

1 Ecological divergence of DNA methylation patterns at distinct spatial scales

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14 effects, landscape genetics, epigenetic polymorphisms, SMPs

15 **ABSTRACT**

16 Adaptive trait divergence between populations is regulated by genetic and non-genetic processes.
17 Compared to genetic change, epigenetic change is unstable and short-lived, questioning its
18 contribution to long-term adaptive potential. However, epigenetic change can accumulate over
19 time, and may result in beneficial epigenetic memories where environments are heterogeneous.
20 Diverging epigenetic memories have been observed across large spatial scales, and can persist
21 through multiple generations even in the absence of the causative environmental stressor. It is
22 unknown, however, how and to what extent epigenetic memories contribute to fine-scale
23 population structure and evolution. Here, we performed whole genome bisulfite sequencing on 30
24 *Fragaria vesca* F1 plants originating from distinct ecological settings and grown in a controlled
25 environment. Specifically, we compared methylation patterns between a steep, altitudinal gradient
26 (<2 km) and a wide spatial gradient (>500 km). If epigenetic variation is random, arising from errors
27 during replication and without evolutionary implications, one would expect similar amounts of
28 epigenetic variation across populations and no spatial scale-effect. Here, we find that epigenetic
29 memories arise even at fine spatial scale, and that both parallel and non-parallel biological processes
30 underpin epigenetic divergence at distinct spatial scales. For example, demethylation of
31 transposable elements consistently occurred at the large but not the small spatial scale, while
32 methylation differentiation for most biological processes were shared between spatial scales. Acute
33 drought stress did not result in significant epigenetic differentiation, indicating that repeated
34 historical stress levels associated with heterogeneous environmental conditions are required for
35 acquiring a stable epigenetic memory and for coping with future environmental change.

36 INTRODUCTION

37 Adaptive genetic variation underlying fitness traits is considered the dominant resource upon which
38 plants depend for evolving under environmental change. Driven by drift, mutation and migration,
39 such genetic variation supplies populations with trait values that support local fitness and adaptive
40 potential. However, although it is widely assumed that adaptive phenotypic variation is mainly
41 regulated by the underlying genetic architecture, only small proportions of the total phenotypic
42 variation observed in many species have been associated with genetic variants (Krishna Kumar *et al.*
43 2016; Wellenreuther & Hansson 2016; Resende *et al.* 2017). The remaining phenotypic variation,
44 typically referred to as missing heritability, can be roughly attributed to (i) the detection limits of
45 rare genetic variants and genetic interactions, and (ii) heritable epigenetic variation (Brachi *et al.*
46 2011; Miska & Ferguson-Smith 2016; Whipple & Holeski 2016; Gienapp *et al.* 2017; Banta & Richards
47 2018).

48 The role of epigenetic variation in governing adaptive evolution remains controversial, yet a growing
49 body of literature demonstrates the ubiquity of transgenerational epigenetic transmission, and
50 consequently considers it as a key evolutionary force (Gugger *et al.* 2016; Miska & Ferguson-Smith
51 2016; Lind & Spagopoulou 2018; Schmid *et al.* 2018; Zhang *et al.* 2018; Danchin *et al.* 2019). Non-
52 random epigenetic variation has been shown to be widespread in natural populations, and to co-
53 vary with a range of environmental stressors, including herbivory, drought, salt and temperature
54 (Foust *et al.* 2016; Jeremias *et al.* 2018; Alonso *et al.* 2019; Gáspár *et al.* 2019). While most stress-
55 induced methylation changes are reset to basal levels after stress relief, part of these modifications
56 can be stably inherited across mitotic and even meiotic cell divisions (Chinnusamy & Zhu 2009; Crisp
57 *et al.* 2016). Such a somatic or transgenerational epigenetic stress memory allows plants to cope
58 more effectively with subsequent stresses, thus evoking considerable fitness benefits in
59 heterogeneous environments (Crisp *et al.* 2016; Hilker *et al.* 2016). Unraveling the relative extent of
60 intra-generational epigenetic change resulting from acute environmental stress vs. transgenerational

61 epigenetic accumulation may contribute to our understanding of how plants rely on their epigenetic
62 machinery for coping with environmental change.

