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Evolution of the selfing syndrome: Anther orientation and herkogamy together determine reproductive assurance in a self-compatible plant

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Capacity for autonomous self-fertilization provides reproductive assurance, has evolved repeatedly in the plant kingdom, and typically involves several changes in flower morphology and development (the selfing syndrome). Yet, the relative importance of different traits and trait combinations for efficient selfing and reproductive success in pollinator-poor environments is poorly known. In a series of experiments, we tested the importance of anther–stigma distance and the less studied trait anther orientation for efficiency of selfing in the perennial herb *Arabis alpina*. Variation in flower morphology among eight self-compatible European populations was correlated with efficiency of self-pollination and with pollen limitation in a common-garden experiment. To examine whether anther–stigma distance and anther orientation are subject to directional and/or correlational selection, and whether this is because these traits affect pollination success, we planted a segregating F2 population at two native field sites. Selection strongly favored a combination of introrse anthers and reduced anther–stigma distance at a site where pollinator activity was low, and supplemental hand-pollination demonstrated that this was largely because of their effect on securing self-pollination. The results suggest that concurrent shifts in more than one trait can be crucial for the evolution of efficient self-pollination and reproductive assurance in pollinator-poor habitats.

KEY WORDS: Adaptive evolution, floral morphology, mating system, natural selection, pollination.

Understanding the mechanisms behind mating system evolution is a central problem in evolutionary biology because the mating system strongly influences the distribution of genetic diversity (Hamrick and Godt 1996), the effective population size, and opportunities for adaptive evolution (Charlesworth 2006; Wright et al. 2013; Hartfield et al. 2017). Most plants are hermaphroditic, but possess traits that promote outcrossing such as self-incompatibility, and spatial and temporal separation of male and female function within flowers (Barrett 2002). This suggests that outcrossing is favored under a wide range of conditions. Yet, the evolutionary shift from outcrossing to self-fertilization is frequent across the plant kingdom (Stebbins 1974; Barrett 2002; Wright et al. 2013). Two main factors promoting the evolution of selfing have been identified. First, when rare, selfing is associated with a 1.5-fold transmission advantage compared to outcrossing (Charlesworth and Charlesworth 2010). This strong advantage may however be countered by inbreeding depression (Charlesworth and Charlesworth 1979) and pollen discounting (i.e., reduced outcross siring success; Holsinger et al. 1984; Harder and Wilson 1998). Empirical studies demonstrate that inbreeding depression is sufficiently strong in many outcrossing populations to more than balance the automatic transmission advantage (reviewed in Husband and Schemske 1996, Winn et al. 2011, Sletvold et al. 2013). Second, the ability to self-fertilize provides a plant with an advantage in environments where mates or pollinators are scarce or unpredictable (Lloyd 1992; Kalisz et al. 2004; Moeller and Geber 2005; Jarne and Auld 2006; Busch and Schoen 2008; Theologidis et al. 2014; Opedal et al. 2016). In plants, reproductive assurance is likely a principal driver of the evolution of autonomous selfing, that is, self-pollination without the assistance of pollinators (Schoen et al. 1996; Busch and Delph 2012).

The transition to selfing is usually associated with a suite of changes in morphology and phenology (i.e., the selfing syndrome). This includes reduced pollen production and reduced allocation to pollinator attraction (floral display, nectar and scent production), which should allow the redirection of resources to seed production (Darwin 1876; Richards 1986; Goodwillie et al. 2010, Sicard and Lenhard 2011). The selfing syndrome also includes morphological changes that should improve the capacity for autonomous self-pollination, for example, reduced spatial and temporal separation of male and female function (Barrett 2003). The evolution of integrated floral phenotypes such as the selfing syndrome should be affected both by the strength and mode of selection and the genetic architecture of the traits involved (Sicard and Lenhard 2011, Slotte et al. 2012, Armbruster et al. 2014, Smith 2016). Yet, the relative importance of different traits for efficient self-pollination and the extent to which traits defining the selfing syndrome are subject to correlational selection are poorly known.

Both distance between anthers and stigma, that is, herkogamy, and their relative orientation should influence the likelihood of autonomous self-pollination, and these effects should not be independent. Herkogamy has long been identified as a major predictor of ability to self-fertilize in self-compatible populations (Darwin 1877; Müller 1883; Webb and Lloyd 1986), and variation in the capacity for autonomous selfing has been associated with differences in herkogamy in several species (e.g., Takebayashi and Delph 2000, Eckert et al. 2009a, Vallejo-Marín and Barrett 2009, de Vos et al. 2012, Brys et al. 2013). Less appreciated is that also anther orientation may influence the likelihood of autonomous self-pollination. In many plants, anthers have a pollen-exposing ventral side where dehiscence occurs, and a dorsal side where pollen grains are few or absent. The likelihood of autonomous self-pollination among open flowers should decrease with increasing angle between the pollen-exposing side of the anther and the stigma, and anther orientation has been identified as a trait covarying with other floral characters associated with mating system variation among taxa in the genus *Leavenworthia* in the Brassicaceae (Rollins 1963; Lloyd 1965). Moreover, the effects of herkogamy and anther orientation on the capacity for autonomous selfing should be interdependent. Reduced herkogamy should increase the likelihood of automatic self-pollination, and this effect should be particularly strong if the pollen-exposing side of the anthers is oriented toward rather than away from the stigma. As a consequence, the two traits are expected to be subject to correlational selection if capacity for autonomous selfing limits female reproductive success.

Because of strong correlations among floral traits related to self-pollination, their relative importance for variation in capacity for autonomous self-pollination among and within populations are challenging to assess (e.g., Dart et al. 2012; Vallejo-Marín et al. 2014; Summers et al. 2015). However, segregating populations derived from crosses between populations can provide a powerful tool to examine the functional and adaptive significance of traits that are otherwise invariant or strongly intercorrelated in natural populations (Schemske and Bradshaw 1999; Lexer et al. 2003; Ågren et al. 2017).

