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Weak response to selection on stigma–anther distance in a primarily selfing population of yellow monkeyflower

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Stebbins hypothesized that selfing lineages are evolutionary dead ends because they lack adaptive potential. While selfing populations often possess limited nucleotide variability compared with closely related outcrossers, reductions in the genetic variability of quantitative characters remain unclear, especially for key traits determining selfing rates. Yellow monkeyflower (*Mimulus guttatus*) populations generally outcross and maintain extensive quantitative genetic variation in floral traits. Here, we study the Joy Road population (Bodega Bay, CA, USA) of *M. guttatus*, where individuals exhibit stigma–anther distances (SAD) typical of primarily selfing monkeyflowers. We show that this population is closely related to nearby conspecifics on the Pacific Coast with a modest 33% reduction in genome-wide variation compared with a more highly outcrossing population. A five-generation artificial selection experiment challenged the hypothesis that the Joy Road population harbours comparatively low evolutionary potential in stigma-anther distance, a critical determinant of selfing rate in *Mimulus*. Artificial selection generated a weak phenotypic response, with low realized heritabilities (0.020–0.028) falling 84% below those measured for floral characters in more highly outcrossing *M. guttatus*. These results demonstrate substantial declines in evolutionary potential with a transition toward selfing. Whether these findings explain infrequent reversals to outcrossing or general limits on adaptation in selfers requires further investigation.

1. Introduction

Transitions from outcrossing to selfing are common, having occurred independently many thousands of times in plant evolution [1,2]. Genetic variability in quantitative traits makes these transitions possible, yet the genetic consequences of selfing are hypothesized to exhaust the evolutionary potential of lineages, causing them to be evolutionary blind alleys [3,4]. The general influence of self-fertilization on quantitative genetic variability has been explored theoretically [5–10] and empirically in many plant populations [11–13]. In populations with low to moderate amounts of selfing, quantitative genetic variability should generally decline in accordance with the loss of heterozygosity expected with single locus theory [6,9,11,14]. In populations with high levels of self-fertilization and correspondingly infrequent recombination, the effects of selection will extend across chromosomes and even whole genomes [7,15,16], directly or indirectly limiting a population's ability to generate novel genetic combinations [17–21]. Consequently, highly selfing populations may exhibit reduced evolutionary potential that limits their longevity over rapid time scales [4,22–24].

There are two major empirical approaches to testing hypotheses on the influence of self-fertilization on quantitative genetic variability. One approach involves quantifying the components of quantitative genetic variability that

are important to evolutionary responses in outcrossing and more highly selfing populations [11,12,25–27]. In randomly mating populations, phenotypic responses to selection on univariate traits are determined by the amount of additive genetic variation in a population [9,26]. With self-fertilization, additional, non-additive components of genetic variation (e.g. dominance (co)variances) contribute to responses to selection, which may be quantified through a combination of line cross-analyses and inference of the genetic structure of the population [25–30]. Given the need to incorporate non-additive components of genetic variation in inbreeding populations, another approach involves subjecting outcrossing and more highly selfing populations to artificial selection and comparing their responses to selection [24]. Using this method, the realized heritability absorbs non-additive components of variance, since they contribute to an inbreeding population's response to selection given a cumulative selection differential [26]. Comparisons of realized heritability avoid the various difficulties with heritability *per se* [31], and permit direct comparisons of evolutionary potential, regardless of a population's position along the continuum from full outcrossing to selfing [24,32].

A primary utility of artificial selection experiments lies in their ability to address questions concerning the nature and magnitude of genetic variation underlying adaptive responses [31,33], which were central to Stebbins' [4] original conception of selfing as an evolutionary dead end. Stebbins' composite hypothesis articulates two claims—that the evolution of higher selfing rates is unidirectional and that the genetic consequences of this reproductive mode compromise the longevity of lineages over time [3,4,24,34,35]. Artificial selection experiments on major phenotypic determinants of the selfing rate, therefore, have the capacity to test both claims in populations spanning the continuum of mating systems, ranging from full outcrossing to nearly complete selfing. Documenting robust responses to artificial selection on quantitative traits influencing the selfing rate, especially in predominantly selfing populations, would reject both the idea that they lack the capacity to re-evolve a more highly outcrossing phenotype and that they generally lack evolutionary potential or the capacity to adapt in response to selection pressure [24,36].

In this article, we test Stebbins' hypothesis that self-fertilization limits evolutionary potential by studying a primarily selfing population's capacity to evolve toward a more highly outcrossing morphology. To reach this goal, we studied a population of yellow monkeyflower (*Mimulus guttatus*) found along Joy Road in Bodega Bay, CA, USA (38.350721° N, 122.96091° W), which has an unusually high selfing rate and a stigma–anther distance (SAD) that readily secures self-pollination [37,38]. While substantial responses to selection are commonplace in highly outcrossing populations of this species [28,39–41], the evolutionary potential of more highly selfing populations remains uncertain [42]. Using the Joy Road population, we first analysed allele frequency differentiation across whole genomes to determine its relation to members of the *M. guttatus* species complex. Second, we confirmed that SAD in the population reflected a shift toward self-fertilization [37]. Third, we quantified standing genetic variation at the nucleotide level to determine whether it is reduced compared with more highly outcrossing *M. guttatus* populations. Finally, we used five generations of artificial selection on SAD to test whether the evolutionary potential of a key character determining selfing ability is attenuated compared with findings in more highly outcrossing *M. guttatus* populations [28].

