

Evolution of flowering time in a selfing annual plant: Roles of adaptation and genetic drift

Laurène Gay^{1,✉}, Julien Dhinaut^{1,*}, Margaux Jullien^{1,*}, Renaud Vitalis², Miguel Navascués², Vincent Ranwez¹, and Joëlle Ronfort¹

¹AGAP, INRAE, CIRAD, IRD, Montpellier SupAgro, Univ Montpellier, Montpellier, France

²CBGP, INRAE, CIRAD, IRD, Montpellier SupAgro, Univ Montpellier, Montpellier, France

*current affiliation: Evolutionary Biology and Ecology of Algae, CNRS, Sorbonne Universités, UPMC, University of Paris VI, UC, UACH, UMI 3614, 29688 Roscoff, France

[✉]current affiliation: GQE - Le Moulon, INRA, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91190, Gif-sur-Yvette, France

1 Resurrection studies are a useful tool to measure how phenotypic traits have changed in populations and they allow testing whether these traits modifications are a response to selection caused by an environmental change. Selfing, through its reduction of effective size, could challenge the ability of a population to adapt to environmental changes. Here, we used a resurrection study to test for adaptation in a selfing population of *Medicago truncatula*, by comparing the genetic composition and flowering times across 22 generations. We found evidence for evolution towards earlier flowering times by about two days and a peculiar genetic structure, typical of highly selfing populations, where some multilocus genotypes (MLGs) are persistent through time. We used the change in frequency of the MLGs through time as a multilocus fitness measure and built a selection gradient that suggests evolution towards earlier flowering times. Yet, a simulation model revealed that the observed change in flowering time could be explained by drift alone, provided the effective size of the population is small enough (<150). These analyses suffer from the difficulty to estimate the effective size in a highly selfing population, where effective recombination is severely reduced.

24 Adaptation | Selfing | Climate change | Selection gradient | Flowering time

25 Correspondence:

26 UMR AGAP, INRAE: Bâtiment ARCAD bureau 207, 10 rue Arthur Young, 34000
27 Montpellier (FRANCE)
28 laurene.gay@inrae.fr

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33 Introduction

34 When facing changing environments, organisms can persist by one of three strategies: fleeing (migration),
35 coping (plasticity) or adapting. If migration and plasticity can lead to rapid and reversible changes in the average phenotype of a population, adaptation proceeds through genetic changes and towards phenotypes with the highest fitness in a given environment. The literature describing adaptation in natural populations is vast (e.g. Bay *et al.* 2017; Côté and Reynolds 2012; Kremer *et al.* 2012; Olson-Manning *et al.* 2012) and the recent rise of next generation sequencing has enabled

tremendous progress in our knowledge about the genetic architecture of adaptation at the species level (Barwick and Lenski 2013; Brown 2012; Fournier-Level *et al.* 2011; Jones *et al.* 2012).

Long term temporal surveys (e.g. Visser 2008), resurrection studies, where ancestors and descendants are compared under common conditions (see Box 1 in Franks *et al.* 2014) or stratified propagule banks (Orsini *et al.* 2013) are powerful tools to reconstruct the evolutionary dynamics of populations that have faced environmental changes. Yet, observing a genetic change through time is not sufficient to claim that it is adaptation. Testing for selection as opposed to drift is one of the essential criteria for demonstrating adaptive responses, but is often overlooked (e.g. overlooked in 34% of the 44 reviewed studies based on phenotypic variation reviewed by Hansen *et al.* 2012). Demonstrating the influence of selection on a phenotypic change can be achieved by one of four methods (detailed in Table 2 in Hansen *et al.* 2012; Merilä and Hendry 2014): reciprocal transplants (Blanquart *et al.* 2013), $Q_{ST}-F_{ST}$ comparisons (Le Corre and Kremer 2012; Rhoné *et al.* 2010), genotypic selection estimates (Morrissey *et al.* 2012; Wilson *et al.* 2010), or tests of neutrality (pattern or rate tests, Lande 1977). **These methods all rely on measuring quantitative traits (fitness traits or traits supposed to be under selection) but require specific experimental settings. In natural populations, unlike in experimental populations, there are no replications, and pPattern tests of neutrality, for example, are based on comparing phenotypic or allele frequency changes across replicates in experimental populations, or across populations, assuming that they are independent replicates of the evolutionary process (same effective size and selective pressure, no migration). Pattern tests can also apply through time in a natural population—rely on time-series to examine the number and ordering of positive and negative allele frequency changes in, if a long sequence of observations is available (Sheets and Mitchell 2001). Alternatively, R rate tests can be useful to—on the other hand examine the rate of genetic change in a population and**

87 compare it to the expectation under a neutral model 144
88 with a given effective population size (Lande 1976). 145
89 The effective population size (thereafter N_e) is defined 146
90 as the size of an ideal Wright-Fisher population expe- 147
91 riencing the same rate of genetic drift as the popula- 148
92 tion under consideration (Crow and Kimura 1970). Un- 149
93 like experimental populations, where N_e can be moni- 150
94 tored, an accurate estimate of N_e is required to perform 151
95 such neutrality tests in natural populations. Temporal 152
96 changes in allele frequency at neutral loci can be used 153
97 to infer the effective size of the population considered 154
98 (Nei and Tajima 1981; Waples 1989). 155

99 The ability for a population to adapt to environ- 156
100 mental changes depends on several factors such as 157
101 genetic variability, generation time, population size or 158
102 mating patterns, in particular self-fertilization rates. 159
103 In plants, a large fraction (40%) of species do, at 160
104 least partially, reproduce through selfing (Goodwillie 161
105 *et al.* 2005; Iqic and Kohn 2006). The young age of 162
106 selfing lineages is often interpreted as evidence that 163
107 selfing species lose the ability to adapt to changing 164
108 environments (Escobar *et al.* 2010; Escobar *et al.* 2001) 165
109 and have therefore higher extinction rates than 166
110 outcrossing lineages (Goldberg *et al.* 2010). Self- 167
111 ing could challenge can indeed have multiple and 168
112 complex effects with heavy repercussions on the 169
113 process of adaptation because it. First, selfing di- 170
114 rectly decreases the effective population size N_e (by 171
115 reducing the number of independent gametes 172
116 sampled for reproduction (Pollak 1987)); Selfing 173
117 also increases homozygosity; and consequently 174
118 reduces the efficacy of recombination (Nordborg 175
119 2000); which can further reduce N_e through increased 176
120 hitchhiking and background selection (Gordo and 177
121 Charlesworth 2001; Hedrick 1980)). Finally, bottlenecks 178
122 are expected to be more frequent in selfing populations 179
123 (Ingvarsson 2002; Ingvarsson 1994). It is therefore 180
124 expected that genetic variability is reduced in selfing 181
125 populations, and empirical measures of diversity from 182
126 molecular markers strongly support this prediction 183
127 (Barrett and Husband 1990; Glémin *et al.* 2006; Hamrick 184
128 and Godt 1996). Furthermore, several theoretical 185
129 models also predict that selfing limits the amount 186
130 of reduces quantitative genetic variation within popu- 187
131 lations (Abu Awad and Roze 2018; Charlesworth and 188
132 Charlesworth 1995; Lande and Porcher 2015), which 189
133 has been recently confirmed by a meta-analysis of 190
134 empirical data (Clo *et al.* 2019). 191

135 We can expect that this depleted quantitative ge- 192
136 netic variation in predominantly selfing populations 193
137 will limit their ability to adapt to changing environ- 194
138 mental conditions and their long-term persistence 195
139 and different theoretical models support this pre- 196
140 diction (Glémin and Ronfort 2013; Hartfield and 197
141 Glémin 2016; Kamran-Disfani and Agrawal 2014). 198
142 Yet, empirical data examining the response of pre- 199
143 dominantly selfing populations to environmental 200

changes remain scarce, especially for data showing short term adaptation in the face of climate change (Qian *et al.* 2020). Some experimental evidences suggest that adaptation to new environments may be fostered by outcrossing (e.g. Morran *et al.* 2009). On the other hand, it has been argued that self-fertilization may have facilitated adaptations to agricultural practices (i.e. domestication) (Glémin and Bataillon 2009; Glémin and Bataillon 2000) and could promote the divergence of ecological niches and speciation processes (Levin 2010). In a recent review focussing on evolutionary and plastic responses to climate change in plants, Franks *et al.* (2014) reported “at least some evidence for evolutionary response to climate change [...] in all of these studies”, and six of these 31 studies considered regardless of the mating system of the focal species. This pleads for an absence of difference in the adaptive potential to environmental change in selfing compared to outcrossing populations, even though only 6 studies focussed on selfing populations. (in respect to 25 outcrossing populations). Similarly, in a review focussing on the effect of the mating system on local adaptation, Hereford (2010) concluded that “despite limited genetic variation in selfing species and greater potential for gene flow in outcrossing species, mating system has little influence on adaptation of populations.”

Because there is no consensus between theoretical predictions, empirical and experimental data, the ability of selfing populations to adapt to environmental changes remains an open question. This calls for further fine scale population genetics analyses, with a focus on the evolutionary mechanisms involved and on the dynamics of adaptation. Here, we present a temporal survey in the barrel medic (*Medicago truncatula*) that enabled us to perform a resurrection study. *M. truncatula* is annual, diploid, predominantly self-fertilizing (>95% selfing, Bonnin *et al.* 2001; Siol *et al.* 2008) and has a circum-Mediterranean distribution. Flowering time is a major heritable trait (>0.5, Bonnin *et al.* 1997) that synchronizes the initiation of reproduction with favourable environmental conditions and could play a role in the adaptation to climate change. In *M. truncatula*, flowering time is highly variable along the distribution range and within some populations (Bonnin *et al.* 1997). It is mainly controlled by two environmental cues: photoperiod and temperature (Hecht *et al.* 2005; Pierre *et al.* 2008). In the Mediterranean region, there has been a significant increase in temperatures between the 80s and nowadays accompanied by a decrease in mean precipitations (<http://www.worldclim.org/>). Most studies about adaptation in *M. truncatula* have so far relied on large collections of individuals representing the whole species with the aim of detecting selection footprints in the genome linked with flowering time (Burgarella *et al.* 2016; De Mita *et al.* 2011) or climatic gradients (Yoder *et al.* 2014). However, the complex popu-

201 lation structure observed at the species level can make 257
 202 it difficult to understand the selective history of those 258
 203 genes (De Mita *et al.* 2007). Indeed, natural populations 259
 204 of *M. truncatula* are composed of a set of highly differ- 260
 205 entiated genotypes that co-occur at variable frequencies 261
 206 (Bonnin *et al.* 2001; Loridon *et al.* 2013; Siol *et al.* 2008), 262
 207 a genetic structure typical for predominantly species. 263
 208 How does this peculiar genetic composition constrain 264
 209 adaptation to changing environments remains unclear, 265
 210 but preliminary results in *M. truncatula* have shown 266
 211 that surveying the multilocus genotypic composition 267
 212 through time could reveal a large variance in the rel- 268
 213 ative contributions of these genotypes to the next gen- 269
 214 erations (Siol *et al.* 2007). Here, we examined the tem- 270
 215 poral change of flowering time at the population level 271
 216 across 22 generations characterised by changing envi- 272
 217 ronmental conditions (temperature and rainfall). We 273
 218 describe the peculiar genetic structure of this highly 274
 219 selfing species and investigate the genetic mechanisms 275
 220 involved in adaptation. In particular, we test for the 276
 221 role of selection as opposed to genetic drift, following 277
 222 four steps. First, we consider the direction of the change 278
 223 in trait value in relation to the environmental change. 279
 224 Second, we estimate the extent of genotypic selection 280
 225 (Morrissey *et al.* 2012; Wilson *et al.* 2010) using selection 281
 226 gradients for flowering time based on several fitness es- 282
 227 timates (taking into account the multilocus genotypic 283
 228 composition of the population through time). Then we 284
 229 estimate the effective population size, test the rate of 285
 230 evolution for neutrality and explore the effect of the im- 286
 231 precision in the estimation of effective size. Finally, we 287
 232 examine the change in flowering time during the same 288
 233 time period at the regional scale, using one individual 289
 234 per population across the distribution range of *M. trun-* 290
 235 *catula* in Corsica. A similar genetic change at the re- 291
 236 gional scale would lend weight to the hypothesis that 292
 237 the change in flowering time occurred in response to 293
 238 selection. 294

239 Materials and Methods 295

240 **Studied population and experimental design.** The foc- 297
 241 us population (F20089 or CO3 according to Jullien 298
 242 *et al.* 2019) is located in Cape Corsica (42°58.406'N - 299
 243 9°22.015'E). In 1987 and 2009, around 100 pods were 300
 244 collected along three transects running across the pop-
 245 ulation, with at least one meter distance between each
 246 pod collected, in order to avoid over-sampling the
 247 progeny of a single individual. Seeds collected in 1987
 248 were stored in a cold room. In 2011, all pods pods col-
 249 lected in 1987 and 2009 were threshed and 100 seeds
 250 per sampling year were replicated through selfing in
 251 standardized greenhouse conditions to control for ma- 301
 252 ternal effects and build families of full-sibs produced 302
 253 by selfing. **64 families collected in 1987 and 96 in 2009** 303
 254 **were successfully multiplied.** In 2012, **55** 304
 255 families for each of the two sampling years were ran- 305
 256 domly chosen in 2012 from the 100 plants replicated. 306

Seeds from the 110 families were scarified to ease ger-
 mination and were transferred in Petri dishes with wa-
 ter at room temperature for six hours. We then used
 two different vernalization treatments (at 5°C during 7
 or 14 days) to compare the vernalization requirement
 between the two years. Five replicates from each ver-
 nalization treatment were transferred back to the green-
 house, according to a randomized block design (five
 blocks and two treatments, adding up to a total of ten
 replicates per family, 1100 plants in total). Data loggers
 were placed on each table to monitor temperature and
 humidity. For each individual, the number of days after
 germination to form the first flower was recorded. In
 addition, the total number of seeds produced by each
 plant throughout its lifetime was measured as a proxy
 for fitness.

Temporal changes in flowering time. Individual flow-
 ering times were converted to thermal times following
 Bonhomme (2000). The thermal time was calculated
 as the sum of the mean daily effective temperatures of
 each day between sowing and the emergence of the first
 flower, where the mean daily effective temperature is
 the day's mean temperature minus the base tempera-
 ture (T_b). We used $T_b = 5^\circ\text{C}$, as reported by Moreau
et al. (2007) for the *Medicago truncatula* reference line
 A17. Plants noted as sick or failing to produce leaves
 were removed from the data sets (22 individuals re-
 moved). Collected measures were tested for normality
 using quantile–quantile (Q-Q) plots (Nobre and Singer
 2007). All analyses were conducted using R version
 2.15.2. We used linear mixed models (lme4 package) to
 test for a significant change in flowering time between
 the sampling years. The model included two fixed ef-
 fects: sampling year (1987 or 2009) and treatment (short
 or long vernalization) as well as their interaction. Block
 (nested in treatment), block \times year and family were ran-
 dom effects. The family effect was nested in years be-
 cause we were interested in estimating the genetic vari-
 ance within population each year of collection. The in-
 teraction between family and treatment was included
 in the family effect as a vectorial random effect. The
 complete model is summarized in equation [1], where
 Y denotes the flowering time, μ the average flowering
 time over the whole sample and ϵ the residuals:

$$Y_{ijkl} = \mu + year_i + treatment_j + year_i \times treatment_j \\ + block_k + year_i \times block_k \\ + family_l | (year_i \times treatment_j) + \epsilon_{ijkl} \quad (1)$$

This maximal model was simplified, using likelihood
 ratio tests (LRT) to compare the models. In addition, we
 tested for a significant change in genetic variance be-
 tween 1987 and 2009 using a LRT between the model [1]
 and a model where family is not nested into year. Stan-
 dard errors for variance components were estimated

using jackknife resampling. We used the variance components estimated for the random effects to calculate broad-sense heritability as $H^2 = \frac{V_G}{V_P}$, where V_G is the genetic variance as estimated by the family effect and V_P is the total phenotypic variance, including block, family and residual variance. Standard errors for H^2 were estimated through jackknife resampling on families (Sokal and Rohlf 1995).

Temporal changes in sensitivity to vernalization. Selection on a trait in an environment can shift both the mean and the plasticity of that trait. Here, we considered the sensitivity to vernalization cues, measured as the slope of the regression line between individual values and the environmental value (estimated as the average phenotype, \bar{Y}) (Falconer and Mackay 1996), for each individual i :

$$\frac{Y_i^{long\ vernalization} - Y_i^{short\ vernalization}}{\bar{Y}^{long\ vernalization} - \bar{Y}^{short\ vernalization}}$$

This coefficient assumes that reaction norms are linear (Gavrilets and Scheiner 1993; Scheiner 1993) and this approximation is expected to work well (Chevin *et al.* 2013). We used a linear mixed model, with sampling year (1987 or 2009) as a fixed effect, a random block effect and its interaction with year, and a family effect (genetic effect) nested into year. As for flowering time, we estimated the broad sense heritability of the vernalization sensitivity.

A genetic correlation between flowering time and sensitivity to vernalization would affect the response to selection in the context of climate change. We therefore used a bivariate model with the sensitivity to vernalization and the flowering time measured in the short vernalization treatment as two dependent variables to estimate their genetic covariance with a random family effect, including block as a random effect, using AsReml (Gilmore *et al.* 2009). We ran an independent model for each sampling year. The significance of genetic covariances was tested by comparing the residual deviance of the final model with that of a model with a fixed covariance of zero in a log-likelihood ratio test.

Selection gradient for flowering date: genetic covariance analysis. In the absence of selection for the trait considered, its observed variation is expected to be independent from fitness. We tested this by measuring the selection gradient, i.e. the statistical relationship between a trait and the fitness. Selection gradients were established for each year (and per treatment) following the Robertson-Price identity that states that ΔZ , the expected evolutionary change in the mean phenotypic trait z per generation is equal to $\Theta_a(z, w)$, the additive genetic covariance of the trait z and the relative fitness w (Price 1970; Robertson 1966):

$$\Delta Z = \Theta_a(z, w) \quad (2)$$

Here, we estimated the broad sense genetic covariance Θ_g . Assuming that the dominance variance is negligible due to the very high levels of homozygosity in selfing populations (Holland *et al.* 2010), genetic covariance should be a good approximation of the additive genetic covariance. As a preliminary step, we checked whether our proxy for fitness, the relative seed production, had significant genetic variance. The relative seed production was measured as the individual seed production standardized by the average seed production of individuals from the same year and treatment. A mixed model was used to analyse the relative seed production, including two random effects for block and family. Then, provided there was significant genetic variance for relative seed production in the population each year, we analysed it in a bivariate model with flowering time to estimate the genetic covariance with a random family effect, including block as a random effect, using AsReml (Gilmore *et al.* 2009). Again, the significance of genetic covariances was estimated by comparing the residual deviance of the final model with that of a model with a fixed covariance of zero in a log-likelihood ratio test.

Genetic analyses. During the multiplication generation in the greenhouse (2011), 200 mg of leaves were sampled from each plant for DNA extraction, using DNeasy Plant Mini Kit (Qiagen). Twenty microsatellite loci were used for genotyping (see the details of amplification reactions and analyses of amplified products in Jullien *et al.* 2019; Siol *et al.* 2007). Briefly, samples were prepared by adding 3 μ l of diluted PCR products to 16.5 μ l of ultrapure water and 0.5 μ l of the size marker AMM524. Amplified products were analyzed on an ABI prism 3130 Genetic Analyzer and genotype reading was performed using GeneMapper Software version 5.

1. Single-locus analyses assuming independence among loci. As a preliminary step, the data was filtered to reduce the percentage of missing data (loci or individuals with >10% missing data were removed), and to discard monomorphic loci. After filtering, the dataset comprised 145 individuals and 16 loci (64 from the year 1987 and 81 from the year 2009). We measured the genetic diversity of the population each year using the allelic richness N_{a-rar} (Hurlbert 1971) and the expected heterozygosity H_e . In this predominantly selfing population, we expect a strong deviation from Hardy-Weinberg heterozygosity expectations. Thus, for each sampling year, we estimated the inbreeding fixation coefficient F_{IS} and its confidence interval using 5,000 bootstraps over loci. Between year differences for N_{a-rar} , H_e and F_{IS} across loci were tested using Wilcoxon signed-rank tests. Analyses were performed in R using the packages adegenet (Jombart 2008) and hierfstat (Goudet 2005) and the program ADZE for rarefaction analyses (Szpiech *et al.* 2008). The percent-

age of pairs of loci showing significant linkage disequilibrium (LD) was calculated using Genepop (Rousset 2008) with a threshold of 0.05. Finally, we measured the temporal variance in allele frequencies using the F_{ST} estimator by Weir and Cockerham (1984). To estimate the effective population size (N_e , measured in number of diploid individuals) from the temporal variance of allele frequencies, we used F_{ST} estimates to account for the correlation of alleles identity within individuals due to selfing (Navascués *et al.* 2020) and followed the method outlined in Frachon *et al.* (2017). We measured a confidence interval for N_e using an approximate bootstrap method (DiCiccio and Efron 1996) over loci.

