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Potential Autonomous Selfing in *Gesneria citrina* (Gesneriaceae), a Specialized Hummingbird Pollinated Species with Variable Expression of Herkogamy

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Abstract

Species with mixed mating systems often demonstrate variable expression of breeding system characteristics and thus represent the opportunity to understand the factors and mechanisms that promote both outcrossed and selfed seed production. Here, we investigate variation in levels of herkogamy (variation in stigma-anther separation distance) in a Puerto Rican population of hummingbird pollinated *Gesneria citrina* Urban. There is significant variation in herkogamy levels among individuals of this species and stigma-anther separation is negatively associated with the ability to set fruits and seeds in the absence of pollinators. The variation in levels of herkogamy may represent a mechanism to ensure the production of some self-fertilized progeny in the absence of hummingbird pollinators. We also describe a novel breeding system in *G. citrina,* where stamens elongate over time to reach stigma height, but stamen elongation is accelerated by pollination. These results suggest that once the flowers are pollinated, stamen elongation may favor increased pollen removal and siring success, while the reduction in stigma-anther distance no longer imposes the risk of interference between male and female functions. We discuss our findings of breeding system variation in the context of pollination system evolution in an island setting (Antillean islands).

Key words: mixed-mating; pollen limitation; pollination system; Puerto Rico; reproductive assurance; stigma-anther separation.

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Reproductive assurance, the ability to set seed in the absence of pollinators, is widespread in the angiosperms (Lloyd and Schoen 1992; Fenster and Martén-Rodríguez 2007). Plant species with reproductive assurance mechanisms often exhibit mixed-mating systems, with seeds derived from both outcrossing and selfing. These species should have both the benefits of outcrossing, producing heterotic offspring when outcross pollen is available (Charlesworth and Charlesworth 1987), and autonomous self-pollination, producing offspring when outcross pollen is limiting (Lloyd 1992). It is likely that fluctuations of environmental variables, most notably pollinator availability (reviewed in Goodwillie et al. 2005), are responsible for the origin and maintenance of breeding systems that facilitate reproductive assurance and consequently promote mixed-mating.

Island ecosystems often exemplify the fluctuations in pollinator availability that should favor the evolution of reproductive assurance mechanisms (Stebbins 1957; Barrett 1996; Anderson et al. 2001). Islands generally have depauperate pollinator faunas and low pollinator service or effectiveness, which often leads to reduced levels of female reproductive fitness (Anderson et al. 2001; Perez-Banon et al. 2007). Consequently, island systems represent a unique opportunity to study how the evolution of pollination systems interacts with the evolution of reproductive assurance mechanisms.

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Within-species variation in the expression of levels of herkogamy or dichogamy is often associated with the expression of reproductive assurance and variation in outcrossing rates (Schoen 1982; Barrett and Shore 1987; Dole 1992; Carr and Fenster 1994; Eckert and Herlihy 2004). Thus, species that exhibit variation in levels of herkogamy or dichogamy are ideal for the study of the selective forces responsible for the evolution and/or maintenance of reproductive assurance.

Here we investigate variation in the expression of herkogamy in a Puerto Rican population of hummingbird pollinated *Gesneria citrina* Urban (Gesneriaceae), as an initial step towards understanding the factors that have promoted the evolution of myriad pollination and breeding systems in *Gesneria*. The genus belongs to a tribe that diversified in the Greater Antilles involving an adaptive radiation of pollination systems (Skog 1976). Previous anecdotal observations by one of us (Martén-Rodríguez) suggested that the expression of herkogamy among plants is variable. Furthermore, fruit production is considerable although the pollinator visitation rate is extremely low (Martén-Rodríguez and Fenster in press). Here we quantitatively examine these previous observations to determine the extent of variation in herkogamy and how it might be related to fruit set. We first describe the expression of herkogamy in *G. citrina* and then test the following hypotheses: (i) there is significant among-plant variation for the expression of herkogamy; and (ii) variation in the expression of herkogamy is associated with differences in levels of potential autonomous selfing (fruit and seed set in the absence of pollinators). We also describe a novel form of herkogamy in *G. citrina* where stamen elongation is accelerated by pollination; we quantify the ontogenetic changes in stigma-anther separation in relation to pollination.

