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Original Article

Ecological and evolutionary drivers of phenotypic and genetic variation in the European crabapple (*Malus sylvestris* (L.) Mill.), a wild relative of the cultivated apple

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Abstract (250 words max with bullet points)

Background and Aims

Studying the relationship between phenotypic and genetic variation in populations distributed across environmental gradients can help us understand the ecological and evolutionary processes involved in population divergence. We investigated the patterns of genetic and phenotypic diversity in the European crabapple, *Malus sylvestris*, a wild relative of the cultivated apple (*Malus domestica*) that occurs naturally across Europe in areas subjected to different climate conditions, to test for divergence among populations.

Methods

Growth rates and traits related to carbon uptake in seedlings collected across Europe were measured under controlled conditions and associated with the genetic status of the seedlings, which was assessed using 13 microsatellite loci and Bayesian clustering method. Isolation-by-distance, -by-climate, and -by-adaptation patterns, which can explain genetic and phenotypic differentiation among *M. sylvestris* populations, were also tested.

Key Results

A total of 11.6% of seedlings were introgressed by *M. domestica*, indicating that crop-wild gene flow is ongoing in Europe. The remaining seedlings (88.4%) belonged to seven *M. sylvestris* populations. Significant phenotypic trait variation among *M. sylvestris* populations was observed. We did not observe significant isolation-by-adaptation; however, the significant association between genetic variation and the climate during the last glacial maximum suggests that there has been local adaptation of *M. sylvestris* to past climates.

Conclusions

This study provides insight into the phenotypic and genetic differentiation among populations of a wild relative of the cultivated apple. This may help us better utilize its diversity and provide options for mitigating the impact of climate change on the cultivated apple through breeding.

Keywords: population structure, isolation-by-distance, isolation-by-ecology, local adaptation, climate change, apple tree, crop wild relatives.

Introduction

Knowledge of the spatial phenotypic and genetic variation among populations is essential for understanding the ecological (biotic and abiotic factors) and evolutionary (gene flow, selection, drift, and mutation) processes involved in population divergence and adaptation (Savolainen *et al.* 2013; Sork 2018). Plant species distributed across climatic gradients typically experience spatial variation in selection, genetic drift, and gene flow, which drive genetic and phenotypic divergences among populations (Svenning *et al.* 2015). Climate indeed influences demographic processes such as population expansion and contraction, the extent of gene flow among populations, and ultimately the degree of genetic divergence among populations (Edwards *et al.* 2022). For instance, changes in the climate since the last glacial maximum (LGM) 20,000 years ago have driven the genetic composition of the European crabapple and many other tree species (Comes and Kadereit 1998; Kremer *et al.* 2002; Pyhäjärvi *et al.* 2008; Cornille, Giraud, *et al.* 2013; Riordan *et al.* 2016; Lander *et al.* 2021; Yamada *et al.* 2021 p. 20221; Parisod 2021). Climate can also shape phenotypic variation among populations. Populations occurring under the same climate may share physiological tolerances to climatic conditions, including plant carbon uptake via photosynthesis. Carbon uptake traits condition plant size and growth, reproduction, and survival under different climatic conditions(Hutyra *et al.* 2007; Nicotra *et al.* 2010; Pasho *et al.* 2012; Way and Montgomery 2015; Mercado *et al.* 2018; Hartmann *et al.* 2020; Kühn *et al.* 2021). Variation in plant carbon uptake in response to climatic conditions can result from phenotypic plasticity, i.e., the ability of individual genotypes to produce different phenotypes when exposed to other environmental conditions, in this case, climate (Dusenge *et al.* 2019). In some cases, local climate can impose divergent selection on carbon uptake traits which leads to reproductive isolation among populations: loci under adaptive divergence act as a local barrier to gene flow (Keller *et al.* 2011; Franks *et al.* 2014; Aitken and Bemmels 2015; Ramírez-Valiente *et al.* 2017; Alexandre *et al.* 2020). Climate can therefore lead to a long-term reduction in gene flow and local adaptation. Whether the phenotypic variation observed in species distributed across large climatic ranges results from their demographic or/and adaptive histories remains an intense topic of investigation (Li *et al.* 2012; Tiffin and Ross-Ibarra 2014; Briscoe Runquist *et al.* 2020). Investigating this question can help us predict how plants may respond to climate change and how species adapt to their environment.

There are multiple ways to investigate whether genetic and phenotypic variation among populations distributed across climatic gradients results from selection, genetic drift, and/or gene flow. A first step is to use a common garden experiment to investigate the genetic basis

of phenotypic variation among populations. Indeed, different populations occurring across a climatic gradient may display clinal variation, i.e., differences in a trait that may result from phenotypic plasticity or local genetic adaptation (Savolainen *et al.* 2013; de Villemereuil *et al.* 2016). Measuring candidate traits for adaptation to climate [e.g., phenology (Brachi *et al.* 2013) or traits related to plant carbon uptake (Savolainen *et al.* 2013; de Villemereuil *et al.* 2016)] in individuals from different populations under the same environmental conditions can help us elucidate the genetic basis of phenotypic variation across populations without the confounding effects of the environment. Ideally, common garden trials should include the main genetic groups across the species' distribution (de Villemereuil *et al.* 2020). The association of neutral genotypic variation with phenotypic variation can also be used as evidence of adaptive divergence among populations (Shafer and Wolf 2013; Wang and Bradburd 2014). The correlation between neutral genetic differentiation and environmental or phenotypic divergence among populations, independent of geographic distance, referred to as isolation-by-ecology (IBE hereafter), is an extension of the isolation-by-distance (IBD hereafter) model (Wright 1943). IBE patterns have been increasingly used as an indicator of adaptive divergence between populations (Shafer and Wolf 2013). In the IBE model, natural selection, which results from several factors, including climate, can indirectly increase neutral genetic and phenotypic differentiation between populations by promoting general barriers to gene flow (Nosil *et al.* 2009; Orsini *et al.* 2013; Shafer and Wolf 2013; Wang and Bradburd 2014). Although it can be challenging to map the processes underlying IBE patterns, testing for it is valuable to understand better how natural selection shapes neutral genetic and phenotypic variation. Therefore, evidence from common garden experiments and IBE patterns can contribute to understanding how genotypes, phenotypes, and the environment interact to influence population divergence and potentially local adaptation.

Fruit trees are a significant component of terrestrial ecosystems (Petit and Hampe 2011). They are grown in managed plantations and orchards to provide a variety of economically important products (Boyd *et al.* 2013). Recent breeding efforts have involved the repeated use of a limited number of commercial cultivars, leading to a reduction in genetic diversity that can lead to the loss of valuable alleles at genes that are not directly targeted by human selection (Myles *et al.* 2011; Warschefsky and von Wettberg 2019; Migicovsky *et al.* 2021). Wild relatives of crop fruit trees (hereafter CWR for crop wild relative) harbor phenotypic and genetic diversity that is potentially highly valuable for future breeding programs in the context of climate change (Zhang *et al.* 2017; Hoban *et al.* 2018; Hübner and Kantar 2021). However, phenotypic variation in CWRs in relation to climate variation has

rarely been studied in fruit trees (Kremer and Hipp 2019). Key traits to study in this context are related to plant carbon uptake. Indeed, climate impacts plant carbon uptake (Aubin *et al.* 2016), which impacts fruit quality characteristics and production (Demestihas *et al.* 2017). These are topical issues as native CWRs can be threatened by crop-to-wild gene flow from nearby domesticated trees (Delplancke *et al.* 2011; Cornille *et al.* 2015; Diez *et al.* 2015; Feurtey *et al.* 2017; Flowers *et al.* 2019; Liu *et al.* 2019). Therefore, studying the genetic and phenotypic variation among CWR fruit tree populations is timely to guide future breeding programs. It may also contribute to understanding the evolutionary and ecological drivers of population divergence, such as climate.

