Evidence for rapid evolution in a grassland biodiversity experiment

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Abstract

Biodiversity often increases plant productivity. In long-term grassland experiments, positive biodiversity effects on plant productivity commonly increase with time. Also, it has been shown that such 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 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*, with epigenetic 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 this epigenetic divergence 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, epiGBS, genetic divergence, epigenetic variation, herbaceous plant species, selection

1. Introduction

Environmental change may result in significant climate-induced range shifts, forcing plant populations into new abiotic and biotic environments (Ouborg, Vergeer, & Mix 2006). The unprecedented rate of environmental change raises the question whether adaptation of natural communities to novel abiotic or biotic conditions is able to match these rates. Biodiversity is known 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 conditions (e.g., Joshi *et al.* 2001) and biotic interactions such as pollinators (e.g., Gervasi & Schiestl 2017) are well studied. Unfortunately, little effort has been devoted to the influence of community diversity on fast evolutionary adaptation (but see Lipowsky, Schmid, & Roscher 2011, Kleynhans, Otto, Reich, & Vellend 2016, van Moorsel *et al.* 2018b). In particular, potential effects of multispecies interactions on the adaptive response of plant species are largely unknown, despite a growing body of evidence showing the importance of multispecies interactions for ecosystem stability (Bastolla *et al.* 2009, Guimarães, Pires, Jordano, Bascompte, & Thompson 2017). It is conceivable that the feedback between

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

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

73 Long-term biodiversity field experiments offer unique opportunities to study effects of
74 community diversity and composition on natural selection. Species mixtures are
75 frequently more productive than average monocultures (Balvanera *et al.* 2006).
76 Interestingly, these biodiversity effects often become more pronounced over time, which
77 has been attributed to increased complementarity among species (Cardinale *et al.* 2007,
78 Fargione *et al.* 2007, Reich *et al.* 2012, Meyer *et al.* 2016). It was suggested that
79 increased complementarity may originate from evenly distributed resource depletion in
80 mixtures or negative plant–soil feedbacks developing in monocultures (Fargione *et al.*
81 2007). Moreover, increased complementarity was also attributed to phenotypic plasticity
82 (Ghalambour, McKay, Carroll, & Reznick 2007) or selection of genotypes that have an
83 advantage to grow in mixtures, i.e., "mixture-type plants". Indeed, recent common-
84 environment experiments with plant material from a grassland biodiversity experiment
85 (the Jena Experiment, Roscher *et al.* 2004) suggest that increased biodiversity effects
86 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, indicating that community composition can lead to phenotypic trans-generational effects (Zuppinger-Dingley *et al.* 2014, Rottstock, Kummer, Fischer, & Joshi 2017, van Moorsel *et al.* 2018b). However, it remains unclear whether the trans-generational effects observed in these studies were due to genetic differentiation, epigenetic differences or (possibly epigenetically-induced) maternal effects (Tilman & Snell-Rood 2014).

While phenotypic changes have been widely linked to genetic variation, an increasing body of evidence suggests epigenetic mechanisms to play an important role in phenotypic variation, and hence ecological processes (e.g., Bird 2007, Bossdorf, Richards, & Pigliucci 2008, Niederhuth & Schmitz 2014, Verhoeven, Vonholdt, & Sork 2016). 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). Schmitz et al. (2013) further found evidence for epigenomic diversity which was potentially independent of genetic diversity in natural *Arabidopsis thaliana* successions. However, the importance of epigenetics in natural populations, in particular of non-model species, and whether it contributes to adaptation remains elusive (Quadrana & Colot, 2016, Richards *et al.* 2017, Groot *et al.* 2018). For example, Dubin et al. (2015) found that differences in DNA methylation between natural populations of *A. thaliana* were largely due to *trans*-acting loci, many of which showed evidence of local adaptation. 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. In an earlier experiment by van Moorsel *et al.* (2018b), phenotypic differences between offspring from plants that were selected in mixtures versus monocultures in the Jena Experiment were recorded when reciprocally grown in monocultures or mixtures. In the present study, we hypothesize that these 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). To assess epigenetic variation across plant individuals, we looked at levels of DNA cytosine methylation, which is a well-studied inheritable epigenetic modification involved in a large number of biological processes (Law & Jacobsen, 2010).

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 (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 for subsequent propagation and reciprocal treatments.

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). Although this does not guarantee close similarity with the original seed pool that was used at the start of the Jena Experiment, it does provide good material to test the difference between plants that have grown in the Jena Experiment and those that experienced different selective environments.

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)

154 monoculture in the fields of the seed supplier until 2014 and 4) monoculture in the fields
155 of the seed supplier until 2002 (only for *V. chamaedrys*). Histories 3) and 4) will be
156 abbreviated as supp2014 and supp2002 (Tab. S1).

157 **2.2. Seed collection in monoculture and mixture histories**

158 Given that all plant species used in the study are perennial plants, it is possible that they
159 reproduced mostly vegetative in the field. Therefore, plants with a selection history in
160 either mixture or monoculture in the Jena Experiment underwent two controlled
161 reproductive cycles in 2010 and 2014. This additional step aimed to increase the genetic
162 diversity, which can then be subject to selection, and to reduce maternal effects and
163 carry-overs of somatic epigenetic marks.

