Structure, stability and ecological significance of natural epigenetic variation: a large-scale survey in *Plantago lanceolata*

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Summary

- Within-species diversity is an important driver of ecological and evolutionary processes. Recent research has found that plants can harbour significant epigenetic diversity, but its extent, stability and ecological significance in natural populations is largely unexplored.
- We analysed genetic, epigenetic and phenotypic variation in a large number of natural grassland populations of *Plantago lanceolata*, covering a broad geographical and environmental range. Within-population diversity and among-population differentiation were calculated from genetic and epigenetic marker data and from measurements of phenotypic traits, both for plants in the field and for the F$_1$ generation grown in a common environment.
- We found weak but significant epigenetic population structure. A large part of the epigenetic population differences observed in the field was maintained in a common environment. Epigenetic differences were consistently related to genetic and environmental variation, and to a lesser degree to phenotypic variation and land use, with more grazed populations harbouring greater epigenetic diversity.
- Our study demonstrates that epigenetic diversity exists in natural populations of a common grassland species, and that at least part of this epigenetic diversity is stable, nonrandom and related to environmental variation. Experimental and more detailed molecular studies are needed to elucidate the mechanistic basis of these observed patterns.

Introduction

Within-species variation is an important level of biological diversity, sometimes with even stronger ecological effects than species-level variation (Des Roches *et al.*, 2018). In studies of natural populations, within-species variation has two main components: (1) the diversity within populations, which serves as the raw material for evolution and adaptation (Barrett & Schluter, 2008), and has been shown to contribute to the resistance and resilience of populations (Hughes *et al.*, 2008); and (2) the genetic differentiation among populations, which reflects local adaptation and other evolutionary processes such as drift and gene flow.

In the past, the study of intraspecific variation was mainly concerned with phenotypic or genetic differences among individuals or populations. In recent years, it has become clear that intraspecific variation also exists at the epigenetic level of DNA methylation or other epigenetic modifications of the genome. Epigenetic variation can be related to variation in phenotype (Cubas *et al.*, 1999; Cortijo *et al.*, 2014; Kooke *et al.*, 2015), and is therefore potentially relevant for ecology and evolutionary biology, as well as plant and animal breeding and conservation. Although much epigenetic variation is under genetic control, there are cases where epigenetic variation is independent of

genetic variation, as a result of spontaneous epimutation (Becker *et al.*, 2011; Van Der Graaf *et al.*, 2015) or environmental induction (Jiang *et al.*, 2014; Quadrana & Colot, 2016), and it is particularly these cases where the study of epigenetic variation has the potential for true discovery of novel intraspecific differences and evolutionary potential (Bossdorf *et al.*, 2008; Richards *et al.*, 2017).

So far, in-depth documentation of intraspecific variation in DNA methylation has been largely restricted to some model plant species (e.g. *Arabidopsis thaliana, Oryza sativa* and *Zea mays*) with extensive genomic and epigenomic resources (Schmitz *et al.*, 2011, 2013; Becker *et al.*, 2011; Li *et al.*, 2012, 2014; Van Der Graaf *et al.*, 2015; Kawakatsu *et al.*, 2016). These studies documented substantial variation in the extent and stability of DNA methylation, both within genomes in different sequence contexts and genomic regions, and among different lines/genotypes and geographical origins. In addition, there has also been a notable increase in research on natural epigenetic variation in nonmodel species (Richards *et al.*, 2017). These studies confirmed that variation in DNA methylation is ubiquitous in natural populations, and that it usually exceeds DNA sequence variation when comparing populations from ecologically contrasting origins (Herrera & Bazaga, 2010; Lira-Medeiros *et al.*,

2010; Richards *et al.*, 2012; Medrano *et al.*, 2014; Schulz *et al.*, 2014). However, most of these previous studies included only few populations, often from very restricted geographical ranges, which makes their conclusions difficult to generalize across larger ranges of populations and environments.

Besides quantifying and describing epigenetic variation in wild populations, another important goal is to clarify and disentangle its relationships with genetic and phenotypic variation. In *A. thaliana*, much of the epigenomic variation appears to mirror underlying genetic patterns (Dubin *et al.*, 2015). However, the structure and dynamics of the *A. thaliana* epigenome are very unusual within the plant kingdom (Mirouze & Vitte, 2014; Alonso *et al.*, 2015) – with exceptionally low overall DNA methylation – and studies from nonmodel plants have reported patterns of epigenetic variation independent of genetic relatedness (Schulz *et al.*, 2014; Foust *et al.*, 2016; Gugger *et al.*, 2016). In addition, phenotypic variation can be caused by epigenetic differences *alone*, as it has been demonstrated, for instance, for flower symmetry, root length and flowering time (Cubas *et al.*, 1999; Cortijo *et al.*, 2014). In natural populations, significant correlations were found between epigenetic markers and several phenotypic traits (Herrera & Bazaga, 2010, 2013; Medrano *et al.*, 2014). However, these phenotypic measurements originated from the field, and so cannot disentangle the plastic and stable components of the relationship between epigenetic and phenotypic variation. In order to do so, epigenetic and phenotypic data must be compared between wild plants and their offspring in a common environment.

Apart from underlying genetic variation, heritable epigenetic variation also can be induced by environmental variability, as documented both in model and nonmodel species (Verhoeven *et al.*, 2010; Wibowo *et al.*, 2016). Because of the multiple sources of epigenetic variation (genetic, environmental and stochastic), its partial inheritance, and the multiple origins of phenotypic variation (genetic and epigenetic), teasing apart the relationships between these processes continues to be a challenge. However, some effects can be separated through experimental designs that combine field-collected and common-environment-derived material with environmental data (Bossdorf *et al.*, 2008). If epigenetic variation is correlated with environmental factors in the field, but this relationship disappears in the common environment, this indicates plastic responses, and their possible drivers might be found by relating the phenotypes from the field to the environmental variables. If the relationships are maintained in a common environment, then this indicates either natural selection acting on stable epigenetic variation or inheritance of environmentally induced epigenetic changes. Because environmentally induced epigenetic changes may be reset after few generations (Wibowo *et al.*, 2016), extending common garden studies across multiple generations can help to distinguish between these two cases. In any case, the environmental and phenotypic correlates of stable epigenetic variation can indicate the underlying drivers and targets of selection.

In summary, to understand the ecological and evolutionary significance of epigenetic variation, it is important to quantify epigenetic variation in large numbers of natural populations also in nonmodel plants and across broad geographical and environmental ranges, to couple epigenetic variation to genetic and phenotypic variation, and to combine field surveys with common garden approaches. Although the need for such studies was already identified a decade ago (Bossdorf *et al.*, 2008), we are not aware of any previous study that has addressed all of the questions above in a comprehensive way.

