Received: 13 January 2020 | Revised: 1 October 2020 | Accepted: 2 October 2020

DOI: 10.1111/mec.15689

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Ecological divergence of wild strawberry DNA methylation patterns at distinct spatial scales

Hanne De Kort[1] | Bart Panis[2] | Dieter Deforce[3] | Filip Van Nieuwerburgh[3] | Ollivier Honnay[1]

[1]Plant Conservation and Population Biology, University of Leuven, Leuven, Belgium

[2]Bioversity International, K.U. Leuven, Leuven, Belgium

[3]Laboratory of Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium

Correspondence
Hanne De Kort, Plant Conservation and Population Biology, University of Leuven, Leuven, Belgium.
Email: hanne.dekort@kuleuven.be

Funding information
Fonds Wetenschappelijk Onderzoek, Grant/Award Number: 12P6517N

Abstract

Epigenetic change is considered relatively unstable and short-lived, raising questions of its contribution to long-term adaptive potential. However, epigenetic modifications can accumulate in the presence of environmental stress, resulting in beneficial epigenetic memories where environments are challenging. Diverging epigenetic memories have been observed across large spatial scales, and can persist through multiple generations. It is unknown, however, to what extent epigenetic variation contributes to fine-scale population structure and evolution. We compared DNA methylation patterns between a steep, altitudinal gradient (<2 km) and a wide spatial gradient (>500 km) using whole genome bisulphite sequencing data from 30 *Fragaria vesca* plants germinated and grown in controlled conditions. To assess the stability of spatial epigenetic variation in the presence of an environmental stressor, we applied acute drought stress to part of the plants and quantified drought-induced changes in DNA methylation signatures. We find that epigenetic memories and genomic islands of epigenetic divergence arise even at fine spatial scale, and that distinct spatial scales are featured by distinct epigenetic patterns. For example, demethylation of transposable elements consistently occurred at the large but not the fine spatial scale, while methylation differentiation for most biological processes were shared between spatial scales. Acute drought stress did not result in significant epigenetic differentiation. Our results indicate that population history, rather than short-term environmental stress, plays a dominant role in shaping epigenetic signatures. Specifically, repeated historical stress levels associated with heterogeneous environmental conditions may be required for acquiring a stable epigenetic memory and for coping with future environmental change.

KEYWORDS

DNA methylation, epigenetic memory, landscape genetics, nongenetic inheritance, phenotypic plasticity

1 | INTRODUCTION

Genetic variation underlying fitness traits is considered the dominant resource upon which plants depend for evolving under environmental change. Driven by drift, mutation and migration, such genetic variation supplies populations with trait values that support local fitness and adaptive potential. However, although it is widely assumed that adaptive phenotypic variation is mainly regulated by

the underlying genetic architecture, only small proportions of the total phenotypic variation observed in many species have been associated with genetic variants (Krishna Kumar et al., 2016; Resende et al., 2017; Wellenreuther & Hansson, 2016). The remaining phenotypic variation, typically referred to as missing heritability, can be roughly attributed to (a) the detection limits of rare genetic variants and genetic interactions, and (b) heritable epigenetic variation (Banta & Richards, 2018; Brachi et al., 2011; Gienapp et al., 2017; Miska & Ferguson-Smith, 2016; Whipple & Holeski, 2016).

The role of epigenetic variation in governing adaptive evolution remains controversial, yet a growing body of literature has demonstrated the ubiquity of transgenerational epigenetic transmission, and consequently considers it as a key evolutionary force (Danchin et al., 2019; Gugger et al., 2016; Lind & Spagopoulou, 2018; Miska & Ferguson-Smith, 2016; Quadrana & Colot, 2016; Schmid et al., 2018; Zhang et al., 2018). Nonrandom epigenetic variation in DNA methylation levels has been shown to be widespread in natural populations, and to covary with a range of environmental stressors, including herbivory, drought, salt and temperature (Alonso et al., 2019; Foust et al., 2016; Gáspár et al., 2019; Jeremias et al., 2018). While most stress-induced methylation changes are reset to basal levels after stress relief, part of these modifications can be stably inherited across mitotic and even meiotic cell divisions (Chinnusamy & Zhu, 2009; Crisp et al., 2016). Such an epigenetic stress memory may allow plants to cope more effectively with subsequent stresses, thereby evoking considerable fitness benefits in heterogeneous environments (Crisp et al., 2016; Hilker et al., 2016). Unravelling the relative extent of intragenerational epigenetic change resulting from acute environmental stress versus relatively stable transgenerational epigenetic memories may contribute to our understanding of how plants rely on their epigenetic machinery for coping with environmental change.

How selection pressures affect genome-wide DNA methylation levels in natural plant populations remains poorly explored in non-model organisms, but considerable advances have been made in *Arabidopsis thaliana*. In plants, DNA methylation typically occurs at cytosines in three sequence contexts: CG, CHG or CHH (where H represents a G, T or A nucleotide) (Law & Jacobsen, 2010). A study involving genome-wide DNA methylation analysis of 122 *A. thaliana* accessions sampled across Eurasia showed that climate characteristics most abundantly covaried with methylation levels of cytosines in the CHH context, with CHH methylation typically indicating the involvement of transposable elements (TEs) (Kawakatsu et al., 2016; Keller et al., 2016). These findings could be related to natural selection at the level of TE-specific methyltransferase genes that facilitate demethylation of transposons when temperatures reach extreme levels, or where populations are genetically impoverished. Stress-induced demethylation of transposons boosts transposon activity and subsequent genetic change, paving the way for rapid genetic replenishment and adaptation to environmental stressors (Ito et al., 2016; Mirouze & Paszkowski, 2011; Rey et al., 2016; Schrader & Schmitz, 2019). The strongest associations between climate and *A. thaliana* methylation levels were, however, found in CG contexts within or near genes related to abiotic stress responses, development and reproduction (Keller et al., 2016). Because (a) DNA methylation has been shown to be meiotically most stable in the CG context, and (b) the majority of reported heritable epimutations occurs at CG sites (Jiang et al., 2014; Mathieu et al., 2007; Stassen et al., 2018), climate–CG methylation associations probably represent solid adaptive signals. A recent study corroborated the evolutionary relevance of CG methylation using a multigenerational *A. thaliana* selection experiment, demonstrating that (a) methylation of differentially methylated cytosines (DMCs) was significantly higher in the CG context after five generations in a selective environment simulating habitat fragmentation, (b) the majority of these DMCs were stably inherited for two or three generations following the selection experiment, (c) selection caused overall reductions in epigenetic diversity and (d) methylation levels of some CG DMCs were associated with phenotypic changes (Schmid et al., 2018).