63 How selection pressures affect genome-wide DNA methylation levels in natural population remains
64 poorly explored in non-model organisms, but considerable advances have been made in *Arabidopsis*
65 *thaliana*. A study involving genome-wide DNA methylation analysis of 122 *A. thaliana* accessions
66 sampled across Eurasia showed that climate characteristics most abundantly co-varied with
67 methylation levels of cytosines in CHH context (where H represents a G, T or A nucleotide), with CHH
68 methylation typically indicating the involvement of transposable elements (TEs) (Keller *et al.* 2016).
69 These findings could be related to natural selection at the level of TE-specific methyltransferase
70 genes that facilitate demethylation of transposons when temperatures reach extreme levels, or
71 where populations are genetically impoverished. Stress-induced demethylation of transposons
72 boosts transposon activity and subsequent genetic change, paving the way for rapid genetic
73 replenishment and adaptation to environmental stressors (Mirouze & Paszkowski 2011; Ito *et al.*
74 2016; Rey *et al.* 2016; Schrader & Schmitz 2019). The strongest associations between climate and *A.*
75 *thaliana* methylation levels were, however, found in CG contexts within or near genes related to
76 abiotic stress responses, development and reproduction (Keller *et al.* 2016). Because (i) DNA
77 methylation has been shown to be meiotically most stable in the CG context, and (ii) the majority of
78 reported heritable epi-mutations occurs at CG sites (Mathieu *et al.* 2007; Jiang *et al.* 2014; Stassen *et*
79 *al.* 2018), climate-CG methylation associations most likely represent solid adaptive signals. A recent
80 study corroborated the evolutionary relevance of CG methylation using a multi-generational *A.*
81 *thaliana* selection experiment, demonstrating that (i) methylation of differentially methylated
82 cytosines (DMCs) was significantly higher in CG context after five generations of selection, (ii) the
83 majority of these DMCs were stably inherited for 2 or 3 generations following the selection
84 experiment, (iii) selection caused overall reductions in epigenetic diversity, and (iv) methylation
85 levels of some CG DMCs were associated with phenotypic changes (Schmid *et al.* 2018).

86 Genome-wide DNA methylation studies in *Quercus* species showed patterns similar to those
87 obtained in *A. thaliana*: DMCs associated with climate dominate in CG context, and these DMCs
88 occur in or near genes (Platt *et al.* 2015; Gugger *et al.* 2016). Using the experimentally more versatile
89 herb *Plantago lanceolata* as a study organism, Gáspár *et al.* (2019) demonstrated that much of the
90 environment-related epigenetic variation is maintained in an F1 common garden. Thus, at least part
91 of the epigenetic variation observed in the field is stable, non-random and of ecological significance.
92 Although these studies considerably increased our understanding of how epigenetic variation is
93 distributed across large spatial scales, it remains unknown to what extent epigenetic variation
94 contributes to population divergence along small-scale environmental gradients, where the interplay
95 between migration, drift and selection can be extremely dynamic (Richardson *et al.* 2014). Highly
96 heterogeneous environments may thus give rise to distinct signatures of epigenetic variation.

97 Evidence is accumulating that an epigenetic memory may be particularly beneficial where genetic
98 diversity is in short supply, e.g. following demographic bottlenecks or in clonal plant species (Latzel
99 *et al.* 2016; Ardura *et al.* 2017; Artemov *et al.* 2017; Thorson *et al.* 2017; Rendina González *et al.*
100 2018; Wibowo *et al.* 2018). More fundamentally, Dapp *et al.* (2015), using epigenetic inbred lines of
101 *Arabidopsis thaliana*, demonstrated that epigenetic diversity can drive hybrid vigour in the absence
102 of genetic diversity. Thus, epigenetic variation may be a crucial element of population persistence
103 where evolutionary trajectories or life history traits limit genetic diversity. Study systems combining
104 strong evolutionary pressure (e.g. expansion fronts or heterogeneous environments) and life history
105 traits that constrain genetic diversity (e.g. asexual reproduction and high levels of self-compatibility)
106 are therefore promising for obtaining more insights in the role of epigenetic variation in adaptation
107 and population persistence.

108 Here, we explore genome-wide epigenetic profiles of F1 plants originating from three natural
109 *Fragaria vesca* populations that were found to harbor strong natural differentiation in terms of traits
110 related to fitness, most likely driven by local topography impacting local soil moisture levels (De Kort

111 *et al. 2019*). Among the most notable patterns observed in a controlled common garden
112 environment were that plants adapted to the stressful conditions at high altitudinal, south-oriented
113 locations were small and produced less flowers than plants originating from low altitudinal, north-
114 oriented locations (*De Kort et al. 2019*). *F. vesca* has also been shown to harbor limited genetic
115 diversity across its range (*Hilmarsson et al. 2017*), presumably as a result of its life history (self-
116 compatible and clonal). Due to this limited genetic diversity in combination with pronounced
117 altitude-dependent phenotypic divergence, we hypothesize substantial adaptive epigenetic signals
118 coinciding with increased stress levels along the studied altitudinal gradient (Fig. 1C). We specifically
119 answer the following questions: (i) do epigenetic memories diverge with altitude, and does
120 methylation increase or decrease with increasing altitude?; (ii) are these small-scale genome-wide
121 methylation patterns comparable to those obtained at a much larger spatial scale (>500 km)?; (iii)
122 are altitudinal DMCs enriched for ecologically relevant gene ontology terms?; and (iv) does acute
123 drought stress induce a detectable epigenetic memory?

