Floral traits and autonomous seed set across populations of a mixed-mating species

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Abstract

1. **Background & Objectives:** Floral traits impact plant mating systems. When traits are correlated, they may facilitate or constrain the evolution of floral morphology and mating systems. In mixed-mating systems, evolution is difficult to project due to self-fertilization, which leads to conflicting outcomes of reproductive assurance and inbreeding depression. Because self-fertilizing reproductive strategies are typically accompanied by a characteristic suite of floral traits, studying the floral morphology of mixed-mating species can be a useful way of predicting whether a population is maintaining outcrossing habits, or whether it may be evolving selfing habits. The two questions I address in this study are: 1) Are floral traits and their correlations conserved across populations of a mixed mating species? and 2) Is herkogamy correlated with autonomous seed set?
2. **Methods:** Seeds of the mixed-mating plant, *Mimulus ringens*, were collected from five natural populations around Virginia. Plants came from populations of different sizes and habitats. Seeds were planted and phenotypes were measured in the greenhouse to minimize environmental effects. Floral characteristics and autonomous seed set were measured and compared across populations.
3. **Results:** Floral traits were significantly correlated within populations, and the strength of correlations differed among populations. Overall flower size and shape varied among populations, but most traits maintained patterns of association. Neither flower size nor herkogamy explained variation in autonomous seed set among individuals.
4. **Synthesis:** Despite divergence in floral traits among populations, most traits retained their associations, suggesting that evolution of individual traits may be constrained by genetic relationships. The lack of relationship between herkogamy and autonomous seed

set suggests a high cost to self-fertilization. The evolution of *M. ringens* mating systems should therefore depend more on the relationship between floral traits and facilitated self-fertilization rather than that between floral traits and autonomous selfing. While pollinators are active and mating opportunities are available, *M. ringens* should maintain a mixed-mating system, as would be predicted with a need for reproductive assurance but a cost to self-fertilization.

Introduction

Mating systems describe the means by which sexual organisms exchange genetic material. They are thus central to evolutionary processes, because they determine the distribution of genetic variation throughout a population. Random cross-fertilization among individuals within a population distributes alleles throughout the population. In contrast, cross-fertilization only among close relatives, or inbreeding, can cause alleles to segregate within family lines. Self-fertilization by hermaphrodites is the most extreme form of inbreeding. It can allow persistence of populations through reproductive assurance under pollen-limited conditions by allowing plants to produce offspring when they otherwise would have produced none (Ruan & da Silva 2012). Alternatively, self-fertilization (as well as other forms of inbreeding) is hypothesized to drive the extinction of populations under changing environmental conditions, due to the inability of genetically depauperate offspring to respond to changing conditions (Igić, Lande & Kohn 2008). The circumstances driving the evolution of mating systems, and the trajectory and consequences of mating system evolution are of immediate concern to plant biologists, because habitat fragmentation and pollinator diseases are causing loss of pollination opportunities for plants (Eckert *et al.* 2010).

While some hermaphroditic species are self-incompatible and can only mate with unrelated partners ('outcrossing'), others mate almost entirely with themselves ('selfing'). Those individuals that regularly produce a combination of self- and cross-fertilized offspring are referred to as 'mixed-mating', and they are more common than genetic theory predicts (Goodwillie, Kalisz & Eckert 2005; Jarne & Auld 2006). Across angiosperm species, primarily selfing species are often characterized by a suite of distinct morphological traits which may include small floral display size, small, scentless, nectarless, non-pigmented flowers, decreased

pollen/ovule ratio, and little physical or temporal separation between anthers and stigma (herkogamy and dichogamy, respectively) (Goodwillie *et al.* 2010; Sicard & Lenhard 2011; Kalisz *et al.* 2012; Duncan & Rausher 2013; de Vos, Wüest & Conti 2014). When these traits appear collectively in a self-fertilizing species, they are referred to as a 'selfing syndrome'.

Genetic correlations can drive or constrain the evolution of floral traits, thus indirectly influencing mating systems. This can occur when two traits are pleiotropic and share a developmental pathway; a single mutation affects both traits at once, and direct selection on one trait indirectly influences expression of the other. Even under different selective regimes, pleiotropy can maintain associations among suites of characters (Arnold & Phillips 1999; Phillips & Arnold 1999; Jones, Arnold & Bürger 2003). Trait correlations can also occur when genes are closely linked and thus regularly inherited together. For example, the cave-dwelling fish, *Astyanax mexicanus*, has surface-dwelling and cave-dwelling forms. The loci that contribute to common cave-dwelling adaptations, such as reduced eyes and vibration-attraction behaviors, are all clustered within the genome of *A. mexicanus* (Yoshizawa, O'Quin & Jeffery 2013). In plants that exhibit distinguishable floral morphs, such as *Primula vulgaris* and *Ophiorrhiza napoensis*, loci that contribute to different morphs are also closely linked with self-incompatibility loci that inhibit mating between similar morphs (Kudoh *et al.* 2001; Li *et al.* 2011). Mutations that alter physical expression of a trait may also cause correlated functional changes. For example, a gene that affects floral morphology may also affect the rate of pollen export or the rate of self-fertilization (Jordan & Otto 2012). Genetic correlations among physical or functional traits can permit mixed-mating systems and inhibit the evolution of primary selfing strategies (Johnston *et al.* 2009; Jordan & Otto 2012). A comparison of phenotypic correlations

among populations can reveal targets of phenotypic selection (Lande & Arnold 1983) and can be used as a proxy for genetic correlations (Cheverud 1988).

The purpose of this study is two-fold. The first objective is to determine whether floral traits and their correlations are conserved across populations of a mixed-mating species. If traits and correlations are similar across populations, the evolution of floral morphological traits may be genetically constrained as well as limited by the genetic variation therein (Charlesworth 2009; Piskol & Stephan 2011; Chevin 2013). Evidence for limited independent evolution of floral traits may indicate limited opportunity for mating systems to diverge among populations and could indicate a species for which mixed-mating may be common. Conversely, if traits and correlations regularly differ among populations, this may indicate a species for which selection can act on independent traits, and the degree of mixed-mating may vary under different selective environments.

The second objective is to determine whether herkogamy can predict potential autonomous seed set without pollinators. Autonomous fertilization refers to unfacilitated within-flower fertilization (i.e. no pollinators required). If herkogamy is correlated with autonomous seed set in the greenhouse, then it likely contributes to total autonomous selfing ability in the field, and it may be a useful character in projecting seed production under pollinator-limited conditions in the field. A strong correlation would suggest there is little cost to self-fertilization and would thus indicate a population or species likely to evolve self-fertilization as a primary sexual reproductive strategy (Fisher 1941; Lloyd 1992). However, if herkogamy is not correlated with autonomous seed set, then selection has not promoted their relationship, and the potential cost of the trait must be considered to be high. The variation in and evolution of the mating system must then be considered in the context of other traits.

Methods

Study species and sites

Mimulus ringens is a clonal, self-compatible species. Flowers open in the morning and typically fall off the plant by early afternoon. A single plant can produce more than 100 flowers, most of which result in fruits with 700-5500 seeds (Karron, Mitchell & Bell 2006). Bumblebees are the most common visiting pollinators, but honeybees, hummingbirds, butterflies, small halictids and hawkmoths have also been seen (Karron *et al.* 1995, 2003; J.D.Karron, D.E.Carr *pers. comm., field obs.*). Selfing rates among populations are known to range from 0.10 to 0.55 but are expected to be higher (Karron *et al.* 1995; J.D.Karron, *pers. comm.*). Outcrossing rates among individuals can range from 0.08 to 1.0, for which complete outcrossing is facilitated by acquisition of a recessive pollen sterility mutation at a single locus (Karron *et al.* 1997, 2003). Outcrossing rates among fruits within individuals (with viable pollen) can range from 0.22 to 0.79 (Karron *et al.* 2009). Herkogamy is heritable and positively correlated with outcrossing rates in the field (Karron *et al.* 1997).