.2. Analyses based on multilocus genotypes. We used the program RMES to estimate selfing rates from the distribution of multilocus heterozygosity (David *et al.* 2007). We tested for a difference in selfing rates between years using a likelihood ratio test between models where the selfing rate was constrained to be constant or not. For each sample (1987 and 2009), we examined the genetic structure by sorting out the number of multilocus genotypes (thereafter called MLG) and measuring their frequency and redundancy through time using GENETHAPLO (available on GitHub at <https://github.com/laugay/GenetHaplo> and described in Supplementary Material S1). GENETHAPLO takes into account the uncertainty of the assignment of a genotype to a MLG group due to missing data: in case of ambiguity, an individual is randomly assigned to one of the candidate MLG group with a probability proportional to the MLG group size. The approach also considers a genotyping error rate: if two individuals differ by less than the error rate, they are considered to belong to the same MLG. After an initial run with an error rate of zero, we checked the distribution of the distances between MLGs. We found an excess of small distances, which could indicate errors in genotype assignment (Arnaud-Haond and Belkhir 2007). We corrected this by re-running the program with an error rate of 1/16 (= one mis-read locus). GENETHAPLO also searches for residual heterozygosity (defined as the proportion of heterozygous loci in the multilocus genotype) and evidence for recombination (S1). To identify putative recombination events between MLGs, it uses the genetic distances: a MLG is a recombinant candidate if the sum of its allele differences with two other MLGs (“parental MLGs”) equals the number of allele differences between these two parental MLGs. If a MLG has a high fitness in a given environment, plants carrying this MLG will produce on average a larger progeny and the frequency of the MLG will rise in the following generations. We therefore propose to use the absolute change in frequency of the fully homozygous MLGs through time as an indicator of their “realised fitness”. As a preliminary step, we **checked whether selection quantified in the greenhouse is**

likely to mirror selection in the field at present and 22 years ago using a linear model to verify whether the change in MLG frequencies covaries positively with and can be predicted by the seed production in the greenhouse. We then measured the selection gradient for flowering time as the slope of the regression of the change in frequency of the MLGs between 1987 and 2009 with flowering time. We compared this pattern with the predictions from the Robertson-Price selection gradient. The MLGs found in 2009 but absent in 1987 may have been undetected in 1987 due to low frequency, or may be recent migrants. Their change in frequency between 1987 and 2009 is thus necessarily positive and may not accurately reflect their realised fitness. We therefore reiterated these analyses using a dataset restricted to the MLGs present in 1987 only. **For each of these models, we verified the normality of the residuals and estimated a confidence interval for the slope using profile likelihood confidence bounds.**

In addition, we tested whether the change in frequencies of the MLGs reflects a response to selection or can be expected by drift alone. This was tested by simulating the effect of 22 generations of drift, using an extension to multiallelic data of the approach described in Frachon *et al.* (2017) and inspired by Goldringer and Bataillon (2004). Again, only the fully homozygous MLGs were kept for this analysis. We assumed complete selfing during the time interval so the whole genome behaves as a single super-locus. Details about the procedure used to simulate individual MLG frequency trajectories are provided in Supplementary Material S2. We simulated each generation of drift by drawing MLG counts from a multinomial distribution parameterized with the effective population size N_e estimated from the temporal F_{ST} , and the MLG frequencies in the previous generation. After 22 simulated generations, we randomly sampled 75 individuals to estimate the frequencies of each MLG and measured the change in MLG frequencies across the 22 generations. This was repeated for 10^4 replicates in order to draw the distribution of the change in MLG frequency expected by drift alone. To account for the potentially large estimation variance for the F_{ST} (as observed in the simulations performed in Supplementary Material S3), we examined the sensitivity of the analysis to the effective population size using a range of values ($10 \leq N_e \leq 500$). Finally, we examined the simulated selection gradient as the relationship between the simulated changes in MLG frequencies through time and the genetic value of flowering time previously measured for each MLG, using a linear model. This provided us with a null distribution of the slopes of the regression between frequency change and flowering time, expected under drift alone. We then tested for the significance of the observed slope against the simulated distribution, by **counting** the proportion of the simulated slopes that were greater than the observed

527 value. 566

528 **Regional analysis.** Finally, we attempted to disentangle 568
 529 selection and drift by considering other populations lo- 569
 530 cated in the same geographical region as the focal pop- 570
 531 ulation and therefore likely submitted to the same selec- 571
 532 tive pressure due to climatic constraints (**pattern test, as** 572
 533 **described in the Introduction**). For this regional anal- 573
 534 ysis, we used 16 populations of *M. truncatula* across 574
 535 Corsica that were sampled twice, once in the 80's and 575
 536 again in the early 2000s (listed in Table S1). Samples 576
 537 consisted of around 100 pods collected along transects 577
 538 running across the populations. Seeds collected were 578
 539 stored in a cold room. In 2010, one pod randomly se- 579
 540 lected from each sample (80's and 2000's) was threshed 580
 541 and one plant per population per year was replicated 581
 542 through selfing in standardized greenhouse conditions. 582
 543 This greenhouse generation allowed suppressing po- 583
 544 tential maternal effects (**as in the experiment with the** 584
 545 **Cape Corsica population**) and resulted in 32 families 585
 546 (16 populations × 2 years) of full-sibs produced by self- 586
 547 ing. In 2011, seeds from the 32 families were germi- 587
 548 nated following the same protocol as described earlier 588
 549 for the intra-population analysis, but with only one ver- 589
 550 nalization treatment at 5°C during seven days. Five 590
 551 plants for each family were then transferred to tables 591
 552 in the greenhouse according to a randomized block de- 592
 553 sign (five blocks). We monitored the temperature and 593
 554 humidity and the flowering time for each plant. 594
 Individual flowering times were converted in thermal 595
 time, in the same way as it was done for the intra- 596
 population analysis. Again, we used linear mixed mod- 597
 els (lme4 package) to test for the effect of sampling year 598
 on flowering time. The model included a single fixed 599
 effect for the sampling year (1980s or 2000s). The block 600
 effect was included as a random effect, along with its 601
 interaction with sampling year. A random population 602
 effect was also included and replaced the "family" ef- 603
 fect of Eq. 1 seen as there was only one family per year 604
 in this regional sample. The resulting model was writ-
 ten as:

$$Y_{ijk} = \mu + year_i + block_j + year_i \times block_j + population_k + \epsilon_{ijk} \quad (3)$$

555 Again, this maximal model was simplified using likeli-
 556 hood ratio tests.

557 Results

558 **Changes in flowering time.** Visual inspection of the
 559 Q-Q plots indicated that the residuals from all the
 560 linear models we used were normally distributed.
 561 We found that flowering time differed significantly
 562 between years: plants sampled in 2009 flowered on
 563 average over two days earlier than plants sampled in
 564 1987 (Table 1, Fig. 1). **This effect remained significant**
 565 **when we analysed flowering time as a number of**

days rather than degree.days (results not shown).
 Longer vernalization also **sped** flowering up (treat-
 ment effect, Table 1). The block effect only explained a
 low proportion of variance (micro-environment) and
 the largest variance component was the family effect,
 for all combinations of years and treatments. The com-
 parison of a model where family was nested in years
 only or in years × treatments showed that the family
 × treatment interaction was significant ($\chi^2 = 66.1$; $df = 7$; $p = 9.10^{-12}$). It means that the reaction norms
 for the different genotypes were not parallel (Fig. 1),
 because the genotypes responded differently when
 exposed for a shorter period to cold temperatures. To
 account for this genotype × environment interaction,
 the heritability for flowering time was estimated in
 each vernalization treatment separately (four compo-
 nents of variance, Table 1). It varied between 0.53 and
 0.77 (Table 2). The genetic variance for flowering time
 in the population remained the same in 1987 and 2009,
 as shown by a LRT between the full model (Eq. 1) and
 a model where family was not nested in year ($\chi^2 = 6.65$;
 $df = 7$; $p = 0.47$). We found no significant year
 effect on the sensitivity to vernalization ($\chi^2 = 1.7$;
 $df = 1$; $p = 0.185$). There was no significant difference
 in the family effect between years (interaction family ×
 year not significant; LRT: $\chi^2 = 1.2$; $df = 2$; $p = 0.552$)
 but the family effect was highly significant ($\chi^2 = 32.6$;
 $df = 1$; $p = 1.10^{-8}$, Table S2) and the heritability of
 the sensitivity to vernalization was 0.19 (+/- 0.04)
 (Table 2). Finally, the multivariate analysis highlighted
 a strong positive genetic correlation between flowering
 time (measured in the short vernalization treatment)
 and the sensitivity to vernalization (in 1987: 0.54 $p = 0.008$;
 in 2009: 0.60 $p < 0.0001$), which means that early
 flowering plants are less sensitive to vernalization
 cues. Using the flowering time measured in the long
 vernalization treatment, we observed the same pattern
 of correlation.

Table 1. Effect of sampling year and treatment on flowering time in the cape Cor-
 sica population, taking into account the family effect (genetic effect). Effect values
 on mean flowering time are given for fixed effects and variance components are
 given for random effects (with standard errors in brackets). The family effect was
 nested into year (1987 or 2009) and treatment (T1: short vernalization treatment;
 T2: long vernalization treatment), leading to four variance components. For each
 component, the degrees of freedom, likelihood ratio (χ^2) and p -values are reported.
 None of the interactions considered in the complete model [1] were significant: be-
 tween year and treatment (LRT $\chi^2 = 1.8$; $df = 1$; $p = 0.178$); between block and year
 ($\chi^2 = 0.0006$; $df = 1$; $p = 0.981$).

Tested effect on flowering time	Mean effect or variance component (SE)	df	χ^2	p
year	-28.76*	1	7.3	0.007
treatment	-162.84	1	42.2	8.10 ⁻¹¹
block	92.34 (9.61)	1	34.5	4.10 ⁻⁹
	1987-T1: 2807.90 (872.97)			
family year × treatment	1987-T2: 1793.51 (500.25) 2009-T1: 5449.80 (1200.16) 2009-T2: 3557.01 (1408.88)	10	850.4	2.10 ⁻¹⁶
error	1500 (38.73)	1081		

* assuming an average daily temperature of 15°C over the time period considered, the difference of 28.76 degree.days corresponds to two days.

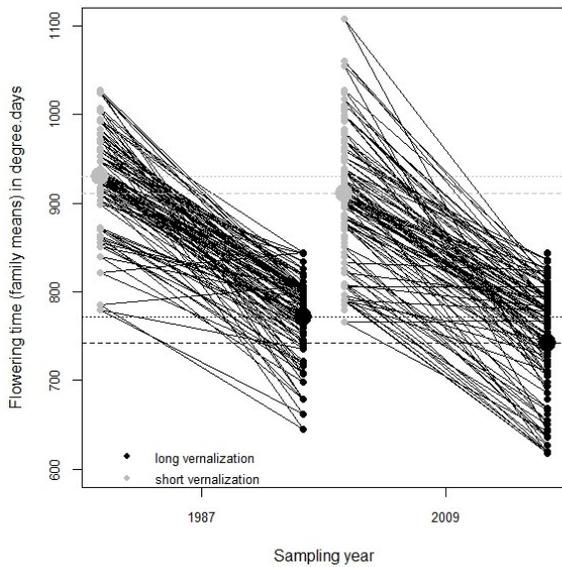


Fig. 1. Average flowering time per family for the two sampling years and the two vernalization treatments. Short vernalization is in grey and long vernalization in black. The large dots and the horizontal lines stand for the average flowering date for each vernalization treatment, for the years 1987 (dotted lines) or 2009 (dashed lines). Black crossing lines indicate that the reaction norms differ between families, as expected if genotype \times environment interactions are significant.

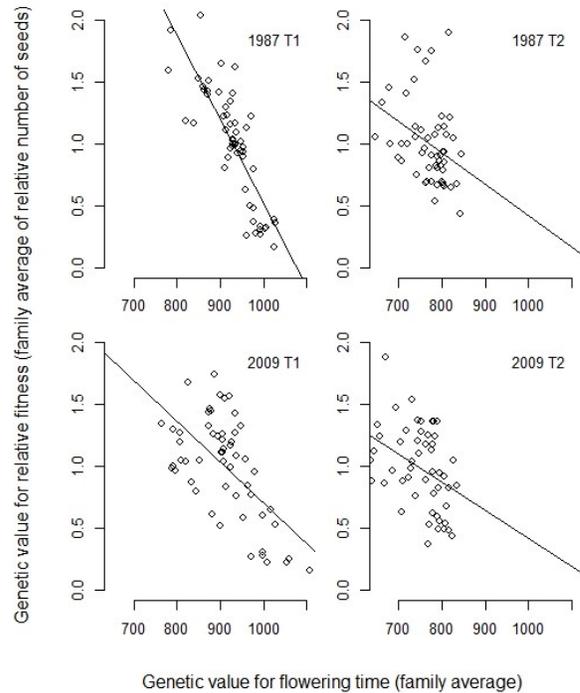


Fig. 2. Selection gradients for flowering time. Established as the relationship between the genetic value for flowering time (family average, in degree.days) and the genetic value for relative fitness (family average of the relative number of seeds), for each sampling year and vernalization treatment. Lines stand for the linear regression.

Selection gradient for flowering date. The relative seed production showed significant genetic variance (family effect, Table S3, heritability of 0.34, Table 2), which enabled us to build multivariate models to examine selection gradients following Eq. 2. In 1987, we found a significant genetic covariance between flowering time and relative fitness: $\Theta_a(z, w) = -20.5$; LRT comparing this model with a model where the genetic covariance was constrained to be zero: $\chi^2 = 60.2$; $df = 1$; $p = 8.10^{-15}$. The covariance remained significantly different from zero when we used the lines derived from the sampling in 2009: $\Theta_a(z, w) = -18.5$; LRT: $\chi^2 = 12.4$; $df = 1$; $p = 6.10^{-7}$. This covariance was a similar negative in both relationship was observed among lines derived from each of the two years, which means that the selection gradients predict an evolution towards early flowering (Fig. 2).

Table 2. Heritabilities (H^2) and coefficients of genetic variance (CV_g) for flowering time in each vernalization treatment (T1: short vernalization; T2: long vernalization) and each sampling year, for sensitivity to vernalization and for relative seed production.

Trait	H^2 (SE)		CV_g	
	1987	2009	1987	2009
Flowering time	T1: 0.64 (0.06) T2: 0.53 (0.07)	T1: 0.77 (0.04) T2: 0.69 (0.07)	5.70 5.49	8.11 8.03
Sensitivity to vernalization	0.19 (0.04)		18.14	
Relative seed production	0.34 (0.03)		30.00	

Changes in the genetic composition of the population.

The analysis of microsatellite data highlighted high levels of genetic diversity for both sampling years, with an increase between 1987 and 2009 only significant for H_e (Table S4). This suggests that the increased diversity between 1987 and 2009 reveals more balanced allele frequencies rather than an increase in the average number of alleles. The temporal differentiation measured using the 16 loci was high ($F_{ST} = 0.226$; 95% confidence interval: 0.182 – 0.269), which translates into a particularly small effective size ($N_e = 19$ diploid individuals; 95% confidence interval: 15-25). According to [Eq. 16] in Nordborg and Donnelly (1997), we predict that $H_e = 1 - \frac{1}{1+4N_e\mu}$, where N_e is the effective size as estimated above. Using mutation rates for dinucleotide microsatellite loci measured in *Arabidopsis thaliana* (5.10^{-5} to 2.10^{-3}) (Marriage *et al.* 2009), and assuming an isolated population at equilibrium, we expect that H_e should lie between 0.004 and 0.134, which is nearly three times lower than the H_e estimated here (Table S4). The observed heterozygosity was particularly low, resulting in large F_{IS} estimates, as expected for a predominantly selfing species. The estimated selfing rate was about 94% in 1987 and rose to 98% in 2009 (statistically significant increase, Table S5). This high selfing rate results in extensive linkage disequilibrium between loci (nearly all pairs of loci are in linkage disequilibrium, Table S4), which makes the analysis of multilocus genotypes particularly relevant.

652 The analysis of MLG identified 60 different MLGs in
 653 this sample of 145 individuals. Out of the 60 MLGs,
 654 48 were fully homozygous at the 16 loci and 12 MLGs
 655 displayed some level of heterozygosity (Fig. S1). We
 656 found no evidence for a link in terms of recombina-
 657 tion or segregation between the heterozygous MLGs
 658 and any of the fully homozygous MLGs. These het-
 659 erozygous MLGs were therefore excluded from the fol-
 660 lowing analyses, leaving us with 48 MLGs (58 indi-
 661 viduals in 1987 and 75 in 2009). The two predom-
 662 inant MLGs represented more than 50% of the pop-
 663 ulation in 1987 and nearly 20% in 2009. These, as
 664 well as ~~two~~three other MLGs, were persistent through
 665 time. The absolute changes in homozygous MLGs fre-
 666 quencies through time tended to covary positively with
 667 the total number of seeds produced by a plant in the
 668 greenhouse (Fig. 3A, regression only significant with
 669 the sample restricted to the MLGs present in 1987),
 670 which provides support to use it as a proxy to esti-
 671 mate the realised fitness. We therefore used the change
 672 in frequency of the 48 MLG (58 individuals in 1987
 673 and 75 in 2009) to build selection gradients for flow-
 674 ering time. Again, we found a gradient with a nega-
 675 tive slope (Fig. 3B), suggesting that the late flower-
 676 ing MLGs have a reduced realised fitness compared to
 677 earlier ones. This confirms the reduced fitness of late
 678 flowering genotypes observed in our greenhouse exper-
 679 iment (Fig. 2). Yet, the effect of flowering date on the
 680 realised fitness was small and only significant when the
 681 dataset was restricted to the MLGs present in 1987 ($n =$
 682 12; Fig. 3B). In addition, the negative slope was mostly
 683 supported by the decreasing frequency of the two late
 684 flowering MLGs that were prevalent in 1987.

685 The simulation of 22 years of drift with an effective pop-
 686 ulation size of 19 showed that the slope of the observed
 687 selection gradient did not deviate significantly from the
 688 distribution expected by drift alone ($p = 0.182$). Yet,
 689 again, when we restricted the dataset to the MLGs that
 690 were present in 1987, the observed selection gradient
 691 deviated significantly from the distribution expected by
 692 drift alone ($p = 0.047$), which suggests that the drift-
 693 alone hypothesis could be rejected.

694 Because selfing reduces the effective recombination, it
 695 reduces the number of independent loci. Measuring
 696 F_{ST} from linked loci therefore amounts to measuring
 697 it from a lower number of markers, and it is known
 698 that F_{ST} estimates based on a few loci suffer from a
 699 large sampling variance (Weir and Hill 2002). Alter-
 700 natively, we could have concatenated the genotypes
 701 at the different loci to compute a diploid version of
 702 the haplotype-based F_{ST} (Mehta *et al.* 2019). Using
 703 the changes of frequencies for 48 homozygous MLGs,
 704 we estimated a temporal F_{ST} of 0.075, which corre-
 705 sponds to an estimated effective size of 136. However,
 706 our simulations (Supplementary Material S3) show
 707 that these haplotype-based F_{ST} estimates are strongly
 708 downwards biased, due to the dependency of F_{ST} with

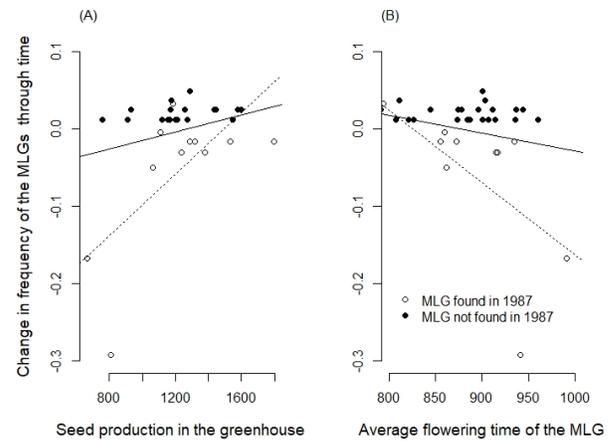


Fig. 3. Analyses of the “realised fitness”, estimated as the absolute change in frequency of the MLGs through time. MLGs with residual heterozygosity were removed from this analysis. (A) Relationship with the average number of seeds produced by plants of a given MLG in the greenhouse. (B) Selection gradient for flowering time. Each point stands for the average flowering date for a given MLG. The black regression lines are estimated using all points ($n = 48$; A: slope = $5.10^{-5}0.029$ points of frequency per seed $p = 0.0940.395$; B: slope = -0.0002 **95% confidence interval: $-0.0006;0.0001$** $p = 0.179$). This includes MLGs that were not observed in 1987 (black dots), for which the change in frequency is necessarily always positive. The dotted lines are the regression lines for the analysis restricted to the MLGs present in 1987 (white dots only; $n = 12$; A: slope = $0.00020.167$ $p = 0.0240.035$; B: slope = -0.0009 **95% confidence interval: $-0.0017;-0.0002$** $p = 0.038$).

