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Adaptive significance of flowering time variation across natural seasonal environments in *Arabidopsis thaliana*

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SUMMARY

- The relevance of flowering time variation and plasticity to climate adaptation requires a comprehensive empirical assessment. We investigated natural

selection and the genetic architecture of flowering time in *Arabidopsis thaliana* through field experiments in Europe across multiple sites and seasons.

- We estimated selection for flowering time, plasticity and canalization. Loci associated with flowering time, plasticity and canalization by genome-wide association studies (GWAS) were tested for a geographic signature of climate adaptation.
- Selection favored early flowering and increased canalization except in the northernmost site, but was rarely detected for plasticity. GWAS revealed significant associations with flowering traits and supported a substantial polygenic inheritance. Alleles associated with late flowering, including functional *FRIGIDA* variants, were more frequent in regions experiencing high annual temperature variation. Flowering time plasticity to fall vs. spring and summer environments was associated with *GIGANTEA SUPPRESSOR 5* which promotes early flowering under decreasing daylength and temperature.
- The finding that late flowering genotypes and alleles are associated with climate is evidence for past adaptation. Real-time phenotypic selection analysis, however, reveals pervasive contemporary selection for rapid flowering in agricultural settings across most of the species range. Response to this selection may involve genetic shifts in environmental cuing compared to the ancestral state.

INTRODUCTION

Phenotypic variation within and among natural populations may reflect adaptive genetic response to natural selection in different local environments, as well as plastic responses across environments (Mimura & Aitken, 2010; Anderson & Gezon, 2015; Kingsolver & Buckley, 2017; Ensing & Eckert, 2019)□. Environmental conditions vary across years and seasons, which may drive the evolution of phenotypic plasticity in response to changing environmental cues (Fournier-Level *et al.*, 2016; Kingsolver & Buckley, 2017; Ensing & Eckert, 2019). Trait values for the same genotype also vary in response to microenvironmental conditions or stochastic developmental noise (Hall *et al.*, 2007; Joseph *et al.*, 2015; Lachowiec *et al.*, 2016; Laitinen & Nikoloski, 2019). Within

populations, selection may favor canalization (decreased stochastic variation) around a phenotypic optimum (Murren *et al.*, 2015) or increase stochastic variation as a bet-hedging strategy in unpredictable environments (Venable, 2007; Childs *et al.*, 2010; Simons, 2011; Gremer & Venable, 2014) or when a population is far from the fitness optimum (Rocabert *et al.*, 2020). Theory predicts that genetic differentiation of trait means, plasticity, and stochastic variation jointly contribute to adaptation to heterogeneous or changing environments (Gavrilets & Scheiner, 1993a,b; Chevin *et al.*, 2010, 2013; Botero *et al.*, 2015; Rocabert *et al.*, 2020). However, empirical studies of phenotypic selection in multiple natural environments are needed to understand how trait means, plasticity, and stochastic variation evolve and to what extent they share a common genetic basis that could constrain their independent adaptive evolution (Ordas *et al.*, 2008; Lachowiec *et al.*, 2016; Kusmec *et al.*, 2017).

The interacting contributions of adaptive genetic differentiation in trait means, phenotypic plasticity, and stochastic variation are particularly relevant for phenological traits, such as the seasonal timing of seed germination, flowering or bud burst. Timing life history events to meet favorable conditions requires an integrated response to multiple environmental cues such as day length and temperature (Wilczek *et al.*, 2009; Burghardt *et al.*, 2016b; Wadgymar *et al.*, 2018; Bonamour *et al.*, 2019). Adaptive differentiation in the response to these cues drives the divergence of phenological traits across a species range (Stinchcombe *et al.*, 2004; Bradshaw & Holzapfel, 2008; Mimura & Aitken, 2010; Blackman, 2017). Here we focus on flowering time, a trait that contributes to local adaptation and exhibits developmental plasticity to several environmental cues (Lowry & Willis, 2010; Wilczek *et al.*, 2010; Colautti & Barrett, 2013; Ågren *et al.*, 2017). Plastic and genetic shifts in flowering time have been documented in many species, and are frequently considered to be a signature of warming climate (Willis *et al.*, 2008; Anderson *et al.*, 2012; Wadgymar *et al.*, 2018). Patterns of local adaptation, phenotypic plasticity, and stochastic variation in flowering time may be critical to population fitness and potential resilience to climate change (Anderson *et al.*, 2012; Colautti *et al.*, 2017; Ehrlén & Valdés, 2020).

To understand the adaptive evolution of flowering phenology, we need to know how selection acts on flowering time and its plastic components in natural environments, and measure the genetic potential for selective response. Many studies have measured natural selection on flowering time in the wild (Ehrlén & Münzbergová, 2009; Munguía-Rosas *et al.*, 2011; Colautti & Barrett, 2013; Ågren et al. 2017; Austen *et al.*, 2017; Ehrlén & Valdés, 2020; Ensing *et al.*, 2021). However, little is known about selection on plasticity or stochastic variation in flowering time in natural environments (Ehrlén, 2015 but see Donohue et al., 2001; Exposito-Alonso et al., 2018). Such studies are needed, because in natural environments with year-to-year variation, selection will act on reaction norms across the environmental range rather than flowering date in one year or season (Ehrlén, 2015). A number of studies have measured phenological plasticity to specific environmental cues (Friedman & Willis, 2013; Burghardt *et al.*, 2016b; Blackman, 2017). However, it is challenging to quantify and interpret multivariate reaction norms to complex natural variation, as in common garden experiments conducted across multiple sites, varying for several environmental factors (De Villemereuil *et al.*, 2016; Johnson *et al.*, 2021). The genetic basis of phenological traits and their plasticity is rarely examined simultaneously in multiple environments (Ågren & Schemske, 2012). If we can identify major axes of variation along multivariate reaction norms to environmental variation, it becomes possible to infer selection and investigate the genetic basis of flowering time variation in the wild.

Here we examine genotype differentiation, phenotypic plasticity, and stochastic variation in the flowering time of *Arabidopsis thaliana* in natural environments across the native European range. *A. thaliana* is an ideal system for investigating flowering time variation because the genetics of flowering response to the environment is well understood. Flowering in this species is promoted by long days and warm ambient temperatures; the response to these promotive cues is regulated by a vernalization requirement that is lifted by winter chilling (Andrés & Coupland, 2012; Bloomer & Dean, 2017). Daily fluctuations in temperature also promote flowering (Burghardt *et al.*, 2016b; Kinmonth-Schultz *et al.*, 2016; Jenkitkonchai *et al.*, 2021). Combinations of these cues vary seasonally and across the species geographic range (Wilczek *et al.*, 2009, 2010) with cue sensitivity

differing among populations (Stinchcombe *et al.*, 2004; Giakountis *et al.*, 2010; Hepworth *et al.*, 2020). The genetic basis of natural variation in flowering time has been well-studied through QTL mapping (El-Assal *et al.*, 2001; Weinig *et al.*, 2002; El-Lithy *et al.*, 2004; Brachi *et al.*, 2010; Ågren *et al.*, 2017) and GWAS (Atwell *et al.*, 2010; Brachi *et al.*, 2010, 2013b,a; Alonso-Blanco *et al.*, 2016; Gould & Stinchcombe, 2017; Zan & Carlborg, 2019; Martínez-Berdeja *et al.*, 2020). However, fewer studies have mapped the genetic basis of plasticity or stochastic variation for flowering time traits (Hall *et al.*, 2007; Brachi *et al.*, 2013a; Sasaki *et al.*, 2015).

Our study leverages a geographically diverse panel of genotypes grown across multiple sites and seasons over the species' native climate range. Fitness data from this panel have previously been used for a genome-wide association study for loci underlying fitness (Fournier-Level *et al.*, 2011) and a demonstration of lagging adaptation to climate (Wilczek *et al.*, 2014). Flowering time data from mutants and recombinant inbred lines in the same experiments has also been used to parameterize photothermal models of flowering phenology (Wilczek *et al.*, 2009; Chew *et al.*, 2012) and measure the effects of allelic variation and loss-of-function mutations at flowering time loci (Fournier-Level *et al.*, 2013; Taylor *et al.*, 2019). Here we use flowering time and fitness data from natural genotypes to measure selection on phenological reaction norms and stochastic variation in different environments, identify loci associated with flowering time variation, and test for a geographic signature of climate adaptation at these loci.

