Title: Changes in pollen production, pollen heteromorphism and ovule production with increased selfing in *Viola arvensis*

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Abstract:

Background and Aims

Pollen:ovule ratios are often lower in species and populations with higher selfing rates. This may be due either to higher pollination efficiency through selfing, or to lower male competition when less allo-pollen is available. Changes in pollination can also impact pollen traits, such as the number of apertures. *Viola arvensis* has experienced a rapid recent increase in selfing rates, and its floral traits have also rapidly evolved towards the selfing syndrome. This study tests the hypothesis that *V. arvensis* is also undergoing a rapid evolution in its pollen:ovule ratio and pollen heteromorphism.

Methods

Using the resurrection ecology methodology, we studied four pairs of populations by comparing an ancestral sample (from ca. 30 years ago) to a descendant sample (from 2021). We counted ovules and pollen and measured the three pollen aperture morphs in 50 individuals per population. We also developed a model linking the number of apertures to mating system to better understand the links between the two.

Key Results

We found no temporal change in pollen or ovule production in our populations. However, populations with the lowest pollen:ovule ratios were also the ones with the highest ancestral selfing rates, suggesting that the pollen:ovule ratio could have evolved on a similar time scale to population differentiation. Our model predicts a positive correlation between number of apertures and selfing rates, if pollination parameters remain constant. However, this positive correlation was not found in our results, neither across populations nor through time.

Conclusions

Unlike floral morphology, pollen and ovule production did have not evolve rapidly with increased selfing rates, suggesting a delayed change of the pollen:ovule ratio compared to other traits of the selfing syndrome. This absence of correlation between pollen heteromorphism and selfing rate can be explained by multiple (non-mutually exclusive) factors: a decrease in allo-pollen deposition correlated with the evolution of the selfing syndrome, pollinator declines, or the absence of selection in this trait.

Key words: rapid evolution, pollen:ovule ratio, pollen heteromorphism, selfing syndrome, resurrection ecology, *Viola arvensis*

Introduction

Pollinators are undergoing worldwide declines (Biesmeijer *et al.*, 2006; Potts *et al.*, 2010; Goulson *et al.*, 2015; Janousek *et al.*, 2023), reducing cross-pollination in plants (Biesmeijer *et al.*, 2006). In some situations, this could act as a selective pressure reinforcing attraction of pollinators, especially for plants unable of autonomous selfing. In others, however, this is likely acting as a selective pressure favoring selfing and the associated selfing syndrome (Lloyd, 1992; Thomann *et al.*, 2013). The selfing syndrome is a collection of traits differing between selfing species and their closely related outcrossing ones (low herkogamy, small floral size or low pollen:ovule ratio; Lloyd, 1965; Ornduff, 1969; Sicard & Lenhard, 2011). The rapid evolution of the selfing syndrome has been demonstrated for several floral traits experimentally in the absence of pollinators (Bodbyl Roels & Kelly, 2011; Panique & Caruso, 2020) and by comparing natural populations in pollinator-rich and pollinator-poor environments (Brys & Jacquemyn, 2012). However, only one previous study investigated the evolution of the pollen:ovule ratio in such conditions (Brys & Jacquemyn, 2012).

Two main mechanisms have been invoked to explain lower pollen:ovule ratios in selfing species. The first is that less pollen grains are needed for self-fertilization due to higher efficiency of self-pollination (Lloyd, 1965; Cruden, 1977). Indeed, autonomous selfing could minimize the exposure of pollen grains to adversity (loss or desiccation during pollen transfer) decreasing the number of pollen grains required to fertilize an ovule. The second mechanism comes from the sex allocation theory, which postulates a trade-off between male and female fitness. As an increase in selfing reduces male competition (fewer individuals competing on stigmas), sex allocation theory predicts that a relative increased investment in the female function compared to the male function increases fitness for higher selfing rates, thus leading to lower pollen:ovule ratios (Charnov, 1982; Zhang, 2000). Whereas lower pollen:ovule ratios have often been used to characterize selfing species, on their own they are not fully informative (Harder & Johnson, 2023). Indeed, lower pollen:ovule ratios in selfing taxa are mainly linked to a decrease in pollen number, rather than to any change in ovule numbers, which does not support the sex allocation mechanism (Brys & Jacquemyn, 2012; Harder & Johnson, 2023).

A third mechanism, the mass action model (Holsinger, 1991), may predict higher pollen:ovule ratio correlated to higher selfing rate. Indeed, if selfing occurs through competition between self- and allo-pollen deposited on the stigma (competitive selfing, Lloyd & Schoen, 1992) then the mass action model predicts that a higher self-pollen deposition on the stigma due to a higher pollen production leads to a higher individual selfing rate. However, this prediction assumes a constant allo-pollen deposition. If instead the higher pollen production translates into an even higher allo-pollen deposition compare to self-pollen deposition, the mass action model predicts that pollen production will be negatively correlated to the mean selfing rate of the population. Given that the decline in allo-pollen deposition is likely responsible for the observed selection of higher selfing rates associated with pollinator declines, a positive correlation between pollen production and selfing rates is unlikely. Accordingly, a decrease in the pollen:ovule ratio likely reflects either direct selection for sex allocation optimization (increase of ovule number and decrease in pollen production) or or else a release of selection on pollen number due to higher efficiency of self-pollination (decrease in pollen production).

Beyond pollen:ovule ratio, reduced pollinator visitation rates and male competition could also impact the traits of pollen grains, such as their size or the number of apertures known to be involved in male competition (Dajoz *et al.*, 1991; McCallum & Chang, 2016). The number of apertures varies from one plant species to another (Walker & Doyle, 1975), but can also vary between individuals (polymorphism), or within individuals, hereinafter called pollen heteromorphism (Fig. **1A**, Till-Bottraud *et al.*, 1995). Despite some genes having been identified as explaining the number of apertures in *Arabidopsis thaliana* mutants (Prieu *et al.*, 2016; Albert *et al.*, 2018), the ontology of pollen heteromorphism remains unclear (but see, Mignot *et al.*, 1995; Ressayre *et al.*, 1998, 2002a,b). Pollen heteromorphism has been proposed as the result of ploidy variation inducing pollen developmental issues (Mignot *et al.*, 1994). However, in *Viola arvensis* and in some other *Viola* species, pollen heteromorphism has been shown to be independent from ploidy level of the sporophyte and the gametophyte (Mignot *et al.*, 1994; Dajoz *et al.*, 1995). A broad-sense heritability has furthermore been demonstrated for *Viola diversifolia* (Dajoz *et al.*, 1993). This suggests that pollen heteromorphism could be under selection, potentially explained by the pollination context (Till-Bottraud *et al.*, 1994; Dajoz, 1999).