Here, we present a survey of natural epigenetic variation across 60 wild populations of *Plantago lanceolata*, a common and ecologically important plant species in Central European grasslands (Sagar & Harper, 1964). The studied populations covered a broad geographical and environmental range. We combined epigenetic with genetic and phenotypic data, and compared plants in the field with their F_1 offspring raised in a common environment to ask three research questions. (1) What is the extent, structure and stability of natural epigenetic variation in *P. lanceolata*? We expected significant epigenetic population structure, with differentiation among geographic regions and populations, and at least a partial persistence of the observed epigenetic variation in the common environment. (2) How is natural epigenetic variation related to genetic and phenotypic variation? We expected significant relationships between all three types of variation, with stronger genetic–epigenetic relationships in the glasshouse than in the field (as stable epigenetic variation is more likely genetically controlled), and stronger epigenetic–phenotypic relationships in the field, reflecting the plastic components of epigenetic and phenotypic variation. (3) How is natural epigenetic variation related to different environmental factors, in particular geographical and environmental distance and the intensities of land use in the studied grasslands? We expected epigenetic variation to show isolation by geographical and environmental distance, and a significant relationship with land-use intensity, all stronger in the field than in the glasshouse.

Materials and Methods

Study system

We worked with *Plantago lanceolata* L. (Plantaginaceae), a short-lived perennial rosette herb that is very common in European grasslands and grows under a wide range of environmental conditions. The species is a wind-pollinated and self-incompatible diploid (Kuiper & Bos, 1992) with a moderately sized genome ($1n \approx 1.28$ Gb). We studied natural populations of *P. lanceolata* within the German research platform Biodiversity Exploratories (www.biodiversity-exploratories.de), a large-scale and long-term project investigating relationships between land use, biodiversity and ecosystem processes (Fischer *et al.*, 2010). Among others, it comprises a hierarchical set of standardized grassland plots, with 50 plots in each of three regions (Fig. 1): the Schorfheide-Chorin Biosphere Reserve in the north, the Hainich National Park and surrounding areas in the middle, and the Schwäbische Alb Biosphere Reserve in the south of Germany, spanning across 600 km in total. In each region, the plots cover a wide range of land-use types and intensities, with precise data for the mowing, fertilization and grazing intensities of each plot, obtained from regular
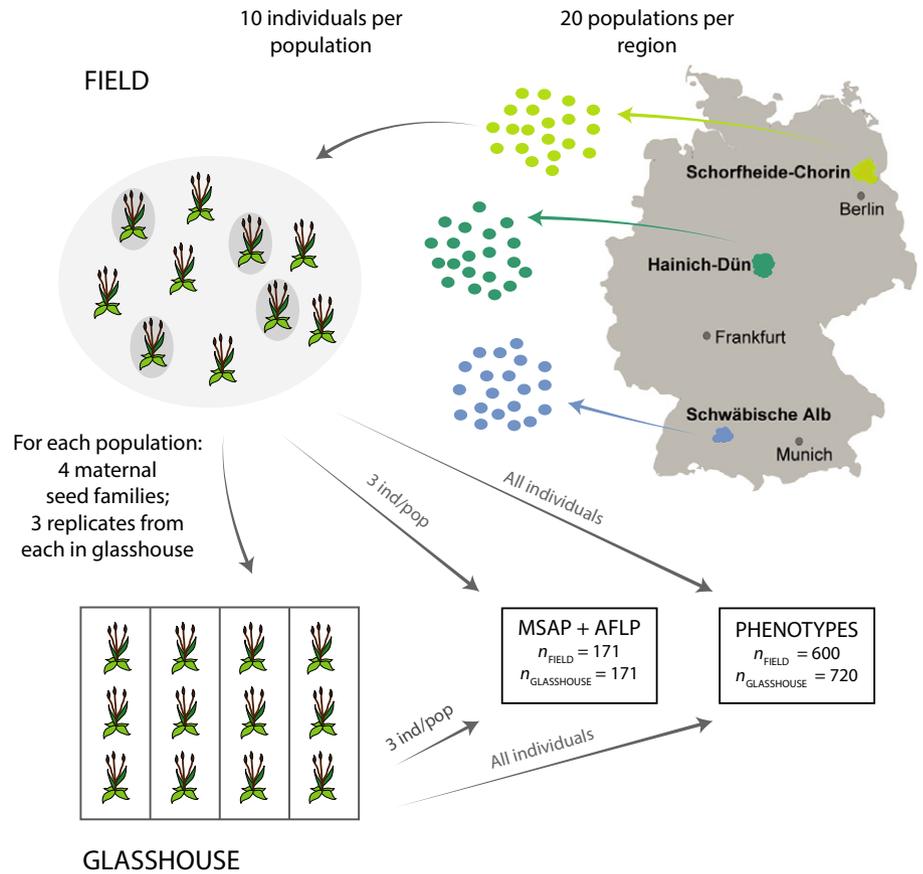
Fig. 1 Schematic of the experimental design of this study. We studied genetic, epigenetic and phenotypic variation in multiple populations of *Plantago lanceolata* in three regions of Germany, on plants growing in the field, as well as on their offspring grown in a common environment. A subset of the field individuals were used as parents for the F_1 generation, and three individuals per population from each environment were analysed for methylation sensitive amplified polymorphism (MSAP) and amplified fragment length polymorphism (AFLP) variation.

land-use inventories (Blüthgen *et al.*, 2012). *Plantago lanceolata* is one of the most common plant species in the Biodiversity Exploratories. Because of their large geographical extent and wealth of environmental data, these plots offer an excellent opportunity for studying epigenetic variation, and its ecological and environmental correlates, in natural plant populations.

Field survey

In September 2015, we collected leaf and seed material, and took phenotypic measurements in at least 20 grassland plots in each of the three regions. According to a vegetation survey from 2014, *P. lanceolata* occurred on 40, 38 and 27 plots in the three regions from south to north. To minimize the probability of management-related direct environmental induction of DNA methylation changes, we sampled only plots where at least 3 wk had passed after the last land-use event (mowing, fertilization or grazing), eventually limiting ourselves to 20 plots per region which maximized the land-use gradients as well as possible. Within regions, the sampled plots were on average 15 km apart from each other (Schwäbische Alb: mean = 11.2 km, range = 0.4–28.9 km; Hainich-Dün: mean = 14.5 km, range = 0.4–36.3 km; Schorfheide-Chorin: mean = 18.4 km, range = 0.4–42.5 km). We generally considered each plot a separate population.

