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Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating

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The evolution of self-fertilization in hermaphrodites is opposed by costs that decrease the value of self progeny relative to that of outcross progeny[1–3]. However, self-fertilization is common in plants[4]; 20% are highly selfing and 33% are intermediate between selfing and outcrossing[5]. Darwin[6] proposed an adaptive benefit of self-pollination in providing reproductive assurance when outcrossing is impossible[6–9]. Moreover, if outcross pollen receipt is inconsistent within or between years, these conditions likewise favour self-pollination[10], and this can result in a mixture of self and outcross seed production (mixed mating). Despite wide acceptance, the reproductive assurance hypothesis has lacked the support of complete empirical evidence to show that variable pollination can create both the ecological and genetic conditions favouring self-pollination. We recently showed in *Collinsia verna* that during periods of infrequent pollinator visits, autonomous self-pollination boosted seed output per flower[11], the key ecological condition. Here we show low inbreeding depression and marker-based estimates of selfing, demonstrating that when the pollination environment in wild populations necessitates reproductive assurance, selfing rates increase. We provide a complete demonstration of reproductive assurance under variable pollination environments and mechanistically link reproductive assurance to intermediate selfing rates through mixed mating.

Populations of flowering plants that lack mates or pollinators, such as those at the edge of a species' range or colonizing species, rapidly evolve autonomous self-fertilization[12,13] (within-flowers selfing without a pollen vector)[8], and this is thought to occur because selfing provides reproductive assurance[6–9]. Other ecological factors, such as unpredictable outcross pollen receipt within or among years[8,10,12,14] may also, in theory, produce conditions that favour autonomous selfing through reproductive assurance[10,15,16]. For autonomous selfing to evolve, its benefits must be balanced against the potential costs. Alleles promoting self-fertilization ability increase in frequency because plants that carry them can serve as pollen parents in two ways: both by fertilizing ovules on other plants (that is, outcrossing) and by fertilizing their own ovules (that is, selfing)[17]. Thus, individuals with alleles that cause more selfing have an advantage in transmission over individuals with alleles for outcrossing[18]. In contrast, selfing is disfavoured when there is inbreeding depression (δ, low vigour of self progeny)[1,2] and/or when the production of selfed progeny pre-empts the production of outcrossed progeny (pollen or seed discounting)[8,16]. Previous investigations have failed to show that when outcross pollen receipt is inconsistent, selfing is favoured and outweighs these costs[19]. We are currently unable to predict when autonomous self-fertilization will provide reproductive assurance. An unequivocal demonstration of reproductive assurance under unreliable pollinators requires several types of data[4,14,19]. Plants must fail to receive outcross pollen, but this failure need not occur every season. During periods of low or no outcross pollen receipt, autonomous selfing must boost seed production. Finally, the combined costs (seed discounting, pollen discounting and inbreeding depression) must not completely negate the fitness gain of selfing.

Costs incurred by autonomous selfing vary depending both upon the timing of self-pollination relative to outcross pollen receipt[8] and the availability of pollinators. When pollinators are present, autonomous self-pollination that occurs after all opportunities for outcross pollen receipt have passed (delayed selfing) incurs no pollen or seed discounting costs[8,10]. Additionally, even if inbreeding depression (δ) is high, the survival of any progeny produced by delayed selfing always provides reproductive assurance[10]. In contrast, if autonomous self-pollination coincides with outcross pollen receipt (competing selfing), then pollen discounting, seed discounting and inbreeding depression can disfavour selfing[8]. In theory, if the fitness of self progeny produced by competing selfing is less than or equal to roughly half the fitness of outcrossed progeny (that is $\delta > 0.5$), then the fitness gain due to the transmission advantage is lost[1,2]. Finally, when pollinators are absent, there are no seed and pollen discounting costs of autonomous selfing[3,8,10,20]. In a field experiment, we previously investigated autonomous self-pollination in the winter annual wildflower, *C. verna* (Plantaginaceae). We showed that this species autonomously self-pollinates[21] in a field experiment that compared fruit set of emasculated versus control flowers (Tables 1 and 2 in ref. 11). Further, we quantified the timing of autonomous selfing by comparing both the timing of pollen deposition and the number of pollen grains on the stigmas of flowers in open-pollinated conditions relative to flowers in pollinator-excluded treatments. Selfing in *C. verna* is autonomous and predominantly delayed, with the potential for some competing selfing (Fig. 2 in ref. 11).

