Ongoing convergent evolution of a selfing syndrome threatens plant-pollinator interactions

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Abstract

Plant-pollinator interactions has evolved early in angiosperm species radiation and is considered as a major trophic link in terrestrial ecosystems. Recent environmental changes however have led to pollinator decline that are susceptible to cause a disruption of plant-pollinator interactions. We used the resurrection ecology methodology to assay ancestral and contemporary descendant phenotypes in natural populations. We assessed patterns of mating system evolution in *Viola arvensis,* over the last three decades in the Parisian regional basin (France), a depauperate pollinator environment. Population genetic analyses revealed about 15% increase in realized selfing rates in the field during the last three decades. Using a two-generation common garden experiment, we documented trait evolution towards small and inconspicuous corollas, reduced nectar production and reduced attractiveness. Trait shifts were found to be convergent over the four studied populations. Our study demonstrates that plant mating system can evolve rapidly in natural populations in the face of recent environmental changes. Such rapid evolution towards a selfing syndrome illustrates the ongoing disruption of a major trophic interaction of natural ecosystems.

Main Text

Introduction

In the last 30 years, insect populations have declined by more than 75% (*1*). More specifically, we are facing a global decline of pollinators, like bees (Anthophila) (*2*, *3*). The astonishing diversification of angiosperms, which started 100 billion years ago, is commonly thought to have been stimulated and hastened by the diversity of plant-pollinator interactions (*4*). More than 80% of extant angiosperms rely on animals for pollination (*5*), thus plants and pollinators have a close relationship. The decline of pollinators is susceptible to affect plant reproduction through the direct lack of pollination, and parallel declines in animal-pollinated plants and insects have been documented, over the last 50 years (*6*). For individual plant species, pollen limitation has been shown to represent a major selective pressure in the evolution of self-fertilization (selfing) in theoretical evolutionary models (*7, 8*). Indeed, hermaphroditic angiosperms predominantly mate via outcrossing *i.e.* with another individual, although recurrent evolution towards selfing is a common macroevolutionary shift (*9, 10*). In recent years, such a shift towards selfing has been experimentally reproduced in the absence of pollinators (*11–13*) and illustrated in natural populations impoverished in pollinators (*14*). This evolution of selfing is commonly associated with changes in reproductive traits such as a decrease in the distance between male and female organs (herkogamy) (*11, 13, 14*) but also a decrease in floral traits mediating plant-pollinator interactions, such as a decrease in corolla and petal size, and decreased scent and nectar production (*12–14*). A shift in traits leading to less conspicuous and attractive flowers has been well described at the phylogenetic level (*15*). This group of trait changes characterizes the evolution of a selfing syndrome (*15,16*).

Faced with pollinator decline, natural populations are expected to evolve towards reduced plant-pollinator interactions and increased autonomous selfing (*8*). Demonstrating trait evolution requires the distinction of the genetic and environmental components of phenotypes. The “resurrection ecology” approach, which involves growing dormant seeds collected several or a few decades previously alongside their descendants (*17,18*), is a powerful methodology to detect such evolution (*18*). Indeed, this approach allows for the measurement of traits of ancestral and recent genotypes in a common environment, thus revealing the genetic components of trait variation.

Using this approach, we analyzed patterns of mating system trait evolution in the field pansy (*Viola arvensis*). To do so, we compared floral traits in ancestral seed population (from the 1990’s and early 2000’s) with the contemporary descendant population collected in 2021 from four localities in the Parisian regional basin (fig. S1 and table S1, *19*). The study species is an annual weed of intensive crops (mostly wheat and oilseed rape) that has a zygomorphic, showy flower and mixed selfing rates (*19*). The length of the nectar spur and observations *in natura* are indicative of pollination by long-tongued bees. Populations were collected across a range of 100 kilometers in the study region. In general the study region has degraded pollination services compared with the rest of France (*20*). The status of pollinators or pollination services in such intensive agricultural areas has been reported as declining in several studies over our timescale (*2,3,21–24*). Parts of Belgium (some 100 to 200 km north of our study area) have experienced similar agricultural dynamics as our study region, and studies there indicate that 32.8% of bee species are threatened or extinct based on comparisons with their presence before and after 1970 (*25*). These studies also highlighted a more severe decline or higher vulnerability of long-tongued bees (*2,6,24,25*).

In this study, we combined genetic and phenotypic approaches. Screening of thirty-two individuals per population with microsatellite markers on the first generation (F1) allowed us to assess population genetic metrics in natural conditions. These metrics provide information on allelic richness of populations and any changes in the mating systems as measured by the inbreeding coefficient, F_{IS} (*26*). The F1 progenies, obtained in common garden, enabled us to establish a half-sib family design for the second generation (F2), with similar maternal environments for the ancestral and descendant populations. Each F2-population is represented by twenty families composed of five half-siblings, for a total of 800 individuals. Phenotypic traits were measured in the second generation (F2) in 2022 in an air-conditioned greenhouse. We quantified the evolution of traits mediating plant-pollinator interactions and morphological traits not directly involved in plant-pollinator interactions. We also performed a plant pollinator preference experiment by monitoring bumblebee visitation in mixed artificial populations containing genotypes of the two different source generations. Together, these approaches document on the evolution of plant-pollinator interactions and evolution of plant mating system induced in recent environmental changes.

Results

Characterization of changes in the genetic structure of populations

Our localities exhibited no significant differences in allelic richness nor genetic diversity between ancestral and descendant populations based on fifteen microsatellites markers (Table 1). In the case of a sampling bias of our populations or a strong demographic bottleneck or genetic drift between the two samples, we predicted a decrease in allelic richness and genetic diversity. Our results rule out these scenarii. On the other hand, we observed a consistent increase in selfing rates in all localities between ancestral and descendant populations (Table 1), with an average increase of around 15%, which is consistent with results found in other locations (*27*).