MATERIALS AND METHODS

Plant materials and flowering time measurement

Between 2006 and 2008, 295 natural genotypes of *Arabidopsis thaliana* from widespread geographical origins were planted in five common gardens across several seasons in Europe (Table S1; Wilczek et al., 2009, 2014; Fournier-Level et al., 2011). Seeds from these genotypes were bulked under common controlled conditions at 20℃ under 14h light (Wilczek *et al.*, 2009). The plantings (site x season) analysed here are Norwich (UK) in

fall 2006 and spring, summer 2007, Cologne (Germany) in spring and fall, Halle (Germany) in fall, Valencia (Spain) in fall 2006 and Oulu (Finland) in fall 2008. For these eight plantings, germination occurred under natural light in unheated greenhouses at each field site, and seedlings were transplanted to the field synchronously with germination flushes of natural *A. thaliana* populations near each field site. Fifteen to 20 replicates of each genotype were transplanted in a randomised block design. Flowering time was determined based on the emergence of the reproductive meristem, referred to as bolting, which was more heritable than the opening of the corolla (Fournier-Level *et al.*, 2013). Flowering time in days was converted into photothermal units (PTU) following Taylor and colleagues (2019b) with the summation starting at gemination. Because our objective was to convert calendar time into phenological units comparable across different seasons rather than to estimate genotype-specific developmental rates as in Wilczek et al. (2009), the PTUs reported here do not include a vernalization modifier or a critical day length response; rather the photoperiodic input here was a linear function of daylength and identical for every genotype. The base temperature was set to 3°C (Granier *et al.*, 2002; Wilczek *et al.*, 2009). Flowering time data in Oulu were analyzed separately as the number of genotypes planted (68) was low compared to other plantings and compromised power in multivariate analysis. Plantings in Cologne were not used for selection analysis as fruit number was not counted for this site. The final dataset, comprising 26,143 observations with an average of 14 genotype replicates for each planting, is available on Figshare (DOI:10.26188/12824765).

Partitioning flowering time and plasticity components

To partition flowering time variation between genotype main effects, genotype-by-planting interaction effects (genetic variation in reaction norms across plantings) and within-planting stochastic variation, we fitted a multi-environment linear-mixed model using the R\MCMCglmm package (Hadfield, 2010) as: $FT_{ijk} = \mu + G_i * E_j + \epsilon_{ikj}$ where the flowering time of replicate *k* of genotype *i* in the environment *j* is modelled using the intercept *μ*, the random genotype effect *G* nested within each planting *E* (defined as the combination of a site and a season, eg. Norwich in Spring) and a residual *ε*. The MCMC simulation was performed with a total of 1,100,000 iterations, a burn-in period of 100,000

iterations and a thinning period of 10,000. The variance/covariance matrix was computed as the means of the posterior distributions, using genotype within planting-based solutions for the genetic structure and individual-based solutions for the residual structure to measure the genetic/residual correlation across plantings. Hierarchical clustering using complete agglomeration and a cluster tree half the height of the initial distance tree was performed on standardised genetic and residual variance/covariance matrices to identify clusters of plantings with similar patterns of environmental plasticity. The residuals for each individually observed plant were extracted from the model solution as the difference between observed and explained PTU to bolting and standardised against the mean for each genotype within planting to compute a coefficient of variation (CV for each genotype and planting combination) as a metric of stochastic variation or conversely, canalization (the smaller the CV, the more canalised the phenotype).

To determine whether displacement of a genotype from its home environment correlated to the amount of stochastic variation around genotype mean flowering time within a common environment, we regressed the CV for each genotype in each planting against the geographic displacement distance from the origin of each genotype to the planting site. The genotype provenances were sourced from the 1001 Genomes Consortium database. The relationship between stochastic variation around the mean and displacement distance was analysed with a cubic smoothing spline using the R\smooth.spline() function with λ=1 and changes in trend were identified for the displacement distance where the first derivative of the smoothed regression curve was nil.

To classify genotypes into discrete flowering time behaviour classes, a hierarchical clustering was performed as described above, using the Euclidean distance in flowering time between pairs of genotypes across plantings (Oulu excepted). Reaction norms were drawn for specific environmental gradients or contrasts using genotype mean flowering time within plantings. To explore the variation in flowering time across plantings with two variables that efficiently captures correlated variation between plantings, we computed the mean flowering time for each of the 295 genotypes within each site by season combination (seven plantings in total) and performed a principal components analysis (PCA) on genotype means with flowering time in each planting corresponding to one

variable.

Selection analysis

Fitness was estimated as the product of total silique number counted on 5 replicates per genotype and planting (except for the Cologne plantings where siliques were not counted), multiplied by the average silique length of the individual plant as previously described in Wilczek et al. (2014)□. Plants that did not survive to flowering or failed to reproduce were given a fitness of zero. The fitness dataset comprises 10,887 observations (41.6% of the flowering time dataset and available on Figshare DOI:10.26188/12824765). We used a general linear model (GLM) to estimate multivariate selection on genotype mean flowering time and stochastic variation around flowering time genotype mean within site and season except for Cologne where fitness data were absent. The GLM regressed fitness, modelled as log-gamma distributed, against the linear effect of mean flowering time and CV. Quadratic or interaction effects did not improve model fit based on Bayesian Information Criteria, so we only report linear, directional selection coefficients. Planting identity was included as a categorical fixed effect when multiple plantings were analysed in the same model (full model, fall gradient model and Norwich seasonal gradient model). Genotypic selection analysis can detect correlated selection acting within an environment on traits expressed in other environments. In particular, a multivariate selection analysis including genotype means from multiple environments can detect selection on cross-environment plasticity independently of the trait mean in the environment where it was measured (Tienderen, 1991)□. This approach was used to test for cost or trade-offs in plasticity that might constrain evolution (DeWitt *et al.*, 1998; Dorn *et al.*, 2000; Auld *et al.*, 2010; Murren *et al.*, 2015)□. We performed such analysis to test for selection on cross-seasonal plasticity and canalization in Norwich, by estimating directional selection gradients for genotype mean flowering time and CV measured in spring, summer, and fall. We also tested for selection on plasticity to environmental variation across the four sites in the fall plantings in Norwich, Valencia, and Halle by estimating selection gradients for flowering time and CV within each focal site, including flowering time means and CV from three non-focal sites (Norwich, Valencia, and Cologne). Although fitness data were not available for Cologne, correlated selection on flowering time in Cologne was

measured in the other sites. We did not include phenotypes from Oulu in these analyses to avoid losing power because of the small sample size in Oulu. Instead, we conducted a separate selection analysis in Oulu, including flowering time and CV in all of the other sites.

We last assessed selection on major axes of reaction norms across all environments by regressing fitness against PC1 and PC2 from the principal component analysis and the genotype CV within planting. We used a GLM where fitness was modelled as log-gamma distributed and alternative models including all possible combinations of linear, quadratic and pairwise interaction effects between the three variables were tested and the best model for each environment was selected based on BIC (the lower the better).

Genome-wide association

GWAS were conducted for three traits (coordinate along PC1, coordinate along PC2 and genotype mean CV across plantings) using published SNP genotype data (Arouisse *et al.*, 2020). Association tests were performed using linear-mixed models (structured association models) which parameterised the random effect of population structure with an identity-by-state matrix between pairs of genotypes computed using the EMMA package (Kang *et al.*, 2008) and the fixed effect of a single SNP, excluding those with a minor allele frequency lesser than 5%. Further to the GWAS using the nominal three traits, alternative models including PC1, PC2 or CV as fixed-effect cofactors were also tested. Significance of the SNP effects were tested using a Wald test between full models and a null model composed only of the random effect of population structure as implemented in GEMMA (Zhou & Stephens, 2012). P-values derived from Wald tests were corrected for multiple testing using a false discovery rate (FDR) correction with a nominal risk α=5% (Benjamini & Hochberg, 1995). The set of genes located within 1Kbp from significantly associated SNPs was tested for enrichment in particular ontologies using the bioprofiling portal (http://bioprofiling.simbioms.org/; Antonov, 2011).

FRIGIDA (FRI) is a vernalization pathway gene for which functional variants are known to contribute to flowering time variation in *A. thaliana* (Johanson *et al.*, 2000; Le Corre *et al.*, 2002; Lovell *et al.*, 2013; Zhang & Jiménez-Gómez, 2020). Functionality of *FRI* was imputed for all genotypes based on the presence of three common deletions in the gene

promoter (Le Corre *et al.*, 2002) for 132 genotypes (C. Dean, pers. comm.). For 2029 genotypes with genome-wide SNP genotypes (Arouisse *et al.*, 2020)□, SNPs located within 10Kbp surrounding *FRI* transcription start site were extracted and used to draw random forest regressions (10 repeated forests of 100 trees each). Models were fitted to maximise training accuracy and validated using 10-fold cross-validation and a confusion matrix approach to build confidence intervals (CI) as implemented in the R\caret package (Kuhn, 2008)□. The cross-validated accuracy for the model predicting *FRI* functionality was 0.92 (95% CI: 0.86-0.96). To avoid genetic redundancy due to linkage, we reduced the list of SNPs to those with minor allele frequency greater than 0.05 and ensured that all SNPs located within 500bp had a pairwise LD (R^2) less than 0.7 using Plink (Purcell *et al.*, 2007). This list of independent SNPs was used to compute partial R^2 for each GWAS interval in a full linear-mixed model including all independent, associated SNP as fixed effects and the population structure as random effect.

