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Rapid divergent evolution of an annual plant across a latitudinal gradient revealed by seed resurrection

Running title: Rapid divergent evolution of plant traits

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Author contributions: P.O.C. and E.I. conceived the study, performed the common garden experiment, and measured growth-related and reproductive traits at the common garden experiment. W.A.V-M. measured performance traits such as seed set and seed predators. E.F. and J.P. genotyped individuals. W.A.V-M. conducted data analysis. W.A.V-M. wrote the paper with discussions and contributions from all authors. P.O.C. supervised the entire project. No conflict of interest to declare.

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1 **Rapid divergent evolution of an annual plant across a latitudinal gradient revealed by seed** 2 **resurrection**

3
4
5 **Abstract** - Global change are expected to drive short-term evolution of natural populations. However,
6 it remains unclear whether different populations are changing in unison. Here, we study contemporary
7 evolution of growth-related and reproductive traits of three populations of *Cyanus segetum* across a
8 latitudinal gradient in France. We resurrected stored seeds sampled up to 24 years apart from
9 northern, central-western, and southern populations and conducted an *in situ* common-garden
10 experiment. To disentangle neutral from selection-driven differentiation, we calculated F_{ST} and Q_{ST}
11 between temporal samples. We found that phenotypic evolution was divergent across populations
12 exhibiting different trends for rosette size, date of flowering, and capitula size. By measuring seed set
13 as a proxy of fitness, we showed that samples with larger mean capitula size outperformed samples
14 with smaller mean capitula size in the western and southern populations. Regression of traits on seed
15 set showed that flowering date and capitula size are the primary determinants of fitness, and $Q_{ST}-F_{ST}$
16 comparisons indicated that natural selection has likely contributed to the shifts in flowering phenology
17 and rosette size. These findings outline the potential for rescue of natural populations through
18 contemporary evolution and emphasize the complex interplay between spatial and temporal variation
19 in species' responses to global change.

20
21
22 **Keywords:** climate change, adaptation, pollinator decline, resurrection study.

23 24 **Introduction**

25 Across the globe, ecosystems are experiencing simultaneous changes in climate, atmospheric
26 composition, pollutant deposition, habitat loss, and fragmentation through land-use intensification (Yu
27 et al. 2016; Zhu et al. 2016). These anthropogenic disturbances have the potential to rapidly modify
28 selection regimes in natural populations, triggering phenotypic change (Lopez-Gallego and O'Neil
29 2010; O'Brien et al. 2017). Although trait changes can occur due to phenotypic plasticity, growing
30 evidence indicates that global change is a strong driver of genetic evolution in plant and animal
31 communities at an ecological time-scale (Franks et al. 2007; Cheptou et al. 2008; Franks and
32 Hoffmann 2012). Adaptation of natural populations to new conditions may buffer the impact of global
33 change through short-term responses of growth-related traits and reproductive strategies (Lopez-
34 Gallego and O'Neil 2010; Brys and Jacquemyn 2012). Yet, to what extent adaptation can rescue
35 populations will depend on the interaction between the local selection regimes, the rate of

36 environmental deterioration, and the standing genetic variation (Franks and Hoffmann 2012;
37 Thomann et al. 2015).

38 Contemporary plant evolution has been documented in response to different components of global
39 change, including temperature increase, droughts, habitat degradation, spread of invasive species,
40 chemical pollutants, and pollinator decline (Antonovics et al. 1971; Franks et al. 2007; Lopez-Gallego
41 and O'Neil 2010; Sultan et al. 2013; Yu et al. 2016). Increased temperatures and severe droughts
42 hinder plant growth and influence phenology by shifting flowering time (Franks et al. 2007; Yu et al.
43 2016; Zhu et al. 2016), while habitat loss and fragmentation result in the loss of genetic diversity and
44 decreased gene flow (Lopez-Gallego and O'Neil 2010; Frei et al. 2014). Beyond detecting phenotypic
45 change over time, a handful of studies have also addressed the role of natural selection in driving
46 rapid evolution under global change through the calculation of selection differentials (Parachnowitsch
47 and Kessler 2010), Q_{ST} - F_{ST} comparisons (Rhoné et al. 2010; Thomann et al. 2015), and more recently,
48 genome-wide scans (Franks et al. 2016; Frachon et al. 2017).

49
50 As part of global change, pollinator decline is a major ecological factor promoting evolution of plant
51 reproductive traits (Thomann et al. 2013). Declines in pollinator visitation reduce plant fitness in
52 allogamous species by inducing strong pollen limitation and hindering pollen transfer (Thomann et al.
53 2013; Thompson et al. 2013). Plants are predicted to respond to pollen limitation either by favouring
54 the evolution of autonomous self-fertilization as a reproductive assurance strategy (Schemske and
55 Lande 1985; Cheptou et al. 2008), or by evolving greater investment in pollinator attraction (Brys and
56 Jacquemyn 2012; Thomann et al. 2015; Ramos and Schiestl 2019). Therefore, strict outcrossing
57 species, such as self-incompatibles, are presumed to reinforce interactions with pollinators to increase
58 visitation rates and efficiency of pollen transfer (Thomann et al. 2013). Traditionally, pollinator-
59 driven selection has been studied for reproductive traits such as flowering phenology, number of open
60 flowers, corolla size, anther-stigma distance or position, and floral morphology (Harder and Johnson
61 2009; Bodbyl Roels and Kelly 2011; Ramos and Schiestl 2019). Among these traits, traits related to
62 corolla size and floral phenology have been shown to be under selection for strict outcrossing species.
63 For instance, Thomann et al. (2015) used a common-garden experiment with stored seeds of the
64 outcrossing *Cyanus segetum* and showed that in an interval of 18 years the studied population
65 flowered earlier and produced larger capitula with longer receptivity and a larger floral display.
66 However, this study was limited to one population whereas different locally adapted populations
67 might show diverse mechanisms to ensure pollinators' visitation (Brys and Jacquemyn 2012).

68
69 In the context of global pollinator decline, differences in selective pressures can result from
70 heterogeneity of populations proximal to forested or disturbed areas affected by pesticides, which
71 translates into higher variance in pollinator abundance and diversity (Brys and Jacquemyn 2012).

72 Hence, although global change components show similar trends across diverse terrestrial ecosystems,
73 pollinator-driven selection can strongly vary at fine-geographic scales (Brys and Jacquemyn 2012).
74 Recent studies have used a resurrection approach to show rapid evolution of populations triggered by
75 climate change and historical droughts across latitudinal gradients (Dickman et al. 2019; Lambrecht et
76 al. 2020; Vtipil and Sheth 2020; Wooliver et al. 2020). However, most of these studies of
77 contemporary evolution in response to global change have focused on showing the direction and
78 tempo of the evolutionary change using only phenotypic data, (Franks et al. 2007; Thomann et al.
79 2015), thus neglecting the role of genetic drift and gene flow in explaining populations' evolutionary
80 responses.

81

82 In this study, we investigated short-term evolution of growth-related and reproductive traits in three
83 populations of the cornflower *Cyanus segetum* Hill (syn. *Centaurea cyanus* L.). We addressed if the
84 direction of the evolutionary change is consistent across different populations throughout a latitudinal
85 gradient in France. We used a combined approach of growing resurrected propagules and common-
86 garden experiments, which allows detection of evolution while excluding plasticity, thus revealing the
87 direction and tempo of the evolutionary process (Franks et al. 2008a; Thomann et al. 2015). We
88 germinated stored seeds sampled 16, 23 and 24 years apart for South, North and Central-West
89 populations, respectively. We conducted common-garden experiments in field conditions and
90 compared growth-related and reproductive traits, as well as seed set between sampled years, to test
91 whether: 1) evolution of growth-related or reproductive traits occurred; 2) if the direction of
92 phenotypic change is the same for the three populations; 3) if natural selection has contributed to trait
93 changes by Q_{ST} - F_{ST} comparisons and selection gradients, 4) and whether the phenotypic changes
94 between temporal samples translate into differences in population performance at the common garden
95 experiment by taking seed set and number of capitula as a proxy of fitness.

