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Abstract

In long-term grassland experiments, positive biodiversity effects on plant productivity commonly increase with time. Subsequent glasshouse experiments showed that these strengthened positive biodiversity effects persist not only in the local environment but also when plants are transferred into a common environment. Thus, we hypothesized that community diversity had acted as a selective agent, resulting in the emergence of plant monoculture and mixture types with differing genetic composition. To test our hypothesis, we grew offspring from plants that were grown for eleven years in monoculture or mixture environments in a biodiversity experiment (Jena Experiment) under controlled glasshouse 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 (i.e. DNA methylation) data. We observed significant divergence in genetic and DNA methylation data according to selection history in three out of five perennial grassland species, namely *Galium mollugo*, *Prunella vulgaris* and *Veronica chamaedrys*, with DNA methylation differences mostly reflecting the genetic differences. In addition, current diversity levels in the glasshouse had weak effects on epigenetic variation. However, given the limited genome coverage of the reference-free bisulfite method epiGBS, it remains unclear how much of the differences in DNA methylation was independent of underlying genetic differences. Our results thus 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.

Keywords

biodiversity, genetic divergence, DNA methylation, epigenetic variation, herbaceous plant species, selection

1. Introduction

Environmental change such as global warming can cause range shifts of species during which new sites are colonized by dispersal while populations go extinct at other sites (Ouborg, Vergeer, & Mix 2006). The speed of environmental change raises the question whether remaining populations are able to adapt fast enough to novel abiotic or biotic conditions and thus be rescued from local extinction by evolutionary change (Schmid, Birrer, & Lavigne 1996, Davis, Shaw, & Etterson 2005, Bell & Gonzalez 2009). Biodiversity is known to buffer ecosystems against negative influences of climatic extremes and novel environmental conditions (Isbell *et al.*, 2015). Additionally, 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 conditions such as temperature and precipitation (e.g., Joshi *et al.* 2001) and biotic interactions such as between pollinators and plants (e.g., Gervasi & Schiestl 2017) are well studied. However, most plants do not occur in pure populations of a single species, i.e. monocultures, but are mixed with other species to which they may adapt in diverse communities, i.e. mixtures. So far, little effort has been devoted to study such adaptation, whether it can occur at ecologically relevant time scales and to what degree it is caused by genetic change in populations (Lipowsky, Schmid, & Roscher 2011, Zuppinger-Dingley *et al.* 2014,

66 Kleynhans, Otto, Reich, & Vellend 2016, van Moorsel *et al.* 2018b). In particular,
67 adaptive responses driven by multispecies interactions of plant species are largely
68 unknown, despite a growing body of evidence showing the importance of multispecies
69 interactions for the stability of ecological communities (Bastolla *et al.* 2009, Guimarães,
70 Pires, Jordano, Bascompte, & Thompson 2017). It is conceivable that the feedback
71 between species interactions and their adaptive responses shapes community-level
72 ecosystem functioning (van Moorsel *et al.* 2018b).

73 In the 1960s it was proposed that evolutionary processes occur at longer time scales than
74 ecological processes (Slobodkin 1961), but now it is commonly believed that micro-
75 evolutionary and ecological processes can occur at the same or at least at similar temporal
76 scales (Hairston, Ellner, Geber, Yoshida, & Fox 2005, Schoener 2011, Hendry 2016). A
77 good understanding of how biodiversity, i.e. the interaction between species, shapes
78 evolutionary responses, is instrumental for predicting ecosystem responses to global
79 change and biodiversity loss.

80 Long-term biodiversity field experiments offer unique opportunities to study effects of
81 community diversity and composition on natural selection. Species mixtures are
82 frequently more productive than average monocultures (Balvanera *et al.* 2006).
83 Moreover, these biodiversity effects often become more pronounced over time, which has
84 been attributed to increased complementarity among species (Cardinale *et al.* 2007,
85 Marquard *et al.* 2009, Reich *et al.* 2012, Meyer *et al.* 2016). Increased complementarity
86 may originate from evenly distributed resource depletion in mixtures or negative plant–
87 soil feedbacks developing in monocultures (Fargione *et al.* 2007). Moreover, increased
88 complementarity can influence phenotypic plasticity (Ghalambour, McKay, Carroll, &

89 Reznick 2007) or selection of genotypes that have an advantage to grow in mixtures, i.e.,
90 “mixture-type plants”. Indeed, recent common-environment experiments with plant
91 material from a grassland biodiversity experiment (the Jena Experiment, Roscher *et al.*
92 2004) suggest that increased biodiversity effects have a heritable component (Zuppinger-
93 Dingley *et al.* 2014, van Moorsel, Schmid, Hahl, Zuppinger-Dingley, & Schmid 2018c).
94 Plants originating from mixed communities showed stronger complementarity effects
95 than plants originating from monoculture communities if they were grown in two-species
96 mixtures in the glasshouse, indicating that community composition can lead to
97 phenotypic trans-generational effects (Zuppinger-Dingley *et al.* 2014, Rottstock,
98 Kummer, Fischer, & Joshi 2017, van Moorsel *et al.* 2018b). However, it remains unclear
99 whether the trans-generational effects observed in these studies were due to genetic
100 differentiation, epigenetic differences or (possibly epigenetically-induced) maternal
101 effects (Tilman & Snell-Rood 2014).

102 While phenotypic changes have been widely linked to genetic variation, an increasing
103 body of evidence suggests epigenetic mechanisms (e.g. DNA methylation) may play an
104 important role in phenotypic variation, and hence ecological processes (e.g., Bird 2007,
105 Bossdorf, Richards, & Pigliucci 2008, Niederhuth & Schmitz 2014, Verhoeven,
106 Vonholdt, & Sork 2016). DNA methylation is a well-studied modification of the DNA
107 sequence involved in a large number of biological processes (Law & Jacobsen, 2010). It
108 was, for example, defined as changes in gene function that cannot be explained by
109 changes in the DNA sequence, which are in some cases mitotically and/or meiotically
110 heritable (Riggs, Russo, & Martienssen 1996, Verhoeven, Vonholdt, & Sork 2016). The
111 term epigenetics is widely defined in different scientific fields, and some definitions call

112 for transgenerational inheritance of epigenetic marks (e.g., see Deans & Maggert 2015;
113 Lind & Spagopoulou 2018). In this study, we look at DNA methylation and use
114 "variation in DNA methylation" interchangeably with "epigenetic variation".

115 Recent work on epigenetic recombinant inbred lines (epiRILs, inbred for DNA
116 methylation variants) of *Arabidopsis thaliana* indeed suggests a considerable contribution
117 of induced epialleles to phenotypic variation, which is independent of genetic variation
118 (Latzel *et al.* 2013, Cortijo *et al.* 2014, Kooke *et al.* 2015). Schmitz et al. (2013) further
119 found evidence for epigenomic diversity, which was potentially independent of genetic
120 diversity in natural *Arabidopsis thaliana* successions. However, the importance of
121 epigenetics in natural populations, in particular of non-model species, and whether it
122 contributes to adaptation, remains elusive (Quadrana & Colot, 2016, Richards *et al.* 2017,
123 Groot *et al.* 2018). For example, Dubin et al. (2015) found that differences in DNA
124 methylation between natural populations of *A. thaliana* were largely due to genetic
125 variation at *trans*-acting loci, many of which showed evidence of local adaptation.
126 Nonetheless, a recent selection experiment with *A. thaliana* suggests that epigenetic
127 variation may indeed contribute to rapid heritable changes and adaptation (Schmid *et al.*
128 2018a).

129 Here, we tested whether community diversity can act as a selective environment resulting
130 in genetic or epigenetic divergence. In an earlier experiment by van Moorsel *et al.*
131 (2018b), phenotypic differences between offspring from plants that were selected in
132 mixtures versus monocultures in the Jena Experiment were recorded when reciprocally
133 grown in monocultures or mixtures. In the present study, we hypothesize that these
134 phenotypic differences between plant populations within several grassland species are

caused by genetic and additional epigenetic differentiation. Genetic differences were quantified as differences in DNA sequence (single nucleotide polymorphisms; SNPs). Epigenetic variation among plant individuals was assessed as the levels of DNA cytosine methylation in three different contexts, CG, CHH and CHG, using epiGBS, a genotyping-by-sequencing approach combined with bisulfite conversion.

2. Material and Methods

2.1. Plant selection histories

To test whether plant communities that were grown in either monocultures or mixtures, showed genetic or epigenetic differentiation, material from plant populations from 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) were used (see also Fig. 1).

