HETEROCHRONY OF FLORAL AND MATING SYSTEM CHARACTERS BETWEEN *NICOTIANA LONGIFLORA* AND *N. PLUMBAGINIFOLIA*

A Thesis
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree

Master of Arts

---

by
JACOB W. SOULE

Dr. Timothy P. Holtsford, Thesis Supervisor

MAY 2007

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

HETEROCHRONY OF FLORAL AND MATING SYSTEM CHARACTERS BETWEEN *NICOTIANA LONGIFLORA* AND *N. PLUMBAGINIFOLIA*

presented by Jacob W. Soule,

a candidate for the degree of Master of Arts,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Timothy P. Holtsford

Professor J. Chris Pires

Professor Bruce A. McClure

ACKNOWLEDGEMENTS

This thesis would not have been possible without my advisor Dr. Tim Holtsford. His insight and thoughtful comments helped me to bring this to completion. He helped me focus my project into something worthwhile. I had a lot of help from my lab mates Rainee Kaczorowski, Sherry Ellberg, Dulce Figueroa-Castro and Chris Lee; their discussions helped me to get past many difficulties for which I am grateful, as well as for their willing help with watering and care of plants. I would like to thank Paul Walker for his enthusiastic help with lab work and for the hours he spent measuring flowers for plastochron calibrations. The terrific feedback given to me by my committee members Dr. J. Chris Pires and Dr. Bruce A. McClure greatly improved the final product. Finally, I never would have made it through this without the love and support of my parents, Robert and Judith Soule, and of my fiancée Danielle A. Sattman.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

HETEROCHRONY OF FLORAL AND MATING SYSTEM CHARACTERS BETWEEN *NICOTIANA LONGIFLORA* AND *N. PLUMBAGINIFOLIA*

Jacob W. Soule

Dr. Timothy P. Holtsford, Thesis Supervisor

ABSTRACT

The evolution of selfing is one the most fascinating and common trends observed in flowering plants. Selfing flowers are often smaller than their outcrossing relatives, with reduced reproductive effort. The selfing species *Nicotiana plumbaginifolia* (plum) follows this pattern with smaller flowers than its outcrossing sister species *N. longiflora* (long). This study focuses on a glasshouse grown population descended from a field collected population with three corolla length morphs: long, medium and plum. Morphological characters likely relating to mating system were measured, as well as glasshouse selfing rate. Anther Stigma Distance, a measure of herkogamy, varies between corolla morphs, with long > 0, medium ≈ 0 and plum ASD negative. Corolla length and anther-stigma distance explained some of the selfing rate variation across the three morphs, but had limited correlation with selfing within morphs. Next, I studied the development of the floral organs in all three morphs. Relative Growth Rates (RGRs) were calculated for eight floral traits. RGR is decreased in plum for all floral organs, while RGRs were the same in long and medium for most organs. Length differences between long and medium result from a shorter period of growth. Medium and plum have different mechanisms resulting in shorter flowers. Medium flowers result from progenesis, while plum flowers are produced through neotony. There is also a decreased herkogamy at anther dehiscence in plum and medium which coincides with greater autogamy. Medium and plum anthers dehisce at the same time, earlier than long, and increase the likelihood of selfing.

Introduction

One of the most intriguing topics in plant biology is the evolution self-fertilization or selfing. Since the majority of plant species are hermaphroditic, the opportunity for selfing is abundant. Since selfing is the most extreme form of inbreeding, leading to inbreeding depression (Lande and Schemske 1985; Willis 1999), plants have developed many different mechanisms to prevent self fertilization from occurring like herkogamy, dichogamy and self incompatibility (SI) (Barrett 2003). While the majority of plants are obligate outcrossers (Goodwillie et al. 2005) we see over and over in diverse angiosperm lineages the evolution of selfing species from outcrossing taxa. In species where SI does not exist, selfing or mixed mating systems can evolve (Uyenoyama and Waller 1991; Barrett 2003). In these self compatible (SC) species there is a clear pattern of the evolution of autogamously selfing species (Lande and Schemske 1985; Schemske and Lande 1985; Fishman 2000; Runions and Geber 2000; Cheptou and Mathias 2001; Ushimaru and Nakata 2002; French et al. 2005). If mutational load can be purged through selection on deleterious alleles, then selfing may have an advantage over outcrossing (Barrett and Charlesworth 1991; Holtsford 1996; Crnokrak and Barrett 2002; Carr and Dudash 2003). In environments where pollinators are scarce or variable in their presence, selfing can provide reproductive assurance (Cheptou 2004; Herlihy and Eckert 2004; Mazer et al. 2004; Sun et al. 2005). Also, for novel selfing genes there is up to a 50% transmission bias, since selfers can contribute 2 copies through their selfed offspring and one as a pollen parent when outcrossed (Charlesworth et al. 1990). Selfing flowers are often smaller, have less nectar, and produce fewer pollen grains (Mayers and Lord 1983; Kaczorowski et al. 2005); making them a cheaper investment for the plant,

allowing for allocation of resources to other needs or sustaining growth when fewer resources are available.

Selfing occurs in many diverse manners. Geitonogamy is a common method of selfing, where pollen is transferred to a separate flower on the same plant (Stebbins 1970; Vogler and Kalisz 2001; Barrett 2003; Armbruster and Rogers 2004). This is frequent in occurrence in plants with large displays, since multiple flowers are often open at the same time (Harder and Barrett 1995). This might be adaptive because it also adds to the attractiveness of the display and frequency of pollination (Klinkhamer and Dejong 1990), or may reduce fitness through increased inbreeding depression or pollen discounting (Dejong et al. 1993; Klinkhamer et al. 1994). Autogamy is automatic selfing, and occurs when no pollination is necessary for selfing to take place, i.e. during floral development pollen is deposited on the stigma. Cleistogamy is the most extreme form of autogamy with flowers never opening and pollination occurring in the bud. Cleistogamous flowers are very small, reducing the investment of the plant in the floral structure (Lord 1982; Mayers and Lord 1983; Minter and Lord 1983; Hill and Lord 1990b). In addition to mechanism promoting selfing in many SC species there are also mechanisms related to the prevention of selfing, the most common being herkogamy and dichogamy (Hill and Lord 1990b; Hill et al. 1992; Fenster et al. 1995; Hill and Malmberg 1996; Runions and Geber 2000; Sherry and Lord 2000). Herkogamy is the spatial separation of the anthers and stigma, while dichogamy is the temporal separation of male and female phases. Reduction in both of these traits allows autogamy to occur. Both herkogamy and dichogamy are controlled by the development of the floral organs. Earlier female phase or reduced herkogamy can result in autogamous selfing.

To understand the evolution of autogamous species it necessary to examine changes in floral development since these changes underlie the mechanisms of divergence in floral morphology (Crone and Lord 1991; Hill et al. 1992; Sherry and Lord 2000). Most selfing species have smaller flowers than their outcrossing relatives. In some species this is the only appreciable difference. A change in the timing of developmental events, heterochrony, has been proposed as the mechanism of divergence in floral form (Gould 1977; Hill and Lord 1990b; Fenster et al. 1995). A slower rate of growth with the same duration, or neotony, would result in phenotypically immature or smaller flowers. There could also be a decrease in time to maturity with unchanged growth rates, progenesis, resulting in mature flowers at smaller size, resembling an immature bud. There are studies of several species that have looked at comparative development in selfing and outcrossing species. These studies fall into to two classes: studies of cleistogamous flowers which are produced as a plastic response to the environment (Lord 1982; Mayers and Lord 1983; Minter and Lord 1983), or in the case of *Lamium* to inflorescence position, and the study of autogamous selfers which are not produced as a plastic response (Solbrig and Rollins 1977; Lyons and Antonovics 1991; Hill et al. 1992; Holtsford and Ellstrand 1992; Lyons 1996; Runions and Geber 2000; Sherry and Lord 2000; Mazer et al. 2004). Certainly these should be cautiously compared, but it is reasonable to assume that the changes that lead to cleistogamy may be similar to those responsible for autogamy if they are developmental in nature. Results of these studies are quite varied with the only common feature in all studies being smaller selfing flowers. Plastochron is defined as the time between the sequential initiations of organs, and is commonly used as a measure of duration of organ growth. Plastochron

