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# Population differentiation in floral characters of *Datura inoxia* Mill.

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## ABSTRACT

**Question:** Is differentiation in single and correlated floral traits among populations of *Datura inoxia* the result of contrasting natural selection?

**Hypothesis:** The pattern and structure of phenotypic variance–covariance matrices of putative adaptive traits differ from those produced by random processes. Population differentiation in quantitative floral traits ($Q_{ST}$) of *D. inoxia* exceeds the magnitude of population differentiation in neutral characters ($F_{ST}$), supporting a selection-driven differentiation scenario.

**Organism:** *Datura inoxia* Mill. (Solanaceae).

**Field sites:** Six natural populations in Mexico (latitudinal range: 18°48′N to 26°41′N).

**Methods:** Using different methods (Mantel and modified Mantel tests, jackknife MANOVA, Flury's method, and $Q_{ST}$–$F_{ST}$ of Whitlock and Guillaume), we contrasted the pattern of phenotypic variance–covariance matrices as well as the magnitude of population differentiation in seven quantitative floral traits, against population structure of *D. inoxia* in neutral loci (molecular markers). We also contrasted floral characters measures and integration indices with local inbreeding values ($F_{IS}$) for each population to contrast selection mediated by pollinators and selection of selfing.

**Conclusions:** *Datura inoxia* populations are genetically structured in both quantitative traits and neutral loci but population differentiation in four of seven floral traits is greater than expected by random differentiation. The strong correlations in characters related to attraction of pollinators and pollen deposition/removal are in agreement with a pattern expected for the adaptive evolution of funnel-shaped corollas in outcrossing species.

*Keywords*: *Datura inoxia*, floral evolution, Flury's method, $F_{ST}$, outcrossing, phenotypic differentiation, phenotypic integration, population genetic structure, $Q_{ST}$, reproductive assurance, selfing, variance–covariance matrix.

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## INTRODUCTION

Flowers are the primary attractors in animal-pollinated plants, and their parts and how these are integrated are commonly targets of selection by pollinators (Bell, 1985). Plant–pollinator interactions constitute an important factor in the maintenance of mixed mating systems in plants as well as the transition from outcrossing to selfing in many species (Goodwillie *et al.*, 2005). Thus, pollinator-mediated selection of floral traits affects the genetic structure of populations, local adaptation, and speciation (Stebbins, 1970; Schemske and Bradshaw, 1999; Fenster *et al.*, 2004).

Since Darwin, studies of the phenotypic variance of floral traits in relation to pollinators have uncovered adaptive evolution in both single characters such as flower size and colour (Schemske and Bradshaw, 1999), shape (Herrera, 1993, 2001), the quantity and/or composition of nectar (Hodges, 1995; Schemske and Bradshaw, 1999), and size of deep corolla tubes (Nilsson, 1988), as well as correlated traits. The latter underscores the evolution of floral phenotypic integration, based on the common observation that separate floral parts do not vary independently (Pérez *et al.*, 2007; Sicard and Lenhard, 2011).

The evolution of integrated floral phenotypes may occur when selection favours a direction (sign) of phenotypic and genetic associations among traits, irrespective of whether selection affects each individual trait independently (Kudho *et al.*, 2001). This is called the selective correlation hypothesis (Delph, 1996). Berg (1960) described the presence of correlations among some traits, but an absence of correlations among others in the same organism, as 'correlation pleiades'. Berg hypothesized that 'correlation pleiades' of plant reproductive traits depends on the level of specificity of their animal pollinators. Selection in plant species associated with specialized pollinators is expected to be stronger than selection exerted, if any, by generalized pollination. Recently, research has supported this hypothesis with the observation that suites of characters that constitute a functional unit are more strongly correlated with one another (Conner and Sterling, 1995; Gómez, 2000; Ordano *et al.*, 2008; Rosas-Guerrero *et al.*, 2011). For instance, traits that improve pollen reception and/or exportation (e.g. stigma, stamen) may be more related to each other than to traits related to the attraction of pollinators (e.g. corolla size, nectary tube) (Berg, 1960; Bell, 1985; Rosas-Guerrero *et al.*, 2011). Thus, the level of covariation and integration among floral characters with different function and with specialized pollination should be more phenotypically integrated than those non-functional traits in species with generalized pollination (Berg, 1960; Nilsson, 1988; Ordano *et al.*, 2008; Rosas-Guerrero *et al.*, 2011).

Empirical studies have validated both Berg's 'correlation pleiades' (Armbruster *et al.*, 1999; Pérez-Barrales *et al.*, 2007; Rosas-Guerrero *et al.*, 2011) and the functional correlation hypothesis. For instance, Pérez-Barrales *et al.* (2007) studied the strength of correlation among floral and vegetative traits in populations of *Narcissus papyraceus*. They found that correlations were stronger among intra-floral traits than among vegetative traits in populations pollinated by moths than populations pollinated by flies, for which correlations were statistically non-significant. Another example is that of Rosas-Guerrero *et al.* (2011), who studied 20 species of *Ipomoea*. They found that self-compatible, pollinator-specialist *Ipomoea* species showed more phenotypic floral integration than generalist/self-incompatible plants.

Despite the relevance of plant–pollinator interactions for floral evolution, trait correlations might be established by selection on other traits and, indeed, show patterns departing from expectations based on Berg's hypothesis. In the absence of pollinators, reproductive assurance would evolve by selection on trait correlations that improve

self-fertilization (Lloyd, 1979). In some populations, reproductive assurance constitutes the starting point of the transition from outcrossing to selfing. Similarly, to optimize resource allocation to reproductive function, the transition in mating system promotes changes in single and correlated floral traits like small flowers and low pollen–ovule ratio, reduction in nectar and floral-scent production, decreased levels of hercogamy (i.e. style–stamen distance), and reduced floral display (i.e. selfing syndrome) (Fenster *et al.*, 2004). This suggests that trait correlations could evolve differentially in selfing and outcrossing species. For instance, Ushimaru and Nakata (2002) found that in three predominantly selfing species (*Mazus japonicus*, *Hosta longissima*, and *Scutellaria dependens*), correlations between filament and stigma height were stronger than those observed in outcrossing species (*Mazus miquelii* and *Hosta sieboldii*), the seed set of which depends mainly on pollinators (Ushimaru and Nakata, 2002). This pattern of correlation could be regarded as a strategy of selfing species to ensure self-fertilization in a scarce-pollinator scenario.

Furthermore, little is known about the relative importance of selection and random processes in the origin and/or maintenance of combinations of functional and morphological floral parts (Kingsolver *et al.*, 2001). The floral phenotype is not determined solely by adaptive processes but by stochastic, historical, and intrinsic developmental, physiological, or genetic constraints (Herrera, 2009). Studies on *Lavandula latifolia* (Herrera, 2001) and *Helleborus foetidus* (Herrera *et al.*, 2002) found no evidence of adaptive selection on floral integration but rather a potential role of genetic, developmental factors or even genetic drift.

Genetic drift, founder events, and inbreeding within populations are expected to reduce the genetic and phenotypic variability in floral traits, and to affect the correlations among traits and the adaptive value of floral functional integration (Pérez-Barrales *et al.*, 2007). Hence, these processes promote population structuring (Hedrick, 2000; Futuyma, 2005) and thus affect floral phenotypic differentiation.

Disentangling random processes and historical constraints from selection affecting flower functional integration (Berg, 1960) helps to understand the adaptive nature of standing phenotypic differentiation among populations in floral traits and their pattern of correlation. This is of particular interest in cases where a plant's mating system or floral evolution depends on animal pollinators.