In the original design at Jena, 16 plant species were present in large 20 x 20 m monoculture and mixture plots from which cuttings were harvested after 8 years of growth in either mono- or mixed cultures. Out of the 16 species, four grew poorly and for several of the remaining 12, seed collection was limited (van Moorsel et al. 2018c). Hence, we were restricted to the following five species for subsequent propagation and reciprocal treatments: 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 up to five years in monocultures with reseeding them every year (van Moorsel *et al.* 2018a). Although this does not guarantee close similarity with the original seed pool that was used at the start of the Jena Experiment (and which was propagated according to the same guidelines by the supplier), it does provide good material to test the difference between plants grown in the garden of the supplier and then in the Jena Experiment and those grown in the garden of the supplier without subsequent selection in the Jena Experiment.

In summary, there were three selection histories for all species and an additional fourth history for *V. chamaedrys* (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 vegetative in the field. Therefore, plants with a selection history in

177 either mixture or monoculture in the Jena Experiment underwent two controlled
178 reproductive cycles in 2010 and 2014. This additional step aimed to increase the potential
179 for evolutionary change—otherwise restricted to the possibility of sorting out genotypes
180 from standing variation—by adding the possibility of recombination and, less likely,
181 mutation. In addition, we hoped to reduce the potential for maternal carry-over effects,
182 which are often stronger in cuttings than seedlings (see e.g. Schmid & Bazzaz 1990) and
183 which tend to wean off after seedling stages (Roach & Wulff 1987). Additionally to
184 reducing this type of maternal carry-over effects, the seed propagation should also have
185 reduced the carry-over of somatic epigenetic marks.

186 In spring 2010, cuttings from all plant communities were collected and transplanted to an
187 experimental garden in Zurich, Switzerland, in an identical plant composition as in the
188 Jena Experiment, for the first controlled pollination and seed production (see also
189 Zuppinger-Dingley *et al.* 2014). In spring 2011, the seedlings produced from these seeds
190 were transplanted back to the same plots of the Jena Experiment from where the parents
191 had originally been collected and in the same community composition (see Tab. S2 for
192 the community compositions of the plots in the Jena Experiment). In March 2014, plant
193 communities of the plots that were re-established in 2011 in the Jena Experiment were
194 again transferred to the experimental garden in Zurich for the second controlled
195 pollination and seed production. For each experimental plot, we excavated several blocks
196 of soil including the entire vegetation (in total one square meter). These blocks were then
197 used to establish the plots in the experimental garden. We added a 30 cm layer of soil
198 (1:1 mixture of garden compost and field soil, pH 7.4, commercial name Gartenhumus,
199 RICOTER Erdaufbereitung AG, Aarberg, Switzerland) to each plot to make sure the

plants established. During the controlled pollination and seed production, plots were surrounded by nets and only left open on top to allow pollinator access. This design did not fully exclude the possibility of cross-pollination between plots containing different plant communities, and such cross-pollination might also have occurred in the field during sexual reproduction events. However, 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 are 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) and an assembly treatment which corresponded to plants being planted in the glasshouse either in monocultures or mixtures as the common test environments. Hence, the full experimental design included five plant species, three selection histories and two assembly treatments. The fourth history of *V. chamaedrys* (supp2002) was an extension of the experiment and plants were grown separately in a glasshouse in the Netherlands at a later time point (see section 2.4 further below).

2.3.1. Setup of the glasshouse experiment

Seeds from monocultures, mixtures and the seed supplier (supp2014) were germinated in December 2014 in germination soil ("Anzuchterde", Ökohum, Herbertingen, Germany) under constant conditions in the glasshouse without additional light. Seedlings were planted as monocultures of four individuals or two-species mixtures (2 x 2 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 replicated in six blocks, each including the full experimental design. Within each block, pots were placed on three different tables in the glasshouse at random 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

The following traits were measured: plant height, leaf thickness, specific leaf area (SLA) and aboveground biomass. These traits were shown to relate to competitive growth and affect plant community productivity in biodiversity experiments (Roscher *et al.* 2015, Cadotte 2017). All traits were measured after twelve weeks 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.

2.3.3. Sampling of plant material

Samples for 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. 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 until further analysis.

2.4. Offspring of the original seed pool (fourth selection history)

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) under natural light conditions (16/8 hrs. day/night). No cold treatment or vernalisation 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 genetic and epigenetic variation using an improved version of a recently developed reference-free bisulfite method ("epiGBS", van Gurp *et al.* 2016). Traditional

reduced representation bisulfite sequencing (RRBS) methods (e.g. Cokus *et al*. 2008) require a reference genome for efficient mapping and variant calling. epiGBS, however, allows for reference-free RRBS of highly multiplexed libraries and is therefore more straightforward and cost-effective. A detailed description of the improvements is given in the supplementary methods. In brief, we used an improved combination of methylation-insensitive restriction enzymes to avoid the bias previously reported in van 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. Given that epiGBS reconstructs the DNA sequence using information from both strands (i.e., also the one with the unconverted guanine), it is possible to infer the sequence context of the cytosines. 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 van Gurp *et al.* (2016) with the pipeline provided by the authors available on https://github.com/thomasvangurp/epiGBS. The short reference sequences (up to 250 bp long) restricted the analysis of linkage disequilibrium in the study 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 variants (SNPs) and epigenetic variants (DMCs) 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. We removed all SNPs located in contigs with more than 1 SNP per 50 base pairs (2 %). First, to avoid that contigs with many SNPs dominate the analysis of genetic differentiation given that SNPs of a contig are linked to each other. Second, to avoid a potentially negative impact of misalignments. 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 contig. Hence, contigs with large number of SNPs may have a higher SNP calling error rate. To assess the impact of this filter, we also performed the analyses described below (section 2.7.2) with all contigs, irrespective of the SNP rate. Even though the filter frequently removed half of all contigs, the results were similar (FDRs are provided in the figures from the analysis with the filter but not discussed further). 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).

To identify individual SNPs that may be directly under selection, we tested for outliers with BayeScan (version 2.1, Foll & Gaggiotti 2008, Fischer, Foll, Excoffier & Heckel 2011). Given that there was no genetic differentiation between assemblies, we treated plants with the same selection histories but different assemblies as a single population. Hence, the tests either included two (monoculture vs. mixture) or three (monoculture, mixture and supp2014) selection histories. For *V. chamaedrys*, we also tested each of the three selection histories (monoculture, mixture and supp2014) against the original seed pool (supp2002). SNPs were identified as significant if the false discovery rate (FDR) was below 0.05 (Tab. S7).

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. Due to the coverage filter, there was a slight bias towards non-CHH sites in four out of five species. On average the fraction of CHH sites was reduced from 73.5 % to 72.9 % and the largest difference was found in *V.*

379 *chamaedrys* (68.7 % instead of 72.4 %). The bias was opposite for *P. vulgaris* (75.0 %
380 instead of 73.15 %). The coverage filter is different from the one applied for the SNP data
381 because the down-stream analyses have different requirements regarding missing data
382 (more flexible for the DNA methylation data). To provide an overview of the genome-
383 wide DNA methylation levels of the five species or each experimental treatment
384 combination per species, we visualized the DNA methylation levels of all cytosines
385 averaged across all individuals with violin plots. We also visualized the average DNA
386 methylation level within genes, transposons, repeats and unclassified reference contigs
387 with heatmaps. Both methods were applied either using all sequence contexts (CG, CHG,
388 CHH) at once or separately for each sequence context.

389 **2.8.2. Identification of differentially methylated cytosines (DMCs)**

390 DNA methylation data were processed and filtered as described above. Variation in DNA
391 methylation at each individual cytosine was then analysed with a linear model in R with
392 the package DSS (version 2.24.0; Y. Park & Wu (2016)), according to a design with a
393 single factor comprising all different experimental treatment combinations as separate
394 levels and using contrasts to compare levels of interest (similar to the approach described
395 for RNA-Seq in Schmid 2017 and the testing procedure described in Schmid, Giraldo-
396 Fonseca, Smetanin & Grossniklaus 2018b). Specific groups were compared with linear
397 contrasts and *P*-values for each contrast were adjusted for multiple testing to reflect false
398 discovery rates (FDR, Benjamini & Hochberg 1995). Taking *P. vulgaris* as an example,
399 we compared the three selection histories across both assemblies and within each
400 assembly to each other. Likewise, we compared the two assemblies across all selection
401 histories and within each selection history to each other. A cytosine was defined as

differentially methylated ("DMC", see also Schmid *et al*. 2018a) 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

To test how much of the genetic differentiation could be attributed to selection history, and, subsequently, how much of the epigenetic (methylation) variation was associated with selection history after controlling for differences in genetic structure that might have been induced by the selection histories, we modelled 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