Both herkogamy and anther orientation vary in the alpine, perennial herb *Arabis alpina* (Brassicaceae), and this offers an excellent system to examine the functional and adaptive significance of these traits. *Arabis alpina* is found in most European mountain ranges and the mating system varies from highly outcrossing self-incompatible populations in Italy to mostly inbreeding populations in northern Europe (Tedder et al. 2011). Anther–stigma distance was recently reported to be larger in three self-incompatible compared to three self-compatible populations sampled in Italy and France (Tedder et al. 2015). In addition, the angle between the pollen-exposing ventral side of anthers and the stigmatic surface (Fig. 1) can vary from 0°, when the ventral side is facing the stigmatic surface (introrse), to 180° when the ventral side is facing outwards (extrorse). Finally, reciprocal transplant experiments have demonstrated that Spanish and Scandinavian populations are locally adapted, and that differences in traits influencing fecundity contribute to adaptive differentiation (Toräng et al. 2015).

Here, we document among-population variation in flower morphology, efficiency of autonomous self-pollination and outcrossing rate in self-compatible populations of *A. alpina*. Moreover, because floral traits covary across populations, we used a segregating F2 population derived from a cross between two locally adapted *A. alpina* populations to examine the independent and combined effects of herkogamy and anther orientation on pollination success and female fitness in contrasting pollination environments.

First, we recorded geographic variation in anther orientation and herkogamy among eight natural populations of *A. alpina* sampled along a latitudinal gradient across its scattered distribution in Europe (from Spain, through the French Alps, to northernmost

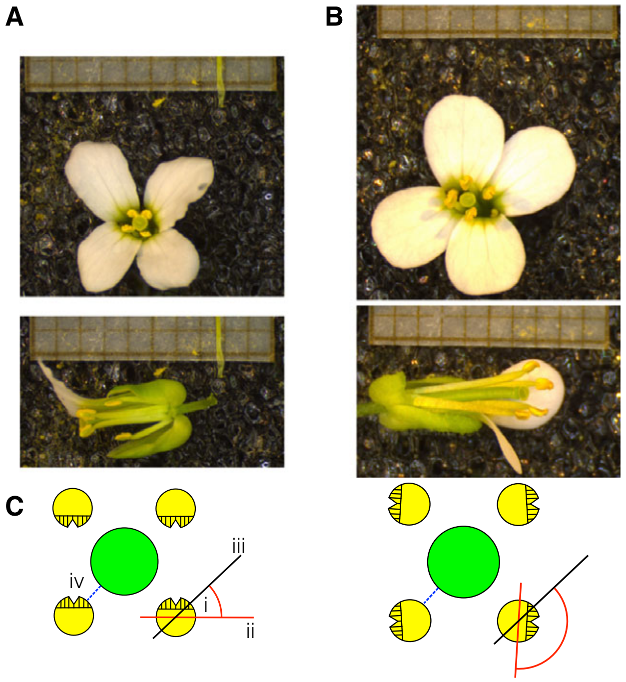


Figure 1. Anther orientation varied considerably among eight self-compatible *Arabis alpina* populations. Representative photos of flowers from plants originating from Scandinavia (A) and Spain (B). The pollen-presenting sides of the anthers are oriented toward the stigma in the Scandinavian flower, and oriented away from the stigma in the Spanish flower. The schematic drawings (C) below the photos illustrate how the angle between the yellow anther's ventral side (from which pollen is dispersed, here illustrated with stripes), and the green stigma was scored. We define the angle (i) as the orientation of the anther's ventral side (ii, red line) relative to its orientation had it been oriented inwards and parallel with the tangent of the stigmatic surface (iii, black line). The anther rotation angle can vary from 0° for completely introrse anthers, to 180° for fully extrorse anthers. The blue dotted line (iv) depicts the anther–stigma distance (herkogamy) scored as the shortest distance between the long anthers and the stigma measured from above in the top panels or from the side in the lower panels. The anther orientation is about 45° in the schematic flower to the left and 135° in the flower to the right.

Scandinavia, Table 1). Second, we examined whether this variation was associated with (1) differences in ability to reproduce through autonomous selfing, (2) pollen limitation (PL) of reproductive output when planted at a native site with low pollinator activity close to the northern range margin, and (3) outcrossing rate. We predicted that capacity for autonomous self-pollination and seed output in environments with low pollinator activity would be negatively related to anther orientation (the angle between the pollen-exposing side of the anther and the stigma) and herkogamy, whereas outcrossing rate would be positively related to these two traits. Third, to examine whether anther orientation and herkogamy are subject to correlational selection, we planted the segregating F2 population at the sites of the two source populations (Angliru, Spain, and Geargevággi, Sweden), and quantified selection through female function on anther orientation, herkogamy, two floral traits reflecting floral display (number of flowers, flower size), and flowering phenology (flowering start). At the site in Sweden, we added supplemental pollen to all flowers of a subset of the F2 plants to test the predictions that reproductive output is pollen limited and that variation in pollination success drives phenotypic selection on anther orientation and herkogamy. Because pollinator activity is higher in Spain, we predicted selection on anther orientation and herkogamy to be less strong in Spain compared to in Sweden.

Methods

STUDY POPULATIONS

The white entomophilous flowers of *A. alpina* are produced in one or several inflorescences that extend from a basal leaf rosette. Like many other species in the Brassicaceae (Al-Shehbaz 1977), *A. alpina* is protogynous. We studied eight self-compatible populations of *A. alpina* sampled from across the European range (four in Scandinavia, two in France, and two in Spain; Table 1). In self-compatible *A. alpina* populations, the stigma is receptive from before the flower opens and for one or a few days thereafter. The anthers dehisce soon after the stigma has become receptive (usually when the flowers open, but we have made a few observations of dehisced anthers and receptive stigmas in closed flowers in Scandinavian populations) and the temporal overlap between stigma receptivity and anther maturity is considerable (one to a few days). When the stigma is no longer receptive, the stamens extend outwards presenting pollen for up to a few days before they wither. Scent is produced by the flowers in all eight populations.