To infer whether selection explains the observed phenotypic differentiation among populations in putatively adaptive quantitative floral characters ($Q_{ST}$), as opposed to genetic drift, one must contrast this hypothesis against a null model of differentiation at adaptively neutral loci ($F_{ST}$) (Spitze, 1993; Guillaume *et al.*, 2008; Whitlock, 2008). The detection of a significant difference between $Q_{ST}$ and $F_{ST}$ (i.e. $Q_{ST}$–$F_{ST}$) in single and correlated traits may imply adaptive differentiation among populations. A common method to contrast the two hypotheses is by means of variance–covariance matrix analysis. Phenotypic variance–covariance matrix analysis addresses the strength of association among traits and the pattern of these relationships (Ordano *et al.*, 2008). For instance, Flury's method assesses the way in which two matrices differ. That is, genetic drift is not expected to change the orientation of the **P** matrix but its overall magnitude (i.e. a proportional change), implying a change in its overall matrix size. In contrast, variation in the orientation of the covariance among traits, or element-specific change, is considered a change in the shape or pattern of the matrix, and these changes are expected to occur due to natural selection (Flury, 1988; Phillips and Arnold, 1999). Nevertheless, using phenotypic data this method is extremely sensitive to variation, and strong correlations among traits are expected to produce proportional changes in the matrices, making it difficult to distinguish among drift and selection or

pleiotropy (Roff and Mousseau, 2005). Another method is to use a Mantel test to compare the neutral genetic distance matrices and pair-wise differences between **P** matrices (Roff and Mousseau, 2005). This test is based on the assumption that genetic drift would affect both neutral and quantitative loci, hence their differentiation among populations or species is expected to be equal.

Roff *et al.* (2012) compared some of the principal statistical approaches to analysing **G** matrices and developed others to contrast $Q_{ST}$ and $F_{ST}$ (Roff and Mousseau, 2005; Santure *et al.*, 2010; Roff *et al.*, 2012). Since each method describes different elements of matrix variation, the use of more than one approach is recommended to better understand the role of evolutionary processes in population differentiation.

In this study, we assessed the extent of phenotypic population differentiation in single and correlated floral traits among populations of *Datura inoxia*, and compared them against a null evolutionary model (genetic drift) by different methods. Hence, we obtained the genetic structure of populations for phenotypic floral characters and for neutral loci. We also wished to distinguish between the population differentiation pattern generated by pollinator-mediated selection and that generated by selection of reproductive assurance.

## METHODS

### Species and study area

*Datura inoxia* Mill. (Solanaceae) is an annual plant found in semi-arid environments in Mexico, mainly in the Chihuahuan Desert (Barclay, 1959). Its white, tubular, nectar- and scent-producing, and crepuscular flowers are self-compatible and pollinated by hawkmoths (*Manduca* spp. and *Hyles* spp.), and visited by non-native pollinators (*Apis mellifera*) (Grant, 1983; Zamudio, 1999). In the Mexican Plateau, *D. inoxia* populations vary in the diversity of their pollinators, i.e. richness and relative abundance (V. Jiménez-Lobato, personal observations), thus suggesting that phenotypic differentiation in floral traits is promoted by selection. Yet, at the same time, the small population size of the species in some localities suggests a potential role of drift and inbreeding on floral phenotypic differentiation. We analysed six natural populations distributed over a broad geographic range in Mexico (Table 1).

**Table 1.** Geographic location and environmental variables[a] of *Datura inoxia* populations

| Population | State | *N* | Latitude | Longitude | Altitude (m, a.s.l.) | Mean annual precipitation (mm) | Mean annual temperature (°C) |
|---|---|---|---|---|---|---|---|
| Cañada de Moreno | Guanajuato | 68 | 21°17′43″ | 100°31′00″ | 1933 | 338.1 | 16.8 |
| Cuautla | Morelos | 19 | 18°48′15″ | 098°57′03″ | 1303 | 842.5 | 21.2 |
| Mapimí | Coahuila | 64 | 26°41′00″ | 103°45′00″ | 1210 | 254.9 | 20.2 |
| Neutla | Guanajuato | 58 | 20°42′21″ | 100°50′10″ | 1825 | 592.6 | 18.9 |
| Querétaro | Querétaro | 38 | 20°34′13″ | 100°22′11″ | 1813 | 521.3 | 18.8 |
| Saltillo | Coahuila | 18 | 25°25′00″ | 101°00′00″ | 1589 | 369.3 | 18.2 |

[a] Data from the National Meteorological Service of Mexico (http://smn.cna.gob.mx/).

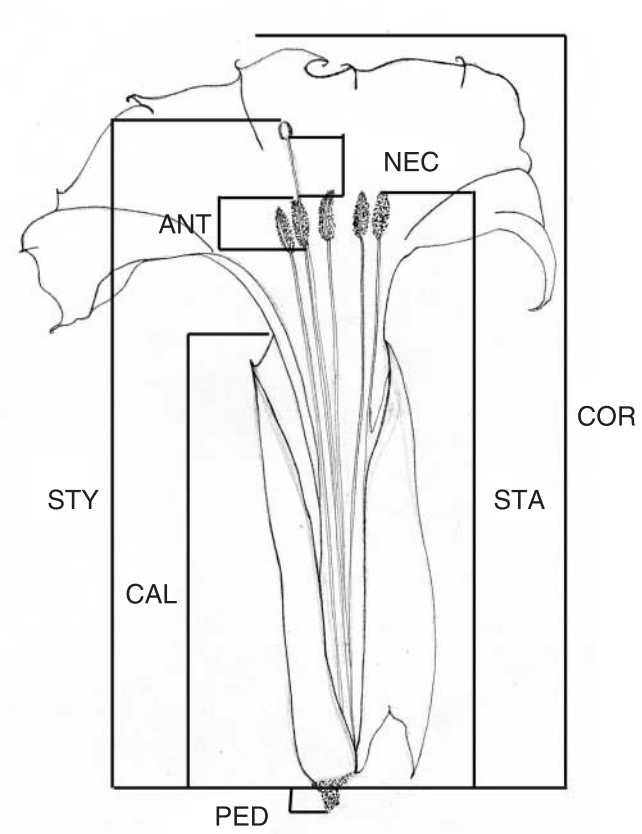



**Fig. 1.** Schematic representation of an opened flower of *Datura inoxia* and the floral traits measured. PED, pedicel; CAL, calyx; STA, stamen; STY, style; ANT, anther; NEC, nectary tube; COR, corolla length.

## Phenotypic analysis of single traits

We characterized the floral phenotype of each population by measuring seven floral traits per flower in each of several individuals per population ($n = 18$–$68$; Table 1). We measured the length of the pedicel (PED), calyx (CAL), stamen (STA), style (STY), anther (ANT), nectary tube (NEC), and corolla (COR) (Fig. 1). Up to six flowers were measured in each individual plant.

Mean floral trait values of each population were computed using individual average values. To obtain the observational components of phenotypic variance among populations, a nested analysis of variance of each floral trait was performed on log-transformed data, utilizing the Satterthwaite correction due to variation in sample size of populations (Sokal and Rohlf, 1995). Individual plants were nested within populations and both factors were considered random variables. The sources of variation included population, plants within population, and flowers within individual plants, within population, as the residual variance (Sokal and Rohlf, 1995). We used JMP 7.0 software for statistical analyses (SAS Institute, 2007).

To determine the magnitude of phenotypic associations among pairs of floral traits, correlation analyses were performed for each population using the average values of all traits of each individual plant.

### Analysis of phenotypic variance–covariance matrices

Although numerous methods have been developed to analyse the phenotypic variance–covariance matrix among taxa, none is completely sensitive to all features of the matrix (Roff *et al.*, 2012). Roff and colleagues (2012) suggest the use of complementary methods to compare matrices and to gain a better understanding of the data.

In this study, we used three different methods to analyse the phenotypic variance–covariance matrix among populations. Whereas the modified Mantel test and jackknife-MANOVA are considered coarser forms of analysis, the Flury or Hierarchical test is considered a more fine-scale test based on the analysis of eigenvalues of the matrices (Roff *et al.*, 2012). The first two analyses were performed with R software using scripts provided by D. Roff (R Development Core Team, 2008; Roff *et al.*, 2012), whereas Flury's test was performed using the software provided by Phillips (1994–1997; http://darkwing.uoregon.edu/~pphil/programs/cpc/cpc.htm).

