Evidence for rapid evolution in a grassland biodiversity experiment

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Summary

- In long-term grassland biodiversity experiments positive effects of biodiversity on plant productivity commonly increase with time. Previously it was shown that differential selection in monoculture and mixed-species grassland communities could lead to the rapid emergence of monoculture and mixture phenotypes. Underlying mechanisms for such rapid phenotypic responses are however still unclear.
- We hypothesized that in biodiversity experiments pre-adapted genotypes or epigenetic variants could be sorted out from the standing genetic or epigenetic variation.
- To test if biodiversity acted as a selective environment, we grew offspring from plants that were exposed for twelve years to a monoculture or mixture environment in a biodiversity experiment (Jena Experiment) under controlled greenhouse conditions. Using epiGBS, a genotyping-by-sequencing approach combined with bisulfite conversion to provide integrative genetic and epigenetic data, we showed that plants with a monoculture or mixture background were genetically distinct.
- Our data reveal strong genetic and epigenetic divergence within species according to selection history and suggest variation in epigenetic variation to be largely under genetic control. This pattern was consistently observed across six perennial grassland species. Our results suggest that selection of genetic variants caused the rapid emergence of monoculture and mixture types in the Jena Experiment.

Key words: biodiversity, epiGBS, epigenetic variation, genetic divergence, herbaceous plant species

Introduction

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

Adaptive responses of plant populations to environmental factors (e.g., Schmid 1985; Joshi *et al*., 2001) and biotic interactions (Gervasi & Schiestl 2017) are well studied, but little effort has been devoted to studying the influence of community diversity on population structure and productivity (but see Lipowsky *et al.,* 2011; Kleynhans *et al.,* 2016). In particular, the influence of multi-species interactions for the adaptive response of a species is largely unknown, despite a growing body of evidence pointing towards the importance of species-interaction networks for the maintenance of ecosystem stability (Bastolla *et al.,* 2009). It is conceivable that the feedback between species interactions and their adaptive responses shapes community-level ecosystem functioning (van Moorsel *et al.*, 2018).

In the 1960s it was proposed that there are large differences in the time scales between ecological and evolutionary processes (Slobodkin 1961), but now it is well known that micro-evolutionary and ecological processes can occur on the same temporal scale (reviewed by Hairston *et al.,* 2005; Schoener 2011). Thus, it appears that micro-evolutionary processes may allow for an evolutionary rescue in a rapidly changing environment. However, evidence of genetic adaptation to mild stresses in natural situations over short time scales of a few generations is still scarce. Understanding how biodiversity, i.e. interactions between species, shapes this evolutionary response will be instrumental to anticipate how ecosystems may change in response to global change.

Adaptation depends on several factors. For organisms with a short generation time and asexual reproduction, such as clonal populations of bacteria, mutations and horizontal gene transfer are the main sources of genetic variation (Anderson *et al.*,

81 2011). However, for species with longer generation times such as perennial plants
82 selection more likely acts on standing genetic variation (Barrett & Schluter 2008),
83 resulting in a sorting-out of suitable genotypes (Fakheran *et al.,* 2010). Furthermore,
84 plants may adjust to a novel environment by phenotypic plasticity (Price *et al.,* 2003).
85 A possible indication for selection in grassland plant communities is the observed
86 strengthening of biodiversity effects in field biodiversity experiments (Cardinale *et*
87 *al.,* 2007; Fargione *et al.,* 2007; Reich *et al.,* 2012; Meyer *et al.,* 2016). An increase in
88 complementarity between species resulted in an increasing positive effect of diversity
89 on productivity over time (e.g. Meyer *et al.,* 2016). The increased complementarity
90 could also have been a result of phenotypic plasticity (Ghalambour *et al.,* 2007).
91 However, recent common garden experiments with plant material from a grassland
92 biodiversity experiment (the Jena Experiment, Roscher *et al.,* 2004) gave a clear
93 indication for genetic divergence between monoculture and mixture types in multiple
94 plant species (Zuppinger-Dingley *et al.,* 2014; van Moorsel *et al.,* 2018b), suggesting
95 that natural selection in response to community diversity had occurred in the field. As
96 a consequence, mixture types showed stronger complementarity effects (Loreau &
97 Hector 2001) than monoculture types when grown in mixture, i.e. community
98 productivity was increased due to increased differences in functional traits between
99 species for mixture types (Zuppinger-Dingley *et al.,* 2014; van Moorsel *et al.,* 2018b).
100 The emergence of such monoculture and mixture types, with different growth
101 performance and plant functional trait variation, thus suggested that community
102 diversity in the field likely acted as a selective environment (Zuppinger-Dingley *et al.,*
103 2014; Rottstock *et al.,* 2017; van Moorsel *et al.,* 2018b). However, direct evidence for
104 a genetic divergence between the different populations in the field experiment is still
105 missing.
106 It should be noted that epigenetics, here defined as meiotically heritable changes in
107 gene expression without changes to the underlying DNA sequence (Verhoeven *et al.,*
108 2016), has also been proposed to play a role (Bird 2007; Bossdorf *et al.,* 2008; Tilman
109 & Snell-Rood 2014). In a comment accompanying the publication of Zuppinger-
110 Dingley *et al.* (2014), Tilman and Snell-Rood wrote: "[…] chance that the differences
111 between the high- and low-diversity selection groups were due to genetic divergence.
112 However, it is possible that epigenetic factors […] could have had a simultaneous
113 role" (Tilman & Snell-Rood 2014). Recent work on epigenetic recombinant inbred
114 lines (epiRILs) of *Arabidopsis thaliana* suggests a considerable contribution of

115 induced epialleles to phenotypic variation, which is independent of genetic variation
116 (Cortijo *et al.*, 2014; Kooke *et al.*, 2015). However, the importance of epigenetics in
117 natural populations and whether it contributes to adaptation remains elusive
118 (Quadrana & Colot 2016), in particular because it is difficult to separate epigenetic
119 from genetic variation. A first step to disentangle genetic from epigenetic variation
120 was achieved with apomictic clones of *Taraxacum officinale*, suggesting that
121 differences in flowering time were mediated by differences in DNA methylation
122 (Wilschut *et al.,* 2016). However, given the fundamental difference between apomixis
123 (apomeiosis, parthenogenesis, autonomous endosperm formation) and sexual
124 reproduction, the results from this study may not be directly transferable to non-
125 apomictic plant species.
126 Here, we tested whether community diversity could act as a selective force leading
127 to a divergence of phenotypes into monoculture and mixture types (Zuppinger-
128 Dingley *et al.*, 2014; van Moorsel *et al.,* 2018b). In particular, we tested the
129 hypothesis that the differentiation of plants into phenotypically distinct mixture
130 (exhibiting stronger biodiversity effects) and monoculture types (exhibiting weaker
131 biodiversity effects) within species was paralleled by genetic differentiation.
132 Anticipating that the differentiation might also have been be due to epigenetic
133 variation, we chose a method which allowed us to analyze genetic and epigenetic
134 variation between plants of six species, all herbaceous perennials of European
135 grassland.
136

137 **Materials and Methods**

138 Plant selection histories

139 To test whether plant types selected over eleven years in mixtures differ genetically or
140 epigenetically from those types selected in monocultures, we chose six species grown
141 in monoculture and mixture plots in the Jena Experiment (Jena, Thuringia, Germany,
142 51˚N, 11˚E, 135 m a.s.l., see Roscher *et al.* (2004) for experimental details). The
143 following species belonging to three functional groups were selected: The three small
144 herbs *Plantago lanceolata* L.*, Prunella vulgaris* L. and *Veronica chamaedrys* L., the
145 tall herb *Galium mollugo* L.*,* and the two legumes *Lathyrus pratensis* L. and
146 *Onobrychis viciifolia* Scop. For the experiment plants from three different selection
147 histories were used (Fig. 1). Plants without a selection history in the experimental

field plots of the Jena experiment were obtained from commercial seed suppliers (Rieger Hoffmann GmbH, Blaufelden-Raboldshausen, Germany and Otto Hauenstein Samen AG, Switzerland), who also provided the seeds for the original set up of the Jena Experiment in 2002. Plants with a selection history in either mixture or monoculture had been growing in the Jena Experiment since 2002. In spring 2010, plant communities of 48 plots (12 monocultures, 12 two-species mixtures, 12 four-species mixtures and 12 eight-species mixtures) of the Jena Experiment were collected as cuttings, multiplied by repeated further cutting and then transplanted into an experimental garden in Zurich, Switzerland, in the identical plant composition as the original experimental plots for the first controlled sexual reproduction among co-selected plants (Zuppinger-Dingley *et al.*, 2014). In spring 2011, the seedlings produced from the seeds of the first controlled sexual reproduction in Zurich were transplanted back into those plots of the Jena Experiment from where the parents had originally been collected. In these newly established plots, plant communities with an identical composition to the original communities were maintained for three years until 2014 (van Moorsel *et al*., 2018c).