96

97 **Material and methods**

98

99 *Studied species, sampling area, and climatic data*

100

101 The cornflower, *Cyanus segetum* (Asteraceae) is a self-incompatible annual plant that used to be
102 common in cereal fields (Le Corre et al. 2014). Nonetheless, *C. segetum*, along with other rare weeds
103 particularly in western Europe, have experienced severe declines since the second half of the
104 twentieth century (Le Corre et al. 2014; Albrecht et al. 2016; Wilson 2017). *C. segetum* is insect-
105 pollinated and produces blue capitula with peripheral sterile florets and central mono-ovulate flowers
106 (Thomann et al. 2015).

107 Three populations were sampled to represent a latitudinal gradient from the northern, central-western

108 and southern regions of France. The northern population is located near Breilly in Picardie (North of
109 France; 49°55'21.24''N, 2°09'25.40''E) (Figure 1A) and is surrounded by very few other cornflower
110 populations (Thomann et al. 2015). Seeds were collected in 1992, 2010 and 2015, thus 18 and 5 years
111 apart by the National Botanical Conservatory of Bailleul (CNBL) from the same agricultural field.
112 Seeds and seedlings from the Western population were collected in an agricultural field situated in Le
113 Mesnil-en-Vallée in Maine-et-Loire (Central West of France; 47°18'27.7463''N, 1°3'28.7636''W)
114 (Figure 1A) at an interval of 24 years (1991 and 2015, CBN of Brest). This area, known as the Loire
115 valley, has experienced a dramatic agricultural intensification in the last 50 years and is the leading
116 horticultural producer in France (Chambres d'agriculture des Pays de la Loire 2013). Seeds from the
117 southern population were collected 16 years apart (1999 and 2015) from the regional park "Parc
118 Naturel Régional de Luberon" close to the Commune Beaumont-de-Pertuis in the Vaucluse
119 department (Southeast of France; 43°46'10.056''N, 5°41'28.68''E) (Figure 1A). The cornflower
120 seeds from this population were recollected in an agricultural field close to a vineyard (CBN
121 Méditerranéen). To explore how climatic variables have changed over time and across sampled
122 populations in France, we gathered data on monthly historical temperature (Tmin, Tmax; °C) and
123 precipitation (cm) from CRU-TS 4.03 (Harris et al. 2014) downscaled with WorldClim 2.1 (Fick and
124 Hijmans 2017) for the locations where we collected seeds. We summarized historical data in
125 equivalent periods according to the time span between the ancestral and recent samples to avoid
126 biases from comparing unequal numbers of years. For instance, the timespan between the Western
127 ancestral (1991) and recent (2015) samples is 24 years, therefore, we defined the relevant period
128 leading to the ancestral population as going back 24 years in time. Hence, for the western samples we
129 compared climatic data within two periods: 1966-1990 (24 years) and 1991-2015 (24 years).
130 Similarly, given that the span between southern ancestral and recent samples is 16 years, we defined
131 the periods 1982-1998 (16 years) and 1999-2015 (16 years). The 1992 and 2010 sample from the
132 northern population are separated by 18 years so we compared the periods from 1973-1991 (18 years)
133 and 1992-2009 (18 years). Finally, we calculated mean climatic variables for the period comprised
134 between northern 2010 and 2015 samples, although we did not perform comparisons in this case
135 because of the difference in variances due to unequal sample sizes. Following Dickman et al. (2019),
136 we also calculated deviations from the expected precipitation between 1975 and 2015 by subtracting
137 the 30-year mean precipitation from the average annual precipitation to identify departures from
138 climate normal, such as droughts (see supplementary material for more information on climatic
139 variables).

140 *F0 Generation* - Seeds were collected from *ex situ* seedbank conservatories following general
141 recommendations summarized in the ESCONET program (ENSCONET 2009). Briefly, seeds were
142 collected randomly from at least 50 mother plants within each population to maximize genetic
143 diversity. All seeds were stored *ex situ* in optimal conditions (low humidity and 5°C), and they were

144 used to generate the F0 generation for the common-garden experiment. On January 2016, we
145 resurrected 300 randomly sampled seeds from each temporal sample from the northern and western
146 1991 populations, and 340 seeds from the southern temporal samples (Table S1). For each temporal
147 sample, we transplanted 50 randomly chosen plants to create the F1 generation. For the western 2015
148 population, we collected 50 randomly sampled seedlings from the exact same locations as the stored
149 seeds from 1991, to harvest seeds for the F1 generation. Hereafter, we refer to the groups of plants
150 from seeds sampled from the northern, central-western, and southern localities, as northern, western,
151 and southern populations (Figure 1A); and we referred to plants sampled in different dates as temporal
152 samples or ‘ancestral’ and ‘recent’ samples.

153

154 *Common-garden experiment*

155

156 The experiment was conducted in a common-garden in Montferrier-sur-Lez (South of France;
157 43°40'52.5468"N, 3°52'31.9188"E) in 2017. To control for maternal and storage effects and to create a
158 family design for analysing plant traits on the F1 generation, we regenerated seeds through random
159 crosses within samples. The 50 plants in the F0 generation were randomly crossed (within samples)
160 using commercial bumblebees (*Bombus terrestris*, Koppert Biological System) in insect-proof cages
161 during a 15-day period (Thomann et al. 2015). We set half-sib families using only one offspring per
162 capitula and assuming that the proportion of full-sibs from different capitula was negligible. We
163 studied 30 families each with 5 half-sibs resulting in circa 150 plants per each sampled year (nested
164 within population). Seeds were sowed on January 25th, 2017 in individual pots in a greenhouse.
165 Seedlings were transferred and planted on March 10th, 2017 for the northern and southern populations
166 and on March 13th for the western population. In order to control for the environmental heterogeneity
167 inherent to a field experiment, the seedlings from different sampling dates were randomized within
168 populations in blocks consisting of 50 individuals (Figure S1-2). To facilitate experimental measures
169 and because we were mostly interested in temporal differentiation, plants were only randomized
170 within the population of origin (i.e., North, West and South).

171 *Plant traits*

172 We recorded growth-related, reproductive, and phenological traits for each plant in the F1 generation
173 to study differences between ancestral and recent samples in three populations. For each plant, we
174 measured rosette size on the day of transplantation (to the nearest 0.5 cm), date of the first flowering,
175 capitula diameter (capitula size), and number of capitula. At the end of the experiment, plants were
176 harvested, and dry biomass was estimated as the total number of seed heads. We estimated number of
177 seeds and number of unpollinated florets to estimate seed set for the first three capitula. Individual
178 fitness was estimated by multiplying seed set by number of seed heads. Finally, to estimate pre-

179 dispersal impact caused by predators. we inspected the harvested capitulas and recorded the presence
180 or absence of arthropods related to seed damage. We summarize this impact by estimating a “seed
181 predators’ ratio” as the number of capitula infested.

