

1 *Letter*

2 Evolutionary consequences of ecological factors: pollinator reliability predicts  
3 mating-system traits of a perennial plant

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24 Data supporting the results will be deposited on Dryad.

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27

## 28 **Abstract**

29 The reproductive-assurance hypothesis predicts that mating-system traits will evolve towards  
30 increased autonomous self-pollination in plant populations experiencing unreliable pollinator  
31 service. We tested this long-standing hypothesis by assessing geographic covariation among  
32 pollinator reliability, outcrossing rates, heterozygosity, and relevant floral traits across  
33 populations of *Dalechampia scandens* in Costa Rica. Mean outcrossing rates ranged from  
34 0.16 to 0.49 across four populations, and covaried with the average rates of pollen arrival on  
35 stigmas, a measure of pollinator reliability. Across populations, genetically based differences  
36 in herkogamy (anther-stigma distance) were associated with variation in stigmatic pollen  
37 loads, outcrossing rates, and heterozygosity. These observations are consistent with the  
38 hypothesis that, when pollinators are unreliable, floral traits promoting autonomous selfing  
39 evolve as a mechanism of reproductive assurance. Extensive covariation between floral traits  
40 and mating system among closely related populations further suggests that floral traits  
41 influencing mating systems track variation in adaptive optima generated by variation in  
42 pollinator reliability.

## 43 **Introduction**

44 A recurrent question in evolutionary biology is how ecological context affects evolutionary  
45 dynamics. For example, most flowering plants rely on animal pollinators for production of  
46 outcrossed seeds, and variation in pollinator communities commonly affects the mating  
47 system of plant populations (e.g. Brunet & Sweet 2006; Moeller 2006). In turn, plant mating  
48 systems (i.e. outcrossing rates) strongly affect the demography and genetic structure of  
49 populations. If we are to predict the consequences of ongoing pollinator declines (Vamosi *et al.*  
50 2006; Potts *et al.* 2010; Gonzalez-Varo *et al.* 2013), we need to understand mating-system  
51 changes that occur in response to reduced pollinator reliability (Eckert *et al.* 2010; Thomann  
52 *et al.* 2013). A long-standing hypothesis is that when pollinators are unreliable, autonomous  
53 self-pollination evolves as a mechanism of reproductive assurance in self-compatible lineages  
54 (Darwin 1876; Baker 1955; Jain 1976; Eckert *et al.* 2006). The ‘reproductive-assurance  
55 hypothesis’ is supported by genetic models predicting that, with strong pollen limitation, the  
56 ability of inbreeding depression to maintain outcrossing will decline, and populations will  
57 evolve towards increased rates of autonomous selfing (Lloyd 1979, 1992; Morgan & Wilson  
58 2005; Porcher & Lande 2005).

59           A corollary of the reproductive-assurance hypothesis is that the topography of the  
60 adaptive landscapes governing the evolution of mating-system traits of plant populations will  
61 depend on the reliability of pollination, thus creating a geographic selection mosaic  
62 (Thompson 2005; Mitchell & Ashman 2008). Variation in mating systems is often  
63 accompanied by variation in floral traits affecting mating systems directly, through the ability  
64 to self-pollinate (herkogamy, dichogamy; Ornduff 1969; Sicard & Lenhard 2011), or  
65 indirectly, through pollinator attraction (e.g. corolla size, floral display, Goodwillie *et al.*  
66 2010). To predict plant population responses to pollinator declines, it is essential to  
67 understand whether mating-system traits are able to rapidly track variation in adaptive optima  
68 ('peaks' on the adaptive landscape) caused by changes in pollinator reliability. When  
69 pollinators are less reliable, we expect stronger selection on traits associated with pollinator  
70 attraction and/or autonomous self-pollination (Moeller & Geber 2005; Fishman & Willis  
71 2008; Mitchell & Ashman 2008), and over time this should forge a correlation between  
72 pollinator reliability and mating-system traits across populations (Fig. 1). Under a  
73 reproductive-assurance scenario, we specifically expect reductions in herkogamy, reward  
74 amount and floral-advertisement traits in populations experiencing unreliable pollination  
75 compared to those experiencing more reliable pollination. These putative adaptations to the  
76 long-term pollination environment are also expected to influence the mating system of  
77 populations in a given year, and contribute to differences in mating system among  
78 populations. Although these predictions appear theoretically sound, studies testing them by  
79 relating geographic variation in pollinator reliability to variation in mating systems have  
80 yielded largely inconclusive results (Herrera *et al.* 2001; Eckert 2002; Molina-Freamer *et al.*  
81 2003; Schueller 2004; Herlihy & Eckert 2005; but see Moeller 2006; Brys *et al.* 2011).

82           Here, we take advantage of geographic variation in pollinator abundance to test  
83 whether populations of the woody vine *Dalechampia scandens* differ in pollinator reliability,  
84 outcrossing rate, floral traits, and heterozygosity in a manner consistent with the reproductive-  
85 assurance hypothesis (Fig. 1). Note that the model represented in Fig. 1 is not specific to our  
86 study system, but applies to any mixed-mating, animal-pollinated plant species. We quantified  
87 pollinator reliability in 14 populations by counting the number of pollen grains on the stigmas  
88 of female-phase blossom inflorescences (when self-pollination is not yet possible) and  
89 assessed geographic covariation among pollinator reliability, outcrossing rates, floral traits,  
90 and heterozygosity. We also assessed the strength of inbreeding depression in four

91 populations by comparing adult inbreeding coefficients to those expected based on  
92 outcrossing rates.

