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

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Summary

- Plant-pollinator interactions have evolved early in the angiosperm radiation. Ongoing environmental changes are however leading to pollinator declines that may cause pollen limitation to plants and change the evolutionary pressures shaping plant mating systems.
- We used the resurrection ecology methodology to contrast ancestries and contemporary descendants in four natural populations of the field pansy (*Viola arvensis*) in the Paris region (France), a depauperate pollinator environment. We combine population genetics analysis and phenotypic measurements and behavorial tests on a common garden experiment.
- Population genetic analyses reveal 27% increase in realized selfing rates in the field during this period. We documented trait evolution towards smaller and less conspicuous corollas, reduced nectar production and reduced attractiveness to bumble bees, with these trait shifts convergent across the four studied populations.
- We demonstrate the rapid evolution of a selfing syndrome in the four studied plant populations, associated with a weakening of the interactions with pollinators over the last three decades. This study demonstrates that plant mating systems can evolve rapidly in natural populations in the face of ongoing environmental changes. The rapid evolution towards a selfing syndrome may in turn further accelerate pollinator declines, in an eco-evolutionary feedback loop with broader implications to natural ecosystems.

Key words: Rapid evolution, mating system, selfing syndrome, plant-pollinator interactions, resurrection ecology, *Viola arvensis*

Introduction

The astonishing diversification of angiosperms, which started 100 billion years ago (Benton et al. 2022), is commonly thought to have been stimulated and hastened by the diversity of plant-pollinator interactions (Whittall and Hodges 2007). More than 80% of extant angiosperms rely on animals for pollination (Ollerton et al. 2011), thus plants and

pollinators have a close relationship. Yet, previous studies have shown a decline of pollinators (including bees, Anthopila) is ongoing across the world (Goulson et al. 2015, Hallmann et al. 2017, Grab et al. 2019). A lack of pollinators can in turn directly affect plant reproduction, and indeed parallel declines in insect-pollinated plants and insects have been documented, over the last 50 years (Biesmeijer et al. 2006). At evolutionary time scales, pollen limitation has been shown to represent a major selective pressure in the evolution of self-fertilization (selfing) in theoretical evolutionary models (Lloyd 1992, Thomann et al. 2013). In recent years, such a shift towards selfing has been experimentally reproduced in the absence of pollinators (Bodbyl Roels and Kelly 2011, Gervasi and Schiestl 2017, Ramos and Schiestl 2019) and illustrated in natural populations impoverished in pollinators (Brys and Jacquemyn 2012). 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) (Bodbyl Roels and Kelly 2011, Brys and Jacquemyn 2012, Ramos and Schiestl 2019) 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 (Brys and Jacquemyn 2012, Gervasi and Schiestl 2017, Ramos and Schiestl 2019). A shift in mating system and traits leading to higher selfing, less conspicuous and attractive flowers has been well described at the phylogenetic level (Stebbins 1957, Sicard and Lenhard 2011). This set of trait changes characterizes the evolution of a selfing syndrome (Ornduff 1969, Sicard and Lenhard 2011).

Faced with pollinator declines, natural populations are expected to evolve towards reduced plant-pollinator interactions and increased autonomous selfing (Thomann et al. 2013). However, demonstrating trait evolution requires distinguishing between the genetic and the environmental components of phenotypes, which is not straightforward in longitudinal studies of natural populations. The “resurrection ecology” approach, which involves growing dormant seeds collected from field populations multiple generations ago alongside their naturally occurring descendants (Franks et al. 2007, Weider et al. 2018), is a powerful methodology to detect such evolution (Weider et al. 2018). Indeed, this approach enables the contrast between traits of ancestral versus recent genotypes in a common environment, thus revealing the genetic components of temporal trait variation. Here, we took advantage of an ancestral seed collection of the field pansy (*Viola arvensis*)

56 collated about two decades ago in the Paris region (France) to test whether this species'
57 mating system has evolved in response to recent pollinator declines.

58 The study species is an annual weed of intensive crops (mostly wheat and oilseed
59 rape) that only produces chasmogamous, zygomorphic, showy flowers and mixed selfing
60 rates (Scoppola and Lattanzi 2012, Cheptou et al. 2022). The length of the nectar spur and
61 observations *in natura* are indicative of pollination by long-tongued bees. For example,
62 *Bombus terrestris* is one of the wild pollinators observed. The study region has degraded
63 pollination services compared with the rest of France (Martin et al. 2019). The status of
64 pollinators or pollination services in such intensive agricultural areas has been reported as
65 declining in several studies over our timescale (Goulson et al. 2015, Grab et al. 2019, Potts
66 et al. 2010, Burkle et al. 2013, Raven and Wagner 2021, Janousek et al. 2023). Parts of
67 Belgium (some 100 to 200 km north of our study area) have experienced similar
68 agricultural dynamics as in our study region, and studies there indicate that 32.8% of bee
69 species are threatened or extinct based on comparisons with their presence before and after
70 1970 (Drossart et al. 2019). These studies also highlighted a more severe decline or higher
71 vulnerability of long-tongued bees (Biesmeijer et al. 2006, Goulson et al. 2015, Drossart
72 et al. 2019, Janousek et al. 2023).

73 In this study, we contrast ancestral (collated in the 1990s to early 2000s) and
74 contemporary descendants (collated in 2021) from four localities across a range of 100
75 kilometres in the study region, combining genetic and phenotypic approaches. After a
76 refresher generation (F1), phenotypic traits were measured in the second generation (F2)
77 in 2022 in an air-conditioned greenhouse. We quantified the evolution of traits mediating
78 plant-pollinator interactions as well as other morphological traits not directly involved in
79 plant-pollinator interactions. We also performed a plant pollinator preference experiment
80 by monitoring bumblebee visitation in mixed artificial populations containing genotypes
81 of the two different source generations. Together, these approaches allowed us to document

82 on the evolution of plant-pollinator interactions and evolution of plant mating system
83 induced in recent environmental changes.

Material and methods

84 Study system and sampling

85 *Viola arvensis* is an annual species of wild pansy (Violaceae). It is commonly found in
86 fields as a weed or in meadows. This species is described as self-compatible with a mixed
87 mating system (Scoppola and Lattanzi 2012, Cheptou et al. 2022). Plants were sampled
88 twice in agricultural fields in four locations of the Parisian Basin (see Table **S1**). The first
89 sampling was done and conserved by the Conservatoire Botanique National de Bailleul for
90 Crouy (Cr) and Lhuys (Lh) in the 1990's and by the Conservatoire Botanique National du
91 Bassin Parisien for Commeny (Co) and Guernes (Gu) in the 2000's. Seeds were collected
92 by sampling fruits on a minimum of 100 mothers picked randomly in each population. The
93 second sampling was performed in exactly the same locations, in an area that overlapped
94 the ancestral sampling area as much as possible, in February 2021 by randomly sampling
95 40 or more seedlings. The minimal sampling areas was of 2000 m^2 and correspond to cases
96 were all the population is circumscribed on this area. This sampling scheme produced four
97 couples, one for each location, of ancestral (A) and descendant (21) populations.