In each population, we randomly selected at least 10 plant individuals along two parallel transects, altogether 615 individuals across the 60 populations. On each individual, we recorded

plant height, length of the longest leaf, and the number of inflorescences, and we collected seeds for later common-garden cultivation (see in the next section 'Common garden study'). We then collected 2–3 undamaged leaves for molecular analyses and stored these at *c.* 5°C in a cooling box until being transferred to −20°C at the end of the day, and later freeze-dried them. We pressed three to eight leaves between blotting paper for later assessment of leaf traits. Upon returning from the field campaign, these leaves were dried in a drying oven at 70°C for at least 72 h, and they were weighed, and scanned with a flatbed photo scanner (Epson V600). The resulting images were analysed with IMAGEJ (Schneider *et al.*, 2012) to calculate average leaf aspect ratio and specific leaf area for each individual. We used the data from the five measured traits to calculate the phenotypic diversity for each population as the mean coefficient of variation (CV) of the five traits.

Common garden study

In order to obtain an F_1 generation of all studied populations, we sowed seeds of four randomly chosen maternal plants per population into seedling trays and stratified them at 5°C and under moist and dark conditions for 3 wk (Pons, 1992). After that, the trays were moved to a glasshouse with a 16 h : 8 h, day : night cycle at 21 : 15°C. The seeds then rapidly germinated, and we transplanted three seedlings per maternal family into 1-l pots filled with a standard potting soil. Altogether, we transplanted

741 seedlings. After 4 wk of growth, the pots were rearranged into a randomized block design, and were allowed to grow for another 6 wk. After that, we took two undamaged leaves per plant for the molecular analyses, flash-froze them in liquid nitrogen and subsequently freeze-dried them. We took the same phenotypic measurements as in the field populations, with five scanned leaves per plant for leaf trait measurements, and calculated the same population-level phenotypic diversities.

Molecular analyses

In order to assess genetic and epigenetic diversity and differentiation, we performed AFLP (amplified fragment length polymorphism) and MSAP (methylation-sensitive amplification polymorphism) analyses, respectively, on a total of 342 individuals (three regions × 19 populations × three individuals × two growing environments). For each population, we randomly chose three of the four maternal families in the glasshouse for the molecular analyses, and the same maternal families were used from the field samples (Fig. 1). In order to increase the accuracy of our analyses and exclude the possibility of a plate effect, we fitted all samples and technical replicates on one 384 multiwell PCR plate, thereby sacrificing one population from each region. Total genomic DNA was extracted from freeze-dried leaf tissue with the peqGOLD Plant DNA Mini Kit (VWR, Darmstadt, Germany). AFLP and MSAP laboratory and scoring procedures followed the protocols described in Schulz *et al.* (2014; Supporting Information Methods S1). We used four and eight selective primer combinations for AFLP and MSAP, respectively (Table S1). Comparison of the *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I reactions of MSAP analyses resulted in four different conditions for each fragment: (I) nonmethylated (band present in both parallel reactions), (II) CG-methylated (band only present in the *Eco*RI/*Msp*I reaction), and (III) CHG-hemimethylated restriction site (band only present in the *Eco*RI/*Hpa*II reaction), and (IV) an uninformative state with fragments absent in both reactions. We re-coded this data matrix – that contains three informative (I–III) and one uninformative condition (IV) at each locus – into three separate presence/absence matrices that correspond to the three informative conditions using the 'Mixed-Scoring 2' approach (Schulz *et al.*, 2013), thus making maximum use of the MSAP information. Furthermore, the two methylated conditions are methylated by two different enzymes – MET1 and CMT3, respectively – that are part of distinct molecular pathways (Law & Jacobsen, 2010) – and have different stabilities (II > III; see (Schmitz *et al.*, 2013), further supporting their separation. Overall error rates for AFLP and MSAP were 4.25% and 3.37%, respectively, based on 40 (12%) replicate samples each.

Data analysis

All analyses were done in R (R Development Core Team, 2008). To quantify genetic and epigenetic diversity, we calculated for each population Shannon's information index, and the number of polymorphic and private loci using the R script MSAP_CALC (Schulz *et al.*, 2013), based on AFLP or MSAP data. We analysed

and visualized population structure through AMOVA and principal coordinates analysis (PCoA), using the POPPR and ADEGENET packages (Jombart & Ahmed, 2011; Kamvar *et al.*, 2015). As a measure of population differentiation, we used the population-level average Nei and Li distances (synonymous to the Soerensen–Dice and Bray–Curtis distances) from other populations calculated with the POPPR package. All analyses of MSAP data were run separately for each of the three MSAP subepiloci (MSAP-n, MSAP-m, MSAP-h), and in parallel for field and common-garden data (referred to as 'growing environment').

In order to assess the stability of epigenetic diversity and differentiation at the population level, we calculated regressions between the parental (field) and offspring (glasshouse) populations in these variables. In addition, we calculated locus-by-locus transmissibility of DNA methylation as described in Herrera *et al.* (2014), except that we excluded shared absences (cases of 'stability' where 0→0) because we considered them uninformative or misleading (there could be changes in other subepiloci at the same locus), and because the same information could otherwise be used multiple times in different datasets. For the AFLP data, we did not exclude the shared absences, considering that in that case there is only one binary data matrix.

In order to test for relationships between epigenetic, genetic and phenotypic variation, we calculated correlations between the respective Shannon diversities, and the population-level CV in the case of the phenotypes. To further explore relationships between these three levels of variation, while at the same time accounting for spatial autocorrelation (Legendre *et al.*, 2015), we employed redundancy analysis (RDA) combined with distance-based Moran's eigenvector maps (dbMEM; originally termed 'principal coordinates of neighbour matrices', PCNM). We used the VEGAN package in R (Oksanen *et al.*, 2017) to perform RDA and to obtain the spatial eigenfunctions. The dbMEM analysis resulted in six spatial eigenfunctions showing a positive spatial autocorrelation (positive eigenvalues), and we retained the three significant ones for further analyses. We then analysed the relationship between genetic and epigenetic variation both with and without correcting for spatial structure. Likewise, we tested the relationship between phenotypic and epigenetic variation in three different ways: first including only epigenetic data as explanatory variables, then including geographical structure, and finally including both geography and genetic variation. The latter allowed us to test whether there were any epigenotype–phenotype relationships independent from genetic variation. When genetic or epigenetic data were used as explanatory variable, we always used the first three PCoA axes of the respective datasets.