Genome-wide DNA methylation studies in *Quercus* species showed patterns similar to those obtained in *A. thaliana*: DMCs associated with environmental stressors dominate in the CG context, and these DMCs occur in or near genes (Gugger et al., 2016; Platt et al., 2015). Using the experimentally more versatile herb *Plantago lanceolata* as a study organism, Gáspár et al. (2019) demonstrated that much of the environment-related epigenetic variation is maintained in a second-generation common garden. Thus, at least part of the epigenetic variation observed in the field is stable, genomically nonrandom and of ecological significance. Although these studies considerably increased our understanding of how epigenetic variation is distributed across large spatial scales, it remains unknown to what extent epigenetic variation contributes to population divergence along fine-scale environmental gradients, where the interplay between migration, drift and selection can be extremely dynamic (Richardson et al., 2014). Highly heterogeneous environments may thus give rise to distinct signatures of epigenetic variation.

Evidence is accumulating that epigenetic variation may be particularly beneficial where genetic diversity is in short supply, such as following demographic bottlenecks or in clonal plant species (Ardura et al., 2017; Artemov et al., 2017; Latzel et al., 2016; Rendina González et al., 2018; Thorson et al., 2017; Wibowo et al., 2018). More fundamentally, Dapp et al. (2015), using epigenetic inbred lines of *A. thaliana*, demonstrated that epigenetic diversity can drive hybrid vigour in the absence of genetic diversity. Thus, epigenetic variation may be a crucial element of population persistence where evolutionary trajectories or life history traits limit genetic diversity. Study systems combining strong evolutionary pressure (e.g., expansion fronts or heterogeneous environments) and life history traits that constrain genetic diversity (e.g., asexual reproduction and high levels of self-compatibility) are therefore promising for obtaining more insights into the role of epigenetic variation in adaptation and population persistence.

Here, we explore genome-wide epigenetic profiles of second-generation common garden plants originating from three natural woodland strawberry (*Fragaria vesca* L.) populations that were found to harbour strong natural differentiation in terms of traits related to fitness, probably driven by local topography impacting local soil moisture levels (De Kort et al., 2020). One of the most notable

patterns observed in a controlled common garden environment was that plants adapted to the stressful conditions at high-altitude, south-oriented locations were smaller and produced fewer flowers than plants originating from low-altitude, north-oriented locations (De Kort et al., 2020). These data illustrate strong fine-scale adaptive divergence along the altitudinal gradient studied here. *F. vesca* has also been shown to harbour limited genetic diversity across its range (Hilmarsson et al., 2017), presumably as a result of its life history (self-compatible and clonal). Due to this limited genetic diversity in combination with pronounced altitude-dependent phenotypic divergence, we hypothesize substantial adaptive epigenetic signals coinciding with increased stress levels along the studied altitudinal gradient (Figure 1c). We specifically ask the following questions: (a) Do epigenetic memories diverge with altitude, and does DNA methylation change with altitude? (b) Are these fine-scale genome-wide methylation patterns comparable to those obtained at a much larger spatial scale (>500 km)? (c) Are altitudinal DMCs enriched for ecologically relevant gene ontology terms? (d) Does acute drought stress induce detectable epigenetic change?

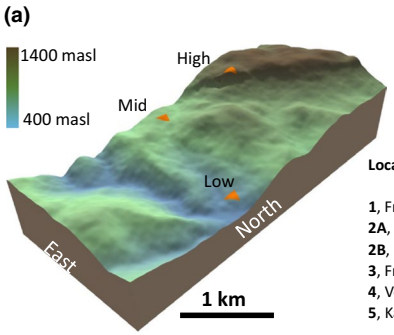
2 | MATERIALS AND METHODS

2.1 | Sample collection

Seeds were collected from five plants at (a) three nearby locations in the French Pyrenees, (b) one location in the French Vosges and (c) one location in Poland (Figure 1). After germination, one seedling per mother plant was randomly selected from every location and grown in controlled conditions in a growth chamber with standardized light and soil moisture regimes. To compare the magnitude of inherited epigenetic memories to intragenerational epigenetic change acquired through acute drought stress, an additional seedling per mother plant was raised for the midaltitude Pyrenean plants, and these seedlings were subjected to reduced soil moisture levels starting 2 months after germination. Specifically, watering stopped until leaves went limp (6–10 days), and this process was repeated consecutively for 4 weeks, after which the plants were allowed to rehydrate for 1 week to remove most drought-induced epigenetic effects that do not result in a relatively stable epigenetic signal. Between each of three cycles of drought, plants were watered for 3 days to allow partial recovery of the soil. The plants were full grown (~3 months of age) prior to the drought treatment and whole genome bisulfite sequencing (WGBS). They were kept in a growth chamber at room temperature and with regular growth lamps. One leaf per plant was then collected in liquid nitrogen prior to DNA extraction, resulting in 30 samples (Figure 1).

