EMBRYONIC INBREEDING DEPRESSION Varies among Populations and by Mating System in *Witheringia solanacea* (Solanaceae)\(^1\)

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- **Premise of the study:** Embryonic inbreeding depression is a key influence on mating system evolution and can be difficult to estimate in self-incompatible species. A pollen chase experiment was used to estimate the magnitude of embryonic inbreeding depression in Costa Rican *Witheringia solanacea*, a species polymorphic for self-incompatibility (SI). In a pollen chase experiment, bud self-pollinations are followed after anthesis by outcross pollinations, with a comparable pair of outcross pollinations used as a control. Lowered seed set for the self-precedence treatment indicates embryonic inbreeding depression.

- **Methods:** Embryonic inbreeding depression was assayed for self-compatible (SC) individuals and for SI plants from two populations that differ quantitatively in the onset and enzymatic activity of their SI response. Microsatellite markers were used to assay the selfing rate of a sample of surviving progeny from the prior self-pollination treatment.

- **Key results:** SC individuals showed no evidence of embryonic inbreeding depression. In SI plants, prior self-pollination reduced seed number by 28–70%, depending on population. Microsatellite genotyping revealed that embryonic inbreeding depression was even more severe than estimated by the phenotypic data: for mature fruits resulting from self-pollination precedence, the majority of the progeny were the result of outcross fertilization. SI populations show contrasting levels of embryonic inbreeding depression, with nearly complete embryonic lethality upon selfing in the Monteverde population. In the face of high embryonic inbreeding depression, an increase in selfing rate can evidently occur only under severe pollen limitation.

**Key words:** Costa Rica; embryonic inbreeding depression; evolution; mating system; self-compatibility; self-incompatibility; Solanaceae; *Witheringia solanacea*.

The transition from genetically enforced self-incompatibility (SI) to a selfing mode of life has been traversed by many plant lineages, perhaps more often than any other evolutionary transition in flowering plants (Stebbins, 1974; Igic et al., 2008). Lineages that make this transition from SI to selfing must cope with initially high inbreeding depression. Early-acting inbreeding depression, that is, inbreeding depression operating during embryogenesis, seed maturation, and germination, is typically much higher in predominantly outcrossing than in predominantly selfing species, whereas late-acting inbreeding depression is similar for species with different breeding systems (Husband and Schemske, 1996). The increased homozygosity caused by selfing can eliminate or purge recessive lethals, reducing inbreeding depression and thereby permitting the spread of selfing variants (Lande and Schemske, 1985; Husband and Schemske, 1996; Crnkak and Barrett, 2002; but see Byers and Waller, 1999). The ability to purge early-acting inbreeding depression is evidently key in the evolution of selfing outcrossing (Glémín et al., 2001; Porcher and Lande, 2005), and estimates of its magnitude in SI populations are therefore of interest.

For self-compatible (SC) species, early-acting inbreeding depression can be estimated simply by comparing seed production resulting from self- and outcross-pollinations. This method cannot be used for species with intact SI systems. Selfed seed can be produced by bud pollinations or other methods used to circumvent SI (de Nettancourt, 2000), but the cause of reduced seed set in these cases is due to an unknown combination of residual SI and embryonic inbreeding depression (e.g., Kärkkäinen et al., 1999). Inbreeding depression can be estimated by comparing inbreeding coefficients of adults to juveniles, but this method is recommended only for species with selfing rates of at least 10% (Ritland, 1990). Another approach to estimating inbreeding depression of SI species is to construct outcross matings among relatives (e.g., Vogler et al., 1999). This method has the virtue of permitting a regression of genetic load over inbreeding coefficient; however, it is infrequently used, perhaps due to labor and space requirements.

A pollen chase experiment can be used to estimate the extent of embryonic inbreeding depression for some species with suspected or confirmed SI systems (Krebs and Hancock, 1990; Barrett, 2002). In a pollen chase experiment, self-pollen is applied in the bud, with outcross pollen being applied after anthesis (the SO treatment). As a control, outcross pollen is applied on a paired flower, both in the bud and after anthesis (the OO treatment). To the extent that self-pollen is successful in fertilization, but generates progeny of lowered embryonic fitness, seed set will be lowered in fruits with self-pollen precedence. The reduction in fruit and seed production for SO as opposed to

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OO treatments can be used as an estimate of lethal inbreeding depression acting at the embryonic stage. If the SI system involves degradation of self-pollen tubes in the style, fluorescence microscopy can be used to observe stylar pollen growth to document the success of bud pollinations in overcoming the SI response. Such data can be used to correct potential errors of interpretation that arise when partial SI persists in the bud (Fig. 1).

