



# Establishment rate of regional provenances mirrors relative share and germination rate in a climate change experiment

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**Abstract.** Climate change and land-use changes are among the major threats to biodiversity as they alter global and local environmental conditions in unprecedented dimensions. Therefore, the investigation of the ability of species and communities to cope with rapidly changing environments as well as the comprehensive understanding of possible evolutionary adaptation processes is urgently needed for their sustainable management and the maintenance of associated ecosystem processes. Here, seminatural grasslands receive special attention, because they are among the most species-rich ecosystems in Central Europe, which are threatened by global change and land-use intensification already since the beginning of the twentieth century. Hence, understanding their potential to respond to rapidly changing environments is important for future management. Here, the Global Change Experimental Facility (GCEF) is an opportunity to investigate the role of microevolution in response to climate change. Two of the land-use regimes in the GCEF are seminatural, extensively used species-rich meadow and pasture grasslands established by sowing common, native, and regionally typical grassland species in 2014. In view of ecological restoration, for each species a seed mixture of up to seven source populations was sown aiming to establish high levels of intraspecific variation from the regional gene pool. Here, we present the first evaluation of genetic and trait variation of source populations and of their establishment in the GCEF two years after sowing for six grassland species. Using AFLP markers, we assessed genetic variation of source populations and tested whether the source gene pools have established in the experiment. Additionally, we investigated phenotypic variation of source populations and performed  $P_{ST}$ - $F_{ST}$  comparisons to test whether trait differentiation is adaptive. Our study revealed that genetic and phenotypic differentiation of source populations is widespread in the grassland species studied, even on small geographic scales. The GCEF populations are highly diverse due to the mixture of the different, often genetically and phenotypically differentiated source populations. They represent a genetically diverse source for both selection among existing and evolution of new genotypes. Thus, the GCEF can be used as experiment to study evolutionary processes in response to the climate change and land-use scenarios.

**Key words:** amplified fragment length polymorphism (AFLP); climate change; ecological restoration; genetic diversity; Global Change Experimental Facility (GCEF); grasslands; intraspecific variation; land-use change; phenotypic differentiation;  $P_{ST}$ - $F_{ST}$  comparison.

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## INTRODUCTION

Climate and land-use change are counted among the major human-induced threats to biodiversity (Cahill et al. 2013). They alter global and local environmental conditions in unprecedented dimensions (Matesanz et al. 2010). Investigating the ability of species and communities to cope with such rapidly changing environmental conditions is urgently needed in order to protect biodiversity and associated ecosystem functions of species and populations (Visser 2008, Cahill et al. 2013). For individual species and populations in general, there are three possible ways to respond to changing environments: (1) migration, the dispersal to a suitable habitat elsewhere; (2) acclimation, the change in the phenotype without changing genotype via phenotypic plasticity; and (3) evolutionary adaptation, the change in the genetic composition by a relative increase in genotypes with higher fitness (Holt 1990, Davis et al. 2005, Gienapp et al. 2008). Although there is evidence for relatively rapid evolutionary responses, for example, to climate change (Franks et al. 2007, Ravenscroft et al. 2015, Warwell and Shaw 2019), there is still a great need to understand evolutionary dynamics for many species and communities, especially to simultaneously changing temperature and precipitation regime (Chevin et al. 2013, Franks et al. 2014).

Seminatural, extensively used grasslands are counted among the most heterogeneous and species-rich ecosystems in Central Europe, which have an esthetic value and provide important ecosystem services. They are threatened by anthropogenic global change and land-use intensification since the beginning of the twentieth century and thus are an important target for biodiversity conservation (Hejcman et al. 2013). To prevent further degradation of existing or to establish species-rich grasslands, there is often a need to introduce seeds from other sources (SER 2004, Hölzel et al. 2012). Different seed sourcing strategies have been adopted for grassland restoration all of which, although with different emphasis, aim both to benefit from local or regional adaptation and to encompass genetic diversity of the seed mixtures to ensure restoration success and adaptability to environmental

changes (Bucharova et al. 2017). This is especially important for seminatural grasslands due to the fact that the different management regimes in those ecosystems, for example, mowing and grazing, may create different sets of biotic and abiotic processes (Merilä and Hoffmann 2016, Bucharova et al. 2019) which in turn may affect the potential of species and communities to respond to climate change (Fischer et al. 2011).

To study the responses of species and communities to climate change and land use in a realistic scenario requires first, an experimental setup allowing the manipulation of environmental conditions. Second, it requires a community large enough to maintain ecological processes similar to natural ecosystems and populations that harbor heritable genetic variation in relevant traits to allow for evolutionary responses. The Global Change Experimental Facility (GCEF) is a long-term and large-scale climate change field experiment that represents an opportunity to investigate the impact of climate change on ecosystem processes including the role of microevolution (Schädler et al. 2019). Two of the land-use types are seminatural, extensively used grasslands, that is, meadow, mown by machine, and pasture, grazed by sheep. Both were established as species-rich grassland by sowing the same seed mixture of 56 common, native, and regionally typical grassland plant species. We aimed to establish genetically diverse plant populations to allow for microevolutionary processes to act on a broad genetic basis. Thus, the choice of seed sources within species was crucial, due to the fact that regional adaptation and genetic differentiation are common across European grassland species (Michalski and Durka 2012, Durka et al. 2017). Consequently, to both reflect the regional gene pool and to encompass high levels of intraspecific variation, for most species a seed mixture from multiple regional natural source populations was sown.