The European crabapple, *Malus sylvestris* (L.) Mill., is a CWR of the cultivated apple, *Malus domestica* (Cornille *et al.* 2012, 2014, 2019; Peace *et al.* 2019). Substantial crop-to-wild gene flow has been observed across *M. sylvestris* populations in Europe [(up to 23.1% of naturally occurring individuals are introgressed by *M. domestica* (Cornille *et al.* 2015)]. Crop-wild hybrids sampled in a forest in France and grown in controlled conditions showed higher growth rates compared to wild seedlings (Feurtey *et al.* 2017). Population genetic analyses also identified five pure (i.e., not introgressed by *M. domestica*) populations in Scandinavia, western France, eastern France, Eastern Europe, and Italy (Cornille *et al.* 2015). These five populations resulted from past contractions and expansions associated with the last glacial maximum (Cornille, Giraud, *et al.* 2013; Cornille *et al.* 2015). Whether these five populations distributed across a large area with different climatic conditions show a phenotypic variation that could result from local adaptation to past and/or present climates is still unknown.

We investigated the spatial phenotypic and genetic variation among populations of a wild contributor to the cultivated apple genome, *M. sylvestris*, to test for adaptive divergence, particularly to climate. Plant growth and traits related to carbon uptake were measured in 584 *M. sylvestris* seedlings grown under controlled conditions and genotyped using 13 microsatellite markers. We first assessed the genetic status of each seedling (pure *vs.* crop-wild hybrid). Then, we compared growth traits and traits related to plant carbon uptake among seedlings from different European genetic groups. We also formally tested the impact of geography (IBD) and ecology (IBE tested with phenotypic traits and climate) on genetic variation observed from 13 microsatellite markers. We investigated the following questions: 1) Do growth rates and carbon uptake traits vary between populations of the European crabapple? Are those traits heritable and therefore be good candidates for the responses to selection?; 2) Is there any association between phenotypic variation and genetic variation, taking into account geographic distance, which would suggest adaptive divergence?; and 3) Do

we detect a pattern of isolation-by-climate in the European crabapple that could suggest local adaptive divergence to climate?

Materials and Methods

Plant material, experimental design, and trait measurements

A total of 584 seeds were collected from 90 *M. sylvestris* mother trees (three-15 seeds per mother tree, Table S1) from 22 geographical sites in Europe representing the five main genetic groups previously detected in Cornille et *al.* 2015: Austria (*N* = 89, two sites), Denmark (*N* = 91, three sites), Spain (*N* = 39, one site), France (*N* = 220, eight sites), Italy (*N* = 32, one site) and Romania (*N* = 117, seven sites) (Table S1). The length, width, and weight of 30 seeds per mother tree were measured with an Opto-Agri (Optomachine, Riom, France).

In mid-April 2019, 584 seeds were washed, sterilized (in 0.5% chlorine for 20 min), and stratified for three months at 4°C in the dark in a mix of damp sand and vermiculite. Then, seeds were sowed in jiffy pellets, and each pellet was randomly placed in a 20-hole array. Seeds were grown in controlled conditions for two months (from mid-July to mid-September 2019: 22 ± 1◦C, 60 ± 5 % relative humidity, a 16:8 (L:D) photoperiod, and a light level of 40–60 µmol $m^{-2}.s^{-1}$). Each 20-hole array was rotated daily in the growth chamber to avoid any micro-environmental variation in plant response, and plants were watered weekly.

During the two-month experiment, the number of leaves and height of each seedling was recorded. Due to the low germination rate, some accessions could not be evaluated (i.e., plant height and number of leaves could not be recorded for 19 seedlings out of 584, resulting in *N* = 565, Table 1). Seedlings were measured every two to three days, starting from days 7-11 after the experiment.

In the last week of the experiment, the superficial flavonol and chlorophyll contents were measured, and the nitrogen balance index (NBI) was calculated for three leaves per seedling. The superficial chlorophyll content is the chlorophyll concentration in the leaf epidermis (µg/cm²), and the superficial flavonol content is an index of the flavonoid concentration (µg/cm²) in this upper layer and is related to phenol accumulation and UV protection. The NBI assesses the leaf's nitrogen status by calculating the ratio of chlorophyll to flavonols (related to nitrogen/carbon allocation) and is currently used as a proxy to estimate the foliar nitrogen content (Demotes-Mainard *et al.* 2008). Leaf chlorophyll, flavonol content, and NBI are parameters correlated with plant carbon uptake via photosynthesis. Flavonol is a phenolic compound that contributes to plant vigor, acclimation, and adaptation to environmental constraints through various mechanisms, including its antioxidant activity. These traits were

measured using a portable Dualex® device (Force-A, Orsay, France), which uses a combination of fluorescence signals at various excitation bands to quantify pigments and chemical compounds, and has already been calibrated on the apple tree (Hamann *et al.* 2018). As carbon uptake-related traits must be measured on the same day, a subsample of 257 seedlings out of the 565 seedlings (Table 1, numbers in brackets) was measured because of time limitations. Seedlings measured for carbon uptake-related traits were selected so that at least one seedling per mother tree and three seedlings per geographical site were sampled.

DNA extraction, microsatellite genotyping, and genetic ancestry of the seedlings

At the end of the experiment, the leaves of each seedling were sampled for microsatellite genotyping. Genomic DNA was extracted using the NucleoSpin Plant DNA Extraction Kit II (Macherey & Nagel, Düren, Germany), according to the manufacturer's instructions. Microsatellites were amplified by multiplex PCR with the Multiplex PCR Kit (QIAGEN, Inc.). We used 13 microsatellite markers, Ch01f02, Ch01f03, Ch01h01, Ch01h10, Ch02c06, Ch02c09, Ch02c11, Ch02d08, Ch03d07, Ch04c07, Ch05f06, GD12 and Hi02c07 in four multiplexes (MP01 to MP04; (Cornille *et al.* 2012)).

PCR was performed in a final reaction volume of 15 µl (7.5 µl of QIAGEN Multiplex Master Mix, 10–20 µM of each primer with the forward primer labeled with a fluorescent dye, and ten ng template DNA). We used a touch-down PCR program (initial annealing temperature of 60°C, decreasing by 1°C per cycle to 55°C). Genotyping was performed at the GENTYANE platform (INRAE Clermont-Ferrand) on an ABI PRISM X3730XL, with 2 ml of GS500LIZ size standard (Applied Biosystems). Alleles were scored with the GENEMAPPER 4.0 software (Applied Biosystems). We retained only multilocus genotypes presenting less than 10% missing data.

Clones or closely related individuals can bias inferences about the population structure. We estimated the kinship coefficient between pairs of individuals (*Fij*) with SPAGeDI 1.5d (Loiselle *et al.* 1995; Hardy and Vekemans 2002), and removed highly genetically related individuals with $Fij > 0.5$.

