

Effects of floral traits and plant genetic composition on pollinator behavior

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Received: 5 February 2009 / Accepted: 13 April 2009 / Published online: 23 April 2009
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Abstract Pollinator-mediated selection plays a major role in floral evolution and speciation. Floral traits that influence animal pollinator behavior are the target of pollinator-mediated selection, but can only evolve if floral phenotypes have underlying genetic variation. Thus, understanding the genetic basis of a floral trait is a crucial step in studying pollinator-mediated selection. In this study I tested the effect of quantitative trait loci (QTL) underlying floral traits on pollinator behavior in recombinant inbred lines (RILs) in the common sunflower, *Helianthus annuus* L. and its crop relative. The indirect effects of QTL on pollinator behavior, mediated by floral phenotypes, were analyzed for six insect visitor types using structural equation modeling (SEM) and path analysis. For three of the six visitor types (large and small bees and non-bee insects) valid models were revealed when all three levels (QTL, floral traits, and pollinator behavior) were incorporated. Nested model without genetics were validated for five of the six visitor types. The results suggest that insect behavior as a reaction to floral phenotypes is affected by the genetic architecture of floral traits.

Keywords Floral evolution · Insect behavior · Path analysis · Pollination ecology · Pollinator-mediated selection · QTL

Introduction

Plant–pollinator interactions have been proposed to play a major role in floral evolution and in plant speciation that is mediated by the preferences of the pollinators to one or a suite of floral traits (Aigner 2006; Grant 1949, 1994; Levin and Kerster 1967; Morgan 2007; van der Pijl 1961). Preferences of pollinators shaped according the association between floral traits and the reward; display size, color, fragrance, and other floral traits are used as cues in the learning process, and pollinator preferences evolve in response to these traits (Chittka and Raine 2006; Gegear 2005; Huber et al. 2005; Kunze and Gumbert 2001; Menzel 2001; Ne’eman and Kevan 2001; Raguso 2004). Differential preference of a pollinator for trait value can exert selection on this trait. For example, numerous studies have shown that larger floral displays attract more visitors, and hence, positive directional selection is exerted on floral display size (e.g., Campbell et al. 1991; Cariveau et al. 2004; Conner and Rush 1996; Fenster et al. 2006; Galen 1989).

Pollinator behavior alone is not enough to produce an evolutionary response of floral traits, because there is an obligatory requirement for genetic variation to underlie a trait for selection to act (Endler 1986). Genetic variation underlying a floral trait can be the result of a single gene/locus, where genetic variation is expressed as allelic variation (e.g., Bradshaw and Schemske 2003; Hoballah et al. 2007). Another possibility is that floral traits are controlled by more than one locus, and genetic interactions, such as pleiotropy or linkage, can affect the phenotype, which in

Handling editor: Heikki Hokkanen

Electronic supplementary material The online version of this article (doi:10.1007/s11829-009-9062-y) contains supplementary material, which is available to authorized users.

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turn affects pollinator behavior (Bouck et al. 2007; Hodges et al. 2002). Other genetic interactions that affect floral traits can be genetic constraints such as negative genetic correlations and trade-offs. These genetic constraints create contrasting evolutionary trends in floral traits (Ashman and Majetic 2006; Caruso 2006). Overall, the genetic basis of floral traits may influence the selective behavior of pollinators and should not be ignored in studies of floral evolution. Surprisingly, the effect of plant genetics on pollinator behavior has been rarely studied in a context of pollination research (but see Bradshaw and Schemske 2003; Hoballah et al. 2007; Martin et al. 2008).

Studies that have shown the effect of plant molecular genetic variation on pollinator behavior are usually restricted to a single gene or locus. In the pioneering work of Bradshaw and Schemske in *Mimulus* (Bradshaw et al. 1998; Bradshaw and Schemske 2003; Schemske and Bradshaw 1999), allele substitution in one locus altered floral pigmentation in *M. cardinalis* versus *M. lewisii*, and as a consequence pollinators shifted from hummingbirds to bees in *M. cardinalis* with *M. lewisii* alleles and vice versa. A similar study used *Petunia*, in which the AN2 gene of the horticultural plant *Petunia hybrida* was transformed with an allele of either the white-flower *P. axillaris* or the violet-reddish *P. integrifolia*. This allele substitution shifted floral pigmentation, and consequently the visitor assemblages (Hoballah et al. 2007). These studies are an example for studying the effect of plant genetics on pollinator behavior based on the variation in a single locus.

While the above studies have clearly shown that allelic variation in one locus/gene affects pollinator behavior, they have not shown the effect of quantitative floral traits that are associated with a few or many loci (quantitative trait loci, QTL). QTL mapping is a method to understand the genetic architecture of floral phenotypes (Baack et al. 2008; Bouck et al. 2007; Bradshaw et al. 1998; Fishman et al. 2002; Goodwillie et al. 2006; Hodges et al. 2002; Juenger et al. 2000; Stuurman et al. 2004). A study on Louisiana irises has used QTL mapping to associate pollinator behavior with genetic variation (Martin et al. 2008). Pollinators of Louisiana irises were treated as plant 'phenotype' for QTL analysis, and indeed few loci were found to be associated with pollinator preferences for three pollinator types (Martin et al. 2008). However, as the pollinators do not share their genome with the plants, such an analysis does not resolve the underlying mechanism in which the phenotype mediates plant genetics and pollinator behavior.

In the study described here, I propose to incorporate genetic composition (QTL), floral traits, and pollinator behavior to investigate the effects of genetic variation on pollinator behavior, represented by a causal model (Shipley 2000). Causal model links variables that are hypothesized

to affect each other. Here the model emphasizes the role of floral traits as a mediator between genetic variation and pollinator behavior, and gives insights into the potential evolutionary response of floral traits. I used this approach and tested for the discrimination of pollinators to genotypes underlying floral phenotypes. The approach used here is an extension of Martin et al.'s (2008) method, because it includes not only plant genetics and pollinator behavior, but also the intermediate path through floral traits.

The goals of this study included (1) to test whether pollinators discriminate among plant genotypes; (2) to test the hypothesis that plant genetic composition affects pollinator behavior through floral traits; and (3) to assess the differences among functional groups of pollinators in discriminating among genotypes and in responding to floral phenotypes.

Methods

Study system

The cultivated sunflower (*Helianthus annuus* L.) is an important crop plant in North America. It is cultivated near its wild progenitor (*Helianthus annuus* var. *annuus*) and crop-wild hybrid zones are frequent (Mercer et al. 2007; Snow et al. 1998). Recombinant inbred lines (RILs) were produced in order to study the dynamics and the genetics of such hybrid zones, as well as to investigate the evolution of domestication (Burke et al. 2002). RILs were developed from an initial cross between the cytoplasmic male sterile elite line cmsHA89 (USDA Ames 3963) and a wild plant grown from seed collected at Cedar Point Biological Station, Keith County, Nebraska, USA (Ann1238). A single F1 plant was self-pollinated to generate F2 plants, and the plants of the next four generations (F3–F6) were reproduced by self-pollination in each generation (Baack et al. 2008). Recurrent selfing events lead to increasing levels of homozygosity, up to a theoretical value of 98.4% of the loci homozygous in these RILs, with a 50% chance of being fixed for wild allele at any locus. RILs offer several advantages for this study. First, using hybrids between the distinct morphologies of crop and wild types (see Burke et al. 2002) increases the variation among plants, and enables continuous scale of phenotype values. Second, the high levels of homozygosity maximize differences at additive loci, and increase the ability to detect genetic effects on phenotypes and fitness. Finally, using RILs provides a system for genetic mapping of loci underlying quantitative traits, such as floral traits (Baack et al. 2008). Nonetheless, the use of RILs precludes direct comparisons with wild plants. Wild plants are self-incompatible and are not readily produce inbred lines. Thus, the exact genotype

of the wild plant used to establish the RIL population was not available. Likewise, genotypic differences, both in terms of the alleles present and the level of heterozygosity, make comparisons between RILs and wild plants of doubtful utility. Wild plants were thus not included, and their pollination was not studied in this experimental design.