### 93 **Methods**

#### 94 *Study species*

95 *Dalechampia scandens* L. (Euphorbiaceae) is a species complex of twining vines occurring in  
96 disturbed habitats throughout most of the lowland Neotropics, from Mexico to Argentina  
97 (Armbruster 1985). The study populations belong to a single species, which has previously  
98 been referred to as ‘large-glanded’ *D. scandens* (Bolstad *et al.* 2014; Opedal *et al.* 2016).  
99 Unisexual staminate and pistillate flowers are aggregated into functionally bisexual blossom  
100 inflorescences (pseudanthia) that function as pollination units (Fig. 2). Each pistillate flower  
101 contains three ovules, so that a blossom can set up to nine seeds. The number of blossoms  
102 open simultaneously varies with plant size, with an average of ca. 4 in most populations (E.  
103 Albertsen & Ø. H. Opedal, unpublished data). The blossoms are functionally protogynous,  
104 with a female phase of ca. two to three days followed by a bisexual phase, during which the  
105 stigmas remain receptive. Pollinator-mediated cross-pollen receipt occurs primarily during the  
106 female phase, while the position of staminate flowers above the pistillate flowers (Fig. 2)  
107 suggests that autonomous and pollinator-facilitated selfing is more common during the  
108 bisexual phase (Armbruster *et al.* 2011). A gland associated with the staminate flowers  
109 secretes a triterpenoid resin attracting apid and megachilid bees that use resin in nest  
110 construction (Armbruster 1984). The two bracts subtending the staminate and pistillate  
111 flowers are usually pale green to white and serve both advertisement and protective functions  
112 (Armbruster 1997).

#### 113 *Population survey*

114 During peak flowering (Oct – Dec) in 2014 and 2015, we studied 22 populations in north-  
115 western Costa Rica (provinces Guanacaste and Puntarenas, Figure S1, Table S1). Most  
116 populations occurred on forest edges or in shrublands along gravel roads. Populations were  
117 generally small (ranging from fewer than ten to around 100 flowering individuals), and  
118 population size was scored on a categorical scale ranging from XS to XL (Table S1). Floral  
119 visitors (Table S1) included worker *Trigona* and *Tetragonisca* (ca. 5 mm), female  
120 *Hypanthidium* (6-7 mm), female *Euglossa dilemma* (11 mm) and female *Eufriesea* cf.  
121 *surinamensis* (17-19 mm). Herbarium specimens from representative populations have been

122 deposited at the herbarium of the University of Costa Rica (USJ), and at the University  
123 Museum, Norwegian University of Science and Technology (TRH).

#### 124 *Field data and materials collection*

125 For populations in bloom ( $n = 17$ ), a single observer (ØHO) measured anther-stigma distance  
126 (ASD, herkogamy; Fig. 2), resin-gland area (GA, the product of gland width and gland  
127 height) and upper bract area (UBA, the product of upper bract length and upper bract width).  
128 Blossoms ( $n = 4 - 55$  per population, median = 22; Table S2) were measured in early bisexual  
129 condition, with one to three male flowers open. Seven populations were measured in both  
130 years, allowing between-year comparisons. Anther-stigma distance covaries negatively with  
131 the rate of autofertility both among *Dalechampia* species, among populations within species,  
132 and within *D. scandens* populations (Armbruster 1988; Opedal *et al.* 2015, 2016). Hence,  
133 there is strong evidence that reduced herkogamy increases autofertility, and provides a  
134 mechanism of reproductive assurance in *D. scandens*. Variation in partial dichogamy (length  
135 of the female phase) did not detectably affect autofertility in previous studies of this species  
136 (Opedal *et al.* 2015, 2016). We therefore focus here on herkogamy as a direct mating-system  
137 trait. Gland size is a good predictor of the amount of reward offered to pollinators (Pélabon *et*  
138 *al.* 2012), and has been shown to predict the size of the largest bees visiting the blossoms in a  
139 population (Armbruster 1988; Armbruster *et al.* 2011). Bract size is a honest signal of reward  
140 quantity, and appears to play a key role in advertisement to pollinators (Pérez-Barrales *et al.*  
141 2013). Leaf samples were collected from all individuals located in small populations (fewer  
142 than ca. 15 individuals), and from ca. 15-20 randomly chosen individuals in larger  
143 populations. Whenever possible, mature fruits were collected for greenhouse cultivation, and  
144 for estimating outcrossing rates.

#### 145 *Pollinator species and reliability*

146 To assess pollinator communities and quantify the reliability of pollination, we combined  
147 several approaches. First, with the aid of a hand lens, we counted the number of pollen grains  
148 deposited on the stigmas of randomly chosen female-phase blossoms, excluding those in the  
149 first day of the female phase. *Dalechampia scandens* pollen grains are large (ca. 75 - 85  $\mu\text{m}$ ;  
150 Webster & Webster 1972) and have a characteristic shape and exine, making them easy to  
151 discriminate from heterogenetic pollen. Because autonomous selfing is not possible during the  
152 female phase, these pollen counts represent exclusively pollinator-deposited pollen. Unlike  
153 visitation rate or similar metrics, this measure of pollinator reliability integrates both the  
154 abundance of pollinators and their efficiency in transferring pollen to stigmas (Engel & Irwin

155 2003). To assess the relative abundance of different bee species, we noted all floral visitors  
156 observed during the time spent in each population (Table S1). We defined pollinators as those  
157 bees that visited blossoms and were large enough to contact anthers and stigmas regularly,  
158 and principle pollinators as the most abundant pollinator(s) observed in populations where at  
159 least three days of observations were made (cf. Armbruster 1985, 1988).

160 Initial field observations suggested that female euglossine bees were the most  
161 abundant pollinators in the study area. To quantify the relative population density of  
162 euglossine bees, we conducted standardized baiting censuses of male euglossine bees (Roubik  
163 & Hanson 2004). Female bees are not attracted to odor baits, but we assumed that the  
164 abundance of male bees would also represent the relative abundance of female bees. Note that  
165 this approach provides a measure of bee abundance that is independent of the data on bee  
166 visits to blossoms. Censuses were always conducted in pairs, with two observers recording  
167 visits to baits at two nearby stations. The baits (eugenol and methyl cinnamate) were dripped  
168 onto 5 cm-diameter filter paper discs attached to tree trunks at a height of 1 – 1.5 m.  
169 Observers recorded the maximum number of bees seen at one time (for both baits combined)  
170 during 30 min census periods, which represents a conservative measure of the number of bees  
171 attracted. Bees were identified to genus, and voucher specimens of hard-to-identify species  
172 (green *Euglossa*) were collected in a subset of populations for later identification.