SNP to environmental variables association

Environmental variables were retrieved from the AraClim database (Ferrero-Serrano & Assmann, 2019). To identify the environmental variables best explaining the turnover of alleles associated with flowering time metrics (Fitzpatrick & Keller, 2015), we used random forest regressions between the set of independent SNPs significantly associated in the GWAS and a set of 196 quantitative environmental variables from the AraClim database as implemented in the R\gradientForest package (Ellis *et al.*, 2012)□. Correlations among environmental variables was controlled using the conditional permutation approach proposed by Strobl and colleagues (2008) with the parameters proposed by Ellis and colleagues (2012)□: a correlation threshold to define partitions of variables of $\rho^*=0.5$ and a maximum number of split values in the out-of-bag sample of $K=\log_2(0.368N_{SNP}/2)$. To assess the inflation in variable importance due to spatial autocorrelation among samples, we computed Moran's Eigenvector Maps (MEM) using the mem function of the R\adespatial package (Dray *et al.*, 2012)□ and re-implemented the gradient forest analysis including the top 10% MEMs (highest eigenvalues) in addition to the environmental variables. To assess the significance of the environmental breakpoints in driving allelic turnover, the cumulative importance over each gradient was

compared to 100 random permutation of the genotypes rows (preserving the pattern of allele frequency and linkage for a more conservative null hypothesis).

RESULTS

Variation in flowering time within and across sites and seasons

Flowering time in photothermal units differed extensively across plantings and genotypes within plantings. Flowering time was primarily plastic to seasons within sites (Fig. 1a and b) and to a lesser extent across sites in fall plantings (Fig. 1c). The broadest range of flowering time variation was observed in plantings where plants received less exposure to vernalizing temperatures, particularly Norwich in summer. Notably, genotypes differed in the magnitude but also in the direction of plasticity to seasonal environments (Fig. 1a). Hierarchical clustering identified five clusters of flowering behaviour. Cluster 2 distinguished the earliest flowering genotypes. Genotypes in Cluster 1 flowered at intermediate times (Fig. 1a-c), and tended to flower earlier in long-day spring and summer plantings relative to short-day fall plantings. Genotypes from Clusters 1 and 2 were geographically widespread (Fig. 1d). The remaining clusters (3-5) distinguished extremely late flowering genotypes that flowered later in spring and summer than in fall, most likely due to unsatisfied vernalization requirements. These genotypes mostly originated from Nordic (Scandinavia and Baltic sea) and mountainous regions (Carpathians, Pyrenees and Sierra Nevada; Fig. 1d).

The major axes of variation in flowering time across the multiple sites and seasons were determined through principal component analysis of the genotype mean flowering times in seven plantings, each planting being used as one variable (Fig. 1e). PC1 (78.3% of variance) distinguished early- and late-flowering genotypes across all plantings (Table S2) with all plantings (i.e. all sites and seasons) contributing equally and in the same direction to the construction of PC1 (Fisher combined probability test, X_{214}=128.95, P=0). It is thus interpreted as an index of mean genotype flowering time that is consistent across all of the plantings. PC2 (10% of variance) contrasted flowering time in fall in Halle, Cologne, and Valencia versus flowering time in spring or summer in Cologne and Norwich (Fig. 1,

Table S2). Thus PC2 is an index of plasticity to fall *vs.* spring or summer environments; genotypes with negative PC2 scores flowered later in fall plantings relative to spring and summer, and genotypes with positive scores flowered later in spring and summer relative to fall.

Variance partitioning across the different levels of plasticity

The general linear-mixed model fitted the flowering time data very well (R^2=0.93; Fig. S1c) with an extremely high proportion of variance explained by main genotype effects (V_G) leading to broad-sense heritabilities ranging from 0.92 to 0.97 (Fig. S1a). This is consistent with PC1 explaining a high proportion of variance, and the equal and collinear contribution of all plantings to PC1. Cross-environment genetic correlation for flowering time was also high and strictly positive (ranging from 0.66 to 1, Fig. S1a) suggesting that most of the variation in flowering time reaction norms across plantings was due to changes in magnitude of the genotype effects rather than changes in ranking. A clustering of the genetic variance-covariance matrix showed that genetic correlations were high between all the fall plantings (0.83-0.99) and between all the spring and summer plantings (0.82-1) but somewhat lower across seasons (0.66-0.81, Fig S1). Thus the evolution of reaction norms to seasonal variation within site may be less constrained in the face of divergent selection than the evolution of reaction norms across sites in fall.

In comparison residual variance, which measured stochastic variation around the genotype mean flowering time within plantings, was small across all plantings (Fig. S1b). The coefficients of variation (CV) of the model residuals were thus used as a standardised measure of stochastic variation for each genotype within planting, whereby higher CV values indicate lower levels of canalization. The average genotype CV was 0.107 units of trait standard deviation (ranging from 0.00139 to 1.67 across the whole dataset). Clustering of the residual variance-covariance matrix showed no obvious seasonal or geographic pattern in contrast to what was observed for the genetic variance-covariance matrix (Fig. S1b). Nonetheless, we observed a correlation of ρ=0.55 between average genotype CV and mean flowering time, indicating that late-flowering genotypes also show higher within-environment plasticity overall (Fig. S1d).

If flowering time were locally adaptive, stabilising selection would favor a specific trait optimum, leading to greater canalization (lower CV) for genotypes originating from close to the planting sites. This would result in canalization decreasing (i.e. CV increasing) with the distance between the planting site and the genotype provenance. Displacement distance indeed significantly correlated with canalization but two changes in trend were identified (Fig. 2): for a distance to planting between 0 and 1584km, stochastic variation increased (i.e. canalization decreased) with distance (GLM, β=3.31x10^{-5}, $F_{1,1049}$=32.18, P=1.81x10^{-8}); for a distance between 1584 and 3279km, stochasticity decreased with distance (β=-2.23x10^{-5}, $F_{1,207}$=4.74, P=0.0306); finally for a distance greater than 3279km, no significant relationship was observed (β=-8.38x10^{-7}, $F_{1,440}$=32.18, P=0.433). The analysis of individual plantings showed that the plantings driving the significant increase in flowering time stochasticity within a 1584km radius were those clustering together in the residual covariance matrix analysis (Valencia in fall, Cologne in spring and fall and Norwich in summer, Fig. S1b and d).

Selection on flowering time and plasticity within and across environments.

Real-time selection on mean genotype flowering time and CV revealed a significant fitness advantage for earlier flowering genotypes in every planting where fitness data were available except Oulu (Table 1). The adaptive value of canalization varied across planting. Selection favored increased CV (greater stochastic variation) in spring in Norwich, and decreased CV (greater canalization) in fall in Norwich and Valencia with no significant selection on CV detected in other plantings. However, all the models where multiple plantings were analysed together (all sites all season, all fall plantings across sites, Norwich plantings across seasons) showed significant selection for both early flowering and increased canalization (Table 1). Selection was essentially directional with no instance of stabilising selection for an optimal value of mean flowering time or stochastic variation within planting (no significant quadratic effects).

We tested for potential selective trade-offs between environments as proposed by van Tienderen (1991). Selection acted on plasticity to seasons in the Norwich summer planting, with selection for early flowering in summer favoring early flowering in spring but later flowering in fall (Table 2). Thus seasonal plasticity led to antagonistic directional

selection between spring or summer and fall. However, no cross-season selection on CV was detected. Across sites in the fall plantings, we detected marginally significant selection: in Norwich, favoring genotypes bolting early in Halle and later in Cologne; in Valencia, favoring canalized flowering in Cologne; in Valencia, favoring early bolting in Norwich and later bolting in Valencia (Table 2).

We finally tested for selection on reaction norms among environments using PC1, PC2 and mean genotype stochastic variation (CV) as cross-environment metrics (Table 3). Directional selection for early flowering (positive coordinates along PC1) was prevalent except in Oulu fall, where selection for later flowering was significant. This analysis also detected stabilizing selection on PC1 in Norwich spring and summer and Oulu fall (Fig. 3). Selection on seasonal plasticity was detected only in Norwich spring, where genotypes with high values of PC2 had greater fitness (i.e. genotypes flowering later under short days in fall than under long days in spring and summer). Finally, selection favored reduced CV (lower stochastic variation, i.e. canalization) in Norwich summer and fall and Valencia fall, but increased CV (higher stochastic variation) in Oulu fall (Fig. 3).

Genome-wide associations

We tested for significant association between single nucleotide polymorphisms (SNPs) and three traits: across-planting flowering time (PC1), seasonal plasticity (PC2) and average stochastic variation across all environments (CV). After correction for multiple testing at FDR=5%, 63 and 42 SNP variants were found to be associated with PC1 and CV (Fig. 4a,c) but none with PC2. However, when using PC1 as cofactor in the model for PC2, 4 SNPs were found to be associated (Fig. 4b). There was no common associated SNPs between PC1, PC2, and CV.

The polymorphisms associated with flowering time traits colocalized within 1Kbp of several relevant candidate genes. Imputed *FRI* functionality showed a strong association with PC1 in the direction of non-functional *fri* alleles accelerating flowering (structured association Wald test, $F_{209,210}$=25.752, P=8.56x10-7; Fig 4a). All genotypes from the three late-flowering clusters that were experimentally genotyped for the *FRI* locus carried a functional allele of the gene (Fig 4d). One of the associations with PC2 colocalized with the *Gigantea Suppressor 5* (*GIS5*) gene involved in the deposition of epigenetic marks

that accelerate flowering in a temperature-dependent manner (Iglesias *et al.*, 2015). The set of genes colocalizing with canalization-associated SNPs showed an enrichment for catatalic activity genes that are neither involved in protein binding, carbohydrate or chloroplast metabolism (7 genes represented out of the 574 genes with the same ontologies in the genome, chi-squared test, *P*=0.01). Among these 7 genes, *peroxisomal 3-ketoacyl-CoA thiolase 4* (*PKT4*) and *Reduced Dormancy 5* (*RDO5*) were noticeable for influencing inflorescence development (Wiszniewski *et al.*, 2014) and seed dormancy (Amiguet-Vercher *et al.*, 2015), respectively.