Sunflower inflorescences are formed by whorls of ray florets that surround the disk florets. Florets are protandrous and open in a pattern of inwardly progressing concentric whorls; inner whorls present the pollen (male phase) while in older, outer whorls, the stigma is presented and receptive (Neff and Simpson 1990). Sunflowers are visited almost exclusively by bees, as has been shown for both crop (Dag et al. 2002; DeGrandi-Hoffman and Watkins 2000; Fell 1986; Parker 1981; Posey et al. 1986; Singh et al. 2001; Skinner 1987) or wild sunflowers (Meynie and Bernard 1997; Neff and Simpson 1990). There are varying results among studies regarding the relative importance and efficiency of wild and honey bees to sunflower pollination, but their combined effect has been certain to improve seed set (DeGrandi-Hoffman and Watkins 2000; Meynie and Bernard 1997; Parker 1981). Display size (i.e., head area) affects positively visitation rate of honey bees (DeGrandi-Hoffman and Watkins 2000), because it is an indicator for the number of open florets and the amount of the reward available for the visitor (Neff and Simpson 1990). Two different groups of bees are attracted to sunflowers with differing reward motivation. The large social bees collect mainly nectar, while smaller nonsocial bees collect both nectar and pollen (Dag et al. 2002; Parker 1981; Tepedino and Parker 1982).

Experimental design

Seeds of the seventh generation of crop \times wild sunflower RILs were obtained from B. Brunick, Oregon State University, Corvallis, OR, USA. 146 lines were used in this experiment alongside the parent crop genotype (cmsHA89). Seeds were planted in late April 2005 at Indiana University Botany Experimental Field, Bloomington, Indiana USA. The vegetation in the field consists of invasive grasses and forbs with patches of native temperate trees. The soil is dark brown silt loam mixed with urban soil above loess with residuals from limestone (Thomas 1981). Seeds were planted in the last week of April 2005 in nine blocks that were adjacent to each other. In each block, each RIL was represented by one plant assigned randomly to a location in the block. Blocks were constructed by 10 rows of 15 plants in each. There was a 50 cm distance between each RIL, and 50 or 100 cm distance between rows alternately to enable passage every other row. Plots were irrigated weekly until the fourth week to ensure

seedlings' establishment. Soil was not fertilized during the experiment.

Morphology scoring

Plants were surveyed weekly until the start of flowering, and then were surveyed daily. Flowering date was recorded as the day of which the florets at the outer whorl of the disk were open. Morphological measurements were performed on the primary flowering head 2 days after the start of flowering. Each plant was scored for the following traits: height of the main stem from the ground to the primary head, diameter of the disk, and diameter of the head including ray florets. Disk and head diameters were measured by two perpendicular axes in each head. The disk and head area were then computed based on the average of these measurements. In preliminary results, I found that head area was highly correlated to disk area (Pearson's $r = 0.880$, $DF = 921$, $P < 0.0001$), consequently only disk area was used in the analyses.

Pollinator observations

During the peak flowering season from 29 June to 26 July, 2005, plants were observed for insect visitors. All observations were conducted in sunny days, at the peak activity hours of bees between 7:00 and 10:00 AM (Posey et al. 1986). Preliminary observations indicated that most of the activity of insect visitors occurred during those hours, showing no significant differences within this time range (data not shown). Thus, I used data within those hours without further separation of smaller time fragments.

An observer watched the target plant and one to four neighboring plants, for 10 min periods, distributed randomly along the observation hours. Observed plants were randomly assigned the second day after flowering. Only primary heads were observed for both target and neighboring plants in order to assure that only measured heads were observed. For each visit to the flowering head, the time of arrival time and departure were recorded. The duration of a visit was calculated as the time spent on the flowering head. If a visitor stayed beyond the observation time, the duration of its visit was also recorded and included until the end of the 10 min period. As visitors to sunflowers are numerous [more than 400 bee species were recorded visiting sunflowers throughout North America (Hurd et al. 1980)], I categorized visitors a priori to any of six groups: (1) large bees (mainly *Bombus* spp., but also *Xylocopa virginica* and *Apis mellifera* were recorded, both in very low numbers); (2) medium-size bees (e.g., *Melissodes trinodis*); (3) small bees (e.g., *Ceratina* and *Augochlora* spp.); (4) Halictidae bees (*Halictus ligatus* and similar species); (5) non-bee insects: flies, butterflies

(including skippers), wasps; (6) beetles (mainly of the family Cantharidae). Although Halictidae bees could have been categorized as small bees (category #3), because they could have been differentiated from other small bees in the field I categorized them as a distinct group. Moreover, field observations, as well as the results (see below) suggest that behavior of Halictidae bees is different from other small bees. I did not check explicitly for pollen on the bodies of all visitor types, but observations suggest that the beetles were not transferring pollen efficiently, because they spent most or all the time of their visit on the ray florets. Insects visiting sunflower heads were haphazardly sampled and specimens of these insects were submitted to the entomological collection at Indiana State University, Terre-Haute, IN, USA for identification (see supplementary Table 1).

Genotypic data

Genetic composition of each plant at the relevant QTL was scored using data from previous research conducted on these plants (Table 2 in Baack et al. 2008). For the analysis I used four loci associated with flowering time (HT913, HT978, ORS398, and ORS735 in Baack et al. 2008 for flowering time QTL #1 to #4, respectively), one locus associated with disk area (HT319), and three loci associated with height of the primary flowering head (HT292, ORS57, and ORS331 for height QTL #1 to #3, respectively) (Baack et al. 2008). See Baack et al. (2008) for detailed description of how these QTL were obtained. Using Windows QTL Cartographer (Wang et al. 2006), a quantitative estimation of the additive effect was obtained for each locus significantly associated with floral phenotypic trait. For each RIL I summarized these additive effect values for all loci associated with each trait. The weight of the additive effect was determined based on the allele content at that locus. Weights were arbitrarily assigned as positive (+1) if the locus was homozygote for wild alleles, and negative (−1) for crop ones. Weights for heterozygote loci or loci that were not genotyped were set to zero. In addition to additive effect size, QTL analysis provides the explanatory power of each locus for the phenotype expressed as the percentage of variance explained (PVE). Additive effect of each locus associated with a trait was weighted by PVE value in each plant. This weight is important because the fraction of the phenotypic variation explained for a locus dictates its relative effect on the floral phenotype.

