pollinators (Viosca 1935; Wesselingh and Arnold 2000b; Bouck et al. 2007). *Iris fulva* possesses a "bird-pollination syndrome," characterized by red flowers, anthers exposed outside the pollination tunnel, and large volumes of diluted nectar (Wesselingh and Arnold 2000a). In contrast, *I. brevicaulis* possesses traits characteristic of a "bee-pollination syndrome": blue flowers marked with prominent white and yellow nectar guides, stiff upright sepals, and small volumes of concentrated nectar. These species also differ in their peak flowering time (Cruzan and Arnold 1994; Martin et al. 2007). In contrast to the expectations from the pollination syndromes, previous experiments using mixed arrays of floral types of the two species and their hybrids discovered that hummingbirds and bumblebees do not restrict themselves to visiting exclusively *I. fulva* and *I. brevicaulis*, respectively (Wesselingh and Arnold 2000b).

In southern Louisiana, hybrid zones between these species are frequently detected (Arnold 1993; Cruzan and Arnold 1993; Johnston et al. 2001). The establishment of these zones has apparently involved pollinator-mediated pollen movement between populations of the two species, rather than through seed movement (Arnold 1993). Furthermore, the plausibility of adaptive trait introgression has been documented in two analyses of survivorship of hybrids in extreme environments (Martin et al. 2005; Martin et al. 2006). Taken together, these findings suggest that the transfer and amalgamation of adaptive floral traits is possible as well. Indeed, numerous recombinant genotypes, and thus floral phenotypes, are observed in natural hybrid populations (e.g., Cruzan and Arnold 1993; Arnold et al., unpubl. data). However, even in hybrid zones, *I. fulva* and *I. brevicaulis* genotypes and phenotypes are present at high frequencies (e.g., Arnold 1993; Johnston et al. 2001).

In the present study we examine the pattern of pollinator behavior, and the *Iris* QTLs that affect this behavior. Using these data we are able to: (1) estimate the directionality of pollinator-mediated selection on the *I. fulva* and *I. brevicaulis* floral traits;

(2) determine the potential for pollinator preference to act as a prezygotic reproductive barrier; (3) identify QTLs that contribute to differential pollinator preferences; and (4) test for co-localization of the "preference" QTLs and previously identified floral trait QTLs (Bouck et al. 2007).

Materials and Methods

EXPERIMENTAL DESIGN AND POLLINATOR OBSERVATIONS

One wild-collected individual each from *I. fulva* (*If*) and *I. brevicaulis* (*Ib*) were used to make two full-sibling F_1 hybrids, one of which was backcrossed to each of the two parental plants to produce reciprocal, interspecific backcross (BCIf and BCIb, respectively) populations (see Bouck et al. 2005; Martin et al. 2007 for details of the crossing design). Two experimental plots, ca. 1 km apart, were established near the Choupique Bayou, located in the U. S. Army Corps of Engineers Atchafalaya Basin Floodway in south-central Louisiana, USA. The plots are in a cypress-mixed hardwood forest in which natural populations of *I. brevicaulis* and *I. fulva* are typically found (Viosca 1935; Cruzan and Arnold 1993; Johnston et al. 2001). The elevation at both of these sites is roughly 5.0 m above mean sea level, and the elevational change within each plot differed by less than 0.5 m. It should be noted that these plots are not the same as those described previously by Martin et al. (2006), as extensive and extended flooding largely destroyed the latter. However, these are the same plots observed for phenology by Martin et al. (2007). Hereafter, plots A and B correspond to the "wet" and "dry" plots, respectively, described in that study.

In October 2005, rhizomes from each BCIf and BCIb plant (172 and 243 individuals, respectively), along with 43 *I. fulva* (clones of five genotypes), 62 *I. brevicaulis* (clones from seven genotypes), and 47 F_1 rhizomes (clones from the same F_1 plants used to generate the BC_1 mapping populations) were transplanted into each plot. *Iris fulva* and *I. brevicaulis* genotypes were collected from the wild as rhizomes and transplanted into the greenhouse where they have been maintained for ca. 15 years. Experimental hybrid plants were grown from seeds and have been cultured in the greenhouse for ca. 10 years.

Within each plot, we randomly assigned positions to the various genotypes, spacing them 0.5 m apart. Plants were marked with a unique number. We noted the date at which each flower opened, and the date at which each flower wilted to the point where it was unattractive to pollinators (ca. 2 days postopening). Up to four flowers/stalk, and multiple stalks/plant can be produced simultaneously on each plant.

Pollinator visitation was recorded intermittently during the peak flowering season, from 30 March to 4 May 2006 (see Table 1). Data were collected at different time intervals to capture the maximum number of daily intervals. The data collection periods also varied from day to day due to weather conditions, pollinator abundance, and amount of time available to the observer. One to three observers were used on the collection days, with each observer tasked to record pollinator movements throughout the entire plot. When a visitor to a flower was detected, the observer recorded the plant number. The pollinator was then observed until it departed the plot, with all flowers visited being noted. Observers recorded only "legitimate" visits. A visit was considered legitimate if the pollinator either touched the anther or the stigma, or its mouth parts (e.g., the tongue of a hummingbird or the proboscis of a butterfly) were inserted into the pollination tunnel. Flowers of the genus *Iris* contain three pollination units, each containing a pollination tunnel and functioning as an advertisement unit (Faegri and van der Pijl 1979; Goldblatt and Manning 2006). Individual flower stalks may produce up to four flowers simultaneously (N. H. Martin, unpubl. data). If the visitor approached more than one flower on the same plant, or more than one unit of the same flower, these were recorded as additional visits. Recordings on any given day were usually stopped when ca. 500 visits had been observed. Visitors were classified as belonging to one of three categories: B—worker *Bombus* sp. bees and queen *Bombus* sp. bees; L—lepidopteran (Only visits from *Phoebis sennae* [Pieridae] were included in these analyses. Other rare lepidopteran visitors included *Danaus plexippus* [Nymphalidae], two unidentified *Papilio* species [Papilionidae], and unidentified members of the family Hesperiidae); and H—hummingbird (*Archilochus colubris*). We have not previously recorded legitimate visits to Louisiana Irises by lepidopterans.