Seed collection

To ensure a second sexual reproductive event for the collection of seed material, entire plant communities from some of the experimental plots replanted in Jena in 2011 were excavated in March 2014 and used to established new 1-m^2 plots in the experimental garden in Zurich. For this purpose, we excavated blocks of soil (0.5 m^2) from plots in Jena including plant vegetation, seedlings and dormant seeds to make sure we transferred the entire plant community. These blocks of soil were then transferred and spread out into 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. As for the first sexual reproduction event in 2010, netting around each plot minimized the possibility of cross-pollination between the same species from different selection histories. Seeds from this second controlled sexual reproduction event were collected throughout the growing season of 2014 from monoculture plots and 4- and 8-species mixture plots. The exact community composition of the plots the seeds originated from is listed in Table S2. Seeds from

181 different mother plants were pooled together. The dry seeds were stored at 5° C for
182 cold stratification until germination.
183

184 Common garden experiment

185 Seeds were germinated in December 2014 with those from the same species being
186 planted within the same day. Germination was done in germination soil
187 ("Anzuchterde", Ökohum, Herbertingen, Germany) under constant conditions in the
188 glasshouse without additional light. Seedlings were planted in monocultures of four
189 individuals and mixtures of two plus two individuals into 2-L pots filled with
190 agricultural soil (50% agricultural sugar beet soil, 25% perlite, 25% sand; Ricoter AG,
191 Aarberg, Switzerland). See van Moorsel *et al*. (2018b) for the experimental design
192 and the planted combinations. In total, we planted 36 monoculture assemblies and 81
193 mixture assemblies from mixture history, 48 monoculture assemblies and 159 mixture
194 assemblies from monoculture history and 33 monoculture assemblies and 100 mixture
195 assemblies from seedlings without a common selection history. Every species
196 combination was replicated, if possible, six times for each selection history (resulting
197 in 457 pots, referred to as "assemblies", and 1828 plants). Seedlings that died within
198 the first two weeks were replaced with seedlings of the same age.
199 The experiment was set up in six blocks with each block representing a replicate.
200 Every block contained ca. 80 pots and within each block, pots were placed in the
201 glasshouse in a randomized fashion without reference to selection history or species
202 assembly. Single pots always contained four plants of a single selection history.
203 During the experiment, plants were watered according to demand and grown at
204 constant temperatures (17-20°C during the day, 13-17°C during the night) with no
205 additional light added. The plants were not fertilized. The fungicide Fenicur (*Oleum*
206 *foeniculi*, Andermatt Biocontrol) was applied on 5 May to control powdery mildew
207 (*Podosphaera* spp.).
208

209 Sampling

210 Samples for subsequent epigenetic and genetic analysis were harvested between 18
211 and 28 May 2015, after twelve weeks of plant growth in the glasshouse. All four
212 plants were sampled in each pot. One young leaf per plant was cut from the living

213 plant and immediately shock-frozen in liquid nitrogen. The samples were then stored
214 at –80° C in Zurich before shipment to the Netherlands for further processing.
215

216 Genetic analysis

217 We measured both genetic and epigenetic variation in monoculture- and mixture-type
218 plants when propagated in monoculture and two-species mixed assemblies using a
219 novel reference-free bisulfite method (van Gurp *et al.*, 2016). This method allows
220 measuring DNA cytosine methylation levels and identifying single nucleotide
221 polymorphisms with merely one lane of sequencing per 96 samples.
222

223 Sample procession and library preparation

224 The epiGBS protocol used is a further developed protocol based on the protocol of
225 van Gurp *et al.* (2016). The main improvements are the used enzyme combination, a
226 "wobble" adapter facilitating the computational removal of PCR duplicates, and a
227 conversion control nucleotide that allows easier Watson / Crick identification. For 348
228 samples, (Csp6I/NsiI) epiGBS libraries were created and sequenced on 4 Hiseq 2500
229 lanes. These samples were divided over six species and three selection histories (see
230 Tables S1, S2).
231

232 DNA extraction

233 Plant material was disrupted by bead-beating frozen leaf tissue in a 2 mL eppendorf
234 tube with 2-3 mm stainless steel beads. No more than 100 mg of fresh tissue was used
235 per sample. DNA isolation was performed using the NucleoSpin® 8 Plant II Core Kit
236 (740669.5 Macherey Nagel). We followed the manufacturers protocol with the
237 following modifications. Cell lysis was performed using Cell lysis buffer PL1 for 30
238 instead of 10 min. After lysis and initial centrifugation, the lysate was carefully
239 pipetted to a fresh 2.5 mL tubes, avoiding the cell debris. An extra centrifugation of 5
240 min at 18.000 g step was performed and the lysate transferred to a 96-well rack for the
241 next steps. At the step were the washed columns were dried, we centrifuged 5 min at
242 4800 g to get rid of the last remaining wash buffer. The restriction enzymes of the first
243 step of the epiGBS are very sensitive to ethanol and other contamination. DNA
244 concentration was determined using Qubit® 2.0 Fluorometric dsDNA HS Assay Kit
245 (Q32851 Life technologies).

246

247 DNA digestion and adapter ligation

248 Per individual, 30–300 ng genomic DNA was digested overnight (17 hrs) at 37° C in a
249 volume of 40 µL containing 1x FD buffer (Thermo Scientific), and 2 µL of both
250 *Csp*6I (FD0214, Thermo Scientific) and *Nsi*I (R0127S, NEB). Following digestion,
251 barcoded "wobble" adapters were ligated to the fragments (Fig. S1). To minimize the
252 possibility of misidentifying samples as a result of sequencing or adapter synthesis
253 error, all pair-wise combinations of barcodes differed by a minimum of three
254 mutational steps. Barcode lengths were modulated from 4 bp to 6 bp to maximize the
255 balance of the bases at each position in the overall set. For the ligation, 4 µL of a
256 sample specific barcode combination of both BA and CO adapters (600 pg/µL), 6 µL
257 T4 DNA ligase buffer, 1 µL T4 DNA ligase (M0202M, NEB) and 5 µL of distilled
258 water were added to the digestion mix to a total volume of 60 µL. Ligation was
259 performed for 3 hours at 22° C followed by 4° C overnight.