Characterization of changes of morphological and reproductive traits

Descriptive multivariate analyses performed on eight floral traits associated with the mating system (corolla length, labellum width, floral area, number of guides, anthesis duration, flowering date, volume of nectar and average floral display) and the ability of plant to set seeds in the absence of pollinators (*i.e.* autonomous self-pollination), corrected for differences between localities, showed differentiation between ancestral and descendant populations. We choose these traits because they are usually associated with shifts in mating systems (*11–13,15*). The descendent populations clustered together below the first bisector while ancestral populations clustered above the first bisector (Fig. 1). This result highlights consistent directional changes of floral traits in all four localities.

Linear models on the nineteen traits measured revealed consistent patterns (Table 2). We detected a significant difference between ancestral and descendant populations for all the floral traits, except sepal length (Table 2, table S2). Plants have evolved a 10% decrease in floral area (Fig. 2A) through a decrease in the labellum width and flower length (Table 2). Flowers have fewer nectar-guides (Fig. 2B), which are assumed to promote recognition of the path to floral rewards. Indeed, a correlation between the number of nectar-guides and the rate of visits inducing pollination has been reported elsewhere (*28*). In contrast, none of the morphological traits supposedly unlinked to pollinator attraction (vegetative traits and sepal length) showed consistent changes (Fig. 2C, fig. S2, Table 2). Based on the resurrection approach, patterns of trait changes between ancestral and descendant populations provide a signature of mating system and associated trait evolution. The lack of changes in vegetative traits rules out the possibility that these changes are due to inbreeding depression, which is highly unlikely to only affect floral traits (*29*). In addition, evolution in petal length and not in sepal (fig. S2) or leaf length (Fig. 2C), despite their homologous origins (*30*), provides strong evidence for specific selection against costly traits that mediate interactions with pollinators.

Regarding reproductive traits, the results revealed a marginally significant increase over time in the ability to set seeds in the absence of pollinators (Fig. 2D) and a significant decrease in seed weight (Table 2). Seed weight represents maternal investment in individual progeny. This shift towards increased reproductive insurance is associated with reduced seed investment, as observed in previous studies (*31*).

Our study also revealed that locality and locality x age interaction are major causes of trait variations. The locality effects indicate spatial differentiation in measured traits and the locality x age interaction reveals a site dependent evolution in time. The latter interaction effects were different in magnitude, but not in sign, between ancestors and descendants for all the traits, except anthesis duration (Table 2). Evolution towards a selfing syndrome in the four localities is in the same direction but differs in magnitude. These effects indicate low gene flow between localities. The consistent evolutionary pattern (Fig. 1) highlights convergent shifts in fairly independent populations.

Characterization of changes of plant-pollinator interactions

Measurements of nectar volume, which is a fundamental rewarding trait, revealed a consistent decrease of 20% on average (Fig. 3A, Table 2). Because nectar production is costly (*32*) and only dedicated to rewarding pollinators, the breakdown of plant-pollinator interactions is expected to be associated with such reduced nectar production. Nectar production is a trait that contributes to the maintenance of stable plant-pollinator interactions (*33*), and its decrease in our study species may loosen the interaction between the plant and its pollinators.

How this evolution impacts plant-pollinator interactions from the point of view of pollinators is addressed in our preference experiment during visits of the long-tongued bumblebees *Bombus terrestris* to ancestral and descendant plants mixture. Bumblebees visited more frequently ancestral than descendant plants *i.e.,* they have a consistent preference of ancestral plants that is associated with their higher attractiveness and reward (Fig. 3B, Table 2). The evolution towards reduced attractiveness is consistent with reduced nectar production and the evolution of a selfing syndrome.

Discussion

Overall, floral display, floral area, floral attractiveness, and nectar production decreases, enhance selfing and increase of realized selfing rates are thus consistent with the ongoing evolution of a selfing syndrome (*15,16*). Importantly, trait evolution is convergent across the four populations investigated. This is a unique demonstrate for such an evolutionary change over a short-time scale. While angiosperm-pollinator interactions have evolved over the long-term, our study thus shows that current environmental changes can drive rapid evolution towards a breakdown of such interactions.

Recurrent evolution towards selfing has been repeatedly documented, but to our knowledge, only over the long-term (*9,34*). This evolutionary transition is classically considered to be irreversible (the “selfing as an evolutionary dead-end” hypothesis, *9,35*) and higher extinction rates of such phyla have been reported (*36*). Evolution towards selfing could thus be driven by natural selection over the short-term but could impede long-term plant population survival (*37*). The increase in selfing rate is indeed expected to have genomic impacts on individuals and populations through inbreeding (homozygosity), reduced effective population sizes and increased genetic drift. Consequently, selfers may have a reduced potential response to natural selection (*38,39*). While we acknowledge that short-term evolution of selfing in *Viola arvensis* is no-doubt facilitated by its initial mixed mating system (see for instance *40*), our results illustrate how mixed-mating species may evolve towards a higher reliance on self-pollination and reduced plant-pollinator interactions in the face of global change, in particular pollinator decline. If a rapid

transition towards selfing syndrome is shared among Angiosperms, many lineages may be in the situation of an extinction debt (*36*).

Direct effect of environmental changes (habitat destruction and fragmentation, agricultural land pollution and species introduction) currently affect pollinator abundance and cause their decline (*2,21*). A decline in floral resources is also caused by these same changes and as a result becomes a driver of pollinator decline (*2*). Over time changes in plant community composition are known to be accompanied by changes in nectar resources (*41*). In addition, as we illustrate here, nectar production can also rapidly evolve within species due to a relaxed selective pressure associated with pollinator decline. Decreases in nectar production may in fact reinforce the pollinator decline if levels fall below those necessary to sustain wild bee populations. The impact on pollinator populations is thus doubled as they become victims of both environmental changes and plant trait evolution (*42*). There is thus a potential eco-evolutionary positive feedback loop that furthers pollinator decline and reinforces plant evolution towards a selfing syndrome. Our results could in fact explain plant-pollinator network degradation (documented in a previous study *22*) and how such degradation may generate cascading effects in trophic networks in general, and not just plant-pollinator interactions.

Pollinator decline is presumably ongoing and, even in the best scenarios, will continue in future (*24*). There is thus an urgent need to investigate the reversibility of plant evolution toward a selfing syndrome and assess whether or not the evolution of the plant-pollinator interactions has reached the threshold below which the breakdown of the interactions is inevitable (*42*).