was shorter for selfing *Viola odorata* and *Clarkia xantia*, resulting in earlier development and faster growth rates (Mayers and Lord 1983; Runions and Geber 2000). The selfing flowers are smaller because they are mature at a younger stage. *V. odorata* has cleistogamous flowers, or flowers which never undergo anthesis (flower opening), but self pollination happens before anthesis would occur in the chasmogamous flower that opens for pollination. Conversely in *Arenaria uniflora* the plastochron is longer in selfing flowers (Wyatt 1984; Hill et al. 1992). Relative growth rates for internodes, pedicels and flower buds are also reduced. This results in fewer smaller flowers in selfers that take longer to develop in *A. uniflora*. This longer development time results in later anther dehiscence and a decrease in protandry. A decrease in dichogamy is also found in *C. xantia*, *Collomia grandiflora's* cleistogamous flowers, and autogamous *Clarkia tembloriensis* (Minter and Lord 1983; Runions and Geber 2000; Sherry and Lord 2000). Dichogamy and herkogamy are likely to be reduced in selfers because this will increase the likelihood of self pollination. Both species of *Clarkia* have reduced protandry and herkogamy in selfers, and also have constant ovary length (Holtsford and Ellstrand 1992; Runions and Geber 2000; Sherry and Lord 2000; Mazer et al. 2004). It is possible that the development of selfers is the same mechanism in both species. Both *A. uniflora* and *Leavenworthia crassa* have fewer flowers in the selfers (Solbrig and Rollins 1977; Wyatt 1984; Lyons and Antonovics 1991; Hill et al. 1992), in *A. uniflora* this is due to a slower growth rate, so it possible that *L. crassa* also shares the reduced growth rate. For there to be a reduced growth rate and plastochron not to increase there would need to be two separate developmental changes since plastochron is affected by growth rate of flowers.

These studies show that both neotony and progenesis appear to be involved in the evolution of selfing in different cases.

The *Nicotiana* Alatae system is perfectly suited for studies of mating system evolution. *Nicotiana* Section Alatae consists of eight species with geographical distribution from Brazil to Mexico (Goodspeed 1954). Pollinator associations in alatae vary from Hymenoptera to Lepidoptera to hummingbirds and Hawk Moths (syphingidae) (Ippolito et al. 2005; Kaczorowski et al. 2005). The mating system of alatae includes two clades with obligate outcrossing through gametophytic self incompatibility (GSI) in one and self compatibility in the other (Ippolito 2000). There are two types of SI, gametophytic and sporophytic. GSI involves the destruction of self pollen tubes, while in sporophytic SI self pollen does not germinate (de Nettancourt 1977; Murfett et al. 1994).

The *Nicotiana* self compatible clade is comprised of two species, *N. longiflora* and *N. plumbaginifolia* which are n = 10, while the other clade is n = 9 and all species have GSI. *N. longiflora* is a facultative outcrosser, with hawk moths as the primary pollinator. *N. plumbaginifolia* is autogamous with self pollination occurring before anthesis. *N. plumbaginifolia* has similar floral morphology to *N. longiflora*, but with shorter corollas 35 mm, versus 120 mm respectively (Fig. 1). Corolla length alone can be used to distinguish *N. plumbaginifolia* from *N. longiflora*, as well as delicate habit, twisted leaf apices and annual character (Goodspeed 1954). Both species have white tubular flowers with five fused petals. The petals reflex at the mouth with the anthers and stigma extruding slightly. Filaments are fused to the corolla in the tube with a portion of free filament before the anthers attach (Fig. 1) (Volskay 2002). This means that any changes in corolla length may also effect position of the anthers and Anther Stigma

Distance (ASD). *N. longiflora* and *N. plumbaginifolia* are probably sister species (Chase et al. 2003).

The plant materials for this study descend from a mixed population containing both *N. longiflora* and *N. plumbaginifolia*. The Mango population, (named for its proximity to a mango orchard), contains three morphs: long (*N. longiflora*), medium (possible hybrid) and plum (*N. plumbaginifolia*). *N. longiflora* is distributed throughout Northern Argentina and Brazil, and overlaps with *N. plumbaginifolia* near Salta, Argentina. The intermediate morphology of the medium morph points to an intermediate mating system which would allows us to examine the introduction of selfing genes into a primarily outcrossing population. The different floral length morphs are due divergent development of floral organs. Studying the mechanisms responsible is necessary for determining the evolution of the divergent traits as well as selfing in this group. It is likely that hybrids might have the same mechanism of divergent morphology (progenesis or neotony), but with intermediate phenotypes.

This study provides quantitative data on the floral morphology and specific mating system characters of a glasshouse grown population descended from a natural population containing diverse phenotypes for corolla length and mating system. Morphological characters may be highly correlated with the mating system of these species and are unknown for the medium phenotype. The mating system traits of medium may shed light on the evolution of selfing in plum and possibly on the origin of medium plants. This study will also investigate the development of floral buds. Measuring the relative growth rates (RGRs) of the floral organs will allow us to compare development of immature buds between morphs. Differences in developmental rates

allow us to investigate the mechanisms (i.e. progenesis, or neotony) underlying the evolution of plum and medium.

Materials and Methods

Plant Materials

Seeds were collected in 2001 from the Mango field population. Six maternal families were germinated in growth chambers at the University of Missouri - Columbia and then transferred to the glasshouse during fall 2003. The glasshouse population consisted of progeny from four long, one medium and one plum families. Fifteen individuals were grown from each line except for the plum family which displayed increased dormancy and reduced germination. Plants were routinely trimmed to allow for ease of measurement and watering. Plants were grown in 3.5 inch pots, and uniformly arrayed over two benches with 5 plants per tray of 15. Day length was supplemented with lights and set to summer long days (~ 16h) so that plants flower more quickly. One tablespoon of Osmocote® was added monthly to the surface of the soil, and plants were watered daily.

Morphology of Mature Flowers and Autogamy Rate Estimation

This glasshouse population was first surveyed in order to quantify mating system and morphological characters. Corolla length and ASD were measured for all plants using calipers. Due to differences in growing conditions (hotter days, wilting, etc.) and age, plants measured after they had been trimmed a second time were excluded from this analysis. Inflorescences initiated later tend to have shorter flowers (personal observation). Flower length was measured as distance from the base of the ovary to the point where the petals reflexed. This represents the total length of the corolla tube. ASD was measured as the distance between the closest anther and the receptive surface of the

stigma, with negative values representing anthers that are distal to the stigma. This represents how far pollen must travel for selfing to occur in mature flowers. This distance is expected to negatively correlate with autogamous fruit production. Each plant was allowed to produce multiple inflorescences and then autogamy was measured. Two mature inflorescences were collected from each plant, and each mature flowering node was scored, at least 35 nodes were scored for each plant. If fruit set had been initiated then that node was scored autogamous, if a flower matured but fruit set was not initiated then that node was scored non-autogamous. Any node were a flower had senesced was counted as non-autogamous, since all fruits remain on the plant even when dry. Each plant was given a value of autogamy calculated as percent of flowering nodes that autogamously set seed. A one-way analysis of variance (ANOVA) with maternal line as the independent variable was run in SAS for ASD, corolla length, and autogamy ratio (SAS 2002-2003). Tukey's HSD was used to compare family means.

Corolla Development

It is impossible to gather growth rates of internal organs such as stigma without destructive sampling of flower buds. Instead, we elected to measure corolla length of growing buds, and simultaneously destructively sample inflorescences sampled from the same growth period (Hill and Malmberg 1991). This allows us to calculate growth rates for all internal floral organs. Fifteen plants were selected to represent long, medium and plum, and were used for both subsequent studies. Ten flowers on each plant involved in the study were measured until anthesis. Each flower bud was measured daily starting when the bud was large enough to distinguish corolla from sepals, and measurements

continued until anthesis and arresting of growth was observed. Plastochron reference length was set at 35 mm for long and medium morphs and 15 mm for plum morphs. Plastochron was calculated using the method of Hill and Lord (1990a), where:

$$t_{Ri} = t_2 - (t_2 - t_1) \left(\frac{\ln [L(n_i, t_2)] - \ln (R)}{\ln [L(n_i, t_2)] - \ln [L(n_i, t_1)]} \right)$$

and

i = the flower number, beginning with first flower to open, and increasing with successively younger flowers;

t_{Ri} = the time when the flower at position i equals the reference length;

t_1 = the time of the initial observation of the flower at position i;

t_2 = the time of the final observation of the flower at position i;

n_i = the flower at position i, which is the youngest flower whose length at t_2 is $\geq$ R;

$L(n_i, t_1)$ = the length of the flower at position i at t_1;

$L(n_i, t_2)$ = the length of the flower at position i at t_2;

R = a reference length chosen to fall in the exponential phase of flower growth (Hill and Lord 1990a; Hill and Malmberg 1996).

