Close clustering of anthers and stigma in *Ipomoea hederacea* enhances prezygotic isolation from *Ipomoea purpurea*

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Summary

- Theory predicts that, for taxa that are already substantially postzygotically isolated but for which hybrid mating is still costly, enhanced prezygotic isolation will be favored. Here, we tested this hypothesis by examining the potential contribution of one particular floral trait, herkogamy, to prezygotic isolation between two species of morning glory, *Ipomoea hederacea* and *Ipomoea purpurea*.
- This trait was experimentally manipulated to determine whether it is a likely prezygotic isolating barrier in naturally pollinated arrays in the field.
- Emasculated *I. hederacea* flowers set significantly fewer seeds than did control flowers, indicating that clustering of anthers and stigma in *I. hederacea* enhances prezygotic isolation from *I. purpurea*. We hypothesize that this occurs through some combination of mechanical protection and increased self-pollination, with the effect of mechanical protection estimated to be 30% greater than the effect of increased selfing.
- Our results identify stigma–anther proximity as a likely prezygotic isolating barrier and target of selection in the presence of heterospecific pollen flow, and provide motivation for further study of the role of floral morphology in reproductive isolation in this system.

Key words: anthers, *Ipomoea hederacea*, *Ipomoea purpurea*, morning glory, prezygotic isolation, stigma.


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Introduction

Theory predicts that, for taxa that are already substantially postzygotically isolated, but between which hybrid matings commonly occur and are costly, the enhancement of prezygotic isolation will be favored (Servedio, 2001; Servedio & Noor, 2003). While several purported examples of such reproductive character displacement in plants are known, only Levin (1985) and Caruso (2000) have characterized how interactions between coflowering species generate selection for the evolution of enhanced prezygotic isolation. In this and subsequent studies, we characterize selection for prezygotic isolation generated by interactions between two species of morning glory, *Ipomoea hederacea* and *Ipomoea purpurea*. These two species commonly grow together throughout much of the south-eastern USA. Although substantial postzygotic barriers between these two species prevent the production of viable hybrids in nature, prezygotic barriers between them are weak (Ennos, 1981; Stucky, 1984; Iwao, 1995). Results from hand-pollination studies suggest that hybrid matings are likely to be costly, in particular for *I. hederacea*, which experiences considerable reductions in viable seed set when exposed to pollen from *I. purpurea* (Guries, 1978; Stucky, 1985).

Characterizing selection for prezygotic isolation requires prior determination of which traits are likely to confer isolation. As a prelude to documenting patterns of selection, we demonstrate here that, in *I. hederacea*, close clustering of
anthers and stigmas contributes to prezygotic isolation between this species and \textit{I. purpurea}, and thus is a likely target of selection for enhanced isolation. Although similar in shape, \textit{I. hederacea} and \textit{I. purpurea} flowers exhibit marked differences in exertion of reproductive parts, primarily as a result of differences in style length and anther position. Whereas in \textit{I. purpurea} the stigma is generally exerted above the anthers, in \textit{I. hederacea} the stigma is commonly found at the same level as, and tightly surrounded by, the anthers (Ennos, 1981). This close clustering (absence of herkogamy) is absent not only in \textit{I. purpurea}, but also in the related species \textit{Ipomoea indica}, \textit{Ipomoea nil}, \textit{Ipomoea tricolor}, and \textit{Ipomoea lindheimeri}. Because these species, along with \textit{I. hederacea}, form a clade in which \textit{I. indica} is sister to \textit{I. hederacea} and the other species are outgroups for this pair (Miller \textit{et al.}, 2004), close clustering appears to be a derived trait in \textit{I. hederacea}. It has previously been hypothesized to play a role in shielding \textit{I. hederacea} from costly heterospecific pollen flow from \textit{I. purpurea} (Ennos, 1981; Stucky, 1984), but until now this hypothesis has not been examined experimentally.