Because competing selfing can occur in *C. verna*, it is important to estimate the magnitude of inbreeding depression. Here we report results from three wild populations (BT, EF and TMC; see Methods) located in southwestern Pennsylvania, USA. We produced both selfed and outcrossed progeny on plants from each population and compared their lifetime performance. Mean trait values of self versus outcross progeny for each population were compared, and indicate that only one of the 15 comparisons was statistically significant (seed weight; BT population, $P < 0.001$). All three populations show markedly low average levels of inbreeding depression (Fig. 1; $\delta < 0.15$ for all traits measured, in all populations), lower than the ~0.5 value that opposes competing selfing. Additionally, previous field estimates of early inbreeding depression in the three study populations revealed no significant difference (for all six comparisons $P > 0.2$) in the fruiting success of selfed versus

outcrossed flowers[11]. Our current results are consistent with both field estimates of δ (Black, B., unpublished data) and previous greenhouse studies of populations from Illinois and Michigan, USA, that used identical methods to quantify inbreeding depression[22] (and unpublished data, S.K., Holtsford, Thiede, Kärkkainen and Black). Moreover, in all our populations, δ estimates for lifetime fitness (R_0 = survival × flower number) are significantly below 0.5[2] (Population $R_0\delta$ (s.e.m.): BT, $R_0\delta = 0.006$ (0.075); EF, $R_0\delta = 0.176$ (0.058); TMC, $R_0\delta = 0.086$ (0.099)). Because selfing in *C. verna* is mostly delayed[11], pollen and seed discounting costs are negligible across all pollinator environments, even for the occasional flowers with competing selfing[10].

Variation in the pollination environments of *C. verna* was previously quantified in a three-year field study in the same populations[11]. We compared fruiting success of open-pollinated, emasculated flowers versus outcrossed intact control flowers that were also open to pollinators (Table 2 in ref. 11) and showed that pollinator visits varied significantly (12 periods had significant pollinator failure ($P < 0.002$) leading to decreased seed production; 15 periods had no significant pollinator failure ($P < 0.06$) and no decrease in seed production) within and among flowering seasons and that this low visitation resulted in significantly reduced fruit set of emasculated flowers (range 0–35% less fruit). Most importantly, we compared the fruiting success of emasculated flowers that were open to pollinators with that of intact flowers that were open to pollinators (Table 2 in ref. 11), to show that during those periods of scarce pollinators autonomous selfing augmented fruit set (up to +30%)[11], the key ecological condition. Thus, ecological circumstances of reproductive assurance—variation in pollinator service and increase in seed set in the absence of pollinators—exist in these *C. verna* populations.

The selfing rate of a population quantifies the proportion of progeny produced through self-pollination[23] and provides an integrated measure of the realized mating system during one flowering season. All else being equal, selfing rates in *C. verna* should mirror the stochasticity of pollinator visitation and increase when pollinator visitation is low because, as we previously showed, proportionately more seeds are sired through autonomous selfing when pollinators fail to visit flowers[11]. However, self-pollination and mixed mating can occur even when pollinators are abundant if pollinators transport self-pollen within a flower (facilitated selfing), or among flowers on the same plant (geitonogamy)[8]. In this way, seeds are sired by a combination of pollinator-mediated movement of outcross pollen, and both pollinator-mediated selfing (geitonogamy + facilitated selfing) and autonomous selfing. When pollinators are abundant, all the above types of selfing suffer the costs of seed and pollen discounting[8], and selfing is disfavoured[14,24]. Conversely, when pollinators are sometimes absent and early inbreeding depression values are low, as in our study, the annual seed production will be a mixture of autonomous selfing and outcrossing. In this case, flowers unvisited by pollinators suffer no discounting costs, mixed mating is favoured and intermediate selfing rates are expected.

