Evidence for rapid evolution in a grassland biodiversity experiment

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Abstract

In long-term grassland biodiversity experiments positive effects of biodiversity on plant productivity commonly increase with time. It has been shown that such increased biodiversity effects persist even if plants are transferred into a common environment. Thus, we hypothesized that community diversity had acted as a selective agent, which resulted in the emergence of plant monoculture and mixture types. To test our hypothesis, we grew offspring from plants that were exposed for eleven years to a monoculture or mixture environment in a biodiversity experiment (Jena Experiment) under controlled greenhouse conditions in monocultures or two-species mixtures. We used epiGBS, a genotyping-by-sequencing approach combined with bisulfite conversion to provide integrative genetic and epigenetic data. We observed significant genetic and epigenetic divergence according to selection history in three out of five perennial grassland species, namely *Galium mollugo*, *Prunella vulgaris* and *Veronica chamaedrys*. For a fourth species, *Lathyrus pratensis*, we found mixed evidence and a fifth one, *Plantago lanceolata*, did not exhibit any divergence. In addition, current diversity levels in the greenhouse also had weak effects on epigenetic variation. Our results suggest that selection of genetic variants, and possibly epigenetic variants, caused the rapid emergence of monoculture and mixture types within plant species in the Jena Experiment. However, given the limited genome coverage, it remains unclear how much of the epigenetic divergence was independent of the genetic divergence.

Keywords

biodiversity, epiGBS, genetic divergence, epigenetic variation, herbaceous plant species, selection

1. Introduction

The Earth's biosphere is currently challenged by the impacts of anthropogenic environmental change and plant populations may encounter new abiotic or biotic environments due to climate-induced range shifts (Ouborg, Vergeer, & Mix 2006). The unprecedented rate of environmental change raises the question whether natural communities can adapt fast enough to novel abiotic or biotic conditions. Biodiversity has been shown to buffer ecosystems against negative influences of climatic extremes and novel environmental conditions (Isbell *et al.*, 2015) and in addition it has been shown that co-evolution among plants comprising a community can dampen the impact of an extreme climatic event (van Moorsel *et al.* 2018a).

Adaptive responses of plant populations to environmental factors (e.g., Joshi *et al.* 2001) and biotic interactions (Gervasi & Schiestl 2017) are well studied, but little effort has been devoted to studying the influence of community diversity on short-time plant evolution (but see Lipowsky, Schmid, & Roscher 2011, Kleynhans, Otto, Reich, & Vellend 2016, van Moorsel *et al.* 2018b). In particular, potential effects of multi-species interactions on the adaptive response of plant species are largely unknown, despite a growing body of evidence pointing towards the importance of species-interaction networks for the maintenance of ecosystem stability (Bastolla *et al.* 2009, Guimarães, Pires, Jordano, Bascompte, & Thompson 2017). It is conceivable that the feedback

between species interactions and their adaptive responses shapes community-level ecosystem functioning (van Moorsel *et al.* 2018b).

In the 1960s it was proposed that evolutionary processes occur at longer time scales than ecological processes (Slobodkin 1961), but now it is commonly believed that micro-evolutionary and ecological processes can occur at the same or at least similar temporal scales (reviewed by Hairston, Ellner, Geber, Yoshida, & Fox 2005, Schoener 2011, Hendry 2016). Thus, it is conceivable that micro-evolutionary processes may allow for an evolutionary rescue in a rapidly changing environment. However, evidence of genetic adaptation to mild stresses in natural situations over short time scales of a few generations is still scarce. Understanding how biodiversity, i.e. interaction between species, shapes this evolutionary response will be instrumental to anticipate how ecosystems may change in response to global change and biodiversity loss.

Long-term biodiversity field experiments offer unique opportunities to study effects of community diversity and composition on natural selection. In general, plant species richness has a positive impact on biomass production. Species mixtures are frequently more productive than average monocultures (Balvanera *et al.* 2006). Interestingly, these biodiversity effects often become more pronounced over time, which has been attributed to increased complementarity among species (Cardinale *et al.* 2007, Fargione *et al.* 2007, Reich *et al.* 2012, Meyer *et al.* 2016). Increased complementarity over time may originate from more equal resource depletion in mixtures or negative plant–soil feedbacks developing in monocultures (Fargione *et al.* 2007). Moreover, increased complementarity could also be due to phenotypic plasticity (Ghalambour, McKay, Carroll, & Reznick 2007) or selection of genotypes that have an advantage to grow in mixtures, i.e.,

"mixture-type plants". Indeed, recent common-environment experiments with plant material from a grassland biodiversity experiment (the Jena Experiment, Roscher *et al.* 2004) suggest that increased biodiversity effects have a heritable component (Zuppinger-Dingley *et al.* 2014, van Moorsel, Schmid, Hahl, Zuppinger-Dingley, & Schmid 2018c). Plants originating from mixed communities showed stronger complementarity effects than plants originating from monoculture communities if they were grown in two-species mixtures in the glasshouse. Hence, community composition can cause trans-generational effects (Zuppinger-Dingley *et al.* 2014, Rottstock, Kummer, Fischer, & Joshi 2017, van Moorsel *et al.* 2018b). However, it remains unclear whether these trans-generational effects observed in these studies were due to genetic differentiation, epigenetic differences or maternal effects (Tilman & Snell-Rood 2014).

Epigenetics has received increased attention over the past years and its contribution to ecological processes has repeatedly been proposed (e.g., Bird 2007, Bossdorf, Richards, & Pigliucci 2008). Recent work on epigenetic recombinant inbred lines (epiRILs) of *Arabidopsis thaliana* indeed suggests a considerable contribution of induced epialleles to phenotypic variation, which is independent of genetic variation (Latzel *et al.* 2013, Cortijo *et al.* 2014, Kooke *et al.* 2015). However, the importance of epigenetics in natural populations and whether it contributes to adaptation remains elusive (Quadrana & Colot, 2016, Richards *et al.* 2017, Groot *et al.* 2018). Nonetheless, a recent selection experiment with *A. thaliana* suggests that epigenetic variation may indeed contribute to rapid heritable changes and adaptation (Schmid *et al.* 2018a).

Here, we tested whether community diversity can act as a selective environment resulting in genetic or epigenetic divergence between offspring from plants grown in monocultures

or mixtures. In particular, we tested whether the phenotypic differences between mixture- and monoculture-type plants described in van Moorsel *et al.* (2018b) were paralleled by genetic or epigenetic differentiation by using these data and overlaying them with genetic and epigenetic variation data. With genetic and epigenetic variation, we here refer to differences in DNA sequence (single nucleotide polymorphisms, SNPs) and differences in DNA cytosine methylation, respectively. We used five different plant species to test our hypothesis.

2. Material and Methods

2.1. Plant selection histories

To test whether plant populations grown for eleven years in either monocultures or mixtures differed genetically or epigenetically from each other, we collected seeds from plant populations grown in monoculture and mixture plots in a large biodiversity field experiment (the Jena Experiment, Jena, Thuringia, Germany, 51 °N, 11 °E, 135 m a.s.l., see Roscher *et al*. 2004 and Weisser *et al*. 2017 for experimental details). In the original design, 16 plant species were present in large 20 x 20 m monoculture and mixture plots. Of these 16 species four grew very poorly and for several of the remaining 12 we had limited seed collections (van Moorsel et al. 2018c). Hence we were restricted to the following five species (van Moorsel et al. 2018c): the three small herbs *Plantago lanceolata* L., *Prunella vulgaris* L. and *Veronica chamaedrys* L., the tall herb *Galium mollugo* L. and the legume *Lathyrus pratensis* L.

To gauge the differences between plants grown in the Jena Experiment and plants that experienced a different selection environment, we obtained seeds from the original seed

supplier of the Jena Experiment (Rieger Hofmann GmbH, in Blaufelden-Raboldshausen, Germany) as outgroups. To test how similar these outgroup seeds were to the original seed pool that was used to set up the Jena Experiment in 2002, we also used seed material from the original seed pool. However, this was only possible for one species, *V. chamaedrys*. According to the seed supplier, all seeds were harvested from plants that were originally collected at different field sites in Germany and then propagated for at least five years in monocultures with reseeding them every year (van Moorsel *et al.* 2018a).

In summary, there were at maximum four different selection histories (see also Fig. 1): 1) monoculture in Jena, 2) mixture in Jena, 3) monoculture in the fields of the seed supplier until 2014 and 4) monoculture in the fields of the seed supplier until 2002 (only for *V. chamaedrys*). Histories 3) and 4) will be abbreviated as supp2014 and supp2002 (Tab. S1).

2.2. Seed collection in monoculture and mixture histories

Given that all plant species used in the study are perennial plants, it is possible that they reproduced mostly vegetatively in the field. To increase the genetic diversity, which can then be subject to selection, and to reduce maternal effects and carry overs if cuttings would have been used instead of seeds, plants with a selection history in either mixture or monoculture in the Jena Experiment underwent two controlled reproductive cycles in 2010 and 2014 as described in the following.

In spring 2010, all plant communities were collected at the same time as cuttings and transplanted in identical plant composition to an experimental garden in Zurich,

Switzerland, for the first controlled sexual reproduction (see also Zuppinger-Dingley *et al.* 2014). In spring 2011, the seedlings produced from the seeds of the first controlled sexual reproduction were transplanted back to the same plots of the Jena Experiment from where the parents had originally been collected and in the same community composition (see Tab. S2 for the community compositions of the plots in the Jena Experiment). In March 2014, plant communities of the plots re-established in 2011 in the Jena Experiment were again transferred to the experimental garden in Zurich for the second controlled sexual reproduction. For each experimental plot, we excavated several blocks of soil including the entire vegetation (in total one square meter). These blocks were then used to establish the plots in the experimental garden. We added a 30 cm layer of soil (1:1 mixture of garden compost and field soil, pH 7.4, commercial name Gartenhumus, RICOTER Erdaufbereitung AG, Aarberg, Switzerland) to each plot to make sure the plants established. Seeds from the second controlled sexual reproduction event were collected throughout the growing season of 2014.

