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The Pollination Biology and Mating System of a Peripheral Population of *Witheringia solanacea* (Solanaceae)

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The Pollination Biology and Mating System of a Peripheral Population
of *Witheringia solanacea* (Solanaceae)

An Honors Thesis

Presented to

The Faculty of the Department of Biology

Colby College

in partial fulfillment of the requirements for the

Degree of Bachelor of Arts with Honors

by

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ABSTRACT

Pollinator visitation rates over the life of a flower are determined by pollinator abundance and floral longevity. If flowers are not visited frequently enough, pollen limitation may occur, favoring the evolution of self-compatibility (SC). In plant species with varying SC levels, central populations often are self-incompatible (SI) and peripheral populations are SC. *Witheringia solanacea* (Solanaceae) is a species that follows this trend with the exception of one population in the Monteverde Cloud Forest Reserve, which is peripheral yet SI. I investigated this population using multiple techniques including floral bagging, pollinator observations, microsatellite analysis, and floral longevity manipulations. My results confirmed the self-incompatibility of the Monteverde population and indicated low but perhaps adequate rates of pollinator visitation per flower per hour. I found reduced genetic diversity at Monteverde and gene flow occurring unidirectionally from San Luis (a central population) to Monteverde. In the greenhouse, there was more of an effect of male than female function on floral longevity, but the largest differences were environmental. Flowers stayed open substantially longer when cool, cloudy weather was simulated and shorter when conditions were hot and sunny. The results indicate that the Monteverde population of *W. solanacea* is SI because 1) it is unable to maximize its fitness due to gene flow from San Luis and its relatively recent colonization of the area and 2) pollen limitation may not be severe because of supplemental pollinator availability from other *Witheringia* species in the area and increased floral longevities due to cool and cloudy conditions.

KEYWORDS

self-compatibility; peripheral populations; floral longevity; pollen limitation; gene flow; Solanaceae; *Witheringia*; Monteverde.

INTRODUCTION

Plant populations at the extremes of the species range (peripheral populations) are often smaller in number and more isolated from one another than central populations (Lammi *et al.* 1999). Therefore peripheral populations are often less genetically diverse and more likely to suffer from inbreeding depression (Lammi *et al.* 1999, Rasmussen & Kollmann 2004). While there may be competition for pollinators at very high densities, a small population will attract fewer pollinators per flower than a large population (Steven *et al.* 2003, Knight *et al.* 2005). Therefore peripheral populations are often pollen limited (Ashman *et al.* 2004, Rasmussen & Kollmann 2004). These factors combined with an unfavorable and often unpredictable environment (Ashman *et al.* 2004) can lead to reduced reproductive success. Peripheral populations, then, can offer interesting case studies in both floral longevity and the evolution of self-compatibility.

The longevity of a flower (the length of time it is open and receptive to pollinators) is determined by three variables: pollinator visitation rates, the metabolic costs of maintenance, and the need for outcrossing (Primack 1985). Unvisited flowers tend to stay open longer than flowers that have been visited (Arathi *et al.* 2002, Ishii & Sakai 2000, Webb & Littleton 1987), but there is some debate over whether floral longevity is set by male or female function. Primack (1985) argues for female function, citing the fact that in monoecious and dioecious plants, female flowers tend to be open longer than male flowers. Some experiments on

hermaphroditic flowers (Webb & Littleton 1987, Arathi *et al.* 2002) support his claim by showing that floral senescence occurs after deposition of pollen on the stigma and not after the pollen has been removed from the anthers. Sutherland and Delph (1984) advance the opposite claim, that male function is more important, by showing that hermaphroditic species have lower fruit set rates as compared to monoecious and dioecious plants. The optimal number of male flowers would be higher than the optimal number of female flowers as shown by the skewed ratios of male to female flowers in monoecious plants. If they could, hermaphroditic plants would have more male flowers than females. But because their biology calls for an even male/female split (all flowers are both), we would expect floral longevity to be optimized for male function. Male function is harder to manipulate experimentally than female function, but work on the hermaphroditic *Erythronium japonicum* showed that its minimum floral longevity is set by the average length of time it takes to disperse most of the pollen grains (Ishii & Sakai 2000). It is alternatively possible that floral longevity is set by either male or female function across species or populations depending on factors such as reproductive assurance, floral physiology, and the relative timing of function.

The relationship between climate and floral longevity is fairly clear. Flowers are longer lived where floral maintenance costs are low (Schoen & Ashman 1995). Flowers are expensive to maintain because of their high evapo-transpirative rates, especially in hot, dry environments. Therefore we expect to see longer-lasting flowers in temperate and montane climates, and this is the case (Primack 1985, Stratton 1989). Longer lasting flowers should also occur when the cost of making a new flower is high in comparison to maintenance costs. Therefore larger flowers should be longer lasting, and again this is true (Stratton 1989). The relationship between the environment and floral longevity responses on the level of an individual plant is less clear. If we

assume that the range limits of a species are set (at least in part) by climatic factors, then peripheral populations are exposed to a climate that is somehow more extreme than central populations. Individuals in peripheral populations may have their average floral longevity affected by these extreme climatic conditions.

Theory would seem to indicate that the greater the need for outcrossing, the longer the flower will stay open (Primack 1985). If a plant is self-incompatible (SI), then by definition it is an obligate outcrosser and must receive pollen from another individual. Self-compatible (SC) individuals have more reproductive assurance and thus can afford to close earlier. Comparing two varieties of an *Impatiens* species, Sato (2002) found the outcrosser did bloom longer than the mixed-mating variety in the absence of pollination.