### 173 *Greenhouse experiment*

174 To assess to what extent the population differences in floral traits were genetically  
175 determined, plants from 16 populations ( $n = 1 - 26$  plants per population, median = 6.5),  
176 representing the full range of phenotypes, were grown from seeds under standardized  
177 greenhouse conditions (13:11 light/dark, 26°C /24°C day/night). Phenotypic traits were  
178 measured on one to three blossoms haphazardly chosen from each plant, following the same  
179 procedure as in the field. We compared field and greenhouse measurements for 10  
180 populations measured in both environments (Tables S2, S3).

### 181 *Microsatellite genotyping and estimation of outcrossing rates and inbreeding depression*

182 Leaf material was collected from seedlings grown from seeds collected in four of the study  
183 populations for which we had a sufficient number of seed families (Table 1). Microsatellite  
184 genotyping followed the procedures outlined in Falahati-Anbaran *et al.* (2013). Maternal  
185 individuals were genotyped at 30 microsatellite loci, and their offspring at 15 loci that were  
186 polymorphic in the parental populations. As a measure of molecular-genetic variation in the  
187 parental populations, observed heterozygosities were computed in GenAEx 6.5 (Peakall &

188 Smouse 2012). Outcrossing rates were estimated with the mixed-mating model implemented  
 189 in MLTR 3.4 (Ritland 2002). Maternal genotypes were inferred from progeny genotypes, with  
 190 pollen and ovule allele frequencies constrained to be equal, and maximum-likelihood  
 191 estimates obtained by Newton-Raphson iterations. Standard errors were obtained from 1000  
 192 bootstrap estimates with seed families as the unit of resampling. Statistical differences in  
 193 outcrossing rates between populations were evaluated by comparing randomly paired  
 194 bootstrap estimates, and calculating the 95% confidence interval of the between-population  
 195 differences (see Herlihy & Eckert 2005).

196 To assess the strength of inbreeding depression, we estimated the relative fitness of  
 197 selfed offspring ( $w$ ) by the equilibrium estimator of Ritland (1990):

$$198 \quad w = 2[(1 - s)F/s(1 - F)]$$

199 where  $s$  is the selfing rate and  $F$  is the adult inbreeding coefficient. Inbreeding coefficients for  
 200 the adult populations were obtained using the Bayesian approach implemented in INEST  
 201 (Chybicki & Burczyk 2009), which accounts for possible null alleles. We estimated  
 202 inbreeding depression ( $\delta$ ) by the relative performance (RP) index of Ågren and Schemske  
 203 (1993):

$$204 \quad \delta = 1 - w \text{ if } w \leq 1, \text{ and } 1/w - 1 \text{ if } w > 1$$

205 The RP index ranges from -1 to 1, with values greater than 0 indicating better performance of  
 206 outbred offspring (inbreeding depression), and avoids the asymmetry of the traditional  
 207 inbreeding depression estimator ( $1 - w_s/w_o$ ). To obtain confidence intervals,  $\delta$  was calculated  
 208 across the bootstrap distribution of  $s$  and posterior distribution of  $F$ , respectively.

#### 209 *Pollen limitation*

210 Reproductive assurance through autonomous selfing becomes important when seed set is  
 211 limited by the amount of cross-pollen arriving on stigmas (pollen limitation, see Ashman *et al.*  
 212 *2004*). To evaluate pollen limitation, we translated female-phase stigmatic pollen loads  
 213 into predicted seed set, using the following relationship between pollen load and seed set in *D.*  
 214 *scandens*:

$$215 \quad S = 9 (\alpha P)/(1 - \alpha P)$$

216 where  $S$  is seed set,  $P$  is pollen load, and  $\alpha$  is a shape parameter describing the relationship  
 217 between pollen load and seed set (see Bolstad *et al.* 2010 and Pérez-Barrales *et al.* 2013 for

218 further discussion of this approach). We estimated  $\alpha$  using empirical data from three of the  
219 study populations, with low (S2), medium (S8) and high (S21) average pollen loads  
220 (Albertsen et al., manuscript in preparation), as the exponent of the intercept of a generalized  
221 linear mixed-effects model with binomial errors, log(pollen load) as fixed factor, and maternal  
222 plant and blossom ID (nested within maternal plant) as random factors. The slope of the  
223 regression was constrained to be 1 (see Pérez-Barrales *et al.* 2013).

#### 224 *Statistical analyses*

225 Statistical analyses were conducted in R version 3.3.1 (R Core Team 2016). To partition the  
226 variances of the blossom traits into among-population, among-family (only in the  
227 greenhouse), among-plant and within-plant components, we fitted linear mixed-effect models  
228 with the ‘lme4’ R package (Bates *et al.* 2014), where population, family (nested within  
229 populations), and plant (nested within families) were modeled as random factors. Models  
230 were fitted by restricted maximum likelihood (REML), and confidence intervals obtained  
231 from 1000 parametric bootstrap estimates.

232 The stigmatic pollen load (on log scale) of female-phase blossoms in each population  
233 was estimated from a mixed-effect model with negative binomial errors (to account for  
234 overdispersion) and log link function, fitted with the ‘glmmADMB’ R package (Fournier *et al.*  
235 *et al.* 2012). Population was treated as a fixed effect, and plant identity as a random effect. The  
236 estimates and standard errors from this model represent the average pollen load in each  
237 population, controlled for the non-independence of blossoms from the same plant, and we  
238 used these estimates of pollen loads in the subsequent analyses. We used likelihood-ratio tests  
239 (LRT) to test whether stigmatic pollen loads, bee numbers on baits and floral traits differed  
240 among populations, and whether stigmatic pollen loads differed among populations of  
241 different sizes.