182

183 *Statistical analyses*

184

185 We evaluated changes in climatic variables between temporal periods by performing paired t-tests. To
186 characterize differences on plant traits between temporal samples, we analysed data separately for
187 each population with linear (LMM) and generalized (GLMM) mixed effect models using the
188 functions “lmer” and “glmer”, implemented in the R package “lme4” (Bates et al. 2014). The models
189 included the family effect as a random factor nested in the fixed effect of sampling date. We
190 performed LMM tests for rosette size, plant biomass, mean capitula size, and days to flowering. We
191 implemented GLMM analyses for number of capitula using a Poisson distribution and a binomial
192 distribution for seed set and presence of seed predators. To evaluate significance of the fixed effects,
193 we used the Satterthwaite's method for linear models as implemented in the *anova.lmerModLmerTest*
194 function in the R package “lmerTest” (Kuznetsova et al. 2017); and likelihood ratio tests for
195 generalized models. We corrected for multiple comparisons using the Benjamini and Hochberg
196 method (Benjamini and Hochberg 1995).

197 Phenotypic selection was estimated using linear (β) and quadratic selection (γ) gradients with classical
198 multiple regression models of standardized traits on relative fitness (Lande and Arnold 1983). The
199 highest-ranking models were used to estimate the selection gradients. We assessed the significance of
200 the inferred β and γ terms by performing t-test with a null hypothesis of β or $\gamma = 0$. All phenotypic
201 values were standardized prior to analyses. We tested phenotypic selection only for three traits: rosette
202 size as a proxy of vegetative growth, days to flowering representing phenology, and mean capitula
203 size for traits associated to pollinator attraction (Renoult et al. 2013).

204

205 All the models were validated by visual inspection of residual structure and homoscedasticity. The
206 statistical analyses were performed in R 3.6.0 (R Core Team 2019).

207

208 *Neutral and quantitative differentiation analysis*

209

210 Leaf material was collected from the mother plants (F0) of the common-garden experiment. DNA was
211 extracted using the CTAB method (Doyle and Doyle 1990) and genotyped at 10 microsatellite
212 markers designed for the cornflower (Le Corre et al. 2014). Neutral genetic differentiation (Nei's F_{ST})

213 between spatial and temporal samples was estimated using the R package “hierfstat” (Goudet 2015).
214 The 95% confidence interval (CI) of the F_{ST} was estimating by bootstrapping 1,000 times across the
215 loci. We inferred a neighbour-joining tree comprising all temporal samples and calculated support for
216 the nodes using R packages “ape” (Paradis et al. 2004) and “hierfstat” (Jerome Goudet 2015). Note
217 that support for the nodes might be low because of factors such as incomplete lineage sorting and
218 gene flow. We calculated measures of genetic diversity such as the observed and expected
219 heterozygosity (Nei’s genetic diversity) per temporal sample using the R package “adegenet” and
220 performed permutation tests to estimate confidence intervals (Jombart 2008).

221 To test whether quantitative trait differentiation was significantly different from neutral genetic
222 differentiation as a signature of natural selection, we compared Q_{ST} and F_{ST} values between temporal
223 samples for each population. The Q_{ST} values for each trait between the temporal samples for each
224 population were defined following Thomann et al. (2015) as:

$$Q_{ST} = \frac{\sigma_{sample}^2}{\sigma_{sample}^2 + 2\sigma_A^2}, \text{ with } \sigma_A^2 = \frac{\sigma_{fam}^2}{2\theta}$$

225
226 where σ_{sample}^2 and $2\sigma_{fam}^2$ are the between-sample and the among-family-nested within-sample
227 variance of the quantitative traits, respectively, σ_A^2 is the pooled additive genetic variance of the two
228 samples, and θ is the coefficient of coancestry. Given that families were composed of half-sibs we set
229 θ to 1/8. We used Linear Mixed Models (LMM) to partition the variance components for the Q_{ST}
230 analyses with both sampling year and family as random effects. The 95% confidence interval of the
231 Q_{ST} values were estimated by bootstrapping 1,000 times over families within each sample. The null
232 hypothesis ($Q_{ST} = F_{ST}$) was tested by comparing their 95% CI’s. Q_{ST} was considered significantly
233 different from F_{ST} when the two 95% CI’s did not overlap or overlapped marginally with point Q_{ST}
234 estimates outside F_{ST} bootstrapping range.

235 **Results**

236

237 *Climatic variation and temporal changes in traits, seed set, and seed predators*

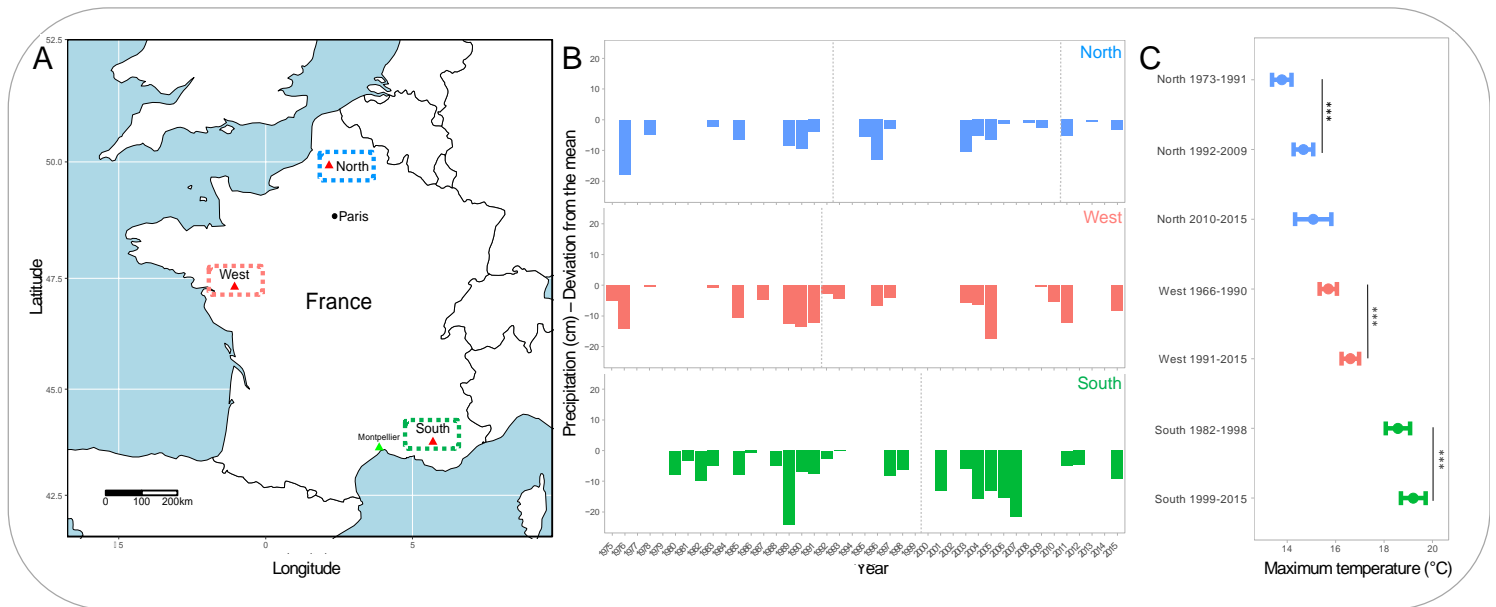
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239 Precipitation was lower than average for the year of collection of all recent samples (2015) across all
240 populations (Figure 1B). The climate leading up to the year of collection for the western 1991
241 ancestral sample, was severely drier than average (Figure 1B). The southern population experienced
242 the greatest variation in precipitation and a long drought from 2002 to 2007 (Figure 1B). Consistent
243 with climate change, temperature increased between periods at the localities of the three populations
244 (Figure 1C, Table S6).