**Materials and Methods**

**Study system**

\textit{Ipomoea hederacea} (L.) Jacquin and \textit{Ipomoea purpurea} (L.) Roth (Convolvulaceae) are self-compatible annual vines that commonly co-occur as weeds of agricultural fields and along roadsides throughout the south-eastern USA. The history of the coexistence of these two species in North America is not fully known. Some authors believe that \textit{I. purpurea} is native to Central America (Gray, 1886; Barkley, 1986; Hickman, 1993), but it has been documented in the eastern USA since at least the early 1700s (Pursh, 1814). There is some disagreement over whether \textit{I. hederacea} is native to the USA (Mohr, 1901; Stevens, 1948) or was introduced from tropical America (Shreve \textit{et al.}, 1910; Strausbaugh & Core, 1964; Long & Lakela, 1971; Wunderlin, 1982; Mahler, 1988); in either case, herbarium specimens show that \textit{I. hederacea} has been in the USA for at least 150 yr (Bright, 1998).

Both species have trumpet-shaped flowers that are showy but short-lived, typically opening before dawn and wilting by the afternoon of the same day. \textit{Ipomoea hederacea} flowers are light to dark blue in color with a white throat. \textit{Ipomoea purpurea} flowers are slightly larger in size and range in color from blue to purple to bright pink to white. Both species have five stamens of variable height and one style (Fig. 1a,b), but whereas in \textit{I. purpurea} the stigma is generally exerted above the anthers, in \textit{I. hederacea} the stigma is commonly found at the same level as, and tightly surrounded by, the anthers (Ennos, 1981). In North Carolina, seeds of both species germinate between May and August. Plants begin flowering c. 6 wk after germination and continue to produce flowers until they are killed by the first hard frost in the fall. Once fertilized, seeds take c. 4 wk to mature. Individual fruits typically contain one to six seeds, although fruits containing as many as eight seeds have been observed (pers. obs.).

Premating barriers between these two species are extremely weak. Although \textit{I. hederacea} typically initiates blooming 1–2 wk earlier than \textit{I. purpurea} (pers. obs.), their flowering phenologies are largely overlapping, and both species are highly attractive to the bumblebees \textit{Bombus pennsylvanicus} and \textit{Bombus impatiens}, their primary pollinators. Results from pollinator preference studies are mixed (Ennos, 1981; Stucky, 1984; Iwao, 1995) and show no consistent tendency for pollinators to prefer either species. Interspecific pollen transfer is thus not only quite possible in natural populations, but also likely to be costly, in particular for \textit{I. hederacea}. Although viable hybrids have not been reported in nature, results from hand-pollination studies indicate that pollen from \textit{I. purpurea} is able to germinate on and grow through the styles of \textit{I. hederacea}, but gives rise to inviable hybrid seeds (Guries, 1978). Competition between conspecific and heterospecific pollen for fertilization
in *I. hederacea* is also strong: when equal amounts of pollen from the two species are placed simultaneously on the stigmas of emasculated *I. hederacea* plants, seed set is reduced by > 50% relative to applications containing *I. hederacea* pollen alone (Stucky, 1985). The consequences of heterospecific pollen transfer are asymmetrical, however, as *I. hederacea* pollen is ineffective at fertilization in *I. purpurea*, and mixed hand-pollinations have no effect on *I. purpurea* seed set (Guries, 1978; Stucky, 1985). This asymmetry is not entirely surprising, as *I. purpurea* styles are generally longer, and in hybrid crosses in plants the longer-styled taxon is generally the more successful pollen parent (Kiang & Hamrick, 1978; Levin, 1978; Williams & Rouse, 1988, 1990; Gore *et al*., 1990).

**Experimental arrays**

Effects of close clustering of anthers and stigma in *I. hederacea* on susceptibility to heterospecific pollen flow from *I. purpurea* were examined using a series of experimental arrays assembled in the Duke University glasshouses in the summer of 2002. *Ipomoea hederacea* and *I. purpurea* plants for these arrays were drawn from a collection of single-seed-descent inbred lines each originally collected as seed from a different individual in natural populations in central North Carolina. Six to eight seeds from each of eight *I. hederacea* lines and six *I. purpurea* lines were planted and grown under identical conditions in a climate-controlled glasshouse for use in the experiment.