Materials and Methods

Study system and sampling

Viola arvensis is an annual species of wild pansy (Violaceae). It is commonly found in fields as a weed or in meadows. This species is described as self-compatible with a mixed mating system (*19*, *27*). Plants were sampled twice in four locations of the Parisian Basin (see table S1). The first sampling was done and conserved by the Conservatoire Botanique National de Bailleul for Crouy and Lhuys in the 1990's and by the Conservatoire Botanique National du Bassin Parisien for Commeny and Guernes in the 2000's. Seeds were collected by sampling fruits on a minimum of 100 mothers picked randomly in each population. The second sampling was performed in exactly the same locations in February 2021 by randomly sampling 40 or more seedlings. This sampling scheme produced four couples, one for each location, of ancestral and descendant populations.

Experimental design

To avoid maternal and potential storage effects, a refresher generation (F1) was grown in common garden for plants issued from the different populations. As we have seeds and seedlings, common conditions started at seedling stage. For ancestral populations, in mid-November 2020, seed lots were placed in germination chambers in petri dishes with vermiculite under light at 15°C for 12h and in the dark at 6°C for 12h. 32 seedlings of ancestral and descendant populations were transplanted in late February 2021 in open ground in the Baillarguet site of the experimental platform "Terrains d'Expériences" of the Labex Cemeb, 10 km north of Montpellier, France (43°40′53.92″N, 3°52′28.74″E). Populations were separated from each other by insect-proof mesh greenhouses (fig. S1). During full blooming, in mid-April, we introduced commercial bumblebee hives, Natupol from Koppert France® (Cavaillon, France), into the greenhouses to produce open-pollinated fruits that we collected.

This refresher generation was used to generate seeds produced in the same environment for the experimental generation (F2) (*43*). This F1 generation allows us to generate a family design in the F2 generation. We randomly selected 20 mothers per population in the F1 generation to construct 20 families in the F2 generation. Each family is composed of five siblings (fig. S1). In December 2021, 30 seeds per family were placed in germination chambers in petri dishes with vermiculite under light at 15°C for 12h and in the dark at 6°C for 12h. Plants were

placed in individual pots (11 × 11 × 11 cm^3) filled with standardized soil in late February and randomized in a greenhouse of the experimental platform “Terrains d'Expériences” of the Labex Cemeb, Montpellier, France. Watering was manual and done when needed. Three families did not germinate enough to obtain 5 plants and thus were composed of one, two or four siblings. In total, 792 plants were followed during this experiment. When all the morphological measurement were performed, plants were moved outside to finish their life cycle.

Population genetics

F1 plants were genotyped using fifteen microsatellite markers, 8 design for *Viola arvensis* (*27*) and 7 design on *Viola tricolor* (*44*), a closely related species (*19*). DNA was extracted from fresh leaves using Dneasy Plant Mini Kit from QIAGEN®. Analyses of metrics such as allelic richness, expected heterozygosity (H_e), using the R package genepop (*45*), were performed to look at a change in genetic diversity over time. A drastic decrease in allelic richness (or H_e) is indicative of genetic drift, strong population bottlenecks or a sampling bias. Heterozygosity deficiency (F_{IS}) analyses were performed, using the R package HierFstats (*46*), on fourteen microsatellite markers. F_{IS} was used to estimate the selfing rate using the following formula: $s_{Fis} = \frac{2F_{IS}}{1 + F_{IS}}$. Confidence intervals were obtained by bootstraps over loci of population (10,000). To perform an overall test on F_{IS}, we pooled individuals of the same location, independently of their age and performed bootstraps to construct again two populations of 32 individuals again. We calculated the F_{IS} of these two reconstruct populations and reproduce the procedure 10 000 times per locality on each locality. Then, we recorded the number of cases where the absolute value of the difference in reconstruct F_{IS} is higher than the true difference in F_{IS} measured. This number divided by 10 000 gives us a *P*-value for H_0: “True difference = 0”. We performed a Fisher's combined probability test on the *P*-values obtained for each locality (*47*).

Measurements of plant traits

Plants traits were measured on the test generation (F2). All the morphometric measurements (fig. S3) were performed by a single experimenter during all the experimentation, to avoid any experimenter effect, using a digital caliper to 0.01 mm.

Corolla length, labellum width, spur length, sepal length, number of guides and anthesis duration were measured on the five first opened flowers of the main shoot of each plant. We chose the first five opened flowers of the main shoot because more exhausted measurements in the F1 showed that they well capture the floral variance of an individual. Measurements were made day+2 after anthesis, or day+3 when falling on weekends. The opening of flowers was followed every working day to assess the anthesis duration. Floral area approximation is the product of corolla length and labellum width. Sepal/corolla lengths ratio is the ratio of sepal to corolla lengths. Around 4,000 flowers were followed. Flowering date is the date of anthesis of the first flower of a plant. The number of flowers opened per plant was counted each working day, from the opening of the first flower, for one month and half, and two more times with one week and then two weeks delay between counts. This number was integrated using trapezoidal rule and divided by the duration between the flowering date of the plant and the last day of measurement, giving the average floral display. Nectar volume was collected and cumulated from three flowers per plant using 0.5 µL microcapillaries. It was measured randomly on individuals of the two populations of the same location simultaneously to avoid differences in nectar production caused by the time of measurement. Thus, for this measure, Locality effect (see “Statistical analysis”) is confounded with the day of measurement. It was measured on half of the plants from each population, representing all the families, thus 400 plants were sampled.

Vegetative traits such as rosette diameter, leaf length and leaf width were measured at the flowering date of the plant. Leaf length and leaf width were measured on the biggest leaf at this time, and the product of these two measures gives leaf area approximation. Biomass was measured on dried naturally dead plants at the end of their life cycle.