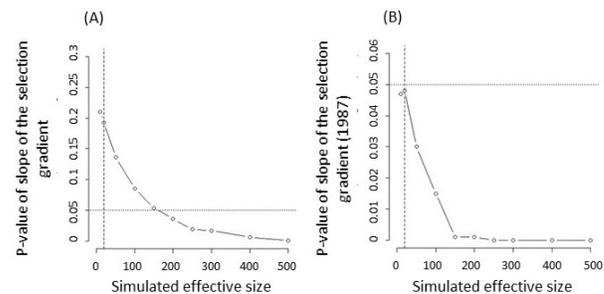


Fig. 4. Test of selection for increasing values of N_e . P -value, defined as the proportion of simulated datasets where the slope of the selection gradient is steeper than the observed slope, for the simulations of drift-alone (A) considering all the homozygous MLGs ($n = 48$) or (B) considering only the MLGs that were already present in 1987 ($n = 12$). The dotted line indicates the 0.05 threshold value for significance. The vertical dashed line is the effective size estimated using the temporal F_{ST} and considering the 16 microsatellite loci as independent ($N_e = 19$; $p = 0.182$ with $n = 48$ (A); $p = 0.047$ with $n = 12$ (B)).

allelic diversity (Alcala and Rosenberg 2017; Edge and Rosenberg 2014; Jakobsson *et al.* 2013) and could therefore overestimate the effective population size. Instead of using this unreliable estimate of 136, we assessed the sensitivity of our neutrality test for MLG frequency changes to the effective population size estimates, using a range of values ($10 \leq N_e \leq 500$). We found that the observed selection gradient can no longer be explained by drift alone if the effective population size exceeds 150 (or even 10 if we consider only the MLGs present in 1987, Fig. 4).

Changes in flowering time at the regional level. At the regional level (Eq. 3), we found no effect of the interaction between block and sampling year (LRT χ^2

= 0; df = 1; $p = 1$). All other effects were significant (Table 3): the random block effect only explained 5% of the total variance whereas the population effect accounted for 34% of variance. The significant year effect showed that the material we collected in 2005 or 2009 in Corsica flowered about five days earlier (78 degree.days, Table 3) compared to the one we collected between 1987 and 1990.

Table 3. Effect of sampling year on flowering time at the regional scale, taking into account the population effect. The effect on the mean flowering time is given for the fixed year effect and variance components are given for random effects (with standard errors in brackets). For each component, the deviance, degrees of freedom, likelihood ratio (χ^2 and p -values are reported.

Tested effect on flowering time	Mean effect or variance component (SE)	df	χ^2	p
year	-78.00*	1	9.3	0.002
block	2379 (1029)	1	5.7	0.017
population	14874 (4423)	1	40.1	$2.10 \cdot 10^{-10}$
error	26971 (8260)	167		
Total variance	44224			

* assuming an average daily temperature of 15°C over the time period considered, the difference of 78.00 degree.days corresponds to five days.

Discussion

Pairing up a resurrection study with population genetic analyses proved highly insightful to understand how flowering time changed through time in *M. truncatula* and to get insights into the mechanisms involved. Growing plants collected in the Cape Corsica population 22 generations apart in a common garden experiment provided evidence for a diminution of flowering times by about two days (i.e. a reduction between 2 and 4% in flowering time). This study also highlighted the peculiar genetic structure of this highly selfing population, where some multilocus genotypes are persistent through time. This enabled us to measure the fitness of a genotype as its frequency change through time and to establish a multilocus selection gradient. We used this multilocus fitness measure as well as a fitness measure based on individual seed production in the greenhouse to estimate the selection gradient for flowering time. Both gradients predict evolution towards earlier flowering but only the selection gradient using seed production as a proxy for fitness was significant. Simulating evolution across 22 generations showed that the observed change in flowering time can be caused by drift alone, provided the effective size of the population is lower than 150. These analyses suffer from the difficulty to estimate the effective size in a highly selfing population, where effective recombination is severely reduced.

Can we use effective population size estimates to test whether the genetic change is caused by selection or drift in a predominantly selfing population?

As pointed out in the Introduction, simulating drift is one of the methods to test whether selection has occurred, but it requires knowledge about the effective

population size. Using changes in allele frequencies between 1987 and 2009 in a natural population, we estimated a temporal F_{ST} of 22.6%, which corresponds to an effective size of 19 (95% confidence interval: 15-25). This estimate is several orders of magnitude lower than the census population size (> 2,000 individuals) **and lower than expected given the observed levels of diversity (Nordborg and Donnelly 1997)**. Similarly low effective population sizes have been estimated previously in other *M. truncatula* populations, based on the temporal variance in allele frequencies (Siol *et al.* 2007), and attributed to the high selfing rate of this species. Yet, the observed levels of polymorphism are often incompatible with such drastically low effective sizes (see Fig. 3c in Hereford 2009; Jullien *et al.* 2019). N_e estimates are likely biased and/or imprecise, because some of the assumptions underlying the temporal method are violated, e.g.: isolation of the populations under scrutiny, absence of selection, independence of marker loci (Jullien *et al.* 2019). For example, the quick change in allele frequency caused by a migration event will be misinterpreted as strong drift because temporal methods estimate N_e using the pace at which allele frequency changes and therefore underestimate it (Wang and Whitlock 2003). In addition, strong selfing affects the precision of temporal F_{ST} estimates because the number of independent loci is reduced (Supplementary Material S3). In our focal population, the whole genome behaves practically as a single locus, which limits the precision of our effective size estimates. Unfortunately, we show in Supplementary Material S3 that inferring effective size from the variation of MLG frequencies (i.e., considering a single, multiallelic super-locus) is unlikely to improve the quality of our estimates.

Finally, if selection occurs in a non-random mating population, it will exacerbate the Hill-Robertson effect and further reduce the effective size (Comeron *et al.* 2007). Indeed, selection will create heritable variance in fitness among individuals, thereby locally reducing N_e (Barton 1995; Charlesworth and Willis 2009; Robertson 1961). In predominantly selfing species, due to drastically reduced effective recombination (Nordborg 2000), selection will extend the reduction in diversity caused by the selective sweep to a larger proportion of the genome compared to a random mating population (Caballero and Santiago 1995; Kamran-Disfani and Agrawal 2014). With selection, the effective size estimated using the temporal variance in allele frequencies can therefore not be considered as a “neutral” effective size but rather reflects the combined effects of inbreeding and selection (Le Rouzic *et al.* 2015). Overall, due to the reduced effective recombination and potential migration, predominantly selfing populations can strongly deviate from the assumptions of the temporal method to estimate effective size and such estimates should be treated with caution (See Fig. 3 in Jullien *et al.* 2019).

823 If highly selfing organisms strongly deviate from the 879
824 general assumptions of population genetics models, a 880
825 major benefit, however, is that the temporal survey of 881
826 MLGs provides a highly integrative measure of fitness, 882
827 which is analogous to measures of genotype-specific 883
828 growth rates in asexual organisms. Our results show 884
829 that changes in frequencies of MLGs through time are 885
830 positively correlated to the fitness measured as the seed 886
831 production in the greenhouse (Fig. 3A). This relation- 887
832 ship is not significant if we consider all the MLGs found 888
833 in 2009, but this is not surprising considering the po- 889
834 tentially strong environmental variance in the field and 890
835 the approximation due to the possibility that a MLG 891
836 that was absent in 1987 appeared within the 22 years 892
837 time period. **A larger sample size in 1987 or addi-** 893
838 **tional temporal samples could help improve this anal-** 894
839 **ysis.** Despite these imprecision, such integrative esti- 895
840 mates of fitness are highly valuable because of the dif- 896
841 ficulty to obtain lifetime measures of fitness in the field 897
842 (Shaw *et al.* 2008), which are generally hindered by per- 898
843 vasive trade-offs between life history traits such as re- 899
844 production and survival (Anderson *et al.* 2014; Ågren 900
845 *et al.* 2013). 901

846 **What selective pressure could have led to this genetic** 903
847 **change in flowering time? Insights from ecophysiol-** 904
848 **ogy.** The evidence that the change in phenology ob- 905
849 served in this population across 22 generations is the 906
850 result of selection as opposed to drift remains equiv- 907
851 ocal. A further step towards evaluating whether sele- 908
852 tion is responsible for the genetic change observed 909
853 is to characterize the potential selective pressure in- 910
854 volved. Phenological changes associated to climate 911
855 change have been reported in a large number of plants 912
856 (Amano *et al.* 2010; Cleland *et al.* 2007; Parmesan and 913
857 Yohe 2003; Root *et al.* 2003). In this context, ecophys- 914
858 iological models of phenology are insightful to under- 915
859 stand how climate change can affect traits such as flow- 916
860 ering time (Chuine 2000; Oddou-Muratorio and Davi 917
861 2014). The phenological response to climate change 918
862 is complex, because the promoting effect of increased 919
863 temperatures opposes the influence of reduced vernal- 920
864 ization (Wilczek *et al.* 2010). Ecophysiological models 921
865 generally predict a plastic shift towards earlier flow- 922
866 ering times, as long as vernalization is sufficient dur- 923
867 ing winter (Morin *et al.* 2009). In agreement with 924
868 these predictions, a meta-analysis exploring the phe- 925
869 nological response to climate change in plant popula- 926
870 tions showed that phenotypic changes are mostly plas- 927
871 tic, while evidence for genetic adaptation remains rel- 928
872 atively scarce (Merilä and Hendry 2014, and other ref- 929
873 erences of Evolutionary Applications special issue, Jan- 930
874 uary 2014). However, a large part of the intraspecific 931
875 variation observed in phenology is genetic (Hendry 932
876 and Day 2005) and the architecture of the network un- 933
877 derlying flowering time variation is well described in 934
878 some species such as *Arabidopsis thaliana* (Sasaki *et al.* 935

2018; Wilczek *et al.* 2010). How climate change will af-
fect the genetic values of phenological traits remains
uncertain. In a first hypothesis, we may assume that
the phenotypic optimum for flowering time is not af-
fected by climate change. We therefore expect a ge-
netic change occurring in the opposite direction than
that of the plastic response (Fig. 5A). This hypothesis re-
sembles counter-gradient variation, which occurs when
the genetic influence on a trait along a gradient op-
poses the environmental influence, resulting in reduced
phenotypic variation across the gradient (Levins 1969).
Counter-gradients are widespread along geographical
gradients, as shown by the meta-analysis by Conover
et al. (2009), who found evidence for counter-gradient in
60 species and for co-gradients in 11 species. Therefore,
assuming that the same mechanism observed across
spatial gradients could occur in temporal gradients, we
would expect the genetic response of flowering time to
counter-balance the plastic response to climate change.
This could be achieved for example with a genetic
change increasing the base temperature T_b (tempera-
ture below which the development is supposed to be
nil).

902 Yet, our temporal survey rejects the counter-gradient
hypothesis, both at the population and at the regional
scale. Instead, we found evidence for a genetic change
towards earlier flowering, in the same direction as the
plastic response to the environmental change (here a
rise in temperatures). Such a co-gradient is expected
if climate change has shifted the phenotypic optimum
towards earlier flowering dates (Fig. 5B). Several hy-
potheses could explain such a shift and the resulting co-
gradient. First, in a plant with undetermined flowering
such as *M. truncatula*, reduced frost risk early in the sea-
son should favour earlier flowering, because plants that
manage to flower early in the season will carry on pro-
ducing flowers until summer drought becomes limit-
ing (end of May-June). We can therefore expect that the
earliest a plant flowers, the highest its fitness. Second,
climate change in the Mediterranean region also tends
to reduce precipitations in spring and early summer
(Goubanova and Li 2007; Schröter *et al.* 2005), thereby
shortening the reproductive period. Severe early sum-
mer drought could therefore create a strong selective
pressure towards earlier flowering. Such a genetic
shift in flowering time in response to extended drought
have been reported before in the literature (Franks *et al.*
2007). **In terms of ecophysiology, it can be caused by**
lower requirements of degree.days, or a reduction of
the base temperature T_b .

Finally, although it is generally assumed that flower-
ing date should be under stabilising selection in order
to avoid frost or drought when flowering occurs re-
spectively too early or too late, a recent meta-analysis
found widespread evidence for frequent directional se-
lection towards early flowering (Munguía-Rosas *et al.*
2011). This could be due to several advantages, among

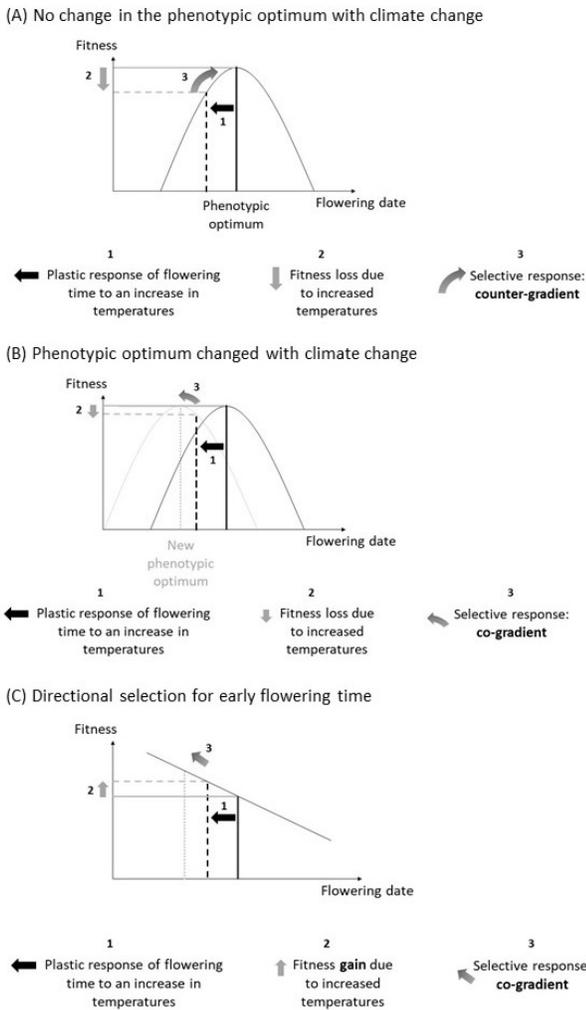


Fig. 5. Hypotheses for the expected selective pressure on flowering time under climate change. (A) Selective response expected under the hypothesis that the phenotypic optimum for flowering date remains the same. The selective response is expected in the opposite direction compared to the plastic response to increased temperatures. This corresponds to the counter-gradient hypothesis. (B) Selective response expected under the hypothesis that the phenotypic optimum for flowering date is displaced with climate change and that it becomes advantageous to flower earlier. The selective response is expected in the same direction as the plastic response to increased temperatures. This corresponds to the co-gradient hypothesis. (C) Selective response expected under the hypothesis that flowering time is under directional selection.

which an increased time for seed maturation in early reproducing plants and a longer period of growth for the progeny issued from seeds that germinate immediately (as reviewed by Elzinga *et al.* 2007; Kudo 2006). Under this scenario of directional selection, we also expect a pattern of co-gradient, as observed in the data (Fig. 5C).

Besides the evidence for a genetic change in flowering date in *M. truncatula* in Corsica, we found no evidence for a change in the sensitivity to vernalization, despite genetic variance for this trait in the population ($H^2 = 0.19$). In the literature, most studies have found at least some genetic variation for plasticity, but corresponding heritabilities were generally low (Scheiner 1993). Our results also suggest that the sensitivity to

vernalization is not independent from flowering date, because the intercept and the slope of the reaction norm to the vernalization treatment are genetically correlated (Gavrilets and Scheiner 1993). Therefore, a lower number of chilling units received during winter (short vernalization treatment) results in higher heritability of flowering date. This correlation could favour the selective response of flowering date to climate warming because warmer winters will inflate the genetic variance of flowering date. Alternatively, if early flowering genotypes are selected for, or arrive in the population by migration, the evolution of the sensitivity to vernalization might be constrained by the positive genetic correlation with flowering time: early flowering genes tend to be associated with genes reducing the sensitivity to vernalization cues.

Conclusions. Because it is difficult to rule out the effect of drift on the observed genetic change in phenology, our results do not entirely answer the question of the adaptive potential in selfing populations raised in the Introduction. Yet, several lines of evidence support the role of selection. First, the observed genetic change is in the direction expected for a response to raising temperatures and reduced rainfalls in the Mediterranean region. Second, the selection gradient measured in the greenhouse suggests that early flowering genotypes produce more seeds. The changes in MLG composition through time provide more equivocal results, but are also compatible with the hypothesis that MLGs with early flowering times had a better reproductive success than later flowering genotypes and replaced them, resulting in the observed genetic change in flowering time. Our simulations of the effect of drift are impacted by uncertainty in effective population size estimations, but the highest effective population size compatible with the observed change caused by drift alone remains relatively low ($N_e \approx 150$, Fig. 4A). Finally, the shift in flowering date observed in the Cape Corsica population was also detected at the regional scale, which suggests that the set of populations studied could be geographic replicates for this response to selection of flowering times in *M. truncatula* in Corsica. Ultimately, only a longer survey of this population combined with a pattern test (Sheets and Mitchell 2001) could provide a definitive answer to the question of adaptation to climate change through a genetic change in flowering time in this predominantly selfing population. Finally, it is worth pointing out that, in contrast with the theoretical predictions presented in the Introduction, this population displays significant genetic variance for a quantitative trait such as flowering time. As suggested before for *M. truncatula* (Jullien *et al.* 2019), it is likely that other evolutionary mechanisms, such as migration, contribute to maintain the adaptive potential of populations in this predominantly selfing species.

ADDITIONAL INFORMATION

Author Contributions. Joëlle Ronfort and Laurène Gay conceived and designed the research. JD conducted the greenhouse experiment in 2012 during his master's project. MN, RV, LG and JR developed the extension to multi-allelic data of the method to detect selection using temporal data. MJ and LG ran the genetic analyses of the CO3 population. VR developed GENETHAPLO, the program to analyse the genome-wide multilocus genetic structure of selfing or clonal populations. LG wrote the article with the help of all authors that critically reviewed and approved the text.

Competing Interests. The authors of this article declare that they have no financial conflict of interest with the content of this article.

Data Availability. Phenotypic data for the intra-population and inter-population experiments and results from the multilocus genetic structure along with the scripts used for the analyses are available on the INRA dataportal <https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/ZY83BE>. The program GENETHAPLO is available at <https://github.com/laugay/GenetHaplo>.

Supplementary data. Supplementary data are available below and consist of the following. Supplementary Material S1: Description of GENETHAPLO, a java program to analyse the genome-wide multilocus genetic structure of predominantly selfing or clonal populations. Table S1: List of the 17 populations sampled in Corsica (France). Table S2: Summary of the GLM on the sensitivity to vernalization. Table S3: Summary of the GLM on the relative seed production. Table S4: Genetic diversity at the 16 microsatellite loci. Table S5: Estimates of the selfing rate. Figure S1: Distribution of residual heterozygosity across MLGs for the two sampling years pooled. Supplementary Material S2: Details about the multi-allelic method to simulate the effect of successive generations of drift. Supplementary Material S3: Comparison of the F_{ST} estimation variance when considering the loci as independent or using the multilocus genotypes as alleles of a single locus. Figure S2 and S3: results of the simulations.

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ii) the list of the loci and individuals that have been removed. The percentage of missing data before and after filtering is also provided.

Supplementary Information

S1: GENETHAPLO: a java program to analyse the genome-wide multilocus genetic structure of predominantly selfing or clonal populations

Multilocus genotypes provide valuable information about mating systems (Jullien *et al.* 2019). Four software packages were previously developed to identify individuals originating from clonal reproduction using their multilocus genotype: MLGSIM (Stenberg *et al.* 2003); GENOTYPE and GENODIVE (Meirmans and Van Tienderen 2004), GENECLONE (Arnaud-Haond and Belkhir 2007) and poppr (Kamvar *et al.* 2014). Yet, none of these programs is specifically designed to identify individuals reproducing by selfing, in particular to detect repeated multilocus genotypes within a population and through time (or space) and recognize potential recombinants, formed by rare outcrossing events. GENETHAPLO is a program written in Java with four modules:

1. A module to convert the format of a dataset
2. A module to filter the dataset
3. A module to analyse the genetic diversity
4. A module to analyse the multilocus genetic structure

Formatting the data file. The first line of the data file is a header line describing the content of each column, i.e. the name of the population, of the sub-population, of the individual and of each locus. Each following line provides the genotype of an individual at the specified loci. The individuals should be sorted so that populations and sub-populations are grouped together in consecutive lines.

