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INFLUENCE OF POLLINATION SPECIALIZATION AND BREEDING SYSTEM ON FLORAL INTEGRATION AND PHENOTYPIC VARIATION IN *IPOMOEA*

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Natural selection should reduce phenotypic variation and increase integration of floral traits involved in placement of pollen grains on stigmas. In this study, we examine the role of pollinators and breeding system on the evolution of floral traits by comparing the patterns of floral phenotypic variances and covariances in 20 *Ipomoea* species that differ in their level of pollination specialization and pollinator dependence incorporating phylogenetic relatedness. Plants with specialized pollination (i.e., those pollinated by one functional group or by few morphospecies) displayed less phenotypic variation and greater floral integration than generalist plants. Self-compatible species also displayed greater floral integration than self-incompatible species. Floral traits involved in pollen placement and pick up showed less variation and greater integration than floral traits involved in pollinator attraction. Analytical models indicate that both breeding system and the number of morphospecies had significant effects on floral integration patterns although only differences in the former were significant after accounting for phylogeny. These results suggest that specialist/self-compatible plants experience more consistent selection on floral traits than generalist/self-incompatible plants. Furthermore, pollinators and breeding system promote integration of floral traits involved in pollen placement and pick up rather than integration of the whole flower.

KEY WORDS: Developmental constraints, floral evolution, floral variation, phenotypic integration, plant-pollinator interaction, self-compatibility.

Traits under long-term directional or stabilizing selection are expected to display reduced genetic and phenotypic variation (Fisher 1958; Lande and Arnold 1983). Pollinator-mediated selection can be an important factor shaping the patterns of variation and covariation of floral traits (Armbruster 1991; Armbruster and Schwaegerle 1996; Cresswell 1998; Medel et al. 2003; Armbruster et al. 2004; Pérez-Barrales et al. 2007; Pérez et al. 2007; Chalcoff et al. 2008; Ordano et al. 2008, but see Strauss and Whittall 2006 and references therein). Natural selection imposed by pollinators should depend on the level of specialization in pollination relationships, being more consistent in plants with specialized pollination than in plants with generalized pollination (Herrera 1988, 1996; Fenster 1991; Armbruster et al. 1999). Several studies have demonstrated that species with floral traits associated with specialized pollination (e.g., species with zygomorphic flowers, species with long corolla tubes) have less phenotypic variation within populations than species with traits associated with generalized pollination (e.g., species with actinomorphic flowers; Fenster 1991; Armbruster et al. 1999; Wolfe and Krstolic 1999; Ushimaru et al. 2006; Meng et al. 2008).

Phenotypic selection studies in plants have usually focused on the effect of single floral traits on plant fitness (reviewed by Fenster et al. 2004). However, flowers are integrated units that may require a precise configuration of floral organs for proper function (Bissell and Diggle 2008). Thus, it is necessary to use multivariate approaches to have a better understanding of the selective pressure of pollinators on floral traits. Several authors proposed that selection imposed by pollinators should favor the integration of floral traits (i.e., correlations among traits within functional units) (Berg 1960; Stebbins 1970; Conner and Via 1993; Armbruster and Schwaegerle 1996; Armbruster et al. 1999, 2004; Murren 2002; Anderson and Busch 2006; Ushimaru et al. 2006; Pérez-Barrales et al. 2007). Also, selection for floral-trait correlations should be more consistent in plants with specialized pollination than in plants with generalized pollination (Berg 1960). However, previous studies that tested the influence of pollination specialization on phenotypic variation and/or integration of floral traits, have inferred pollination specialization either without direct observations of pollinators or without distinguishing between legitimate pollinators and nonpollinating flower visitors, which could lead to underestimate the role of true pollinators as selective agents on floral traits (e.g., Berg 1960; Fenster 1991; Armbruster et al. 1999; Wolfe and Krstolic 1999; Ushimaru et al. 2006; Meng et al. 2008).

The magnitude of floral correlations could differ among floral traits with different functions. For example, it is expected that floral traits that interact to favor accurate placement of pollen grains on pollinators (e.g., stamen and style lengths), and thence on stigmas, should be more correlated than floral traits that interact to advertise floral rewards to pollinators (e.g., corolla diameter) (Conner and Via 1993; Armbruster et al. 1999; Ordano et al. 2008). Floral traits related to efficient pollen delivery on stigmas are more likely to directly affect plant fitness than floral traits related to visitor attraction that may not result in legitimate pollination. Additionally, floral traits related to advertisement could attract both pollinators and floral antagonists (e.g., nectar robbers, florivores), which could result in opposing selection on floral display (Strauss and Whittall 2006), causing less consistent selection on attraction traits than on traits related to pollen placement and pick up.

Floral-trait correlations could also differ among plant species with different breeding system (Anderson and Busch 2006; Pérez et al. 2007). Anderson and Busch (2006) found that self-compatible *Leavenworthia* species have weaker stamen–pistil or pistil–petal correlations, but similar stamen–pistil correlations than self-incompatible *Leavenworthia* species. They suggested that similar stamen–pistil correlations could be maintained by selection to ensure self-pollination. Recently, Pérez et al. (2007) showed that pollinator-dependent *Schizanthus* species exhibited lower corolla integration than their autonomous selfing congeners, as well as a decoupling among nonfunctional traits and the rest of the corolla traits. These authors suggested that species that do not experience strong pollinator selection (i.e., autonomous selfing species), should display high integration between all floral traits due to their genetic and developmental constraints and due to a relaxed pollinator-mediated selection on nonfunctional traits. If high covariation among all floral traits was the ancestral condition, and nonfunctional traits are not under selection mediated by pollinators (Berg 1960; Pérez et al. 2007), we could predict that self-compatible (SC) plants should exhibit stronger correlations and greater floral integration among floral parts related to pollinator attraction (i.e., "non functional traits") than self-incompatible (SI) plants. Additionally, it could be expected that SI plants should experience more consistent selection against self-pollination than SC plants, promoting herkogamy (spatial separation of anthers from stigma). These changes in positions of stigmas or anthers can increase adaptive inaccuracy (deviation of the population mean from its adaptive optimum) in pollen placement on pollinators relative to stigma contact (Armbruster et al. 2004). Furthermore, it seems that higher inaccuracy in flowers is related with lower levels of integration (Armbruster et al. 2009). Therefore, it is expected that SI plants should display lower floral integration than SC plants in both floral traits related to pollinator attraction and floral traits related to pollen deposition.

Several studies have reported strong phenotypic correlations among corolla tube, gynoecium, and androecium (Conner and Via 1993; Conner and Sterling 1995; Faivre and McDade 2001; Kudoh et al. 2001; Mayr and Weber 2006; Bissell and Diggle 2008; Smith and Rausher 2008), but these patterns of phenotypic correlations may be caused by pleiotropy or linkage disequilibrium rather than correlational selection. For instance, Conner (2002) demonstrated that after nine generations of enforced random mating, correlations between six floral traits in wild radish plants were unchanged, showing that pleiotropy generates these correlations. Smith and Rausher (2008) found that the predicted response to selection for several floral traits was substantially constrained by their genetic correlations. Thus, developmental-genetic factors can slow or constrain the evolution of the most advantageous combinations of traits by natural selection (Lande and Arnold 1983; Cheverud 1984; Smith et al. 1985; Via and Lande 1985; Futuyma 1986; Zeng 1988; Arnold 1992; Mitchell-Olds 1996; Conner 2002; Herrera et al. 2002; Hansen et al. 2003; Caruso 2004; Anderson and Busch 2006; Bissell and Diggle 2008; Smith and Rausher 2008).