Relative time was calculated by setting zero equal to the day when plastochron reference length was reached for each flower. Then all flowers from a plant were stacked using time as a reference for developmental stage to create a growth curve for each plant (see Fig. 2). Proc Reg was used in SAS to estimate regression coefficients for each plant (SAS 2002-2003).

Floral Organ Development

Organ development was studied using two inflorescences collected from the same individuals. Inflorescences were collected when the second flower reached anthesis so that a sample of buds from throughout floral development was present. Inflorescences were collected and stored in 70% ethanol. Each bud was dissected and measured for the floral traits: corolla length, ovary length, pistil length, stamen length, short stamen length, free filament length, mouth circumference and anther dehiscence was scored as +/- for each bud. Since a smaller mouth opening would force anthers closer the stigma in a lateral direction mouth circumference was examined. Free filament length was included because greater free filament allows for freedom of movement of anthers, and increases the likelihood of contact between anthers and stigma. We also looked at short stamen length (length of the proximal anther) due to my observation that some of the plum flowers had the proximal anther touching the stigma at anthesis. We used these data to estimate herkogamy (pistil length – stamen length), and style length (pistil length – ovary length). Regression coefficients for each trait were estimated by SAS with floral organ length as the dependent variable and time as the independent variable. We used the corolla length of the buds to determine the relative age (time) of buds using the regression coefficients generated by the previous plastochron calibration. For all traits excluding herkogamy the natural log was used making growth curves linear. Herkogamy contains negative values and therefore no natural log was used so the regression coefficients compared were third order polynomials. The regression coefficients generated for each trait are relative growth rates (RGR) and can be statistically analyzed. A homogeneity of slope ANOVA model with time as the dependent variable and length

class (plum, medium, long) as the main effect was run with each trait as the independent variable in PROC GLM in SAS (SAS 2002-2003). This is necessary to determine whether an analysis of covariance (ANCOVA) or a separate slope model is appropriate (Hill and Lewicki 2006). If there is a significant interaction between length class and time then a separate slope model is necessary otherwise ANCOVA is used. Both ANCOVA and the separate slope models determine whether the slopes of the growth curves differ between length classes. Anther dehiscence was scored as presence/absence for all buds. Using these data mean corolla length at anther dehiscence was calculated for each inflorescence collected and the average was plotted for each length class. The relative time at anther dehiscence was determined in the same manner as all other traits.

Results

Morphology of Mature Flowers and Autogamy Rates

Corolla lengths. In the glasshouse, corolla lengths were distributed into three length classes (plum, medium, and long), as were observed in the natural population. Corolla length is distributed as a discontinuous trait in the glasshouse population with a bimodal distribution. Long flowers were 90 to 120 mm, medium flowers were 65 to 85 mm, and plum flowers were 40 to 50 mm (Fig. 3).

Anther Stigma Distance. Anther stigma distances are continuous in distribution, with overlap between long and medium maternal lines (Fig. 4). Long lines ASD range from 0 to 3.5 mm while 32% of long plants are approximately 1.5 mm at anthesis, while medium lines have ASD of approximately 0. ASD for plum lines is very different at anthesis; all plants have negative ASD; the anthers extend past the stigma (see Fig. 1).

Maternal Effects. There were significant maternal line effects at the $p<0.05$ level for all traits. Maternal phenotype consistently predicts the phenotype of the offspring in medium and plum families (Fig. 5). However, long mothers produce both long and medium offspring. Since pollinations occurred naturally paternity cannot be taken into account in this analysis. Plum maternal lines had higher autogamy and shorter corolla length (1.00, 44.52 mm) than both long and medium lines (Fig. 5). ASD for plum lines were negative, while long and medium lines were near to zero and mostly positive. No differences in autogamy between long (0.207, 0.427, 0.283, 0.207) and medium lines (0.445) were detected due to high variances (0.08). Plants from medium lines had shorter corollas (72.32 mm) than plants from long lines (99.91, 82.16, 83.35, 99.30 mm).

Autogamy and ASD. The ASD/autogamy relationship (Fig. 6) was tri-phasic

essentially corresponding with plum medium and long. ASD at anthesis had no affect on the level of autogamy among plum lines because autogamy was 1.0 for all lines. When ASD is close to zero, autogamy is highly variable. This was the case for all medium plants and with four long plants from two families (Fig. 6). Plants with the medium phenotype (60 – 80 mm, ASD ≈ 0) are highly variable in their level of autogamy (mean = 0.463, SE = 0.074, range = 0.862), while long plants are less autogamous and less variable (mean = 0.257, SE = 0.034, range = 0.723). In long lines, autogamy has a negative relationship with ASD. Corolla length predicts autogamy in long lines (r^2 = 0.2575, $p < 0.01$). This relationship is negative, with shorter flowers having greater autogamy, more than 1% per mm (slope = -0.0114 autogamy/mm).

Floral Organ Development

Corolla Growth Curves. Corolla growth curves from each bud were compiled into one growth curve for the entire plant (Fig. 2). The regression coefficients of the growth curves were used to estimate the developmental rate (RGR) of organs of preserved buds.

Floral Organ Growth. All traits had significant regressions for all three classes, excluding herkogamy for medium plants (Table 1). Medium plants have a slope of approximately zero for herkogamy making any regression non-significant. Floral organ growth was plotted over time with the shortest bud measured set as day one (Fig. 7). Long flowers grew for 10.5 days while medium flowers grew for 8.5 days, and plum flowers grew for 9.5 days. RGRs for long and medium were greater than plum for all traits, however this is not significant in ovary length and mouth circumference.

Homogeneity of slopes revealed no significant interaction between length class and time for ovary length, mouth circumference, and free filament allowing the use of analysis of covariance to test for slope differences (Table 2). Interactions with time were detected for pistil length, stamen length, style length, short stamen length and herkogamy necessitating the use of a separate slopes model. All traits had significant differences among length classes (Table 2). Contrasts were used to test the differences between pairs of classes. Most traits showed the same pattern: long and medium RGRs were different from plum, while long and medium were indistinguishable. Exceptions to this pattern were ovary length ($p < 0.01$) and mouth circumference (nearly significant, $p = 0.0584$), which were different for all length classes. We also found that the slopes of plum and medium were different for all traits except herkogamy. This is a product of the non-significant slope (no change over time) of herkogamy in the medium group.

Herkogamy and Anther Dehiscence. Herkogamy is a composite of pistil length and stamen length. Positive values for herkogamy indicate that the stigma is exerted beyond the anthers. Herkogamy changes throughout the development of the buds, except in medium (regression non-significant, but see Fig. 8), with the anthers and stigma moving further apart from prior to anthesis until fertilization. Herkogamy was plotted as actual length in mm since there were negative values which cannot be transformed to natural log and this gives us a more relevant description of the actual developmental processes. Herkogamy becomes negative prior to anthesis in plum while both long and medium remain positive (Fig. 8). Some medium flowers also show a negative or near zero herkogamy and the regression is non-significant, but most buds have positive herkogamy at anthesis. Long anthers dehisced later than plum and medium (8.68, 6.90

and 6.21 days respectively). There were no significant differences in time at anther dehiscence between plum and medium ($p < 0.01$). Long flowers had a much greater herkogamy at anther dehiscence (2.53 mm) than both plum (0.04 mm) and medium (1.18 mm).

Discussion

Possible Origin of Medium Phenotype

The original Mango population was comprised of three corolla morphs: long, medium and plum. Medium flowered plants could be smaller *N. longiflora* flowers or a hybrid between *N. longiflora* and *N. plumbaginifolia.* Medium flowers have intermediate phenotypes for corolla length, ASD and glasshouse autogamy rate (Fig 4). Medium phenotypes are on the short tail of the long distribution for these traits, with a pronounced discontinuity between long/medium and plum distributions (Fig. 3, 4). Further, the developmental trajectories of medium plants are indistinguishable from long (Table 2, Fig. 7). Due to the similarity of growth for the long and medium, it is possible that medium is not a hybrid, but instead just a shorter morph of long.