DATA ANALYSES

Comparing visitation rates of different cross-types

Numbers of visits by individuals of each pollinator class were pooled for each day, in each plot. We then totaled the number of visits each plant received from each visitor type during each observation day, and the plants were then pooled by their genotypes (often several replicates per plot) and by their cross-type (*Ib*, *If*, F_1, BCIb, and BCIf). We also used phenological data from Martin et al. (2007) to obtain the number of flowers of each genotype/cross-type that were open each day, in each plot. These data provided the relative frequency of each genotype/cross-type that was open each day, and thus an expected frequency of visits for each flower type if pollinators were visiting flowers at random. We then calculated the expected number of visits to each cross-type by simply multiplying the expected frequency of visits (based on the frequency of flowers produced by each cross-type) and the total observed visits. For each day of pollinator observations (see Table 1 for days included in these analyses), we used 2×2, 2×3, and 2×4 χ^2 contingency tables (depending on the number of cross-types that were flowering on a particular day) to

Table 1. In each data-cell, the number of observed visits/expected visits (rounded to the nearest whole number) are reported, followed by this calculated ratio (based on nonrounded expected visits) for three different pollinator classes in two different experimental plots (Plot A – Table 1A and Plot B – Table 1B). Expected visits are calculated based on the number of flowers of each cross-type that were open on a given day. In plot A, no pollinator observations were made when *I. brevicaulis* plants were flowering. Data in bold represent days in which significant differences (based on χ^2 tests) in pollinator preference were observed. Letters following the observed/expected ratio represent significant pairwise differences between the attractiveness of different genotypic classes. Genotypic classes sharing a letter were not found to be significantly different (and those letters in parentheses were not significant when Bonferroni corrections were applied). Note: in some cases sample sizes were too low for a meaningful test and thus were not carried out.

Plot (A)

	31-Mar	1-Apr	3-Apr	5-Apr	6-Apr	7-Apr	9-Apr	12-Apr
Bee								
BCIB	–	–	–	0/0, 0.00	–	0/0, 0.00	0/4, 0.00	–
F1	–	–	–	0/0, 0.00	–	2/1, 2.78	7/12, 0.60	–
BCIF	–	–	–	5/4, 1.29	–	10/10, 1.03	81/71, 1.13	–
IF	–	–	–	0/1, 0.00	–	0/1, 0.00	5/5, 0.93	–
Butterfly								
BCIB	–	–	–	0/0, 0.00	–	2/1, 3.13	–	–
F1	–	–	–	0/0, 0.00	–	1/1, 0.69	–	–
BCIF	21/22, 0.97	3/2, 1.21	8/7, 1.10	6/5, 1.11	–	19/19, 0.98	–	–
IF	16/15, 1.04	1/2, 0.66	2/3, 0.73	1/1, 0.78	–	2/3, 0.78	–	–
Hummingbird								
BCIB	–	–	–	**0/3, 0.00 B**	0/0, 0.00	**0/1, 0.00 A,B**	**0/2, 0.00 A,B**	3/3, 1.12
F1	–	–	–	**9/3, 3.15 A**	2/1, 2.56	**0/1, 0.00 A,B**	**1/5, 0.19 B**	19/17, 1.14
BCIF	**9/4, 1.54 A**	–	**89/115, 0.77 B**	**107/108, 0.99 B**	8/12, 0.68	**15/18, 0.85 B**	**40/32, 1.24 A**	73/75, 0.97
IF	**1/6, 0.24 B**	–	**70/43, 1.61 A**	**24/26, 0.93 B**	5/2, 2.40	**7/2, 2.98 A**	**1/2, 0.41 A,B**	4/5, 0.85

	13-Apr	14-Apr	15-Apr	16-Apr	17-Apr	18-Apr	**19-Apr**
Bee							
BCIB	0/0, 0.00	**51/67, 0.77 B**	**90/95, 0.95 A**	**24/16, 1.49 A**	75/65	**158/148, 1.07 B**	**313/377, 0.83 B**
F1	2/1, 5.50	**107/85, 1.25 A**	**114/103, 1.10 A**	**22/18, 1.20 A,B**	46/44	**127/92, 1.38 A**	**197/110, 1.79 A**
BCIF	0/0, 0.00	**245/246, 1.00 A,B**	**321/318, 1.01 A**	**34/45, 0.76 B**	104/114	**160/204, 0.78 C**	275/298, 0.92 B
IF	0/0, 0.00	**5/10, 0.48 B**	**0/8, 0.00 B**	**0/1, 0.00 A,B**	0/1	–	–
Butterfly							
BCIB	0/2, 0.00	1/0, 2.04	0/1, 0.00	**1/4, 0.21 B**	–	–	–
F1	2/3, 0.58	0/1, 0.00	1/1, 0.85	**0/6, 0.00 B**	–	–	–
BCIF	14/13, 1.04	2/2, 1.11	5/4, 1.37	**23/13, 1.72 A**	–	–	–
IF	3/0, 6.95	0/0, 0.00	0/0, 0.00	**0/0, 0.00 A,B**	–	–	–
Hummingbird							
BCIB	**39/30, 1.31 A,B**	28/27, 1.03	**53/73, 0.72 B**	**65/76, 0.86 A,B**	55/66, 0.83	**94/115, 0.82 B**	63/60, 1.06
F1	**49/64, 0.77 B**	28/35, 0.81	**96/80, 1.20 A**	**61/87, 0.70 B**	54/45, 1.19	**61/71, 0.85 B**	13/17, 0.75
BCIF	**247/249, 0.99 A,B**	107/100, 1.07	**250/246, 1.02 A,B**	**246/210, 1.17 A**	121/118, 1.03	**191/159, 1.20 A**	48/47, 1.02
IF	**15/8, 1.89 A**	3/4, 0.71	**7/6, 1.08 A,B**	**5/4, 1.15 A,B**	1/2, 0.66	–	–

Continued

Table 1. Continued.