From the initial list of significantly associated SNPs, we identified a set of independent, LD block-tagging SNPs (index SNPs) defining 18 loci associated with PC1, 4 loci associated with PC2 and 14 loci associated with canalization. In total, the proportion of variation explained by these loci was R^2=0.36 for PC1, R^2=0.33 for PC2 and R^2=0.34 for canalization (partial R^2 for each index SNP reported in Table S3) which suggests a polygenic basis for each of these traits.

SNP to environment associations

Out of the 295 genotypes used in this study, 51% were sourced from anthropogenically perturbed habitat (cropland, artificial and cultivated areas) according to Global Land Cover 2000 database (Bartholomé & Belward, 2005) and the UMD Land Cover classification data (Hansen *et al.*, 2000). We identified an enrichment for non-functional *fri* alleles among genotypes from perturbed habitats using both Global Land Cover 2000 and UMD Land cover classification data (G-test, *P*=0.038 and *P*=6.53x10^{-4}, respectively). We next used a gradient forest analysis to detect correlations between SNPs associated with the PC1, PC2 and CV phenotypes, including *FRI* genotype for PC1, and a set of 196 environmental variables. For PC1, the climate variables predicted the turnover for the 18 index SNPs plus the *FRI* genotype with an average out-of-bag accuracy of 66% ranging from 25 to 92% (Fig. 5a). For PC2, the accuracy for the 4 SNPs ranged from 32% to 42%. For CV, the climate variables predicted the turnover for the 14 index SNPs with an average accuracy of 70% ranging from 46 to 88%. The permutation analysis showed that the cumulative importance (R_f^2) of these environmental gradients, were much greater than expected by chance (grey lines in Fig. 5a-c) and controlling for spatial autocorrelation

through MEMs did not change the ranking of climate variables importance.

The distribution of alleles associated with mean flowering time and canalization was consistently correlated with the yearly temperature variation (seasonality and annual range) and distance to the coast (Fig. 5a and 5c). The analysis of the gradient of cumulative importance for the most important environmental variable of each analysis showed that 14 out of the 18 alleles associated with late flowering (PC1) were more frequent in regions of high temperature seasonality (Fig. 5a) and that 10 out of 14 alleles associated with high flowering time stochastic variation (CV) were more frequent in regions of high temperature annual range (Fig. 5c). This result further consolidates a model where strong seasonal cueing and particularly temperature is required for the consistent flowering of genotypes having retained a winter annual strategy. Results were very distinct for the 4 SNPs associated with seasonal plasticity (PC2), with a strong influence of water equivalent anomaly in spring and summer, an index of seasonal variation in terrestrial water storage (Fig. 5b).

DISCUSSION

Plastic and genetic shifts in flowering time over the last few decades have been documented in many species, and are considered to be a widespread population response to a warming climate (Parmesan et al. 2003; Cleland et al. 2007; CaraDonna et al. 2014). Our common garden experiments revealed substantial genotypic variation in flowering time, plasticity and stochastic variation, as well as natural selection on these traits in different sites and seasons. The assessment of evolutionary potential for adaptation to future climate can be taken further with the search for genomic signatures of climate adaptation at loci influencing trait variation (Capblancq *et al.*, 2020). Our study identified specific loci associated with flowering time variation showing such genomic signal of climate adaptation.

Characterizing flowering time variation within and across natural environments

Quantifying variation in phenological traits across multiple natural environments presents

two challenges. First, developmental rates depend upon local photothermal environments, so measurements in calendar time are not comparable across sites or seasons. By measuring flowering time in photothermal time, we draw reaction norms across environments using comparable units (Wilczek *et al.*, 2009; Fournier-Level *et al.*, 2013; Taylor *et al.*, 2019) without the heterogeneity introduced by heterogeneous development across plantings. A second challenge is that multi-site common gardens capture many environmental factors, resulting in complex, multivariate reaction norms. To address this challenge, we used principal component analysis to identify major axes of variation across plantings. Mean flowering time varied dramatically among genotypes, as previously reported (Stinchcombe *et al.*, 2004; Shindo *et al.*, 2005; Lempe *et al.*, 2005) showing a very high heritability and was strongly genetically correlated across environments, a potential constraint on the evolution of plasticity (Via & Lande, 1985; Gavrilets & Scheiner, 1993b)□. Nearly 90% of all the variation was captured by two dimensions of the PCA: early *vs*. late flowering, and plasticity to long-day *vs.* short-day seasonal environments.

The variation in reaction norms among genotypes (Fig 1a-c) may reflect genetic differences in the response to seasonal cues such as vernalization and daylength (Alonso-Blanco *et al.*, 1998; Koornneef *et al.*, 2004; Giakountis *et al.*, 2010; Andrés & Coupland, 2012). Genotypes from high altitude or latitude typically require vernalization as conditioned by functional *FRIGIDA* alleles (Stinchcombe *et al.*, 2004; Méndez-Vigo *et al.*, 2011; Montesinos-Navarro *et al.*, 2012; Suter *et al.*, 2014; Hepworth *et al.*, 2020). This requirement prevents the induction of flowering in late spring or summer unless plants have undergone a prolonged chilling period (Amasino, 2010). The delayed flowering of genotypes carrying *FRI*-functional alleles (open circles in Fig. 4d) in spring and summer is therefore attributable to this unfulfilled vernalization requirement. In contrast, the acceleration of flowering in fall plantings by several early-flowering genotypes, often with non-functional *fri* alleles that dampen vernalization sensitivity (Fig. 4d), is likely the response to decelerating photoperiodic and thermal cues in fall. We hypothesise that this ecological strategy corresponds to the new evolutionary frontier in *Arabidopsis thaliana*: plants exploiting warming fall/winter conditions through short cycling and escaping the spring and summer drought.

Natural selection on flowering time variation in the field

Evidence of adaptive differentiation in flowering time along altitudinal or latitudinal gradients is often reported (Hall & Willis, 2006; Kooyers *et al.*, 2015; Wadgymar *et al.*, 2018; Mercer & Perales, 2019; Qian *et al.*, 2020; Coughlan *et al.*, 2021; Ryan & Cleland, 2021 but see Ensing *et al.*, 2021). In *A. thaliana*, later flowering genotypes typically originate from higher latitudes or elevations (Stinchcombe *et al.*, 2004; Montesinos-Navarro *et al.*, 2010; Méndez-Vigo *et al.*, 2011; Ågren & Schemske, 2012; Suter *et al.*, 2014; Lewandowska-Sabat *et al.*, 2017), so we expected later flowering to be selected for in cooler sites and seasons. This prediction was true in Oulu (Finland) at the northern range limit but in all the other plantings, we observed pervasive directional selection favoring early flowering. We also found little evidence of stabilizing selection around different optima in different sites or seasons in the Norwich summer planting (Fig. 3). This only partially overlap with our previous findings analyzing recombinant inbred lines in an early flowering background planted in fall where early flowering was selected for in Cologne and Norwich, but later flowering in Halle (Fournier-Level *et al.*, 2013). This inconsistency in Halle may be due to environmental differences between the years in which the experiments were conducted or to the genetic composition of the sample.

Only a few field experiments have measured selection on flowering time in *A. thaliana* (Korves *et al.*, 2007; Ågren et al. 2017; Samis *et al.*, 2019; Exposito-Alonso *et al.*, 2019). A common garden experiment in Rhode Island (USA) found selection favoring late flowering in a fall cohort, but early flowering in a spring cohort (Korves *et al.*, 2007). However, a provenance test with North American genotypes revealed selection for early flowering in both northern (Ontario) and southern (South Carolina) sites (Samis *et al.*, 2019). Another provenance trial with north-western Iberian genotypes planted in two sites in southern Spain detected selection for early flowering in six of eight experiments (Exposito-Alonso *et al.*, 2018). Later flowering can occasionally be favored, especially in cold climates as shown through a reciprocal transplant with recombinant inbred lines between Swedish and Italian genotypes (Ågren & Schemske, 2012; Ågren *et al.*, 2013; Dittmar *et al.*, 2014). Overall, the emerging picture in *A. thaliana* is that of selection favoring early flowering across the native European range, except the Northern edge.

Early flowering is thought to be the result of recent positive selection, associated with *A.*

thaliana's evolution as an opportunistic weed of perturbed agricultural environments after the end of the last glaciation (Toomajian *et al.*, 2006; François *et al.*, 2008; Lee *et al.*, 2017; Martínez-Berdeja *et al.*, 2020). Our common gardens were indeed agricultural fields where rapid cycling may be favored. However, frequent selection for early flowering time has been observed across many species (Munguía-Rosas *et al.*, 2011; Austen *et al.*, 2017; Ehrlén & Valdés, 2020; Ensing *et al.*, 2021). Austen et al. (2017) suggest several underlying mechanisms for this pattern, some of which may have contributed to our findings.