Determining when and how self-compatibility evolves has been the subject of much debate and modeling (Lloyd 1979, Solbrig 1976, Holsinger *et al.* 1984, Sutherland 1986, Carr *et al.* 1986, Zhang 2000). At the heart of these models are the assumptions that being SC conveys more reproductive assurance, but at the same time lowers overall health of the offspring due to inbreeding depression (Lloyd 1979). Solbrig (1976) predicts that under normal circumstances when outcrossing can be achieved with some assurance, self-incompatibility is favored. Self-compatibility is only favored in small populations with clumped distributions, and where pollen limitation is occurring. As previously mentioned, peripheral populations often meet these SC-favoring requirements. In response to pollen limitation, these populations may experience a breakdown in their self-incompatibility mechanisms (Lloyd 1979, Stone & Pierce 2006). Indeed there are numerous examples of species with central populations that are SI and peripheral populations that are SC (Stebbins 1957, Rick *et al.* 1979, Cabrera & Dieringer 1992, Barrett & Shore 1987).

An interesting compromise between being SI and SC is delayed selfing. Delayed selfing differs from either prior or normal (competing) selfing in that a flower is pollinated by itself only after it has been open and has not received any out-crossed pollen (Lloyd 1992). It could be achieved either by a breakdown in the SI mechanism over the life of the flower, or by a physical mechanism such as corolla abscission (Dole 1990). Delayed selfing, because it provides reproductive assurance without compromising the potential for out-crossed offspring, should be advantageous in any population able to achieve it, especially when pollen is limiting (Lloyd 1992). Interestingly, delayed selfing, unlike competing or prior selfing, is not predicted to affect floral longevity (Lloyd 1992, Arathi *et al.* 2002).

There is an alternative response to pollinator limitation other than evolving self-compatibility. Individuals may bet-hedge—that is overproduce flowers to compensate for the unpredictability of the pollinator availability (Ashman *et al.* 2004, Stephenson 1979, Stone & Jenkins 2008). Bet-hedging predicts a low fruit set rate under normal circumstances with a dramatic increase in fruit-set occurring after supplemental pollination (Stone & Jenkins 2008), but this overproduction could also be the result of pollen limitation caused by a recent ecological perturbation (Ashman *et al.* 2004) making it difficult to show experimentally.

Interestingly, the strategies of bet-hedging and the breakdown of SI predict opposite floral longevity responses. Bet-hedging would predict that, if unpollinated, flowers should be maintained as long as possible to maximize the potential for pollinator visitation. Breakdown of SI would predict a reduction in floral longevity to avoid the high maintenance costs of a flower that probably will not be pollinated anyway. However, if the self-compatibility were in the form of delayed selfing, theory would predict no change in floral longevity.

As a cautionary note, both the bet-hedging and breakdown of SI models assume that individuals in a population are able to optimize their fitness in their specific environment via natural selection. This assumption may not be met if enough gene flow is occurring from central populations (Garcia-Ramos & Kirkpatrick 1997) that it keeps directional selection from achieving optimization. The maladapted population, despite limited reproductive success, could persist as a sink population as long as it is infrequently replenished from the central source population. Only a disruption in gene flow would cause a change and lead either to population extinction or rapid divergence and the evolution of SC or a bet-hedging strategy (Garcia-Ramos & Kirkpatrick 1997).

To investigate the roles of floral longevity and the evolution of SC in a peripheral population I studied an unusual *Witheringia solanacea* population. *Witheringia solanacea* is a Solanaceous shrub that is typically self-incompatible. Several populations have been found, however, in which some or all of the individuals show a complete or partial breakdown in SI (Bohs 2000a, Stone & Pierce 2006, Stone & Jenkins 2008). With one exception, the peripheral populations are the ones with at least some SC plants (Stone & Pierce 2006). That exception is a population growing inside the Monteverde Reserve in Costa Rica. The Monteverde population is small and at the altitudinal extreme of its range, yet seems to be entirely SI (Stone & Pierce 2006). My study attempted to explain this anomaly by investigating the pollination biology of *W. solanacea* and in particular the Monteverde population. I looked at the interplay of self-incompatibility, gene flow, male versus female floral function, and environmental influences on floral longevity using a variety of techniques: flower bagging, pollinator observations, comparison to related species, genetic analysis, and floral longevity manipulations. I offer a

hypothesis involving a synthesis of theory to explain the existence of SI of the Monteverde population.

MATERIALS AND METHODS

Study Species and Site:

Witheringia solanacea (L'Her.) is a shrub that grows on the Pacific slope of southern Mexico and Central America from sea-level to 1500 m. Originally a rainforest light-gap specialist, it has become common in disturbed locations such as along roadsides or in banana plantations. The 1-cm wide, greenish-yellow flowers are pendant and axillary, and are produced continuously throughout the year. Their main pollinator is a halictid bee which collects the pollen reward on the ventral part of its abdomen and thorax. Flowers are open under normal conditions for about two days after which the corolla dehisces. The bird-dispersed (Murray 1988) red berries ripen after a month, but it is possible to determine if pollination was achieved within about a week of flowering by observing a swelling of the ovary (for further description see Stone & Pierce 2006, Stone & Jenkins 2008, Bohs 2000a, and for the floral biology of the related *W. asterotricha* (Standl.), Bohs 2000b).

All field work was conducted at the Monteverde Cloud Forest Reserve, a lower montane site located at an elevation of 1550 meters near the continental divide in northwest Costa Rica. The study population there consisted of roughly 75 individual plants which grew exclusively along the Sendero El Camino, the widest trail in the park. The trail is maintained by frequently cutting the vegetation along its sides with a machete. Unfortunately, the machete had been vigorously applied just prior to my arrival, so few of the plants were in bloom. Conspecifics also occur as scattered individuals along the road leading up to the reserve, the nearest being about

one kilometer away. The closest large population is found at the University of Georgia San Luis Research Station which is three kilometers away and about 400 meters lower in elevation.