164 In spring 2010, cuttings from all plant communities were collected and transplanted to an
165 experimental garden in Zurich, Switzerland, in an identical plant composition as in the
166 Jena Experiment, for the first controlled pollination and seed production (see also
167 Zuppinger-Dingley *et al.* 2014). In spring 2011, the seedlings produced from these seeds
168 were transplanted back to the same plots of the Jena Experiment from where the parents
169 had originally been collected and in the same community composition (see Tab. S2 for
170 the community compositions of the plots in the Jena Experiment). In March 2014, plant
171 communities of the plots that were re-established in 2011 in the Jena Experiment were
172 again transferred to the experimental garden in Zurich for the second controlled
173 pollination and seed production. For each experimental plot, we excavated several blocks
174 of soil including the entire vegetation (in total one square meter). These blocks were then
175 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. During the controlled pollination and seed production plots were surrounded by nets and only left open on top to allow pollinator access. This did not fully exclude the possibility of cross-pollination between plots containing different plant communities, and such cross-pollination may 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 includes 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

198 planted as monocultures of four individuals or two-species mixtures (2 x 2 individuals)
199 into 2-L pots filled with agricultural soil (50 % agricultural sugar beet soil, 25 % perlite,
200 25 % sand; Ricoter AG, Aarberg, Switzerland). Species pairs in the mixtures were chosen
201 according to seedling availability and single pots always contained four plants of the
202 same selection history (i.e., there was no competition between different selection
203 histories).

204 The experiment was replicated in six blocks, each including the full experimental design.
205 Within each block, pots were placed on three different tables in the glasshouse at random
206 without reference to selection history or assembly treatment. During the experiment the
207 pots were not moved. The plants were initially kept at day temperatures of 17–20 °C and
208 night temperatures of 13–17°C without supplemental light. To compensate for
209 overheating in summer, an adiabatic cooling system (Airwatech; Bern, Switzerland) was
210 used to keep inside temperatures constant with outside air temperatures.

211 **2.3.2. Phenotype measurements**

212 The following traits were measured: plant height, leaf thickness, specific leaf area (SLA)
213 and aboveground biomass. These traits were shown to relate to competitive growth and
214 affect plant community productivity in biodiversity experiments (Roscher *et al.* 2015,
215 Cadotte 2017). All traits were measured after twelve weeks from 18 May to 4 June 2015.
216 Leaf thickness was measured for three representative leaves using a thickness gauge.
217 Specific leaf area (SLA) of up to 20 representative leaves (depending on the leaf size of
218 the species) of each species in a pot was measured by scanning fresh leaves with a Li-
219 3100 Area Meter (Li-cor Inc., Lincoln, Nebraska, USA) immediately after harvest and

220 determining the mass of the same leaves after drying. All four individuals in a pot were
221 sampled.

222 **2.3.3. Sampling of plant material**

223 Samples for epigenetic and genetic analysis were harvested between 18 and 28 May
224 2015, after twelve weeks of plant growth in the glasshouse. We chose to sequence all
225 individuals from the first three experimental blocks. All four plants were sampled in each
226 pot. One young leaf per plant was cut from the living plant and immediately shock-frozen
227 in liquid nitrogen. The samples were then stored at –80 °C until further analysis.

228 **2.4. Offspring of the original seed pool (fourth selection history)**

229 For the species *V. chamaedrys*, seeds from offspring of the original seed pool used to set
230 up the Jena Experiment (supp2002) were stored since 2002 at –20 °C and germinated in
231 the glasshouse as described above. Seedlings were then transferred to an experimental
232 garden and seeds were collected one year later. The additional generation in the
233 experimental garden was used to overcome potential maternal effects due to the old age
234 of the stored seeds. The collected seeds were then stored at 5 °C, transported to Nijmegen
235 and germinated in the glasshouse of Radboud University Nijmegen. Individual plants
236 were grown in individual 2 x 2-cm squares in a potting tray filled with a potting soil
237 consisting of “Lentse potgrond” (www.lentsepotgrond.nl) under natural light conditions
238 (16/8 hrs. day/night). No cold treatment or vernalisation was applied for germination.
239 Individual plants were harvested and quick frozen in liquid nitrogen after 5 weeks of
240 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). 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. 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 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. 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. 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

353 combination per species, we visualized the DNA methylation levels of all cytosines
354 averaged across all individuals with violin plots. We also visualized the average DNA
355 methylation level within genes, transposons, repeats and unclassified reference contigs
356 with heatmaps. Both was done either using all sequence contexts (CG, CHG, CHH) at
357 once or separately for each sequence context.

358 **2.8.2. Identification of differentially methylated cytosines (DMCs)**

359 DNA methylation data were processed and filtered as described above. Variation in DNA
360 methylation at each individual cytosine was then analysed with a linear model in R with
361 the package DSS (version 2.24.0; Y. Park & Wu (2016)), according to a design with a
362 single factor comprising all different experimental treatment combinations as separate
363 levels and using contrasts to compare levels of interest (similar to the approach described
364 for RNA-Seq in Schmid 2017 and the testing procedure described in Schmid, Giraldo-
365 Fonseca, Smetanin & Grossniklaus 2018b). Specific groups were compared with linear
366 contrasts and *P*-values for each contrast were adjusted for multiple testing to reflect false
367 discovery rates (FDR, Benjamini & Hochberg 1995). Taking *P. vulgaris* as an example,
368 we compared the three selection histories across both assemblies and within each
369 assembly to each other. Likewise, we compared the two assemblies across all selection
370 histories and within each selection history to each other. A cytosine was defined as
371 differentially methylated ("DMC", see also Schmid *et al*. 2018a) if the FDR was below
372 0.01 for any of the contrasts.