245

246 Germination rates for the different accessions of the F0 and F1 plants where >70% and >91%,
 247 respectively. Germination date in the F0 generation did not differ significantly between dates for the
 248 northern and southern samples (Table S2, Figure S3). However, plants from the recent western sample
 249 germinated on average one and half day later compared to the 1991 sample (Table S3, Figure S3).

250 Ancestral and recent samples showed significant and divergent differences across populations for
 251 rosette size, flowering date, capitula size, and seed set (Table S2, Figure 2). Rosette size was different
 252 between dates for the northern and southern populations, and the trend of the evolutionary change was
 253 directional, although following opposite directions: while plants in recent northern population are
 254 smaller, rosette size in the southern population increased over time (Table S2, Figure 2A, C). In
 255 contrast, days to flowering was different between temporal samples only for the western populations,
 256 whereby recent individuals flowered on average 6 days earlier (Figure 2E).



257

258 **Figure 1.** Sample locations and climatic variables; precipitation showed no trend with time across populations' localities,
 259 while temperature has increased over time in all localities. **A.** Map of seed collection sites from northern, western, and
 260 southern populations of *Cyanus segetum* in France. **B.** Precipitation (cm) at the northern, western, and southern locations
 261 over the 30 years preceding the year of collection of the most recent temporal sample. Vertical bars correspond to
 262 precipitation deviation (cm) from the 30-year average. **C.** Maximum temperatures (°C) at the northern, western, and southern
 263 locations summarized over periods of time according to the time span between the ancestral and recent temporal samples.
 264 Significance codes: p-value < 0.0001 '***', < 0.01 '**', 0.01-0.05 '*'. Horizontal lines correspond to the standard error.

265 We found divergent evolution of capitula size in western and southern populations: the recent sample
 266 from the South exhibited larger capitula size and outperformed the ancestral sample having a higher
 267 seed set and lower infestation by seed predators (Table S2, Figure 2I). Conversely, the recent sample
 268 from the West had smaller capitulas and lower fitness in terms of seed set and biomass compared to

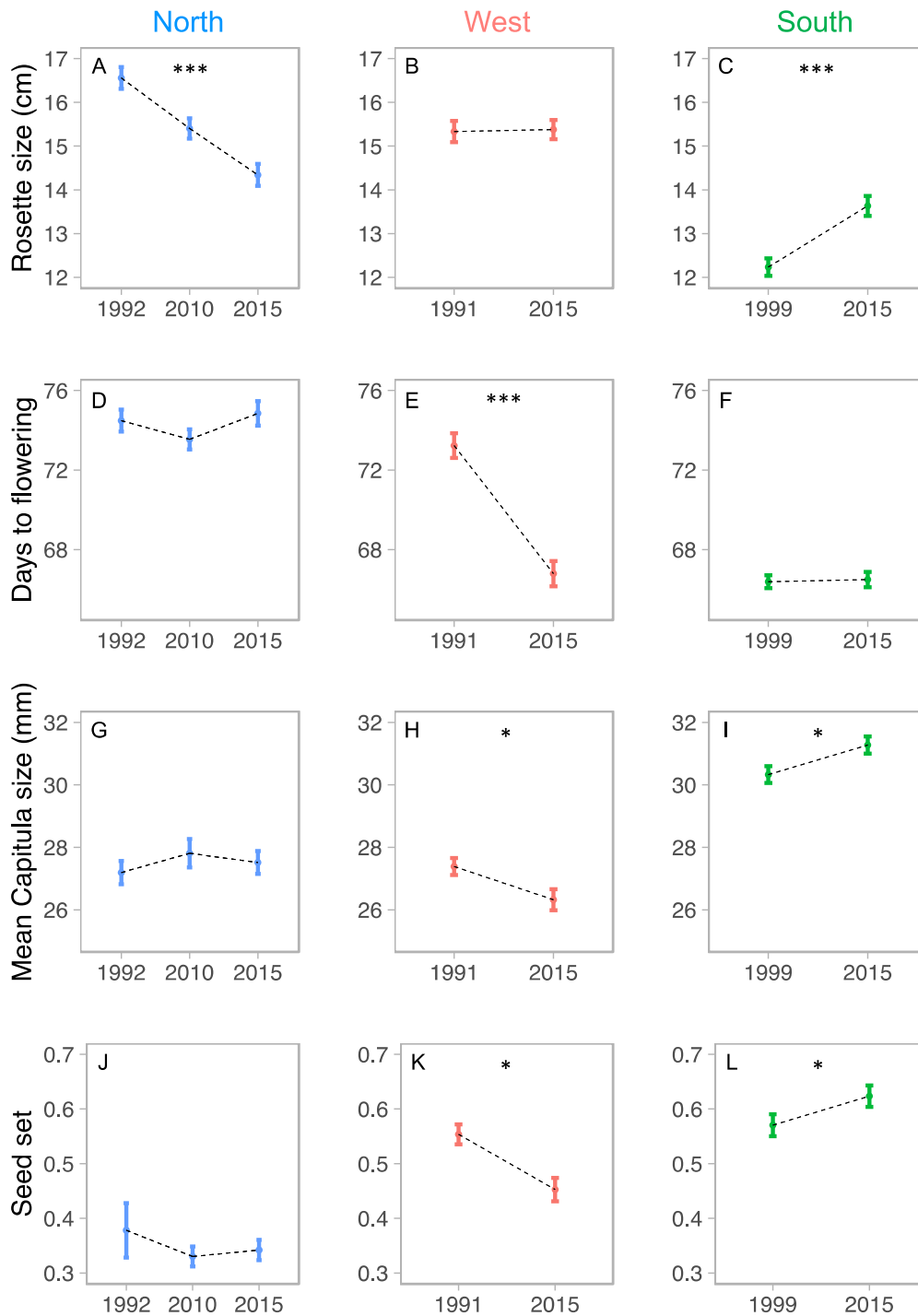
269 the ancestral, yet it showed a lower proportion of attacks by seed predators (Table S2, Figure 2).
270 Similarly to groups of seed predators found in other cornflower populations (Koprdoва et al. 2015),
271 the most abundant seed predators were larvae of Cecidomyiidae (gall midges). We also found species
272 of Tephritidae (fruit flies), Tortricidae (leafroller moths), Curculionidae (weevils), and parasitoid
273 wasps.

274 The spatial block had a pervasive effect on most measured traits (Table S2, Figure S2, S4). In
275 particular, the block, as well as the interaction between sampling date and block, had a strong effect
276 on condition-related estimates such as number of capitula, biomass, seed set, and seed predators ratio
277 across all populations (Table S2, Figure S2). On average, blocks closer to the edges in the northern
278 and southern populations presented higher seed set (Figure S2), while biomass was lower in the edges
279 of the experimental field (Figure S2-3). Since we measured rosette size before transplantation, we
280 were able to compare values across the three different latitudinal samples. We found that the
281 population of origin had a significant impact on rosette size; the southern population showed smaller
282 rosette size (12.89 ± 0.15 cm) than the northern (15.43 ± 0.14 cm) and western (15.35 ± 0.16 cm)
283 populations ($F = 75.4$, $DF = 2$, p -value < 0.0001), which did not diverge among them. Although days
284 to flowering was affected by our block design primarily within the western population, we were able
285 to contrast this trait throughout latitudinal populations (Table S2, Figure S2). We showed that date of
286 flowering is different for all the populations with southern populations blooming first, followed by the
287 western, and finally by the northern samples (Figure S2).