Example:

```
temp, pop, Individu, ENPB1, MTIC59L, MTIC37C, MTIC126, FMTBN, MTIC243, MTIC40, MTIC86
pop, 1987, F20089-1987-001, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 157157
pop, 1987, F20089-1987-003, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 157157
pop, 1987, F20089-1987-004, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 149149
...
pop, 2009, F20089-2009-006, 274280, 097110, 086086, 099099, 166166, 118118, 134134, 126126
pop, 2009, F20089-2009-007, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 149149
pop, 2009, F20089-2009-008, 274280, 097097, 095095, 099099, 188188, 118118, 128128, 155155
```

Module 1: format conversion. This module takes a dataset in the read2snp (Uricaru *et al.* 2014) format and converts it to a format suitable for GENETHAPLO, as detailed above.

Module 2: data filter. This module allows to filter out the loci, and individuals, having a percentage of missing data exceeding a specific threshold (given by the user). The two output files are i) a reduced dataset and

Module 3: genetic diversity. This module computes the key descriptors of genetic diversity classically used in population genetics studies. A first table summarizes the average number of individuals, alleles, the expected and observed heterozygosity and the F_{IS} . The selfing rate is also calculated from the F_{IS} for each sub-population. The module also provides these descriptors of diversity per locus and a table of allele frequencies for each sub-population.

Module 4: multilocus genetic structure. This module comprises three steps:

1. Grouping individuals according to their multilocus genotypes (thereafter called MLG): This module is based on a graph algorithm, where each node is an individual and nodes are connected when the individuals share the same MLG. An error rate can be specified by the user to allow grouping MLGs that differ at less than a given proportion of loci. This avoids over-splitting the MLGs due to genotyping errors or recent mutations. The module also takes into account missing data that can generate uncertainties. In case of missing data, it is possible for an individual to have a genotype compatible with several MLG. In such a case, the individual is randomly assigned to one of the possible MLG groups based on a random draw where each MLG group has a probability of being chosen that is proportional to its size. The output files provide i) the list of all individuals with the MLG to which they are assigned ii) the list of all identified MLGs with their frequency in each sub-population, their residual heterozygosity, defined as the proportion of heterozygous loci out of the total number of loci without missing values, and the number of missing values in each MLG.

2. Estimating genetic distance between MLGs: The genetic distance between two MLGs is estimated as the number of alleles that differ between the two synthetic MLGs divided by the total number of alleles without missing data in these two MLGs. This module generates a distance matrix as well as a histogram depicting the pairwise distance distribution.

3. Identifying recombinant MLGs: This module uses the genetic distances to rapidly identify putative recombination events between MLGs. A MLG is a candidate recombinant between two other MLGs (thereafter called “parental MLGs”) if the sum of the allele differences between it and its two putative parents equals the number of allele differences between these two parental MLGs. Only the MLGs that are represented by at least two individuals can be considered as potential parents. The output file provides a list of potential families, with the details of pairwise genetic distances.

1450 **Running the program.** This java program can be 1462
 1451 launched from a command prompt, in the folder where 1463
 1452 the modules are stored, using the command java 1464
 1453 -jar module.jar, where module.jar should be
 1454 replaced by the corresponding module name.

1455
1456

Module	Function	Module name	Arguments	Example
Module 1	format conversion	atgcFol2_v1.jar	infile	java -jar atgcFol2_SNP_EPO_seuil_160.csv
Module 2	data filter	popFilter_viblw.in.jar	infile + threshold of missing data for individuals and loci	java -jar popFilter_viblw.in.jar F20089.csv 25 35
Module 3	genetic diversity	popDiversity_viblw.in.jar	infile	java -jar popDiversity_viblw.in.jar F20089.csv
Module 4	multilocus genetic structure	genetHaplo_viblw.jar	infile + error rate + type of analysis* + random seed	java -jar atgcFol2_F20089.csv 5 d 0

* the type of analysis for the module 4 can be:
 - only MLG groups (no argument)
 - MLG groups and distances (d as an argument, as shown in the example)
 - MLG groups + distance + potential recombinants (r as an argument, as shown in the example)

1457 If no argument (infile or option) is added in the com-
 1458 mand, a short description of the script is displayed.
 For example:

```

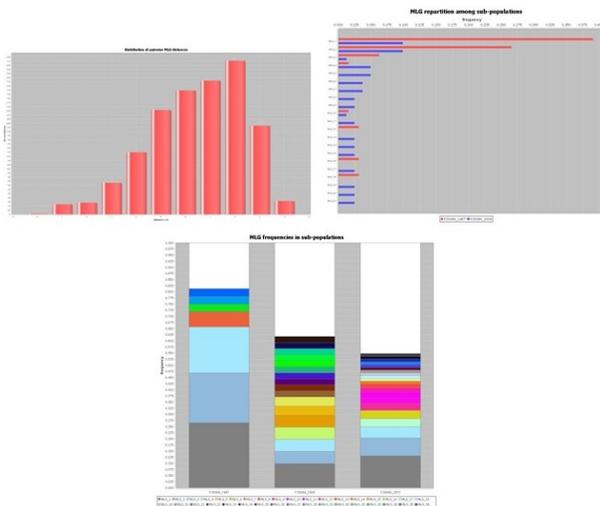
2 /drives/c/Users/gayl/DONNEES/
[2020-08-21 14:42:03] /drives/c/Users/gayl/DONNEES/DATA_2/Medicago_systemerepro/Intrapop/pop_F20089/haplo_java_OK
(gayl.arcard@pop-gayl) > java -jar genetHaplo_viblw.jar
>This application generates mlg groups structure, distances and recombinants of a given SNP or microsatellite input population.
>The MLG groups research can allow percent of error when matching individuals in a group.
>structure, distance and recombinants are interdependent, but you can choose to do only structure by typing 's' ('d' for structure+distance, 'r' for total) as a parameter on program calling
>Some situations due to marker errors are managed by a random jet. You can choose your own seed if you need the exact same result.
>Program calling: java -jar hAnalysis.jar input.csv seed s
input.csv --> input file name with .csv
error --> error tolerance for matching MLGs, in percent (e.g. 10)
s --> (optional) type of analysis you want
seed --> (optional) integer value (ex: -234154)
  
```

1459 Example of output of the console:

```

2 /drives/c/Users/gayl/DONNEES/
[2020-08-21 14:42:22] /drives/c/Users/gayl/DONNEES/DATA_2/Medicago_systemerepro/Intrapop/pop_F20089/haplo_java_OK
(gayl.arcard@pop-gayl) > java -jar genetHaplo_viblw.jar F20089_3years_CORRECTED_java_filtered_2POP.csv 6 r 0
Create haplotypes with a tolerance of 6%
.\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_infos.csv
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_infos.csv
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_distances.csv
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_repartition_in_subPopulations.png
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_repartition_in_subPopulations.png
20 50
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_composition.png
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_distances.csv
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_nb_differences.csv
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_MLG_distances.png
file created: .\analysis_F20089_3years_CORRECTED_java_filtered_2POP_possible_hybridizations.csv
  
```

1460 Example of output figures:



1461

GENETHAPLO is a program freely available at <https://github.com/laugay/GenetHaplo>. Source codes are available from authors upon request.

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 1469 Jullien, M., M. Navascués, J. Ronfort, K. Loridon, and L. Gay. 2019.
 1470 Structure of multilocus genetic diversity in predominantly selfing
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 1473 R package for genetic analysis of populations with clonal, partially
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 1476 genodive: two programs for the analysis of genetic diversity of
 1477 asexual organisms. *Molecular Ecology Notes* 4:792-794.
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 1480 Notes 3:329-331.
 1481 Uricaru, R., G. Rizk, V. Lacroix, E. Quillery, O. Plantard, R. Chikhi,
 1482 C. Lemaître, and P. Peterlongo. 2014. Reference-free detection of
 1483 isolated SNPs. *Nucleic Acids Research* 43:e11.
 1484

Supplementary tables

Table S1. List of the 16 populations sampled in Corsica (France) with geographic coordinates and sampling years

Population	Latitude	Longitude	Altitude	Sampling years
FRA20025	42.756332397	9.4508333206	60	1985-2005
FRA20031	42.182167053	9.3766670227	130	1985-2005
FRA20035	42.361167908	9.4963331223	280	1985-2009
FRA20039	42.021499634	8.7348337173	410	1985-2005
FRA20043*	42.462001801	8.6848335266	40	1987-2009
FRA20044	42.551166534	8.7391662598	250	1985-2005
FRA20046	42.592441559	8.9075956345	240	1985-2005
FRA20049	42.444000244	9.4596662521	120	1985-2005
FRA20050	42.165782928	9.546872139	10	1985-2009
FRA20051	42.199165344	9.4646663666	100	1987-2005
FRA20056	41.41350174	9.1668329239	60	1985-2009
FRA20058	41.405334473	9.1265001297	195	1985-2009
FRA20069	42.591667175	8.9081668854	410	1987-2005
FRA20087	42.901668549	9.470000267	15	1987-2005
FRA20088	42.958332062	9.3950004578	160	1987-2005
FRA20089†	42.970500946	9.3668336868	380	1987-2009

Table S2. Effect of sampling year on sensitivity to vernalization, taking into account the family effect (genetic effect). For each effect, the variance component (with standard errors in brackets), the deviance, degrees of freedom, likelihood ratio (χ^2) and *p*-values are reported.

Tested effect on sensitivity to vernalization	Variance component (SE)	df	χ^2	<i>p</i>
block	0.01 (0.003)	1	21.0	5.10 ⁻⁶
family	0.03 (0.008)	1	32.6	1.10 ⁻⁸
error	0.13 (0.012)	542		

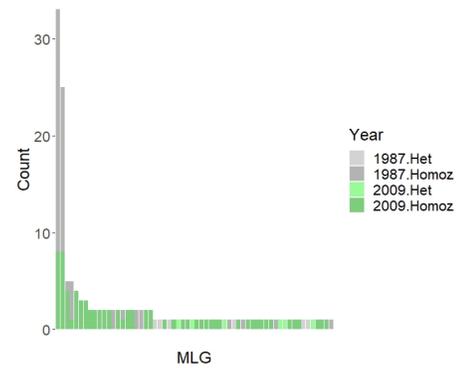
Table S3. Analysis of the family effect (genetic effect) on relative seed production (seed production standardized by year and treatment), taking into account the block effect. For each random effect, variance components (with standard deviations in brackets), degrees of freedom, likelihood ratio (χ^2) and p -values are reported.

Tested effect on relative production	Variance component (SD)	df	χ^2	p
block	0.024 (0.15)	1	116	$< 2.10^{-16}$
family	0.090 (0.30)	1	291	$< 2.10^{-16}$
error	0.153 (0.39)	1094		

Table S4. Genetic diversity at the 16 microsatellite loci for the Cape Corsica population in 1987 and 2009. n stands for the sample size, N_a and N_{arar} are the average number of alleles per locus and the allelic richness (after correction using a rarefaction method), H_e is the expected heterozygosity, F_{IS} is the heterozygote deficiency (both with 95% confidence interval in brackets, estimated by bootstrapping the individuals) and LD is the percentage of loci under significant linkage disequilibrium (for a type I error fixed at 5% when rejecting the equilibrium hypothesis). Multilocus diversity is described by n_{MLG} , the number of multilocus MLGs and n_{MLG}^h , the number of fully homozygous MLGs. Wilcoxon signed rank tests were performed across loci for N_{a-rar} , H_e and F_{IS} to test for a significant difference between the two years and the p -values are given.

Sampling year	n	N_a	N_{arar}	H_e (CI95)	F_{IS} (CI95)	LD	n_{MLG}	n_{MLG}^h
1987	64	3.6	3.4	0.351 (0.252-0.424)	0.942 (0.913-0.966)	89%	18	12
2009	81	3.9	3.7	0.623 (0.599-0.627)	0.967 (0.957-0.976)	96%	47	41
Total	145						60	48
p -value			0.211	6.10-5	0.090			

Fig. S2. Distribution of MLGs in the population and through time. The four most frequent MLGs are shared between years. MLGs with residual heterozygosity are shown in light grey (for the year 1987) and light green (for the year 2009).



S2: Details about the multiallelic method to simulate the effect of successive generations of drift

We simulated the effect of 22 generations of drift, using an extension to multiallelic data of the approach described in Frachon *et al.* (2017) and inspired by Goldringer and Bataillon (2004). In order to account for the sampling variance in initial MLG frequencies, we simulated individual MLG frequency trajectories as follows: suppose that we observe a vector \mathbf{y} of MLG counts, out of n total counts, in the 1987 sample. We assume that these observed counts are drawn from a multinomial distribution $\text{Mult}(n, \mathbf{x})$ where \mathbf{x} is the vector of (unknown) MLG frequencies in the 1987 population. Assuming a Dirichlet $\text{Dir}(1)$ prior distribution for \mathbf{x} , and using the Bayes inversion formula, the posterior distribution of \mathbf{x} is distributed as $\text{Dir}(\mathbf{y} + 1)$. For each simulation, we therefore randomly draw the initial MLG frequencies in the 1987 sample π_{1987} , from a $\text{Dir}(\mathbf{y} + 1)$ distribution. We then draw “pseudo-observed” MLG counts using a random draw from $\text{Mult}(n, \pi_{1987})$.

References. Frachon, L., C. Libourel, R. Villoutreix, S. Carrère, C. Glorieux, C. Huard-Chauveau, M. Navascués, L. Gay, R. Vitalis, E. Baron, L. Amsellem, O. Bouchez, M. Vidal, V. Le Corre, D. Roby, J. Bergelson, and F. Roux. 2017. Intermediate degrees of synergistic pleiotropy drive adaptive evolution in ecological time. *Nature Ecology Evolution* 1:1551-1561. Goldringer, I., and T. Bataillon. 2004. On the distribution of temporal variations in allele frequency: consequences for the estimation of effective population size and the detection of loci undergoing selection. *Genetics* 168:563-568.

S3: Comparison of the F_{ST} estimation variance when considering the loci as independent or using the multilocus genotypes as alleles of a single locus

Due to reduced effective recombination, the entire genome of a predominantly selfing population can behave as a giant supergene. This violates the hypoth-

Supplementary Figures

Fig. S1. Distribution of residual heterozygosity across MLGs for the two sampling years pooled. Residual heterozygosity is defined here as the proportion of heterozygous loci in the multilocus genotype (over 16 loci) of each individual.

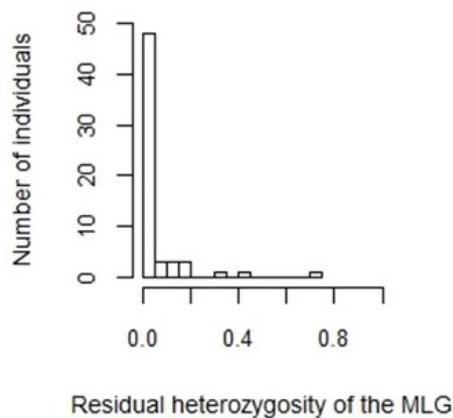


Table S5. Estimates of the selfing rate in the Cape Corsica population for each sampling year obtained using the program RMES by maximizing the log-likelihood of the whole multilocus heterozygosity structure of the sample. The 95% confidence intervals and the log-likelihood are given for the two unconstrained models and the constrained model, along with the p -value of the likelihood ratio test comparing constrained and unconstrained models.

Sampling year	Selfing rate [CI95]	Log-likelihood
(Unconstrained) 1987	0.944 [0.902-0.966]	-90.896
(Unconstrained) 2009	0.980 [0.974-0.986]	-84.592
Constrained	0.970 [0.960-0.978]	-177.952
p -value LRT		0.026

1527 esis of independence between loci that is commonly 1583
1528 assumed in population genetics, in particular for F_{ST} 1584
1529 estimation. One solution to this violation could be to 1585
1530 take the linkage disequilibrium into account by con- 1586
1531 catenating all loci and considering the distinct multi- 1587
1532 locus genotype (thereafter MLG) as different alleles of 1588
1533 a single (mega) locus. Here we use simulations to com- 1589
1534 pare estimates of genetic differentiation measured using 1590
1535 all loci considered independent or using MLGs as 1591
1536 alleles of a single locus, and compare bias and estima-
1537 tion variance (MSE).

1538 **Methods.** We used the individual-based simulations of 1594
1539 diploid hermaphroditic populations performed using 1595
1540 SLiM 2.5 (Haller and Messer 2017) by Jullien *et al.* 1596
1541 (2019). Briefly, we simulated the evolution of 20 inde- 1597
1542 pendent loci (with a recombination rate of 0.5) in an iso- 1598
1543 lated population with a given selfing rate and effective 1599
1544 population size. Each simulation comprised two peri- 1600
1545 ods. A first period of 25 N generations (with N the de- 1601
1546 mographic population size, measured as the number of 1602
1547 diploid individuals) allowed the populations to reach 1603
1548 the mutation-drift equilibrium. At this stage (time t_0 1604
1549 = 0), 100 diploid individuals were randomly sampled. 1605
1550 Twenty generations later (t_{20}), a second sample of 100 1606
1551 individuals was drawn. This matches the sampling de- 1607
1552 sign performed on the focal population in Cape Cor- 1608
1553 sica. Further details can be found in Jullien *et al.* (2019). 1609
1554 We simulated 1,000 replicates for populations with a 1610
1555 selfing rate ranging between 0.8 and 1 and a population 1611
1556 size N of 250 individuals. For each simulated dataset, 1612
1557 we assessed the relative temporal differentiation be- 1613
1558 tween the two samples using Weir and Cockerham's 1614
1559 (1984) estimator of F_{ST} , as implemented in the R pack- 1615
1560 age hierfstat (Goudet 2005). We then grouped individ- 1616
1561 uals with identical combinations of alleles (multilocus 1617
1562 genotypes, MLG) using the program GENETHAPLO 1618
1563 (Supplementary Material S1 above and as detailed in 1619
1564 the main text, except for the error rate, which was set 1620
1565 to zero). MLGs with residual heterozygosity were re- 1621
1566 moved for the multilocus assessment of temporal dif- 1622
1567 ferentiation. We considered each MLG as an allele of a 1623
1568 single locus and computed the allele frequency for each 1624
1569 temporal sample (t_0 , t_{20}). We used the function hap- 1625
1570 loDiv of the R package diversity (Keenan *et al.* 2013) 1626
1571 to estimate the F_{ST} on this haploid locus using Weir 1627
1572 and Cockerham's method (1984). We also reiterated this 1628
1573 analysis without removing the MLGs with residual het- 1629
1574 erozygosity to assess the effect of this step on the bias 1630
1575 and variance of F_{ST} estimation. 1631
1576 For each simulated selfing rate, we calculated the ex- 1632
1577 pected F_{ST} using the relationship established in Fra- 1633
1578 chon *et al.* (2017): $F_{ST} = \tau / (4N_e + \tau)$, where N_e is the 1634
1579 number of haploid individuals (or gene copy number) 1635
1580 and τ the number of generations between the two tem- 1636
1581 poral samples. Selfing reduces the number of independ- 1637
1582 ent gametes sampled for reproduction, so that the ef- 1638
1639
1640

effective size is reduced to $N_e \sim N(2 - \sigma) / 2$ (Wright 1969,
Pollak 1987) with σ the selfing rate and N the popula-
tion size. As a result, $F_{ST} = \frac{\tau}{4N_e(2-\sigma) + \tau}$.

We measured the bias as the difference between this
reference F_{ST} and the F_{ST} we estimated assuming in-
dependent loci or the F_{ST} we estimated using MLGs
as alleles of a single locus. We measured the mean
square error as the sum of the bias and the variance:
$$MSE = \sum_{i=1}^{1000} (\widehat{F_{ST} \text{ indep or MLG}}(i) - F_{ST \text{ expected}}(i))^2.$$

1592 **Results and discussion.** When using MLGs as alleles
1593 of a single locus, the estimated F_{ST} suffers from an
increased negative bias compared to the method as-
suming that all loci are independent (Fig. S3). The bias
decreases with increasing selfing rate but is always neg-
ative for the selfing rates we considered (>0.8), which
will tend to overestimate the effective population size
(Fig. S4). This bias is likely due to the dependence
of F_{ST} on the frequency of the most frequent allelic
type (Jakobsson *et al.* 2013, Edge and Rosenberg 2014,
Alcala and Rosenberg 2017): as the number of alleles
increases, the frequency of the most frequent allele nec-
essarily decreases, which sets an upper bound to the
 F_{ST} estimates (Fig. 2 in Jakobsson *et al.* 2013). Remov-
ing MLGs with residual heterozygosity reduces the
bias, because heterozygous MLGs are generally unique
and therefore form new alleles of the single "MLG"
locus. In addition, the precision of the F_{ST} estimates us-
ing the MLG method is expected to decrease when the
number of loci considered increases, because genetic
diversity is influenced by haplotype length (Mehta *et al.*
2019). As already shown (Navascués *et al.* 2020), the
variance of the F_{ST} estimation assuming independent
loci increases with high selfing rates (Fig. S3), due to
the linkage disequilibrium that reduces the number of
effective loci (Golding and Strobeck 1980, Nordborg
2000). Surprisingly, the MLG method seems to limit
the estimation variance. This is most probably arti-
ficial, because the upper-bound on the F_{ST} estimates
also constrains its variance. Altogether, despite the
high sampling variance due to the low number of
effective loci available under strong selfing, our re-
sults suggest that it is preferable to assume that all loci
are independent instead of using MLGs to estimate F_{ST} .