To minimize cross-pollination during the controlled sexual reproduction events, plots were surrounded by nets and only left open on top to allow pollinator access. However, it should be noted that this does not fully exclude the possibility of cross-pollination. Cross-pollination might also have occurred in the field during sexual reproduction events. It is important to note that such cross-pollination would have resulted in the populations becoming more similar to each other and hence, would have reduced the possibility to find genetic or epigenetic divergence.

The experimental set up and design is schematically shown in Fig. 1.

2.3. Glasshouse experiment

The glasshouse experiment included three of the four selection histories described above (monoculture, mixture and supp2014; for the fourth history, supp2002, see further below) and an assembly treatment which corresponds to plants being planted in the glasshouse either in monocultures or mixtures as the common test environments. Hence, the full experimental design includes five plant species, three selection histories and two assembly treatments.

2.3.1. Setup of the glasshouse experiment

To set up the experiment, seeds from monocultures, mixtures and the seed supplier (supp2014) were germinated in December 2014 with those from the same species being planted within the same day. Germination was done in germination soil ("Anzuchterde", Ökohum, Herbertingen, Germany) under constant conditions in the glasshouse without additional light. Seedlings were planted in monocultures of four individuals and mixtures of two plus two individuals into 2-L pots filled with agricultural soil (50 % agricultural sugar beet soil, 25 % perlite, 25 % sand; Ricoter AG, Aarberg, Switzerland). Species pairs in the mixtures were chosen according to seedling availability and single pots always contained four plants of the same selection history (i.e., there was no competition between different selection histories).

The experiment was split into six blocks (replicates), each including the full experimental design. Within each block, pots were placed on three different tables in the glasshouse in a randomized fashion without reference to selection history or assembly treatment. During the experiment the pots were not moved. The plants were initially kept at day

temperatures of 17–20 °C and night temperatures of 13–17°C without supplemental light. To compensate for overheating in summer, an adiabatic cooling system (Airwatech; Bern, Switzerland) was used to keep inside temperatures constant with outside air temperatures.

2.3.2. Phenotype measurements

We measured the following traits commonly measured in biodiversity experiments and shown to relate to competitive growth and affect plant community productivity (Roscher *et al.* 2015, Cadotte 2017): plant height, leaf thickness, specific leaf area (SLA) and aboveground biomass. All traits were measured after twelve weeks of the experiment from 18 May to 4 June 2015. Leaf thickness was measured for three representative leaves using a thickness gauge. Specific leaf area (SLA) of up to 20 representative leaves (depending on the leaf size of the species) of each species in a pot was measured by scanning fresh leaves with a Li-3100 Area Meter (Li-cor Inc., Lincoln, Nebraska, USA) immediately after harvest and determining the mass of the same leaves after drying. All four individuals in a pot were sampled. Research assistants, who were not informed about the specific experimental treatments, assisted in the regular measurements and harvesting of plants at the end of the experiment.

2.3.3. Sampling of plant material

Samples for subsequent epigenetic and genetic analysis were harvested between 18 and 28 May 2015, after twelve weeks of plant growth in the glasshouse. We chose to sequence all individuals from the first three experimental blocks (for more details, see van Moorsel *et al.* 2018b). All four plants were sampled in each pot. One young leaf per plant was cut from the living plant and immediately shock-frozen in liquid nitrogen. The

samples were then stored at –80 °C in Zurich before shipment to the Netherlands for further processing.

2.4. Offspring of the original seed pool

For the species *V. chamaedrys*, seeds from offspring of the original seed pool used to set up the Jena Experiment (supp2002) were stored since 2002 at –20 °C and germinated in the glasshouse as described above. Seedlings were then transferred to an experimental garden and seeds were collected one year later. The additional generation in the experimental garden was used to overcome potential maternal effects due to the old age of the stored seeds. The collected seeds were then stored at 5 °C, transported to Nijmegen and germinated in the glasshouse of Radboud University Nijmegen. Individual plants were grown in individual 2 x 2-cm squares in a potting tray filled with a potting soil consisting of "Lentse potgrond" (www.lentsepotgrond.nl) in a greenhouse under natural light conditions (March). No cold treatment or vernalization was applied for germination. Individual plants were harvested and quick frozen in liquid nitrogen after 5 weeks of growth.

2.5. Measuring genetic and epigenetic variation with epiGBS

We measured both genetic and epigenetic variation using a recently developed reference-free bisulfite method (Gurp *et al.* 2016). We used an improved version of the protocol based on the original one. A detailed description is given in the supplementary methods. In brief, we used another combination of methylation-insensitive restriction enzymes to avoid the bias previously reported in Gurp *et al.* 2016, a "wobble" adapter facilitating the computational removal of PCR duplicates and a conversion-control nucleotide that

allowed for a more efficient identification of the Watson/Crick strand. The epiGBS libraries were sequenced on 4 Illumina HiSeq 2500 lanes at the facilities of Wageningen University & Research Plant Research International. Samples from different selection histories and species were distributed among lanes to prevent lane effects. An exception were the supp2002 samples from *V. chamaedrys* which were sequenced at a later time point.

2.6. Data processing

De-multiplexing, *de novo* reference construction, trimming, alignment, strand-specific variant calling and methylation calling were done for each species as described in Gurp *et al.* (2016) with the pipeline provided by the authors available on https://github.com/thomasvangurp/epiGBS. It is important to note that the reference sequences are only up to 250 bp long, which restricted the analysis of linkage disequilibrium in the studies species because these had no reference genomes available. *De novo* reference sequences were annotated with DIAMOND (protein coding genes; NCBI non-redundant proteins as reference; version 0.8.22; (Buchfink, Xie, & Huson 2015)) and RepeatMasker (transposons and repeats; Embryophyta as reference species collection; version 4.0.6; (Smit, Hubley, & Green, 2013–2015)). We summarized the transposable element and repeat classes into “transposons” comprising DNA, LTR, LINE, SINE and RC transposon, and “repeats” including satellite, telomeric satellite, simple, rRNA, snRNA, unknown and unclassified repeats. The annotation was then used to classify the genetic and epigenetic variants into the different feature contexts (e.g., to identify whether a single nucleotide polymorphism was located in a gene or a transposon). A summary of the reference sequences is given in Tab. S3. The total

reference sequence length in Tab. S3 ranges from 3 to 11% of the entire genome for the five test species.

2.7. Genetic variation

2.7.1. Visualization of genetic distances with single nucleotide polymorphisms (SNPs)

Individuals with a SNP calling rate below 90 % were *a priori* removed from the analysis of genetic variation. These were three, eleven, five, nine, and five individuals of *G. mollugo*, *P. lanceolata*, *L. pratensis*, *P. vulgaris* and *V. chamaedrys*, respectively (Tab. S1). These samples were well distributed across the experimental treatment combinations, i.e., one or two for a single experimental group, except for the seed-supplier history by monoculture assembly combination of *P. lanceolata* for which four individuals were removed. For each species, we filtered the genetic-variation data for single nucleotide polymorphisms (SNPs) sequenced in all individuals with a total coverage between 5 and 200. SNPs homozygous for either the reference or the alternative allele in more than 95 % of all individuals were removed as uninformative SNPs. To reduce the impact of false positive SNP calls, we removed all SNPs located in contigs with more than 1 SNP per 50 base pairs (2 %) because higher rates appear unlikely (for example: in *Arabidopsis thaliana*, Col-0 compared to other ecotypes has a median SNP rate of 0.36 % (Lutz & Schwechheimer, 2017) and human compared to chimpanzee has an average SNP rate of 1.23 % (The Chimpanzee Sequencing and Analysis Consortium 2005)). In addition, higher rates may also originate from spurious alignments to the wrong reference contig (considering that the reference contigs represent only a minor

fraction of the entire genome, there may be many reads originating from other locations not represented with a reference contig, which are still similar enough to (wrongly) align to the reference). The removal of contigs with many SNPs also avoids that few contigs with high SNP rates drive the tests for genetic differentiation. SNP allele frequencies were scaled with the function “scaleGen” from adegenet (version 2.0.1; Jombart (2008)) and genetic distances between the individuals were visualized with t-SNE (Maaten & Hinton 2008, Maaten 2014). We calculated 100 maps starting from different random seeds and selected the map with the lowest final error. Individual maps were calculated in R with the package Rtsne (version 0.13; Maaten & Hinton 2008, Maaten 2014). Parameters for the function Rtsne were pca = FALSE, theta = 0, perplexity = 10.

2.7.2. Test for genetic differentiation between populations with single nucleotide polymorphisms (SNPs)

SNP data were processed and filtered as described above. The study design included the factors “current assembly” and “selection history” with two and three levels, respectively. However, this design was incomplete in all species except *P. vulgaris* (see Fig. 1D). In addition, *V. chamaedrys* had a fourth level of selection history, the supp2002 plants, which were grown separately from all others. Given these imbalances and the most interesting comparison being between monoculture and mixture selection histories, we did not use a full factorial model (selection history crossed with assembly and species) to test for genetic differentiation. Instead, we tested for each species each factor within all levels of the other factor for genetic differentiation. Taking *P. vulgaris* as an example, we tested for genetic differentiation between selection histories within monoculture and mixture assemblies (between all three histories and between monoculture and mixture

types), and between assemblies within the supp2014, monoculture- and mixture-type selection histories. For each test, we extracted the corresponding individuals and tested for genetic differentiation with the G-statistic test (Goudet, Raymont, Meeûs, & Rousset 1996, function gstat.randtest implemented in the package hierfstat, version 0.04-22, Goudet & Jombart 2015). P-values were corrected for multiple testing to reflect false discovery rates (FDR) and the significance threshold was set to an FDR of 0.01. This analysis was carried out with (1) all SNPs, (2) SNPs located within genes, and (3) SNPs located within transposons. We chose to separately test SNPs in genes and transposons because we expected that selection more likely acted on genes and that selection of transposons would primarily occur due to genetic linkage to an advantageous gene. In addition, we expected that SNP calls are more reliable within genes because many transposon families tend to be highly repetitive. To estimate the extent to which the genetic variation was caused by the differentiation between populations we calculated average (i.e., across all tested SNPs) pairwise F_{ST} values with the function pairwise.fst from the package adegenet (version 2.0.1, Jombart 2008, Tab. S4). Because many SNPs had F_{ST} values close to zero, we assumed that only few SNPs with F_{ST} values clearly larger than zero were under selection. To estimate the maximal divergence between the populations, we therefore also calculated the F_{ST} of each individual SNP and extracted the 99th percentiles (we chose the 99th percentile because this is more robust to outliers than the highest value, Tab. 1, S5 and S6).