The evolution of flowering time plasticity

Plasticity to seasonal environments is often considered a critical component of local adaptation. By allowing organisms to express phenotypes closer to the optimum for each season, adaptive plasticity may facilitate population persistence in changing environments (Chevin *et al.*, 2010). However, the evolution of plasticity may be constrained by genetic correlations across environments (Via & Lande, 1985; Auld *et al.*, 2010; Murren *et al.*, 2015)□, or fitness trade-offs (Tikhonov *et al.*, 2020)□. Despite calls for investigations of selection on flowering time plasticity in the field (Ehrlén, 2015) such studies have been rare (Donohue *et al.*, 2000; Exposito-Alonso *et al.*, 2018). We observed strong cross-environment genetic correlations which constrain the evolution of reaction norms across sites and to some extent across seasons (Tables 2). The adaptive evolution of flowering time plasticity to season is most likely to occur in populations where multiple seasonal cohorts occur with different fitness optima (Donohue *et al.*, 2005; Burghardt *et al.*, 2016a). Our observations of selection on seasonal plasticity in Norwich, where multiple overlapping germination cohorts are common (Burghardt *et al.*, 2016a)□ illustrate how selection on reaction norms in spring (Table 3) and summer (Table 2) may antagonise direct selection on flowering time in fall, potentially constraining adaptive evolution. However, response to this selection may depend upon the relative contribution of different cohorts to population fecundity and the degree of genetic differentiation among cohorts (Fournier-Level et al. 2016). We also found some evidence for selection on flowering time plasticity across sites, which is a space-for-time proxy for plasticity to variation in fall conditions within sites across years.

Relatively few studies have tested whether natural selection acts on stochastic variation within genotypes (Franks *et al.*, 2007). In most of our plantings, selection favored reduced stochastic variation (CV) within genotypes, suggesting that canalization is adaptive in most environments. The notable exception was Oulu, where selection favored a combination of stabilizing selection on mean genotypic flowering time (PC1) with directional selection for greater stochastic variation. One possible explanation is that most genotypes flowered on average much earlier than the late optimum, so that a greater within-genotype CV could allow some later individuals to flower closer to the optimum.

The Genetic basis of flowering time adaptation

A major goal of evolutionary biology is to understand the genomic basis of adaptive variation for ecologically important traits. In *A. thaliana*, the genetic pathways controlling flowering time has been extensively studied and QTL mapping has identified major genes that may contribute to natural variation (Alonso-Blanco *et al.*, 1998; El-Assal *et al.*, 2001; Weinig *et al.*, 2002; El-Lithy *et al.*, 2004; Balasubramanian *et al.*, 2009). However, our GWAS found very few associations with known flowering time genes. The notable exceptions were the strong association of *FRI* loss-of-function alleles with flowering time (PC1) and a novel association of *GIS5* with natural variation in seasonal plasticity (PC2). *GIS5* encodes the catalytic subunit of the DNA Polymerase δ which mediates the deposition of transcriptionally active epigenetic marks influencing flowering; *gis5* mutants display accelerated flowering independently of the photoperiod pathway and additively to the thermosensing pathway (Iglesias *et al.*, 2015). Our findings set ecological context and evolutionary relevance to the gene's function by pointing to its involvement in seasonal plasticity. Otherwise, SNPs associated with flowering time in our field experiments did not tag *a priori* candidates, and explained only 33 to 36% of the genetic variance. Thus flowering time traits appear to be largely polygenic. However, the lack of GWAS signal for known candidate genes does not rule out the possibility that functional variants at these loci contribute to variation in flowering time in natural conditions. The effect of rare alleles may be undetectable in our geographically broad sample. For example, genotypes with extreme flowering time behavior such as *Cvi* or *An-1* are known to carry functional variants of flowering time genes (*CRYPTOCHROME2* for

Cvi, El-Assal et al., 2001□; or *FLM* and *HUA2* for *An-1,* El-Lithy et al., 2006) that could contribute to early fall flowering but remained undetectable in the GWAS. Associations may also be intractable if the phenotypic variation is driven by multiple independent mutations termed allelic heterogeneity, or if different genes mediate flowering time variation in different geographic regions (Brachi *et al.*, 2011). Finally, the effect of the population structure and the statistical method employed to control it can produce false positives and negatives, respectively.

The SNPs associated with PC1 and CV did not overlap, yet were both associated with similar climate variables, specifically temperature seasonality. In contrast, the distribution of polymorphism associated with seasonal plasticity (PC2) was predominantly driven by summer water equivalent anomaly (an index of variation in water storage through snowpack, soil moisture, or groundwater), suggesting a different mode of climate adaptation compared to PC1 and CV. The identification of *GIS5,* a novel and plausible candidate gene, builds confidence that the adaptive plasticity in flowering time in response to seasonal variation has a genetic basis in this species.

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AUTHOR CONTRIBUTION

JS and AMW conceived, designed, and carried out the experiment. AMW, JLR, and MDC supervised data collection. AF-L, MAT, JFP, AM-B, and MCS analysed the data and AF-L and JS wrote the manuscript with input from all authors.

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SUPPLEMENTARY FILE HEADERS:

Fig. S1: Correlation between estimates based on linear-mixed modelling of flowering time.

Table S1: Accession used in the study and mean flowering time across plantings.

Table S2: Contribution of plantings to the construction of the principal components.

Table S3: Summary of associated SNP markers and candidate loci.

TABLE HEADERS

Table 1: Effect of flowering time and flowering time canalization (CV) on *Arabidopsis thaliana*'s fecundity (seed proxy) within individual plantings or set of plantings. *: only 68 accessions were planted in Oulu.

Table 2: Effect of flowering time and flowering time canalization across *Arabidopsis thaliana*'s plantings on the fecundity (seed proxy) within individual plantings.

Table 3: Effect of mean flowering time (PC1) and seasonal plasticity in flowering time (PC2) and canalization of flowering time (CV) measured across all plantings on

Arabidopsis thaliana's fecundity (seed proxy) within individual plantings. NA: non-applicable as the effect was not retained in the best model.

FIGURE LEGENDS

Fig. 1: (a-c) Reaction norms for *Arabidopsis thaliana* genotype mean flowering time expressed in photothermal units to bolting along environmental gradients where plantings were sorted by mean flowering time. **(d)** Geographic origin of the genotypes clustered based on their flowering time behaviour. **(e)** Principal component analysis of mean genotype flowering time behaviour across 7 plantings. PC1 discriminates early- and late-flowering genotypes (positive and negative values, respectively). Positive values on PC2 discriminates higher flowering time plasticity in long-days (positive values) than in short-days (negative values) for late flowering genotypes (PC1<0) and higher flowering time plasticity in short-days (positive values) than in long-days (negative values) for early flowering genotypes (PC1>0). The clusters were determined using hierarchical clustering based on the Euclidean distance between mean flowering time within planting for each pair of genotypes. HF: Halle (Germany) in fall, VF: Valencia (Spain) in fall, KF: Cologne (Germany) in fall, NF: Norwich (UK) in fall, NSp: Norwich (UK) in spring KSp: Cologne (Germany) in Spring and NSu: Norwich (UK) in summer.

Fig. 2: Effect of the displacement distance on the level of stochastic variation for each *Arabidopsis thaliana* genotype across the seven plantings. The red curve represents the spline regression lines; the vertical dashed lines represent positions where the trend stochastic variation changed direction; the solid black lines represent the regression lines for segments of monotonous stochastic variation trend. HF: Halle (Germany) in fall, VF: Valencia (Spain) in fall, KF: Cologne (Germany) in fall, NF: Norwich (UK) in fall, NSp: Norwich (UK) in spring KSp: Cologne (Germany) in Spring and NSu: Norwich (UK) in summer.

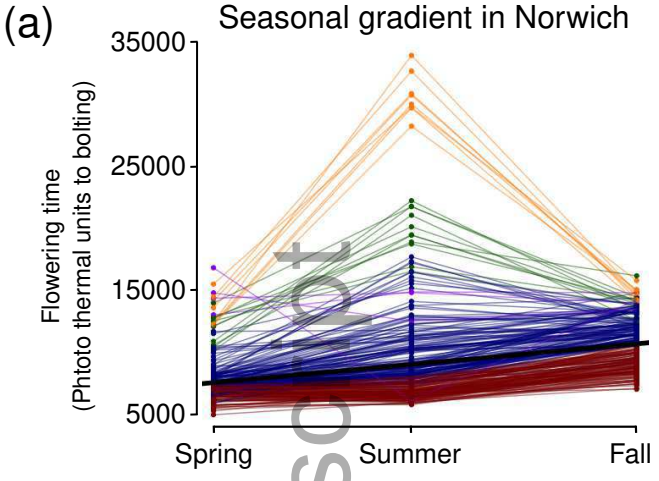
Fig. 3: Fitness landscapes for the 271 *Arabidopsis thaliana* genotypes along PC1 and

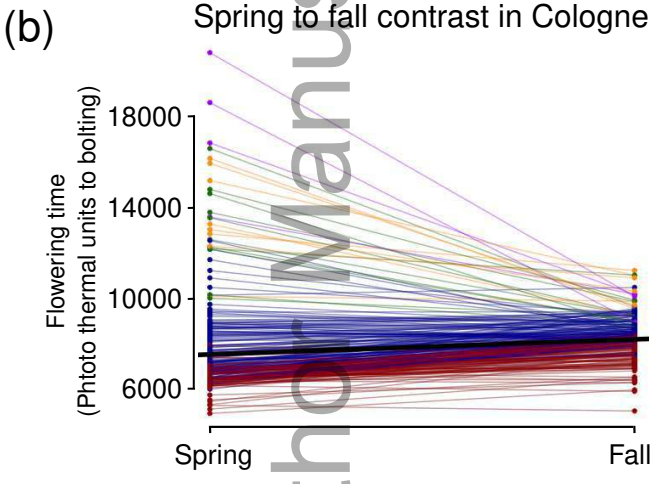
CV. No fitness data were collected in Cologne, Germany and fitness was measured for a subset of the genotypes in Oulu, Finland. The colour scale represents estimated seed number (product of the plant total silique number by the average silique length). Color on the contour represents predicted fitness from the general linear model and color filling the dots represents observed fitness from the empirical data (averaged by genotype within planting).