242

## 243 **Results**

#### 244 *Phenotypic traits*

245 In the field, average anther-stigma distance (ASD) varied nearly threefold among populations  
246 (range = 2.35 – 6.32 mm, Table S2), with 48.6% of the variance due to differences among  
247 populations (Table S4). Average gland size (17.43 - 34.62 mm<sup>2</sup>) and average bract size  
248 (254.96 – 526.32 mm<sup>2</sup>) varied approximately twofold among populations (Table S2), with  
249 differences among populations accounting for 28.1% and 19.9% of the variance, respectively.  
250 Among-population differences were statistically significant for ASD [LRT,  $\chi^2(16) = 59.08$ ,  $P$



251 < 0.001], gland size [ $\chi^2(16) = 42.19, P < 0.001$ ], and bract size [ $\chi^2(16) = 41.99, P < 0.001$ ].  
 252 Among populations, ASD correlated positively with gland size [ $r = 0.74$  (95% CI = 0.39,  
 253 0.90),  $n = 17$ ], and bract size [ $r = 0.53$  (0.06, 0.80),  $n = 17$ ]. The between-year correlation in  
 254 trait means was high for ASD [ $r = 0.91$  (0.36, 0.99),  $n = 6$ ], gland size [ $r = 0.72$  (-0.07, 0.96),  
 255  $n = 7$ ], and bract size [ $r = 0.84$  (0.25, 0.98),  $n = 7$ ]. We used population grand means across  
 256 years in the subsequent analyses.

257         Among plants within populations, ASD correlated weakly and inconsistently with  
 258 gland size (mean  $r = 0.04$ , range = -0.74 – 0.75,  $n = 13$  populations) and bract size (mean  $r =$   
 259 0.14, range = -0.79 – 0.83,  $n = 13$ ). In contrast, bract size and gland size were strongly  
 260 correlated within populations (mean  $r = 0.69$ , range = 0.30 – 0.96,  $n = 13$ ).

261         Gland size [ $\chi^2(1) = 12.34, P < 0.001$ ] and bract size [LRT,  $\chi^2(1) = 124.77, P < 0.001$ ]  
 262 differed significantly between environments (greenhouse vs. field). Although blossoms were  
 263 larger in the greenhouse (population-mean bract and gland size was, on average, 69.3% and  
 264 15.9% larger), field and greenhouse measurements were correlated across populations [gland  
 265 size,  $r = 0.85$  (0.44, 0.96), bract size,  $r = 0.61$  (-0.05, 0.89),  $n = 10$ ], confirming that the  
 266 population differences were largely genetically determined. In the greenhouse, gland area [ $\chi^2$   
 267 (15) = 45.58,  $P < 0.001$ ], but not bract area [ $\chi^2(15) = 24.49, P = 0.057$ ], varied significantly  
 268 among populations. ASD varied significantly among populations [ $\chi^2(15) = 54.81, P < 0.001$ ],  
 269 but not between environments [ $\chi^2(1) = 0.60, P = 0.437$ ]. Furthermore, population averages  
 270 correlated strongly between environments [ $r = 0.93$  (0.73, 0.98),  $n = 10$ ], suggesting limited  
 271 influence of environmental variation on ASD. We were therefore able in subsequent analyses  
 272 to use greenhouse measurements of ASD for the few populations for which field  
 273 measurements were lacking (Table S3).

#### 274 *Pollinator species, pollinator reliability, and pollen limitation*

275 In all populations for which we had at least three days of pollinator observations, the clear  
 276 principle pollinators were female euglossine bees (*Euglossa dilemma* and/or *Eufriesea* cf.  
 277 *surinamensis*), except in the S1 population, where no pollinators were observed (Table S1)  
 278 and in the S21 population, where 44% of observed pollinator visits ( $n = 34$ ) were made by  
 279 female *Hypanthidium* sp. (Megachilidae). During a total of 60 baiting censuses in 16  
 280 populations, the average number of male euglossine bees recorded on baits ranged from 0.5 to  
 281 12, and the vast majority of these were *Euglossa dilemma*. The number of bees attracted

282 differed among populations [LRT,  $\chi^2(15) = 85.98$ ,  $P < 0.001$ ], and there was a tendency for  
 283 more bees to be attracted in small populations (Figure S2).

284 Average stigmatic pollen loads of female-phase blossoms ranged from 0.21 to 45.1  
 285 pollen grains, and differed significantly among all populations [LRT,  $\chi^2(14) = 46.14$ ,  $P <$   
 286  $0.001$ ] and among the four core populations in which outcrossing rates were estimated [ $\chi^2(3)$   
 287  $= 14.49$ ,  $P = 0.002$ ]. These female-phase pollen loads translated into predicted seed sets  
 288 ranging from 0.24 to 7.69 seeds per blossom (Fig. 3), indicating that seed set was limited by  
 289 female-phase pollen arrival in most or all populations. Stigmatic pollen loads were not  
 290 detectably dependent on population size [ $\chi^2(4) = 5.2$ ,  $P = 0.158$ ].

#### 291 *Outcrossing rates and inbreeding depression*

292 Outcrossing rates estimated in our four core populations ranged from 0.16 to 0.49 (Table 1).  
 293 All bootstrap distributions were unimodal, indicating a single most likely outcrossing rate in  
 294 each population. Outcrossing rates differed significantly between populations S2 and S8  
 295 [mean difference = 0.34 (95% CI = 0.08, 0.63)], but not between populations with  
 296 intermediate outcrossing rates (Table 1). Parental individuals in all four populations were less  
 297 inbred than expected based on the outcrossing rate, with inbreeding depression ( $\delta$ ) ranging  
 298 from 0.30 to 0.90 (Table 1).

#### 299 *Relationships among pollen load, outcrossing rate, herkogamy and heterozygosity*

300 Populations with more reliable pollinator service (larger average pollen loads) had higher  
 301 outcrossing rates [ $r = 1.00$  (0.95, 1.00),  $n = 4$ , Fig. 4B], and greater anther-stigma distances [ $r$   
 302  $= 0.65$  (0.18, 0.88),  $n = 14$ , Fig. 4C]. Consequently, anther-stigma distance and outcrossing  
 303 rates tended to be positively correlated [ $r = 0.93$  (-0.29, 1.00),  $n = 4$ ]. The link between  
 304 pollinator reliability and anther-stigma distance was further supported by the positive  
 305 correlation between male-bee abundance and anther-stigma distance [ $r = 0.79$  (0.48, 0.92),  $n =$   
 306  $16$ ]. Populations with a greater abundance of male bees also tended to have more pollen on  
 307 their stigmas, although this relationship was not statistically significant [ $r = 0.46$  (-0.15, 0.82),  
 308  $n = 12$ ]. Greater anther-stigma distance was associated with greater heterozygosity [ $r = 0.62$   
 309 (0.22, 0.85),  $n = 18$ , Fig. 4D]. Heterozygosity did not differ detectably between populations of  
 310 different size (ANOVA,  $F_{4,13} = 0.97$ ,  $P = 0.46$ ). The S21 population deviated from the overall  
 311 pattern by having large stigmatic pollen loads, yet low anther-stigma distance and few male  
 312 bees on baits (shown as an open symbol in Fig. 4). Nevertheless, we included the S21  
 313 population in all analyses.