Three congeners to *W. solanacea* also occur in the Monteverde reserve. *Witheringia meiantha* (Donn. Sm.) is the most common of the four species and grows in sometimes dense patches along the Sendero El Camino as well as in light gaps along the smaller trails (for a description, see Bohs 2000a). Two additional species, *W. maculata* (C. V. Morton and Standl.) and *W. coccoloboides* (Dammer) occur as scattered individuals. *Witheringia maculata* is densely pubescent all over with larger fruits than the other four. None of the individuals were in flower during the study period, although I found one fruit. *Witheringia coccoloboides* looks similar to *W. meiantha* but has larger leaves and a larger, 4-merous, orange-tinged corolla. It was found at higher elevations in the reserve.

Bagging manipulations and pollinator observations:

All work at the Monteverde Reserve was conducted during the rainy season from August 19-31, 2007. To confirm prior research that plants were completely self-incompatible, I conducted a bagging experiment. I selected 40 buds that were close to opening (based on size and color) on 40 separate *W. solanacea* individuals, and marked them by looping a piece of thread around the selected pedicel and attaching a piece of numbered lab tape. I also flagged marked plants with flagging tape. I bagged 20 of these buds (every other as I moved down the trail) with a piece of mosquito netting that was stapled around the bud. Mosquito netting was selected to be permeable to sunlight and rain but not to bees. After 12 days, I removed the bags and looked at all the tagged flowers. Fertilization was deemed “successful” if I could observe any swelling of the ovary, “unsuccessful” if the entire flower (pedicel, etc.) looked yellow or limp, or had fallen off, and “undetermined” if the flower was still a bud, in bloom, or an

unswollen ovary. I repeated this procedure with 40 *W. meiantha* individuals that were also growing along the Sendero El Camino.

Pollinator observations were conducted on individuals of both *W. solanacea* and *W. meiantha* in half-hour intervals. I observed from 1 to 12 flowers at a time, the number being dependent on how many I could see from one location. In accordance with the observations of Stone and Jenkins (2008), I focused my observations from 0800 h to 1200 h, although I did observe at times at all hours of the day. The weather at the time of each observation was recorded, as well as the number and description of all visitors and the number of flowers each visited. I collected a specimen of the common visitors to be identified later. I also briefly observed flowers of *W. coccoloboides* and two other Solanaceous plants—*Solanum acerifolium* (Dunal) a large thorny shrub, and the other an unidentified small vine. I compared visitation rates in visits per flower per hour for *W. solanacea* versus *W. meiantha*, and then within the *W. meiantha* observations, sunny versus cloudy conditions and small (1-6 flowers) versus large (7-12 flowers) floral displays.

On the last day, I collected the seeds of about two ripe fruits and a tissue sample from 12 *W. solanacea* individuals. I also went to San Luis Research Station and collected the samples from 19 plants there. I brought the samples back with me in order to estimate out-crossing rates.

Genetic analysis:

I planted the collected seeds in seedling trays filled with plug mix, and allowed them to germinate and grow for 2 months on a misting bench in the Colby College greenhouse. Then, I harvested and dried the plants. In total, the DNA from 14 parents and 21 offspring was extracted from the San Luis population, and 12 parents and 41 offspring from the Monteverde population. Additionally, fresh tissue from four previously collected plants growing in the Colby greenhouse

was analyzed. The greenhouse plants (MV 2, 4, 6, and 11) were collected from areas roughly in between San Luis and Monteverde and so represented a possible path of gene flow between the two main populations. All samples from the collected maternal tissue as well as the progeny were frozen in liquid nitrogen and DNA was extracted using a DNeasy kit from Qiagen. The extracted genomic DNA was then quantified using a Nanodrop Spectrophotometer and diluted to 20 nanograms per microliter (μl). One μl of the DNA was added to a PCR cocktail containing 17.6 μl of water, 2.5 μl of 10X Taq buffer with 1.5 mM magnesium, 0.5 μl of 40mM dNTPs, 1 μl of Taq polymerase, and 1.2 μl each of 10 μM left and right primers. The microsatellite primers, developed especially for this species using Primer3 software, amplify a GATA repeat. The fragment was 204 base pairs long in the genetic library created from the original sample. This length was expected to vary among my samples in increments of four as mutations caused different numbers of the repeating GATA unit in different individuals. A specific microsatellite length will be referred to in this paper as an allele. The right primer was fluorescently marked and its sequence was <TTTTCTCGGTTGCAATGGTT>, and the unmarked left primer's sequence was <CTGCTACCCATGGCTCAACT>. I ran a touch-down PCR sequence with an initial denaturing step at 95° C. This was followed by a touch-down annealing sequence of 20 cycles of denaturing (20 seconds), annealing (20 seconds), and extension (30 seconds), the annealing temperature starting at 60° and decreasing 0.5° per cycle. Then there were 15 more cycles run with an annealing temperature of 50° followed by a final extension of 1 minute at 72°. One μl of the PCR product per sample was then added to 9 μl of an 11:1 mixture of Hi-Dye Formamide and ROX 500 size standard and sent to an ABI3130 Genetic Analyzer.

Progeny permitted rough estimates of the out-crossing rates of both populations (Ritland 1986). Out-crossing rates can indicate the degree of self-incompatibility within a population

(Murawski *et al.* 1994). If the number of offspring that had both maternal alleles is low, then outcrossing rates are high and perhaps obligatory outcrossing (SI) is occurring. By comparing the allelic frequencies between the Monteverde and San Luis populations, I was able to make a determination of relative genetic diversity using the Shannon diversity index ($H' = -\sum p_i \ln p_i$) and the direction of gene flow between the populations. Finally, I sent the genotypes of unrelated plants to Judy Stone to run in the Migrate software program (Beerli 2004). She ran the program on the Rocket Calc computer using maximum likelihood estimation of the parameters, the stepwise mutation model, and defaults for the number of long and short chains. The program was able to estimate relative population size and gene flow between populations.