However, the genetic composition and phenotypic trait variation of the sown seed material and the source populations were not known at the time of sowing, and while almost all sown species have established in the experiment (H. Auge, *personal communication*), it is also not known to which extent the sown source populations established successfully in the GCEF.

Here, we present the first evaluation of genetic and trait variation of source populations and of their establishment in the GCEF representing a baseline two years after sowing for six grassland species. For this, we analyze genetic marker variation of the used seed sources and of the established populations in the GCEF for six plant species. We use AFLP markers to assess genetic variation within and among the natural source populations. We then address phenotypic variation within and among natural source populations using a common garden experiment and perform  $P_{ST}$ - $F_{ST}$  comparisons in order to understand whether expressed trait differentiation is potentially adaptive. Finally, we assess whether the gene pools representing the source populations have established in the experiment and whether established genetic variation is concordant with random expectations. In particular, we ask the following: (1) “Are source populations genetically differentiated?” (2) “Are source populations phenotypically differentiated from each other, and if so, are phenotypic differences due to divergent selection or in line with neutral expectations?” (3) “Which of the sown gene pools did establish in the experimental plots and did they establish homogeneously across experimental blocks and treatments?” and (4) “Are experimental plots genetically more than or at least as diverse as source populations?”

## MATERIALS AND METHODS

### *Study sites and species*

The Global Change Experimental Facility (GCEF), established in 2014, is a field experiment located in Bad Lauchstädt near Halle (Saale), Saxony-Anhalt, Germany (51.391667, 11.880278, 116 m a.s.l.). The GCEF has a split-plot design with climate (future versus ambient) as main plot factor and five land-use types as subplot factors, replicated five times for each climate  $\times$  land-use treatment combination (Schädler et al. 2019). Subplot sizes (16 m  $\times$  24 m each) allow realistic agricultural regimes. The climate manipulation consists of increased mean annual temperatures of about 2°C and an altered precipitation regime (minus  $\sim$  20% in summer, plus  $\sim$  10% in spring and autumn) compared with the plots under ambient climate conditions. This treatment is a consensus scenario across several models of

climatic conditions in Central Germany predicted for the years between 2070 and 2100. Beside conventional farming, organic farming and intensively used grassland managed by frequent mowing (4  $\times$  each year), the land-use regimes include extensively used grassland managed by either moderate mowing (2  $\times$  each year) or moderate sheep grazing (2  $\times$  each year). Both extensively used grassland types were established by sowing 56 plant species where the original seed mixture was obtained from a professional seed producer of regional seeds (Saale-Saaten, Halle (Saale), Germany). For more detailed information, refer to Schädler et al. (2019). For each species, the seed mixture consisted of one to three main sources of propagated seeds originating from the local seed transfer zone. This main share was complemented by seeds that were manually collected from multiple natural source populations located in Central Germany each representing a small share ( $\sim$ 10%). Across all species, on average, seeds from 2.8 source populations were sown.

Out of the 56 native grassland species, we selected six perennial species from different functional groups, including grasses, nonlegume herbs, and legumes: *Achillea millefolium* L. (Asteraceae), *Arrhenatherum elatius* P. Beauv. ex J. Presl & K. Presl (Poaceae), *Bromus erectus* Huds. (Poaceae), *Galium album* Mill. (Rubiaceae), *Leucanthemum vulgare* Lam. s. str. (Asteraceae), and *Trifolium pratense* L. (Fabaceae). All six species are native, common, and regionally typical for extensively managed grasslands and established abundantly in the experimental plots. The sowing mixture for these species contained seeds from between three and six source populations all located in Central Germany with an average distance of 44 km (max. 211 km) from the experimental site (Appendix S1: Table S1). The seed contribution of individual source populations in the seed mixture differed and ranged between 10% and 80% (Appendix S1: Table S1).

### *Genetic analyses*

Leaf material of source populations was collected from specimens grown in the common garden (see Phenotypic trait analysis), except for *T. pratense* accession TRIF1 which was collected at the original location in the field. In the GCEF, we sampled the four treatments ambient climate

meadow, ambient climate pasture, future climate meadow, and future climate pasture, each with five replicate plots in May 2016, that is, two years after sowing. Per species, leaf material of four individuals was collected randomly on each plot, resulting in 20 individuals per treatment, henceforward referred to as population. All leaf material was immediately freeze-dried after collection.

For each species, amplified fragment length polymorphism analysis (AFLP) was performed, following the protocol of Kloss et al. (2011). DNA was extracted using DNeasy 96 kits (Qiagen, Hilden, Germany). Restriction and ligation were performed in 11  $\mu\text{L}$  with 6  $\mu\text{L}$  DNA (corresponding on average to 38 ng/ $\mu\text{L}$  DNA for *G. album*, 10 ng/ $\mu\text{L}$  DNA for *T. pratense*, 13 ng/ $\mu\text{L}$  DNA for *A. millefolium*, 15 ng/ $\mu\text{L}$  DNA for *L. vulgare*, 21 ng/ $\mu\text{L}$  DNA for *A. elatius*, and 17 ng/ $\mu\text{L}$  DNA for *B. erectus*) and MseI and EcoRI restriction enzymes at room temperature overnight. 4  $\mu\text{L}$  was used for preselective amplification and 2.2  $\mu\text{L}$  for selective amplification. After screening 16 primer combinations, four primer combinations were selected for each species for genotyping (Appendix S1: Table S2). The fragments were separated on an ABI 3130 genetic analyzer and binned manually in GENEMAPPER 5.0 (Applied Biosystems, Foster City, California, USA). Peak-height data were exported, and a peak-specific definition of the threshold and error rate (based on 16 double extractions of DNA per species) was implemented. Only suitable peaks with a bimodal peak-height distribution and an error rate <5% were selected, and the resulting individual fragment information was exported. Finally, between 113 and 516 AFLP loci per species with a mean genotypic error rate of 2.3% were obtained (Appendix S1: Table S3).