Here we test the relationship between pollinator failure rate and selfing rate over two years (1999 and 2000) in the same three field populations in which we estimated inbreeding depression. To quantify pollinator failure rates, we compare the proportion of open-pollinated, emasculated flowers that failed to set fruit, with the fruit set failure of paired, hand-pollinated control flowers in both years. None of the study populations experienced significant pollinator failure in 1999. In contrast, both the BT and EF populations experienced significant pollinator failure in 2000, whereas the TMC population again did not. Concordant with expectations, selfing rates vary significantly among populations and years

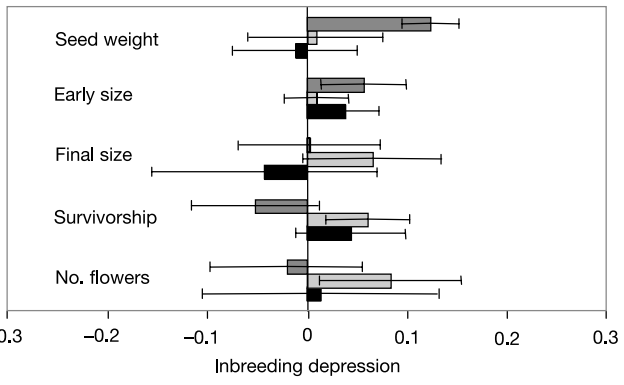

Figure 1 Populations of the annual species, *C. verna*, have low values of inbreeding depression, δ. Mean levels of δ for all populations and traits are <0.15, below the theoretical value of 0.50 that disfavours competing selfing. Traits were measured across the entire lifespan of the plants. Population-level mean and s.e.m. of δ for each trait were calculated by averaging (family-level difference in trait value between selfed and outcrossed progeny/trait value of the outcrossed progeny). Populations: BT, dark grey; EF, light grey; TMC, black (±1 s.e.m.). Population differences in mean δ were non-significant (one-way analysis of variance for each trait, $P > 0.2$).

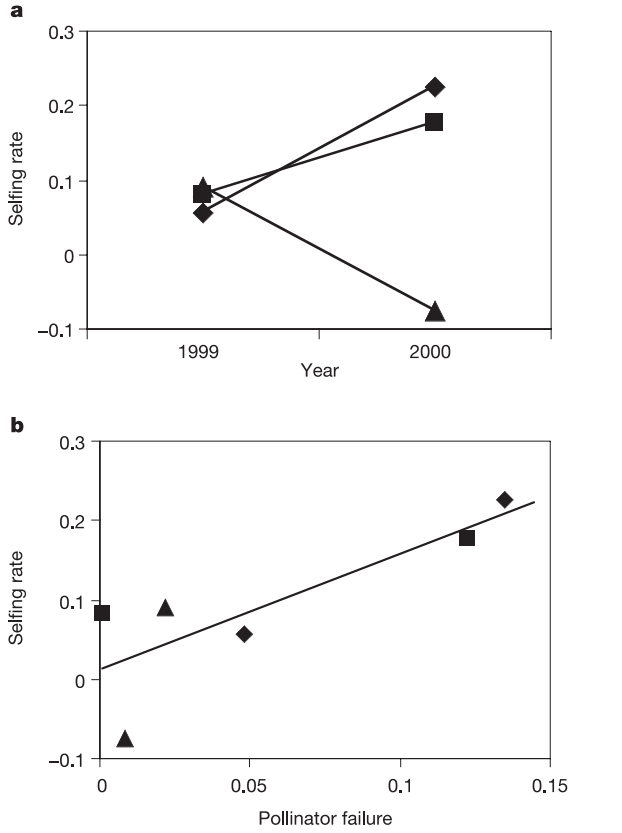

Figure 2 Annual variation in selfing rates in *C. verna* populations results from an increase in autonomous self pollination when pollinators fail to visit flowers. **a**, Selfing rates vary significantly among populations and years. Annual mean selfing rates for each population (BT, diamond; EF, square; TMC, triangle) were determined using multi-locus data. Bootstrapped s.e.m. values are all less than ±0.01 and are hidden by the symbols. **b**, Selfing rates increase as pollinator visitation rates decrease because more seeds are sired by autonomous selfing. Population selfing rates are significantly and positively correlated with field estimates of pollinator failure ($P < 0.05$, $R^2 = 0.67$, $y = 1.46 + 0.01$). Bootstrapped s.e.m. values are all less than ±0.01 and are hidden by the symbols.