Estimates of inbreeding depression in species where variation in SI has been documented can test whether variation in SI corresponds with variation in inbreeding depression (e.g., Vogler et al., 1999; Busch, 2005). We have found both qualitative and quantitative variation in the SI response of *Witheringia solanacea* L’Her, a tropical shrub occurring in Central America. Most individuals are SI, but autogamous SC variants have been found in two very small isolated Costa Rican populations (Stone et al., 2006). An undescribed SC variant occurs sympatrically with a large SI population in the Monteverde region of Costa Rica and in at least one small isolated population farther west (Stone and Jenkins, 2008). In addition, two large SI populations differ quantitatively in the strength of their SI response, with the Monteverde population producing lower levels of stylar RNase and permitting more self-pollen tubes to grow to the base of the style in bud pollinations than the Las Cruces population (Stone et al., 2006).

To gain insight into the mating system evolution of this species, we used a pollen chase experiment to compare the level of early-acting inbreeding depression for plants that varied in SI response. We compared fruit and seed set for SO vs. OO pollinations for SC plants and for plants from the Las Cruces and Monteverde populations that differ quantitatively in the strength of their SI response. For SI plants, we compared germination rates for seeds resulting from the two treatments. We also genotyped seedlings at five microsatellite loci to estimate selfing rate of surviving progeny, which we used in concert with previously published pollen tube data to estimate the relative fitness of selfed and outcrossed embryos. We hypothesized that selfing lineages would show decreased inbreeding depression due to purging of genetic load. We also hypothesized that the two SI populations might show differences in inbreeding depression corresponding to quantitative variation in the SI response. If the less robust SI system in Monteverde has led to a history of selfing, that population might show lower inbreeding depression due to partial purging of genetic load.

**MATERIALS AND METHODS**

*Study species and populations*—*Witheringia solanacea* is a small shrub that extends from southern Mexico and the Caribbean to South America (D’Arcy, 1973). It possesses the gametophytic SI system characteristic of the Solanaeae, where allele-specific S-RNases expressed in the style cause the degradation of RNA, and therefore the death of pollen tubes bearing the cognate S-allele (McClure et al., 1990). The floral biology of *W. solanacea* is described in detail by Bohs (2000). Its flowers are pollinated by small bees and its fleshy fruits are bird-dispersed. *Witheringia solanacea* also reproduces prolifically by vegetative reproduction in gardens and along trailsides. Cuttings were collected in the field from Costa Rican populations and maintained in the greenhouse at Colby College. Populations and locations have been described by Stone and Pierce (2005) and Stone et al. (2006). Plants were sampled from populations in three geographic regions. Self-incompatible plants were sampled from large populations in the Las Cruces region, at 1180–1600 m a.s.l. in southwestern Costa Rica, and in the Monteverde region in northwestern Costa Rica, extending from 1100 m elevation in the San Luis Valley to 1500 m a.s.l. in the village of Monteverde. The SC individuals came from the very small population at Vara Blanca at 1360 m a.s.l. in central Costa Rica and from the undescribed but morphologically distinct variety at lower elevations in the Monteverde region. Treatments were carried out on seven SI individuals from Las Cruces and 13 from Monteverde, in addition to the two strongly SC individuals available from Vara Blanca and three from Monteverde. The low sample size of SC plants was a consequence of the relative scarcity of the SC phenotype in Monteverde and the tiny overall size of the population in Vara Blanca, which over a decade of monitoring has never been found to contain more than six individuals.

**Pollen chase experiment and pollen tube growth**—For each trial, we conducted 3–6 pairs of SO and OO treatments over a period of 1 week to 10 days. During the summers of 2004–2007, 48 trials were carried out: seven on SC plants, 14 on Las Cruces plants, and 27 on Monteverde plants. Pollen was collected in clean vials and applied using clean toothpicks to styles of unopened flowers, 24 h before anthesis. Buds at this stage can be identified because they are still tightly closed but have developed the green splotches that decorate the corolla of mature flowers. Pairs of buds were chosen on each recipient, the bud for the SO treatment receiving self-pollen, and the bud for the OO treatment receiving outcross pollen. Twenty-four hours later, outcross pollen from a single compatible donor was applied to both flowers. Previous work in our laboratory has shown that although bud self-pollinations produce fewer pollen tubes at the base of the style than do bud outcross-pollinations, both types of pollinations produce tubes that grow past midstyle at 24 h and reach the base of the style by 48 h. Therefore, we assume that 24 h priority is sufficient to ensure that bud self-pollen tubes reach the base of the style before the subsequent outcross pollen tubes do. In total, 852 pollinations were done, half of which were bud pollinations. Fruits were allowed to ripen, and seeds were counted.