The individual-based Bayesian clustering method implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000) was used to estimate the admixture between *M. domestica* and *M. sylvestris*, and the population genetic structure of *M. sylvestris*. STRUCTURE uses Markov Chain Monte Carlo (MCMC) simulations to infer the proportion of ancestry of genotypes from *K* distinct clusters. The underlying algorithm minimizes deviations from Hardy–Weinberg, and

linkage disequilibria. *K* ranged from 1 to 10. Ten independent runs were carried out for each *K* and 500,000 MCMC iterations after a burn-in of 50,000 steps were used. CLUMPAK (Greedy algorithm) (Kopelman *et al.* 2015) was used to identify distinct modes in the ten replicated runs for each *K*. STRUCTURE analyses were run for the entire dataset (N = 584), plus 40 *M. domestica* genotypes from Western Europe (Table S2) included as a reference for the cultivated apple gene pool (Cornille, Gladieux, *et al.* 2013). The *R* package pophelper v2.3.0 was used (Francis 2016) to visualize bar plots. The amount of additional information explained by increasing *K* was determined using the ΔK statistic (Evanno *et al.* 2005), as implemented in Structure Harvester (Earl and vonHoldt 2012). However, ΔK provides statistical support for the strongest but not the finest population structure (Puechmaille 2016). Natural populations can display a hierarchical genetic structure with a fine-scale population structure. Visual inspection of the bar plots was used to identify the *K* value for which all clusters have well-assigned individuals, and where additional clusters at higher *K* values do not have well-assigned individuals. Therefore, the *K* value corresponded to the finest one, which can be higher than the *K* value of the strongest population structure identified by ΔK.

We used the membership coefficients at the best *K* value (inferred with STRUCTURE) for the crop and wild seedlings and defined the genetic status of each seedling. We first separated cultivated apples (seedlings from mother trees that were misidentified in the field, i.e., with a membership proportion to the *M. domestica* gene pool, P_{dom}, > 0.9, referred to as "*dom*" hereafter), crop-wild hybrids (seedlings with $0.1 > P_{dom} > 0.9$, referred to as "*cw*" hereafter) and pure *M. sylvestris* (seedlings with a membership coefficient to a given wild apple cluster > 0.9 and with $P_{dom} < 0.1$, referred to as "*pure*" hereafter). We then separated "pure" wild seedlings (i.e., seedlings with membership coefficients to a given wild gene pool > 0.9) from wild-wild admixed seedlings (i.e., seedlings with a membership coefficient to a given wild apple cluster < 0.9, "*ww*", hereafter). Two effects were then tested using the statistical models described below: the genetic status effect (i.e., *dom*, *cw*, *ww*, *pure*), and the wild apple population effect (i.e., corresponding to the "*pure*" populations detected with STRUCTURE). A seedling that could not be assigned to any cluster (with a membership coefficient to any cluster < 0.5) was removed for further analysis.

We computed descriptive population genetic estimates for each population (i.e., each cluster inferred with STRUCTURE, excluding admixed individuals with a membership coefficient < 0.9). Heterozygosity (expected and observed), Weir and Cockerham *F*-statistics, and deviations from Hardy–Weinberg equilibrium were calculated with Genepop v4.2 (Raymond and Rousset 1995; Rousset 2008).

Phenotypic traits and correlations

Height, leaf growth rate, and carbon-related traits were estimated for each seedling. The absolute height and leaf growth rates (*AGR*, (Radford 1967)), relative growth rates (*RGR*, (Briggs *et al.* 1920)), and whole *AGRs* were estimated as follows:

$$AGR(cm/day) = \frac{(trait_{t+1} - trait_t)}{(date_{t+1} - date_t)} \quad (1)$$

$$RGR(cm/day/day) = \frac{AGR}{date_t} \quad (2)$$

$$WholeAGR(cm/day) = \frac{trait_{end} - trait_{beginning}}{date_{end} - date_{beginning}} \quad (3)$$

Note that for whole AGRs, the beginning of the experiment corresponded to days 7 and 11 for leaf and height measurements, respectively, while the last measurement was performed on day 60. The *internode* ratio, which represents the ratio between the number of leaves and the height of the seedling at day 60, was also considered a fitness trait, as this value plays an essential role in apple tree architecture (Ripetti *et al.* 2008).

Seven phenotypic traits were therefore calculated for the entire dataset (565 seedlings, Table 1): *height_AGR*, *height_RGR*, *whole_height_AGR*, *leaf_AGR*, *leaf_RGR*, *whole_leaf_AGR*, and *internode*. In addition, chlorophyll (*Chl*) content, flavonol (*Flav*) content, and *NBI* were measured in a subsample of 257 seedlings (Table 1).

A preliminary exploration of the variation among phenotypic traits was performed. We used the cor R function to assess the correlation 1) between phenotypic traits and seed sizes (length, width, and weight) of the mother trees and 2) between phenotypic traits. The corrplot R package to visualize the correlations (Wei and Simko 2017). Principal component analyses of the phenotypic traits of the seedlings (PCA) were conducted with the FactoMineR R package (Lê *et al.* 2008).

Statistical analyses of phenotypic variation

A previous study demonstrated that crop-to-wild gene flow impacts early-stage growth rate (Feurtey *et al.* 2017). The effect of the genetic status of seedlings (*dom*, *cw*, *ww*, *pure*; Table 1) on fitness variation among seedlings was therefore tested. A linear mixed model was fitted to the data as follows:

$$Y_{ijkl} \sim \mu + \text{wild population of origin}_i + \text{genetic status}_j + \text{wild population of origin}_i * \text{genetic status}_j + \text{mother}_k + e_{ijkl} \quad (4)$$

Where Y_{ijk} is the phenotypic trait (height or leaf growth rate or a carbon uptake-related trait) of a seedling from mother *k* from population *i* with the genetic status *j, wild population of origin* is the fixed effect of the population of origin *i* of the seedling inferred with STRUCTURE, *genetic status* is the fixed effect of the genetic status of the seedling (i.e., *pure*, *dom*, *ww*, *cw*), the interaction between the two fixed effects, *mother tree* is a normally distributed random effect with its own mean and variance parameters and e_{ijkl} is the residual. The mother tree of each seedling was used as a random factor to avoid pseudo-replication due to the presence of multiple half-siblings (i.e., from the same mother tree). We ran the model (4), but replaced the *genetic status* effect with the *Pdom* fixed effect. We gradually removed interactions and effects depending on their significance. In addition, we evaluated the differences in the effect on trait variation using a contrast analysis. We fitted the data to the model using the *lme4* R package (Bates *et al.* 2015). The statistical significance of an effect was assessed with a type II Wald chi-square test.

For phenotypic traits estimated from the number of leaves (*leaf_AGR, leaf_RGR* and *whole_leaf_AGR),* a log-link function was used and the residual distribution was fitted to a negative binomial distribution (function *glm.nb* in R package lme4). For phenotypic traits defined from the height of the seedling (*height_AGR, height_RGR, whole_height_AGR),* and for chlorophyll content, flavonol content, and NBI, a similar linear mixed model was run, but with a residual term that was assumed to be normally distributed.

Heritability estimates

The level of heritability estimate provides an indication of the potential extent of the response to the selection of a trait. Therefore, computing heritability can allow us to determine whether the traits measured in this study could be good candidates to respond to selection. Heritability estimates were calculated using only pure and wild-wild *M. sylvestris* seedlings (Table 1). We fitted each fitness proxy with a linear mixed model as follows: $Y_{ijk} = \mu + F_i + C_j + e_{ijk}$ (5), where Y_{ijk} is the fitness proxy (growth rate or carbon uptake-related trait) of the k^{th} seedling from mother tree *i*, member of the j^{th} genetic cluster, μ the overall fixed mean of the population, F_i the random effect of the i^{th} mother tree, C_j is the fixed effect of the j^{th} genetic cluster and e_{ijk} the random error term. The model was fitted using REML (restricted maximum likelihood). Calculations were performed by the *lme*-function of the *R*-library *nlme* (Pinheiro *et al.* 2022). The output of *lme* provides estimates for the variance components, the corresponding standard

deviations (sd), and the best unbiased linear predictors (BLUP) for random effects. Genetic parameters were then calculated as follows:

- the additive genetic variance: $VA = 4\sigma_F^2$ *w*ith σ_F^2representing the between-mother tree variance
- the corresponding coefficient of variation: $CVA = \frac{\sqrt{VA}}{\mu}$
- the phenotypic variation: $VP = \sigma_F^2 + \sigma_E^2$, with σ_E^2 representing the residual variance
- the corresponding coefficient of variation: $CVP = \frac{\sqrt{VP}}{\mu}$
- Narrow-sense heritability: $h^2 = \frac{VA}{VP}$
- Dickerson's approximation of standard deviation: $sd(h^2) \approx \frac{4sd(\sigma_F^2)}{VP}$

Test for isolation-by-ecology

Only pure and wild-wild hybrid *M. sylvestris* seedlings were sampled for the IBE analysis (*N* = 449, 21 sites, Table 1). The IBE pattern, i.e., the contribution of climate (isolation-by-climate) and phenotypic (isolation-by-adaptation) distances to the genetic structure, taking into account geographical distance, was evaluated using distance-based redundancy analysis (db-RDA). Db-RDA can be used when the response variable is a distance matrix - here is a genetic distance matrix (F_{ST}) across 21 sampled sites - and the explanatory variables are in vector form as follows. The geographical distance between sampled sites, estimated with SPAGeDI 1.5d (Hardy and Vekemans 2002), that underlies an IBD process, was represented by vectors with positive Eigenvalues of a principal coordinate of a neighbor matrix (*PCNM*) (Borcard and Legendre 2002). A total of 19 bioclimatic variables, downloaded from the Worldclim2 database (30s resolution, https://www.worldclim.org/data/worldclim21.html) and representing annual and seasonal trends and extremes averaged over the years 1970-2000 and averaged for the Pleistocene period (20,000 years ago) (Gamisch 2019), were used to test for an isolation-by-climate pattern. Growth rate (*N* = 551, Table 1) and carbon uptake-related traits (*N* = 239, Table 1) averaged per site were used to test for an isolation-by-adaptation pattern, referred to as IBA hereafter.

To identify the variables that explained the genetic structure of *M. sylvestris*, a db-RDA using the "capscale" function (Oksanen *et al.* 2014) was run on a complete model that included all investigated variables (i.e., PCNM components, growth rates, carbon uptake-related traits, and 19 bioclimatic variables). The best variables were selected for an optimum model with the function "step" based on the Akaike Information Criterion (AIC). Because db-RDA does not

provide information on the relative contribution of each variable of the model, a variance partitioning analysis was run using the "varpart" function from the R-package "vegan" (Peres-Neto *et al.* 2006).

Results

Genetic status of seedlings

No clones or closely related individuals were detected (Figure S1); therefore, 584 seedlings were included in the STRUCTURE analyses.

STRUCTURE revealed a clear spatial population genetic structure of *M. sylvestris* in Europe and crop-wild admixture (Figures 1 and S2). From K=2 to K=8, several clusters appeared. When $K > 8$, STRUCTURE did not reveal any further substructures but only additional clusters with highly admixed individuals (Figure S2). Therefore, although the ΔK indicated that the most likely K value was five (Figure S3), $K = 8$ was the finest population structure and was retained in subsequent analyses. For $K = 8$, we found that the *M. domestica* reference varieties were admixed with Italian and Western French *M. sylvestris*. Note that cultivars used as references originated from Western Europe (Table S2). Conversely, we detected 68 *M. sylvestris* seedlings with $P_{dom} > 0.1$ (considered to be *cw* hybrids), corresponding to 11.6% of the seedlings ($N = 584$, Figures 1, S4, S5, S6, Table 1). We also found 21 seedlings with a membership coefficient to the *M. domestica* gene pool > 0.9, corresponding to 4% of the seedlings. Nearly all Spanish seedlings were assigned to the *M. domestica* gene pool with membership coefficients > 0.1 (i.e., 26 *cw* hybrids and 13 individuals assigned to the *M. domestica* gene pool) and showed admixture only with the wild Italian purple gene pool (Figures S4 and S6). A total of 33 individuals could not be assigned to any cluster (i.e., individuals with a membership coefficient to any cluster < 0.5).

We, therefore, identified 68 *cw*, 21 *dom*, 167 *ww*, and 282 *pure* seedlings (Table 1, $N = 551$). After removing *cw* hybrids ($N = 68$), seedlings sampled from misidentified mother trees ($N = 21$), the *M. domestica* reference samples ($N = 40$), and individuals with a membership coefficient to any cluster < 0.5, seven wild apple populations (i.e., groups of seedlings with a membership coefficient > 0.5 to a wild apple cluster) were defined: French Western (*FR-W* hereafter, $N = 77$), French Eastern (*FR-E*, $N = 50$), French Lorraine (*FR-Lor*, $N = 28$), Danish (*DA*, $N = 78$), Italian (*IT*, $N = 27$), Austrian (*AUT*, $N = 81$) and Romanian (*RO*, $N = 108$) (Figure S6). Each *M. sylvestris* population exhibited a high level of genetic variation (Table S3). The Romanian population was the most genetically differentiated and was close to the Austrian population; the Danish and French Western populations were the closest genetically (Figure

S7), which is congruent with previous results from 26 microsatellite markers (Cornille et *al.* 2015).

No effect of seedling genetic status on phenotypic variation

Variations and correlations among phenotypic traits are presented in Figures S8 to S12. Significant correlations between seed length and weight of the mother tree and height of the seedling were observed ($P<0.01$). Leaf growth rates (*leaf_RGR*, *leaf_AGR*, *leaf_whole AGR*) were significantly correlated, as were height growth rates (*height_RGR*, *height_AGR*, *height_whole_AGR*). Chlorophyll content was positively correlated with NBI, whereas flavonol content was negatively correlated with NBI. Heritability estimates were moderate to high for all traits except growth rates based on leaf number (*AGR_leaf*, *RGR_leaf*, *whole_AGR_leaf*; Table S4). Given the limited sample size, these estimates must be taken cautiously, as reflected by the large standard deviations.

We did not find any significant effect of the genetic status of the seedlings (i.e., *pure*, *ww*, *cw*, *dom*) (Table S5, Figure S13) or P_{dom} (Table S6, Figure S14) on phenotypic traits, except a marginally significant effect ($P = 0.04$, Table S6) of P_{dom} on the whole height AGR: seedlings with higher levels of introgression by *M. domestica* had higher whole height AGRs. We, therefore, removed the seedling genetic status and *Pdom* effects from the model 4, as well as *dom* and *cw* individuals. Thus, the final model only included wild apple seedlings (i.e., *pure* and *ww*, $N = 449$) and focused on the *wild population of origin* effect (Table 2).

Significant variation in growth rates and chlorophyll content among populations

The mean height variation during the experiment among seedlings from different populations is shown in Figure 2. There was significant variation among seedlings from other populations in certain growth-related traits (Table 2). On average, seedlings belonging to the Austrian population were taller (+11 cm, $P = 0.047$) whereas Romanian (-14.9 cm, $P = 0.008$) and Italian (-18.7 cm, $P = 0.044$) seedlings were shorter (Figures 2 and S15) than seedlings from other populations. Seedlings from other populations did not show a significant height difference. In addition, the number of leaves and height traits were negatively correlated, $r = -0.3$, $P < 0.001$). The Austrian population presented the lowest number of leaves (average = 5, sd = 4), whereas seedlings belonging to the Romanian population had the highest number of leaves (average = 8, sd = 7, Figure S16). The Romanian population also had the largest *internode* (+ 0.02 leaf/cm, $P = 0.024$).

Chlorophyll content differed among populations, with seedlings from the Italian population producing, on average, more chlorophyll (+4.14 µg/cm², $P = 0.039$, Figure S17) than seedlings from other populations. Flavonol content and NBI did not differ significantly among populations.