424 in this order (percent methylation ~ CTXT + AS + SNP + CTXT:SNP + SH +
425 CTXT:SH). We then compared this result to an alternative model in which SH and SNP
426 were switched (percent methylation ~ CTXT + AS + SH + CTXT: SH + SNP +
427 CTXT:SNP). Hence, whereas the second model tests for epigenetic differentiation
428 between selection histories irrespective of the underlying genetics, the first model tests
429 whether there was epigenetic differentiation between selection histories that could not be
430 explained by the underlying genetics. We only used reference sequences which passed
431 the coverage filters described above. We further only included the monoculture and
432 mixture histories from the Jena field because only these two were fully factorially crossed
433 with assembly in all species. Models were calculated with the functions lm() and anova()
434 in R (version 3.5.1). Results from all reference sequences were collected and *P*-values for
435 each term were adjusted for multiple testing to reflect false discovery rates (FDR,
436 Benjamini & Hochberg 1995). Note that because of different distribution and testing
437 procedure, results from this model with an average level of DNA methylation across
438 several cytosines cannot be directly compared with the results from the model used to test
439 for differential DNA methylation at individual cytosines. This model can detect
440 dependency of epigenetic variation on genetic variation within our reference contigs with
441 a maximal size of 250 bp. Most associations between DNA sequence variation and
442 methylation loci decay at relatively short distances (i.e., after 200 bp in *A. thaliana* or 1
443 kb in *A. lyrata*; Hollister *et al*. 2010). This model may thus provide good proxy for close-
444 *cis* associations (close to each other at the same location in the genome, i.e., close enough
445 to be on the same 250 bp reference sequence). However, far-*cis* associations (for example
446 a transposon insertion variant which is close to the place of origin of the reference

447 sequence but not represented in the reference sequence, i.e., too far to be on the same 250
448 bp reference sequence) or *trans* dependencies (effects from other loci that are not linked
449 to the place of origin of the reference sequence) cannot be detected. As a result, by using
450 this model, we might have potentially overestimated the proportion of epigenetic
451 variation that is unlinked to genetic variation.

452 **2.10. Relation between genotype/epigenotype and phenotype**

453 **2.10.1. Overall correlation**

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

461 **2.10.2. Association of genotypes/epigenotypes with phenotypes**

462 To test whether individual reference sequences correlated with phenotypic variation, we
463 separately modelled the variation in the four phenotypic traits (biomass, height, leaf
464 thickness and SLA) in response to the genotype (SNP) and the percent DNA methylation
465 (METH) for a given sequence context with the same data previously used to test linkage
466 of genetic and epigenetic variation (see section 2.9.2. above). Models were calculated
467 with the function lm() and anova() in R (version 3.5.1). We tested both fitting orders with
468 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 separated them according to their selection history in three out of five species, namely *G. mollugo*, *P. vulgaris* and *V. chamaedrys* (Fig. 2). As expected, populations did not separate according to the assembly treatment, because plants were assigned randomly to the assembly treatment. 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

491 exhibit any significant genetic differentiation. Also, the test including only the
492 monoculture and mixture types within the mixture assemblies was not significant for *L.*
493 *pratensis*. However, in *L. pratensis* statistical power was limited because there were only
494 nine individuals available (Tab. S2, almost all other experimental groups from
495 monoculture and mixture selection history had at least 10 individuals each). In contrast,
496 the tests comparing the monoculture and mixture assemblies within each of the selection
497 histories were never significant at the critical level of FDR = 0.01 (Fig. 3, bottom rows).

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

509 Within *V. chamaedrys*, comparisons between supp2002 plants and the other populations
510 were all significant (FDR < 0.01 in all comparisons). The average pairwise F_{ST} values
511 between the supp2002 plants and the other populations (Tab. S4) were between 0.010 and
512 0.015. In comparison, pairwise F_{ST} values between any of the supp2014-, monoculture-,
513 or mixture-history populations were between 0.027 and 0.038 for this species. Likewise,

514 the 99th percentiles of the SNP-wise F_{ST} values were consistently lower in the
515 comparisons between the supp2002 plants and the other populations than among those
516 (i.e., supp2014, monoculture and mixture histories populations, Tab. 1, S5 and S6). This
517 confirmed the previous observation that supp2002 individuals, which could be considered
518 as "parental" to the others, were genetically intermediate between the other selection
519 histories (Fig. 2).

520 To identify individual SNPs that may be directly under selection, we tested for outliers
521 with BayeScan (Tab. S7). While we could not find any outliers in *G. mollugo*, *P.*
522 *lanceolata* and *V. chamaedrys*, we could identify several significant SNPs in both tests of
523 *P. vulgaris*. 13 SNPs were significant if the three selection histories were compared with
524 each other and 7 SNPs were significant if the monoculture and mixture selection histories
525 were compared with each other. We could also identify a significant SNP in *L. pratensis*
526 between the monoculture and mixture selection histories, but only if tested with all
527 reference contigs, including the ones with a SNP rate above 2 %. These results are in
528 parallel to the results with the 99th percentiles for which *P. vulgaris* exhibited the highest
529 F_{ST} values (Tab. 2). However, it is difficult to assess the functional relevance of these
530 SNPs because all of them were annotated as either unknown, repeat or transposable
531 element (data not shown).

532 **3.2. Epigenetic variation**

533 To get an overview of the DNA methylation data, we visualized DNA methylation levels
534 in percent at individual cytosines for each plant species, sequence context (CG, CHG,
535 CHH) and genomic feature context (genes, transposons, repeats and unclassified contigs,
536 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 (e.g., Law & Jacobsen 2010, Niederhuth *et al.* 2016, Paun, Verhoeven, & Richards 2019, but see Gugger *et al.* 2016), however, between-species differences in DNA methylation levels were previously shown to be large (van Gurp et al. 2016, SI).

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 highly 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. S8, S9, and S10 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

560 0.05% and 0.21% on average across all species and between 0.05% and 0.40% per
561 individual species. Thus, the fraction of differentially methylated cytosines between the
562 selection histories was generally larger than differences between the two assemblies
563 (Tables S8, S9, S10).

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

574 To further characterize the differences in DNA methylation, we calculated the average
575 change in DNA methylation at the DMCs for each contrast, across and within all
576 sequence contexts (CG, CHG and CHH) and feature types (genes, transposons, repeats
577 and unclassified) and visualized these differences (Fig. 5). We could not identify clear
578 patterns between the different comparisons with one exception: differences in the
579 comparisons between plants from Jena and the supp2014 plants within genes (all
580 sequence contexts) were mostly biased towards a higher methylation in the supp2014
581 plants. Thus, plants in the Jena Experiment showed an overall loss of DNA methylation
582 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. S11). 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 supp2002 plants grew in a markedly different environment (glasshouse in the Netherlands vs. Switzerland) and that their ancestors had been stored as seeds for 12 years, effects of underlying genetic differences might have been confounded with effects of the storage and glasshouse environment. Nonetheless, the results suggest that there was considerable epigenetic variation within *V. chamaedrys*.

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 and 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 reference sequences) and *P. lanceolata* (1 out of 314 reference sequences) 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). Only one species, *G. mollugo*, did not show significant correlation between genetic or epigenetic variation with phenotypic traits. For example, leaf thickness was significant in *L. pratensis* and SLA was significant in *P. vulgaris* and

626 *V. chamaedrys*. However, the coefficients of determination (R^2) were with 0.02 to 0.06
627 relatively low, indicating that only a small fraction of the genome was correlated to the
628 measured phenotypic traits. This was not surprising considering that we only measured
629 few traits and that these might not have been so highly polygenic to be covered by the <
630 2% of the genome assessed with our reduced representation sequencing approach (i.e.,
631 epiGBS; van Gurp *et al*. 2016).

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

645 Epigenetic variation was rarely significantly associated with phenotypic traits if fitted
646 after genetic variation (Tab. 6). However, if the epigenotype was fitted first, the number
647 of reference contigs with a significant association between the epigenotype and
648 phenotypic traits was almost identical to the number of significant associations found

649 previously between the genotype and the phenotypic traits if the genotype was fitted first.
650 This suggests, that DNA methylation was under genetic control. In line with the previous
651 results, both genetic and epigenetic variation were significantly associated to phenotypic
652 traits but at the same time they were also well correlated with each other.

653 **4. Discussion**

654 For three out of five test species, namely *G. mollugo, P. vulgaris* and *V. chamaedrys*, we
655 found genetic differences between monoculture and mixture types in a large number of
656 SNPs. In a fourth species, *L. pratensis*, we found evidence for genetic divergence among
657 plants grown in monoculture assemblies in the glasshouse. The comparison, however,
658 was insignificant for plants grown in mixture assemblies, as we could only test nine
659 individuals in total. In the fifth species, *P. lanceolata*, we could not identify significant
660 genetic differentiation between plants with different selection histories. This finding was
661 unexpected because *P. lanceolata* has recently been shown to exhibit clear genetic
662 divergence after 15 years of simulated climate change (Ravenscroft, Whitlock & Fridley,
663 2015). It is conceivable that we could not detect genetic divergence in *P. lanceolata*
664 because of the low number of reference sequences that passed our filter: there were only
665 50 sequences corresponding to 6 kb and 61 SNPs left. Thus, we might have missed
666 regions under selection.