Plot (B)										
	30-Mar	31-Mar	1-Apr	3-Apr	4-Apr	7-Apr	**8-Apr**	9-Apr	12-Apr	**13-Apr**
Bee										
IB	–	–	–	–	–	–	–	–	–	–
BCIB	–	–	–	–	0/0, 0.00	4/3, 1.33	**4/1, 3.01 A**	0/0, 0.00	0/0, 0.00	**11/3, 3.19 A**
F1	–	–	–	–	–	14/15, 0.93	**4/4, 1.13 A,B**	2/1, 2.24	0/0, 0.00	**21/5, 3.85 A**
BCIF	–	–	11/9, 1.20	1/1, 1.18	4/3, 1.23	323/321, 1.00	**46/46, 1.01 B**	9/9, 0.99	2/1, 1.39	**1/23, 0.04 B**
IF	–	–	1/3, 0.35	0/0, 0.00	1/1, 0.00	35/36, 0.97	**1/4, 0.23 B**	0/1, 0.00	0/0, 0.00	**0/1, 0.00 (A),B**
Butterfly										
BCIB	–	–	–	–	**11/4, 2.45 A**	1/0, 8.93	–	0/0, 0.00	–	–
F1	–	–	–	–	**–**	1/1, 1.79	–	0/1, 0.00	–	–
BCIF	3/5, 0.66	27/27, 1.00	6/7, 0.88	–	**95/99, 0.96 B**	11/12, 0.92	–	7/6, 1.21	–	–
IF	5/3, 1.46	9/9, 1.00	3/2, 1.40	–	**15/18, 0.84 B**	1/1, 0.74	–	0/0, 0.00	–	–
Hummingbird										
IB	–	–	–	–	–	–	–	–	–	–
BCIB	–	–	–	–	10/5, 2.00	0/0, 0.00	1/0, 2.95	–	7/11, 0.65	**1/10, 0.10 B**
F1	–	–	–	–	–	0/1, 0.00	2/1, 2.21	–	16/10, 1.61	**5/16, 0.31 B**
BCIF	**18/26, 0.68 B**	**18/14, 1.33 A**	0/1, 0.00	–	107/110, 0.97	20/18, 1.11	10/12, 0.86	–	56/60, 0.94	**91/69, 1.32 A**
IF	**28/20, 1.42 A**	**0/5, 0.00 B**	1/0, 4.20	–	18/20, 0.90	1/2, 0.50	1/1, 0.89	–	4/3, 1.48	**2/3, 0.58 A,B**

	14-Apr	**15-Apr**	**16-Apr**	**17-Apr**	**18-Apr**	**19-Apr**	**1-May**	3-May	4-May
Bee									
IB	**–**	**–**	**–**	**–**	**–**	**–**	**38/16, 2.38 A**	58/46, 1.26	10/10, 1.00
BCIB	**75/42, 1.80 A**	**81/38, 2.11 (A)**	**92/65, 1.42 A**	**142/72, 1.97 A**	**167/95, 1.75 A,(D)**	**133/86, 1.54 A,C**	**851/817, 1.04 B**	390/390, 1.00	104/113, 0.92
F1	**84/47, 1.78 A**	**118/77, 1.54 (A)**	**130/87, 1.49 A**	**152/96, 1.59 A,(C)**	**159/87, 1.83 A,(C)**	**84/59, 1.43 A,C**	**0/16, 0.00 C**	–	–
BCIF	**146/209, 0.70 B**	**126/207, 0.61 B**	**198/262, 0.76 (B)**	**158/281, 0.56 B**	**105/246, 0.43 B**	**42/112, 0.37 B**	**115/156, 0.74 D**	72/84, 0.86	26/18, 1.49
IF	**1/8, 0.13 B**	**1/4, 0.23 (A), B**	**0/6, 0.00 (B)**	**0/3, 0.00 B,(C)**	**0/3, 0.00 B (C), (D)**	**0/2, 0.00 B,C**	**–**	–	–
Butterfly									
BCIB	**5/15, 0.34 B**	**4/9, 0.45 B**	**–**	**0/5, 0.00 B**	**0/23, 0.00 D**	–	–	–	–
F1	**2/17, 0.12 B**	**3/18, 0.17 B**	**–**	**1/7, 0.15 B**	**7/21, 0.34 C**	–	–	–	–
BCIF	**95/75, 1.27 A**	**64/48, 1.35 A**	**–**	**29/20, 1.46 A**	**93/59, 1.58 B**	–	–	–	–
IF	**7/3, 2.60 A**	**4/1, 4.08 A**	**–**	**2/0, 8.25 A**	**3/1, 4.34 A**	–	–	–	–
Hummingbird									
IB	–	–	–	**–**	–	–	0/0, 0.00	**1/9, 0.11 B**	0/2
BCIB	24/28, 0.85	18/28, 0.65	24/34, 0.71	**22/38, 0.57**	**43/58, 0.74 B**	11/13, 0.83	10/10, 1.02	**93/74, 1.25 A**	27/23
F1	36/32, 1.12	57/56, 1.03	48/46, 1.05	**58/51, 1.13 A**	**73/71, 1.37 A**	12/9, 1.32	0/0, 0.00	**–**	–
BCIF	146/143, 1.02	157/150, 1.05	143/137, 1.04	**153/150, 1.02 A**	**143/151, 0.95 B**	17/17, 0.98	2/2, 1.75	**5/16, 0.31 B**	1/4
IF	3/5, 0.58	4/3, 1.30	5/3, 1.69	**8/2, 4.38**	**5/2, 2.82 A**	0/0, 0.00	–	–	–

test whether observed visitation differed from that expected. This was performed on each day separately. On days in which these contingency tests proved significant ($P < 0.05$), we performed all possible post hoc 2×2 χ^2 contingency tests to examine whether pollinators preferred one cross-type over another. *P*-values were adjusted using Bonferroni step-down corrections for each day. For all post hoc tests, expected values were recalculated assuming that only those two cross-types examined were present.