373 **2.9. Correlation between genetic and epigenetic data**

374 **2.9.1. Overall correlation**

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

385 **2.9.2. Linkage of genetic and epigenetic variation**

386 To test how much of the genetic differentiation could be attributed to selection history,
387 and, subsequently, how much of the epigenetic (methylation) variation was associated
388 with selection history after controlling for differences in genetic structure that may have
389 been induced by the selection histories, we modelled the average DNA methylation level
390 of a given reference sequence in response to the sequence context (CTXT), the assembly
391 treatment (AS), the genotype of the reference sequence (SNP), the interaction between
392 the sequence context and the genotype (CTXT:SNP) and the selection history (SH) fitted
393 in this order (percent methylation ~ CTXT + AS + SNP + CTXT:SNP + SH +
394 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). Note that because of different distribution and testing procedure, results from this model with an average level of DNA methylation across several cytosines cannot be directly compared with the results from the model used to test for differential DNA methylation at individual cytosines. This model can detect dependency of epigenetic variation on genetic variation within our reference contigs with a maximal size of 250 bp. Most associations between DNA sequence variation and methylation loci decay at relatively short distances (i.e., after 200 bp in *A. thaliana* or 1 kb in *A. lyrata*; Hollister *et al.* 2010). This model may thus provide good proxy for close-*cis* associations (close to each other at the same location in the genome, i.e., close enough to be on the same 250 bp reference sequence). However, far-*cis* associations (for example a transposon insertion variant which is close to the place of origin of the reference sequence but not represented in the reference sequence, i.e., too far to be on the same 250 bp reference sequence) or *trans* dependencies (effects from other loci that are not linked

418 to the place of origin of the reference sequence) cannot be detected. As a result, by using
419 this model, we may have potentially overestimated the proportion of epigenetic variation
420 that is unlinked to genetic variation.

421 **2.10. Relation between genotype/epigenotype and phenotype**

422 **2.10.1. Overall correlation**

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

430 **2.10.2. Association of genotypes/epigenotypes with phenotypes**

431 To test whether individual reference sequences correlated with phenotypic variation, we
432 separately modelled the variation in the four phenotypic traits (biomass, height, leaf
433 thickness and SLA) in response to the genotype (SNP) and the percent DNA methylation
434 (METH) for a given sequence context with the same data previously used to test linkage
435 of genetic and epigenetic variation (see section 2.9.2. above). Models were calculated
436 with the function lm() and anova() in R (version 3.5.1). We tested both fitting orders with
437 either SNP or METH fitted first. Hence, the formulas were TRAIT ~ SNP + METH and
438 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 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

484 comparisons between the supp2002 plants and the other populations than among those
485 (i.e., supp2014, monoculture and mixture histories populations, Tab. 1, S5 and S6). This
486 confirmed the previous observation that supp2002 individuals, which could be considered
487 as "parental" to the others, were genetically intermediate between the other selection
488 histories (Fig. 2).

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

501 **3.2. Epigenetic variation**

502 To get an overview of the DNA methylation data, we visualized DNA methylation levels
503 in percent at individual cytosines for each plant species, sequence context (CG, CHG,
504 CHH) and genomic feature context (genes, transposons, repeats and unclassified contigs,
505 Fig. 4). For all species, DNA methylation was generally highest in the CG context
506 (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, Gugger *et al*. 2016, Niederhuth *et al.* 2016, Paun, Verhoeven, & Richards 2019).

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 0.05% and 0.21% on average across all species and between 0.05% and 0.40% per

529 individual species. Thus, the fraction of differentially methylated cytosines between the
530 selection histories was generally larger than differences between the two assemblies.

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

541 To further characterize the differences in DNA methylation, we calculated the average
542 change in DNA methylation at the DMCs for each contrast, across and within all
543 sequence contexts (CG, CHG and CHH) and feature types (genes, transposons, repeats
544 and unclassified) and visualized these differences (Fig. 5). We could not identify clear
545 patterns between the different comparisons with one exception: differences in the
546 comparisons between plants from Jena and the supp2014 plants within genes (all
547 sequence contexts) were mostly biased towards a higher methylation in the supp2014
548 plants. Thus, plants in the Jena Experiment showed an overall loss of DNA methylation
549 at DMCs within genes. However, it remains unclear what functional consequences this
550 might have had because the function of gene body methylation remains to be elucidated
551 (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 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

574 methylation level even if an explanatory term for genotype (SNP, see section 2.9.2) was
575 fitted first. We compared this to a model with the opposite fitting order (Tab. 4). If
576 selection history was fitted first, its model terms SH and CTXT:SH were significant in
577 2.01 % of all reference sequences (average across species). However, if fitted after SNP,
578 the effect of selection history was only significant in 0.85 % of all cases. This varied
579 between species. For example, almost no significant effects of selection history were
580 found in *L. pratensis* (2 out of 5,554 reference sequences) and *P. lanceolata* (1 out of 314
581 reference sequences) whereas up to 2.01 % of the reference sequences of *V. chamaedrys*
582 exhibited a significant effect of selection history on DNA methylation after fitting the
583 explanatory term for genotype first. Hence, overall and at most individual reference
584 sequences, epigenetic variation was likely linked to genetic variation. Nonetheless, in up
585 to 2.01 % of the reference sequences of individual species, genetic variation on the same
586 reference sequence could not explain epigenetic variation.