Floral Longevity Manipulations:

Due to the time constraints of the project, I was unable to grow my collected specimens to the size where they would begin to flower. Instead, I used the greenhouse specimens of *W. solanacea* which had been previously collected by Judy Stone. These specimens came from two places: two SC individuals from Vara Blanca located in central Costa Rica (see Bohs 2000a for site description), and nine individuals from the area around Monteverde, four of which were SI, one was SC, and three were “leaky” or secretly self compatible (SSC). The SSC plants have a quantitative breakdown in their stylar RNase activity, allowing the occasional self-fertilization to occur (Stone & Pierce 2006). In addition to these twelve plants, I also experimented on 17 individuals that were the result of a cross between a SC Vara Blanca individual (VB3) and a SI Monteverde individual (MV1). Seven of the crosses were SI and 10 were SC. Thus in total, the manipulations were done on 29 individuals, 11 of which were SI, 4 were SSC, and 14 were SC. The manipulated plants are shown in Table 1.

For each treatment, I marked five ready-to-open buds per plant with lab tape attached to a loop of thread. I then observed each flower twice a day (once mid-morning, once mid-afternoon) until the corolla abscised. Abscission was induced by gently pulling on the corollas of open flowers to simulate field conditions such as wind or biotic disturbance. Flowers were rated as: bud, half-open, open, half closed, closed, dried, or abscised. The length of time the flower remained open was recorded in half-day intervals and measured beginning at the first observed “open” and ending at the last observed “open.” For the control condition, flowers were left unpollinated under a normal greenhouse environment.

I performed manipulations on both the role of gender and of climate on floral longevity. Two of these were pollination treatments designed to manipulate female function. One was a bud pollination where after tagging a bud, I gently opened the corolla with a pair of forceps and rubbed the anther of another plant on the stigma. The second was an open pollination where I waited until the flower opened on its own to apply the foreign anther. This was to account for the possibility that the stigma was unreceptive in the bud. I also ran a forceps control manipulation on 11 of the crossed plants. For each plant I tagged 6 buds, two of which I opened with forceps, but did not pollinate, two I bud pollinated, and two I left as controls. This manipulation was to determine if the physical disturbance to the bud affected floral longevity. Finally I performed an anther removal experiment designed to affect male function. Here I waited until the flower first opened and then removed all four of the anthers with forceps.

The environmental manipulations were designed to alter temperature, light, and moisture. The marked flowers were left alone, as in the control condition. To simulate a cold, cloudy environment, the temperature in the greenhouse was set to 14° C, near the coldest the daily temperature averages in Monteverde. The windows of the greenhouse were covered with

construction paper which considerably reduced light levels. Additionally, I aeriually watered the plants twice a day making sure all leaves were wetted. For the sunny, dry condition the temperature was set to 23° C, near the high for Monteverde. All aerial watering was stopped, the construction paper was removed, and the UV lights in the greenhouse set to a 12-on, 12-off cycle. (In the control, the lights were only turned on when light levels fell below a set level). The exact light levels in both treatments varied considerably due to weather conditions outside, but the sunny day condition averaged about 5 times the light levels as the cloudy day condition, with the control falling somewhere in between. For a summary of the floral manipulation conditions, see Table 2.

RESULTS

Bagging manipulations:

Out of the 40 *W. solanacea* individuals in the bagging experiment, 10 had progressed to the point of being able to tell if fertilization was successful or not. For the four of these individuals in the control (unbagged) treatment, fertilization was successful, and for the six in the bagged treatment, fertilization was unsuccessful. Of the 40 *W. meiantha* individuals, 23 could have their fertilization success determined. Ten of ten had swollen ovaries in the control, and zero of 13 in the bagged treatment.

Pollinator observations:

For both *W. solanacea* and *W. meiantha*, by far the most common visitor was a halictid bee, in the genus *Lasioglossum*, subgenus *Evyllaesus* (103/119 visits or 87%). As described by Stone and Jenkins (2008), the bee actively collected pollen in its scopae (modified, brush-like hairs that hold pollen). It seemed to prefer younger flowers and I occasionally observed it biting

the petals of half-open buds to facilitate opening. All other species of visitors were flies (Diptera) which did not collect pollen and thus were most likely not effective pollinators. The pollinator visitation rates described below, then, were only taken from the *Evyllaesus* visits. I also observed *Evyllaesus* pollinating *W. coccoloboides* flowers. The other two Solanaceous plants I observed had different species of pollinators. *Solanum acerifolium* was visited by another *Evyllaesus* species, distinguished from the first by red on the abdomen and tips of the legs and antennae (the *Evyllaesus* visiting *Witheringia* was all black). The unidentified Solanaceous vine was buzz pollinated by bumble bees (*Bombus* sp.).

The mean visitation rate per hour was 1.1 bees for *W. meiantha* and 0.65 bees for *W. solanacea* although the standard deviations were very high (1.1 and 1.2 respectively). The difference between species was marginally significant (Mann-Whitney U = 184, $N_1 = 32$, $N_2 = 17$, $P = 0.052$). There were fewer *W. solanacea* observations because of a shortage of flowers in bloom. The size of the floral display (1-6 flowers versus 7-12 flowers) among the *W. meiantha* observations did not significantly affect visitation rates (U = 85, $N_1 = 21$, $N_2 = 11$, $P = 0.238$). The weather did show a significant effect on visitation rates (U = 32, $N_1 = 8$, $N_2 = 24$, $P = 0.004$). There were more bee visitations when it was sunny or partly sunny than during cloudy conditions, and no visitations were observed at all during rain harder than a drizzle.