In 2013, 10 - 24 seed families were collected from each of five Virginian populations of *M. ringens* (Fig. 1). BLEF is an isolated population of approximately 300 plants in an open field, SKYM is located approximately 12 km from BLEF and includes a population of approximately 100 plants in a field with patchy canopy cover, P-227 is a population of approximately 20 plants along a shallow, rocky shelf in the Moorman's River and shaded by trees along the bank, F-3322 and F-MR1 are distinct subpopulations approximately 0.45 km from each other in a metapopulation including thousands of plants in a wetland field with patches of young sycamores and cedars.

Data collection

Plants from the five populations were grown and measured in the greenhouse to minimize developmental differences due to environmental effects. Two plants from each collected seed family were planted in a greenhouse with supplemental sodium lights for 16 hour days. Flower traits were measured through destructive sampling. Digital calipers were used to obtain petal length, width, and height, and corolla length and width. Each flower was then dissected, and the anthers and stigma pressed between two glass slides. A photo was taken, and the lengths of the male and female parts were analyzed using ImageJ (Fig. 2; Rasband 2014). At the time of morphological sampling, well-developed buds on the same plant were also tagged. This was to allow total autonomous seed set to be estimated in flowers of similar ages to those for which herkogamy was measured. This protocol minimized variance in either measurement that would be caused by age of the plant (Ries 2004) or a drastic change in greenhouse conditions (*pers. obs*.). Between one and three flowers were measured for each individual, and at least one week was allowed to elapse between repeated measurements. Total seed mass of a fruit was used as a proxy for total seed set in a fruit. Fruits were collected from a plant when the seeds were brown and before the fruit dehisced. The fruit was then opened to expose the seeds, and then set inside of a coin envelope for a minimum of two days to allow the seeds to dry out. The seeds were weighed on an analytical balance to the nearest 0.1 mg.

Data analyses

To determine whether floral trait correlations differed among populations, I created a correlation matrix for each population, using a Pearson correlation and determined significance of correlations with a Bonferroni adjustment. I used a principle components analysis to assess the

multivariate relationships among the traits that were measured. I first examined the data with a traditional principal components analysis using one flower per plant (because further analyses showed that flower measurements were consistent within plants). Then, for each population and the populations inclusively, I extracted two principal components using the function 'principal' in the 'psych' package for R (Revelle 2014). The function computes the data in a manner similar to factor analysis, such that all variation in the data are explained by the specified number of components (i.e. two). I then used the two PCs derived by this constrained analysis in an ANOVA to determine whether populations differed in each principal component.

I used two different methods to determine whether trait relationships among populations differed significantly from each other. Method 1: First, I performed pairwise comparisons of correlation matrices using a method outlined by Steiger (1980) and implemented with 'cortest' in the 'psych' package for R (Revelle 2014). The method computes the z-score equivalents of the correlation data to test for the equality of matrices, and it is meant to be accommodating to small matrices and small sample sizes (Steiger 1980). Due to the differentiating effects of selection and drift, matrices are expected to differ from each other unless the relationships between the involved traits are highly conserved within the species. Conserved relationships among traits could happen if they share a developmental pathway or are closely linked (Jones *et al.* 2003).

Method 2: Correlation matrices are subject to extreme differences in variance when small sample sizes are used, even when correlation coefficients are standardized (Cheverud 1988; Steppan 1997). Therefore, I also conducted comparisons of covariance matrices using Common Principal Components (CPC; Phillips 1998). It is a method based on Flury's Hierarchy of Tests (1988) and adapted specifically to account for the non-independence of data when comparing G-matrices (Phillips & Arnold 1999). This method is robust to sampling error and has the added

benefit of describing a range of possible relationships among the matrices (Steppan 1997; Phillips & Arnold 1999). CPC analysis uses randomization tests to compare the structure of two or more covariance matrices using their eigenvalues and eigenvectors. It proceeds through a series of tests in a hierarchical fashion by first testing matrix similarities assuming no relationship between the matrices, then by testing the similarities along principle components, the proportionality of the matrices, and finally the equality of the matrices (Phillips & Arnold 1999). There are two different ways to interpret the results of the CPC analysis. The first is through a step-up and model-building approach, where each comparison in the hierarchy is made to the one below it. It provides a decomposition of the log-likelihood ratio test, but because each step is based on the one previous, the tests are non-independent, and *p*-values provided can thus be difficult to interpret. Due to the nature of the test, Flury (1988) advocates using the AIC value to determine the best-fitting model for the test. The second approach to CPC interpretation is termed the "jump-up" approach and is advocated by Phillips and Arnold (1999). This is still a hierarchical series of comparisons, but it tests each new hypothetical structure against the hypothesis of unrelated structure (as opposed to similarity of structure to a matrix in the previous step). A relatively unbiased level of matrix association can be ascertained by choosing the highest level in the hierarchy for which the null hypothesis of common structure can be accepted at p=0.05 (Phillips & Arnold 1999).

I used a MANOVA to determine whether populations exhibited variation in floral traits. All data were normal but for characteristics related to length, including corolla length, flower length, and style length, which were strongly left-skewed and could not be transformed to normality. I then used an analysis of variance (ANOVA) to determine whether the composite variable herkogamy or the total mass of seeds differed among populations. I used a cosine

transformation to coerce data for total seed mass into normality. To determine whether herkogamy or total seed mass were consistent across flowers within a plant through time, I used an ANOVA with ‘time’ as the independent variable. I used all available data in these analyses.

I used a linear regression to determine whether herkogamy predicts autonomous seed set in the greenhouse, using all data for which seed mass and herkogamy could be paired with flowers of the same age on the same plant. To determine the inclusive variables that would be most likely to predict autonomous seed set, I performed a multiple linear regression, including floral traits and native population as factors, and used stepwise elimination of predictors based on the AIC value of the resulting model.

Results

Analyses included flowers measured and tagged between June 25, 2014 and August 10, 2014. This resulted in 127 plants sampled at least once, and 56 plants sampled multiple times across five populations. For the five populations, the strongest, significant correlation was that between long anthers and short anthers ($0.87 < r < 0.93$; $p<0.01$). Beyond this, however, the strength of correlations and their significance varied among populations (Table 1). Due to the high and consistent correlation between lengths of long and short anthers, the length of short anthers was removed as a variable from all further analyses.

The strength and association of correlations among floral traits differed among populations. The Steiger (1980) method of matrix comparison showed that most populations were not equal in matrix structure (i.e. flower structure was not consistent across populations). This was shown by the low p-values for most pairwise comparisons, indicating significant differences between the matrices (Table 2). The results suggest that P-227 is an outlier population, because its correlation matrix is *equal to* matrices of three other populations. The CPC analysis identified the underlying structure of the matrices and thus the important components of flower structure (Table 3). Read from the bottom up, the highest test in the hierarchy for which the null hypothesis of similarity was not rejected, indicated the degree of common structure. In the "jump-up" approach, each higher test was an independent test that assumed that criteria for the one below were fulfilled. When acceptable p-values were adjusted for the number of comparisons made, the data showed that most population pairs shared at least a few common principal components. The alternative "step up" approach, which was a model-fitting approach, is displayed for comparison purposes and showed that the two types of tests were generally in agreement: most populations shared multiple principal components.