587 **3.4. Relation between genetic/epigenetic variation and phenotype**

588 To assess the relation between genetic or epigenetic variation and phenotypic variation,
589 we tested whether phenotypic traits could explain the genetic and epigenetic distances
590 between individuals (Tab. 5). Except for *G. mollugo*, all species had at least one
591 phenotypic trait that could significantly ($P < 0.05$) explain genetic and sometimes also
592 epigenetic differences. For example, leaf thickness was significant in *L. pratensis* and
593 SLA was significant in *P. vulgaris* and *V. chamaedrys*. However, the coefficients of
594 determination (R^2) were with 0.02 to 0.06 relatively low, indicating that only a small
595 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 (i.e., epiGBS; van Gurp *et al*. 2016).

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 in models that already included epigenetic variation (Tab. 6). Hence, in line with

619 the previous results, genetic and epigenetic variation that were significantly associated to
620 phenotypic traits were both also well correlated with each other.

621 **4. Discussion**

622 For three out of five test species, namely *G. mollugo, P. vulgaris* and *V. chamaedrys*, we
623 found genetic differences between monoculture and mixture types in a large number of
624 SNPs. In a fourth species, *L. pratensis*, we found evidence for genetic divergence among
625 plants grown in monoculture assemblies in the glasshouse. The comparison, however,
626 was insignificant for plants grown in mixture assemblies, as we could only test nine
627 individuals in total. In the fifth species, *P. lanceolata*, we could not identify significant
628 genetic differentiation between plants with different selection histories. This finding was
629 unexpected because *P. lanceolata* has recently been shown to exhibit clear genetic
630 divergence after 15 years of simulated climate change (Ravenscroft, Whitlock & Fridley,
631 2015). It is conceivable that we could not detect genetic divergence in *P. lanceolata*
632 because of the low number of reference sequences that passed our filter: there were only
633 50 sequences corresponding to 6 kb and 61 SNPs left. Thus, we might have missed
634 regions under selection.

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

639 Besides the genetic divergence, we could also identify differences in methylation levels
640 between the selection histories, which were generally below 1% of all tested cytosines.

For *V. chamaedrys*, we observed pronounced differences in methylation levels 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 differences in methylation levels 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 differences 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, variation in methylation levels 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 provides evidence for epigenetic divergence between selection histories that is independent and additional to genetic divergence, 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 associations 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, *G. mollugo* exhibited the highest number of associations, even though 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.

A previous selection experiment with plants 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 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 had found more epigenetic than genetic divergence, we could not have been certain that this epigenetic divergence was unrelated to genetic divergence as we may have missed genomic regions that contain genetic loci that control for methylation. For example, in genome-wide studies with single base-pair resolution in *A. thaliana,* which revealed extensive epigenetic variation between different populations and accession, most of this variation was linked to underlying genetic differences in *cis* as well as *trans*-acting loci (Dubin *et al.* 2015,

687 Kawakatsu *et al.* 2016, but see Schmitz et al. 2013). Such *trans*-acting loci make it
688 difficult to separate genetics from epigenetics in non-model species because few such loci
689 can alter large parts of the epigenome despite being only a tiny fraction of the entire
690 genome. Hence, even though reduced representation sequencing approaches like epiGBS
691 allow for high resolution estimates of genetic and epigenetic divergence, these techniques
692 cannot, unfortunately, provide conclusive answers to the question whether the observed
693 epigenetic variation has a genetic basis or not. Full exploration of the evolutionary and
694 ecological relevance of epigenetic mechanisms may only be possible with whole-genome
695 bisulfite sequencing and for species with high quality reference genomes (Niederhuth &
696 Schmitz 2014; Schmid *et al.* 2018a; Paun, Verhoeven, & Richards 2019), which currently
697 is still restricting more conclusive tests of how epigenetic variation can influence plant
698 adaptation to natural selection.

699 **5. Conclusion**

700 Our study supports the hypothesis that the phenotypic differences observed between plant
701 populations within several grassland species derived from the Jena Experiment, a long-
702 term biodiversity field experiment (Zuppinger-Dingley *et al.* 2014, Hahl 2017, van
703 Moorsel *et al.* 2018c) were caused by genetic divergence and additional epigenetic
704 divergence. This suggests that these species can evolve rapidly in response to their biotic
705 environment, i.e. monoculture or mixed-species communities. However, due to
706 limitations of the novel reference-free reduced representation bisulfite sequencing method
707 that was used to measure differences in genetic variation and levels and patterns of
708 methylation, it was not possible to fully disentangle the genetic and epigenetic

709 determinants of the observed rapid evolution in this grassland biodiversity experiment.
710 Thus, despite much excitement about its potential consequences (Bossdorf *et al.* 2008,
711 Jablonka & Raz 2009, Richards *et al.* 2010, Balao, Paun, & Alonso 2018), there is still a
712 lack of clear evidence for the relative roles of genetic and epigenetic variation in rapid
713 plant adaptation in nature. Further progress will be possible once more high-quality
714 reference genomes become available to enable ecologically relevant experiments with
715 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 analysed all data and produced the figures. S.J.V.M and M.W.S. wrote the manuscript with contributions from all authors.