We also devised a global measure of pollinator "choice" across the entire study period separately for both sites. This was simply the ratio of the observed visits for a particular cross-type (summed across all days) to that expected (summed across all days). A ratio larger than one indicates a higher proportion of visits to a cross-type relative to that expected, a ratio less than one indicates a smaller proportion of visits relative to that expected, whereas a ratio approaching one indicates that pollinators were visiting a particular cross-type at a frequency equal to that of the frequency of that cross-type's flowers produced throughout the season. We performed all possible post hoc 2×2 χ^2 contingency tests (comparing observed vs. expected visits, *P*-values adjusted using Bonferroni step-down corrections) to statistically compare each cross-type. Only days in which both cross-types were present could be included for these post hoc comparisons, and in those days, observed visits were summed across all days. Expected values were thus recalculated assuming only those two cross-types were present, and summed across all relevant days. *Iris fulva* and *I. brevicaulis* could not be statistically compared because their flowering dates did not overlap.

QTL ANALYSIS

To infer the genetic basis of "pollinator choice" (i.e., in terms of plant loci affecting this trait), we had to calculate "preference" at the level of the individual genotype separately for the two mapping populations, BCIb and BCIf, and separately for each pollinator class. We calculated preference for each genotype that flowered throughout the experiment (116 different BCIF individuals, and 124 different BCIB individuals) in the following manner. Only individuals of the same backcross mapping population were included in the calculations. Observed visits were recorded for each census date for each genotype (summed across all genotypes flowering in the plot, i.e., up to three replicates were included in the plot spaced at random, and all flowers that occurred on all replicates were counted equally). Expected visits were calculated by simply counting the total number of flowers produced by plants of a particular genotype. The ratio "observed:expected" visits was calculated for each plant separately for each census date, and for each date the genotype with the highest calculated "observed:expected" ratio was set at one, and all other genotypes were scaled relative to that maximum. Thus, all flowering plants received a score ranging from zero (no visits) to one (the maximum observed:expected ratio) on each day. A mean score for each genotype was calculated across all days in which a particular genotype was censused. Days in which more than half of the plants did not receive a visitor (i.e., were assigned a score of zero) were eliminated from the analyses. The mean score (ranging from zero to one) was used as the phenotypic data for the QTL analyses described below. In total, we performed QTL analyses separately for both plots for bee "choice," hummingbird "choice," and butterfly "choice." For the butterflies, only plot B was included in the analysis because insufficient visits were observed in plot A.

QTL analysis was performed on pollinator "preference" data for the backcross populations (BCIb and BCIf), using Windows QTL Cartographer version 2.5 (Wang et al. 2006). Each genotype was scored for three preferences—one for each visitor type (*Bombus* bees, lepidopterans, and hummingbirds). The genetic maps used for these analyses were those described by Martin et al. (2007). We performed Composite Interval Mapping (CIM; Zeng 1994) separately for each trait, and separately for each mapping population. CIM tests, using a forward and backward regression method, were performed at 2-cM intervals along both maps. A 10-cM window size was used to exclude closely linked cofactors, with the number of control markers set to five (the default setting). Experiment-wise threshold values for declaring the significance of a QTL ($\alpha = 0.05$) were determined using 1000 permutation tests (as suggested by Churchill and Doerge 1994; Doerge and Churchill 1996). A drop below the permutation threshold (often accompanied by a change in the directionality of the QTL effect) was used as an indicator of a boundary between multiple QTL peaks on the same linkage group. We report significant QTLs as determined from the permutation-test criteria. We calculated two-LOD support limits for each significant QTL.

In a previous study, QTL mapping was used in the same mapping populations described here to identify the genetic architecture of a large number of floral structure and color traits (Bouck et al. 2007). In that study, morphological characters examined included flower stalk height, anther extension, stylar branch length, total sepal length, sepal stalk length, sepal blade length, and the ratio sepal stalk length:sepal blade length. Color traits examined included the calculation of "nectar guide area" (*I. brevicaulis* plants have a yellow nectar guide on the sepals.). Sepal blade color was also quantified by calculating a standard reflectance spectrum of the adaxial surface of the sepals. From this calculation, "sepal blade chroma," "sepal blade hue," and "sepal blade brightness" were calculated using standard methodologies described by Endler (1990). QTL mapping revealed a number of QTLs that affect these traits (See fig. 3 in Bouck et al. 2007). We compared those floral QTLs identified by Bouck et al. (2007) to the pollinator-QTLs identified in the current study to determine whether there was any significant overlap (i.e., the confidence intervals of pollinator-QTLs overlapped with those of

flower-morphology/color QTLs). If such overlap is detected, this would be consistent with the hypothesis that pollinators are responding to the visual cues coded for by the genes underlying the structure/color QTLs.

Results

POLLINATOR PREFERENCES

A total of 11,834 visits were recorded from the two field sites, 5201 from plot A and 6633 from plot B. Most of the visits were by hummingbirds (47.8% in plot A and 26.6% in plot B) and *Bombus* (49.6% in plot A and 65.6% in plot B). Lepidopterans accounted for 2.6% and 7.7% of the visits in plots A and B, respectively. The observed number and expected number (based on flowering phenological data published in Martin et al. 2007) of visits to each genotypic class on each census date are given in Table 1. None of the *I. brevicaulis* plants in plot A flowered during the study period.

Pollinator preferences for the various genotypic classes differed among the three pollinator classes (Table 1; Fig. 1). There were a series of preferences and lack of preferences for various genotypic classes that were consistent between both of the experimental plots. Hummingbirds showed a consistent preference for *I. fulva* flowers, but significantly under-visited both BCIb and F_1 genotypes. Like hummingbirds, lepidopteran pollen vectors demonstrated a lack of preference for BCIb genotypes. These insects also significantly under-visited *I. brevicaulis* flowers. The lepidopteran visitation patterns did, however, reflect a preference for BCIf genotypes (Table 1; Fig. 1). In contrast to both the bird and lepidopteran pollinators, bumblebees preferred the *I. brevicaulis* genotypes in both environmental settings. They also preferred F_1 hybrids as well. The one class consistently under-visited by bees was *I. fulva*.

There were also significant visitation patterns by the three pollinator classes that were only found in one of the two environments (i.e., Plot B; Table 1; Fig. 1). The majority of these supported the above findings indicating that pollinators tended to favor either *I. fulva*-like or *I. brevicaulis*-like genotypes/phenotypes. For example, lepidopterans preferred BCIf in experimental Plot B. Also in Plot B, bumblebees significantly over visited BCIb, but significantly under-visited backcrosses toward *I. fulva*. Finally, hummingbirds demonstrated a preference for F_1 hybrids in Plot B.