#### Phenotypic trait analysis

To assess the genetic and phenotypic variation within and among the source populations, we established a common garden in 2016 using the original seed material, which had been used for sowing the GCEF and had been stored at  $-24^{\circ}\text{C}$  (Appendix S1: Table S1). Seeds were germinated on moistened filter paper in Petri dishes in a growth chamber with a 12 h/22 $^{\circ}\text{C}$  and 12 h/12 $^{\circ}\text{C}$  day–night regime. When reaching the cotyledon stage, the seedlings were pricked into a soil–sand

substrate (3:1) in multipot plates and kept within the chamber for three more weeks. Afterward, a maximum of 25 individuals per source population and species were potted individually into three-liter plastic pots, containing about 1.5 kg of a peat-free soil–sand substrate (3:1). Pots were placed outside May 2016 on a layer of bark mulch, with a distance of 50 cm between each other and patch edges. Individuals of each species were arranged randomly in one block and watered on demand. For all individuals, a set of phenotypic traits was quantified in August 2016: above-ground biomass (g), plant height (cm), leaf area ( $\text{cm}^2$ ), leaf width (cm), leaf length (cm), leaf length–width ratio (cm), specific leaf area (SLA,  $\text{mm}^2 \times \text{g}^{-1}$ ), leaf dry matter content (LDMC,  $\text{mg} \times \text{g}^{-1}$ ), number of inflorescences ( $n$ ), and flowering time (d). Additionally, for *A. millefolium* and *L. vulgare* the leaf perimeter–area ratio ( $\text{cm} \times \text{cm}^{-2}$ ) was determined. One source population (*T. pratense*, TRIF1) did not germinate and hence was not represented in the common garden and in the analyses of phenotypic traits. For *B. erectus*, only very few individuals came into flower in 2016. Consequently, the number of inflorescences and the flowering time were quantified in 2017.

#### Data analysis

Using the AFLP data, we quantified overall and pairwise genotypic differentiation ( $F_{\text{ST}}$ ) among source and GCEF populations (among blocks and treatments) using a band-based approach (Bonin et al. 2007) of an analysis of molecular variance (AMOVA; Excoffier et al. 1992), as implemented in GenALEX 6.5 (Peakall and Smouse 2012).

To investigate the relationships between individuals, natural sources, and GCEF populations, we applied a Bayesian clustering approach using STRUCTURE 2.3.4 (Falush et al. 2007) in the recessive allele mode as recommended for dominant markers such as AFLP. The most probable number of genetic groups ( $K$ ) was determined by doing 10 iterations for each  $K$  from 1 to 10, always performed with a burn-in period of 50.000 followed by 100.000 Markov Chain Monte Carlo (MCMC) steps. STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used for determining the most probable number of genetic groups based on the  $\Delta K$  approach (Evanno et al. 2005; Appendix S1: Fig. S1). For



each species, consensus STRUCTURE plots were obtained with CLUMPP 1.1.2. (Appendix S1: Fig. S1).

Genetic variability within populations was assessed as band richness, Br, based on a rarefaction approach and calculated with AFLPdiv v. 1.0 (Coart et al. 2005) with rarefaction samples sizes of 13, 12, 7, 19, 19, and 19, respectively, for the six species listed above, and as unbiased heterozygosity,  $H_{e_{uv}}$ , calculated with GenALEX 6.5 (Peakall and Smouse 2012). We compared genetic variability between GCEF populations and source populations by means of *t* test. Additionally, we compared observed proportions of individual gene pools in the GCEF with expected proportions, considering gene pool proportions in source populations, seed contribution in the seed mixture, and germination rate. Similarly, to assess whether genetic variability of GCEF populations was within the range expected we performed a randomization test by assembling 100 populations by randomly drawing, without replacement, genotypes from a rarefied sample of individuals of the source populations, weighted by germination percentage and by seed contribution (Appendix S1: Table S1), calculating Br and  $H_{e_{u}}$ , and testing whether observed values were within the 95% percentile of the expectation. We compared source populations and GCEF plots with respect to the proportion of individuals showing admixed gene pools, that is, consisting of at least two gene pools with a share of >25%.

For the analysis of phenotypic data, we log- or sqrt-transformed data to ensure normal distribution or errors if necessary (Appendix S1: Table S4). To determine significant differences between the source populations, we implemented an analysis of variance (ANOVA) combined with Tukey's honest significant difference (HSD) test for each phenotypic trait. The distribution of the data for each population was visualized in box plots (Appendix S1: Fig. S2). To investigate the degree of phenotypic divergence ( $P_{ST}$ ) per considered trait and to compare the intensity of differentiation among traits and species, we quantified  $P_{ST}$  as  $P_{ST} = \sigma_{GB}^2 / (\sigma_{GB}^2 + 2 \times (h^2 \times \sigma_{GW}^2))$ , where  $\sigma_{GB}^2$  and  $\sigma_{GW}^2$  reflect the phenotypic variances between and within populations, respectively (Leinonen et al. 2006). As a reasonable estimate of  $h^2 = 0.3$  was

adapted from Geber and Griffen (2003), representing the mean heritability across various traits of outcrossing and mixed mating plant species. Variance components were estimated using a mixed-effect model implemented in the R-package MCMCglmm, performed with a burn-in period of 40.000 followed by 200.000 MCMC steps and a thinning interval of 80.