In order to test for relationships between epigenetic and genetic diversity and land-use intensity, we used general linear models that included genetic or epigenetic diversity as dependent variables, and tested for the effects of the different land-use components (mowing, fertilization and grazing), the effects of the regions, the growing environments (field vs glasshouse), and their interactions. Finally, we used RDA to test whether epigenetic variation was related to land-use intensity or other environmental variables, respectively, both with or without correcting for geographical structure via the dbMEM approach. For the

environmental variables, we included the following standardized environmental descriptors of the study plots: elevation, slope, aspect, mean height of vegetation, biomass per area, plant species richness and Shannon-diversity, as well as the Ellenberg indicator values for moisture, soil acidity and nutrients (F, R, N). For land-use intensity, we included the three land-use intensity components: mowing, fertilization and grazing, all taken from the Biodiversity Exploratories database (www.bexis.uni-jena.de). In order to account for multiple testing in the case of the RDAs, we report the false discovery rate-corrected P-values.

Results

Extent, structure and stability of epigenetic variation

The MSAP analysis of 326 individuals yielded 606 polymorphic epiloci, which were resolved into 1481 polymorphic subepiloci (560 n-type, 430 m-type and 491 h-type). AFLP analysis resulted in 545 polymorphic loci. The population-level epigenetic diversity was lower than genetic diversity, and decreased from n- to m- to h-subepiloci (Fig. 2a; Table S2). Population differentiation showed an opposite pattern, with the lowest interpopulation distances for AFLP, larger distances for MSAP n- and m-subepiloci, and strongest differentiation for MSAP h-subepiloci (Fig. 2b). These patterns also were visible in the PCoA, with increasing scatter of individuals from AFLP to MSAP n-, m- and h-subepiloci (Fig. 3). Although there was much overlap between the regions, there was a small degree of segregation, often with the Schwäbische Alb region most distinct from the other two. AMOVA confirmed that there was significant genetic and epigenetic differentiation between regions and populations in most cases, explaining around 2% of genetic variation (Fig. 3, Table S3). However, we found no significant differentiation for AFLP and MSAP-m in the glasshouse with AMOVA. The RDA/dbMEM analysis showed that there was significant spatial structure in all datasets; the amount of variance explained (ranging from 2.14% to 3.56%) generally decreased from the more stable epiloci towards the more unstable ones, and it was generally lower in the glasshouse than in the field (Table 1). In line with the AMOVA and PCoA results, one of the significant spatial eigenvectors separated the Schwäbische Alb region from the other two regions.

In order to assess the stability of the epigenetic differences observed in the field, we related the field-derived dataset to the glasshouse-derived data. The PCoA showed that the overall spread of individuals decreased in the glasshouse, but remained larger in MSAP-n, MSAP-m and particularly MSAP-h than in AFLP data. Moreover, differences among regions disappeared in AFLP and MSAP-m (Fig. 3). Comparison of descriptive parameters showed that the glasshouse-derived diversities followed the pattern of field data ($H'_{AFLP} > H'_{MSAP-n} > H'_{MSAP-m} > H'_{MSAP-h}$; Table S2). AFLP diversity was higher and MSAP-h diversity was lower in the glasshouse, and interpopulation distances were significantly lower in glasshouse in all cases (Fig. 2). In AMOVA, the regional components of variance were generally maintained in the glasshouse, albeit at a slightly lower level, whereas population components were not significant anymore for AFLP and
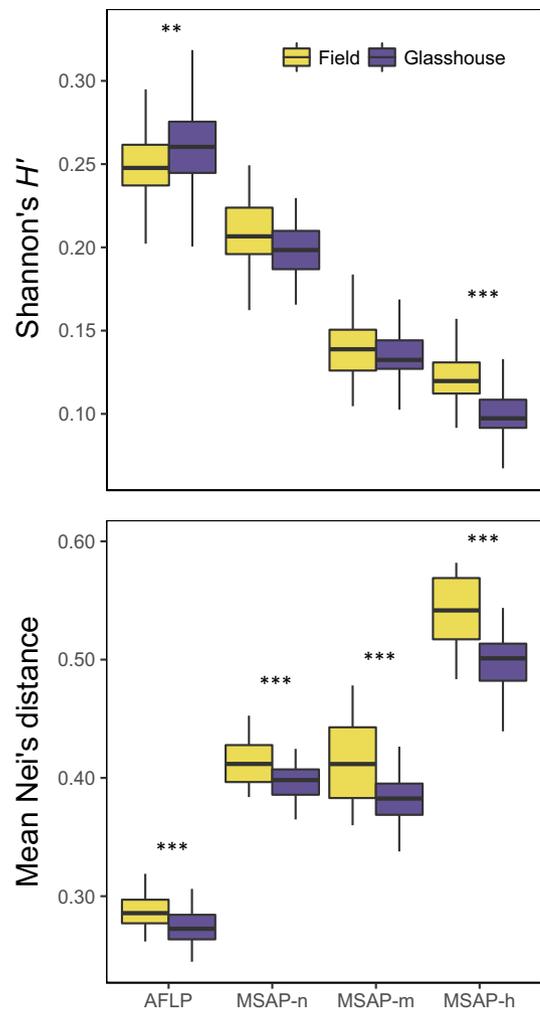

Fig. 2 Magnitudes of epigenetic and genetic variation among 60 natural *Plantago lanceolata* populations. Shannon diversity (H', upper panel) and mean Nei's distances between populations (lower panel) were compared between amplified fragment length polymorphism (AFLP) markers and the three methylation sensitive amplified polymorphism (MSAP) conditions (nonmethylated, methylated and hemimethylated subepiloci), for plants in the field and their offspring grown in a common glasshouse environment. The boxplots indicate medians, 25th/75th percentiles, and the 1.5 × interquartile range. Significant differences between field and glasshouse plants, based on permutation tests with 10^4 replications: **, $P < 0.01$; ***, $P < 0.001$.

MSAP-m (Fig. 3). The comparison of parent and offspring populations for genetic and epigenetic diversity and differentiation data showed significant stability in all MSAP conditions but not in AFLP (Fig. 4; Table S4a). Locus-by-locus transmissibility was highest in the AFLP markers (86%) and decreased from MSAP-n (57%) to MSAP-m (52%) and MSAP-h (40%) (Table S4b).

