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Research

Evolution of plant drought strategies and herbivore tolerance after two decades of climate change

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Summary

- Ongoing global warming, coupled with increased drought frequencies, together with other biotic drivers may have resulted in complex evolutionary adaptation. The resurrection approach, comparing ancestors raised from stored seeds with their contemporary descendants under common conditions, is a powerful method to test for recent evolution in plant populations.
- We used 21–26-yr-old seeds of four European plant species – *Matthiola tricuspidata*, *Plantago crassifolia*, *Clinopodium vulgare* and *Leontodon hispidus* – stored in seed banks together with re-collected seeds from their wild populations. To test for evolutionary changes, we conducted a glasshouse experiment that quantified heritable changes in plant responses to drought and simulated insect herbivory.
- In three out of the four studied species, we found evidence that descendants had evolved shorter life cycles through faster growth and flowering. Shifts in the osmotic potential and leaf dry matter content indicated that descendants also evolved increased drought tolerance. A comparison of quantitative genetic differentiation (Q_{ST}) vs neutral molecular differentiation (F_{ST}) values, using double digest restriction-site associated DNA (ddRAD) genotyping data, suggested that directional selection, and therefore adaptive evolution, was underlying some of the observed phenotypic changes.
- In summary, our study revealed evolutionary changes in plant populations over the last decades that are consistent with adaptation of drought escape and tolerance as well as herbivory avoidance.

Introduction

Global change involves multiple abiotic and biotic changes that have affected European ecosystems over the last decades (Vitousek, 1992; Matesanz *et al.*, 2010; IPCC, 2018). For plant populations, climate change is particularly challenging as it includes both increased temperatures and changes in precipitation (IPCC, 2021). Their interaction can lead to an increased frequency and duration of drought events, as is the case for instance in Southern and Central Europe (Ruosteenoja *et al.*, 2018; Samaniego *et al.*, 2018; Spinoni *et al.*, 2018). Under current scenarios, such novel conditions pose significant challenges to plant persistence (Shaw & Etterson, 2012; Fleta-Soriano & Munné-Bosch, 2016), and many plant populations are under increased risk of local extinction (Thomas *et al.*, 2004; Urban, 2015). Plant populations are already responding to environmental changes through migration (Parmesan & Yohe, 2003; Lenoir *et al.*, 2008), phenotypic plasticity and adaptive evolution (Holt, 1990; Hoffmann & Sgrò, 2011; Franks *et al.*, 2014).

A powerful method to test for recent evolution is the resurrection approach in which ancestors raised from stored seeds (e.g. from seed banks) are compared in common garden experiments to newly sampled descendants from the same populations (Franks *et al.*, 2007, 2018; Franks & Weis, 2008; Orsini *et al.*, 2013; Merilä & Hendry, 2014; Everingham *et al.*, 2021; Rauschkolb *et al.*, 2022). On its own, the resurrection approach only reveals whether evolutionary changes occurred; it cannot answer to which degree these resulted from natural selection, genetic drift,

immigration of new genotypes or mutations (Niklas, 1997; Leinonen *et al.*, 2008). However, with additional data from neutral molecular markers, comparisons between the neutral molecular differentiation (F_{ST}) and quantitative genetic differentiation (Q_{ST}) of phenotypic characters can help to better understand the importance of selective vs random evolutionary forces (Merilä & Crnokrak, 2001; McKay & Latta, 2002).

Plant responses to climate change can be classified into 'escape', 'avoidance' or 'tolerance' mechanisms (Levitt, 1987; Barton & Koricheva, 2010). For example, advanced flowering can help to escape future droughts (Franks *et al.*, 2007; Kigel *et al.*, 2011; Metz *et al.*, 2020), whereas increased root biomass (Martin & Stephens, 2006; Villagra & Cavagnaro, 2006) or rooting depth (Padilla & Pugnaire, 2007) can help to avoid droughts. Finally, plants can resist drought stress by physiological adjustments such as changes in their osmotic potential (Kolb & Sperry, 1999; Bartlett *et al.*, 2014; Májeková *et al.*, 2019). Over recent years, some resurrection studies have already demonstrated the rapid evolution of plants in response to climate change, including adaptation to increased drought intensities and frequencies, for example through shifts in flowering onset and growth (Franks *et al.*, 2007; Vigouroux *et al.*, 2011; Nevo *et al.*, 2012; Thomann *et al.*, 2015; Dickman, 2016).

In addition to the impact of climatic changes, another important stress and driver of evolutionary changes in plants is insect herbivory. The dynamics of invertebrate herbivory is also strongly affected by climate change (Futuyma & Agrawal, 2009; Turcotte *et al.*, 2014; Hamann *et al.*, 2021). Ectothermic organisms such as insect herbivores are directly influenced by climate warming, with increased performance until a temperature optimum, and decreased performance beyond it (Bale *et al.*, 2002; Cornelissen, 2011). Increased drought could lead to insect outbreaks as relative nitrogen content in leaves is elevated (White, 1984). However, how changes in temperature and precipitation interact with insect herbivory in their effects on plants is still not well understood (Schoonhoven *et al.*, 2005; Prasch & Sonnewald, 2013; Pandey *et al.*, 2017; Descombes *et al.*, 2020; Hamann *et al.*, 2021). One problem is that we know much less about long-term trends in insect herbivory than about trends in climate (Turcotte *et al.*, 2014). Some interesting evidence comes from recent herbarium studies that have found increased levels of insect damage on herbarium specimens during the 20th century (Meineke *et al.*, 2019, 2021), suggesting that insect herbivory may have changed in parallel with climate in some geographic regions.

Another link between climate change and insect herbivory could be that the advancement of flowering could be an adaptive strategy to escape both (Pilson, 2000; Kawagoe & Kudoh, 2010; Meineke *et al.*, 2021), although this also depends on the temporal dynamics of the insects (Pilson, 2000). For instance, Kawagoe & Kudoh (2010) found that an *Arabidopsis halleri* population with intensive floral herbivory advanced flowering in comparison to a population without herbivory, so responses may be synergistic under climate change. By contrast, Pilson (2000) showed for *Helianthus annuus* that delayed flowering is favoured under herbivory, indicating a possible trade-off between adaptations to herbivory and climate change. Synergisms or trade-offs between adaptations to climate change and insect herbivory can also be expected for strategies of herbivore avoidance. For instance, under drought plants may reduce metabolically costly investment into chemical defences (Purrington, 2000; Strauss *et al.*, 2002; Jander, 2018), which could make insect outbreaks particularly detrimental for plant populations already impacted by climate change (Haugen *et al.*, 2008; Gutbrodt *et al.*, 2011).