The modified Mantel test explains differences in shape and eliminates differences in size between matrices. The null hypothesis assumes that the two matrices are identical. It uses a randomization approach to calculate the probability of the test. In this study, we used 10,000 randomizations. The jackknife-MANOVA compares the variation in structure between matrices. The method uses a jackknife procedure to create a set of pseudo-values that are then related to the predictor variable by a MANOVA test. In our case, the predictor variable is the population (POP) (Roff *et al.*, 2012). Finally, the hierarchical Flury's analysis is based on common principal components (CPC) (Flury, 1988) and determines the way in which two matrices differ. This analysis was performed using only the set of traits (COR, NEC, STY, STA, and CAL) whose paired correlations resulted in $r \geq 0.5$. The comparison between two matrices is performed in a hierarchical manner, beginning with unrelated matrices, followed by partial common principal components (PCPC), a full common principal component model (full CPC), proportionality, and an equality matrix (Flury, 1988). Specifically, unrelated matrices do not share any principal component. When the matrices share some axes but others have different orientation (i.e. partial CPC), the matrices differ in orientation, shape, and size. Next, a full CPC model occurs when two matrices have the same orientation in the multivariate space (i.e. they have common eigenvectors, or principal components), but differ in the amount of variation encompassed along the axes. They differ in shape. Proportionality occurs when the matrices have the same eigenvector but their eigenvalues differ by a constant (i.e. differing in size). Finally, equality occurs when two matrices have the same eigenvalues and eigenvectors (i.e. the same orientation, shape, and size) (Phillips and Arnold, 1999; Ashman, 2003).

To test the proportionality hypothesis, we used the covariance instead of correlation matrices (Phillips and Arnold, 1999). We performed the analyses using the jump-up procedure to facilitate interpretation.

### Patterns of phenotypic integration

The phenotypic integration index (INT) of each population was calculated following Wagner (1984) and Cheverud *et al.* (1989). The INT was estimated using the variance of the eigenvalues of the correlation matrix obtained by principal component analysis (PCA). Because the sample size differed among populations, we used the correction proposed by Wagner (Herrera *et al.*, 2002). The INT values were expressed as the maximum possible value (MAX INT) given by the number of traits we used (Wagner, 1984; Cheverud *et al.*, 1989). Confidence intervals for the INT values were obtained by bootstrapping ($n = 10{,}000$ permutations) in S-Plus Software (MathSoft, 1999).

### Construction of the null model ($F_{ST}$) and local inbreeding values

The genetic structure of populations of *D. inoxia* was obtained using allozyme variation through starch gel electrophoresis (Cheliak and Pitel, 1984). Fresh leaves were collected from 30 individuals in each population, bagged, labelled, and stored in an ultra-freezer at –97°C until electrophoresis. Two grams of tissue of each individual were macerated with 250 ml of extraction buffer (Cheliak and Pitel, 1984). The supernatant was collected on wicks of filter paper and kept in the ultra-freezer at –97°C for later use. In total, 12 enzymes were screened in each individual plant, five of them polymorphic. The gel buffer and electrode protocols are given in the Appendix.

The apportioning of genetic variation, within and among populations, was obtained using Weir and Cockerham's (1984) estimator, equivalent to Wright's *F*-statistic (Wright, 1978). A 95% confidence interval of *F*-values was obtained by bootstrapping over loci (Weir and Cockerham, 1984). To determine whether $F_{IS}$ and $F_{IT}$ were significantly different from zero, we used the $\chi^2$ test proposed by Li and Horvitz (1953), computed as $\chi^2 = F(2N)(k-1)$, with $k(k-1)/2$ degrees of freedom, where $F$ stands for $F_{IS}$ or $F_{IT}$. In the case of $F_{ST}$, $\chi^2$ was computed as $\chi^2 = (2N)F_{ST}(k-1)$, with $(k-1)(s-1)$ degrees of freedom (Li and Horvitz, 1953; Workman and Niswander, 1970). In all cases, $k$ is the total number of alleles, and $s$ is the number of sub-populations sampled. All calculations were performed with TFPGA 1.3 software (Miller, 2000).

To determine the exact probability in the observed differences in allele frequencies among populations, we applied the exact test of population differentiation (Raymond and Rousset, 1995) using TFPGA 1.3 software (Miller, 2000). To obtain the level of indirect gene flow ($Nm$) among populations, we calculated the average value of $Nm$ among all pairs of populations as

$$Nm = \frac{\frac{1}{F_{ST}} - 1}{4}$$ (Wright, 1951).

To determine whether the floral phenotypic pattern is in agreement with the expectation of a transition from outcrossing to selfing, we regressed the local fixation index of each population on the population average value of each trait and on their levels of floral integration. If any, we expect a significant relation between the level of self-fertilization and changes in some floral traits. The local fixation index ($F_{IS(L)}$) was calculated as $F_{IS(L)} = 1 - (H_O/H_E)$ (Hedrick, 2000), assuming that all inbreeding results from the level of self-fertilization in the population. We used JMP 7.0 software (SAS Institute, 2007).

## Analysing for drift

To determine whether the pattern of population differentiation is determined mainly by drift or by natural selection, different methods have been developed (Whitlock and Guillaume, 2009; Santure *et al.*, 2010). In this study, we applied three tests to single and first PC scores, as well as to whole **P** matrices in order to strengthen our results.

## $\approx Q_{ST}$–$F_{ST}$ comparisons: an approximation

The first method by Whitlock and Guillaume (2009) was applied to single traits and the first PC scores. The comparison between $Q_{ST}$ and $F_{ST}$ is often performed using the mean value of each estimator. However, bias and sampling errors produce heterogeneity in both $F_{ST}$ and $Q_{ST}$ among neutral loci and phenotypic traits, respectively (Whitlock, 2008). Thus, the comparison of average values of both estimators of population structure is insufficient to quantify, if any, local adaptation of traits. To test if quantitative phenotypic characters show neutral differentiation among populations, it is necessary to determine if $Q_{ST}$ of a given character falls within the probability distribution of differentiation at neutral loci (O'Hara and Merilä, 2005; Whitlock, 2008; Whitlock and Guillaume, 2009).

A null distribution of the $\approx Q_{ST}$–$F_{ST}$ difference was parametrically simulated using random values of $F_{ST}$, $V_A$, and $V_B$, under the null hypothesis that $\approx Q_{ST} = F_{ST}$ (Whitlock and Guillaume, 2009). Then, the observed $\approx Q_{ST}$–$F_{ST}$ values were compared with the simulated $\approx Q_{ST}$–$F_{ST}$ distributions to determine the probability of accepting/rejecting the null hypothesis. Computations were performed using the script provided by Whitlock and Guillaume (2009) in R (R Development Core Team, 2008) and modified for unbalanced data.

## $\approx Q_{ST}$ calculations

The $\approx Q_{ST}$ value of each floral character ought to be calculated from genetic variance of traits. In the case of *D. inoxia*, since the relationship among individuals within populations is unknown, we estimated $Q_{ST}$ by considering the relatedness among individuals conservatively as full-sibs. Hence, we considered $Q_{ST}$ as $\approx Q_{ST}$, since it is an approximation to the genetic value. $\approx Q_{ST}$ was calculated from the estimated phenotypic variance components as $\sigma_W^2 = (1 - F_{ST})\sigma_0^2$, and $\sigma_B^2 = 2F_{ST}\sigma_0^2$, where $\sigma_W^2$ is the genetic variance within populations, $\sigma_B^2$ is the genetic variance among populations, and $\sigma_0^2$ the genetic variance that would exist if populations were under panmixia (Lande, 1992). When the total genetic variance is $\sigma_T^2 = (1 + F_{ST})\sigma_0^2$, the parameter that measures population differentiation in a quantitative trait (i.e. $Q_{ST}$, analogous to the $F_{ST}$ for a neutral locus in the case of diploid organisms) is obtained as (Wright, 1951):

$$Q_{ST} = \frac{\sigma_B^2}{\sigma_B^2 + 2\sigma_W^2} = \frac{1}{1 + 2\left(\frac{\sigma_W^2}{\sigma_B^2}\right)}.$$

Depending on the breeding design $\sigma_W^2 = V_A = 4V_{IND}$ for half-sibs, $\sigma_W^2 = V_A = 2V_{IND}$ for full-sibs, and $V_A = V_{IND}$ for clones; where $V_A$ is the estimated variance among individuals within populations, and $\sigma_B^2 = V_B$ is the variance among populations.