Relationships between epigenetic, genetic and phenotypic variation

At the level of aggregated, population-level measures of diversity, there were no significant relationships between epigenetic diversity and genetic or phenotypic diversity, respectively (Table S5).
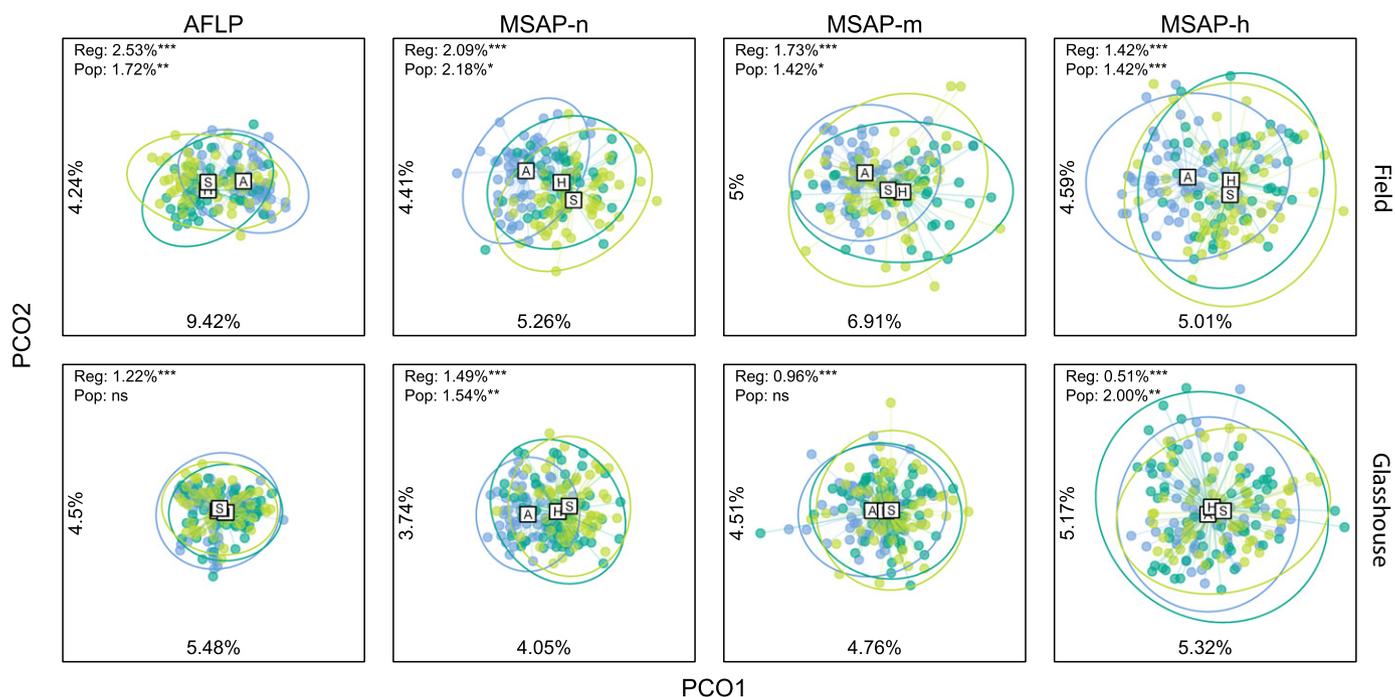
Fig. 3 Epigenetic and genetic variation among *Plantago lanceolata* individuals in the field (upper row) and glasshouse (lower row). We show principal coordinates analyses (PCoA) for genetic (AFLP, amplified fragment length polymorphism) and epigenetic (MSAP, methylation sensitive amplified polymorphism) markers, separately for nonmethylated (MSAP-n), methylated (MSAP-m) and hemimethylated (MSAP-h) subepiloci. The three regions are distinguished by colour (A-Alb, blue; H-Hainich, green; S-Schorfheide, yellow), with their centroid marked by the respective abbreviation, and the coloured ellipses delineating the 95% bivariate confidence interval around their mean. The percentages on the axes indicate the amount of variance explained by each PCoA axis. In addition, the amounts of variance assigned by AMOVA to region and populations are given in the upper left corner of each panel, with significances marked as: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Table 1 Results of redundancy analyses (RDA) relating epigenetic variation in *Plantago lanceolata* to genetic (GEN) or phenotypic (PHEN) variation, as well as to spatial geographic (GEO) variation, environmental (ENV) or land-use intensity (LUI) variation, separately for the three different methylation sensitive amplified polymorphism (MSAP) epiloci types, and for field vs glasshouse data.

	Field						Glasshouse					
	MSAP-n		MSAP-m		MSAP-h		MSAP-n		MSAP-m		MSAP-h	
	Var %	P	Var %	P	Var %	P	Var %	P	Var %	P	Var %	P
EPI vs GEO	3.56	0.003	3.26	0.003	3.09	0.003	3.00	0.003	2.48	0.008	2.14	0.010
EPI vs GEN	3.48	0.003	3.62	0.003	3.28	0.003	2.11	0.003	2.17	0.008	1.95	0.158
EPI vs GEN (GEO)	2.80	0.003	3.12	0.003	2.75	0.003	2.00	0.034	2.15	0.008	1.99	0.158
PHEN vs EPI	4.81	0.320	3.20	0.736	7.43	0.031	3.48	0.494	2.45	0.831	4.47	0.235
PHEN vs EPI (GEO)	4.26	0.420	3.99	0.587	8.52	0.023	3.68	0.494	2.51	0.831	4.44	0.235
PHEN vs EPI (GEO + GEN)	5.15	0.320	4.27	0.587	8.41	0.023	4.36	0.349	3.42	0.791	4.50	0.235
EPI vs ENV	8.28	0.003	7.94	0.003	7.71	0.003	6.79	0.003	6.50	0.008	6.35	0.040
EPI vs ENV (GEO)	7.13	0.072	7.27	0.030	6.96	0.170	6.03	0.386	5.94	0.791	6.20	0.235
EPI vs LUI	2.26	0.012	2.28	0.030	2.26	0.023	1.97	0.023	1.77	0.791	1.84	0.235
EPI vs LUI (GEO)	2.11	0.320	2.12	0.326	2.25	0.043	1.88	0.245	1.74	0.791	1.89	0.235

(GEO) or (GEO + GEN) indicate whether the effects of spatial or genetic structure were accounted for before testing a specific relationship. The values are the % variances explained by each model, followed by their FDR-corrected significance levels. *P*-values < 0.05 highlighted in grey. MSAP-n, nonmethylated; MSAP-m, methylated; MSAP-h, hemimethylated.