Each experimental array consisted of a 6 × 6 arrangement of potted plants, assigned to random positions within the array and separated by 0.75 m. Within each array, 18 randomly placed target *I. hederacea* plants were interspersed checkerboard fashion with 18 *I. purpurea* plants. Arrays contained two to six plants from each of four to eight of the *I. hederacea* lines already described, and four to eight plants from each of three to six of the *I. purpurea* lines, depending on availability of flower buds on any given day. Two types of array were constructed: one in which all 18 *I. purpurea* plants in an array were emasculated the evening before flower opening and were thus incapable of exporting pollen (− *purpurea* pollen), and one in which all 18 *I. purpurea* plants were left intact (− *purpurea* pollen). Within each array, each *I. hederacea* plant was subjected to one of two treatments: half of the plants in each *I. hederacea* line in an array were emasculated before anther dehiscence by cutting a small slit in the corolla tube and pulling out the anthers with forceps (hederacea emasculated), and half were left intact as controls (hederacea control). Per flower seed set of emasculated and control *I. hederacea* flowers was measured in two different types of array: one in which all 18 *I. purpurea* plants were emasculated and thus incapable of exporting pollen (− *purpurea* pollen), and one in which all 18 *I. purpurea* plants had intact anthers (+ *purpurea* pollen). Each array was run for 24 h for a total of 10 separate runs per treatment.

This array design allowed us to manipulate the potential for heterospecific pollen flow while keeping the species composition, patch size, and plant density constant. To standardize floral display size, all but one flower bud was removed from each plant the evening before that plant was placed in an array. To avoid resource competition, experimental plants were left in their pots rather than being planted in the soil. The relative proportions of the different *I. purpurea* flower color types were kept constant throughout the experiment. No *Ipomoea* plants were known to occur within 1 km of the site. Arrays were assembled at a rate of one per day and with treatments in random order between 8 July and 2 August 2002, as weather permitted. Each array was run for 24 h for a total of 10 replicates per treatment.

To account for any differences in pollinator visits to *I. hederacea* plants in the different treatment combinations, we also recorded pollinator visitation. Every plant in an array was numbered and labeled for easy identification from a distance. All bumblebee foraging bouts that occurred in the arrays were observed between 06:00 and 08:00 h on each of 13 mornings between 8 July and 1 August 2002 (six replicates for the + *purpurea* treatment and seven replicates for the − *purpurea* treatment). A foraging bout consisted of all the visits by a single bee to plants in an array. For each foraging bout we recorded the sequence and identity of each plant visited in the array. Because we were interested not in pollinator preferences during individual foraging bouts *per se* but rather
in differences in the total number of pollinator visits received by individual plants and potential effects of differential pollinator visitation on seed set, our observations of pollinator visitation were translated into number of visits per *I. hederacea* flower per hour.

In the afternoon of each run, each *I. hederacea* flower in an array was tagged to indicate date and treatment. Once the resulting seed capsules were mature we collected them, counted the number of seeds in each capsule, and then estimated the average number of *I. hederacea* seeds produced per flower for each treatment combination. Tagged flowers that ultimately did not produce any fruits were recorded in our analyses as having zero seed set. The data on per flower seed set were analyzed via analysis of variance (ANOVA) with a split plot design, with ‘+ purpurea pollen’ and ‘– purpurea pollen’ as the whole-plot treatment, and ‘hederacea emasculated’ and ‘hederacea control’ as the subplot treatment (Sokal & Rohlf, 1995). The full model included the heterospecific pollen flow treatment (+ purpurea/– purpurea) and *I. hederacea* emasculation treatments as fixed effects, line and replicate as random effects, and all two- and three-way interactions. After running the full model all nonsignificant interaction terms were eliminated. Results reported here are from a reduced model running the full model all nonsignificant interaction terms.