POPULATION DIFFERENTIATION IN ANTHER ORIENTATION AND HERKOGAMY

To document among-population variation in floral traits, we grew plants originating from the eight study populations (Table 1) under controlled conditions in the greenhouse. We planted seeds collected in the field from each of 22–48 mother plants per source population in $5 \times 5 \times 6$ cm^3 pots filled with standard commercial potting soil (Yrkesplantjord, Weibulls Horto, Sweden) and a 0.5 cm nutrient-poor topsoil layer (S-jord, Hasselfors garden, Sweden). The pots were stratified for one week at 4°C in the dark, and then transferred to a greenhouse (18°C/16°C, 18-h day [150 μE/m^{2}s]/6 h night), where they were distributed randomly on six benches. After six weeks of vegetative growth, one plant per seed family was vernalized for 12 weeks at 4°C receiving low-intensity light (50 μE/m^{2}s) for 12 h per day. After vernalization, temperature and light condition were restored to 18°C 18-h long days as above and the plants were watered three times per week

Table 1. Location, number of established plants sampled, number of families included in the mother–offspring arrays, number of polymorphic SNP loci, number of distinct multilocus genotypes, observed (H_O) and expected heterozygosity (H_E), and estimated inbreeding coefficients (F_{IS}) and outcrossing rates in the eight *Arabis alpina* populations.

Population	Region	Location	No. established plants sampled	No. families in mother–offspring arrays	No. polymorphic SNP loci	No. distinct multilocus genotypes	H_O	H_E	F_{IS}	Outcrossing rate ± SE (established plants)	Outcrossing rate ± SE (mother–offspring arrays)
S1	Northern Scandinavia	68° 24′ N, 18° 19′ E	69	31	9	34	0.034	0.309	0.890	0.0619 ± 0.0083	0.095 ± 0.020
S2	Northern Scandinavia	68° 21′ N, 18° 43′ E	50		12	22	0.090	0.251	0.728	0.139 ± 0.029	
S4	Central Scandinavia	62° 50′ N, 11° 44′ E	56	23	23	19	0.026	0.384	0.933	0.0134 ± 0.0024	0.109 ± 0.042
S5	Central Scandinavia	63° 12′ N, 12° 19′ E	57		8	22	0.024	0.290	0.915	0.0972 ± 0.022	
Fr1	French Alps	45° 03′ N, 06° 24′ E	131	30	70	119	0.115	0.281	0.585	0.266 ± 0.0031	0.192 ± 0.041
Fr2	French Alps	44° 57′ N, 06° 36′ E	63		84	30	0.051	0.263	0.806	0.139 ± 0.0029	
E3	Northwest Spain	43° 14′ N, 05° 56′ W	42	25	83	32	0.133	0.323	0.588	0.231 ± 0.0047	0.178 ± 0.022
E4	Northwest Spain	43° 03′ N, 06° 06′ W	50		105	48	0.138	0.330	0.584	0.297 ± 0.0044	

Outcrossing rates were estimated from multilocus structure among established plants and from marker segregation in mother–offspring arrays.

with a small dose of nutrients added. To reduce attacks by pollen feeding thrips (Thysanoptera), we applied *Amblyseius cucumeris* mites (Biobasiq Sverige AB) on all plants every second week.

To score anther orientation and herkogamy with precision, we photographed individual flowers from two directions using a Leica MZ8 stereomicroscope and a Leica DFC450 microscope camera connected to a PC (Leica Microsystems Inc., USA). For calibration, each flower was photographed together with a piece of millimeter paper (Fig. 1). To ensure that sampled flowers were in the same developmental stage, we marked emerging buds and photographed the flowers as soon as the anthers dehisced. Anthers dehisced on the day of flower opening, except in the flowers of a few plants of Scandinavian origin, whose anthers dehisced in the late bud stage. To record anther orientation and herkogamy of the latter plants, petals were removed and flowers photographed at that late bud stage. Photographs were analyzed using ImageJ (Schneider et al. 2012). We recorded herkogamy to the nearest 0.1 mm and anther orientation of the long lateral stamens closest to the stigma to the nearest 5° as described in Figure 1. For each plant, we calculated a mean value for each trait based on measurements of two flowers.

Population differentiation in anther orientation and herkogamy was examined with one-way ANOVA, where population was included as a fixed factor. Post hoc Tukey tests were used to identify statistically significant differences between populations.

POPULATION DIFFERENTIATION IN THE EFFICIENCY OF AUTONOMOUS SELF-POLLINATION

To document among-population variation in the efficiency of autonomous self-pollination and self-compatibility, we grew 13–25 plants from each of the eight study populations under controlled conditions in a pollinator-free greenhouse (each plant representing a different maternal family collected in the field). To examine whether plants were self-compatible, and to quantify the efficiency of autonomous self-pollination, emerging buds were marked and randomly assigned to one of the three treatments. Buds assigned to the outcrossing and selfing treatments were emasculated (i.e., anthers were removed before they dehisced) using forceps. Buds assigned to the autogamy treatment were left intact. On each plant, two to six (median = 3) buds were assigned to each of the three treatments. In addition, to check whether emasculated flowers were able to produce seeds without hand-pollination, we marked and emasculated one flower on each plant. None of these flowers produced any seeds.

Flowers assigned to the outcrossing treatment were hand-pollinated with pollen from flowers of another experimental plant originating from the same source population, and flowers assigned to the selfing treatment with pollen from other flowers on the same plant.

When the fruits were fully mature, they were put in individual paper bags and stored in the office for three months, after which the seeds were counted and weighed. Individual seed mass was calculated as the total seed mass per fruit divided by the number of seeds.

We estimated for each plant the efficiency of autonomous self-pollination as the mean number of seeds produced per flower in the autogamy treatment divided by the mean number of seeds produced per flower in the selfing treatment. Population differences in the efficiency of autonomous self-pollination were examined with one-way ANOVA and a post hoc Tukey test.