A traditional PCA using data from across populations revealed that the first four PCs explained 72% of the variation among variables (Table 4). The loadings of each PC were relatively small and exhibited little variance, making it difficult to separate the importance of individual traits along each axis. When the analysis was constrained to only two PCs, most flower size traits were incorporated into the first PC, while peduncle or style length weighted most heavily into the second PC (Table 5). Post-hoc Tukey tests revealed that BLEF and F-3322 differed in their flower shape, while BLEF and SKYM appeared to differ in both shape and ovary length (Table 5, Table 6).

A multivariate analysis of variance including all floral traits demonstrated that overall flower size varied among populations (Table 7). A one way analysis of variance indicated that herkogamy also differed among the populations ($F(4,177)=8.015$, $p<0.0001$; Table 8), and a post-hoc Tukey test indicated that negative values for population 227 drove this difference (i.e. length of long anthers exceeded that of the composite lengths of ovary and style; Fig. 3). When the absolute value of herkogamy was used in the analysis, there was no difference among populations ($F(4,177)=1.572$, $p<0.184$), indicating no difference in the magnitude of the distance between anthers and stigma (i.e. it did not matter whether anthers were located above or below the stigma). Autonomous seed set per fruit also differed among the populations ($F(4,57)=5.34$, $p=<0.001$), and seed set appeared much more variable among populations than was herkogamy (Fig. 4). For plants for which multiple flowers were measured, both herkogamy and seed mass per fruit were consistent across time (herkogamy: $F(2,180)=1.839$, $p=0.162$; seed mass: $F(2,61)=1.34$, $p=0.269$).

Analysis of the relationship between herkogamy and total seed mass indicated that there was no correlation between relative position of anthers and stigma and total seed mass

(β = 0.001, $F(61)=0.494$, n.s.; Fig. 5A), nor did the magnitude of distance between anthers and stigma improve the relationship (β = 0.001, $F(61)=2.07$, n.s.; Fig. 5B). A model which included floral characteristics, plant base width, and population, showed that the model that best explained total seed mass included population as a variable but did not include any measured floral traits (Table 9).

Discussion

Trait correlations and mating systems

This study shows that floral traits were correlated within populations. Furthermore, the analyses suggest that while univariate traits may diverge (Table 7), the multivariate relationships determining flower size and shape are complex and traits may retain their associations with each other (Table 3). Plants in this study came from populations of different sizes and habitats, and the trends seen here may be indicative of important evolutionary processes.

The natural processes of establishment and drift, as well as environmental differences among habitats may all cause differentiation of populations (Mitchell-Olds, Willis & Goldstein 2007). A population's response to selection pressures will be limited by the variation contained within reproductive individuals, and small populations tend to have less genetic and phenotypic variation than large populations (Charlesworth 2009). As are most *M. ringens* populations, three of the populations used in this study were small (< 100 genotypes), and drift could have caused or limited their phenotypic differentiation. Inbreeding can also reinforce genetic and phenotypic differentiation, particularly in small or structured populations. For example, in *Erysimum mediohispanicum*, serially inbred plants have larger flowers than plants produced through frequent outcrossing, and researchers hypothesize that it is due to the exposure and elimination of deleterious alleles ('purging') in the inbred plants (Abdelaziz *et al.* 2014). The BLEF population used in this study is thought to be highly inbred and largely purged of deleterious alleles (Ries 2004; Rohde & Carr 2004). In this study, the BLEF population possessed the largest flowers, and a project is underway to assess individual heterozygosity and breeding histories of the BLEF (purged) and F populations.

Comparisons of G-matrices and P-matrices can be difficult, due to the non-independence and high variability of the data (Phillips & Arnold 1999). When multiple approaches with different strengths are used to analyze data sets, one can confidently draw conclusions about general patterns from the data. In this study, I used three approaches to compare matrices. The Steiger method and two CPC analyses could both accommodate small data sets, and they utilized different types of data manipulation (e.g. z-transformations and random permutations of matrix elements). While the Steiger method determined whether there was support for overarching similarity of flower structures, the CPC analyses determined the degree to which flower structures were similar. Furthermore, the CPC analysis returned two types of results. Although the structural nature of the "step up" analysis can be difficult to interpret (Phillips & Arnold 1999), the value of using it in conjunction with the "jump up" approach in this study was that it showed that parametric and maximum-likelihood methods of analysis were usually in agreement. Used in conjunction with each other, the Steiger method and CPC analyses provided both a comprehensive and decomposed analysis of structural similarites of floral traits in small and large populations.

Despite divergence in individual size characters of populations in this study, traits largely remained associated (Table 3). Because phenotypic correlations are frequently determined by the developmental relationship among traits, the genetic relationships among traits will be reflected in the physical manifestation of those traits, regardless of their heritability or the environmental influence on their expression (Cheverud 1988). An elegant example lies in the comparison of two sexually dimorphic populations of garter snake in northern California that diverged nearly two million years ago. Despite phenotypic and ecological divergence, analyses of trait matrix structures revealed that the principal components of the populations and the sexes were still

nearly equivalent (Arnold & Phillips 1999). Thus, if the underlying genetic relationship is strong enough, trait associations will be maintained despite different selection pressures. The populations used in my study were all derived from environments with different levels of water, light, and competition. In *M. ringens*, each element can impose stress on different suites of traits (Hutchings 1932; Ries 2004; Griffin 2010). In this study, individual trait values did vary among populations, yet analyses of underlying matrix structure suggested that traits did not evolve independently within the populations. Thus, despite differences among environments, the evolution of each character in a population seems constrained by its relationship with others. If mating systems rely on floral structure, then the evolution of mating systems should also be constrained by the multivariate relationship among floral traits.

Herkogamy and mating systems

For *Mimulus ringens*, there is a negative relationship between herkogamy and self-fertilization in the field (Karron *et al.* 1997), but my study showed no relationship between these characters in the greenhouse. Furthermore, neither the value nor magnitude of herkogamy (anthers above or below stigma and distance between anthers and stigma, respectively) showed a relationship with autonomous self-fertilization. The relationship between herkogamy and self-fertilization is a contested issue in the mating system literature, with few consistent trends. Greenhouse studies show that the correlation between herkogamy and autonomous seed set can be positive (Roels & Kelly 2011) or weak to non-existent (e.g. Carr & Fenster 1994; Kalisz *et al.* 2012). Interestingly, the weak to non-existent relationships are characteristic of plants derived from open-pollinated seed in the field, while the positive relationship in Roels and Kelly's (2011) study is derived from plants having undergone five generations of autonomous selfing or

bee pollinations in the greenhouse. In the field, herkogamy can associate with autonomous seed set (Brys & Jacquemyn 2011), but it more frequently correlates with outcrossing rates in open-pollinated settings (e.g. Karron *et al.* 1997; Chen *et al.* 2009). Still, some studies show little evidence for herkogamy's influence on rates of outcrossing among populations or years (Medrano *et al.* 2012).

My data provide multiple lines of evidence that herkogamy in *M. ringens* mediates pollinator interactions in the field but is not a mechanism to provide reproductive assurance through autonomous selfing. First, ecological patterns suggest that self-fertilization likely persists as a form of reproductive assurance for *M. ringens*, because the species is an early colonizing species characterized by many small, isolated populations (*pers. obs.*). In the greenhouse, autonomous seed production per fruit in unmanipulated *M. ringens* was consistent and reasonably high; however, in the field, there was observable variation in the number of seeds per fruit within individuals (*pers. obs.*). Because flowers only last one day, within-individual variation in seed set in the field suggests that seed set is influenced by fluctuation in an environmental component. Historical variation in the pollination environments may have led to the evolution of a high number of ovules per *M. ringens* ovary (Burd *et al.* 2009). Contemporary variation in the pollination environment may now be responsible for variation in seed set.