Results

Variation in the expression of herkogamy among flowers and plants

In *Gesneria citrina* the stigma develops above the anthers (approach herkogamy), but stigma-anther separation varies considerably among plants. At the flower level, stigma-anther separation varied from 0 mm to 6 mm and averaged 3.51 ± 0.09 (± *SE* and henceforth) mm ($n = 205$ flowers) for early second-day flowers. At the plant level, stigma-anther separation ranged from 1.1 mm to 5.1 mm and averaged 3.06 ± 0.16 mm ($n = 42$) for early second-day flowers. Although there was variation of levels of herkogamy within plants, we detected significant variation of stigma-anther separation among plants for these early measurements ($F_{[43,118]} = 7.17$, $P < 0.001$). Among-plant variation accounted for 66% of the overall variation in the first measurement of stigma-anther separation, while intra-plant variation and error contributed the remaining percentage.

Variation in the expression of herkogamy within flower

Stamen filaments continue to elongate throughout the flower's life and may come into contact with the stigma in the later stages of floral development. Stigma-anther separation in fourth day flowers decreased 0.39 ± 0.14 mm ($n = 36$ flowers) for bagged flowers, and decreased 1.49 ± 0.23 mm ($n = 30$ flowers) for hand self-pollinated flowers (Figure 1). The elongation of stamen filaments of hand self-pollinated flowers is significantly greater than that of bagged flowers (Mann-Whitney *U*-test, $P < 0.001$). The final separation between stigma and anther was significantly correlated with initial separation in both bagged ($r = 0.82$, $P < 0.001$) and hand self-pollinated flowers ($r = 0.68$, $P < 0.001$).

Levels of herkogamy and autonomous fruit-set

The fruit set of bagged flowers was 16.7% and the fruit set of hand self-pollinated flowers was 65.8%. Mean seed number of bagged flowers including flowers that set no seed was 5.70 ± 1.93 seeds ($n = 102$) while hand self-pollinated flowers produced 91.57 ± 13.50 seeds ($n = 76$). The mean seed number of bagged flowers that set seeds autonomously (34.18 ± 8.98, $n = 17$) was significantly lower than the seed number of fruits from hand self-pollinated flowers that set seeds (137.22 ± 17.20, $n = 50$) (Mann-Whitney *U*-test, $P < 0.001$).

Fruit set and seed number per fruit of bagged flowers was significantly correlated with stigma-anther separation at the flower and individual plant level (Table 1). Fruit set and seed number per fruit of hand self-pollinated flowers was not significantly

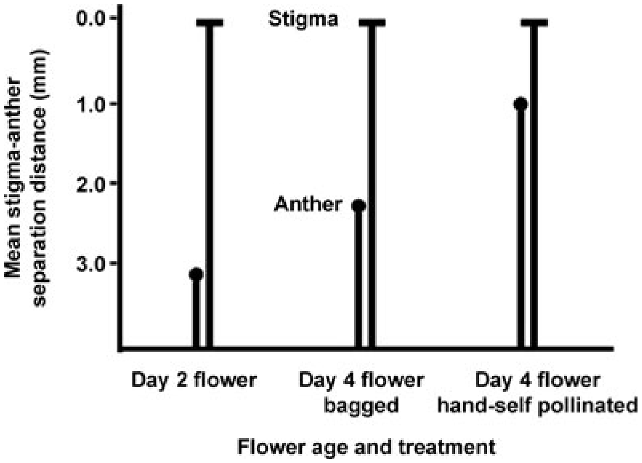

Figure 1. Stigma-anther separation distance decreases across the life-span of a flower of *Gesneria citrina* depending on the pollination status of the stigma.