To assess the strength of the population effect, two models were compared for each trait, with and without "population" as random effect. A difference in the deviance information criterion (DIC) between models of  $\Delta DIC \geq 2$  was considered to indicate a significant population effect for the specific phenotypic trait. Finally, to examine whether phenotypic differences are likely due to natural selection or in line with neutral expectations, an  $F_{ST}$ - $P_{ST}$  comparison (Leinonen et al. 2013) was executed for each species by testing whether the 95% Bayesian credible intervals of phenotypic divergence  $P_{ST}$  for each individual trait overlapped with the overall neutral genetic differentiation value  $F_{ST}$  attained from the molecular analysis (AMOVA). If not stated otherwise, all analyses were performed with R-3.3.2. (R Core Team 2018).

## RESULTS

### Population differentiation among natural source populations

Marker-based genetic differentiation among source populations was significant in all species as revealed by significant overall  $F_{ST}$  values derived from AMOVA (Table 1). Genetic differentiation was largest for *A. millefolium* ( $F_{ST} = 0.18$ ), with decreasing values for *G. album* ( $F_{ST} = 0.14$ ), *L. vulgare* ( $F_{ST} = 0.12$ ), *B. erectus* ( $F_{ST} = 0.08$ ), *T. pratense* ( $F_{ST} = 0.06$ ), and *A. elatius* ( $F_{ST} = 0.06$ ). Pairwise differentiation among source populations was significant ( $P < 0.05$ ) for all species and all population pairs (Appendix S1: Table S5). The Bayesian cluster analysis implemented with STRUCTURE revealed species-specific patterns with the most likely number of gene pools ranging between  $K = 2$  and  $K = 5$  (Fig. 1; Appendix S1: Fig. S1). While in three species each source population represented a unique gene pool (*B. erectus*  $K = 5$ , *G. album*  $K = 4$ , *L. vulgare*  $K = 3$ ), only two gene pools were found in the other species.

Table 1. Number of source populations; overall  $F_{ST}$  among source populations as derived from AMOVA; most likely number of gene pools revealed with the Bayesian structure analysis; and overall proportions of gene pools and admixed individuals for GCEF plots.

Species	No. source populations	Overall $F_{ST}$	Number of gene pools	GCEF, overall gene pool proportions (%)	GCEF, source populations (% admixed individuals)
<i>Achillea millefolium</i>	4	0.18***	2	39:61	0.0 (1.4)
<i>Arrhenatherum elatius</i>	6	0.06***	2	11:89	9.5 (13.5)
<i>Bromus erectus</i>	5	0.08***	5	5:6:33:3:53	2.5 (13.6)
<i>Galium album</i>	4	0.14***	4	94:1:3:2	0.0 (6.3)
<i>Leucanthemum vulgare</i>	3	0.12***	3	22:64:14	8.8 (8.5)
<i>Trifolium pratense</i>	4	0.06***	2	46:54	13.9 (5.0)

\*\*\*  $P \leq 0.001$ .

Phenotypic differentiation among source populations was common both across species and traits with nearly identical results for the ANOVA (Appendix S1: Table S6, Fig. S2) and the  $P_{ST}$  approach (Appendix S1: Table S7). Across species, *A. millefolium* showed the highest number of significantly differentiated traits (eight traits) among populations ( $P_{ST} > 0$ ), followed by *G. album* (seven traits), *A. elatius* and *B. erectus* (five traits), *L. vulgare* (five traits in the ANOVA approach, but only three in the  $P_{ST}$  approach), and *T. pratense* (three traits in the ANOVA and four in the  $P_{ST}$  approach). The most commonly differentiated traits were plant height, leaf width (five species), and flowering time (even six species in the  $P_{ST}$  approach). SLA was the only trait not differentiated in any of the species investigated.

The results of the  $P_{ST}$ - $F_{ST}$  comparison are visualized in Fig. 2 (Appendix S1: Table S7). Out of the 32 species- and trait-specific significant differentiation patterns among populations in the  $P_{ST}$  approach, 14 were also significantly more differentiated than expected from neutral genetic markers ( $F_{ST}$ ).

Across species, *G. album* showed the highest number of traits (four) that were significantly more differentiated, whereas *A. elatius* and *L. vulgare* exhibited only one trait significantly more differentiated than neutral expectations. Trait differentiation most commonly exceeded expectations from  $F_{ST}$  for leaf width (four species). Biomass, perimeter/area ratio, SLA, and LDMC showed no significant deviations between  $P_{ST}$  and  $F_{ST}$ .

#### Population differentiation among GCEF plots

In the Bayesian cluster analysis, the GCEF populations were clearly mixed from several gene pools (>10% contribution) in five species (*A. millefolium*, *A. elatius*, *B. erectus*, *L. vulgare*, and *T. pratense*), while for one species (*G. album*) a single gene pool contributed nearly 95% to the experimental populations (Table 1; see Appendix S1: Table S8 for detailed account of gene pool proportions in source populations and GCEF plots). The observed proportions of the gene pools in general matched expectations, except for two source populations each in *Bromus* and *Trifolium*, which contributed considerably more or less than predicted (Fig. 3).

The intraindividual admixture of gene pools did hardly differ between source populations and GCEF plots, except for *T. pratense* in which there was an increase of 9% admixed individuals, likely representing first-generation hybrids among gene pools.