2. Methods

(a) Study system

In the genus *Mimulus*, the degree of outcrossing is strongly associated with SAD. In primarily outcrossing taxa, stigma height exceeds stamen height (i.e. positive SAD), while primarily selfing taxa exhibit the reverse orientation of negative SAD [37,38,42]. While anther height exceeds the stigma surface in selfing taxa, there is close overlap securing self-pollination [43,44]. *M. guttatus* is a common species in western North America and displays broad variation in floral morphology [38,40]. While most populations primarily outcross, several notable populations of the *M. guttatus* species complex self-fertilize to a greater degree [45,46]. Crosses between *M. guttatus* and the more highly self-fertilizing *M. nasutus* and *M. platycalyx* suggest a polygenic basis for variation in SAD [37,43,47]. Throughout this article, the use of *Mimulus* taxonomic names is retained to maximize clarity regarding references to historical work [48].

M. platycalyx is one of several varieties of *M. guttatus* [38]. This variety, to which the Joy Road population belongs, was proposed because its plants exhibit unusually wide calyces and negative SADs typical of primarily selfing *Mimulus* lineages [37,44]. Surveys of genetic variability in the Joy Road population have established large inbreeding coefficients (F_{IS}), with this deviation from the Hardy–Weinberg equilibrium providing an estimate of the selfing rate (S) in an equilibrium population ($S_{eq} = 2F_{IS}/(1 + F_{IS})$ [49]. These inferred selfing rates (S = 0.53–0.90) [37,38,45] are higher than those reported in most populations of *M. guttatus* (S = 0.24–0.34) [50,51]. Using adult genotypes to infer the selfing rate underestimates the true selfing rate when there is inbreeding depression (i.e. when selfed progeny are less fit than outcrossed progeny [52]). It is, therefore, reasonable to assume that the Joy Road population reproduces primarily through selfing.

(b) DNA sequencing, relations within *M. guttatus* and DNA polymorphism

Fruits were collected from maternal plants sampled in May 2019 at 1 m intervals from the Joy Road population (figure 1). This natural population harbours hundreds of individuals and occurs at the intersection of Joy Road and the Bodega Bay Highway (38.350721° N, 122.96091° W [45]. Randomly chosen seeds sampled from four different maternal plants in nature were grown in a greenhouse. DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol [54], with the double-stranded DNA concentration of each sample estimated using a Qubit fluorometer. Large molecular weight DNA