Initial separation distance found in day two flowers is 3.1 mm, and then 2 d later, is 2.3 mm in unpollinated flowers or 1.0 mm in pollinated flowers.

correlated with stigma-anther separation at the flower or plant level (Table 1). The autofertility index averaged 14.7% and was significantly negatively correlated with stigma-anther separation ($r = -0.51$, $P < 0.01$, $n = 31$; Table 1). The mean distance of stigma-anther separation (2.09 ± 0.23 mm, $n = 17$) of early second-day flowers which set autonomous fruits is significantly smaller than for flowers that did not set autonomous fruits (3.15 ± 0.12 mm, $n = 85$) ($t = -3.64$, degrees of freedom [d.f.] $= 100$, $P < 0.001$).

Table 1. The correlation between herkogamy (stigma-anther separation) with fruit set and seed number in the absence of pollinators in a Puerto Rican population of *Gesneria citrina*

Parameter	Stigma-anther separation	
	Flower	Plant
Autonomous fruit set	-0.34^{***} ($n = 102$)	-0.51^{**} ($n = 38$)
Autonomous seed number	-0.35^{***} ($n = 102$)	-0.50^{**} ($n = 38$)
Hand-selfed fruit set	-0.09 ($n = 76$)	-0.19 ($n = 41$)
Hand-selfed seed number	-0.13 ($n = 76$)	-0.23 ($n = 41$)
Autofertility index	–	-0.51^{**} ($n = 31$)

$^{***}P < 0.001$; $^{**}P < 0.01$.

The autofertility index was calculated by dividing autonomous fruit set by hand-selfed fruit set (after Lloyd and Schoen 1992). Sample size (n) in parentheses.

Discussion

Gesneria citrina exhibits considerable variation in stigma-anther separation and has the potential to set fruits and seeds in the absence of pollinators. Anther filaments may elongate and consequently lessen the distance between stigma and anther in late floral stages, especially after pollination. Autonomous fruit and seed set were significantly correlated with stigma-anther separation, but not with flower size. Here, we present several hypotheses that might explain these correlations in the context of pollen limitation and reproductive assurance through delayed autonomous selfing.

Herkogamy and reproductive assurance

Spatial separation of anthers and stigmas (herkogamy) within blossoms of an individual plant has long been considered as an adaptive feature that functions to promote outcrossing by either reducing the probability of self-pollination or reducing interference between male and female parts (Webb and Lloyd 1986; Barrett and Harder 1996; Medrano et al. 2005). For self-compatible, non-dichogamous species, small stigma-anther separation distance or absence of herkogamy may cause autonomous selfing. Within species variation in the expression of herkogamy is frequently negatively correlated with potential autonomous selfing rate (Carr and Fenster 1994). The advantages of autonomous selfing depend on when self pollination occurs, levels of inbreeding depression and pollinator abundance (Goodwillie et al. 2005). Delayed autonomous selfing, which occurs when movement of flower parts at the end of anthesis leads to pollen-stigma contact, may be advantageous because it provides reproductive assurance (Lloyd and Schoen 1992).

We document a negative relationship between herkogamy and potential autonomous selfing in *G. citrina*; thus, it seems likely that herkogamy is acting as an inbreeding avoidance mechanism. Self-compatible *G. citrina* usually has high population density and exhibits a highly specialized hummingbird pollination system. However, because pollinator visitation is extremely low, the fate of cross-fertilization for many plants is likely to be unpredictable (Martén-Rodríguez and Fenster 2008). Although *G. citrina* has protogynous flowers, overlap between female and male phases occurs at the beginning of the second-day of stigma receptivity and continues throughout the life-span of the flower, allowing autonomous self-pollination. Thus, plants with reduced herkogamy may benefit from having the possibility to self when pollinator visits are scarce.