Genetic Analysis:

In total, 76 *W. solanacea* tissue samples were successfully extracted and analyzed: 28 from the San Luis population (9 parents and 19 progeny), 44 from the Monteverde population (8 parents and 36 progeny) and four plants from the greenhouse collected from in between the two populations. From these samples I could construct 11 progeny arrays, four from San Luis and seven from Monteverde, which contained from one to seven offspring (See Table 3). In total,

eight alleles (eight different lengths of the GATA microsatellite repeat) were found, only three of which were represented in the Monteverde population. The four greenhouse samples only had two alleles, both of which were also in the Monteverde population. (See Table 4 for their sizes and relative proportions). There was a more even distribution of alleles in the Monteverde population, but nevertheless, allelic diversity was higher in San Luis (Shannon Value of 1.62 for San Luis, 1.09 for Monteverde). In all progeny arrays there was evidence for outcrossing; that is, at least one of the offspring had an allele not found in the maternal tissue sample. Fourteen of 17 individual offspring (82.3%) were definitely outcrossed in the San Luis Population and 15 of 30 (50%) were at Monteverde. If the population was entirely outcrossed, based on the number of alleles in the San Luis population, we would expect about 12.5% (1 in 8) of offspring to receive a paternal allele that was the same as one of the maternal alleles. That number would climb to 33.3% (1 in 3) for the Monteverde population simply due to reduced allelic diversity.

The Migrate software generated a value for θ , which is four times the effective population size times the mutation rate. If we assume that mutation rate is constant between populations, then θ will give the relative population size. At San Luis, θ was 0.026 and at Monteverde it was 0.013. Therefore the San Luis population is about twice as large as Monteverde. Migrate also estimated that there was no gene flow from Monteverde to San Luis, but there was gene flow from San Luis to Monteverde. The immigration rate from the larger to the smaller population was about 4.4 plants per generation.

Floral Longevity Manipulations:

A summary of the longevity treatments is given in Figure 1. The mean length of time that the flowers remained open without pollination was 2.46 days (sd = 0.60). In this control treatment, as with the other treatments, neither origin of the plant (Monteverde or Vara Blanca)

nor its self-compatibility (SI, secretly SC, or SC) had any significant effect (Location: $U = 275$, $N_1 = 39$, $N_2 = 15$, $P = 0.734$; Self-compatibility: Kruskal-Wallis $\chi^2 = 2.01$, $df = 2$, $P = 0.367$). Flowers that were bud pollinated remained open for 2.15 days ($sd = 0.55$) which was a significantly shorter length of time than the control ($U = 6428$, $N_1 = 136$, $N_2 = 134$, $P < 0.001$). The open pollination treatment (mean = 2.22, $sd = 0.58$) also had shorter floral longevities than the control ($U = 7202$, $N_1 = 136$, $N_2 = 135$, $P = 0.001$) but was not significantly different than the bud pollination ($U = 8208$, $N_1 = 134$, $N_2 = 135$, $P = 0.161$). In the forceps control manipulation, I found no differences between the forceps only, bud pollination, or control conditions ($\chi^2 = 2.01$, $df = 2$, $P = 0.922$). Interestingly, the control flowers in this manipulation were open significantly shorter (mean = 2.06, $sd = 0.66$) than the overall controls ($U = 992$, $N_1 = 136$, $N_2 = 22$, $P = 0.009$) although the bud pollination treatment was statistically the same as before ($U = 1437$, $N_1 = 134$, $N_2 = 22$, $P = 0.841$). Finally, mean floral longevity for the anther removal treatment was 1.24 ($sd = 0.40$) which was significantly shorter than both the control ($U = 946$, $N_1 = 136$, $N_2 = 117$, $P < 0.001$) and even the bud pollination treatments ($U = 1576$, $N_1 = 134$, $N_2 = 117$, $P < 0.001$).

The environmental manipulations proved to have much larger effects than the floral manipulations. The “cloudy day” condition resulted in floral longevities with a mean of 3.55 days ($sd = 1.23$), a full day longer than the control ($U = 4161$, $N_1 = 136$, $N_2 = 137$, $P < 0.001$). Flowers in “sunny day” condition had mean a longevity of only 0.86 days ($sd = 0.43$), more than a day and a half shorter than the control ($U = 344$, $N_1 = 136$, $N_2 = 131$, $P < 0.001$).

DISCUSSION

Self compatibility:

The bagging experiments and progeny arrays both indicate that the Monteverde population of *W. solanacea* is self-incompatible (SI) as suspected. None of the bagged flowers

were able to set fruit and all of the unbagged flowers that I was able to make a determination about were able to do so. The trend was the same for *W. meiantha* indicating that it is also SI—not surprising because of the abundance of that species in the area. The progeny arrays show that every plant (four from San Luis, seven from Monteverde) must have received out-crossed pollen because at least one offspring showed an allele not found in the maternal DNA. There was a slightly higher percentage of offspring having both of the maternal alleles than would be expected by chance (17.7% versus 12.5% in the San Luis population, 50% versus 33% in the Monteverde population). This higher-than-chance occurrence of maternal alleles is not convincing evidence for self-pollination, however, because we can not assume a random spatial distribution of genes in the population. Offspring may fall and germinate more frequently near their parent if the birds that consume the berries remain in the same area to defecate. Therefore pollination may more frequently occur between related individuals.

Pollinator Availability:

The visitation rate of 0.65 visits per flower per hour for *W. solanacea* was higher than the very low visitation rates that Stone and Jenkins (2008) recorded for the site in 2004 and 2006, although it was still lower than the rates they recorded for their other three sites including San Luis. One explanation for why I observed pollinators and they did not is a seasonal difference in pollinator abundance (their field work was conducted in January). More convincingly, Stone and Jenkins observed the scattered individuals along the roadside outside the park. These individuals, unlike my plants did not have congeners growing nearby which may have enticed more pollinators.