98 Experimental design

99 To avoid maternal and potential storage effects (Franks et al. 2018), a refresher generation
100 (F1) was grown in common garden for plants issued from the different populations. As we
101 have seeds and seedlings, common conditions started at seedling stage. For ancestral
102 populations, in mid-November 2020, seed lots were placed in germination chambers in
103 petri dishes with vermiculite under light at 15°C for 12h and in the dark at 6°C for 12h.
104 Germination rates was of 50% to 65%. 32 seedlings of ancestral and descendant
105 populations were transplanted in late February 2021 in open ground in the Baillarguet site
106 of the experimental platform "Terrains d'Expériences" of the Labex Cemeb, 10 km north
107 of Montpellier, France (43°40′53.92″N, 3°52′28.74″E). A single event of mortality was

108 observed in Cr21 population. Populations were separated from each other by insect-proof
109 mesh greenhouses (Fig. **S1**). During full blooming, in mid-April, we introduced
110 commercial bumblebee hives, Natupol from Koppert France® (Cavaillon, France), into the
111 greenhouses to produce open-pollinated fruits that we collected.

112 This refresher generation was used to generate seeds produced in the same
113 environment for the experimental generation (F2). This F1 generation allows us to generate
114 a family design in the F2 generation. We randomly selected 20 mothers per population in
115 the F1 generation to construct 20 families in the F2 generation. Each family is composed
116 of five siblings (Fig. **S1**). In December 2021, 30 seeds per family were placed in
117 germination chambers in petri dishes with vermiculite under light at 15°C for 12h and in
118 the dark at 6°C for 12h. Germination rates per population range between 75% and 90%.
119 Plants were placed in individual pots (11 × 11 × 11 cm^3) filled with standardized soil in
120 late February and randomized in a greenhouse of the experimental platform "Terrains
121 d'Expériences" of the Labex Cemeb, Montpellier, France. Watering was manual and done
122 when needed. Three families did not germinate enough to obtain 5 plants and thus were
123 composed of one, two or four siblings. In total, 792 plants were followed during this
124 experiment, no early mortality was observed. When all the morphological measurement
125 were performed, plants were moved outside to complete their life cycle.

126 Population genetics

127 32 F1-plants per populationwere genotyped using eleven microsatellite markers, 6 design
128 for *Viola arvensis* (Cheptou et al. 2022) and 5 design on *Viola tricolor* (Latron et al. 2018),
129 a closely related species (Scoppola and Lattanzi 2012). DNA was extracted from fresh
130 leaves using Dneasy Plant Mini Kit from QIAGEN®. Analyses of metrics such as allelic
131 richness, expected heterozygosity (H_e), using the R package genepop (Rousset 2023), were
132 performed to look at a change in genetic diversity over time. A drastic decrease in allelic
133 richness (or H_e) is indicative of genetic drift, strong population bottlenecks or a sampling
134 bias. Heterozygosity deficiency (F_{IS}) analyses were performed, using the R package
135 HierFstats (Goudet et al. 2022), on fourteen microsatellite markers. F_{IS} was used to
136 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 test for a difference in F_{IS}, we pooled individuals of the same location, independently of their age and performed bootstraps to reconstruct two populations of 32 individuals. 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 (Fisher 1970) to test a global increase in selfing rate between A and 2021 populations.

Measurements of plant traits

Plants traits were measured on the test generation (F2). All the morphometric measurements (Fig. **S2**) 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 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

166 caused by the time of measurement. Thus, for this measure, Locality effect (see "Statistical
167 analysis") is confounded with the day of measurement. It was measured on half of the
168 plants from each population, representing all the families, thus 400 plants were sampled.

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

173 Reproductive traits were obtained while counting and weighing all the seeds
174 contained by fruits to the nearest 0.1 mg using a precision balance. When floral and leaf
175 traits had been scored, plants were placed by groups of twenty in insect-proof mesh
176 greenhouses which contained commercial *Bombus terrestris* bumblebee hives, Natupol
177 from Koppert France® (see Bumblebee preferences experiment below). After 2 hours
178 minimum, plants were moved outside in open pollination context. Youngest receptive
179 flower and oldest non-receptive ones on the same shoot were marked before the exposition
180 to pollinator to differentiate fruits issued from flower in zero-pollinator context (self-
181 pollination) versus fruits produced in open pollination. Thus, open pollination fruits are
182 issued from flowers produced later in the shoot. For each plant, just before the dehiscence
183 of the fruit produced by self-pollination, we sampled the two fruits at the same time and
184 recorded the difference in the flower position number separating collected fruits (minimum
185 1, by definition, to maximum 5, with a mean of 1.6). The sampling of the two fruits was
186 performed at the same time because of the practical difficulty to follow 1,600 fruits
187 independently. Thus, open pollination fruits are not fully developed and comparing weights
188 between open pollination and self-pollination fruits has a slight bias. However, comparing
189 each measure between populations is fully possible while differences in floral positions are
190 distributed randomly between populations. The seed weight reported is the measure of the
191 weight of seeds contained by each fruit divided by the number of seeds counted in the fruit.
192 The ratio of the number of seeds produced by self-pollination to the number of seeds
193 produced by open pollination is a proxy of pollen limitation due to the absence of
194 pollinators, the less this limitation is the more the self-pollination ability is.

195 <u>Bumblebee preferences experiment</u>

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

214 Statistical analysis

215 Changes in traits were evaluated using linear discriminant analysis (LDA) and univariate
216 linear mixed models (LMMs). We performed the LDA over nine traits supposed to be
217 linked with reproductive system; corolla length, labellum width, floral area, number of
218 guides, anthesis duration, flowering date, volume of nectar, average floral display and ratio
219 of seeds produced in self-pollination to open pollination. For traits with repeated measures,
220 we used the means of the trait per individual. We normalized values by locality in order to
221 focus on Age effect. We predefined only the population (8 groups) in the LDA and not the
222 age. LDA gives linear combinations of traits (discriminant functions) that attempt to
223 capture differences among groups. In our case, it produced seven discriminant functions.
224 The two first functions represent 55% and 22% of the variation.