Although distinguishing between the relative contribution of natural selection and developmental-genetic constraints is a recurrent question in evolutionary biology, most studies of phenotypic variation and covariation of floral traits have focused on only one plant species (e.g., Armbruster 1991; Conner and Via 1993; Herrera 2001; Williams and Conner 2001; Conner 2002; Herrera et al. 2002; Sanchez-Lafuente 2002; Brock and Weinig 2007; Frey 2007; Hansen et al. 2007; Pérez-Barrales et al. 2007) or on several unrelated species (e.g., Berg 1960; Fenster 1991; Armbruster et al. 1999; Wolfe and Krstolic 1999; Ushimaru et al. 2006; but see Armbruster et al. 2004). Genetic correlations caused by pleiotropy are expected to be relatively similar across closely related species, while selective pressures are expected to be species-, or even population-specific (Conner and Sterling 1995; Kudoh et al. 2001; Herrera et al. 2002). Thus, comparing the correlation structure of flower traits in phylogenetically related species with different breeding system or levels of pollination specialization should clarify the degree to which the correlation structure among flower traits is influenced by developmental-genetic versus ecological factors. Specifically, if developmental-genetic factors generate patterns of phenotypic integration, then we expect to see a strong phylogenetic signal in the distribution of integration indices across species. Alternatively, if pollinator-mediated selection largely generates patterns of phenotypic integration, then we expect to detect considerable evolutionary lability and a weak phylogenetic signal in degree of integration.

To determine whether pollinator-mediated selection or developmental–genetic correlations alter patterns of floral phenotypic variance and integration, we studied 20 tropical species of *Ipomoea* that differ in their degree of pollinator dependence (i.e., self-compatible vs. self-incompatible) and in their pollinator specialization (i.e., specialist vs. generalist, estimated as the number of functional groups of pollinators and as the number of pollinator morphospecies). In particular, we addressed the following questions: (1) How much do these plant species differ in their degree of pollinator specialization and pollinator dependence?, (2) Do specialist species show less floral variation than generalist species?, (3) Are there differences in patterns of correlation and integration of floral traits among species with different degree of pollinator specialization and pollinator dependence?, (4) Are floral traits that influence pollen removal and deposition ("efficiency-function traits") less variable, more strongly correlated and more integrated than floral traits that relate to advertisement or accessibility to rewards ("attraction-function traits")?, and (5) Are present patterns of similarity in variation, correlation, and integration related to phylogenetic distance? To our knowledge, this is the first study that tests in several closely related plant species across a molecular phylogeny, the association of floral variation and/or floral integration patterns with breeding system and pollination specialization estimated through direct observation of legitimate pollinators.

Materials and Methods

STUDY SPECIES AND STUDY SITE

The cosmopolitan genus *Ipomoea* (Convolvulaceae) comprises approximately 650 species (Austin and Huáman 1996), with the majority of species being tropical (Austin and Bianchini 1998). *Ipomoea* species commonly have pentamerous, actinomorphic, and gamopetalous flowers (McDonald 1991). Several types of animals have been considered as pollinators, including beetles, flies, bees, butterflies, moths, hummingbirds, and bats (McDonald 1991; Chemás-Jaramillo and Bullock 2002; Galetto and Bernardello 2004; Wolfe and Sowell 2006; Rosas-Guerrero et al. unpubl. ms.).

Twenty species of *Ipomoea* were sampled in the tropical dry forest of Chamela–Cuitzmala Biosphere Reserve on the Pacific coast of Mexico (19°30′N, 105°03′W). These plant species show great variation in flower color, time of flower opening, floral shape, and breeding system (Chemás-Jaramillo and Bullock 2002; Rosas-Guerrero et al. unpubl. ms.).

POLLINATION SPECIALIZATION

To estimate the level of pollination specialization, floral visitors were recorded during three consecutive flowering seasons (from August to March 2005–2008) using camcorders (Sony Digital Handycam DCR-PC 100, DCR-TRV80E, DCR-SR42) during six intervals of 30 m uniformly distributed along flower duration (i.e., from flower opening to flower wilt). We considered pollinators those floral visitors that made contact with both anthers and stigmas. Each floral visitor was classified into a functional group (*sensu* Armbruster et al. 2000) according to similarities in their fit to, and behavior on, flowers. Members of a functional group are expected to exert similar selection pressures on floral traits (Fenster et al. 2004). Through filming, we were able to identify the functional group of pollinators and differentiate between several morphospecies of pollinators. On average, we filmed 14 flowers (∼40 h) per species. Morphospecies-accumulation curves indicated that pollinator morphospecies leveled off after 29 h of observation for all species (results not shown).

We use two approaches to estimate the level of pollination specialization: the number of pollinator morphospecies (as a continuous variable) and as specialist/generalist (as a categorical variable). We considered arbitrarily plant species with specialized pollination as those that were legitimately pollinated exclusively by one functional group of pollinators, and plant species with generalized pollination as those pollinated legitimately by more than one functional group of pollinators.

FLORAL VARIATION AND COVARIATION

To determine floral phenotypic variation and covariation, the following floral traits were measured in situ using a digital caliper (Mitutoyo Corp., Kawasaki, Japan) with 0.1 mm precision, using one fresh flower per individual in approximately 45 individuals per species chosen at random: corolla diameter (CD), corolla length (CL), throat diameter (TD), tube length (TL), style length (SL), longest stamen (LS), and shortest stamen (SS) (see Fig. 1). All flower measurements were performed by one person and without prior knowledge of the expected pattern of trait covariation. The coefficient of variation (CV) for each trait was calculated and used as a standardized measure of floral-size variation. To test for differences in floral variation among plants with different pollination specialization, we compared the average CV of each floral trait in specialized and generalized plant species using a Mann–Whitney two-sample test. We also compared the mean CV of each floral trait across all species, to test if those floral traits related in pollen deposition and pick up (i.e., SL, LS, SS) have lower variation than floral traits related to pollinator advertisement or floral traits related to accessibility to rewards by floral visitors (i.e., CD, CL, TL, TD) using a Wilcoxon matched pairs test.