## 314 Discussion

315 To predict the long-term plant-demographic consequences of pollinator declines, we need to  
316 understand the potential of plant mating systems to evolve in response to changes in pollinator  
317 reliability. Consistent with the reproductive-assurance hypothesis, geographic variation in  
318 pollinator reliability covaried with variation in the mating system of *D. scandens* populations  
319 both on a short time-scale represented by outcrossing rates (Fig. 4B), and on longer time-  
320 scales represented by variation in herkogamy and heterozygosity (Fig. 4C, D). Because our  
321 measure of pollinator reliability, stigma pollen count, was a population average, we interpret  
322 the among-population correlation between pollinator reliability and outcrossing rate as a  
323 population-level property, not just the automatic outcome of individual blossoms with more  
324 pollen on their stigmas being more outcrossed. This interpretation is supported by the strong  
325 correlation between pollinator reliability and herkogamy, a correlation assumed to represent  
326 an evolved relationship (Webb & Lloyd 1986; Herlihy & Eckert 2007). Variation in  
327 herkogamy strongly predicts population-specific rates of autofertility in *D. scandens*  
328 (Armbruster 1988; Opedal *et al.* 2015), and most other species tested previously (e.g.  
329 Holtsford & Ellstrand 1992; Herlihy & Eckert 2007; Dart *et al.* 2012). Here, we provide  
330 empirical evidence suggesting that the often-observed relationship between outcrossing rate  
331 and herkogamy, and in turn autofertility, is due to all three variables responding to pollinator  
332 reliability (Fig. 4). Herkogamy correlated positively with gland size and bract size across  
333 populations, placing populations along an axis of increasing trait values. Because all three  
334 traits are expected to be reduced in populations and species with greater rates of selfing (e.g.  
335 Ornduff 1969; Goodwillie *et al.* 2010; Dart *et al.* 2012), we interpret this axis as a mating-  
336 system gradient ranging from high selfing rates and reduced trait values associated with  
337 unreliable pollination, to higher outcrossing rates and greater trait values associated with more  
338 reliable pollination.

339 Because our data are observational, we cannot completely exclude the possibility that  
340 the inferred causal links reflects spurious correlations or are indeed causal but operate in the  
341 opposite direction. For example, more pollinators could be attracted to populations with large  
342 blossoms. If this was the case, however, we would expect pollinator reliability also to depend  
343 on population size, because larger populations should be more attractive to pollinators. This  
344 was not the case: stigmatic pollen loads did not depend detectably on population size, nor did  
345 we attract more male euglossine bees to baits in large populations than in smaller ones (Figure  
346 S2). Furthermore, phenotypic-selection studies conducted in three of the study populations

347 have shown that pollinators consistently exert selection on floral traits (Albertsen et al.,  
348 manuscript in preparation). Based on these observations, it appears most likely that pollinators  
349 respond to overall habitat quality (e.g. the availability of nectar sources or nesting sites), and  
350 plants then evolve towards the ‘optimal’ phenotype in their pollination environment.

351 Experimental studies in natural populations have demonstrated the adaptive value of  
352 reproductive assurance, i.e. self-fertilization when seed set is limited by cross-pollen arrival  
353 (Herlihy & Eckert 2002; Elle & Carney 2003; Kalisz & Vogler 2003; Eckert & Herlihy 2004;  
354 Kalisz *et al.* 2004; Weber & Goodwillie 2009; Brys *et al.* 2011), and some of these studies  
355 have further suggested that the relative importance of autonomous selfing increases with  
356 reduced pollinator reliability. However, studies attempting to link mating-system variation  
357 with geographic variation in pollinator reliability have yielded less conclusive results. Perhaps  
358 the most convincing case to date comes from work on the California endemic *Clarkia*  
359 *xantiana* (Fausto *et al.* 2001; Moeller & Geber 2005; Moeller 2006; Moeller *et al.* 2012),  
360 where variation in the abundance of specialist pollinators across populations predicts variation  
361 in herkogamy (Moeller 2006). Similar to our results, this finding supports the hypothesis that  
362 reduced herkogamy evolves as a mechanism of reproductive assurance. In contrast, variation  
363 in floral traits was not associated with mating-system divergence between central and  
364 peripheral populations of *Aquilegia canadensis* (Herlihy & Eckert 2005), and variation in  
365 autofertility was not associated with variation in pollinator service among regions in  
366 *Helleborus foetidus* (Herrera *et al.* 2001), or between mainland and island populations of  
367 *Nicotiana glauca* (Schueller 2004; but see Ollerton *et al.* 2012). Finally, despite clear  
368 differences in pollinator communities between southern and northern populations of *Decodon*  
369 *verticillatus*, outcrossing rates were similar (Eckert 2002).

370 Measures of current pollinator abundance may not always accurately predict plant  
371 mating systems because of temporal fluctuations in pollinator communities (e.g. Brunet &  
372 Sweet 2006). The strong relationships among stigmatic pollen load, herkogamy, and  
373 heterozygosity in *D. scandens* (Fig. 4C, D) suggests that the pollinator reliability in the study  
374 populations is fairly consistent over periods sufficiently long to allow local adaptation. Data  
375 on variation in stigmatic pollen loads across years are available for only two populations; in  
376 both of these the average stigmatic pollen loads were comparable across years (20.7 vs. 17.3  
377 pollen grains in population S8, and 4.03 vs. 0.17 pollen grains in population S2). Temporal  
378 variation in pollinator service could, however, explain our observation of reliable pollinators  
379 yet low herkogamy in the S21 population. An alternative explanation is that this population is

380 adapted to pollination by smaller bees (see Results), and therefore inhabits a different peak on  
381 the adaptive landscape than most of our study populations (see Armbruster 1988).