All flowers in the cross- and self-pollination treatments produced seeds. To test whether the number of seeds per fruit or seed mass differed between the two treatments, we used ANOVA where treatment and population were included as fixed factors, and plant nested within population as a random factor. Number of seeds per fruit and mean seed mass did not differ between flowers pollinated with self- and cross-pollen, respectively (seeds per fruit, treatment $F_{1,1} = 0.097$, $P = 0.76$, treatment × population, $F_{1,7} = 0.77$, $P = 0.61$; seed mass, treatment $F_{1,1} = 3.0$, $P = 0.083$, treatment × population, $F_{1,1} = 0.21$, $P = 0.98$; Fig. S1), suggesting that all study populations were self-compatible.

POLLEN LIMITATION UNDER NATIVE FIELD CONDITIONS

To determine whether PL varied among populations planted at a site with low pollinator activity, we performed supplemental hand-pollinations in a common-garden experiment established in the S1 population in northern Sweden that included seven of the eight populations (Geargevággi, 68°24′ N, 18°19′ E, 950 m.a.s.l.; Toräng et al. 2015). In early July before the onset of flowering, we marked two plants from each of 20 seed families per population and randomly assigned one plant per seed family to either the supplemental hand-pollination or the open-pollinated control treatment. Due to low flowering propensity in some populations, the number of experimental plants was in the end reduced to 196 in total (range = 7–40 plants per population). Plants assigned to the hand-pollination treatment were repeatedly visited throughout the flowering season. All open flowers on these plants were pollinated by hand with a mixture of pollen from the same flower and pollen from other plants of the same origin not included in the experiment. At the end of the growing season in early September, all inflorescences of the experimental plants were collected and brought to the laboratory. For each plant, we recorded the total number of fruits, and the number of seeds produced in three fruits sampled across the inflorescence. Total seed output of each plant was estimated by multiplying the number of fruits with mean number of seeds per fruit. For each population, PL was quantified as 1 – mean seed production of open-pollinated control plants/mean seed production of plants receiving supplemental hand-pollination. Confidence intervals for PL estimates were obtained through bootstrapping by resampling seed production estimates for plants receiving supplemental hand-pollination and controls, and calculating PL 10,000 times. Because of considerable mortality, and therefore an unbalanced design at the end of the experiment, individuals were resampled regardless of family of origin.

NATURAL SELECTION ON FLORAL TRAITS IN A SEGREGATING F2 POPULATION

To examine the independent and combined effects of anther orientation and herkogamy, and other floral traits on plant fecundity, we planted a segregating F2 population derived from a reciprocal cross between a plant from Spain (Angliru, 43°14′ N, 5°56′ W, 1500 m.a.s.l.) and a plant from Sweden (Geargevággi, 68°24′ N, 18°19′ E, 950 m.a.s.l.) at the sites of the two source populations using similar methods and the same field sites as in a previous transplant study described in Toräng et al. (2015). In spring 2013 (early May in Spain and early June in Sweden), F2 seeds were sown and germinated in the greenhouse to ensure high establishment success. After about four weeks in the greenhouse, the seedlings were transplanted to the experimental sites (early June in Spain and early July in Sweden). The plants were watered on their first day in the field, but were after that left without care. In Spain, the planting was fenced to exclude grazing ungulates, and weeds were removed in the following spring.

In 2014, the experiment was visited every day throughout the flowering season (beginning in mid-April in Spain and in mid-July in Sweden) and for each plant, we recorded flowering start (days after snowmelt) and total number of flowers produced. In addition, we sampled and photographed one newly opened flower on each individual plant (one of the first 10 flowers was sampled avoiding the very first flower which occasionally has an atypical morphology: style may be bent and petals wrinkled). We did not observe dehisced anthers in closed flowers in any plant in the segregating F2 population. In Spain, the flowers were photographed in the field using a Canon EOS350 camera with a 100 mm f/2.8 macrolens mounted on a tripod. In Sweden, the flowers were put in individual 1.5 mm Eppendorf tubes and brought to the nearby laboratory at Abisko Scientific Research Station (sampling was conducted in the mornings when the flowers were typically moist from dew and temperatures low). Photos were taken within 5 h using a Leica MZ8 stereomicroscope and a Leica DFC450 microscope camera connected to a PC. A handful of flowers (out of about 400) had begun to wilt during the transfer and were discarded. Anther orientation and herkogamy were scored from the digital images as described above, and flower diameter was quantified as the distance between the tips of two opposite petals. The means and SDs of these three floral traits were similar at the two sites (Table S2), suggesting that the flowers were little affected by the short transfer to the laboratory. To test whether reproductive output was pollen limited and whether variation in pollination success drove phenotypic selection on anther orientation and herkogamy (cf. Sandring and Ågren 2009), we added supplemental pollen to all flowers of a subset of the F2 plants ($n = 59$) at the site in Sweden. The total number of flowering F2 plants in the year of the experiment was about 500 in Sweden, of which 450 were included in the experiment, and 55 in Spain.

At the end of the growing season in August, the inflorescences of all experimental plants were put in paper bags and brought to the laboratory where the number of fruits produced was recorded. Many plants had fruits that had opened at the time of fruit collection and it was therefore not possible to determine seed production. Among plants with intact fruits however, fruit and total seed production were strongly correlated (Pearson's correlation coefficient $r = 0.90$, $P < 0.001$, $n = 142$), and we therefore used fruit production as an estimate of female fitness.