However, when we analysed the individual-level relationships between the three types of variation through RDA, we found that except for the hemimethylated loci in the glasshouse, epigenetic and genetic variation were generally significantly related (Table 1). When spatial autocorrelation was included, the amounts of variance explained decreased, but the relationships remained significant. Moreover, the variance explained was generally lower in the glasshouse than in the field.

In contrast to epigenetic–genetic relationships, there was little evidence of relationships between epigenetic and phenotypic variation. Only for MSAP-h loci in the field, phenotypic variation was significantly related to epigenetic variation, and this
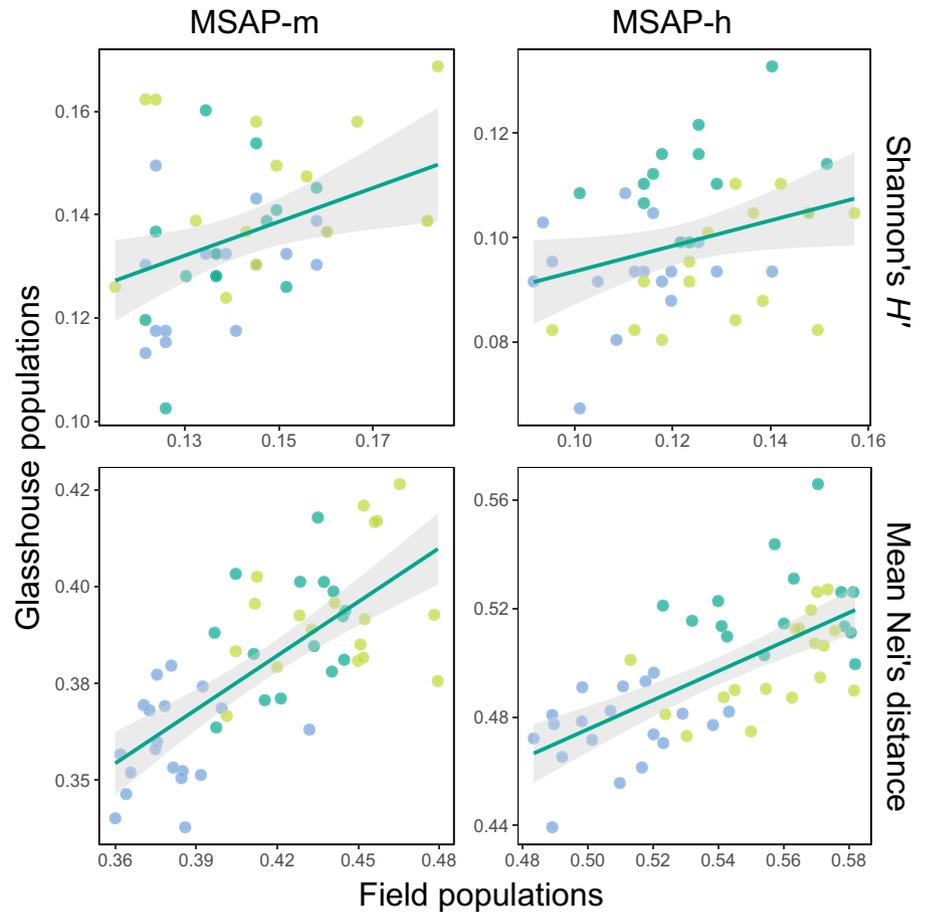
Fig. 4 Stability of epigenetic variation in natural populations of *Plantago lanceolata*. We show relationships between the epigenetic diversities and mean epigenetic distances of 60 wild populations and their glasshouse-grown offspring, separately for methylated (MSAP-m) and hemimethylated (MSAP-h) loci. The three regions are distinguished by colour (Alb, blue; Hainich, green; Schorfheide, yellow). The fitted generalized linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively. All four regressions are significant at $P \leq 0.01$.

relationship remained significant also after incorporating spatial and genetic structure into the model.

Environmental correlates

We found a significant positive relationship between grazing intensity and MSAP-m diversity in both field and glasshouse plants (grazing main effect: $F = 11.6$, $P = 0.001$), and a significant grazing-by-growing-environment interaction for MSAP-h diversity ($F = 7.35$, $P = 0.008$), where a positive correlation was present in the field but disappeared in the glasshouse (Fig. 5; Table S6). In addition, there also was a significant main effect of mowing on MSAP-h diversity ($F = 5.20$, $P = 0.025$), and a mowing-by-region interaction for MSAP-n diversity ($F = 5.98$; $P = 0.004$) (Table S6). We found no significant land-use effects in the analysis of genetic diversity.

Epigenetic variation was not only related to land use, it also was significantly related to other environmental factors for all MSAP loci types in the field and glasshouse. In the RDA analyses, environmental descriptors explained some 6–8% of the epigenetic variation, following the same pattern as before: the variation explained decreased from the more stable towards the more unstable epiloci and was lower in the glasshouse than in the field (Table 1). However, when spatial structure was included in the model, only the relationship with MSAP-m in the field remained significant. Epigenetic variation was also related to land

use in the RDAs, with significant relationships in the field for all MSAP epiloci and in the glasshouse for MSAP-n. When geographical structure was included in the models, the only remaining significant relationship was the one including the MSAP-h epiloci in the field.

Discussion

The ecological and evolutionary role of epigenetic variation in natural plant populations has received much attention in recent years. Here, we studied the extent, structure and stability of epigenetic diversity and differentiation, and its genetic, phenotypic and environmental correlates, in a large number of natural populations of *Plantago lanceolata*. We found low levels of epigenetic variation and population structure, and a partly stable transmission of the signal into the next generation. The heritable part of the epigenetic variation was consistently related to genetic and environmental variation, and to the land-use intensity in the studied grasslands, whereas the nonheritable part was associated also with plant phenotype.