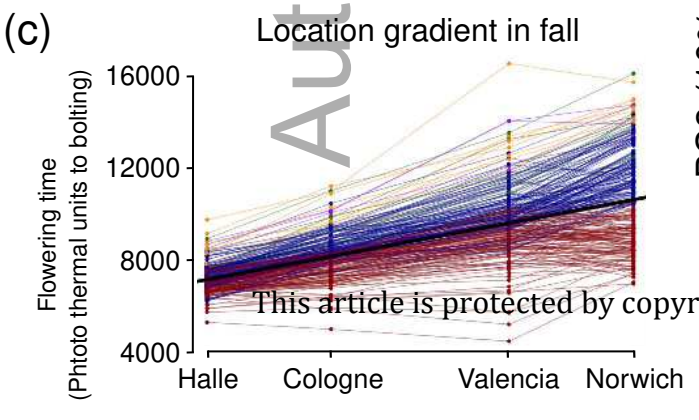
Fig. 4: Genetic associations with flowering time in *Arabidopsis thaliana*. **(a-c)** Manhattan plots presenting the level of association across the genome to mean flowering time (PC1), seasonal plasticity in flowering time (PC2) and canalization of flowering time (CV), respectively. The red horizontal line indicates the FDR=5% threshold, the orange dots highlight single nucleotide polymorphism (SNP) position located within 10Kbp of top candidate genes. **(d)** Functional class of genotype for *FRI* (FRI: functional, fri: mutant) and *GIS5* (A: late flowering in short days, T: early flowering in short days) among the genotypes used in the principal component analysis. Accessions experimentally genotyped at the FRI locus are marked with a cross (+).

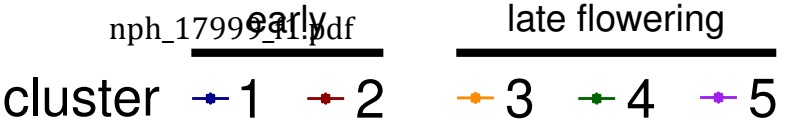
Fig. 5: (a-c) Gradient Forest analysis of the environmental drivers of *Arabidopsis thaliana*'s allele turnover for single nucleotide polymorphisms (SNPs) associated with mean flowering time (PC1), seasonal plasticity in flowering time (PC2) and canalization of flowering time (CV), respectively. The main panels report the overall variable importance for each of the 197 variables included in the analysis and classified in 6 color-coded categories. The secondary panels report the cumulative importance along the environmental gradient for the eight most important environmental variables for each of the SNP sets in colored lines against the null hypothesis drawn through random permutation in grey lines.