In a study that evaluated the pollen limitation and reproductive assurance in various *Gesneria* species, fruit set in *G. citrina* was highly pollen-limited (fruit set of hand-outcrossed plants 66% vs. 43% fruit set of unmanipulated plants) (Martén-Rodríguez and Fenster 2008). Furthermore, autonomous self-pollination significantly contributed to fruit set, although the contribution of selfing varied considerably among plants (Martén-Rodríguez and Fenster 2008). The variability in stigma-anther separation reported here could thus explain the variability in reproductive assurance documented by Martén-Rodríguez and Fenster (2008). Large stigma-anther separation apparently prevents self-pollination and may not be advantageous when pollinator visitation is low or absent. Therefore continuous variation in the expression of herkogamy in the population may be maintained by the unpredictability of the hummingbird pollinators; shorter stigma-anther separation distances may be favored at times of low visitation and increased stigma-anther separation distance may be favored when there is adequate hummingbird service. A number of studies conducted on other species support this conjecture. Autonomous selfing contributes more to seed production in years of low pollinator activity in bee-pollinated *Collinsia verna* (Kalisz and Voglar 2003; Kalisz et al. 2004) and hummingbird and bee pollinated *Aquilegia canadensis* (Herlihy and Eckert 2002, 2004). In addition, Moeller (2006) found that population level variation in the expression of herkogamy in bee pollinated *Clarkia xantiana* is associated with the abundance of the specialist bee pollinators. Although the visitation rates of *G. citrina* among years do not show significant variation, visitation rates are consistently low; thus, some plants may receive adequate visitation while others may not (Martén-Rodríguez and Fenster 2008). Furthermore, since delayed selfing in a flower may affect resource allocation to future flowers so that seed discounting is expressed through future lost outcrossing opportunities, intermediate levels of delayed selfing may be favored (Holsinger 1996; Kliber and Eckert 2004). Further work documenting heritable variation in herkogamy (e.g., Carr and Fenster 1994; Motten and Stone 2000), variation in pollinator visitation frequency across individuals and populations, and quantifying inbreeding depression will help elucidate why herkogamy variation is maintained in populations of *G. citrina*.

Reduction of herkogamy after pollination

To our knowledge, the observation that stamen filaments elongate more after pollination has not been previously documented. It does not appear that the pollen source, self or outcross, affects the elongation of the stamen filaments, since application of outcross pollen also led to a reduction in levels of herkogamy (Martén-Rodríguez pers. obs., 2006). This leads to the question: what is the functional significance of the decrease in stigma-anther distance after pollination? We believe this phenomenon reflects that herkogamy may also function to reduce interference between male and female sexual function (Webb and Lloyd 1986; Routley et al. 2004). Pollen export may be higher if the anthers are at the same level as the stigma, but having both anthers and stigma at similar positions in the floral tube may result in interference with pollen deposition on the stigmas, in addition to contributing to increased selfing. However, if the stamen filaments reach stigma height only after pollen has been deposited on the stigmas, then the opportunity for both interference and selfing is reduced. We describe this phenomenon as pollination-induced reduced herkogamy. It is the opposite of movement herkogamy, where separation of male and female function occurs with the displacement of anthers from the stigma as often occurs with protandry, or where the stigma closes after pollination, for example, *Mimulus* (Barrett 2002). In *G. citrina*, following successful pollination there is pollination-induced reduced herkogamy, as the distance between anthers and stigma becomes reduced.

Conclusion

The present study demonstrates that it is much more illuminating to study the evolution of both breeding and pollination systems within the context of each other. Mating system expression is clearly dependent on both the floral structures determining breeding systems and the activity of pollinators experienced by a plant population (Holsinger 1996; Harder and Barrett 1996; Fenster and Martén-Rodríguez 2007). The flower is a complex structure that represents selection on both pollination and breeding system characters resulting in suites of traits that together function as an adaptation (Stebbins 1950). Several evolutionary responses are likely to occur in the face of reduced pollinator activity including shifts towards other specialized pollinators, evolution of generalized pollination systems and/or the evolution of reproductive assurance mechanisms (Fenster et al. 2004; Fenster and Martén-Rodríguez 2007). The seemingly simple tubular flower of *G. citrina* has an exquisitely complex breeding system that may reflect consistently low and unpredictable patterns of hummingbird visitation. Consequently, the reduction of herkogamy in the absence of pollination is suggestive of a reproductive assurance mechanism. However, the nearly threefold further decrease in levels of herkogamy following pollination may reflect selection for both efficient pollen transport and reduction of interference with female function. Thus, the degree of spatial separation between stigma and anther and the placement of the anthers relative to the corolla tube opening may reflect natural selection on both breeding and pollination systems in *G. citrina* in the context of reduced activity by hummingbird pollinators in an island ecosystem.