We estimated phenotypic selection gradients for the floral traits recorded following Lande and Arnold (1983), using regression analyses with relative fitness (number of fruits produced divided by the mean number of fruits) as the response variable and standardized trait values (anther orientation, herkogamy, flower diameter, number of flowers, and flowering start) as explanatory variables. We estimated directional selection gradients (β_i) from multiple regression models including only linear terms, and quadratic (γ_{ii}) selection gradients from a regression model that also included the quadratic terms. The reported (γ_{ii}) were obtained by doubling the extracted regression coefficients (Lande and Arnold 1983). To test the prediction that anther orientation and herkogamy are subject to correlational selection, we also analyzed models that in addition to linear and quadratic terms included the anther orientation × herkogamy interaction. To limit model complexity, we did not include additional cross-product (γ_{ij}) terms. Relative fitness and standardized trait values were calculated separately for each site × treatment combination (open-pollinated control and plants receiving supplemental hand-pollination in Sweden, and open-pollinated control in Spain). We examined whether selection gradients differed between sites (control in Sweden vs. control in Spain) and between pollination treatments in Sweden using ANCOVA models, which included site or treatment as a categorical variable, and standardized traits and interactions between traits and the categorical variable as independent variables and relative fitness as the dependent variable. In the F2 population, low variance inflation factors (< 2) indicated that collinearity was not a problem in the multiple regression analyses.

Including cross-direction in the selection analyses did not affect the results (data not shown) and it was excluded from the final models. Statistical analyses were conducted using the R 3.2.1 software (R Core Team 2015), with the car (Fox and Weisberg 2011) and contrast (Kuhn 2013) packages.

INBREEDING COEFFICIENT AND OUTCROSSING RATES

To examine the relationship between floral morphology and outcrossing rate, we estimated both the historical outcrossing rate from the multilocus structure of established plants, and the current outcrossing rate from marker segregation in maternal progeny arrays. To estimate the inbreeding coefficient, and the outcrossing rate from multilocus structure of established plants, we sampled leaf material from 42–131 plants in each of the eight study populations (518 plants in total, Table 1). To estimate outcrossing rates in mother–offspring arrays, we sampled and genotyped 10 offspring from each of 23–31 plants (109 plants in total) in each of four populations. Ten seeds per family were planted in pots filled with commercial potting soil, and grown for four weeks in the greenhouse until leaf harvest. Two to five fresh leaves per plant were collected, dried, and stored on silica gel until used for DNA extraction. DNA was extracted using the DNeasy plant mini kit (Qiagen, Inc.) following the manufacturer's protocol for dried samples. Genotypes were scored using the Illumina Golden Gate Assay for 253 SNP (single nucleotide polymorphism) loci, of which eight to 105 were polymorphic within populations (Table 1).

We used GenAlEx (version 6.5, Peakall and Smouse 2006, 2012) to estimate observed and expected heterozygosity (H_O and H_E, respectively), and the population inbreeding coefficient (F_{IS}). We used the RMES software (David et al. 2007) to estimate selfing rates (S) from the multilocus structure among established plants, and we calculated the outcrossing rate of each population as $1 - S$. This represents a historical outcrossing rate, as it integrates the effect of selfing and any biparental inbreeding over several generations, and is affected by any inbreeding depression expressed from seed formation until plant establishment. Current outcrossing rates in four of the populations were estimated from the mother–offspring arrays using the software MLTR for Windows (Ritland 2002). SEs were estimated through resampling of progenies. Estimates of outcrossing based on multilocus structure among established plants and in mother–offspring arrays were correlated in the four populations where both estimates were obtained (Pearson's correlation coefficient, $r = 0.96$, $P = 0.038$).

Anther orientation and herkogamy may be related to both historical and current outcrossing rates in self-compatible populations, but partly for different reasons: They are expected to be positively related to the historical outcrossing rate to the extent that the latter reflects the strength of past selection for more efficient selfing mechanisms. They are expected to be positively related to the current outcrossing rate to the extent that this is determined by flower morphology.

Results

POPULATION DIFFERENTIATION IN ANTHER ORIENTATION, HERKOGAMY, AND EFFICIENCY OF AUTONOMOUS SELF-POLLINATION

Both anther orientation and herkogamy varied among populations. Anther orientation was introrse in the four Scandinavian populations (population mean angle between the pollen-exposing ventral side of the anther and the stigma ranged between 28.4° and 45.5°) and extrorse with the ventral sides oriented away from the stigma in the French and Spanish populations (population means ranged from 103.5° to 131.7°; Fig. S2a). Anther–stigma distance was shorter in the four Scandinavian populations (population means, 0.14–0.23 mm) than in the four other populations (population means, 0.37–0.68 mm; Fig. S2b).

The efficiency of autonomous selfing was lower among the populations originating from Spain (E3 and E4; Efficiency = 0.38–0.39) and France (Fr1 and Fr2; Efficiency = 0.17–0.43) than among the populations from Scandinavia (S1, S2, S4, S5; Efficiency = 0.71–0.84; Fig. S2c), and was negatively related to anther orientation and herkogamy (Fig. 2A and B).

POLLEN LIMITATION UNDER NATIVE FIELD CONDITIONS

In the common-garden experiment conducted at the site of a native Scandinavian population, seed production was pollen limited among Spanish and French plants, but not in the Scandinavian plants. Supplemental hand-pollination increased seed output per plant compared with open-pollinated controls among Spanish (E3 and E4; PL = 0.55-0.94) and French (Fr1 and Fr2; PL = 0.36-0.61), but not among Scandinavian plants (S1, S2, S4; PL = −0.06 to 0.19; Fig. S3), and among-population variation in PL was positively correlated with anther orientation and herkogamy (Fig. 2C and D). The among-population variation in PL recorded at the Scandinavian field site was negatively correlated with efficiency of autonomous self-pollination determined in the greenhouse ($r = -0.772$; $P = 0.042$; $n = 7$).

On average, the Scandinavian plants produced almost 12 times more seeds than did Spanish plants (561 vs. 48 seeds per plant), and four times more seeds than did French plants (561 vs. 144 seeds per plant) when grown at the Scandinavian site (Fig. S3). Differences in PL explained a substantial share of this local advantage. After supplemental hand-pollination, Scandinavian plants produced on average 2.5 more seeds compared to Spanish plants (614 vs. 242 seeds per plant), and about twice as many seeds as French plants did (614 vs. 300 seeds per plant).