667 On average, only 1.7% to 11% of genetic variation was explained by selection histories.
668 However, at individual SNP-level, selection histories explained up to 40% of the genetic
669 variation. This indicates that these loci were under selection (i.e., high divergence)
670 whereas other parts of the genome segregated randomly (i.e, low divergence).

671 Besides the genetic divergence, we could also identify differences in methylation levels
672 between the selection histories, which were generally below 1% of all tested cytosines.
673 For *V. chamaedrys*, we observed pronounced differences in methylation levels between
674 offspring of the original seed pool of the Jena Experiment (supp2002) and the three other
675 selection histories. Given that these plants grew in a different glasshouse environment
676 and that their ancestors had been stored as seeds for 12 years, we could not be sure if the
677 differences in methylation levels were due to underlying genetic or environmental
678 differences. Nonetheless, with 7.4% of all tested cytosines being significantly differently
679 methylated between supp2002 and the other populations (supp2014, monoculture and
680 mixture history), there was a substantial amount of epigenetic differences within *V.*
681 *chamaedrys*. Given that the genetic variation of the supp2002 population was overlapping
682 with the other groups (see Fig. 2) but that the epigenetic variations did not overlap, there
683 was probably a considerable amount of environmentally-induced epigenetic variation that
684 was independent of genetic divergence between groups.

685 Overall, variation in methylation levels of each individual sequence context were
686 significantly correlated with genetic variation in four out of five species (Tab. 3). When
687 we tested each reference sequence for epigenetic variation that could not be explained by
688 genetic variation in close-*cis*, we found that up to 2.01 % of all sequences exhibited
689 epigenetic variation that was unlinked to such genetic variation. Although this provides
690 evidence for epigenetic divergence between selection histories that is independent and
691 additional to genetic divergence, our analysis could not account for potential correlations
692 between epigenetic variation and genetic variation in far-*cis* or *trans*. For example,
693 genome-wide studies with *A. thaliana* revealed extensive epigenetic variation between

694 different populations and accession, which was mostly linked to underlying genetic
695 differences in cis as well as trans-acting loci (Dubin et al. 2015, Kawakatsu et al. 2016,
696 but see Schmitz et al. 2013). Trans-acting loci make it difficult to separate genetics from
697 epigenetics in non-model species because they can alter large parts of the epigenome
698 despite being only a tiny fraction of the entire genome.
699 We further tested to which extent the genetic and epigenetic variation was related to
700 variation in phenotypic traits. For the genetic variation this was significant for at least one
701 phenotypic trait in four out of five species, including leaf thickness, plant height and SLA
702 (see Table 5). Epigenetic variation could significantly explain differences in phenotypic
703 traits, leaf thickness or SLA, in three out of five species. In all cases, these traits were
704 also significantly explained by genetic variation. Only for one species, *G. mollugo*, we
705 could not find any significant correlation between genetic or epigenetic variation and
706 phenotypic traits. When we tested for associations of genetic and epigenetic variation
707 with phenotypic traits in individual reference sequences, we could identify multiple
708 significant associations (see Table 6). Interestingly, here *G. mollugo* was the species with
709 the highest number of associations. The number of significant associations between
710 epigenetic variation and phenotypic traits were always much smaller than for genotypic
711 variation. However, given that epigenetic variation was fitted after genetic variation,
712 these remaining associations suggest that they were not linked to genetic variation and
713 thus that some phenotypic differences were indeed due to epigenetic variation.
714 We only know of one previous selection experiment with plants that found evidence for
715 epigenetic differentiation within genotypes after few generations in *Arabidopsis thaliana.*
716 In this study, DNA methylation could be assessed genome-wide in a genetically uniform

717 background. Thus, the authors were able to show that the selected epigenetic variation
718 was independent of genetic variation (Schmid *et al.* 2018a). However, clear evidence
719 from non-model plant species is still lacking and also the present analysis can only
720 indirectly provide it. Our results suggest that epigenetic differences mostly reflect genetic
721 differences and that the heritable phenotypic differences clearly have a genetic rather than
722 an epigenetic basis.
723 A caveat of the novel reference-free reduced representation bisulfite sequencing method
724 (van Gurp *et al.* 2016) is the low genome coverage (about 2 %). Thus, even if we had
725 found more epigenetic than genetic divergence, we could not have been certain that this
726 epigenetic divergence was unrelated to genetic divergence as we might have missed
727 genomic regions that contain genetic loci that control for methylation. Further,
728 methylation in the CHH context is often not transgenerational, even though it can be
729 environmentally induced. It is possible that we have under-studied this one type of
730 methylation that may influence phenotypes, given the sampling of only a small fraction
731 of the genome combined with its low heritability. Hence, even though reduced
732 representation sequencing approaches like epiGBS allow for high resolution estimates of
733 genetic and epigenetic divergence, these techniques cannot, unfortunately, provide
734 conclusive answers to the question whether the observed epigenetic variation has a
735 genetic basis or not. Full exploration of the evolutionary and ecological relevance of
736 epigenetic mechanisms may only be possible with whole-genome bisulfite sequencing
737 and for species with high-quality reference genomes (Niederhuth & Schmitz 2014;
738 Schmid *et al.* 2018a; Paun, Verhoeven, & Richards 2019), 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 observed between plant populations within several grassland species derived from the Jena Experiment, a long-term biodiversity field experiment (Zuppinger-Dingley *et al.* 2014, Hahl 2017, van Moorsel *et al.* 2018c) were caused largely by genetic divergence and additional some 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 novel reference-free reduced representation bisulfite sequencing method that was used to measure differences in genetic variation and levels and patterns of methylation, it was not possible to fully disentangle the genetic and epigenetic determinants of the observed rapid evolution in this grassland biodiversity experiment. Thus, despite much excitement about its potential consequences (Bossdorf *et al.* 2008, Jablonka & Raz 2009, Richards *et al.* 2010, Balao, Paun, & Alonso 2018), there is still a lack of clear evidence for the relative roles of genetic and epigenetic variation in rapid plant adaptation in nature.

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References

Balao, F., Paun, O., & Alonso, C. (2018). Uncovering the contribution of epigenetics to plant phenotypic variation in Mediterranean ecosystems. *Plant Biology, 20*, 38–49.

Balvanera, P., Pfisterer, A. B., Buchmann, N., He, J.-S., Nakashizuka, T., Raffaelli, D., & Schmid, B. (2006). Quantifying the evidence for biodiversity effects on ecosystem functioning and services. *Ecol Lett 9*, 1146–1156.

Bastolla, U., Fortuna, M., Pascual-García, A., Ferrera, A., Luque, B., & Bascompte, J. (2009). The architecture of mutualistic networks minimizes competition and increases biodiversity. *Nature*, *458*, 1018–1020.

Bell, G. & Gonzalez, A. (2009). Evolutionary rescue can prevent extinction following environmental change. *Ecol Lett, 12*, 942–948.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R STAT SOC B*, *57*, 289–300.

Bird, A. (2007). Perceptions of epigenetics. *Nature*, *447*, 396–398.

Bossdorf, O., Richards, C., & Pigliucci, M. (2008). Epigenetics for ecologists. *Ecol Lett*, *11*, 106–115.

Buchfink, D., Xie, C., & Huson, D. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat Methods*, *12*, 59.60.

Cadotte, M.W. (2017). Functional traits explain ecosystem function through opposing mechanisms. *Ecol Lett 29*, 989–996.

Cardinale, B., Wright, J., Cadotte, M., Carroll, I., Hector, A., Srivastava, D., … Weis, J. (2007). Impacts of plant diversity on biomass production increase through time because of species complementarity. *Proc Natl Acad Sci U S A*, *104*, 18123–18128.

Charlesworth, D., Barton, D.H. & Charlesworth, B. (2017). The sources of adaptive variation. *Proc. R. Soc. B, 284,* 20162864.

Cortijo, S., Wardenaar, R., Colomé-Tatcheé, M., Gilly, A., Etcheverry, M., Labadie, K., … Johannes, F. (2014). Mapping the epigenetic basis of complex traits. *Science*, *343*, 1145–1148.

Davis, M. B., Shaw R. G., & Etterson, J. R. (2005). Evolutionary responses to changing climate. *Ecology, 86(7),* 1704–1714.

Deans, C. & Maggert, K.A. (2015). What Do You Mean, "Epigenetic"? *Genetics*, *199*, 887–896.