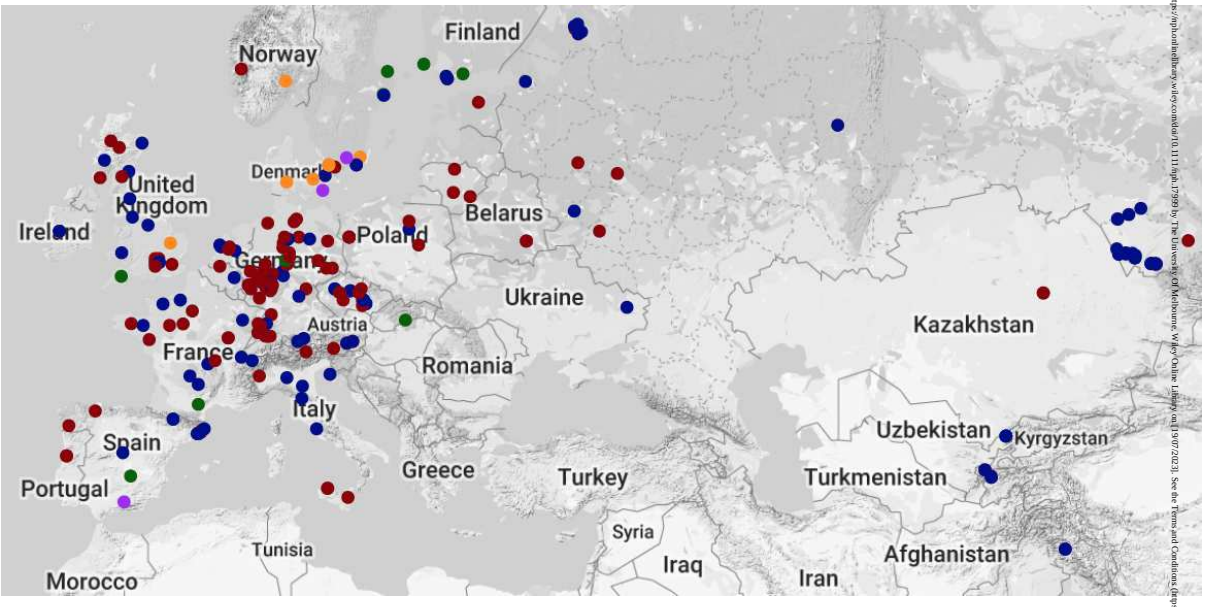
Fig. 1











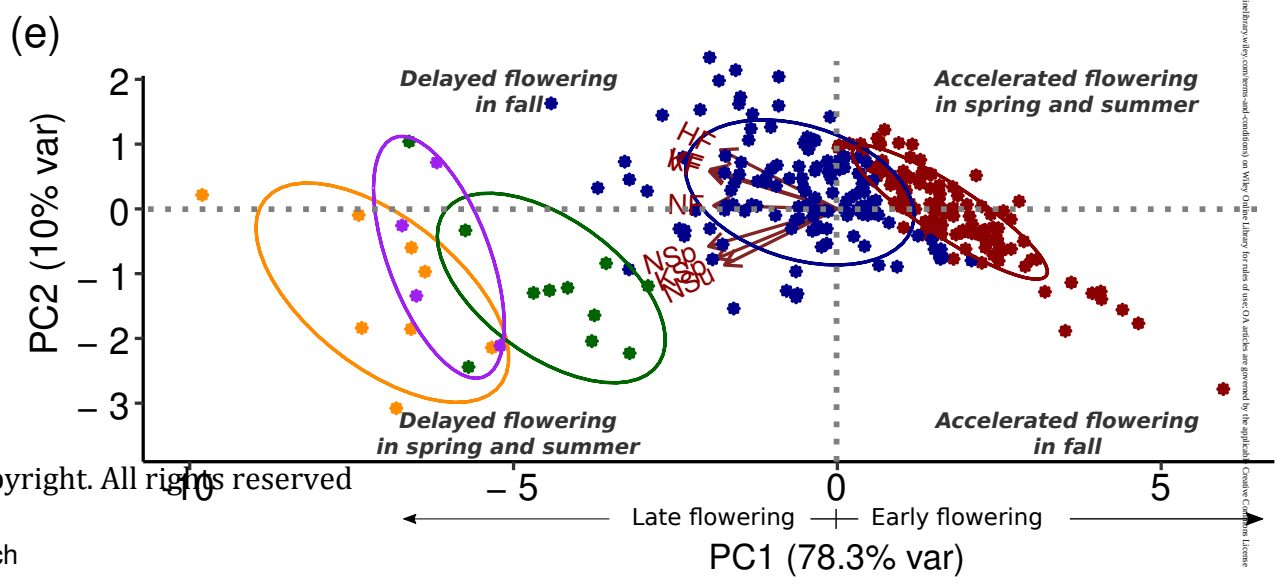


Fig. 2

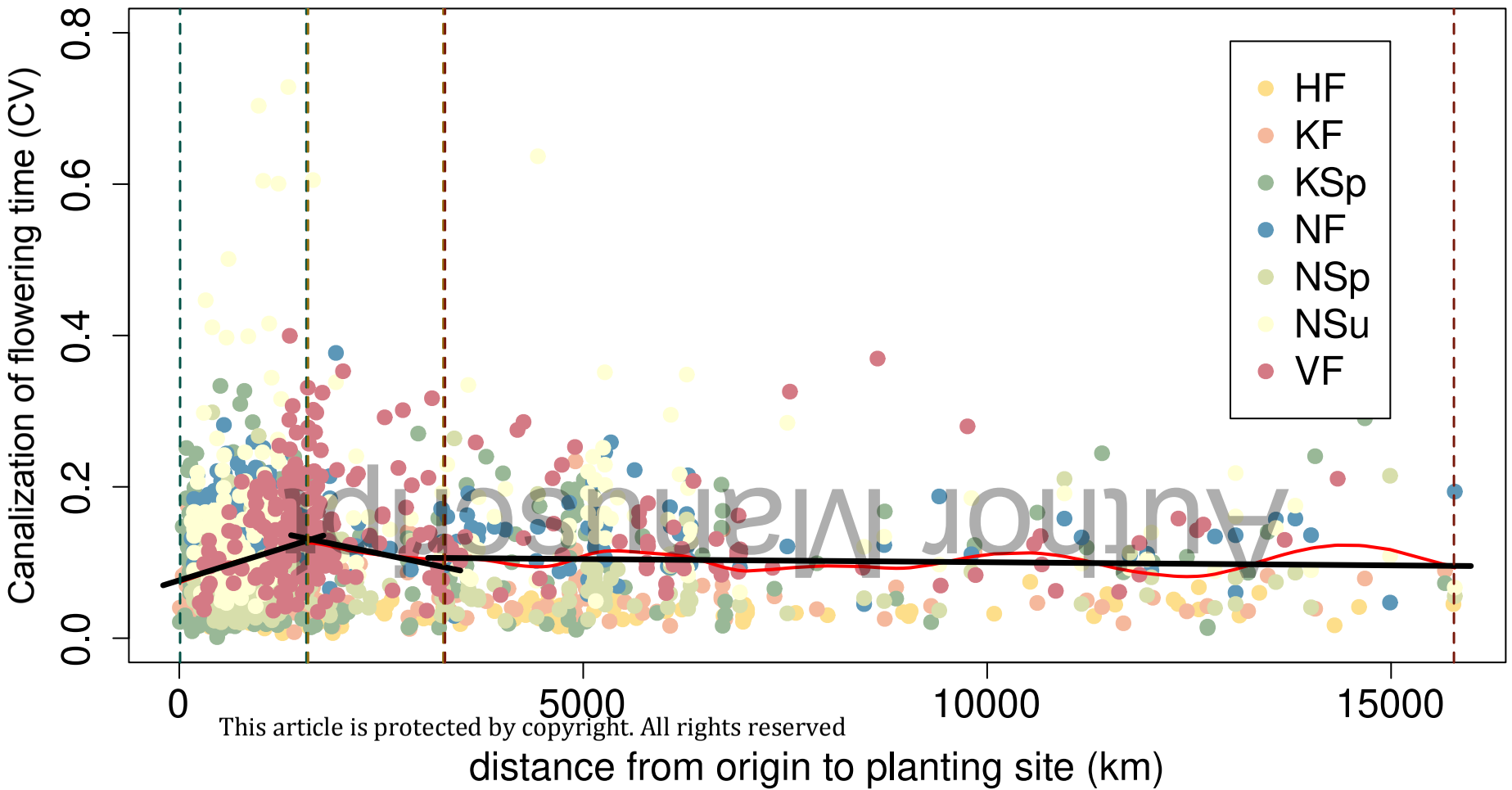


Fig. 3

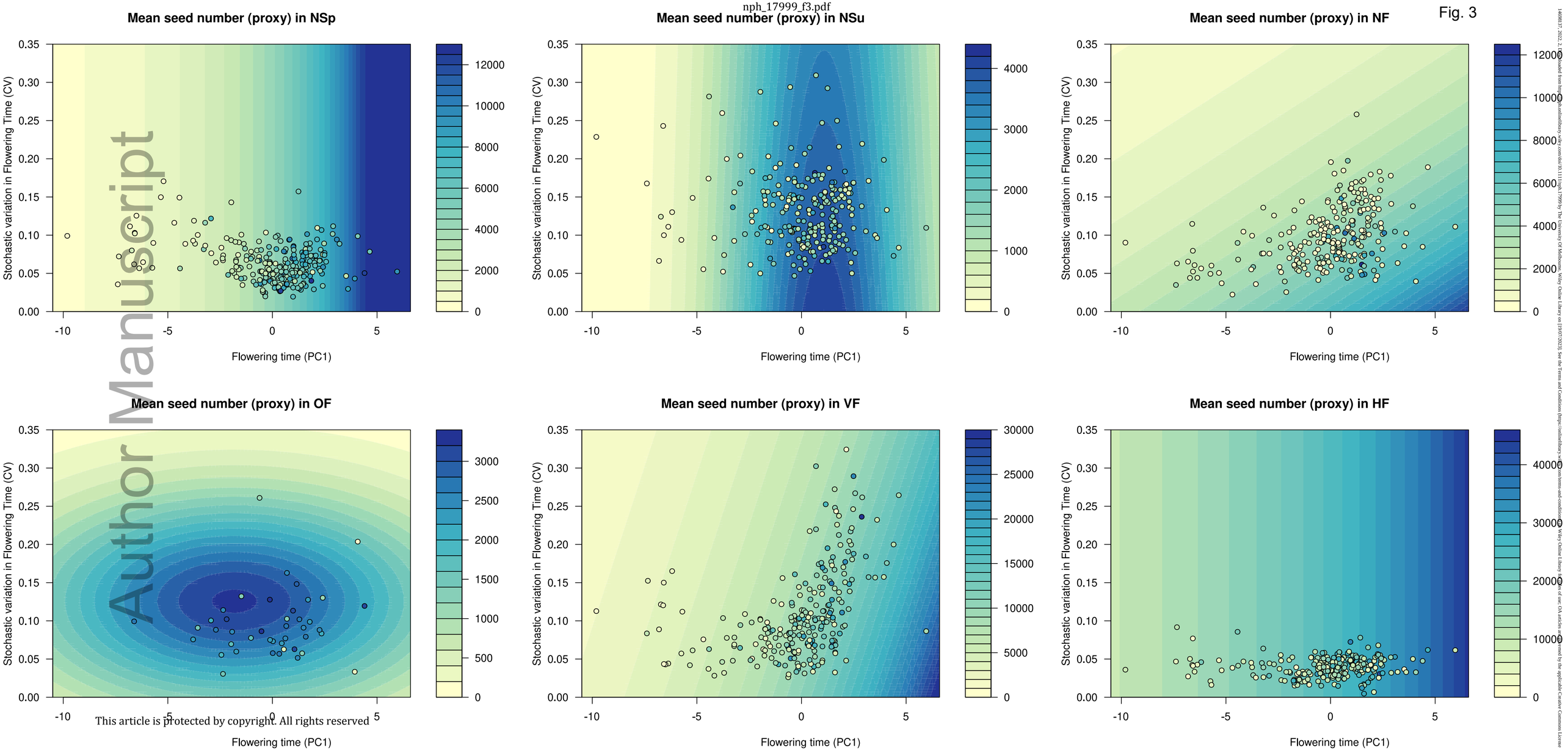
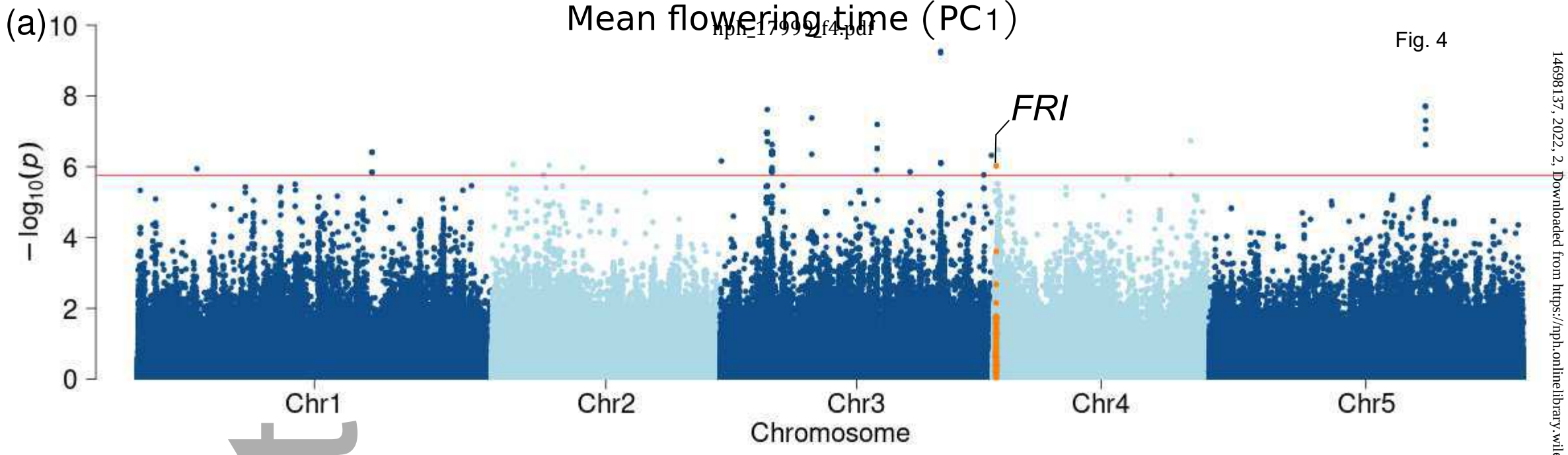
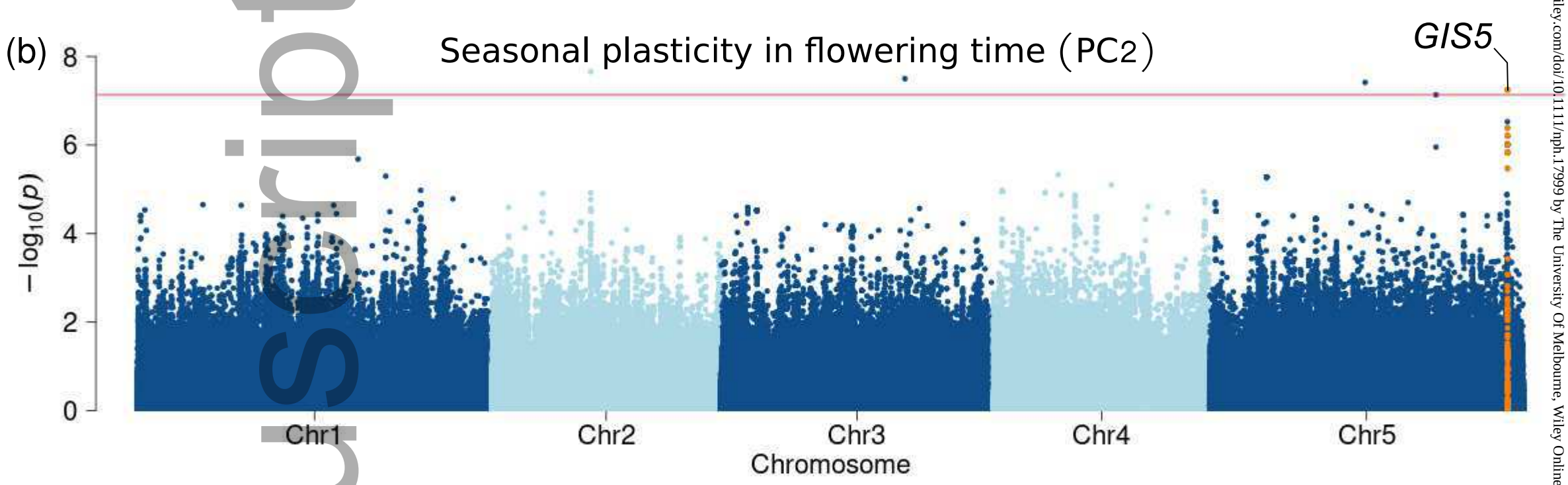