Another possible explanation for the same growth rates for long and medium is that growth rate genes from long are completely dominant to those of plum. Other evidence for hybridization between *N. longiflora* and *N. plumbaginifolia*, includes two medium flowered plants germinating from seed collected from two different plum mothers (not shown). Two of the long flowered lines contained medium and long offspring, while two had only long flowered offspring. This result is consistent with a hybrid origin of those medium offspring. Autogamy is highly variable within medium lines, but also in two long lines containing medium offspring. Plum parents have only autogamous offspring, except for two medium, almost assuredly hybrid offspring from separate plum lines (not shown). This is strong evidence for gene flow between the two species -- although the hybrid status of the original medium Mango plant remains uncertain until genetic marker data are available. *Nicotiana plumbaginifolia* does receive

hawkmoth visits in natural populations, but visitation has so far only been observed in populations that also contain *N. longiflora* (D. Figueroa-Castro, pers. comm).

Developmental Basis of Autogamy

Floral organs final position within the flower can be altered through changes in developmental growth rates (Crone and Lord 1991; Hill et al. 1992; Runions and Geber 2000; Sherry and Lord 2000). This heterochrony can also change relative positions of organs during bud formation. Since herkogamy and dichogamy can both affect selfing, these should be considered throughout bud development. Herkogamy is well supported as a mechanism preventing selfing in self compatible species (Webb and Llyod 1986; Takebayashi and Delph 2000; Stone and Motten 2002). Some recent attention has been given to the idea that herkogamy may instead have evolved to reduce pollen discounting (Fishman 2000; Barrett 2002, 2003). Regardless of the actual adaptive processes involved, herkogamy has been shown to be correlated with selfing rate in many species (Holtsford and Ellstrand 1992; Belaoussoff and Shore 1995; Karron et al. 1997; Runions and Geber 2000; Stone and Motten 2002; Herlihy and Eckert 2004). Corolla length does have an effect on ASD, but most of that effect is caused by highly differentiated development of ASD between plum and long, with ASD becoming negative before anthesis in plum. This is the result of greater growth of the corolla (with inserted filaments), not to free filament growth, since free filament length does not increase in plum (Fig. 1, 7). The medium length flowers show great variation in ASD, and the effect of length is lost (Fig. 6). ASD predicts autogamy only for long flowered individuals where reduced ASD increases autogamy. Plum flowers show no relationship between

ASD and level of autogamy; all flowers have negative ASD and are autogamous. ASD is highly variable, but always negative, at anthesis in plum (Fig. 5). Medium flowers have ASD near zero but a large variance in level of autogamy. The level of autogamy is affected by both corolla length and ASD for long flowers; neither trait is correlated with autogamy in medium. It is also important to consider the role of these values in natural conditions. Since all the morphs are capable of outcrossing the role of selfing might be reduced. However, with the presence of natural pollinators the amount of geitonogamy would almost certainly increase. It is also important to consider how variable these traits are. It is possible that environmental variation could alter the expression of dichogamy in *N. longiflora*. Anthesis and growth are known to be plastic in expression and the same might be true in this case. Dissection of autogamy under field conditions with the addition of pollinators will likely be much more complicated, but ongoing marker development could help quantify this.

There was an obvious pattern that emerged when examining floral organ RGR. Long and medium had the same RGR, while plum flowers were different, growing for the same amount of time, but at a slower rate. This pattern of reduced rate of growth would make neotony the mechanism responsible for shorter flowers in plum. However, this contrasts with medium, where progenesis is responsible for shorter flowers. Medium flowers discontinue growth nearly 2 days before long flowers, and are therefore shorter. This is surprising since the simplest scenario to imagine is one in which corolla length is controlled by the same developmental mechanism in all floral morphs, and therefore medium plants would be expected to have an intermediate developmental phenotype. Traits cannot always be assumed to have homologous origins, and this instance

demonstrates the importance of determining how final phenotypes are reached through an ontogenetical study.

Free filaments do not increase in length in plum meaning any growth occurs in the corolla, not the filament. The most striking finding is that the relative growth rate of the pistil is slower than that of the stamen in plum. This means that the herkogamy will become negative over time and is the major developmental difference between the plum and long. This is due to a reduced style length RGR. In plum, style length and stamen length have the same RGR. However, both long and medium have a greater style length RGR than stamen length.

Here herkogamy is a composite measure, based on the measurements of pistil and stamen length. This may not represent the actual physical distance between anther and stigmatic surface in the same way as ASD does. This measure is a conservative estimate of ASD, since it is one dimensional and anthers could actually be closer to the stigma in the three dimensional flower because of their free filament. Long and medium have similar growth curves for herkogamy. However, herkogamy for plum flowers becomes negative as the anthers grow past the stigma. This striking difference could easily explain the complete autogamy for plum flowers if the anthers have dehisced when anthers and stigma are touching (herkogamy is zero). Looking at anther dehiscence in all three morphs we see that anthers dehisce more than 2 days later in long than in plum and medium (Fig. 8). Herkogamy increases with time in long making selfing less likely later in development. There is no difference between medium and plum for dehiscence time indicating a possible similar origin (plum = 6.90 SE = 0.548, medium = 6.21 SE = 0.194). Since gene flow is likely present between plum and long, it is possible that a dichogamy

reducing gene might be responsible for increased autogamy in medium. Herkogamy is near zero when medium and plum anthers dehisce. Therefore, the increased autogamy seen in medium flowers is due to a reduction in time to male phase (dichogamy), and a simultaneous reduction in herkogamy. Study of the developmental differences between long and plum reveal the mechanism responsible for the reduced floral form of *N. plumbaginifolia*, but when dichogamy is also considered, the evolution of mating system can be properly investigated. With the discovery of the traits directly responsible for selfing rate it is possible perform comparative phylogenetic analyses, investigate quantitative genetics, and quantify selection on the traits. It is clear that in further studies of mating system evolution it is necessary to include the analysis of phenology and dichogamy with other morphological characters.

Figures

Trait	Relative Growth Rates			R^2		
	Long	**Med**	**Plum**	**Long**	**Med**	**Plum**
Ovary length	0.0658	0.0567	0.0531	0.781***	0.773***	0.760**
Circumference	0.0692	0.0905	0.0672	0.872***	0.865***	0.931***
Free filament	0.1356	0.1943	0.0226	0.480**	0.450*	0.025
Pistil length	0.3034	0.3053	0.1906	0.980***	0.963***	0.915***
Stamen length	0.3067	0.3059	0.2252	0.981***	0.963***	0.933***
Style length	0.3359	0.3600	0.2276	0.975***	0.960***	0.910***
Short Stamen	0.3290	0.3350	0.2440	0.976***	0.960***	0.936***
Herkogamy (Pistil – Stamen)	-	-	-	0.681**	0.308	0.653*
3rd order	-0.0450	-0.0029	0.0029	-	-	-
2nd order	0.0804	0.0349	-0.1228	-	-	-
1st order	0.7884	0.3123	-0.5046	-	-	-

Table 1. Relative growth rate regressions for floral traits. Regression coefficients are the estimated slope for three length morphs: Long (*N. longiflora*), Med (putative hybrid), and Plum (*N. plumbaginifolia*). Herkogamy is a third order polynomial and therefore has three estimated regression coefficients, none of which are strictly RGR but are useful for comparisons between morphs. *** = $p < 0.0001$, ** = $p < 0.001$, * = $p < 0.05$.

Character	Homogeneity of slope	Separate Slope	Covariance	Long/Medium vs. Plum	Long vs. Medium	Medium vs. Plum
Ovary length	NS	na	***	***	**	***
Mouth circumference	NS	na	***	***	0.0584	***
Pistil length	***	***	na	***	NS	***
Stamen length	**	***	na	***	NS	***
Style length	***	***	na	***	NS	***
Free filament	0.0782	***	***	***	NS	***
Short stamen length	**	***	na	**	NS	**
Herkogamy (Pist.-Stam)	**	*	na	*	NS	NS

Table 2: ANCOVA of RGR for 8 floral traits (Table 1) from a time series of floral buds. For herkogamy tests are only for the first order term. Homogeneity of slope (HoS) model tests for significant interactions of trait values with time. If HoS model is significant ($p \leq 0.05$) then the separate slopes model is used to test for differences in slopes among the three floral types. If HoS model is <u>not significant</u> then the covariance model is appropriate. Contrasts for the appropriate model (SS or Cov) are also shown. Significant differences indicate that the growth curves have different slopes. NS : not significant, * : $p \leq 0.05$, ** : $p \leq 0.01$, *** : $p \leq 0.0001$.