2.8. Epigenetic variation

2.8.1. Characterization of genome-wide DNA methylation levels

For each species, we filtered the epigenetic variation data for cytosines sequenced in at least three individuals per population (i.e., experimental treatment combination) with a total coverage between 5 and 200. Note that this filter is different from the one applied for the SNP data because the down-stream analyses have different requirements regarding missing data (more flexible for the DNA methylation data). To provide an overview of the genome-wide DNA methylation levels of the five species or each experimental treatment combination per species, we visualized the DNA methylation levels of all cytosines averaged across all individuals with violin plots. We also visualized the average DNA methylation level within genes, transposons, repeats and unclassified reference contigs with heatmaps. Both was done either using all sequence contexts (CG, CHG, CHH) at once or separately for each sequence context.

2.8.2. Identification of differentially methylated cytosines (DMCs)

DNA methylation data were processed and filtered as described above. Variation in DNA methylation at each individual cytosine was then analyzed with a linear model in R with the package DSS (version 2.24.0; Y. Park & Wu (2016)), according to a design with a single factor comprising all different experimental treatment combinations as separate levels and using contrasts to compare levels of interest (similar to the approach described for RNA-Seq in Schmid 2017 and the testing procedure described in Schmid, Giraldo-Fonseca, Smetanin & Grossniklaus 2018b). Specific groups were compared with linear contrasts and *P*-values for each contrast were adjusted for multiple testing to reflect false

discovery rates (FDR, Benjamini & Hochberg 1995). Taking *P. vulgaris* as an example, we compared the three selection histories across both assemblies and within each assembly to each other. Likewise, we compared the two assemblies across all selection histories and within each selection history to each other. A cytosine was defined as differentially methylated (DMC) if the FDR was below 0.01 for any of the contrasts.

2.9. Correlation between genetic and epigenetic data

2.9.1. Overall correlation

To assess the correlation between genetic and epigenetic data, we calculated between-individual distances for both data sets and tested for correlation between the distances with Mantel tests. Genetic distances between two individuals were calculated as the average distance of all per-SNP differences. Per SNP, the distance was set to 0 if all alleles were identical, 1 if all alleles were different and 0.5 if one allele was different. Epigenetic distances between two samples were calculated as the average difference in DNA methylation across all cytosines. The tests were conducted in R with the package vegan (version 2.4-4, function mantel() with 9999 permutations; Oksanen *et al.* 2017). *P*-values were corrected per species for multiple testing to reflect false discovery rates (FDR).

2.9.2. Linkage of genetic and epigenetic variation in close-*cis*

To estimate how much of the epigenetic variation was potentially unlinked to the genetic variation we modeled the average DNA methylation level of a given reference sequence in response to the sequence context (CTXT), the assembly treatment (AS), the genotype of the reference sequence (SNP), the interaction between the sequence context and the

genotype (CTXT:SNP) and the selection history (SH) fitted in this order (percent methylation ~ CTXT + AS + SNP + CTXT:SNP + SH + CTXT:SH). We then compared this result to an alternative model in which SH and SNP were switched (percent methylation ~ CTXT + AS + SH + CTXT: SH + SNP + CTXT:SNP). Hence, whereas the second model tests for epigenetic differentiation between selection histories irrespective of the underlying genetics, the first model tests whether there was epigenetic differentiation between selection histories that could not be explained by the underlying genetics. We only used reference sequences which passed the coverage filters described above. We further only included the monoculture and mixture histories from the Jena field because only these two were fully factorially crossed with assembly in all species. Models were calculated with the functions lm() and anova() in R (version 3.5.1). Results from all reference sequences were collected and *P*-values for each term were adjusted for multiple testing to reflect false discovery rates (FDR, Benjamini & Hochberg 1995). It is important to note that results from this model with an average level of DNA methylation across several cytosines cannot be directly compared to the results from the model used to test for differential DNA methylation at individual cytosines, because of different distribution and testing procedure. Also, whereas this model can detect dependency of epigenetic variation on genetic variation in close-*cis* (close to each other at the same location in the genome), it cannot detect far-*cis* (for example a transposon insertion variant 200 bp away from the place of origin of the reference sequence) or *trans* (effects from other loci that are not linked to the place of origin of the reference sequence) dependencies. Thus, this model potentially overestimates the proportion of epigenetic variation unlinked to genetic variation.

2.10. Relation between genotype/epigenotype and phenotype

2.10.1. Overall correlation

To assess whether variation in phenotypic traits could be related to variation in genetic and epigenetic data we used a multivariate ANOVA with genetic or epigenetic distances between individuals (DIST) as a dependent variable and phenotypic traits as explanatory variables with 9999 permutations (package vegan, version 2.4-4, function adonis(); Oksanen *et al.* 2017). The formula was DIST ~ biomass + thickness + height + sla. An in-depth analysis of the phenotypes in response to the experimental design has already been presented in van Moorsel *et al.* 2018c.

2.10.2. Association of genotypes/epigenotypes with phenotypes

To test whether individual reference sequences correlated with phenotypic variation, we separately modeled the variation in the four phenotypic traits (biomass, height, thickness and SLA) in response to the genotype (SNP) and the percent DNA methylation (METH) for a given sequence context with the same data previously used to test linkage of genetic and epigenetic variation (see section 2.9.2. above). Models were calculated with the function lm() and anova() in R (version 3.5.1). We tested both fitting orders with either SNP or METH fitted first. Hence, the formulas were TRAIT ~ SNP + METH and TRAIT ~ METH + SNP. Results from all reference sequences were collected and *P*-values for each term were adjusted for multiple testing to reflect false discovery rates (FDR, Benjamini & Hochberg 1995).

3. Results

3.1. Genetic variation

Visualization of genetic distances between the plant individuals clearly separated the individuals according to their selection history in three out of five species, namely *G. mollugo*, *P. vulgaris* and *V. chamaedrys* (Fig. 2). Populations did not separate according to the assembly treatment, which was expected because plants were assigned randomly to the assembly treatment. As expected, offspring of plants from the original seed pool (supp2002) of *V. chamaedrys* showed greater variability than plants of the same species derived from the original seed pool but with 11 years of monoculture or mixture history in the Jena Experiment. In addition, the supp2002 individuals were interspersed between these two histories, indicating that individuals with a selection history in the field had undergone differential evolution away from the original seed pool. The supp2014 plants differed from the other two selection histories in *V. chamaedrys* as well as in *G. mollugo* and *P. vulgaris*, confirming their status as "outgroups" at least in these three species. To see whether the separation observed in the visualization were significant, we tested for genetic divergence between the selection histories and the assemblies with the G-statistics test (Fig. 3, S1 and S2, Goudet *et al.* (1996)). We first focus on the results without the supp2002 plants.

Genetic differentiation was consistently significant (FDR < 0.01) in three of the five plant species (Fig. 3, top and middle rows). The selection histories of *P. lanceolata* did not exhibit any significant genetic differentiation. Also, the test including only the monoculture and mixture types within the mixture assemblies was not significant for *L. pratensis*. However, in *L. pratensis* statistical power was limited because there were only

nine individuals available (Tab. S2, almost all other experimental groups from monoculture and mixture selection history had at least 10 individuals each). In contrast, the tests comparing the monoculture and mixture assemblies within each of the selection histories were never significant at the critical level of FDR = 0.01 (Fig. 3, bottom rows).

To estimate the amount of genetic variation explained by the selection histories, we calculated average pairwise F_{ST} values (Tab. S4) and the 99th percentiles of the SNP-wise F_{ST} values (Tab. 1, S5 and S6). Average pairwise F_{ST} values for the different selection histories were between 0.017 (supp2014 *vs.* monoculture type within the monoculture assemblies of *L. pratensis*) and 0.111 (supp2014 *vs.* mixture type within monoculture assemblies of *P. vulgaris*). With the exception of *P. lanceolata*, the 99th percentiles were markedly higher and between 0.084 (monoculture *vs.* mixture types within monoculture assemblies of *L. pratensis*) and 0.398 (all selection histories within mixture assemblies of *P. vulgaris*). Thus, overall, 1.7% to 11% of the genetic variation were explained by selection histories. However, for individual SNPs, selection histories could explain up to 40% of the genetic variation.

Within *V. chamaedrys*, comparisons between supp2002 plants and the other populations were all significant (FDR < 0.01 in all comparisons). The average pairwise F_{ST} values between the supp2002 plants and the other populations (Tab. S4) were between 0.010 and 0.015. In comparison, pairwise F_{ST} values between any of the supp2014-, monoculture-, or mixture-history populations were between 0.027 and 0.038 for this species. Likewise, the 99th percentiles of the SNP-wise F_{ST} values were consistently lower in the comparisons between the supp2002 plants and the other populations than among those (i.e., supp2014, monoculture and mixture histories populations, Tab. 1, S5 and S6). This

confirmed the previous observation that supp2002 individuals, which could be considered as “parental” to the others, were genetically intermediate between the other selection histories (Fig. 2).