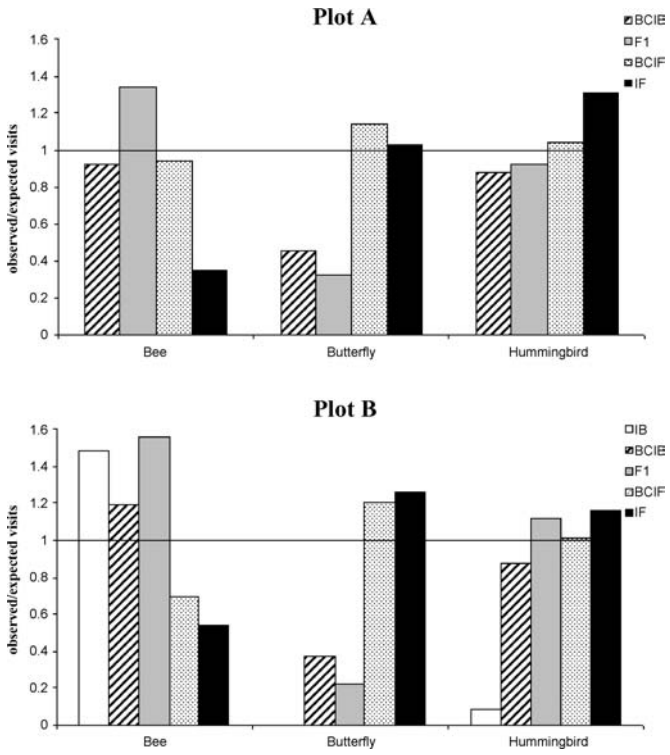

Figure 1. Frequency of observed pollinator visits : expected pollinator visits summed throughout the experiment in two different plots (A) Plot A, (B) Plot B. A ratio larger than one indicates a higher proportion of visits relative to that expected, a ratio less than one indicates a smaller proportion of visits relative to that expected, whereas a ratio approaching one indicates that observed and expected visits were relatively equivalent.

PREFERENCE QTL

CIM detected six significant QTLs that exceeded significance thresholds (ranging from Likelihood Ratio [LR] = 12.2 – 14.5) for the preference phenotypes in the BCIb mapping population and nine significant QTLs exceeded LR thresholds ranging from 12.6 to 13.1 for the preference phenotypes in the BCIf mapping population (Table 2, Fig. 2). Because butterfly visitation to BCIb individuals was extremely infrequent, we did not perform QTL analyses in this population for butterfly preference.

As many as three QTLs were detected for each trait. In the BCIb mapping population, a single QTL was detected on LG6 that influenced *Bombus* preferences in plot A, whereas one QTL each on LG1 and LG2 influenced *Bombus* preferences in plot B. Significant QTL were also detected on LG3, LG6, and LG7 that influenced hummingbird preferences in plot A. In the BCIf mapping population, there was evidence for a single QTL influencing *Bombus* preference in plot A on LG13, and one QTL influencing *Bombus* preference in plot B on LG1. Significant QTLs were also detected for hummingbird preferences on LG4, LG9, and LG15 for plot A, and two QTLs were detected on the same linkage group (LG3) that significantly affected hummingbird preference in plot B. Only two QTLs that affected butterfly preference were detected (plot B) on LG1 and LG7. For each of the traits, the number of QTLs detected should be considered a minimum, because sample sizes were small for several of the traits examined (34–48 separate BCIf genotypes, and 33–75 BCIb genotypes were scored

Table 2. QTL underlying the preference behavior of four visitor types to Louisiana Irises, in two experimental plots. The plots are denoted following the visitor name as (A) or (B). QTL locations are denoted by linkage group (LG), nearest marker, confidence intervals, the most likely location (in cM), effect size, and percentage of variation explained (PVE) are also given. Effect size is positive when the introgressed allele increases the preference and negative when the introgressed allele decreased the preference. Trait means are also given in the final column. BCIb – QTL for *I. fulva* alleles introgressed into an *I. brevicaulis* genetic background; BCIf – QTL for *I. brevicaulis* alleles introgressed into an *I. fulva* genetic background.

	QTL#	LG	Nearest marker	Location (most likely)	Effect	PVE	Trait mean
BCIb							
Bombus (A)	1	6	aCTAC8	0–24 cM (8)	0.205	0.450	0.217
Hummingbird (A)	2	3	aCTCA3	88–127 cM (101)	0.238	0.233	0.272
	3	6	aCTAC8	0–26 cM (14)	0.201	0.182	–
	4	7	cACGT1	0–16 cM (4)	−0.278	0.316	–
Bombus (B)	5	1	aAGAA3	121–145 cM (134)	−0.206	0.202	0.417
	6	2	cCTTA2	39–73 cM (57)	−0.191	0.165	–
Hummingbird (B)	–	–	–	–	–	–	0.253
BCIf							
Bombus (A)	1	13	cACTGN3	0–10 cM (10)	−0.156	0.270	0.243
Hummingbird (A)	2	4	aACGA21	25–58 cM (43)	0.173	0.261	0.253
	3	9	cACATN5	10–39 cM (32)	0.142	0.189	–
	4	15	aACAA15	9–28cM (18)	−0.126	0.136	–
Butterfly (A)	–	–	–	–	–	–	0.081
Bombus (B)	5	1	aCTTC17	96–135 cM (117)	0.224	0.405	0.172
Hummingbird (B)	6	3	cCTCT15	49–72 cM (66)	−0.284	0.386	0.238
	7	3	aCTTA11	76–102 cM (88)	0.225	0.195	–
Butterfly (B)	8	1	aCTTC20	60–94 cM (76)	0.143	0.178	0.156
	9	7	cCTAA5	0–24 cM (6)	0.162	0.231	–

for pollinator choice). When sample sizes are small, the ability of QTL mapping programs to detect significant QTLs diminishes, although the QTLs that are detected are in fact significant. Likewise, the small sample sizes may lead to an overestimate in both the magnitude of effect and the proportion of variance explained by individual loci. We therefore do not focus our results on the magnitude of effects or the proportion of variance explained (although we report these values in Table 2).