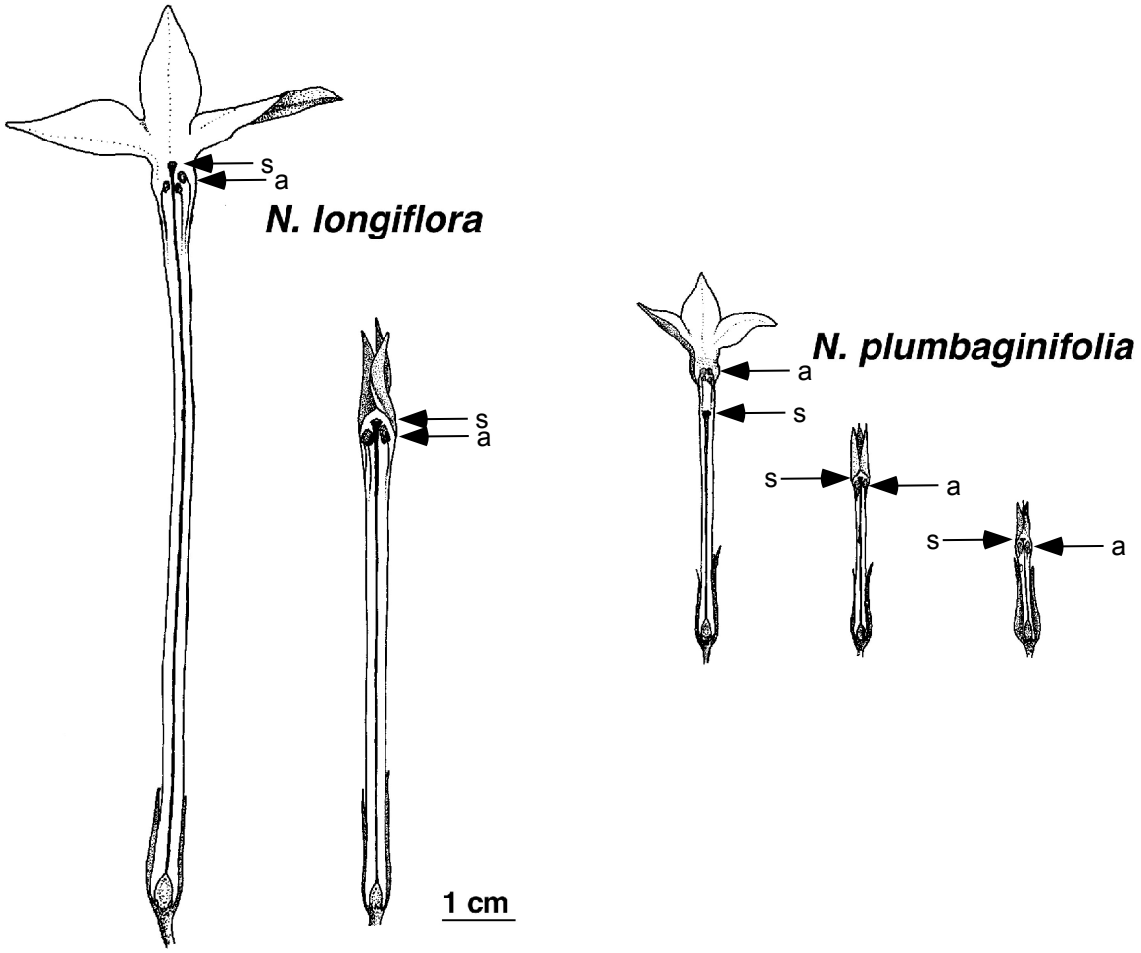

Figure 1: *N. longiflora* and *N. plumbaginifolia* before and after anthesis. Cut-away reveals relative position of anthers and stigma during development. Anther position is indicated by a, and stigma position is indicated by s. Modified from Volskay 2002.

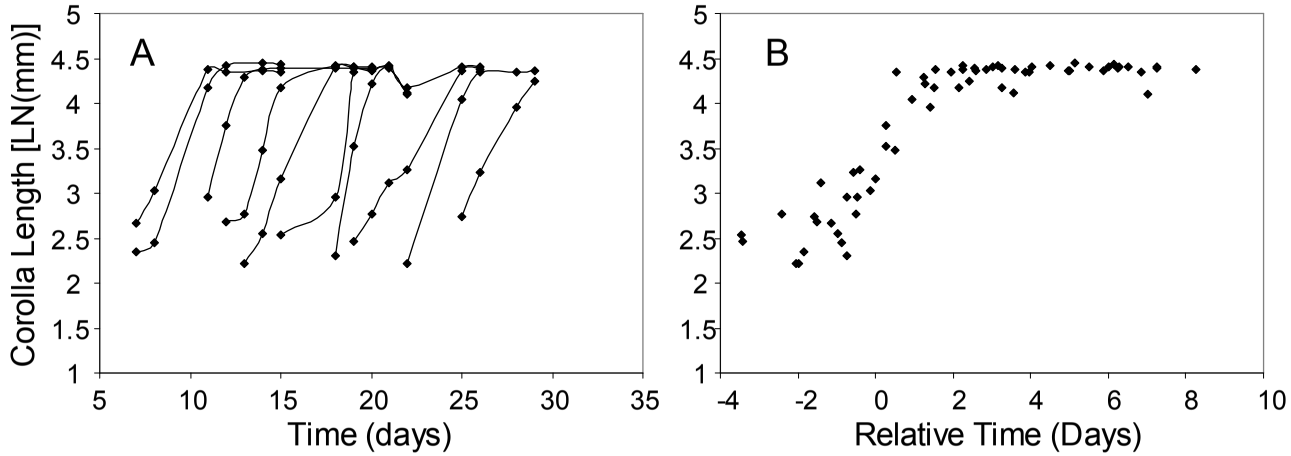

Figure 2: Construction of individual plant flower bud growth rates. (A) Individual bud growth, each line represents one bud. Each bud was measured daily until after anthesis. (B) Composite growth curve for a single plant $r^2 = 0.8635$. The time at which each bud reached the plastichron reference length was set to zero to compress the curves. All buds are aligned to one point and one curve is then constructed. Negative time values are days previous to reaching plastichron reference length.

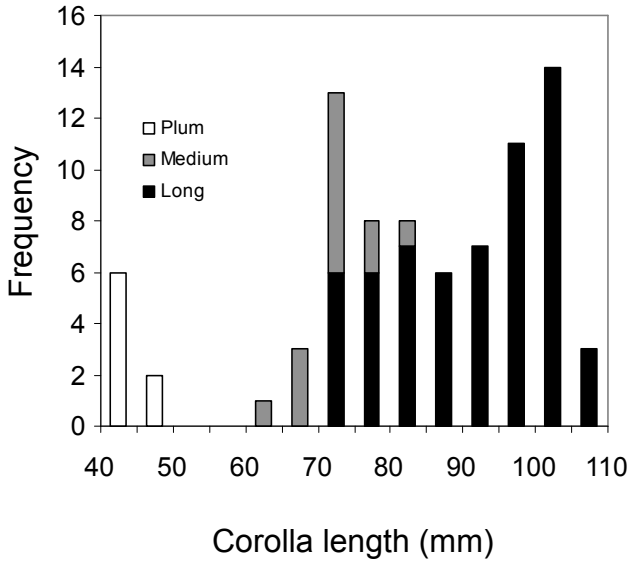

Figure 3: Distribution of corolla length for three corolla morphs from the Mango population. Plum, medium and long was assigned to maternal lines in the field, progeny were subsequently grown in the glasshouse. Length class of maternal lines are indicated by different colored bars. Plum flowers are 40 - 50 mm, medium flowers are 60 - 90 mm, and long flowers are 90 - 110 mm.