288

289

290



291

292 **Figure 2.** Phenotypic changes between ancestral and recent samples exhibited divergent trends for rosette size, days to
 293 flowering, and mean capitula size across three populations along a latitudinal gradient in France. Seeds were resurrected
 294 from a northern (North), central-western (West), and southern (South) population and traits were measured in a common
 295 garden experiment. **A-C.** Rosette size (cm). **D-F.** Mean Capitula size (mm). **G-I.** Days to flowering from germination. **J-L.**
 296 Mean seed set (a proxy for fitness). Significance codes: p-value < 0.0001 '***', < 0.01 '**', 0.01-0.05 '*'. Vertical lines
 297 correspond to the standard error.

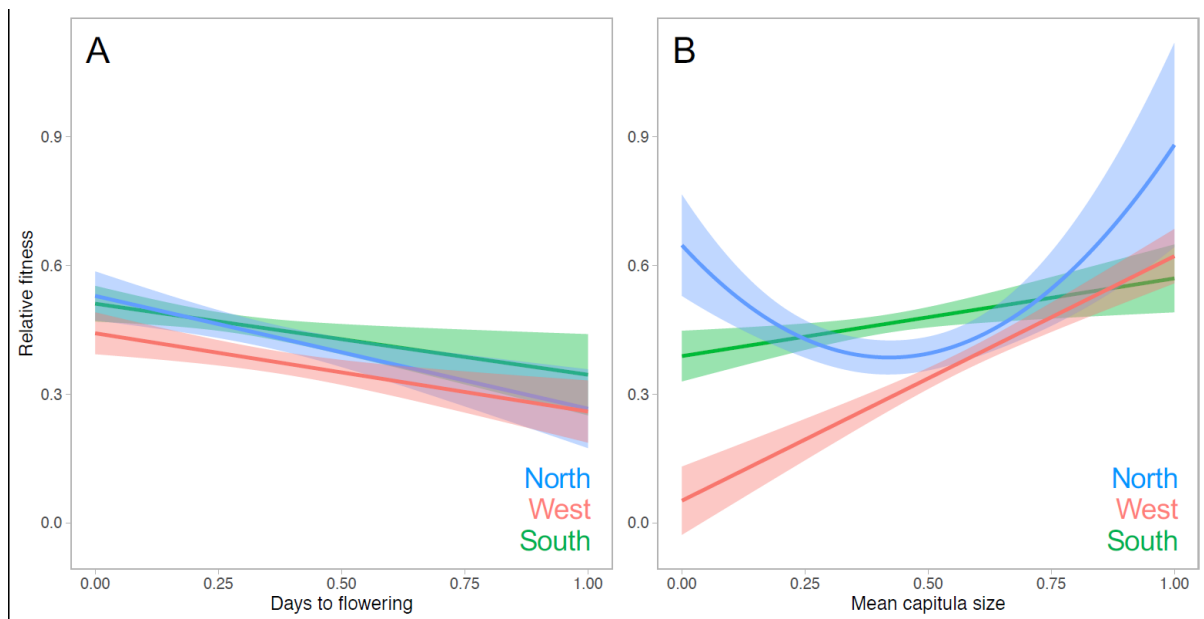
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299 We found moderate correlations between most of the traits for the temporal samples within
300 populations, including plant size, mean capitula size, biomass, and number of capitula (Table S4). The
301 strongest
302 phenotypic correlation was between biomass and number of capitula throughout all populations, with
303 values ranging from 0.821 to 0.902.

304

305 Finally, all significant effects remained significant after Benjamini and Hochberg correction for
306 multiple testing except mean capitula size in the southern population, which was marginally
307 significant (p-value =0.052, Table S2) suggesting moderate evidence for this shift.

308



309
310

311 **Figure 3.** Earlier flowering and larger capitula size are correlated with higher fitness. Graphs depict the relationship between
312 average flowering date and mean capitula size versus fitness measured in the common garden experiment in southern
313 France. Selection gradients were inferred estimating fitness as mean seed set multiplied by number of capitulas and by fitting
314 regression models using maximum likelihood methods. Shaded areas indicate the standard deviation.

315

316 *Strength of selection on phenotypic traits*

317

318 Phenotypic selection analysis at the experiment location with a natural community of pollinators
319 indicated that reproductive traits were significantly correlated with fitness (Table 1). We found
320 negative linear selection gradients for days to flowering across all populations indicating that earlier
321 flowering led to higher fitness (Figure 3A). Similarly, plants with larger capitula sizes showed higher
322 fitness, though selection is disruptive on the northern population ($\gamma = 0.518$, $p < 0.0001$, Figure 3B).
323 Within populations, we found directional selection for larger capitulas in the ancestral samples from

324 the southern 1999 sample, while for the recent 2015 sample, selection on capitula size was not
325 significant (Table 1). In contrast, in the western population, there was strong positive selection for
326 bigger flowers on both temporal samples with the strength of selection being higher on the recent
327 2015 population, which shows a lower mean capitula size compared to the ancestral 1991 (Table 1).
328 Notably, selection for larger capitulas was stronger for the sampling dates with smaller capitulas in
329 both western and southern populations.

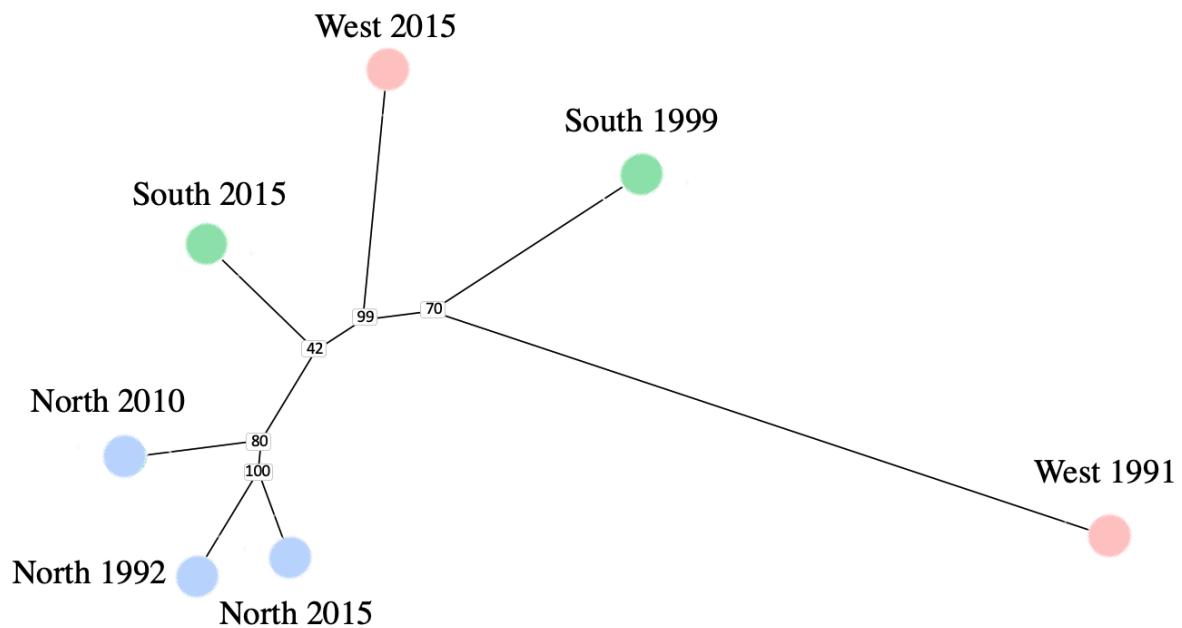
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331 In all our regression models, the inclusion of multiple predictors had only a slight influence on the β
332 and γ terms, with no change in the estimated direction of selection, suggesting that selection is mostly
333 acting on individual traits.