260

261 Pooling, clean-up and nick translation

262 In order to assess the quality of libraries, the pooling was performed per species level
263 in batches of around 12 samples per pool. When pooled, the total volume of the pool
264 was reduced by Qiaquick PCR cleanup (28104, Qiagen) to 40 µL. The libraries were
265 size selected by a 0.8x Agencourt AMPure XP (A63880, Beckman coulter)
266 purification favouring > 200 bp DNA fragments and eluted in a total volume of 22
267 µL. To prevent the formation of adapter dimers, the barcoded adapters were not
268 phosphorylated. Therefore, after the ligation, the insert DNA fragment–adapter
269 connection was nicked at the 3' positions of the insert DNA fragment (Fig. S1). This
270 nick is repaired by Nick translation that recreates the total non-(5mC) methylated
271 adapter strand and during that process also "unwobbles" the adapters since the
272 removed nucleotides are replaced by complementing nucleotides. This nick repair
273 prevents the partial loss of the adapter during bisulfite treatment. The nick translation
274 reaction (1 hour at 15° C) was performed in a reaction of 25 µL containing 19.25 µL
275 of the purified library, 2.5 uL of 10 mM 5-methylcytosine dNTP Mix (D1030 Zymo
276 research), 2.5 uL NEBuffer 2 and 0.75 uL DNA polymerase I (M0209, NEB).

277

278 Bisulfite conversion

279 For bisulfite conversion of non-methylated cytosines, 20 µL of the nick-translated
280 library was used. Bisulfite treatment was performed using the EZ DNA Methylation-
281 Lightning™ Kit (Zymo Research) with the following program according to the
282 manufacturers protocol: 8 min at 98° C, 1 h at 54 °C followed by up to 20 h at 4° C.
283

284 epiGBS PCR

285 Library amplification was performed in four individual 10 µL reactions containing 1
286 µL ssDNA template, 5 µL KAPA HiFi HotStart Uracil+ ReadyMix
287 (Kapabiosystems), 3 pmol of each illumina PE PCR Primer (5'-
288 AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTT
289 CCGATCT-3' and 5'-
290 CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGC
291 TCTTCCGATCT-3'). Temperature cycling consisted of 95° C for 3 min followed by
292 18 cycles of 98° C for 10 s, 65° C for 15 s, 72° C for 15 s with a final extension step
293 at 72° C for 5 min. Replicate PCR products were pooled and quantified using a
294 Qubit® dsDNA HS Assay Kit (Life technologies). The quality of the Libraries was
295 assessed by analyzing 1 µL on a High Sensitivity DNA chip on a 2100 Bioanalyzer
296 system (Agilent). Libraries were considered suitable for sequencing if the majority of
297 DNA fragments were between 150–400 bp. When the libraries passed quality control,
298 they were pooled according to concentration and number of samples in the species
299 pool, so that each individual sample was expected to yield an equal number of clusters
300 on the Illumina flow cell. Before sequencing, the libraries were spiked with 10% PhiX
301 control to increase the complexity of the libraries.
302

303 Sequencing

304 Finally, Paired-End sequencing was performed on a Hiseq2500 sequencer using the
305 HiSeq v4 reagents and the latest version of the HiSeq Control Software (v2.2.38),
306 which optimizes the sequencing of low-diversity libraries
307 (http://res.illumina.com/documents/products/technotes/technote-hiseq-low-
308 diversity.pdf). As the first five cycles of a sequencing run are used to calculate the
309 color matrix, our barcode design achieves almost perfect balance of the first five
310 nucleotides when equal numbers of sequences are obtained per "A" barcode. The "B"
311 barcodes do not have this requirement; hence same length barcodes were used.

312 Sequencing reads were demultiplexed and deposited at SRA (SRA accession ID
313 SRP132258). The barcode files can be accessed on Zenodo (DOI
314 10.5281/zenodo.1167563). In total, we generated 132'884'678, 130'721'280,
315 81'376'294, 140'883'779, 215'473'088, and 137'868'869 short reads for *G. mollugo*,
316 *L. pratensis*, *O. viciifolia*, *P. lanceolata*, *P. vulgaris*, and *V. chamaedrys*, respectively.
317

318 Statistical analysis

319 De-multiplexing, *de novo* reference construction, trimming, alignment, strand-specific
320 variant calling, and methylation calling were done for each species as described in van
321 Gurp *et al.* (2016). *De novo* reference sequences were annotated with DIAMOND
322 (protein coding genes; NCBI non-redundant proteins as reference; version 0.8.22;
323 Buchfink, Xie, and Huson 2015) and RepeatMasker (transposons and repeats;
324 *Embryophyta* as reference "species"; version 4.0.6; Smit, Hubley, and Green 2013–
325 2015). We summarized the transposable element and repeat classes into "transposons"
326 comprising DNA, LTR, LINE, SINE, and RC transposon, and "repeats" including
327 satellite, telomeric satellite, simple, rRNA, snRNA, unknown, and unclassified
328 repeats. The annotation was then used to classify the genetic and epigenetic variants
329 into the different feature contexts (e.g., to identify whether a single nucleotide
330 polymorphism is located in a gene or a transposon). A summary of the reference
331 sequences is given in Table S3.
332

333 Genetic variation: visualization of genetic distances with single
334 nucleotide polymorphisms (SNPs)

335 The following individuals with a SNP calling rate below 30% were *a priori* removed
336 from the analysis of genetic variation: "pool_pla_lan_15", "pool_pla_lan_46",
337 "pla_lan_81", "pla_lan_82", "pla_lan_52", "pla_lan_83", "pla_lan_95",
338 "pla_lan_111", "pru_vul_60", "pru_vul_79", "pru_vul_80", "pru_vul_87",
339 "pool_pru_vul_22", "pool_pru_vul_24", "ono_vic_15", and "ver_cha_73". These
340 samples were well distributed across the experimental group, i.e., one or two for a
341 single experimental group, except for the mixture-type mixture assembly of *P.*
342 *lanceolata* for which three individuals were removed. For each species, we filtered the
343 genetic variation data for single nucleotide polymorphisms (SNPs) sequenced in all

344 individuals with a total coverage between 5 and 200. SNPs homozygous for either the
345 reference or the alternative allele in more than 90 % of all individuals were removed
346 as uninformative SNPs. To reduce the impact of false positive SNP calls, we removed
347 all SNPs located in contigs with more than 1 SNP per 50 basepairs because higher
348 rates appear unlikely and may also originate from spurious alignments to the wrong
349 reference contig (considering that the reference contigs represent only a minor
350 fraction of the entire genome, there may be many reads originating from other
351 locations not represented with a reference contig which are still similar enough to
352 (wrongly) align to the reference). This also avoids that few contigs with high SNP
353 rates drive the tests for genetic differentiation.

354 SNP allele frequencies were scaled with the function "scaleGen" from adegenet
355 (version 2.0.1; Jombart 2008) and genetic distances between the individuals were
356 visualized with t-SNE (van der Maaten and Hinton 2008, van der Maaten 2014). We
357 calculated 100 maps starting from different random seeds and selected the map with
358 the lowest final error. Individual maps were calculated in R with the package Rtsne
359 (version 0.13; van der Maaten and Hinton 2008, van der Maaten 2014). Parameters
360 for the function Rtsne were pca = FALSE, theta = 0, perplexity = 10 (except for *O.*
361 *viciifolia* for which perplexity = 5). To select the SNPs with the highest differentiation
362 between the populations, we calculated Jost's D (Jost 2008) with the function
363 "basic.stats" from hierfstat (version 0.04-22; Goudet & Jombart 2015) and only
364 included the top 5% in the visualization.