225 Univariate analyses were conducted using LMMs with each trait as response
226 variable, Age (ancestral or descendant), Locality (4 localities), and the interaction between
227 Age and Locality as fixed factors, and Family as random factor. In order to account for
228 repeated measures on the same individual for floral measurement, we add Individual as
229 random factor nested in Family. For seeds traits, we accounted for the difference in flower
230 position between the two fruits collected adding this difference as random factor nested in
231 family. When significant Age effect, analysis was followed by T-test comparison, on the
232 means of the trait per individual for repeated measures, of ancestral and descendant
233 populations of each locality. *p*-values were corrected using Bonferroni correction. Rosette
234 diameter, leaf length, width and area, nectar volume and ratio of seeds without/with
235 pollinators were log-transformed to approach normal distribution of residuals. In these
236 analyses, Age significant effect represents a signal of global evolution of traits between
237 ancestral and descendant populations, Locality significant effect, represents a difference in
238 traits between populations. A significant effect of the interaction between Age and Locality
239 represents a difference in the magnitude or in the sign of the evolution between localities.
240 In the case of a significant Age effect and a significant interaction effect, if evolution is
241 consistent (same sign of evolution) among localities and only the magnitude change, we
242 can hypothesize a directional selective force that selects a trait in a direction convergently
243 in every locality. In other cases, a significant interaction between Age and Locality
244 indicates local differences in selective pressures on the traits or alternatively genetic drift.
245 For bumblebee preference analyses, we first performed a GLMM to test the effect
246 of Age on the number of unvisited plants (proportions: CoA: 0.24, Co21: 0.35;
247 CrA: 0.26, Cr21: 0.26; GuA: 0.15, Gu21: 0.18; LhA: 0.13, Lh21: 0.12). We modelled it
248 with Age and Locality as fixed effect, Family, Greenhouse, Position nested in Greenhouse
249 and Date as random effects, with binomial distribution and logit link, as response variable
250 was a Boolean variable with TRUE for visited plants and FALSE otherwise. There was no
251 significant effect of Age in our dataset (N = 580 plants, estimates: AgeA-Age21 = 0.223,
252 $P = 0.296$). We performed further analysis with and without unvisited plants (N = 124) and
253 found similar pattern. To avoid non-normal distribution, we excluded unvisited plants
254 (N = 124) in the rest of the analysis. We performed a LMM with log-proportion of visits
255 per plant as response variable (N = 456), and the same structure as for other traits with Age,

256 Locality, and the interaction between Age and Locality as fixed effect, Family as random
257 effect and we added Greenhouse, Position nested in Greenhouse and Date as random
258 effects. We then performed T-test comparisons between Age for given Locality and
259 corrected *p*-values using Bonferroni correction.

260 All our statistical analyses were performed using the R software (R Core Team,
261 Version 4.2.1). LDA was performed using the "lda" function implemented in the package
262 "MASS" (Venables and Ripley 2002). LMMs and GLMM were performed using the
263 "lmer" and "glmer" functions implemented in the package "lme4" (Bates et al. 2015).

Results

264 The localities exhibited no significant differences in allelic richness nor genetic
265 diversity between ancestral and descendant populations based on fifteen microsatellites
266 markers (Table **1**). In the case of a resampling bias of the populations or a strong
267 demographic bottleneck or genetic drift between the two samples, we predicted a decrease
268 in allelic richness and genetic diversity. Our results do not support these *scenarii*. In
269 contrast, we did observe a consistent and significant increase in selfing rates in all four
270 localities between ancestral and descendant populations (Table **1**), with an average and
271 significant increase of 27% (Fisher test, $\chi^2_8 = 40.36$, $P < 0.001$).

272 Descriptive multivariate analyses performed on eight floral traits associated with
273 the mating system (corolla length, labellum width, floral area, number of guides, anthesis
274 duration, flowering date, volume of nectar and average floral display) and the ability of
275 plant to set seeds in the absence of pollinators (*i.e.* autonomous self-pollination), corrected
276 for differences between localities, showed differentiation between ancestral and
277 descendant populations. We choose these traits because they are usually associated with
278 shifts in mating systems (Bodbyl Roels and Kelly 2011, Sicard and Lenhard 2011, Gervasi
279 and Schiestl 2017, Ramos and Schiestl 2019). The descendent populations and ancestral
280 populations form two distinct clusters in the trait space (Fig. **1**), revealing convergent
281 directional changes of floral traits in all four localities.

282 **Table 1. Allelic richness, genetic diversity and selfing rates in our populations (mean**
283 **± SD or [CI]).** Average allelic richness and genetic diversity are based on fifteen

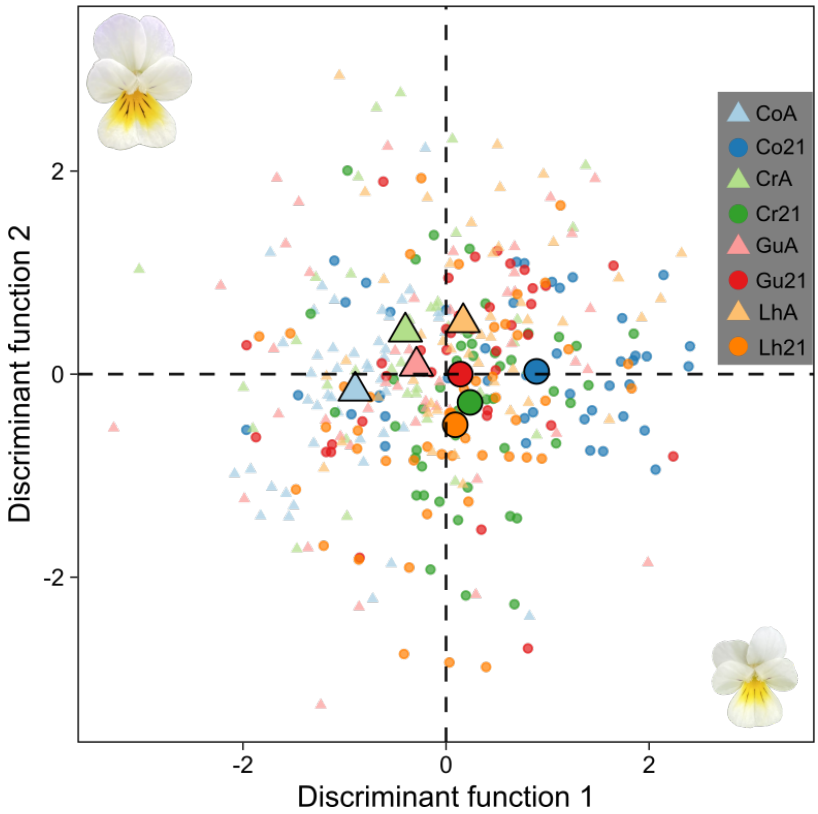
microsatellites markers and selfing rate on fourteen. These markers were genotyped on the thirty-two individuals per population of the F1 generation issued from natural populations. For selfing rate, confidence intervals (CI) were obtained by bootstraps on loci (10,000). Fisher's combined probability test gave a significant overall increase in F_{IS} ($\chi^2_8 = 40.36$, $P < 0.001$, see Material and methods).