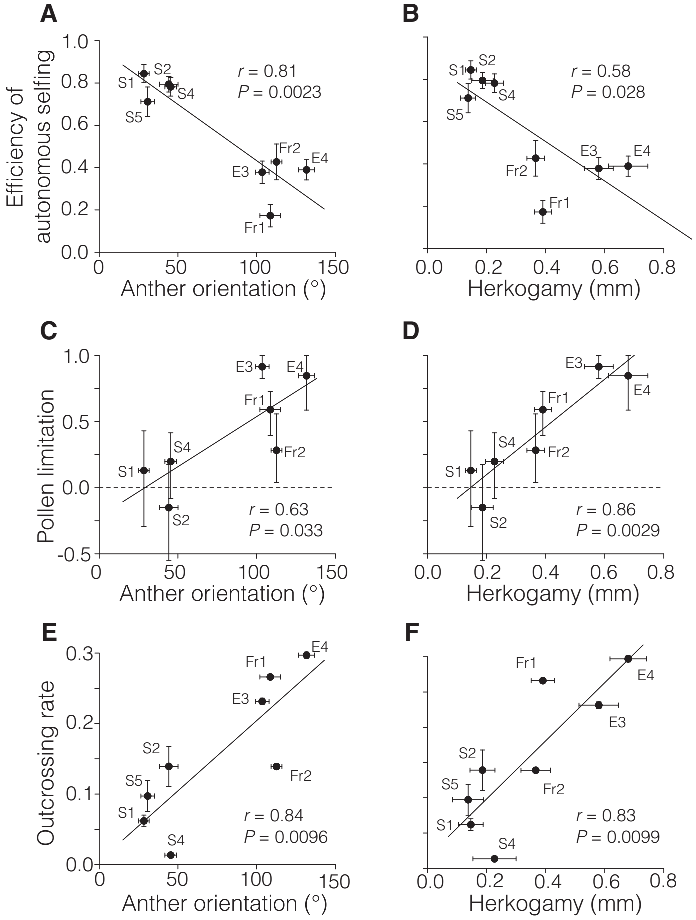


Figure 2. Among-population variation in anther orientation and herkogamy was correlated with the efficiency of autonomous selfing (A and B), PL (C and D), and outcrossing rate estimated from multilocus structure of established plants (E and F). The efficiency of autonomous self-pollination was tested in the greenhouse and was estimated as the number seeds produced per flower in the absence of pollinators relative to that of flowers pollinated by hand with self-pollen. PL was tested in a common-garden experiment conducted at the site of a native population with low pollinator activity in northern Scandinavia and was estimated as 1 – mean number of seeds produced by open-pollinated control plants/mean number of seeds produced by plants receiving supplemental pollen by hand. Error bars depict the SEs in all panels except in (C) and (D) where vertical error bars depict 95% confidence intervals. Confidence intervals not including zero (indicated with dashed line) imply statistically significant PL. S1, S2, S4, and S5, Scandinavian populations; Fr1 and Fr2, French populations; E3 and E4, Spanish populations. Pearson's correlation coefficients are indicated.

INBREEDING COEFFICIENTS AND OUTCROSSING RATES

The two Spanish populations and the French population Fr1 had higher observed heterozygosity and outcrossing rates, and lower inbreeding coefficients than had the four Scandinavian populations and the French population Fr2 (Table 1). The number of polymorphic SNP loci was markedly lower in the Scandinavian (eight to 23 loci) compared to the French and Spanish populations (70–105 loci), but a large proportion of the sampled plants could still be assigned to unique multilocus genotypes (Table 1).

Outcrossing rate estimates were low for all populations (range 0.0134–0.297, based on multilocus genotypes of established plants; 0.095–0.192, based on mother–offspring arrays), but higher in populations E3, E4, and Fr1 compared to the other five populations (Table 1). Both anther orientation and herkogamy were positively correlated with outcrossing rate estimated from multilocus structure of established plants (Pearson's correlation coefficient, r, 0.84 and 0.83, respectively, $n = 8$; Fig. 2E and F), whereas only anther orientation was significantly correlated with outcrossing rate estimates based on mother–offspring arrays, which were available for four populations only (anther orientation, $r = 0.99$, $P = 0.0036$; herkogamy, $r = 0.86$, $P = 0.14$).

NATURAL SELECTION ON FLORAL TRAITS IN THE SEGREGATING F2 POPULATION

Anther orientation and anther–stigma distance were strongly positively correlated among populations, but in the F2

Table 2. Pearson's correlations between floral traits in the *Arabis alpina* F2 population planted at the sites of the parental populations in Sweden (below the diagonal, $n = 485$) and Spain (above the diagonal, $n = 55$).

	Anther orientation	Herkogamy	Flower diameter	Flowering start	Number of flowers
Anther orientation		**−0.287**	0.237	0.142	−0.0604
Herkogamy	−0.0474		0.0795	0.0458	0.147
Flower diameter	0.134	−0.0710		−0.0345	−0.107
Flowering start	0.127	0.00311	**0.181**		−0.170
Number of flowers	0.00905	0.121	0.108	**−0.411**	

The three correlations that were statistically significant at $P < 0.05$ are in bold.

population the correlation between these two traits was much reduced ($r = -0.0474$, $P = 0.39$, $n = 332$ in Sweden; $r = -0.287$, $P = 0.033$, $n = 55$ in Spain) and other pairwise correlations among floral traits were weak ($|r| < 0.2$ except for the negative correlation between flowering start and number of flowers in Sweden, $r = -0.411$, $P < 0.001$; Table 2). The weak correlation between anther orientation and anther–stigma distance in the F2 suggests that the genetic basis of the two traits is at least partly different, and makes this population suitable for assessing their independent and combined effects on measures of reproductive success.