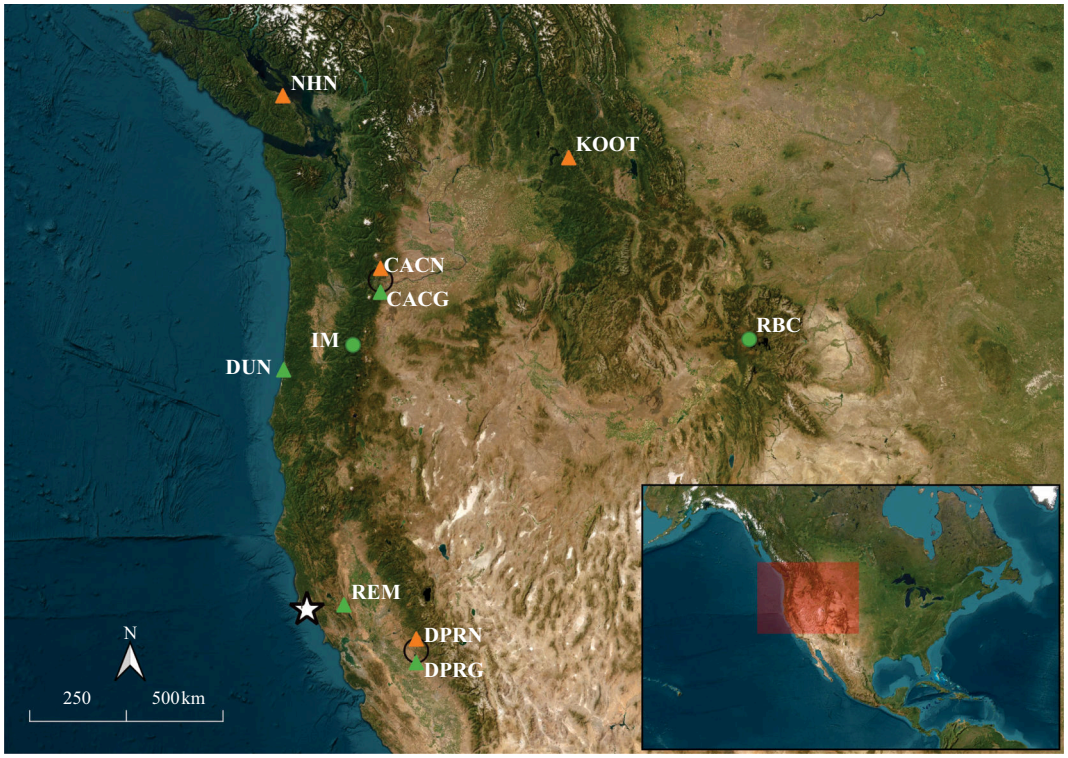

Figure 1. The location of the primarily selfing Joy Road population (white star). Whole-genome sequences of individuals from nearby populations of *Mimulus guttatus* (green triangles) and *Mimulus nasutus* (orange triangles) were considered to evaluate the relation of Joy Road to close relatives. Pairs of sympatric samples are joined by dark circles. Two additional *M. guttatus* populations (IM = Iron Mountain, RBC = Rabbit Creek, green circles) are shown because their floral phenotypes and genetic variability are also compared with the Joy Road population. Other population abbreviations follow those reported by Brandvain *et al*. [53].

was fragmented, and a barcoded library of 350 bp inserts was produced. Paired-end 150 bp sequences were generated on the Illumina NovaSeq platform to an expected depth of 20× per individual. Library preparation and sequencing was conducted by Novogene, Inc.

Whole-genome sequences of Joy Road plants were compared with previously analysed samples of *M. guttatus* and *M. nasutus* individuals sampled across the geographic range of the species complex [53; figure 1]. Individuals of *M. nasutus* were included given evidence of introgression with *M. guttatus* [53]. An outgroup sample of *Mimulus dentilobus* was included to facilitate interpretation of divergence. Sequences were aligned to *M. guttatus* TOL v. 5.0 reference with BWA v. 0.7.17 [55], and polymerase chain reaction (PCR) duplicates were removed with Picard tools v. 2.21.4 [56]. Individual samples were genotyped with the GATK haplotype caller v. 4.1.4.1 (GATK4 [57]), enforcing a minimum read depth of 10 and the removal of sites with excess heterozygosity (hard filtering script available in the Dryad repository). Biallelic single nucleotide polymorphisms (SNPs) were retained. SNPs were excluded if genotypes were absent for more than 50% of all individuals or the total read depth at the SNP exceeded the 95% cumulative density. A total of 6615 SNPs remained, with an average read depth of 24.89 per individual. The relations among individuals were visualized using Treemix v. 1.13, which plots genetic differentiation under models that include varying numbers of migration events [58].

We used synonymous variants to determine the standing genetic diversity present in the genome using four individuals from the Joy Road population. As mentioned above, individual samples were genotyped with the GATK haplotype caller and filtered on a minimum read depth of 10 and a maximum read depth of 95% cumulative density, removing sites with excess heterozygosity and those with more than two segregating bases. SnpEff [59] was used to identify synonymous positions in the genome. After limiting positions to those that were genotyped in all four Joy Road individuals, this filtering resulted in a dataset containing 191 988 bp. Nucleotide diversity (π) was calculated using sliding windows of 1000 bp. Each window-specific π was then averaged to obtain average nucleotide diversity in the Joy Road population. Joy Road π was then compared with the synonymous nucleotide diversity found in the IM population of *M. guttatus* [60]. Tajima's *D* was also calculated in each window containing polymorphism to evaluate departures from neutral expectations [61].

(c) An experimental test of Stebbins' hypothesis: the source population

Joy Road seeds collected from unrelated maternal plants (the G_0 generation) were grown to flowering and a source population was synthesized from these 50 plants from the natural population. Specifically, a total of 50 families were established by randomly pairing plants, with each serving once as an outcross seed and outcross pollen parent. A single randomly outcrossed fruit and a single selfed fruit were collected from the 50 plants. Seed families from this large source (G_1) population were split into two experimental groups, with each containing two replicate populations (figure 2). Two replicates of the control treatment (C_1, C_2) and the selection treatment (S_1, S_2) were established as experimental populations. In the first generation of the experiment (G_1), two selfed and two randomly outcrossed seeds were grown from each family, yielding 200 plants in each population and 800 total plants. Plants experienced a 14 h day length (20°C days, 15°C nights) with supplemental lighting in

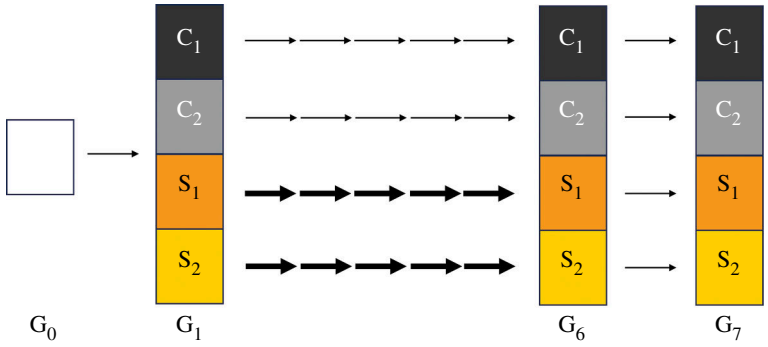

Figure 2. Design of the artificial selection experiment. An offspring from 50 plants (G_0) gathered from a single natural population was selfed and randomly outcrossed to generate a large ancestor population (G_1). G_1 seed families were split into isolated populations that evolved for five generations in a pollinator-free greenhouse. In each generation, two outcrossed and two selfed seeds from 50 parents were grown, yielding populations with 200 individuals. In control populations (C_1, C_2), parents were chosen randomly with respect to stigma–anther distance (SAD, light arrows). In selection populations (S_1, S_2), parents with the most positive SADs were chosen (heavy arrows). G_1 and G_7 plants were then grown in a common greenhouse to test hypotheses of phenotypic evolution and estimate realized heritability.