**Germination and genotyping**—To compare germination rates among treatments for SI populations, we planted seeds from fruits representing all available mothers and cross types. In most instances, 20 seeds were planted per cross, but in some cases all seeds per fruit were planted. We planted five seeds per cell in a 128-cell flat filled with Metro-Mix 200 (Sun-Gro Horticulture, Vancouver, British Columbia, Canada), alternating the position of cells occupied by seeds from SO and OO fruits. Seeds were covered with a fine layer of vermiculite, and the flats were placed in a mist bench under 12-h light/12-h dark in the greenhouse at Colby College. Liquid fertilizer was applied weekly once initial sprouts appeared. Germination was recorded at 6 wk, at which time seedlings were transplanted to individual cells and randomly placed on the bench. Seedlings were harvested by clipping with scissors at ground level at 12 wk and air dried.

To detect the level of self-fertilization resulting from the SO treatment, we used five microsatellite loci to genotype progeny and parental plants. We genotyped 73 progeny from 13 SO pollinations on eight maternal plants. Microsatellite-enriched libraries were developed by Craig Newton of ATG genetics (Vancouver, Washington, USA). We amplified inserts using universal m13 primer, and witheringia solanacea also reproduces prolifi-
primers, sequenced the inserts using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, California, USA), and designed primers using the program Primer3 (Rozen and Skaletsky, 2000). Primer sequences appear in Table 1.

We extracted genomic DNA using the Qiagen (Germantown, Maryland, USA) DNeasy Plant Mini Kit on 40 mg dried leaf material, using dry ice to keep the tissue brittle for grinding in the Qiagen tissue lyser. PCR was carried out in 25 µL volumes in standard buffer with 1.5 mM MgCl₂, 0.8 mM dNTPs, 0.5 µM each primer, 2 U Taq polymerase, and 10 ng genomic DNA. In each reaction, the left primer was fluorescently tagged with 6-FAM. A touchdown PCR program was used, with the annealing temperature decreasing from 60°C to 50°C by 0.5°C each cycle for the first 20 cycles and kept at 50°C thereafter. Other PCR conditions were an initial denaturation at 95°C for 2.5 min; and 35 cycles with 95°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s; with a final extension at 72°C for 10 min. PCR products were suspended in denatured formamide with ROX-500 size standard (Applied Biosystems) and run under standard conditions on an ABI 3130 genetic analyzer. Peaks were visualized using GeneScan and scored manually.

Data analysis—For the two SI populations, we used a two-way ANOVA to compare fruit and seed set of SO and OO treatments, with population as a random factor and treatment as a fixed factor. Maternal plant was nested within population. Treatment and the population × treatment interaction were tested over treatment × maternal plant nested within the population. To meet assumptions of ANOVA, proportion fruit set was arcsine-transformed, and seed number per plant per treatment was averaged and square-root transformed. The low number of available SC plants made it impossible to evaluate assumptions of parametric tests. For analyses involving SC plants, we used a Wilcoxon’s matched pairs signed rank test for paired comparisons, a Mann–Whitney test for unpaired comparisons, and Kruskal–Wallis tests for comparisons involving all three populations. Nonparametric tests were also used for germination data. Mann–Whitney tests were done by hand, and the program Stata 10.1 (StataCorp, College Station, Texas, USA) was used for all other analyses. The cumulative embryonic fitness decline associated with self-precedence was calculated for each individual according to the formula: $\delta = 1 - (w_T / w_{SO} \cdot w_{OO})$, where $w_T$ = proportion of SO pollinations producing fruit, $w_{SO}$ = proportion of OO pollinations producing fruit, and $w_{OO}$ = average seed number resulting from SO pollinations, $w_{SO}$ = average seed number resulting from OO pollinations.

To estimate the selfing rate for SO fruits, we calculated the probability, at each locus, that each offspring was a product of self-fertilization. If any locus revealed a definitive outcrossing event, the progeny was recorded as an outcross. If no loci revealed definitive outcrossing, the likelihood of selfing was found by multiplying the probabilities across loci.