Extent, structure and stability of epigenetic variation

We found that overall levels of within-population epigenetic diversity were rather moderate in natural populations of *P. lanceolata*, and that the values for epigenetic diversity were
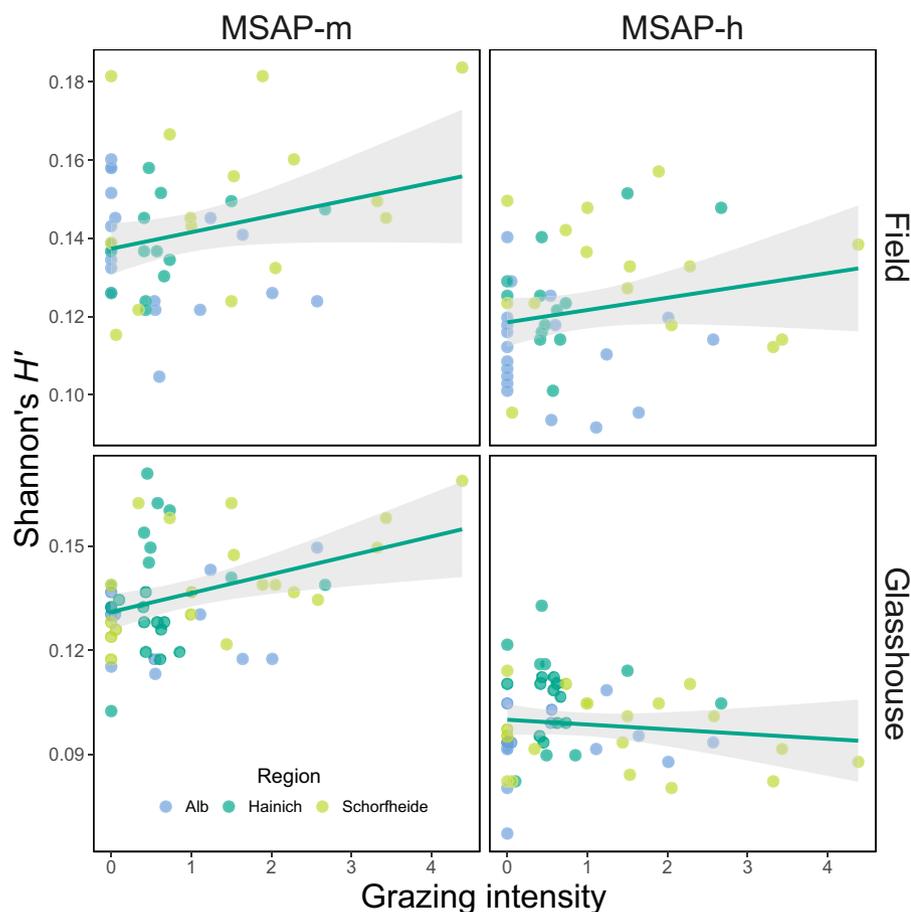
Fig. 5 Relationships between grazing intensity and epigenetic diversity in 60 grassland populations of *Plantago lanceolata*, separately for the methylated (MSAP-m) and hemimethylated (MSAP-h) methylation sensitive amplified polymorphism (MSAP) conditions. Epigenetic diversity increases with grazing intensity, and the pattern is stable in the methylated, but not in the hemimethylated condition. The fitted generalized linear models (GLMs) and 95% confidence intervals are shown as solid lines and grey shading, respectively.

generally lower than for genetic diversity. This is in contrast to several previous studies which found higher epigenetic than genetic diversity (Herrera & Bazaga, 2010; Lira-Medeiros *et al.*, 2010; Richards *et al.*, 2012, 2017; Medrano *et al.*, 2014; Schulz *et al.*, 2014). It is possible that this is mainly driven by the relatively high within-population genetic diversity. *Plantago lanceolata* is wind-pollinated and an obligate outcrosser, and these characteristics, together with enhanced dispersal through livestock and vehicles in the studied semi-agricultural landscapes, most probably result in high gene flow (which also is indicated by the low levels of population differentiation, the high number of polymorphic loci and extremely low number of private loci in populations). This in turn maintains higher diversity in the more stable amplified fragment length polymorphism (AFPL) loci, whereas the less stable methylation-sensitive amplification polymorphism (MSAP) loci are partly homogenized within populations by the common environmental conditions. This idea is supported by the differences between MSAP subepiloci types, where within-population diversity decreases from the more stable nonmethylated to the less stable methylated and hemimethylated conditions.

Surprisingly, we found that genetic diversity (within populations) was significantly higher and genetic differentiation (among populations) was lower in the glasshouse than in the field. A possible explanation is that we established the glasshouse experiment from randomly selected seedlings, which, unlike their mother

plants, had not undergone any selection, thus resulting in a higher diversity in the F_1 generation.

Epigenetic differentiation between regions and populations was generally low but nevertheless significant in all cases except for population differentiation of MSAP-m in the glasshouse. In contrast to the results with within-population diversity, population differentiation was generally larger at the epigenetic level than at the genetic level, with highest values for the least stable (MSAP-h) markers. Again, these results are consistent with the idea that epigenetic variation is generally more responsive to environmental conditions, which on the one hand decreases diversity within populations but at the same time increases divergence between natural populations, relative to genetic variation.

The epigenetic differentiation observed in the field was not just a result of short-term environmental induction, but much of it was stably transmitted to the F_1 offspring, as shown by the analysis of glasshouse data, the parent–offspring population comparisons, the locus-by-locus transmissibility analyses, and the redundancy analyses including spatial eigenvectors. Although population differentiation generally decreased in glasshouse plants, it remained substantially larger at the epigenetic than the genetic level, with strongest differentiation in MSAP-h markers. In general, hemimethylated MSAP-h loci (reflecting CHG sequence context) appear to be more responsive to the environment than methylated MSAP-m loci, but they also lose their differences again more rapidly in a common environment. The

transmissibility of DNA methylation observed in the different conditions was in concordance with previous results from *A. thaliana* (Schmitz *et al.*, 2013; Van Der Graaf *et al.*, 2015).

To our knowledge, our study constitutes the first rigorous test of the stability of natural epigenetic variation through comparison of wild plants and their common-garden offspring in a sexually reproducing nonmodel plant. Gao *et al.* (2010) compared field and common-garden populations of the invasive alligator weed (*Alternanthera philoxeroides*), but with vegetatively propagated material originating from only three contrasting habitats. The authors found a very low level of variation (*c.* 5% polymorphic MSAP loci), but nevertheless 22% of the polymorphic loci were transmitted from field to common garden. Other studies quantified the heritability of stress-induced changes in a controlled environment (Verhoeven *et al.*, 2010), or sporophyte-to-pollen transmissibility of DNA methylation in the field (Herrera *et al.*, 2014), all without including both field and common garden populations. In summary, we found weak but significant natural epigenetic population structure, and part of the population differences in epigenetic diversity were maintained in a common environment.

Relationships between epigenetic, genetic and phenotypic variation

Besides characterizing the extent, structure and stability of natural epigenetic variation in itself, another major goal is to understand the (genetic and environmental) origins of this variation and its ecological and evolutionary consequences. Here, we found consistent significant relationships between epigenetic and genetic variation, as well as some – albeit weaker – evidence for a relationship between epigenetic and phenotypic variation.