3.2. Epigenetic variation

To get an overview of the DNA methylation data, we visualized overall DNA methylation levels for each plant species, sequence context (CG, CHG, CHH) and genomic feature context (genes, transposons, repeats and unclassified contigs, Fig. 4). For all species, DNA methylation was generally highest in the CG context (82.6%), lower in the CHG context (59.2%), and lowest in CHH context (12.2%). Differences between species were most pronounced in the CHG context in which *L. pratensis* (71.6%) and *P. lanceolata* (75.3%) exhibited markedly higher methylation levels than the other three species (54.6%, 44.4%, and 52.5% in *G. mollugo*, *P. vulgaris* and *V. chamaedrys*, respectively). Within each species and context, DNA methylation was highest in transposons and lowest in genes (Fig. 4B). Overall, these patterns are within the range of what has been reported previously for other angiosperms (Niederhuth *et al.* 2016).

For an initial comparison between the experimental treatment combinations, we visualized the overall DNA methylation levels as we did for the different species, but for each experimental treatment combination separately (Fig. S3). Given that the overall methylation levels appeared to be similar between the experimental treatment combinations within species, we tested for significant differences in DNA methylation levels at each individual cytosine (Tab. 2 for all contexts and Tab. S7, S8, and S9 for each context separately). We first focused on the results excluding the supp2002 plants from *V. chamaedrys*. On average, 1.6% of all tested cytosines were significant in at least one of

the tested contrasts (FDR < 0.01, “DMCs” for differentially methylated cytosine). Relative to the total number of cytosines tested, differences between selection histories (tested within or across both assemblies) were between 0.18% and 1.02% on average across all species and between 0.07% and 1.02% per individual species. Differences between the two assemblies (tested within or across all selection histories) were between 0.05% and 0.21% on average across all species and between 0.05% and 0.40% per individual species. Thus, the fraction of differentially methylated cytosines between the selection histories was generally larger than differences between the two assemblies.

Within the selection histories, differences between the monoculture types and the supp2014 plants were between 0.16% and 1.01% within species. Differences between mixture types and supp2014 plants were between 0.21% and 1.02% within species. Differences between monoculture and mixture types were between 0.06% and 0.80% within species. However, if compared within each species separately, there were always more DMCs in the comparisons between plants from Jena and the supp2014 plants than in the comparison between monoculture and mixture types. It is possible that this was at least partly due to the underlying genetic differences, given that the genetic distances between supp2014 and the other two selection histories were generally larger than the distances between the monoculture and mixture history (Tab. S4).

To further characterize the differences in DNA methylation, we calculated the average change in DNA methylation at the DMCs for each contrast, across and within all sequence contexts (CG, CHG and CHH) and feature types (genes, transposons, repeats and unclassified) and visualized these differences (Fig. 5). We could not identify clear patterns between the different comparisons with one exception: differences in the

comparisons between plants from Jena and the supp2014 plants within genes (all sequence contexts) were mostly biased towards a higher methylation in the supp2014 plants. Thus, plants in the Jena Experiment showed an overall loss of DNA methylation at DMCs within genes. However, it remains unclear what functional consequences this might have had because the function of gene body methylation remains to be elucidated (Zilberman 2017).

For *V. chamaedrys*, we also compared the supp2002 to the other experimental treatment combinations (Tab. S10). Relative to the total number of cytosines tested, differences between supp2002 plants and the other populations were between 0.82% (supp2002 *vs.* mixture history in mixture assembly) and 4.17% (supp2002 *vs.* monoculture history in both assemblies). In total, 7.4% of all cytosines tested were significant in at least one of the comparisons. Thus, even though genetically intermediate, supp2002 differed epigenetically more from the other populations than these did between each other. However, considering that these plants grew in a markedly different environment (and their ancestors had been stored as seeds for 12 years), it was not possible to separate effects of underlying genetic differences from effects of the environment. Nonetheless, it suggests that there was considerable epigenetic variation within *V. chamaedrys*, even though all plants were likely at least distantly related.

3.3. Correlation between genetic and epigenetic variation

To assess the correlation between genetic and epigenetic variation, we tested whether there was a significant correlation between the genetic and epigenetic distance matrices (Tab. 3). This correlation was significant (FDR < 0.05) in all species except for *G.*

mollugo. Correlation to the genetic variation in these four species was highest for the CG-methylation (0.30 on average), intermediate for CHG-methylation (0.25 on average) and lowest for CHH-methylation (0.20 on average).

To better estimate how much epigenetic variation was unlinked to genetic variation in close-*cis* (i.e., on the same reference sequence), we calculated the percentage of reference sequences that exhibited a significant effect of the selection history on the DNA methylation level even if an explanatory term for genotype (SNP, see section 2.9.2) was fitted first. We compared this to a model with the opposite fitting order (Tab. 4). If selection history was fitted first, its model terms (SH & CTXT:SH) were significant in 2.01 % of all reference sequences (average across species). However, if fitted after SNP, the effect of selection history was only significant in 0.85 % of all cases. This varied between species. For example, almost no significant effects of selection history were found in *L. pratensis* (2 out of 5,554) and *P. lanceolata* (1 out of 314) whereas up to 2.01 % of the reference sequences of *V. chamaedrys* exhibited a significant effect of selection history on DNA methylation after fitting the explanatory term for genotype first. Hence, overall and at most individual reference sequences, epigenetic variation was likely linked to genetic variation. Nonetheless, in up to 2.01 % of the reference sequences of individual species, genetic variation on the same reference sequence could not explain epigenetic variation.

3.4. Relation between genetic/epigenetic variation and phenotype

To assess the relation between genetic or epigenetic variation and phenotypic variation, we tested whether phenotypic traits could explain the genetic and epigenetic distances

between individuals (Tab. 5). Except for *G. mollugo*, all species had at least one phenotypic trait that could significantly ($P < 0.05$) explain genetic and sometimes also epigenetic differences. For example, thickness was significant in *L. pratensis* and specific leaf area (SLA) was significant in *P. vulgaris* and *V. chamaedrys*. However, the coefficients of determination (R^2) were with 0.02 to 0.06 relatively low, indicating that only a small fraction of the genome was correlated to the measured phenotypic traits. This was not surprising considering that we only measured few traits and that these may not have been so highly polygenic to be covered by the < 2% of the genome assessed with our reduced representation sequencing approach.

To identify reference sequences that were linked to the phenotypic differences, we tested for significant associations of their genotype and epigenotype with the phenotypic traits (Tab. 6). We first focused on the model in which the genotype was fitted first. All species had a trait that was at least once significantly related to genetic variation assessed with the epiGBS method (FDR < 0.05). For example, 18 and 49 reference sequences were associated with biomass in *P. vulgaris* and *V. chamaedrys*, respectively. Interestingly, *G. mollugo*, which had no significant correlations in the previous test (see Tab. 5), had a considerable amount of sequences associated with biomass or leaf thickness (429 and 320 out of 12,279, respectively). To ensure that the genetic differences in the reference sequences of *G. mollugo* were indeed also associated with the selection history, we visualized the genetic distances between the individuals (Fig. 6). The clear separation of the individuals by the factor selection history confirmed that these reference sequences were associated with the phenotype as well as the selection history.

Epigenetic variation was rarely significantly associated with phenotypic traits if fitted after genetic variation (Tab. 6). However, if the epigenotype was fitted first, the number of reference contigs with a significant association between the epigenotype and phenotypic traits was almost identical to the number of significant associations found previously between the genotype and the phenotypic traits if the genotype was fitted first. Likewise, genetic variation was rarely significantly associated with phenotypic traits if fitted after epigenetic variation (Tab. 6). Hence, in line with the previous results, genetic and epigenetic variation that were significantly associated to phenotypic traits were both also well correlated to each other.

4. Discussion

We aimed to estimate the extent of genetic and epigenetic divergence between plants selected for eleven years in either monocultures or mixtures in the Jena Experiment, a long-term grassland biodiversity field experiment (Weisser *et al.* 2017). The plants we used had previously been shown to exhibit heritable phenotypic and performance differences between monoculture and mixture types when grown in monoculture or two-species mixture in the glasshouse (Hahl 2017, van Moorsel *et al.* 2018c). In the present study we could show that in three out of five test species, namely *G. mollugo, P. vulgaris* and *V. chamaedrys*, there were indeed genetic differences between the two plant types in a large number of SNPs. In a fourth species, *L. pratensis*, we found evidence for genetic divergence among plants grown in monoculture assemblies in the glasshouse, but the comparison was insignificant for plants grown in mixture assemblies, for which we could only test nine individuals in total. In the fifth species, *P. lanceolata*, we could not identify

significant genetic differentiation between plants with different selection histories. This was unexpected because this species has recently been shown to exhibit clear genetic divergence after 15 years of simulated climate change (Ravenscroft, Whitlock & Fridley, 2015). It is conceivable that we could not detect genetic divergence in this species because of the low number of reference sequences that passed our filter: there were only 50 sequences corresponding to 6 kb and 61 SNPs left. Thus, we might have missed regions under selection.

Overall, only 1.7% to 11% of genetic variation was explained by selection histories. However, for individual SNPs, selection histories could explain up to 40% of the genetic variation. This may indicate that these loci were under selection (high divergence) whereas other parts of the genome segregated randomly (low divergence).

Besides the genetic divergence, we could also identify epigenetic differences between the different selection histories, which were generally below 1% of all tested cytosines. For *V. chamaedrys*, we observed pronounced epigenetic differences between offspring of the original seed pool of the Jena Experiment (supp2002) and the three other selection histories. Given that these plants grew in a different environment and that their ancestors had been stored as seeds for 12 years, we could not be sure if the epigenetic differences were due to underlying genetic or environmental differences. Nonetheless, with 7.4% of all tested cytosines being significantly differently methylated between supp2002 and the other populations (supp2014, monoculture and mixture history), there was a substantial amount of epigenetic variation within *V. chamaedrys*. Given that the supp2002 were genetically overlapping with the other groups (see Fig. 2), there was probably a

considerable amount of environmentally induced epigenetic variation that was independent of genetic divergence between groups.