Reproductive traits were obtained while counting and weighing all the seeds contained by fruits to the nearest 0.1 mg using a precision balance. After all the measurements plants were placed by groups of twenty in insect-proof mesh greenhouses which contained commercial bumblebee hives, Natupol from Koppert France® (see Bumblebee preferences experiment below). After 2 hours minimum, plants were moved outside in open pollination context. Youngest receptive flower and oldest non-receptive ones on the same shoot were marked before the exposition to pollinator to differentiate fruits issued from flower in zero-pollinator context (self-pollination) versus fruits produced in open pollination. Thus, open pollination fruits are issued from flowers produced later in the shoot. For each plant, just before the dehiscence of the fruit produced by self-pollination, we sampled the two fruits at the same time and recorded the difference in the flower position number separating collected fruits (minimum 1, by definition, to maximum 5, with a mean of 1.6). The sampling of the two fruits was performed at the same time because it was too complicated to follow 1,600 fruits independently. Thus, open pollination fruits are not fully developed and comparing weights between open pollination and self-pollination fruits has a slight bias. However, comparing each measure between populations is fully possible while differences in floral positions are distributed randomly between populations. The seed weight reported is the measure of the weight of seeds contained by each fruit divided by the number of seeds counted in the fruit. The ratio of the number of seeds produced by self-pollination to the number of seeds produced by open pollination is a good proxy of the self-pollination ability and is comparable among plants if we consider that in open pollination treatment we have a full seed set and an incomplete seed set after self-pollination treatment.

Bumblebee preferences experiment

Plants were exposed to commercial bumblebee hives, Natupol from Koppert France®, in insect-proof mesh greenhouses once all the trait measures had been made. In each insect-proof greenhouse, we placed, for the entire experiment, a bumblebee hive of around 30 workers with a queen, with nutrient reserves. We randomly disposed plants from the same location in a group of 20 plants, composed of 10 plants from each ancestral and descendant population randomly selected in one of the two insect-proof greenhouses. Pots were equally spaced at 35 cm. The observer, stationed in the entrance of the insect-proof greenhouses, selected a foraging worker bumblebee, and recorded each flower visit during 10 to 15 minutes. Greenhouse number (1, 2), position in the greenhouse (1-20) and plant identities were also recorded. Experiments were performed between the 2nd and the 19th of May 2022. We performed the experiment on 8 groups for each locality but just 6 and 7 observations were kept for Lhuys and Crouy respectively, because no pollinators foraged enough time in the discarded observations. On the 580 plants in our dataset, 3 are individuals exposed two times for experimental purpose. During a 10-15 minutes test, 4.28 plants were not visited in average and 124 in total for all the tests. In the analyses, we sorted out plants visited and unvisited (see below). We calculated the proportion of visits per plant as the ratio between the number of visits on a given plant divided by the total number of visits during a flight.

Statistical analysis

Changes in traits were evaluated using linear discriminant analysis (LDA) and univariate linear mixed models (LMMs). We performed the LDA over nine traits supposed to be linked with reproductive system; corolla length, labellum width, floral area, number of guides, anthesis duration, flowering date, volume of nectar, average floral display and ratio of seeds produced in self-pollination to open pollination. For traits with repeated measures, we used the means of the trait per individual. We normalized values by locality in order to focus on Age effect. We predefined only the population (8 groups) in the LDA and not the age. LDA gives linear combinations of traits (discriminant functions) that attempt to capture differences among groups.

In our case, it produced seven discriminant functions. The two first functions represent 55% and 22% of the proportion of the trace.

Univariate analyses were conducted using LMMs with each trait as response variable, Age (ancestral or descendant), Locality (4 localities), and the interaction between Age and Locality as fixed factors, and Family as random factor. In order to account for repeated measures on the same individual for floral measurement, we add Individual as random factor nested in Family. For seeds traits, we accounted for the difference in flower position between the two fruits collected adding this difference as random factor nested in family. When significant Age effect, analysis was followed by T-test comparison, on the means of the trait per individual for repeated measures, of ancestral and descendant populations of each locality. *p*-values were corrected using Bonferroni correction. Rosette diameter, leaf length, width and area, nectar volume and ratio of seeds without/with pollinators were log-transformed to approach normal distribution of residuals. In these analyses, Age significant effect represents a signal of global evolution signal of traits between ancestral and descendant populations, Locality significant effect, represents a difference in traits between populations and so population polymorphism spread in this species. A significant effect of the interaction between Age and Locality represents a difference in the magnitude or in the sign of the evolution in time between localities. In the case of a significant Age effect and a significant interaction effect, if evolution is consistent (same sign of evolution) among localities and only the magnitude change, we can hypothesize a directional selective force that selects a trait in a direction convergently in every locality. In other cases, a significant interaction between Age and Locality indicates a local selective pressure on the traits or, perhaps more conservatively, an evolutionary drift of the trait. These cases are beyond the scope in this study.

For bumblebee preference analyses, we first performed a GLMM to test the effect of Age on the number of unvisited plants (proportions: CoA:_0.24, Co21: 0.35; CrA: 0.26, Cr21: 0.26; GuA: 0.15, Gu21: 0.18; LhA: 0.13, Lh21: 0.12). We modelled it with Age and Locality as fixed effect, Family, Greenhouse, Position nested in Greenhouse and Date as random effects, with binomial distribution and logit link, as response variable was a Boolean variable with TRUE for visited plants and FALSE otherwise. There was no significant effect of Age in our dataset (N = 580 plants, estimates: AgeA-Age21 = 0.223, P = 0.296). Thus, we excluded unvisited plants (N = 124) in the rest of the analysis. We performed a LMM with log-proportion of visits per plant as response variable (N = 456), and the same structure as for other traits with Age, Locality, and the interaction between Age and Locality as fixed effect, Family as random effect and we added Greenhouse, Position nested in Greenhouse and Date as random effects. We then performed T-test comparisons between Age for given Locality and corrected *p*-values using Bonferroni correction.

All our statistical analyses were performed using the R software (R Core Team, Version 4.2.1). LDA was performed using the “lda” function implemented in the package “MASS” (*48*). LMMs and GLMM were performed using the “lmer” and “glmer” functions implemented in the package “lme4” (*49*).