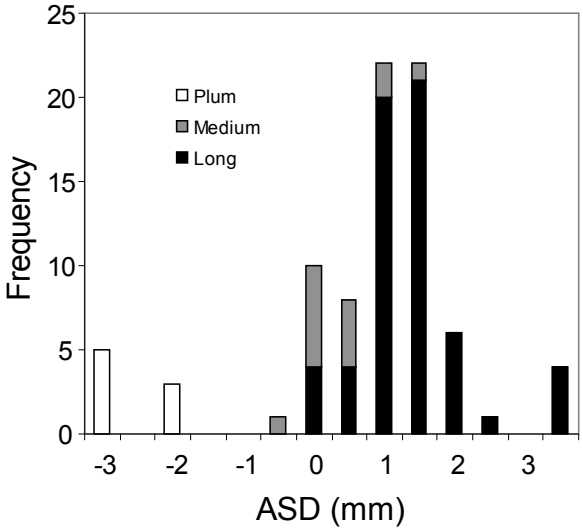

Figure 4: Anther stigma distance (ASD) for three corolla length morphs from Mango Population. Colored bars represent different maternal phenotypic classes assigned in the field. Plum flowers have negative ASD, medium ASD is near 0 mm, while long flowers have larger ASD.

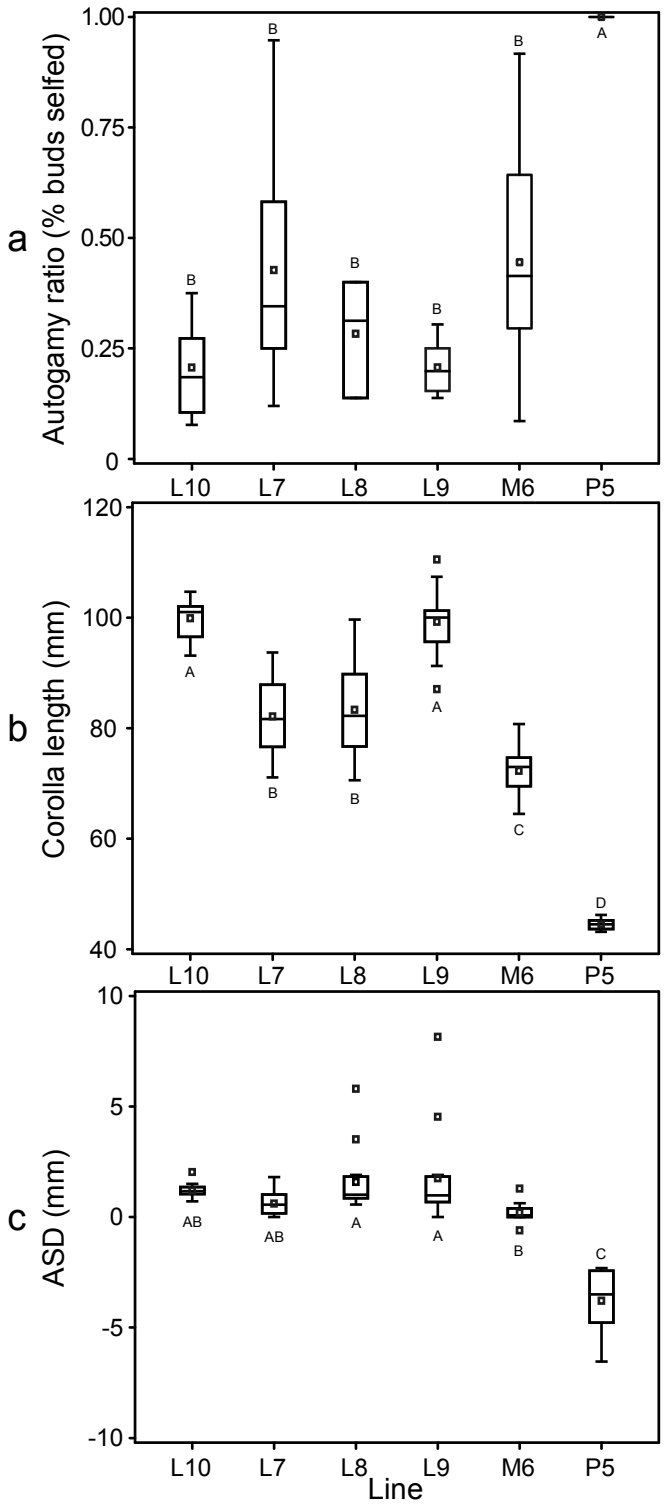

Figure 5: Family means for (a) autogamy, (b) corolla length and (c) anther stigma distance (ASD) from ANOVA. within boxes represent means. Horizontal lines represent medians. Boxes represent the first to third quantile or IQR. Whiskers are ± 1.5 the IQR. Tuckey's HSD was used to determine family differences. Families with the same letter are not significantly different at $p<0.05$.

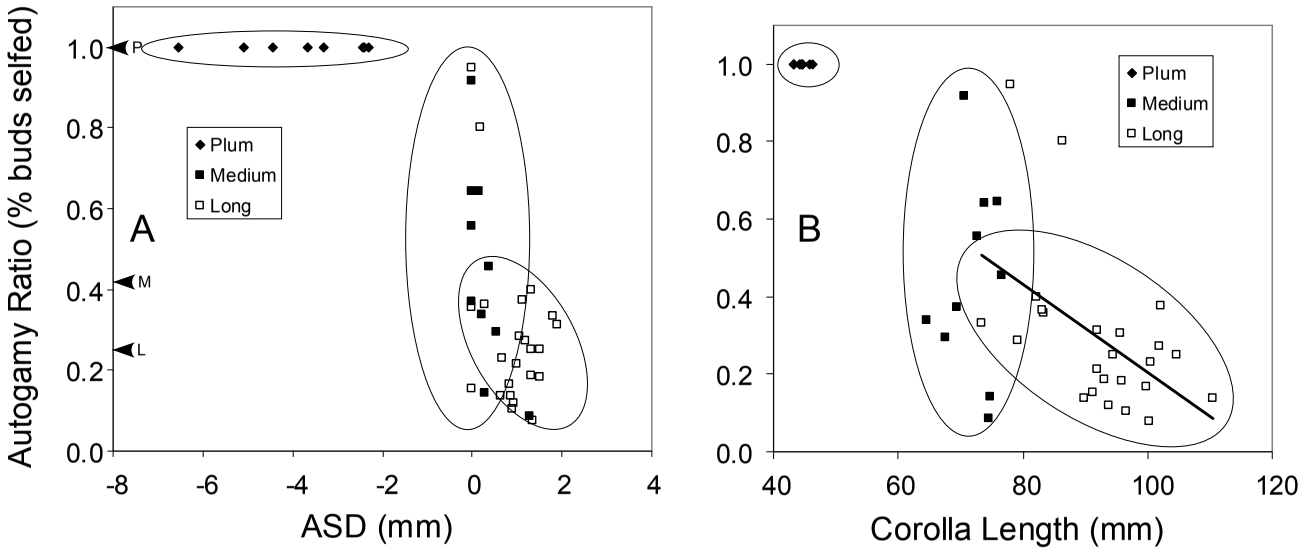

Figure 6: Percent autogamous fruit set by (A) anther stigma distance (ASD) and (B) corolla length for three maternal length classes. Ovals group maternal classes and represent trends. ASD is negative for short flowers, while medium flowers have ASD near zero. Autogamy is negatively correlated with corolla length for long flowers $r^2 = 0.2575$. Plum and medium flowers have no relationship between corolla length and autogamy. P = mean autogamy for plum, M = mean autogamy for medium, and L = mean autogamy for long.