We report results of analyses using the appropriate means squares as error terms (Sokal & Rohlf, 1995). Assumptions of normality were tested using the Shapiro–Wilk statistic (Sokal & Rohlf, 1995). The full model included the heterospecific pollen flow treatment (+ purpurea/– purpurea) and *I. hederacea* emasculation treatments as fixed effects, line and replicate as random effects, and all two- and three-way interactions. After running the full model all nonsignificant interaction terms were eliminated. Results reported here are from a reduced model containing only main effects and significant interaction terms. We report results of analyses using the appropriate means squares as error terms (Sokal & Rohlf, 1995). Assumptions of normality were tested using the Shapiro–Wilk statistic (Sokal & Rohlf, 1995). Tests for equality of the subplot variances were performed using the PROC TTEST in SAS (SAS Institute, Cary, NC, USA), and showed no significant deviation from equality ($F = 1.16$; $P = 0.57$).

### Results

**Pollinator visitation**

Plants in our experimental arrays were visited almost exclusively by *Bombus* bumblebees, although rare visits by hummingbirds and butterflies were also observed. A total of 286 bumblebee foraging bouts were observed in arrays over the 13 mornings in which pollinator visitation was observed, 78% of which included visits to both *I. hederacea* and *I. purpurea*. Pollinators visited an average of 5.0 ($\pm 0.19$ standard error (SE)) flowers per foraging bout, comprising an average of 2.80 ($\pm 0.11$ SE) visits to *I. hederacea* flowers and 2.20 ($\pm 0.11$ SE) visits to *I. purpurea* flowers.

The rate of pollinator visitation to *I. hederacea* flowers differed significantly among treatments (Table 1). *Ipomoea hederacea* plants in the + purpurea pollen treatment were visited at the same rate as those in the – purpurea pollen treatment ($P = 0.52$), although in both treatments emasculated *I. hederacea* flowers received only two-thirds to three-quarters of the number of visits per hour as did control flowers (Table 1). This result is not entirely surprising, as bumblebees are known to be both nectar and pollen foragers (Spencer-Booth, 1965). While emasculated *I. hederacea* flowers received significantly fewer visits per hour than control flowers, this effect was no more or less severe for *I. hederacea* in the + purpurea pollen treatment than for those in the – purpurea pollen treatment, as indicated by a nonsignificant interaction term between interspecific pollen flow treatment and *I. hederacea* treatment (Table 1b). Pollinator visitation to *I. hederacea* was also influenced by day-to-day fluctuations in abiotic conditions, as indicated by a significant replicate effect on visitation rate (Table 1b).

### Table 1

Effect of emasculation and interspecific pollen flow treatments on rates of pollinator visits to *Ipomoea hederacea* in experimental arrays of *I. hederacea* and *Ipomoea purpurea* ($n = 225$). (a) Mean rates of pollinator visits to *I. hederacea* flowers in each treatment combination; (b) analysis of variance of rates of pollinator visits to *I. hederacea* in experimental arrays of *I. hederacea* and *I. purpurea*.

(a)

<table>
<thead>
<tr>
<th></th>
<th>Hederacea control, purpurea pollen absent</th>
<th>Hederacea control, purpurea pollen present</th>
<th>Hederacea emasculated, purpurea pollen absent</th>
<th>Hederacea emasculated, purpurea pollen present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visits h$^{-1}$</td>
<td>1.97 ± 0.16</td>
<td>1.79 ± 0.12</td>
<td>1.55 ± 0.15</td>
<td>1.12 ± 0.14</td>
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</tbody>
</table>

(b)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Type III SS</th>
<th>Mean square</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterospecific pollen flow (+ purpurea/– purpurea)</td>
<td>1</td>
<td>4.30</td>
<td>4.30</td>
<td>0.44</td>
<td>0.52</td>
</tr>
<tr>
<td>Replicate</td>
<td>11</td>
<td>107.46</td>
<td>9.77</td>
<td>7.26</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><em>I. hederacea</em> treatment (emasculated/control)</td>
<td>1</td>
<td>17.90</td>
<td>17.90</td>
<td>13.30</td>
<td>0.0003</td>
</tr>
<tr>
<td>Inbred line</td>
<td>7</td>
<td>8.09</td>
<td>1.16</td>
<td>0.86</td>
<td>0.54</td>
</tr>
<tr>
<td>Heterospecific pollen flow * I. hederacea treatment</td>
<td>1</td>
<td>1.58</td>
<td>1.58</td>
<td>1.18</td>
<td>0.28</td>
</tr>
<tr>
<td>Error</td>
<td>203</td>
<td>273.14</td>
<td>1.35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in (a) are mean ± standard error. d.f., degrees of freedom; SS, sums of squares.
Table 2  Effect of emasculation and interspecific pollen flow treatments on Ipomoea hederacea seed set in experimental arrays. (a) Mean seed set of both control and emasculated I. hederacea plants in the presence and absence of pollen flow from Ipomoea purpurea (n = 358); (b) analysis of variance of I. hederacea seed set (seeds per flower) in the presence and absence of pollen flow from I. purpurea (n = 358)