916

917 **Tables**

918 **Table 1** 99th percentile of F_{ST} values in the data set with all SNPs. AS, assembly, SH,
919 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

920

921

922 **Table 2** Number of cytosines with significant differences (FDR < 0.01) in DNA
923 methylation between selection-history treatments and assemblies. AS, assembly, SH,
924 selection history. For data on separate sequence contexts see Tab. S7 (CG), S8 (CHG),
925 and S9 (CHH). For the results of the comparisons with the supp2002 plants (*V.*
926 *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	

927

928 **Table 3.** Correlation between genetic and epigenetic variation (Pearson correlation
929 coefficients of distance matrices). Non-significant correlations (Mantel test, FDR ≥ 0.05)
930 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

931

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

939

940 **Table 5.** Coefficients of determination (R^2) from multivariate ANOVAs to test whether
941 phenotypic traits could explain genetic and epigenetic variation. Only significant ($P <$
942 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.

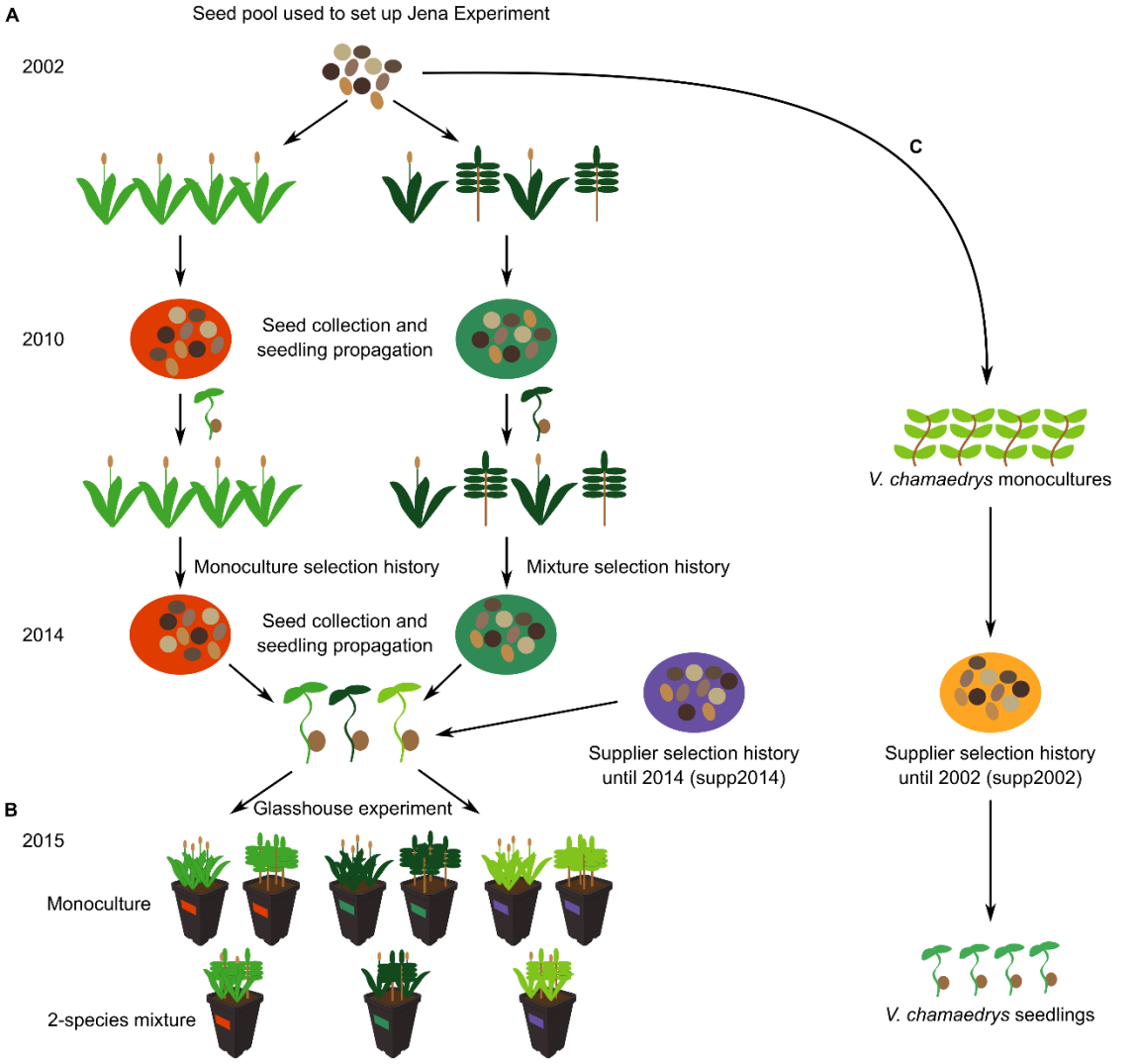
	CHH meth.	n.s.	n.s.	0.022	n.s.
V. chamaedrys	Genetic	n.s.	n.s.	0.059	n.s.
	CG meth.	n.s.	n.s.	0.036	n.s.
	CHG meth.	n.s.	n.s.	0.033	n.s.
	CHH meth.	n.s.	n.s.	0.029	n.s.