1627 **References.** Alcala, N., and N. A. Rosenberg. 2017. Mathematical
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1629 tions. *Genetics* 206:1581-1600.
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1630 in terms of the frequency of the most frequent allele and total
1631 homozygosity: The case of a specified number of alleles. *Theoretical*
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Frachon, L., C. Libourel, R. Villoutreix, S. Carrère, C. Glorieux, C.
1633 Huard-Chauveau, M. Navascués, L. Gay, R. Vitalis, E. Baron, L. Am-
1634 sellem, O. Bouchez, M. Vidal, V. Le Corre, D. Roby, J. Bergelson, and
1635 F. Roux. 2017. Intermediate degrees of synergistic pleiotropy drive
1636 adaptive evolution in ecological time. *Nature Ecology Evolution*
1637 1:1551-1561.
1638 Golding, G. B., and C. Strobeck. 1980. Linkage disequilibrium in a
1639
1640

Fig. S3. Estimates of temporal differentiation (F_{ST}) using all loci and assuming independence (in black) or using the MLG (concatenated genotype) as alleles of a single locus, with (green) or without (blue) exclusion of the MLGs with residual heterozygosity. The red line stands for the expected value for the F_{ST} where $F_{ST} = \frac{\tau}{4N\sigma^2 + \tau}$ with τ the number of generations between the two temporal samples, σ^2 the selfing rate and N the simulated population size.

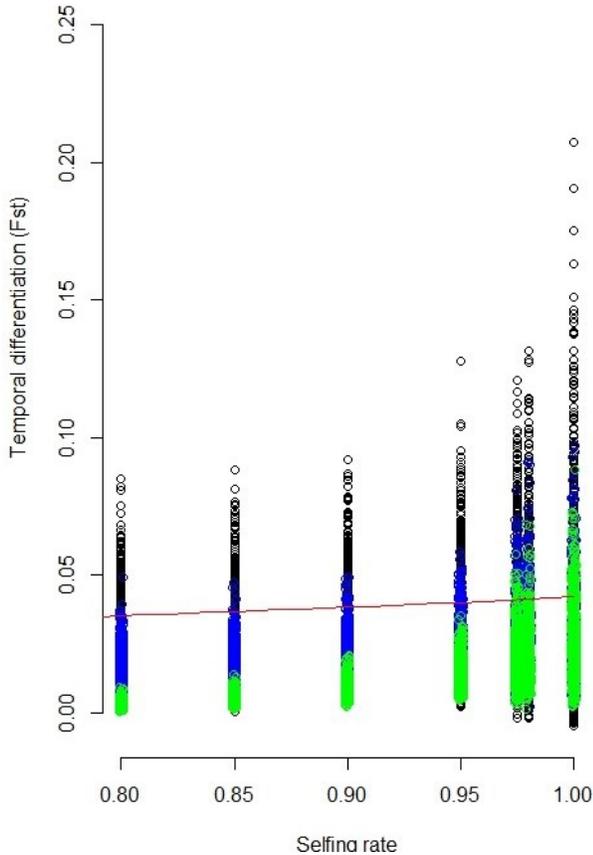
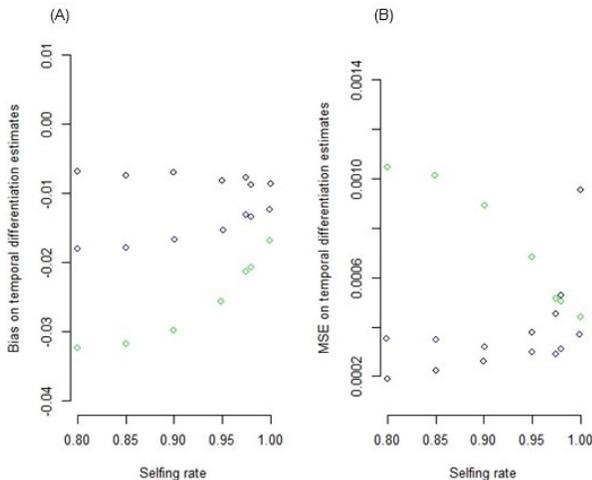


Fig. S4. Average bias (A) and MSE (B) for the estimation of temporal differentiation (F_{ST}) using all loci and assuming independence (in black) or using the MLG (concatenated genotype) as alleles of a single locus, with (green) or without (blue) exclusion of the MLGs with residual heterozygosity.



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 Keenan, K., P. McGinnity, T. F. Cross, W. W. Crozier, and P. A. Prodöhl. 2013. diveRsity: An R package for the estimation and exploration of population genetics parameters and their associated errors. *Methods in Ecology and Evolution* 4:782-788.
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1641 finite population that is partially selfing. *Genetics* 94:777-789.
 1642 Goudet, J. 2005. Hierfstat, a package for R to compute and test

Evolution of flowering time in a selfing annual plant: Roles of adaptation and genetic drift

Laurène Gay^{1,✉}, Julien Dhinaut^{1,*}, Margaux Jullien^{1,*}, Renaud Vitalis², Miguel Navascués², Vincent Ranwez¹, and Joëlle Ronfort¹

¹AGAP, INRAE, CIRAD, IRD, Montpellier SupAgro, Univ Montpellier, Montpellier, France

²CBGP, INRAE, CIRAD, IRD, Montpellier SupAgro, Univ Montpellier, Montpellier, France

*current affiliation: Evolutionary Biology and Ecology of Algae, CNRS, Sorbonne Universités, UPMC, University of Paris VI, UC, UACH, UMI 3614, 29688 Roscoff, France

[✉]current affiliation: GQE - Le Moulon, INRA, Univ. Paris-Sud, CNRS, AgroParisTech, Université Paris-Saclay, 91190, Gif-sur-Yvette, France

Resurrection studies are a useful tool to measure how phenotypic traits have changed in populations and they allow testing whether these traits modifications are a response to selection caused by an environmental change. Selfing, through its reduction of effective size, could challenge the ability of a population to adapt to environmental changes. Here, we used a resurrection study to test for adaptation in a selfing population of *Medicago truncatula*, by comparing the genetic composition and flowering times across 22 generations. We found evidence for evolution towards earlier flowering times by about two days and a peculiar genetic structure, typical of highly selfing populations, where some multilocus genotypes (MLGs) are persistent through time. We used the change in frequency of the MLGs through time as a multilocus fitness measure and built a selection gradient that suggests evolution towards earlier flowering times. Yet, a simulation model revealed that the observed change in flowering time could be explained by drift alone, provided the effective size of the population is small enough (<150). These analyses suffer from the difficulty to estimate the effective size in a highly selfing population, where effective recombination is severely reduced.

Adaptation | Selfing | Climate change | Selection gradient | Flowering time

Correspondence:

UMR AGAP, INRAE: Bâtiment ARCAD bureau 207, 10 rue Arthur Young, 34000 Montpellier (FRANCE)
laurene.gay@inrae.fr

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Introduction

When facing changing environments, organisms can persist by one of three strategies: fleeing (migration), coping (plasticity) or adapting. If migration and plasticity can lead to rapid and reversible changes in the average phenotype of a population, adaptation proceeds through genetic changes and towards phenotypes with the highest fitness in a given environment. The literature describing adaptation in natural populations is vast (e.g. Bay *et al.* 2017; Côté and Reynolds 2012; Kremer *et al.* 2012; Olson-Manning *et al.* 2012) and the recent rise of next generation sequencing has enabled

tremendous progress in our knowledge about the genetic architecture of adaptation at the species level (Barick and Lenski 2013; Brown 2012; Fournier-Level *et al.* 2011; Jones *et al.* 2012).

Long term temporal surveys (e.g. Visser 2008), resurrection studies, where ancestors and descendants are compared under common conditions (see Box 1 in Franks *et al.* 2014) or stratified propagule banks (Orsini *et al.* 2013) are powerful tools to reconstruct the evolutionary dynamics of populations that have faced environmental changes. Yet, observing a genetic change through time is not sufficient to claim that it is adaptation. Testing for selection as opposed to drift is one of the essential criteria for demonstrating adaptive responses, but is often overlooked (e.g. overlooked in 34% of the 44 reviewed studies based on phenotypic variation reviewed by Hansen *et al.* 2012). Demonstrating the influence of selection on a phenotypic change can be achieved by one of four methods (detailed in Table 2 in Hansen *et al.* 2012; Merilä and Hendry 2014): reciprocal transplants (Blanquart *et al.* 2013), $Q_{ST}-F_{ST}$ comparisons (Le Corre and Kremer 2012; Rhoné *et al.* 2010), genotypic selection estimates (Morrisey *et al.* 2012; Wilson *et al.* 2010), or tests of neutrality (pattern or rate tests, Lande 1977). These methods all rely on measuring quantitative traits (fitness traits or traits supposed to be under selection) but require specific experimental settings. Pattern tests of neutrality, for example, are based on comparing phenotypic or allele frequency changes across replicates in experimental populations, or across populations, assuming that they are independent replicates of the evolutionary process (same effective size and selective pressure, no migration). Pattern tests can also apply through time in a natural population, if a long sequence of observations is available (Sheets and Mitchell 2001). Alternatively, rate tests can be useful to examine the rate of genetic change in a population and compare it to the expectation under a neutral model with a given effective population size (Lande 1976). The effective population size (thereafter N_e) is defined as the size of an ideal Wright-Fisher population experiencing the same rate of genetic drift as

the population under consideration (Crow and Kimura 1970). Unlike experimental populations, where N_e can be monitored, an accurate estimate of N_e is required to perform such neutrality tests in natural populations. Temporal changes in allele frequency at neutral loci can be used to infer the effective size of the population considered (Nei and Tajima 1981; Waples 1989).

The ability for a population to adapt to environmental changes depends on several factors such as genetic variability, generation time, population size or mating patterns, in particular self-fertilization rates. In plants, a large fraction (40%) of species do, at least partially, reproduce through selfing (Goodwillie *et al.* 2005; Iqic and Kohn 2006). Selfing could challenge the process of adaptation because it directly decreases the effective population size N_e (reduced number of independent gametes sampled for reproduction (Pollak 1987); increased homozygosity; reduced efficacy of recombination (Nordborg 2000); increased hitchhiking and background selection (Gordo and Charlesworth 2001; Hedrick 1980)). It is therefore expected that genetic variability is reduced in selfing populations, and empirical measures of diversity from molecular markers strongly support this prediction (Barrett and Husband 1990; Glémin *et al.* 2006; Hamrick and Godt 1996). Furthermore, several theoretical models also predict that selfing reduces quantitative genetic variation within populations (Abu Awad and Roze 2018; Charlesworth and Charlesworth 1995; Lande and Porcher 2015), which has been recently confirmed by a meta-analysis of empirical data (Clo *et al.* 2019).

We can expect that this depleted genetic variation in predominantly selfing populations will limit their ability to adapt to changing environmental conditions and their long-term persistence and different theoretical models support this prediction (Glémin and Ronfort 2013; Hartfield and Glémin 2016; Kamran-Disfani and Agrawal 2014). Yet, empirical data examining the response of predominantly selfing populations to environmental changes remain scarce, especially for data showing short term adaptation in the face of climate change (Qian *et al.* 2020). In a recent review focussing on evolutionary and plastic responses to climate change in plants, Franks *et al.* (2014) reported “at least some evidence for evolutionary response to climate change [...] in all of these studies”, and six of these 31 studies considered selfing populations.

Because there is no consensus between theoretical predictions, empirical and experimental data, the ability of selfing populations to adapt to environmental changes remains an open question. This calls for further fine scale population genetics analyses, with a focus on the evolutionary mechanisms involved and on the dynamics of adaptation. Here, we present a temporal survey in the barrel medic (*Medicago truncatula*) that enabled us to perform a resurrection study. *M. truncatula* is annual, diploid, predominantly self-fertilizing (>95%

selfing, Bonnin *et al.* 2001; Siol *et al.* 2008) and has a circum-Mediterranean distribution. Flowering time is a major heritable trait (>0.5, Bonnin *et al.* 1997) that synchronizes the initiation of reproduction with favourable environmental conditions and could play a role in the adaptation to climate change. In *M. truncatula*, flowering time is highly variable along the distribution range and within some populations (Bonnin *et al.* 1997). It is mainly controlled by two environmental cues: photoperiod and temperature (Hecht *et al.* 2005; Pierre *et al.* 2008). In the Mediterranean region, there has been a significant increase in temperatures between the 80s and nowadays accompanied by a decrease in mean precipitations (<http://www.worldclim.org/>). Most studies about adaptation in *M. truncatula* have so far relied on large collections of individuals representing the whole species with the aim of detecting selection footprints in the genome linked with flowering time (Burgarella *et al.* 2016; De Mita *et al.* 2011) or climatic gradients (Yoder *et al.* 2014). However, the complex population structure observed at the species level can make it difficult to understand the selective history of those genes (De Mita *et al.* 2007). Indeed, natural populations of *M. truncatula* are composed of a set of highly differentiated genotypes that co-occur at variable frequencies (Bonnin *et al.* 2001; Loridon *et al.* 2013; Siol *et al.* 2008), a genetic structure typical for predominantly species. How does this peculiar genetic composition constrain adaptation to changing environments remains unclear, but preliminary results in *M. truncatula* have shown that surveying the multilocus genotypic composition through time could reveal a large variance in the relative contributions of these genotypes to the next generations (Siol *et al.* 2007). Here, we examined the temporal change of flowering time at the population level across 22 generations characterised by changing environmental conditions (temperature and rainfall). We describe the peculiar genetic structure of this highly selfing species and investigate the genetic mechanisms involved in adaptation. In particular, we test for the role of selection as opposed to genetic drift, following four steps. First, we consider the direction of the change in trait value in relation to the environmental change. Second, we estimate the extent of genotypic selection (Morrissey *et al.* 2012; Wilson *et al.* 2010) using selection gradients for flowering time based on several fitness estimates (taking into account the multilocus genotypic composition of the population through time). Then we estimate the effective population size, test the rate of evolution for neutrality and explore the effect of the imprecision in the estimation of effective size. Finally, we examine the change in flowering time during the same time period at the regional scale, using one individual per population across the distribution range of *M. truncatula* in Corsica. A similar genetic change at the regional scale would lend weight to the hypothesis that the change in flowering time occurred in response to

selection.

Materials and Methods

Studied population and experimental design. The focus population (F20089 or CO3 according to Jullien *et al.* 2019) is located in Cape Corsica (42°58.406'N - 9°22.015'E). In 1987 and 2009, around 100 pods were collected along three transects running across the population, with at least one meter distance between each pod collected, in order to avoid over-sampling the progeny of a single individual. Seeds collected in 1987 were stored in a cold room. In 2011, pods collected in 1987 and 2009 were threshed and seeds were replicated through selfing in standardized greenhouse conditions to control for maternal effects and build families of full-sibs produced by selfing. 64 families collected in 1987 and 96 in 2009 were successfully multiplied. Out of these, 55 families for each of the two sampling years were randomly chosen in 2012. Seeds from the 110 families were scarified to ease germination and were transferred in Petri dishes with water at room temperature for six hours. We then used two different vernalization treatments (at 5°C during 7 or 14 days) to compare the vernalization requirement between the two years. Five replicates from each vernalization treatment were transferred back to the greenhouse, according to a randomized block design (five blocks and two treatments, adding up to a total of ten replicates per family, 1100 plants in total). Data loggers were placed on each table to monitor temperature and humidity. For each individual, the number of days after germination to form the first flower was recorded. In addition, the total number of seeds produced by each plant throughout its lifetime was measured as a proxy for fitness.

Temporal changes in flowering time. Individual flowering times were converted to thermal times following Bonhomme (2000). The thermal time was calculated as the sum of the mean daily effective temperatures of each day between sowing and the emergence of the first flower, where the mean daily effective temperature is the day's mean temperature minus the base temperature (T_b). We used $T_b = 5^\circ\text{C}$, as reported by Moreau *et al.* (2007) for the *Medicago truncatula* reference line A17. Plants noted as sick or failing to produce leaves were removed from the data sets (22 individuals removed). Collected measures were tested for normality using quantile–quantile (Q-Q) plots (Nobre and Singer 2007). All analyses were conducted using R version 2.15.2. We used linear mixed models (lme4 package) to test for a significant change in flowering time between the sampling years. The model included two fixed effects: sampling year (1987 or 2009) and treatment (short or long vernalization) as well as their interaction. Block (nested in treatment), block \times year and family were random effects. The family effect was nested in years because we were interested in estimating the genetic vari-

ance within population each year of collection. The interaction between family and treatment was included in the family effect as a vectorial random effect. The complete model is summarized in equation [1], where Y denotes the flowering time, μ the average flowering time over the whole sample and ϵ the residuals:

$$Y_{ijkl} = \mu + year_i + treatment_j + year_i \times treatment_j + block_k + year_i \times block_k + family_l | (year_i \times treatment_j) + \epsilon_{ijkl} \quad (1)$$

This maximal model was simplified, using likelihood ratio tests (LRT) to compare the models. In addition, we tested for a significant change in genetic variance between 1987 and 2009 using a LRT between the model [1] and a model where family is not nested into year. Standard errors for variance components were estimated using jackknife resampling. We used the variance components estimated for the random effects to calculate broad-sense heritability as $H^2 = \frac{V_G}{V_P}$, where V_G is the genetic variance as estimated by the family effect and V_P is the total phenotypic variance, including block, family and residual variance. Standard errors for H^2 were estimated through jackknife resampling on families (Sokal and Rohlf 1995).

Temporal changes in sensitivity to vernalization. Selection on a trait in an environment can shift both the mean and the plasticity of that trait. Here, we considered the sensitivity to vernalization cues, measured as the slope of the regression line between individual values and the environmental value (estimated as the average phenotype, \bar{Y}) (Falconer and Mackay 1996), for each individual i :

$$\frac{Y_i^{long\ vernalization} - Y_i^{short\ vernalization}}{\bar{Y}^{long\ vernalization} - \bar{Y}^{short\ vernalization}}$$

This coefficient assumes that reaction norms are linear (Gavrilets and Scheiner 1993; Scheiner 1993) and this approximation is expected to work well (Chevin *et al.* 2013). We used a linear mixed model, with sampling year (1987 or 2009) as a fixed effect, a random block effect and its interaction with year, and a family effect (genetic effect) nested into year. As for flowering time, we estimated the broad sense heritability of the vernalization sensitivity.

A genetic correlation between flowering time and sensitivity to vernalization would affect the response to selection in the context of climate change. We therefore used a bivariate model with the sensitivity to vernalization and the flowering time measured in the short vernalization treatment as two dependent variables to estimate their genetic covariance with a random family effect, including block as a random effect, using AsReml (Gilmore *et al.* 2009). We ran an independent model for

each sampling year. The significance of genetic covariances was tested by comparing the residual deviance of the final model with that of a model with a fixed covariance of zero in a log-likelihood ratio test.

Selection gradient for flowering date: genetic covariance analysis. In the absence of selection for the trait considered, its observed variation is expected to be independent from fitness. We tested this by measuring the selection gradient, i.e. the statistical relationship between a trait and the fitness. Selection gradients were established for each year (and per treatment) following the Robertson-Price identity that states that ΔZ , the expected evolutionary change in the mean phenotypic trait z per generation is equal to $\Theta_a(z, w)$, the additive genetic covariance of the trait z and the relative fitness w (Price 1970; Robertson 1966):

$$\Delta Z = \Theta_a(z, w) \quad (2)$$

Here, we estimated the broad sense genetic covariance Θ_g . Assuming that the dominance variance is negligible due to the very high levels of homozygosity in selfing populations (Holland *et al.* 2010), genetic covariance should be a good approximation of the additive genetic covariance. As a preliminary step, we checked whether our proxy for fitness, the relative seed production, had significant genetic variance. The relative seed production was measured as the individual seed production standardized by the average seed production of individuals from the same year and treatment. A mixed model was used to analyse the relative seed production, including two random effects for block and family. Then, provided there was significant genetic variance for relative seed production in the population each year, we analysed it in a bivariate model with flowering time to estimate the genetic covariance with a random family effect, including block as a random effect, using AsReml (Gilmore *et al.* 2009). Again, the significance of genetic covariances was estimated by comparing the residual deviance of the final model with that of a model with a fixed covariance of zero in a log-likelihood ratio test.

Genetic analyses. During the multiplication generation in the greenhouse (2011), 200 mg of leaves were sampled from each plant for DNA extraction, using DNeasy Plant Mini Kit (Qiagen). Twenty microsatellite loci were used for genotyping (see the details of amplification reactions and analyses of amplified products in Jullien *et al.* 2019; Siol *et al.* 2007). Briefly, samples were prepared by adding 3 μ l of diluted PCR products to 16.5 μ l of ultrapure water and 0.5 μ l of the size marker AMM524. Amplified products were analyzed on an ABI prism 3130 Genetic Analyzer and genotype reading was performed using GeneMapper Software version 5.