To estimate the within ($\sigma_W^2$) and among ($\sigma_B^2$) populations components of phenotypic variance, we used the unbiased mean squares ($MS$) estimates from the nested analysis of

variance described above. The mean squares for an unbalanced design were estimated as $MS_W = (\sigma^2_E + n_0\sigma^2_{IND\subset POP})$ and $MS_B = (\sigma^2_E + n'_0\sigma^2_{IND\subset POP}) + (nb)_0\sigma^2_B$ (Sokal and Rohlf, 1995), where $\sigma^2_E$ is the error variance, $\sigma^2_{IND\subset POP}$ is the within-population variance ($\sigma^2_W$), and $n_0$, $n'_0$, and $(nb)_0$ are the corrected sample sizes (Sokal and Rohlf, 1995). $\sigma^2_W$ and $\sigma^2_B$ were estimated as

$$\sigma^2_W = \frac{(MS_W - MS_E)}{n_0} \qquad \text{and} \qquad \sigma^2_B = \frac{(MS_B - MS_E - n'_0\sigma^2_{IND\subset POP})}{(nb)_0},$$

respectively. $\approx Q_{ST}$ was calculated for each floral trait. The standard error and confidence interval of $\approx Q_{ST}$ were obtained from a parametric bootstrap with a confidence level of 95% (O'Hara and Merilä, 2005), using R Software (R Development Core Team, 2008).

### Mantel test

The second method to test drift is the Mantel test. Using a Mantel test with 10,000 randomizations, we compared a matrix composed of the genetic distance (neutral molecular markers) between pairs of populations (i.e. Reynolds genetic distance) versus a matrix composed of mean differences among pair-wise populations of each floral trait and first PC scores, and versus a matrix of mean percentage difference between elements of two **P** matrices:

$$T\% = 100\left[\frac{\sum_{i=1}^{c} \left|\rho_{i1} - \rho_{i2}\right|^{\frac{1}{c}}}{(\overline{\rho}_1 - \overline{\rho}_2)^{\frac{1}{2}}}\right],$$

where $\rho_{ij}$ is the $i$th element of the $j$th matrix, $c$ is the number of entries of each matrix, and $\overline{\rho}_j$ is the overall average of the elements of the $j$th matrix (Roff and Mousseau, 2005). If the correlation among neutral and quantitative matrices is significant, genetic drift may be implied in phenotypic differentiation patterns observed among populations.

### Flury's test

The third method was that of Flury, which was explained above. Change in proportionality between two matrices is expected to occur when genetic drift is the main evolutionary force acting on **P** matrices, implying a change in the overall matrix size.

## RESULTS

### Phenotypic analysis of single traits

All floral traits of *D. inoxia* varied significantly among populations (Table 2). In general, the Mapimí population had the largest flowers and Cañada de Moreno population the smallest flowers (Table 2). The among- and within-populations components accounted for a significant fraction of phenotypic variance in all floral traits (Table 3). The population component of variance explained up to 60% of phenotypic variance (style); the lowest percentage was for the corolla (~20%, Fig. 2).

**Table 2.** Population mean and coefficient of variation (CV, %) of seven floral traits of *Datura inoxia* from Mexico

| Character | Cañada de Moreno | Cuautla | Mapimí | Neutla | Querétaro | Saltillo |
|---|---|---|---|---|---|---|
| Anther | 12.169$^{b}$ (5.336) | 11.892$^{bc}$ (8.368) | 12.765$^{a}$ (8.720) | 9.137$^{e}$ (12.246) | 10.030$^{d}$ (13.588) | 11.235$^{c}$ (13.806) |
| Calyx | 104.950$^{b}$ (7.681) | 98.840$^{bc}$ (11.997) | 118.216$^{a}$ (12.004) | 107.178$^{b}$ (8.288) | 98.559$^{c}$ (10.089) | 117.359$^{a}$ (8.577) |
| Corolla | 171.358$^{b}$ (5.258) | 162.329$^{bc}$ (5.411) | 179.977$^{a}$ (7.789) | 163.086$^{c}$ (5.968) | 170.646$^{b}$ (5.091) | 167.506$^{bc}$ (9.622) |
| Nectary tube | 73.964$^{c}$ (9.839) | 85.609$^{b}$ (6.243) | 98.881$^{a}$ (9.360) | 86.087$^{b}$ (8.072) | 86.587$^{b}$ (7.960) | 92.269$^{b}$ (9.455) |
| Pedicel | 16.476$^{a}$ (15.469) | 9.053$^{c}$ (22.696) | 12.840$^{b}$ (18.568) | 10.811$^{c}$ (18.478) | 15.609$^{a}$ (31.348) | 10.561$^{c}$ (24.514) |
| Stamen | 135.857$^{d}$ (3.715) | 153.408$^{bc}$ (5.789) | 172.893$^{a}$ (7.606) | 152.574$^{c}$ (6.518) | 158.016$^{bc}$ (4.354) | 162.723$^{b}$ (7.261) |
| Style | 131.028$^{d}$ (4.625) | 147.234$^{bc}$ (10.327) | 176.397$^{a}$ (9.295) | 144.347$^{c}$ (7.426) | 144.938$^{c}$ (5.212) | 156.835$^{b}$ (12.467) |

*Note:* Different superscripts indicate significant differences among populations ($P < 0.05$).

**Table 3.** Nested ANOVA of seven floral traits among populations of *Datura inoxia*

| Character | Source of variation | d.f. | SS | *F* | *P* |
|---|---|---|---|---|---|
| Anther | Population | 5 | 1572.651 | 101.205 | < 0.0001 |
| | Individual | 259 | 951.098 | 6.450 | < 0.0001 |
| Calyx | Population | 5 | 48535.311 | 28.080 | < 0.0001 |
| | Individual | 259 | 106461.093 | 7.949 | < 0.0001 |
| Corolla | Population | 5 | 35154.602 | 18.294 | < 0.0001 |
| | Individual | 259 | 116289.162 | 4.803 | < 0.0001 |
| Nectary tube | Population | 5 | 94613.389 | 101.090 | < 0.0001 |
| | Individual | 259 | 56929.154 | 5.431 | < 0.0001 |
| Pedicel | Population | 5 | 5650.469 | 51.119 | < 0.0001 |
| | Individual | 259 | 6709.206 | 5.153 | < 0.0001 |
| Stamen | Population | 5 | 208122.891 | 150.083 | < 0.0001 |
| | Individual | 259 | 85506.791 | 8.399 | < 0.0001 |
| Style | Population | 5 | 315429.871 | 140.762 | < 0.0001 |
| | Individual | 259 | 137148.947 | 6.442 | < 0.0001 |

Five floral characters related to flower size showed strong and positive correlations among themselves (calyx, corolla, style, stamen, and nectary tube) (Fig. 3), whereas correlations involving the pedicel or anther length were the weakest and only five of them were statistically significant (Fig. 3).

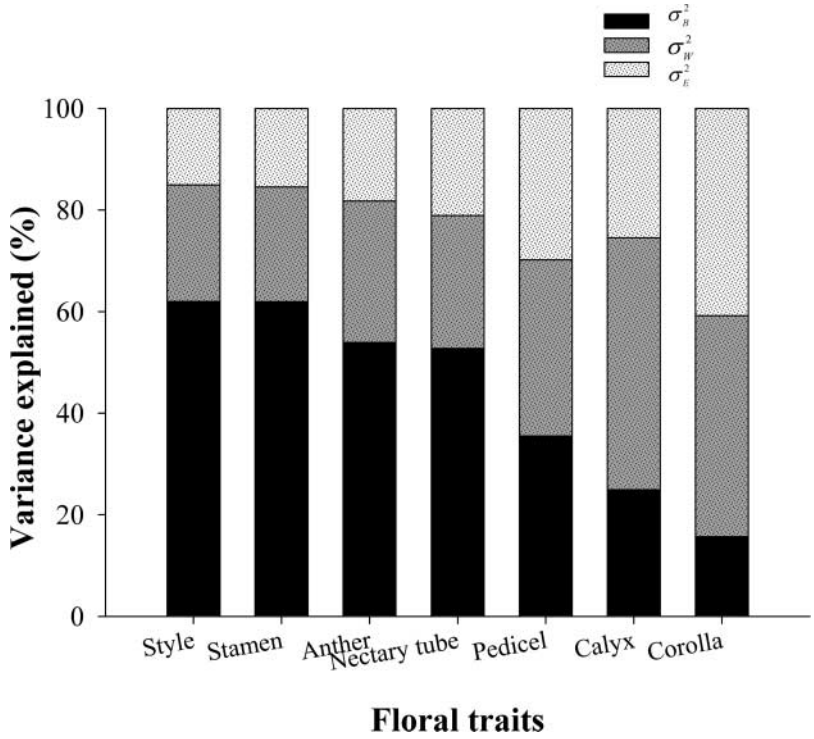



**Fig. 2.** Percentage of phenotypic variance of seven floral traits accounted for by populations ($\sigma^2_B$), individuals within population ($\sigma^2_W$), and residual variance ($\sigma^2_E$) in *Datura inoxia*.