QTLs, for the most part, did not overlap with each other. However, examining the BCIf lod-score Figure 2, QTL 6 on LG3 (a hummingbird preference QTL showing positive additive effects) overlaps perfectly with a nearly significant peak associated with bee preference (interestingly of opposite effect, data not shown). Similarly, QTL 7 on LG1 (a butterfly preference QTL showing positive additive effects, Table 2) overlaps with a nearly significant peak associated with bee preference (again of opposite effect, data not shown). In the BCIb mapping population, QTL 1 and 3 on LG6 (a bee QTL and hummingbird QTL, respectively) showed almost a complete overlap, with effects in the same positive direction (Table 2). Furthermore, QTL 6 on LG2 (Fig. 2, a bee preference QTL of negative effect, Table 2) revealed strong overlap with a nearly significant hummingbird QTL (Fig. 2 also of negative effect, data not shown).

No clear pattern in the direction of the phenotypic effects (+ or – additive effects in Table 2) was detected. The pollination syndrome of *I. fulva* seems to be preferred by both butterflies and hummingbirds (Fig. 1), whereas that of *I. brevicaulis* seems to be preferred by bees. If this is the case, we might expect that for BCIf individuals, which are 75% *I. fulva*, hummingbird, and butterfly preference QTLs would reveal negative additive effects (i.e., the introgression of *I. brevicaulis* alleles would decrease the likelihood that a hummingbird or butterfly might find the flower attractive). Conversely, for BCIb individuals, we might expect that hummingbird and butterfly QTLs would largely have positive phenotypic effects (i.e., the introgression of *I. fulva* alleles would increase the chance for hummingbird or butterfly visitation). For bees foraging on BCIf individuals, we might expect that QTLs would largely be positive in their additive effects (i.e., the introgression of *I. brevicaulis* alleles increases the probability of a bee visiting the flower), and reveal negative effects in BCIb individuals. In reality, no clear pattern was found for the directionality of bee, hummingbird, or butterfly QTLs. In the BCIf mapping population, the butterfly QTLs detected were both positive, whereas three of five hummingbird QTLs also revealed positive additive effects (expectations being negative). Examining *Bombus* QTLs in the BCIf mapping population, one of the two was

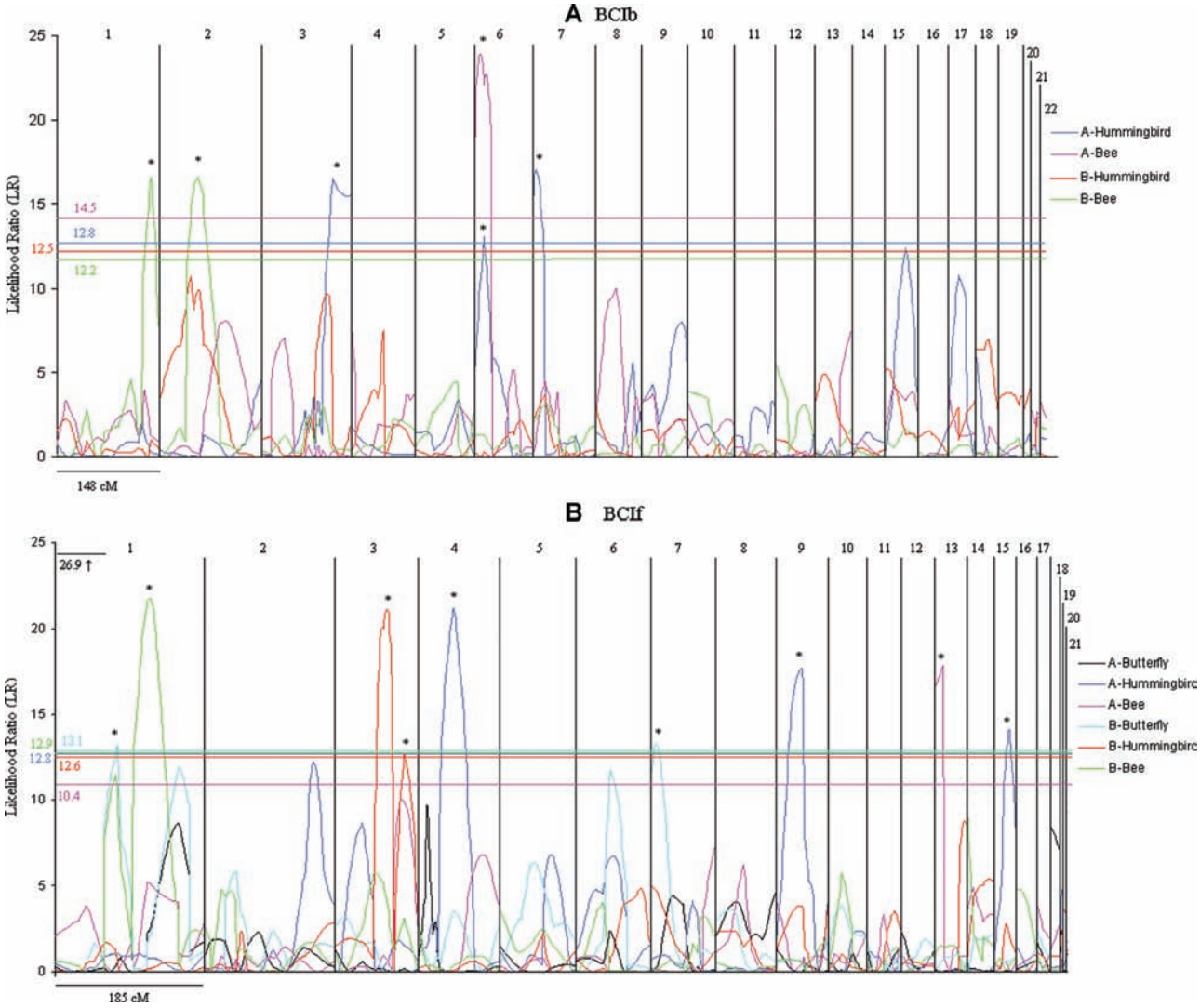

Figure 2. **QTL locations for pollinator preference traits in BCIb (Fig. 1A) and BCIf (Fig. 1B) mapping populations. Likelihood-ratio plots are given for all preference traits across the map. Map distances on the x-axis are given in centiMorgans. Asterisks indicate significant QTLs based on a permutation threshold (horizontal lines). Trait names denote the plot designation (A or B) followed by the visitor type.**

positive (expectations being positive). In the BCIb mapping population, two of three hummingbird QTLs detected were positive (expectations were positive), whereas two of three *Bombus* QTLs were negative (expectations were negative).