The visitation rate for *W. meiantha* (1.1) was closer to what Stone and Jenkins recorded for the San Luis population of *W. solanacea* (1.8 visits/flower/hour in 2004, 1.2 in 2006). I do

not believe the marginally significant difference I recorded between the visitation rates of the two species was due to a preference of *Evylaeus* for *W. meiantha* over *W. solanacea*. The flowers had very similar morphologies and were worked the same way (personal observation). I think the difference was simply due to the fact that *W. meiantha* was far more common and my observations of *W. solanacea* were limited to one or two flowers at a time. The abundance of other *Witheringia* species in the area could support a large *Evylaeus* population which would be available to visit *W. solanacea*. Also the population of *W. solanacea* is concentrated in a small enough area (only along a part of Sendero El Camino) that it becomes likely that bees would occasionally fly from one *W. solanacea* individual to another and not to another species.

Genetic diversity and gene flow:

Assuming the microsatellite I used is representative of the genome as a whole, the Monteverde population is much less genetically diverse than the San Luis population. Moreover, the Monteverde population probably originated from dispersal from the San Luis population. The alleles of the Monteverde plants are a subset of the San Luis alleles (3 of 8) with no new alleles appearing. The alleles shared between the populations are not necessarily the most common ones at San Luis. Allele 201 was most common at both sites (44.7% at San Luis, 38.5 at Monteverde) and the third most common allele (185) for the San Luis population was also found at Monteverde. But allele 197, the second most common at San Luis (18.4%) was missing from Monteverde. Also, allele 193, rare at San Luis (2.6%) was fairly common at Monteverde (32.7%). The changes in relative allelic abundance could represent a founder effect in the Monteverde plants (Mayr 1949). By chance, the first plants to colonize Monteverde from San Luis were individuals with allele 193 and not 197.

The four plants sampled that occurred in between the two populations provide evidence that dispersal from San Luis to Monteverde occurred in a series of dispersal events over small to medium distances rather than one large jump dispersal. They showed two of the same alleles as the Monteverde population and could have been its immediate source, although the small sample size prevents me from drawing any firm conclusions. Birds are the seed-dispersal mechanism for this species, and while birds can disperse the seeds to distances greater than 500 meters (Murray 1988), dispersal events of over several kilometers would be rare and due to a nonstandard means of dispersal (Higgins et al 2003). If this rare long distance dispersal event did occur, perhaps because of an anthropogenic event or an altitudinal avian migration, the scattered plants between the populations could represent secondary dispersal from the Monteverde population. Further genetic analysis of a larger sample combined with mapping the individuals and their alleles could distinguish between the two hypotheses. In either case, *W. solanacea* probably only colonized Monteverde fairly recently, after the construction of the Sendero El Camino opened up a large enough light gap for the plants to compete in a sub-optimal climate.

The result from the genetic analysis that the San Luis population is about twice as large as the Monteverde population seems approximately accurate as based on field surveys (personal observation). Assuming the populations are about 75 and 150 individuals respectively, an immigration rate of 4.4 plants per generation represents a non-trivial number. Of course, “immigration” for plants does not mean individuals are leaving one location in favor of another, but rather that seeds from one population are germinating at the location of the other. Also, because the generation time is unknown and probably extremely variable, the rate of 4.4 only indicates that significant gene flow is occurring, not that a certain number of seeds are arriving at

Monteverde from San Luis over a given period of time. It is important to note that the results of this study do not indicate that there is no gene flow into the San Luis population, just that there is no gene flow from Monteverde. In fact, it is probably likely that gene flow is occurring from scattered populations around San Luis into the main population. Nevertheless, from the perspective of these two populations, San Luis represents a source and Monteverde a sink population (Pulliam 1988). The gene flow from San Luis could continuously maintain the existence of the Monteverde population, but it also could prevent directional selection within the Monteverde population for traits such as self-compatibility, thus keeping the plants from evolving to optimal fitness.

Delayed Selfing:

The fact that no differences in floral longevity occurred across the location or self-compatibility variables may provide evidence for delayed selfing in the SC plants. If prior or competitive SC were occurring, when unpollinated, SC plants would stay open for a shorter time than SI plants because they have assured reproductive success and flowers are costly to maintain (Lloyd 1992). A delayed selfer, however would stay open just as long as a SI plant and then pollinate itself only after not receiving out-crossed pollen. A possible physical mechanism for delayed selfing in *W. solanacea* would be to have the corolla close around the anthers before abscising, bringing residual pollen into contact with the stigma (as in Dole 1990). However I did not observe any differences between SC and SI plants in corolla retention length or structure upon abscising. Therefore perhaps the cause of delayed selfing is the breakdown of the SI mechanism over the life of the flower.

Alternatively, selfing in *W. solanacea* could in fact be competing and not delayed if SC has evolved very recently. If this is the case, the adaptive advantage of closing earlier despite the

assured pollination may not have had time to arise in some or all of the populations. Further pollination and floral manipulations would be necessary to truly distinguish between the possibilities of delayed or competing selfing.

Floral longevity and male versus female function:

The results seem to indicate that floral longevity is affected by both male and female function, but I am skeptical about the latter. Though the differences between the control and bud, and control and open pollination treatments were significant, the difference in means (2.46 days versus 2.15 or 2.22 days) was less than my observation interval of half a day. The standard deviations of the treatments were also larger than the difference. More importantly, in the forceps control manipulation, I found no differences between conditions (control, forceps only, bud pollination) at all. The control here was significantly lower than the overall control. I believe the difference between the two controls was caused by environmental variation, which seemed to have more of an effect than I thought it would (see next section). During the course of the floral manipulation treatments, temperature and humidity were kept constant, but light levels fluctuated according to the outside weather. If the original control was conducted over several cloudy days, and the pollination and forceps manipulation control over more sunny days, this could account for the differences in means.