2.2 | Whole genome bisulphite sequencing and DMC calling

DNA of 30 freeze-dried samples was extracted with a QIAGEN kit. Up to 200 ng of DNA was fragmented to 400 bp with a Covaris S2 sonicator prior to whole-genome library preparation (NEBNExt Ultra II kit), ligation of methylated adaptors and size selection on 2% E-gel (450–650 bp). Bisulphite conversion was performed using the EZ DNA methylation gold kit (Zymo Research). The low percentage of unconverted CHH cytosines gives an indication that the bisulphite conversion step was successful and adequate (Figure S1). An enrichment PCR (polymerase chain reaction) was performed using KAPA Hifi hotstart Uracil + mastermix in a 12-cycle PCR. Paired-end 75-bp sequencing of the library fragments was performed on eight lanes of an Illumina Hiseq4000 sequencer, generating 76,417,704 ± 13,837,490 reads (mean ± *SD*) per sample. An extensive quality control was performed on the sequencing data using FASTQC version 0.11.5 (Babraham Bioinformatics), showing a mean quality value across each base position in the reads, average quality scores of the reads and average GC content of reads expected from a high-quality Illumina sequencing run (see Figures S1–S3 for quality and coverage stat). TRIMMOMATIC (Bolger et al., 2014) version 0.36 was used to trim reads for sequences with a Phred score lower



Location (N)	Altitude	Space	Latitude	Longitude
1, French Pyrenees (5)	High		42.93007	1.151337
2A, French Pyrenees (5)	Mid(Dry)		42.927851	1.165021
2B, French Pyrenees (5)	Mid(Moist)		42.927851	1.165021
3, French Pyrenees (5)	Low	West	42.942821	1.170258
4, Vosges (eastern France) (5)		Central	48.197752	5.884375
5, Karkonosze (Poland) (4)		East	50.883056	15.559722

FIGURE 1 Geographical location of altitudinal WGBS samples (a and b) and of the broader spatial WGBS (b). Samples originate from southwestern France (Pyrenees), eastern France (Vosges) and southwestern Poland (Karkonosze). N represents the number of successfully sequenced samples (one Polish sample failed post-sequencing quality checks). Samples from location 2 are used for comparisons within the altitudinal gradient (Mid) as well as between the soil moisture treatments (Dry vs. Moist). Samples from location 3 are used for comparisons within the altitudinal (Low) as well as the spatial (West) gradient. High, mid- and low altitude correspond to 1, 200, 750 and 450 m asl, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

than 33 and sequences corresponding to Illumina TruSeq adapters. Sequences shorter than 75 bp after trimming were discarded using default filtering parameters. One sample from Poland ("East") showed an increased level of duplicate reads and was excluded from further analysis (Figure S3).

FASTQ SCREEN version 0.11.1 (Babraham Bioinformatics) was used to remove genomic sequences originating from other species. The trimmed reads were mapped against the Fragaria_vesca_v4.0.a1 genome using BISMARK version 0.17.0. (Krueger & Andrews, 2011). Average sequencing depth after mapping and deduplication was 30×.

Averaged across the 30 samples, 45.4%, 15.0% and 3.6% of CG, CHG and CHH cytosines were methylated after filtering and mapping, and thus were considered for downstream DMC calling, using the R package METHYLKIT version 1.10.0 with conventional DMC parameters (Akalin et al., 2012). Specifically, only cytosines with at least 5 × coverage in at least three samples per group were retained (Walker et al., 2015; Wan et al., 2016). To reduce bias due to outlier depth, bases with a read depth above the 99.9th percentile of coverage were filtered out. The filtered data were used to test for DMCs, considering a 25% difference and q-values <0.05 as significant. Significant DMCs were identified between (a) low-, mid- and high-altitude samples (hereafter "altitudinal DMCs"), (b) the three distance European samples (hereafter "spatial DMCs"), and (c) the two soil moisture treatments (hereafter "drought DMCs").

To test the robustness of our results depending on the DMC settings, we reconstructed DMC density and clustering plots (see "Methylation profiling of altitude, space and drought") with the following parameters (bold indicates deviation from conventional DMC parameters):

- min sequencing depth of 5 in at least three samples, q-value < 0.05, methylation difference 10%
- min sequencing depth of 5 in at least three samples, q-value < 0.005, methylation difference 25%
- min sequencing depth of 10 in at least three samples, q-value < 0.05, methylation difference 25%

These alternative parameters merely altered comparative methylation patterns and downstream results (Figures S4 and S5).

2.3 | Methylation profiling of altitude, space and drought

All DMCs (q < 0.01, Table S1) were assumed to be ecologically divergent (i.e., resulting from drift or fitness differences). Because the epigenetic signals observed here have persisted in the second generation (hereafter "common garden generation"), they may represent stable epigenetic changes. However, because the long-term transgenerational stability of these DMCs is uncertain, we further refer to ecological rather than evolutionary divergence of methylation patterns. Cytosines that were significant along the altitudinal gradient ("altitude DMCs") were thus considered to be ecologically divergent along this gradient. We similarly defined "spatial DMCs" and "drought DMCs" as ecologically divergent DMCs along the spatial gradient and the soil moisture treatment, respectively. We further assumed that clusters of nearby DMCs have a more important role in genome function and ecological processes because individual DMCs have been suggested to contribute little to gene expression (Paun et al., 2019; Teschendorff & Relton, 2018). We thus defined DMC blocks of 500 bp with a maximum distance of 50 bp between individual DMCs, using the R package "bumphunter v1.12" (see Table S2 showing that alternative clustering parameters do not strongly affect clustering behaviour). For each DMC, we then defined "ClusterSize" as the number of DMCs in the cluster to which the respective DMC belongs. A higher cluster size thus represents a higher tendency of a DMC to cluster with other DMCs. The relationship between DMC differentiation and cluster size was tested using a linear mixed model (DMC differentiation ~ ClusterSize + 1 | Cluster_ID), with Cluster_ID accounting for genomic linkage within clusters. If DMCs involved in large genomic clusters also are significantly more differentiated than solitary DMCs, then these DMCs are strong potential candidates for adaptive processes (isolation-by-environment).