Although the climate change adaptation of plants has been studied intensively during the last years, we know of only one resurrection study that has focused on evolution of herbivory defences (Bustos-Segura *et al.*, 2014) and none that have addressed interactions between climate change adaptation and adaptation to herbivory. We attempted to do this in our study, and used the resurrection approach to test whether individual populations of four plant species had undergone evolutionary changes in their drought responses over the last two decades and whether there were simultaneous evolutionary changes in their responses to insect herbivory. To broaden the climatic scope of our study we included two species from the Mediterranean coastal habitat and two from temperate European grasslands, both from regions where temperatures have been increasing and in which herbivory plays a key role. We grew ancestor and descendant lines in a common environment and subjected the plants to a full-factorial combination of drought and simulated herbivory. We further used double digest restriction-site associated DNA (ddRAD) genotyping data to compare F_{ST} and Q_{ST} values between ancestors and descendants to understand the adaptive significance of observed phenotypic changes.

Specifically, we addressed the following questions: (1) Do ancestral and contemporary populations differ in their phenotypes? (2) Does this differentiation result from selective or random processes? and (3) Do ancestral and contemporary populations differ in their responses to drought and simulated herbivory, and if yes, are there synergies or trade-offs between these two types of responses?

Materials and Methods

Study species and seed origins

We investigated four plant species, *Matthiola tricuspidata* (L.) R.Br. (Brassicaceae) and *Plantago crassifolia* Forssk. (Plantaginaceae) from the French Mediterranean coast and *Clinopodium vulgare* L. (Lamiaceae) and *Leontodon hispidus* L. (Asteraceae) from temperate Belgian grasslands (Table 1). The two Mediterranean species are halophytic herbaceous species originating from sandy beaches; *M. tricuspidata* is an insect-pollinated annual and *P. crassifolia* a wind-pollinated perennial. The two temperate species are both insect-pollinated perennial, mesophilic forbs and typically found on dry calcareous soils. Although *L. hispidus* occurs mainly in dry grasslands and *C. vulgare* prefers thermophile woodland margins, both study populations had originated from calcareous grasslands prone to drought. The perennial study species are all hemicryptophytes and reached maturity under glasshouse conditions within the first year of cultivation; all species except *L. hispidus* are self-compatible.

Table 1 Species used in our study, with information on locations, collection years, germination rates and sampling sizes for ancestors (A) and descendants (D).

Species	Locations	Collection years (A/D)	Germination rates (A/D)	No. of plants (A/D)
Matthiola tricuspidata (Brassicaceae)	Hyères (France), 43°02′40″N, 6°07′50″E	1994/2018	73%/84%	386/393
Plantago crassifolia (Plantaginaceae)	Hyères (France), 43°02′40″N, 6°07′50″E	1997/2018	96%/88%	396/398
Clinopodium vulgare (Lamiaceae)	Couvin (Belgium), 50°03′55″N, 4°26′40″E	1992/2018	42%/40%	281/251
Leontodon hispidus (Asteraceae)	Bassenge (Belgium), 50°47′35″N, 5°40′25″E	1995/2018	59%/77%	179/189

The source populations of all species underwent significant climate changes during the last decades. For the Mediterranean species, average temperatures in March–July have increased by 1.6°C, and precipitation summed to a small increase of *c.* 5.5 mm per year in 1991–2020 compared with the baseline (1961–1990) leading to a slight increase in drought. In Belgium, average temperature between 1991 and 2020 have increased by *c.* 0.9°C in the area of origin of *C. vulgare* and by 1.3°C in the area of *L. hispidus*, and precipitation in spring and summer decreased by 80 and 38 mm per year, respectively (data from CRU; Camarillo-Naranjo *et al.*, 2019; Harris *et al.*, 2020). Our species selection allowed us to study the response of several species from two very different independent European bioclimatic systems, which differ in the increase of drought during the last decades. This gave us the possibility to study interesting evolutionary trajectories caused by the interaction of climate change with different evolutionary strategies.

For all four species, we collected seeds in 2018 from the same wild populations as did the seed collectors > 20 yr ago for the seed bank collections. For the original seed bank collections (1992–1997 depending on the species; please refer to Table 1), large numbers of seeds of a representative number of individuals were collected in the populations and bulked, dried and stored at 5°C (Mediterranean species) or at −20°C (temperate species). We obtained the stored (ancestral) seeds from the seed banks at the Conservatoire Botanique National Méditerranéen de Porquerolles (CBNMed, Hyères, France) and at Meise Botanic Garden (Belgium). For the descendants, we re-collected seeds from all populations in the spring (Mediterranean species) and summer (temperate species) of 2018. We sampled 10–47 individuals per population (Supporting Information Table S1) and bulked their seeds to have a seed mix comparable with that of the ancestors.

Although the seed bank materials we used were not collected with resurrection experiments in mind, they can be used for this purpose, for several reasons: (1) the previous collectors aimed to maximise the number of sampled individuals, and (2) the numbers of seeds stored in the seed bank lots were high (> 800; Table S1), which together means that the risks of bottleneck effects should be low. Moreover, (3) analyses of single nucleotide polymorphism (SNP) markers showed similar levels of relatedness among ancestors and descendants in all four study species, which further supports the idea that sampling procedures were similar and therefore the samples equally representative of the studied populations in both sampling periods (Methods S1; Fig. S1).

To disentangle evolutionary changes from possible storage and maternal effects (Franks & Weis, 2008), we cultivated a refresher generation before the main experiment in spring 2019 in a glasshouse at the University of Tübingen. For this, we first dark stratified the ancestor and descendant seeds of the Mediterranean species at 5°C for 1 wk and of the temperate species for 2 months. For each species and temporal origin, we used 100–300 seeds, and we observed germination rates of at least 29% (Table S1). We transplanted 15 seedlings per temporal origin into 9 × 9 × 9 cm pots filled with a 1 : 3 mixture of sand (0–2 mm play sand; WECO GmbH, Leer, Germany) and potting soil (Bio Topfsubstrat Öko torffrei, Einheitserde®, Sinntal-Altengronau, Germany). The glasshouse was set to a 12 h : 12 h, light : dark cycle and temperatures of 20°C : 15°C as upper and lower limits, respectively. To prevent unintentional cross-pollination between ancestors and descendants, we grew the plants in net cages and hand pollinated the plants within temporal origins, with random crosses within each set of 15 individuals. From these plants, we then harvested the ripe seeds for use in the subsequent experiments.