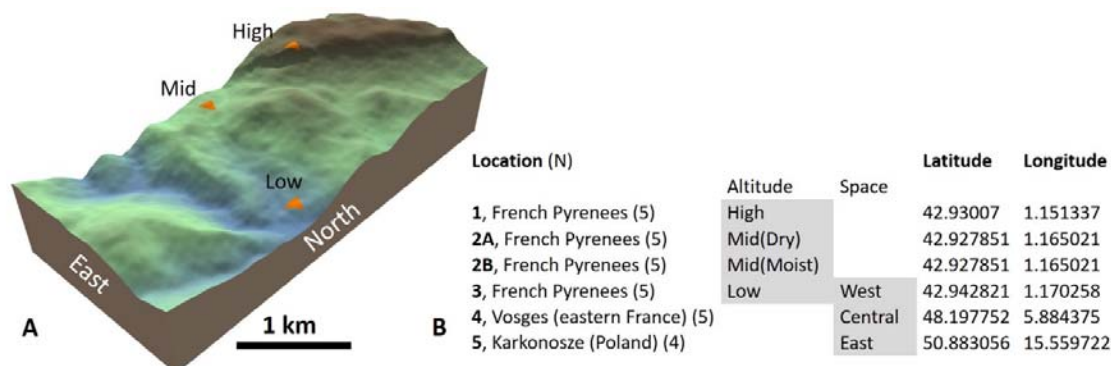
124 **METHODS**

125 **Sample collection**

126 Seeds were collected from five plants at (i) three nearby locations in the French Pyrenees, (ii) one
127 location in the French Vosges, and (iii) one location in Poland (Fig. 1). After germination, one
128 seedling per mother plant was randomly selected from every location and grown in humid soil. To
129 compare the magnitude of inherited epigenetic memories to intra-generational epigenetic change
130 acquired through acute drought stress, an additional seedling per mother plant was raised for the
131 mid-altitudinal Pyrenean plants, and these seedlings were subjected to reduced soil moisture levels
132 starting two months after germination. Specifically, watering stopped until leaves went limp, and
133 this process was repeated consecutively for four weeks, after which the plants were allowed to
134 rehydrate during one week to remove most drought-induced epigenetic effects that do not result in

135 a relatively stable epigenetic memory. DNA was then extracted from one leaf per plant, resulting in
 136 30 samples (Fig. 1).

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138
 139 **Fig. 1.** Geographical location of altitudinal WGBS samples (A,B) and of the broader spatial WGBS (B). Samples
 140 originate from south western France (Pyrenees), eastern France (Vosges) and south western Poland
 141 (Karkonosze). N represents the number of successfully sequenced samples (one Polish sample failed post-
 142 sequencing quality checks). Samples from location 2 are used for comparisons within the altitudinal gradient
 143 (Mid) as well as between the soil moisture treatments (Dry vs. Moist). Samples from location 3 are used for
 144 comparisons within the altitudinal (Low) as well as the spatial (West) gradient. High, mid and low altitude
 145 correspond to 1200, 750 and 450 meter asl, resp.

146 **Whole genome bisulfite sequencing and DMC calling**

147 DNA of 30 freeze-dried samples was extracted with a QIAGEN kit. Up to 200ng of DNA was
 148 fragmented to 400bp with a Covaris S2 sonicator prior to whole-genome library preparation
 149 (NEBNext Ultra II kit), ligation of methylated adaptors and size selection on 2% E-gel (450-650 bp).
 150 Bisulfite conversion was performed using the EZ DNA methylation gold kit (Zymo Research, Irvine,
 151 CA, USA). An enrichment PCR was performed using KAPA Hifi hotstart Uracil+ mastermix in a 12
 152 cycles PCR reaction. Paired-end 75 bp sequencing of the library fragments was performed on eight
 153 lanes of an Illumina HiSeq4000 sequencer, generating $76,417,704 \pm 13,837,490$ reads (mean \pm
 154 standard deviation) per sample. An extensive quality control was performed on the sequencing data
 155 using FastQC version 0.11.5 (Babraham Bioinformatics), showing a mean quality value across each
 156 base position in the reads, average quality scores of the reads and average GC content of reads that
 157 is to be expected from a high-quality Illumina sequencing run (see Supporting Figs. 1-5 for quality
 158 and coverage stat). Trimmomatic (Bolger et al. 2014) version 0.36 was used to trim reads for

159 sequences with a Phred score lower than 33 and sequences corresponding to Illumina TruSeq
160 adapters. Sequences shorter than 50bp after trimming were discarded. One sample from Poland
161 (“East”) showed an increased level of duplicate reads and was excluded from further analysis
162 (Supporting Fig. 3).