Significant IBD and IBC

Correlation plots between bioclimatic variables are provided in Figures S18 and S19; however, all variables were included in the analysis, as db-RDA can cope with correlated variables. The optimal model was chosen according to its best AIC value. The optimal model explained up to 25.9% of the genetic structure ($Adj\text{-}R^2 = 69.9\%$, $P = 0.001$) and contained seven variables (four geographic and three bioclimatic variables) (Table 3): the geographical distance is represented by the *1st, 2nd, 3rd* and *6th* axis of the PCNM analysis and three past climatic variables (Bio3: isothermality; Bio6: minimum temperature of the coldest month; Bio 9: mean temperature of the driest quarter). In total, IBD explained 47% of the variance of the wild apple tree population genetic structure, whereas IBC explained 22% (Figure S20). Taking geographical distance into account, we did not find a pattern of IBA, i.e., covariation between phenotype and genetic divergences.

Discussion

This study assessed the relationship between phenotypic and genetic variation among populations of a wild contributor to the cultivated apple genome (Cornille *et al.* 2012), *M. sylvestris*, a CWR species that occurs naturally along a climatic gradient in Europe. Bayesian clustering revealed a substantial number of seedlings introgressed by *M. domestica*. With the hybrids removed, seven populations of *M. sylvestris* distributed across Europe were detected and showed phenotypic variation in growth and chlorophyll content. Based on the IBA pattern estimated from the phenotypic traits measured in this study, this phenotypic variation was not adaptive. However, the significant association between population genetic variation and the LGM climate suggests that the European crabapple may be locally adapted to the past climate conditions of the LGM. The results of this study, therefore, indicate the occurrence of adaptive divergence related to climate in a wild contributor to the cultivated apple genome. This may help us to utilize its diversity better, providing options for mitigating the impact of climate change on the cultivated apple through breeding (Warschefsky *et al.* 2014; Prohens *et al.* 2017; Satori *et al.* 2022).

Ongoing crop-to-wild gene flow in the European crabapple

We showed substantial gene flow from *M. domestica* to the European crabapple gene pool, with 11.6% of seedlings, mainly from Western Europe, introgressed by *M. domestica*. Introgression rates were lower compared to previous studies (i.e., 37% in Cornille *et al.* (2013b) and 23.1% in Cornille *et al.* (2015)). However, these studies genotyped more mother trees (i.e., $N = 756$ and $N = 1{,}889$, respectively), which could explain the difference in estimates of crop-to-wild gene flow. Crop-to-wild gene flow is therefore still ongoing in the European crabapple. The higher number of seedlings from Western Europe that are introgressed by *M. domestica* can be explained by the use of reference Western European cultivated apple varieties. Alleles specific to Eastern and Northern cultivated apple varieties can be missed and may decrease the probability of detecting crop-to-wild introgression events in non-Western wild populations (from Eastern and Northern Europe). Note that the Spanish seedlings sampled in this study were the progeny of trees growing in a location known to have high levels of introgression by *M. domestica* (pers. comment. G. Alins). It is even possible that the mother trees of these seedlings were *M. domestica* and not *M. sylvestris*.

The consequences of crop-wild introgression on phenotypic variation between crop and wild individuals are poorly understood in perennial fruit trees. One study has shown that crop-wild hybrid apple seedlings have higher growth rates and germinate earlier than wild apple seedlings (Feurtey *et al.*, 2017). We did not detect any effect of the genetic status of a seedling (*pure*, *ww*, *cw*, *dom*) or the level of introgression (*Pdom*) on growth and carbon uptake-related fitness proxies. This could be due to the low number of samples from the *cw* and *dom* categories.

Signs of local adaptation to past climate in the European crabapple

Under controlled conditions, seedlings from different populations exhibited significantly different growth and physiology. Seedlings belonging to the Austrian population were the tallest, with the highest absolute growth rate and the lowest number of leaves; by contrast, Romanian seedlings were the shortest and had the lowest absolute growth rate and the highest number of leaves. Italian seedlings had the highest chlorophyll content. We therefore tested whether this phenotypic variation was adaptive. For instance, the seedlings from the Austrian population may be fitter in the climate conditions simulated in this controlled condition experiment. However, considering the geographic distance, we found no significant covariation between genetic and phenotypic variation. This suggests a lack of divergent selection on carbon uptake or growth traits, which are yet often associated with plant responses

to climate (Bussotti *et al.* 2015). Therefore, the phenotypic variation we observed among populations under controlled conditions may result from genetic drift alone and not from divergent selection. Alternatively, although the specific traits we selected are among the traits that are generally considered to be related to responses to climate (Kühn *et al.* 2021), they might not be perfect candidates for investigating divergent selection by climate. Another explanation for the lack of observation of divergent selection on carbon uptake or growth traits could be that we did not phenotype enough seedlings from each genetic group. Indeed, we observed a high variation in each phenotypic trait and their heritability estimates, suggesting that the traits we studied may be relevant but that a larger number of seedlings could be phenotyped and analyzed. However, some studies have found that even with large sample sizes, the standard error of heritability estimates can still be large and vary significantly between experimental designs (Visscher and Goddard 2015). The reasonably high heritability estimates for most traits considered here could be consistent with relatively weak within-population selection, enabling the maintenance of ample additive genetic variation (Wheelwright *et al.* 2014). Furthermore, high variation in seedling traits combined with high heritability estimates could suggest a substantial amount of genetic variation for adaptation to work on. Altogether, the effect of the population of origin on phenotypic traits suggests a genetic basis for this variation. However, we need to increase the sampling size and measure new traits to draw more precise conclusions on the occurrence of adaptive phenotypic variation in the European crabapple.

As *M. sylvestris* is distributed across various climatic conditions, we further investigated the role of climate in shaping the genetic variation among populations of the European crabapple without considering phenotypic trait variation. While the association between phenotypic and genetic variation was not significant (see paragraph above), we observed a significant association between LGM climate and genetic variation in the European crabapple suggesting local adaptation. We tested for an IBE pattern, where the pattern of neutral genetic variation covaries with ecological variables (here, climate). There was no combined effect of geographic and climatic distance (IBD ∩ IBC), which allowed us to assess the contribution of these processes separately (Wang and Bradburd 2014). We showed that IBD and IBC played a significant role (R^2_{adj} = 47% and R^2_{adj} = 22%, respectively) on the genetic differentiation of European crabapple populations. Weak but significant IBD has been previously identified in wild apple relatives of the cultivated apple (i.e., *M. sylvestris, M. orientalis,* and *M. sieversii*) (Cornille, Giraud, *et al.* 2013; Cornille, Gladieux, *et al.* 2013; Cornille *et al.* 2015). Here we used 13 out of the 26 microsatellite markers used in previous

studies; the lack of resolution of the 13 SSRs may explain the lack of IBD. However, this is unlikely as the Bayesian inferences method was previously able to detect genetic clusters in wild apples (Cornille, Giraud, *et al.* 2013; Cornille, Gladieux, *et al.* 2013; Cornille *et al.* 2015). Weak IBD pattern suggests that *M. sylvestris* has high dispersal capacities (Coart *et al.* 2006; Larsen *et al.* 2006; Cornille, Giraud, *et al.* 2013; Cornille *et al.* 2015; Reim *et al.* 2017; Feurtey *et al.* 2017). A weak IBD is explained by a self-incompatibility system that prevents self-fertilization (Brown 1992), pollen dispersal by bees, beetles, and flies, and endozoochorous seed dispersal by large mammals such as ungulates, wild boars, brown bears or humans (Larsen *et al.* 2006).