334

335

336



337

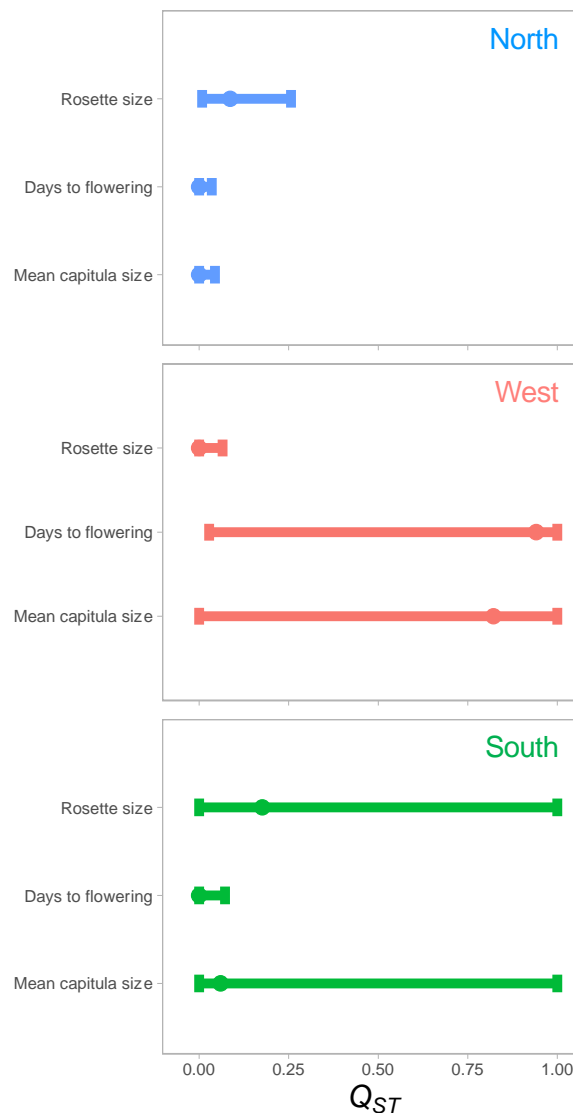
338 **Figure 4.** Neighbour-joining tree based on raw pairwise F_{ST} estimates from microsatellite markers among temporal samples
339 from the different populations. Numbers at nodes denote bootstrap support. Blue, northern population; red, western
340 population; and green southern population.

341

342 *Neutral genetic differentiation between temporal samples and among populations*

343 All populations and temporal samples exhibit high levels of genetic diversity, which did not
344 significantly differ across temporal samples and populations (Table S1). Global F_{ST} values suggested
345 moderate differentiation between populations (Figure 4, Table S5). Comparisons between the Northern
346 temporal samples showed lower F_{ST} values (F_{ST} ranged from 0.015 to 0.036) compared to any other

347 western or southern samples (F_{ST} ranged from 0.042 to 0.1391), supporting genetic continuity of the
 348 northern samples across the three sampling dates. In contrast, ancestral and recent samples from the
 349 West and the South clustered together and showed a higher genetic affinity by temporal sample than
 350 by sampling origin (Figure 4, Table S5). Unexpectedly, the western 2015 sample showed the lowest
 351 differentiation with the southern 2015 ($F_{ST}= 0.067$ [0.004, 0.094] 95% CI). By contrast, although the
 352 western 1991 sample showed the lowest F_{ST} with the northern 1992 ($F_{ST}= 0.1232$ [0.083, 0.167] 95%
 353 CI), it seemed highly differentiated from all the other temporal samples. Thus, suggesting high levels
 354 of ancestral as well as ongoing asymmetrical gene-flow across populations (Figure 4, Table S5).
 355



356

357 **Figure 5.** Natural selection played a role shaping some of the observed distributions of traits of ancestral and recent samples.
 358 Graphs depict comparisons between Q_{ST} and F_{ST} for growth-related and reproductive traits between temporal samples. Dots
 359 represent the estimated Q_{ST} between the ancestral and recent samples from northern, western, and southern populations with
 360 their 95% confidence intervals (CI). The grey shade represents the F_{ST} 95% CI estimates.

361

363

364 Comparisons of F_{ST} and Q_{ST} estimates for the different traits indicated a significant role of selection
 365 for some traits between the ancestral and recent samples (Figure 5). Q_{ST} point estimates were higher
 366 than the F_{ST} 95% CI for rosette size in the North and the South, and days to flowering and mean
 367 capitula size in the West, thus suggesting moderate evidence for divergent selection on these traits
 368 (Figure 5). Contrariwise, the Q_{ST} for rosette size in the West was significantly lower than the F_{ST} ,
 369 pointing towards stabilizing selection maintaining similar values of this trait (Figure 5). Of note, Q_{ST}
 370 point estimates for days to flowering ($Q_{ST} = 0.969$) and capitula size ($Q_{ST} = 0.902$) for the western
 371 population were remarkably high (Q_{ST} values > 0.7 are considered high, see (O’Hara 2005)) although
 372 the CI basically spanned the entire range of possible values. In the South, the empirical Q_{ST} point
 373 estimate for mean capitula size was slightly higher than the point F_{ST} estimate, yet this trait showed a
 374 wide confidence interval, similarly to other traits with higher Q_{ST} point estimates. All remaining traits
 375 showed lower Q_{ST} point values than the F_{ST} estimates (Figure 4).

376

377 **Table 2.** The linear (β) selection gradients and quadratic (γ) selection gradients for plant traits per temporal sample and
 378 population. (ns: not significant $p > 0.010$, † $p < 0.10$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

379

Traits	North			West		South	
	1992	2010	2015	1991	2015	1999	2015
Rosette size	0.270ns	0.138ns	-0.097ns	-0.204**	0.00001ns	0.093ns	0.087ns
Days to flowering	-0.430†	-0.090ns	-0.395**	-0.286**	-0.164*	-0.069ns	-0.148ns
Mean capitula size	0.038ns	-0.002ns	-0.132ns	0.273**	0.532***	0.211*	0.008ns
		$\gamma = 0.518***$					

380

381

382

383 **Discussion**

384 We resurrected seeds from ancestral and recent samples of *Cyanus segetum* across populations at
 385 different latitudes to document plant trait evolution. The stored and recent seeds were separated by a
 386 span of about two decades, characterized by a relentless escalation of various global change
 387 components. By growing ancestral and descendant seeds in a common environment, we showed
 388 divergent evolution in plant size and capitula size for different locally adapted populations and a shift
 389 in flowering date between ancestral and recent samples from western France. Importantly, the
 390 similarity between neutral genetic diversity estimates across temporal samples suggests that genetic
 391 drift is not the sole driver of the shifts observed. We documented selection gradients for earlier
 392 flowering and bigger capitula size, which along with Q_{ST} estimates for rosette size and days to

393 flowering, indicate that natural selection may have played a role in triggering some of these
394 phenotypic changes over time. Thus, our results outline the rapid pace of evolution shaping the
395 trajectories of natural populations across local spatial gradients and the potential for adaptation of
396 populations facing global change through contemporary evolution.