943

944 **Table 6.** Number of reference sequences with patterns of genetic (top) or epigenetic
945 (bottom) variation that are significantly (FDR < 0.05) associated with phenotypic traits.
946 The genotype (SNP) was fitted prior to the epigenotype (percent DNA methylation).
947 Numbers in parenthesis correspond to the model with the inverted fitting order (TRAIT ~
948 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.

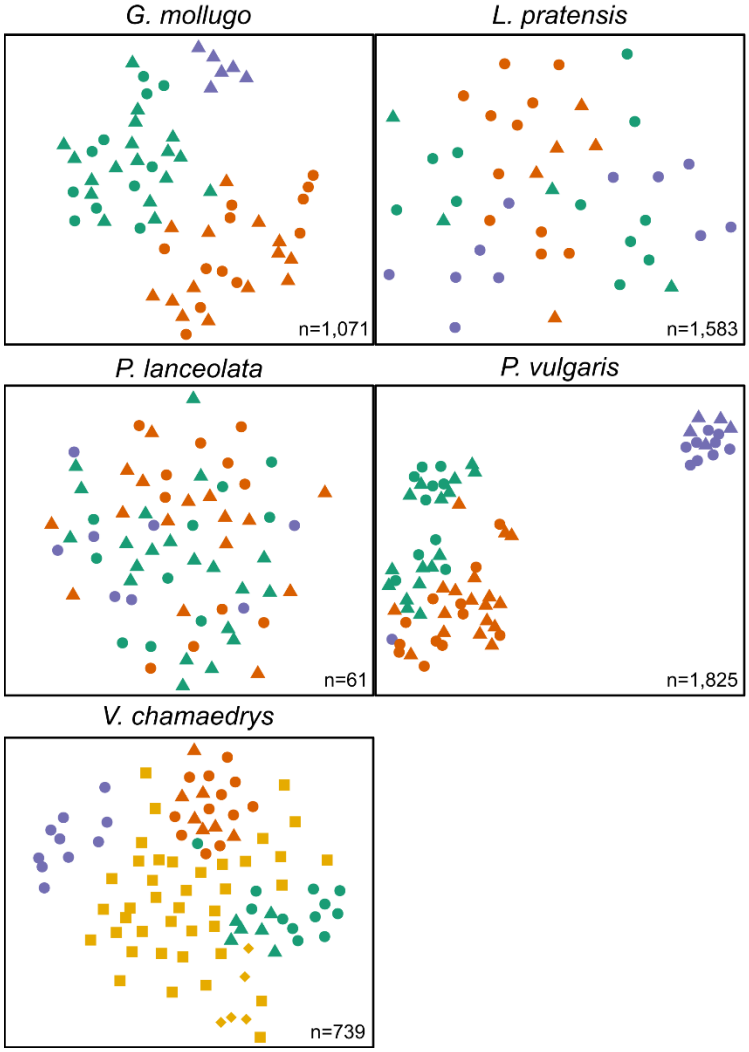


Figure 2. Genetic distance between individuals of the different populations for the five species. Green: selection history in mixture, orange: selection history in monocultures, blue: selection history in the field of the original seed supplier, seeds bought in 2014 (supp2014), yellow: offspring from 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.