Experimental design

In spring and summer 2020, we conducted a common garden experiment, using the seeds from the refresher generation, in the same glasshouse and with the same climatic settings as above. Following the natural phenology of the species, we split the experiment into two parts: an experiment with the two Mediterranean species from January to April 2020 and an identical experiment with the two temperate species from May to August 2020. For the F2 experiments, we used 10 seed families (i.e. maternal lines) per temporal origin for *M. tricuspidata* and *P. crassifolia,* nine ancestor and seven descendant seed families for *C. vulgare* and five seed families for ancestors and descendants of *L. hispidus*. The numbers of seed families were lower than in the refresher generation because the pollination rates of some mother plants were too low to produce sufficient seeds. After 1 wk of dark stratification at 5°C, we germinated 100 seeds per seed family in 54-cell QuickPot® trays filled with germination soil (Bio Pikiersubstrat, Einheitserde®, Sinntal-Altengronau, Germany). All germination rates were > 40% and did not differ substantially between ancestors and descendants (Table 1). We transplanted 24–40 seedlings per seed family into 9 × 9 × 9 cm pots with a 1 : 3 mixture of sand (0–2 mm play sand; WECO GmbH) and potting soil (Bio Topfsubstrat Öko torffrei, Einheitserde®, Sinntal-Altengronau, Germany). After 2 wk of seedling

establishment, we randomly assigned 6–10 replicates per seed family to each of four treatment combinations: control, drought, herbivory or drought plus herbivory. The watering treatments were as follows: the control plants were watered twice a week, with 100 ml in weeks 3–6, 150 ml in weeks 6–7 and to 200 ml in weeks 7–13. The drought plants received only half of the amount of water as the control plants throughout the experiment. Herbivory was simulated by clipping three holes in one leaf using a standardised hole puncher and pouring 15 µl jasmonic acid solution (1 mM) over this leaf (van Kleunen *et al.*, 2004). The control group did not receive physical damage and was treated with a solution of the solvents (water and methanol) without jasmonic acid. The herbivory treatment was applied twice, at 3 and 5 wk after seedling establishment. We ran the experiment until > 80% of the individuals of each species and temporal origin had flowered (10–13 wk after transplanting).

Measurements

At 2 wk after seedling establishment, and before the first treatments were applied, we estimated initial plant size as a covariate through vertical top-down photographs of all pots in a standardised photo box using a high-resolution digital camera. The amounts of green pixels per picture, calculated with a custom script in Python, were used as estimates of plant size. Throughout the experiment, we recorded the flowering of plants as the days when the first open flowers (*M. tricuspidata*, *C. vulgare*, *L. hispidus*) or anthers (*P. crassifolia*) were visible. To assess resource investment into aboveground biomass at the time of flowering we measured plant height for *M. tricuspidata* and *C. vulgare*, the length of the longest leaf for *P. crassifolia*, and the rosette diameter for *L. hispidus*. From week 10, we successively harvested the plants separately by species, with 1 wk of harvesting for each, and random order of harvesting within species.

In addition to these morphological and phenological characteristics, we also estimated two functional leaf traits, leaf dry matter content (LDMC) and osmotic potential. To obtain fully hydrated leaves for this, we watered the pots and covered them with plastic bags overnight before harvesting. On the next day we weighed the fresh biomass of one randomly selected, well developed leaf for LDMC, and we took an additional leaf of the same size from five replicate plants per treatment and seed family for osmotic potential analyses, following the protocol of Májeková *et al.* (2019) and Boyer (1995). We cut leaf laminae, placed them in a sealed 1-ml syringe (Carl Roth GmbH+Co. KG, Karlsruhe, Germany) and froze them at −20°C until February 2021, when we thawed the samples for 30–60 min before determining the osmotic potential at full hydration using a Vapro5600 osmometer (ELITechGroup Benelux, Zottegem, Belgium).

Before cutting the plants, we counted the numbers of inflorescences of each plant, and afterwards separated vegetative aboveground biomass and reproductive biomass. We then dried all biomass samples for 3 d at 60°C and determined the dry weight of each. We calculated LDMC by dividing the dry biomass of the target leaf by its fresh biomass. The dry weight of this leaf was added to calculate the total vegetative aboveground biomass (mg), and the reproductive investment as the fraction of the reproductive biomass to the total aboveground biomass. In total we measured nine plant traits: (1) initial plant size, (2) flowering onset, (3) size at flowering, (4) aboveground vegetative biomass, (5) reproductive biomass, (6) reproductive investment, (7) number of inflorescences, (8) LDMC and (9) osmotic potential. We chose these traits because they represented different functions and processes: traits (4), (5) and (7) were considered performance traits, whereas traits (1), (2), (3) and (6) informed us about changes in life-history strategies, and traits (8) and (9) about changes in functional (morphological–physiological) traits, in response to drought and simulated herbivory.

Statistical analyses

For all statistical analyses we square-root transformed initial plant size, aboveground vegetative biomass and reproductive biomass to improve normality and homoscedasticity of the model residuals. We analysed the variation in the nine measured traits for each species separately. We used linear mixed-effects models for all analyses except for the number of inflorescences, for which we used a generalised linear mixed-effects model with Poisson error distribution. All models included temporal origin (ancestor vs descendant), watering treatment (control vs drought), herbivory treatment (control vs damaged) and all possible interactions as fixed explanatory variables, as well as seed family and the spatial block within the glasshouse as random variables. In all models except for the analysis of early size we further included early size as a covariate. We analysed the generalised linear mixed-effects models for the number of inflorescences using model comparisons by stepwise adding the fixed factors and their interactions. Because of the large numbers of traits, species and model factors, we adjusted our *P*-values for the false discovery rate (FDR) following Benjamini & Hochberg (1995). All analyses were done in R (v.4.0.2) using the packages PLYRfor data structuring (Wickham, 2011), and LME4 (Bates *et al.*, 2015) and LMERTEST (Kuznetsova *et al.*, 2017) for the analyses.