382         The observation of moderate to strong inbreeding depression coupled with a  
383 reproductive-assurance mechanism based on delayed selfing lead us to conclude, at least  
384 tentatively, that the mixed mating system of *D. scandens* is evolutionary stable. Inbreeding  
385 depression is assumed to be the key genetic factor opposing selfing (Lande & Schemske  
386 1985; Charlesworth & Charlesworth 1987; Lloyd 1992). Although inbreeding depression in  
387 *D. scandens* appears to be very low for early-life fitness when measured in the greenhouse  
388 (Opedal *et al.* 2015), the present results suggest it is much stronger in nature. This is not an  
389 unusual finding (e.g. Goodwillie *et al.* 2005; Herlihy & Eckert 2005; Dart & Eckert 2013),  
390 and can be attributed either to increased environmental stress in natural populations, or to the  
391 fact that the population-genetic method used here incorporates fitness costs expressed  
392 throughout the life cycle. Although this method is based on several assumptions, including  
393 inbreeding equilibrium and consistent outcrossing rates across years (Ritland 1990), the  
394 estimated average strength of inbreeding depression in Costa Rican *D. scandens* populations  
395 ( $\delta = 0.53$ ) is sufficient to explain the maintenance of outcrossing when pollinators are reliable.  
396 Furthermore, because *Dalechampia* blossoms are partially protogynous, evolution of reduced  
397 anther-stigma distances to facilitate autonomous selfing does not preclude outcrossing during  
398 periods of reliable pollination. Delayed selfing is indeed expected to be favored in self-  
399 compatible plants under nearly all conditions (Lloyd 1992), and was termed a ‘best-of-both-  
400 worlds’ scenario by Becerra and Lloyd (1992).

401         As noted above, mating-system variation among populations of *D. scandens*, and  
402 many other species, appears to be tightly linked to evolutionary divergence in herkogamy and  
403 related floral traits. To predict plant extinction risk associated with future declines in  
404 pollinator reliability, we need to understand whether conditions are favorable for herkogamy  
405 to evolve (Thomann *et al.* 2013). Rapid evolution of mating-system traits have recently been  
406 shown experimentally in *Mimulus guttatus* (Bodbyl Roels & Kelly 2011) and *Centaurea*  
407 *cyanus* (Thomann *et al.* 2015), and using comparisons of populations in stable vs. human-  
408 disturbed habitats (Brys & Jacquemyn 2012; Brys *et al.* 2013). For *D. scandens*, selection on  
409 herkogamy has been documented in natural populations (Pérez-Barrales *et al.* 2013; Albertsen  
410 and Opedal, unpublished results), and herkogamy has a high evolvability (i.e. a high expected  
411 response to selection; Hansen *et al.* 2003). Herkogamy also appears to be more buffered from  
412 environmental variation (canalized) than are other traits (Opedal *et al.* 2016, and see Results).

413 Gene flow among populations could restrict evolutionary divergence, but gene flow appears  
414 to be limited in this system (Opedal *et al.*, manuscript in revision). If we assume a constant  
415 evolvability of 1.71% (Hansen *et al.* 2003), and a mean-standardized selection gradient of -  
416 12.67% (Pérez-Barrales *et al.* 2013), we obtain a 50% reduction in herkogamy in ca. 320  
417 generations ( $t = [\ln(0.5) / 0.0171 \times 0.1267]$ ). However, in the event of a drastic reduction in  
418 pollinator reliability, selection on herkogamy might be much stronger (Moeller & Geber  
419 2005; Fishman & Willis 2008; Mitchell & Ashman 2008). If selection on herkogamy is as  
420 strong as selection on fitness (i.e. a selection gradient of 100%; Hereford *et al.* 2004), the  
421 expected time to a 50% reduction in herkogamy would be only 40.5 generations.

422 In summary, substantial trait evolvabilities combined with limited long-distance gene  
423 flow suggests that conditions are favorable for evolutionary divergence in herkogamy and  
424 other floral traits, and we expect populations experiencing differences in the direction or  
425 strength of selection to diverge accordingly. The present data do not allow us to ascertain  
426 whether the among-population correlations among herkogamy, gland size and bract size are  
427 caused by correlated selective pressures (selective covariance), or genetic constraints causing  
428 traits to evolve together in a correlated fashion. Still, herkogamy varied nearly threefold  
429 among populations, and within-population correlations between herkogamy and the other  
430 traits were inconsistent and much weaker than the among-population correlations. These  
431 observations suggest that genetic constraint is unlikely to be the main explanation of the  
432 observed patterns of covariance (see Armbruster & Schwaegerle 1996 and Bolstad *et al.* 2014  
433 for further discussion).

#### 434 *Conclusions*

435 The mating system of *D. scandens* appears to be highly evolvable, and able to track variation  
436 in adaptive ‘peaks’ caused by changes in pollinator reliability. Populations experiencing  
437 unreliable pollinator service appear to have evolved reduced anther-stigma distances as a  
438 mechanism of reproductive assurance, and might be more buffered against periods of  
439 unreliable pollination while maintaining the opportunity for outcrossing during periods of  
440 reliable pollination. Indeed, as long as pollinators are reliable, the highest fitness may be  
441 achieved by plants that invest in the attraction of pollinators, because these avoid the  
442 potentially high cost of inbreeding depression, or perhaps because plants investing in  
443 outcrossing also increase their fitness through the male function. More generally, our findings  
444 suggest that mating-system traits are able to track variation in adaptive optima, and that the  
445 often-observed correlations among mating-system traits, autofertility and outcrossing rate

446 across plant populations represents local adaptation to the long-term average pollination  
447 environment.

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- 634
- 635

Table 1. Mating-system and inbreeding depression estimates in four *Dalechampia scandens* populations from Costa Rica.