397 *Is the evolution of plant traits across populations consistent with global change?*

398 Rosette size evolved in different directions in the northern and southern populations: while it steadily
399 decreased over time in the North, it increased in the South. We found moderate evidence that these
400 divergent shifts were driven by natural selection with point Q_{ST} estimates higher than the F_{ST}
401 confidence intervals. These idiosyncratic changes in face of warming are likely related to the
402 inconsistent impact of global change across latitudes. Increases in leaf size in southern warmer
403 habitats have frequently been demonstrated (Royer et al. 2005; Niinemets et al. 2007; Tng et al.
404 2013). These studies agree with predictions from simulation experiments suggesting that variations in
405 leaf size along a climatic gradient result from greater evaporative demand of larger leaves due to
406 enhanced thickness of the boundary layer for energy and gaseous exchange (Niinemets et al. 2007).
407 Yet, reduced plant size and leaf size have also been observed in response to pronounced droughts
408 (Eziz et al. 2017). An increase in plant size in the South is nonetheless consistent with a mechanism to
409 cope with the escalation of high temperatures in the Mediterranean (Harpaz et al. 2014).

410 Additionally, severe droughts in southern France in the first decade of the twenty-first century may
411 have hindered increase in plant size (Figure 1B), suggesting that the observed increase is likely the
412 result of recent selection over the last ten years when precipitation has been, overall, higher than
413 average (Figure 1B). Larger plant size might constitute a fast-growing strategy to reach minimum
414 sizes for early flowering, as apparently indicated by the negative correlation between onset of
415 flowering and plant size we found only for the southern population. Similarly to this result, Sun and
416 Frelich (Sun and Frelich 2011) showed a negative correlation between growth rate and flowering
417 onset across 25 herbaceous plants, hinting at a fast-growth, flower-early strategy; which might also be
418 the case of the cornflower in the southern population. The steady decrease in rosette size in the
419 northern population can be interpreted as the result of warming with non-limiting precipitation. In
420 such context, selection for a fast-growing strategy is relaxed, resulting in reduced size of seedlings.
421 This is consistent with the positive and non-significant selection gradient for rosette size in the
422 northern population.

423
424 We observed a latitudinal gradient for flowering onset with northern populations flowering later than
425 southern ones, closely resembling the temporal patterns of the plant communities in these latitudes
426 (Rhoné et al. 2010). In annual plant species as the cornflower, flowering time is a major adaptive trait

427 because it synchronizes the initiation of reproduction with favourable environmental conditions and is
428 expected to respond rapidly to latitudinal changes (Rhoné et al. 2010). In northern populations, where
429 winters are colder and frost may still occur during spring, plants flowering too early are exposed to
430 frost damage, whereas in southern populations, plants flowering exceedingly late could suffer from
431 heat and drought stress in early summer (Rhoné et al. 2010). Besides the expected latitudinal gradient
432 in floral phenology, we found heterogeneous flowering date shifts among temporal samples.
433 Flowering has become earlier only for the western population whereby the recent sample flowered, on
434 average, six days before the ancestral. Remarkably, Q_{ST} estimates moderately supported the role of
435 divergent selection triggering this shift in the western population.

436 Regardless of the population and dates, selection gradients showed that earlier flowering resulted in
437 higher fitness at the warmer southern location where the experiment was carried out. The earlier
438 blooming of spring-flowering species is a well-characterized response to climate warming because
439 flowering is especially sensitive to temperature changes (Abu-Asab et al. 2001; Fitter and Fitter
440 2002). Explicitly, advances in first flowering is directly correlated with an increase in minimum
441 temperatures (Abu-Asab et al. 2001; Fitter and Fitter 2002; Nevo et al. 2012; Dijk and Hautekèete
442 2014) and minimum temperatures have significantly increased for all locations (Table S6).
443 Nevertheless, the widespread trend of selection for earlier flowering, although consistent with global
444 change and life history theory, can be misleading if the trait is condition-dependent, flowering
445 duration is unaccounted for, or not all fitness elements are considered (Austen et al. 2017). Flowering
446 date in self-incompatible species like the cornflower can also be constrained by pollinator emergence,
447 which is expected to be a primary factor shaping the evolution of early flowering. In addition, given
448 the low level of genetic differentiation between the western 2015 and Southern 2015 samples, and
449 their similitude in mean flowering date, we cannot rule out a contribution of migration from
450 populations locally adapted to southern latitudinal selection regimes into the advance in flowering
451 date observed for the western population. Likewise, a shift towards earlier floral phenology could
452 have contributed to greater synchronization between western 2015 and southern 2015 populations,
453 thus facilitating gene-flow. This hypothesis is also supported by studies showing that human-
454 transportation of seeds is a major factor reshuffling genetic variation in natural populations of *C.*
455 *segetum* in France (Le Corre et al. 2014).

456 Capitula size exhibited shifts over time and was the main trait associated with fitness across temporal
457 samples and populations. Selection gradients between temporal samples measured in southern France
458 indicated that selection was stronger for ancestral samples, as well as for samples with smaller
459 capitulas. Capitula size (flower size) plays a key role in pollinator attraction and seed set
460 (Parachnowitsch and Kessler 2010; Gervasi and Schiestl 2017). Because seed set has been shown to
461 positively correlate to capitula size in the cornflower (Thomann, 2014), we assume that the strong

462 positive relationship between larger capitulas and fitness observed in our experiment was driven by
463 pollinators. Indeed, pollinators can drive adaptive evolution for traits that maximize their attraction
464 and subsequent pollen delivery (Gervasi and Schiestl 2017). Particularly, pollinators select for larger
465 flowers because they are more conspicuous and may be associated with greater rewards (Blarer et al.
466 2018). For instance, Parachnowitsch and Kessler (2010) manipulated the effect of pollinator on fitness
467 through hand pollination and compared phenotypic selection in open- and hand-pollinated plants.
468 They found that natural selection was significantly stronger in the open-pollinated plants and that
469 hand pollination removed directional selection for larger flowers (Parachnowitsch and Kessler 2010).
470 It is thus likely that decreases in pollinators abundance, namely pollinator decline, have driven the
471 evolution of capitula size in the cornflower; although this remains to be tested empirically via
472 experimental manipulation of pollinator availability. Martin et al (2019) have reported declines in
473 pollination services in France based on reductions in pollinator-dependant crop yield. Surprisingly, in
474 the face of pollinator decline, temporal evolution of this trait was not convergent across populations,
475 although bigger capitulas were correlated with higher fitness. In fact, we found moderate evidence
476 that mean capitula size has decreased in the western population while increased over time in the
477 southern population. Conversely, in the northern population, while Thomann et al. (2015) reported a
478 significant increase in capitula size between 1992 and 2010 (using the same seed lots but conducting
479 an *in situ* greenhouse experiment), we report the same trend here although no statistically significant.
480 The failure to recover the changes reported by Thomann et al. (2015) is likely associated with the
481 higher variance in the phenotypic traits introduced by our field experiment due to an *a priori* bias in
482 the plastic responses of the non-locally adapted populations from northern and western France
483 (Whitlock 2008a; Rhoné et al. 2010).