As a measure of epigenetic diversity (ED) that is insensitive to sample size (Schmid et al., 2018; Vellend et al., 2010), we calculated the mean pairwise methylation difference between the five individuals originating from each location (Schmid et al., 2018). High ED thus reflects high variation in the degree of methylation within a population. Because ED represents a proportion, a beta regression model was used to test whether ED differs significantly between populations depending on DMC clustering behaviour and genomic context (ED ~ population + Clustering + Context), with ED averaged across DCMs within clusters. The fixed effect "Clustering" was added to the model to specifically test whether (a) clustered (i.e., potentially adaptive) DMCs have lower ED than solitary DMCs, as would be expected when natural selection causes diverging methylation patterns (Schmid et al., 2018), and (b) clustering behaviour is a more important determinant of ED than genomic context. The term "Clustering" represents a binary variable distinguishing between solitary DMCs and clustered DMCs (at least 10 DMC in a cluster). The modelling was performed using the R package "betareg," which also provides an effect size (pseudo R^2) indicating the explanatory power of the model. For each population, we also provided epigenetic fixation rate defined as the number of DMCs that were either fully methylated or fully demethylated across all samples.

2.4 | DMC enrichment analysis

Gene ontology terms (GOs) were retrieved from the Genome Database for Rosaceae (GDR, Jung et al., 2019). To test which biological processes were over-represented in sequences containing DMCs (as compared to the full *Fragaria vesca* genome), we performed a Fisher's exact test with false discovery rate (FDR) correction as implemented in OMICSBOX, using DMC GOs as test data and *F.*

vesca GOs as reference data. The same analysis was performed for the altitudinal and spatial DMCs, and in CG and non-CG (CHG and CHH) contexts separately.

To address the role of non-CG methylation in transposon regulation, we aligned genes in which we found one or more DMCs to all TEs known in *F. vesca*, using the GDR search function. Specifically, we extracted all genes related to the keyword "transpos" (referring to, for example, transposase, transposon, TE), and grouped our transposon-related DMCs into the two major TE classes (DNA transposons and retrotransposons).

3 | RESULTS

Apart from slight increases in genome-wide methylation levels from high to low soil moisture, and from east to west (Figure S6), no systematic genome-wide differences in methylation were observed between the 29 samples. Nonetheless, to further explore whether specific genomic regions may be affected, we compared the methylation levels at single cytosines between samples. We accordingly detected a total of 82,839, 699 and 23 DMCs along the spatial gradient, the altitudinal gradient and between the soil moisture treatments, respectively (Figures 2a and 3; Table S1). These DMCs often clustered together in genomic islands of differential methylation (Figures 2a, 3 and 4).

Methylation divergence was most pronounced along the spatial gradient, in terms of both frequency (Figure 2a) and strength (Figure 2b), followed by the altitudinal gradient (Figure 2b). A total of 59 altitudinal DMCs (8.5%) were also found along the spatial gradient, and another 153 altitudinal DMCs (21.9%) were located within 500 bp of a spatial DMC, indicating shared methylation patterns among distinct spatial scales.

DMC density profiles, showing cumulative loss and gain in methylation percentages, were substantially different between the altitudinal and the spatial gradient (Figure 2c,d). For altitudinal DMCs, the most dominant shift in methylation was observed in CG contexts, with considerable methylation gain as altitude increased (Figure 3c). However, the most extreme shifts in DMC methylation level (i.e., toward 100% methylation loss or gain) were observed along the spatial gradient (Figure 3d). Thus, fixation of methylation patterns occurred at both fine and large spatial scale, but was more frequent along the spatial gradient.

A total of 247 out of 698 DMCs (35.4%) systematically gained (113 CG, 21 CHG and 20 CHH) or lost (38 CG, 35 CHG and 20 CHH) methylation from low to high altitude. Methylation gains along the altitudinal gradient thus predominantly occurred in the CG context (see also Figure 3c). Along the spatial gradient, a total of 56,795 out of 82,839 DMCs (68.6%) systematically lost (18,099 CG, 8,731 CHG and 3,609 CHH) or gained (17,038 CG, 7,680 CHG and 1,638 CHH) methylation from east to west (see also Figure 3d). The plants from