(Fig. 2a). More importantly, annual selfing rates are positively correlated with pollinator failure rate (Fig. 2b). Our data suggests that a 10% reduction in pollinator visits leads to a 14% increase in the selfing rate. Because only autonomous selfing can occur when pollinators fail to visit a flower, our data mechanistically link reproductive assurance to intermediate selfing through context-dependent changes in the proportion of seed produced by mixed mating.

Our data provide a complete demonstration of reproductive assurance under unpredictable pollinator environments[4] and an empirical demonstration that selfing rates of populations directly respond to the pollinator environment (Fig. 2). These results indicate that when pollinator visits decrease, populations shift towards intermediate selfing rates through an increase in the proportion of autonomously selfed seeds. This field study provides only a snapshot of the evolutionary process of *C. verna*'s mating system, but brings into focus surprising results. Outcross pollination events generated the majority of the seeds in all populations over both years (73–100% outcrossing, Fig. 2a). However, these selfed progeny express lower early inbreeding depression (Fig. 1) than is typically seen in outcrossing species[25], and the magnitude of inbreeding depression increases from early to late stages, as is typically seen in selfing species[22,25]. Unpredictable pollination environments are the norm in wild plant populations, and can include periods of total pollinator failure[12,26]. Within the evolutionary history of these *C. verna* populations, years of complete pollinator failure must undoubtedly have occurred. Such extreme pollination environments can both favour autonomous self-pollination[8,12,13] and reduce genetic load[25,27]. Species with floral developmental mechanisms that promote outcrossing when pollinators are present, but ensure self-pollination if they are not[8,11], can have different annual selfing rates as a functional response to pollinator environments, assuring reproduction and providing a 'best of both worlds' mating system[12]. Mating system models have shown that an intermediate level of selfing can be evolutionarily stable[10,28]. The observation of intermediate selfing rates in many other animal-pollinated species[5,29] may in part reflect similar fluctuating, context-dependent benefits to selfing and outcrossing in a variable world. □

Methods

Study species and populations

C. verna is a self-compatible winter annual herb, native to the eastern half of North America, that flowers with the spring ephemeral flora. In this species, autonomous self-pollination occurs relatively late in a flower's lifetime and ranges from competing (coincident with outcross pollen receipt) to delayed (after the opportunity for outcrossing has passed)[11]. Approximately 4–5 days after anthesis begins, the style elongates and brings the receptive stigma into contact with the pollen-bearing anthers[30], which can result in self-pollination. The three populations of *C. verna* used in this study are located in different counties and/or watersheds in Pennsylvania, USA: Braddock Trail (BT), Ohio River watershed (Westmoreland Co.); Enlow Fork (EF), Monongahela River watershed (Washington Co.); and Ten Mile Creek (TMC), Ohio River watershed (Washington Co.). These populations differ significantly in their pollinator communities and their dates of first flowering differ by as much as 20 days[11]. Pollinators include native bumble bees and solitary bees as well as the introduced European honey bee[11].

Inbreeding depression

At the end of the 1999 flowering season, 30 plants bearing seeds were collected in the field from each population. All seeds were individually planted and placed under growth chamber conditions that cue germination. One seedling was randomly chosen from each of the original 30 plants, per population, until a sample size of 20 parents was achieved for each population. These parents were grown to flowering in a greenhouse. Eight flowers per parent were emasculated at the bud stage. Four of the emasculated flowers were hand self-pollinated with pollen from other flowers on the same parent plant, whereas the remaining four flowers were outcross-pollinated with a pollen mixture from three to six donor plants from the same population in the experiment. As in a previous study on inbreeding depression in *C. verna*[22], the resulting seeds were individually weighed, and six self-pollinated seeds and six outcrossed seeds per parent were germinated in a Conviron controlled environment growth chamber, transplanted and grown to maturity in a greenhouse. Imbalances in the number of progeny per parent reduced the number of parents that could be used in the analyses to 16–18 per population. Traits measured on all progeny were: seed weight (mg), early size (cotyledon diameter, mm), final size (number of branches + number of whorls at flowering), survival to flowering, number of flowers and lifetime reproductive output (R_0). The difference in mean trait value (fitness) between self-pollinated progeny (w_s) and outcrossed progeny (w_o) estimates the magnitude of inbreeding depression (δ values) calculated as in ref. 2: $\delta = (w_o - w_s)/w_o$. Family-level δ were determined and used to calculate the population-level mean and standard error in δ (Fig. 1). Population mean δ values were compared using a one-way analysis of variance for each trait. Finally, we compared the mean traits values for each population using selfed and outcrossed family means in a two-tailed, paired *t*-test.