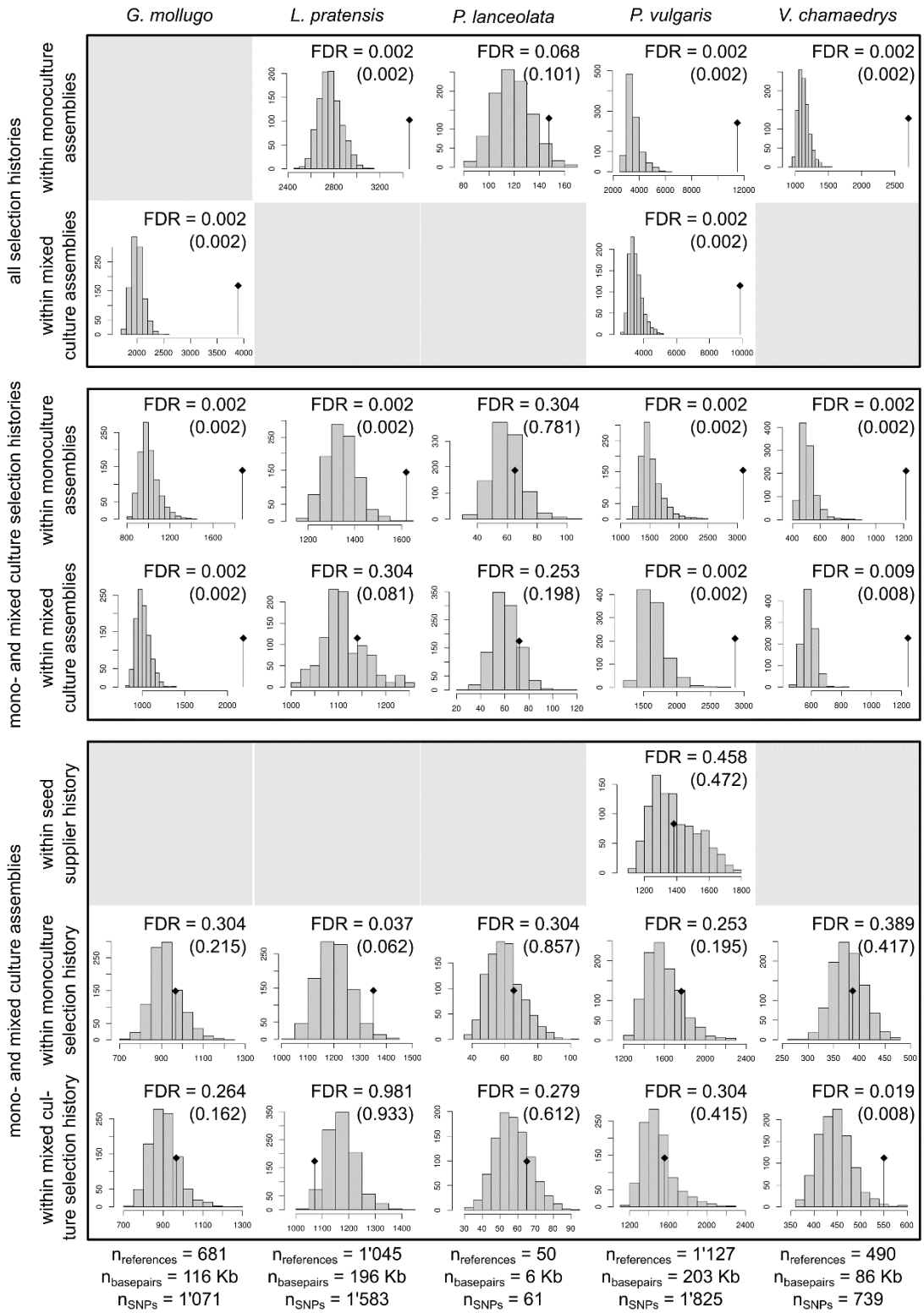


Figure 3. Results from the G-statistic tests given all SNPs. Each panel shows a histogram of permuted test statistics (999 permutations) and indicates the observed statistics by a black dot and a segment. Test statistics are on the x-axis, frequencies on the y-axis. Grey

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 %.

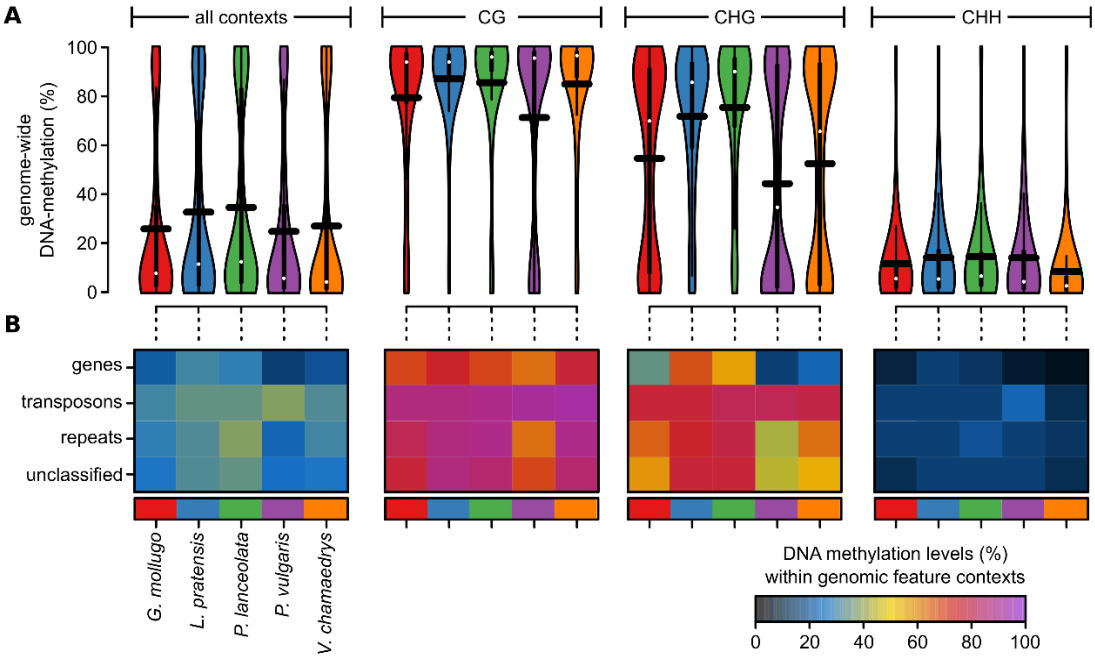


Figure 4. (A) DNA methylation levels in percent at individual cytosines across all or within each individual sequence context (CG, CHG, CHH) for each species used in this study shown as violin plots. The horizontal black bars correspond to the means. (B) Average DNA methylation levels in percent for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