Marker-based genetic differentiation among GCEF blocks, among climate change treatments and among land-use treatments, was nonsignificant in most cases (Appendix S1: Tables S9–S11). However, low levels of differentiation were detected among GCEF blocks in *A. millefolium* (2%,  $P = 0.007$ ) and *A. elatius* (1%,  $P = 0.094$ ; Appendix S1: Table S9), among land-use treatments for *A. millefolium* (2%,  $P = 0.003$ ) and for *B. erectus* (1%,  $P = 0.026$ ; Appendix S1: Table S11). All other species did not show any differentiation among blocks, climate change treatments, and land-use types (Appendix S1: Tables S9–S11).

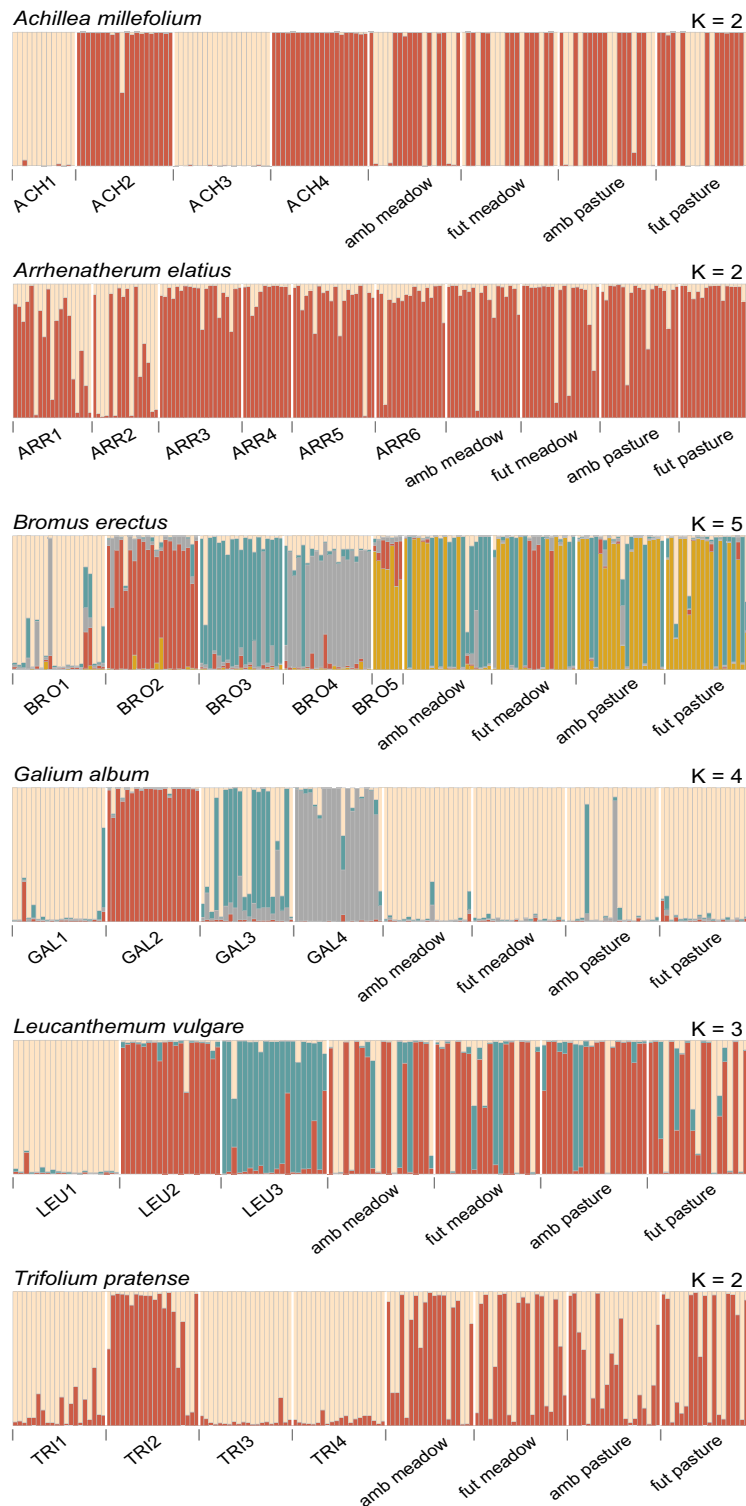


Fig. 1. Bayesian cluster analysis of AFLP data with STRUCTURE for six species. Between three and six source populations are shown left and the GCEF treatments (ambient vs. future climate and meadow vs. pasture) to the right of each panel. See Appendix S1: Fig. S1 for details.