Pollen tube data, together with selfing rate estimates, allowed us to account for residual SI in the estimate of embryonic inbreeding depression. Bud self-pollination of SI plants from the Monteverde population yielded an average of 37 pollen tubes at the base of the style, whereas bud self-pollination of SI plants from Las Cruces yielded an average of only 13 pollen tubes at the base of the style (Stone et al., 2006). The number of pollen tubes at the base of the style after bud self-pollinations provides the maximum number of selfed seeds that could be expected under bud self-pollination. Any seeds in excess of that expectation should be due to the outcross pollination done on the second day of the pollen chase experiment. An expected selfing rate can therefore be obtained by comparing the number of self-pollen tubes with the total number of seeds. The observed selfing rate of surviving progeny as obtained by microsatellite genotyping was compared with the expected rate to estimate the relative postzygotic fitness of selfed progeny.

RESULTS

Fruit set for the OO treatment averaged 52% and did not differ across populations ($H = 3.020, df = 2, P = 0.22$). For the SI plants, mean fruit set for SO was 24%, significantly less than for the OO treatment (Fig. 2A, $F_{1,18} = 34.2, P < 0.001$). For the SC plants, fruit set for the SO treatments averaged 67%, which was not significantly different from the OO treatment ($z = 0.54, N = 5$ pairs, $P = 0.60$). For those fruits that set seeds, seed set was reduced by self-pollen precedence in SI plants (Fig. 2B, $F_{1,12} = 6.25, P < 0.03$) but not in SC plants ($U = 18, n_1n_2 = 24, P = 0.20$). There was an interaction between population and treatment, with Monteverde plants having a greater reduction than Las Cruces plants in seeds per fruit with self-pollen precedence ($F_{1,13} = 6.25, P < 0.03$). Loss of cumulative fitness at the embryonic stage resulting from self-bud pollination averaged
significantly differ by population (progeny from SO pollinations were likely to be self-fertilized. For plants from the Monteverde population, 15% of surviving progeny from SO vs. OO (Table 2). Germination rate was just over 50% and did not differ by treatment or population (Table 3).

The great majority of the progeny genotyped from the SO treatment showed definitive evidence of outcrossing by displaying a paternal allele that was not present in the mother (Table 4). For plants from the Las Cruces population, 28% of surviving progeny from SO pollinations were likely to be self-fertilized. For plants from the Monteverde population, 15% of surviving progeny from SO pollinations were likely to be self-fertilized. The estimated outcrossing rates of surviving progeny did not significantly differ by population ($U = 30, n_1 n_2 = 40, P > 0.05$). Individual mothers were not always consistent in the outcrossing rates of replicate progeny arrays. For example, the SO treatment of plant MV4 yielded outcross progeny in one trial and predominantly selfed progeny in another.

Estimates of cumulative inbreeding depression increased greatly when controlled for residual SI (Table 5). Bud self-pollinations were not completely successful in overcoming the SI response, so that we could expect selling rates of only 0.32 and 0.85 for the Las Cruces and Monteverde populations, respectively. The observed selling rate of surviving embryos at Las Cruces was similar to the expected, but the observed selling rate of surviving embryos at Monteverde was much lower than expected. Once residual SI was taken into account, embryonic inbreeding depression was estimated to be 0.49 for the Las Cruces population and 0.97 for the Monteverde population. Inbreeding depression for the Monteverde population was significantly greater than for the Las Cruces population ($U = 82.5, n_1 n_2 = 91, P < 0.01$).

**DISCUSSION**

The pollen chase experiment demonstrated that SC individuals had markedly lower embryonic inbreeding depression than did SI individuals (Table 2). Such a contrast in inbreeding depression is widely found in interspecific comparisons between SI and SC species (Husband and Schemske, 1996). Correlations between selling rate and inbreeding depression have also been observed in intraspecific comparisons among populations or lineages that differ in selling rate (Chang and Rausher, 1999; Vogler et al., 1999; Takebayashi and Delph, 2000; Fishman, 2001; Stone and Motten, 2002; Busch, 2005; Goodwillie and Knight, 2006; Kennedy and Elle, 2008), although the extent to which we should expect to see such lineage-specific inbreeding depression has been controversial (Schultz and Willis, 1995).

**Table 2.** Cumulative fitness decline through both fruit and seed set of flowers receiving bud self-pollinations (SO) relative to those receiving bud-outcross pollinations (OO).