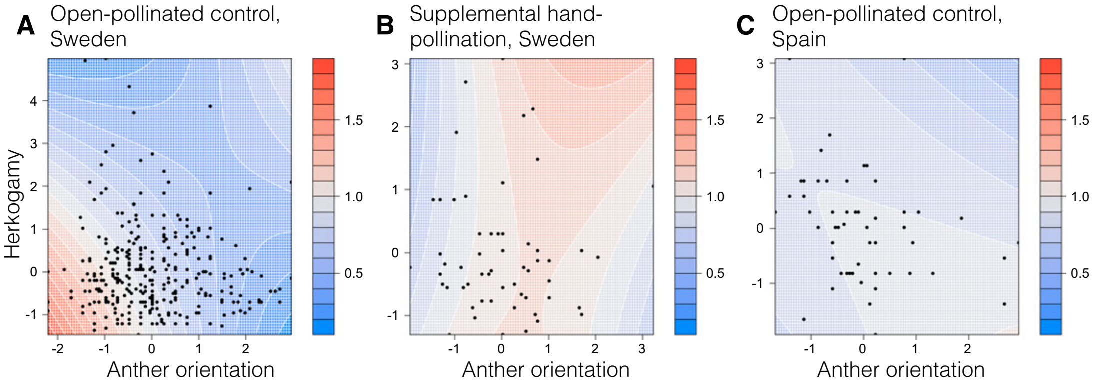
In Sweden, fruit set (the proportion of flowers forming a mature fruit) was strongly pollen limited in the F2 population. Supplemental hand-pollination increased fruit set twofold (from 0.41 ± 0.017 to 0.84 ± 0.016; Table S1), that is, to a level similar to that recorded among open-pollinated plants at the site in Spain (0.88 ± 0.078; Table S1).

Selection on floral traits differed between the field sites in Sweden and Spain, and between pollination treatments in Sweden. In the open-pollinated control treatment in Sweden there was directional selection for reduced anther orientation (angle between the pollen-exposing ventral side of the anther and the stigma), reduced herkogamy (anther–stigma distance) and later flowering (Table S2). Moreover, anther orientation and herkogamy were subject to strong correlational selection favoring a combination of introrse anthers and short anther–stigma distance (Fig. 3A; Table S2). In contrast, relative female fitness was positively related to anther orientation and not significantly related to herkogamy or flowering time among hand-pollinated plants (Fig. 3B; Table S2). The estimate of correlational selection had the same sign and was of similar magnitude as that obtained in the open-pollinated control, but was not statistically significant and reflected a fitness surface with the highest relative fitness recorded among individuals with extrorse anthers and high herkogamy (Fig. 3B). In both treatments, there was strong selection for more flowers. In Spain, relative female fitness was not related to any of the floral traits recorded, except number of flowers (Fig. 3C, Table S2).

Discussion

Reproductive assurance has been identified as a major factor favoring the evolution of selfing in many lineages (Darwin 1876; Pannell and Barrett 1998; Eckert et al. 2006), yet the relative importance of different floral traits and their integration for the efficiency of autonomous self-pollination and reproductive success in environments with low pollinator activity is poorly known. This study has identified anther orientation and herkogamy as two key traits strongly influencing the capacity for autonomous selfing in self-compatible populations of the perennial herb *A. alpina*. Among-population variation in efficiency of autonomous self-pollination and PL in a common garden experiment were correlated with differences in anther orientation and anther–stigma distance. In addition, a field experiment using a segregating F2 population derived from a cross between a Spanish and Swedish population demonstrated that anther orientation and anther–stigma distance synergistically affect reproductive success when pollinator activity is low. The results suggest that concurrent shifts in anther orientation and herkogamy can be crucial for the evolution of efficient self-pollination and reproductive assurance in environments with low pollinator activity, and that correlational selection on these traits should contribute to the evolution of the selfing syndrome.

The results of the supplemental hand-pollination showed that selection favored introrse anthers (reduced angle between the pollen-exposing side of the anther and the stigma) in combination with reduced herkogamy in the F2 population planted in Sweden because of the effects of these traits on PL. In contrast, anther orientation and herkogamy appeared to be selectively neutral in Spain where the fruit set of *A. alpina* was high. Field observations suggest that the differences in fruit set and phenotypic selection between the two sites are related to differences in pollinator activity: Despite many hours of field work in the Scandinavian *A. alpina* populations (more than 400 h across five flowering seasons), we have made only very few observations of pollinating insects visiting this species, whereas bees, flies, and thrips have been regularly observed foraging on flowers in Spain. The growing season is much shorter and colder in Scandinavia compared to



A Open-pollinated control, Sweden

B Supplemental hand-pollination, Sweden

C Open-pollinated control, Spain

Figure 3. Phenotypic selection on anther orientation and herkogamy in an F2 population planted in Sweden and Spain illustrated with added-variable contour plots. The residuals from a regression of relative fitness on traits other than the focal traits are plotted against the residuals from a regression of the focal traits on the other traits included in the analysis of selection gradients (cf. Fox and Weisberg 2011). Panels depict relative female fitness as a function of standardized anther orientation and herkogamy among open-pollinated control plants (A) and among plants receiving supplemental hand-pollination (B) at a native field site in Sweden with low pollinator activity, and among open-pollinated plants in Spain (C). Red colors depict high, and blue colors low, relative fitness. Residuals were derived from models that also included standardized flower diameter, number of flowers, and flowering start as independent variables. Statistically significant correlational selection on anther orientation and herkogamy was detected only among open-pollinated plants in Sweden.

Spain (Toräng et al. 2015), which should negatively affect insect activity and insect-mediated pollen transfer. An association between pollination intensity and selection on herkogamy has been documented also in other systems. The strength of selection for reduced herkogamy was correlated with rates of pollinator visitation in experimental populations of *Clarkia xantiana* (Moeller and Geber 2005). In a field experiment with *Mimulus guttatus,* selection for reduced herkogamy was observed when pollinators were excluded (Fishman and Willis 2008). Moreover, in the absence of pollinators, reduced herkogamy and increased autonomous seed set had evolved after five generations in a greenhouse experiment with the same species (Bodbyl Roels and Kelly 2011). There is thus some experimental support for the prediction that pollination intensity affects the strength of selection on herkogamy. However, the present study is the first to demonstrate that anther orientation can be as important as herkogamy for reproductive success in environments with low pollinator activity.