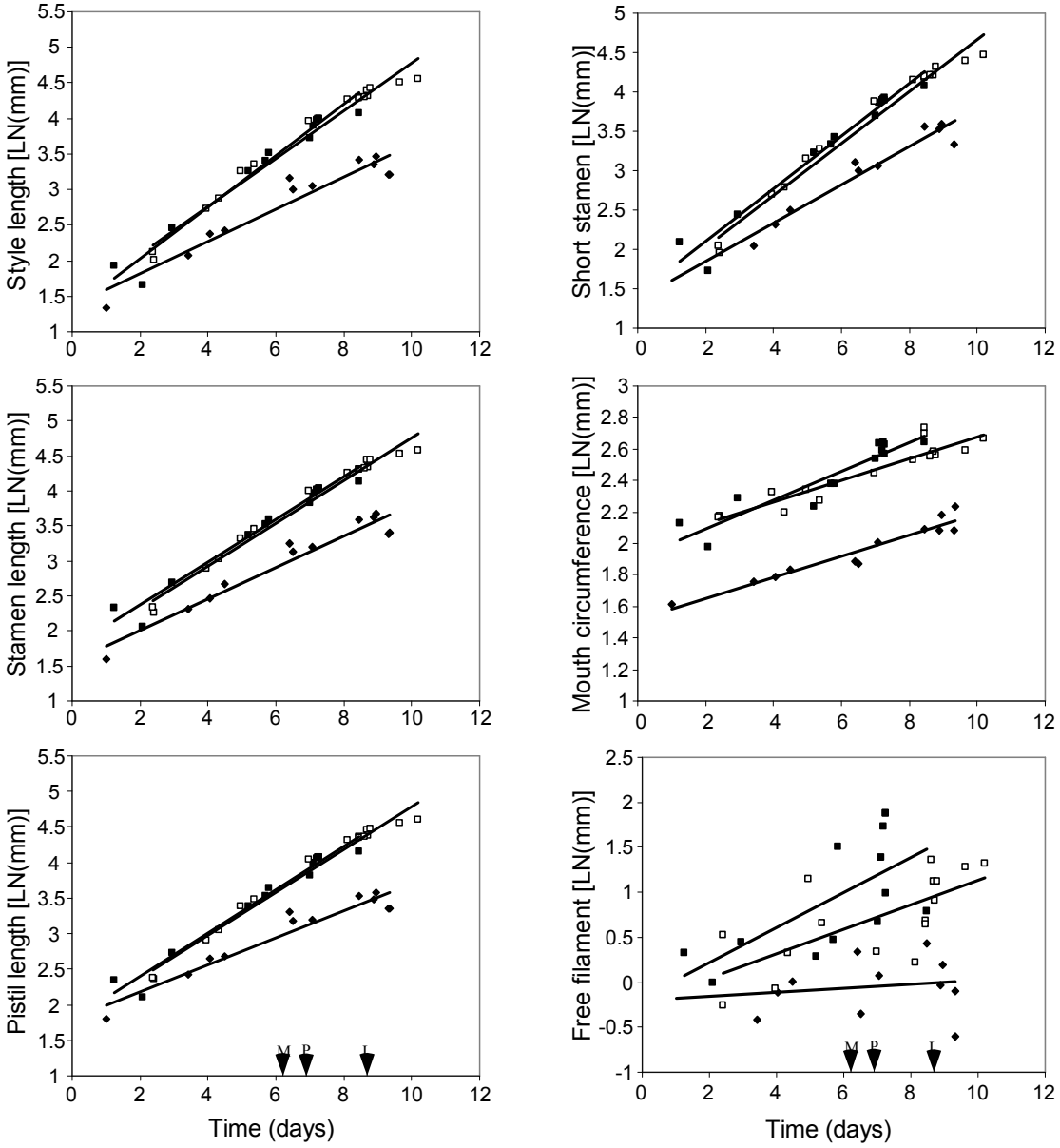

Figure 7: Floral organ growth curves in three corolla length morphs for style length, stamen length, pistil length, short stamen length, mouth circumference, and free filament length. Long = open boxes, medium = closed boxes, plum = closed diamonds. Anther dehiscence is indicated by arrows: L = long, M = medium, and P = plum.

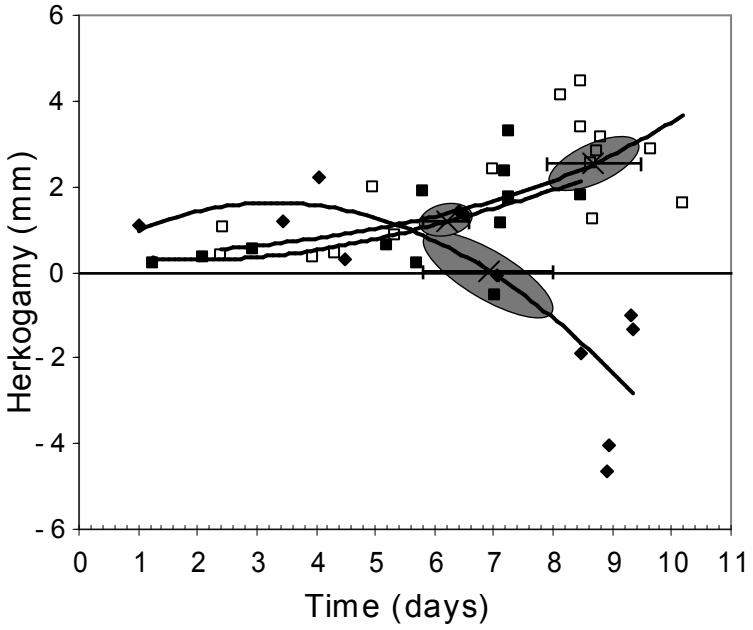

Figure 8: Herkogamy growth curves for three morphs with mean anther dehiscence time. Mean time of anther dehiscence for each morph is indicated by X. Error bars are two standard errors, and ellipses indicate herkogamy at time of anther dehiscence based on error bars. Long and medium have similar growth rates with medium ending its growth at about 8.5 days, while long continues growing until about 10.5 days. Plum has a very different growth curve with herkogamy becoming negative after 7 days. Plum =◆, medium =■, long =□.

References Cited

Armbruster, W. S., and D. G. Rogers. 2004. Does pollen competition reduce the cost of inbreeding? American Journal of Botany 91:1939-1943.

Barrett, S. C. H. 2002. Sexual interference of the floral kind. Heredity 88:154-159.

Barrett, S. C. H. 2003. Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 358:991-1004.

Barrett, S. C. H., and D. Charlesworth. 1991. Effects of a Change in the Level of Inbreeding on the Genetic Load. Nature 352:522-524.

Belaoussoff, S., and J. S. Shore. 1995. Floral Correlates and Fitness Consequences of Mating-System Variation in Turnera-Ulmifolia. Evolution 49:545-556.

Carr, D. E., and M. R. Dudash. 2003. Recent approaches into the genetic basis of inbreeding depression in plants. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 358:1071-1084.

Charlesworth, D., M. T. Morgan, and B. Charlesworth. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing rates in a multilocus system with no linkage. Evolution 44:1469-1489.

Chase, M. W., S. Knapp, A. V. Cox, J. J. Clarkson, Y. Butsko, J. Joseph, V. Savolainen, and A. S. Parokonny. 2003. Molecular systematics, GISH and the origin of hybrid taxa in Nicotiana (Solanaceae). Annals of Botany 92:107-127.

Cheptou, P. O. 2004. Allee effect and self-fertilization in hermaphrodites: Reproductive assurance in demographically stable populations. Evolution 58:2613-2621.

Cheptou, P. O., and A. Mathias. 2001. Can varying inbreeding depression select for intermediary selfing rates? Am Nat 157:361-373.

Crnokrak, P., and S. C. H. Barrett. 2002. Perspective: Purging the genetic load: A review of the experimental evidence. Evolution 56:2347-2358.

Crone, W., and E. M. Lord. 1991. A Kinematic Analysis of Gynoecial Growth in *Lilium longiflorum*: Surface Growth Patterns in All Floral Organs Are Triphasic. Developmental Biology 143:408-417.

de Nettancourt, D. 1977. Incompatibility in Angiosperms. Springer-Verlag, Berlin; New York.

Dejong, T. J., N. M. Waser, and P. G. L. Klinkhamer. 1993. Geitonogamy - the Neglected Side of Selfing. Trends Ecol. Evol. 8:321-325.

Fenster, C. B., P. K. Diggle, S. C. H. Barrett, and K. Ritland. 1995. The Genetics of Floral Development Differentiating Two Species of *Mimulus* (Scrophulariaceae). Heredity 74:258-266.

Fishman, L. 2000. Pollen Discounting and the Evolution of Selfing in *Arenaria Uniflora* (Caryophyllaceae). Evolution 54:1558 -1565.

French, G. C., R. A. Ennos, A. J. Silverside, and P. M. Hollingsworth. 2005. The relationship between flower size, inbreeding coefficient and inferred selfing rate in British Euphrasia species. Heredity 94:44-51.