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 Co21	CrA Cr21	GuA Gu21	LhA Lh21
Average allelic richness	2.55 ± 1.21 1.73 ± 0.79	3.09 ± 1.81 3.36 ± 1.86	2.64 ± 1.29 3.27 ± 1.56	2.82 ± 1.78 2.18 ± 1.08
Genetic diversity (H_e)	0.302 ± 0.275 0.099 ± 0.133	0.435 ± 0.263 0.437 ± 0.254	0.315 ± 0.211 0.327 ± 0.255	0.315 ± 0.291 0.245 ± 0.225
Selfing rate [95% CI] derived from heterozygosity deficiency, F_{IS}	0.77 [0.68-0.82] 0.89 [0.85-1]	0.66 [0.60-0.76] 0.70 [0.65-0.76]	0.44 [0.34-0.61] 0.81 [0.73-0.86]	0.33 [0.21-0.47] 0.88 [0.81-0.94]

Figure 1. Multivariate differences between traits of ancestral and descendant populations of *Viola arvensis*. Results of a multivariate analysis (linear discriminant analysis) performed on nine traits and 357 plants (almost equally distributed among groups; N_{min} = 36 individuals; 7 points not represented because out of scale). The first two functions represent 55% and 22% of the variation 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.



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 combined decrease in the labellum width and flower length (Table **2**). Flowers also have fewer nectar-guides (Fig. **2b**), which are assumed to promote recognition of the path to floral rewards (a correlation between the

309 number of nectar-guides and the rate of visits inducing pollination has been reported for
310 the South African iris, *Lapeirousia oreogena* (Hansen et al. 2012)). In contrast, none of the
311 morphological traits presumed to be unlinked to pollinator attraction (vegetative traits and
312 sepal length) showed consistent changes (Fig. **2c**, Fig. **S3**, Table **2**). Based on the
313 resurrection approach, patterns of trait changes between ancestral and descendant
314 populations provide a signature of mating system and associated trait evolution. The lack
315 of changes in vegetative traits reduces the likelihood that these changes are due to
316 inbreeding depression (unlikely that it would affect only floral traits), especially because
317 late acting traits such as biomass exhibited no changes (Winn et al. 2011). In addition,
318 evolution in petal length but not in sepal (Fig. **S3**) or leaf length (Fig. **2c**), despite their
319 homologous origins (Breuninger and Lenhard 2010), provides strong evidence for a
320 specific selection against costly traits that mediate interactions with pollinators.

321 **Table 2. Linear models: effects of time, locality, and their interaction on the changes**
322 **of floral, vegetative and reproductive traits.** Values in parentheses are sample sizes.
323 All traits are shown and the degree of significance of each effect on each trait is reported
324 (LMM, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, • $P < 0.1$, n.s. $P > 0.1$, see Table **S2** for
325 full statistical values). (+) a positive effect (increase), (−) a negative effect (decrease), (~)
326 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.

327 Regarding reproductive traits, our results revealed a marginally significant
328 (χ_1^2 = 3.201, P = 0.074) increase over time in the ability to set seeds in the absence of
329 pollinators (Fig. **2d**) and a significant decrease in seed weight (Table **2**). Seed weight
330 represents maternal investment in individual progeny. This shift towards increased
331 reproductive insurance is associated with reduced seed investment, as observed in
332 previous studies (De Jong et al. 2005).
333 Our study also reveals that both locality and the interaction between locality and
334 age (ancestors or descendants) have a strong effect on trait variations. The locality effects
335 indicate spatial differentiation in measured traits, whereas the locality×age interaction
336 reveals a site dependent evolution. The latter interaction effects were different in
337 magnitude, but not in sign, between ancestors and descendants for all the traits, except
338 anthesis duration (Table **2**). Hence, evolution towards a selfing syndrome in the four
339 localities exhibits the same directional change *i.e.* evidence of convergent shifts in the four
340 independent populations (Fig. **1**) while differing in magnitude (indicative of slight
341 differences in selective pressures between localities).

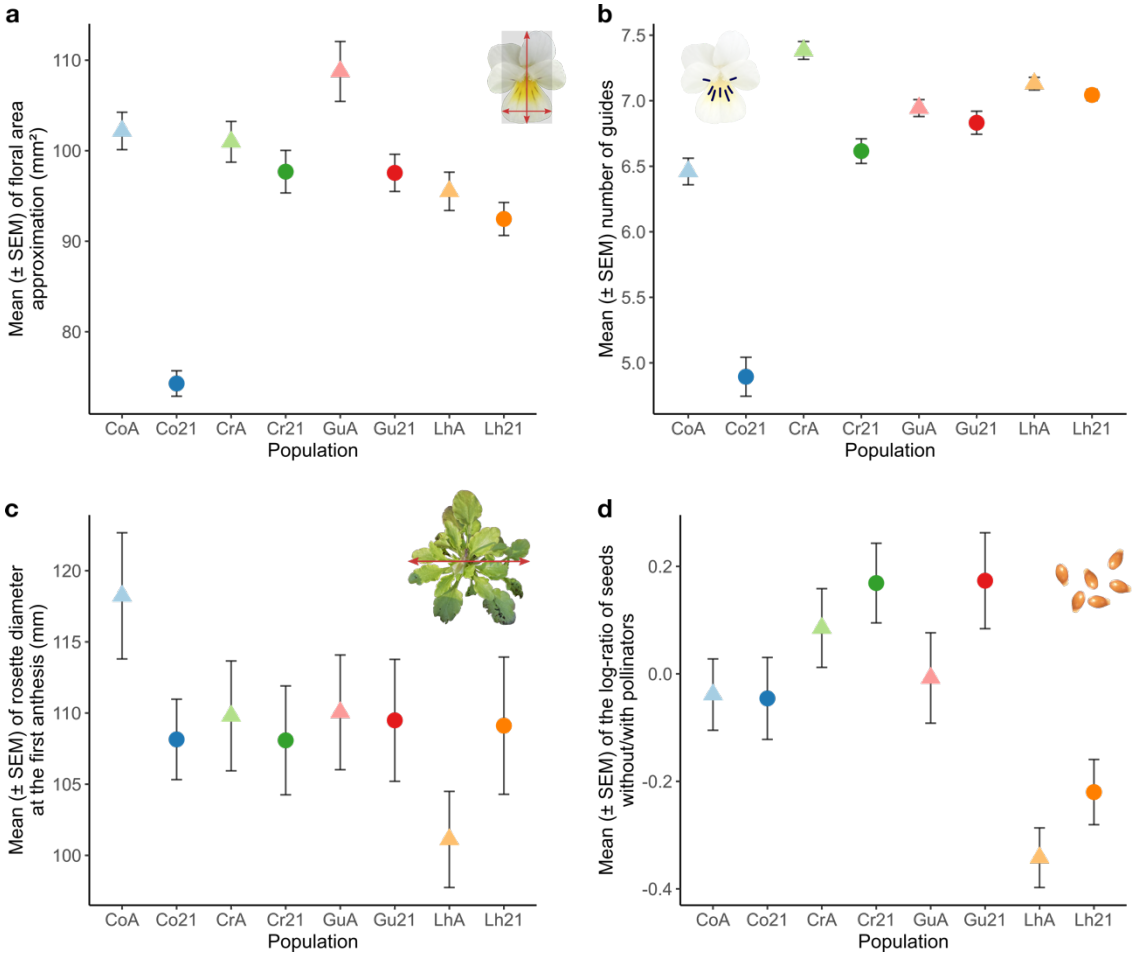


Figure 2. Evolutionary changes in floral, reproductive and vegetative traits. We measured floral traits (**a** and **b**) in the first five developed flowers per individual; N ≈ 4,000). (**a**) Floral area (multiplication of labellum width × corolla length). (**b**) Number of attractive guides. (**c**) Rosette diameter, measured on each plant at the start of flowering. (**d**) Log-ratio of seeds produced in self-pollination compared to open pollination is a proxy of selfing ability, measured by collecting one fruit in self-pollination and one in open pollination per plant (N = 693).