Pollen apertures are areas with thinner or absent exine, which favor exchanges with the environment, and therefore hydration or dehydration. Previous studies have shown that pollen with a higher number of apertures germinates faster but has lower survival, likely due to a faster rehydration capacity but higher dehydration risk, inducing a tradeoff between longevity and speed of germination (Dajoz *et al.*, 1991, 1993; Prieu *et al.*, 2016). Comparing *Arabidopsis thaliana* mutant lines, pollen with 3 apertures has been shown siring more seeds than those with no aperture (Albert *et al.*, 2018). A mass-action model, incorporating this tradeoff through three parameters has been proposed: delay between anthesis and pollination, lower survival in time of pollen grains with more apertures, but also advantage due to their faster germination (Till-Bottraud *et al.*, 1994). It shows that polymorphism, which involves a pure alternative strategy in different individuals, is not stable and leads to monomorphisms in the population, while pollen heteromorphism is an evolutionary stable strategy with morph proportions given by the three parameters (Till-Bottraud *et al.*, 1994). In the case of pollinator declines, we expect to see an increased delay between anthesis and pollination, favoring pollen with fewer apertures. However, this model assumes panmixia and introducing mixed mating could modify the prediction.

Because pollen heteromorphism has never been studied in correlation with mixed mating system, we first develop a mathematical model incorporating competitive selfing to shed light on the links between pollen heteromorphism and the mating system. The resulting hypothesis is that an increase in selfing rates is correlated to an increase in proportion of pollen grains with more apertures, if other parameters remain constant. Whereas the evolution of pollen and ovule production and of the pollen:ovule ratio have been widely studied in interaction with the mating system over long-time scales (Lloyd, 1965; Cruden, 1977; Plitmann & Levin, 1990; Mione & Anderson, 1992; Sicard & Lenhard, 2011; Brys & Jacquemyn, 2012; Harder & Johnson, 2023), no study to our knowledge has tried to observe their evolution during rapid changes of the mating system. In the species *Viola arvensis*, we previously showed a rapid evolution of floral traits toward selfing syndrome and an increase in selfing rates (Table **1**; Cheptou *et al.*, 2022; Acoca-Pidolle *et al.*, 2024). Using the same populations, we will test the hypothesis that there should be concomitant rapid changes in the pollen:ovule ratio. The experimental design is a resurrection ecology experiment with 4 pairs of ancestral and descendant natural populations of *Viola arvensis*, separated by 20 to 29 years, grown altogether in a common garden. In this pollen heteromorphic plant (Dajoz *et al.*, 1995; Mignot *et al.*, 1995; Dajoz, 1999; Scoppola & Magrini, 2019), we test the following hypotheses:

1– In line with the pollination efficiency hypothesis, pollen production is decreasing in natural populations experiencing evolution toward selfing, inducing a decrease in pollen:ovule ratio.

2– The mean number of pollen apertures is increasing in correlation to the increase in selfing rates.

Material and methods

Study system and sampling — *Viola arvensis* Murray is an annual species of wild pansy (Violaceae). It is commonly found in fields as a weed or in meadows. The species is self-compatible with a mixed mating system (Scoppola & Lattanzi, 2012; Cheptou *et al.*, 2022; Acoca-Pidolle *et al.*, 2024). The flower possesses a tricarpellary syncarpous ovary, each carpel containing several ovules with parietal placentation. The species presents pollen heteromorphism; a single individual produces, within each anther, different pollen morphs (Till-Bottraud *et al.*, 1995; Scoppola & Magrini, 2019). *Viola arvensis* produces a majority of 5-aperturate pollen grains but can also produce 4- and 6-aperturate morphs (Fig. **1A**, Scoppola & Magrini, 2019).

Plants were sampled twice in agricultural fields in four locations of the Parisian Basin (see Table **1**). The first sampling was done by the Conservatoire Botanique National de Bailleul in 1992 for Lhuys (Lh) and 1993 for Crouy (Cr), and by the Conservatoire Botanique National du Bassin Parisien in 2000 for Guernes (Gu) and 2001 for Commeny (Co). Seeds were collected by sampling fruits on a minimum of 100 mothers picked randomly in each population. The second sampling was performed in the same locations, in an area that overlapped the ancestral sampling area as much as possible, in February 2021 by randomly sampling 40 or more seedlings. The minimal sampling area was of 2,000 m^2 and corresponds to a case where all the population is circumscribed to this area. This sampling scheme produced eight populations in total: four pairs, one for each location, each pair including an ancestral (A) and a descendant (D) population. A population, hereafter, corresponds to one locality at one sampling period, thus there are two populations per locality and four populations per sampling period.

Experimental design — In accordance with the resurrection ecology methodology (Franks *et al.*, 2018), we corrected for maternal effects by first growing a refresher generation (F0), by bringing into a common garden plants that originated from the field for both ancestral and descendant populations. As ancestral populations had been stored as seeds and descendant populations were collected at the seedling stage (due to time constrains), common conditions for the F0 started at the seedling stage. For ancestral populations, in mid-November 2020 we placed seed lots in germination chambers in petri dishes with vermiculite under light at 15°C for 12h and in the dark at 6°C for 12h. Germination rates were of 50% to 65%. We transplanted 32 seedlings of ancestral and descendant populations in late February 2021 into the field 10 km north of Montpellier, France (Baillarguet site of the experimental platform “Terrains d’Expériences” of the Labex Cemeb; 43°40′53.92′′N, 3°52′28.74′′E). Two individuals died, one in the CrD and another in the LhD population. Populations were separated in insect-proof mesh cages. During full blooming, in mid-April, we introduced commercial bumblebee hives, Natupol from Koppert France® (Cavaillon, France) into the cages, avoiding gene flow between populations, to produce a second generation of open pollinated individuals with similar maternal effects. We collected one to three fruits per individual produced in this pollination context were collected.