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M.-S., Osborne, E. J., Casale, F. P., … Nordborg, M. (2015). DNA methylation in *Arabidopsis* has a genetic basis and shows evidence of local adaption. *eLIFE*, *4*, e05255.

Fargione, J., Tilman, D., Dybzinski, R., Lambers, J., Clark, C., Harpole, W., … Loreau, M. (2007). From selection to complementarity: Shifts in the causes of biodiversity-productivity relationships in a long-term biodiversity experiment. *P R Soc B*, *274*, 871–876.

807 Fischer, M.C, Foll, M., Excoffier, L. & Heckel, G. (2011). Enhanced AFLP genome
808 scans detect local adaptation in high-altitude populations of a small rodent (*Microtus*
809 *arvalis*). *Molecular Ecology, 20*, 1450–1462.

810 Foll, M. & Gaggiotti, O.E. (2008). A genome scan method to identify selected loci
811 appropriate for both dominant and codominant markers: A Bayesian perspective.
812 *Genetics,* 180, 977-993.

813 Gervasi, D., & Schiestl, F. (2017). Real-time divergent evolution in plants driven by
814 pollinators. *Nat Comm*, *8*, 14691.

815 Ghalambour, C., McKay, J., Carroll, S., & Reznick, D. (2007). Adaptive versus non-
816 adaptive phenotypic plasticity and the potential for contemporary adaptation in new
817 environments. *Funct Ecol*, *21*, 394–407.

818 Goudet, J., & Jombart, T. (2015). *Hierfstat: Estimation and tests of hierarchical F-*
819 *statistics.* Retrieved from https://CRAN.R-project.org/package=hierfstat

820 Goudet, J., Raymont, M., Meeûs, T., & Rousset, F. (1996). Testing differentiation in
821 diploid populations. *Genetics*, *144*, 1933–1940.

822 Gugger, P. F., Fitz-Gibbon, S., Pellegrini, M., & Sork, V. L. (2016). Species-wide
823 patterns of DNA methylation variation in *Quercus lobata* and their association with
824 climate gradients. *Molecular Ecology*, 25, 1665–1680.

825 Guimarães Jr, P.R., Pires, M., Jordano, P., Bascompte, J., & Thompson, J.N. (2017).
826 Indirect effects drive coevolution in mutualistic networks *Nature*, *550*, 511–514.

827 Gurp, T. van, Wagemaker, N., Wouters, B., Vergeer, P., Ouborg, J., & Verhoeven, K.
828 (2016). epiGBS: Reference-free reduced representation bisulfite sequencing. *Nat*
829 *Methods*, *13*, 322–234.

830 Groot, M.P., Wagemaker, N., Ouborg, N.J., Verhoeven, K.J.F., Vergeer, P. (2018)
831 Epigenetic population differentiation in field- and common garden-grown *Scabiosa*
832 *columbaria* plants. *Ecol Evol, 8(6)*, 3505–3517.

833 Hahl, T. (2017). Testing for co-adaptation of plants and soil organisms in a biodiversity
834 experiment. PhD Thesis. University of Zurich. Retrieved from Zurich Open Repository
835 and Archive: https://doi.org/10.5167/uzh-145156
836 Hairston, N., Ellner, S., Geber, M., Yoshida, T., & Fox, J. (2005). Rapid evolution and
837 the convergence of ecological and evolutionary time. *Ecol Lett*, *8*, 1114–1127.

838 Hendry, A.P. (2016) *Eco-evolutionary Dynamics*, Princeton, New Jersey, Princeton
839 University Press.

840 Hollister, J. D., Smith, L. M., Guo, Y. L., Ott, F., Weigel, D., & Gaut, B. S. (2011)
841 Transposable elements and small RNAs contribute to gene expression divergence
842 between *Arabidopsis thaliana* and *Arabidopsis lyrata*. *Proceedings of the National*
843 *Academy of Sciences*, *108*, 2322–2327.

844 Isbell, F., Craven, D., Connolly, J., Loreau, M., Schmid, B., Beierkuhnlein, C., …
845 Eisenhauer, N. (2015). Biodiversity increases the resistance of ecosystem productivity to
846 climate extremes. *Nature*, *526*, 574–577.

847 Jablonka, E., & Raz, G. (2009). Transgenerational Epigenetic Inheritance: Prevalence,
848 Mechanisms, and Implications for the Study of Heredity and Evolution. *The Quarterly*
849 *Review of Biology, 84*, 131–176.

850 Jombart, T. (2008). Adegenet: A R package for the multivariate analysis of genetic
851 markers. *Bioinformatics*, *24*, 1403–1405.

852 Joshi, J., Schmid, B., Caldeira, M., Dimitrakopoulos, P., Good, J., Harris, R., … Lawton,
853 J. (2001). Local adaptation enhances performance of common plant species. *Ecol Lett*, *4*,
854 536–544.

855 Kawakatsu, T., Huang, S.-S., Jupe, F., Sasaki, E., Schmitz, R., Urich, M., … Ecker, J.
856 (2016). Epigenomic diversity in a global collection of *Arabidopsis thaliana* accessions.
857 *Cell*, *166*, 492–505.

858 Kleynhans, E., Otto, S., Reich, P., & Vellend, M. (2016). Adaptation to elevated CO2 in
859 different biodiversity contexts. *Nat Comm*, *7*, 12358.

860 Kooke, R., Johannes, F., Wardenaar, R., Becker, F., Etcheverry, M., Colot, V., …
861 Keurentjes, J. (2015). Epigenetic basis of morphological variation and phenotypic
862 plasticity in *Arabidopsis thaliana*. *Plant Cell*, *27*, 337–348.

863 Latzel, V., Allan, E., Bortolini Silveira, A., Colot, V., Fischer, M., & Bossdorf, O.
864 (2013). Epigenetic diversity increases the productivity and stability of plant populations.
865 *Nat Comm*, *4*, 2875.

866 Law, J. A. & Jacobsen, S.E. (2010). Establishing, maintaining and modifying DNA
867 methylation patterns in plants and animals. *Nature Rev Gen 11(3),* 204–220.

868 Lind, M.I. & Spagopoulou, F. (2018). Evolutionary consequences of epigenetic
869 inheritance. *Heredity, 121*, 205–209.

870 Lipowsky, A., Schmid, B., & Roscher, C. (2011). Selection for monoculture and mixture
871 genotypes in a biodiversity experiment. *Basic Appl Ecol*, *12*, 360–371.

872 van der Maaten, L. (2014). Accelerating t-SNE using tree-based algorithms. *J Mach Lern*
873 *Res*, *15*, 1–21.

874 van der Maaten, L., & Hinton, G. (2008). Visualizing data using t-SNE. *J Mach Lern Res*,
875 *9*, 2579–2605.

876 Marquard, E., Weigelt, A., Temperton, V.M., Roscher, C., Schumacher, J., Buchmann,
877 N., Fischer, M., Weisser, W.W., & Schmid, B. (2009). Plant species richness and
878 functional composition drive overyielding in a 6-year grassland experiment. *Ecology, 90,*
879 3290–3302.

880 Meyer, S., Ebeling, A., Eisenhauer, N., Hertzog, L., Hillebrand, H., Milcu, A., …
881 Weisser, W. (2016). Effects of biodiversity strengthen over time as ecosystem
882 functioning declines at low and increases at high biodiversity. *Ecosphere*, *7*, e01619.

883 van Moorsel S.J., Hahl, T., Petchey, O., Ebeling, A., Eisenhauer, N., Schmid, B., &
884 Wagg, C. (2018a). Evolution increases ecosystem temporal stability and recovery from a
885 flood in grassland communities. *bioRxiv*. Retrieved from
886 https://dx.doi.org/10.1101/262337.

van Moorsel, S. van, Hahl, T., Wagg, C., De Deyn, G., Flynn, D., Zuppinger-Dingley, D., & Schmid, B. (2018b). Community evolution increases plant productivity at low diversity. *Ecol Lett*, *21*, 128–137.

van Moorsel, S., Schmid, M. W., Hahl, T., Zuppinger-Dingley, D., & Schmid, B. (2018c). Selection in response to community diversity alters plant performance and functional traits. *Perspect Plant Ecol Evol, 33*, 51–61.

Niederhuth, C. E. & Schmitz, R.J. (2014*).* Inheritance of DNA methylation in plant genomes. *Molecular Plant 7(3),* 472–480.

Niederhuth, C., Bewick, A., Ji, L., Alabady, M., Kim, K., Li, Q., … Schmitz, R. (2016). Widespread natural variation of DNA methylation within angiosperms. *Genome Biol*, *17*, 194.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., … McGlinn, D. (2017). Vegan: Community ecology package. Retrieved from https://CRAN.R-project.org/package=vegan.