.1. Single-locus analyses assuming independence among loci. As a preliminary step, the data was filtered to reduce the percentage of missing data (loci or individuals with >10% missing data were removed), and to discard monomorphic loci. After filtering, the dataset comprised 145 individuals and 16 loci (64 from the year 1987 and 81 from the year 2009). We measured the genetic diversity of the population each year using the allelic richness N_{a-rar} (Hurlbert 1971) and the expected heterozygosity H_e . In this predominantly selfing population, we expect a strong deviation from Hardy-Weinberg heterozygosity expectations. Thus, for each sampling year, we estimated the inbreeding fixation coefficient F_{IS} and its confidence interval using 5,000 bootstraps over loci. Between year differences for N_{a-rar} , H_e and F_{IS} across loci were tested using Wilcoxon signed-rank tests. Analyses were performed in R using the packages *adegenet* (Jombart 2008) and *hierfstat* (Goudet 2005) and the program ADZE for rarefaction analyses (Szpiech *et al.* 2008). The percentage of pairs of loci showing significant linkage disequilibrium (LD) was calculated using Genepop (Rousset 2008) with a threshold of 0.05. Finally, we measured the temporal variance in allele frequencies using the F_{ST} estimator by Weir and Cockerham (1984). To estimate the effective population size (N_e , measured in number of diploid individuals) from the temporal variance of allele frequencies, we used F_{ST} estimates to account for the correlation of alleles identity within individuals due to selfing (Navascués *et al.* 2020) and followed the method outlined in Frachon *et al.* (2017). We measured a confidence interval for N_e using an approximate bootstrap method (DiCiccio and Efron 1996) over loci.

.2. Analyses based on multilocus genotypes. We used the program RMES to estimate selfing rates from the distribution of multilocus heterozygosity (David *et al.* 2007). We tested for a difference in selfing rates between years using a likelihood ratio test between models where the selfing rate was constrained to be constant or not. For each sample (1987 and 2009), we examined the genetic structure by sorting out the number of multilocus genotypes (thereafter called MLG) and measuring their frequency and redundancy through time using GENETHAPLO (available on GitHub at <https://github.com/laugay/GenetHaplo> and described in Supplementary Material S1). GENETHAPLO takes into account the uncertainty of the assignment of a genotype to a MLG group due to missing data: in case of ambiguity, an individual is randomly assigned to one of the candidate MLG group with a probability proportional to the MLG group size. The approach also considers a genotyping error rate: if two individuals differ by less than the error rate, they are considered to belong to the same MLG. After an initial run with an error rate of zero, we checked the distribution of the distances between MLGs. We found an excess of small distances,

which could indicate errors in genotype assignment (Arnaud-Haond and Belkhir 2007). We corrected this by re-running the program with an error rate of 1/16 (= one mis-read locus). GENETHAPLO also searches for residual heterozygosity (defined as the proportion of heterozygous loci in the multilocus genotype) and evidence for recombination (S1). To identify putative recombination events between MLGs, it uses the genetic distances: a MLG is a recombinant candidate if the sum of its allele differences with two other MLGs (“parental MLGs”) equals the number of allele differences between these two parental MLGs.

If a MLG has a high fitness in a given environment, plants carrying this MLG will produce on average a larger progeny and the frequency of the MLG will rise in the following generations. We therefore propose to use the absolute change in frequency of the fully homozygous MLGs through time as an indicator of their “realised fitness”. As a preliminary step, we checked whether selection quantified in the greenhouse is likely to mirror selection in the field at present and 22 years ago using a linear model to verify whether the change in MLG frequencies covaries positively with and can be predicted by the seed production in the greenhouse. We then measured the selection gradient for flowering time as the slope of the regression of the change in frequency of the MLGs between 1987 and 2009 with flowering time. We compared this pattern with the predictions from the Robertson-Price selection gradient. The MLGs found in 2009 but absent in 1987 may have been undetected in 1987 due to low frequency, or may be recent migrants. Their change in frequency between 1987 and 2009 is thus necessarily positive and may not accurately reflect their realised fitness. We therefore reiterated these analyses using a dataset restricted to the MLGs present in 1987 only. For each of these models, we verified the normality of the residuals and estimated a confidence interval for the slope using profile likelihood confidence bounds.

In addition, we tested whether the change in frequencies of the MLGs reflects a response to selection or can be expected by drift alone. This was tested by simulating the effect of 22 generations of drift, using an extension to multiallelic data of the approach described in Frachon *et al.* (2017) and inspired by Goldringer and Bataillon (2004). Again, only the fully homozygous MLGs were kept for this analysis. We assumed complete selfing during the time interval so the whole genome behaves as a single super-locus. Details about the procedure used to simulate individual MLG frequency trajectories are provided in Supplementary Material S2. We simulated each generation of drift by drawing MLG counts from a multinomial distribution parameterized with the effective population size N_e estimated from the temporal F_{ST} , and the MLG frequencies in the previous generation. After 22 simulated generations, we randomly sampled 75 individuals to

estimate the frequencies of each MLG and measured the change in MLG frequencies across the 22 generations. This was repeated for 10^4 replicates in order to draw the distribution of the change in MLG frequency expected by drift alone. To account for the potentially large estimation variance for the F_{ST} (as observed in the simulations performed in Supplementary Material S3), we examined the sensitivity of the analysis to the effective population size using a range of values ($10 \leq N_e \leq 500$). Finally, we examined the simulated selection gradient as the relationship between the simulated changes in MLG frequencies through time and the genetic value of flowering time previously measured for each MLG, using a linear model. This provided us with a null distribution of the slopes of the regression between frequency change and flowering time, expected under drift alone. We then tested for the significance of the observed slope against the simulated distribution, by computing the proportion of the simulated slopes that were greater than the observed value.

Regional analysis. Finally, we attempted to disentangle selection and drift by considering other populations located in the same geographical region as the focal population and therefore likely submitted to the same selective pressure due to climatic constraints (pattern test, as described in the Introduction). For this regional analysis, we used 16 populations of *M. truncatula* across Corsica that were sampled twice, once in the 80’s and again in the early 2000s (listed in Table S1). Samples consisted of around 100 pods collected along transects running across the populations. Seeds collected were stored in a cold room. In 2010, one pod randomly selected from each sample (80’s and 2000’s) was threshed and one plant per population per year was replicated through selfing in standardized greenhouse conditions. This greenhouse generation allowed suppressing potential maternal effects (as in the experiment with the Cape Corsica population) and resulted in 32 families (16 populations \times 2 years) of full-sibs produced by selfing. In 2011, seeds from the 32 families were germinated following the same protocol as described earlier for the intra-population analysis, but with only one vernalization treatment at 5°C during seven days. Five plants for each family were then transferred to tables in the greenhouse according to a randomized block design (five blocks). We monitored the temperature and humidity and the flowering time for each plant.

Individual flowering times were converted in thermal time, in the same way as it was done for the intra-population analysis. Again, we used linear mixed models (lme4 package) to test for the effect of sampling year on flowering time. The model included a single fixed effect for the sampling year (1980s or 2000s). The block effect was included as a random effect, along with its interaction with sampling year. A random population effect was also included and replaced the “family” ef-

fect of Eq. 1 seen as there was only one family per year in this regional sample. The resulting model was written as:

$$Y_{ijk} = \mu + year_i + block_j + year_i \times block_j + population_k + \epsilon_{ijk} \quad (3)$$

Again, this maximal model was simplified using likelihood ratio tests.

Results

Changes in flowering time. Visual inspection of the Q-Q plots indicated that the residuals from all the linear models we used were normally distributed. We found that flowering time differed significantly between years: plants sampled in 2009 flowered on average over two days earlier than plants sampled in 1987 (Table 1, Fig. 1). This effect remained significant when we analysed flowering time as a number of days rather than degree.days (results not shown). Longer vernalization also sped flowering up (treatment effect, Table 1). The block effect only explained a low proportion of variance (micro-environment) and the largest variance component was the family effect, for all combinations of years and treatments. The comparison of a model where family was nested in years only or in years \times treatments showed that the family \times treatment interaction was significant ($\chi^2 = 66.1$; $df = 7$; $p = 9.10^{-12}$). It means that the reaction norms for the different genotypes were not parallel (Fig. 1), because the genotypes responded differently when exposed for a shorter period to cold temperatures. To account for this genotype \times environment interaction, the heritability for flowering time was estimated in each vernalization treatment separately (four components of variance, Table 1). It varied between 0.53 and 0.77 (Table 2). The genetic variance for flowering time in the population remained the same in 1987 and 2009, as shown by a LRT between the full model (Eq. 1) and a model where family was not nested in year ($\chi^2 = 6.65$; $df = 7$; $p = 0.47$). We found no significant year effect on the sensitivity to vernalization ($\chi^2 = 1.7$; $df = 1$; $p = 0.185$). There was no significant difference in the family effect between years (interaction family \times year not significant; LRT: $\chi^2 = 1.2$; $df = 2$; $p = 0.552$) but the family effect was highly significant ($\chi^2 = 32.6$; $df = 1$; $p = 1.10^{-8}$, Table S2) and the heritability of the sensitivity to vernalization was 0.19 (+/- 0.04) (Table 2). Finally, the multivariate analysis highlighted a strong positive genetic correlation between flowering time (measured in the short vernalization treatment) and the sensitivity to vernalization (in 1987: 0.54 $p = 0.008$; in 2009: 0.60 $p < 0.0001$), which means that early flowering plants are less sensitive to vernalization cues. Using the flowering time measured in the long vernalization treatment, we observed the same pattern

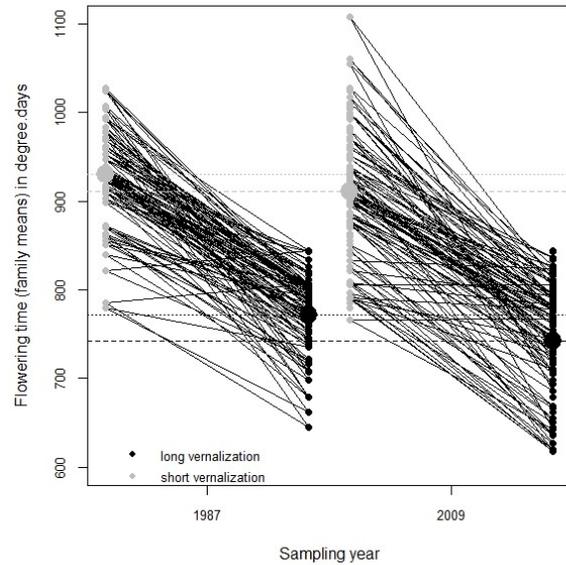


Fig. 1. Average flowering time per family for the two sampling years and the two vernalization treatments. Short vernalization is in grey and long vernalization in black. The large dots and the horizontal lines stand for the average flowering date for each vernalization treatment, for the years 1987 (dotted lines) or 2009 (dashed lines). Black crossing lines indicate that the reaction norms differ between families, as expected if genotype \times environment interactions are significant.

of correlation.

Table 1. Effect of sampling year and treatment on flowering time in the cape Corsica population, taking into account the family effect (genetic effect). Effect values on mean flowering time are given for fixed effects and variance components are given for random effects (with standard errors in brackets). The family effect was nested into year (1987 or 2009) and treatment (T1: short vernalization treatment; T2: long vernalization treatment), leading to four variance components. For each component, the degrees of freedom, likelihood ratio (χ^2) and p -values are reported. None of the interactions considered in the complete model [1] were significant: between year and treatment (LRT $\chi^2 = 1.8$; $df = 1$; $p = 0.178$); between block and year ($\chi^2 = 0.0006$; $df = 1$; $p = 0.981$).

Tested effect on flowering time	Mean effect or variance component (SE)	df	χ^2	p
year	-28.76*	1	7.3	0.007
treatment	-162.84	1	42.2	8.10^{-11}
block	92.34 (9.61)	1	34.5	4.10^{-9}

family year \times treatment	1987-T1: 2807.90 (872.97)	10	850.4	2.10^{-16}
	1987-T2: 1793.51 (500.25)			
	2009-T1: 5449.80 (1200.16)			
	2009-T2: 3557.01 (1408.88)			
error	1500 (38.73)	1081		

*assuming an average daily temperature of 15°C over the time period considered, the difference of 28.76 degree.days corresponds to two days.

Selection gradient for flowering date. The relative seed production showed significant genetic variance (family effect, Table S3, heritability of 0.34, Table 2), which enabled us to build multivariate models to examine selection gradients following Eq. 2. In 1987, we found a significant genetic covariance between flowering time and relative fitness: $\Theta_a(z, w) = -20.5$; LRT comparing this model with a model where the genetic

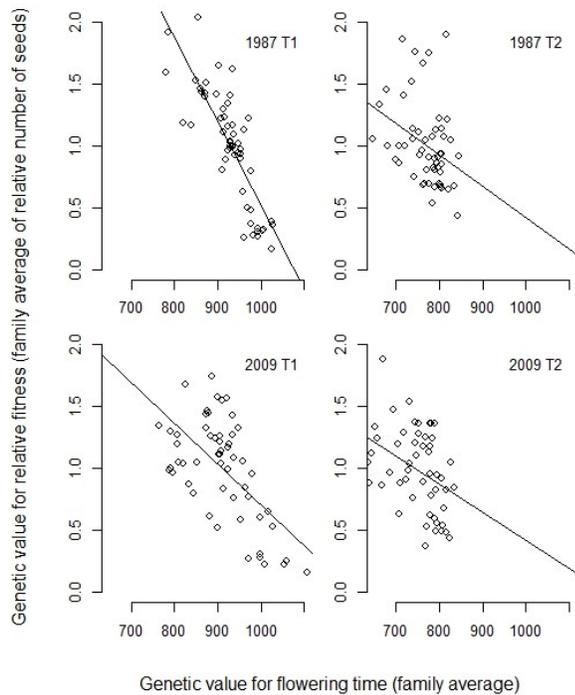


Fig. 2. Selection gradients for flowering time. Established as the relationship between the genetic value for flowering time (family average, in degree.days) and the genetic value for relative fitness (family average of the relative number of seeds), for each sampling year and vernalization treatment. Lines stand for the linear regression.

covariance was constrained to be zero: $\chi^2 = 60.2$; $df = 1$; $p = 8.10^{-15}$. The covariance remained significantly different from zero when we used the lines derived from the sampling in 2009: $\Theta_{\alpha}(z, w) = -18.5$; $LRT: \chi^2 = 12.4$; $df = 1$; $p = 6.10^{-7}$. A similar negative relationship was observed among lines derived from each of the two years, which means that the selection gradients predict an evolution towards early flowering (Fig. 2).

Table 2. Heritabilities (H^2) and coefficients of genetic variance (CV_g) for flowering time in each vernalization treatment (T1: short vernalization; T2: long vernalization) and each sampling year, for sensitivity to vernalization and for relative seed production.

Trait	H^2 (SE)		CV_g	
	1987	2009	1987	2009
Flowering time	T1: 0.64 (0.06)	T1: 0.77 (0.04)	5.70	8.11
	T2: 0.53 (0.07)	T2: 0.69 (0.07)	5.49	8.03
Sensitivity to vernalization	0.19 (0.04)		18.14	
Relative seed production	0.34 (0.03)		30.00	

Changes in the genetic composition of the population.

The analysis of microsatellite data highlighted high levels of genetic diversity for both sampling years, with an increase between 1987 and 2009 only significant for H_e (Table S4). This suggests that the increased diversity between 1987 and 2009 reveals more balanced allele frequencies rather than an increase in the average number of alleles. The temporal differentiation mea-

sured using the 16 loci was high ($F_{ST} = 0.226$; 95% confidence interval: 0.182 – 0.269), which translates into a particularly small effective size ($N_e = 19$ diploid individuals; 95% confidence interval: 15-25). According to [Eq. 16] in Nordborg and Donnelly (1997), we predict that $H_e = 1 - \frac{1}{1+4N_e\mu}$, where N_e is the effective size as estimated above. Using mutation rates for dinucleotide microsatellite loci measured in *Arabidopsis thaliana* (5.10^{-5} to 2.10^{-3}) (Marriage *et al.* 2009), and assuming an isolated population at equilibrium, we expect that H_e should lie between 0.004 and 0.134, which is nearly three times lower than the H_e estimated here (Table S4). The observed heterozygosity was particularly low, resulting in large F_{IS} estimates, as expected for a predominantly selfing species. The estimated selfing rate was about 94% in 1987 and rose to 98% in 2009 (statistically significant increase, Table S5). This high selfing rate results in extensive linkage disequilibrium between loci (nearly all pairs of loci are in linkage disequilibrium, Table S4), which makes the analysis of multilocus genotypes particularly relevant.

The analysis of MLG identified 60 different MLGs in this sample of 145 individuals. Out of the 60 MLGs, 48 were fully homozygous at the 16 loci and 12 MLGs displayed some level of heterozygosity (Fig. S1). We found no evidence for a link in terms of recombination or segregation between the heterozygous MLGs and any of the fully homozygous MLGs. These heterozygous MLGs were therefore excluded from the following analyses, leaving us with 48 MLGs (58 individuals in 1987 and 75 in 2009). The two predominant MLGs represented more than 50% of the population in 1987 and nearly 20% in 2009. These, as well as three other MLGs, were persistent through time (Fig. S2). The absolute changes in homozygous MLGs frequencies through time tended to covary positively with the total number of seeds produced by a plant in the greenhouse (Fig. 3A, regression only significant with the sample restricted to the MLGs present in 1987), which provides support to use it as a proxy to estimate the realised fitness. We therefore used the change in frequency of the 48 MLG (58 individuals in 1987 and 75 in 2009) to build selection gradients for flowering time. Again, we found a gradient with a negative slope (Fig. 3B), suggesting that the late flowering MLGs have a reduced realised fitness compared to earlier ones. This confirms the reduced fitness of late flowering genotypes observed in our greenhouse experiment (Fig. 2). Yet, the effect of flowering date on the realised fitness was small and only significant when the dataset was restricted to the MLGs present in 1987 ($n = 12$; Fig. 3B). In addition, the negative slope was mostly supported by the decreasing frequency of the two late flowering MLGs that were prevalent in 1987. The simulation of 22 years of drift with an effective population size of 19 showed that the slope of the observed selection gradient did not deviate significantly from the distribution

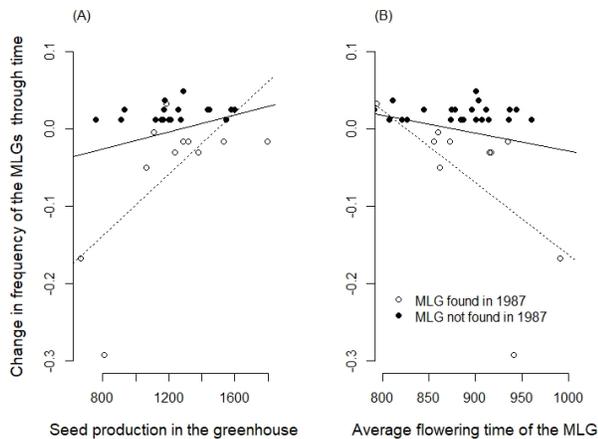


Fig. 3. Analyses of the “realised fitness”, estimated as the absolute change in frequency of the MLGs through time. MLGs with residual heterozygosity were removed from this analysis. (A) Relationship with the average number of seeds produced by plants of a given MLG in the greenhouse. (B) Selection gradient for flowering time. Each point stands for the average flowering date for a given MLG. The black regression lines are estimated using all points ($n = 48$; A: slope = 5.10^{-5} points of frequency per seed $p = 0.094$; B: slope = -0.0002 95% confidence interval: $-0.0006; 0.0001$ $p = 0.179$). This includes MLGs that were not observed in 1987 (black dots), for which the change in frequency is necessarily always positive. The dotted lines are the regression lines for the analysis restricted to the MLGs present in 1987 (white dots only; $n = 12$; A: slope = 0.0002 $p = 0.024$; B: slope = -0.0009 95% confidence interval: $-0.0017; -0.0002$ $p = 0.038$).

expected by drift alone ($p = 0.182$). Yet, again, when we restricted the dataset to the MLGs that were present in 1987, the observed selection gradient deviated significantly from the distribution expected by drift alone ($p = 0.047$), which suggests that the drift-alone hypothesis could be rejected.