We then used these collected seeds produced by the F0 generation, corrected for maternal effects, to grow a new family-designed test generation (F1). We randomly selected 20 mother plants per population in the F0 generation to construct 20 families in the F1 generation. In December 2021, we placed 30 seeds per family in germination chambers in petri dishes with vermiculite under light at 15°C for 12h and in the dark at 6°C for 12h, to obtain enough plants for measurements. Germination rates per population ranged from 75% to 90%. We then randomly selected five plants per family for this experiment. Thus, each family was, at the end, composed of five siblings, except for three families that did not germinate enough to obtain five plants and were composed of one, two and four siblings. We placed plants in individual pots ($11 \times 11 \times 11$ cm^3) filled with standardized soil in late February and randomized their position in a greenhouse of the experimental platform “Terrains d'Expériences”. Watering was manual and was done when needed. In total, F1 generation was constituted of 792 plants, distributed across the eight populations. We did not observe early mortality.

Individuals used in this study are the same as those used in the previous study of Acoca-Pidolle *et al.*, 2024.

Measurements of ovule and pollen productions — We measured ovule and pollen productions in the F1 generation. In each population, constituted of approximatively 100 individuals, we selected 50 individuals randomly such that it included at least one member per family (giving 1 to 5 individuals per family with a mean of 2.5 individuals). We collected one bud per plant close to anthesis (state determined empirically) and froze it at -20 °C in a labelled tube. The collection was performed between 26 April and 12 May 2022, only in the main stem, recording floral position (number of flowers already in bloom on the main stem). We defrosted and dissected the buds 17 months after collection, between September and October 2023. Under a binocular magnifier, we dilacerated one of the five anthers in a drop of lactic acid on a microscope slide. Placing the microscope slide under a microscope then enabled us to count all the pollen grains while determining their number of apertures (4-, 5-, or 6-aperturate, Fig. **1A**) using a mounted bank of three counters. The motivation to count the whole pollen grain assemblage on a single flower came from previous results showing no significant variation between flowers and more robust estimates with the entire assembly rather than a few hundred of pollen grains (Magrini & Scoppola, 2015). We isolated the ovary, incised it under a binocular magnifier and manually counted the ovules. A single experimenter dissected all the buds and counted the ovules, while pollen grains were counted by another single experimenter.

Data analysis — The pollen:ovule ratio was calculated by multiplying the number of pollen grains contained in one anther by five to obtain a flower-wide estimate, divided by the number of ovules.

We computed the proportion of each morph as the number of counted grains of the given morph divided by the total pollen count. As long as more than two morphs exist, pollen heteromorphism can be divided in two components: the first the mean number of apertures of pollen grains within an individual; and the second the diversity of the number of apertures within an individual. We calculated the mean number of apertures by multiplying proportions of morphs by the number of apertures they exhibit. Pollen diversity was calculated using Simpson's D diversity index, which measures the probability to sample, by chance, two pollen grains of different morphs, thus:

$$D = 1 - \sum_i \frac{N_i(N_i-1)}{N(N-1)}$$

with N the total number of pollen grains and N_i the number of pollen grains of i th morph. An individual producing only five-aperturate pollen grains has a mean number of apertures of 5 and a diversity of 0. An individual producing an equal mix of four- and six-aperturate pollen grains also has a mean number of apertures of 5 but a diversity around 0.5. Finally, an individual producing an equal mix of four-, five- and six-aperturate pollen grains also has a mean number of apertures of 5 but a diversity around $\frac{2}{3} \approx 0.66$. In case of only two morphs a and b produced per individual, and considering a sufficiently large number of pollen grains to reach $N_i - 1$ approximatively equal to N_i for any i morph and thus $N - 1$ approximatively equal to N, $D_{a,b} = 1 - \frac{N^2(\mathrm{f}_\mathrm{a}^2+\mathrm{f}_\mathrm{b}^2)}{N^2}$, with f_a and f_b the proportions of morph a and b respectively; and the mean number of apertures, $\overline{x_{a,b}} = n_a f_a + n_b f_b$, with n_a and n_b the number of apertures of morph a and b respectively. Then because $f_a = 1 - f_b$:

$$D_{a,b} = 2f_a(1 - f_a) \text{ and } f_a = \frac{\overline{x_{a,b}} - n_b}{n_a - n_b}$$

$$\Rightarrow \; D_{a,b} = 2\frac{\overline{x_{a,b}} - n_b}{n_a - n_b}\left(1 - \frac{\overline{x_{a,b}} - n_b}{n_a - n_b}\right)$$

linking pollen diversity and mean number of apertures by a second-degree function (parabola). These theoretical parabolas were drawn in Figure 3 to help interpretation of data.

We conducted statistical analyses using linear mixed models with each trait as response variable, age (ancestral or descendant), locality (four localities), and the interaction between age and locality as fixed factors, and family, date of collection and floral position as random factors. Mean pollen aperture, ovule and pollen numbers were analyzed without any transformation given the normality of residuals, while pollen:ovule ratio was transformed using the log transformation, and pollen diversity using the square root transformation to improve the normality of residuals. We did not test directly pollen proportions because their variations are totally captured by mean pollen aperture and pollen diversity variations. All our statistical analyses were performed using the R software (R Core Team, Version 4.2.1). LMMs were performed using the "lmer" function implemented in the package "lme4" (Bates *et al.*, 2015).