The difference between anther removal and the control was much larger than the differences with the female function manipulations (1.24 versus 2.45, a difference of more than a day). Moreover, I happened to do anther removal treatment over two different time periods. Therefore it is highly doubtful that the shortened floral longevities observed in the anther removal treatment were simply the result of a series of very sunny days. Interestingly, the anthers of *W. solanacea* are closed in a fresh flower and pollen can only be obtained from the

very top. As the flower matures, the anthers gradually open longitudinally, making the pollen more and more accessible, as well as more likely to be knocked off by wind or other disturbances. The process of the anthers opening usually occurs over the course of two days, or the average length of time a flower is open. After two days, even an unvisited flower may have little pollen left because it has all been knocked off. When the anthers are removed, or if pollinator visitation has been frequent, the pollen would be gone in a much shorter time. Therefore the process of anther maturation may determine floral longevity for an unvisited flower and pollen depletion may shorten it in a frequently visited one. A possible molecular mechanism of how the presence or absence of a flower's own pollen could alter floral longevity would be that the pollen sends a molecular signal that inhibits corolla dehiscence. Its removal would cause a cessation of the signal and cause dehiscence to proceed.

There is an alternative explanation that my results do not exclude. It is possible that the floral longevities in the anther removal treatment were shorter because of damage to the floral tissue. I removed the four anthers with forceps by twisting them off. The anther broke off at the top of the very short filament, so the surface area of tissue damage was minimal, but existent. A plant may prematurely close any damaged flower under the assumption that a damaged flower will not successfully yield offspring. A true test for male floral longevity would be to somehow cover the style to prevent fertilization and then expose the flower to different numbers of pollinator visitations. If male function determined floral length, a flower that was visited more and thus lost its pollen faster, would stay open for a shorter period of time.

If floral longevity is determined by neither male nor female function, each flower may be allotted a fixed energy budget no matter if it is visited or not. The energy spent per flower would be optimized for overall reproductive output per cost. This strategy might make sense for a

relatively short-lived flower such as *W. solanacea* where closing early may be measured in hours rather than days. Under the fixed-energy budget hypothesis, floral longevity would be completely determined by environmental factors.

Floral longevity and climate:

Of the treatments, the “cloudy day” condition had by far the longest floral longevity (3.55 days) and “sunny day” condition by far the shortest (0.86 days). To my knowledge, dramatic variation in the floral longevity of individual plants purely due to environmental conditions has never been described in the literature, although it makes inherent sense. Cool temperatures, low light, and supplemental moisture would greatly reduce transpirative losses making it much less energetically costly to maintain a flower for several days. The reverse would be true for warm temperatures and high light. Interestingly, Monteverde by virtue of its higher elevation, has on average, cooler temperatures and more cloudy days than San Luis. Therefore based on the greenhouse manipulations, I would predict that plants at Monteverde have, on average, longer floral longevity than those at San Luis. The difference would not be due to genetic variation between the populations, but simply to environmental factors.

Keeping flowers open on cloudy days may be adaptive for another reason. My pollinator observations showed that *Evyllaesus* was more active in sunny weather. Therefore a flower that was open for two straight days of rain could greatly improve its chances of being visited if it could open on a third, sunny day. Being able to be open longer at cooler Monteverde may help compensate for the lower pollinator visitation rates that Monteverde plants experience.

General Conclusions:

In light of the results of this study, I offer a hypothesis to explain the persistence of self-incompatibility in the Monteverde population of *W. solanacea*. The hypothesis has two different aspects, and either of them could stand alone, or they could work in concert. The first, broadly, is that the Monteverde population is not able to maximize its fitness. There are three potential reasons that prevent the realization of optimal fitness. One is gene influx from the San Luis population. My results show that significant immigration is occurring from San Luis, where it is evolutionarily advantageous to be SI. Directional selection could not occur in the Monteverde population because these SI individuals are constantly entering the population. SI individuals do not have reduced survivorship at Monteverde but they have a reduced chance at reproductive success if pollen limitation is occurring. Therefore as long as their numbers are being replenished, SC will never become a fixed trait. Also, the population is almost certainly young. Monteverde was only settled about 50 years ago, and while I don't know the exact date of the construction of the Sendero El Camino, it must have been more recently than that. *Witheringia solanacea* would not have been able to colonize this site without the help of the light gap that this large trail created. Therefore even if SC would eventually evolve in the population, it may not have had time to do so. Finally there is the phenomenon of vegetative reproduction from cuttings. To keep the Sendero El Camino clear, the staff of Monteverde uses machetes to cut back the vegetation. The parts of the plants that are hacked off are not picked up. *Witheringia solanacea* has the capacity to clone itself from these cuttings by growing roots from the cut-off stem (personal observation). These clones would have a competitive advantage over plants grown from seed because first, they would have a considerable head start in size, and second,

they would avoid inbreeding depression caused by crossing with the genetically similar individuals that make up the Monteverde population. A particular genome, then, could theoretically live for a very long time by simply being cloned. The end result is a large increase in generation time and the lessening of the evolutionary pressure for SC.