163 FastQ Screen version 0.11.1 (Babraham Bioinformatics) was used to check whether the library is
164 consisting of *F. vesca* genomic sequences to rule out contamination with DNA originating from
165 genomes of other species. The trimmed reads were mapped against the *Fragaria vesca*_v4.0.a1
166 genome using bismark version 0.17.0. (Krueger and Andrews 2011). One sample from Poland
167 (“East”) showed suspicious duplication levels and was expelled from further analysis (see Supporting
168 Figs. 1-5 for quality and coverage stats). Average sequencing depth after mapping and deduplication
169 was 30x.

170 The resulting mapped dataset was used for downstream analyses using R package methylKit version
171 1.10.0 (Akalin *et al.* 2012). Only CpGs with at least 5x coverage in at least 3 samples per group were
172 retained (Walker *et al.* 2015; Wan *et al.* 2016). To reduce bias due to outlier depth, bases with a read
173 depth above the 99.9th percentile of coverage are filtered out. The filtered data were used to test
174 for differentially methylated CpGs (DMCs), considering a 25% difference and q-values <0.01 as
175 significant. Significant differentially methylated cytosines (DMCs) were identified between (i) low,
176 mid and high altitudinal samples (hereafter “altitudinal DMCs”), (ii) the three distance European
177 samples (hereafter “spatial DMCs”), and (iii) the two soil moisture treatments (hereafter “drought
178 DMCs”).

179 **Methylation profiling of topography, space and drought**

180 All DMCs were assumed to be ecologically divergent (i.e. resulting from drift or fitness differences).
181 Because the epigenetic signals observed here have persisted in the second generation (F1), they may
182 represent stable epigenetic changes. However, because we cannot corroborate the long-term
183 transgenerational stability of these DMCs, we further refer to ecological rather than evolutionary
184 divergence of methylation patterns. Cytosines that were significant along the altitudinal gradient,

185 were thus considered to be ecologically divergent and potentially adaptive. On the other hand,
186 spatially divergent cytosines that were not significant along the altitudinal gradient, were assumed
187 to be ecologically neutral along the altitudinal gradient. Similarly, the altitudinal DMCs that were not
188 significant along the spatial gradient were assumed to be neutral along the spatial gradient, while
189 spatial DMCs were considered divergent and potentially adaptive along the spatial gradient. The
190 drought DMCs were not significant along the two gradients and were thus considered neutral along
191 both gradients.

192 As a measure of epigenetic structure (ES), a principal component analysis (PCA) was performed to
193 reveal to what extent DMCs clustered samples according to topography, space and drought. As a
194 measure of epigenetic diversity (ED) that is insensitive to sample size (Vellend *et al.* 2010; Schmid *et*
195 *al.* 2018), we calculated the average pairwise methylation difference between the five individuals
196 originating from each location. High ED thus reflects high variation in the degree of methylation
197 within a population. Because ED represents a proportion, a quasibinomial model (logit link) was used
198 to test whether ED differs significantly between populations. The resulting effect size (R^2) was
199 estimated using the R package “Rsq” (default function), and pairwise comparisons were assessed
200 Tukey-wise (R package “Multcomp”). Methylation shifts, ED and ES were examined for ecologically
201 neutral and potentially adaptive DMCs separately.