Model linking pollen heteromorphism and selfing rates

Our model is inspired by the one developed by Till-Bottraud *et al.* (1994) where pollen heteromorphism is characterized by the mean number of apertures treated as a quantitative trait. Our model considers that all the individuals produce the same amount of pollen and ovules. An individual produces a proportion z of the first pollen morph and a proportion $1 - z$ of the second morph with fewer apertures. These proportions vary among individuals and the average proportion of the morph with more apertures in the population is $\bar{z}$. The morph with more apertures germinates faster than the one with less apertures, giving it an advantage a, but also suffers a decrease in survival during the pollination waiting time ν. All the plants can autonomously deposit the same amount of self-pollen on their own stigma. All the plants also receive the same amount of allo-pollen compared to self-pollen, q_p, dependent on pollinator abundance/diversity. Geitonogamy is not considered here. Received allo-pollen is separated into two categories, fresh and aged. The fraction of aged allo-pollen deposited on a stigma, $t_{p,}$ is the same for all the individuals and is dependent on pollinator abundance/diversity. The links between pollinator abundance/diversity and proportion of aged pollen t_p and amount of allo-pollen q_p are not explicit in this model, but the higher pollinator abundance and/or diversity are, the lower the proportion of aged pollen t_p and the higher the amount of allo-pollen compared to self-pollen q_p. By definitions, z, $\bar{z}$, t_p and ν vary between 0 and 1, a is strictly superior to 1 and q_p can take any positive value (no limit in pollen load).

Self-pollen can fertilize ovules through competition with allo-pollen (competitive selfing). Competitive selfing is likely in *Viola arvensis* given absence of differences between seed sets of autonomously set fruits and open pollinated fruits ruling out prior selfing (Acoca-Pidolle *et al.*, 2024), and early stamen dehiscence rendering delayed selfing unlikely (personal observations). Then, the selfing rate for an individual with a proportion z of pollen with more apertures in a population with a proportion $\bar{z}$ of pollen with more apertures is given by:

$$s(z) = \frac{az + (1 - z)}{az + (1 - z) + q_p\left(t_p \nu a\bar{z} \, + \, (1 - t_p)a\bar{z} \, + \, (1 - \bar{z})\right)} \tag{1}$$

The selfing rate is the proportion of the amount of self-pollen (numerator) to the total amount of pollen in the stigma (denominator), with pollen categories weighted by their relative advantages and disadvantages. Using this equation (1), it gives the approximation for the mean selfing rate within the population (see Appendix):

$$\bar{s} = \frac{a\bar{z} + (1 - \bar{z})}{a\bar{z} + (1 - \bar{z}) + q_p\left(t_p \nu a\bar{z} \, + \, (1 - t_p)a\bar{z} \, + \, (1 - \bar{z})\right)} \tag{2}$$

Whereas a and ν are considered fixed parameters in our model, other terms are variable. The sign of the derivation of $\bar{s}$ by $\bar{z}$ is positive (see Appendix), which means that an increase in population selfing rate could lead to an increase in mean number of apertures if the pollination parameters remain constant. The signs of the derivative of $\bar{s}$ with respect to t_p and with respect to q_p are positive and negative respectively (see Appendix), which means that an increase in population selfing rate could be due to a decrease in pollinator abundance/diversity (increase of time before pollination and decrease of allo-pollen deposition) while mean number of apertures remains constant.

The values of the parameters are difficult to estimate. In the literature, we found that, for *Arabidopsis thaliana,* in competition in equal proportions, pollen with more apertures sires 60% of seeds (Albert *et al.*, 2018), so 1.5 times more seeds than pollen with fewer apertures, giving a equal to 1.5. In another *Viola* species, pollen with fewer apertures represents approximatively 25% of fresh germinating pollen, while it is approximately 50% of 48h-aged germinating pollen (Dajoz *et al.*, 1993). Hence, the survival of pollen with more apertures after 48h is 1/3 of those of pollen with fewer apertures, giving ν equal to 1/3. Finally, *Viola arvensis* has an anthesis duration of approximatively 6 days, if pollen is taken uniformly during the anthesis of the flower, we can assume that 2/3 of the pollen is taken after 48h and considered as aged pollen, giving t_p equal to 2/3. We found no values in the literature allowing us to estimate the quantity of allo-pollen deposited in comparison to self-pollen q_p. These parameters are the ones used in Figure **2A**, while Figure **2B** presents results where pollen ages faster or is exported later and survival of aged pollen with more apertures is lower.

Results

Pollen and ovule production — Mean pollen:ovule ratios varied from 44.2 to 49.1 (Table **2**). No difference between ancestral and descendant populations were observed. We found differences across localities (spatial) in all the traits with Commeny and Crouy exhibiting the lowest pollen:ovule ratios with highest ovule production and lowest pollen production respectively (Table **2**). Pollen production exhibited some temporal x spatial interaction, with a decrease between ancestral and descendant population in Guernes.

Pollen heteromorphism — All individuals produced at least two pollen morphs (no zero diversity, Fig. **3**). The curves in Figure **3** represent the expected relation between pollen diversity and mean number of apertures if individuals produce only two pollen morphs (see Material and methods). The greater the deviation from these curves, the greater the deviation from this expectation. Of the 400 individuals observed, 72 were perfectly aligned along the solid line curves, and therefore produced only two out of three morphs (14 without 4-aperturate morph and 58 without 6-aperturate morph; Fig. **3**). The dominant morph was the 5-aperturate morph followed by the 4-aperturate morph and finally the 6-aperturate morph, rare, but present in all the populations (Fig. **1B**, Table **2**). Interestingly, no point was aligned along the dashed predicted curve (Fig. **3**), meaning that no individual presented only 4- and 6-aperturate morphs, nor high proportions of both morphs together. The highest proportions of both types were found in an individual with 24% 4-aperturate morph and 5% 6-aperturate morph, while each morph can be present up to 80% individually (see dots with extreme values of mean apertures in Fig. **3**). Individuals with lower mean aperture number tended to be closer to the predicted curves (Fig. **3**), thus to exhibit only 4- and 5-aperturate morphs, revealing a positive correlation between the absence of the 6-aperturate morph and the proportion of the 4-aperturate one (glmm estimate = 20.72, z = 4.85, $P < 0.001$). Individuals with three morphs possessed pollen diversity far below the maximum of 2/3, expected when the three morphs are in equal proportion, and several points were close to the solid line predicted curves (Fig. **3**). Thus, individuals possess almost only two morphs, the 5-aperturate morph with either 4- or 6-aperturate ones and a very low proportion of the third morph. Therefore, pollen diversity and mean number of apertures were correlated measures.