Calculation of Q_{ST} and F_{ST}

The comparison of Q_{ST} with F_{ST} is a useful tool for understanding the relative importance of selective vs random processes in trait differentiation (Merilä & Crnokrak, 2001; McKay & Latta, 2002). When comparing these indices, three outcomes are possible: $Q_{ST} > F_{ST}$ suggests that natural selection is the main cause of differentiation, $Q_{ST} \approx F_{ST}$ that genetic drift could be the sole driver of it (but contributions of drift and selection remain unclear), and $Q_{ST} < F_{ST}$ indicates the influence of stabilising selection (Leinonen *et al.*, 2008). As all study species were outcrossing, and because we implemented a half-sibling experimental design using seed families, we used the approach of Petit *et al.* (2001) to calculate Q_{ST} for each trait, except for osmotic potential in which the small numbers of replicates did not permit this. Q_{ST} was estimated as $Q_{ST} = V_{POP}/(2 \times V_A + V_{POP}) = V_{POP}/(8 \times V_{FAM} + V_{POP})$, where V_{POP} is the phenotypic variance between the two temporal origins and V_A the genetic variance within temporal

origins (Wright, 1951). We calculated V_{POP} by first running a linear model with early size and the four treatment combinations, then extracting the residuals and using these in a linear mixed-effects model including temporal origin and its treatment interactions ($V_{POP} = V_{temporal\ origin} + V_{temporal\ origin \times treatments}$) as genetic factors and seed families (V_{FAM}) as random factors. We resampled data 999 times from the original dataset to estimate a mean value and bootstrapped standard error for the Q_{ST} of every measured trait.

We estimated neutral genetic differentiation (F_{ST}) between the two temporal origins based on 2257–5785 biallelic SNP markers per species (W. Durka *et al.*, unpublished), using the function *stamppFst* from the R package STAMPP (Pembleton *et al.*, 2013). A more detailed description of the SNP genotyping can be found in Methods S1. The raw data have been deposited in the European Nucleotide Archive under the accession no. PRJEB47887 (https://www.ebi.ac.uk/ena/browser/view/PRJEB47887).

Results

Differentiation between ancestral and contemporary plants

The frequency and strength of genetic differentiations between ancestors and descendants strongly differed among the studied traits. Genetic differentiation was particularly common in flowering onset, with significantly accelerated flowering in descendants of *Matthiola tricuspidata* ($P = 0.006$) and *Clinopodium vulgare* ($P = 0.017$), but the opposite change for *Leontodon hispidus* ($P = 0.031$; Fig. 1b; Table 2; please refer to also Table S2; Figs S2–S4 for mean values and Table S3 for detailed model results). In two species, *M. tricuspidata* ($P = 0.03$) and *C. vulgare* ($P = 0.025$), we found that the reproductive investment was significantly higher in the descendant plants (Figs 1g, S2, S3; Tables 2, S2, S3). Ancestors and descendants of two species differed in their size at flowering, with larger descendants in *L. hispidus* ($P = 0.03$) but smaller ones in *C. vulgare* ($P = 0.03$, Figs 1c, S3, S4; Tables 2, S2, S3). For the remaining traits we only found differences in single species (Fig. 1; Tables 2, S2, S3).

Past selection within the studied populations

Molecular marker differentiation based on SNP data ranged from $F_{ST} = 0.005$ (*L. hispidus*) to $F_{ST} = 0.148$ (*P. crassifolia*; Fig. 2). Our mixed-effects model results showed significant phenotypic differentiation between the two temporal origins in 12 out of 32 trait × species combinations (Table 2; except the osmotic potential). For 10 of these, Q_{ST} was higher than the corresponding F_{ST}, and only two showed a lower Q_{ST} (Fig. 2). The most consistent results were for the time of flowering onset and size at flowering, where Q_{ST} was higher than F_{ST} in three out of four species. In addition, the LDMC showed consistent results but the other way around. We found no differentiations for this trait between ancestors and descendants for *M. tricuspidata*, *P. crassifolia* and *C. vulgare* and also a lower Q_{ST} in comparison with the F_{ST} (Fig. 2a–c). Across all traits, phenotypic differentiation was strongest, and Q_{ST} always above F_{ST}, in *L. hispidus* (Fig. 2d), whereas in *M. tricuspidata* and *P. crassifolia* the Q_{ST} values were generally much lower, and in most cases below the estimated F_{ST} (Fig. 2a,b).

Plasticity of ancestral vs contemporary plants

The drought treatment strongly influenced plant traits in all four species (Tables 2, S2, S3), whereas the effects of simulated herbivory were much more moderate, with significant effects only on six traits in *M. tricuspidata* and on one trait in *P. crassifolia* (Tables 2, S2, S3; Fig. S5). In 10 cases, the plastic responses of plant traits to our treatments depended on the temporal origin (treatment × origin interactions in Table 2), again mostly with regard to drought (9 out of 10 interactions) and in *M. tricuspidata* (6 out of 10 interactions). For vegetative biomass and LDMC the observed interactions were respectively consistent across two species (*P. crassifolia*, *C. vulgare* and *M. tricuspidata, C. vulgare*), indicating that descendants decreased their vegetative biomass and their LDMC less under drought than their ancestors.

The overall most plastic and differentiated trait was the number of inflorescences in *M. tricuspidata,* with significant effects of both experimental treatments, and all possible two- and three-way interactions between drought, herbivory and temporal origin (Tables 2, S2, S3; Fig. 3b). For example, descendants decreased the number of inflorescences more in response to drought and to herbivory, whereas the decrease in their ancestors was smaller (Fig. 3a).

Discussion

Differentiation between ancestral and contemporary plants

The potential for rapid evolutionary changes not only depends on the strength of selection exerted by environmental changes, but also on the numbers of generations that have passed. All of our study species can reproduce within 1 yr, so we assume that the studied plant populations underwent approximately the same number of sexual generations over the 21–26 yr period since the ancestral seed were collected. We found that flowering onset was significantly advanced in the descendant compared with the ancestral plants in two species, and that there was a similar trend in a third species (*P. crassifolia*, $P = 0.07$). Accelerated reproduction is often considered an adaptation to increasingly drier and warmer conditions (Franks *et al.*, 2007; Kigel *et al.*, 2011; Metz *et al.*, 2020), as early-flowering plants may have a better chance to escape summer droughts.