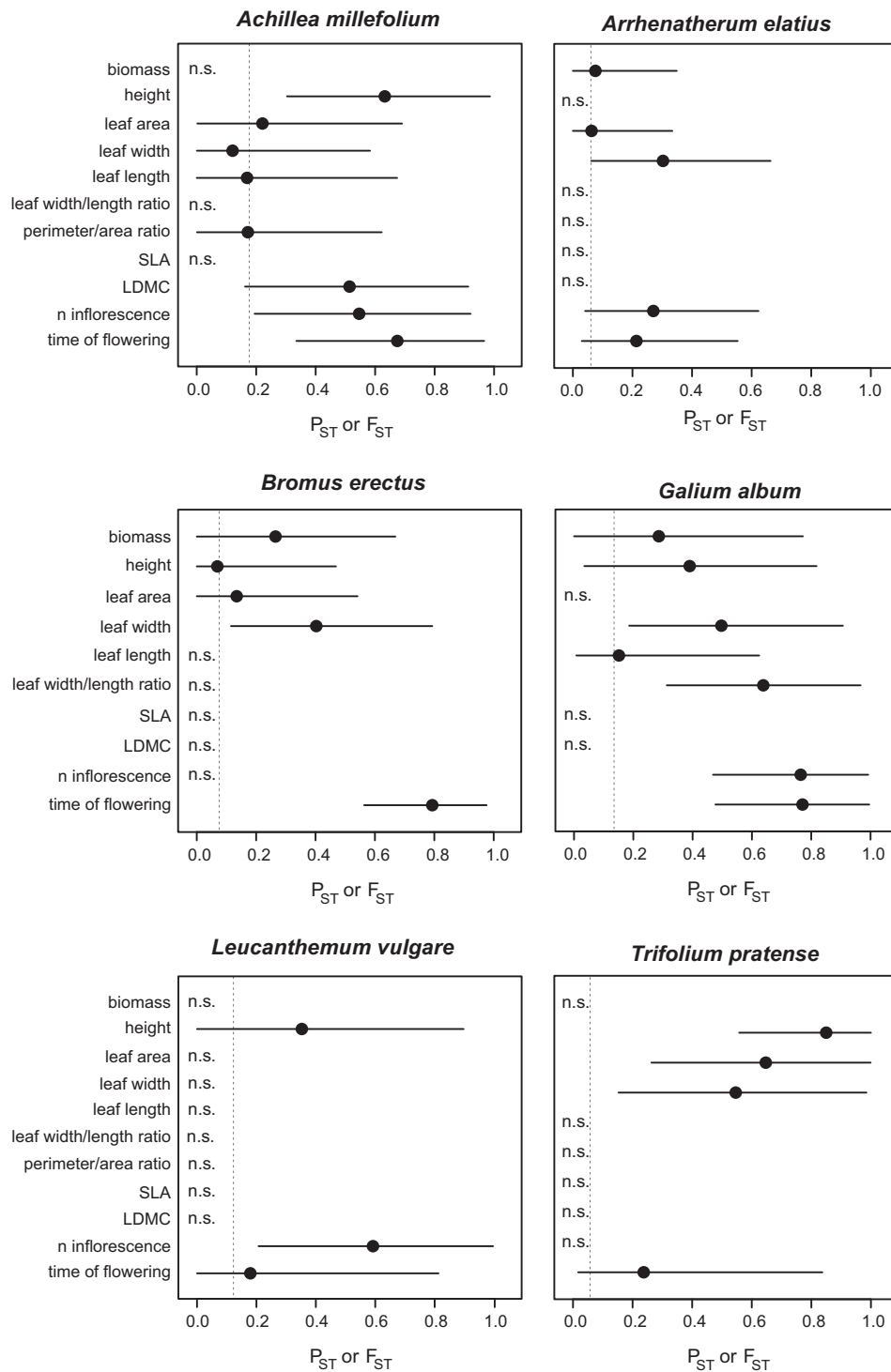


Fig. 2.  $F_{ST}$ - $P_{ST}$  comparisons for each species and eleven traits. The vertical dashed line represents the  $F_{ST}$  value; the black dots represent trait-specific  $P_{ST}$  values; and the horizontal black lines indicate appropriate 95% Bayesian credible intervals. Traits are considered under selection when credible intervals of  $P_{ST}$  do not overlap with  $F_{ST}$  values. n.s. indicates nonsignificant  $P_{ST}$  value.



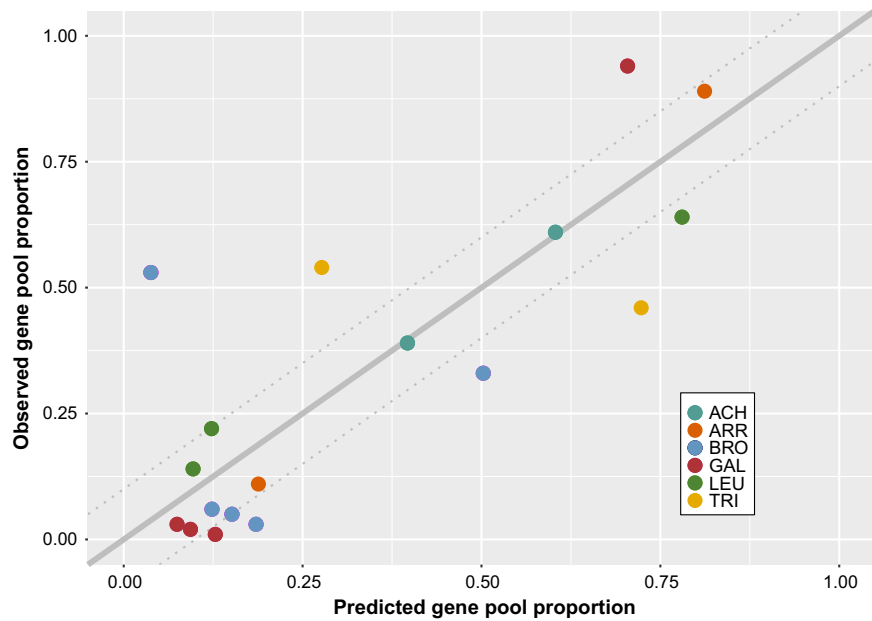


Fig. 3. Observed proportions of gene pools in GCEF plot as a function of predicted proportions, considering gene pool proportions in source populations, seed contribution in seed mixture, and germination rate. The solid line indicates exact match of observed and predicted gene pool proportions, and the dashed lines, a 10% deviation.