Ouborg, N., Vergeer, P., & Mix, C. (2006). The rough edges of the conservation genetics paradigm for plants. *J Ecol*, *94*, 1233–1248.

Paun, O., Verhoeven, K. J., & Richards, C. L. (2019). Opportunities and limitations of reduced representation bisulfite sequencing in plant ecological epigenomics. *New Phytologist*, 221, 738–742.

Park, Y., & Wu, H. (2016). Differential methylation analysis for BS-seq data under general experimental design. *Bioinformatics*, *32*, 1446–1453.

Quadrana, L., & Colot, V. (2016). Plant transgenerational epigenetics. *Annu Rev Genet*, *50*, 467–491.

Ravenscroft, C., Whitlock, R., & Fridley, J. (2015). Rapid genetic divergence in response to 15 years of simulated climate change. *Glob Change Biol*, *21*, 4165–4176.

Reich, P., Tilman, D., Isbell, F., Mueller, K., Hobbie, S., Flynn, D., & Eisenhauer, N. (2012). Impacts of biodiversity loss escalate through time as redundancy fades. *Science*, *336*, 589–592.

Richards, C., Bossdorf, O., & Verhoeven, K. (2010) Understanding natural epigenetic variation. *New Phytologist*, 187, 562–564.

Richards, C., Alonso, C., Becker, C., Bossdorf, O., Bucher, E., Colomé-Tatché, M., … Verhoeven, K. (2017). Ecological plant epigenetics: Evidence from model and non-model species, and the way forward. *Ecol Lett*, *20*, 1576–1590.

Riggs, A.D., Russe, V.E.A., & Martienssen, R.A. (1996) Epigenetic Mechanisms of Gene Regulation. *Cold Spring Harbour Monographs*.

Roach, D.A. & Wulff, R.D. (1987). Maternal effects in plants. *Annual Review of Ecology and Systematics, 18,* 209–235.

Roscher, C., Schumacher, J., Baade, J., Wilcke, W., Gleixner, W., Weisser, W., … Schulze, E.-D. (2004). The role of biodiversity for element cycling and trophic

interactions: An experimental approach in a grassland community. *Basic Appl Ecol*, *5*, 107–121.

Roscher, C., Schumacher, J., Schmid, B., & Schulze, E.-D. (2015). Contrasting effects of intraspecific trait variation on trait-based niches and performance of legumes in plant mixtures. *PLOS ONE, 10, e01197986*.

Rottstock, T., Kummer, V., Fischer, M., & Joshi, J. (2017). Rapid transgenerational effects in *Knautia arvensis* in response to plant community diversity. *J Ecol*, *105*, 714–725.

Schmid, B. (1985). Clonal growth in grassland perennials: III. genetic variation and plasticity between and within populations of *Bellis perennis* and *Prunella vulgaris*. *J Ecol*, *73*, 819–830.

Schmid, B. & Bazzaz, F.A. (1990). Plasticity in plant size and architecture in rhizome-derived vs. seed-derived *Solidago* and *Aster*. *Ecology, 71,* 523–535.

Schmid, B., Birrer, A., & Lavigne, C. (1996). Genetic variation in the response of plant populations to elevated CO2 in a nutrient-poor, calcareous grassland. In: Körner, C., Bazzaz, F.A. (Eds.), Carbon dioxide, populations, and communities. Academic Press, San Diego, California, pp. 31-50.

Schmid, M. W. (2017). RNA-Seq data analysis protocol: Combining in-house and publicly available data. In A. Schmidt (Ed.), *Plant germline development: Methods and protocols* (pp. 309–335). New York, NY: Springer New York.

Schmid, M. W., Heichinger, C., Coman Schmid, D. Guthörl, D., Gagliardini, V., Bruggmann, R., … Grossniklaus, U. (2018a). Contribution of epigenetic variation to adaptation in *Arabidopsis*. *Nat Commun, 9*: 4446.

Schmid, M. W., Giraldo-Fonseca, A., Smetanin, D., & Grossniklaus, U. (2018b). Extensive epigenetic reprogramming during the life cycle of *Marchantia polymorpha*. *Genome Biol*, *19*, 9.

Schmitz, R.J., Schultz, M.D., Urich, M.A., Nery, J.R., Pelizzola, M., Libiger, O., … Ecker, J. (2013). Patterns of population epigenomic diversity. *Nature 495(7440),* 193–198.

Schoener, T. (2011). The newest synthesis: Understanding the interplay of evolutionary and ecological dynamics. *Science*, *331*, 426–429.

Slobodkin, L. (1961). *Growth and regulation of animal populations*. Holt, Rinehart; Winston, New York.

Smit, A. F. A., Hubley, R., & Green, P. (2013–2015). *RepeatMasker Open-4.0*. Retrieved from http://www.repeatmasker.org/

The Chimpanzee Sequencing and Analysis Consortium. (2005). Initial sequence of the chimpanzee genome and comparison with the human genome. *Nature*, *437*, 69–87.

Tilman, D., & Snell-Rood, E. (2014). Ecology: Diversity breeds complementarity. *Nature*, *515*, 44–45.

Verhoeven, K. J., Vonholdt, B. M., & Sork, V. L. (2016). Epigenetics in ecology and evolution: what we know and what we need to know. *Molecular Ecology*, 25, 1631–1638.

Weisser, W. W., Roscher, C., Meyer, S. T., Ebeling, A., Luo, G., Allan, E., … Eisenhauer, N. (2017). Biodiversity effects on ecosystem functioning in a 15-year grassland experiment: Patterns, mechanisms, and open questions. *Basic and Applied Ecology*, *23(Supplement C)*, 1–73.

Zilberman, D. (2017). An evolutionary case for functional gene body methylation in plants and animals. *Genome Biol* 18:87.

Zuppinger-Dingley, D., Schmid, B., Petermann, J., Yadav, V., De Deyn, G., & Flynn, D. (2014). Selection for niche differentiation in plant communities increases biodiversity effects. *Nature*, *515*, 108–111.

Data accessibility

Data is available on Zenodo (DOI 10.5281/zenodo.1167563) and SRA (accession ID SRP132258).

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 analysed 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	

999

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:SH and SNP & CTXT: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: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:SNP	1.40	1.01	6.69	5.10	1.30	3.10
SNP & CTXT:SNP	3.86	1.19	7.64	9.54	1.89	4.82
SH & CTXT: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: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:SH	0.30	0.02	0.32	0.68	1.42	0.55
SNP & CTXT:SNP	4.37	1.42	7.64	10.19	2.78	5.28
SH & CTXT:SH	0.85	0.04	0.32	1.01	2.01	0.85

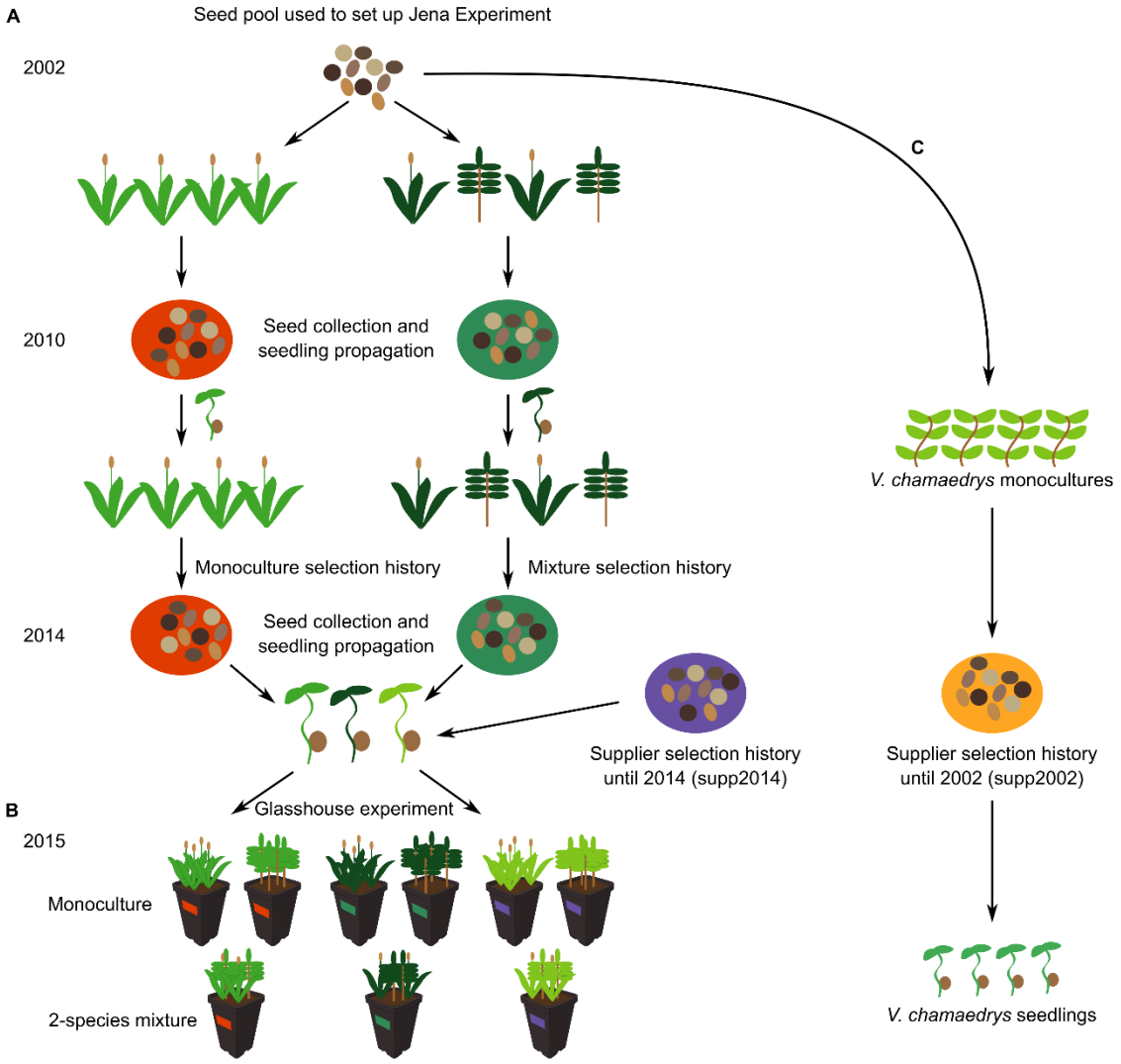
Table 5. Coefficients of determination (R^2) from multivariate ANOVAs to test whether phenotypic traits could explain genetic and epigenetic variation. Only significant ($P < 0.05$) results are shown. n.s.: not significant.