Because selfing reduces the effective recombination, it reduces the number of independent loci. Measuring F_{ST} from linked loci therefore amounts to measuring it from a lower number of markers, and it is known that F_{ST} estimates based on a few loci suffer from a large sampling variance (Weir and Hill 2002). Alternatively, we could have concatenated the genotypes at the different loci to compute a diploid version of the haplotype-based F_{ST} (Mehta *et al.* 2019). Using the changes of frequencies for 48 homozygous MLGs, we estimated a temporal F_{ST} of 0.075, which corresponds to an estimated effective size of 136. However, our simulations (Supplementary Material S3) show that these haplotype-based F_{ST} estimates are strongly downwards biased, due to the dependency of F_{ST} with allelic diversity (Alcala and Rosenberg 2017; Edge and Rosenberg 2014; Jakobsson *et al.* 2013) and could therefore overestimate the effective population size. Instead of using this unreliable estimate of 136, we assessed the sensitivity of our neutrality test for MLG frequency changes to the effective population size estimates, using a range of values ($10 \leq N_e \leq 500$). We found that the observed selection gradient can no longer be explained by drift alone if the effective population size exceeds 150 (or even 10 if we consider only the MLGs

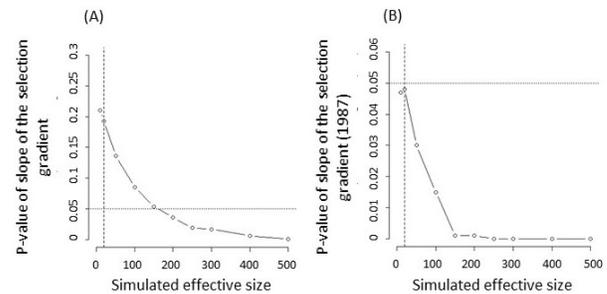


Fig. 4. Test of selection for increasing values of N_e . P -value, defined as the proportion of simulated datasets where the slope of the selection gradient is steeper than the observed slope, for the simulations of drift-alone (A) considering all the homozygous MLGs ($n = 48$) or (B) considering only the MLGs that were already present in 1987 ($n = 12$). The dotted line indicates the 0.05 threshold value for significance. The vertical dashed line is the effective size estimated using the temporal F_{ST} and considering the 16 microsatellite loci as independent ($N_e = 19$; $p = 0.182$ with $n = 48$ (A); $p = 0.047$ with $n = 12$ (B)).

present in 1987, Fig. 4).

Changes in flowering time at the regional level. At the regional level (Eq. 3), we found no effect of the interaction between block and sampling year (LRT $\chi^2 = 0$; $df = 1$; $p = 1$). All other effects were significant (Table 3): the random block effect only explained 5% of the total variance whereas the population effect accounted for 34% of variance. The significant year effect showed that the material we collected in 2005 or 2009 in Corsica flowered about five days earlier (78 degree.days, Table 3) compared to the one we collected between 1987 and 1990.

Table 3. Effect of sampling year on flowering time at the regional scale, taking into account the population effect. The effect on the mean flowering time is given for the fixed year effect and variance components are given for random effects (with standard errors in brackets). For each component, the deviance, degrees of freedom, likelihood ratio (χ^2) and p -values are reported.

Tested effect on flowering time	Mean effect or variance component (SE)	df	χ^2	p
year	-78.00*	1	9.3	0.002
block	2379 (1029)	1	5.7	0.017
population	14874 (4423)	1	40.1	2.10^{-10}
error	26971 (8260)	167		
Total variance	44224			

* assuming an average daily temperature of 15°C over the time period considered, the difference of 78.00 degree.days corresponds to five days.

Discussion

Pairing up a resurrection study with population genetic analyses proved highly insightful to understand how flowering time changed through time in *M. truncatula* and to get insights into the mechanisms involved. Growing plants collected in the Cape Corsica population 22 generations apart in a common garden experiment provided evidence for a diminution of flowering times by about two days (i.e. a reduction between 2 and 4% in flowering time). This study also highlighted the peculiar genetic structure of this highly selfing population, where some multilocus genotypes are persistent

through time. This enabled us to measure the fitness of a genotype as its frequency change through time and to establish a multilocus selection gradient. We used this multilocus fitness measure as well as a fitness measure based on individual seed production in the greenhouse to estimate the selection gradient for flowering time. Both gradients predict evolution towards earlier flowering but only the selection gradient using seed production as a proxy for fitness was significant. Simulating evolution across 22 generations showed that the observed change in flowering time can be caused by drift alone, provided the effective size of the population is lower than 150. These analyses suffer from the difficulty to estimate the effective size in a highly selfing population, where effective recombination is severely reduced.

Can we use effective population size estimates to test whether the genetic change is caused by selection or drift in a predominantly selfing population?

As pointed out in the Introduction, simulating drift is one of the methods to test whether selection has occurred, but it requires knowledge about the effective population size. Using changes in allele frequencies between 1987 and 2009 in a natural population, we estimated a temporal F_{ST} of 22.6%, which corresponds to an effective size of 19 (95% confidence interval: 15-25). This estimate is several orders of magnitude lower than the census population size (> 2,000 individuals) and lower than expected given the observed levels of diversity (Nordborg and Donnelly 1997). Similarly low effective population sizes have been estimated previously in other *M. truncatula* populations, based on the temporal variance in allele frequencies (Siol *et al.* 2007), and attributed to the high selfing rate of this species. Yet, the observed levels of polymorphism are often incompatible with such drastically low effective sizes (see Fig. 3c in Hereford 2009; Jullien *et al.* 2019). N_e estimates are likely biased and/or imprecise, because some of the assumptions underlying the temporal method are violated, e.g.: isolation of the populations under scrutiny, absence of selection, independence of marker loci (Jullien *et al.* 2019). For example, the quick change in allele frequency caused by a migration event will be misinterpreted as strong drift because temporal methods estimate N_e using the pace at which allele frequency changes and therefore underestimate it (Wang and Whitlock 2003). In addition, strong selfing affects the precision of temporal F_{ST} estimates because the number of independent loci is reduced (Supplementary Material S3). In our focal population, the whole genome behaves practically as a single locus, which limits the precision of our effective size estimates. Unfortunately, we show in Supplementary Material S3 that inferring effective size from the variation of MLG frequencies (i.e., considering a single, multiallelic super-locus) is unlikely to improve the quality

of our estimates.

Finally, if selection occurs in a non-random mating population, it will exacerbate the Hill-Robertson effect and further reduce the effective size (Comeron *et al.* 2007). Indeed, selection will create heritable variance in fitness among individuals, thereby locally reducing N_e (Barton 1995; Charlesworth and Willis 2009; Robertson 1961). In predominantly selfing species, due to drastically reduced effective recombination (Nordborg 2000), selection will extend the reduction in diversity caused by the selective sweep to a larger proportion of the genome compared to a random mating population (Caballero and Santiago 1995; Kamran-Disfani and Agrawal 2014). With selection, the effective size estimated using the temporal variance in allele frequencies can therefore not be considered as a “neutral” effective size but rather reflects the combined effects of inbreeding and selection (Le Rouzic *et al.* 2015). Overall, due to the reduced effective recombination and potential migration, predominantly selfing populations can strongly deviate from the assumptions of the temporal method to estimate effective size and such estimates should be treated with caution (See Fig. 3 in Jullien *et al.* 2019).

If highly selfing organisms strongly deviate from the general assumptions of population genetics models, a major benefit, however, is that the temporal survey of MLGs provides a highly integrative measure of fitness, which is analogous to measures of genotype-specific growth rates in asexual organisms. Our results show that changes in frequencies of MLGs through time are positively correlated to the fitness measured as the seed production in the greenhouse (Fig. 3A). This relationship is not significant if we consider all the MLGs found in 2009, but this is not surprising considering the potentially strong environmental variance in the field and the approximation due to the possibility that a MLG that was absent in 1987 appeared within the 22 years time period. A larger sample size in 1987 or additional temporal samples could help improve this analysis. Despite these imprecision, such integrative estimates of fitness are highly valuable because of the difficulty to obtain lifetime measures of fitness in the field (Shaw *et al.* 2008), which are generally hindered by pervasive trade-offs between life history traits such as reproduction and survival (Anderson *et al.* 2014; Ågren *et al.* 2013).

What selective pressure could have led to this genetic change in flowering time? Insights from ecophysiology.

The evidence that the change in phenology observed in this population across 22 generations is the result of selection as opposed to drift remains equivocal. A further step towards evaluating whether selection is responsible for the genetic change observed is to characterize the potential selective pressure involved. Phenological changes associated to climate change have been reported in a large number of plants

(Amano *et al.* 2010; Cleland *et al.* 2007; Parmesan and Yohe 2003; Root *et al.* 2003). In this context, ecophysiological models of phenology are insightful to understand how climate change can affect traits such as flowering time (Chaine 2000; Oddou-Muratorio and Davi 2014). The phenological response to climate change is complex, because the promoting effect of increased temperatures opposes the influence of reduced vernalization (Wilczek *et al.* 2010). Ecophysiological models generally predict a plastic shift towards earlier flowering times, as long as vernalization is sufficient during winter (Morin *et al.* 2009). In agreement with these predictions, a meta-analysis exploring the phenological response to climate change in plant populations showed that phenotypic changes are mostly plastic, while evidence for genetic adaptation remains relatively scarce (Merilä and Hendry 2014, and other references of Evolutionary Applications special issue, January 2014). However, a large part of the intraspecific variation observed in phenology is genetic (Hendry and Day 2005) and the architecture of the network underlying flowering time variation is well described in some species such as *Arabidopsis thaliana* (Sasaki *et al.* 2018; Wilczek *et al.* 2010). How climate change will affect the genetic values of phenological traits remains uncertain. In a first hypothesis, we may assume that the phenotypic optimum for flowering time is not affected by climate change. We therefore expect a genetic change occurring in the opposite direction than that of the plastic response (Fig. 5A). This hypothesis resembles counter-gradient variation, which occurs when the genetic influence on a trait along a gradient opposes the environmental influence, resulting in reduced phenotypic variation across the gradient (Levins 1969). Counter-gradients are widespread along geographical gradients, as shown by the meta-analysis by Conover *et al.* (2009), who found evidence for counter-gradient in 60 species and for co-gradients in 11 species. Therefore, assuming that the same mechanism observed across spatial gradients could occur in temporal gradients, we would expect the genetic response of flowering time to counter-balance the plastic response to climate change. This could be achieved for example with a genetic change increasing the base temperature T_b (temperature below which the development is supposed to be nil).

Yet, our temporal survey rejects the counter-gradient hypothesis, both at the population and at the regional scale. Instead, we found evidence for a genetic change towards earlier flowering, in the same direction as the plastic response to the environmental change (here a rise in temperatures). Such a co-gradient is expected if climate change has shifted the phenotypic optimum towards earlier flowering dates (Fig. 5B). Several hypotheses could explain such a shift and the resulting co-gradient. First, in a plant with undetermined flowering such as *M. truncatula*, reduced frost risk early in the sea-

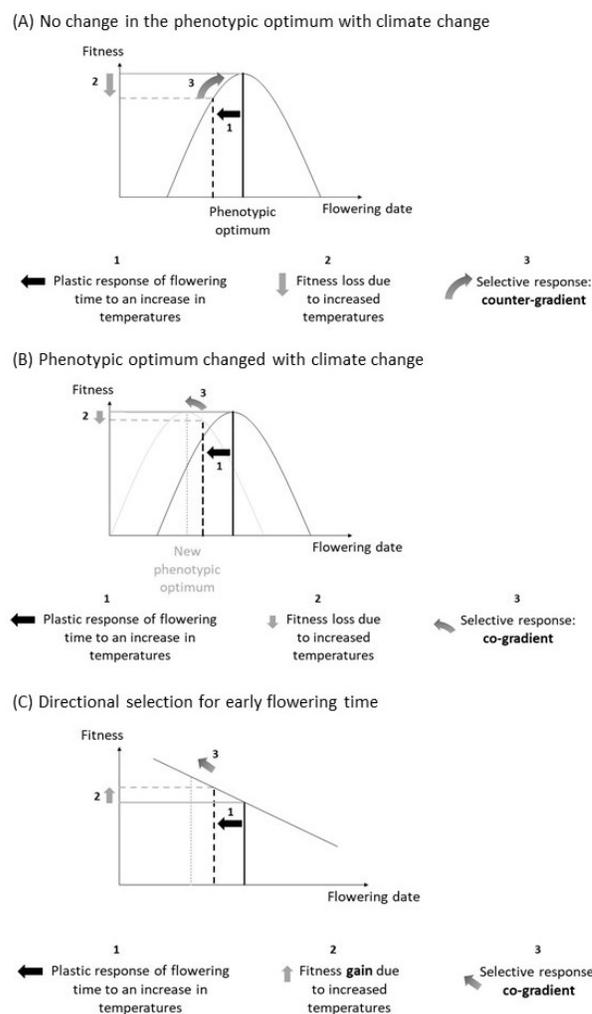


Fig. 5. Hypotheses for the expected selective pressure on flowering time under climate change. (A) Selective response expected under the hypothesis that the phenotypic optimum for flowering date remains the same. The selective response is expected in the opposite direction compared to the plastic response to increased temperatures. This corresponds to the counter-gradient hypothesis. (B) Selective response expected under the hypothesis that the phenotypic optimum for flowering date is displaced with climate change and that it becomes advantageous to flower earlier. The selective response is expected in the same direction as the plastic response to increased temperatures. This corresponds to the co-gradient hypothesis. (C) Selective response expected under the hypothesis that flowering time is under directional selection.

son should favour earlier flowering, because plants that manage to flower early in the season will carry on producing flowers until summer drought becomes limiting (end of May-June). We can therefore expect that the earliest a plant flowers, the highest its fitness. Second, climate change in the Mediterranean region also tends to reduce precipitations in spring and early summer (Goubanova and Li 2007; Schröter *et al.* 2005), thereby shortening the reproductive period. Severe early summer drought could therefore create a strong selective pressure towards earlier flowering. Such a genetic shift in flowering time in response to extended drought have been reported before in the literature (Franks *et al.* 2007). In terms of ecophysiology, it can be caused by lower requirements of degree.days, or a reduction of

the base temperature T_b .

Finally, although it is generally assumed that flowering date should be under stabilising selection in order to avoid frost or drought when flowering occurs respectively too early or too late, a recent meta-analysis found widespread evidence for frequent directional selection towards early flowering (Munguía-Rosas *et al.* 2011). This could be due to several advantages, among which an increased time for seed maturation in early reproducing plants and a longer period of growth for the progeny issued from seeds that germinate immediately (as reviewed by Elzinga *et al.* 2007; Kudo 2006). Under this scenario of directional selection, we also expect a pattern of co-gradient, as observed in the data (Fig. 5C).

Besides the evidence for a genetic change in flowering date in *M. truncatula* in Corsica, we found no evidence for a change in the sensitivity to vernalization, despite genetic variance for this trait in the population ($H^2 = 0.19$). In the literature, most studies have found at least some genetic variation for plasticity, but corresponding heritabilities were generally low (Scheiner 1993). Our results also suggest that the sensitivity to vernalization is not independent from flowering date, because the intercept and the slope of the reaction norm to the vernalization treatment are genetically correlated (Gavrilets and Scheiner 1993). Therefore, a lower number of chilling units received during winter (short vernalization treatment) results in higher heritability of flowering date. This correlation could favour the selective response of flowering date to climate warming because warmer winters will inflate the genetic variance of flowering date. Alternatively, if early flowering genotypes are selected for, or arrive in the population by migration, the evolution of the sensitivity to vernalization might be constrained by the positive genetic correlation with flowering time: early flowering genes tend to be associated with genes reducing the sensitivity to vernalization cues.

Conclusions. Because it is difficult to rule out the effect of drift on the observed genetic change in phenology, our results do not entirely answer the question of the adaptive potential in selfing populations raised in the Introduction. Yet, several lines of evidence support the role of selection. First, the observed genetic change is in the direction expected for a response to raising temperatures and reduced rainfalls in the Mediterranean region. Second, the selection gradient measured in the greenhouse suggests that early flowering genotypes produce more seeds. The changes in MLG composition through time provide more equivocal results, but are also compatible with the hypothesis that MLGs with early flowering times had a better reproductive success than later flowering genotypes and replaced them, resulting in the observed genetic change in flowering time. Our simulations of the effect of drift are impacted

by uncertainty in effective population size estimations, but the highest effective population size compatible with the observed change caused by drift alone remains relatively low ($N_e \approx 150$, Fig. 4A). Finally, the shift in flowering date observed in the Cape Corsica population was also detected at the regional scale, which suggests that the set of populations studied could be geographic replicates for this response to selection of flowering times in *M. truncatula* in Corsica. Ultimately, only a longer survey of this population combined with a pattern test (Sheets and Mitchell 2001) could provide a definitive answer to the question of adaptation to climate change through a genetic change in flowering time in this predominantly selfing population. Finally, it is worth pointing out that, in contrast with the theoretical predictions presented in the Introduction, this population displays significant genetic variance for a quantitative trait such as flowering time. As suggested before for *M. truncatula* (Jullien *et al.* 2019), it is likely that other evolutionary mechanisms, such as migration, contribute to maintain the adaptive potential of populations in this predominantly selfing species.

ADDITIONAL INFORMATION

Author Contributions. Joëlle Ronfort and Laurène Gay conceived and designed the research. JD conducted the greenhouse experiment in 2012 during his master's project. MN, RV, LG and JR developed the extension to multi-allelic data of the method to detect selection using temporal data. MJ and LG ran the genetic analyses of the CO3 population. VR developed GENETHAPLO, the program to analyse the genome-wide multilocus genetic structure of selfing or clonal populations. LG wrote the article with the help of all authors that critically reviewed and approved the text.

Competing Interests. The authors of this article declare that they have no financial conflict of interest with the content of this article.

Data Availability. Phenotypic data for the intra-population and inter-population experiments and results from the multilocus genetic structure along with the scripts used for the analyses are available on the INRA dataportal <https://data.inrae.fr/dataset.xhtml?persistentId=doi:10.15454/ZY83BE>. The program GENETHAPLO is available at <https://github.com/laugay/GenetHaplo>.

Supplementary data. Supplementary data are available below and consist of the following. Supplementary Material S1: Description of GENETHAPLO, a java program to analyse the genome-wide multilocus genetic structure of predominantly selfing or clonal populations. Table S1: List of the 17 populations sampled in Corsica (France). Table S2: Summary of the GLM on the sensitivity to vernalization. Table S3: Summary of the GLM on the relative seed production. Table S4: Genetic diversity at the 16 microsatellite loci. Table S5: Estimates of the selfing rate. Figure S1: Distribution of residual heterozygosity across MLGs for the two sampling years pooled. Supplementary Material S2: Details about the multiallelic method to simulate the effect of successive generations of drift. Supplementary Material S3: Comparison of the F_{ST} estimation variance when considering the loci as independent or using the multilocus genotypes as alleles of a single locus. Figure S2 and S3: results of the simulations.

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Supplementary Information

S1: GENETHAPLO: a java program to analyse the genome-wide multilocus genetic structure of predominantly selfing or clonal populations

Multilocus genotypes provide valuable information about mating systems (Jullien *et al.* 2019). Four software packages were previously developed to identify individuals originating from clonal reproduction using their multilocus genotype: MLGSIM (Stenberg *et al.* 2003); GENOTYPE and GENODIVE (Meirmans and Van Tienderen 2004), GENECLONE (Arnaud-Haond and Belkhir 2007) and poppr (Kamvar *et al.* 2014). Yet, none of these programs is specifically designed to identify individuals reproducing by selfing, in particular to detect repeated multilocus genotypes within a population and through time (or space) and recognize potential recombinants, formed by rare outcrossing events. GENETHAPLO is a program written in Java with four modules:

1. A module to convert the format of a dataset
2. A module to filter the dataset
3. A module to analyse the genetic diversity
4. A module to analyse the multilocus genetic structure

Formatting the data file. The first line of the data file is a header line describing the content of each column, i.e. the name of the population, of the sub-population, of the individual and of each locus. Each following line provides the genotype of an individual at the specified loci. The individuals should be sorted so that populations and sub-populations are grouped together in consecutive lines.

Example:

```
t emp, pop, individu, ENPB1, MTIC59L, MTIC37C, MTIC126, FMTBN, MTIC243, MTIC40, MTIC86
pop, 1987, F20089-1987-001, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 157157
pop, 1987, F20089-1987-003, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 157157
pop, 1987, F20089-1987-004, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 149149
...
pop, 2009, F20089-2009-006, 274280, 097110, 086086, 099099, 166166, 118118, 134134, 126126
pop, 2009, F20089-2009-007, 278278, 110110, 086086, 099099, 198198, 118118, 128128, 149149
pop, 2009, F20089-2009-008, 274280, 097097, 095095, 099099, 188188, 118118, 128128, 155155
```

Module 1: format conversion. This module takes a dataset in the read2snp (Uricaru *et al.* 2014) format and converts it to a format suitable for GENETHAPLO, as detailed above.

Module 2: data filter. This module allows to filter out the loci, and individuals, having a percentage of missing data exceeding a specific threshold (given by the user). The two output files are i) a reduced dataset and ii) the list of the loci and individuals that have been removed. The percentage of missing data before and after filtering is also provided.

Module 3: genetic diversity. This module computes the key descriptors of genetic diversity classically used in population genetics studies. A first table summarizes the average number of individuals, alleles, the expected and observed heterozygosity and the F_{IS} . The selfing rate is also calculated from the F_{IS} for each sub-population. The module also provides these descriptors of diversity per locus and a table of allele frequencies for each sub-population.