(a)

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>Type III SS</th>
<th>Mean square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replicate</td>
<td>18</td>
<td>165.5</td>
<td>9.19</td>
<td>4.44</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Inbred line</td>
<td>7</td>
<td>50.14</td>
<td>7.16</td>
<td>3.46</td>
<td>0.002</td>
</tr>
<tr>
<td>Heterospecific pollen flow × I. hederacea treatment</td>
<td>1</td>
<td>0.03</td>
<td>0.03</td>
<td>0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>Replicate × I. hederacea treatment</td>
<td>18</td>
<td>761.22</td>
<td>761.22</td>
<td>367.38</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Replicate × inbred line</td>
<td>94</td>
<td>250.37</td>
<td>2.66</td>
<td>1.29</td>
<td>0.07</td>
</tr>
<tr>
<td>Inbred line × heterospecific pollen flow</td>
<td>7</td>
<td>29.24</td>
<td>4.18</td>
<td>2.02</td>
<td>0.054</td>
</tr>
<tr>
<td>Error</td>
<td>210</td>
<td>435.12</td>
<td>2.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In (a), values are means (± 1 standard error) from pooling across all 10 replicates for each I. purpurea pollen flow treatment. 

d.f., degrees of freedom; SS, sums of squares.

Seed set

Ipomoea hederacea seed set in each treatment combination also differed significantly among treatments (Table 2). Emasculated I. hederacea flowers set significantly fewer seeds (only 5–14% as many) than control flowers (Tables 2, I. hederacea treatment effect), indicating that insect-mediated pollen transfer to emasculated flowers was insufficient for full seed set, and that close clustering of anthers and stigma may provide a degree of reproductive assurance in the absence of pollinator visits. Emasculated I. hederacea flowers were also more vulnerable than control flowers to pollen flow from I. purpurea whereas addition of pollen flow from I. purpurea reduced seed set of emasculated flowers by more than 50% (1.18 vs 0.54 seeds per flower, respectively), seed set of control flowers was unaffected (3.67 vs 3.79 seeds per flower, respectively; Table 2a; see Table 2b for the interspecific pollen flow × I. hederacea treatment interaction term). This interaction effect remained significant when visitation rates were included as a covariate to account for possible differences in visitation rates among the treatments (pollen flow × treatment interaction, F = 12.10, degrees of freedom (d.f.) = 1, 127, P < 0.0007; covariate, F = 0.92, d.f. = 1, 127, not significant).

Discussion

In cases where temporal isolation and behavioral isolation are incomplete, morphological features that mechanically prevent interspecific pollen transfer may be especially important prezygotic isolating barriers. This has been a suggested function of anther–stigma clustering in a number of plant species (Webb & Lloyd, 1986), including I. hederacea (Ennos, 1981; Stucky, 1984), although there is little direct experimental evidence to support this suggestion. Our experiment provides such evidence by demonstrating that close clustering of anthers around the stigma in I. hederacea enhances prezygotic isolation from I. purpurea. Protection from heterospecific pollen is not necessarily the only advantage of clustering. In a number of taxa, close clustering of anthers around the stigma is believed to mechanically prevent pollinators from coming into direct contact with the stigma and to maximize the number of self pollen grains deposited on the stigma, both limiting contact with outcross pollen and increasing deposition of self pollen (Webb & Lloyd, 1986). Differences between species in the placement of sexual structures may also reduce interspecific pollen transfer by ensuring that each species applies pollen to a unique part of a pollinator’s body. It is not clear to what extent this enhancement of prezygotic isolation is caused by increased self-pollination vs some form of mechanical protection. We believe that, with respect to selfing rate, the treatment in which I. hederacea is emasculated approximates a situation in which anthers are not clustered around the stigma. In this situation, seed set is reduced by > 50% in the presence of I. purpurea pollen. By contrast, when clustered anthers are present, seed set is not reduced at all in the presence of I. purpurea pollen. Comparison of intact and emasculated flowers in the absence of I. purpurea indicates that the increased selfing conferred by clustering also substantially increases seed set. Moreover, Stucky (1985) reported that the number of pollen grains on stigmas in the
early morning (before the onset of pollinator activity, thus representing self- rather than cross-pollination) was substantially higher for *I. hederacea* than for *I. purpurea*, in which anthers are not clustered (101.0 ± 31.54 vs 4.0 ± 3.61 grains per stigma, respectively).