We found no difference in pollen heteromorphism between ancestral and descendant populations, however there were significant locality effects in all the traits (Table **2)**. Commeny and Lhuys showed the highest mean numbers of apertures, and thus the lowest pollen diversities, mainly driven by the lowest 4-aperturate morph proportions. Pollen diversity and mean number of apertures showed interactions between locality and age. Mean number of apertures decreased between ancestral and descendant populations in Commeny and Guernes, while it increased in Crouy and Lhuys.

Discussion

This study investigates whether pollen and ovule productions and pollen heteromorphism have changed concurrently with the rapid increase of selfing and the evolution of floral traits toward a selfing syndrome in *Viola arvensis*. Overall, the results did not support our hypotheses. We found no differences in pollen:ovule ratios between ancestral and descendant populations. Despite the expected correlation between selfing rate and pollen heteromorphism predicted by our model, we found no difference between ancestral and descendant populations in pollen heteromorphism.

Population differences in pollen:ovule ratio — Overall, the pollen:ovule ratios measured are consistent with the high selfing rates of this species (Table **1**, Cruden, 1977). Contrary to our hypothesis, there was no change through time in pollen or ovule production and pollen:ovule ratio in those populations. However, it is interesting to note that populations with the lowest pollen:ovule ratios were those which also possessed the highest ancestral selfing rates. This could be due to the joint evolution of selfing rate and pollen:ovule ratio in these populations followed by a recent increase in selfing rate with a lag in pollen:ovule ratio evolution. This lag could be explained by the high positive genetic correlation between pollen and ovule production found in selfing species (Mazer *et al.*, 2007). Indeed, in case of selection for a lower pollen production, it would induce a lower ovule production which would in turn decrease fitness if there is no pollen limitation, as it seems to be the case in *Viola arvensis* (Acoca-Pidolle *et al.*, 2024). This lower pollen:ovule ratio is due to lower pollen production in Crouy, but to higher ovule production in Commeny. An increase in ovule number is not consistent with previous results and the pollination efficiency model (Brys & Jacquemyn, 2012; Harder & Johnson, 2023). An increase in ovule number without a decrease in pollen number is also not consistent with the sex allocation model alone (Charnov, 1982), but could be explained by the joint evolution of reproductive allocation and sex allocation with increased selfing (Zhang, 2000). Indeed, an increase in reproductive allocation and a relative decrease in male allocation are expected with increased selfing, resulting in an increase of the resources allocated to female function without a decrease in the male ones (Zhang, 2000) .

The lack of change in pollen:ovule ratios could also indicate weak selection, or even absence of selection for this trait with increased selfing rates. The absence of selection is unlikely given the broad record of the correlation between lower pollen:ovule ratios and higher selfing rates between outcrossed and selfed closely related species or populations (Lloyd, 1965; Cruden, 1977; Plitmann & Levin, 1990; Mione & Anderson, 1992; Sicard & Lenhard, 2011; Brys & Jacquemyn, 2012; Harder & Johnson, 2023). A first explanation is the relatively high plasticity of these traits, especially of pollen production (De Storme & Geelen, 2014), which could mask genotypes to selection. A second explanation is that pollen:ovule ratio is not directly involved in selfing and rather secondarily optimized in response to the mating system itself. Therefore, this trait would suffer weakened selection compare to traits directly involved in mating such as herkogamy and to a lesser extent floral size (Bodbyl Roels & Kelly, 2011). This raises the question of the pace and sequence of development of the selfing syndrome, which is a set of traits correlated to higher selfing rates with likely very different functional links with selfing.

Individual and population differences in pollen heteromorphism — All plants were heteromorphic. The absence of monomorphism is consistent with the model predicting that all individuals should be heteromorphic under conditions for stability of pollen heteromorphism (Till-Bottraud *et al.*, 1994). The distribution of pollen morphs was not random and despite the usual occurrence of the three morphs together, not all plants presented all morphs of pollen, while 4- and 6-aperturate morphs excluded each other. There were often mainly two morphs, the 5-aperturate accompanied by one of the two others. Thus, despite the potential two dimensions of pollen heteromorphism, mean number of apertures and pollen diversity, pollen diversity was fully described by the mean number of apertures. Therefore, only mean number of apertures seems relevant to describe pollen heteromorphism. Pollen grains with 4- and 6-apertures were never found in high proportion together, which could in theory be selected given that they should respectively represent the fittest morph to two exclusive strategies, late and early pollination (Dajoz *et al.*, 1993; Till-Bottraud *et al.*, 1994). This could be due to developmental constrains on aperture number. Studying the meiotic process and tetrad organization in these populations of *Viola arvensis* could help to understand the production of the three morphs and the absence of coexistence at high proportions of 4- and 6-aperturate morphs (Mignot *et al.*, 1995; Ressayre *et al.*, 1998, 2002a).

At the population level, the 6-aperturate morph was infrequent but never absent, which could correspond to the rate of sporadic problems or constrains in pollen development, or be the signature of a selective advantage of the rare (negative-frequency dependent selection). Indeed, we also found that individuals producing high mean number of apertures were highly competitive in other stigma, whereas receptive individuals did not produce high mean number of apertures themselves (Acoca-Pidolle & Cheptou, unpubl. res.). Therefore, if the morph with 6-apertures is too frequent in the population, the higher probability of pollen deposition in receptive individuals producing themselves pollen grains with 6-apertures reduces the advantage of this morph during early pollination. If so, the cost on delayed pollination is selecting against it, which could explain the low proportions of this morph.

Spatial differentiation was not correlated to differences in selfing rates and could be caused by drift or local adaptation. The latter could be due to local conditions affecting pollination, such as pollination intensity and timing, or intensity of pollen competition due to population demography (Dajoz, 1999) affecting the relative amount of allo-pollen deposited q_p and/or the fraction of aged pollen deposited t_p, or local environmental conditions affecting pollen survival ν or male meiosis (De Storme & Geelen, 2014).