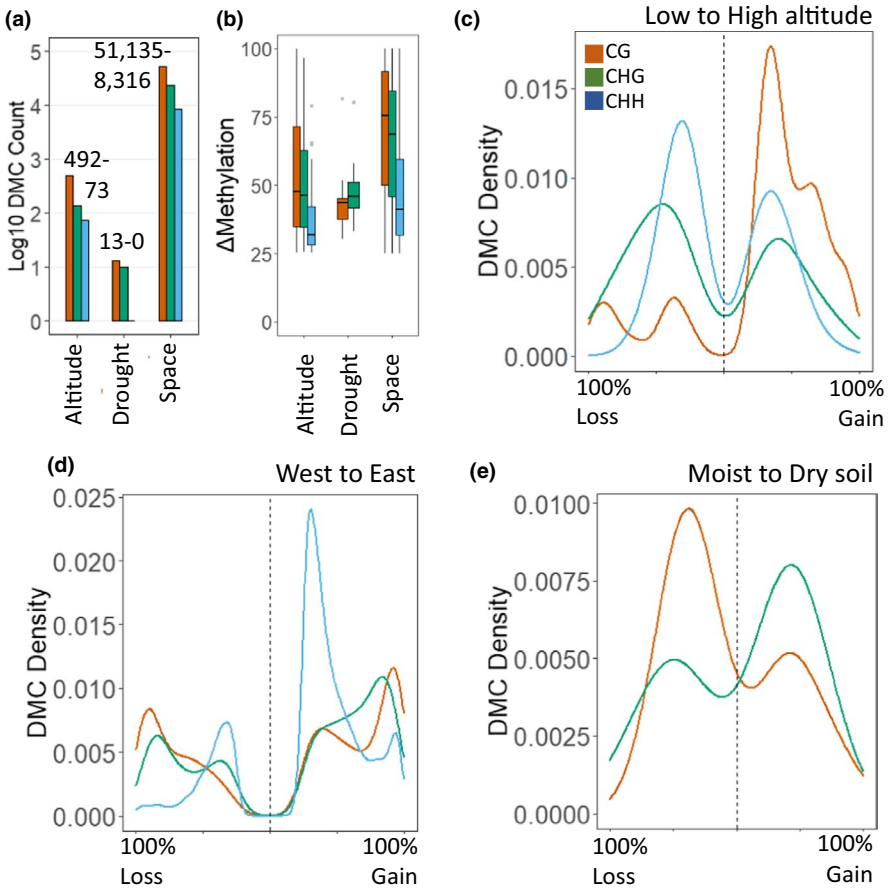


FIGURE 2 Distribution of methylation patterns among the studied gradients, including DMC counts (a), average DMC differentiation (b), and DMC density of change in methylation level along the altitudinal gradient (c), the spatial gradient (d) and between soil moisture treatments (e). Zero in density plots represents no change in methylation percentage from low to high altitude, east to west, and moist to dry, respectively. Dotted lines represent the average change in methylation level. All patterns were visualized for each sequence context separately (CG in orange, CHG in green and CHH in blue). Note that panel (a) shows the range in number of DMCs (e.g., the number of altitudinal DMCs varied from 73 in the CHH context to 492 in the CG context) [Colour figure can be viewed at wileyonlinelibrary.com]

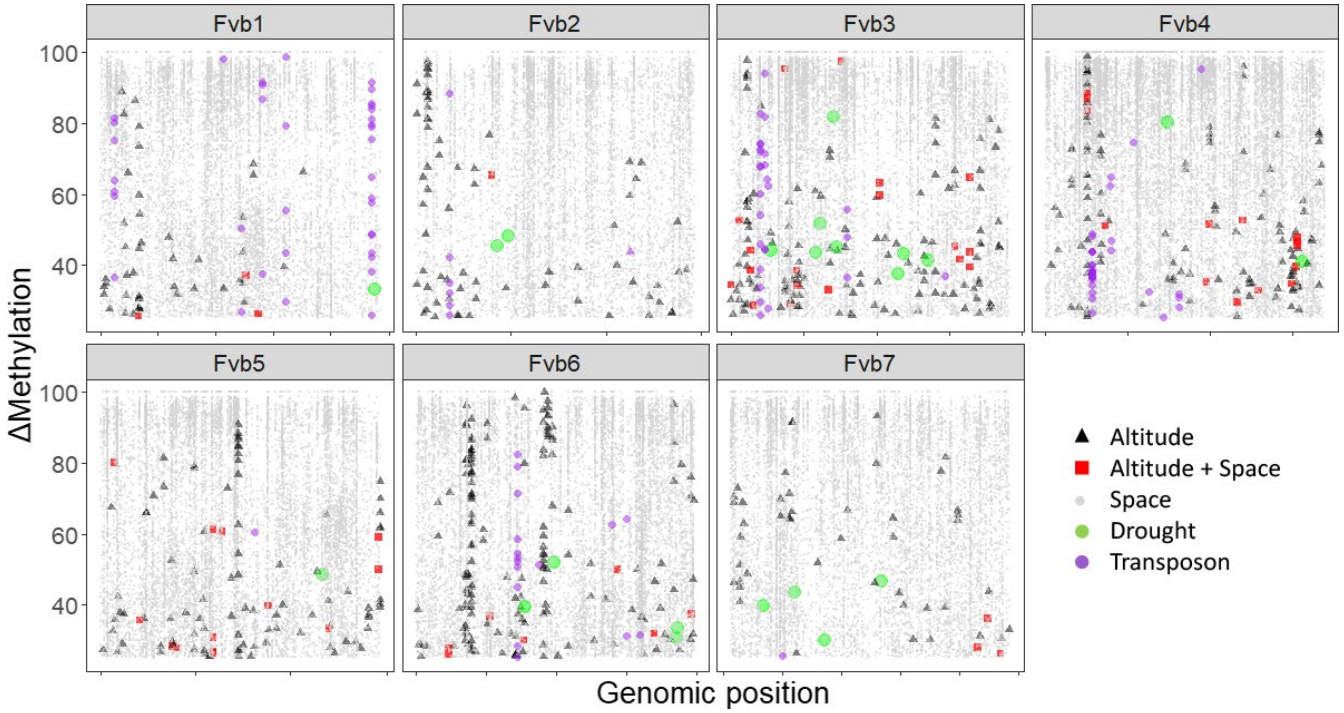


FIGURE 3 Genome-wide differentiation in methylation for the DMCs in all sequence contexts along the spatial gradient (grey), along the altitudinal gradient (red) and between soil moisture treatments (green). Methylation differentiation represents the average difference in methylation percentage between groups (e.g., between west, central and east for the spatial gradient) [Colour figure can be viewed at wileyonlinelibrary.com]

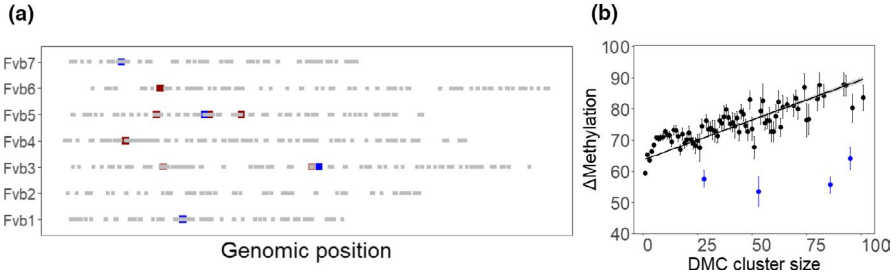


FIGURE 4 Clustering behaviour of DMCs, including the genomic position of DMC clusters (grey = spatial DMC clusters, red = altitudinal DMC clusters and blue = spatial DMC clusters with exceptionally low DMC differentiation) (a) and the relationship between cluster size (number of DMCs in a cluster) and DMC differentiation (blue = DMC clusters with exceptionally low DMC differentiation) (b). See Figure S5 for similar clustering results when different DMC calling parameter settings are chosen [Colour figure can be viewed at wileyonlinelibrary.com]

western Europe thus particularly differ from central Europe in the amount of demethylated CHH sites.