We show that in addition to IBD, IBC persisted after considering the geographical distance. Climate can impose divergent selection pressures on different locations and thus reduce gene flow between populations. For instance, divergent selection imposed by climate can limit the reproductive success of individuals moving between different climates from which they are adapted, so that IBC contributes to genetic differentiation among populations (Wang and Bradburd 2014). The main variables explaining genetic differentiation in the European crabapple were related to temperature during the LGM. This suggests that the European crabapple may be adapted to its past climate but not to its current one. In wind-dispersed trees, local adaptation to the current climate has been demonstrated (Savolainen *et al.* 2013; Kremer and Hipp 2019; Pyhäjärvi *et al.* 2020), but to our knowledge, no study has shown local adaptation to past climate conditions in an insect-pollinated tree species.

Factors other than climate can also shape the adaptive divergence between populations. Local adaptation to biotic factors, such as the presence of other species, is possible. *Malus sylvestris* is a species that needs high levels of light and is not very competitive. Some of the observed variations of the seedlings in growth rate, leaf number, and chlorophyll content may derive from adaptations that would be advantageous within the local niche. In Romania, the populations sampled were in forest edges, middle succession woodlands (with hawthorn and wild pear), or grasslands; no wild apples were found in mature forests. There, *M. sylvestris* does not compete with other woody species but with grasses and shrubs in the seedling phase; this might explain the slower growth in height and the larger number of leaves (leading to a larger leaf area and more shading of the competing grasses). In France, the trees are present at the edge of mature forests, and in Austria, in mature forests. It is hard to draw clear conclusions; further investigations of the ecology of the wild populations *in situ* are needed. The rhizosphere composition among seedlings from different populations can impact the phenotypic variation and potentially plant fitness in response to climate (Trivedi *et al.* 2022). Here seeds were

cleaned with chlorine to avoid the effect of local micro-organism community and seed growth. Still, investigations of phenotypic variation among cleaned and uncleaned seeds can also help assess the role of the rhizosphere in the divergence of the wild apple population. Local adaptation of fruit trees to biotic factors, including parasites (Olvera-Vazquez *et al.* 2021) also deserves further investigation. Besides selection, the role of phenotypic plasticity in enabling growth and optimal fitness in changing environments also needs to be carefully evaluated (Benito Garzón *et al.* 2011).

Further investigations are needed on local adaptation and phenotypic plasticity in response to climate in the European crabapple

Our study raises questions regarding the response of wild apple populations to climate change. More data is needed to draw clear conclusions. The adaptation of tree species to climate is complex (Bussotti *et al.* 2015). For instance, in *Eucalyptus camaldulensis*, variation in leaf traits and performance was unrelated to the climate of genotype provenance (Asao *et al.*, 2020), while in Australia, the same species displays variation in several photosynthetic traits that were related to the climate of genotype provenance (Dillon *et al.* 2018). By contrast, collective differences in leaf morphology and photosynthetic physiology associated with the length of the growing season, temperature, and the level of insolation in several *Populus* species may be adaptive (Keller *et al.* 2011; Kaluthota *et al.* 2015). Further investigations on local adaptation and phenotypic plasticity in response to climate in the European crabapple are needed. Genomic data will help determine the relative influence of adaptive and neutral processes on climate-driven divergence by scanning the genomes of trees from different populations in Europe. Comparing the fitness of seedlings from different populations in reciprocal transplants (under controlled or natural conditions) will also allow us to investigate local adaptation and phenotypic plasticity. Further studies using additional genetic markers (single nucleotide polymorphism) and measuring phenotypic traits in different climate conditions are therefore needed. This study is nevertheless a starting point for future breeding and conservation programs of a CWR of an emblematic temperate fruit tree, as it characterized the phenotypic and genetic variation of seedlings in the wild that can be used as *ex-situ* sources to enrich the crop gene pool. Indeed, some of the seedlings included have sequenced genomes and have been planted in several orchards in France (France, https://www.ideev.universite-paris-saclay.fr/en/the-orchard) and are measured each year for several phenotypic traits. Information and samples from these orchards can be requested from the corresponding author. Genetic variation of trees in these *ex-situ* orchards can help enrich the cultivated apple gene pool.

Certain traits related to climate change adaptation can be introduced into future apple varieties (Warschefsky *et al.* 2014; Prohens *et al.* 2017; Satori *et al.* 2022).

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Authors contributions

AC, GV, AR, SB, and TU conceived and designed the experiments; AC, GV, AT, TU, and TK obtained the funding; AC, GV, AT, TU, KAO, SV, RR, XC, TK, CR sampled the material; XC, CR, AV, AR, GL, KAO, RR, MLG, HB, VC, HC, SV, MF performed the molecular biology analyses; AC, AF, KomAvi and XC analyzed the data. The manuscript was written by AC, KomAvi and AF, with essential input from other co-authors.

Figures and Tables

Figure 1. Bayesian clustering of *Malus sylvestris* seedlings sampled in this study (*N* = 584) and reference samples of *Malus domestica* (*N* = 40) inferred with STRUCTURE at *K* = 8 and its associated map of mean membership per sampled site. Each individual is represented by a vertical bar partitioned into clusters. Visualization was improved by sorting genotypes by country. Countries are separated by a white line. The *M. domestica* reference samples are shown on the far left of the map in the Atlantic. Circle size is proportional to the number of individuals within the cluster (scale shown in the top right-hand corner).

Figure 2. Cumulative height growth over time in apple seedlings under controlled conditions. Seedlings measured were pure and wild-wild hybrid *Malus sylvestris* seedlings, *N* = 449, and seedlings assigned to the *M. domestica* gene pool, *N* = 21, as detected with STRUCTURE for *K*=8**.** The 40 reference *M. domestica* individuals were not measured under controlled conditions and are therefore not shown here. Vertical lines represent the standard deviation. Populations: Austria (AUT, *N* = 81), Denmark (DA, *N* = 78), *M. domestica* (DOM, *N* = 21), which includes 13 Spanish genotypes and seedlings from other countries), Eastern France (FR-E, *N* = 50), Lorraine in France (FR-Lor, *N* = 28), Western France (FR-W, *N* = 77), Italy (IT, *N* = 27) and Romania (RO, *N* = 108).

Table 1. Number of *Malus sylvestris* seedlings used in this study for population genetic analyses inferred with STRUCTURE for *K* = 8, with 13 microsatellite markers and phenotyping (growth and carbon uptake-related traits).

Clusters	N_{pure}	N_{ww}	N_{cw}	N_{dom}	$N_{no\ cluster}$	Total measured for phenotypic traits	Population name
Q1 (light green)	32	46	7	0	10	92	FR-W
Q2 (yellow)	0	52	5	0	4	57	FR-E
Q3 (lor)	28	1	0	0		28	FR-Lor
Q4 (blue)	61	21	1	0	6	85	DA
Q5 (purple)	23	4	3	0	4	34	IT
Q6 (dark green)	66	17	1	0	1	83	AUT
Q7 (red)	77	34	5	0	0	113	RO
Q8 (black – *M. domestica*)	40	0	46	21	8	73	DOM
Total	287	175	68	21	33	551 (584)	

Total measured for height and number of leaves	282	167	63	21	32	533 (565)
Total measured for leaf chlorophyll and flavonol contents, NBI	129	82	22	6	18	239 (257)

N_{pure}: the number of seedlings assigned to a wild gene pool with a membership coefficient > 0.9; N_{ww}: the number of wild-wild hybrids (i.e., seedlings with a membership coefficient > 0.1 to a wild gene pool other than its own wild gene pool and a membership coefficient < 0.1 to the *M. domestica* gene pool); N_{cw}: the number of crop-wild hybrids (i.e., seedlings assigned to the *M. domestica* gene pool with a membership coefficient > 0.1). $N_{no\ cluster}$: seedlings that could not be assigned to any defined gene pool; Total measured for phenotypic traits : the number of individuals measured for each phenotypic trait and included in the statistical analyses, the number in brackets represents the initial sample size before data were filtered for statistical analyses. Wild population name: populations defined with STRUCTURE for K=8 excluding crop-wild hybrids and seedlings from misidentified mother trees (i.e., including only wild pure and wild-wild hybrids). Populations: Austria (AUT), Denmark (DA), Eastern France (FR-E), Lorraine in France (FR-Lor), Western France (FR-W), Italy (IT), Romania (RO), *M. domestica* (DOM).