Overall, we observed that epigenetic variation was significantly correlated with genetic variation in four out of five species. When we tested each reference sequence for epigenetic variation that could not be explained by genetic variation in close-*cis*, we found that up to 2.01 % of all sequences exhibited epigenetic variation that was unlinked to such genetic variation. Although this does provide evidence for epigenetic divergence between selection histories that is independent and additional to genetic divergence, it is important to note that our analysis could not account for potential correlations between epigenetic variation and genetic variation in far-*cis* or *trans*.

We further tested to which extent the genetic and epigenetic variation was related to variation in phenotypic traits. For the genetic variation, this was significant for at least one phenotypic trait in four out of five species. Epigenetic variation could also significantly explain differences in phenotypic traits in three out of four species. However, in all cases, these traits were also significantly explained by genetic variation. When we tested for association of genetic and epigenetic variation with phenotypic traits in individual reference sequences, we could identify multiple significant associations in four out of five species. Interestingly, the highest number of associations was found in *G. mollugo*, for which the overall tests did not detect significant correlations between genetic, epigenetic or phenotypic variation. The number of significant associations between epigenetic variation and phenotypic traits were always much smaller than for genotypic variation. However, given that epigenetic variation was fitted after genetic

variation, these associations suggest that some phenotypic differences might be due to epigenetic variation that is unlinked with genetic variation.

There has been one previous selection experiment with plants that found evidence for epigenetic differentiation within genotypes after few generations in *Arabidopsis thaliana* (Schmid *et al.* 2018a), but clear evidence from non-model plant species is still lacking. Our present results suggest that epigenetic differences mostly reflect genetic differences and that the heritable phenotypic differences rather have a genetic than an epigenetic basis. A caveat of the novel reference-free reduced representation bisulfite sequencing method (van Gurp *et al.* 2016) is the low genome coverage (about 2 %). Thus, even if we would have found more epigenetic than genetic divergence, we could not have been completely certain that this epigenetic divergence was unrelated to genetic divergence. For example, genome-wide studies with single base-pair resolution in *A. thaliana*, which revealed extensive epigenetic variation between different populations and accession, found that most of this variation was both linked to underlying genetic differences in *cis* as well as *trans*-acting loci (Dubin *et al*. 2015, Kawakatsu *et al*. 2016). Such *trans*-acting loci make it difficult to separate genetics from epigenetics in non-model species because few such loci can alter large parts of the epigenome despite being only a tiny fraction of the entire genome. Hence, even though reduced representation sequencing approaches like the epiGBS method used in this study allow for estimates of genetic and epigenetic divergence, they cannot clearly reveal whether the observed epigenetic variation has a genetic basis or not. This may only be possible with high quality reference genomes and whole-genome bisulfite sequencing (as recently shown in *Arabidopsis thaliana*, Schmid

et al. 2018a), which currently is still restricting more conclusive tests of how epigenetic variation can influence plant adaptation to natural selection.

5. Conclusion

Our study supports the hypothesis that the phenotypic differences recently observed between plant populations within several grassland species derived from a long-term biodiversity field experiment (Zuppinger-Dingley *et al.* 2014, Hahl 2017, van Moorsel *et al.* 2018c) were caused by genetic divergence and possibly additional epigenetic divergence. This suggests that these species can evolve rapidly in response to their biotic environment, i.e. monoculture or mixed-species communities. However, due to limitations of the method chosen to measure the genetic and epigenetic variation, it was not possible to fully disentangle whether there was genetically independent epigenetic variation. We note that despite much excitement about its potential consequences (Bossdorf *et al.* 2008, Jablonka & Raz 2009, Richards *et al.* 2010, Balao, F., Paun, O., & Alonso, C. 2018), there is still a lack of clear evidence for the role of epigenetic variation in rapid plant adaptation in nature, which is also possible based purely on genetic variation as demonstrated here. Further progress will be possible once more high-quality reference genomes become available for non-model species.

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Data accessibility

Data will be made publicly available on Zenodo (DOI 10.5281/zenodo.1167563) and SRA (accession ID SRP132258) at time of acceptance.

Authors' contributions

S.J.V.M, P.V. and B.S. planned and designed the study, S.J.V.M. carried out the pot experiment and collected plant material, C.A.M.W. performed the lab work and created the sequencing library and T.V.G. initially processed the sequencing data. M.W.S. processed and analyzed all data and produced the figures. S.J.V.M and M.W.S. wrote the manuscript with contributions from all authors.

Tables

Table 1 99th percentile of F_{ST} values in the data set with all SNPs. AS, assembly, SH, selection history. For SNPs within genes or transposons see Tab. S5 and S6.

Populations included	*G. mollugo*	*L. pratensis*	*P. lanceolata*	*P. vulgaris*	*V. chamaedrys*
SH within monoculture AS		0.131	0.045	0.346	0.188
SH within mixture AS	0.227			0.398	
Monoculture vs mixture SH within in monoculture AS	0.154	0.084	0.029	0.167	0.179
Monoculture vs mixture SH within mixture AS	0.130	0.174	0.035	0.113	0.215
AS within supp2014 SH				0.115	
AS within monoculture SH	0.067	0.171	0.030	0.066	0.039
AS within mixture SH	0.062	0.123	0.034	0.064	0.082
Comparison to supp2002 (only *V. chamaedrys*)					
Supp2014 within monoculture AS					0.120
Monoculture SH					0.098
Monoculture SH within monoculture AS					0.118
Monoculture SH within mixture AS					0.132
Mixture SH					0.073
Mixture SH within monoculture AS					0.098
Mixture SH within mixture AS					0.123

Table 2 Number of cytosines with significant differences (FDR < 0.01) in DNA methylation between selection-history treatments and assemblies. AS, assembly, SH, selection history. For data on separate sequence contexts see Tab. S7 (CG), S8 (CHG), and S9 (CHH). For the results of the comparisons with the supp2002 plants (*V. chamaedrys*) see Tab. S10.

	G. mollugo	*L. pratensis*	*P. lanceolata*	*P. vulgaris*	*V. chamaedrys*	average %
SH: mixture vs. monoculture	5734 (0.55%)	397 (0.07%)	160 (0.08%)	5240 (0.27%)	8473 (0.8%)	0.35%
% in genes	9.57	1.51	7.5	9.81	9.21	
% in transposons	10.85	31.99	12.5	10.73	10.39	
% in repeats	3.82	5.04	6.25	6.26	3.76	
% in unclassified contigs	75.76	61.46	73.75	73.21	76.64	
>> within monoculture AS	2484 (0.24%)	414 (0.07%)	107 (0.06%)	2093 (0.11%)	4397 (0.42%)	0.18%
% in genes	8.9	1.93	10.28	6.93	7.69	
% in transposons	11.43	28.99	11.21	11.71	9.96	
% in repeats	3.78	4.59	5.61	6.64	4.21	
% in unclassified contigs	75.89	64.49	72.9	74.73	78.14	
>> within mixture AS	4039 (0.39%)	502 (0.08%)	1049 (0.54%)	6797 (0.35%)	4085 (0.39%)	0.35%
% in genes	7.65	2.19	1.91	8.3	7.81	
% in transposons	12.01	29.68	14.59	14.34	11.8	
% in repeats	4.43	5.38	5.24	6.02	3.89	
% in unclassified contigs	75.91	62.75	78.27	71.34	76.5	
SH: mixture vs. supp2014	-	-	-	19746 (1.02%)	-	(1.02%)
% in genes				6.36		
% in transposons				11.14		
% in repeats				6.63		
% in unclassified contigs				75.87		
>> within monoculture AS	-	1285 (0.21%)	464 (0.24%)	8352 (0.43%)	6612 (0.63%)	0.38%
% in genes		1.71	4.53	6.41	6.79	
% in transposons		31.36	12.28	10.21	10.41	
% in repeats		4.12	3.45	6.68	4.05	
% in unclassified contigs		62.8	79.74	76.7	78.75	
>> within mixture AS	6139 (0.59%)	-	-	13749 (0.71%)	-	0.65%
% in genes	7.27			6.11		
% in transposons	11.86			11.95		
% in repeats	4.71			6.5		
% in unclassified contigs	76.17			75.44		
SH: monoculture vs. supp2014	-	-	-	19550 (1.01%)	-	(1.01%)

% in genes				6.15		
% in transposons				11.52		
% in repeats				6.66		
% in unclassified contigs				75.67		
>> within monoculture AS	-	1555 (0.26%)	315 (0.16%)	6625 (0.34%)	6249 (0.59%)	0.34%
% in genes		1.74	8.25	6.19	6.75	
% in transposons		29.9	13.33	10.17	9.44	
% in repeats		4.37	4.76	6.93	4.35	
% in unclassified contigs		63.99	73.65	76.71	79.45	
>> within mixture AS	4874 (0.47%)	-	-	15861 (0.82%)	-	0.65%
% in genes	7.2			6.56		
% in transposons	11.74			12.14		
% in repeats	3.8			6.3		
% in unclassified contigs	77.27			75		
AS: mixture vs. monoculture	-	-	-	883 (0.05%)	-	(0.05%)
% in genes				5.55		
% in transposons				14.5		
% in repeats				8.27		
% in unclassified contigs				71.69		
>> within supp2014 SH	-	-	-	1762 (0.09%)	-	(0.09%)
% in genes				7.89		
% in transposons				14.36		
% in repeats				7.95		
% in unclassified contigs				69.81		
>> within monoculture SH	1286 (0.12%)	300 (0.05%)	255 (0.13%)	2081 (0.11%)	1308 (0.12%)	0.11%
% in genes	6.38	3.33	15.69	6.01	3.44	
% in transposons	16.87	28.33	12.16	14.66	17.74	
% in repeats	4.98	5	8.24	7.4	3.21	
% in unclassified contigs	71.77	63.33	63.92	71.94	75.61	
>> within mixed culture SH	4143 (0.40%)	833 (0.14%)	460 (0.24%)	1522 (0.08%)	1956 (0.19%)	0.21%
% in genes	6.4	1.08	3.7	5.39	5.62	
% in transposons	13.71	31.21	10.43	16.1	13.85	
% in repeats	5.14	3.84	6.09	6.44	3.83	
% in unclassified contigs	74.75	63.87	79.78	72.08	76.69	
Total (percentage DMCs of tested cytosines)	19774 (1.91%)	3905 (0.65%)	2223 (1.15%)	45231 (2.34%)	20407 (1.93%)	1.60%
Total cytosines tested	1034753	598609	193844	1929089	1056852	

Table 3. Correlation between genetic and epigenetic variation (Pearson correlation coefficients of distance matrices). Non-significant correlations (Mantel test, FDR ≥ 0.05) are indicated by "n.s.".