### Analysis of phenotypic variance–covariance matrices

The modified Mantel test and the jackknife-MANOVA analyses showed significant population differences among the variance–covariance matrices of floral characters (Mantel test: $P = 0.001$; Wilks' $\lambda = 0.2456$, $F_{140,\ 1150.4} = 2.7034$, $P < 0.00001$; Pillai's trace = 0.63, $F_{140,\ 4560} = 4.696$, $P < 0.01$).

Flury's analysis indicated that the neighbouring populations of Saltillo and Mapimí are equal. The principal component was determined mainly by the style and corolla length. Querétaro, Neutla, and Mapimí differed in shape, sharing the full CPC model determined mainly by the style, stamen, and corolla lengths (Table 4). Saltillo showed proportional divergence from Querétaro and Neutla (eigenvector 1: style, stamen, and corolla lengths) and Cuautla (eigenvector 1: style length) as expected under the null hypothesis. Finally, Cañada de Moreno and Cuautla did not show any relationship (except Cuautla–Saltillo) with the remaining populations (Table 4).

### Patterns of phenotypic integration

The magnitude of phenotypic integration was significantly different from zero (INT ranged from 0.928 to 1.915) (Table 5). The MAX INT ranged from 0.287 to 0.141. The Mapimí and Cañada de Moreno populations were the most and less integrated population, respectively (Mapimí: INT = 1.915; Cañada de Moreno: INT = 0.928) (Table 5).

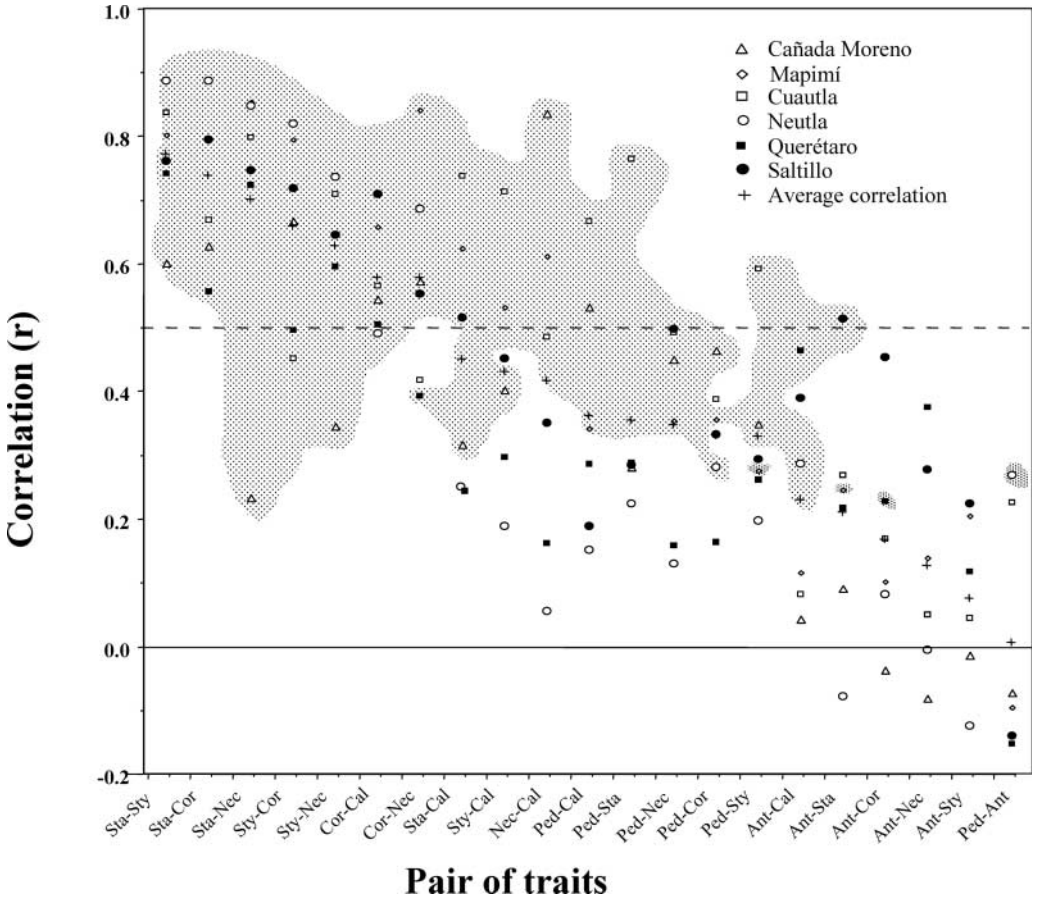



**Fig. 3.** Correlation coefficient for pairs of floral characters in six populations of *Datura inoxia*. PED, pedicel; CAL, calyx; STA, stamen; STY, style; ANT, anther; NEC, nectary tube; COR, corolla length. Significant values are highlighted by grey tint.

### Null model ($F_{ST}$) and local inbreeding values

Analysis of the genetic structure of *D. inoxia* indicates a deficiency of heterozygous individuals both at the whole ($F_{IT}$) and local ($F_{IS}$) population levels (Table 6). High and statistically significant inbreeding at the local population level was detected ($F_{IS} = 0.33$, Table 6), although variation among loci was noticeable (range 0.17–0.48). There is evidence of population differentiation at all but one (*SDH*) enzymatic locus in *D. inoxia*. Differences among populations in gene frequencies accounted for a significant amount of total genetic variation ($F_{ST} = 0.125$). The exact test of population differentiation showed that only the populations Cañada de Moreno and Cuautla did not differ in allele frequencies ($\chi^2 = 7.911$, $P = 0.638$), whereas all other pairs of populations differed significantly ($P < 0.05$; results not shown). The mean gene flow among all pairs of populations was $Nm = 2.64$ (range 0.5–9.34).

The fixation index varied among populations (range $F_{IS(L)} = 0.114$–$0.561$). Cañada de Moreno had the lowest fixation index, while Saltillo and Mapimí had the highest index

**Table 4.** Comparisons of phenotypic covariance matrices among six populations of *Datura inoxia* using the jump-up procedure of Flury's analysis

| Comparison | Hierarchy | Accepted | $\chi^2$ | d.f. | *P* |
|---|---|---|---|---|---|
| Cañada de Moreno–Mapimí | CPC(1) | Unrelated | 34.418 | 4 | 0.0000 |
| Cañada de Moreno–Saltillo | CPC(1) | Unrelated | 18.245 | 4 | 0.0011 |
| Cañada de Moreno–Cuautla | CPC(1) | Unrelated | 30.221 | 4 | 0.0000 |
| Cañada de Moreno–Neutla | CPC(1) | Unrelated | 38.682 | 4 | 0.0000 |
| Cañada de Moreno–Querétaro | CPC(1) | Unrelated | 10.605 | 4 | 0.0314 |
| Cuautla–Mapimí | CPC(1) | Unrelated | 10.484 | 4 | 0.0330 |
| Cuautla–Neutla | CPC(1) | Unrelated | 14.48 | 4 | 0.0059 |
| Cuautla–Querétaro | CPC(1) | Unrelated | 12.692 | 4 | 0.0129 |
| Cuautla–Saltillo | CPC(1) | Unrelated | 12.414 | 4 | 0.0145 |
| Neutla–Mapimí | CPC(1) | Unrelated | 10.463 | 4 | 0.0333 |
| Neutla–Saltillo | Proportionality | Full PC | 24.184 | 14 | 0.0435 |
| Neutla–Querétaro | Proportionality | Full PC | 32.977 | 14 | 0.0029 |
| Querétaro–Saltillo | Equality | Proportionality | 39.117 | 15 | 0.0006 |
| Querétaro–Mapimí | Proportionality | Full PC | 37.19 | 14 | 0.0007 |
| Saltillo–Mapimí | Equality | Equality | 22.2 | 15 | 0.1027 |

**Table 5.** Phenotypic integration index (INT) of seven floral traits of *Datura inoxia*

| Population | INT | Confidence interval | % max |
|---|---|---|---|
| Cañada de Moreno | 0.928 | 0.680–1.216 | 17.65 |
| Cuautla | 1.851 | 1.414–2.324 | 25.67 |
| Mapimí | 1.915 | 1.674–2.135 | 28.79 |
| Neutla | 1.504 | 1.342–1.666 | 21.36 |
| Querétaro | 1.085 | 0.817–1.392 | 14.12 |
| Saltillo | 1.686 | 1.229–2.192 | 21.67 |

*Note:* % max is the maximum possible value of INT. The confidence interval (at 95%) was obtained by bootstrapping.