484

485 In addition to the variance introduced by the field experiment, there were phenotypic differences
486 between the spatial blocks, which suggest that pollination, competition, or resources may have
487 differed at a fine scale in the field (Table S3, Figure S2, S4). Higher seed set closer to the edges might
488 reflect increased accessibility to foraging pollinators. Contrariwise, since competition is expected to
489 have played a minor role given that plants were separated by a meter, decreases in plant size towards
490 the edges may be the result of higher wind exposure. Besides germination and rosette size, which
491 were measured before transplantation, we were not able to make comparisons of trait values across
492 different populations to explore for signatures of local adaptation since we only randomized within
493 populations (Figure S1). Several studies have shown that heterogeneities among blocks mostly alter
494 plastic responses but not the direction of the evolutionary change, which can still be detected. For
495 instance, through a resurrection experiment, Frachon et al. (2017) grew *Arabidopsis thaliana* seeds
496 collected across an 8-year period in six micro-habitats with different soil and competition conditions

497 representative of the local populations (Frachon et al. 2017). They found that evolution of the
498 phenotypic traits towards either higher or lower values was consistent across micro-habitats, though
499 the underlying genetic basis and the strength of the significance varied (Frachon et al. 2017). We
500 therefore regard as unlikely that the microhabitat conditions of the terrain have affected the direction
501 of the evolutionary change that we report but presumably only its significance. Within our findings of
502 increased capitula size in the South, and the previous experiment using seeds from the northern
503 locality (Thomann et al. 2015), is nested the possibility of a general trend towards larger floral display
504 over time. A reciprocal transplant design, instead of a common garden experiment restricted to one
505 locality, would have allowed us to test population-specific adaptation across the sample locations to
506 assess whether fitness of the recent samples was indeed higher in their habitats. Additional refinement
507 of the experiment to include a pollinator community under controlled conditions across all the studied
508 localities might improve our understanding of the influence of local factors into the characterization
509 of convergent/divergent evolution of the phenotypic traits and the adaptive value of these changes.

510 Self-incompatible plant species facing pollinator decline, are expected to increase flower size or other
511 traits related to floral display to enhance pollinator visitation (Thomann et al. 2013). Gervasi et al.
512 (2017) conducted real-time experiments showing that populations pollinated by bumblebees evolved
513 larger plants and flowers in only six generations. Since the cornflower is a self-incompatible that
514 entirely relies upon pollinators for fertilization, evolution towards showier capitulas might be
515 associated with the pollinator-poor environments caused by the widespread increase in habitat
516 destruction, pesticide use, light pollution, and other components of global change fostering declines of
517 pollinator communities (Brys and Jacquemyn 2012; Thomann et al. 2015). Given that short-term
518 evolution towards self-compatibility is rare among plants, the evolution of reinforced interactions with
519 pollinators can be considered as one of the main adaptive mechanisms for outcrossing species like *C.*
520 *segetum* under pollinator decline (Thomann et al. 2013). However, Ramos and Schiestl (2019) have
521 revealed that higher degrees of self-compatibility in *Brassica rapa* can evolve by experimentally
522 manipulating pollinator abundance, which highlights differences in evolutionary trajectories
523 depending on species and the genetic architecture of the self-incompatible system. Moreover, floral
524 signals used for pollinator attraction can also be perceived and used by other organism making
525 reproductive traits particularly vulnerable to conflicting selection (Strauss and Irwin 2004; Raguso
526 2018). For example, antagonists such as herbivores and pre-dispersal seed predators can also exert
527 strong selection on floral traits (Strauss and Irwin 2004). Notably, in the northern population, bigger
528 capitulas are seemingly related to higher fitness after intermediate capitula sizes, and there seems to
529 be a trade-off at further increases in capitula size, which might be explained in part by a higher extent
530 of seed predators. Moreover, conflicting selection may contribute to explain the fact that the recent
531 western population evolved towards smaller capitula size but had a lower rate of infestation compared
532 to the ancestral one. Accordingly, we do not rule out that seed predators may have played a part

533 triggering this seemingly maladaptive shift towards smaller capitulas resulting in lower rates of seed
534 predator infestation in spite of compromising pollinator visitation.

535

536 A common finding is that measures of genetic variance are increased in novel environments causing
537 Q_{ST} estimates to be too low (O'Hara 2005a; Whitlock 2008b). Consequently, identifying traits for
538 which Q_{ST} is significantly different from F_{ST} has proven to be extremely difficult for studies that
539 consider less than ten populations (O'Hara 2005a; Whitlock 2008b; Leinonen et al. 2013). The impact
540 of the number of sampled populations in the estimation of Q_{ST} seems to be particularly problematic
541 for its use to test for natural selection in *in situ* resurrection experiments, whereby usually two
542 temporal samples of a population are compared (Franks et al. 2008b). Furthermore, the bias in the
543 estimation of Q_{ST} confidence intervals is appreciable when Q_{ST} true values are high ($Q_{ST} > \sim 0.7$)
544 (O'Hara 2005b). Therefore, we do not rule out that selection has played a role in the changes on
545 rosette size, date of flowering, and capitula size across populations where the Q_{ST} true estimate was
546 higher than the F_{ST} CI, especially for days to flowering and capitula size in the western population
547 whereby $Q_{ST} > 0.9$.

548

549 *Can evolution contribute to population viability of species facing global change?*

550 On-going resurrection projects represent an exciting opportunity to test whether contemporary
551 evolution is mitigating the impact of global change on plant demography, possibly rescuing
552 populations from extinction (Franks et al. 2008a). For outcrossing species, shifts in flowering time
553 and evolution towards reinforced interactions with pollinators have been predicted as the primary
554 evolutionary changes rescuing populations facing global pollinator decline (Thomann et al. 2013). We
555 uncovered evolutionary shifts in pollinator attractive traits such as capitula size, which was linked to
556 higher fitness for some populations. However, we did not observe a clear signature of fitness
557 maximization over time despite evolution across all studied populations. Migration load due to high
558 gene flow between western and southern populations might help explain the maladaptation of the
559 western 2015 sample. This recent western sample not only showed later germination and earlier
560 flowering but also a smaller capitula size, lower biomass, and seed set compared to the ancestral; thus,
561 seemingly evolving away from a fitness peak. Theoretical models and empirical evidence show that
562 gene-flow imposes a limit on local adaptation, and may foster evolutionary change in a direction
563 opposite to natural selection, resulting in a population sub-optimally adapted (Lenormand 2002).
564 However, the experiment was conducted in the South of France, which undermines the unique role of
565 gene-flow shaping this pattern.

566 We further hypothesize that frequency-dependent selection may have played a role hampering fitness

567 optimization. In particular, traits for which we report evolutionary change are presumably under
568 frequency-dependent selection, whereby there is no optimization principle to predict the outcome of
569 adaptive evolution (Ferriere and Legendre 2012). For instance, early flowering individuals will be rare
570 since most conspecific plants have not yet flowered, and given that many pollinators prefer to forage
571 in common phenotypes, these earliest bloomers can be easily skipped (Forrest and Thomson 2009).
572 Contrastingly, the advantage of phenotypes with increased plant or flower size is higher when rare or
573 in low-density populations (Jacquemyn et al. 2012). Nonetheless, it is hard to disentangle the effects
574 of frequency-dependent selection from migration load and drift, and temporal data along with
575 theoretical models will provide insights into the evolutionary dynamics of these reproductive traits in
576 *C. segetum*.

577 In summary, we have shown rapid evolution of growth-related and reproductive traits. Notably,
578 patterns of evolution are not convergent across traits and latitudes. Although compelling evidence of
579 the adaptive nature of most of these trait shifts remains elusive, they are consistent with widespread
580 documented responses to global change and might represent putative cases of evolutionary rescue.
581 Nonetheless, testing for evolutionary rescue would entail linking these observed rapid changes to
582 populations escaping the fate of extinction, which involves collecting additional demographic data
583 through long-term monitoring of the populations in the field. Indeed, there is a wealth of possible
584 feedback scenarios that can only be assessed by studying evolution *in situ*. Therefore, field
585 experiments testing for contemporary evolution should be higher on the research agenda due to the
586 importance of predicting evolutionary trajectories of natural populations in realistic environments
587 under the pressing impacts of global change.

588

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