365

366 Genetic variation: test for genetic differentiation between populations 367 with single nucleotide polymorphisms (SNPs)

368 SNP data were processed and filtered as described before. Considering that our design
369 only included two factors with two and three levels (assembly in the glasshouse and
370 selection history respectively; and incomplete for all species except *P. vulgaris*), we
371 did not use a hierarchical model (with assembly nested within) to test for genetic
372 differentiation. Instead, we tested each factor within all levels of the other factor for
373 genetic differentiation. Taking *P. vulgaris* as an example, we tested for genetic
374 differentiation between plant histories within monoculture and mixture assemblies

375 (between all three histories and between monoculture and mixture types), and
376 between assemblies within the naïve, monoculture, and mixture-type selection
377 histories. For each test, we extracted the corresponding individuals and tested for
378 genetic differentiation with the G-statistic test (Goudet *et al.,* 1996, function
379 gstat.randtest implemented in the package hierfstat, version 0.04-22; Goudet &
380 Jombart 2015). We did not correct for multiple testing because *P*-values were derived
381 from 999 simulations and were thus limited to a minimal value of 0.001. Instead, we
382 used a significance threshold of 0.01 and provide plots showing histograms of
383 permuted G-statistics and the observed G-statistics. This analysis was carried out with
384 the (1) entire data set, (2) SNPs located within genes, and (3) SNPs located within
385 transposons. We chose to separately test SNPs in genes and transposons because we
386 expected that selection more likely acted on genes and that selection of transposons
387 would primarily occur due to genetic linkage to an advantageous gene. In addition, we
388 expected that SNP calls are more reliable within genes because many transposon
389 families tend to be highly repetitive.

390 To estimate the extent to which the genetic variation was caused by the
391 differentiation between populations, we calculated average (i.e., across all tested
392 SNPs) pairwise F_{ST} values with the function pairwise.fst from the package adegenet
393 (version 2.0.1; Jombart 2008). Assuming that only few loci were under selection,
394 many SNPs have F_{ST} values close to zero (only SNPs under selection have F_{ST} values
395 clearly larger than zero). To estimate the maximal divergence between the
396 populations, we therefore also calculated the F_{ST} of each individual SNP and extracted
397 the 99th percentile (we chose the 99th percentile because this is more robust than the
398 highest value).

399

400 Epigenetic variation: characterization of genome-wide DNA methylation 401 levels

402 For each species, we filtered the epigenetic variation data for cytosines sequenced in
403 at least three individuals per population (i.e., experimental group) with a total
404 coverage between 5 and 200. To provide an overview of the genome-wide DNA
405 methylation levels of the six species or each experimental group per species, we

406 visualized the DNA methylation levels of all cytosines averaged across all individuals
407 with violin plots. We also visualized the average DNA methylation level within
408 genes, transposons, repeats, and unclassified reference contigs with heat maps. Both
409 were done either using all sequence contexts (CG, CHG, CHH) at once or separately
410 for each sequence context.

411

412 Epigenetic variation: identification of differentially methylated cytosines 413 (DMCs)

414 DNA methylation data was processed and filtered as described before. Variation in
415 DNA methylation at each individual cytosine was then analyzed with a linear model
416 in R with the package DSS (version 2.24.0; Park and Wu 2016), according to a design
417 with a single factor comprising all different experimental groups (similar to the
418 approach described for RNA-Seq, Schmid 2017 and the testing procedure in Schmid
419 *et al.,* 2018). Specific groups were compared with linear contrasts and *P*-values for
420 each contrast were adjusted for multiple testing to reflect false discovery rates (FDR).
421 Taking *P. vulgaris* as an example, we compared the three plant histories across both
422 assemblies and within each assembly to each other. Likewise, we compared the two
423 assemblies across all plant histories and within each to each other. A cytosine was
424 defined as differentially methylated (DMC) if the FDR was below 0.01 for any of the
425 contrasts.

426

427 Results

428 Visualization of genetic distances between the plant individuals using 5% of the loci
429 with the strongest divergence between the populations clearly separated the
430 individuals according to their selection history in five out of six species (Fig. 2). In
431 addition to the separation by the selection history, individuals from *L. pratensis* also
432 clustered according to the current diversity level (i.e., assembly). However,
433 considering that the plants were assigned randomly to the assembly treatment, we
434 expected a genetic differentiation according to the selection history but not the
435 assembly. We therefore tested for a significant genetic divergence between the

436 selection histories and the assemblies with the G-statistics test (Fig. 3, S3 and S4,
437 Goudet *et al.,* 1996).
438 Genetic differentiation was significant in all data sets ($P <= 0.001$), with two
439 exceptions. First, the plant histories of *P. lanceolata* did not exhibit any significant
440 genetic differentiation (only the test with all plant histories within monoculture
441 assemblies in the data set with all SNPs was significant). Second, the test including
442 only the monoculture and mixture types within the mixture assemblies was not
443 significant for *L. pratensis*. In contrast, the tests comparing the monoculture and
444 mixture assemblies within each of the plant histories were never significant ($P >$
445 0.01).
446 To estimate the amount of genetic variation explained by the plant histories, we
447 calculated average pairwise F_{ST} values (Table S4) and the 99th percentiles of the SNP-
448 wise F_{ST} values (Table 1 and Tables S5, S6). Average pairwise F_{ST} values for the
449 different plant histories were between 0.014 (naïve *vs.* monoculture type within the
450 monoculture assemblies of *L. pratensis*) and 0.1 (naïve *vs.* monoculture or mixture
451 type within monoculture assemblies of *P. vulgaris*). The 99th percentiles were
452 markedly higher and between 0.13 (monoculture vs. mixture types within mixture
453 assemblies of *G. mollugo*) and 0.67 (all plant histories within mixture assemblies of *P.*
454 *vulgaris*). Thus, overall, 1.4% to 10% of the genetic variation was explained by plant
455 histories. However, for individual SNPs, plant histories could explain up to 67% of
456 the genetic variation.
457 To get an overview of the DNA methylation data, we visualized overall DNA
458 methylation levels for each plant species, sequence context (CG, CHG, CHH), and
459 genomic feature context (genes, transposons, repeats, and unclassified contigs, Fig. 4).
460 For all species, DNA methylation was generally highest in the CG context (80.3%),
461 lower in the CHG context (59.7%), and lowest in CHH context (13.2%). Differences
462 between species were most pronounced in the CHG context in which *L. pratensis*
463 (77.1%) and *P. lanceolata* (85.8%) exhibited markedly higher methylation levels than
464 the other four species (56.6%, 52.3%, 43.6%, and 53.1% in *G. mollugo*, *O. viciifolia*,
465 *P. vulgaris*, and *V. chamaedrys*, respectively). Within each species and context, DNA
466 methylation was highest in transposons and lowest in genes (Fig. 4B). Overall, these
467 patterns are within the range of what was reported previously for other angiosperms
468 (Niederhuth *et al.,* 2016).

469 For an initial comparison between the experimental groups, we visualized the
470 overall DNA methylation levels as we did about for the genetic variation, but for each
471 experimental group separately (Fig. S4). Given that the overall methylation levels
472 appeared to be similar, we tested for significant differences in DNA methylation
473 levels at each individual cytosine. On average, 1.8% of all tested cytosines were
474 significant in at least one of the tested contrasts (FDR < 0.01, "DMCs" for
475 differentially methylated cytosines, Table 1 for all contexts and Tables S5, S6, and S7
476 for each context separately). Relative to the total number of cytosines tested,
477 differences between plant histories (tested within or across both assemblies) were
478 between 0.14% and 1.22% on average across all species and between 0.03% and
479 1.99% per individual species. Differences between the two assemblies (tested within
480 or across all plant histories) were between 0.04% and 0.15% on average across all
481 species and between 0.03% and 0.35% per individual species. Thus, the fraction of
482 differentially methylated cytosines between the plant histories was generally larger
483 than differences between the two assemblies.
484 Within the plant histories, differences between the monoculture types and the naïve
485 plants were between 0.20% and 1.22% within species. Differences between mixture
486 types and naïve plants were between 0.13% and 1.27% within species. Differences
487 between monoculture and mixture types were between 0.03% and 1.99% within
488 species. However, if compared within each species separately, there were always
489 more DMCs in the comparisons between plants from Jena and the naïve plants than in
490 the comparison between monoculture and mixture types. The sole exception was *O.*
491 *viciifolia* for which there were more differences between the monoculture and mixture
492 types than between these and the naïve plants.
493 To further characterize the differences in DNA methylation, we calculated the
494 average change in DNA methylation at the DMCs for each contrast, across and within
495 all sequence contexts (CG, CHG, and CHH) and feature types (genes, transposons,
496 repeats, and unclassified) and visualized these differences (Fig. 5). We could not
497 identify clear patterns between the different comparisons with one exception:
498 differences in the comparisons between plants from Jena and the naïve plants within
499 genes (all sequence contexts) were mostly biased towards a higher methylation in the
500 naïve plants. Thus, plants in the Jena Experiment lost on average DNA methylation at
501 DMCs within genes (*O. viciifolia* was the exception with changes close to zero).