**Table 6.** Wright's *F*-statistics of six populations of *Datura inoxia* in Mexico

| Locus | $F_{IT}$ | $F_{ST}$ | $F_{IS}$ |
|---|---|---|---|
| *6PGD* | 0.3389* | 0.0902* | 0.2733* |
| *GOT3* | 0.5664* | 0.1594* | 0.4841* |
| *GOT1* | 0.4102* | 0.1972* | 0.2654* |
| *PGI* | 0.3349* | 0.1983* | 0.1704* |
| *SDH* | 0.4876* | 0.005 | 0.485* |
| Average (SD) | 0.4115 (0.05) | 0.1253 (0.044) | 0.33 (0.079) |
| 95% CI | 0.336–0.517 | 0.047–0.194 | 0.204–0.478 |

*$P < 0.0001$.

(Table 7). Variation in fixation index among populations did not show any significant relationship with variation in floral traits (results not show) or with the level of floral integration ($F = 0.218$, $P = 0.664$).

### $\approx Q_{ST}$–$F_{ST}$ comparisons

With the Whitlock and Guillaume method, differentiation in single floral traits ($Q_{ST}$ obs) ranged from 0.08 for corolla length to 0.42 for stamen length (Table 8). Comparison between the observed and simulated $Q_{ST}$–$F_{ST}$ difference indicated a statistically significant difference in four of seven characters (STA, STY, NEC, and ANT), indicating higher differentiation in these four quantitative characters than expected under the null hypothesis (Table 8; Fig. 4).

**Table 7.** Local fixation index ($F_{IS(L)}$) of six populations of *Datura inoxia*

| Population | $F_{IS(L)}$ | $\chi^2$ | $P$ |
|---|---|---|---|
| Cañada de Moreno | 0.140 | 9.366 | >0.05 |
| Cuautla | 0.344 | 160.729 | <0.001 |
| Mapimí | 0.362 | 59.250 | <0.001 |
| Neutla | 0.341 | 60.518 | <0.001 |
| Querétaro | 0.309 | 48.759 | <0.001 |
| Saltillo | 0.561 | 66.714 | <0.001 |

**Table 8.** Simulated and observed values of $F_{ST}$ (S.D.) and $Q_{ST}$ (S.D.) and their difference for floral traits of *Datura inoxia*

| Character | $Q_{ST}$ sim | $F_{ST}$ sim | $Q_{ST}$–$F_{ST}$ sim | $Q_{ST}$ obs | $F_{ST}$ obs | $Q_{ST}$–$F_{ST}$ obs |
|---|---|---|---|---|---|---|
| Anther | 0.115 (0.065) | 0.113 (0.041) | 0.001 (0.077) | 0.3245* | 0.115 | **0.209** |
| 95% CI | 0.021 to 0.259 | 0.033 to 0.187 | −0.125 to 0.168 | | | |
| Calyx | 0.111 (0.064) | 0.112 (0.040) | −0.001 (0.075) | 0.1107* | 0.115 | −0.005 |
| 95% CI | 0.018 to 0.259 | 0.033 to 0.187 | −0.1242 to 0.1617 | | | |
| Corolla | 0.108 (0.063) | 0.114 (0.040) | −0.006 (0.076) | 0.082* | 0.115 | −0.033 |
| 95% CI | 0.015 to 0.258 | 0.033 to 0.187 | −0.132 to 0.159 | | | |
| Nectary tube | 0.109 (0.063) | 0.114 (0.0340) | −0.004 (0.074) | 0.3333* | 0.115 | **0.218** |
| 95% CI | 0.015 to 0.263 | 0.036 to 0.187 | −0.130 to 0.156 | | | |
| Pedicel | 0.115 (0.065) | 0.114 (0.039) | 0.000 (0.077) | 0.2025* | 0.115 | 0.087 |
| 95% CI | 0.018 to 0.264 | 0.033 to 0.184 | −0.136 to 0.158 | | | |
| Stamen | 0.111 (0.063) | 0.114 (0.040) | −0.003 (0.075) | 0.424* | 0.115 | **0.309** |
| 95% CI | 0.019 to 0.265 | 0.033 to 0.187 | −0.128 to 0.168 | | | |
| Style | 0.114 (0.066) | 0.114 (0.039) | 0.000 (0.076) | 0.4013* | 0.115 | **0.286** |
| 95% CI | 0.015 to 0.266 | 0.033 to 0.187 | −0.125 to 0.169 | | | |

*Note:* CI is the confidence interval (at 95%). The significant observed $Q_{ST}$–$F_{ST}$ are highlighted in bold typeface.
*Significant estimate at $P < 0.0001$; sim, simulated; obs, observed.

### Mantel test

Results obtained by Mantel comparisons did not show any correlation between the matrix of genetic distance and the matrix of differences in trait means of pairs of populations, either among genetic distances and the first PC scores or $T$% matrices (results not shown).

### Flury's test

As mentioned above, only Saltillo showed proportional divergence from Querétaro, Neutla, and Cuautla, as expected under the null hypothesis.