Materials and Methods

Study site and organism

This study was conducted in the karst hills surrounding the Arecibo Observatory (18°20′36.6″ N, 66°45′11.1″ W), Puerto Rico from December 2006 to March 2007. *Gesneria citrina* Urban is a small shrub with trailing growth, endemic to the Puerto Rican karst region. The plant produces several branches usually less than 1 m long and grows in monospecific patches of high density on steep limestone cliffs. Flowering and fruiting occurs year round. The yellow tubular flowers are protogynous and self-compatible and on average produce 11 μL of nectar per day with a sucrose concentration of 13%, mainly in the early morning (Martén-Rodríguez and Fenster 2008). The corolla can be maintained on the plant for up to 7 d, whether the flower has been pollinated or not; however, hydrogen peroxide tests of stigma viability (Kearns and Inouye 1993) demonstrate that the stigma is usually not receptive beyond the fourth day. The plants are fully self-compatible (Martén-Rodríguez and Fenster 2008). The fruit is a capsule that matures about 2 months after fertilization and contains hundreds of tiny seeds. The hummingbirds *Anthracothorax viridis* and *Chlorostilbon maugaeus* are the main pollinators; the visitation rate by both is low (one visit per flower every 2 d) (Martén-Rodríguez and Fenster 2008).

Experimental design

In order to quantify the variation in the expression of herkogamy we first bagged large flower buds with bridal veil on 44 marked plants. We measured the shortest distance between anthers and stigma to the nearest 0.1 mm with calipers early in the morning of second-day flowers, when anthers started dehiscing (205 flowers on 42 plants, two to seven flowers per plant). In the cases where stigma-anther separation was very small, we used the mean stigma-anther separation of open flowers to infer the separation of bagged flowers in the same plant to avoid causing accidental self-pollination during measurement. Bagging was used to obtain estimates of the potential for autonomous self pollination in relation to stigma-anther separation. Thus, on the same plant, some bagged flowers ($n = 1$–7) were left unmanipulated to quantify the potential for autonomous fruit set while other flowers ($n = 1$–5) were hand-pollinated with self pollen to be used as the control. Self pollen was used instead of outcross pollen to prevent potential conflicts of resource allocation between autonomously selfed and outcrossed flowers within plants. Hand-selfed flowers were re-bagged after hand pollination. To reduce variability of microenvironmental conditions, all flowers remained covered throughout fruit development.

In independent experiments to quantify pollen limitation in *G. citrina*, one of us (SMR) anecdotally observed that the stamens elongate following controlled hand pollination. In order to quantify this observation, we assessed ontogenetic changes in stigma-anther separation distance across the life-span of the flower by measuring stigma-anther separation again 2 d later (day 4 of the flower's life-span). We did this for a subset of flowers (66 flowers on 32 plants). Fruits were collected 1–2 months later and oven dried for 48 h at 37 °C. We counted seed number per fruit under a microscope.

Statistical analyses

Among-plant variation for stigma-anther separation was tested using ANOVA in SAS v 9.1 (SAS Institute 2004). Because many of the variables were not normally distributed or had unequal variances between treatments even after data transformation, we mostly resorted to non-parametric tests for the remaining analyses. The associations between stigma-anther separation and autonomous fruit set and seed number per fruit were estimated using Spearman rank correlations. We averaged stigma-anther separation and fruit set and seed for the plant and repeated the Spearman rank correlations at the individual plant level. We compared the ontogenetic change in the mean stigma-anther separation distance between bagged and hand self-pollination treatments using the Mann-Whitney *U*-test. We compared the mean stigma-anther separation distance of flowers which set autonomous fruits with that of flowers which did not set autonomous fruits using a *t*-test. We defined an autofertility index as the mean seed of autonomous fruits divided by the mean of hand self-pollinated fruits (after Lloyd and Schoen 1992). We compared the seed of autonomous fruits with that of fruits from hand self-pollination using the Mann-Whitney *U*-test to determine whether autonomous selfing in *G. citrina* can yield full seed-set. In this case, we excluded plants that did not set fruit after hand pollination.

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