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.

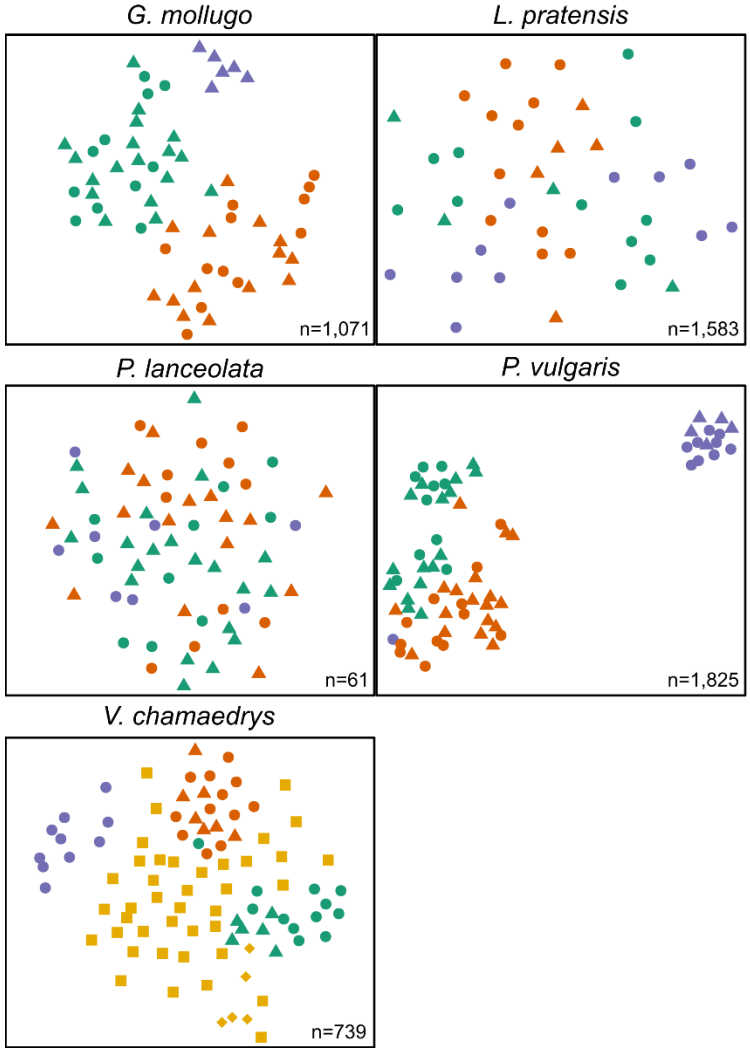


Figure 2. Genetic distance between individuals of the different populations for the five species. Green: selection history in mixture, orange: selection history in monocultures, blue: selection history in the field of the original seed supplier, seeds bought in 2014 (supp2014), yellow: offspring from 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. Assembly refers to the diversity level in the glasshouse. Note that t-SNE projection axes are arbitrary and dimensions are therefore not shown.

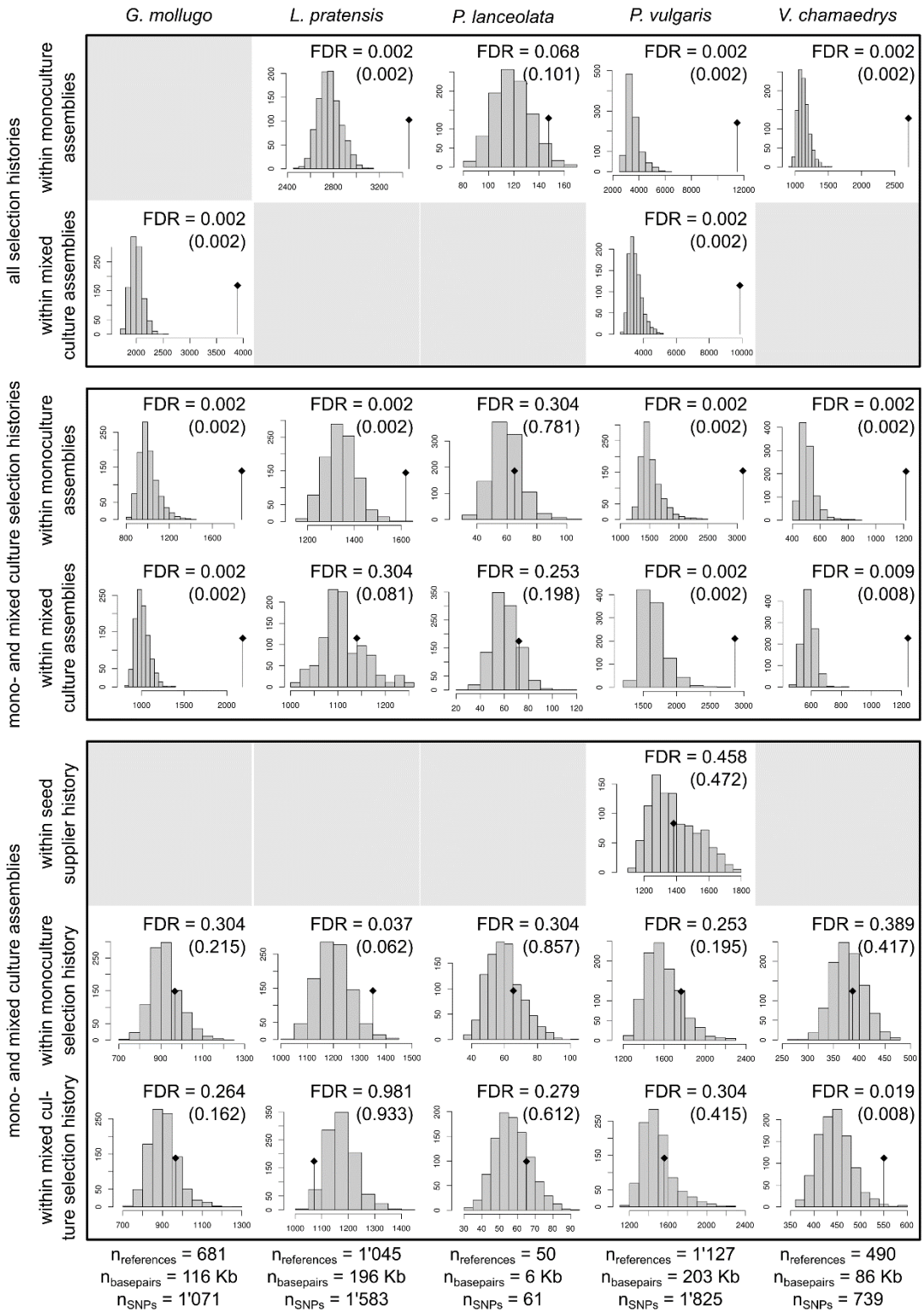


Figure 3. Results from the G-statistic tests given all SNPs. Each panel shows a histogram of permuted test statistics (999 permutations) and indicates the observed statistics by a

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). Numbers in parentheses correspond to FDRs of the same test using all reference sequences, including sequences with a SNP rate greater than 2 %.