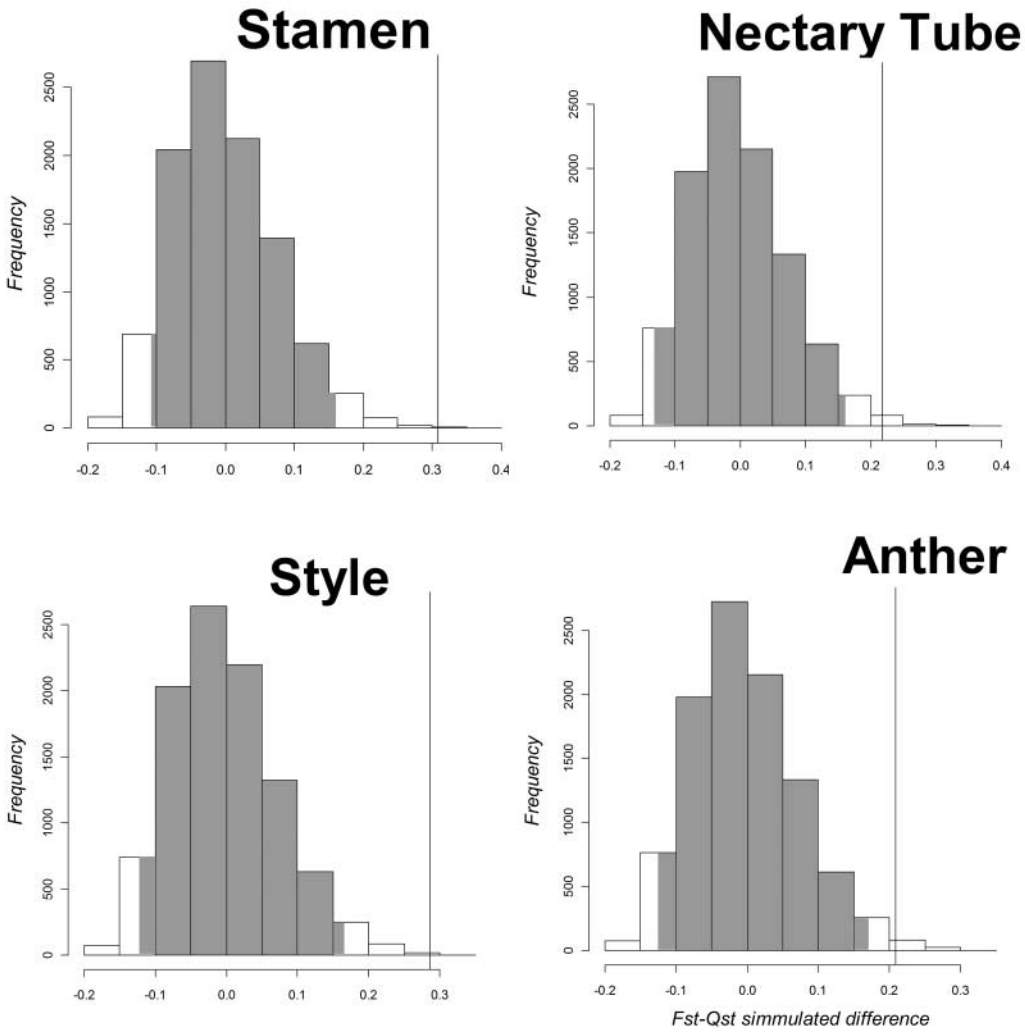
## DISCUSSION

Population differentiation in quantitative trait values or gene frequencies is a ubiquitous result of evolution. However, to what extent population differentiation is the result of stochastic or deterministic evolutionary processes cannot be ascertained without contrasting the expectations derived from each hypothesis. *Datura inoxia* shows genetic structure in both quantitative phenotypic traits and neutral loci. By using three complementary methods of analysis, the present study has demonstrated that population differentiation in four of seven floral traits of *D. inoxia* (nectary tube, anther, style, and stamen lengths) is greater than that expected by random differentiation. Furthermore, the pattern of correlation observed between filaments and corolla length is in line with a functional hypothesis of stronger correlations among intra-floral parts, which can partly be explained by Berg's hypothesis (Berg, 1960; Conner and Sterling, 1995). The strong correlations (i.e. filaments and corolla length) found in *D. inoxia* are in line with the pattern expected for the evolution of outcrossing species, especially those related to the evolution of funnel-shaped corollas due to selection mediated by pollinators (Darwin, 1862; Nilsson, 1988; Conner and Sterling, 1995; Ushimaru and Nakata, 2002).

### Single floral traits

*Datura inoxia* populations are genetically structured for both nearly neutral genetic loci (allozymes) and phenotypic floral traits of putative adaptive value. In accordance with the $\approx Q_{ST}$–$F_{ST}$ method, the degree of phenotypic differentiation found in single traits (e.g. nectary tube, anther, style, and stamen length) among populations of *D. inoxia* exceeds differentiation in neutral genetic loci. In line with Mantel tests, none of seven traits shows a neutral differentiation pattern. These results suggest that divergent selection may be implicated in the observed phenotypic differentiation in floral traits of *D. inoxia* (Spitze, 1993). Gene flow observed in *D. inoxia* could be considered extensive ($Nm = 2.646$) (Ellstrand, 1992). Data on gene flow in plants reveals that $Nm \geq 1$ in each generation is enough to counteract genetic drift and moderate intensities of natural selection. However, gene flow is highly variable (Ellstrand, 1992). Although the average magnitude of $Nm$ among populations of *D. inoxia* is high, we detected significant population differentiation in both quantitative traits and in allelic frequencies; hence, it is possible that the indirect estimate of gene flow accounts for historic rather than contemporaneous gene flow.

Studies of *Clarkia dudleyana* (Podolsky and Holtsford, 1995) have reported higher $Q_{ST}$ values of single floral traits (range 0.190–0.518) than $F_{ST}$ values, suggesting a role of natural selection




in population differentiation. $Q_{ST}$ values of flower size differentiation in *Scabiosa columbaria* and *S. canescens* expressed were higher (0.485 and 0.217, respectively) than $F_{ST}$ values, implying divergent selection as the main force shaping large-scale patterns of variation in these species (Waldmann and Andersson, 1998). Analysis of phenology and floral traits in *Senecio vulgaris* resulted in higher $Q_{ST}$ (range 0.27–0.71) than $F_{ST}$ values also (Steinger *et al.*, 2002).

The different results obtained by $\approx Q_{ST}$–$F_{ST}$ and Mantel tests could be because Mantel tests use a single value around which a confidence interval is constructed. In contrast, the $\approx Q_{ST}$–$F_{ST}$ method recalculates $Q_{ST}$ and $F_{ST}$ values each time, such that the confidence interval of each parameter could be larger than that estimated by a Mantel test. Thus, it is likely that the probability distribution of differentiation in $\approx Q_{ST}$–$F_{ST}$ is more sensitive and conservative to those differences.

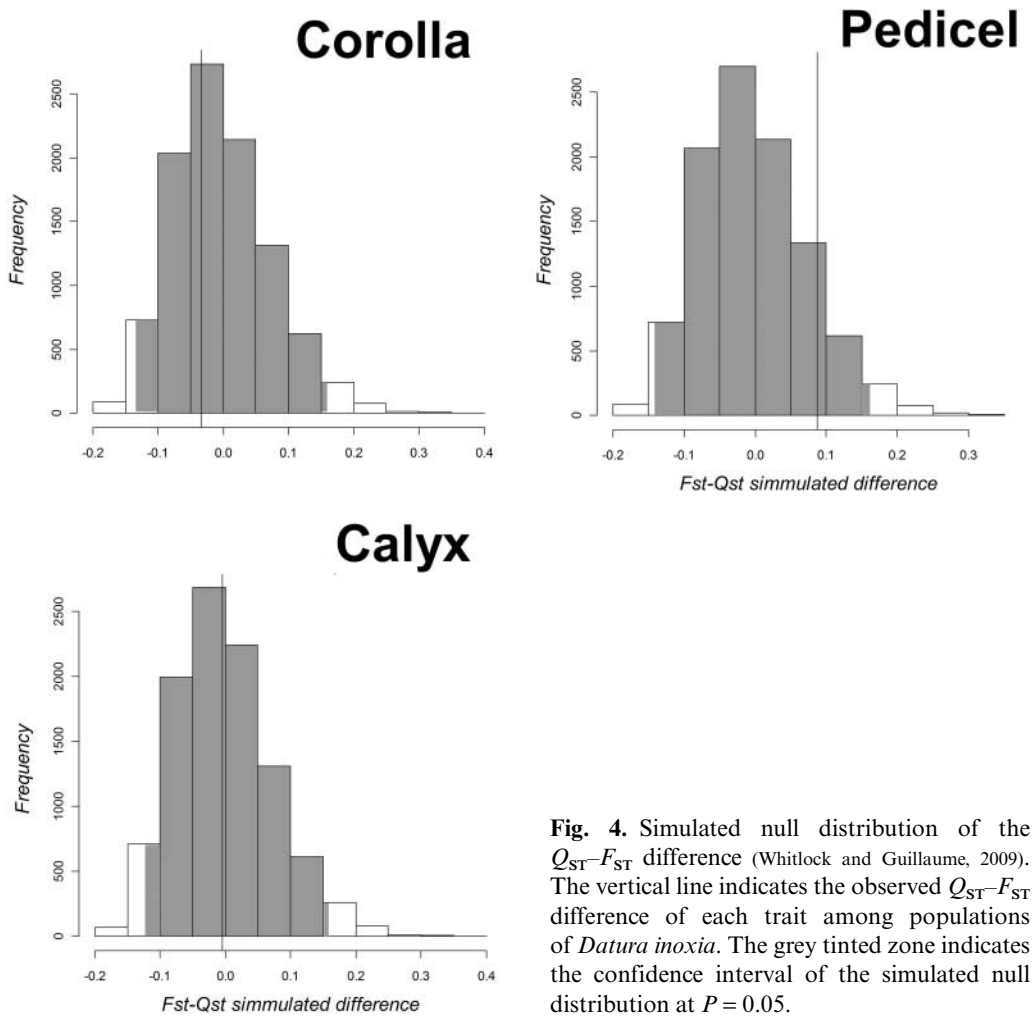



**Fig. 4.** Simulated null distribution of the $Q_{ST}$–$F_{ST}$ difference (Whitlock and Guillaume, 2009). The vertical line indicates the observed $Q_{ST}$–$F_{ST}$ difference of each trait among populations of *Datura inoxia*. The grey tinted zone indicates the confidence interval of the simulated null distribution at $P = 0.05$.