Pollinator failure rate and selfing rate

In all three populations in 1999 and 2000, groups of 200 flowers were emasculated at the bud stage and paired with groups of 200 intact flowers. Pollinators do not discriminate against emasculated flowers of *C. verna*[30]. Four to five days later, intact flowers were hand outcrossed with pollen from three to six pollen donors located at least 1 m away, whereas emasculated flowers received pollen only from natural pollinator visits (open-pollinated). This experiment was repeated three times across the peak flowering period each year ($N = 600$ flowers per treatment per population per year). We define pollinator failure rate as: 1 − (% fruit set of emasculated flowers)/% fruit set of hand outcrossed intact flowers.

To determine the selfing rate, all seeds from 50 randomly chosen plants per population were placed under germination conditions in growth chambers and 20–35 maternal sibships per population with at least five seedlings were used to determine annual selfing rate per population. Tissue was ground and electrophoresed on an 11% starch gel and stained for seven polymorphic enzyme systems: MDH1, MDH2, UGPP1, UGPP2, UGPP3, DIA, and PGI. Maximum likelihood estimation techniques with 500 bootstraps using family resampling methods were used to calculate the mean and standard error of the selfing rate for each population and year using the MLTR program[23].

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A barley cultivation-associated polymorphism conveys resistance to powdery mildew

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Barley (*Hordeum vulgare*) has played a pivotal role in Old World agriculture since its domestication about 10,000 yr ago[1]. Barley plants carrying loss-of-function alleles (*mlo*) of the *Mlo* locus are resistant against all known isolates of the widespread powdery mildew fungus[2]. The sole *mlo* resistance allele recovered so far from a natural habitat, *mlo*-11, was originally retrieved from Ethiopian landraces and nowadays controls mildew resistance in the majority of cultivated European spring barley elite varieties[2]. Here we use haplotype analysis to show that the *mlo*-11 allele probably arose once after barley domestication. Resistance in *mlo*-11 plants is linked to a complex tandem repeat array inserted upstream of the wild-type gene. The repeat units consist of a truncated *Mlo* gene comprising 3.5 kilobases (kb) of 5′-regulatory sequence plus 1.1 kb of coding sequence. These generate aberrant transcripts that impair the accumulation of both *Mlo* wild-type transcript and protein. We exploited the meiotic instability of *mlo*-11 resistance and recovered susceptible revertants in which restoration of *Mlo* function was accompanied by excision of the repeat array. We infer *cis*-dependent perturbation of transcription machinery assembly by transcriptional interference in *mlo*-11 plants as a likely mechanism leading to disease resistance.

Barley *Mlo* encodes the prototype of a plant-specific family of seven-transmembrane domain proteins[3–5]. The protein interacts with the Ca^{2+} sensor calmodulin and seems to inhibit a vesicle-associated and SNARE-protein-dependent resistance to the barley powdery mildew fungus (*Blumeria graminis* f. sp. *hordei*; *Bgh*) at the cell periphery[6–8]. Each of 17 molecularly characterized *mlo* mutants was derived from chemical-induced or radiation-induced mutagenesis, invariably affecting coding or intron splice junction sequences[3,9]. Some primitive cultivars (landraces) collected from the granaries of local farmers in Ethiopia during expeditions in 1937 and 1938 possess strong resistance against all tested *Bgh* isolates[2,10] that genetic analysis has attributed to the presence of *mlo* alleles (designated *mlo*-11; ref. 11). The frequency of this naturally occurring broad-spectrum resistance to powdery mildew was 0.2–0.6% in total Ethiopian landrace material but in a particular locality was up to a level of 24% (refs 2, 12).