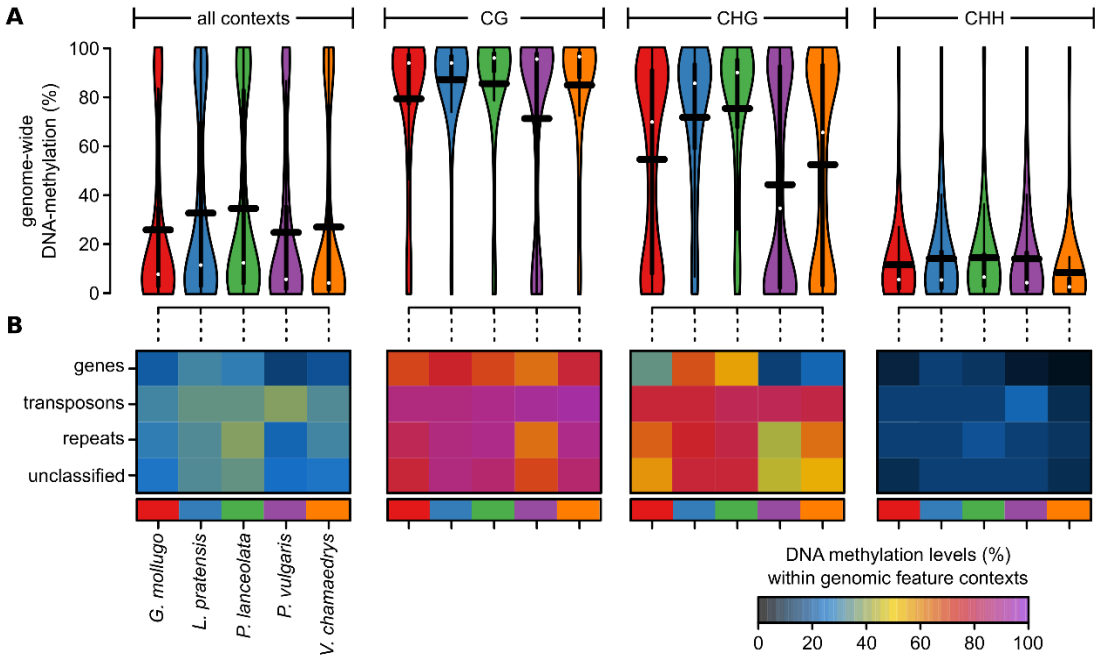
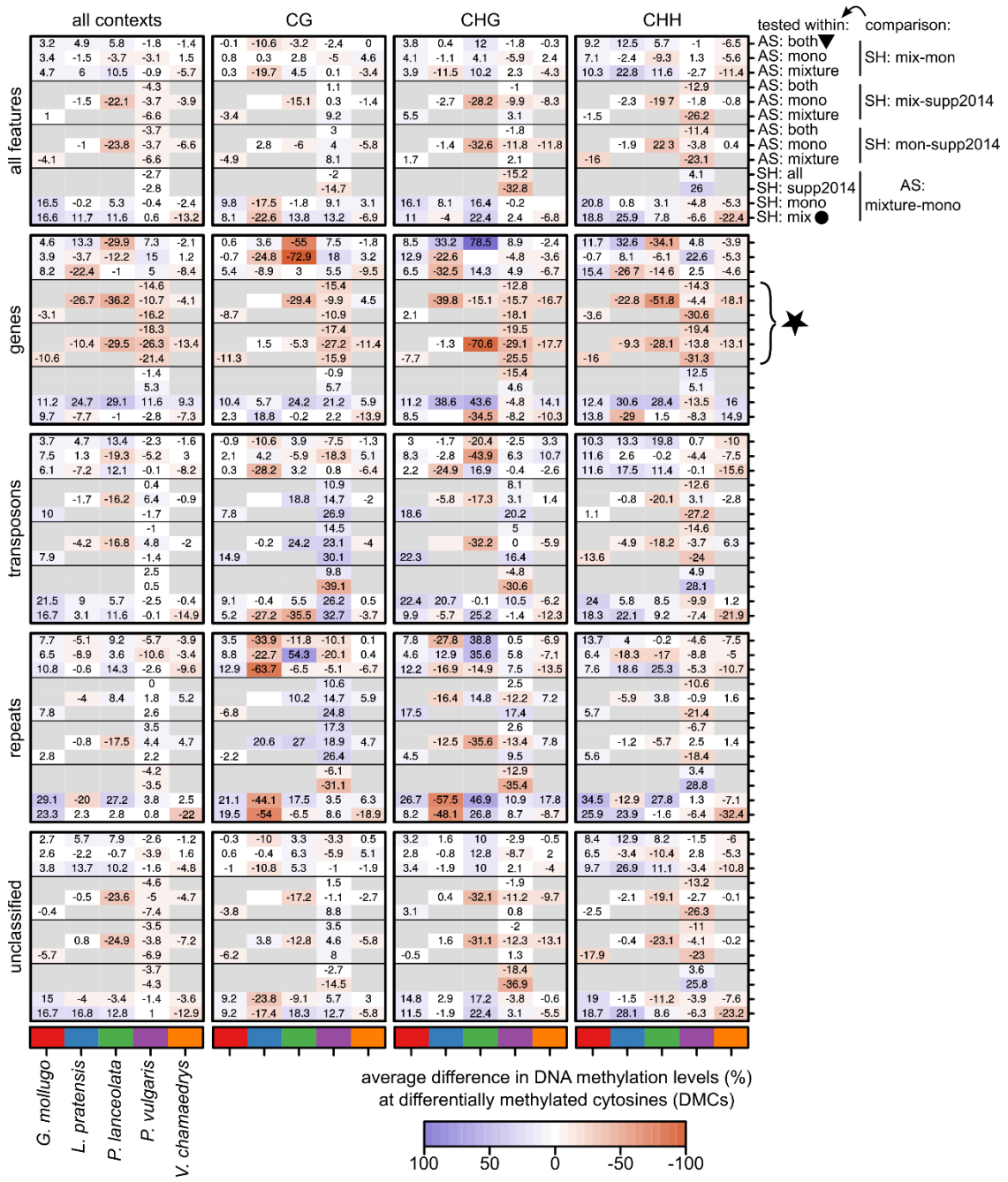


Figure 4. (A) DNA methylation levels in percent at individual cytosines across all or within each individual sequence context (CG, CHG, CHH) for each species used in this study shown as violin plots. The horizontal black bars correspond to the means. (B) Average DNA methylation levels in percent for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



1086

1087 **Figure 5.** Average differences in DNA methylation at significantly differentially
1088 methylated cytosines (DMCs; FDR < 0.01) within a given sequence (all, CG, CHG, and
1089 CHH) and feature context (all, genes, transposons, repeats, unclassified) are shown for all
1090 contrasts. The comparisons and within which levels of the other factor they were tested
1091 are given in the first panel row on the right (same for all features). AS: assembly, PH:
1092 selection history. For example, the row marked with a triangle corresponds to the
1093 comparison between mixture and monoculture selection histories across both
1094 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).

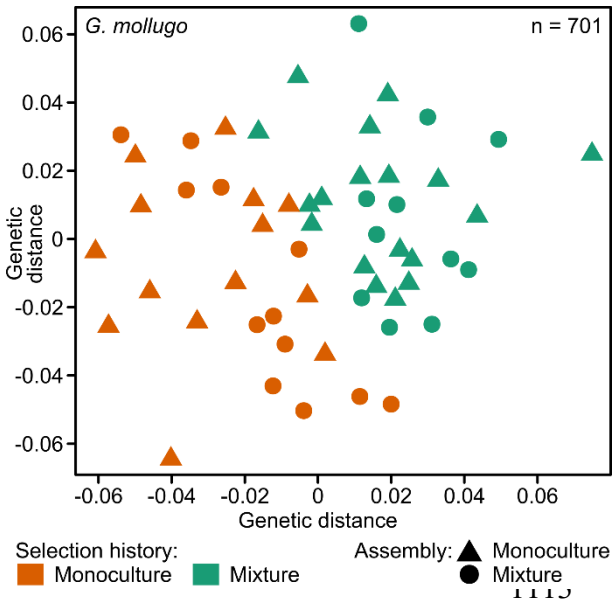


Figure 6. Genetic distance between the 701 reference sequences that were significantly (FDR < 0.05) associated with the phenotype in *G. mollugo*. Selection histories in this analysis were limited to the two histories in the Jena Experiment (monoculture and mixture). Distances were visualized with the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

Supplemental information

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