Species	CG methylation	CHG methylation	CHH methylation
G. mollugo	n.s.	n.s.	n.s.
L. pratensis	0.23	0.18	0.16
P. lanceolata	0.17	0.13	0.12
P. vulgaris	0.41	0.34	0.22
V. chamaedrys	0.40	0.36	0.30

Table 4. Percentage of reference sequences that exhibit a significant effect (FDR) in the models to test for epigenetic variation that is unlinked to genetic variation in close-*cis* (model at the bottom in which the genotype is fitted first). CTXT: sequence context of DNA methylation, AS: assembly, SH: selection history, SNP: genotype. SH & CTXT x SH and SNP & CTXT x SNP indicate the percentage of reference sequences that exhibit a significant effect in the main effect or in the interaction (union).

Species	*G. mollugo*	*L. pratensis*	*P. lanceolata*	*P. vulgaris*	*V. chamaedrys*	average
# Tests	4,351	5,554	314	6,330	1,692	
Selection history fitted first						
CTXT	94.69	82.54	98.41	88.63	98.58	92.57
AS	0.39	0.00	0.32	0.25	0.00	0.19
SH	1.06	0.02	0.00	1.53	2.84	1.09
CTXT x SH	1.17	0.00	1.59	1.64	2.42	1.36
SNP	3.33	0.54	3.82	7.95	1.12	3.35
CTXT x SNP	1.40	1.01	6.69	5.10	1.30	3.10
SNP & CTXT x SNP	3.86	1.19	7.64	9.54	1.89	4.82
SH & CTXT x SH	1.86	0.02	1.59	2.43	4.14	2.01
Genotype fitted first						
CTXT	95.06	83.72	98.41	88.67	98.82	92.94
AS	0.39	0.00	0.32	0.25	0.00	0.19
SNP	3.68	0.79	5.10	8.50	1.71	3.96
CTXT x SNP	1.79	1.10	6.37	5.48	1.65	3.28
SH	0.64	0.04	0.00	0.52	1.06	0.45

CTXT x SH	0.30	0.02	0.32	0.68	1.42	0.55
SNP & CTXT x SNP	4.37	1.42	7.64	10.19	2.78	5.28
SH & CTXT x SH	0.85	0.04	0.32	1.01	2.01	0.85

Table 5. Coefficients of determination (R^2) from multivariate ANOVAs to test whether phenotypic traits could explain genetic and epigenetic variation. Only significant ($P < 0.05$) results are shown. n.s.: not significant.

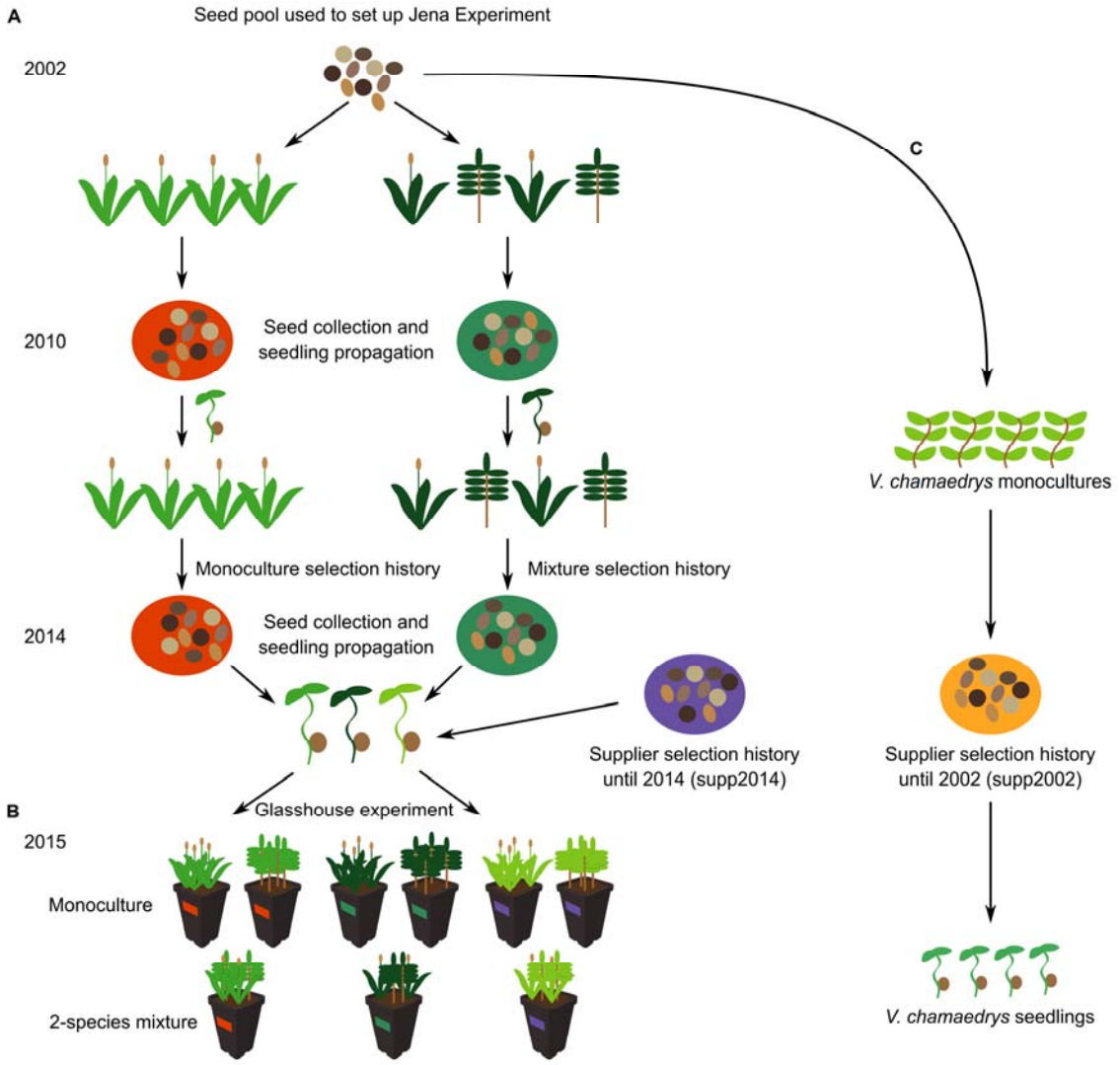
Species	Variation	Biomass	Height	SLA	Thickness
G. mollugo	Genetic	n.s.	n.s.	n.s.	n.s.
	CG meth.	n.s.	n.s.	n.s.	n.s.
	CHG meth.	n.s.	n.s.	n.s.	n.s.
	CHH meth.	n.s.	n.s.	n.s.	n.s.
L. pratensis	Genetic	n.s.	n.s.	n.s.	0.030
	CG meth.	n.s.	n.s.	n.s.	n.s.
	CHG meth.	n.s.	n.s.	n.s.	n.s.
	CHH meth.	n.s.	n.s.	n.s.	0.031
P. lanceolata	Genetic	n.s.	0.028	n.s.	n.s.
	CG meth.	n.s.	n.s.	n.s.	n.s.
	CHG meth.	n.s.	n.s.	n.s.	n.s.
	CHH meth.	n.s.	n.s.	n.s.	n.s.
P. vulgaris	Genetic	0.043	n.s.	0.028	n.s.
	CG meth.	n.s.	n.s.	0.021	n.s.
	CHG meth.	n.s.	n.s.	0.020	n.s.
	CHH meth.	n.s.	n.s.	0.022	n.s.
V. chamaedrys	Genetic	n.s.	n.s.	0.059	n.s.
	CG meth.	n.s.	n.s.	0.036	n.s.
	CHG meth.	n.s.	n.s.	0.033	n.s.

	CHH meth.	n.s.	n.s.	0.029	n.s.

Table 6. Number of reference sequences with patterns of genetic (top) or epigenetic (bottom) variation that are significantly (FDR < 0.05) associated with phenotypic traits. The genotype (SNP) was fitted prior to the epigenotype (percent DNA methylation). Numbers in parenthesis correspond to the model with the inverted fitting order (TRAIT ~ percentMethylation + SNP).