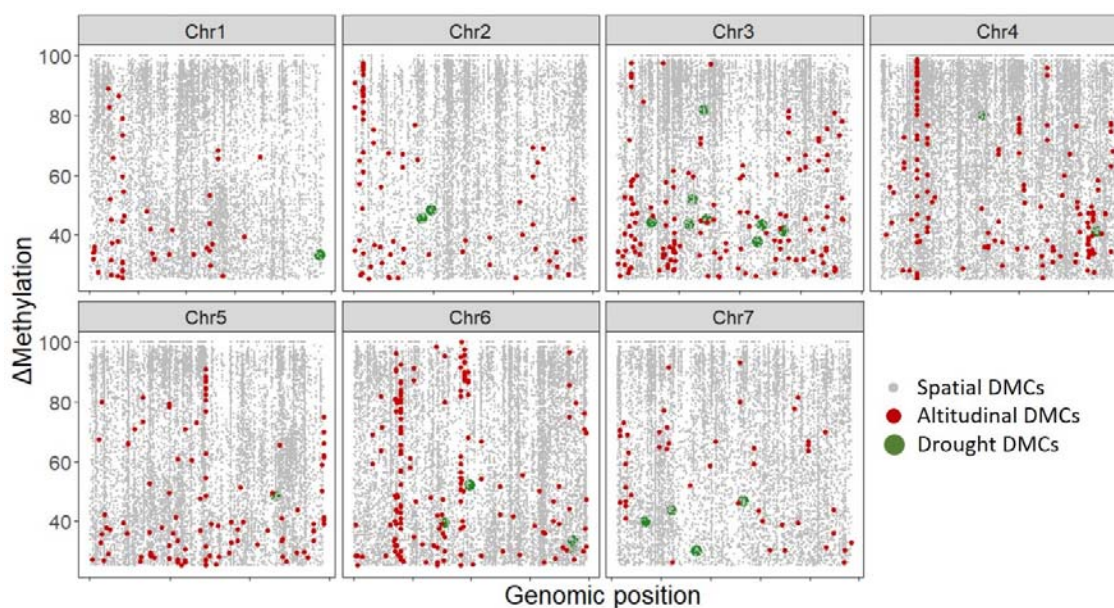
202 **DMC enrichment analysis**

203 Gene ontology terms (GOs) were retrieved from the Genome Database for Rosaceae (GDR, Jung *et*
204 *al.* 2019). To test which biological processes were overrepresented in sequences containing DMCs
205 (as compared to the full *F. vesca* genome), we performed a Fisher’s exact test with FDR correction as
206 implemented in OmicsBox, using altitudinal DMC GOs as test data and *F. vesca* GOs as reference
207 data. The same analysis was performed for the spatial DMCs, and in CG and non-CG context
208 separately.

209 To address the role of non-CG methylation in transposon regulation, we aligned genes in which we
210 found one or more DMCs to all transposable elements known in *F. vesca*, using the GDR search
211 function. Specifically, we extracted all genes related to the keyword “transpos” (referring to. e.g.
212 transposase, transposon, transposable element).

213 RESULTS

214 Apart from slight increases in genome-wide methylation levels from high to low soil moisture, and
215 from east to west (Supp. Fig. 6), no systematic genome-wide differences in methylation were
216 observed between the 29 samples. This suggests that the environmental conditions historically
217 encountered by our *F. vesca* populations target specific genomic regions or cytosine sites. We
218 accordingly detected a total of 82 839, 699 and 23 DMCs along the spatial gradient, the altitudinal
219 gradient and between the soil moisture treatments, respectively (Supp. Table 1). These DMCs often
220 clustered together in genomic islands of differential methylation (Fig. 1, Fig. 2A). A Mann-Whitney U-
221 test confirmed this clustering, showing that DMCs in all sequence contexts were significantly more
222 proximate to one another than expected based on the genome size (240 Mbp) and on the genome-
223 wide number of DMCs (varying from 13 altitudinal DMCs in CG context to 51135 spatial DMCs in CG
224 context) (Supp. Fig. 7, Supp. Table 2).