COLOCALIZATION OF FLORAL AND PREFERENCE QTL

A floral QTL map (Bouck et al. 2007) was compared to the data from the present analysis to test for colocalization of the floral trait and preference QTLs. Two preference QTLs were colocalized with floral QTLs in the BCIb map. Specifically, hummingbird preference QTL 2 detected in plot A colocalized with a positive floral color-QTL that affected "hue" (Bouck et al. 2007), whereas the *Bombus* QTL 6 detected in plot B colocalized with a different floral color-QTL that affected "brightness" (Bouck et al. 2007). The direction of effect for the preference QTLs differed among the pollinator classes (hummingbird vs. bee). For the *Bombus* QTL, bees generally avoided those plants that had introgressed *I. fulva* alleles (the additive effect was negative, Table 2, QTL 2), whereas for the hummingbird QTL, birds favored those plants that had introgressed *I. fulva* alleles (i.e., the additive effect was positive, Table 2, QTL 6). No colocalization of floral and pollinator-choice QTLs occurred in the BCIf mapping population.

Discussion

In general, our results support the hypothesis that pollinator behavior does not greatly limit gene flow between the Louisiana Iris species, *I. brevicaulis* and *I. fulva*. These species differ significantly in their peak flowering times (Cruzan and Arnold 1994; Martin et al. 2007). However, once formed, the F_1 and

backcross generation plants can act as a bridge for the introgressive hybridization detected in natural hybrid zones (Arnold et al. 1992; Arnold 1993; Cruzan and Arnold 1993; Johnston et al. 2001).

All three pollinator classes present during this study demonstrated a similar visitation pattern. The insect and avian vectors thus visited one of the parental species and at least one of the hybrid classes at similar frequencies. This observation suggests that the formation and evolution of hybrid zones between *I. brevicaulis* and *I. fulva* will be facilitated by pollinator preferences. This stands in opposition to the expectation derived from the divergent pollination syndromes exhibited by these species.

In this study we defined not only the ecological component of pollinator preference, but also defined some of the genomic regions, in the plant species of interest, which affect these preferences. To our knowledge, species of *Mimulus* reflect the only other plant system for which such a definition has been accomplished. For *Mimulus*, the analyses of Schemske and Bradshaw (1999) and Bradshaw and Schemske (2003) demonstrated the role of a single Mendelian factor. In contrast, the present analysis reflects the role that quantitative genetic factors may play in pollinator-mediated genetic exchange.

POLLINATION SYNDROMES

Iris brevicaulis and *I. fulva* possess a suite of characteristics that traditionally place them into bee- or bird-pollination syndromes, respectively. The various floral traits include coloration, nectar, shape, and size (Viosca 1935; Wesselingh and Arnold 2000a; Bouck et al. 2007). The hypotheses concerning pollination syndromes and pollinator-mediated speciation have an assumption that pollinators tend to specialize on the plant they visit, and visit it more frequently than other floral types (Proctor et al. 1996; Wolfe and Sowell 2006). This constancy would result in some measure of prezygotic reproductive isolation between the different floral types. However, pollination syndromes have been described as "classes with bad boundaries, but with a clear center" (van der Pijl 1961, p. 44). Therefore, in spite of the general acceptance of the pollination syndrome paradigm, it has come under increased criticism during the past decade (Herrera 1996; Waser 1998; but see Fenster et al. 2004).

Our data suggest that the apparent pollination syndromes displayed by *I. brevicaulis* and *I. fulva* do not result in exclusive preferences. However, these divergent pollination syndromes do apparently contribute to a partial discrimination by pollen vectors. Indeed, our results suggest that bees and birds/butterflies are differentially attracted to their predicted pollination syndromes. Butterflies and hummingbirds are thus attracted by *I. fulva*-like floral attributes, whereas bumblebees are attracted by *I. brevicaulis*-like phenotypes. Yet, none of these pollen vectors completely avoided flowers belonging to the alternate end of the scale of phenotypes/genotypes. This indicates a relaxed correspondence between a given pollinator class and a Louisiana Iris floral genotype/phenotype. Although our findings do not strongly support the pollination syndrome paradigm, we must emphasize the need to examine the relative efficiency of the different pollinators, and thus their effect on the fitness of the different plant genotypes (Campbell et al. 1998; Castellanos et al. 2003; Fenster et al. 2004). Furthermore, pollinator isolation is perhaps more appropriately viewed as the extent to which pollinators make interspecific visits. Yet, the pure-species plants placed in these experimental plots were completely isolated during this study period due to flowering phenology alone (i.e., *I. brevicaulis* and *I. fulva* flowered at different times). Thus, although there is a degree of prezygotic isolation due to pollinator behavior between those classes (parental and hybrid) that did overlap, the strongest barrier for gene flow is flowering phenology (Martin et al. 2007). However, as observed in the present study, pollinators do sequentially visit the various hybrid and parental classes that flowered at the same time, but they do so with marked preferences. Thus, the pollinators act as a strong yet incomplete barrier to introgressive hybridization.

DO POLLINATOR PREFERENCES ACT AS A REPRODUCTIVE BARRIER?