Statistical analysis

The plants included in the analysis were only those with a complete measured set of morphological and pollination traits, i.e. the data set was orthogonal. All statistical

analyses were performed in R (R development Core Team 2006). The response variables for each analysis were checked for normality and transformed where needed using the Box-Cox transformation family (MASS package for R; Box and Cox 1964; Venables and Ripley 2002).

In order to test the response of insect visitors to plant genetic composition, I tested the differences in both visitation rates and the duration of the visit between genetic classes. Genetic classes for all loci were defined by the allele content of each locus as either homozygous for wild alleles, crop alleles, or heterozygous. Two-way analysis of variance was performed, with genetic class and visitor type as categorical factors.

In order to test the mechanisms that underlie the response of pollinators to plant genetics, I used path analysis and structural equation modeling. The path model was constructed for a set of hierarchical effects, where the effect of plant genetics on insect visitation behavior is mediated by floral traits. Path analysis is a powerful tool to test specific hypotheses of causality in an evolutionary-ecology framework (Kingsolver and Schemske 1991; Mitchell 1992, 2001; Scheiner et al. 2000). It allows partitioning the covariation between a set of variables according to a specific model of causal relationships. Path analysis can test a structure of interactions among variables in addition to measure both direct and indirect effects on response variables (Mitchell 1992; Shipley 2000).

A path diagram was constructed as a working hypothesis (Fig. 1) to test the indirect effect of plant genetics on pollinator behavior. There are few hypotheses that support this model. The genetic composition in a set of QTL associated with a floral trait is expected to affect the phenotype of that trait (Burke et al. 2002; Erickson et al. 2004). Linear effects of the QTL allele combination on the single trait were assumed in this model. Potential genetic interactions between QTL were incorporated by testing for correlations between QTL variables as well as the correlations between the underlying floral traits. Floral traits are expected to affect pollinator visitation rates and duration of visits. The number of visits is affected by the size of the visual attraction of the inflorescence. This cue is also supported by the height of the stem with the assumption that higher stems create a more prominent signal above the competitor plants. It can also be affected by the date of flowering, because height of competing flowering species increases and the relative attraction of the sunflowers decreases (Moeller and Geber 2005). Duration of a visitor on the flowering head is affected by the number of florets which is associated with disk area (Neff and Simpson 1990), and by number of florets visits.

Model testing in path analysis involves partitioning of the path diagram to a set of linear multiple regression models for each response variable. The benefit of path

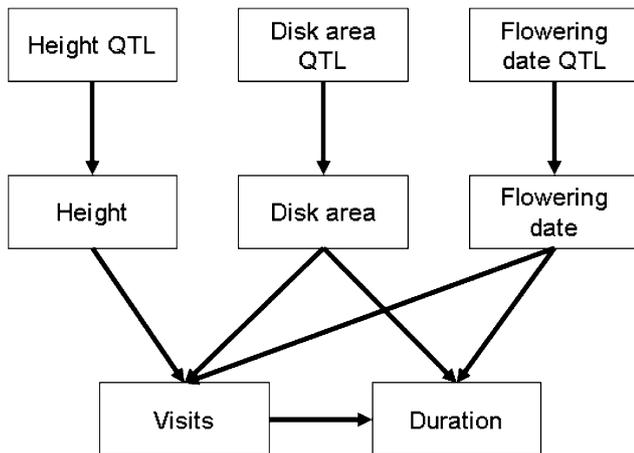


Fig. 1 Hypothesized path diagram for the effects of plant genetics and floral traits on pollinator behavior. Variables (in rectangles) are grouped by their position: genetic architecture of floral traits (QTL values) at the top, floral traits at the middle, and insect behavior (number of visits and duration of visits) at the bottom. Arrows leading from variables to other variables denote statistical effect (analogue of partial regression coefficient). See text for the methods used to evaluate the variables and their quantitative effects on other variables. Correlations among QTLs and among floral traits were also hypothesized in the model, but are not shown in the diagram for graphical simplicity

analysis over regular multiple regression models is that standardized path coefficients (partial regression coefficients) provide a measure of both direct and indirect effects on each dependent variable (Li 1975). The total effect on a response variable is the summary of all the paths (i.e. sequences of effects) leading from any of the explanatory factors to the response variable. Direct effects are the values of path coefficients, which are the standardized partial regression coefficients of the displayed arrow that directly connect two variables. Indirect effects are the product of path coefficients along a specific path. Structural Equation Modeling (SEM) was used to solve the path models (Mitchell 1992; Shipley 2000). This approach enables to test the deviation of the hypothetical model from the observed data (expressed by the standardized covariance matrix) by means of maximum likelihood estimates which generate a test statistic that is distributed approximately as χ^2 (Mitchell 1992). Good fit of the hypothesized model to the data will result in low χ^2 values and non-significant P value. SEM package for R (Fox 2006) was used for solving the structural equation models.

Significance of the path coefficients was tested using randomization simulations. The null distribution of each path coefficient was obtained by 100 iterations of random sampling (with replacement) of all the variables. SEM analysis was run for each iteration and path coefficients were obtained for each path. Significance of a path was determined if the value obtained from the real data was

outside 95% of the null distribution obtained from the randomization.

Various species of visitors can differ in their behavior on the flowers, as well as their efficiency as successful pollinators (Fell 1986; Michener 2000; Sahli and Conner 2007). To address these potential differences and to test whether different types of visitors are affected differently by floral traits, I analyzed separate path models for each visitor type.

In order to partition the effect of genetics (QTL) and floral traits on pollinator behavior, analyses on a nested model that contain only the effect of floral traits on pollinator behavior, without testing for genetic effect, were performed. Nested models were compared to the complete model (Fig. 1) using the differences in χ^2 and DF values (Mitchell 1992).

Results

A total of 361 plants that belong to 148 RILs were observed between June 29th and July 26th, 2005. A total of 2,314 insect visitors were recorded within 1,800 min of observations, over 19 days, 6–12 observations per day. A total of 23 plants did not receive any insect visits. The spectrum of insect visitors to the sunflowers was relatively broad as expected (Hurd et al. 1980). Significance differences were found among visitor types in mean number of visits (ANOVA; $F_{5,890} = 12.4$; $P < 0.001$; Fig. 2, top). Both nectar (large bees) and pollen foragers (medium size and *Halictus* spp. bees) were the dominant visitors in the observations. Mean duration of visit to an inflorescence was also different among visitor types (ANOVA; $F_{5,890} = 14.6$; $P < 0.001$; Fig. 2, bottom). Beetles and *Halictus* bees had the highest average duration time on the plants, but the observations suggest that beetles' role as pollinators is negligible because most of the time they were on the rays not touching the florets, as opposed to *Halictus* bees that actively collected pollen from florets. The mean, standard error and median for the pollinator behavior and floral traits are shown in Table 1. Correlations and covariances among all traits are listed in supplementary Table 2.