However, during the 20th century not only the climate has changed, but also insect herbivory may have increased (Meineke *et al.*, 2019, 2021). This may be more than a coincidence, because climate change can facilitate insect outbreaks (Bale *et al.*, 2002) and foliar damage (Hamann *et al.*, 2021) through changes in the C : N ratio of plant leaves (White, 1984; Robinson *et al.*, 2012) and a positive influence of increasing temperatures on insect performance (Cornelissen, 2011). Our findings may

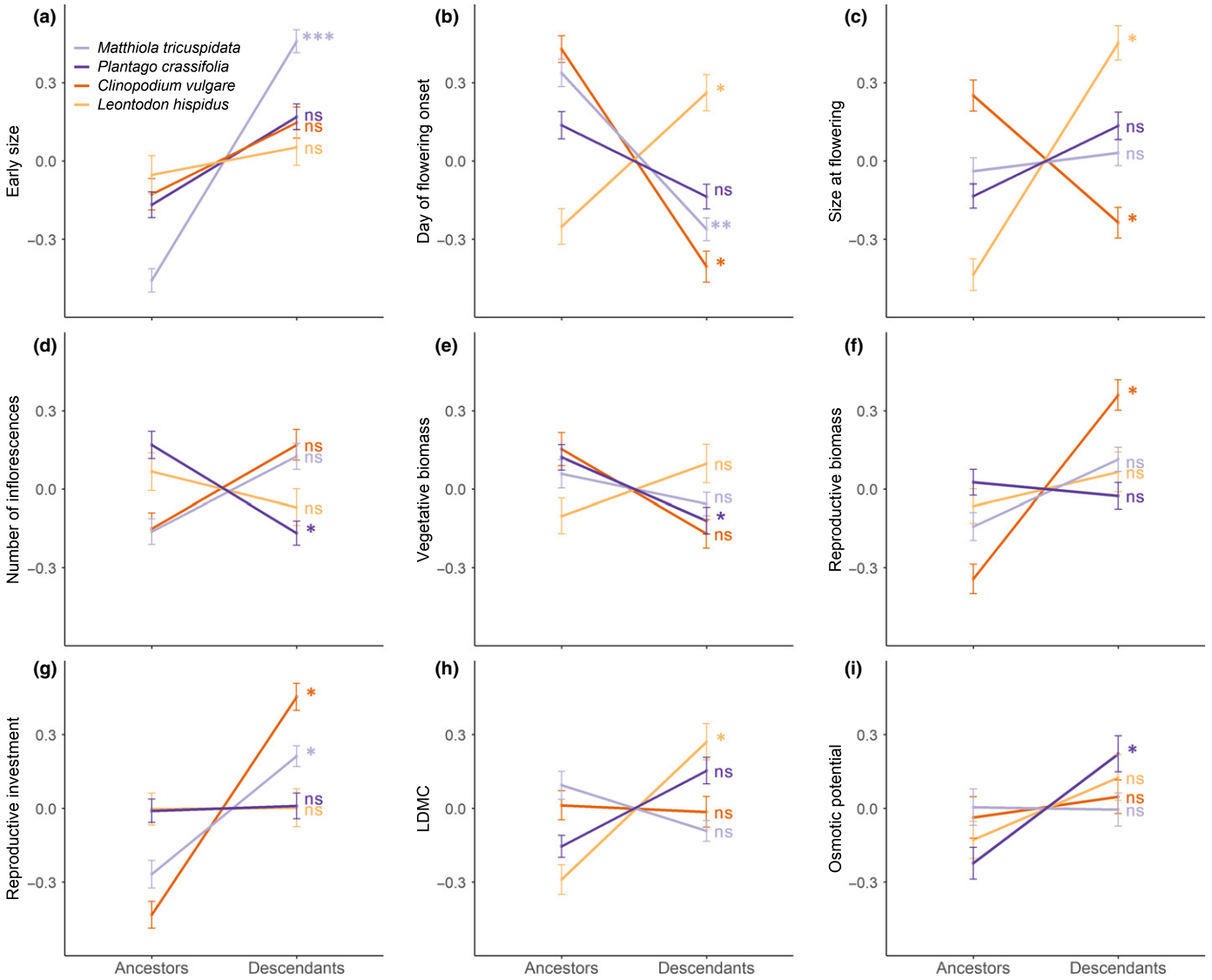

Fig. 1 (a–i) Changes from ancestors to descendants in the nine measured phenotypic traits for each of the four individual species (coloured lines), with asterisks indicating significance levels of ancestor-descendant comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). All data are standardised; the error bars are standard errors. LDMC, leaf dry matter content.

therefore also partly reflect responses to changes in herbivory, or synergistic responses to both climate change and herbivory as shortened life cycles may help to escape from both (Pilson, 2000; Kawagoe & Kudoh, 2010). Observational studies have found that plants advance their phenology more strongly than insects in response to climate warming (Forrest & Thomson, 2011), so escape may indeed be possible. Conversely, generalist herbivores often dominate in Mediterranean and temperate regions (Forister *et al.*, 2015), and acceleration of plant life cycles as observed in our study may lead to escape from some summer herbivores, but could increase exposure to herbivores that are already present in the spring (Meineke *et al.*, 2021).

The phenotypic differentiation between ancestors and descendants depended strongly on the species. In contrast with the three study species with accelerated life cycles, descendants of *L. hispidus* flowered later than their ancestors (Table 2; Fig. 1b). A possible explanation for this is that climate change elongates the growing season in temperate regions, which could also favour a delay in flowering time in some species (Johansson *et al.*, 2013; Weis *et al.*, 2014). However, if this explains later flowering of *L. hispidus*, then why do we not find the same for the other temperate species? Moreover, the studied population of *L. hispidus* is a dry calcareous grassland where reduced precipitation in spring and early summer combined with higher temperatures should rather lead to shorter growing seasons, favouring earlier flowering. An alternative explanation for the delayed flowering in *L. hispidus* descendants could be that this species originated from a site unmanaged in the 1990s and since 2007 it has been sheep grazed in spring and early summer. Such alteration of management might have selected for later flowering, to escape damage by large herbivores (Völler *et al.*, 2013) and therefore potentially counteracted the opposing selection exerted by climate change. Finally, an invisible fraction (Weis, 2018), that is selection during the seed storage and germination stages, could have contributed

Table 2 Results of statistical models testing the effects of temporal origin (ancestors vs descendants), simulated drought and herbivory, and their interactions, on the growth, fitness and functional traits of *Matthiola tricuspidata* (*Mt*), *Plantago crassifolia* (*Pc*), *Clinopodium vulgare* (*Cv*) and *Leontodon hispidus* (*Lh*).