Table 1. Models of phenotypic evolution in descendant populations (G_7) compared with an ancestor population (G_1). All populations were raised simultaneously in the same greenhouse conditions. The number of parameters (k) reflects the number of unique normal distributions which have their own means yet share a common s.d. Each population mean (e.g. μ_0, μ_1, etc.) was calculated from individual phenotypes assigned to the same underlying normal distribution.

model	evolutionary process	*k*	model parameters
0	genetic drift	2	μ_0: ancestor, C_1, C_2, S_1, S_2
1	response to selection	3	μ_0: ancestor, C_1, C_2
			μ_1: S_1, S_2
2	adaptation to greenhouse	3	μ_0: ancestor
			μ_1: C_1, C_2, S_1, S_2
3	responses to selection	4	μ_0: ancestor, C_1, C_2
			μ_1: S_1
			μ_2: S_2
4	idiosyncratic adaptation	6	μ_0: ancestor
			μ_1: C1
			μ_2: C2
			μ_3: S1
			μ_4: S2

a greenhouse. Individuals were watered twice daily, randomly moved weekly and populations were propagated without gene flow using this scheme throughout the selection experiment (G_1–G_6).

To directly compare SAD in Joy Road with a more highly outcrossing population in the yellow monkeyflower species complex, an otherwise identical G_1 population was initiated from 50 field-collected maternal families from the RBC population of *M. guttatus*, which occurs within Yellowstone National Park [50,62]. In comparison to the Joy Road population, plants in the RBC population have inbreeding coefficients that suggest comparatively lower selfing rates (S = 0.30–0.32) [50], which resemble those reported in the IM population of *M. guttatus* (S = 0.24–0.34) [51]. Both the Joy Road and *M. guttatus* G_1 populations were raised simultaneously under the same conditions in the same greenhouse. A two-sample t-test was used to test the hypothesis of a significant difference in SAD between the populations. The RBC population was not subjected to artificial selection because of its much longer time to flowering [62].

(d) The selection experiment: control and selection treatments

A five-generation selection experiment was conducted, using 200 plants raised from seed in each population (C_1, C_2, S_1 and S_2) per generation (28; figure 2). Plants were labelled with random numbers and moved weekly to minimize environmental effects. During the generations in which treatments were enforced (G_1–G_5), each individual had floral traits measured on two flowers after they opened in the morning. On each flower, the following traits were measured using digital callipers: corolla length, corolla width, tube length, throat width, stigma height and maximum anther height. Landmarks for these traits were established using the approach employed in similar studies [39,43]. Maximum leaf width and days to flowering were also measured. Trait means of individual plants were calculated, with SAD = stigma height – anther height.

Once all floral traits were measured, parents were chosen within each population. In the control populations, parents were randomly chosen for each generation. In the selection populations, truncation selection was used to choose the 50 plants with

the most positive SAD [28,40]. Consequently, the evolution of SAD should reflect random genetic drift in control populations and the additional influence of directional selection in selection populations. For each of the 50 parents, a single randomly chosen pollen donor was selected as an outcross pollen parent. An outcrossed fruit and a selfed fruit were then generated on each of the 50 parents. Two outcrossed and two selfed offspring were raised from each parent to yield the expected number of 200 plants that were grown in each generation of the selection experiment. Effects of treatment on SAD were tested during the initial generation and after each of the five generations of selection (G_1–G_6). To test this hypothesis, populations were grouped according to a history without selection (C_1, C_2) or with selection (S_1, S_2). A nested ANOVA was conducted using these treatment groups and population(group) as factors. The critical p-value for these tests was reduced to $\alpha = 0.01$, given multiple comparisons.

(e) Evolutionary response to selection and realized heritability

At the end of the artificial selection experiment, the distributions of SAD in the ancestor and descendant populations were directly compared in a common greenhouse [39]. To generate G_7 individuals, 50 random plants in each population were used to generate selfed and randomly outcrossed offspring (figure 2). Two hundred plants from the G_1 ancestor and each descendant G_7 (C_1, C_2, S_1 and S_2) were grown in the same greenhouse and had floral traits measured using the protocols described above. To test whether differences between selection and control lines persisted, a nested ANOVA was conducted on the G_7 populations, as described above. The cumulative response to selection and selection differential were then calculated. The total response to selection (R_{total}) was calculated by directly subtracting the mean SAD of the ancestor (G_1) from the mean SAD in each descendant (G_7) population. Selection differentials (S_t) from each generation (t) of the experiment (G_1–G_5) were summed to yield the cumulative selection differentials (S_{total}) in the S_1 and S_2 populations.