Response of pollinators to genotypes

Nine loci were tested for the response of insect visitors to the genetic composition of crop homozygous, wild homozygous, or heterozygous (Fig. 3). For both number of visits and duration the visitor type was highly significant factor affecting behavior in all analyses (Tables 2, 3). Genetic composition was a significant ($P < 0.05$) factor in the

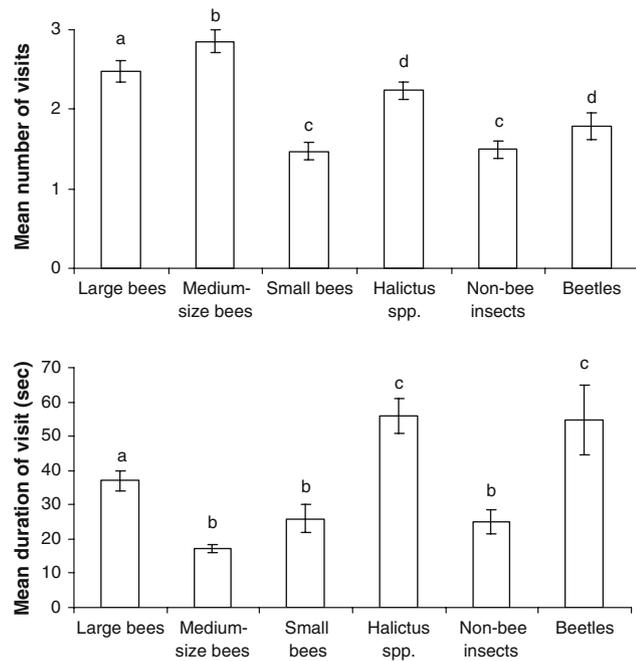


Fig. 2 Behavior of six insect visitor types to sunflowers. *Top*: mean (\pm SE) of visits per flowering head in 10-min observation intervals. *Bottom*: mean (\pm SE) of duration of visits. Bars with identical letters are not significantly different (contrast analysis; $P > 0.05$)

Table 1 Mean, standard error of the mean, and median for pollinator behavior, floral and fitness traits measured in this study

	Visits	Duration	Flowering time	Head area	Disk area	Height
Mean	5.46	195.24	17.11	6809.84	1390.17	86.11
SE	0.22	13.25	0.43	220.58	52.13	0.99
Median	4.5	120	15	6221	1195	84

Visits number of visits per flowering head per observation; *Duration* time spent by the visitor on the flowering head (in seconds); *Flowering time* time elapsed to flowering (in days) after the first flower started; *Head area* area (in mm²) of the flowering head, includes ray florets; *Disk area* area (in mm²) of the disk florets; *Height* the height (in cm) of the primary head above ground. $N = 361$ plants

analysis for flowering time QTL #1 and height QTL #2, and had a marginally significant effect for flowering time QTL #2 and disk area QTL (Table 2). Genetic composition was a significant ($P < 0.05$) factor in the analyses of duration of visits for flowering time QTL #4 and had a marginally significant effect for flowering time QTL #1 (Table 3). Contrast analyses for genetic composition that were significant at a level of $P < 0.1$ are shown in Fig. 4. Visitation rates were higher in loci homozygous for the crop alleles in all four loci tested. Heterozygous plants had values that were not significantly different than either crop or wild heterozygotes in all four loci. Duration was higher

for crop homozygotes in comparison with heterozygotes in flowering time QTL #1, and lower in flowering time QTL #4. In both loci, wild homozygotes showed intermediate values of duration.

Indirect effects and path analysis

The full hypothesized path model that incorporates all three levels (QTL, floral traits, and pollinator behavior) was significantly deviated from the observed data when all pollinator types were pooled together (Table 4). However, separate analyses of visitor types revealed models that did not deviate significantly from observed data for large bees, small bees and non-bee insects. The model for large bees revealed significant effects on visitation rates by all three floral traits measured, but only the disk area affected the duration of the visit (Fig. 5a). Visitation rates of small bees were affected by the height of the flowering head, and visitation rates of non-bee insects were affected by disk area as well as the time of flowering. No significant effect on the duration was found for small bees and non-bee insects (Fig. 5b, c).

Nested models that did not incorporate QTL effect on floral traits revealed a significant deviation from the observed data only in non-bee insects (Table 4). Nested models were significantly more accurate for medium size and *Halictus* bees and beetles, but were inconclusive for the data for small bees and non-bee insects (Table 4). Models for the large bees showed marginally significant deviation from the observed data ($P = 0.052$; Table 4), and similarly the difference between the full and nested model was close to the margin of significance ($P = 0.049$). According to the nested models, visitation rates of large bees were affected positively by all three floral traits. The visitation rates of all other insects were affected only by one of the three traits: height (small bees), disk area (medium-size and *Halictus* bees) or date of flowering (beetles; Fig. 6d, c, e, & f respectively). Duration of visits of small bees were not affected by measured floral traits, whereas disk area affected the duration of visits in large bees, and date of flowering affected duration in medium-size bees and beetles. Interestingly, while the latter effects were all positive, date of flowering had negative effect on duration of visits in *Halictus* bees (Fig. 6e).

Indirect effects of QTL on visitation rate and duration were calculated as the product of standardized path coefficients along each path, and summarizing all paths (Table 5). For example, the indirect effect of height QTL on number of visits has few paths in large bees: (1) the effect of Height QTL on height, multiply by the effect of height on visitation rate; (2) the effect of Height QTL on height, multiply by the correlation of height and disk area, multiply by the effect of disk area on visitation

Fig. 3 Response of insect visitors to genotypic composition of eight QTL. *Bars* represent mean (\pm SE) of number of visits (*left*) or duration of visits (*right*), as a function of the allelic content of QTL. *Crop* homozygous for crop alleles, *Wild* homozygous for wild alleles, *H* heterozygous locus. No heterozygotes found for flowering time QTL 2. Duration of visits are not shown for beetles (visitor type #6) because their values were in an order of magnitude higher than other insects, and probably not associated with potential pollination (see text)