	Early size	Flowering onset	Size at flowering	Number of inflorescences	Vegetative biomass	Reproductive biomass	Reproductive investment	Leaf dry matter content	Osmotic potential
Origin									
Mt	↑	↓					↑		
Pc				↓	↓				↑
Cv		↓	↓			↑	↑		
Lh		↑	↑					↑	
Drought									
Mt		↓	↓	↓	↓	↓	↓	↓	↑
Pc		↓	↓	↓	↓	↓	↓	↓	↑
Cv			↓	↓	↓	↓	↓		
Lh		↓	↓	↓	↓	↓		↓	
Herbivory									
Mt	↑	↑	↑	↓		↓	↓		
Pc					↓				
Cv									
Lh									
Origin × Drought									
Mt		↗	↘	↘			↙	↙	
Pc					↙				↗
Cv					↙			↙/↗	
Lh									
Origin × Herbivory									
Mt				↘					
Pc									
Cv									
Lh									
Drought × Herbivory									
Mt									
Pc									
Cv									
Lh									
Origin × Drought × Herbivory									
Mt									
Pc									
Cv									
Lh									

Significant results are indicated by shading: light green $P < 0.05$, green $P < 0.01$, dark green $P < 0.001$, with arrows indicating their directions: ↑ or ↓ if descendants have larger/smaller values in origin effect or the treatments (drought or herbivory) lead to larger/smaller values; ↗ or ↘ if descendants responded stronger to treatments than ancestors, and values increased/decreased compared with the control treatment; ↖ or ↙ if ancestors responded stronger to treatments than descendants, and values increased/decreased compared with the control; ↙/↗ is a special case in which both temporal origins responded to the treatment, with decreasing values in the ancestors, but increasing values in descendants.

to the observed pattern. Although the germination rates of *L. hispidus* ancestors and descendants were generally fairly high, the germination rate of the ancestors was 18% lower than that of the descendants. If the seeds of later flowering individuals were collected prematurely during the seed collection of the ancestors, these might have not survived the seeds storage and therefore the surviving seeds may have been 'selected' for earlier flowering. While we cannot exclude this possibility, we consider it unlikely as the seed collectors for the Meise seed bank are trained staff who understand the importance of seed maturity for the longevity of stored seeds.

The faster development in terms of advanced flowering is also accompanied by faster early growth in the annual *M. tricuspidata*, with similar (nonsignificant) tendencies in the three other study species, possibly affecting the time when plant size thresholds for flowering are reached (Bolmgren & Cowan, 2008; Sun & Frelich, 2011). However, for the size at flowering results were inconsistent across species (Table 2; Fig. 1c), maybe reflecting differences in life-history strategies among the species, or differences in habitat conditions across the population origins. The descendants of the two Mediterranean species flowered earlier but did not differ in their size at flowering compared with their ancestors, whereas the descendants of *C. vulgare* flowered earlier and were smaller at the time of flowering. As the two traits are generally only weakly to moderately correlated (Pearson's r from −0.13 to 0.40; Table S4), we are confident that these observations are not merely an artefact of dependent variables. Therefore, we conclude that the descendants of *M. tricuspidata* and *P. crassifolia* grew faster and therefore reached size thresholds for flowering earlier (Sun & Frelich, 2011), and that the descendants of *C. vulgare* evolved flowering onset at an earlier developmental stage. With both strategies, life cycles are completed faster,

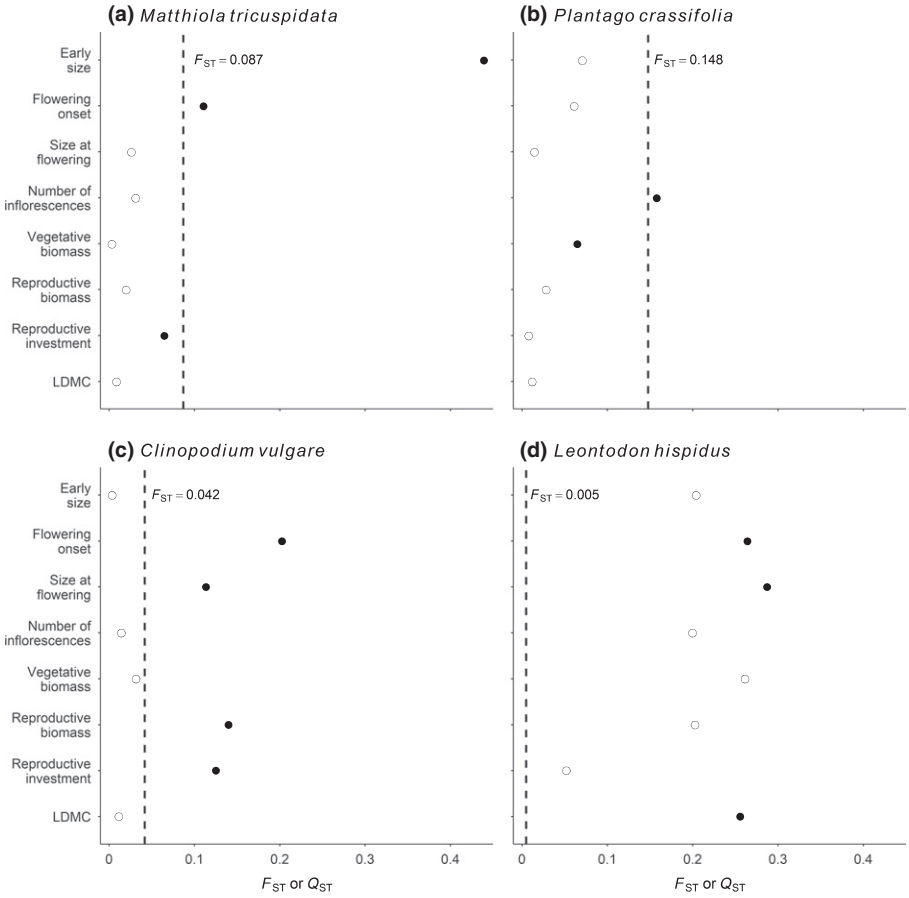

Fig. 2 (a–d) Quantitative genetic differentiation (Q_{ST}) values describing the quantitative phenotypic differentiation between ancestral and descendant plants (filled/empty circles), and how they compare to the respective neutral molecular differentiation (F_{ST}) values (neutral molecular differentiation) based on molecular data (dashed vertical lines), for each of the four studied species. Filled circles indicate traits with significant 'origin' effects in the mixed models (Table 2). The standard errors of Q_{ST} values are too small for displaying. LDMC, leaf dry matter content.