Table 2. Final model depicting the effects of the *Malus sylvestris* population to which each seedling belonged (i.e., cluster inferred with STRUCTURE for *K*=8) on phenotypic traits (*i.e.*, height, number of leaves, internode, chlorophyll and flavonol contents, NBI) measured on 533 individuals.

Explanatory variable	Population			Mother tree		Model			Mother tree
Fitness	X^2	*P-value*	df	REML	Standard Deviation	AIC	R^2	Corrected R^2	R^2
Height_AGR	17.863	0.007***	6	1,229	0.204	1,248	0.047	0.091	0.044
Height_RGR	12.846	0.045*	6	-2,264	0.006	-2,245	0.041	0.147	0.106
Leaf_AGR	4.302	0.6359	6	134	0.036	152	0.01	0.028	0.018
Leaf_RGR	4.302	0.6359	6	134	0.036	152	0.01	0.028	0.018
Whole height AGR	22.243	1.00e-03***	6	630	0.175	650	0.074	0.192	0.118

Whole leaf AGR	36.326	2.38e-06***	6	-1,277	0.009	-1,258	0.09	0.118	0.028
Height	31.623	1.93e-05***	6	4,113	11.69	4,131	0.119	0.301	0.182
Number of leaves	22.285	0.001** *	6	-	0.084	2,659	0.052	0.064	0.012
Chlorophyll	14.418	0.025*	6	1,181	1.352	1,199	0.074	0.171	0.097
Flavonol	6.7752	0.342	6	-124	0.091	-105	0.043	0.299	0.256
NBI	1.838	0.934	6	1,735	7.6617	1,754	0.011	0.224	0.213
Internode (nbleaf/height)	17.768	0.007** *	6	-1,328	0.009	-1,309	0.044	0.073	0.029

***: P-value <0.001; **: 0.01< P-value <0.001; *: 0.05< P-value <0.01; AIC: Akaike Information Criterion; - Models without any significant effect.

Table 3. Contribution of geography and climate to the genetic variation observed among *Malus sylvestris* seedlings. Distance-based redundancy analyses tested the effects of geography, climate, and phenotype on the genetic differentiation (from 13 microsatellites) among 21 sites in the European crabapple. Only the significant variables are listed.

	db-RDA			
	% of variance explained	**d.f.**	**p-value**	**Adj-R^2**
Global analysis	25.9	7	0.001	69.9
Residuals	11.3	13	-	
Geography (IBD, PCNM 1-2-3-6*)*	14.9	4	<0.015	
Environment (IBC_LGM: BIO3_LGM, BIO6_LGM, BIO9_LGM*)*	11.04	3	<0.015	
Residuals	11.3%	-	-	

BIO3_LGM: isothermality (BIO2/BIO7) (×100)); BIO6_LGM: minimum temperature of the coldest month; BIO9_LGM: mean temperature of the driest quarter; IBD: isolation-by-distance; IBC_LGM: isolation-by-climate during the last glacial maximum.