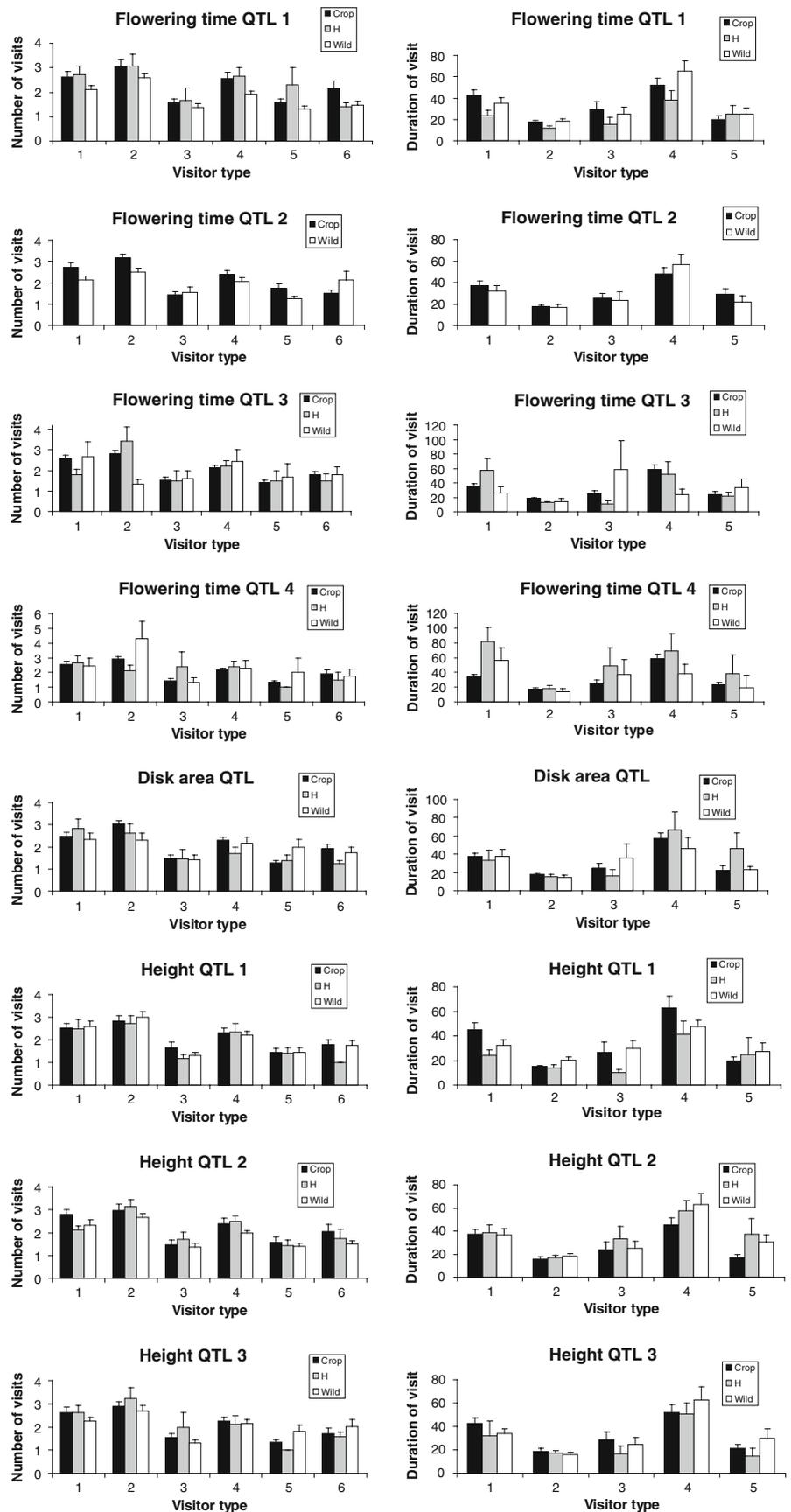


Table 2 Analysis of variation of number of visits as a function of visitor type and genotypic composition of QTL for floral traits

Factor	DF	SS	MSS	F	Sig. (P value)
Flowering time QTL 1					
Visitor type	5	7.82	1.56	15.7	<0.0001
Genetic composition	2	1.22	0.61	6.1	0.002
Residuals	837	83.35	0.10		
Flowering time QTL 2					
Visitor type	5	8.23	1.65	16.2	<0.0001
Genetic composition	1	0.35	0.35	3.4	0.065
Residuals	750	76.14	0.10		
Flowering time QTL 3					
Visitor type	5	7.19	1.44	14.1	<0.0001
Genetic composition	2	0.06	0.03	0.3	0.74
Residuals	781	77.94	0.1		
Flowering time QTL 4					
Visitor type	5	8.34	1.67	16.8	<0.0001
Genetic composition	2	0.21	0.11	1.1	0.35
Residuals	759	75.4	0.1		
Disk area QTL					
Visitor type	5	8.81	1.76	17.57	<0.0001
Genetic composition	2	0.58	0.29	2.89	0.056
Residuals	881	88.4	0.1		
Height QTL 1					
Visitor type	5	8.56	1.71	17.0	<0.0001
Genetic composition	2	0.01	0.01	0.1	0.94
Residuals	750	75.45	0.1		
Height QTL 2					
Visitor type	5	8.93	1.79	17.9	<0.0001
Genetic composition	2	0.75	0.38	3.8	0.024
Residuals	888	88.82	0.1		
Height QTL 3					
Visitor type	5	8.24	1.65	16.2	<0.0001
Genetic composition	2	0.1	0.05	0.5	0.606
Residuals	847	86.16	0.1		

Table 3 Analysis of variation of duration of visits as a function of visitor type and genotypic composition of QTL for floral traits

Factor	DF	SS	MSS	F	Sig. (P value)
Flowering time QTL 1					
Visitor type	4	83.85	20.96	18.2	<0.0001
Genetic composition	2	6.45	3.22	2.8	0.062
Residuals	749	864.32	1.15		
Flowering time QTL 2					
Visitor type	4	67.03	16.76	15.1	<0.0001
Genetic composition	1	2.56	2.56	2.3	0.13
Residuals	669	744.31	1.11		
Flowering time QTL 3					
Visitor type	4	77.77	19.44	16.7	<0.0001
Genetic composition	2	1.4	0.7	0.6	0.547
Residuals	708	824.06	1.16		
Flowering time QTL 4					
Visitor type	4	79.43	19.86	17.2	<0.0001
Genetic composition	2	11.28	5.64	4.9	0.008
Residuals	685	790.32	1.15		
Disk area QTL					
Visitor type	4	89.39	22.35	19.5	<0.0001
Genetic composition	2	1.52	0.76	0.7	0.517
Residuals	789	905.71	1.15		
Height QTL 1					
Visitor type	4	74.28	18.57	15.9	<0.0001
Genetic composition	2	1.97	0.99	0.8	0.429
Residuals	671	781.36	1.16		
Height QTL 2					
Visitor type	4	87.75	21.94	19.1	<0.0001
Genetic composition	2	2.18	1.09	0.9	0.389
Residuals	795	914.01	1.15		
Height QTL 3					
Visitor type	4	78.27	19.57	16.6	<0.0001
Genetic composition	2	1.03	0.51	0.4	0.648
Residuals	759	896.89	1.18		

rate; and (3) the correlation of height and flowering date QTLs multiply by the effect of flowering date QTL on flowering date, multiply by the effect of flowering date on visitation rate. Summary of paths 1–3 gives the total indirect effect.

QTLs associated with date of flowering had significant positive indirect effects on the number of visits in all three models that were not deviating from the observed data. QTLs associated with disk area were affected negatively with visitation rates of large bees, but did not have indirect effect on visitation rate for small bees and non-bee insect visitors. Visit duration was indirectly affected only by the flowering date and height QTL, and only for large bees with their effects in contrasting directions (Table 5).

Discussion

The study presented here shows that plant genetic composition can have an indirect effect on pollinator behavior that are mediated by floral genotype for different groups of visitors. There are currently only a handful of studies that used plant molecular genetics or QTL mapping to study pollinator behavior (e.g., Bradshaw et al. 1998; Bradshaw and Schemske 2003; Dyer et al. 2007; Hoballah et al. 2007; Martin et al. 2008). In some studies, particularly in those on *Mimulus*, *Antirrhinum* and *Petunia*, the effect of allelic variation in a single locus on the behavior of the major pollinator group was tested. These plant species are becoming a model system for floral evolution (Gerats and

Fig. 4 Response of insect visitors to genotypic composition in loci with effect of genotypic composition in level $P < 0.1$. Bars represent average values (\pm SE) of number of visits (graphs **a–d**) or duration (graph **e**). Bars with identical letters are not significantly different (contrast analysis; $P > 0.05$). Differences in graph **b** are in a level of $P = 0.064$. **a** Flowering time QTL 1, **b** Flowering time QTL 2, **c** Disk area QTL, **d** Height QTL 2, **e** Flowering time QTL 1

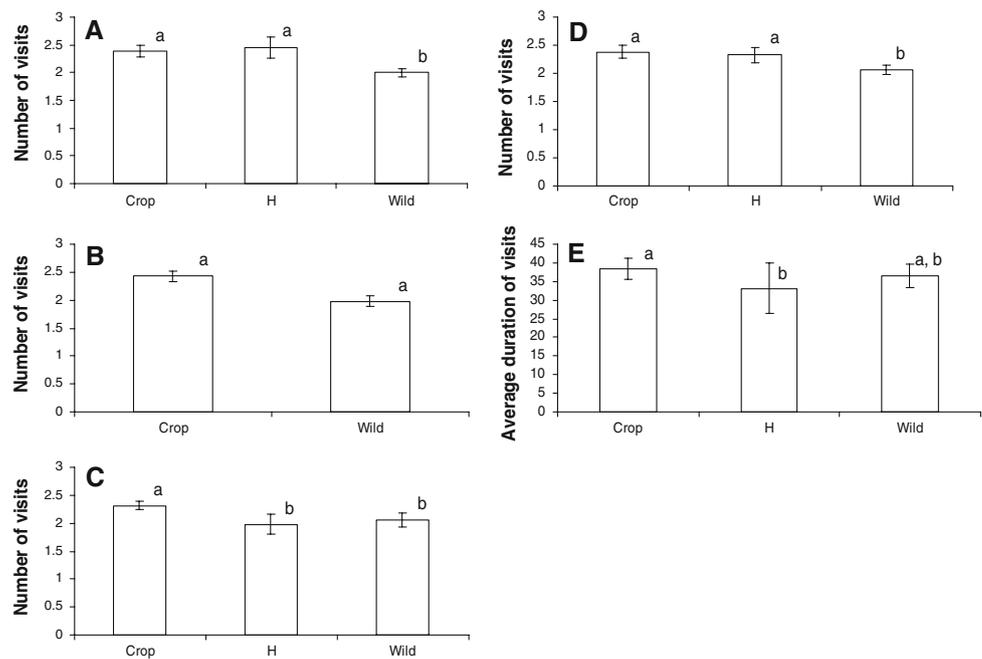


Table 4 Alternative hypothesized path models tested for the effect of genetic composition and floral traits on pollinator behavior and seed set of recombinant inbred lines of crop \times wild sunflower plants

Visitor type	Full model		Nested model		Nested compared to full model	
	χ^2 (DF = 13)	<i>P</i> value	χ^2 (DF = 1)	<i>P</i> value	χ^2 (DF = 12)	<i>P</i> value
All visitors pooled	83.56	<0.001	0.76	0.384	82.8	<0.001
Large bees	22.18	0.052	1.09	0.296	21.09	0.049
Medium-size bees	34.79	<0.001	0.70	0.404	34.09	<0.001
Small bees	6.77	0.914	0.11	0.741	6.66	0.879
<i>Halictus</i> spp.	31.4	0.003	0.38	0.538	31.02	0.002
Non-bee insects	19.06	0.121	4.57	0.032	14.49	0.271
Beetles	27.64	0.010	1.91	0.167	25.73	0.012

Full model includes all three levels of QTL, floral traits and pollinator behavior (Fig. 1); nested model includes only floral traits and pollinator behavior. Nested models compared to full models by subtracting χ^2 and DF values

Vandenbussche 2005; Hileman et al. 2003; Wu et al. 2007), and it was feasible to pinpoint candidate genes responsible for variation in floral traits. However, studies in non-model systems are needed for drawing generalizations on the role of plant genetics in pollination systems. In this study I have proposed a method that uses the genetic architecture of floral traits (i.e., QTL mapping) to connect plant genetics with pollinator behavior. I propose that the variation in floral traits can mediate between genetic composition of the plant and pollinator behavior. While a single-gene approach is promising for identifying the genes that affect pollination systems, the approach proposed here is additionally beneficial for plants with no gene annotations and for quantitative traits that are controlled by multiple genes.

The variation in floral characteristics has a genetic component that may indirectly affect a pollinator's

behavior, which, in turn, can affect the evolutionary response of the traits due to fitness differences. Evolutionary response requires that the trait under selection will be heritable and this depends on genetic basis of the trait. Therefore, in order to understand the evolutionary response to pollinator-mediated selection, it is essential to test the relationship between pollinator behavior and the genetic compounds in the floral traits. The majority of studies on the evolution of flower–pollinator interactions do not link the full path from gene to floral traits and pollinator behavior. This is a missing link in the abundant amount of research that has been conducted on genes, or QTL, controlling pollination-relevant floral traits (e.g., Baack et al. 2008; Bouck et al. 2007; Comba et al. 2000; Fishman et al. 2002; Goodwillie et al. 2006; Hodges et al. 2002; Juenger et al. 2000). Some of the studies that connected variation in

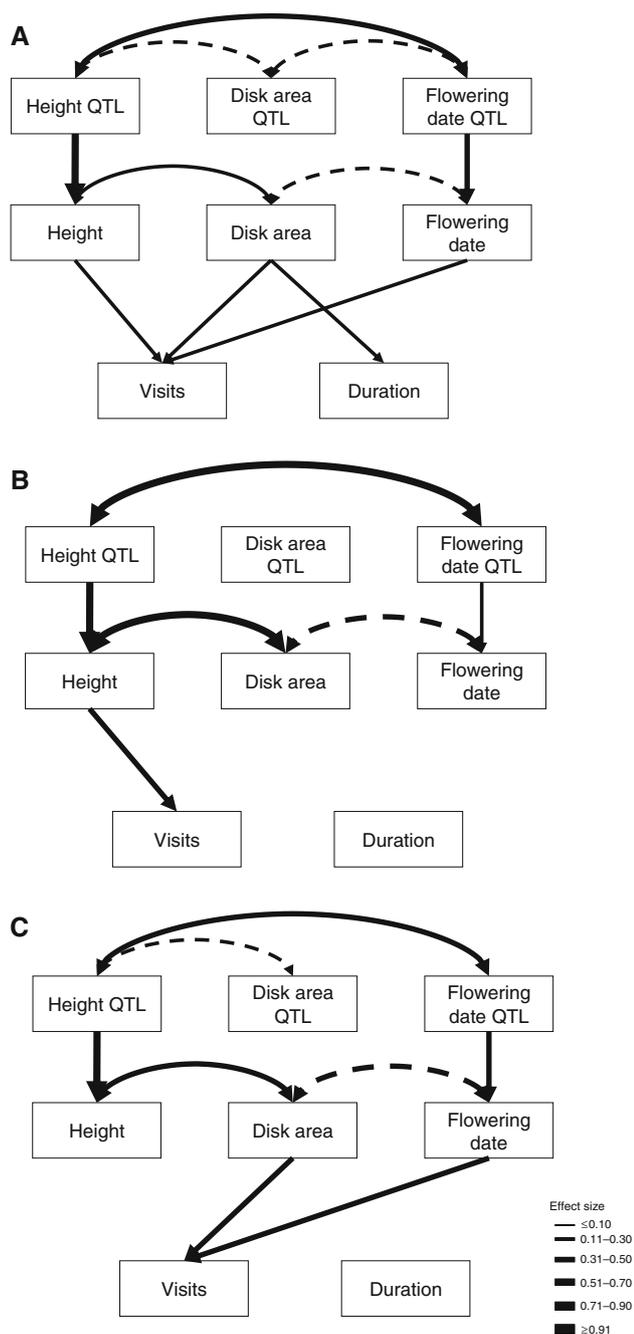


Fig. 5 Path models for the effect of floral trait QTLs on pollinator behavior in recombinant inbred lines of crop \times wild sunflower plants. Only models that did not deviated significantly from observed data are presented (see Table 4). Variables are in rectangles (see Fig. 1 and text for description), and arrows denote effects found among variables. *One-headed lines* denote linear effect (analogue of partial regression coefficient); *solid lines* indicate positive effects, and *dashed lines* indicate negative effects. The width of the arrow represents the size of the effect in standardized path coefficient units. *Curved double-headed arrows* represent correlations between variables. Only significant effects and correlations are presented in this path diagram. For example, non-significant effects were found affecting duration of visits in the models for small bees and non-bee insects (models **b** and **c**, respectively). **a** Large bees, **b** Small bees, **c** Non-bee insects

floral traits and pollinator behavior also investigated the fitness effects of pollinator behavior and estimated the extent of pollinator-mediated selection on the measured floral traits (e.g., Campbell et al. 1991; Cariveau et al. 2004; Lau and Galloway 2004; Medel et al. 2003). However, there are strikingly few studies that connect specific plant genes or loci with both floral traits and pollinator behavior (Dyer et al. 2007; Hoballah et al. 2007; Martin et al. 2008; Schemske and Bradshaw 1999). Here I show that this connection is a promising method for understanding the evolutionary context of pollination ecology.

It is important to note that in this study, I did not analyze the full path from gene to seeds, mediated by floral traits and seed-set. Plants used in this experiment are able to self, as required by the method of producing RILs (see “Methods” and Baack et al. 2008 for details). Indeed, there is still a shortage of systems where the full path can be studied, but molecular methods of paternity analysis would fill this gap. This was beyond the scope of the current study.

Genetic architecture has been explored in Louisiana Irises to study pollinator behavior. The visitation of pollinators to Louisiana Irises was considered as a plant phenotypic “trait” and was genetically mapped as a QTL (Martin et al. 2008). The analysis resulted in pollinator behavior QTL that were subsequently compared to floral trait QTL map. It revealed some co-localization of pollinator behavior traits with floral traits. Although the approach of Martin et al. (2008) is intriguing, it does not resolve the mechanism involved in these effects. The advantage of the method here is that an explicit hypothesis was tested for the mechanism that underlies the effect of plant genome on pollinator behavior. This is the mediation by the variation in floral phenotypes. I propose that such an approach enables better understanding of plant–pollinator co-evolution.

Insect visitors to the sunflowers in this study have shown discriminating behavior among genotypes, at least in some of the loci presented (Fig. 4). Interestingly, heterozygote genotype received similar number of visits to one of the homozygote genotypes (Fig. 4a, c, d). On the other hand, duration of visits were intermediate for heterozygotes in the two loci that showed visitor discrimination (Fig. 4e, f). This suggests that there is dominance of either the crop or wild alleles. However, given that these plants are a synthetic population produced by recurrent selfing, the expectation was that there will be hardly any heterozygote loci as in flowering time QTL #2 (Fig. 4b). Thus, it is plausible that average advantage of the heterozygote, and average disadvantage of homozygotes, maintained the existence of heterozygote loci. This can happen if many heterozygote loci in one plant will give it advantage over plant carrying many recessive homozygote loci (Hedrick 2005). This is also evident in the two loci that affected

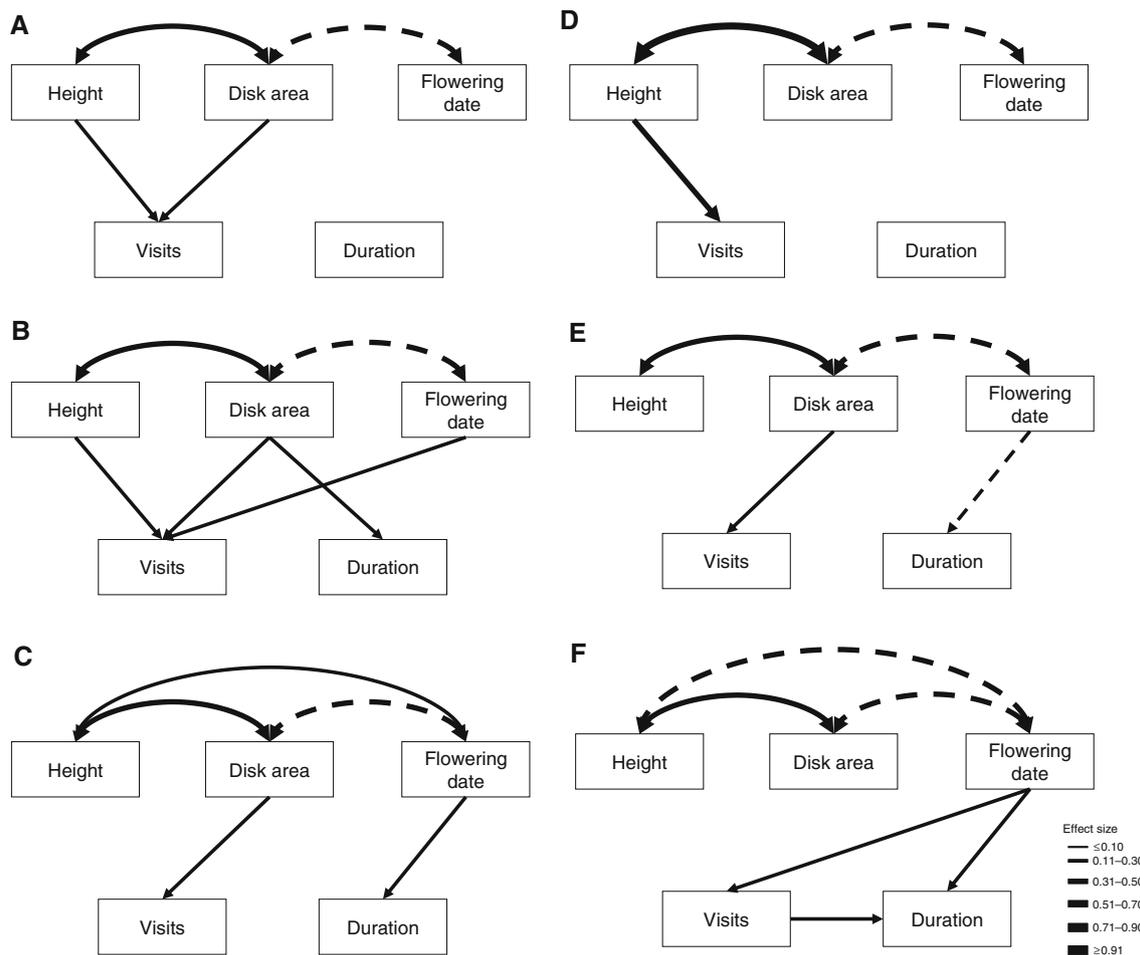


Fig. 6 Nested path models for the effect of floral traits on pollinator behavior in recombinant inbred lines of crop × wild sunflower plants. Only models that did not deviated significantly from observed data are presented (see Table 4). Variables are in rectangles (see Fig. 1 and text for description), and arrows denote effects found among variables. One-headed lines denote linear effect (analogue of partial regression coefficient); solid lines indicate positive effects, and dashed lines indicate negative effects. The width of the arrow

represents the size of the effect in standardized path coefficient units. Curved double-headed arrows represent correlations between variables. Only significant effects and correlations are presented in this path diagram. For example, non-significant effects were found affecting duration of visits in the models for all visitor pooled and for small bees (models a and d, respectively). a All visitors pooled, b Large bees, c Medium-size bees, d Small bees, e *Halictus* bees, f Beetles

Table 5 Indirect effects (in units of standardized path coefficients) of floral trait QTL on pollinator behavior

Visitor type	Effect on	Flowering time QTL	Disk area QTL	Height QTL
Large bees	Visit	0.070	-0.024	0.136
	Duration	-0.025	-	0.020
Small bees	Visit	0.042	-	0.082
	Duration	-	-	-
Non-bee insects	Visit	0.101	-	0.151
	Duration	-	-	-

“-” denotes no significant path. Indirect effects are sum of all indirect paths (including correlations) between the explaining and response variables. Indirect effects of more than three steps were ignored

duration that showed that the heterozygote is either disadvantageous (Fig. 4e) or beneficial (Fig. 4f), which can be due to dominant/recessive alleles in this loci.

Visitation rates as a function of genotype at the single locus (Fig. 4) suggest that crop alleles have an advantage over wild alleles. For example, crop alleles at flowering

time QTL revealed higher visitation rate for the plant homozygote than for the crop allele in these loci (Fig. 4a, b). Crop plants have earlier flowering time than the wild varieties, their stem height is shorter, and they have larger disk diameter (Burke et al. 2002). Earlier flowering expressed by crop plants can be beneficial if pollinators are in competition for floral food resources at the start of the season. In addition, the larger disk area of the crop plants can be more prominent and attract pollinators better than the wild phenotypes. Higher attraction for pollinators can increase the fitness of the plant by enhancing pollen export (male function), or import (female function) (Campbell et al. 1991; Caruso 2001; Conner et al. 1996a, b; Morgan 1992; Stanton et al. 1986). The results of this study, although limited to a few loci, suggest that pollinator behavior may be altered for plants carrying crop alleles in certain loci, and thus, selection will favor plants that are carrying more crop alleles. Natural selection against crop alleles in a crop–wild hybrid zone was proposed to act as a mechanism to prevent the spread of genetically-modified weeds. The mechanism uses a tandem construct containing a selectively unfit gene from the crop repertoire (Al-Ahmad et al. 2006; Al-Ahmad and Gressel 2006; Gressel 1999). This method was tested for sunflowers, and some loci were identified as a potential for such construct based on fitness measurements and their association with QTL (Baack et al. 2008). Nonetheless, the results presented in this study on the same system suggest that selection may favor the crop alleles instead of eliminating them as a result of pollinator behavior. As alleles can be spread through male function (pollen export), and the study conducted by Baack et al. (2008) tested for maternal fitness only, their conclusions on using these loci for tandem construct should be viewed with caution.

The results of this study suggest that trait-mediated genetic effects on pollinator behavior are complex and depend not only on genetic variation and its effect on floral phenotype, but also on the type of pollinator. For three out of six visitors types (large and small bees and non-bee insects), floral trait QTL affected insect behavior. For the other three types (medium size and *Halictus* bees and beetles) the nested model that did not incorporate QTL had a better fit to the observed data, and as it is the more parsimonious model, it is likely that for these insects genetic variation is less affecting pollinator behavior. Hence, these pollinator types are probably less important for floral evolution in sunflowers.

The three floral traits studied here—flowering date, stem height and disk area—had a significant effect on the behavior of large bees. In contrast, the number of visits of other visitor types were affected by only one or two of the traits (Figs. 5, 6). Visitation rate of the other two main visitor groups, medium size bees and *Halictus* spp. bees,

were only affected (positively) by the size of the disk. The group of large bees consisted almost exclusively of social bees, mainly *Bombus* spp., which are nectar foragers in sunflowers (Tepedino and Parker 1982). Observations in the experimental field suggest that medium size bees and the *Halictus* spp. bees were mainly pollen foragers in sunflowers. This implies that different reward types can create different foraging behavior based on different suite of floral traits. The mechanism by which these traits affect the behavior of the visitors to sunflowers are yet to be studied, as this study did not include other variables that might affect pollinator behavior, such as nectar volume and concentration, floral color, floral fragrance, or visitor abundance in the field (Mitchell 1994; Price et al. 2005).

Visitation rate is theoretically affected by the correlation between display size and the quantity of reward (Blarer et al. 2002; Cohen and Shmida 1993). In sunflowers, the quantities of both nectar and pollen rewards are positively correlated with disk area, which determines the number of florets (Neff and Simpson 1990). As presented here, the effect of disk area on visitation rate was positively correlated for all visitor types. Nectar volume was not measured in this study, but assuming that nectar volume in the single floret is not associated with number of florets, I hypothesize that the reward to the visitor is the total volume of nectar available in the inflorescence (nectar standing crop). This is directly associated with the number of open florets, determined by the disk area (Neff and Simpson 1990). Further study is required on the relationships among number of open florets, nectar standing crop, and pollinator behavior (Neff and Simpson 1990), as well as on the genetic basis of nectar secretion in sunflowers.

Generalist flowers such as the inflorescence of sunflowers are expected to attract many types of visitors. However, as shown in previous studies, not all of them will exert selection on floral traits (Fenster et al. 2004; Sahli and Conner 2007; Waser 1998; Waser et al. 1996). There is, however, a generally accepted theory that there is directional pollinator-mediated selection on floral display traits such as stem height and display area (e.g., Campbell et al. 1991; Cariveau et al. 2004; Conner and Rush 1996; Ehrlén et al. 2002; Fenster et al. 2006; Galen 1989; Torang et al. 2006). In this study the basis for selection (expressed in pollinators visitation rate and duration) seems not uniform, exhibiting a difference among functional groups of visitors. The variety of visitor types observed on the plants (>15 species of 4 orders), as well as the general shape of the sunflower flowering head (open radial display, pollen exposed, reward in short-tube florets) suggest that pollinator-mediated selection on floral traits is weak in sunflowers. Nonetheless, this study suggests that studying pollination ecology in an evolutionary context can be beneficial if the genetic architecture of floral traits is incorporated.

Acknowledgments I thank E. Baack for a great collaboration and for help in all aspects of this work. I thank A. Richardson for assistance in the field, and S. Barnes, N. Jones and J. Williams for assistance in post-harvest processing of the sunflowers. R. Jean and P. Scott for identified the bees. M. Arnold, A. Dafni, L. Hadany, M. Lynch and R. Mitchell provided useful ideas and discussions, J. Fox and N. Lazar advised with the statistical analyses, R. Irwin and R. Mitchell gave valuable comments on early versions of the manuscripts, and R. Kirschenbaum helped improving the English. The work was done as a post-doc research in the lab of L. Rieseberg, and funding for this work was provided by a US Department of Agriculture Biotechnology Risk Assessment Program grant to L. R. (03-39210-13958). I was supported by a post-doctoral fellowship (FI-353-2004) from US-Israel Binational Agricultural Research and Development foundation.

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