which is thought to benefit plants in disturbed and/or unpredictable environments (Grime, 1977).

In summary, we find evolutionary changes towards accelerated life cycles and increased reproduction or reproductive allocation in several species, for example higher reproductive biomass of descendants in *C. vulgare*, or a higher reproductive investment of descendants in *M. tricuspidata* and *C. vulgare*. The consistency of the changes across the studied species suggests that they are driven by adaptive evolutionary processes instead of random processes such as drift, most likely in response to increased environmental stress during summertime, especially summer droughts, within the last decades (Franks *et al.*, 2007; Kigel *et al.*, 2011; Metz *et al.*, 2020).

Evolutionary processes

Comparisons of Q_{ST} (phenotypic differentiation) and F_{ST} (neutral molecular differentiation) can help to understand the importance of natural selection vs other, nonselective evolutionary processes such as genetic drift or gene flow as causes of population differentiation. We found F_{ST} values between 0.01 to 0.15 in our study species, with higher F_{ST} values in the two Mediterranean species, in particular *P. crassifolia*, but small values in the two temperate species *C. vulgare* and the self-incompatible *L. hispidus*. In contrast with the temperate species, the Mediterranean species came from frequently disturbed habitats, which might increase chances for nonadaptive, random processes through bottlenecks and/or immigration (Banks *et al.*, 2013; Davies *et al.*, 2016) and lead to stronger differentiation between ancestors and descendants. However, many other factors can influence molecular differentiation between populations, and its differences between species, such as mating system, pollination mode, seed dispersal (Gamba & Muchhala, 2020), connectivity (Rousset, 1997; Durka *et al.*, 2017) and population size (van Treuren *et al.*, 1991). Notwithstanding these uncertainties, our F_{ST} measurements are in the expected range. For example, Summers and colleagues resurrected seeds from the soil seed bank of the perennial *Schoenoplectus americanus*, ranging from 1900 to 1998, and collected new plant material from the same population in 2002 and found a maximum F_{ST} of 0.19 (Summers *et al.*, 2018). Our F_{ST} results generally also support our study design and sampling strategy. With strong bottleneck events, or different sampling strategies, we would probably have found much stronger molecular differentiation between ancestors and descendants (Rucińska & Puchalski, 2011; Lauterbach *et al.*, 2012).

The Q_{ST} of onset of flowering was larger than the F_{ST} in three species (*M. tricuspidata*, *C. vulgare* and *L. hispidus*), suggesting directional selection as the most likely responsible evolutionary process (Leinonen *et al.*, 2013). Conversely, for *M. tricuspidata*, *P. crassifolia* and *C. vulgare*, the Q_{ST} values of vegetative biomass and LDMC were smaller than the F_{ST} values, indicating stabilising selection on these traits. These observations are in line

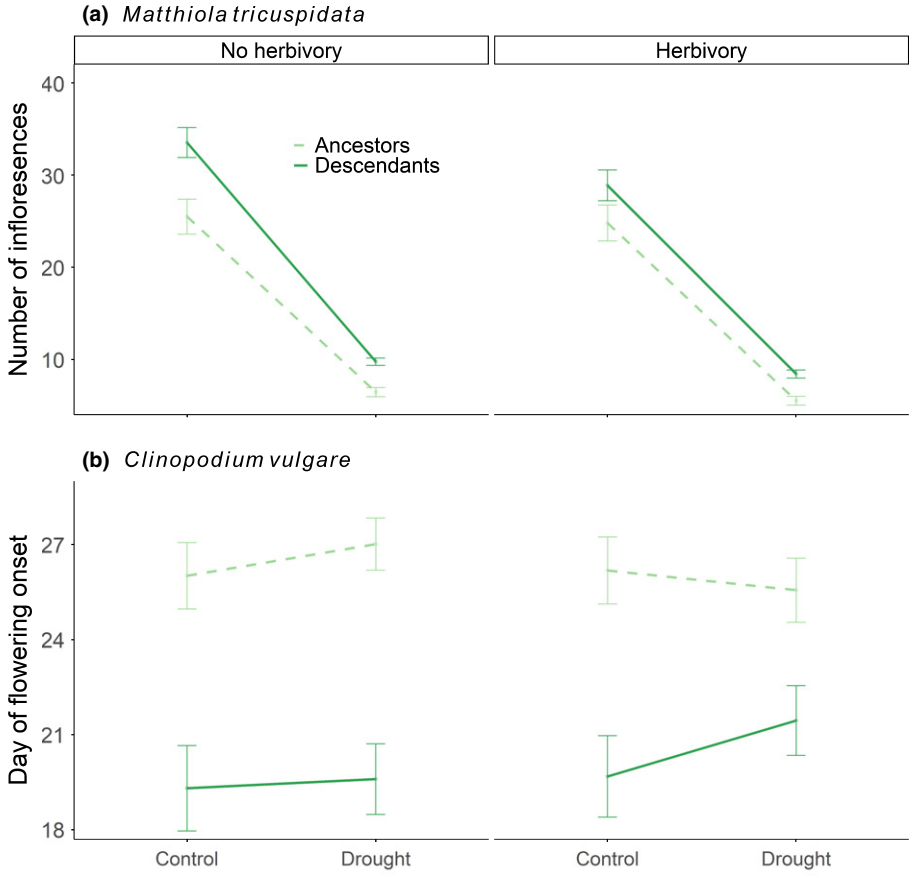

Fig. 3 Significant three-way interactions between drought, herbivory and temporal origin for the number of inflorescences in *Matthiola tricuspidata* (a) and day of flowering onset in *Clinopodium vulgare* (b). The data are mean values and their standard errors.

with other studies, which also found directional selection for flowering-related traits and stabilising selection for vegetative traits (Chun *et al.*, 2011; Kesselring *et al.*, 2015), and it supports our idea described earlier that the acceleration of life cycles is a key evolutionary adaptation in response to climate change, to escape from drought stress.