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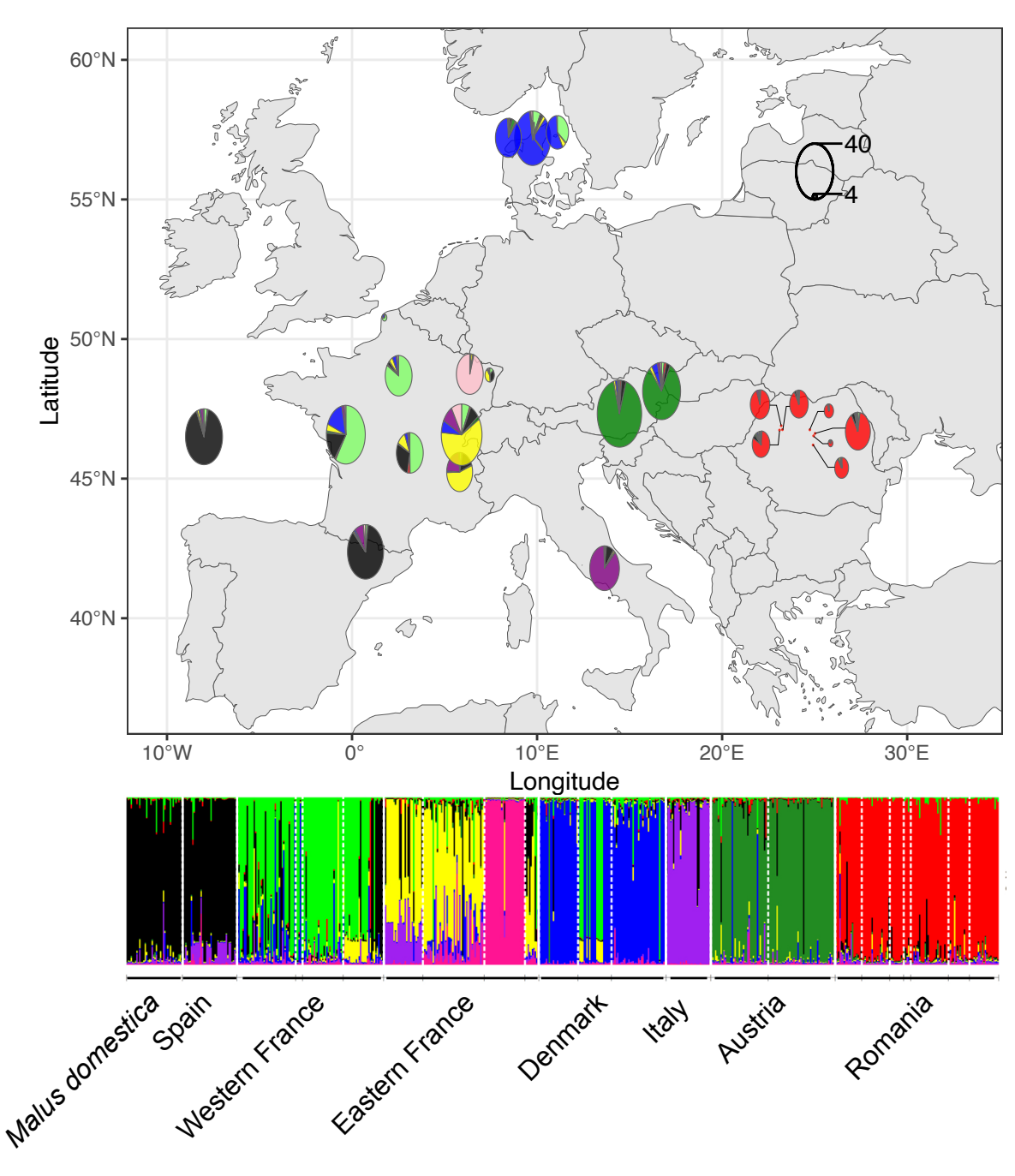
60°N
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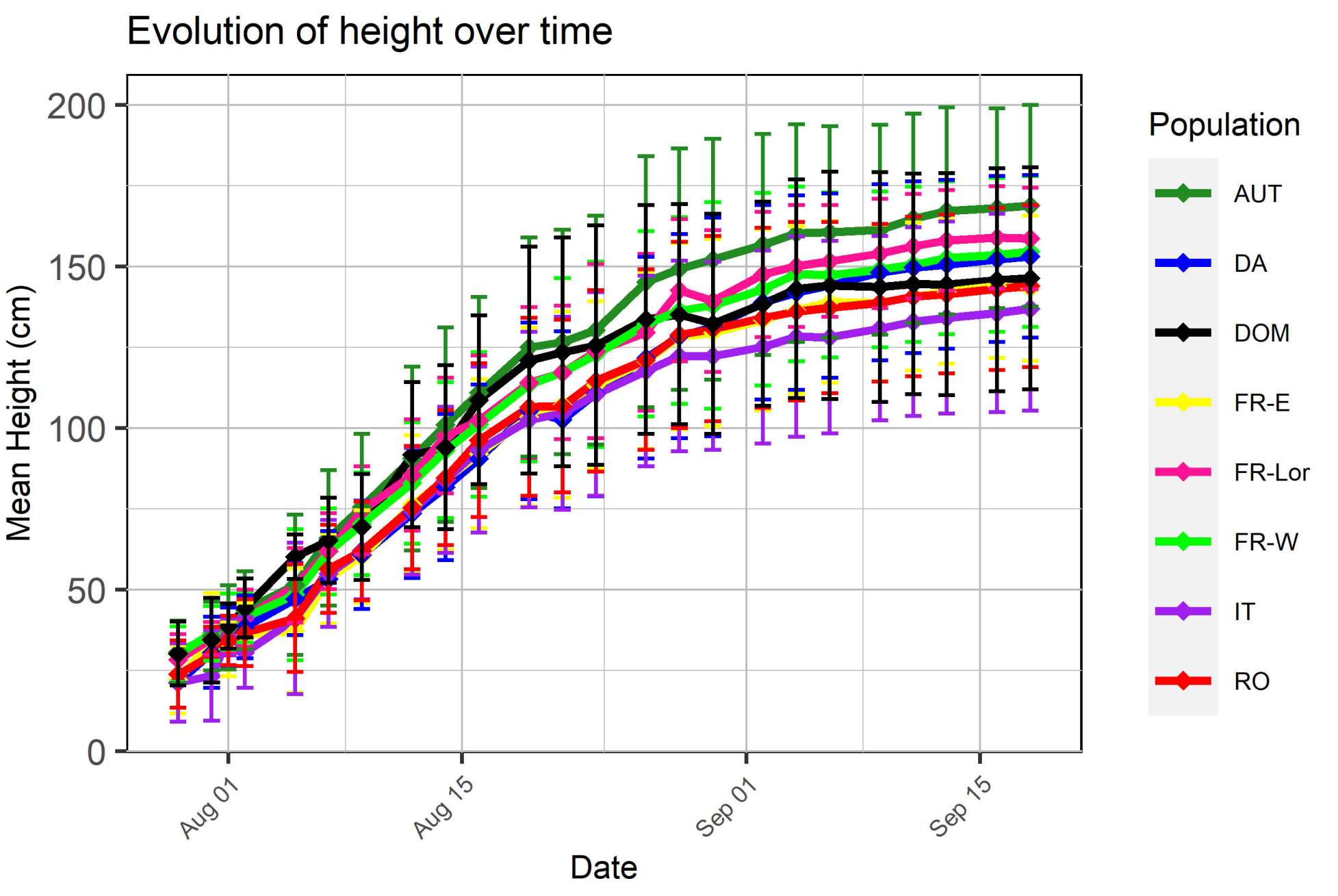
Evolution of height over time
Mean Height (cm)
200
150
100
50
0
Aug 01
Aug 15
Sep 01
Sep 15
Date
Population
AUT
DA
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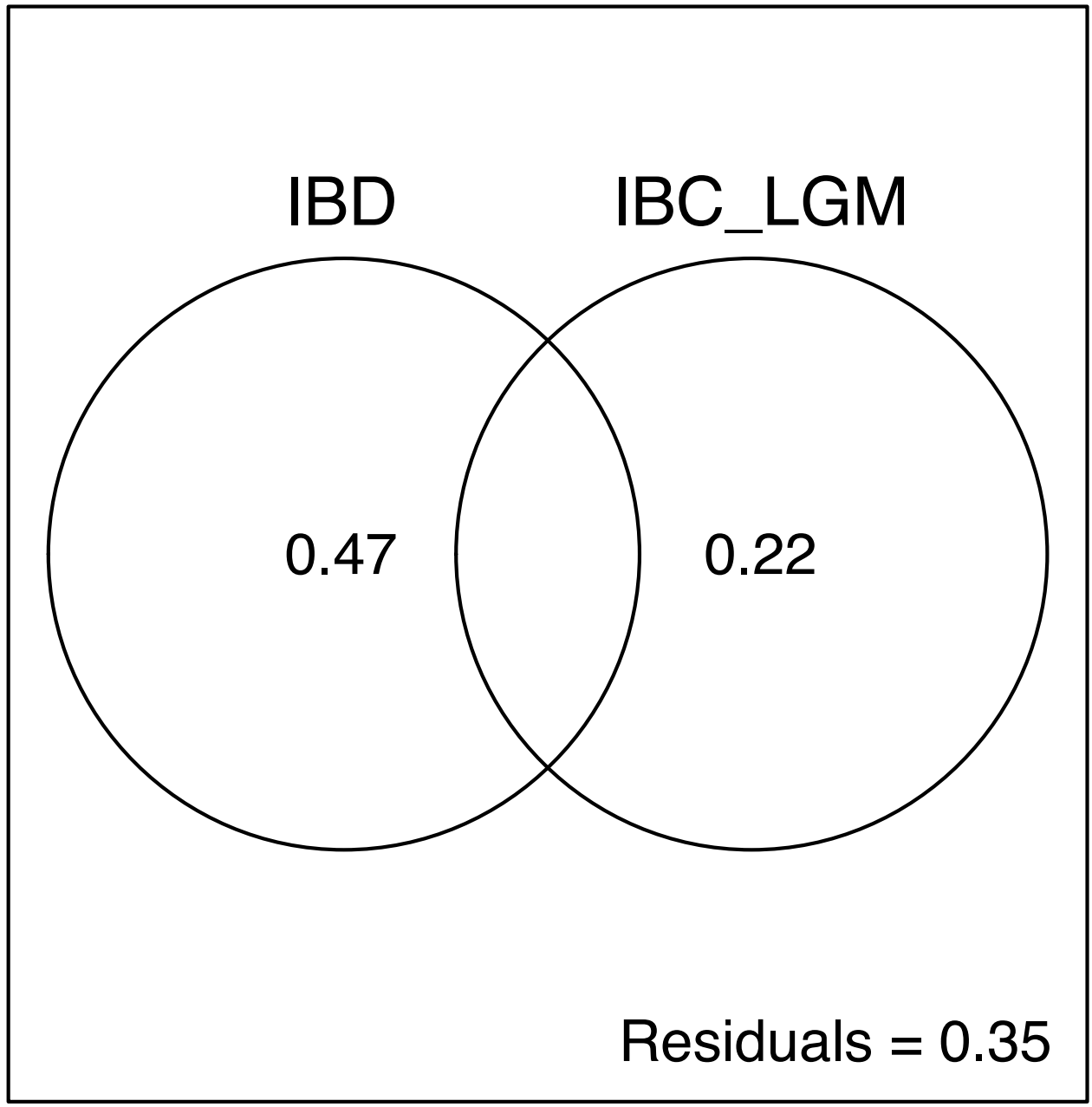
FR-W
IT
RO

Variance partitioning of the db–RDA results



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