Genotype	Biomass	Height	SLA	Thickness	# Tested
G. mollugo	429 (79)	0 (0)	0 (0)	320 (25)	12,279
L. pratensis	1 (1)	2 (1)	0 (0)	76 (15)	15,797
P. lanceolata	0 (4)	0 (0)	1 (0)	0 (0)	904
P. vulgaris	18 (0)	0 (0)	0 (0)	0 (0)	17,563
V. chamaedrys	49 (3)	0 (0)	7 (1)	0 (0)	4,992
DNA methylation	Biomass	Height	SLA	Thickness	# Tested
G. mollugo	18 (425)	0 (0)	0 (0)	16 (320)	12,279
L. pratensis	0 (1)	0 (0)	0 (0)	9 (73)	15,797
P. lanceolata	4 (0)	0 (0)	0 (1)	0 (1)	904
P. vulgaris	0 (16)	0 (0)	0 (0)	0 (0)	17,563
V. chamaedrys	1 (41)	0 (0)	0 (7)	0 (0)	4,992

Figures



D

Seed origin		From Supplier 2002					From Supplier 2014	
Selection history (SH)		2002-2014 in monoculture plots of Jena Experiment		2002-2014 in mixture plots of Jena Experiment		2002-2014 in cold storage in Jena Laboratory	Cultivated by supplier until 2014	
Assembly (AS)		Monoculture pots	2-species mixture pots	Monoculture pots	2-species mixture pots	Monoculture pots	Monoculture pots	2-species mixture pots
Plant species	*G. mollugo*	12 (12)	14 (16)	11 (12)	18 (18)	0	0	6 (6)
	L. pratensis	11 (12)	5 (6)	11 (12)	4 (5)	0	11 (12)	0
	P. lanceolata	10 (12)	16 (18)	10 (12)	21 (23)	0	8 (12)	0
	P. vulgaris	10 (12)	18 (20)	10 (12)	16 (18)	0	10 (12)	5 (6)
	V. chamaedrys	12 (12)	7 (7)	12 (12)	6 (6)	45 (47)	10 (12)	0

Figure 1. Overview of the experiment. Details are provided in the Material and methods section. (A) The origin of seeds used for the glasshouse experiment and genetic analysis. Seedlings were planted in mixtures and monocultures in the Jena Experiment in the year 2002 (Weisser *et al.* 2017). Two reproduction events occurred when seeds were collected, and subsequently new seedlings were produced and planted again in the same community composition. (B) Schematic representation of the glasshouse experiment. Monoculture assemblies and two-species mixture assemblies were planted with either plants with mixture selection history (green), monoculture selection history (orange) or supp2014 plants originating from a commercial seed supplier (blue). (C) Seeds from offspring of the original seed pool of the Jena Experiment (supp2002) were grown in an experimental garden. Figure modified after van Moorsel *et al.*, 2018c. (D) Table with the experimental design. Numbers in parenthesis equal to the number of sequenced individuals. Smaller numbers in front of the parenthesis correspond to the number of individuals used during all analyses.

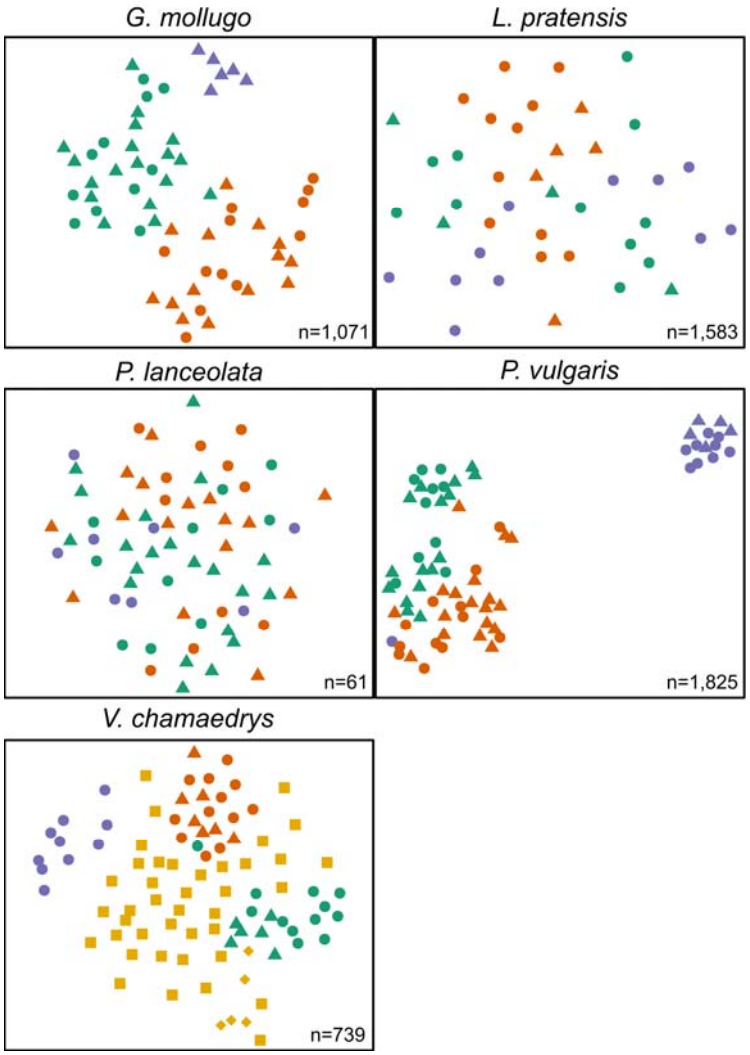
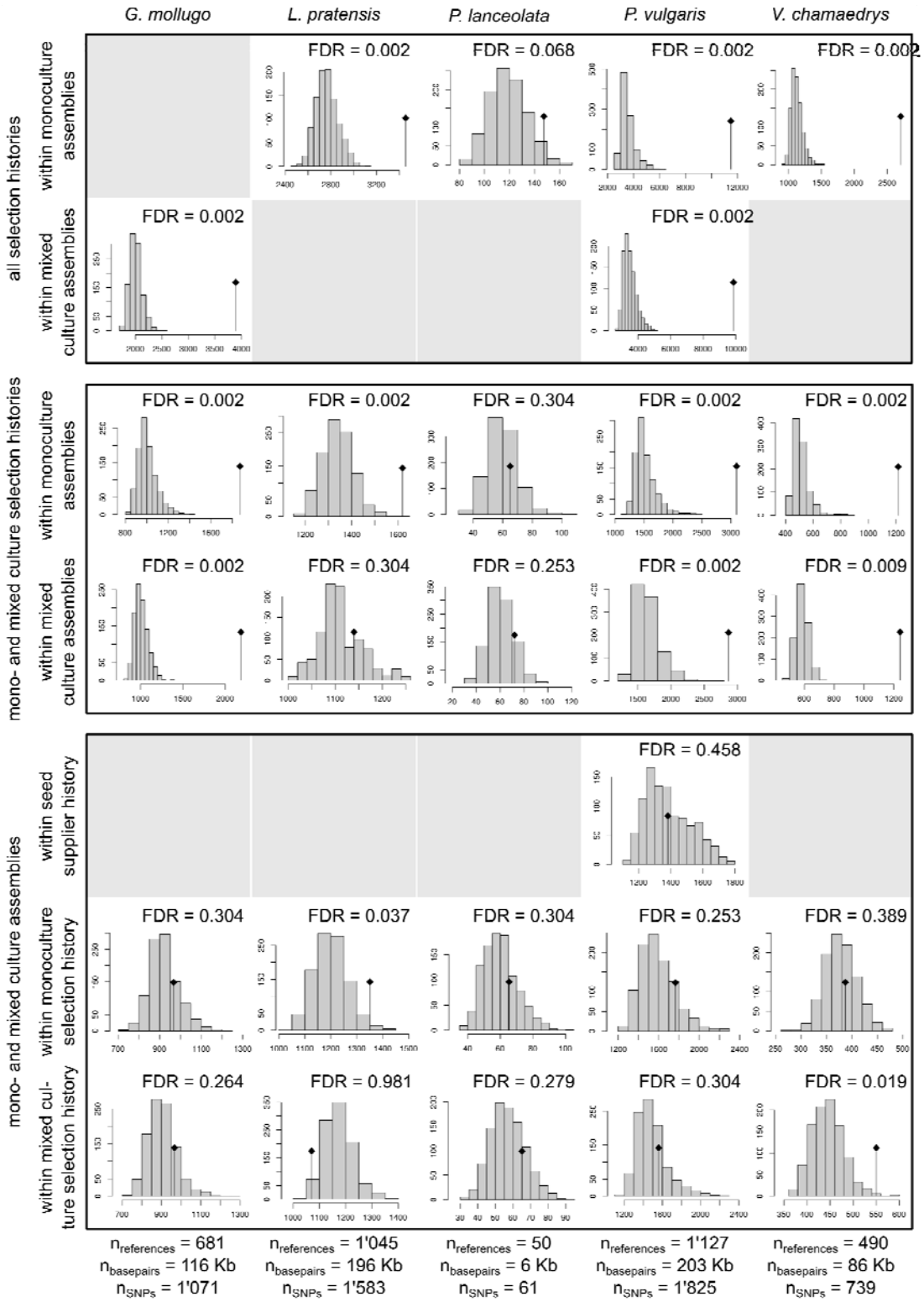


Figure 2. Genetic distance between individuals of the different populations for the five species. Green: selection history in mixture, orange: selection history in monocultures, blue: selection history in the field of the original seed supplier, seeds bought in 2014 (supp2014), yellow: offspring from original Jena seed pool supp2002. Triangles: monoculture assembly, circles: mixture assembly, squares: supp2002 grown in the garden, diamonds: supp2002 individuals collected from a single seed pod to qualitatively show the similarity between siblings. Note that assembly refers to the diversity level in the glasshouse.