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).

We found a consistent decrease of 20% on average in rewarding traits, as represented by measurements of nectar volume (Fig. **3a**, Table **2**). Because floral nectar production is costly (Pyke 1991) and only dedicated to rewarding pollinators, it is key to maintenance of stable plant-pollinator interactions(Brandenburg et al. 2012). A decrease in nectar production in our study species is thus evidence of a weakening of the interaction between the plant and its pollinators.

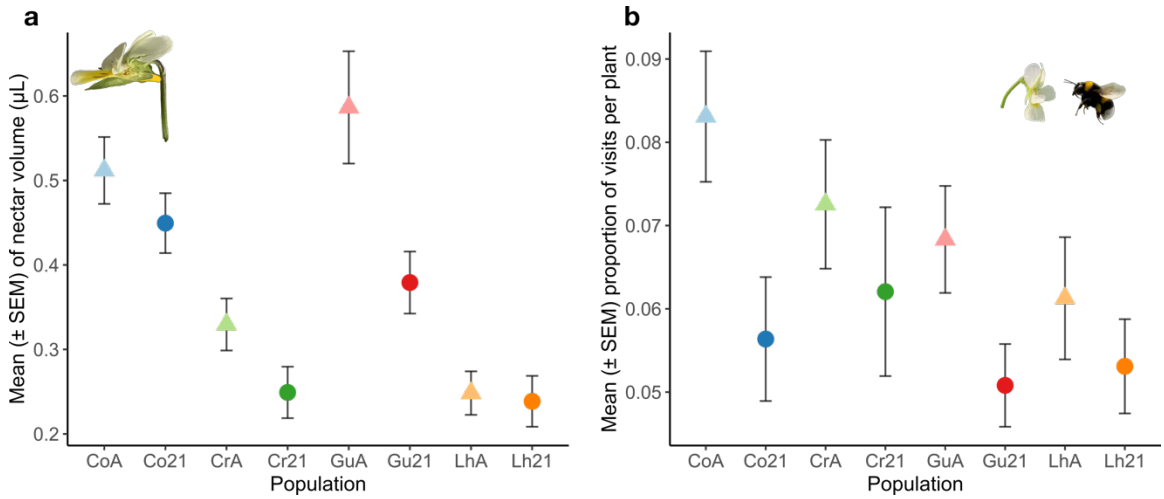


Fig. 3. Change in plant-pollinator interactions. (**a**) Nectar production measured as the sum of the volume in three flowers per plants on fifty plants per population (N = 400). (**b**) Bumblebee preferences, measured as proportion of visits per plant to a mixed plantation of 10 plants of the ancestral and 10 of the descendant population of a single locality in a greenhouse, exposed together to bumblebees. We recorded the number of visits to 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 plants visited once are represented).

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).

We assessed how this evolution impacts plant-pollinator interactions from the point of view of pollinators through a greenhouse preference experiment involving visits of the

375 long-tongued bumblebees *Bombus terrestris* to a mixture of ancestral and descendant
376 plants. Bumblebees visited ancestral plants more frequently than they visited descendant
377 plants, showing a preference towards the former that is consistent with their higher
378 attractiveness and reward traits (Fig. **3b**, Table **2**).

Discussion

379 Overall, we found decreases in floral display, in floral area, in floral attractiveness,
380 and in nectar production, as well as evidence for enhanced selfing and an increase in
381 realized selfing rates that are all consistent with the ongoing evolution of a selfing
382 syndrome (Ornduff 1969, Sicard and Lenhard 2011). Furthermore, we found such patterns
383 to be convergent across the four populations investigated. This study thus provides a unique
384 demonstration for an evolutionary change in plant mating systems over a short-time scale
385 in natural populations. While angiosperm-pollinator interactions have evolved over the
386 long-term, our study shows that current environmental changes can drive a rapid evolution
387 towards a breakdown of such interactions.

388 Recurrent evolution towards selfing has been repeatedly documented in the wild,
389 but to our knowledge, only over the long-term (Stebbins 1957, Barrett 2002). This
390 evolutionary transition is classically considered to be irreversible (the "selfing as an
391 evolutionary dead-end" hypothesis (Stebbins 1957, Igic and Busch 2013)) and higher
392 extinction rates of such phyla have been reported (Goldberg et al. 2010). Evolution towards
393 selfing could thus be driven by natural selection over the short-term but could impede long-
394 term plant population survival (Cheptou 2019). The increase in selfing rate is indeed
395 expected to have genomic impacts on individuals and populations through inbreeding
396 (homozygosity), reduced effective population sizes and increased genetic drift.
397 Consequently, selfers may have a reduced potential response to natural selection (Glémin
398 and Ronfort 2013, Noël et al. 2017). The short-term evolution of selfing in *Viola arvensis*
399 we document here is no-doubt facilitated by its initial mixed mating system (contrary to
400 self-incompatible plants for example (Thomann et al. 2015)), nonetheless our results
401 illustrate how mixed-mating species may evolve towards a higher reliance on self-
402 pollination and reduced plant-pollinator interactions in the face of global change, in

403 particular pollinator declines. If this rapid transition towards a selfing syndrome reflects a
404 broader trend among Angiosperms, it may reflect a concerning extinction debt (Goldberg
405 et al. 2010).

406 Ongoing environmental changes – such as habitat destruction and fragmentation,
407 agricultural land pollution and species' introductions – have been documented as causes of
408 pollinator declines, both by affecting pollinator populations directly (Potts et al. 2010,
409 Goulson et al. 2015), and indirectly through their impacts on plant community composition,
410 affecting the quantity and diversity of nectar resources (Goulson et al. 2015, Baude et al.
411 2016). In addition, and as we illustrate here, floral nectar production can also evolve rapidly
412 in plant species in response to a relaxed selective pressure associated with pollinator
413 declines, prompting declines in nectar resources of a magnitude similar to those associated
414 with changes in plant community composition (Baude et al. 2016). These decreases in
415 nectar production may then reinforce pollinator declines if nectar levels fall below those
416 necessary to sustain wild bee populations. Environmental changes may thus a double
417 jeopardy to pollinator populations, as they become victims of both the changes themselves
418 and of plant trait evolution (Weinbach et al. 2022). This in turn may result in an eco-
419 evolutionary positive feedback loop that furthers pollinator declines, further reinforcing
420 plant evolution towards a selfing syndrome. This can explain plant-pollinator network
421 degradation as documented in a previous study (Burkle et al. 2013) and raises the
422 concerning prospect of cascading effects in trophic networks in general, beyond plant-
423 pollinator interactions.