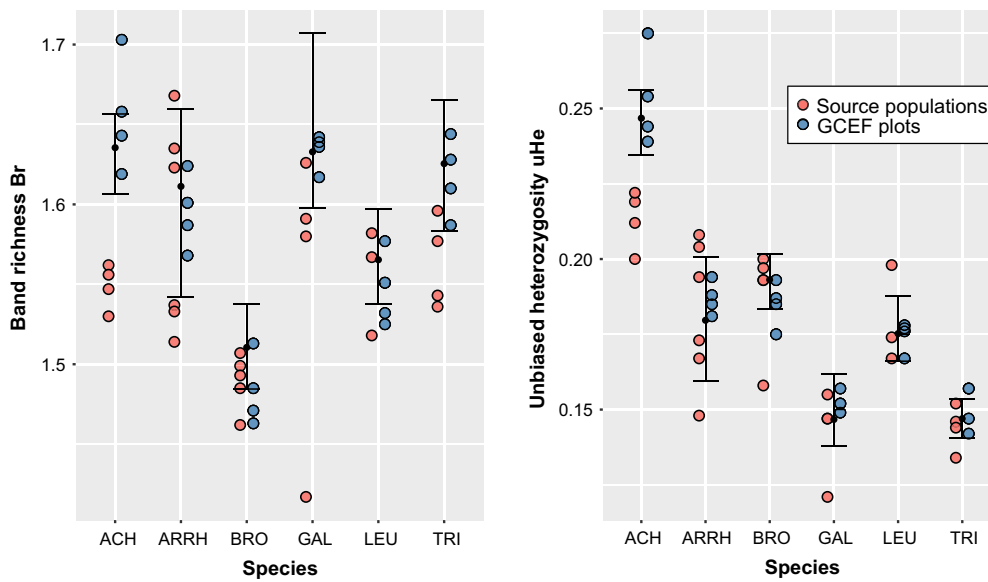


Fig. 4. Genetic diversity (band richness and unbiased heterozygosity) of source and GCEF populations. A random expectation (mean and 95% confidence interval, black dot, and error bar, respectively) is added for comparison for GCEF populations based on the assumption of establishment according to germination rate and relative seed amount of the seed sources.

### Genetic variability within populations compared between sources and GCEF

Estimates of genetic variability within natural source and GCEF populations are shown in Fig. 4. On average, genetic diversity of GCEF populations was higher than that of the source populations for *A. millefolium* (both Br and  $H_{e\_u}$ ) and for *T. pratense* (Br, all  $t$  test  $P < 0.05$ ). For the majority of species, however, within-population genetic diversity in GCEF populations, on average, was as high as in source populations. When comparing genetic variation of GCEF populations to expected values based on randomly assembled populations weighted by source-specific seed contribution and germination rates, almost all populations were in the range of expected diversity for both Br and  $H_{e\_u}$ , however with eight exceptions in 48 tested combinations. In detail, one GCEF population of *A. millefolium* (Br and  $H_{e\_u}$ ) and *T. pratense* ( $H_{e\_u}$ ) had higher and some populations of *B. erectus* (two for Br, one for  $H_{e\_u}$ ) and *L. vulgare* (two for Br) revealed lower diversity than expected.

## DISCUSSION

### Genetic variation in natural source populations

Establishing plant populations or plant communities for ecological experiments can be challenging due to differential establishment or initial mortality of the used source populations and/or species. For example, Hahn et al. (2017) report on initial mortality between 16% and 63% among species. Thus, established experimental communities may differ from target communities, for example, with respect to species richness (Weisser et al. 2017). Therefore, it can be similarly expected for the intraspecific genetic level that not the complete gene pool sown will establish. As a precondition to address this question, we first showed that in all investigated species the source populations represented different gene pools being significantly genetically differentiated ranging from  $F_{ST} = 0.06$  to 0.18 in *A. elatius*/*T. pratense* and *A. millefolium*, respectively. This range goes in line with the literature with in general lower differentiation for wind-pollinated than for the insect-pollinated species (Reisch and Bernhardt-Römermann 2014, Durka et al. 2017). Within species, the number of genetically differentiated gene pools ranged between

two and five. Thus, using a mixture of seeds from multiple populations from the same geographic region was a successful strategy to maximize genetic variation in the seed mixture. This high genetic variability in turn likely will reduce risks such as inbreeding depression and negative effects of genetic drift in established populations, and increase the probability that at least parts of the diverse seed material are regionally adapted to the prevalent environmental conditions and finally ensures for a high adaptability to future environmental conditions such as climate change (Bucharova et al. 2017).

### Trait variation in natural source populations

The ability for evolutionary reaction to environmental change depends on heritable trait variation. Thus, it is important to prove that plant populations used in the GCEF harbor variation in traits that are potentially under selection by climate change or land use. We found flowering phenology to be the most sensitive plant trait showing significant among-population differentiation in all investigated species, with  $P_{ST}$  ranging from 0.19 to 0.79. Moreover, for three species, *A. millefolium*, *B. erectus*, and *G. album*, flowering time was significantly more differentiated than expected from neutral processes alone ( $P_{ST} > F_{ST}$ ), indicating divergent selection as driver for the phenotypic differentiation. This corroborates previous findings indicating flowering phenology to be among the evolutionary most responsive plant traits, for example, Bucharova et al. (2017) found significant differentiation of flowering time among regional populations across Germany in six out of seven investigated grassland species ( $P_{ST}$  ranging from 0.10 to 0.45). Flowering time is known to respond to microclimate and land use. Brunet and Larson-Rabin (2012) showed that populations flower earlier with increasing temperature and increasing water availability. Reisch and Poschlod (2009) demonstrated that *Scabiosa columbaria* flowers earlier in mown than in grazed sites.