While most DMCs (n = 31,417) clustered together into DMC islands, many DMCs occurred alone (n = 23,578) (Tables S1 and S2). Interestingly, we found a significant positive relationship between DMC differentiation and cluster size, also after correcting for cluster_ID (Figure 4; Table S3, Figure S5). Thus, DMCs that tend to cluster together are also more differentiated. Based on this relationship, we identified four DMC clusters with exceptionally low DMC differentiation (Figure 4). While gene function of three of these DMC clusters is unknown, the fourth cluster lies in a gene involved in histone acetylation required for transcription (FvH4_1g16810). In this gene, all DMCs (n = 27) behaved congruently, with increased methylation levels in Poland, but complete demethylation in other populations (Table S1). Decreasing methylation levels towards the Pyrenees appears to be the rule for clustered DMCs (Figure S7), while solitary DMCs behave more randomly irrespective of spatial direction (Figure S7). Within the Pyrenees, however, both clustered and solitary DMCs systematically increase methylation levels towards higher altitude (Figure S7).

Clustered DMCs were significantly less diverse than solitary DMCs, but the extent of this difference depended on the population (Table S4; Figure 5). Particularly at high elevation, clustered DMCs had significantly lower epigenetic diversity than solitary DMCs (Table S4; Figure 5). The difference in ED between clustered and solitary DMCs also increased from east to west along the spatial

gradient (Figure 5). In addition, there was a marked reduction in ED from east to west for both clustered and solitary DMCs (Table S4; Figure 5). Finally, genomic context did not affect ED, indicating that clustering behaviour is more important than genomic context in explaining differences in ED among the sampled populations of *Fragaria vesca* (Table S4).

Out of 138 and 25 GO terms that were associated with the altitudinal DMCs in CG and non-CG contexts, respectively, 54 (39.1%) and two (8.0%) were significantly enriched in comparison to the full *F. vesca* GO compilation (Tables S5 and S6). A similar proportion of enriched GO terms was observed for the spatial DMCs in the CG context (39.7%, i.e., 578 out of 1,456 GOs). In contrast to the altitudinal DMCs, however, a high proportion of enriched GOs was also found in the non-CG context (48.0%, i.e., 290 out of 604 GOs).

DMCs in CG versus. non-CG contexts represented a distinct set of enriched biological processes (Table S5). Where non-CG DMCs were particularly enriched for regulatory functions (e.g., regulation of gene expression and protein dephosphorylation), biological processes more directly related to environmental stressors were over-represented only in the CG context (e.g., circadian rhythm, antibiotic metabolism and response to light). GOs related to cell division and reproduction (e.g., spindle organization and sexual reproduction) were enriched in both CG and non-CG DMCs. No pronounced differences in GO composition were found between altitudinal and spatial DMCs, and most altitudinal GOs (69%) were also part of the spatial GO distribution (Tables S5 and S6,) Supek et al. 2011.

A total of 30 TEs were found to contain one or more DMCs (Tables S1 and S7). The most heavily differentiated transposon was a putative retrotransposon harbouring not fewer 21 DMCs, of which 15 were in the non-CG and six in the CG context. All differentially methylated transposons except one were found at the large spatial scale, and were dominated by DMCs in the non-CG context. However, there was a clear distinction in DMC context between DNA transposons and retrotransposons, with CHG and CG DMCs only found in retrotransposons (Figure 6). We also found that DNA transposons contained much fewer DMCs ($n = 2.4$) than retrotransposons ($n = 7.1$) (Table S7). On average, transposon DMCs lost 18% and 32% of methylation from east to west in the CG and non-CG context, respectively.

The negligible proportion of genome-wide cytosines that was differentially methylated between soil moisture treatments (Figure 2a,b,e) suggests that short-term acute soil dryness does not result in a pronounced epigenetic signature, and does not seriously impact historically obtained epigenetic memories. Drought stress only generated solitary DMCs (Table S1).

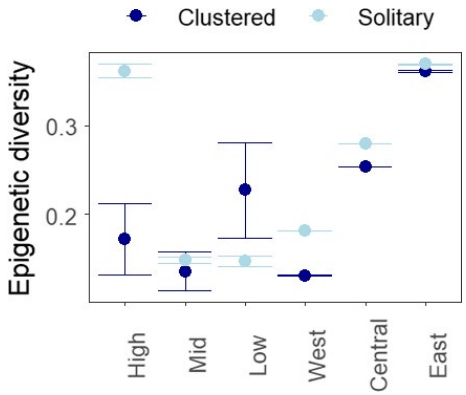


FIGURE 5 Epigenetic diversity (ED) of clustered versus solitary DMCs. Error bars represent 95% confidence intervals of the means (fitted model means). See Table S4 for corresponding statistics [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Epigenetic variation in natural populations is probably key to their survival, particularly when they are genetically depleted and environmentally challenged. In such systems, it may be favourable for individuals to acquire an epigenetic memory that allows efficient responses to fluctuating environmental stressors. Here, we shed light on the prevalence of natural epigenetic variation along a steep environmental gradient, and put these findings into a much wider geographical context. Collectively, our results indicate that epigenetic memories develop at both fine and large spatial scales, each associated with distinct epigenetic signatures. Specifically, methylation in the non-CG context gains in importance as the spatial scale increases, and this translates into more methylation differentiation of genes with regulatory functions and TEs. Conversely, divergence of CG methylation was more pronounced at the fine-scale altitudinal