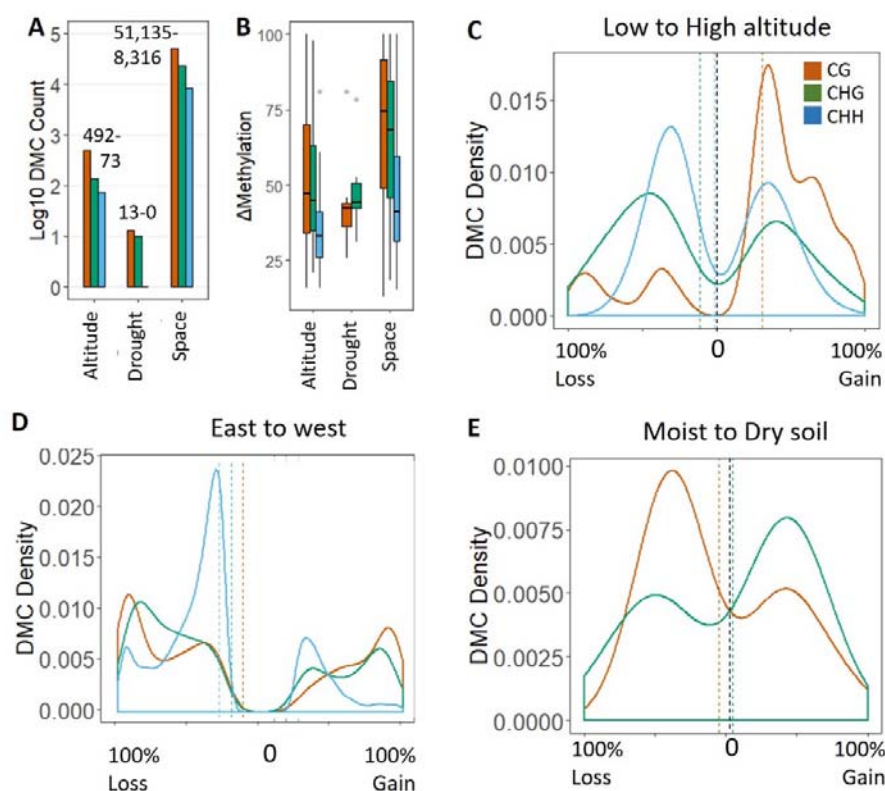


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226 **Fig. 1. Genome-wide differentiation in methylation** for the DMCs detected along the spatial gradient (grey),
227 along the altitudinal gradient (red), and between soil moisture treatments (green).

228 Methylation divergence was most pronounced along the spatial gradient, followed by the altitudinal
229 gradient and the soil moisture treatments (Fig. 2B). A total of 59 altitudinal DMCs (8.5%) were also
230 significantly divergent along the spatial gradient, and another 153 altitudinal DMCs (21.9%) were
231 located within 500 bp of a spatial DMC, indicating shared methylation patterns among distinct
232 spatial scales. The negligible proportion of genome-wide cytosines that was differentially methylated
233 between soil moisture treatments suggests that short-term acute soil dryness does not constitute a
234 pronounced epigenetic memory.

235 DMC density profiles were substantially different between the altitudinal gradient, the spatial
236 gradient and the soil moisture treatments (Fig. 2C-E). For altitudinal DMCs, the most dominant shift
237 in methylation was observed in CG context, with substantial methylation gain as altitude increased
238 (Fig. 2C). However, the most extreme shifts in DMC methylation level (i.e. toward 100% methylation
239 loss or gain) was observed along the spatial gradient (Fig. 2D). Thus, fixation of methylation patterns
240 occurred both at small and large spatial scale, but was more frequent along the spatial gradient.



241

242

243 **Fig. 2. Distribution of methylation patterns among the studied gradients**, including DMC counts (A), DMC
 244 differentiation (B), and DMC density of change in methylation level along the topographical gradient (C), the
 245 spatial gradient (D) and between soil moisture treatments (E). All patterns were visualized for each sequence
 context separately (CG in orange, CHG in green and CHH in blue).

246

247 A total of 247 out of 698 DMCs (35.4%) systematically gained (113 CG, 21 CHG and 20 CHH) or lost
 248 (38 CG, 35 CHG and 20 CHH) methylation from low to high altitude. Methylation gains along the

248

249 altitudinal gradient thus predominantly occurred in the CG context (see also Fig. 2C). Along the
 250 spatial gradient, a total of 56 795 out of 82 839 DMCs (68.6%) systematically lost (18 099 CG, 8731

250

251 CHG and 3609 CHH) or gained (17 038 CG, 7680 CHG and 1638 CHH) methylation from east to west
 252 (see also Fig. 2D). The plants from western Europe thus particularly differ from central Europe in the

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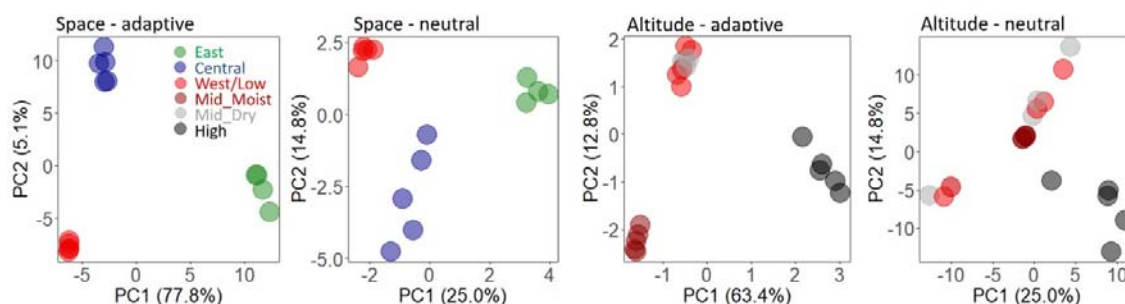
253 amount of demethylating CHH sites. Given that the plants raised here were genetically variable, we
 254 assume that the observed drastic and systematic shifts in methylation from low to high altitude and

254

255 from east to west are (i) not solely driven by genetic differences, and/or (ii) underpinned by epistatic
 effects.