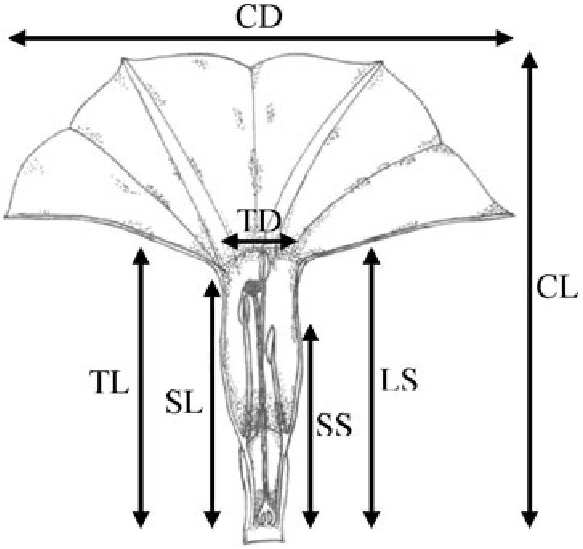

Figure 1. Schematic representation of a typical flower of *Ipomoea*. Measured floral traits: CD = corolla diameter, CL = corolla length, TD = throat diameter, TL = tube length, SL = style length, LS = longest stamen, SS = shortest stamen.

To assess whether the covariance architecture of flowers more likely reflects developmental-genetic constraints or natural selection, we compared the patterns of floral trait correlations and floral integration among species with different pollination specialization and different breeding system. After being natural-log transformed, we calculated Pearson product-moment correlations among floral traits for each plant species. We compared the average correlation coefficient of species with specialized pollination with plants with generalized pollination with a two-sample t-test. Estimates of means and 95% confidence intervals were obtained by bootstrapping ($n = 20{,}000$ permutations in each test). Using these same procedure, we compared the average correlation coefficient of floral traits related with pollen deposition and pick up (i.e., TL, SL, LS, SS; hereafter named "efficiency correlation coefficient"), with the average correlation coefficient of floral traits related with the attraction/restriction of floral visitors (i.e., CD, CL, TL, TD; hereafter named "attraction correlation coefficient"). Besides comparing correlation patterns with the categorical variables of pollination specialization, we tested if correlation coefficients of floral traits were correlated with the number of pollinator morphospecies using Spearman rank correlation.

Phenotypic integration was estimated using the variance of the eigenvalues of a correlation matrix through a principal component analysis (Wagner 1984; Cheverud et al. 1989) of the floral traits mentioned previously. Prior to analysis, all measurements were log-transformed to approach normality and homoscedasticity more closely. As species differed in sample size, a corrected integration index was estimated (see Wagner 1984; Pérez-Barrales et al. 2007). The integration index was expressed as a percentage of the maximum possible value. We compared the average integration index of floral traits for plants with specialized pollination with plants with generalized pollination with a two-sample t-test. The significance of differences between means and confidence intervals were also obtained by bootstrapping ($n = 20{,}000$ permutations in each test). Additionally, we tested if floral integration indices were correlated with the number of pollinator morphospecies using Spearman rank correlation.

To detect differences between floral traits involved in pollen placement and pick up (TL, SL, LS, SS) with floral traits related to attraction or restriction to floral visitors (CD, CL, TL, TD), we compared the two integration indices (hereafter named "efficiency integration index" and "attraction integration index," respectively) with a two-sample t-test as described previously.

Hereafter, all bootstrapping estimations were calculated with Data Pilot version 1.03 (Anonymous 2003), while additional statistical analyses were performed with SAS version 9.1.3 (SAS 2002) and MATLAB 7.7 (The Mathworks, Natick, MA).

TESTING THE EFFECTS OF BREEDING SYSTEM ON CORRELATION AND INTEGRATION PATTERNS

Our study includes five self-incompatible species (*Ipomoea ampullacea*, *I. bracteata*, *I. pes-caprae*, *I. trifida*, and *I. wolcottiana*), and 10 self-compatible species (*Ipomoea alba*, *I. chamelana*, *I. clavata*, *I. hederifolia*, *I. meyeri*, *I. muricata*, *I. nil*, *I. pedicellaris*, *I. quamoclit*, and *I. triloba*) (Chemás-Jaramillo and Bullock 2002; Rosas-Guerrero V., unpubl. ms.). Because self-compatible plants could experience strong selection on floral traits involved in pollen placement and pick up, we compared the average coefficients of correlation and integration of efficiency-, and attraction-function traits of plants with self-compatible system ($n = 10$) with those plants with self-incompatible breeding system ($n = 5$).

To test for the effect of breeding system, pollination specialization and number of pollinator morphospecies on attraction or efficiency integration, we performed a generalized linear model using a Poisson distribution for the dependent variables and the logarithmic function as a link function using the GENMOD procedure (SAS 2002). The model used attraction or efficiency integration index as response variables and breeding system, pollination specialization, and number of pollinator morphospecies as independent variables.

PHYLOGENETIC ANALYSES OF FLORAL VARIATION, CORRELATION AND INTEGRATION

To determine whether pollinator-mediated selection versus developmental–genetic correlations have stronger effects on patterns of floral phenotypic variance and integration, we conducted analyses that accounted for the shared phylogenetic history while estimating the effects of pollination specialization and breeding system on floral trait variation and covariation. Many approaches have been proposed for incorporating phylogenetic effects into statistical analyses (e.g., Felsenstein 1985; Garland et al. 1993; Martins and Hansen 1997), and the choice of method depends on factors including the type of data to be analyzed (e.g., discrete or continuous), the parameter(s) to be estimated (e.g., relationships among traits, ancestral trait values), and the evolutionary models to be considered (e.g., Brownian motion, Ornstein–Uhlenbeck process). Here we employ phylogenetic generalized least squares (PGLS) to estimate whether differences in pollination specialization and breeding system across *Ipomoea* species affect variation in individual floral traits (CV), correlation among floral traits (CC), and floral integration indices (II). PGLS was chosen for this application because it allows for easy comparison of a range of evolutionary models and can be used to consider both discrete and continuous variables (Martins and Hansen 1997).

As with all comparative methods, PGLS requires an estimate of the phylogeny with branch lengths. For this purpose, we compiled ITS sequences for 78 species and 2 outgroups (Table S1) from previous studies of *Ipomoea* and its relatives (Miller et al. 1999, 2004; Manos et al. 2001). This sampling included 13 of the 20 species studied (Table S1); sequences were not available for the remaining 7 species. Sequences were aligned with MEGA 4.0 (Tamura et al. 2007) and subsequently corrected manually. Poorly aligned positions were identified using Gblocks software version 0.91b (Castresana 2000) and eliminated. Phylogenetic analysis was performed using likelihood in RAxML-7.0.4 (Stamatakis 2006). A general time-reversible model of sequence evolution with gamma-distributed rate heterogeneity and a proportion of invariant sites ($\mathrm{GTR} + \Gamma + \mathrm{I}$) was used, and the single maximum likelihood phylogeny was ultrametricized with nonparametric rate smoothing in TreeEdit (http://tree.bio.ed.ac.uk/software/treeedit). Finally, the tree was pruned to include only the 13 taxa for which trait data was collected and transformed in a variance–covariance matrix for PGLS using the R package ape (Paradis et al. 2004).