502

503 **Discussion**

504 We aimed at measuring to what extent differential selection in monoculture and
505 mixed species grassland communities had influenced the genetic and epigenetic
506 make-up of six grassland species and whether these mechanisms could have mediated
507 the rapid phenotypic responses that were previously observed in plants with different
508 diversity backgrounds (see Zuppinger-Dingley *et al*., 2014; van Moorsel *et al.*,
509 2018b). Strong genetic divergence within species in response to selection history was
510 found in five out of six species, suggesting that in these five species different
511 genotypes may have been sorted out from standing genetic variation that was present
512 at the start of the diversity experiment. Less likely, recombination and mutation could
513 also have contributed.

514 For *P. lanceolata,* no significant differentiation was found between monoculture
515 and the mixture types within mixture assemblies. This may be due to the panmictic
516 nature of *P. lanceolata*. Movement of pollen and seeds between plots together with
517 high germination and establishment rates in this species may have leveled out genetic
518 differentiation between plots of different species diversity. Furthermore, coverage was
519 low and only a small proportion of the genome was covered, resulting in only 202
520 SNPs in the dataset. We therefore cannot exclude the possibility that genetic variation
521 was present but that our epiGBS screening missed the genomic regions under
522 selection. For future research, we propose that increasing the sequencing coverage
523 should be prioritized over adding more samples.

524 Only in *O. viciifolia* we found more genetic differences between the monoculture
525 and mixture types than between these and naïve plants. This may be due to the fact
526 that some subplots that were transplanted from the field site in Jena to the
527 experimental plots contained only few individuals of *O. viciifolia*. The results indicate
528 that *O. viciifolia* plant material originating from monoculture and mixture selection
529 history could have originated from a singly ancestor, which resulted in a high degree
530 of relatedness within each population. In contrast, the seeds sourced from the
531 commercial supplier likely originated from many mother plants.

532 Overall, only 1.4% to 10% of genetic variation was explained by plant histories.
533 However, for individual SNPs, plant histories could explain up to 67% of the genetic
534 variation. This may indicate that only few loci were under selection (high

535 differentiation) and that most of the genome segregated randomly (low
536 differentiation). Interestingly, this would suggest that the traits underlying the
537 differentiation within species into monoculture and mixture types are not highly
538 polygenic, thus making them accessible to breeding.
539 Our work supports the hypothesis that perennial grassland species in field
540 experiments evolve as a response to their biotic environment (Zuppinger-Dingley *et*
541 *al.*, 2014; van Moorsel *et al.*, 2018b; van Moorsel *et al.*, 2018c). Recent experiments
542 showed stronger complementarity effects (Loreau & Hector 2001) in communities
543 with a selection history in species mixtures as opposed to plant individuals with a
544 selection history in monocultures (Zuppinger-Dingley *et al*., 2014; van Moorsel *et al*.,
545 2018b). It has been under debate whether epigenetic or genetic mechanisms are the
546 main driver of observed phenotypic variation in such scenarios (Rapp & Wendel
547 2005; Bird 2007; Bossdorf *et al.*, 2008; Tilman & Snell-Rood 2014). In our
548 experiment, we found genetic and epigenetic variation. However, genetic variation
549 can have a major effect on epigenetic variation not only in *cis* but also in *trans* (Dubin
550 *et al.,* 2015; Kawakatsu *et al.,* 2016). However, *cis* associations can also evolve
551 independently of underlying genetic variation, when spontaneous epimutations are
552 inherited through epigenetic inheritance (Taudt *et al.,* 2016). Thus, given the observed
553 and the additional unobserved (only parts of the genome were sequenced) genetic
554 variation, it seems plausible that most of the observed epigenetic variation was caused
555 by the underlying genetic variation. This is in line with quantitative genetics studies in
556 *A. thaliana* in which DNA methylation differences were found to be mostly associated
557 with underlying genetic variation (e.g., Li *et al.,* 2014).
558 We did, however, also observe methylation differences between the assemblies in
559 absence of genetic differentiation, even though these differences were small when put
560 into context. A recent study using the same statistical method but a more stringent
561 significance threshold (FDR < 0.001 instead of FDR < 0.01) compared DNA
562 methylation profiles of different tissue types of the liverwort *Marchantia polymorpha*,
563 covering important stages of the life cycle (Schmid *et al.,* 2018). With less than 1%
564 DMCs, the differences between the two assemblies of this study correspond to the
565 difference between two individual plants in Schmid *et al.* (2018). However, around
566 42% of all cytosines varied significantly across the entire life cycle in that study.
567 Thus, differences within individuals were much more pronounced than between
568 individuals. Several other studies provide direct or indirect evidence for clear

differences in DNA methylation between different tissue, cell types or developmental stages (Jullien *et al.,* 2012; Calarco *et al.,* 2012; Ibarra *et al.,* 2012; Park *et al.,* 2016; Ingouff *et al.*, 2017; Bouyer *et al.,* 2017). Clearly, this does not exclude the possibility that epigenetics may play a role in adaptation (see Hauser *et al.,* 2011, Kronholm & Collins 2016 and Quadrana & Colot 2016 for examples of natural epialleles), but it makes it less likely that the strengthening of the biodiversity effect in the populations assessed in this study was due to epigenetic differences that were not a consequence of the underlying genetic differentiation.

Conclusions

We used novel genomic tools to study the importance of epigenetics for adaptation to novel biotic conditions in six non-model plant species differentially selected in a field biodiversity experiment. Future research should continue to integrate molecular tools with studies on non-model species in order to push the field forward (Richards *et al*., 2017; Heer *et al.*, 2018). Our findings suggest that selection on standing genetic variation is a powerful driver of evolution even in the absence of many generations of plant growth. In addition, we propose that community diversity had the selective power to differentiate plant populations within species into mixture and monoculture types in only a few generations. Molecular tools and the integration of evolutionary concepts into plant community ecology can open up new alleys of research, which should be exploited in order to understand the community evolutive processes (Shafer *et al.*, 2015) that lead to the plant community compositions and structures as we see them today.

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Author 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. created the bioinformatics pipeline. M.W.S. analyzed the data and produced the figures. S.J.V.M and M.W.S. wrote the first draft of the manuscript. All authors contributed to revisions.