424 In summary, our study highlights the potential of natural populations to respond
425 quickly to environmental changes. However, such evolutionary responses may have
426 impacts on ecological interactions, here plant-pollinator interactions, and potentially
427 cascading trophic consequences in ecosystems. There is thus an urgent need to investigate
428 whether these results are symptomatic of a broader pattern among angiosperms and their
429 pollinators, and if so understanding whether there is a possibility to reverse this process
430 and break this eco-evolutionary positive feedback loop.

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Competing interests

Authors declare that they have no competing interests.

Author contributions

Conceptualization: POC, SAP; methodology: SAP, POC; lab work: LD, SAP; data collection: SAP, PG, VP, POC; analysis: SAP, LD; writing – original draft: SAP; writing – review & editing: POC, SAP

Data availability

All data are available on (*access will be available before publication*).

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568

Supporting information

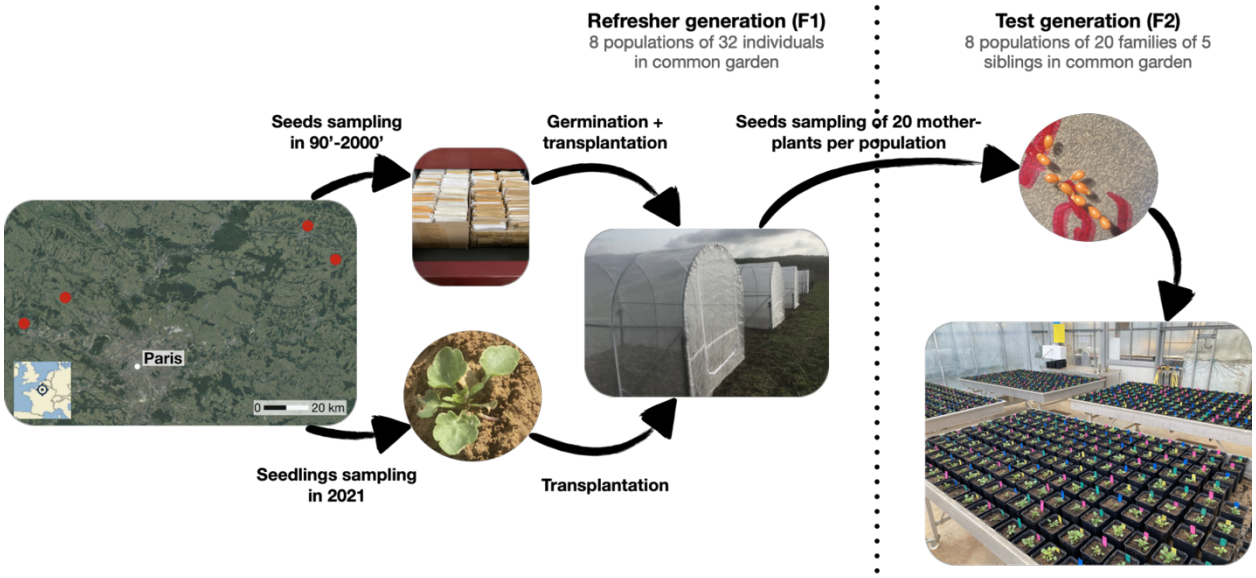

Fig. S1. Experimental design. Four localities were sampled two times, with a minimum 20 years between the two samplings. A first generation (F1), the refresher generation, was performed in common garden. It was used to analyze population genetics and to produce a family design of 20 families of 5 siblings per population for a second generation (F2), the test generation, on which we measured traits.

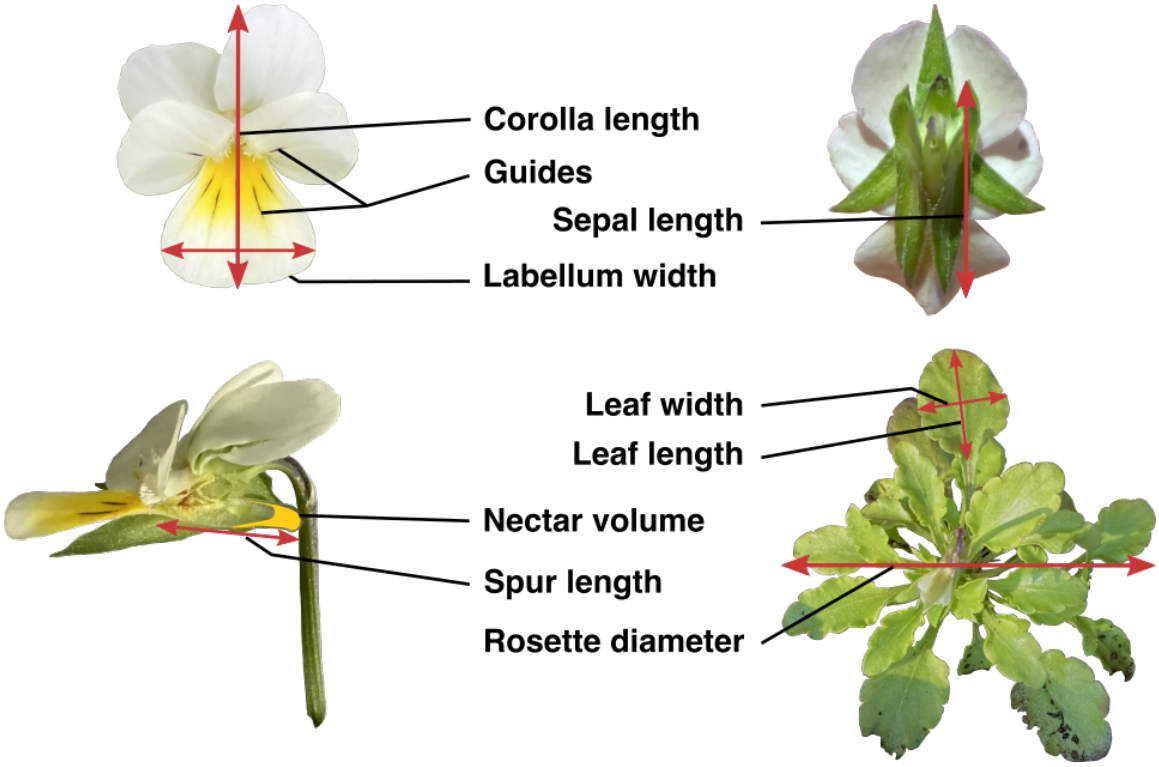

Fig. S2. Trait measures. List of traits measured and delimitation of their measurements. Traits represented in this figure are only traits which were directly measured on plants.