The second part of my hypothesis is that pollen limitation occurs to a lesser extent than might first be assumed. The fact that the common *W. meiantha* shares the same *Evyllaesus* species as a pollinator with *W. solanacea* means there probably is a large population of *Evyllaesus* in Monteverde. Other peripheral populations may not have the availability of so many pollinators and therefore would be faster to evolve SC. Although a visitation rate of 0.65 visits per flower per hour is not high, it was only marginally lower than the very successful *W. meiantha*. The low visitation rate may be partially compensated over the floral lifespan by longer floral longevities. The cool, cloudy weather of Monteverde may mean fewer bees flying around, but it also means less water loss. And when it is sunny, visitation rates for *W. meiantha* jump to over two visits per flower per hour. The floral visitation rates over the entire duration of a *W. solanacea* flower may actually be similar between San Luis and Monteverde. In fact, Stone and Jenkins (2008) found the fruit set rates to be very similar between the two sites.

These results indicate that in a species with varying self-compatibilities, it is not simply that central populations are SI and peripheral ones are SC. Many factors are involved in determining where and why SC evolves including gene flow, population size, generation times, and time scales, as well as pollen limitation. Pollen limitation itself is not inevitable in peripheral populations. Pollinator supplementation from other plant species combined with increased floral longevities due to climatic factors may serve to mediate some of the effects of isolation and small population size.

Floral longevities for this species seem to be more determined by male function than female. The extent to which this can be generalized to other species and the importance it has to individual flowers in the field remains unclear. More convincingly, *W. solanacea* floral longevities can be greatly influenced by the weather. It would be very interesting to run the same environmental manipulations on a wide selection of plant species from different habitats, including ones with longer overall floral longevities. If other species show similar variation, this finding could have a large impact on our understanding of floral physiology and pollination biology.

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Table 1. Individual plants used in floral longevity manipulations. SC = self-compatible. SI= self-incompatible. SSC= secretly self-compatible. VB2a and b, and MV12a and b are genetically identical—one is a cutting of the other. Monteverde individuals were collected along the roads outside the actual reserve. The experimental cross was between MV1 and VB3.

ID Number	Location	Mating System
VB2a	Vara Blanca	SC
VB2b	Vara Blanca	SC
VB3	Vara Blanca	SC
MV1	Monteverde	SI
MV9	Monteverde	SI
MV10	Monteverde	SI
MV14	Monteverde	SI
MV39	Monteverde	SC
MV2	Monteverde	SSC
MV11	Monteverde	SSC
MV12a	Monteverde	SSC
MV12b	Monteverde	SSC
X1	exp. cross	SI
X2	exp. cross	SI
X8	exp. cross	SI
X10	exp. cross	SI
X16	exp. cross	SI
X17	exp. cross	SI
X19	exp. cross	SI
X3	exp. cross	SC
X4	exp. cross	SC
X5	exp. cross	SC
X6	exp. cross	SC
X9	exp. cross	SC
X12	exp. cross	SC
X13	exp. cross	SC
X14	exp. cross	SC
X15	exp. cross	SC
X18	exp. cross	SC

Table 2. Summary of conditions for the floral longevity manipulations. Each floral and environmental manipulation was done with plants from both locations and all three compatibility levels. The control for the floral and environmental manipulations was the same treatment. Numbers in parentheses = sample sizes.

Variable	Conditions
Location (12 plants)	Monteverde (39), Vara Blanca (15)
Self-compatibility	Self-compatible (73), Secretly SC (15), Self-incompatible (50)
Floral Manipulation	Control (136), Bud Pollinated (134), Open Pollinated (135), Anther Removal (117)
Environmental Manipulation	Control (136), "Sunny" Day (131), "Cloudy" Day (137)
Forceps	Control (22), Forceps Only (22), Bud Pollinated (22)

Table 3. Progeny arrays from the San Luis and Monteverde populations. All progeny arrays show evidence of outcrossing.

San Luis			Monteverde		
Aarrays (4)	Alleles		Arrays (7)	Alleles	
Parent 1	201	220	Parent 20	193	201
1.1	201	220	20.1	185	193
1.2	216	220	20.2	193	201
1.3	197	201	20.4	185	201
Parent 2	197	201	20.5	185	193
2.1	197	201	20.6	193	201
2.2	197	201	Parent 21	185	201
2.3	197	205	21.1	185	201
2.4	201	201	21.3	193	201
2.5	197	205	Parent 22	193	201
2.6	185	197	22.1	193	193
Parent 15	185	201	22.2	193	201
15.1	201	201	22.4	193	193
Parent 19	193	197	Parent 24	193	201
19.1	193	220	24.1	193	201
19.2	193	201	24.2	193	193
19.3	193	201	24.3	193	201
19.4	197	201	Parent 27	193	201
19.5	193	201	27.1	201	201
19.6	197	201	27.2	201	201
19.7	193	201	27.3	193	201
			27.4	193	193
			27.5	193	201
			27.6	185	201
			27.7	193	193
			Parent 30	185	185
			30.1	185	201
			30.2	185	185
			30.3	185	201
			30.4	185	185
			Parent 31	185	201
			31.1	185	185
			31.3	185	201
			31.4	185	201
			31.5	185	201
			31.6	185	201

Table 4. Allelic Diversity of a GATA microsatellite repeat in the San Luis and Monteverde populations. Greenhouse plants originally came from an area roughly in between the two larger populations. Alleles named by their length in number of base pairs. The San Luis population had 8 alleles and the Monteverde population only 3. The tested greenhouse plants only had 2 alleles, both also found in the Monteverde population.

allele	San Luis		Monteverde		Greenhouse	
	number	percent	number	percent	number	percent
201	17	44.7	20	38.5	4	66.7
197	7	18.4				
185	5	13.2	15	28.8	2	33.3
220	4	10.5				
205	2	5.3				
193	1	2.6	17	32.7		
208	1	2.6				
216	1	2.6				

Figure 1: The floral longevity of *W. solanacea* after different floral and environmental manipulations. Error bars equal one standard deviation. All treatments shown below are significantly different except between the bud and open pollination. There were no differences across the location of origin or self-compatibility variables.