Evolutionary potential was extracted from study populations using the realized heritability. The realized heritability of SAD was independently estimated for the S_1 and S_2 populations [28]. Realized heritability equalled the ratio of R_{total}/S_{total} [23]. This parameter absorbs both the additive and non-additive components of genetic variations that contribute to evolutionary responses in populations reproducing through a mixture of outcrossing and selfing [24,26]. To quantify uncertainty in estimates of realized heritability, we generated 1000 bootstrapped datasets. To make each replicate dataset, 200 individuals were sampled with replacements from the ancestor and selection populations (S_1, S_2) that were raised together in the G_7 generation. The response to selection (R_{total}) was calculated for each replicate, yielding sampling distributions of realized heritability in the selection populations. The standard error (s.e.) of realized heritability was computed from the standard deviation (s.d.) of each sampling distribution.

(f) Selection and phenotypic divergence between the ancestor and descendant populations

We analysed the distribution of SADs generated by the experiment to evaluate additional hypotheses of selection. Specifically, we evaluated the fit of models to the SAD distributions of the ancestor and descendant populations, measured in a common greenhouse. In a simple model of genetic drift, all individuals share a common mean SAD (μ_0). Models of selection take on various forms (models 1–4, table 1). Given the experiment, we considered a model where individuals from the ancestor and control populations (C_1, C_2) share the same SAD mean (μ_0), while individuals from the selection populations (S_1, S_2) have a distinct SAD mean induced by the treatment (μ_1). Models in which selection produces similar (i.e. adaptation to greenhouse) or distinct responses (i.e. responses to selection, idiosyncratic adaptation) in all descendant populations were also considered. For each model, the probability of each individual phenotype was calculated assuming one or more normal distributions of SAD, depending on the evolutionary scenario. After applying a natural log to these data, their sum yielded the model log-likelihood (LL). In all models, the s.d. of SAD within the ancestor population was used for all distributions to simplify model construction, given very similar s.d. in each population (electronic supplementary material, figure S1). This s.d. was the largest among the five populations and is, therefore, conservative with respect to testing hypotheses of selection.

Support for each model was calculated using AIC = $2k$ – 2LL, with k denoting the number of parameters (table 1). The best-fitting selection model with the lowest AIC was identified, and a likelihood ratio test (LRT) of this hypothesis was conducted with genetic drift as a null model. The LRT statistic was calculated using twice the difference between the selection model LL and the null model LL. LRT statistics are approximately χ^2 distributed with degrees of freedom equal to the number of additional parameters in the best-fitting selection model compared with the null model. All statistical analyses were conducted in R ([63]; v. 4.3.1).

3. Results

(a) The Joy Road population, relation to *M. guttatus* and DNA polymorphism

As is typical in *M. guttatus* populations (e.g. the IM population) [39], individuals in the RBC population had positive SADs, where anthers were shorter than the length of the stigma. In the Joy Road population, SADs were negative and significantly different (figure 3; $t_{df\ =\ 1118} = 17.098$, $p < 0.001$). Whole-genome sequence data show strong genetic similarity and recent coalescence of alleles among Joy Road individuals (figure 4). These individuals are closely related to individuals sampled nearby in coastal Oregon and California (figure 1).

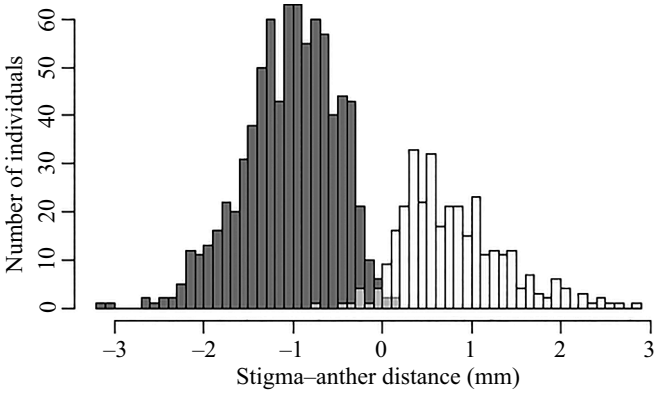

Figure 3. A comparison of SAD in the Joy Road population (dark bars) and the Rabbit Creek population (white bars) of *M. guttatus,* which reproduces through a mixed mating system. Within each population, naturally collected seeds were grown and then crossed, using a combination of random outcrossing and selfing (see figure 2, G_1). The SAD of individuals is significantly reduced in Joy Road ($t_{df=1118} = 17.098$, $p < 0.001$).