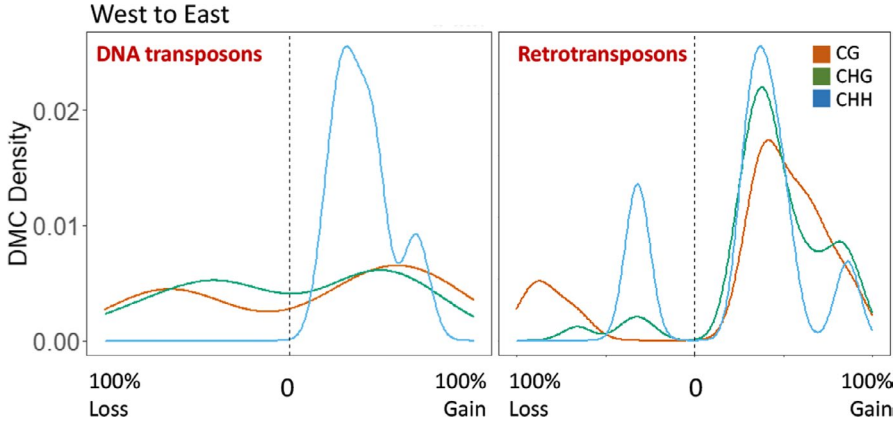


FIGURE 6 DMC density plot showing methylation gain and loss for transposable elements, in CG, CHG and CHH contexts [Colour figure can be viewed at wileyonlinelibrary.com]

gradient, where it may guide adaptive gene expression in response to environmental variability. In addition, we are the first to demonstrate an intense correlation between the clustering behaviour of DMCs and their differentiation between natural populations. Together with low epigenetic diversity at clustering DMCs in the most stressful environment (high altitude), this finding suggests that clustering DMCs may be more involved in adaptive processes than solitary DMCs. This does not rule out, however, that solitary DMCs may have large effects on gene expression.

We found that epigenetic memories, predominantly in the CG context, can diverge even at very fine spatial scale (<2 km), indicative of epigenetic isolation-by-ecology. This finding is in strong contrast to an earlier study that was unable to detect significant epigenetic differences between alpine herb populations originating from three elevations and grown in a common garden using 150 methylation-sensitive amplified fragment-length polymorphisms (MS-AFLPs) (Nicotra et al., 2015). In our study, methylation in the CG context increased from low to high altitude, suggesting that high-altitude environments may trigger activation of genes predominantly underpinning amino acid metabolism, intracellular transport, responses to light conditions and cellulose catabolism (Table S5). However, predicting transcriptional activity from levels of DNA methylation remains a point of discussion. We furthermore observed a markedly lower epigenetic diversity for clustered DMCs than for solitary DMCs at high altitude (Figure 5), potentially representing signatures of natural selection favouring clustered CG methylation (Figure 2) in gene bodies. Given that the altitudinal gradient studied here features intense phenotypic divergence (De Kort et al., 2020), at least part of the potentially adaptive epigenetic signatures observed here is probably attributed to topographical variation along the gradient. Our finding of reduced epigenetic diversity in the CG context and increased CG methylation at higher altitudes is in line with *Arabidopsis thaliana* studies showing that increased stress levels at the location of origin are associated with higher CG gene body methylation (Dubin et al., 2015; Keller et al., 2016; Schmid et al., 2018). In parallel, several studies found increased up-regulation and CG gene body methylation of genes involved in environmental responses to novel and/or stressful conditions through gene body methylation (Artemov et al., 2017; Dixon et al., 2018; Dubin et al., 2015).

Not only the high-altitude population, but also the "west" population was characterized by very low ED for clustered as compared to solitary DMCs (Figure 5). This may indicate increasing environmental stress towards the edge of the species' distribution (defined by the Pyrenees in the southwest of its distribution), where CG DMCs in particular have increased methylation rates (Figure 2). Moreover, because roughly 30% of altitudinal DMCs were shared with the spatial DMCs, part of the adaptive epigenetic divergence along both spatial scales may be driven by parallel ecological processes. This spatial ecological parallelism underlying methylation patterns is corroborated by the strong overlap in enriched GO processes between both spatial scales (Tables S5 and S6). Interestingly, the "east" population was characterized by strongly increased methylation (Figure 2) as well as high ED (Figure 5), indicating high but very variable methylation levels in this population. High gene flow in the core of the species' range may counteract natural selection that would otherwise reduce ED at clustered DMCs.

Whereas the suggested impact of environmental stress on selection towards increased CG methylation may be ubiquitous (i.e., at fine and large spatial scale, and in *F. vesca* and *A. thaliana*), fine-scale epigenetic patterns differed substantially from large-scale epigenetic patterns. As compared to the fine-scale altitudinal gradient, the large spatial gradient was characterized by (a) an increase in the number of DMCs with two orders of magnitude, (b) more intense methylation differentiation (on average 70% versus. 40%), (c) a more prominent role for non-CG differentiation and (d) more pronounced differences in epigenetic diversity across populations. Although the CG context constituted the most divergent methylation patterns irrespective of spatial scale (Figure 3), the proportion of non-CG DMCs increased considerably from fine to large spatial scale (Figure 3d). This finding is in agreement with a large-scale study on *A. thaliana* showing that non-CG demethylation associated with transposon activity was abundant where temperature reached extreme levels (Keller et al., 2016). It is unclear, however, to what extent demographic history and range dynamics have contributed to this relationship between extreme temperatures and non-CG methylation. Indeed, temperatures become more extreme towards range edges, and the increased transposon activity at *A. thaliana's* range edges, where its distribution becomes more scattered, may have partially resulted from range dynamics (for range-wide genetic population structure, see Alonso-Blanco et al., 2016; Beck et al., 2007). Here, non-CG DMCs lost methylation and were less diverse (Figures 2 and 5) from east to west, which, hypothetically, results from ecology-driven activation of transposons towards the edge of the distribution range of *F. vesca*, where transposon activation through demethylation may provide opportunities for genetically impoverished populations to boost genetic change. Although this could point to an evolutionary rescue mechanism for transposons during range expansions (see also Rey et al., 2016; Stapley et al., 2015), more research on the spatial distribution of transposon activity and its role in evolution is required to validate this assumption. Nevertheless, the observation that transposons are differentially suppressed at large spatial scale and not along a steep gradient, suggests that differential suppression of transposon activity follows biogeographical or phylogeographical storylines rather than fine-scale environmental clines. If environmental stress drives transposon demethylation, then this should result in systematic transposon demethylation towards the high-altitude population while only one transposon-related DMC was found along the altitudinal gradient. Our findings thus favour intraspecific biogeography as the main determinant of transposon demethylation. In addition, the (a) decrease in CG epigenetic diversity from Poland to the Pyrenees, corresponding to increasing inbreeding and/or decreasing gene flow towards the distribution edge, and (b) increase in methylation at DMC islands towards Poland (range centre) (Figure S7) where stress levels are expected to be less pronounced as compared to the range edge, corroborate the role of intraspecific biogeography in shaping spatial epigenetic patterns.