Also, traits related to growth and reproduction were differentiated among populations in most species investigated here. Among the traits related to plant architecture and leaf size, plant height and leaf width showed the strongest population effects in five out of six species. This is in line with the close relation of

these traits to water availability and temperature (Westoby and Wright 2006, DeWoody et al. 2015). Plants show smaller leaf dimensions with decreasing water availability (DeWoody et al. 2015) and larger leaf dimensions with increasing temperature (Baruah et al. 2017). Reisch and Poschlod (2011) demonstrated that plants from mown sites were larger than the ones on grazed sites. In our analysis, sexual reproduction, that is, number of inflorescences, showed strong population effects, significantly exceeding neutral genetic differentiation in three species (*A. millefolium*, *G. album*, and *L. vulgare*). In contrast, for total biomass, which is the most general global fitness correlate, trait divergence among populations never exceeded neutral expectations. Similarly, Bucharova et al. (2017) found that biomass was less differentiated than, for example, phenology-related traits hypothesizing that it is under selection in the same direction across populations (Kingsolver and Diamond 2011).

Nevertheless, results of  $P_{ST}$ - $F_{ST}$  comparisons should be treated with caution. First, we used a single heritability estimate for all species and traits, although it is known that heritability is known to vary across traits, species, and environments (Falconer 1989, Hoffmann and Merilä 1999, Geber and Griffen 2003). Actually, using a half-sib family design, we estimated heritabilities for multiple traits in two of the study species (*B. erectus* and *T. pratense*) ranging from 0.05 to 0.25 (mean 0.16) in *Bromus* and from 0.04 to 0.38 (mean 0.19) in *Trifolium*, which, moreover, were dependent on environmental conditions (*unpublished data*). Accordingly, by using a constant value for  $h^2$  of 0.3, our estimates of  $P_{ST}$  are conservative, rather underestimating adaptive differentiation in most of the investigated traits. Second, while minimizing the effects of environmental variation, the  $P_{ST}$ - $F_{ST}$  approach disregards the importance of other sources of phenotypic variation. Nongenetically inherited traits, that is, maternal effects via epigenetic inheritance, leading to transgenerational plasticity, are well known to influence plant phenotype over generations (Herman and Sultan 2011, Richards et al. 2017, Donelson et al. 2018, Gáspár et al. 2019). Thus, any contribution of genetically independent transgenerational plasticity to plant

phenotype in our common garden experiment would have lowered the contribution of adaptive genetic variation to trait variation.

#### *Genetic variation in experimental plots*

Using a mixture of seeds from multiple populations from the same geographic region proved to be a successful strategy to maximize genetic variation in the seed mixture. However, not all gene pools established equally well on the GCEF. While for most species all identified gene pools established according to expectations based on experimentally obtained germination rates and seed contribution of source populations, species-specific exceptions were observed, for example, in *B. erectus* one gene pool was not represented and in *G. album* only one out of four gene pools was successful. Similarly, observed genetic diversity for established GCEF populations was in the expected range for most species-treatment combinations, with only *B. erectus* and *L. vulgare* exhibiting some lower than expected values.

Apart from differences in seed contribution to the total seed mixture and differential germination percentage among accessions, for which we accounted for in our analyses, differential establishment can also be caused by maladaptation of certain genotypes or accessions (Lofflin and Kephart 2005), or by source-specific differences in plant-soil feedbacks (van Grunsven et al. 2010) leading to differences in germination rates in the field compared with controlled laboratory conditions (see Appendix S1: Table S1). Thus, selecting multiple source populations turns out to be a suitable approach to establish multiple genetically diverse plant populations on the GCEF, while selecting only one source population would have been risky.

Our sampling of the GCEF plots took place two vegetation periods after initial sowing. At that time, most individuals could be affiliated to a single gene pool and admixture was rare, indicating that sexual reproduction and establishment from seed have not yet led to a large number of F2 individuals.

For the experiment, it was not only important to verify the establishment of diverse gene pools, but also important to determine whether gene pools established homogeneously across experimental blocks and treatments. All species showed no or only low genetic differentiation

among blocks indicating homogenous establishment of gene pools across the experiment. Also, the treatments (mowing/grazing and ambient/future climate) did not affect gene pools differently. Hence, environmental conditions on the GCEF did not induce selective establishment or mortality after two years. It is very likely that differences among treatment conditions based on differences in seed production, germination, and establishment will only show up when sexual reproduction has taken place. Recent studies (e.g., Lima et al. 2017) modeled future climate scenarios predicting a significant loss of genetic diversity over longer time periods. Diversity loss is one of the major threats for species survival and finally may lead to local extinction (Lima et al. 2017). In conclusion, finding no or only low effects of climate change and land-use treatments on the genetic diversity even emphasizes the importance and justification of establishing the GCEF as long-term field experiment lasting for at least 15 yr. A distinct genetic response to anthropogenic environmental changes will be most probably measurable after a longer time period, by investigating subsequent plant generations.

In conclusion, genetic and phenotypic differentiation is widespread in grassland species on rather small geographic scales. Sowing a mixture of seeds from genetically and phenotypically differentiated source populations resulted in a large amount of molecular and phenotypic variation in the experimental plant populations in the GCEF. This variation represents the fundamental source for evolutionary responses to climate change and land-use scenarios that are expected for the GCEF.

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