Still, we should keep in mind that, in our study, we calculated Q_{ST} values for ancestral vs descendant plants of a single population per species. The generality of our results is therefore unclear and will require further testing across multiple populations. Another caveat is the small number of seed families per temporal origin for *L. hispidus*, which may have contributed to low F_{ST} estimates for the species, and our conclusions of directional selection from Q_{ST}–F_{ST} comparisons.

Plasticity of ancestral vs contemporary plants in response to drought and herbivory

The drought treatment generally had much stronger effects on the measured traits than the herbivory treatment, and there were also many more significant drought × origin interactions than herbivory × origin interactions. This could either be because herbivory was a weaker driver of natural selection in the studied populations during the last decades, or it could be because the simulated herbivory in our experiment was too weak to provoke stronger plastic plant responses. This disparity of our two treatments in impacting the studied species could be the reason that we found hardly any evidence for trade-offs in adaptations to drought and herbivory (Pilson, 2000; Sthultz *et al.*, 2009; Nelson *et al.*, 2017).

We generally observed the most plastic responses in *M. tricuspidata,* including the only significant herbivory main effects, most drought × origin interactions and the only significant herbivory × origin interaction. As *M. tricuspidata* was the only strictly annual species in our study it is possible that ancestors and descendants were stronger differentiated because of the higher effective number of generations available for evolutionary changes. We found for *M. tricuspidata* that descendants delayed flowering significantly less under drought than did their ancestors. This could be interpreted as evolution of a stronger ability to tolerate increasingly drier summers (Grene *et al.*, 2011; De Kort *et al.*, 2020) or as a more opportunistic phenology if water becomes available later in the growing season (Dyer *et al.*, 2012). In addition to differences in the plasticity of flowering time, we also found that the descendants decreased their number of inflorescences more strongly under both drought, as well as herbivory, conditions. Whether this represents the evolution of a more opportunistic reproduction, or plants just suffered more under more stressful environmental conditions (Dyer *et al.*, 2012; De Kort *et al.*, 2020), cannot be answered by our experiment, but the first explanation is supported by the weaker decreases of LDMC in *M. tricuspidata* descendants, a morphological trait that

is positively related to plant tolerance against drought and herbivory (Gardarin *et al.*, 2014; Blumenthal *et al.*, 2020). A similar pattern was found in *C. vulgare*, in which descendants increased and ancestors decreased LDMC in response to drought.

We found that the descendants of *P. crassifolia* increased their osmotic potential more strongly under drought than did their ancestors. The osmotic potential is directly related to the molar concentration of solutes in plant cells, which is tightly linked to the plant wilting point (Bartlett *et al.*, 2012; Meinzer *et al.*, 2016) and therefore drought tolerance (Kolb & Sperry, 1999; Lenz *et al.*, 2006; Májeková *et al.*, 2019). At the same time, ancestors of *P. crassifolia* and also *C. vulgare* showed significantly greater decreases in biomass in response to drought. Our results therefore strongly indicate that descendants of *P. crassifolia* have evolved greater plasticity in a functional trait that allows them to better cope with drought (Ackerly *et al.*, 2000; Richards *et al.*, 2006).

Conclusion

We studied four plant species from two independent biogeographic regions in Europe in a resurrection experiment, and we found evidence that the descendant populations of three species evolutionarily shortened their life cycles, presumably in response to climate change, during a period of only 21–26 yr. Shortened life cycles may allow plants to escape increasingly frequent summer droughts and potential insect outbreaks. In our study the plants realised this through rapid seedling growth, earlier flowering onset and/or shifts in resource allocation. In addition to these evolutionary 'escape strategies', we also detected evolutionary changes in the osmotic potential in one species, and in LDMC in three species, which indicate evolution of greater drought and herbivory tolerance through increased phenotypic plasticity in the descendant plants. Our quantitative genetic analysis indicated directional selection on several functional traits, supporting our hypothesis of adaptive evolutionary changes in the studied plant populations.

In spite of including several species, the power to generalise from our study remains limited, because we included only four plant species that came from two biogeographic regions, and each species was represented by a single population. True multispecies studies usually cover a broader range of species (van Kleunen *et al.*, 2014), and it should in principle be possible to repeat our study with many more species to achieve such greater generality. However, this must necessarily come at the cost of less within-species and experimental detail, which is required, for example, for testing phenotypic plasticity or calculating quantitative genetic metrics. In our study we were particularly interested in the latter.

Our study demonstrates the power of historical comparisons between banked seeds and current populations for studying rapid evolutionary changes. To gain deeper insights into evolutionary changes future studies should conduct transplantations of ancestors and descendants into their original habitat and include longer-term fitness measures.

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Author contributions

RR, AE and JFS conceived the experiment. RR, ZL, AE and JFS designed the experiment with input from MM. RR, SG and LD conducted fieldwork and RR and ZL performed the experiment. RR and ZL collected data and performed data analysis with input from OB, AE and JFS. WD performed the molecular and bioinformatics analyses. RR wrote the manuscript with input from all coauthors.

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Data availability

All generated and analysed data from this study are included in the published article and its Supporting Information. Raw data from the ddRAD-SNP sequencing have been deposited in the European Nucleotide Archive (ENA) at EMBL under accession no. 409 number PRJEB47887 (https://www.ebi.ac.uk/ena/browser/view/PRJEB47887).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Population genomic analyses.

Figs S2–S5 Graphs of all measured traits separated by the treatments and the four species.

Methods S1 ddRAD library preparation and SNP genotyping.

Table S1 Information about the original seed material, which was used for the refresher generations.

Table S2 Mean values and standard errors for all measured traits separated by the treatments.

Table S3 Parameters of the statistical models.

Table S4 Pairwise comparisons of the nine measured traits.

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