Most of the epigenetic differences observed in this study are caused inevitably by genetic change, for example because cytosine to thymine or adenine substitutions can be erroneously read as a DMC following bisulphite sequencing and PCR, or, more likely, because genetic polymorphisms epistatically drive methylation changes nearby or in other genomic regions (Paun et al., 2019). It is unlikely that many DMCs in the same DMC cluster are erroneous, so we are confident that our clustered DMCs in particular reflect epigenetic rather than genetic divergence. In addition, studies that do combine genetic and epigenetic markers show that relationships between methylation patterns and environmental stress are stronger than gene–environment associations, and that epigene–environment interactions remain after controlling for genetic structure (e.g., Foust et al., 2016; Meröndun et al., 2019; Schmid et al., 2018; Wogan et al., 2020). In the same vein, we suspect that at least part of our epigenetic differentiation arose from epigenetic isolation-by-environment rather than genetic isolation-by-distance because we found (a) biologically meaningful GO terms for many DMCs, (b) many DMCs at a fine-scale gradient harbouring adaptive phenotypic divergence as shown previously for our study species, (c) nonrandom genomic clustering of DMCs within genomic regions, particularly for very divergent DMCs, and (d) low epigenetic diversity in stressful environments (high altitude and range edge). Together, these findings suggest that many DMCs did not merely arise randomly/neutrally but at least partially resulted from ecological differentiation. However, whether this ecological divergence of methylation is largely gene-independent remains an open question.

While the origin-dependent epigenetic memories (i.e., altitudinal and spatial DMCs) were stably transmitted to the common garden generation, acute drought stress-induced epigenetic signatures were weak (Figures 2 and 3). Although the low number of DNA methylation differences induced by our drought stress treatment may reflect the experimental conditions, our plants probably suffered more stress (as we allowed them to go limp) than *F. vesca* plants usually experience in their natural environment (buffered forest conditions). We therefore argue that the observed lack of methylation is conservative for naturally occurring reductions in soil moisture. We suspect that repeated exposure to stressful conditions is required for acquiring a detectable epigenetic signature, and emphasizes the importance of historical stress experience for the generation of an epigenetic memory. Vice versa, our results suggest that the loss of an epigenetic memory requires long-term release of stressful conditions, and that multiple generations without stress exposure are required for completely resetting the epigenetic machinery. Multigenerational persistence of epigenetic signatures (i.e., epigenetic carryover) and thus slow transgenerational loss of epigenetic variation is a typical epigenetic mechanism observed in common gardens quantifying epigenetic variation across generations (Miska & Ferguson-Smith, 2016; Paszkowski & Grossniklaus, 2011; Proulx et al., 2019). Given the natural ubiquity of transgenerational epigenetic inheritance, as indicated by the former studies, at least part of the epigenetic patterns observed in our common garden generation is expected to reflect such epigenetic carryover. Nevertheless, the precise extent of multigenerational methylation inheritance requires additional generations of epigenetic profiling, as other mechanisms such as catastrophic events, other timescales and different types of stress may affect the efficacy of transgenerational epigenetic inheritance. Collectively, our findings provide novel insights into the natural prevalence of adaptive epigenetic divergence and the processes driving epigenetic memories at distinct spatial scales. We showed that significantly different epigenetic memories, primarily in CG context and thus presumably in or near gene bodies, arise at fine spatial scales. At large spatial scale, epigenetic memories also diverge at the level of regulatory genes and transposons. We hypothesize that genetic and epigenetic responses support fitness in heterogeneous environments, and that non-CG demethylation increases in importance as genetic variation becomes depleted. Further research involving higher resolution sampling and a multigenerational common garden is required to shed more light on the role of epigenetic variation at distinct spatial scales. This would particularly increase our understanding of epigenetic memory acquisition and divergence as an adaptive strategy of natural populations that could enhance their ability to cope with global change stressors. We also argue that disentangling epigenetic signatures and DMC clustering linked to phylogeography versus adaptive evolution represent interesting research avenues that are fundamental to our understanding of large-scale epigenetic divergence.

ACKNOWLEDGEMENTS

H.D.K. holds a postdoctoral fellowship funded by FWO (Research Foundation Flanders, 12P6517N). Dr Kenny Helsen and Kasper Van Acker helped in raising the plants. Dr Martin Diekmann and Josef Müller performed the sampling in Poland. Dr Filip Rolland provided the cultivation room for raising our plants.

AUTHOR CONTRIBUTIONS

H.D.K. coordinated the research, performed data analyses of DMCs and wrote the manuscript. F.V.N. performed all bio-informatics analyses. B.P. and O.H. helped in shaping the project. All co-authors provided comments and suggestions on the first version of the manuscript.

DATA AVAILABILITY STATEMENT

All raw data are provided in the Supporting Information. Sequence reads are stored in GenBank under accession no. PRJNA635047. Filtered DMCs used for all analyses are stored in Dryad (doi.org/10.5061/dryad.zs7h44j6r).

ORCID

Hanne De Kort https://orcid.org/0000-0003-2516-0134

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: De Kort H, Panis B, Deforce D, Van Nieuwerburgh F, Honnay O. Ecological divergence of wild strawberry DNA methylation patterns at distinct spatial scales. *Mol Ecol*. 2020;29:4871–4881. https://doi.org/10.1111/mec.15689