<table>
<thead>
<tr>
<th>Population</th>
<th>$N$</th>
<th>Embryonic inbreeding depression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Las Cruces</td>
<td>10</td>
<td>0.28 ± 0.86</td>
</tr>
<tr>
<td>Monteverde</td>
<td>21</td>
<td>0.70 ± 0.51</td>
</tr>
<tr>
<td>SC plants</td>
<td>4</td>
<td>0.02 ± 0.68</td>
</tr>
</tbody>
</table>

*Notes:* Open flowers were outcross-pollinated in both treatments. Values are means ($±$SD). $N =$ Number of trials for which both fruit and seed set were recorded. A trial consisted of 3–6 pairs of SO and OO treatments.

**Table 3.** Mean germination rates ($±$SD) at 6 wk.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fruits</th>
<th>Seeds</th>
<th>Las Cruces</th>
<th>Fruits</th>
<th>Seeds</th>
<th>Monteverde</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO</td>
<td>4</td>
<td>173</td>
<td>0.48 ± 0.27</td>
<td>4</td>
<td>11</td>
<td>0.53 ± 0.32</td>
</tr>
<tr>
<td>OO</td>
<td>9</td>
<td>266</td>
<td>0.51 ± 0.32</td>
<td>15</td>
<td>296</td>
<td>0.59 ± 0.27</td>
</tr>
</tbody>
</table>

*Notes:* SO, self-pollen was applied in the bud the day before outcross pollination; OO, outcross-pollen was applied in the bud the day before outcross pollination.

Key to the distinction between inter- vs. intraspecific purging of genetic load is the degree of reproductive isolation that may occur at the intraspecific level. In the current study, three of the selfing individuals derived from an undescribed variety of *W. solanacea*. It is not yet known whether the two varieties are interfertile, but the morphological distinctness suggests that reproductive barriers are present. The other two selfing individuals originated from the Vara Blanca population, which segregates for morphologically indistinguishable fully SI and SC individuals (Bohs, 2000; Stone et al., 2006), suggesting the possibility of lineage-specific inbreeding depression in the absence of any reproductive isolation other than mating system.

Plants from the two SI populations differed in the extent to which the SO treatment reduced seed set, with plants from the Monteverde population showing a 70% loss in production, whereas the plants from Las Cruces declined only ~30%. Estimates of inbreeding depression increased for both populations when pollen tubes and genotype data were taken into account, with embryonic inbreeding depression at 97% for Monteverde and 49% for Las Cruces (Table 5). The incorporation of pollen tube and genotype data probably results in an overestimate of inbreeding depression because the expected selling rate does not account for attrition of pollen tubes from the base of the style to the ovule. The true level of inbreeding depression should lie between the first and second estimates, which are both quite high, as well as distinct by population. The observation of more-severe inbreeding depression in the Monteverde population contradicts our initial hypothesis that the quantita-
Table 5. Embryonic inbreeding depression in self-incompatible populations, corrected for residual self-incompatibility in bud pollinations by incorporating pollen tube data and microsatellite results.

<table>
<thead>
<tr>
<th>Population</th>
<th>Expected selfing rate a</th>
<th>Observed selfing rate b</th>
<th>Relative postzygotic fitness of selfed embryos c</th>
<th>Relative fitness of SO for fruit set</th>
<th>Cumulative embryonic inbreeding depression d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Las Cruces</td>
<td>0.32 ± 0.06</td>
<td>0.28</td>
<td>1.08 ± 0.25</td>
<td>0.48 ± 0.13</td>
<td>0.49 ± 0.18</td>
</tr>
<tr>
<td>Monteverde</td>
<td>0.85 ± 0.11</td>
<td>0.15</td>
<td>0.07 ± 0.03</td>
<td>0.34 ± 0.10</td>
<td>0.97 ± 0.01</td>
</tr>
</tbody>
</table>

Notes: Values are means (for selfing rate) or means ± standard error. N = 7 plants from Las Cruces and 13 plants from Monteverde.

a Estimated proportion of self-pollen tubes reaching ovary in self-pollen precedence (SO) treatment = number of pollen tubes that reach the base of the style in self-bud pollinations/number of seeds produced in bud outcross-pollinations (OO).

b Population average from microsatellite data.

c Ratio of observed/expected selfing and outcrossing rates, (OIE)_{selfed}/(OIE)_{outcrossed}.

Cumulative relative fitness is the product of relative fitness for fruit set and relative fitness for seed set.

References:


