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Rapid evolution of selfing syndrome traits in *Viola arvensis* revealed by resurrection ecology

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Abstract:

Premise of the study : As part of global change, climate warming and pollinator decline are expected to affect plant phenology and plant-pollinator interactions. This paper aims at characterizing rapid evolution of life history traits and floral traits over two decades in the wild pansy (*Viola arvensis*), a common weed in agrosystems.

Methods: we used a resurrection ecology approach with genotypes sampled in 1991 and 2012 from a population in Burgundy (France). The species has a mixed mating system (hereafter: mixed selfer) and presents a floral polymorphism. In order to correct for maternal effects, plant traits were measured in the second generation in a common garden (after a refreshing generation) to characterize plant evolution during the two decades. In addition, historical population selfing rates in the 1991 and 2012 were inferred from microsatellites markers through heterozygote deficiency and identity disequilibrium.

Key results: Phenotypic data revealed a significant advance in flowering date, reduced flower sizes and a higher propensity of plant to set seed by autonomous selfing. Moreover, we detected a change in color morph frequency with an increase of the pale morph frequency. In accordance with phenotypic data, the neutral genetic data revealed an increase in historical selfing rates from $s=0.68$ in 1991 to $s=0.86$ in 2012.

Conclusions: Taken together, such data suggest that the wild pansy, as mixed selfer, is evolving a selfing syndrome that may be the consequence of reduced pollinator activity in agrosystems.

Key words: resurrection ecology-selfing syndrome-pollinator decline-*Viola arvensis*-rapid adaptation

INTRODUCTION

Plant-pollinator interactions are major trophic networks in ecosystems. More than 70 % of angiosperms rely on insects for their reproduction. Such interactions range from generalist to specialist and plant-pollinator interactions have co-evolved during the radiation of angiosperms (Van der Niet et al. 2014). In hermaphroditic plants, the dependence of plants on pollinators is associated with mating system traits such as showy flowers, large corolla, or rewards. While fully outcrossing species require pollinators to set seeds, full selfers can set seeds autonomously. Between these two extremes, mixed selfers partly depend on pollinators for their sexual reproduction. Macroecological studies have established that the evolution of selfing appears as a recurrent pattern in plant phylogenies (Goldberg et al. 2010). While practicing selfing may appear as a rampart against the risk of pollinator failure, phylogenetic patterns of evolution support the hypothesis that selfing is disadvantageous in the long run (Cheptou 2019), the so called “dead-end hypothesis” (Takebayashi and Morell 2001). Plant traits favoring selfing encompass a variety of plant traits termed the “selfing syndrome” including inconspicuous flowers and a reduced pollen–ovule ratio. The evolution of selfing syndromes has been described in several genera as reported in the genus *Ipomoea*, (Duncan and Rausher 2013), in *Collinsia* (Kalisz et al. 2012) or in *Clarkia* (Sicard and Lenhard, 2011). The authors described reduced flower size, reduced anther-stigma distance, and dissolution of self-incompatibility, consistent with diverging selection among outcrossing vs. selfing taxa.

Modern plant mating system theory has been intensively studied since the 1980's , following the publication of Lloyd's seminal work (Lloyd, 1979, Morgan and Schoen,

1997). Mating system theory relies on population genetics models typically adapted to microevolutionary predictions. Factors governing the evolution of self-fertilisation have been identified (Lloyd 1979). Among them, pollination scarcity is expected to select for self-fertilisation through the reproductive assurance it confers. In contrast, macroevolutionary patterns have been described empirically (Goldberg et al., 2010) but theoretical arguments at this scale are mostly verbal (Stebbins, 1950; see however Glemin and Ronfort, 2013).

Recently, plant experimental evolution has shown that plant mating system traits can evolve rapidly, in fewer than ten generations. In the self-compatible species *Mimulus guttatus*, Roels and Kelly (2011) showed that only five generations of evolution without pollinators were sufficient to produce a reduction in anther-stigma distance, resulting in a higher propensity for evolved plants to set seeds autonomously by selfing. Also, Gervasi and Schiestl (2017) demonstrated in the fast cycling race of *Brassica rapa* that different pollination treatment (Bumblebee, Syrphid, hand pollination) produced a shift in floral plant traits, in particular floral scent, in nine generations. Interestingly, plant height -ie. trait indirectly related to mating system- has significantly changed in the Bumblebee treatment pointing out that, beyond the floral traits, adaptation to pollination environment encompasses a variety of plant traits, which justifies the notion of syndrome.

Recent environmental changes are likely to have affected plant pollinator interaction and consequently plant mating systems. Hallmann et al. (2017) reported that more than 75% of insect biomass has declined during the last thirty years, potentially endangering plant reproduction and plant demography (Biesmeijer et al. 2006). Moreover, for some crops, yields depend on pollinators and a decrease in crops yields in the recent years has been

attributed to pollinator decline (Martin et al. 2019). Beyond the direct effect of pollinator loss, habitat fragmentation, land use change (e.g. loss of grasslands), and global warming are also likely to affect plant pollinator interactions. For instance, shifts in pollinator and plant phenology (Valencia-Montoya et al. 2021) have the potential to desynchronize plant-pollinator interactions. While it is difficult to identify selection pressures on plant mating systems in the face of global change, there is a body of information suggesting that plant-pollinator interactions may be at risk in ecosystems (Thomann et al, 2013). For instance, weeds in agrosystems have experienced major declines in pollinator abundance since the Second World War due to land use change and chemicals (Fried et al, 2009). Such a situation is expected to generate pollen limitation for weeds dependent on pollinators as a consequence of pollinator limitation and/or plant mate limitation (Cheptou, 2021).

While plant-pollinator interactions are assumed to have played a role in angiosperms radiation (Van der Niet et al. 2014), it is reasonable to hypothesize that current environmental changes can lead to plant-pollinator disruption, through short-term evolution. For mixed selfing plants, we may expect pollinator decline to select for higher selfing (Eckert et al. 2010, Thomann et al. 2013). Detecting evolutionary changes requires controlling for environmental factors affecting phenotypes. Indeed, without a control for environmental changes, differences in plant traits and realised selfing rates detected in longitudinal studies might result from plastic changes in response to environmental differences rather than evolutionary changes. Recent resurrection ecology methodology offers the possibility of filling this gap. Resurrection ecology takes advantage of dormant propagules (e.g. seeds for plants) to compare old phenotypes sampled a few decades ago to recent phenotypes sampled in the same population (Weider et al. 2018). The

comparison of old and recent genotypes in common environment allows disentangling plastic and evolutionary responses and thus inferring rapid evolution of traits (Weider et al. 2018).

In this study, we examined shifts in floral and phenological traits in the annual weed *Viola arvensis* (Violaceae) over two decades in agrosystems in Burgundy, France. In Burgundy, comparative surveys of weed communities from 1970 to 2005 have revealed that entomophilous plants were declining while anemophilous and self-fertilizing plants showed no temporal trends (Fried et al, 2016). Also, indirect estimates of pollination services have revealed a decrease in pollination in this region (Martin et al, 2019). In addition, temperature has increased in the last 50 years (WorldClim 2.1; Fick and Hijmans, 2017). In this context, we hypothesize (1) that flowering phenology has evolved towards earlier flowering because of climate warming and (2) that increased selfing has evolved because of pollinator decline. Using old and current genotypes of our studied population, we first inferred realised selfing rates using microsatellites markers. Second, we measured floral traits, plant phenology, and the ability of plants to produce seeds without pollinators in a common garden to characterize trait shifts. Overall, our study showed that traits have shifted in the directions predicted over a twenty-year period.

MATERIAL AND METHODS

Studied species, sampling area and sampling population

The field pansy (*Viola arvensis*, Violaceae) is an annual diploid crop weed (Clausen 1926) widely distributed in northern Europe. It occurs mainly in fields with C3 crops such as oilseed rape (*Brassica napus*). Flowers are zygomorphic and predominantly cream-colored with a yellow spot in the middle lower petal. The two upper petals may also

exhibit purple spots. In Burgundy (France), three different floral morphs can be found. The purple morph exhibits a purple spot in the upper petals. The white morph lacks purple spots, and the intermediate morph exhibits light purple spots (Figure 1). According to Espeut (1996), phenotypes of the studied population refer to *Viola arvensis* subsp *megalantha*. The field pansy is described as self-compatible with mixed mating system (Scoppola & Lattanzi 2012) but there is no estimate of the selfing rate based on molecular markers reported in the literature. The flowers produce nectar and are visited by Apoids (BiolFlor database). The studied focal population of *V. arvensis* was sampled near Fauverney, Côte-d'Or, Bourgundy, France (47°15'35.96"N ; 5° 9'0.84"E and 47°15'8.25"N ; 5° 8'34.14"E). Seeds sampled in 1991 were obtained from the Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement, Dijon. Seeds were sampled from fruits mass collected on 50 mother plants. In February 2012, we randomly sampled 100 seedlings in the same plot. Because the plots were several hectares wide, it is possible that the recently sampled plants were located a few hundred meters away from the initial sampling.

Common garden experimental design

In order to minimize maternal effects, a refresher generation was grown for plants from the historical and recent samples, the refresher generation, were cultivated in a common garden to generate seed materials in the same environment for the test generation for which phenotypic traits were measured (Appendix S1; see the Supplementary Data with this article). Because the recent plants were sampled as seedlings, the common conditions for both samples started at seedling stage. On December 2011, 1991 seed lots were placed in germination chambers in trays with vermiculite. Following Baskin & Baskin (2014), germination conditions were dark-6°C

144 during 11h followed by light-15°C during 13h. Seedlings from 1991 and 2012 were placed
145 outdoor in the experimental platform “Terrains d’Experiences” platform of the Labex
146 Cemeb, Montpellier, south of France (43°38'19.97"N, 3°51'43.69"E). 140 seeds of the
147 1991 sample were sown in seed trays and 51 seeds successfully germinated (36%) but 11
148 seedlings died. Of the 100 transplanted from the field site in 2012, one seedling died.
149 Seedlings were transplanted in 0.5L pots. In spring 2012, we regenerated seeds by open
150 pollination within the 1991 and within the 2012 samples to maintain the genetic integrity
151 of each sample. After transplantation of the 1991s seedlings in 0.5L pots, all plants from
152 both samples were randomly distributed and grown in pots in common garden
153 conditions. Because we used seeds for the 1991 sample but seedlings for the 2012 sample,
154 plants from 2012 started to flower about ten days earlier than plants from 1991. 80 plants
155 from the 2012 sample successfully flowered and were naturally pollinated to produce
156 seeds for the test generation. When plants started to flower, they were pollinated within
157 population by bumblebees introduced into insect-proof greenhouses to prevent crosses
158 between the 1991 and 2012 samples.

159 For the test generation, twenty seeds were randomly picked from each of the 27 mother
160 plants for the historical sample and from 38 mother-plants for the recent sample. These
161 seeds were sown in germination chambers following the first generation germination
162 protocol. Germination occurred after ca. 20 days and germination success was high ($85 \pm$
163 2% and $74 \pm 4 \%$ for 1991 and 2012 samples respectively). All seedlings were then
164 transferred to seedling trays and placed in the greenhouse under natural photoperiod and
165 without temperature control. One month later, six randomly chosen individuals per
166 mother-plant were transferred in 0.5 L pots and grown outside in a random arrangement
167 of pots. The test generation represents thus a family design with 382 plants in total.

168 *Population genetics using microsatellites markers*

169 Plants produced from old and recent samples were genotyped using eight microsatellite
170 markers (Devresse et al, submitted). Tissue from individual plants was dried at 40°C and
171 stored in granular silica gel (Carlo Erba cobalt free). DNA was extracted with the CTAB
172 protocol (Doyle and Doyle 1987).

173 *Genetic diversity and selfing rate estimates*

174 We analyzed allelic richness and expected heterozygosity (H_e) in the two samples for the
175 height microsatellites loci to check that genetic diversity did not change over time. A
176 drastic change in allelic diversity (or H_e) would suggest either a strong bottleneck or drift
177 in natural populations (low N_e) and/or a bias caused by sampling. In addition,
178 microsatellite loci allow inference of historical selfing rates according to two methods. We
179 used the classical method based on heterozygote deficiency (F_{is}). At equilibrium, F_{is} can
180 be related to selfing rate through the formula $s = \frac{2.F_{is}}{1+F_{is}}$. F_{is} was estimated using the R
181 package HierFstat issued from genepop software (Rousset, 2008) and confidence intervals
182 were calculated by bootstraps (9000). We also used the method developed by David et al.
183 (2007) based on identity disequilibrium. Identity disequilibrium is the relative excess in
184 double heterozygous genotypes for pairs of loci. Contrary to the methods based on F_{is} ,
185 identity disequilibrium is relatively insensitive to the non-detection of heterozygotes (null
186 alleles), which is a quite common scoring artifact in the use of molecular markers (e.g.
187 microsatellites). The Rmes software was used to estimate historical selfing rates from
188 identity disequilibrium (David et al. 2007).

189 *Phenotypic traits : phenology, floral traits and autonomous selfing*

190 Phenotypic traits were measured on the test generation. Five measures were taken in the
191 three first flowers of each individual plant: the date of anthesis, the date of senescence,

the maximum height, the maximum width and the floral morph (white, light purple, purple spotted morphs). From these measures, we derived the following measures at the scale of the individual: the date of the initiation of flowering, the mean floral longevity, the mean floral height, the mean floral width and the floral color. The mean was calculated as the average of the three measures. Concerning floral morph, if at least one of the three flowers had a purple spot, the individual was referenced as “purple spot”; if at least one of the three flowers had light diffuse purple (but no flowers had a purple spot), the individual was referenced as “light purple”; if the three flowers were white, the individual was referenced as “white”. Finally for each individual plant, the floral display (the total number of flower simultaneously opened) was recorded at 15 days after initiation of flowering, and the total flower production was recorded at 30 days after initiation of flowering.

In addition, we estimated the ability of autonomous selfing for plants. Autonomous selfing was estimated as: $A = \frac{N_A}{N_O}$ where N_A is the number of seeds from a flower under autonomous selfing and N_O the number of seeds from a flower under open pollination. N_O was obtained under open pollination in the experimental field, while plants were placed in the insect-proof greenhouse to obtain N_A . The N_A flowers opened and senesced in the greenhouse to ensure that seed production was due only to autonomous selfing. Both N_A and N_O were estimated from one flower per plant, chosen at random.

Statistical analysis of the phenotypic traits

All reproductive traits except floral color were analyzed with a linear mixed effect models (LMM, nlme R package) with sampling year (either 1991 or 2012) as a fixed effect and identity of the mother-plant as a random effect. As floral color is an ordinal variable with

3 categories, we first considered the floral color as a binomial variable, coding “white” as 0 and non-white (“light purple” and “purple spot”) as 1. Secondly, we compared the non-purple spotted morphs (“white” and “light purple”, coded as 0) to the “purple spot” (coded as 1). For these two analyses, we used a generalized linear mixed effect model (GLMM, glmmPQL R package) with a binomial distribution and fixed and random effects as above. Finally, in a third analysis, we performed a quantitative analysis of color by assigning score to each floral color morphs: “white” as 1, “light purple” as 2 and “purple spot” as 3, and we analyzed this variable with a GLMM with a poisson distribution. In this analysis, we assumed equal distances between each pair of consecutive categories. Autonomous selfing was analyzed with a LMM with sampling year (either 1991 or 2012) as a fixed effect and mother plant as a random effect. Autonomous selfing was square root transformed to improve the normality of residuals. All statistical analyses were conducted using R statistical software (R Development Core Team (2012); 2.15.0 version).

RESULTS

Genetic diversity in the 1991 and 2012 samples, and comparison between temporal and spatial F_{ST}

Mean allelic richness and mean genetic diversity (H_e) at the 8 putative neutral markers were not statistically different between the 1991 and the 2012 samples, and there were private alleles in the samples (Table 1). Estimated F_{IS} and 95% confidence intervals revealed a significantly higher F_{IS} for the 2012 sample compared with the 1991 sample, which corresponds to selfing rates of 0.68 and 0.86 for the 1991 and the 2012 samples respectively (Table 2). The selfing rates estimated from the analysis of multilocus correlation of heterozygosity exhibited the same trend as those derived from the F_{IS} but the difference between 1991 and 2012 samples was lower than the difference from the F_{IS}

analysis (0.71 vs 0.84), although the estimates were within the confidence intervals for the Fis values (Table 2).

Patterns of evolution of reproductive traits

Compared with 1991 sample, plants from the 2012 sample started to flower 1.15 ± 0.51 days earlier (Figure 1A, $p < 0.05$), this pattern was conserved if we calculated the mean date of flowering of the three first flowers (*not shown*). Regarding floral traits, floral height and floral width were significantly smaller in the 2012 than in the 1991 sample (Figure 2 B, $p < 0.001$, 1C, $p < 0.01$). Similarly, floral longevity was significantly shorter in the 2012 sample than in the 1991 sample (Figure 2D, $p < 0.001$). There was a significant decrease in both the frequency of colored floral morphs (“light purple” + “purple spot” versus “white”), and in the frequency of purple-spotted (“purple spot” versus “white” + “light purple”) in the 2012 compared with the 1991 sample (Figure 3A,B, $p < 0.001$). When a numeric value was assigned to the floral color as described above, we detected significantly lower color score in the 2012 sample compared with the 1991 sample (Figure 3 A, C, $p < 0.01$). Finally there was no difference in floral display or in total floral production (Figure 2 E, F, $p > 0.05$).

Autonomous selfing ability was greater for the 2012 than for the 1991 samples of field pansy (Figure 4, $p < 0.001$).

Discussion

In this study, we analyzed how the weed *Viola arvensis* has evolved over two decades in an agrosystems. The resurrection methodology allows detection of trait shifts and minimizes the effects of plasticity, and the use of microsatellites allows inference of realized selfing rates in the past and present. Taken together, our findings allows us to propose an

evolutionary scenario for the evolution of *Viola arvensis* experiencing environmental changes in agrosystems.

Resurrection ecology: potential bias

While resurrection ecology appears as a powerful methodology to detect short-term evolution, several potential biases have been identified (Weider et al, 2018). Indeed, there is the possibility that seed preservation affects phenotypic traits, which implies that trait shifts between old and recent phenotypes could be an artefact of seed storage. According to Franks et al. (2019), there is the possibility that non-random seed mortality during conservation modifies traits at the adult stages as measured in this study. This phenomenon, named “the invisible fraction,” results from the association of seed mortality with traits expressed later in the life cycle through genetic correlations. By artificially aging seed of *Brassica rapa* under high temperature conservation, Franks et al. (2019) have shown that flowering phenology estimates were biased by seed mortality during conservation. In a mixed selfer like *V. arvensis*, a likely mechanism for differential survival during seed storage is inbreeding depression (δ). If inbreeding depression affects seed survival, we expect that selfing rate estimates to be biased according to the relationship $s' = \frac{s(1-\delta)}{s(1-\delta)+(1-s)}$ (Barrett and Harder 1996). To account for a decrease in selfing rates from 0.88 to 0.68 (our estimates) caused by inbreeding depression on seed mortality during seed storage, a value of $\delta=0.7$ would be necessary. Given inbreeding depression values reported for seed survival (Winn et al, 2011), it is unlikely that inbreeding depression in itself is the cause of selfing rate changes. Also, according to Baskin and Baskin (2014), *Viola arvensis* is highly dormant and *in situ* estimates revealed high survival for dormant seeds in the soil (Pluntz et al. 2018). Moreover, genotyping was

performed on seedlings from natural populations in the 2012 sample but from lab-germinated seedlings the 1991 sample, the latter minimizing inbreeding depression. We thus expect inbreeding depression on seedling mortality to decrease the selfing rate estimate in the 2012 sample (conservative with respect to our hypothesis). Overall, we conclude that selfing rate changes estimated by molecular markers provide evidence for higher selfing rates in 2012 compared to 1991 in natural conditions. Nevertheless, it is still possible that non-random mortality (independent from inbreeding depression) have bias our phenotypic results. However, the low germination rate in the 1991 sample (refreshing generation) is likely higher than the germination rate in the field where the 2012 seedlings of the 2012 have been sampled. If evolution is driven by non-random mortality, evolving a set of trait consistent with a selfing syndrome would require very specific genetic correlation (*e.g.* seed mortality linked to flower size, seed mortality linked to duration of anthesis....). This interpretation is unlikely, though not it cannot be totally ruled out.

Patterns of trait evolution

While the increase in selfing rates over time revealed by microsatellites may be the sole result of reduced pollinator activity without evolution (plasticity), common garden allows detection of trait shifts via evolution. Regarding mating system traits, we have a body of evidence for ongoing evolution towards a selfing syndrome in *Viola arvensis*. The floral size decrease (from 16 mm to 14.5 mm in height, from 13.5 mm to 12.5 mm in width) and the reduction of floral longevity (from 5.7 days to 5.3 days) are consistent with a shift towards the smaller, shorter-lived flowers characteristic of selfing species (Duncan and Rausher, 2013). In addition, the floral morph frequencies have changed, with an increase of the pale morphs. While we have no direct evidence of lower pollinator attractiveness of the pale morph, such color patterns are in line with the evolution of a selfing syndrome.

The higher autonomous selfing ability in the 2012 sample compared to the 1991 sample shows that the recent sample has higher ability to set seed in depauperate pollination environments. The pollinator decline in Burgundy (Martin et al, 2019; Fried et al, 2016) is a putative selective factor for the pattern observed.

Our resurrection experiment also shows a shift towards earlier flowering phenology. This pattern has been reported in several studies (Franks et al, 2007; Valencia-Montoya et al, 2021). Climate warming and late season drought are considered to be the driving forces selecting for earlier flowering (Root et al, 2003). A non-exclusive explanation for this pattern may be indirect consequences of synchrony with early pollinator emergence caused by warming. Overall, the shift towards earlier flowering time is consistent with direct or indirect effect of climate warming.

Short-term evolution of selfing syndrome and plant-pollinator interactions in ecosystems.

The evolution of selfing syndrome from outcrossing ancestors is a recurrent pattern reported in several plant phyla on a macroevolutionary timescale (Stebbins, 1950; Goldberg et al. 2010). The recognition that such a pattern can evolve on the short-term, in response to environmental changes, is new in plant mating system studies. This was first reported in experimental evolution studies (Gervasi and Shiestl, 2017; Roels and Kelly, 2011). However, to our knowledge, the rapid evolution in nature towards a selfing syndrome in twenty years reported in this study is a novel finding. Our results for *Viola arvensis* show that current environmental changes can potentially drive evolutionary change that cause plant populations to evolve as fast as those under experimental evolution. Our results emphasize that the current ecological crisis generates substantial selection pressures leading to rapid evolution, in contrast to the traditional view that

evolution was always very slow (Darwin, 1859). This study supports the idea that the signature of natural selection on floral morphology and plant mating systems caused by environmental changes is visible, even in a few generations.

The pattern of evolution observed in *Viola arvensis* has the potential to disrupt plant-pollinator interactions. Assuming that pollinator decline has caused the evolution of a selfing syndrome, this evolutionary change may reinforce pollinator decline and leads to a positive feedback loop and ultimately to the breakdown of a major trophic relationship in ecosystems (Cheptou, 2021). In this context, there is an urgent need to analyze patterns of mating system evolution in floras to see if the pattern observed in *Viola arvensis* is general or not. Resurrection ecology appears as a powerful methodology to analyse such patterns. In Europe, the large number of seed banks (Ensconet network) has the potential to reveal patterns of plant evolution in the last decades.

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Author Contributions: P.-O. C and E.I designed the study. M. T. performed the experiment and lab work. MT did the statistical analysis and wrote a first version of the results section. P.-O. C. wrote the manuscript. P.-O.C, E.I and M.T provides comments and corrections.

Data Availability Statement: Data are available from the Dryad Digital Repository: doi: 10.5061/dryad.pvmcvdnp6

Additional Supporting Information may be found online in the supporting information section at the end of the article” Appendix S1: Experimental design.

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Tables

Table 1. Genetic richness and genetic diversity (He) in the temporal samples of *V. arvensis*. Private alleles in 1991 and 2012 samples are shown in brackets.

locus	Allelic richness (private alleles)			Genetic diversity (He)	
	1991	2012	Total	1991	2011
Varv03	7 (1)	8 (2)	9	0,74	0,77
Varv06	5	6 (1)	6	0,64	0,71
Varv08	2	4 (2)	4	0,24	0,47
Varv09	2 (1)	2 (1)	3	0,40	0,14
Varv10	8 (2)	6	8	0,83	0,81
Varv15	2	3 (1)	3	0,07	0,14
Varv37	3 (1)	2	3	0,32	0,26
Varv42	2	2	2	0,51	0,18
moy \pm ES	3,87 \pm 0,87	4,12 \pm 0,81	4,75 \pm 0,92	0,47 \pm 0,09	0,43 \pm 0,10

445 Table 2. Selfing rates estimated by hierFstat (Fis [95% CI] in italic) and by RMES (\pm SE)
 446 in the 1991 and the 2012 samples of *Viola arvensis*.

447

448

	Selfing rate in natural conditions	
	Genepop	RMES
Sample age		
1991	0.68	0,71 \pm 0,07
	<i>0,51 [0.36-0.67]</i>	
2012	0.86	0,84 \pm 0,04
	<i>0,76 [0.67-0.85]</i>	

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Figure legends.

Figure 1. Floral morphs for *Viola arvensis* subsp *megalantha* in the study population.

Figure 2. Shifts in phenotypic traits between 1991 and 2012 in *Viola arvensis* estimated from historical and recent samples. Estimates \pm SE are adjusted to account for the random effect. Significance level: n.s., $P > 0.1$; †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 3. Change in floral morph frequencies between 1991 and 2012 in *Viola arvensis* estimated from historical and recent samples. (A) spineplot of the row data for the floral morphs : white for white morph, light gray for light purple morph and dark gray for purple spot morph (see Figure 1). (B) statistical comparison of floral morph frequencies. (C) Numerical score for historical and recent population : 1, 2, 3 encoded for white, light purple and purple spot morphs respectively. The horizontal full bar and the horizontal dotted bars represent the estimated score of each sample with its \pm SE, respectively. Significance level: n.s., $P > 0.1$; †, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. Shift in autonomous selfing between 1991 and 2012 in *Viola arvensis* estimated from historical and recent samples. Autonomous selfing ability is estimated from the ratio (square root transformed in the statistical analysis) between seed set in the absence of pollinators and seed set under natural pollination. Back-transformed estimates \pm SE are presented.

475 **Fig1**

476

Scale :1 cm



White
morph



Light purple
morph



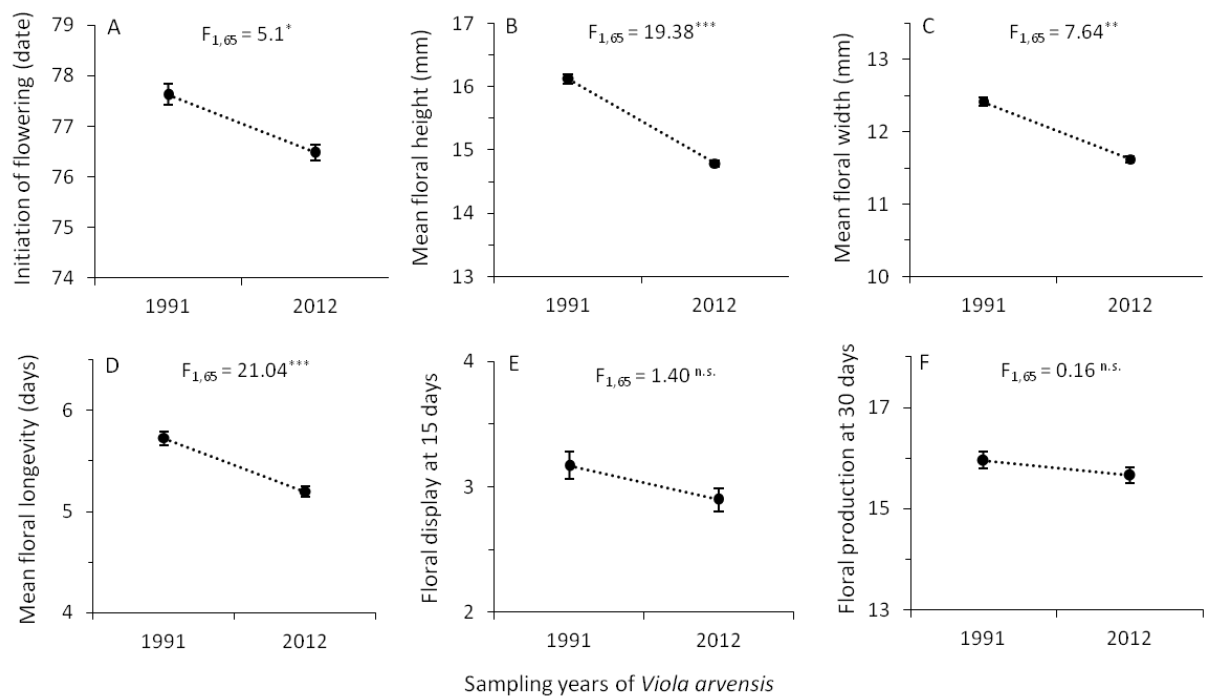
Purple spotted
morph

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480 **Fig2**

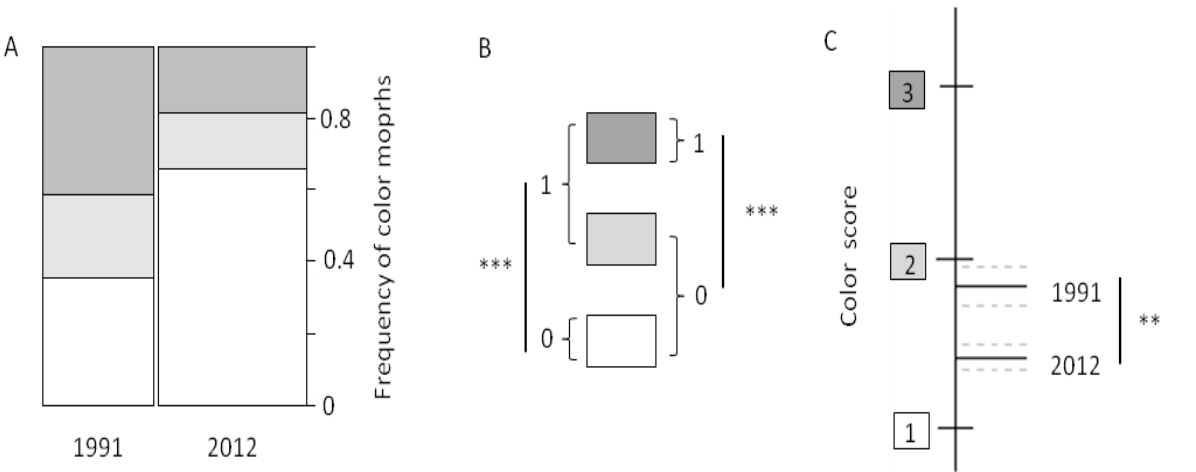


481

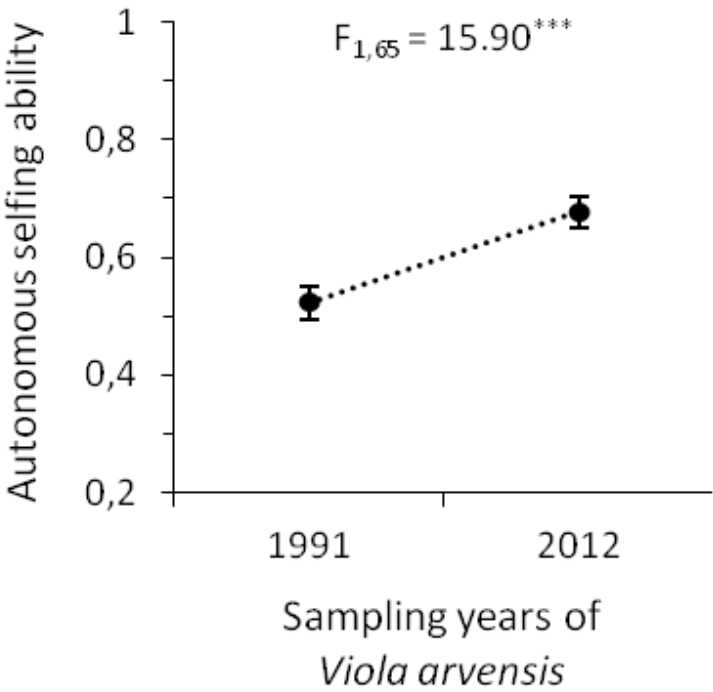
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484 **Fig3**



487 Fig 4



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