As an assessment of phylogenetic signal in the trait data, we calculated the K statistic (Blomberg et al. 2003) for all of the continuous measures of floral trait variation using the picante package (Kembel et al. 2010) in R. The K statistic uses the variance of the standardized phylogenetically independent contrasts as a measure of how well the tree fits the data given a Brownian motion model of trait evolution. K values of 1 correspond to the amount of similarity predicted by the phylogeny under Brownian motion while lower values indicate less signal, that is, less similarity in trait values among closely related species. The significance of K can be tested by permuting the data across the tree and recomputing K from the shuffled values (Blomberg et al. 2003). We used the phylosignal function in picante with 1000 permutations to conduct a one-tailed test for signal. However, as phylogenetic signal is difficult to detect with fewer than 20 species (Blomberg et al. 2003), we did not consider the results definitive and conducted GLS analyses both with and without incorporating the phylogeny.

PGLS analyses were conducted using the program MERegPHYSIG developed by Ives et al. (2007) for use in MATLAB. The effects of pollination specialization (coded as 0/1, specialized/generalized), number of morphospecies and breeding system (coded as 0/1, self-incompatible, or compatible) on continuously varying values of CV, CC and II were estimated individually and jointly using two models, ordinary least squares (OLS) and PGLS. The OLS regression model is a standard nonphylogenetic analysis and is equivalent to PGLS using a completely unresolved (star) phylogeny. PGLS incorporates the variance–covariance matrix built from the ML phylogeny and assumes that traits have evolved along the phylogeny via Brownian motion (BM). PGLS using a BM model and including an intercept (as in MERegPHYSIG) is equivalent to independent contrasts (Rohlf 2001). Although other

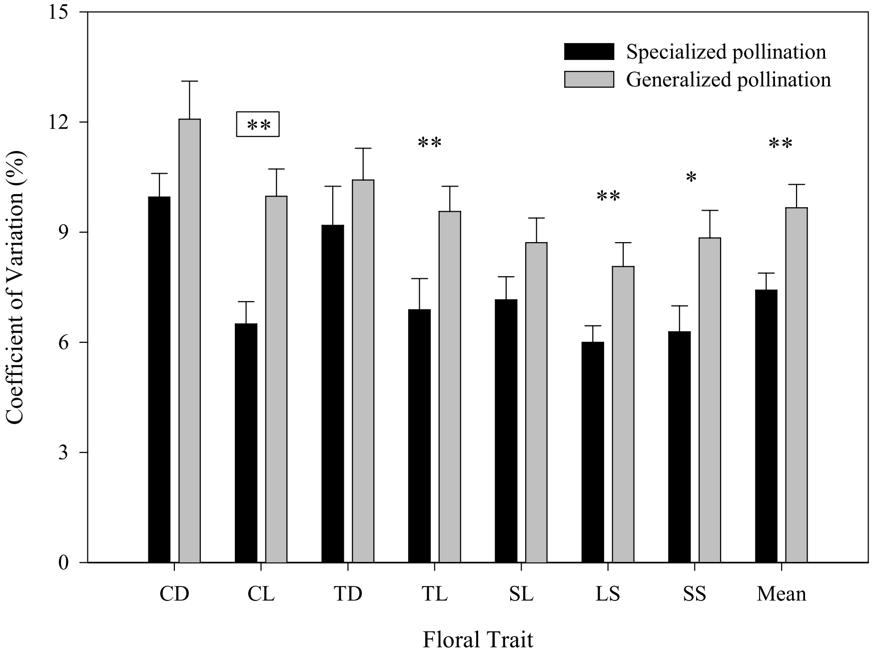

Figure 2. Coefficients of variation of seven floral traits for 20 species of *Ipomoea* with different pollination specialization. Floral trait abbreviations as in Figure 1. Mean = average of the coefficients of variations of the seven floral traits. Lines above bars indicate +1SE. Asterisks indicate significant differences after Mann–Whitney two sample test. $*P < 0.10$, $**P < 0.05$. Asterisks in square indicate significant differences after phylogenetic corrected data.

models of trait evolution can be used with PGLS, the number of species available for these analyses (13) reduces the capacity to accurately measure phylogenetic signal and thus to select among models (Blomberg et al. 2003). For each model, the statistical significance of the effects of pollination and breeding system on the floral trait variables was estimated by parametric bootstrapping using 2000 simulated datasets (Ives et al. 2007). Model fit was compared using a likelihood ratio test with one degree of freedom (Lavin et al. 2008). By comparing models with no phylogeny (OLS) to models assuming strong effects of phylogeny (PGLS with BM), we aim to assess the extent to which our results depend on assumptions about the phylogenetic effect.

Results

FLORAL VISITORS AND POLLINATION SPECIALIZATION

Many species of *Ipomoea* were pollinated primarily by one functional group of pollinator. However, only five species were pollinated by exclusively one functional group and were classified as pollinator specialists (Table S2). Three species were pollinated exclusively by moths, one by bees, and one by hummingbirds.

We were able to differentiate between 56 morphospecies of floral visitors that made contact with the reproductive parts of flowers. The number of morphospecies registered per plant species varied widely, from one to 15 (Table S2).

Visitation rates of pollinators also varied widely, from 0.04 in *I. alba* to 22.23 visits/flower/h in *I. pes-caprae*. We found that self-compatible plants received, in average, fewer pollinator visits than self-incompatible plants (mean = 0.96 vs. 5.87 visits/flower/h) (see Table S2 for values for each species).

PHENOTYPIC VARIATION IN RELATION TO POLLINATION SPECIALIZATION

As predicted, all measured floral traits showed less phenotypic variation (lower CVs) in plants pollinated by one functional group than in plants pollinated by more than one functional group (Fig. 2). However, these differences were significant (without phylogenetic correction) only for corolla length ($U = 65$, $P = 0.016$), tube length ($U = 60$, $P = 0.049$), and longest stamen ($U = 60$, $P = 0.049$), and marginally significant for shortest stamen ($U = 57$, $P = 0.088$). We also observed a significant difference between specialized (one functional group) and generalized (more than one functional group) species when the mean CVs of all floral traits were taken together ($U = 60$, $P = 0.049$).

Pooling all study species, corolla and throat diameters were more variable (larger CVs) than were the length of the styles, longest stamens and shortest stamens ($Z > 2.02$, $P < 0.050$ for all comparisons; no phylogenetic correction). In plants with one functional group of pollinator, corolla and throat diameters showed more variation than corolla length and the shortest-stamen length ($Z > 2.02$, $P < 0.050$ for all comparisons), while in plants with more functional groups of pollinators, corolla and throat diameters were more variable than style length and the longest-stamen length ($Z > 2.21$, $P < 0.03$ for all comparisons). These results indicate less variation in floral traits involved in pollen placement and pick up than in traits involved in attraction, as predicted from theory.

Comparisons incorporating phylogenetic effects showed similar trends as reported previously, although these findings were significant less often (note reduction in sample size). Across analyses comparing floral trait CVs between generalized and specialized species, OLS (nonphylogenetic) models consistently fit the data better than PGLS in line with the generally low phylogenetic signal detected in the data (Table S3). Generalized species were found to have significantly greater corolla length (CL) variation than specialized species under the OLS model (Table S4). The other traits also had greater CVs in generalized species (as indicated by the positive slope values in the OLS models, Table S4) as in the preceding analyses (see Fig. 2) although these effects were not significant.