G. mollugo
L. pratensis
P. lanceolata
P. vulgaris
V. chamaedrys
all selection histories
within monoculture assemblies
within mixed culture assemblies
FDR = 0.002
FDR = 0.068
FDR = 0.002
FDR = 0.002
FDR = 0.002
FDR = 0.002
mono- and mixed culture selection histories
within monoculture assemblies
within mixed culture assemblies
FDR = 0.002
FDR = 0.002
FDR = 0.304
FDR = 0.002
FDR = 0.002
FDR = 0.002
FDR = 0.304
FDR = 0.253
FDR = 0.002
FDR = 0.009
mono- and mixed culture assemblies
within seed supplier history
within monoculture selection history
within mixed culture selection history
FDR = 0.458
FDR = 0.304
FDR = 0.037
FDR = 0.304
FDR = 0.253
FDR = 0.389
FDR = 0.264
FDR = 0.981
FDR = 0.279
FDR = 0.304
FDR = 0.019
n_references = 681
n_basepairs = 116 Kb
n_SNPs = 1'071
n_references = 1'045
n_basepairs = 196 Kb
n_SNPs = 1'583
n_references = 50
n_basepairs = 6 Kb
n_SNPs = 61
n_references = 1'127
n_basepairs = 203 Kb
n_SNPs = 1'825
n_references = 490
n_basepairs = 86 Kb
n_SNPs = 739

935

936

Figure 3. Results from the G-statistic tests given all SNPs. Each panel shows a histogram of permuted test statistics (999 permutations) and indicates the observed statistics by a black dot and a segment. Test statistics are on the x-axis, frequencies on the y-axis. Grey boxes occur where data were not available (experimental treatment combination missing).

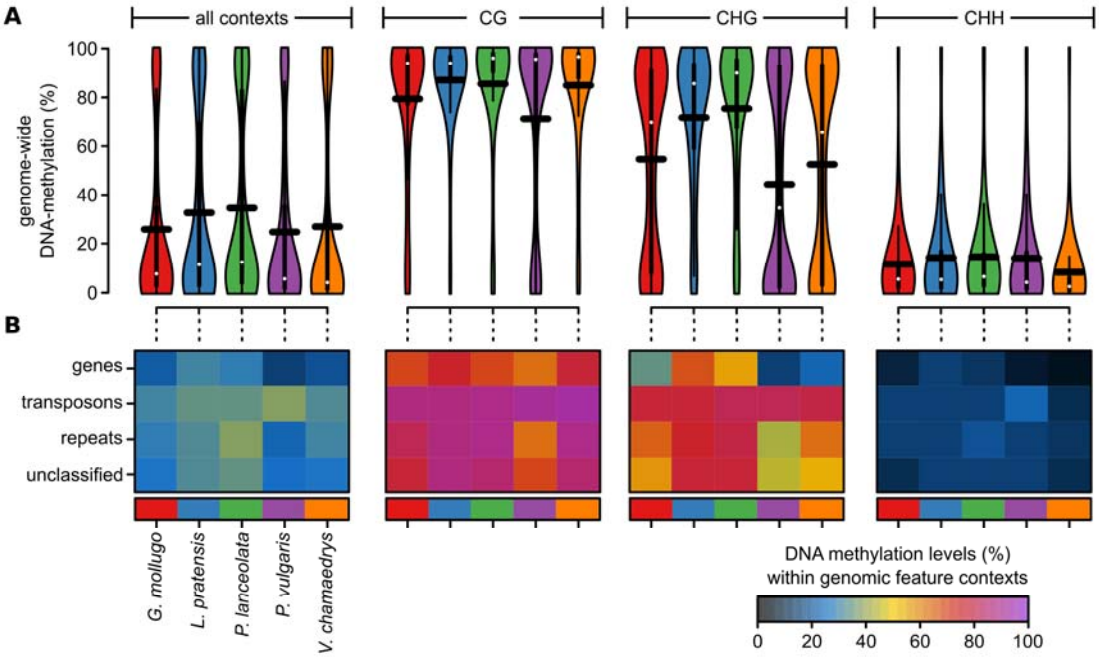


Figure 4. (A) DNA methylation levels in percent at individual cytosines across all or within each individual sequence context (CG, CHG, CHH) for each species used in this study shown as violin plots. The horizontal black bars correspond to the means. (B) Average DNA methylation levels in percent for each sequence context, genomic feature, and species shown as a heat map.

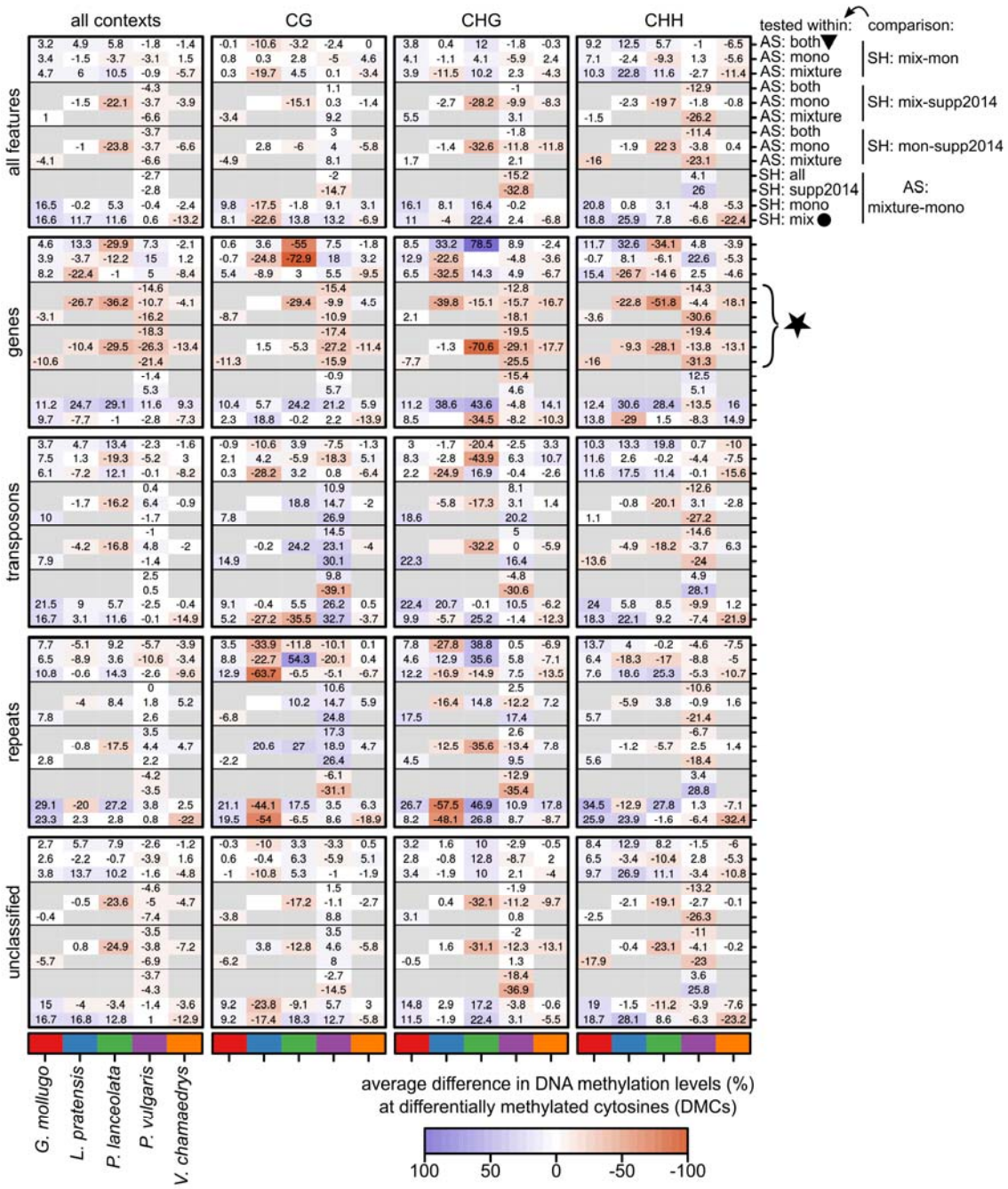


Figure 5. Average differences in DNA methylation at significantly differentially methylated cytosines (DMCs; FDR < 0.01) within a given sequence (all, CG, CHG, and CHH) and feature context (all, genes, transposons, repeats, unclassified) are shown for all contrasts. The comparisons and within which levels of the other factor they were tested are given in the first panel row on the right (same for all features). AS: assembly, PH: selection history. For example, the row marked with a triangle corresponds to the comparison between mixture and monoculture selection histories across both monoculture and mixture assemblies. In contrast, the row marked with a dot corresponds

to the comparison between mixture assembly and monoculture assembly within the mixture selection history. The average differences are shown as colour gradient. The numbers within the heat map are the average differences. The asterisk marks the rows which show that plants in the Jena field lost on average DNA methylation at DMCs within genes compared to supp2014 plants (the two comparisons SH mix – supp2014 and SH mon – supp2014; within and across monoculture and mixture assemblies).

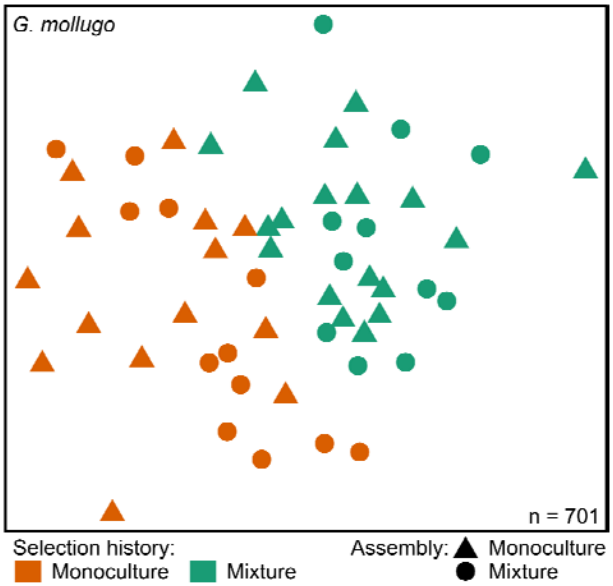


Figure 6. Genetic distance between the 701 reference sequences that were significantly (FDR < 0.05) associated with the phenotype in *G. mollugo*. Selection histories in this analysis were limited to the two histories in the Jena Experiment (monoculture and mixture). Distances were visualized with the function isoMDS of the R-package MASS.

Supplemental information

The supplementary information contains supplementary methods, four supplemental figures and ten supplemental tables and can be accessed online.