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References

1. C. A. Hallmann, M. Sorg, E. Jongejans, H. Siepel, N. Hofland, H. Schwan, W. Stenmans, A. Müller, H. Sumser, T. Hörren, D. Goulson, H. de Kroon, More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE* **12**, e0185809 (2017).
2. D. Goulson, E. Nicholls, C. Botías, E. L. Rotheray, Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957 (2015).
3. H. Grab, M. G. Branstetter, N. Amon, K. R. Urban-Mead, M. G. Park, J. Gibbs, E. J. Blitzer, K. Poveda, G. Loeb, B. N. Danforth, Agriculturally dominated landscapes reduce bee phylogenetic diversity and pollination services. *Science* **363**, 282–284 (2019).
4. J. B. Whittall, S. A. Hodges, Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**, 706–709 (2007).
5. J. Ollerton, R. Winfree, S. Tarrant, How many flowering plants are pollinated by animals? *Oikos* **120**, 321–326 (2011).
6. J. C. Biesmeijer, S. P. M. Roberts, M. Reemer, R. Ohlemüller, M. Edwards, T. Peeters, A. P. Schaffers, S. G. Potts, R. Kleukers, C. D. Thomas, J. Settele, W. E. Kunin, Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* **313**, 351–354 (2006).
7. D. G. Lloyd, Self- and cross-fertilization in plants. II. The selection of self-fertilization. *Int. J. Plant Sci.* **153**, 370–380 (1992).
8. M. Thomann, E. Imbert, C. Devaux, P.-O. Cheptou, Flowering plants under global pollinator decline. *Trends Plant Sci.* **18**, 353–359 (2013).
9. G. L. Stebbins, Self fertilization and population variability in the higher plants. *Am. Nat.* **91**, 337–354 (1957).
10. B. Igic, L. Bohs, J. R. Kohn, Ancient polymorphism reveals unidirectional breeding system shifts. *Proc. Natl. Acad. Sci.* **103**, 1359–1363 (2006).
11. S. A. Bodbyl Roels, J. K. Kelly, Rapid evolution caused by pollinator loss in *Mimulus guttatus*: rapid evolution after pollinator loss. *Evolution* **65**, 2541–2552 (2011).
12. D. D. L. Gervasi, F. P. Schiestl, Real-time divergent evolution in plants driven by pollinators. *Nat. Commun.* **8**, 14691 (2017). doi:10.1038/ncomms14691.
13. S. E. Ramos, F. P. Schiestl, Rapid plant evolution driven by the interaction of pollination and herbivory. *Science* **364**, 193–196 (2019).
14. R. Brys, H. Jacquemyn, Effects of human-mediated pollinator impoverishment on floral traits and mating patterns in a short-lived herb: an experimental approach. *Funct. Ecol.* **26**, 189–197 (2012).
15. A. Sicard, M. Lenhard, The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptation in plants. *Ann. Bot.* **107**, 1433–1443 (2011).
16. R. Ornduff, Reproductive biology in relation to systematics. *TAXON* **18**, 121–133 (1969).
17. S. J. Franks, S. Sim, A. E. Weis, Rapid evolution of flowering time by an annual plant in response to a climate fluctuation. *Proc. Natl. Acad. Sci.* **104**, 1278–1282 (2007).
18. L. J. Weider, P. D. Jeyasingh, D. Frisch, Evolutionary aspects of resurrection ecology: Progress, scope, and applications-An overview. *Evol. Appl.* **11**, 3–10 (2018).
19. A. Scoppola, E. Lattanzi, *Viola* section *Melanium* (Violaceae) in Italy. New data on morphology of *Viola tricolor*-Group. *Webbia* **67**, 47–64 (2012).

20. G. Martin, C. Fontaine, F. Accatino, E. Porcher, New indices for rapid assessment of pollination services based on crop yield data: France as a case study. *Ecol. Indic.* **101**, 355–363 (2019).
21. S. G. Potts, J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, W. E. Kunin, Global pollinator declines: trends, impacts and drivers. *Trends Ecol. Evol.* **25**, 345–353 (2010).
22. L. A. Burkle, J. C. Marlin, T. M. Knight, Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. *Science* **339**, 1611–1615 (2013).
23. P. H. Raven, D. L. Wagner, Agricultural intensification and climate change are rapidly decreasing insect biodiversity. *Proc. Natl. Acad. Sci.* **118**, e2002548117 (2021).
24. W. M. Janousek, M. R. Douglas, S. Cannings, M. A. Clément, C. M. Delphia, J. G. Everett, R. G. Hatfield, D. A. Keinath, J. B. U. Koch, L. M. McCabe, J. M. Mola, J. E. Ogilvie, I. Rangwala, L. L. Richardson, A. T. Rohde, J. P. Strange, L. M. Tronstad, T. A. Graves, Recent and future declines of a historically widespread pollinator linked to climate, land cover, and pesticides. *Proc. Natl. Acad. Sci.* **120**, e2211223120 (2023).
25. M. Drossart, P. Rasmont, P. Vanormelingen, M. Dufrêne, M. Folschweiller, A. Pauly, N. Vereecken, S. Vray, E. Zambra, J. D'Haeseleer, D. Michez, *Belgian Red List of Bees*. (Presse universitaire de l'Université de Mons, Mons, Belgique, 2019).
26. D. L. Hartl, A. G. Clark, *Principles of Population Genetics*. (Oxford University Press, 2007).
27. P. Cheptou, E. Imbert, M. Thomann, Rapid evolution of selfing syndrome traits in *Viola arvensis* revealed by resurrection ecology. *Am. J. Bot.* **109**, 1838–1846 (2022).
28. D. M. Hansen, T. Van der Niet, S. D. Johnson, Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proc. R. Soc. B Biol. Sci.* **279**, 634–639 (2012).
29. A. A. Winn, E. Elle, S. Kalisz, P.-O. Cheptou, C. G. Eckert, C. Goodwillie, M. O. Johnston, D. A. Moeller, R. H. Ree, R. D. Sargent, M. Vallejo-Marín, Analysis of inbreeding depression in mixed-mating plants provides evidence for selective interference and stable mixed mating. *Evolution* **65**, 3339–3359 (2011).
30. H. Breuninger, M. Lenhard, "Control of tissue and organ growth in plants" in *Current Topics in Developmental Biology* (Elsevier, 2010), pp. 185–220.
31. T. J. De Jong, H. Van Dijk, P. G. L. Klinkhamer, Hamilton's rule, imprinting and parent-offspring conflict over seed mass in partially selfing plants: Parent-offspring conflict over seed mass. *J. Evol. Biol.* **18**, 676–682 (2005).
32. G. H. Pyke, What does it cost a plant to produce floral nectar? *Nature* **350**, 58–59 (1991).
33. A. Brandenburg, C. Kuhlemeier, R. Bshary, Hawkmoth pollinators decrease seed set of a low-nectar *Petunia axillaris* line through reduced probing time. *Curr. Biol.* **22**, 1635–1639 (2012).
34. S. C. H. Barrett, The evolution of plant sexual diversity. *Nat. Rev. Genet.* **3**, 274–284 (2002).
35. B. Igic, J. W. Busch, Is self-fertilization an evolutionary dead end? *New Phytol.* **198**, 386–397 (2013).
36. E. E. Goldberg, J. R. Kohn, R. Lande, K. A. Robertson, S. A. Smith, B. Igić, Species selection maintains self-incompatibility. *Science* **330**, 493–495 (2010).
37. P.-O. Cheptou, Does the evolution of self-fertilization rescue populations or increase the risk of extinction? *Ann. Bot.* **123**, 337–345 (2019).
38. S. Glémin, J. Ronfort, Adaptation and maladaptation in selfing and outcrossing species: new mutations versus standing variation. *Evolution* **67**, 225–240 (2013).
39. E. Noël, P. Jarne, S. Glémin, A. MacKenzie, A. Segard, V. Sarda, P. David, Experimental evidence for the negative effects of self-fertilization on the adaptive potential of populations. *Curr. Biol.* **27**, 237–242 (2017).
40. M. Thomann, E. Imbert, R. C. Engstrand, P.-O. Cheptou, Contemporary evolution of plant reproductive strategies under global change is revealed by stored seeds. *J. Evol. Biol.* **28**, 766–778 (2015).
41. M. Baude, W. E. Kunin, N. D. Boatman, S. Conyers, N. Davies, M. A. K. Gillespie, R. D. Morton, S. M. Smart, J. Memmott, Historical nectar assessment reveals the fall and rise of floral resources in Britain. *Nature* **530**, 85–88 (2016).