The results from the present study indicate that the three pollinator classes differ significantly in their floral preferences. Hummingbirds are traditionally associated with a strong preference for red flowers (Proctor et al. 1996). This analysis did indeed detect such a preference by hummingbirds. Thus, in keeping with the accepted pollination syndrome, these vectors demonstrated a higher visitation frequency to *I. fulva*-like plants and a significant avoidance of *I. brevicaulis*-like plants. However, the high-visitation frequency to hybrid genotypes as well suggests that hummingbirds can serve as a vector for pollen flow between the species and their hybrids, thereby facilitating introgression.

As stated above, *Bombus* individuals were defined by a preference for *I. brevicaulis*-like and a lack of preference for *I. fulva*-like genotypes. Their strong preferences for *I. brevicaulis*- or *I. brevicaulis*-like traits should contribute to reproductive isolation between the two species. Yet, the fact that this pollinator class also visits F_1 and backcross individuals at high frequencies (Fig. 1) indicates their potential role in the production of additional, introgressed genotypes. Wesselingh and Arnold (2000b) found that queen, but not worker, *Bombus* showed a definite preference for *I. brevicaulis*-like phenotypes/genotypes. They also found that F_1 flowers were the most visited by worker *Bombus*. In the present analysis, *Bombus* visited the F_1 genotypes at high frequencies as well (Fig. 1). Given the high frequency of visitations to both parental (especially *I. brevicaulis*) and hybrid genotypes, foraging *Bombus* should fuel the evolution of hybrid zones and act as an avenue for gene transfer between *I. brevicaulis* and *I. fulva*.

Butterflies, like hummingbirds, exhibited a strong preference for *I. fulva*-like genotypes and morphologies (Fig. 1). These findings suggest that the lepidopteran pollen vectors represent a stringent barrier for gene flow between these two Louisiana Iris species. Indeed, if lepidopterans were the main, or only, pollinator class present, interspecific gene flow would be expected to be very limited. However, even this pollen vector visited classes of genotypes from the alternate floral spectrum (i.e., *I. brevicaulis*-like). Furthermore, of the three studies of pollinator behavior carried out by our group, this is the first occasion that we have observed legitimate visits by lepidopterans. Given this, it is most likely that hybridization and reproductive isolation in this system will be most affected by bee and bird vectors.

The diversity of pollinators associated with the Louisiana Irises, and their preference patterns, supports the hypothesis that interspecific pollen flow is likely (Emms and Arnold 2000; Wesselingh and Arnold 2000b; the present study). Thus, although the insect and avian pollen vectors display significant preferences for a specific range of the floral traits that define the *I. brevicaulis* and *I. fulva* pollination syndromes, they do not limit their visitation to these classes. One caveat for our results is the lack of information on the relative efficiency of each visitor (i.e., the amount of pollen removal and deposition associated with different genotypic classes). Differential efficiencies in either of these functions may increase (or decrease) the effect of the different visitor types as pollinators, and thus alter the likelihood of hybrid formation and introgression.

POLLINATOR-MEDIATED SELECTION ON FLORAL TRAITS IN LOUISIANA IRISES

Our results lead to the inference that pollinator-mediated selection on floral traits may impact Louisiana Iris genotypes. The alternate preferences demonstrated by bees and hummingbirds/butterflies should result in selection coefficients that push floral phenotypes in opposite directions. In particular, if either bees or hummingbirds/lepidopterans predominate in a given space or time, selection would favor *I. brevicaulis* or *I. fulva* floral traits, respectively.

By examining the direction of effect of QTLs that affect pollinator preferences, one can test for evidence of past pollinator-mediated selection on floral traits (Orr 1998). For example, the pollination syndrome paradigm leads to the prediction that hummingbirds and butterflies would have exerted selective pressure on *I. fulva* to maintain its floral phenotype (e.g., red flowers, large volume of nectar), whereas bees would have exerted selective pressure on *I. brevicaulis* to maintain its floral phenotype (e.g., blue flowers, nectar guides). If this were the case, we would expect that the effect of butterfly and hummingbird preference QTLs would largely be in the direction of *I. fulva* (i.e., *I. fulva* alleles would increase hummingbird and butterfly preference in both mapping populations). Conversely, we would predict that the effects of QTLs for bumblebee preference would largely be in the direction of *I. brevicaulis* (i.e. *I. brevicaulis* alleles would increase bee preference in both mapping populations). These predictions were not consistently supported by our data. Instead, we found that the introgressed alleles in both mapping populations had a mixture of positive and negative effects on pollinator preferences. Due to the low sample size of QTLs detected for each trait, we did not have sufficient power to test for evidence of selection by examining direction of QTL effects (Orr 1998).

Notwithstanding the above findings, *I. brevicaulis* and *I. fulva* maintain distinctive floral phenotypes in nature, even in areas of sympatry. This suggests that pollinator-mediated selection may play some role, in spite of interspecific gene flow. We would suggest that this conclusion reflects the paradox of directional selection in spite of generalized pollinators (Ollerton 1996). In this regard, Waser (1998) suggested that pollinator-mediated selection might be effective, even when weak. One possible scenario for the Louisiana Irises is that (1) the significant preference of hummingbirds and butterflies for *I. fulva*-like flowers and (2) the significantly higher preference by *Bombus* for *I. brevicaulis*-like flowers (Fig. 1) may create a selection differential that contributes to the maintenance of these floral types.

We detected little evidence for colocalization of QTLs affecting floral traits (Bouck et al. 2007) with those that affected pollinator behaviors. Exceptions were two preference QTLs that colocalized with floral trait QTLs that contributed to the color-traits "hue" and "brightness" (Bouck et al. 2007). There is a possibility that such colocalizations are coincidental, and do not necessarily represent cases in which pollinators are "choosing" flowers based on those particular genomic regions. However, because pollinators have been shown to discriminate between flowers on a variety of visual cues (i.e., Duffield et al. 1993; Martin 2004), it is certainly reasonable that pollinators are in fact perceiving and responding to the underlying QTLs affecting floral morphology. We are continuing to test for floral trait QTLs that occupy overlapping genomic regions with those detected in the present study. In particular, we are currently examining the genetic architecture of the nectar characteristics that form the basis of the "reward" for pollen vectors. Such ongoing studies should yield an even greater definition of the genomic characteristics contributing to prezygotic isolation and introgression between *I. fulva* and *I. brevicaulis*.

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