One caveat is that if self-fertilization is associated with seed discounting and inbreeding depression, this may reduce or even outweigh the fitness benefits of increased seed production from autonomous selfing (Herlihy and Eckert 2002; Layman et al. 2017). In the present study, we found no difference in seed output per fruit or mean seed mass between flowers pollinated with cross- and self-pollen in any of the eight populations. There was thus no evidence that inbreeding depression at these early life-history stages would negate the fecundity advantage of plants with high autonomous self-pollination. Moreover, estimates of selfing rates based on multilocus structure among established plants were not consistently lower than those obtained with mother–offspring arrays (Table 1), indicating that inbreeding depression may be limited, as would be expected in highly selfing populations (cf. Husband and Schemske 1996; Winn et al. 2011).

Another caveat is that the present study considered effects of floral traits on female reproductive success only. Introrse anthers and reduced herkogamy may interfere with successful pollen transfer to other plants (male function; Harder and Wilson 1998; Barrett 2002), and optimal arrangement of stigma and anthers may reflect a trade-off between efficient pollen export and efficiency of self-pollination. A full evaluation of the fitness consequences of variation in anther orientation and herkogamy should thus consider also possible effects on offspring quality beyond the seed stage and effects on male reproductive success.

Finally, our analyses focused on the effects of variation in two floral traits (anther orientation and herkogamy). Efficiency of self-pollination and PL could in addition be influenced by the degree of temporal separation of male and female function within flowers (dichogamy), and differences in traits influencing attractiveness to pollinators. However, in the present study, all populations showed large overlap between stigma receptivity and anther dehiscence suggesting that differences in dichogamy are not likely to be important for variation in pollination success. Moreover, all populations produced scented flowers, and effects of anther orientation and herkogamy on PL and female reproductive success in the F2 population planted in the field were observed in models including in addition two traits influencing floral display (flower size and number of flowers) and flowering time as independent variables. Taken together, this suggests that the detected effects of anther orientation and herkogamy on efficiency of self-pollination, and on PL and fecundity in the field represent causal relationships rather than the effects of traits correlated with the two focal traits. In general, the relative importance of changes in anther orientation, herkogamy, and dichogamy for the evolution of efficient selfing mechanisms should vary among systems, and depend on trait expression in the evolving population.

Estimates both of the historical outcrossing rate assessed from the multilocus structure of established plants, and of the current outcrossing rate based on maternal progeny arrays were low in all study populations (range 0.013 – 0.297 and 0.095 – 0.192, respectively), but tended to be positively correlated with anther orientation and herkogamy. Despite markedly lower variation in outcrossing rate, the present results are consistent with a recent study that documented reduced herkogamy in three highly selfing compared to three outcrossing *A. alpina* populations in the Mediterranean part of the range (Tedder et al. 2015). Anther–stigma distances recorded in the present study tended to be even shorter than those reported for the three selfing populations studied by Tedder et al. (2015), but variation in the age of flowers examined (day of opening in the present study vs. up to third day of flowering in the previous study) may have contributed to the difference. In the present study, fruit set and selfing rates in the Spanish populations were high despite rather low capacity for autonomous self-pollination (Fig. S2c), suggesting that pollinators contributed substantially to the transfer of self-pollen in these populations.

Anther orientation is correlated with the mating system also in other members of the Brassicaceae. In the 1960s, Rollins (1963) and Lloyd (1965) identified introrse anthers as one of several floral traits distinguishing selfing from outcrossing taxa of *Leavenworthia*. Two later studies have confirmed this notion and shown that anthers are more introrse in highly selfing populations than in populations with low or intermediate selfing rates in *Leavenworthia crassa* (Lyons and Antonovics 1991) and in *L. alabamica* (Busch 2005). To evaluate the generality of these findings, anther orientation should be documented in additional taxa varying in the capacity for autonomous selfing and outcrossing rate in the Brassicaceae and other plant families.

Taken together, the results show that concurrent shifts in more than one trait can be crucial for the evolution of efficient self-pollination and reproductive assurance in pollinator-poor habitats. They further suggest that directional and correlational selection can operate on standing genetic variation and potentially lead to rapid assembly of the selfing syndrome and thus contribute to major shifts in the mating system over relatively short time periods. Here, we have demonstrated that anther orientation and herkogamy may synergistically influence the capacity for autonomous self-pollination and fecundity in habitats with low pollinator activity. Information about the corresponding relationships in additional systems would be of interest both for an understanding of the mechanisms underlying the evolution of self-pollination in natural populations, and from an applied perspective when it comes to developing crops with a high capacity for autonomous selfing. It has been proposed that current declines in pollinator abundance may favor traits that make plants less dependent on insect pollinators (Eckert et al. 2009b; Thomann et al. 2013; Ushimaru et al. 2014). The present study suggests that this should include not only reduced dichogamy and herkogamy, but also introrse anthers with the pollen-presenting side oriented toward the stigma.

AUTHORS' CONTRIBUTION

PT, JW, GC, and JÅ contributed to the design of the research. PT and LV performed the research with the aid of field assistants. SW extracted DNA and coordinated sample preparation for genotyping. PT and LV analyzed the data, and PT and JÅ wrote the paper.

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DATA ARCHIVING

Data is stored in Dryad public archive, https://doi.org/10.5061/dryad.0s63q

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Number of seeds per fruit and seed mass of flowers pollinated with cross- and self-pollen, respectively.
Figure S2. Among-population differentiation in anther orientation, herkogamy, and efficiency of autonomous self-pollination in *Arabis alpina* documented in a greenhouse experiment.
Figure S3. Supplemental hand-pollination indicated that seed production was strongly pollen limited in the Spanish and French populations, but not in the Scandinavian populations grown in a common-garden experiment at the site of a native population with low pollinator activity in northern Sweden.
Table S1. Plant traits and reproductive performance of the *Arabis alpina* F2 population planted at the sites of the parental populations in Sweden and Spain.
Table S2. Phenotypic selection gradients for floral traits in the *Arabis alpina* F2 population planted at the sites of the parental populations in Sweden and Spain.