256 Methylation patterns grouped the plants according to their location of origin (Fig. 3, Supp. Table 3),
257 thus convincingly indicating that diverging methylation levels persisted through the F1 generation.
258 Our results further show that neutrally behaving cytosines can align with spatial organization,
259 particularly at large spatial scales (Fig. 3B, 3D), as expected under isolation-by-distance. This finding
260 is concordant with the behavior of neutral genetic markers that spatially cluster as a result of drift or
261 gene flow. Clustering was more pronounced for adaptive than for neutral DMCs (Fig. 3A, 3C),
262 indicating epigenetic isolation-by-ecology.

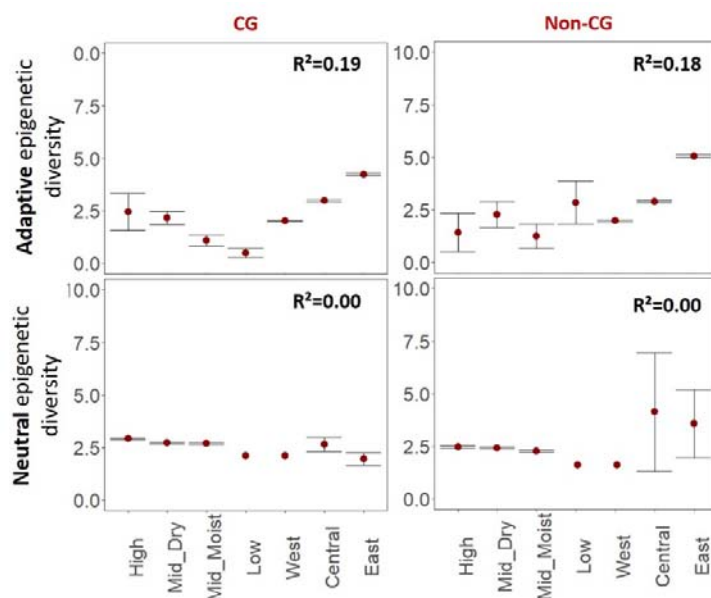
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265 **Fig. 3. Principal components analysis** of methylation levels at large and small spatial scale, and for ecologically
266 neutral vs. potentially adaptive methylation marks. See supporting Table 3 for eigenvalues and variable
267 contributions.

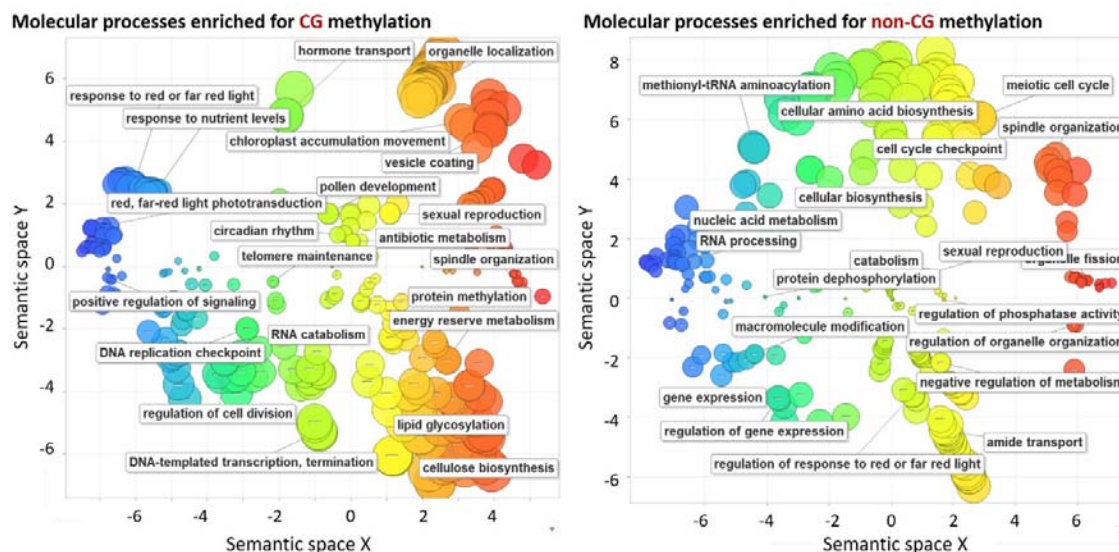
268 Adaptive epigenetic diversity (ED) significantly increased from low to high altitude in CG context, but
269 not in non-CG context (Fig. 4A-B, Supp. Table 4). There also was a significantly lower adaptive ED in
270 the west than in the mid-center and east of the sampling area, both in CG and non-CG context (Fig. 4A-B,
271 Supp. Table 4). As opposed to adaptive ED, neutral ED did not significantly change with altitude or
272 space (Fig. 4C-D, Supp. Table 4).