Fig. 4

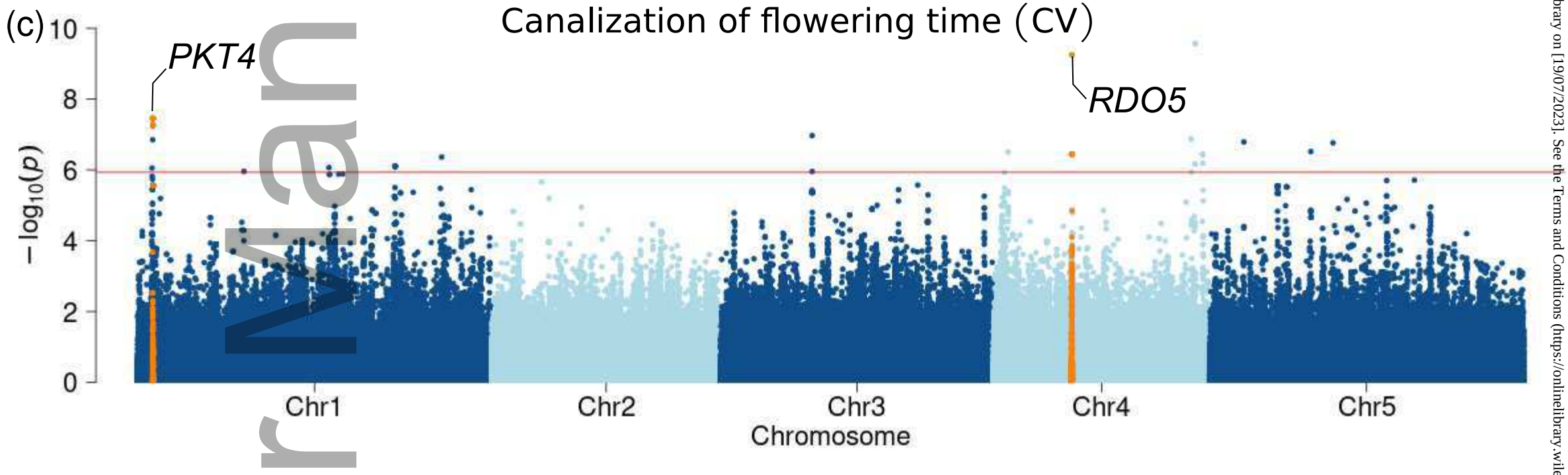
(a) Mean flowering time (PC1)



(b) Seasonal plasticity in flowering time (PC2)



(c) Canalization of flowering time (CV)



(d)

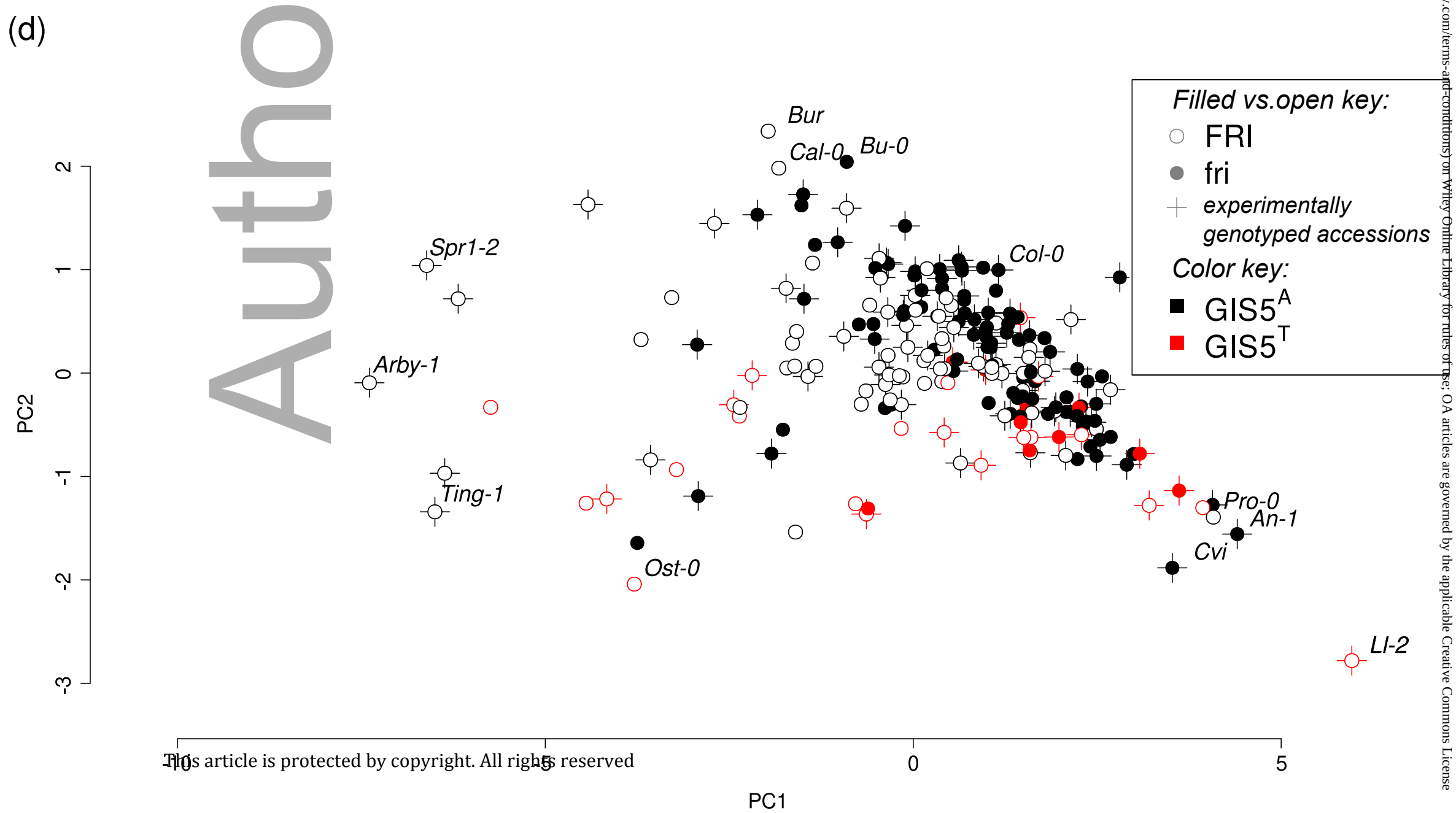


Fig. 5

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