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793
794 **Data Accessibility Statement**
795 Data will be made publicly available on Zenodo (DOI 10.5281/zenodo.1167563) and
796 SRA (accession ID SRP132258) at time of acceptance.
797
798 **Supporting information**
799 Additional Supporting Information may be found online in the Supporting
800 Information tab for this article:

801 **Fig. S1** Adapter overview.
802 **Fig. S2** Results from the G-statistic tests given all SNPs within genes.
803 **Fig. S3** Results from the G-statistic tests given all SNPs within transposons.
804 **Fig. S4** DNA methylation levels in % at individual cytosines across all or within each
805 individual sequence context (CG, CHG, CHH) for each experimental group of each
806 species used.
807 **Table S1** Sample overview.
808 **Table S2** Community diversity and composition of the plots the seeds originated
809 from.
810 **Table S3** Reference sequences generated in this study.
811 **Table S4** Average pairwise F_{ST} values.
812 **Table S5** 99th percentiles of F_{ST} values in the SNPs within genes.
813 **Table S6** 99th percentiles of F_{ST} values in the SNPs within transposons.
814 **Table S7** Number of cytosines with significant differences (FDR < 0.01) in DNA
815 methylation between selection-history treatments and assemblies in the CG sequence
816 context.
817 **Table S8** Number of cytosines with significant differences (FDR < 0.01) in DNA
818 methylation between selection-history treatments and assemblies in the CHG
819 sequence context.
820 **Table S9** Number of cytosines with significant differences (FDR < 0.01) in DNA
821 methylation between selection-history treatments and assemblies in the CHH
822 sequence context.
823
824

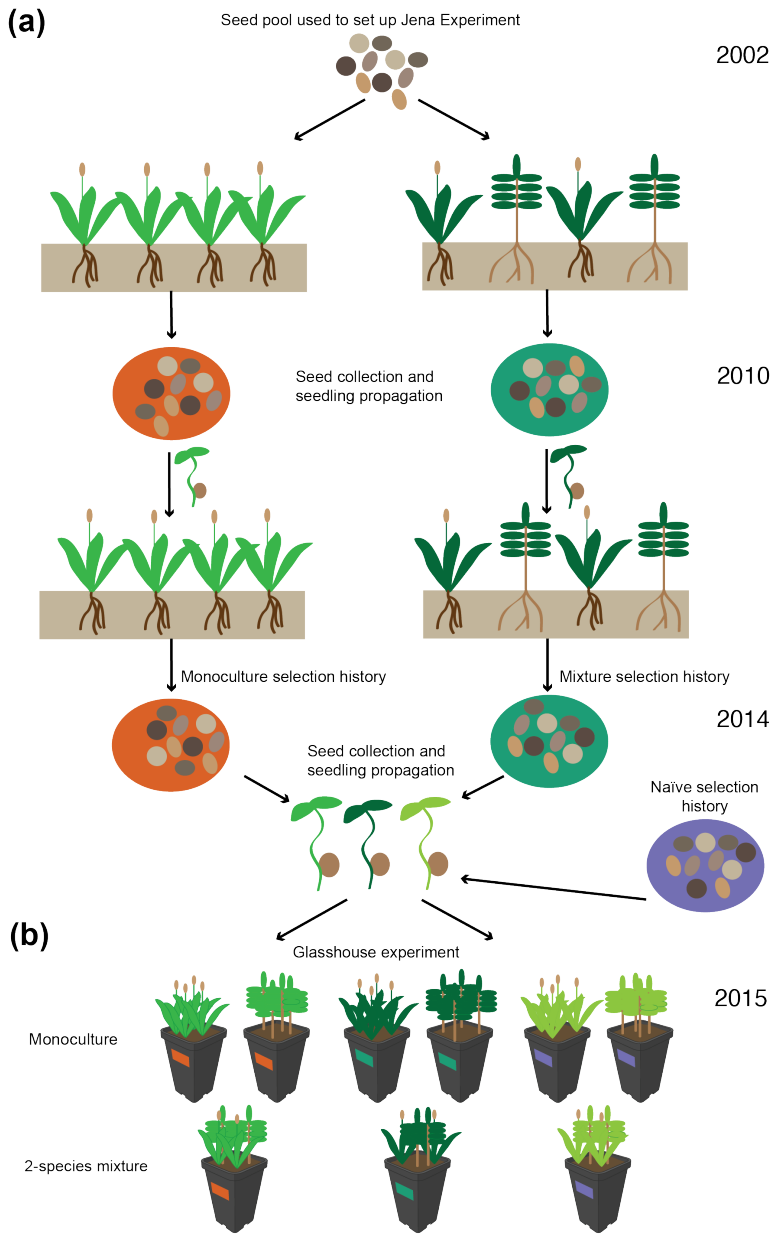


Fig. 1 (a) The origin of seeds used for the glasshouse experiment and genetic analysis. Seedlings were planted in mixtures and monocultures in Jena in the year 2002. Two reproduction events occurred when seeds were collected and subsequently new seedlings were produced and planted again in the same community composition. (b) Schematic representation of the glasshouse experiment. Monoculture assemblies and 2-species mixture assemblies were planted with either plants with mixture selection history (green), monoculture selection history (orange) or naïve plants originating from a commercial seed supplier (blue). Figure modified after van Moorsel *et al.* (2018b).

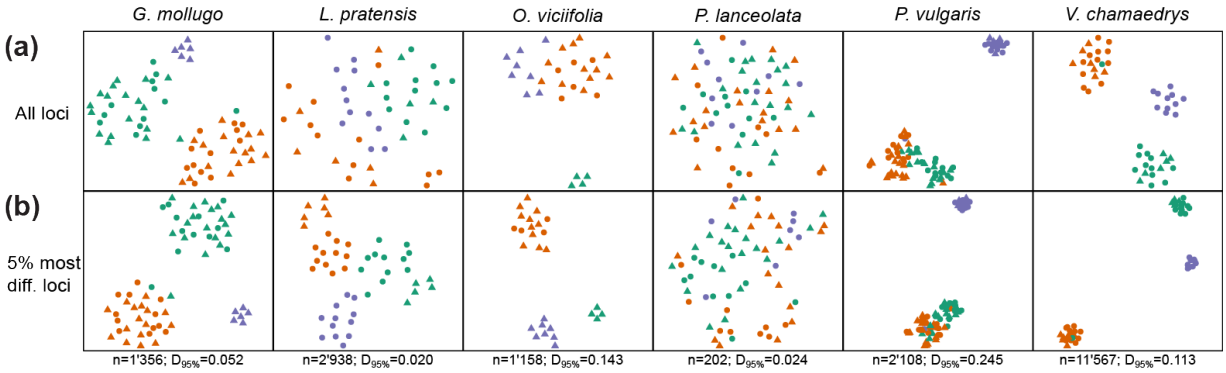


Fig. 2 Genetic distance between individuals of the different populations for the six species. (a) All loci included in the analyses. (b) Only the 5% most differentiated loci between the experimental groups included. Green: selection history in mixture, orange: selection history in monocultures, blue: naïve selection history. Triangles: current assembly monoculture, circles: current assembly mixture. $D_{95\%}$ is the threshold to choose the 5% most differentiated loci based on Jost's D (the 95% percentile in the entire data set, Jost 2008).