Despite the theoretical correlation between pollen heteromorphism and selfing rate given by the model, we found no difference in pollen heteromorphism between ancestral and descendant populations. Trait stasis (the absence of change) may be the result of the short timescale observed or of other selective pressures acting on pollen heteromorphism. Indeed, while selfing rates are predicted to be positively correlated to mean number of apertures in our model, fitness could be negatively correlated to mean number of apertures. For example, if the aged pollen proportion t_p is large, the failure in exporting viable pollen suppresses the outcross component of male fitness, so the automatic advantage for individual with higher mean number of apertures.

The model proposed in this study predicts that an increase in selfing rate, like the one observed from approximately 0.5 to 0.8, could be correlated to a gain of almost one aperture in average if other parameters were fixed (Fig. **2B**). However, this increase can also solely be due to a decrease of 75% in the relative amount of allo-pollen deposited q_p, from 1 to 0.25 (Fig. **2A**). A higher self-pollen deposition or a decrease in visitation number and/or efficiency can explain a decrease in the relative amount of allo-pollen deposited. In our case, the decrease in visitations is expected by the evolution of the selfing syndrome for floral traits (Acoca-Pidolle *et al.*, 2024). However, pollinator declines, likely responsible for the evolution of the selfing syndrome for floral traits, could also decrease directly the visitation frequency and/or efficiency. Looking at changes in autonomous self-pollen deposition could be very informative and measures of the relative amount of allo-pollen deposited in several pollination conditions could be useful to better understand the prediction of this model.

In conclusion, we observed no evolution of pollen or ovule production or pollen heteromorphism in these four natural populations over 20-29 years. However, some indications suggest that low pollen:ovule ratio could evolve later or slower during evolution toward selfing syndrome. This stasis may be due to positive genetic correlation and opposite selective forces on pollen and ovule production. Despite prediction of positive correlation between pollen heteromorphism and selfing rate, selection operating on this trait in link with the selfing system is still unknow and further investigations are needed to understand if and how this trait is linked with mating system.

Appendix

Because $s(z)$ is not linear, the expected value of $s(z)$ is not directly equal to $s(\bar{z})$. However, using the Taylor-Young formula, as z tends to $\bar{z}$, with $s'(\bar{z})$ being the first derivative of s in $\bar{z}$ and $\epsilon(z)$ negligeable in front of $z-\bar{z}$, we have:

$$s(z) = s(\bar{z}) + s'(\bar{z})(z-\bar{z}) + \epsilon(z)$$

Thus, the expected value of $s(z)$, $\mathrm{E}(s)$, is approximately equal to:

$$\mathrm{E}(s) = \mathrm{E}(s(\bar{z}) + s'(\bar{z})(z-\bar{z}))$$

Because $s(\bar{z})$, $s'(\bar{z})$, and $\bar{z}$ are constant and $\mathrm{E}(z) = \bar{z}$, it finally gives:

$$\mathrm{E}(s) = s(\bar{z})$$

Higher-degree development requires a hypothesis about the higher moments of the variable z. An exploration of a second-degree development and a conservative hypothesis on variance showed that the given equation is a very close approximation (differences only appear at the third decimal place).

Sign of the derivation of $\bar{s}$ by $\bar{z}$, by t_p and by q_p. By definitions $\bar{z}$, t_p and ν are comprised between 0 and 1, a is strictly superior to 1 and q_p can take any positive value, given equation (2):

$$\frac{\partial \bar{s}}{\partial \bar{z}} = \frac{q_p a t_p (1 - \nu)}{\left(a\bar{z} + (1 - \bar{z}) + q_p\left(t_p \nu a \bar{z} + \left(1 - t_p\right) a \bar{z} + (1 - \bar{z})\right)\right)^2} > 0$$

$$\frac{\partial \bar{s}}{\partial t_p} = \frac{q_p a \bar{z} (1 - \nu)(a\bar{z} + 1 - \bar{z})}{\left(a\bar{z} + (1 - \bar{z}) + q_p\left(t_p \nu a \bar{z} + \left(1 - t_p\right) a \bar{z} + (1 - \bar{z})\right)\right)^2} > 0$$

$$\frac{\partial \bar{s}}{\partial q_p} = \frac{-(a\bar{z} + 1 - \bar{z})\left(t_p \nu a \bar{z} + \left(1 - t_p\right) a \bar{z} + (1 - \bar{z})\right)}{\left(a\bar{z} + (1 - \bar{z}) + q_p\left(t_p \nu a \bar{z} + \left(1 - t_p\right) a \bar{z} + (1 - \bar{z})\right)\right)^2} < 0$$

AUTHOR CONTRIBUTIONS

S.A.-P.: Conceptualization, investigation, data collection, formal analysis, visualization, initial writing. P.G.: Investigation, data collection, writing. P.-O.C.: Conceptualization, writing, supervision.

DATA AVAILIBILITY STATEMENT

The data that support the findings of this study will openly be available in data.InDoRES after acceptance.

CONFLICT OF INTEREST STATEMENT

Authors declare that they have no competing interests.