42. A. Weinbach, N. Loeuille, R. P. Rohr, Eco-evolutionary dynamics further weakens mutualistic interaction and coexistence under population decline. *Evol. Ecol.* **36**, 373–387 (2022).
43. S. J. Franks, E. Hamann, A. E. Weis, Using the resurrection approach to understand contemporary evolution in changing environments. *Evol. Appl.* **11**, 17–28 (2018).
44. M. Latron, J.-F. Arnaud, H. Ferla, C. Godé, A. Duputié, Polymorphic nuclear markers for coastal plant species with dynamic geographic distributions, the rock samphire (*Crithmum maritimum*) and the vulnerable dune pansy (*Viola tricolor subsp. curtisii*). *Mol. Biol. Rep.* **45**, 203–209 (2018).
45. F. Rousset, genepop: Population Genetic Data Analysis Using Genepop.
46. J. Goudet, hierfstat: Estimation and Tests of Hierarchical F-Statistics.
47. R. A. Fisher, *Statistical Methods for Research Workers*. (Hafner Pub. Co., 1970).
48. W. N. Venables, B. D. Ripley, *Modern applied statistics with S*. (Springer, 2002).
49. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. *J. Stat. Soft.* **67**, (2015).

Figures and Tables

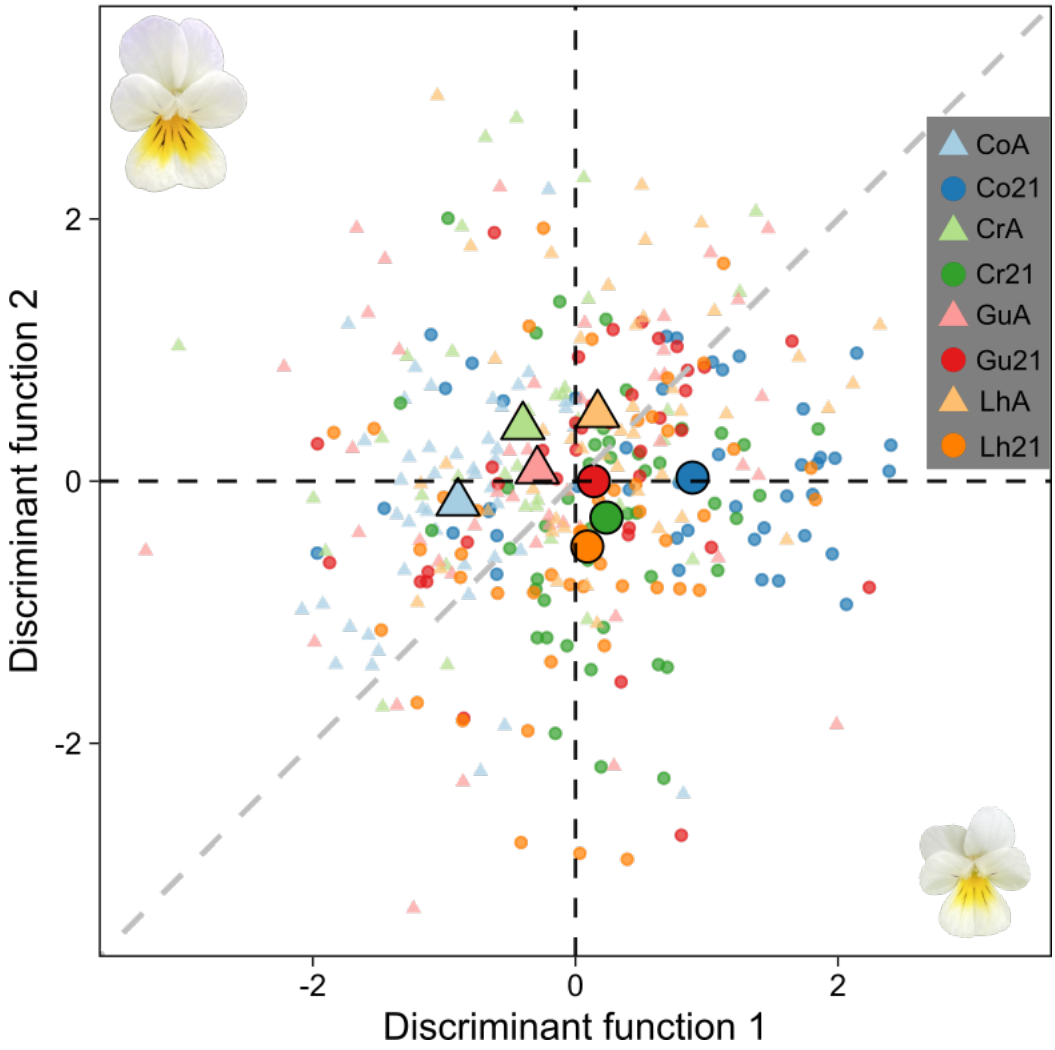

Figure 1. Multivariate differences between our population. Multivariate analysis (linear discriminant analysis performed on nine traits; N = 357 plants, almost equally distributed among groups, N_{min} = 36 individuals; 7 points are not represented because out of scale) used to explore differences between ancestral and descendant populations. In the analysis, we predefined the eight different groups but not the status of the age factor. The first two functions represent 55% and 22% of the proportion of the trace respectively.