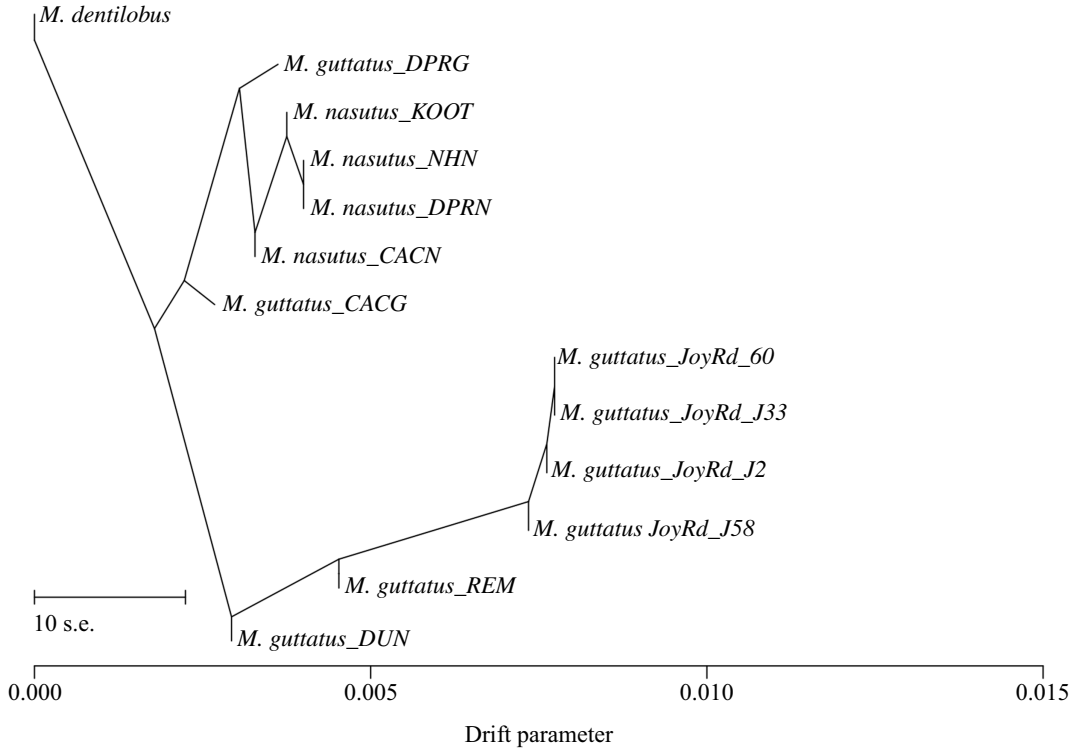

Figure 4. Allele frequency differentiation among individuals in the *M. guttatus* species complex. Four Joy Road individuals are closely related to each other and share recent ancestry with populations in the southern clade of *M. guttatus* (DUN, REM), which are found in close geographic proximity (see figure 1). Joy Road individuals are less closely related to northern *M. guttatus* (CACG, DPRG) or *M. nasutus*.

Of the 191 988 bp analysed in the Joy Road population, there were 8161 SNPs. Nucleotide diversity (π) in the Joy Road population equalled 0.022 (s.e. = 0.001), a reduction of 33% in comparison to the well-characterized and genetically variable IM population of *M. guttatus* (table 2). The mean Tajima's *D* equalled 1.563 (s.e. = 0.044), consistent with a deficit of relatively rare variants in the population.

(b) The selection experiment and evolutionary potential

Artificial selection did not generate a significant difference between the control and selection treatments in a nested ANOVA until the G_5 and G_6 generations (table 3), when selection populations expressed significantly more positive SADs (figure 5). When the G_1 ancestor was grown in the same greenhouse environment with G_7 descendants, the distinction between selection and control populations persisted, with control and selection treatments differing significantly in a nested ANOVA (figure 6; $F_{1,2} = 12.880$; $p < 0.001$). These data support a phenotypic response in populations experiencing selection compared with the ancestor and control populations (model 1, table 3; electronic supplementary material, table S1; LRT = 15.131; $p < 0.001$).

Cumulative selection differentials of 3.158 and 3.189 were generated in the S_1 and S_2 populations, respectively. Much smaller cumulative selection differentials of −0.073 and 0.0078 were generated in the C_1 and C_2 populations. Given the evolutionary responses measured in the selection populations when compared with their G_1 ancestor (figure 6), the realized heritabilities of SAD equalled 0.028 (s.e. = 0.013) and 0.020 (s.e. = 0.013) in the S_1 and S_2 populations.

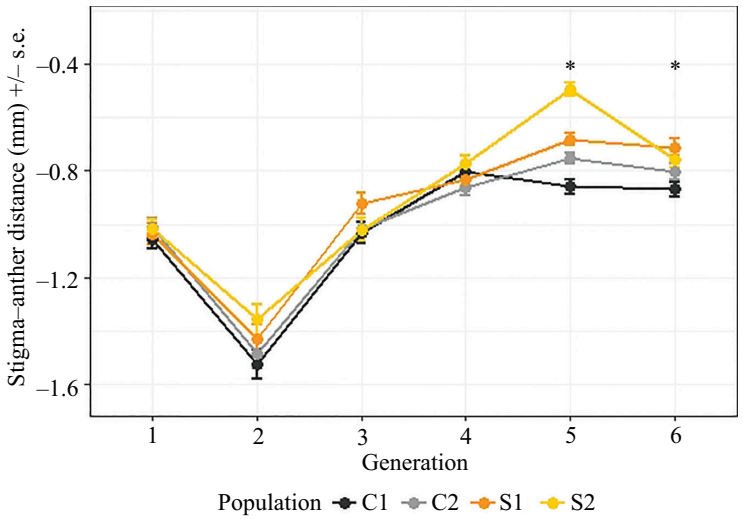

Figure 5. Evolution of SAD during the experiment. Control and selection treatments were compared in each generation using a nested ANOVA. Stars denote significant differences among treatments (see table 3).