Population	Code	ASD $\pm$ SE (mm)	N	$t_m$ (95% CI)	$F_{IS}$ (95% CI)	$\delta$ (95% CI)
Palo Verde A	S8	4.50 $\pm$ 0.19	17/32/144	0.49 (0.30, 0.78)	0.231 (0.119, 0.391)	0.41 (-0.55, 0.81)
Palo Verde B	S9	3.88 $\pm$ 0.17	9/12/87	0.36 (0.17, 0.50)	0.396 (0.252, 0.527)	0.30 (-0.37, 0.79)
Punta Casique	S11	3.16 $\pm$ 0.15	9/13/84	0.23 (0.03, 0.49)	0.455 (0.266, 0.654)	0.51 (-0.48, 0.95)
Puente la Amistad	S2	3.41 $\pm$ 0.18	14/22/108	0.16 (0.04, 0.34)	0.222 (0.095, 0.368)	0.90 (0.64, 0.98)

ASD is anther-stigma distances (herkogamy) measured in the field. N is given for number of plants/number of infructescences/number of offspring.  $t_m$  is the multilocus outcrossing rate,  $F_{IS}$  is the adult inbreeding coefficient and  $\delta$  is inbreeding depression. See Table S1 for further details about the study populations.

637 **Figure legends**

638 Fig. 1. Conceptual model illustrating the expected relationships among variables related to  
 639 plant mating systems under the reproductive-assurance hypothesis. Single-headed arrows  
 640 indicate hypothesized causal links, double-headed arrows indicate correlations. In populations  
 641 with more abundant and efficient pollinators, more reliable pollination is expected. In the  
 642 short term, more reliable pollination is expected to translate into higher outcrossing rates,  
 643 which is in turn expected to affect population heterozygosity. In the long term, variation in  
 644 pollinator reliability is expected to be associated with variation in traits directly (herkogamy,  
 645 dichogamy) or indirectly (floral advertisements, reward amount) related to outcrossing rates.  
 646 These traits are, in turn, expected to be positively correlated, because they all respond to  
 647 variation in pollinator reliability. Finally, variation in floral traits is expected to affect short-  
 648 term mating patterns by affecting pollinator attraction (advertisement and reward traits) and/or  
 649 self-pollination rate (herkogamy, dichogamy).

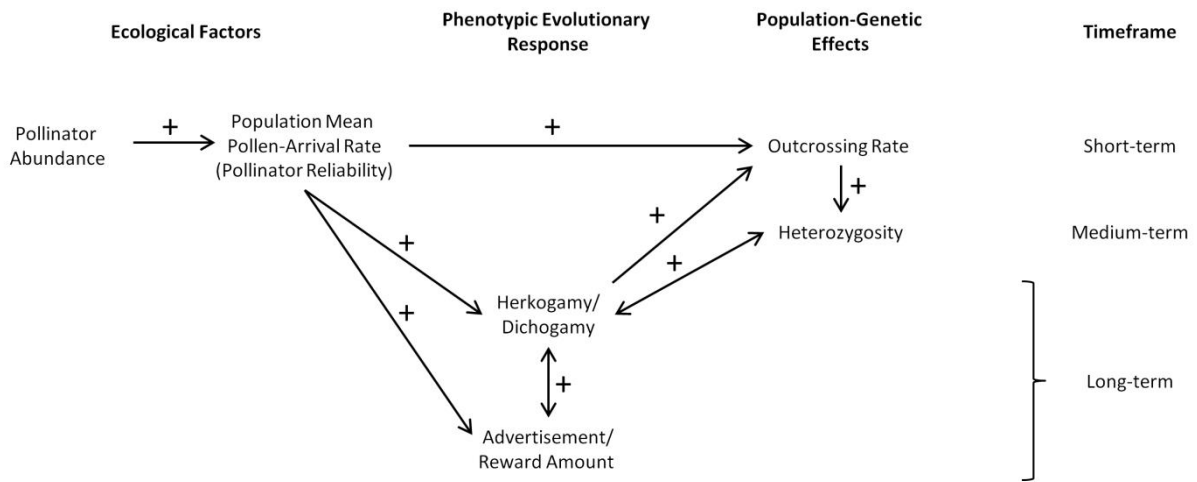
650 Fig. 2. Blossom inflorescence of *Dalechampia scandens* with indications of traits measured.  
 651 The area of the upper floral bract (UBA) was calculated as the product of its length and width,  
 652 and the area of the resin gland as the product of its width and height. Herkogamy (ASD,  
 653 anther-stigma-distance) was measured as the minimum distance between anthers and stigmas.  
 654 Drawing by M. Carlson.

655 Fig. 3. Response curve showing predicted seed set as a function of stigmatic pollen loads of  
 656 female-phase blossom inflorescences, given by the asymptotic function  $S = \alpha P / (1 + \alpha P)$ , for  $\alpha$   
 657 = 0.130. Predicted seed set is shown for the average pollen load in each population.

658 Fig. 4. Relationships among unobserved (A) and observed (B-D) variables at the population  
 659 level. Panel A shows a female *Eufriesea* collecting resin from a blossom inflorescence of  
 660 *Dalechampia scandens*. Larger stigmatic pollen loads in the female phase (on log scale) were  
 661 associated with greater outcrossing rates (B), and greater herkogamy (C). The number of male  
 662 euglossine bees on odor baits was positively correlated with stigmatic pollen loads, and  
 663 herkogamy. Herkogamy was positively associated with observed heterozygosity (D). The four  
 664 populations for which outcrossing rates were estimated are shown as square symbols. All  
 665 error bars indicate standard errors.