EFFECTS OF POLLINATION SPECIALIZATION ON CORRELATION AND INTEGRATION PATTERNS

All floral traits measured, with exception of throat diameter, were highly inter-correlated in most plant species (Table 1), particularly the longest stamen with the shortest stamen (mean value for all species $r^2 = 0.86$), and corolla length with tube length ($r^2 = 0.71$). Consistent with our expectations, the efficiency correlation coefficient of plants pollinated by one functional group was greater than in plants pollinated by more than one functional group ($P = 0.023$); while the attraction correlation coefficients did not differ between plants with different levels of pollination specialization (Fig. 3A). However, the effect of level of pollination specialization on efficiency correlation coefficient disappeared when phylogenetic effects were incorporated for (Table S5). We also found that across all species, and in plants within the same level of pollination specialization, floral traits related to pollen placement and pick up were more highly inter-correlated than floral traits related to attraction/restriction of floral visitors ($P < 0.0001$ for all comparisons).

We did not detect correlations between the number of morphospecies and the attraction correlation coefficient ($r_s = 0.15$, $P = 0.541$) or the efficiency correlation coefficient ($r_s = -0.24$, $P = 0.305$) in either nonphylogenetic or phylogenetic analyses (Table S5).

The magnitude of efficiency integration index varied widely between species, from 8% in *Ipomoea ampullacea* to 92% in *I. clavata* (Table S2, see Fig. 5). Species pollinated by one functional group had greater efficiency integration than species pollinated by several functional groups ($P = 0.0179$, nonphylogenetic comparison; Fig. 3B). However, when phylogenetic effects were incorporated, no effect of functional-group specialization on integration was detectable (Table S6). After pooling species with the same degree of pollination specialization, the efficiency integration index was significantly larger than the attraction integration index ($P < 0.002$ for both comparisons; see Fig. 3B). As expected, the number of pollinator morphospecies also had a strong influence on the integration index for efficiency traits. The efficiency integration index was significantly negatively correlated with the number of morphospecies ($r_s = -0.62$, $P = 0.004$; Fig. 4) and

Table 1. **Mean values of phenotypic correlation coefficients among flower traits in 20 species of *Ipomoea* calculated as the Pearson product–moment correlations of log transformed flower size traits. Floral traits: CD = corolla diameter, CL = corolla length, TD = throat diameter, TL = tube length, SL = style length, LS = longest stamen, SS = shortest stamen. Numbers above and below the diagonal are mean coefficients of correlations of species with specialized ($n = 5$) and generalized ($n = 15$) pollination, respectively. Numbers in parenthesis indicate percentage of species that showed significant correlation between traits at $P < 0.05$.**

	CD	CL	TD	TL	SL	LS	SS
CD		0.491	0.510	0.554	0.636	0.679	0.646
		(80%)	(80%)	(100%)	(100%)	(100%)	(100%)
CL	0.518		0.275	0.687	0.498	0.700	0.663
	(67%)		(40%)	(100%)	(100%)	(100%)	(80%)
TD	0.464	0.327		0.281	0.301	0.378	0.386
	(73%)	(53%)		(40%)	(40%)	(40%)	(40%)
TL	0.527	0.714	0.246		0.597	0.733	0.722
	(87%)	(93%)	(47%)		(80%)	(100%)	(100%)
SL	0.517	0.552	0.169	0.557		0.724	0.649
	(60%)	(80%)	(40%)	(80%)		(100%)	(100%)
LS	0.485	0.606	0.239	0.675	0.572		0.896
	(87%)	(80%)	(33%)	(87%)	(87%)		(100%)
SS	0.411	0.547	0.160	0.640	0.563	0.848	
	(67%)	(73%)	(40%)	(87%)	(73%)	(100%)	