### *Selection mediated by pollinators in single floral traits*

Natural selection exerted by pollinators might explain the observed phenotypic pattern of differentiation found in this study. It has been shown that attraction characters and rewards offered by plants to pollinators modify pollinators' behaviour (Harder and Barrett, 1995; Conner and Rush, 1996). Nectar and pollen constitute the most frequent rewards offered by plants to hawkmoths and hummingbirds (Scobell and Scott, 2002), and bumblebees and honeybees, respectively (Robertson *et al.*, 1999). Thus, an adaptive role of the nectary tube, in this regard, has been proposed (Rowley, 1980; Wolf and Stiles, 1989; Aigner, 2005), similar to anther length (Grant, 1983; Robertson *et al.*, 1999), especially in species with tubular flowers (Hodges, 1995; Scobell and Scott, 2002; Riffell *et al.*, 2008). Although we did not conduct natural selection experiments, *D. inoxia* is commonly pollinated by hawkmoths (Grant, 1983). Bees are frequent floral visitors in some

populations (V. Jiménez-Lobato, unpublished data) and their role as selective agents on the floral phenotype cannot be ruled out. Natural selection experiments are urgently required to test this hypothesis.

### *Selection of selfing in single floral traits*

Evolution towards selfing is another hypothesis to explain the phenotypic pattern of floral differences in *D. inoxia*. Selfing constitutes an advantage for plant reproduction when pollinators are absent or scarce (Lloyd, 1979; Schoen and Brown, 1991). The transition from outcrossing to selfing is expected to spur changes in floral traits by reallocating resources to the reproductive function (Charlesworth and Morgan, 1991).

Empirical evidence indicates that selfers have smaller flowers (Lyons and Antonovics, 1991; Worley and Barrett, 2000; Armbruster *et al.*, 2002; Goodwillie and Ness, 2005), little hercogamy (Ritland and Ritland, 1989; Lyons and Antonovics, 1991; Belaoussoff and Shore, 1995; Armbruster *et al.*, 2002; Brunet and Sweet, 2006; Vallejo-Marin and Barrett, 2009), smaller pollen–ovule ratios (Cruden, 1977; Ritland and Ritland, 1989; Lyons and Antonovics, 1991), reduced nectar and floral scent production (Cruden, 1977; Rainne *et al.*, 2005), and smaller floral displays (Goodwillie *et al.*, 2010). Thus, if some populations of *D. inoxia* are in transition to increased self-fertilization, changes in floral traits would be expected. We did not find any relationship between the level of self-fertilization and floral traits in each population. One explanation for the observed phenotypic differences among populations in single floral traits of *D. inoxia* could be related to differential selection exerted by pollinators and not due to selection for self-fertilization as reproductive assurance. However, the theory of reproductive assurance predicts that changes in floral traits are a consequence of modifications in the mating system. Thus, it is possible that in some populations of *D. inoxia* selection of selfing is occurring but changes in floral phenotype and their relation with local inbreeding is not apparent, as yet.

## Patterns of phenotypic covariances and correlations

Phenotypic correlations might result from developmental associations among characters (Herrera, 2001; Merilä and Björklund, 2004; Smyth, 2005), by selection, or by stochastic processes like genetic drift (Conner and Sterling, 1995).

The correlation matrices among populations of *D. inoxia* showed that correlations among style, stamen, and corolla lengths were the highest in all six populations. Ushimaru and Nakata (2002) found that the pattern of correlation evolves differently among selfing and outcrossing species. They predict that correlations among anther and stigma positions should be stronger in selfers than in outcrossers, whereas higher correlations between filaments and corolla tube are expected in outcrossing than in selfing species. Most populations of *D. inoxia* showed a stronger correlation between corolla tube and filaments than between style and stamen, in line with Ushimaru and Nakata's hypothesis. Thus, this suggests that in *D. inoxia* this set of traits is related to outcrossing, both to attract pollinators and to ensure pollen reception/deposition functions (Nilsson, 1988; Ushimaru and Nakata, 2002).

### *Genetic drift hypothesis in trait covariances*

To detect the potential effect of genetic drift in the patterns of floral covariances in *D. inoxia* we performed several different analyses. Our results (i.e. modified Mantel tests, jackknife MANOVA) showed that matrices differ among populations. Both tests showed that the

matrices differed in structure instead of size (Roff *et al.*, 2012). The Mantel test was also non-significant when we compared the matrix of genetic distances with the matrix of distances in mean percentage difference ($T\%$). Furthermore, the finer analyses of floral differentiation among populations of *D. inoxia* (Flury's method) showed that genetic drift and natural selection are both in part plausible explanations of the observed patterns. Flury's method found that one pair of populations (Querétaro–Saltillo) has proportional variance–covariance matrices resulting in differences in the size of floral traits, as expected by genetic drift. Other pairs of populations had equal (Saltillo–Mapimí) or unrelated matrices (Cañada de Moreno and Cuautla vs. all others populations), which could be due to natural selection, genetic drift, or environmental variation (Armbruster and Schwaegerle, 1996; Pérez *et al.*, 2007). The remaining populations (Querétaro, Mapimí, Neutla, and Saltillo) shared the full CPC model showing a consistent pattern of covariation mainly determined by style, stamen, and corolla lengths (cf. correlation analyses above). All tests seem to indicate that selection instead of genetic drift is modelling population differentiation of floral phenotype in at least four of the six populations of *D. inoxia*.

### *Selection mediated by pollinators or by reproductive assurance*

The pattern of floral trait correlation between corolla and filaments first described by Darwin (1862) and Nilsson (1988) is supported by studies in *Mirabilis longiflora* (Grant, 1983), *Raphanus raphanistrum* (Conner and Via, 1993), and *Ophiorrhiza napoensis* (Kudho *et al.*, 2001). The hypothesis suggests that funnel-shaped corollas pollinated by hawkmoths need to maintain the relationship between style, stamen, and corolla tube to ensure pollination efficiency. Whenever the stigma and anthers are positioned near the mouth of the corolla tube, to obtain nectar insects will make contact with both structures, promoting a more efficient deposition and removal of pollen (Nilsson, 1988; Scobell and Scott, 2002).

Although we did not conduct correlative selection experiments, the pattern of correlation found in four of six populations may represent a functional integration unit as expected by Berg's hypothesis and for functional intra-floral integration (Fenster, 1991; Ordano *et al.*, 2008). The fact that INT values and any other character were not related to local self-fertilization indexes does not support the hypothesis of selection for selfing, and is in agreement with the hypothesis for the evolution of funnel-shaped flowers due to selection mediated by pollinators (Darwin, 1862; Nilsson, 1988).

Single floral traits and the floral covariation pattern of *D. inoxia* populations seem to be affected by both natural selection and genetic drift. The outcome of the evolution of single floral traits, particularly attraction traits (i.e. anther and nectary tube) may have an adaptive value in association with pollinators. On the other hand, similar patterns of floral trait covariation composed of filaments and corolla length found in four of six populations seem to play both functions: deposition and removal of pollen, and the attraction of pollinators. Selection for selfing does not appear, as yet, to be a significant evolutionary process in the evolution of floral phenotype of *D. inoxia* populations in Mexico.

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## APPENDIX

| Allozyme | E.C. number | Locus abbreviation | Buffer* |
|---|---|---|---|
| Phosphogluconate dehydrogenase | E.C. 1.1.1.44 | *6-PGD* | Poulik |
| Aspartate transaminase | E.C. 2.6.1.1 | *GOT-3, GOT-1* | M-C |
| Glucose-6-phosphate isomerase | E.C. 5.3.1.9 | *PGI* | M-C |
| Shikimate dehydrogenase | E.C. 1.1.1.25 | *SDH* | Poulik |
| Peroxidase | E.C. 1.11.1.7 | *APX-1, APX2, APX3* | Poulik |
| Malate dehydrogenase | E.C.1.1.1.37 | *MDH* | M-C |
| Ribulose bisphosphate carboxylase | E.C. 4.1.1.39 | *RUB* | M-C |
| Isocitrate dehydrogenase | E.C. 1.1.1.42 | *IDH* | M-C |
| Glutamate dehydrogenase | E.C.1.4.1.2 | *GDH* | Poulik |

*M-C: pH 6.7, gel 12%, 200 volts, 40 mA, 8 h; Poulik: pH 8.5, 8.6, gel 13%, 300 volts, 50 mA, 6 h.
Data of the IUBMB Enzyme Nomenclature List.