Depending on MSAP epilocus type and growing environment, the genetic variation among *Plantago* individuals explained 2–3% of the epigenetic variation in our study. Other field studies in nonmodel plants (Herrera & Bazaga, 2010; Schulz *et al.*, 2014; Foust *et al.*, 2016) found no relationships between epigenetic and genetic variation at all, whereas (usually controlled-environment) studies in the model plant *A. thaliana* generally showed strong genetic control of DNA methylation variation (Dubin *et al.*, 2015; Kawakatsu *et al.*, 2016), which led to debate about the true epigenetic nature of DNA methylation. Although the previous nonmodel studies might have missed true genetic–epigenetic relationships because of the few MSAP and AFLP markers they used, or because of their less controlled environmental conditions which created additional stochastic and environmentally induced epigenetic 'noise' and thereby made it more difficult to detect such relationships, another explanation could be that epimutation rates in these species also could be several orders of magnitude greater than genetic mutation rates, as it has been shown in *A. thaliana* (Schmitz *et al.*, 2011), and thus the two types of variations diverged. However, the strong epigenetic–genetic association in *A. thaliana* could be due to its unusual genomic and epigenomic characteristics (i.e. small genome size and low global DNA methylation (Alonso *et al.*, 2015), transposable elements (TEs) and DNA methylation concentrated around the centromeres). Most other plants have larger genomes with more TEs and DNA methylation along the whole chromosomes (Mirouze & Vitte, 2014) making it very difficult to extrapolate from *A. thaliana* to other species. It seems plausible that in the majority of plants the truth lies somewhere in between, with a strong genetic control of epigenetic variation but also some level of independence of it. Here, we found some genetic–epigenetic associations, but the total amount of epigenetic variation explained by genetic variation remained low, most likely a consequence of the extremely high degree of heterozygosity of the *P. lanceolata* genome (A.-L. Laine, pers. comm.), mirrored by the high polymorphism of AFLP loci and low variation among populations, and of the low resolution of MSAP and AFLP markers, even though the number of markers used was close to the upper limit of feasibility for these methods.

We also found some association between epigenetic and phenotypic variation. In the field data, some 8% of the combined variation in five of the phenotypic traits could be explained by variation in MSAP-h, the most unstable type of MSAP epiloci, even after correcting for geographical and genetic variation. However, these patterns were absent in glasshouse data, which suggests that some of the phenotypic responses by which these plants respond to environmental variation in the field might be associated with underlying reversible DNA methylation changes. Other studies in wild nonmodel populations also found natural epigenetic and phenotypic variation to be related (Herrera & Bazaga, 2010, 2013; Medrano *et al.*, 2014), and studies with *A. thaliana* epiRILs demonstrated a mechanistic relationship between epigenetic variation and phenotypic variation (Cortijo *et al.*, 2014; Kooke *et al.*, 2015). In the former ones, no common-garden measurements were part of the design, whereas in the *Arabidopsis* studies the epigenetic–phenotypic relationships proved to be heritable over several generations. From our data this does not seem to be the case in *P. lanceolata*, which is infamous for its high phenotypic plasticity (Warwick & Briggs, 1979), as well as high gene flow and heterozygosity.

Environmental correlates

Whether epigenetic variation is plastic, environmentally induced, or stable, ultimately only a significant relationship with the environment is proof that the observed variation is ecologically significant (Bossdorf *et al.*, 2008). However, testing for such environmental correlates in a large population sample requires high-quality environmental data for all studied populations. We were fortunate to be able to use the rich metadata from the Biodiversity Exploratories, which allowed us not only to relate epigenetic to geographical and environmental variation, but also to test the effects of mowing, fertilization and grazing intensity – land-use processes that play a key role in the studied grasslands (Fischer *et al.*, 2010; Blüthgen *et al.*, 2012). We found a consistent and stable relationship between epigenetic and environmental variation that was maintained in the F_1 generation in a common environment, suggesting that at least part of the observed epigenetic variation might be related to environmental adaptation of *P. lanceolata*. The epigenetic-environmental

relationships mostly disappeared after spatial structure was incorporated into the models which indicates that not only epigenetic variation, but also environmental factors were spatially autocorrelated and likely co-varied in space.

Epigenetic variation also was related to land use, albeit to a much lesser degree than to other environmental variables. We found grazing intensity to be positively related to epigenetic diversity in the field. A possible explanation for this is that grazing creates environmental heterogeneity which results in variable epigenetic signatures of plant individuals. In contrast to mowing and fertilization, which are applied rather homogeneously within managed grasslands, grazing is a spatially heterogeneous process, with irregular trampling patterns, selective removal of biomass, and patchy deposition of nutrients from animal droppings (Bakker *et al.*, 1984; Adler *et al.*, 2001; Socher *et al.*, 2013). The relationship was plastic – not maintained in the glasshouse – for the hemimethylated MSAP loci, but it was stable for the methylated loci, consistent with the different stabilities of the two subepilocus types. Together, these results suggest that the grazing-related MSAP-h variation might reflect plastic phenotypic responses of *Plantago lanceolata* to land use, whereas the MSAP-m variation might reflect past selection on stable epigenetic variation, and thus adaptive epigenetic differentiation in these plant populations.

Of course, we only studied one offspring generation, so we cannot distinguish between environmentally induced, transient heritability – as has been found, for example, in *A. thaliana* (Wibowo *et al.*, 2016) – from truly stable epigenetic variation. Nevertheless, our study is the first demonstration of stable environment–epigenetics relationship in natural populations of a sexually reproducing nonmodel plant, and it is particularly intriguing that we observed this relationship only between grazing intensity and epigenetic but not genetic variation, demonstrating that at least sometimes epigenetic variation has the potential to provide truly novel insights.

Author contributions

OB and WD planned and designed the research; BG conducted fieldwork, performed experiments and laboratory work; and all authors analysed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Methods S1 AFLP and MSAP protocol, adapted from Schulz *et al.* (2014).

Table S1 Adaptor- and primer sequences used for AFLP and MSAP analyses.

Table S2 Measures of genetic and epigenetic diversity in *Plantago lanceolata*.

Table S3 AMOVA results.

Table S4 (a) Regressions comparing epigenetic variation of parent and offspring populations. (b) Locus-by-locus transmissibility.

Table S5 Correlation analyses between epigenetic and genetic diversity, and between epigenetic and phenotypic diversity.

Table S6 Results of GLM analyses of land-use effects on epigenetic and genetic diversity.

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