Self-fertilization is costly in terms of inbreeding depression, resource allocation for traits used for pollinator attraction, and if selfing does not provide reproductive assurance it also results in lost opportunities for cross-fertilization (pollen and seed discounting) (Fisher 1941; Harder, Richards & Routley 2008; Sicard & Lenhard 2011; Ruan & da Silva 2012). If the benefit of self-fertilization outweighs these collective costs, we would expect an increase in the amount of selfing, and we expect that increase to be correlated with some floral morphological trait

associated with autonomous selfing. In this study, autonomous seed and many floral traits differed among populations; however, a mixed linear model showed that 'population' was the best explanatory factor of seed set. This suggests that there is some population-level trait influencing autonomous seed set, but it is due to a trait that was not measured in this study.

That herkogamy is meant to prevent self-fertilization in *M. ringens*, rather than promote it, is supported by a study showing that in the field, facilitated self-fertilization incurs a greater cost than is gained through autonomous self-fertilization (Karron & Mitchell 2012). Evidence against herkogamy's primary role in autonomous fertilization is further supported by studies in other species showing that herkogamy contributes more to mate diversity than selfing rate (Medrano *et al.* 2012) and herkogamy delays the probability of self-fertilization until opportunities for cross-pollination have occurred (Chen *et al.* 2009). Because herkogamy is correlated with selfing rates in the field but not with autonomous seed set, it is also probable that autonomous selfing is not the primary determinant of rates of self-fertilization in the field.

When investigating the evolution of mating systems and mating system traits, it is important to remember that self-fertilization in greenhouse and field environments occurs due to different processes and that studies in these environments, while they can be complimentary, are not directly comparable. Self-fertilization in the greenhouse typically occurs via autonomous, non-facilitated self-pollination, and whether it occurs early or late in the life of the flower is usually considered to be of little importance to final seed set. In contrast, self-fertilization in field environments can occur autonomously or due to facilitated self-pollination within or between flowers by some other mechanism (e.g. pollinators). Under these circumstances, the timing of self-fertilization can significantly affect the number and type of seeds (inbred or outbred) produced in a fruit (Lloyd 1979; Morgan & Wilson 2005). When self-fertilization incurs few

costs (such as in successive generations without pollinators), mechanisms that ensure cross-fertilization begin to be lost, and often flowers develop the morphology characteristic of a 'selfing syndrome' (e.g. Sicard & Lenhard 2011; Kalisz *et al.* 2012; Duncan & Rausher 2013). When self-fertilization incurs high costs, to the extent that they can, plants develop or maintain mechanisms to maintain cross-fertilization (Pannell 2010). The environment in which we measure both herkogamy and seed set can thus influence our understanding of herkogamy's true influence on mating systems.

Future directions

Neither herkogamy nor flower size predicted autonomous self-fertilization in this study. These data and field observations suggest that future studies should consider that some traits may influence mating systems through methods other than autonomous fertilization, or may only influence mating systems in the context of pollinators (Kalisz *et al.* 2012; Medrano *et al.* 2012). Furthermore, reproductive traits beyond those studied here should be considered in future mating system studies. For example, in this study, there was no opportunity to examine variation in stigma sensitivity, nectar production, or the timing of autonomous fertilization. However, these are all traits shown to influence the quantity and quality of offspring produced, and they warrant further study in *M. ringens*. Stigma sensitivity, a heritable trait in *M. ringens* (J.D.Karron, *pers. comm.*.), is known to increase the rate of pollen export in *M. aurantiacus* (Fetscher 2001). Stigma sensitivity seems to incur few costs in terms of the number of ovules that cannot be fertilized once the stigma closes (Fetscher & Kohn 1999), suggesting that variation in stigma sensitivity may explain variation in mating systems among individuals and environments. Nectar abundance and nectar secondary compounds influence patterns of pollinator visitation and rates

of pollen deposition (Hodges 1985; Irwin & Adler 2008). The petals and occasionally corollas of *M. ringens* flowers in the greenhouse typically ooze with nectar, and this may indicate a critical mechanism by which to encourage pollinator visitation and cross-fertilization. In *Collinsia* species, dichogamy (the timing of autonomous fertilization), is correlated with the amount of autonomous fertilization (Kalisz *et al.* 2012). *Mimulus ringens* exhibits prior and competing fertilization (prior to and simultaneous with opportunities for cross-fertilization, respectively), the levels of which have important implications for evolution of mating systems (Lloyd 1979; Morgan & Wilson 2005). Finally, this study was conducted in the greenhouse, but field studies should also be used to assess the potential costs and benefits of floral traits to both male and female reproduction when plants are accessible to pollinators (Duncan & Rausher 2013).

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Figures and Tables

Fig. 1. Map of locations of study populations. Purple stars represent locations with a single study population (SKYM, BLEF, P-227), green star represents the location of the metapopulation in which two subpopulations were phenotyped (F-3322, F-MR1).

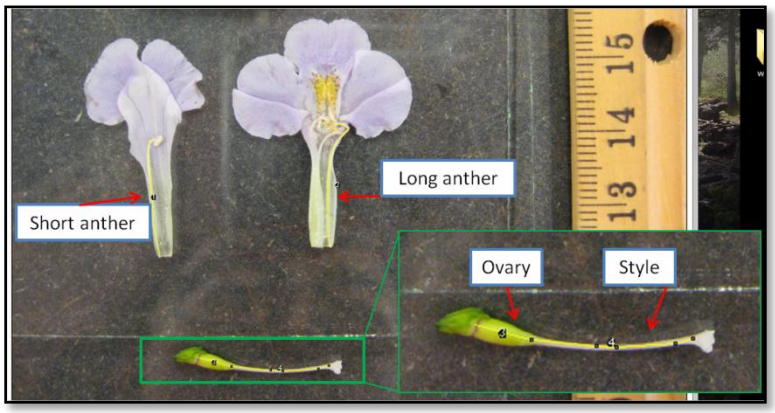

Fig. 2. Measurements of male and female reproductive parts were digitally analyzed using ImageJ software (Rasband 2014).

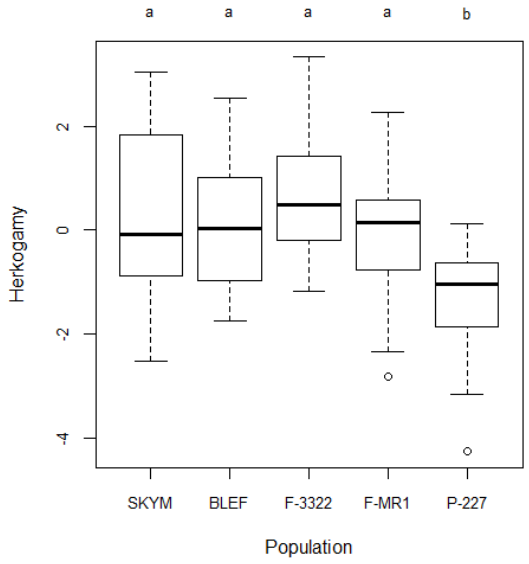

Fig. 3. Box and whisker plot of herkogamy. Values above 0 indicate stigma positioning above anthers, values below 0 indicate stigma positioning below anthers. Letters indicate significant differences by Tukey HSD.