Table 2. Nucleotide diversity (π) in the Joy Road population and the Iron Mountain population of *M. guttatus* (from Ref. [60]). Both estimates are based on genome-wide samples of synonymous SNPs.

population	synonymous π
Iron Mountain	0.033
Joy Road	0.022 (s.e. ± 0.001)

4. Discussion

Stebbins [4] hypothesized that a primary reason for the limited longevity of predominantly self-fertilizing plants followed from their lack of evolutionary potential. Populations of *M. guttatus* generally reproduce through outcrossing and mixed mating [51], harbour high levels of standing genetic variation in their genomes [60,62,64] and produce rapid phenotypic responses to artificial selection [28,39–41]. The primarily selfing Joy Road population, known as a variety of *M. guttatus* (i.e. *M. platycalyx*), shares close ancestry with nearby *M. guttatus* populations on the Pacific Coast, which belong to the southern clade of the *M. guttatus* species complex [65]. Compared with more outcrossing *M. guttatus*, reductions in nucleotide diversity in the Joy Road population are relatively modest in light of the losses that are typically found in selfing populations compared twith closely related outcrossers in nature [66–68]. While more extensive declines in genetic variation have been documented in highly selfing populations of *M. nasutus* [47,53], even these populations maintain considerable genetic variation at the nucleotide level.

Genetic variability at the nucleotide level, however, often provides little information on the genetic variance of quantitative traits [69,70]. Artificial selection was applied to approximate the strength of selection in an experiment conducted in the more highly outcrossing IM population, where the top 25% of individuals were chosen to reproduce each generation [28]. After four generations of selection on an index based on the corolla width and flowering times of individuals, Holeski & Kelly [28] found realized heritabilities ranging from 0.18 to 0.33. In the current study, realized heritabilities ranged from 0.020 to 0.028, representing a decline of at least 84% in evolutionary potential in the more highly selfing lineage. This difference in heritability cannot be attributed to smaller population sizes in the current study, since populations were 40% smaller in the historical study [28]. Similarly, large reductions in the total genetic variance of SAD were found in highly selfing populations of *M. micranthus* when compared with *M. guttatus* [42].

Artificial selection presents an effective means to study genetic variability in quantitative characters, yet the results of this study are not free from potential bias. Given that a history of directional selection on SAD in the Joy Road population appears likely [37], this process is expected to reduce evolutionary potential in theory, although evolutionary potential often persists for such characters [71]. While meta-analyses on the heritability of general quantitative traits show declines with selfing [11,12], a large study on herkogamy revealed surprisingly minor reductions in its evolvability in highly selfing lineages [13], so it is by no means clear that key determinants of the selfing rate have depressed evolutionary potential. The crossing design used in the current study may also have introduced additional selection pressures beyond the control of the experimenter. In highly selfing natural populations with distinct multi-locus genotypes, crosses between genotypes may increase fitness upon masking of deleterious recessive alleles or reduce fitness upon the disruption of beneficial epistatic interactions [72–74]. The potential influence of these selective processes depends heavily on the genetic architecture of quantitative traits that were not tested directly [8].

In other studies, artificial selection has been applied directly to compare the evolutionary responses of natural populations that vary in their degree of self-fertilization. Artificial selection on autogamy (i.e. selfed seed set) in