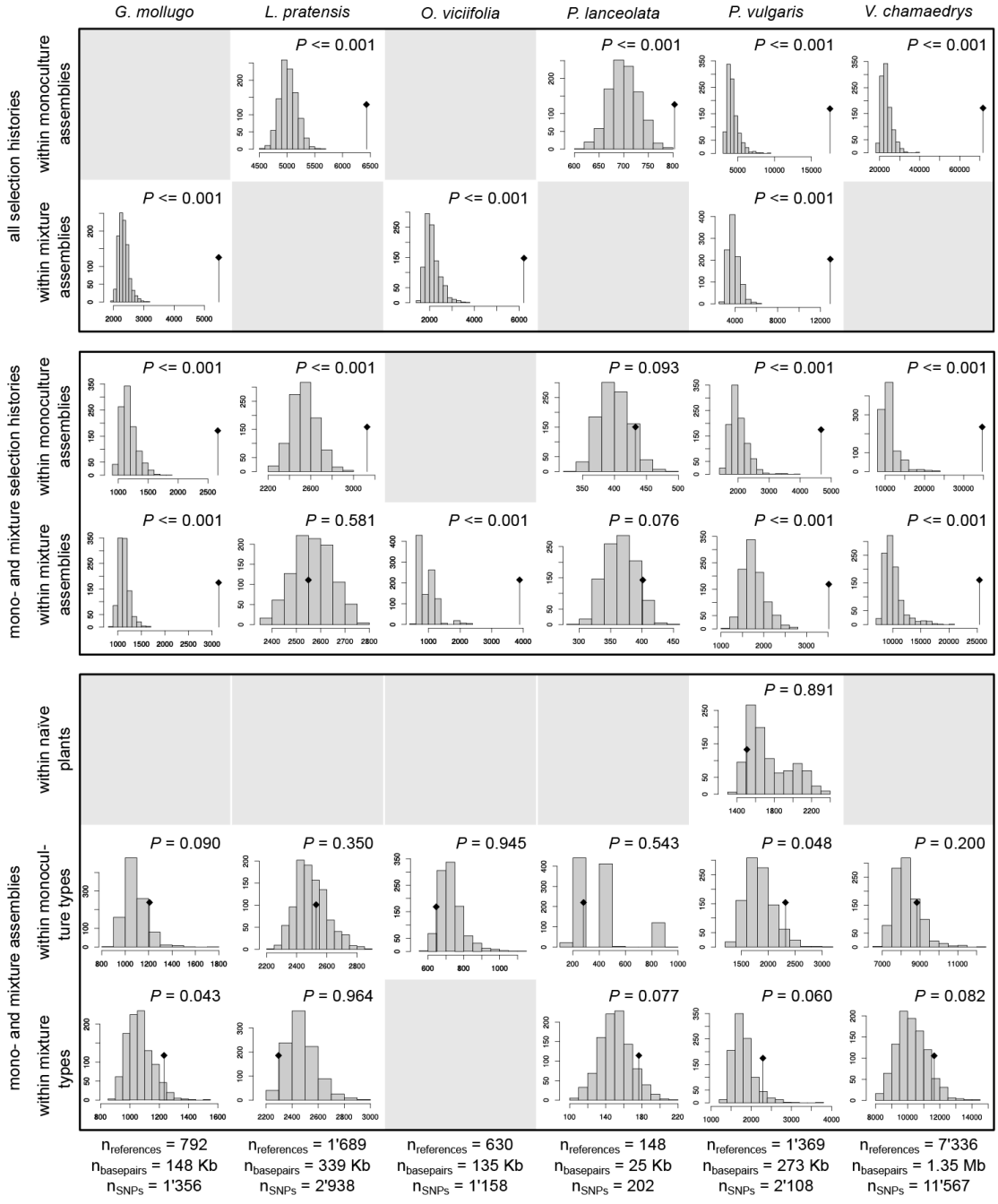
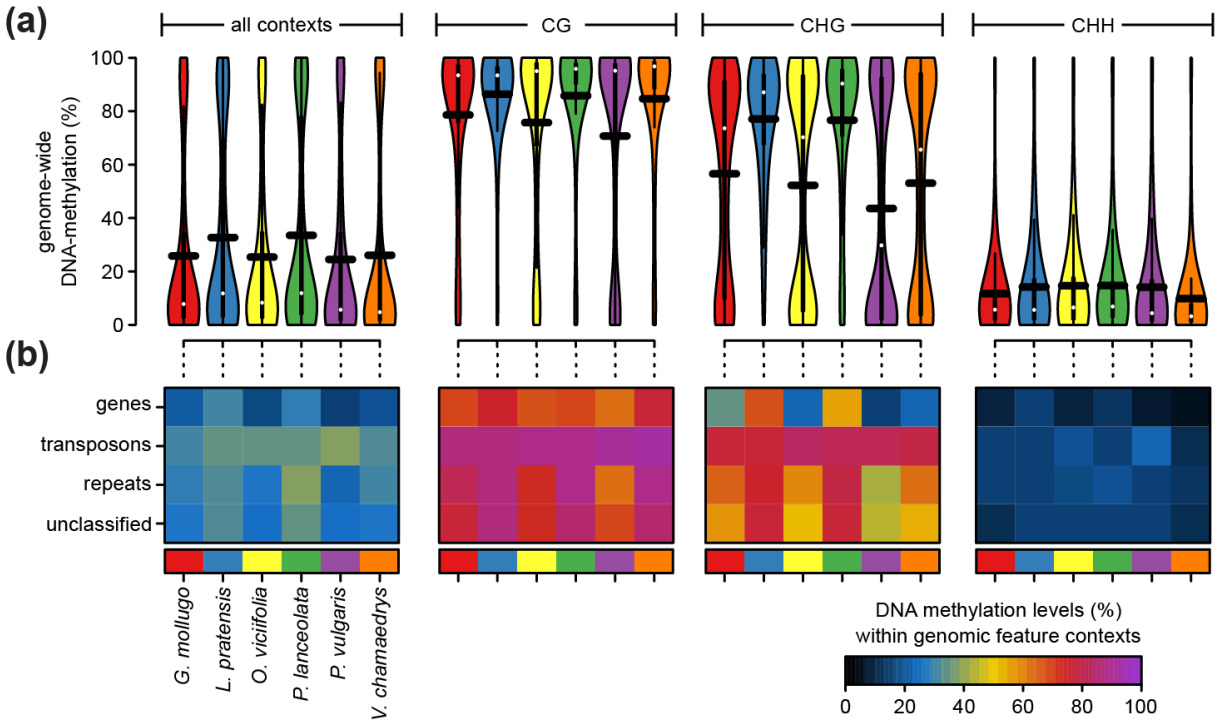


Fig. 3 Results from the G-statistic tests given all SNPs. Each panel shows a histogram of permuted test statistics (999 permutations) and indicates the observed statistics by a black dot and a segment. Test statistics are on the x-axis, frequencies on the y-axis. Grey boxes where data was not available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