Our results can be used to estimate the relative magnitude of these two advantages under the conditions of our experiment. In the absence of *I. purpurea*, seed set per fruit is increased on average by 2.49 by anther clustering (3.67 – 1.18), whereas in the presence of *I. purpurea* this increase is 3.25 seeds per capsule (3.79 – 0.54) (Table 1). The ratio of these two increases (3.25/2.49 = 1.3) indicates that the effect of protection from heterospecific pollen on seed set is approx. 30% greater than the effect from increased selfing. The increased seed set of nonemasculated flowers in the absence of *I. purpurea* indicates that emasculated flowers were pollen limited under the conditions of our experiment, probably because of the small number of plants in the arrays. Under more natural conditions, populations are substantially larger, often numbering in the thousands of individuals (M. D. Rausher, pers. obs.), and we would expect pollen limitation to be much less severe. Under these conditions we would expect the protective effect of clustering on seed set to be substantially larger relative to the effect of selfing, per se. Thus, clustering enhances seed production in the presence of *I. purpurea* by more than can be explained by just increased selfing.

While our experimental manipulations were designed to test the effects of proximity of anthers and stigma on susceptibility to heterospecific pollen transfer, another possible explanation for the reduced seed set measured in emasculated *I. hederacea* flowers in the presence of pollen flow from *I. purpurea* is reduced pollinator visitation to these flowers. Our analyses were based on the assumption that the reduced seed set we measured in emasculated *I. hederacea* in our *+ purpurea* pollen treatment was a result of heterospecific pollen transfer, and not of inadvertent effects of our experimental floral manipulations on pollinator visitation rate. Two lines of evidence indicate that this latter explanation can be ruled out: First, while emasculated *I. hederacea* flowers received significantly fewer visits per hour than control flowers, this effect was statistically no more severe for *I. hederacea* in the *+ purpurea* pollen treatment than in the – purpurea pollen treatment, as indicated by a nonsignificant interaction term between heterospecific pollen flow treatment and *I. hederacea* treatment in our ANOVA of pollinator visitation. Secondly, accounting for potential effects of differential visitation on seed set did not change our results, as emasculated *I. hederacea* flowers were more vulnerable than control flowers to pollen flow from *I. purpurea* even when pollinator visitation rate was included as a covariate in our models.

Our demonstration that anther–stigma clustering enhances prezygotic isolation does not necessarily imply that anther–stigma clustering is the only barrier in this system. It is possible that other aspects of floral architecture or display in addition to anther–stigma proximity also contribute to reproductive isolation between these two species. For example, other modes of self-pollination (such as geitonogamous selfing, i.e. fertilization of flowers by other flowers on the same plant) could play a role. Nevertheless, our results identify stigma–anther proximity as an important prezygotic isolating barrier and target of selection in the presence of heterospecific pollen flow, and provide motivation for further study of the role of floral morphology in reproductive isolation in this system. While our results suggest that the interactions between *I. hederacea* and *I. purpurea* have the potential to generate selection favoring close clustering of anthers and stigma in *I. hederacea*, however, they do not demonstrate that such selection occurs; nor do they indicate the potential for a response to such selection, because we have not here estimated the amount of genetic variation for anther–stigma separation. Future studies will also address these issues.

Acknowledgements

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References


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