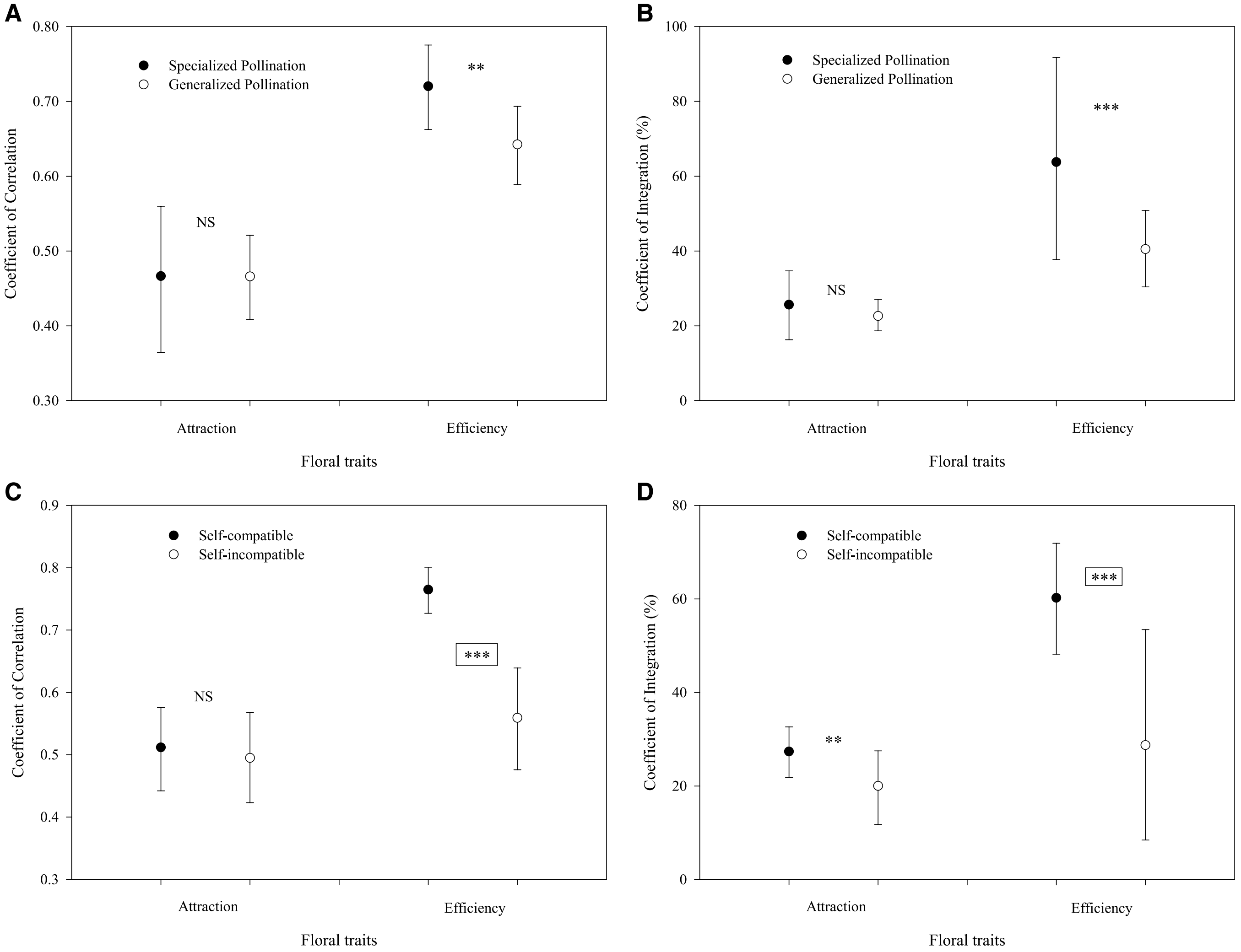

Figure 3. Mean values and confidence intervals (95%) of A attraction and efficiency correlation coefficients of plants with specialized and generalized pollination, B attraction and efficiency integration coefficients of plants with specialized and generalized pollination, C attraction and efficiency correlation coefficients of plants with different breeding system, and D attraction and efficiency integration coefficients of plants with different breeding system. See methods for calculation of each coefficient. Asterisks indicate significant differences after two sample *t*-test. $^{**}P < 0.05$, $^{***}P < 0.01$, NS = no statistical differences. Confidence intervals and differences between mean values were estimated by bootstrapping (20000 runs). Asterisks in square indicate significant differences after phylogenetic corrected data.

this result was supported after phylogenetic effects were included (Table S6). In contrast, pollinator morphospecies did not affect the integration of traits associated with attraction ($r_s = -0.19$, $P = 0.422$, see also Table S6).

EFFECTS OF BREEDING SYSTEM AND POLLINATION SPECIALIZATION ON ATTRACTION AND EFFICIENCY TRAITS

Univariate analyses indicated significant effects of breeding system (self-compatible vs. self-incompatible) on floral traits associated with efficiency. We found that the efficiency correlation coefficient in self-compatible species was greater than in self-incompatible species ($P < 0.0001$; Fig. 3C). No effect of breeding system on attraction correlation coefficients was found ($P = 0.3722$; Fig. 3C). These same patterns were found in analyses incorporating phylogeny (Table S5).

In contrast, the integration indices for both efficiency and attraction traits were significantly affected by breeding system. Self-compatible species had higher integration for both types of traits ($P = 0.0005$ for efficiency and $P = 0.0261$ for attraction; Fig. 3D). Phylogenetical analyses confirmed the effect of breeding system on the efficiency integration index, but not on the attraction integration index (Table S6).

We employed analytical models including both breeding system and specialization as independent variables to determine the relative importance of each in shaping floral covariation. When

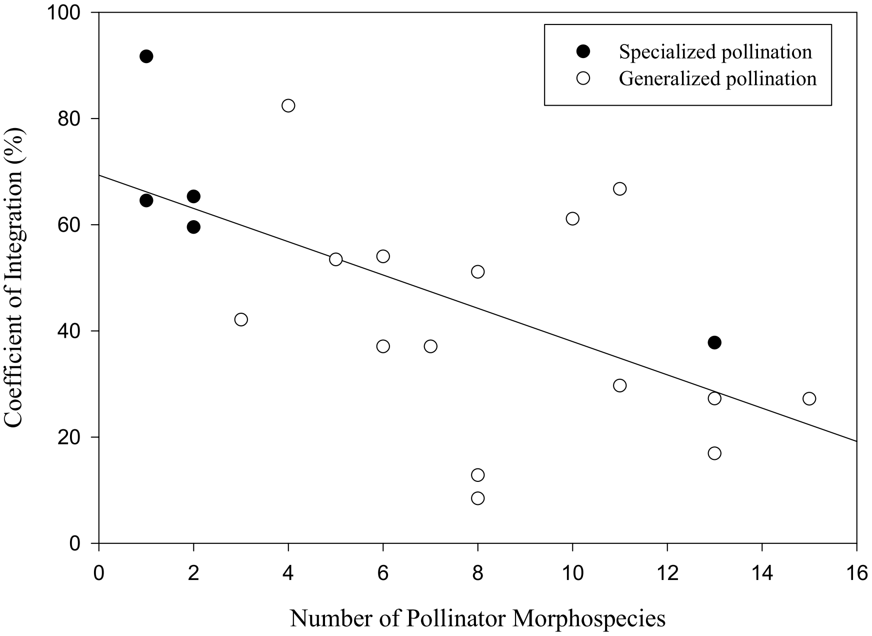

Figure 4. Spearman rank correlation for the relationship between the number of pollinator morphospecies and the efficiency integration coefficient of 20 species of *Ipomoea* ($r_s = -0.62$, $P = 0.004$). Best fitted line is showed.

we coded pollination specialization as a discrete (0/1) trait in multiple regression analyses, only breeding system appeared to be a significant predictor of variation in efficiency and attraction integration indices ($P = 0.008$ and $P = 0.006$, respectively; Table S7). However, when we characterized pollination system as the number of morphospecies, both variables (number of morphospecies and breeding system) had a significant effect on the efficiency integration index ($P = 0.0373$ and $P = 0.0162$, respectively; Table S7).

We conducted similar two-independent-variable analyses to assess how breeding system and pollination specialization affect floral integration after accounting for phylogeny of the 11 species for which both breeding-system and sequence data were available. These analyses generally supported the finding that breeding system affects integration of both efficiency and attraction related traits. In most analyses, the effect of breeding system was significant only with one of the two models (OLS or PGLS), suggesting that is only marginally significant. However, we failed to detect any effect of either measure of specialization on either integration index after incorporating both breeding system and phylogeny.

Discussion

PATTERNS OF PHENOTYPIC VARIATION

As predicted, plant species with specialized pollination presented lower phenotypic variation in flower size than species with generalized pollination in both phylogenetic and nonphylogenetic analyses. A few other studies have found similar trends. Wolfe and Krstolic (1999) and Ushimaru et al. (2006) found that plant species with radially symmetrical flowers (which supposedly experienced generalized pollination) were more variable in size than species with bilaterally symmetrical flowers (which supposedly experienced specialized pollination). Similarly, Armbruster et al. (1999) and Meng et al. (2008) found lower variation in species with specialized pollination in comparison to species with generalized pollination. In our study only a subset of floral traits (i.e., corolla length, tube length, and the longest and shortest stamens), showed significantly less phenotypic variation between plants with different level of specialization. Interestingly, across all species (data pooled), corolla diameter and throat diameter (traits related to attraction and restriction of floral visitors, respectively), were significantly more variable than the lengths of the style and the longest and shortest stamens (traits involved in pollen placement and pick up). Taken together, these results suggest that: (1) species with specialized pollination, experience stronger or more consistent stabilizing or directional selection on floral traits than species with generalized pollination, and (2) pollinators exert stronger selective pressures on floral traits involved in the pollen transfer process than floral traits related to attraction/restriction. Even when pollinators seemed to be the main selective agents on efficiency-function traits, floral traits related to attraction can be subject of nonpollinating agents (e.g., nectar robbers or florivores), which could result in less consistent or even opposite selective pressures on those traits (Brody 1992a,b; Brody and Mitchell 1997; Gomez 2003).

Several authors have found strong directional selection on floral tube length (Miller 1981; Roubik et al. 1985; Nilsson 1988; Robertson and Wyatt 1990; Fenster 1991; Johnson and Steiner 1997; Boyd 2002, 2004). It is therefore expected that plants with long corolla tubes have experienced stronger and more consistent selection by pollinators on floral length than species with shorter corollas, resulting in less phenotypic variance in the former than in the latter (Fenster 1991). Consistent with this idea, we found that tube and corolla length were the floral traits whose CVs differed most between plants with different degrees of specialization (see Fig. 2), suggesting that these floral traits are among the main targets of selection in plants with specialized pollination.

PATTERNS OF PHENOTYPIC COVARIATION AND FLORAL INTEGRATION

Berg (1960) proposed that plants with specialized pollination should exhibit greater phenotypic integration among floral traits than do plants with generalized pollination. Analyses of phylogenetically uncorrected data in the present study indicated that plant species with specialized pollination had greater phenotypic integration and larger correlations among those floral traits related to pollen transfer than did species with generalized pollination. However, we failed to find a significant effect of pollination specialization on floral correlation or integration patterns using two types of phylogenetic regression (OLS and PGLS) on a subset of these data and two models. The OLS model uses a star (completely unresolved) phylogeny and thus is expected to return results identical to phylogenetically uncorrected analyses since OLS regressions were similar to the *t*-tests used with the full data set of 20 species. Thus, the discrepancy between the results from the *t*-tests and OLS for pollination specialization is attributable to the reduced sample size in the latter. Despite the reduced sample size, both sets of analyses detected significant negative correlations between the number of pollinator morphospecies and the integration of pollen-transfer traits. Thus, it seems that the number of pollinator morphospecies, and to a lesser degree the number of functional groups, affects the pattern of floral integration (see Fig. 4). These results indicate that plants pollinated by a small number of morphospecies that belong to one or a few functional groups of pollinators experience strong selection for certain combinations of floral traits.

Several studies have provided support for the hypothesis that pollinators modify phenotypic correlations of floral traits, selecting for floral integration (e.g., Berg 1960; Schemske and Horvitz 1984; Campbell et al. 1991; Conner and Sterling 1995). For example, Pérez et al. (2007) found different patterns of phenotypic integration among eight *Schizanthus* species that differed in their degree of pollinator dependence. Pérez-Barrales et al. (2007) found that populations of *Narcissus papyraceus* pollinated by moths (whose morphology closely matched flower shape) showed stronger inter-correlation of floral traits than populations pollinated by hoverflies (whose morphology did not closely fit floral shape). Nevertheless, some studies have reported absence of evidence for pollinator-mediated selection on correlated traits (e.g., Herrera 2001; Conner 2002; Herrera et al. 2002; Bissell and Diggle 2008; Meng et al. 2008).

Correlations between floral traits could be caused by natural selection, developmental constraints, or both (Cheverud 1984; Armbruster 1991; Conner and Via 1993; Armbruster and Schwaegerle 1996). The larger correlations between the lengths of longest and shortest stamen and between corolla and tube lengths, as found in all *Ipomoea* species, may be caused by developmental-genetic relationships. Indeed, it is expected that floral traits belonging to the same floral whorl experience stronger phenotypic integration because of their shared developmental precursors (Conner and Sterling 1995).

Nevertheless, several floral trait combinations differed in their correlation patterns. For example, only half of the *Ipomoea* species showed a significant correlation between corolla length and throat diameter. Similarly, while five *Ipomoea* species showed high correlations between throat diameter and the rest of the flower traits, another five species did not show any correlations between those traits. These results suggest that natural selection affects the relationship between throat diameter and the other flower traits.

If developmental-genetic correlations were the main cause of floral trait covariation, then we should find that closely related *Ipomoea* species generally have similar floral-integration values. Our results showed that the pattern of floral integration differed among the 20 *Ipomoea* species (from 8% to 92% in efficiency integration index). Some of the greatest differences in integration index existed between closely related taxa, for example, between *I. clavata* and *I. pedicellaris* and between *I. ampullacea* and *I. nil* (see Fig. 5). As might be expected from this pattern, models assuming phylogenetic structure often fit poorly compared to models assuming no phylogenetic effect in comparative analyses of integration indices (Tables S3–S6). These findings suggest that floral integration is evolutionarily labile and that the variation in integration across species is likely due to differences in their selective environments.

Several studies have found stronger phenotypic correlations between floral traits than between floral and vegetative traits, that is, decoupling of floral from vegetative traits ("floral modularity" Berg 1960; Conner and Sterling 1995; Armbruster et al. 1999; Murren 2002; Ashman and Majetic 2006; Brock and Weinig 2007; Hansen et al. 2007; Pérez-Barrales et al. 2007; but see Meng et al. 2008). Similarly, we found greater integration of floral traits involved in pollen transfer than of floral traits related to attraction/restriction to floral visitors (46% vs. 23%, respectively), and decoupling of traits involved in pollen transfer from the other flower parts. Similarly, Conner and Via (1993) showed that the correlations between the length of the filaments and the lengths of the corolla tube was larger than the correlations between the rest of the floral traits. Armbruster et al. (1999) and Ordano et al.

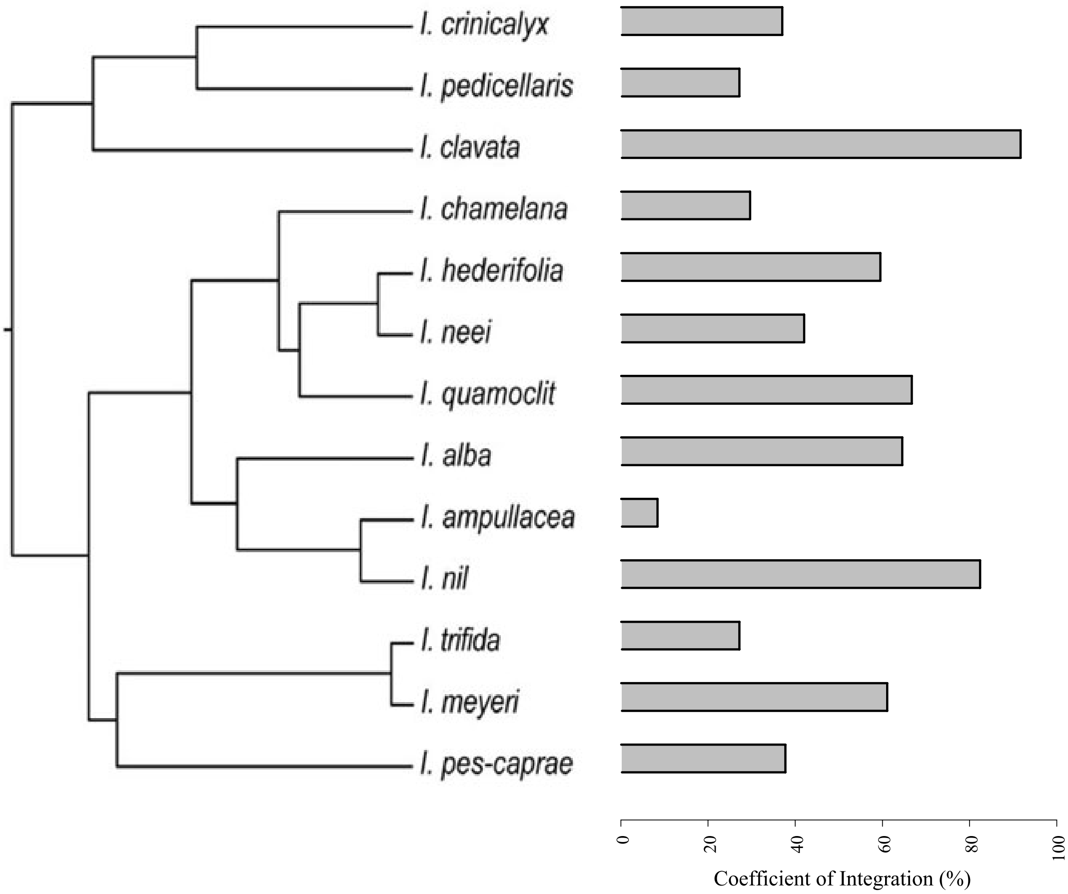

Figure 5. ITS phylogeny of the 13 species of *Ipomoea* used in phylogenetical comparative analyses. Values of "efficiency integration index" for each species are included.

(2008) suggested that natural selection generally favors, not integration of the whole flower, but instead "intrafloral" integration; that is, integration of only certain floral parts.

In brief, our results indicate that: (1) particular suites of floral traits (i.e., traits that belong to the same floral whorl, e.g., longest stamen and shortest stamen, corolla and tube lengths) exhibit high integration as a result of developmental constraints; (2) some combinations of floral traits, mainly those involved in pollen transfer, have responded to selection imposed by pollinators; and (3) natural selection apparently favors decoupling of floral traits involved in pollen transfer from those involved in attraction.

INFLUENCE OF BREEDING SYSTEM ON FLORAL INTEGRATION

Self-compatible (SC) plants can produce seeds via self-fertilization in the absence of pollinators if there is some mechanism of autonomous self-pollination. Even though rates of self-fertilization were not estimated, several data suggest that it is very likely that most SC species of *Ipomoea* produce many seeds via self-fertilization: (1) the close proximity of anthers and stigma (Ennos 1981; Brown and Clegg 1984, personal observation); (2) the relative low pollen-ovule ratios (mean = 141.6; data from Chemás-Jaramillo and Bullock 2002) of most (7 of 9) of SC *Ipomoea* species, which suggest facultative autogamy (*sensu* Cruden 1977); (3) the high production of autogamous fruits in *Ipomoea alba* (fruit set in autogamous pollination = 0.75 vs. 0.70 from exogamous pollination; unpublished data); and (4) the relatively low visitation rate of pollinators in SC species (mean = 0.96 vs. 5.87 visits/flower/h in SC and SI plants, respectively).

Self-compatible plants with autonomous selfing are more likely to produce inbred progeny with greater genetic correlations on floral traits due to linkage disequilibrium because of a reduction in the efficiency of recombination (Kelly 1999; Kalisz et al. 2004). Therefore, we expected that phenotypic integration of floral traits should be greater in SC than in SI species (Pérez et al. 2007; but see Anderson and Busch 2006). Our results support this hypothesis, SC plants exhibited stronger correlations and greater floral integration among floral parts related to attraction and pollen placement and pick up than self-incompatible (SI) plants. There is apparently greater consistency of selection on style and stamen lengths to maximize self-pollination in the face of environmental variance (e.g., unpredictability of pollinator service). Additionally, compared to SC plants, SI plants should experience more consistent selection against self-pollination, promoting spatial separation between anthers and stigma. These changes in the relative position of stigmas with respect to anthers can increase inaccuracy of pollen placement of pollinators to stigmas (Armbruster et al. 2004) causing lower levels of integration on floral traits in SI plants (Armbruster et al. 2009).

Greater floral integration and correlations among floral traits in SC plants were strongly supported after accounting for phylogeny in comparative analyses using only the subset of the species for which phylogenetic information was available (Tables S3 and S4). Analyses (not including phylogeny) of the number of pollinator morphospecies and breeding system (used as independent variables simultaneously), indicated that the integration patterns of floral traits involved in pollen placement and pick up were affected by both variables. Although similar analyses incorporating phylogeny found only the effect of breeding system on the integration of efficiency-related traits, the sample size for these analyses was much smaller. Additional studies will be needed to obtain more accurate estimates of the relative importance of breeding system and pollination biology in shaping patterns of floral variation and covariation.

CONCLUSIONS

Plants exhibit a continuum from extreme generalization to total specialization in pollination systems (Waser et al. 1996; Johnson and Steiner 2000). Here, our results show that plants with specialized pollination exhibit lower phenotypic variation and greater floral integration than plants with generalized pollination. It is therefore crucial to know the degree of floral specialization of plant species to understand the evolution of floral traits. Although, we still do not know precisely the degree to which interspecific variation and covariation of floral traits reflect genetic or developmental constraints versus response to natural selection generated by effective pollination (either by pollinators or self-fertilization), it seems that natural selection maintains a nonrandom associations of certain subsets of floral traits, rather than whole-flower integration, particularly for those floral traits that influence pollen transfer (Armbruster et al. 2004, 2009). Studies of pollinator-mediated selection on particular combinations of floral traits are crucial to understand the role of pollinators on floral evolution even in plants that do not depend entirely on pollinators to reproduce successfully.

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Supporting Information

The following supporting information is available for this article:

Table S1. GenBank numbers for *Ipomoea* ITS sequences used in phylogenetic analyses.
Table S2. Coefficients of variation of floral traits, number of functional groups of pollinators, main pollinators, number of morphospecies of pollinators, visitation rate of pollinators (VR), breeding system (BS), and integration indices (II) for 20 species of *Ipomoea*.
Table S3. Phylogenetic signal (*K*) for continuously-valued measures of floral trait variation.
Table S4. Effects of pollination specialization (generalized or specialized) on coefficients of variation (CV) for seven floral traits using phylogenetic generalized least squares.
Table S5. Effects of pollination specialization, number of pollinator morphospecies and breeding system on floral trait correlation coefficients (CC) estimated using phylogenetic generalized least squares.
Table S6. Effects of pollination specialization, number of pollinator morphospecies and breeding system on floral trait integration indices (II) estimated using phylogenetic generalized least squares.
Table S7. Analytical analyses estimating the effects of pollination specialization (PS), number of pollinator morphospecies (MS) and breeding system (BS) on the integration of floral traits related to pollinator attraction (Attraction II) and of traits associated with pollen placement and pick up (Efficiency II).

Supporting Information may be found in the online version of this article.