The first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys). “A” (▲) ancestral population (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and “21” (●) descendant population (all collected in 2021). Enlarged symbols are centroids of groups. The gray dashed line represents the first bisector.

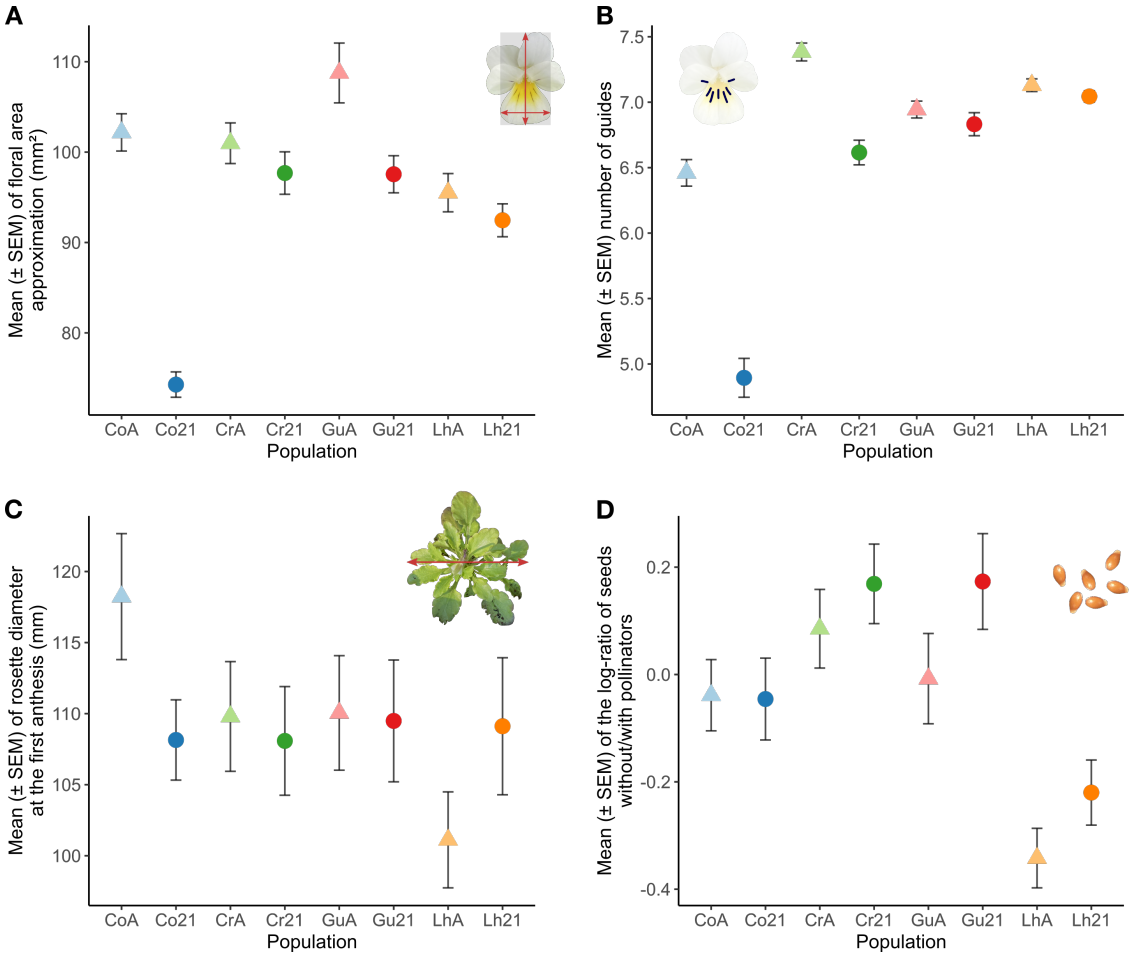

Figure 2. Evolutionary changes in floral, reproductive and vegetative traits. (**A** and **B**) Evolution of floral traits. We measured the first five developed flowers per individuals (N ≈ 4,000). (A) Floral area is the multiplication of labellum width and corolla length. It showed an overall decrease between ancestors and descendants (Table 2). (B) The number of attractive guides showed an overall decrease in time (Table 2). (**C**) Rosette diameter, measured on each plant at the start of flowering, shows no consistent or significant differences between ancestors and descendants, like other vegetative traits (Table 2). (**D**) The log-ratio of seeds produced in self-pollination compared to open pollination is a proxy of selfing ability. We measured it by collecting one fruit in self-pollination and one in open pollination per plants (N = 693). This showed a trend towards an increase in selfing ability between ancestors and descendants (Table 2).
The first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys). "A" (▲) ancestral population (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and "21" (●) descendant population (all collected in 2021).