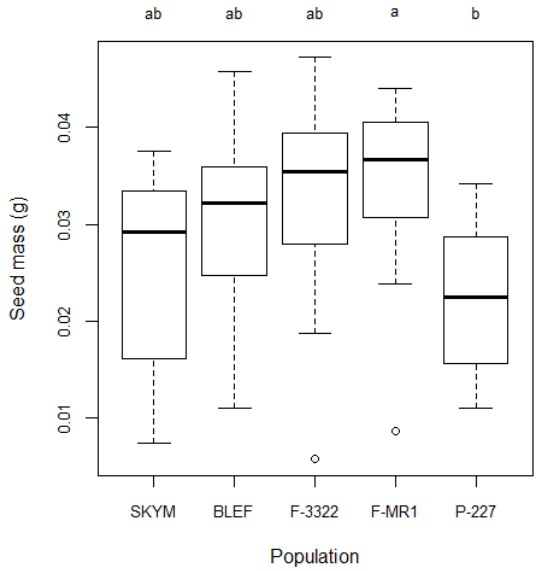

Fig. 4. Box and whisker plot of autonomous seed set per fruit. Letters indicate significant differences of transformed data by Tukey HSD.

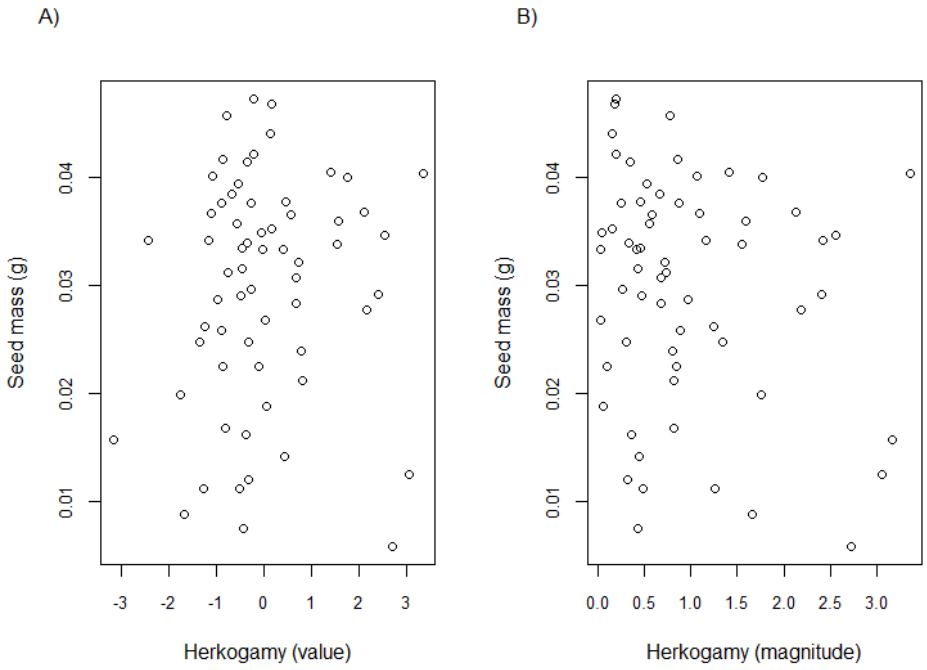

Fig. 5. Relationship between herkogamy and autonomous seed set per fruit. A) Value of herkogamy: negative values along x-axis indicate anthers positioned above stigmas, positive values along x-axis indicate anthers positioned below stigmas. B) Magnitude of herkogamy: absolute value of herkogamy to indicate distance between anthers and stigma. Data include measurements from plants throughout the five populations.

Table 1. Correlations among measured floral components. "Anther length" refers to length of the pair of long anthers. Correlations above 0.5 or below -0.5 are portrayed on a gray background. Text colors indicate populations: Bold=Inclusive, blue=SKYM, red=BLEF, purple=F-3322, orange=F-MR1, light blue=P-227.

	Flower height	Flower length	Flower width	Corolla length	Corolla width	Long anther	Ovary length	Style length
Peduncle	**0.15**	**0.24***	**0.34*****	**0.43*****	**0.3*****	**0.28****	**0.36*****	**-0.10**
	-0.02	0.17	0.2	0.53***	0.18	0.25	0.38	-0.27
	0.27	0.09	-0.12	0.48	0.24	-0.12	0.26	-0.25
	0.19	0.29	0.57***	0.57***	0.44	0.35	0.28	0.16
	0.15	0.38	0.47	0.47	0.13	0.53**	0.52*	0.11
	0.2	-0.03	0.37	-0.11	0.33	0.36	0.05	0.11
Flower height		**0.33*****	**0.24***	**0.33*****	**0.40*****	**0.34*****	**0.35*****	**0.22**
		0.27	-0.17	0.31	0.45*	0.34	0.45*	0.18
		-0.1	0.47	0.17	0	0.21	0.11	0.15
		0.6***	0.5*	0.57***	0.56***	0.55***	0.58***	0.42
		0.32	0.32	0.45	0.29	0.18	0.01	0.32
		-0.05	0.08	0.1	0.62	-0.02	-0.06	0.2
Flower length			**0.35*****	**0.39*****	**0.34*****	**0.63*****	**0.46*****	**0.26****
			0.45*	0.41	0.31	0.81***	0.63***	0.27
			-0.3	0.64***	0.13	0.53	0.47	0.23
			0.61***	0.62***	0.64***	0.56***	0.38	0.38
			0.29	0.32	0.25	0.44	0.15	0.25
			-0.11	0.03	0.09	0.66	0.42	0.81***
Flower width				**0.36*****	**0.28****	**0.36*****	**0.25***	**0.1**
				0.31	0.29	0.46*	0.37	0.04
				-0.21	-0.06	0.27	-0.19	0.25
				0.5*	0.5*	0.5*	0.26	0.27
				0.57***	-0.08	0.28	0.19	0.32
				0.01	0.28	0.07	-0.12	0.11
Corolla length					**0.41*****	**0.29*****	**0.37*****	**0.17**
					0.56***	0.57***	0.64***	0.1
					0.19	0.36	0.66***	0.29
					0.63***	0.50*	0.46	0.26
					0.04	0.32	0.24	0.44
					0.39	0.01	0.02	0.09
Corolla width						**0.22**	**0.34*****	**0.08**
						0.32	0.44	0.08
						-0.08	-0.01	-0.08
						0.45	0.51*	0.33
						0	0.09	-0.29
						0.21	0.15	0.44
Anther length							**0.53*****	**0.3*****
							0.71***	0.13
							0.15	0.61
							0.48*	0.63
							0.44	0.54**
							0.50	0.63
Ovary length								**0.16**
								0.08
								0.2
								0.33
								0.3
								0.35

* p<0.05, ** p<0.01, *** p< 0.001

Table 2. Pairwise comparison of matrices that calculates the χ^2 distribution of z-transformed correlations (Steiger 1980). P-values indicating significant differences after Bonferroni corrections are highlighted in bold.

Matrix 1	**Matrix 2**	χ^2	***p***
SKYM	BLEF	80.04	**<0.001**
SKYM	F-3322	64.16	**0.003**
SKYM	F-MR1	76.83	**<0.001**
SKYM	P-227	56.53	0.016
BLEF	F-3322	108.22	**<0.001**
BLEF	F-MR1	58.05	**0.011**
BLEF	P-227	45.39	0.14
F-3322	F-MR1	71.61	**<0.001**
F-3322	P-227	68.75	**0.001**
F-MR1	P-227	47.51	0.095

Table 3. Analysis of covariance matrix structure based on Flury's Hierarchy of Tests (1988) and adapted to account for non-independence of data (Phillips and Arnold 1999). "CPC(_#_)" refers to the number of principal components being tested in the model, "CPC" refers to a model that includes all principal components, "Proport" refers to a proportional model for which eigenvectors are similar but eigenvalues differ, "Equality" refers to a model for which all eigenvalues and eigenvectors are similar. Tests highlighted in bold indicate matrix similarity according to the highest level in the hierarchy for which the null hypothesis of common structure can be accepted at p=0.005 (following a Bonferroni correction for multiple comparisons between populations). Step-up models that would be accepted based on AIC values are indicated with * in the AIC column.