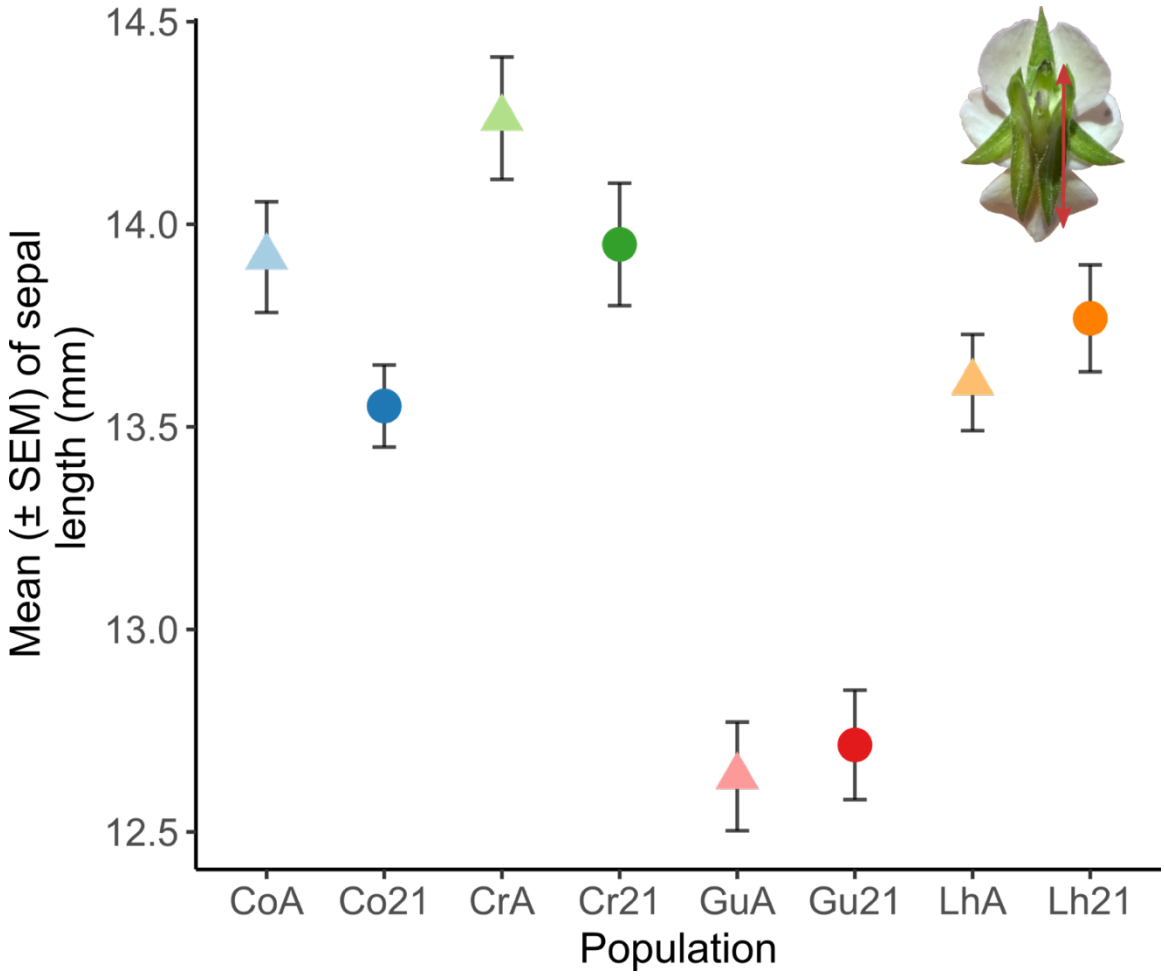

Fig. S3. Absence of consistent change in sepal length between 1990's-2000's-ancestors and 2021-descendants. We measure sepal length in 3,955 flowers over 792 individuals. The first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys). "A" (▲) ancestral population (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and "21" (●) descendant population (all collected in 2021).

586 **Table S1. Position and year of the sampling of our populations.**

587

Location	**Commeny** (49°7'37.11"N, 1°53'46.30"E)	**Crouy** (49°24'24.31"N, 3°22'20.73"E)	**Guernes** (49° 1'10.75", 1°38'46.82"E)	**Lhuys** (49°16'35.40"N, 3°32'58.52"E)
Year of first sampling	2001	1993	2000	1992
Year of second sampling	2021	2021	2021	2021

Table S2. Trait differences (mean ± SD) among plants of our populations. Statistical analyses were performed using LMMs with Age, Locality and the interaction between the two as fixed effects and family and, when necessary, replication of measures on the same plant as random effects (see Material and methods). When the Age effect was significant (bold traits), analyses were followed by two-sides T-Tests with a Bonferroni correction performed between the ancestral and descendant populations by locality. Significant differences in traits between ancestors and descendants ($P < 0.05$) was highlighted by bold measures and sign of changes are given using arrows (↘ for a decrease in time and ↗ for an increase in time).

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).

Trait	*N*	CoA Co21	CrA Cr21	GuA Gu21	LhA Lh21	Factor	Chisq	Chi df	*P*
Floral traits									
Corolla length (mm)	3955	**14.03 ± 1.09** **12.09 ± 1.13** ↘	13.28 ± 1.45 13.03 ± 1.88	14.06 ± 2.18 13.66 ± 1.35	13.23 ± 1.47 12.99 ± 1.46	**Age** **Locality** **Age × Locality**	**15.314** **13.384** **16.638**	**1** **3** **3**	**< 0.001** **0.004** **< 0.001**
Labellum width (mm)	3955	**7.16 ± 0.94** **6.03 ± 0.59** ↘	7.45 ± 0.99 7.30 ± 0.92	**7.51 ± 1.16** **7.01 ± 0.88** ↘	7.04 ± 0.76 6.94 ± 0.67	**Age** **Locality** **Age × Locality**	**17.872** **30.047** **14.725**	**1** **3** **3**	**< 0.001** **< 0.001** **0.002**
Floral area approximation (mm²)	3955	**102.2 ± 19.9** **74.3 ± 14.0** ↘	101.0 ± 22.5 97.7 ± 23.5	**108.8 ± 33.1** **97.6 ± 20.5** ↘	95.5 ± 21.2 92.5 ± 18.2	**Age** **Locality** **Age × Locality**	**17.965** **17.979** **14.989**	**1** **3** **3**	**< 0.001** **< 0.001** **0.002**
Spur length (mm)	3954	**4.25 ± 0.22** **4.00 ± 0.23** ↘	4.30 ± 0.24 4.27 ± 0.27	4.30 ± 0.28 4.23 ± 0.31	4.37 ± 0.19 4.38 ± 0.23	**Age** **Locality** **Age × Locality**	**8.373** **42.226** **12.388**	**1** **3** **3**	**0.004** **< 0.001** **0.006**
Sepal length (mm)	3955	13.92 ± 1.32 13.55 ± 1.01	14.26 ± 1.51 13.95 ± 1.51	12.64 ± 1.34 12.71 ± 1.35	13.61 ± 1.19 13.77 ± 1.32	Age **Locality** Age × Locality	0.550 **63.509** 2.7607	1 **3** 3	0.459 **< 0.001** 0.430
Sepal/corolla lengths ratio	3955	**0.998 ± 0.078** **1.132 ± 0.099** ↗	1.088 ± 0.117 1.093 ± 0.125	0.918 ± 0.120 0.941 ± 0.107	**1.044 ± 0.102** **1.077 ± 0.083** ↗	**Age** **Locality** **Age × Locality**	**14.137** **93.828** **15.302**	**1** **3** **3**	**< 0.001** **< 0.001** **0.002**