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Figures

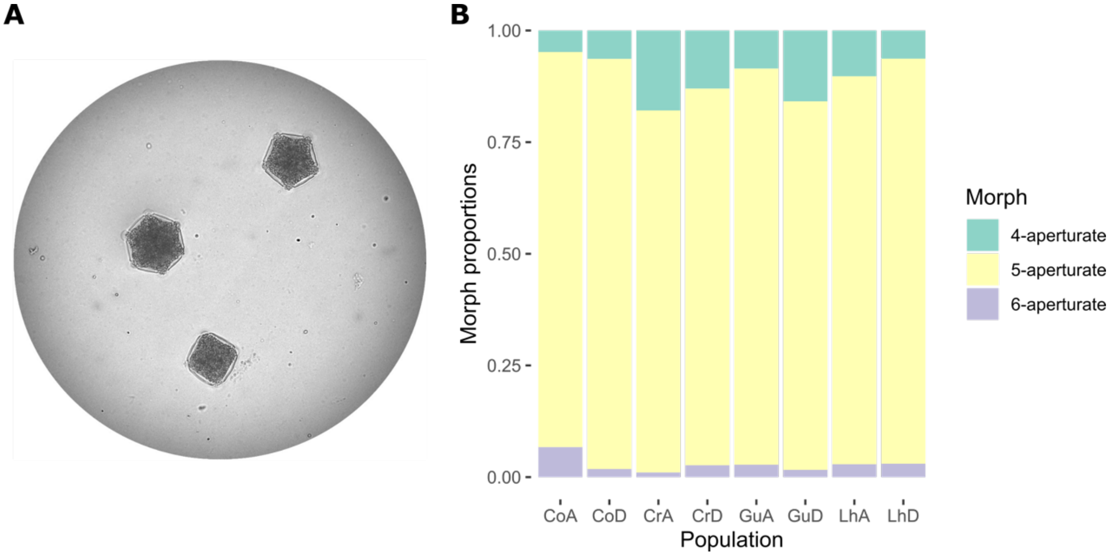

Figure 1. Pollen grain heteromorphism and population morph proportions in *Viola arvensis*. (A) Picture of three pollen grains issued from a single anther of *Viola arvensis* (optical microscope, x400). Apertures are part of the pollen grain with a thinner exine wall, corresponding to the angles. This image shows the three different types of pollen grains produced in our populations: 4-aperturate pollen (square-shaped), 5-aperturate pollen (pentagon-shaped) and 6-aperturate pollen (hexagon-shaped). **(B)** Morph proportions in each population. 400 individuals representing 160 families were measured. For exact values, see Table **2.** The population first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys), "A" is for ancestral (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and "D" for descendant (all collected in 2021).

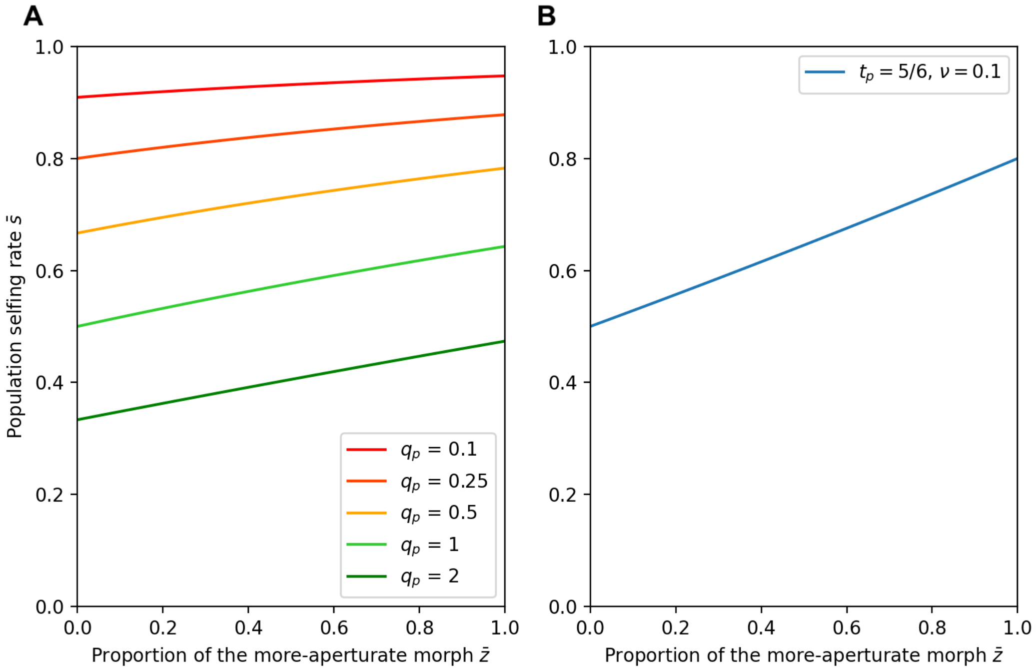

Figure 2. Theoretical relationships between pollen heteromorphism and selfing rate. These curves are graphic representation of equation (2) obtained with the constructed model. **(A)** This panel represent a set of parameters with low changes in selfing rate correlated to pollen heteromorphism changes, while changes in the relative amount of allo-pollen deposited q_p greatly affect selfing rates. Unspecified parameters are: $a = 1.5$, $t_p = 2/3$ and $\nu = 1/3$. **(B)** This panel represent a set of parameters with high changes in selfing rate correlated to pollen heteromorphism changes. Unspecified parameters are: $a = 1.5$ and $q_p = 1$.