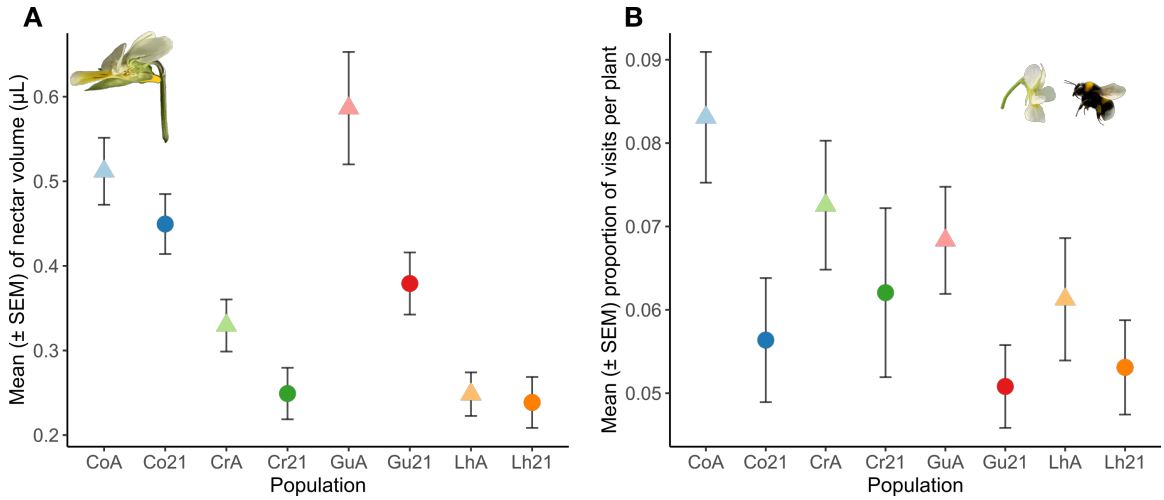

Fig. 3. Decrease of plant-pollinator interactions. (**A**) Decrease of nectar production. We measured nectar volume as the sum of the volume in three flowers per plants on fifty plants per population (N = 400). There is an overall trend of a decrease in the volume of nectar over time (Table 1), which is significant for Cr and Gu (table S2). (**B**) Test of the preference of bumblebees. 10 plants of the ancestral and the descendant populations of a single locality were exposed together to bumblebees. We recorded the number of visits on each plant by a unique flying bumblebee for 10 to 15 minutes in 6 to 8 replicates per location and divided it by the total number of visits during the flight. Only visited plants are represented. There is an overall preference of ancestral plants (Table 1), significant for Co (table S2).
The first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys). “A” (▲) ancestral population (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and “21” (●) descendant population (all collected in 2021).

Table 1. Allelic richness, genetic diversity and selfing rates in our populations (mean ± SD or [CI]). Average allelic richness and genetic diversity are based on fifteen microsatellites markers and selfing rate on fourteen. These markers were genotyped on the thirty-two individuals per population of the F1 generation issued from natural populations. For selfing rate, confidence intervals (CI) were obtained by bootstraps on loci (10,000). Fisher's combined probability test gave a significant overall increase in F_{IS} (χ^2_8 = 35.04, P < 0.001).

The first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys). "A" ancestral population (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and "21" descendant population (all collected in 2021).

Population	CoA	CrA	GuA	LhA
	Co21	Cr21	Gu21	Lh21
Average allelic richness	2.47 ± 1.36	3.07 ± 1.67	2.73 ± 1.39	2.53 ± 1.60
	1.80 ± 0.86	3.20 ± 1.90	3.33 ± 1.68	2.00 ± 1.13
Genetic diversity (H_e)	0.274 ± 0.278	0.411 ± 0.265	0.311 ± 0.218	0.257 ± 0.269
	0.095 ± 0.122	0.399 ± 0.272	0.322 ± 0.251	0.193 ± 0.211
Selfing rate [95% CI] derived from heterozygosity deficiency, F_{IS}	0.75 [0.69-0.81]	0.73 [0.64-0.83]	0.54 [0.40-0.71]	0.44 [0.29-0.55]
	0.85 [0.81-0.94]	0.77 [0.70-0.84]	0.77 [0.74-0.84]	0.87 [0.77-0.94]

Table 2. Linear models: effects of time, locality, and their interaction on the changes of floral, vegetative and reproductive traits. Values in parentheses are sample sizes. All traits are shown and the degree of significance of each effect on each trait is reported (LMM, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, • $P < 0.1$, n.s. $P > 0.1$, see table S2 for full statistical values). (+) a positive effect (increase), (−) a negative effect (decrease), (~) a difference of effect sign between localities (see table S2 for exact values).

Trait (sample size)	Age	Locality	Interaction of Locality and Age
Floral traits			
Corolla length (3955)	***(—)	**	***
Labellum width (3955)	***(—)	***	**
Floral area approximation (3955)	***(—)	***	**
Number of guides (3993)	***(—)	***	***
Average floral display (789)	**(—)	***	*
Anthesis duration (3991)	**(~)	***	***
Spur length (3954)	**(—)	***	**
Sepal length (3955)	n.s.	***	n.s.
Sepal/corolla lengths ratio (3955)	***(+)	***	**
Flowering date (792)	n.s.	n.s.	n.s.
Vegetative traits			
Rosette diameter (792)	n.s.	n.s.	n.s.
Leaf length (792)	n.s.	***	*
Leaf width (792)	n.s.	***	*
Leaf area approximation (792)	n.s.	***	*
Biomass (777)	n.s.	n.s.	n.s.
Reproductive traits			
Log-ratio of the number of seeds without/ with pollinators (693)	•(+)	***	n.s.
Weight per seed with pollinators (656)	*(—)	***	n.s.
Weight per seed without pollinators (701)	**(—)	***	***
Plant-pollinator interaction traits			
Nectar volume (400)	***(—)	***	n.s.
Bumblebee visit preferences (456)	***(—)	n.s.	n.s.