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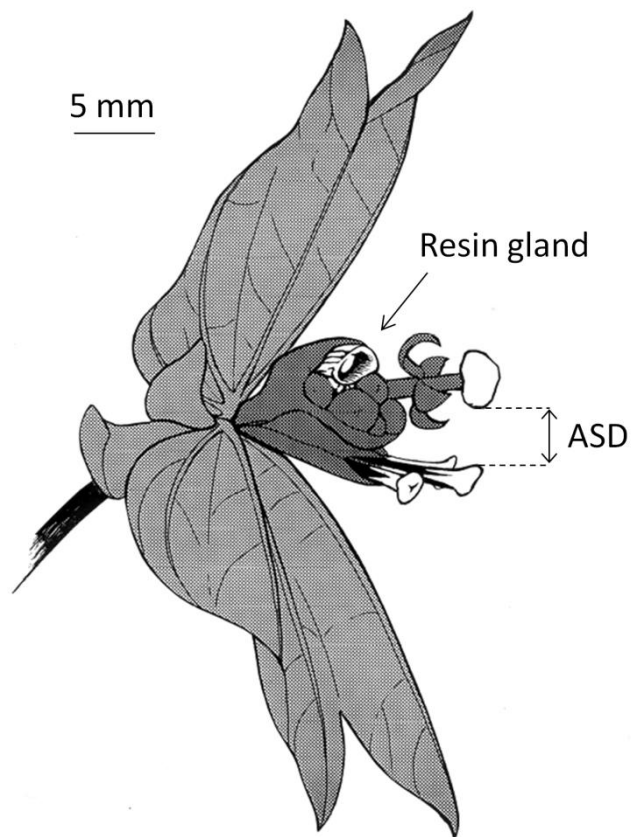
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669 Figure 1

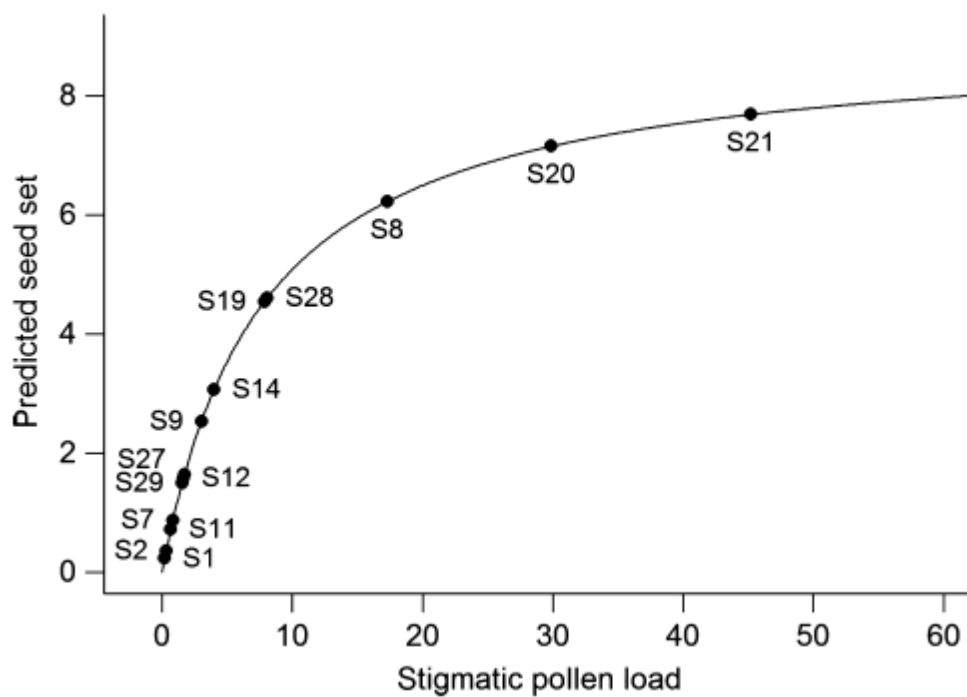
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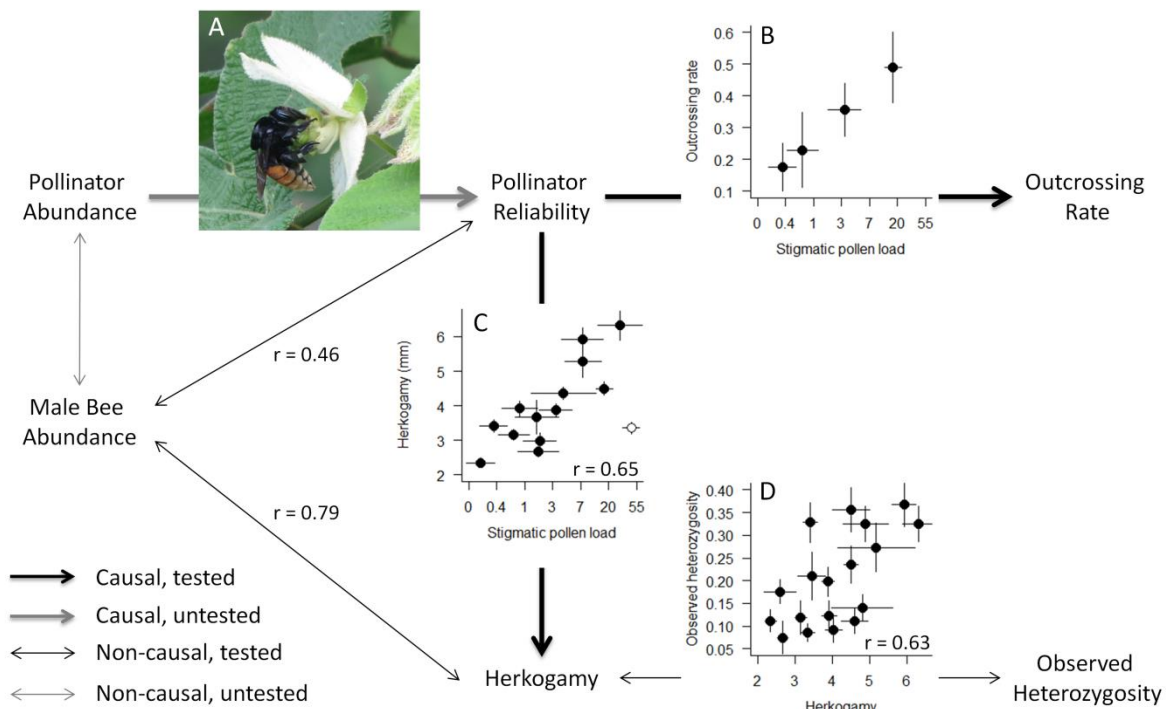
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672 Figure 2

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674 Figure 3



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676 Figure 4

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