Module 4: multilocus genetic structure. This module comprises three steps:

1. Grouping individuals according to their multilocus genotypes (thereafter called MLG): This module is based on a graph algorithm, where each node is an individual and nodes are connected when the individuals share the same MLG. An error rate can be specified by the user to allow grouping MLGs that differ at less than a given proportion of loci. This avoids over-splitting the MLGs due to genotyping errors or recent mutations. The module also takes into account missing data that can generate uncertainties. In case of missing data, it is possible for an individual to have a genotype compatible with several MLG. In such a case, the individual is randomly assigned to one of the possible MLG groups based on a random draw where each MLG group has a probability of being chosen that is proportional to its size. The output files provide i) the list of all individuals with the MLG to which they are assigned ii) the list of all identified MLGs with their frequency in each sub-population, their residual heterozygosity, defined as the proportion of heterozygous loci out of the total number of loci without missing values, and the number of missing values in each MLG.
2. Estimating genetic distance between MLGs: The genetic distance between two MLGs is estimated as the number of alleles that differ between the two synthetic MLGs divided by the total number of alleles without missing data in these two MLGs. This module generates a distance matrix as well as a histogram depicting the pairwise distance distribution.
3. Identifying recombinant MLGs: This module uses the genetic distances to rapidly identify putative recombination events between MLGs. A MLG is a candidate recombinant between two other MLGs (thereafter

called “parental MLGs”) if the sum of the allele differences between it and its two putative parents equals the number of allele differences between these two parental MLGs. Only the MLGs that are represented by at least two individuals can be considered as potential parents. The output file provides a list of potential families, with the details of pairwise genetic distances.

Running the program. This java program can be launched from a command prompt, in the folder where the modules are stored, using the command `java -jar module.jar`, where `module.jar` should be replaced by the corresponding module name.

Module	Function	Module name	Arguments	Example
Module 1	format conversion	atcgToL2_v1.jar	infile	java -jar atcgToL2_SNP_EPO_seuil_160.csv
Module 2	data filter	popFilter_vtblwin.jar	infile + threshold of missing data for individuals and loci	java -jar popFilter_vtblwin.jar F20089.csv 25 35
Module 3	genetic diversity	popDiversity_vtblwin.jar	infile	java -jar popDiversity_vtblwin.jar F20089.csv
Module 4	multilocus genetic structure	genethaplo_vtbl.jar	infile + error rate + type of analysis* + random seed	java -jar atcgToL2_F20089.csv 5 d 0

- * the type of analysis for the module 4 can be:
 - only MLG groups (no argument)
 - MLG groups and distances (d as an argument, as shown in the example)
 - MLG groups + distance + potential recombinants (r as an argument, as shown in the example)

If no argument (infile or option) is added in the command, a short description of the script is displayed. For example:

```

[2020-08-21 14:42:03] /drives/c/Users/gayl/DONNEES/DATA_2/Medicago_systemerepro/Intrapop/pop_F20089/haplo_java_OK
[Gayl.arcad-aggp-gayl] * java -jar genethaplo_vtbl.jar
>This application generates mlg groups structure, distances and recombinants of a given SNP or microsatellite input population:
>The MLG groups research can allow percent of error when matching individuals in a group.
>structure, distance and recombinants are interdependent, but you can choose to do only structure by typing 's' (d: for structure+distance, 'r' for total) as a parameter on program calling.
>Some situations due to marker errors are managed by a random jet. You can choose your own seed if you need the exact same result.
>Program calling: java -jar hAnalysis.jar input.csv seed s
input.csv --> input file name with .csv
error --> error tolerance for matching MLGs, in percent (e.g. 10)
s --> (optional) type of analysis you want
seed --> (optional) integer value (ex: -234154)

```

Example of output of the console:

```

[2020-08-21 14:42:22] /drives/c/Users/gayl/DONNEES/DATA_2/Medicago_systemerepro/Intrapop/pop_F20089/haplo_java_OK
[Gayl.arcad-aggp-gayl] * java -jar genethaplo_vtbl.jar F20089_3years_CORRECTED_java_filtered_2POP.csv 6 r 0
Create haplotypes with a tolerance of 6%
Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_infos.csv
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_infos.csv
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_distances.csv
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_repartition_in_subPopulations.png
20 50
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_subPopulation_MLG_composition.png
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_distances.csv
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_diff_differences.csv
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_VMG_distances.png
file created: \Analysis_F20089_3years_CORRECTED_java_filtered_2POP_possible_hybridizations.csv

```

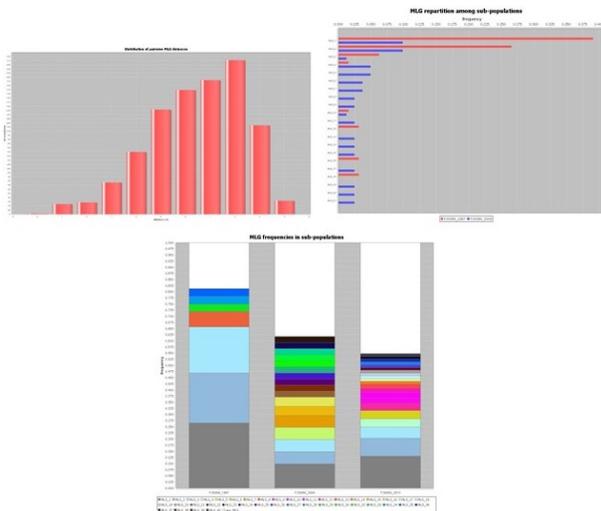
Example of output figures:

GENETHAPLO is a program freely available at <https://github.com/laugay/Genethaplo>. Source codes are available from authors upon request.

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Supplementary tables

Table S1. List of the 16 populations sampled in Corsica (France) with geographic coordinates and sampling years

Population	Latitude	Longitude	Altitude	Sampling years
FRA20025	42.756332397	9.4508333206	60	1985-2005
FRA20031	42.182167053	9.3766670227	130	1985-2005
FRA20035	42.361167908	9.4963331223	280	1985-2009
FRA20039	42.021499634	8.7348337173	410	1985-2005
FRA20043*	42.462001801	8.6848335266	40	1987-2009
FRA20044	42.551166534	8.7391662598	250	1985-2005
FRA20046	42.592441559	8.9075956345	240	1985-2005
FRA20049	42.444000244	9.4596662521	120	1985-2005
FRA20050	42.165782928	9.546872139	10	1985-2009
FRA20051	42.199165344	9.4646663666	100	1987-2005
FRA20056	41.41350174	9.1668329239	60	1985-2009
FRA20058	41.405334473	9.1265001297	195	1985-2009
FRA20069	42.591667175	8.9081668854	410	1987-2005
FRA20087	42.901668549	9.470000267	15	1987-2005
FRA20088	42.958332062	9.3950004578	160	1987-2005
FRA20089†	42.970500946	9.3668336868	380	1987-2009

Table S2. Effect of sampling year on sensitivity to vernalization, taking into account the family effect (genetic effect). For each effect, the variance component (with standard errors in brackets), the deviance, degrees of freedom, likelihood ratio (χ^2) and p -values are reported.

Tested effect on sensitivity to vernalization	Variance component (SE)	df	χ^2	p
block	0.01 (0.003)	1	21.0	5.10^{-6}
family	0.03 (0.008)	1	32.6	1.10^{-8}
error	0.13 (0.012)	542		

Table S3. Analysis of the family effect (genetic effect) on relative seed production (seed production standardized by year and treatment), taking into account the block effect. For each random effect, variance components (with standard deviations in brackets), degrees of freedom, likelihood ratio (χ^2) and p -values are reported.

Tested effect on relative seed production	Variance component (SD)	df	χ^2	p
block	0.024 (0.15)	1	116	$< 2.10^{-16}$
family	0.090 (0.30)	1	291	$< 2.10^{-16}$
error	0.153 (0.39)	1094		

Table S4. Genetic diversity at the 16 microsatellite loci for the Cape Corsica population in 1987 and 2009. n stands for the sample size, N_a and N_{arar} are the average number of alleles per locus and the allelic richness (after correction using a rarefaction method), H_e is the expected heterozygosity, F_{IS} is the heterozygote deficiency (both with 95% confidence interval in brackets, estimated by bootstrapping the individuals) and LD is the percentage of loci under significant linkage disequilibrium (for a type I error fixed at 5% when rejecting the equilibrium hypothesis). Multilocus diversity is described by n_{MLG} , the number of multilocus MLGs and n_{MLG}^h , the number of fully homozygous MLGs. Wilcoxon signed rank tests were performed across loci for N_{a-rar} , H_e and F_{IS} to test for a significant difference between the two years and the p -values are given.

Sampling year	n	N_a	N_{arar}	H_e (CI95)	F_{IS} (CI95)	LD	n_{MLG}	n_{MLG}^h
1987	64	3.6	3.4	0.351 (0.252-0.424)	0.942 (0.913-0.966)	89%	18	12
2009	81	3.9	3.7	0.623 (0.599-0.627)	0.967 (0.957-0.976)	96%	47	41
Total	145						60	48
p -value			0.211	6.10 ⁻⁵	0.090			

Supplementary Figures

Fig. S1. Distribution of residual heterozygosity across MLGs for the two sampling years pooled. Residual heterozygosity is defined here as the proportion of heterozygous loci in the multilocus genotype (over 16 loci) of each individual.

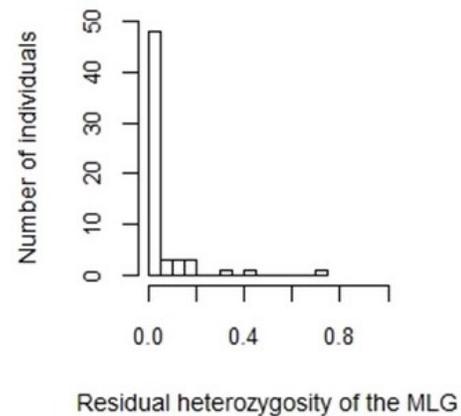
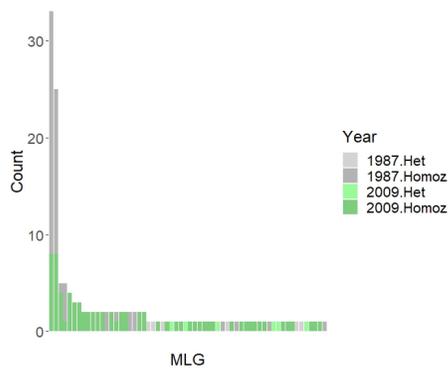


Table S5. Estimates of the selfing rate in the Cape Corsica population for each sampling year obtained using the program RMES by maximizing the log-likelihood of the whole multilocus heterozygosity structure of the sample. The 95% confidence intervals and the log-likelihood are given for the two unconstrained models and the constrained model, along with the p -value of the likelihood ratio test comparing constrained and unconstrained models.

Sampling year	Selfing rate [CI95]	Log-likelihood
(Unconstrained) 1987	0.944 [0.902-0.966]	-90.896
(Unconstrained) 2009	0.980 [0.974-0.986]	-84.592
Constrained	0.970 [0.960-0.978]	-177.952
p -value LRT		0.026

Fig. S2. Distribution of MLGs in the population and through time. The four most frequent MLGs are shared between years. MLGs with residual heterozygosity are shown in light grey (for the year 1987) and light green (for the year 2009).



S2: Details about the multiallelic method to simulate the effect of successive generations of drift

We simulated the effect of 22 generations of drift, using an extension to multiallelic data of the approach described in Frachon *et al.* (2017) and inspired by Goldringer and Bataillon (2004). In order to account for the sampling variance in initial MLG frequencies, we simulated individual MLG frequency trajectories as follows: suppose that we observe a vector \mathbf{y} of MLG counts, out of n total counts, in the 1987 sample. We assume that these observed counts are drawn from a multinomial distribution $\text{Mult}(n, \mathbf{x})$ where \mathbf{x} is the vector of (unknown) MLG frequencies in the 1987 population. Assuming a Dirichlet $\text{Dir}(1)$ prior distribution for \mathbf{x} , and using the Bayes inversion formula, the posterior distribution of \mathbf{x} is distributed as $\text{Dir}(\mathbf{y} + 1)$. For each simulation, we therefore randomly draw the initial MLG frequencies in the 1987 sample π_{1987} , from a $\text{Dir}(\mathbf{y} + 1)$ distribution. We then draw “pseudo-observed” MLG counts using a random draw from $\text{Mult}(n, \pi_{1987})$.

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S3: Comparison of the F_{ST} estimation variance when considering the loci as independent or using the multilocus genotypes as alleles of a single locus

Due to reduced effective recombination, the entire genome of a predominantly selfing population can behave as a giant supergene. This violates the hypoth-

esis of independence between loci that is commonly assumed in population genetics, in particular for F_{ST} estimation. One solution to this violation could be to take the linkage disequilibrium into account by concatenating all loci and considering the distinct multilocus genotype (thereafter MLG) as different alleles of a single (mega) locus. Here we use simulations to compare estimates of genetic differentiation measured using all loci considered independent or using MLGs as alleles of a single locus, and compare bias and estimation variance (MSE).

Methods. We used the individual-based simulations of diploid hermaphroditic populations performed using SLiM 2.5 (Haller and Messer 2017) by Jullien *et al.* (2019). Briefly, we simulated the evolution of 20 independent loci (with a recombination rate of 0.5) in an isolated population with a given selfing rate and effective population size. Each simulation comprised two periods. A first period of 25 N generations (with N the demographic population size, measured as the number of diploid individuals) allowed the populations to reach the mutation-drift equilibrium. At this stage (time $t_0 = 0$), 100 diploid individuals were randomly sampled. Twenty generations later (t_{20}), a second sample of 100 individuals was drawn. This matches the sampling design performed on the focal population in Cape Corsica. Further details can be found in Jullien *et al.* (2019). We simulated 1,000 replicates for populations with a selfing rate ranging between 0.8 and 1 and a population size N of 250 individuals. For each simulated dataset, we assessed the relative temporal differentiation between the two samples using Weir and Cockerham’s (1984) estimator of F_{ST} , as implemented in the R package hierfstat (Goudet 2005). We then grouped individuals with identical combinations of alleles (multilocus genotypes, MLG) using the program GENETHAPLO (Supplementary Material S1 above and as detailed in the main text, except for the error rate, which was set to zero). MLGs with residual heterozygosity were removed for the multilocus assessment of temporal differentiation. We considered each MLG as an allele of a single locus and computed the allele frequency for each temporal sample (t_0, t_{20}). We used the function haploDiv of the R package diversity (Keenan *et al.* 2013) to estimate the F_{ST} on this haploid locus using Weir and Cockerham’s method (1984). We also reiterated this analysis without removing the MLGs with residual heterozygosity to assess the effect of this step on the bias and variance of F_{ST} estimation.

For each simulated selfing rate, we calculated the expected F_{ST} using the relationship established in Frachon *et al.* (2017): $F_{ST} = \tau / (4N_e + \tau)$, where N_e is the number of haploid individuals (or gene copy number) and τ the number of generations between the two temporal samples. Selfing reduces the number of independent gametes sampled for reproduction, so that the ef-

fective size is reduced to $N_e \sim N(2 - \sigma)/2$ (Wright 1969, Pollak 1987) with σ the selfing rate and N the population size. As a result, $F_{ST} = \frac{\tau}{4N\frac{2-\sigma}{2} + \tau}$.

We measured the bias as the difference between this reference F_{ST} and the F_{ST} we estimated assuming independent loci or the F_{ST} we estimated using MLGs as alleles of a single locus. We measured the mean square error as the sum of the bias and the variance: $MSE = \sum_{i=1}^{1000} (\widehat{F_{ST} \text{ indep or MLG}}(i) - F_{ST \text{ expected}}(i))^2$.

Results and discussion. When using MLGs as alleles of a single locus, the estimated F_{ST} suffers from an increased negative bias compared to the method assuming that all loci are independent (Fig. S3). The bias decreases with increasing selfing rate but is always negative for the selfing rates we considered (>0.8), which will tend to overestimate the effective population size (Fig. S3). This bias is likely due to the dependence of F_{ST} on the frequency of the most frequent allelic type (Jakobsson *et al.* 2013, Edge and Rosenberg 2014, Alcalá and Rosenberg 2017): as the number of alleles increases, the frequency of the most frequent allele necessarily decreases, which sets an upper bound to the F_{ST} estimates (Fig. 2 in Jakobsson *et al.* 2013). Removing MLGs with residual heterozygosity reduces the bias, because heterozygous MLGs are generally unique and therefore form new alleles of the single “MLG” locus. In addition, the precision of the F_{ST} estimates using the MLG method is expected to decrease when the number of loci considered increases, because genetic diversity is influenced by haplotype length (Mehta *et al.* 2019). As already shown (Navascués *et al.* 2020), the variance of the F_{ST} estimation assuming independent loci increases with high selfing rates (Fig. S4), due to the linkage disequilibrium that reduces the number of effective loci (Golding and Strobeck 1980, Nordborg 2000). Surprisingly, the MLG method seems to limit the estimation variance. This is most probably artificial, because the upper-bound on the F_{ST} estimates also constrains its variance. Altogether, despite the high sampling variance due to the low number of effective loci available under strong selfing, our results suggest that it is preferable to assume that all loci are independent instead of using MLGs to estimate F_{ST} .

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Fig. S3. Estimates of temporal differentiation (F_{ST}) using all loci and assuming independence (in black) or using the MLG (concatenated genotype) as alleles of a single locus, with (green) or without (blue) exclusion of the MLGs with residual heterozygosity. The red line stands for the expected value for the F_{ST} where $F_{ST} = \frac{\tau}{4N\frac{2-\sigma}{2} + \tau}$ with τ the number of generations between the two temporal samples, σ the selfing rate and N the simulated population size.

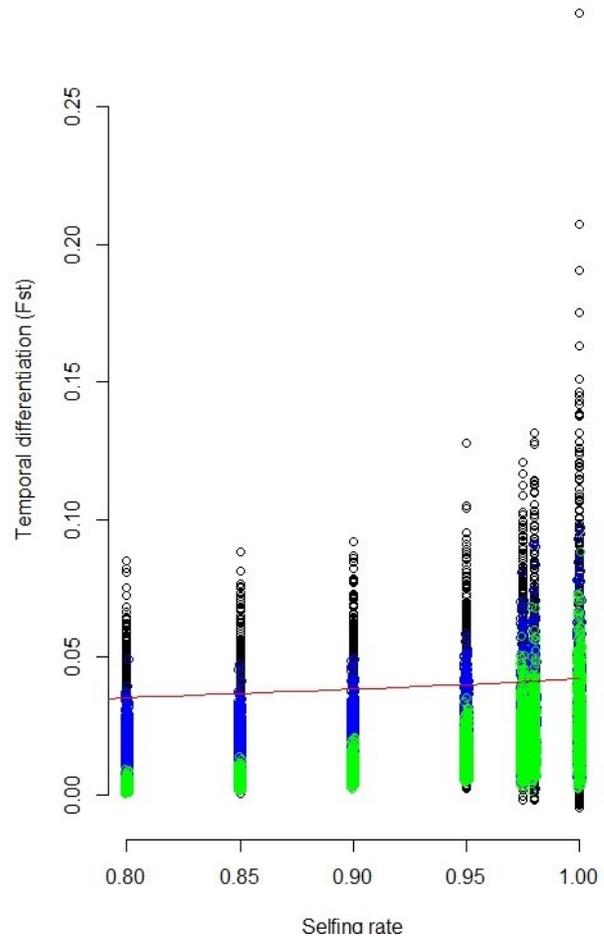
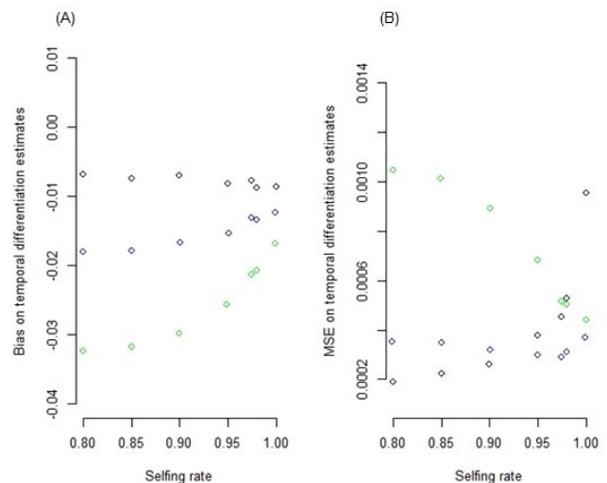


Fig. S4. Average bias (A) and MSE (B) for the estimation of temporal differentiation (F_{ST}) using all loci and assuming independence (in black) or using the MLG (concatenated genotype) as alleles of a single locus, with (green) or without (blue) exclusion of the MLGs with residual heterozygosity.



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