Inclusive comparison of five population matrices

"Jump-up" approach					"Step-up" and model-building approach					
_____Model_____					_____Model_____					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	433.762	180	0	Equality	Proport	21.292	4	0.0003	433.762
Proport	Unrelated	412.47	176	0	Proport	CPC	153.622	32	0	420.47
CPC	Unrelated	258.848	144	0	CPC	CPC(7)	3.305	4	0.5082	330.848
CPC(7)	Unrelated	255.543	140	0	CPC(7)	CPC(6)	16.585	8	0.0347	335.543
CPC(6)	Unrelated	238.958	132	0	CPC(6)	CPC(5)	43.285	12	0	334.958
CPC(5)	Unrelated	195.673	120	0	CPC(5)	CPC(4)	22.777	16	0.1198	315.673*
CPC(4)	Unrelated	172.896	104	0	CPC(4)	CPC(3)	22.896	20	0.294	324.896
CPC(3)	Unrelated	150.001	84	0	CPC(3)	CPC(2)	36.558	24	0.0484	342.001
CPC(2)	Unrelated	113.443	60	0	CPC(2)	CPC(1)	67.684	28	0	353.443
CPC(1)	**Unrelated**	**45.758**	**32**	**0.0546**	**CPC(1)**	**Unrelated**	**45.758**	**32**	**0.0546**	**341.758**
					Unrelated	---				360

Pairwise comparisons of matrices

SKYM, BLEF

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	121.166	45	0	Equality	Proport	2.253	1	0.1334	121.166
Proport	Unrelated	118.914	44	0	Proport	CPC	62.611	8	0	120.914
CPC	**Unrelated**	**56.303**	**36**	**0.0168**	**CPC**	**CPC(7)**	**0.796**	**1**	**0.3721**	**74.303***
CPC(7)	Unrelated	55.506	35	0.0152	CPC(7)	CPC(6)	1.79	2	0.4086	75.506
CPC(6)	Unrelated	53.717	33	0.0128	CPC(6)	CPC(5)	2.656	3	0.4477	77.717
CPC(5)	Unrelated	51.06	30	0.0096	CPC(5)	CPC(4)	7.711	4	0.1028	81.06
CPC(4)	Unrelated	43.35	26	0.0177	CPC(4)	CPC(3)	8.4	5	0.1355	81.35
CPC(3)	Unrelated	34.95	21	0.0286	CPC(3)	CPC(2)	13.045	6	0.0423	82.95
CPC(2)	Unrelated	21.905	15	0.1103	CPC(2)	CPC(1)	11.879	7	0.1046	81.905
CPC(1)	Unrelated	10.026	8	0.2632	CPC(1)	Unrelated	10.026	8	0.2632	84.026
					Unrelated	---				90

Table 3. Continued (2/4)

SKYM, F-3322

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	119.935	45	0	Equality	Proport	3.581	1	0.0584	119.935
Proport	Unrelated	116.354	44	0	Proport	CPC	51.952	8	0	118.354
CPC	Unrelated	64.402	36	0.0025	CPC	CPC(7)	1.206	1	0.2721	82.402*
CPC(7)	Unrelated	63.196	35	0.0024	CPC(7)	CPC(6)	0.918	2	0.6318	83.196
CPC(6)	Unrelated	62.278	33	0.0015	CPC(6)	CPC(5)	1.798	3	0.6153	86.278
CPC(5)	Unrelated	60.479	30	0.0008	CPC(5)	CPC(4)	12.02	4	0.0172	90.479
CPC(4)	Unrelated	48.459	26	0.0048	CPC(4)	CPC(3)	3.815	5	0.5764	86.459
CPC(3)	Unrelated	44.644	21	0.0019	CPC(3)	CPC(2)	7.75	6	0.257	92.644
CPC(2)	Unrelated	36.894	15	0.0013	CPC(2)	CPC(1)	22.142	7	0.0024	96.894
CPC(1)	**Unrelated**	**14.752**	**8**	**0.0641**	**CPC(1)**	**Unrelated**	**14.752**	**8**	**0.0641**	**88.752**
					Unrelated	---				90

SKYM, F-MR1

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	137.93	45	0	Equality	Proport	2.654	1	0.1033	137.93
Proport	Unrelated	135.276	44	0	Proport	CPC	52.795	8	0	137.276
CPC	Unrelated	82.481	36	0	CPC	CPC(7)	1.385	1	0.2393	100.481
CPC(7)	Unrelated	81.096	35	0	CPC(7)	CPC(6)	2.774	2	0.2499	101.096
CPC(6)	Unrelated	78.322	33	0	CPC(6)	CPC(5)	25.389	3	0	102.322
CPC(5)	**Unrelated**	**52.933**	**30**	**0.006**	CPC(5)	CPC(4)	9.512	4	0.0495	82.933
CPC(4)	Unrelated	43.422	26	0.0174	CPC(4)	CPC(3)	7.933	5	0.1599	81.422
CPC(3)	Unrelated	35.488	21	0.0249	CPC(3)	CPC(2)	19.814	6	0.003	83.488
CPC(2)	Unrelated	15.674	15	0.4041	**CPC(2)**	**CPC(1)**	**8.792**	**7**	**0.2679**	**75.674***
CPC(1)	Unrelated	6.882	8	0.5494	CPC(1)	Unrelated	6.882	8	0.5494	80.882
					Unrelated	---				90

SKYM, P-227

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	118.086	45	0	Equality	Proport	2.218	1	0.1364	118.086
Proport	Unrelated	115.868	44	0	Proport	CPC	66.274	8	0	117.868
CPC	**Unrelated**	**49.593**	**36**	**0.0652**	**CPC**	**CPC(7)**	**0.18**	**1**	**0.6718**	**67.593***
CPC(7)	Unrelated	49.414	35	0.0539	CPC(7)	CPC(6)	3.05	2	0.2176	69.414
CPC(6)	Unrelated	46.364	33	0.0613	CPC(6)	CPC(5)	2.645	3	0.4497	70.364
CPC(5)	Unrelated	43.72	30	0.0506	CPC(5)	CPC(4)	7.73	4	0.102	73.72
CPC(4)	Unrelated	35.989	26	0.0919	CPC(4)	CPC(3)	3.212	5	0.6673	73.989
CPC(3)	Unrelated	32.777	21	0.0488	CPC(3)	CPC(2)	17.864	6	0.0066	80.777
CPC(2)	Unrelated	14.913	15	0.4577	CPC(2)	CPC(1)	2.937	7	0.8907	74.913
CPC(1)	Unrelated	11.976	8	0.1523	CPC(1)	Unrelated	11.976	8	0.1523	85.976
					Unrelated	---				90

Table 3. Continued (3/4)