Goodspeed, T. H. 1954. The genus Nicotiana; origins, relationships, and evolution of its species in the light of their distribution, morphology, and cytogenetics. Chronica Botanica Co., Waltham, Mass.

Goodwillie, C., S. Kalisz, and C. G. Eckert. 2005. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. Annual Review of Ecology Evolution and Systematics 36:47-79.

Gould, S. J. 1977. Ontogeny and Phylogeny. Belknap Press of Harvard University Press, Cambridge, Mass.

Harder, L. D., and S. C. H. Barrett. 1995. Mating Cost of Large Floral Displays in Hermaphrodite Plants. Nature 373:512-515.

Herlihy, C. R., and C. G. Eckert. 2004. Experimental dissection of inbreeding and its adaptive significance in a flowering plant, *Aquilegia canadensis* (Ranunculaceae). Evolution 58:2693-2703.

Hill, J. P., and E. M. Lord. 1990a. A Method For Determining Plastochron Indices During Heteroblastic Shoot Growth. American Journal of Botany 77:1491-1497.

Hill, J. P., and E. M. Lord. 1990b. The role of developmental timing in the evolution of floral form. Seminars in Developmental Biology 1:281-287.

Hill, J. P., E. M. Lord, and R. G. Shaw. 1992. Morphological and growth rate differences among outcrossing and self-pollinating races of *Arenaria uniflora* (Caryophyllaceae). Journal of Evolutionary Biology. 5:559-573.

Hill, J. P., and R. I. Malmberg. 1991. Rates of corolla growth in tobacco determined with the plastochron index. Planta 185:472-478.

Hill, J. P., and R. L. Malmberg. 1996. Timing of morphological and histological development in premeiotic anthers of *Nicotiana tabacum* cv Xanthi (Solanaceae). American Journal of Botany 83:285-295.

Hill, T., and P. Lewicki. 2006. STATISTICS Methods and Applications. Statsoft, Tulsa, OK.

Holtsford, T. P. 1996. Variation in inbreeding depression among families and populations of Clarkia tembloriensis (Onagraceae). Heredity 76:83-91.

Holtsford, T. P., and N. C. Ellstrand. 1992. Genetic and Environmental Variation in Floral Traits Affecting Outcrossing Rate in Clarkia-Tembloriensis (Onagraceae). Evolution 46:216-225.

Ippolito, A. 2000. Systematics and Floral Evolution of Nicotiana. Pp. 109. Division of Biological Sciences. University of Missouri - Columbia, Columbia, MO.

Ippolito, A., G. W. Fernandes, and T. P. Holtsford. 2005. Pollinator preferences for *Nicotiana alata*, *N. forgetiana*, and their F-1 hybrids. Evolution 59:253-253.

Kaczorowski, R. L., M. C. Gardener, and T. P. Holtsford. 2005. Nectar traits in Nicotiana section Alatae (Solanaceae) in relation to floral traits, pollinators, and mating system. American Journal of Botany 92:1270-1283.

Karron, J. D., R. T. Jackson, N. N. Thumser, and S. L. Schlicht. 1997. Outcrossing rates of individual Mimulus ringens genets are correlated with anther-stigma separation. Heredity 79:365-370.

Klinkhamer, P. G. L., and T. J. Dejong. 1990. Effects of Plant Size, Plant-Density and Sex Differential Nectar Reward on Pollinator Visitation in the Protandrous Echium-Vulgare (Boraginaceae). Oikos 57:399-405.

Klinkhamer, P. G. L., T. J. Dejong, and J. A. J. Metz. 1994. Why Plants Can Be Too Attractive - a Discussion of Measures to Estimate Male Fitness. J. Ecol. 82:191-194.

Lande, R., and D. W. Schemske. 1985. The Evolution of Self-Fertilization and Inbreeding Depression in Plants 1. Genetic Models. Evolution 39:24-40.

Lord, E. M. 1982. Floral Morphogenesis in Lamium-Amplexicaule L (Labiatae) with a Model for the Evolution of the Cleistogamous Flower. Botanical Gazette 143:63-72.

Lyons, E. E. 1996. Breeding system evolution in Leavenworthia .2. Genetic and nongenetic parental effects on reproductive success in selfing and more outcrossing populations of Leavenworthia crassa. Am Nat 147:65-85.

Lyons, E. E., and J. Antonovics. 1991. Breeding System Evolution in Leavenworthia - Breeding System Variation and Reproductive Success in Natural-Populations of Leavenworthia-Crassa (Cruciferae). American Journal of Botany 78:270-287.

Mayers, A. M., and E. M. Lord. 1983. Comparative flower development in the cleistogamous species *Viola odorata* 1 a growth rate study. American Journal of Botany. 70:1548-1555.

Mazer, S. J., H. Paz, and M. D. Bell. 2004. Life history, floral development and mating system in Clarkia xantiana (Onagraceae): Do floral and whole-plant rates of development evolve independently. Am. J. Bot. 91:2041-2050.

Minter, T. C., and E. M. Lord. 1983. A Comparison of Cleistogamous and Chasmogamous Floral Development in Collomia-Grandiflora Dougl Ex Lindl (Polemoniaceae). American Journal of Botany 70:1499-1508.

Murfett, J., T. L. Atherton, B. Mou, C. S. Gasser, and B. A. McClure. 1994. S-Rnase Expressed in Transgenic Nicotiana Causes S-Allele-Specific Pollen Rejection. Nature 367:563-566.

Runions, C. J., and M. A. Geber. 2000. Evolution of the self-pollinating flower in *Clarkia xantiana* (Onagraceae). I. Size and development of floral organs. American Journal of Botany 87:1439-1451.

SAS, I. 2002-2003. SAS for Windows. SAS Institute Inc., Cary, NC USA.

Schemske, D. W., and R. Lande. 1985. The Evolution of Self-Fertilization and Inbreeding Depression in Plants 2 Empirical Observations. Evolution 39:41-52.

Sherry, R. A., and E. M. Lord. 2000. A comparative developmental study of the selfing and outcrossing flowers of Clarkia tembloriensis (Onagraceae). International Journal of Plant Sciences 161:563-574.

Solbrig, O. T., and R. C. Rollins. 1977. Evolution of Autogamy in Species of Mustard Genus Leavenworthia. Evolution 31:265-281.

Stebbins, G. L. 1970. Adaptive Radiation Of Reproductive Characteristics In Angiosperms, I: Pollination Mechanisms. Annual Review of Ecology & Systematics 1:307-326.

Stone, J. L., and A. F. Motten. 2002. Anther-stigma separation is associated with inbreeding depression in Datura stramonium, a predominantly self-fertilizing annual. Evolution 56:2187-2195.

Sun, S. G., Y. H. Guo, R. W. Gituru, and S. Q. Huang. 2005. Corolla wilting facilitates delayed autonomous self-pollination in *Pedicularis dunniana* (Orobanchaceae). Plant Systematics & Evolution 251:229-237.

Takebayashi, N., and L. F. Delph. 2000. An Association Between a Floral Trait and Inbreeding Depression. Evolution 54:840–846.

Ushimaru, A., and K. Nakata. 2002. The evolution of flower allometry in selfing species. Evolutionary Ecology Research 4:1217-1227.

Uyenoyama, M. K., and D. M. Waller. 1991. Coevolution of Self-Fertilization and Inbreeding Depression I. Mutation-Selection Balance at One and Two Loci. Theor Popul Biol 40:14-46.

Vogler, D. W., and S. Kalisz. 2001. Sex among the flowers: The distribution of plant mating systems. Evolution 55:202-204.

Volskay, A. R. 2002. Morphology and Development of Key Floral Traits in *Nicotiana* Section *Alatae*. Pp. 48. Division of Biological Sciences. University of Missouri-Columbia, Columbia, MO.

Webb, C. J., and D. G. Llyod. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms. II. Herkogamy. New Zealand Journal of Botany 24:163-178.

Willis, J. H. 1999. Inbreeding Load, Average Dominance and the Mutation Rate for Mildly Deleterious Alleles in *Mimulus guttatus*. Genetics 153:1885–1898.

Wyatt, R. 1984. The Evolution of Self-Pollination in Granite Outcrop Species of Arenaria (Caryophyllaceae) .1. Morphological Correlates. Evolution 38:804-816.