850

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

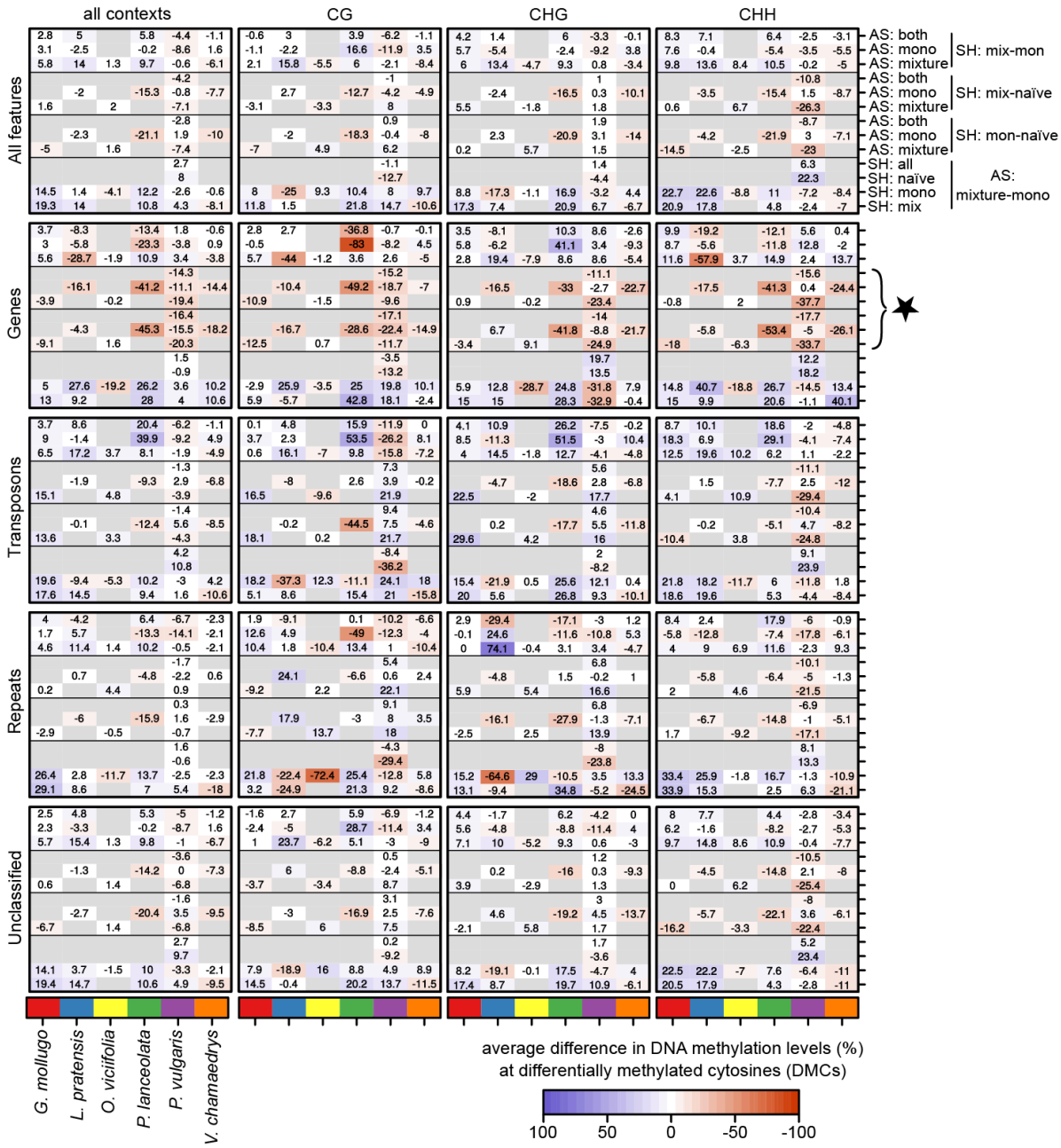


Fig. 5 Average differences in DNA methylation at significantly differentially methylated cytosines (DMCs; FDR < 0.01) within a given sequence (all, CG, CHG, and CHH) and feature (all, genes, transposons, repeats, unclassified) context are shown for all contrasts. The average differences are shown as colour gradient. The numbers within the heat map are the average differences. The asterisk marks the rows showing that plants in the Jena field lost on average DNA methylation at DMCs within genes compared to naïve plants.

Table 1 99th percentile of FST values in the data set with all SNPs.

	G. mollugo	*L. pratensis*	*O. viciifolia*	*P. lanceolata*	*P. vulgaris*	*V. chamaedrys*
SH in monoculture	NA	0.10641	NA	0.1413	0.6032	0.29453
SH in mixture	0.2354	NA	0.4	NA	0.67471	NA
SH contrast mono vs. mix in monoculture	0.1579	0.1	NA	0.09282	0.25	0.2571
SH contrast mono vs. mix in mixture	0.13332	0.1462	0.3333	0.05899	0.15592	0.3333
AS for naïve history	NA	NA	NA	NA	0.13182	NA
AS for mono history	0.0755	0.0909	0.08469	0.0762	0.10173	0.14305
AS for mix history	0.08355	0.0926	NA	0.09214	0.12	0.1586

AS = assembly, SH = selection history. For SNPs within genes or transposons see Tables S5 and S6.

868 **Table 2** Number of cytosines with significant differences (FDR < 0.01) in DNA

869 methylation between selection-history treatments and assemblies.

	G. mollugo	*L. pratensis*	*O. viciifolia*	*P. lanceolata*	*P. vulgaris*	*V. chamaedrys*	average %
SH: mixture vs. monoculture	6365 (0.55%)	239 (0.03%)	-	582 (0.11%)	6578 (0.28%)	12966 (0.60%)	0.31%
% in genes	13.68	4.6		4.47	12.82	11.51	
% in transposons	10.65	30.54		8.25	11.28	8.18	
% in repeats	4.41	4.18		8.93	6.67	4.63	
% in unclassified contigs	71.25	60.67		78.35	69.23	75.68	
>> within monoculture assembly	2332 (0.20%)	360 (0.05%)	-	216 (0.04%)	2507 (0.11%)	6507 (0.30%)	0.14%
% in genes	12.22	4.72		6.48	10.33	10.7	
% in transposons	11.11	28.61		7.87	11.61	8.16	
% in repeats	3.82	4.72		12.04	5.78	4.84	
% in unclassified contigs	72.86	61.94		73.61	72.28	76.3	
>> within mixture assembly	4617 (0.40%)	288 (0.04%)	10353 (1.99%)	2573 (0.47%)	8875 (0.37%)	5538 (0.25%)	0.59%
% in genes	11.63	4.17	12.13	3.26	11.14	9.73	
% in transposons	10.92	34.38	15.94	11.31	14.6	8.22	
% in repeats	4.7	5.21	6.93	7.5	6.49	4.66	
% in unclassified contigs	72.75	56.25	65.01	77.92	67.76	77.39	
SH: mixture vs. naïve	-	-	-	-	28970 (1.21%)	-	(1.21%)
% in genes					9.69		
% in transposons					11.84		
% in repeats					6.73		
% in unclassified contigs					71.73		
>> within monoculture assembly	-	893 (0.13%)	-	1040 (0.19%)	18439 (0.77%)	14692 (0.68%)	0.44%
% in genes		4.26		7.69	9.75	10.22	
% in transposons		26.32		9.71	12.55	7.83	
% in repeats		4.82		5.1	6.84	4.27	
% in unclassified contigs		64.61		77.5	70.87	77.68	
>> within mixture assembly	7012 (0.61%)	-	6574 (1.27%)	-	15019 (0.63%)	-	0.84%
% in genes	11.52		12.64		9.54		
% in transposons	10.95		16.06		11.85		
% in repeats	4.08		6.77		6.78		
% in unclassified contigs	73.45		64.53		71.83		
SH: monoculture vs. naïve	-	-	-	-	29052 (1.22%)	-	(1.22%)
% in genes					9.25		
% in transposons					12.52		
% in repeats					6.83		
% in unclassified contigs					71.4		
>> within monoculture assembly	-	1388 (0.20%)	-	620 (0.11%)	15877 (0.67%)	13614 (0.63%)	0.40%
% in genes		5.04		6.29	9.01	10.13	

% in transposons		28.6		7.1	12.36	7.28	
% in repeats		6.63		8.06	7.26	4.73	
% in unclassified contigs		59.73		78.55	71.37	77.86	
>> within mixture assembly	5336 (0.46%)	-	5464 (1.05%)	-	18419 (0.77%)	-	0.76%
% in genes	10.91		11.79		9.98		
% in transposons	9.07		15.98		12.24		
% in repeats	3.77		6.9		6.59		
% in unclassified contigs	76.26		65.34		71.19		
AS: mixture vs. monoculture	-	-	-	-	937 (0.04%)	-	(0.04%)
% in genes					8		
% in transposons					11.31		
% in repeats					8.86		
% in unclassified contigs					71.82		
>> within naïve selection history	-	-	-	-	2988 (0.13%)	-	(0.13%)
% in genes					9.64		
% in transposons					14.63		
% in repeats					7.83		
% in unclassified contigs					67.9		
>> within monoculture selection history	948 (0.08%)	191 (0.03%)	216 (0.04%)	506 (0.09%)	2264 (0.09%)	1588 (0.07%)	0.06%
% in genes	9.07	7.85	8.8	10.87	7.91	5.79	
% in transposons	10.34	31.94	18.52	6.92	14.31	12.22	
% in repeats	5.38	3.14	3.7	11.86	7.99	6.11	
% in unclassified contigs	75.21	57.07	68.98	70.36	69.79	75.88	
>> within mixture selection history	4032 (0.35%)	708 (0.10%)	-	944 (0.17%)	1857 (0.08%)	1624 (0.07%)	0.15%
% in genes	6.92	4.38		3.6	7.7	9.3	
% in transposons	12.7	29.66		11.55	16.05	8.37	
% in repeats	6.42	6.36		9	6.41	5.11	
% in unclassified contigs	73.96	59.6		75.85	69.84	77.22	
Total (percentage DMCs of tested cytosines)	21086 (1.8%)	3291 (0.5%)	16544 (3.2%)	5122 (0.9%)	65507 (2.7%)	36076 (1.7%)	1.80%

AS = assembly, SH= selection history. For data on separate sequence contexts see Tables S7 (CG), S8 (CHG), and S9 (CHH).