BLEF, F-3322

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	81.962	45	0.0006	**Equality**	**Proport**	**0.022**	**1**	**0.8831**	**81.962**
Proport	Unrelated	81.94	44	0.0005	Proport	CPC	21.118	8	0.0068	83.94
CPC	Unrelated	60.823	36	0.006	CPC	CPC(7)	2.77	1	0.096	78.823
CPC(7)	Unrelated	58.052	35	0.0085	CPC(7)	CPC(6)	3.847	2	0.1461	78.052*
CPC(6)	Unrelated	54.205	33	0.0114	CPC(6)	CPC(5)	5.323	3	0.1496	78.205
CPC(5)	Unrelated	48.883	30	0.0162	CPC(5)	CPC(4)	2.4	4	0.6626	78.883
CPC(4)	Unrelated	46.483	26	0.0081	CPC(4)	CPC(3)	4.577	5	0.4696	84.483
CPC(3)	Unrelated	41.905	21	0.0043	CPC(3)	CPC(2)	7.267	6	0.2969	89.905
CPC(2)	Unrelated	34.639	15	0.0028	CPC(2)	CPC(1)	15.468	7	0.0304	94.639
CPC(1)	**Unrelated**	**19.171**	**8**	**0.014**	CPC(1)	Unrelated	19.171	8	0.014	93.171
					Unrelated	---				90

BLEF, F-MR1

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	82.611	45	0.0005	**Equality**	**Proport**	**0.018**	**1**	**0.8934**	**82.611***
Proport	Unrelated	82.593	44	0.0004	Proport	CPC	16.359	8	0.0375	84.593
CPC	Unrelated	66.234	36	0.0016	CPC	CPC(7)	3.355	1	0.067	84.234
CPC(7)	Unrelated	62.878	35	0.0026	CPC(7)	CPC(6)	1.758	2	0.4152	82.878
CPC(6)	Unrelated	61.121	33	0.0021	CPC(6)	CPC(5)	9.102	3	0.028	85.121
CPC(5)	Unrelated	52.019	30	0.0076	CPC(5)	CPC(4)	0.857	4	0.9306	82.019
CPC(4)	Unrelated	51.161	26	0.0023	CPC(4)	CPC(3)	3.329	5	0.6494	89.161
CPC(3)	Unrelated	47.832	21	0.0007	CPC(3)	CPC(2)	17.796	6	0.0068	95.832
CPC(2)	**Unrelated**	**30.037**	**15**	**0.0118**	CPC(2)	CPC(1)	15.889	7	0.0261	90.037
CPC(1)	Unrelated	14.147	8	0.078	CPC(1)	Unrelated	14.147	8	0.078	88.147
					Unrelated	---				90

BLEF, P-227

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	104.097	45	0	Equality	Proport	3.572	1	0.0588	104.097
Proport	Unrelated	100.525	44	0	Proport	CPC	39.29	8	0	102.525
CPC	**Unrelated**	**61.235**	**36**	**0.0054**	**CPC**	**CPC(7)**	**3.755**	**1**	**0.0527**	**79.235**
CPC(7)	Unrelated	57.48	35	0.0097	CPC(7)	CPC(6)	1.587	2	0.4523	77.48
CPC(6)	Unrelated	55.893	33	0.0077	CPC(6)	CPC(5)	5.653	3	0.1298	79.893
CPC(5)	Unrelated	50.24	30	0.0117	CPC(5)	CPC(4)	9.33	4	0.0534	80.24
CPC(4)	Unrelated	40.911	26	0.0317	CPC(4)	CPC(3)	10.101	5	0.0724	78.911
CPC(3)	Unrelated	30.81	21	0.0769	CPC(3)	CPC(2)	15.391	6	0.0174	78.81
CPC(2)	Unrelated	15.419	15	0.4217	CPC(2)	CPC(1)	3.047	7	0.8806	75.419*
CPC(1)	Unrelated	12.372	8	0.1354	CPC(1)	Unrelated	12.372	8	0.1354	86.372
					Unrelated	---				90

Table 3. Continued (4/4)

F-3322, F-MR1

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	**Unrelated**	**69.58**	**45**	**0.0108**	**Equality**	**Proport**	**0**	**1**	**0.9888**	**69.58***
Proport	Unrelated	69.58	44	0.0083	Proport	CPC	13.072	8	0.1094	71.58
CPC	Unrelated	56.508	36	0.016	CPC	CPC(7)	0.001	1	0.9762	74.508
CPC(7)	Unrelated	56.507	35	0.0121	CPC(7)	CPC(6)	6.383	2	0.0411	76.507
CPC(6)	Unrelated	50.124	33	0.0285	CPC(6)	CPC(5)	2.316	3	0.5094	74.124
CPC(5)	Unrelated	47.808	30	0.0207	CPC(5)	CPC(4)	5.704	4	0.2224	77.808
CPC(4)	Unrelated	42.104	26	0.024	CPC(4)	CPC(3)	5.339	5	0.376	80.104
CPC(3)	Unrelated	36.765	21	0.0179	CPC(3)	CPC(2)	8.589	6	0.198	84.765
CPC(2)	Unrelated	28.176	15	0.0205	CPC(2)	CPC(1)	14.69	7	0.0402	88.176
CPC(1)	Unrelated	13.486	8	0.0962	CPC(1)	Unrelated	13.486	8	0.0962	87.486
					Unrelated	---				90

F-3322, P-227

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	91.169	45	0.0001	Equality	Proport	4.245	1	0.0394	91.169
Proport	Unrelated	86.924	44	0.0001	Proport	CPC	42.237	8	0	88.924
CPC	**Unrelated**	**44.688**	**36**	**0.1518**	**CPC**	**CPC(7)**	**0.905**	**1**	**0.3414**	**62.688**
CPC(7)	Unrelated	43.782	35	0.1466	CPC(7)	CPC(6)	5.858	2	0.0534	63.782
CPC(6)	Unrelated	37.924	33	0.2548	CPC(6)	CPC(5)	7.295	3	0.0631	61.924
CPC(5)	Unrelated	30.629	30	0.4338	CPC(5)	CPC(4)	3.352	4	0.5007	60.629*
CPC(4)	Unrelated	27.277	26	0.3949	CPC(4)	CPC(3)	8.554	5	0.1282	65.277
CPC(3)	Unrelated	18.723	21	0.6029	CPC(3)	CPC(2)	1.975	6	0.922	66.723
CPC(2)	Unrelated	16.748	15	0.3341	CPC(2)	CPC(1)	10.974	7	0.1397	76.748
CPC(1)	Unrelated	5.774	8	0.6725	CPC(1)	Unrelated	5.774	8	0.6725	79.774
					Unrelated	---				90

F-MR1, P-227

"Jump-up" approach					"Step-up" and model-building approach					
Higher	**Lower**	**χ^2**	**df**	***p***	**Higher**	**Lower**	**χ^2**	**df**	***p***	**AIC**
Equality	Unrelated	95.112	45	0	Equality	Proport	5.252	1	0.0219	95.112
Proport	Unrelated	89.86	44	0.0001	Proport	CPC	35.86	8	0	91.86
CPC	**Unrelated**	**53.999**	**36**	**0.0274**	CPC	CPC(7)	0.813	1	0.3671	71.999
CPC(7)	Unrelated	53.186	35	0.0251	CPC(7)	CPC(6)	0.981	2	0.6123	73.186
CPC(6)	Unrelated	52.205	33	0.018	CPC(6)	CPC(5)	15.99	3	0.0011	76.205
CPC(5)	Unrelated	36.215	30	0.2011	**CPC(5)**	**CPC(4)**	**3.749**	**4**	**0.441**	**66.215***
CPC(4)	Unrelated	32.466	26	0.1782	CPC(4)	CPC(3)	5.921	5	0.3139	70.466
CPC(3)	Unrelated	26.545	21	0.1864	CPC(3)	CPC(2)	9.641	6	0.1406	74.545
CPC(2)	Unrelated	16.904	15	0.3246	CPC(2)	CPC(1)	8.472	7	0.2928	76.904
CPC(1)	Unrelated	8.432	8	0.3924	CPC(1)	Unrelated	8.432	8	0.3924	82.432
					Unrelated	---				90

Table 4. Traditional PCA incorporating data from across populations.