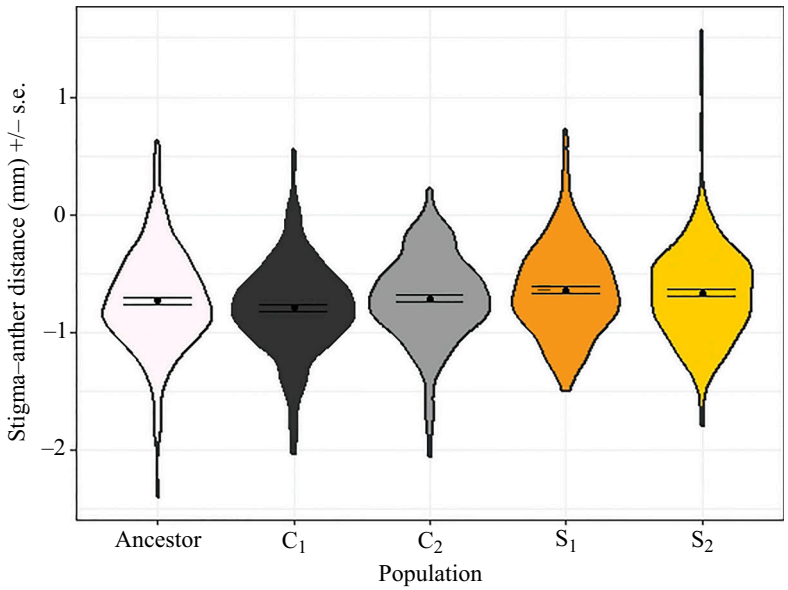

Figure 6. SAD in the ancestor (G_1) and its four descendant populations (G_7), raised in a common greenhouse environment. Control and selection treatments differed significantly in a nested ANOVA ($F_{1,2} = 12.880$; $p < 0.001$). The data support a phenotypic response in populations experiencing selection compared with the ancestor and control populations (model 1, table 3; electronic supplementary material, table S1; LRT = 15.131; $p < 0.001$). Samples sizes (n): ancestor = 198, $C_1 = 198$, $C_2 = 196$, $S_1 = 200$, $S_2 = 195$.

Table 3. SAD in the ancestor generation (G_1) and after each episode of artificial selection (G_2–G_6). The average SAD is reported in each population, with the s.d. in parentheses followed by the sample size (n). Differences between control (C_1, C_2 populations) and selection treatments (S_1, S_2 populations) were evaluated with nested ANOVA. F ratios that reject the null hypothesis of no treatment effect are denoted by bold p values lower than $\alpha = 0.01$.

generation	C_1	C_2	S_1	S_2	treatment effect ($F_{1,2}$)	p
G_1	−1.055 (0.520); $n = 198$	−1.012 (0.486); $n = 197$	−1.036 (0.540); $n = 200$	−1.019 (0.513); $n = 200$	0.029	0.864
G_2	−1.526 (0.776); $n = 200$	−1.486 (0.759); $n = 200$	−1.430 (0.800); $n = 199$	−1.356 (0.812); $n = 199$	4.139	0.042
G_3	−1.032 (0.576); $n = 200$	−1.019 (0.565); $n = 200$	−0.922 (0.568); $n = 198$	−1.020 (0.583); $n = 199$	1.781	0.182
G_4	−0.804 (0.316); $n = 199$	−0.861 (0.384); $n = 198$	−0.834 (0.391); $n = 197$	−0.773 (0.369); $n = 195$	1.173	0.279
G_5	−0.860 (0.361); $n = 200$	−0.753 (0.314); $n = 200$	−0.682 (0.310); $n = 200$	−0.495 (0.360); $n = 200$	83.61	**<0.001**
G_6	−0.869 (0.398); $n = 200$	−0.804 (0.409); $n = 200$	−0.711 (0.453); $n = 198$	−0.756 (0.459); $n = 198$	11.34	**<0.001**

the primarily outcrossing *Phlox drummondii* and selfing *P. cuspidata* demonstrated significant declines in the realized heritability of this trait in the more highly selfing lineage [23]. Both self-compatible and primarily selfing lineages have shown the capacity to respond to selection in the known cases where more highly outcrossing phenotypes were favoured by artificial selection [23,75]. It remains unclear, however, whether small evolutionary responses in quantitative traits, as reported in this study, demonstrably shift outcrossing rates in natural populations. For one, the relationship between SAD and selfing rate is likely nonlinear, with little change in the mating system once anthers are sufficiently close to the stigma surface. Such functional evidence would be necessary before concluding that populations with morphological adaptations securing self-pollination may re-evolve higher outcrossing rates and, therefore, escape the blind alleys envisioned by Stebbins [23,42,76,77].

On a more general note, informative tests of the idea that transitions from outcrossing to selfing are irreversible heavily depend on the mechanisms prohibiting the re-evolution of higher outcrossing rates in nature [34,35]. While Stebbins drew attention to the drawbacks of exhausted genetic variability in selfing populations [4], the adaptive evolution of higher outcrossing rates in these contexts requires natural selection. There are at least two major reasons why selection of quantitative traits that predispose individuals to outcross may fail to produce evolutionary responses in highly selfing natural populations. First, the reduced fitness of selfed relative to outcrossed offspring (i.e. inbreeding depression) is necessary to counteract the transmission advantage of selfing [35,78], yet this genetic load is generally reduced in highly selfing populations [79]. Second, selection follows from a covariance between individual outcrossing rates and fitness, which requires variance in the mating system to be expressed among individuals in a natural population [80]. If highly selfing populations inhabit environments with few pollinators or plants self-fertilize ovules well before outcross pollen grains are available (i.e. prior selfing [81]), opportunities for selection may be compromised because outcrossing is rarely realized. Limited genetic variation in quantitative traits may, therefore, only partially explain why selfing is a dead end, especially when natural selection is weak relative to other causes of evolutionary change.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Joy Road DNA sequences are available at SRA under the following BioProject ID: PRJNA1043204. Data are available on Dryad [82].

Supplementary material is available online [83].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. S.L.T.: data curation, formal analysis, investigation, methodology, software, writing—review and editing. J.W.B.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, writing—original draft, writing—review and editing.

Both authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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