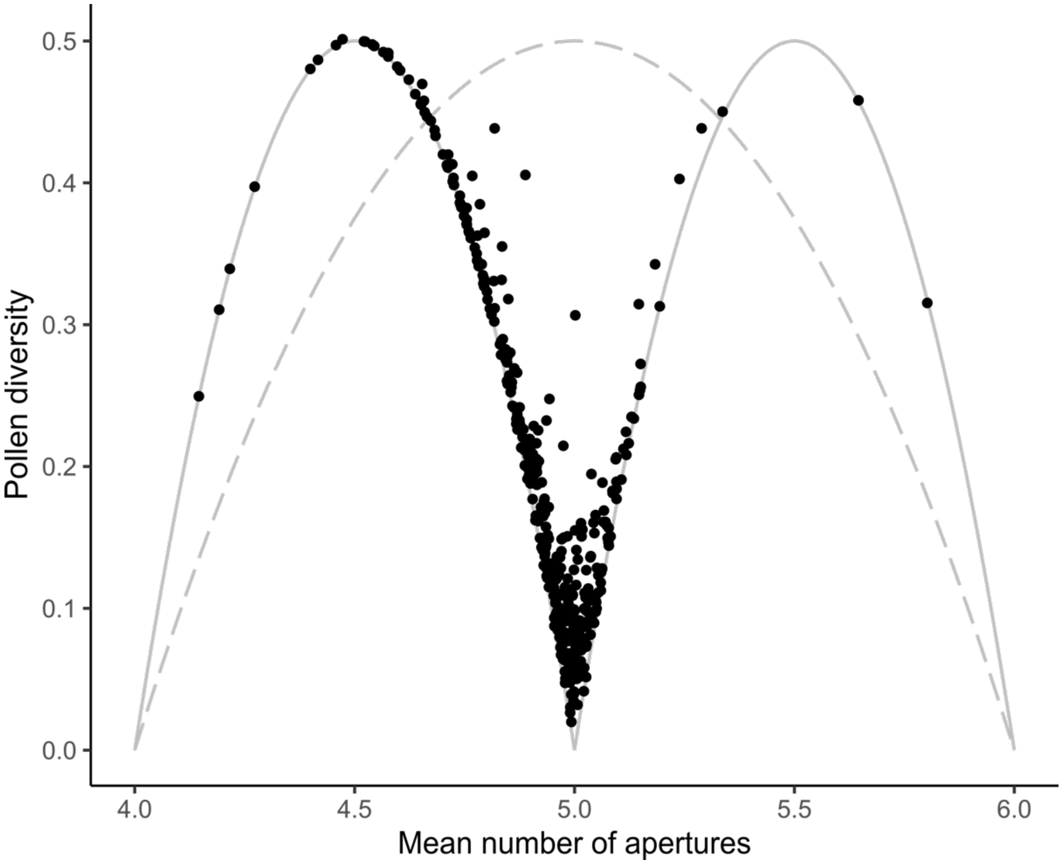

Figure 3. Patterns of pollen heteromorphism in individuals of *Viola arvensis*. Pollen diversity as a function of the mean number of apertures observed in a single anther of *Viola arvensis*. All population are pooled together and each point corresponds to a single individual (N = 400). The solid line is the expected pollen diversity in function of the mean number of apertures given only two morphs, 4- and 5-aperturate or 5- and 6-aperturate (see Material and methods). The dashed line is the expected pollen diversity in function of the mean number of apertures given only 4- and 6-aperturate morphs. Points not aligned in the grey lines are individuals presenting 4-, 5- and 6-aperturate morphs together. The highest pollen diversity could be reach for an equal balanced proportion of 1/3 of each morph, given a mean value of 5 apertures and a pollen diversity of 0.66.

Tables

Table 1. Coordinates, year of sampling and estimated selfing rates of the eight population of *Viola arvensis*. Estimation of selfing rates and 95% confidence intervals are from Acoca-Pidolle *et al.* (2024).

	Coordinates	**Year of first sampling (ancestors) and selfing rate estimated (95% CI)**	**Year of second sampling (descendants) and selfing rate estimated (95% CI)**
Commeny	49°7'37.11"N, 1°53'46.30"E	2001 0.74 (0.57–0.85)	2021 0.89 (0.66–1)
Crouy	49°24'24.31"N, 3°22'20.73"E	1993 0.66 (0.46–0.79)	2021 0.70 (0.55–0.80)
Guernes	49° 1'10.75"N, 1°38'46.82"E	2000 0.44 (0.22–0.64)	2021 0.80 (0.71–0.88)
Lhuys	49°16’35.40"N, 3°32’58.52"E	1992 0.33 (0.09–0.48)	2021 0.88 (0.78–0.95)

Table 2. Mean (SEM) of ovule and pollen traits measured. We collected a single bud per individual (*N*) just before anthesis and dissected it to count ovules and pollen grains contained inside. Statistical analyses were conducted using linear mixed models with family, date of collection and bud position as random factors. For additional information, see “Material and methods” section. The population first two letters are the name of the locality (Co = Commeny; Cr = Crouy; Gu = Guernes; Lh = Lhuys), “A” is for ancestral (collected in 2000 for Co, 1993 for Cr, 2001 for Gu and 1992 for Lh) and “D” for descendant (all collected in 2021).

Trait	***N***	**CoA** **CoD**	**CrA** **CrD**	**GuA** **GuD**	**LhA** **LhD**	**Factor**	χ^2	**df**	***P***
Number of ovules	400	80.6 (1.7) 81.7 (0.8)	75.3 (1.4) 77.9 (1.1)	79.9 (1.2) 75.3 (1.2)	76.1 (0.9) 76.3 (0.8)	Age **Locality** Age × Locality	0.117 **5.601** 2.397	1 **3** 3	0.733 **0.004** 0.090
Number of pollen grains per anther	400	746 (18) 765 (13)	681 (16) 687 (14)	779 (15) 709 (16)	722 (14) 743 (12)	Age **Locality** **Age × Locality**	0.003 **29.557** **9.502**	1 **3** **3**	0.958 **< 0.001** **0.023**
Pollen:ovule ratio	400	46.4 (0.8) 46.8 (0.7)	45.5 (0.9) 44.2 (0.8)	49.1 (1.1) 47.2 (0.9)	47.6 (0.9) 48.8 (0.7)	Age **Locality** Age × Locality	0.087 **19.357** 3.371	1 **3** 3	0.768 **< 0.001** 0.338
Pollen diversity	400	0.16 (0.02) 0.14 (0.01)	0.26 (0.02) 0.22 (0.02)	0.17 (0.02) 0.25 (0.02)	0.21 (0.02) 0.16 (0.01)	Age **Locality** **Age × Locality**	0.282 **29.049** **15.042**	1 **3** **3**	0.596 **< 0.001** **0.002**
Mean number of apertures	400	5.02 (0.03) 4.95 (0.01)	4.83 (0.02) 4.90 (0.03)	4.94 (0.02) 4.86 (0.02)	4.93 (0.02) 4.97 (0.01)	Age **Locality** **Age × Locality**	0.495 **26.470** **12.108**	1 **3** **3**	0.482 **< 0.001** **0.007**
Proportion of 4-aperturate morph	400	0.05 (0.01) 0.06 (0.01)	0.18 (0.02) 0.13 (0.02)	0.09 (0.02) 0.16 (0.02)	0.10 (0.01) 0.06 (0.01)				
Proportion of 5-aperturate morph	400	0.88 (0.02) 0.92 (0.01)	0.81 (0.02) 0.84 (0.02)	0.89 (0.02) 0.83 (0.02)	0.87 (0.01) 0.91 (0.01)				
Proportion of 6-aperturate morph	400	0.07 (0.02) 0.02 (0.00)	0.01 (0.00) 0.03 (0.01)	0.03 (0.01) 0.02 (0.00)	0.03 (0.01) 0.03 (0.01)				