In contrast to fully resistant mutagen-induced *mlo*-null mutants[3], *mlo*-11 plants allow the low-level growth of sporulating *Bgh* colonies (Fig. 1a). When homozygous *mlo*-11 resistant plants were self-pollinated (selfing), fully susceptible individuals were recovered with a frequency of about $(0.5–1) \times 10^{-4}$ (designated 'revertants'; Fig. 1a, Supplementary Table 1), indicating a possible meiotic instability of the *mlo*-11 allele. In contrast, no susceptible individual was found in about 125,000 progeny obtained after selfings of *mlo* resistant lines containing various mutation-induced lesions in *Mlo* (Supplementary Table 1). Extensive genetic analysis of the susceptible *mlo*-11 revertants indicated that either the *Mlo* susceptibility allele was restored or that susceptibility was the result of a heritable change in a tightly linked locus (Supplementary Table 2).

DNA sequencing of the *Mlo* coding region in *mlo*-11 resistant plants failed to detect differences from the *Mlo* wild-type sequence. However, genomic Southern blots probed with full-size *Mlo* complementary DNA (cDNA) detected expected fragment sizes of wild-type *Mlo* and additional strongly hybridizing fragments (Fig. 1b). Similarly, six of seven Ethiopian broad-spectrum powdery-mildew-resistant accessions of the Centre for Genetic Resources of The Netherlands (Supplementary Table 3), included in the haplotype analysis described below, showed a genomic Southern pattern identical to *mlo*-11 plants (not shown). The additional hybridizing signals were absent from both homozygous susceptible *mlo*-11 revertant progeny and susceptible *Mlo* wild-type control plants (Fig. 1b), indicating a causal link between the presence of these additional *Mlo*-homologous fragments in *mlo*-11 plants and resistance. Relative signal intensities suggested that the extra sequences were present in multiple copies. Polymerase chain reaction (PCR) analysis of *mlo*-11 genomic DNA showed that these were arranged as tandem repeat units, consisting of 1.1 kb of *Mlo* coding sequence (exon 1 to intron 5) flanked by 3.5-kb upstream sequences (Fig. 2a, b). Juxtaposed repeats were separated by a GT dinucleotide (Fig. 2b) not present in wild-type *Mlo*. Quantitative real-time PCR analysis revealed 9.4 ± 4.2 copies of the repeat unit in cultivar Ingrid BC *mlo*-11, 1.0 ± 0.3 copies in the tested homozygous susceptible revertant line, and 7.2 ± 4.3 copies in the tested homozygous resistant revertant sibling (all values relative to cultivar Ingrid *Mlo*, set as 1.0). The tandem repeat structure in *mlo*-11 is reminiscent of concatemers generated by the 'rolling-circle' DNA replication used by some viruses and transposons present in plants, for example the geminiviruses[13] and *Helitron* transposons[14]. Chance use of a section of the *Mlo* gene by the rolling-circle DNA replication machinery offers a possible explanation for the presence of the *mlo*-11 repeat array.

We constructed a genomic cosmid library from *mlo*-11 plants and isolated four cosmid clones with the use of a *Mlo* 5′-terminal cDNA probe. DNA sequencing from the clones identified the 5′ end of the repeat structure, consisting of a severely truncated repeat unit (Fig. 2d). Two low-copy loci located 5′ of the repeat array were anchored to three of four *Mlo*-containing yeast artificial chromosome (YAC) clones, thereby unambiguously locating the *mlo*-11 repeat structure upstream of and adjacent to wild-type *Mlo* (Fig. 2e). None of the cosmid clones contained the 3′ end of the repeat structure, and PCR amplification of this region from genomic DNA failed. However, the 3′ end is likely to be located at least 1.8 kb upstream of the wild-type *Mlo* copy because identically sized fragments (representing wild-type *Mlo*) were detected by Southern