Number of guides	3993	**6.46 ± 0.98** **4.89 ± 1.48** ↘	**7.38 ± 0.68** **6.62 ± 0.94** ↘	6.94 ± 0.64 6.83 ± 0.88	7.13 ± 0.48 7.04 ± 0.42	**Age** **Locality** **Age × Locality**	**33.591** **112.889** **30.675**	**1** **3** **3**	**< 0.001** **< 0.001** **< 0.001**
Anthesis duration (days)	3991	**6.39 ± 0.85** **5.07 ± 0.85** ↘	6.20 ± 1.06 6.07 ± 0.91	**7.85 ± 1.56** **7.27 ± 1.29** ↘	**7.00 ± 1.04** **7.41 ± 0.81** ↗	**Age** **Locality** **Age × Locality**	**9.261** **137.555** **24.407**	**1** **3** **3**	**0.002** **< 0.001** **< 0.001**
Flowering date (days after the first flowering)	792	20.31 ± 6.78 18.54 ± 4.58	17.48 ± 8.78 17.57 ± 7.82	19.58 ± 10.81 20.23 ± 12.69	19.30 ± 7.57 19.69 ± 9.13	Age Locality Age × Locality	0.027 3.783 1.007	1 3 3	0.870 0.286 0.800
Average floral display (open flowers per day)	789	**6.37 ± 2.39** **4.64 ± 1.99** ↘	6.85 ± 2.59 6.89 ± 2.87	7.89 ± 3.47 6.95 ± 3.26	6.64 ± 2.31 6.43 ± 2.17	**Age** **Locality** **Age × Locality**	**8.190** **32.182** **7.855**	**1** **3** **3**	**0.004** **< 0.001** **0.049**
Vegetative traits									
Rosette diameter (mm)	792	118.23 ± 42.75 108.15 ± 28.11	109.80 ± 38.60 108.08 ± 38.24	110.05 ± 40.29 109.49 ± 42.85	101.12 ± 33.77 109.11 ± 48.21	Age Locality Age × Locality	0.194 5.601 2.397	1 3 3	0.660 0.133 0.494
Leaf length (mm)	792	26.30 ± 6.38 22.42 ± 3.95	24.10 ± 5.63 24.28 ± 5.65	22.34 ± 5.18 23.17 ± 6.07	21.57 ± 4.85 21.94 ± 5.63	Age **Locality** **Age × Locality**	1.226 **19.895** **11.311**	1 **3** **3**	0.268 **< 0.001** **0.010**
Leaf width (mm)	792	19.84 ± 3.77 17.96 ± 2.56	18.67 ± 3.03 18.94 ± 3.01	17.02 ± 2.76 16.66 ± 2.91	17.58 ± 3.07 17.66 ± 3.07	Age **Locality** **Age × Locality**	2.499 **40.014** **8.024**	1 **3** **3**	0.114 **< 0.001** **0.046**
Leaf area approximation (mm^2)	792	541.3 ± 231.9 410.5 ± 128.7	461.3 ± 173.6 472.4 ± 177.6	391.3 ± 152.8 398.5 ± 173.7	390.2 ± 147.1 401.6 ± 173.0	Age **Locality** **Age × Locality**	1.910 **27.414** **10.559**	1 **3** **3**	0.167 **< 0.001** **0.014**
Biomass (g)	777	7.243 ± 2.515 8.034 ± 2.553	8.220 ± 2.712 7.789 ± 2.747	8.027 ± 2.624 7.606 ± 2.904	8.090 ± 2.901 8.386 ± 3.066	Age Locality Age × Locality	0.029 3.490 4.850	1 3 3	0.864 0.322 0.183
Reproductive traits									
Log-ratio of the number of seeds without/with pollinators	693	-0.039 ± 0.619 -0.046 ± 0.711	0.085 ± 0.659 0.169 ± 0.687	-0.008 ± 0.775 0.173 ± 0.757	-0.342 ± 0.546 -0.220 ± 0.601	Age **Locality** Age × Locality	3.201 **38.344** 1.671	1 **3** 3	0.074 **< 0.001** 0.643
Weight per seed with pollinators (mg)	656	0.446 ± 0.199 0.385 ± 0.161	0.421 ± 0.153 0.369 ± 0.146	0.301 ± 0.154 0.300 ± 0.143	0.541 ± 0.146 0.546 ± 0.142	**Age** **Locality** Age × Locality	**4.744** **212.885** 5.999	**1** **3** 3	**0.029** **< 0.001** 0.112

Weight per seed without pollinators (mg)	701	**0.689 ± 0.206** **0.565 ± 0.200** ↘	0.633 ± 0.169 0.585 ± 0.164	0.517 ± 0.187 0.501 ± 0.189	0.764 ± 0.137 0.788 ± 0.117	**Age** **Locality** **Age × Locality**	**9.3644** **226.2555** **18.3624**	**1** **3** **3**	**0.002** **< 0.001** **< 0.001**
Plant-pollinator interaction traits									
Nectar volume (µL)	400	0.512 ± 0.280 0.450 ± 0.251	**0.330 ± 0.218** **0.249 ± 0.215** ↘	**0.586 ± 0.470** **0.379 ± 0.260** ↘	0.248 ± 0.182 0.239 ± 0.213	**Age** **Locality** Age × Locality	**12.605** **75.120** 4.636	**1** **3** 3	**< 0.001** **< 0.001** 0.200
Bumblebee visit preferences (proportion of visit per plant)	456	**0. 083 ± 0. 008** **0. 056 ± 0. 007** ↘	0.073 ± 0.008 0.062 ± 0.010	0.068 ± 0.006 0.051 ± 0.005	0.061 ± 0.007 0.053 ± 0.006	**Age** Locality Age × Locality	**14.722** 2.027 1.950	**1** 3 3	**< 0.001** 0.567 0.583

596