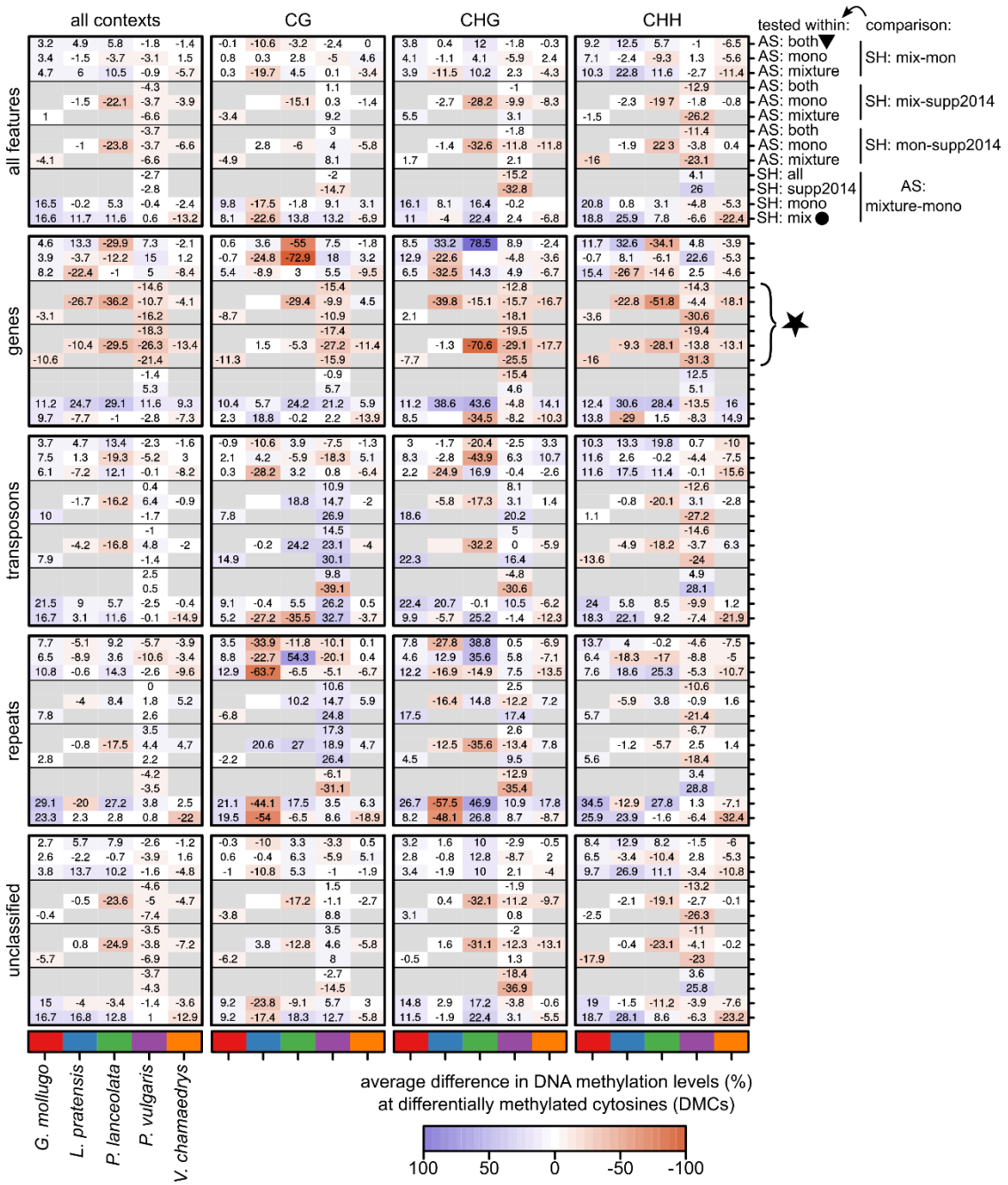


Figure 5. Average differences in DNA methylation at significantly differentially methylated cytosines (DMCs; FDR < 0.01) within a given sequence (all, CG, CHG, and CHH) and feature context (all, genes, transposons, repeats, unclassified) are shown for all contrasts. The comparisons and within which levels 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).

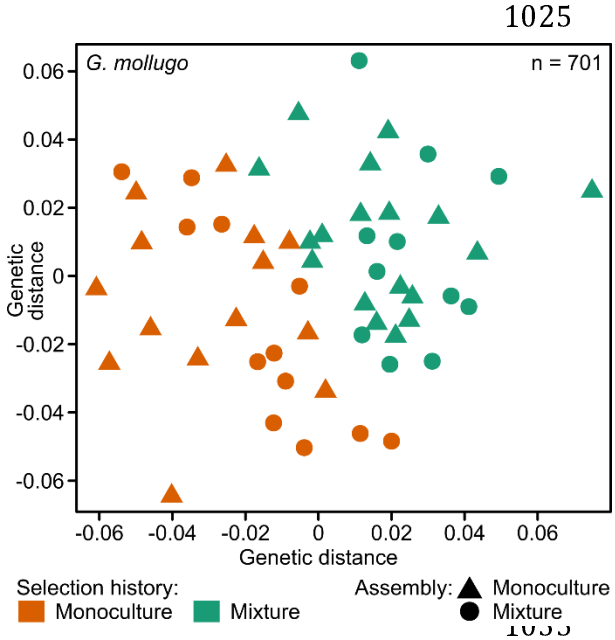


Figure 6. Genetic distance between the 701 reference sequences that were significantly (FDR < 0.05) associated with the phenotype in *G. mollugo*. Selection histories in this analysis were limited to the two histories in the Jena Experiment (monoculture and mixture). Distances were visualized with the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