	PC1	PC2	PC3	PC4
Peduncle	-0.288	0.547	0.232	
Flower height	-0.316	-0.148	-0.548	-0.168
Flower length	-0.397	-0.214	0.235	
Flower width	-0.313	0.170	0.180	0.680
Corolla length	-0.363	0.225	-0.197	0.262
Corolla width	-0.322	0.208	-0.547	-0.113
Anther length	-0.389	-0.276	0.415	-0.162
Ovary length	-0.380		0.175	-0.495
Style length	-0.175	-0.659	-0.131	0.383
Proportional variance	**0.391**	**0.138**	**0.102**	**0.086**
Cumulative variance	**0.391**	**0.530**	**0.632**	**0.719**

Table 5. A PCA constrained to explain all variance in two principal components, conducted on all populations ("Inclusive") and each population separately. Variables with loadings between 0.5 and -0.5 were omitted for visualization purposes.

	Inclusive		**SKYM**		**BLEF**		**F-3322**		**F-MR1**		**P-227**	
	n =119		*n* =30		*n* =21		*n* =32		*n* =26		*n* =10	
Variable	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
Peduncle		0.64		0.83		-0.52	0.6		0.71			0.88
Flower height	0.58			-0.55			0.75		0.55	0.51		0.81
Flower length	0.75		0.79		0.83		0.79		0.65		0.76	
Flower width	0.67		0.67			0.78	0.76		0.71		0.61	
Corolla length	0.6		0.74		0.84		0.77		0.71			
Corolla width	0.59		0.64				0.8			0.82		0.6
Long anther	0.74		0.86		0.76		0.73		0.75		0.93	
Ovary length	0.73		0.86		0.62	-0.51	0.71		0.62		0.64	
Style length		-0.71	0.59	-0.64	0.68	0.62		0.71		-0.62	0.83	
Proportional variance	**0.71**		**0.71**		**0.57**		**0.8**		**0.68**		**0.59**	

Table 6. Pairwise comparisons of the two principal components from the constrained PCA, using an ANOVA and Tukey's HSD: A) PC1 and B) PC2. Significant differences are highlighted in bold.

A) PC1

Pop 1	Pop 2	Estimate	Error	*t*	*p*
SKYM	BLEF	-0.7549	0.27387	-2.756	0.0504
SKYM	F-3322	0.1991	0.24462	0.814	0.9241
SKYM	F-MR1	-0.04372	0.25791	-0.17	0.9998
SKYM	P-227	-0.21404	0.35148	-0.609	0.9727
BLEF	**F-3322**	**0.954**	**0.27032**	**3.529**	**0.0052****
BLEF	F-MR1	-0.71118	0.28241	-2.518	0.0912
BLEF	P-227	0.54086	0.36983	1.462	0.5828
F-3322	F-MR1	0.24282	0.25415	0.955	0.8715
F-3322	P-227	-0.41314	0.34872	-1.185	0.7556
F-MR1	P-227	-0.17032	0.35817	-0.476	0.9891

B) PC2

Pop 1	Pop 2	Estimate	Error	*t*	*p*
SKYM	**BLEF**	**-0.8041**	**0.2757**	**-2.916**	**0.0329***
SKYM	F-3322	-0.2144	0.2463	-0.871	0.9049
SKYM	F-MR1	-0.4268	0.2597	-1.644	0.4666
SKYM	P-227	0.1796	0.3539	0.508	0.9861
BLEF	F-3322	0.5896	0.2722	2.166	0.1955
BLEF	F-MR1	-0.3772	0.2843	-1.327	0.6698
BLEF	P-227	0.9837	0.3724	2.642	0.0675
F-3322	F-MR1	0.2124	0.2559	0.83	0.9189
F-3322	P-227	0.394	0.3511	1.122	0.7904
F-MR1	P-227	0.6064	0.3606	1.682	0.443

* p<0.05, ** p<0.01, *** p< 0.001

Table 7. Multivariate analysis to test for differences of flower traits among populations.

	Pillai's Trace	Hypoth df	Error df	*F*	*p*
Multivariate effect	0.714	4	176	4.68	< 0.0001***

Univariate decomposition	Hypoth df	Error df	*F*	*p*
flower height	4	176	2.11	0.082
flower length	4	176	3.198	0.015*
flower width	4	176	11.652	< 0.0001***
corolla length	4	176	7.41	< 0.0001***
corolla width	4	176	4.92	< 0.0001***
anther length	4	176	6.62	< 0.0001***
ovary length	4	176	6.71	< 0.0001***
style length	4	176	0.324	0.861

* p<0.05, ** p<0.01, *** p< 0.001

Table 8. Means and standard deviations for flower traits.

	SKYM			BLEF			F-3322			F-MR1			P-227		
	n	**mean**	**s.d.**	***n***	**mean**	**s.d.**	***n***	**mean**	**s.d.**	***n***	**mean**	**s.d.**	***n***	**mean**	**s.d.**
Peduncle	31	34.28	10.05	21	38.58	6.75	33	31.65	7.82	27	34.36	8.65	11	32.18	6.18
Flower height	31	20.17	2.03	21	20.9	1.79	33	19.95	2.05	27	19.77	2.29	11	20.27	1.36
Flower length	31	32.18	2.63	21	33.09	2.44	33	31.76	2.29	27	31.91	2.38	10	33.66	2.63
Flower width	31	23.42	3.95	21	27.39	2.54	33	24.42	2.91	27	24.49	2.39	11	25.53	1.96
Corolla length	31	17.48	1.66	21	19.4	1.24	33	18.74	1.74	27	18.94	1.25	11	16.95	3.05
Corolla width	31	4.48	0.34	21	4.69	0.36	33	4.38	0.57	27	4.5	0.44	11	4.23	0.45
Anther length	31	21.48	1.65	21	21.54	1.67	33	20.56	1.24	27	21.16	1.42	11	22.16	1.2
Ovary length	31	6.38	0.71	21	6.73	0.7	33	6.06	0.64	27	6.3	0.5	11	6.58	0.71
Style length	31	14.9	2.85	21	14.82	1.07	33	15.25	1.16	27	15.09	1.2	11	14.38	1.19
Herkogamy	31	-0.2	2.97	21	0.01	1.4	33	0.75	1.15	27	0.23	1.13	11	-1.2	1.33

Table 9. Mixed linear model to explain variation in seed mass among populations. The best model is the one for which only 'population' remains as an explantory variable.

Initial Model:

Total seed mass ~ Population + Flower length + Flower height + Flower width + Corolla length + Corolla width + Ovary + Style + Anther + Plant diameter

Final Model:

Total seed mass ~ Population

	Step	Df	Deviance	Resid. Df	Resid. deviance	AIC
1				**49**	**0.005454195**	**-561.334**
2	- Plant diameter	1	1.80E-08	50	0.005454212	-563.334
3	- Corolla width	1	9.19E-06	51	0.0054634	-565.228
4	- Flower length	1	8.87E-06	52	0.005472271	-567.125
5	- Flower height	1	1.95E-05	53	0.005491745	-568.902
6	- Flower width	1	2.39E-05	54	0.00551569	-570.628
7	- Ovary	1	2.72E-05	55	0.005542933	-572.317
8	- Anther	1	1.69E-05	56	0.005559853	-574.125
9	- Corolla length	1	6.06E-05	57	0.005620454	-575.442
10	- Style	1	5.97E-05	58	0.005680151	-576.777