273
274 **Fig. 4. Epigenetic diversity (ED)** for ecologically neutral vs. putatively adaptive methylation, both in CG and
275 non-CG context. Effect sizes are shown as R^2 retrieved from generalized linear models testing for differences in
276 ED between locations. Error bars represent 95% confidence intervals of the means. See Supp. Table 4 for
277 corresponding statistics.

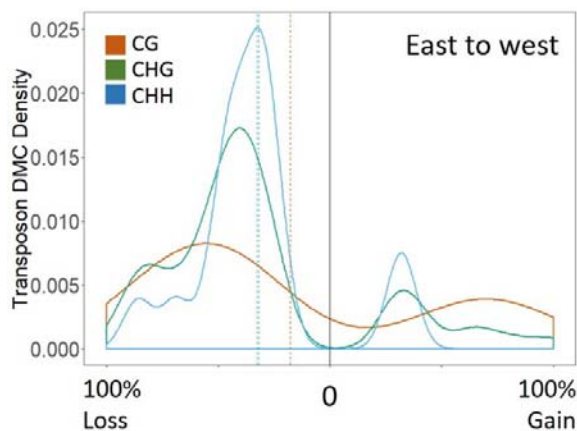
278 Out of 138 and 25 GO terms that were associated with the altitudinal DMCs in CG and non-CG
279 context, respectively, 54 (39.1%) and 2 (8.0%) were significantly enriched in comparison to the full *F.*
280 *vesca* GO compilation (Supp. Table 5, 6). A similar proportion of enriched GO terms was observed for
281 the spatial DMCs in CG context (39.7%, i.e. 578 out of 1456 GOs). As opposed to the altitudinal
282 DMCs, however, a high proportion of enriched GOs was also found in non-CG context (48.0%, i.e.
283 290 out of 604 GOs).

284 DMCs in CG vs. non-CG context represented a distinct set of enriched biological processes (Fig. 5).
285 Where non-CG DMCs were particularly enriched for regulatory functions (e.g. regulation of gene
286 expression and protein dephosphorylation), biological processes more directly related to
287 environmental stressors were overrepresented only in CG-context (e.g. circadian rhythm, antibiotic
288 metabolism and response to light). GOs related to cell division and reproduction (e.g. spindle
289 organization and sexual reproduction) were enriched in both CG and non-CG DMCs. No pronounced
290 differences in GO composition were found between altitudinal and spatial DMCs, and most
291 altitudinal GOs (69%) were also part of the spatial GO distribution (Supp. Table 5).



292
 293 **Fig. 5. GO enrichment graphs** showing the significantly enriched GO terms in CG (Left) and non-CG context for
 294 the spatial DMCs. Terms were visualized using REVIGO (Supek *et al.* 2011), which cluster GO terms according
 295 to their semantic similarities (e.g. response to abiotic stimulus and response to stimulus are clustered
 296 together). In each cluster, one or two GOs representing their corresponding cluster are shown. These graphs
 297 represent enrichment in spatial DMCs. Please see Supp. Table xx for GOs enriched in altitudinal DMCs.

298 A total of 31 transposable elements were found to contain one or more DMCs (Supp. Table 1). The
 299 most heavily differentiated transposon harbored no less than 21 DMCs, of which 15 in non-CG and 6
 300 in CG context. All but one differentially methylated transposon were found at the large spatial scale,
 301 and were dominated by DMCs in non-CG context (Fig. 6). On average, transposon DMCs lost 18%
 302 and 32% of methylation from east to west in CG and non-CG context, respectively.



303
 304 **Fig. 6. DMC density plot** showing methylation gain and loss for transposable elements, in CG, CHG and CHH
 305 context.

306

307 **DISCUSSION**

308 Epigenetic variation in natural populations probably is key to their survival, particularly when they
309 are genetically depleted and environmentally challenged. In such systems, it may be favorable for
310 individuals to acquire an epigenetic memory that allows efficient responses to fluctuating
311 environmental stressors. Here, we shed light on the prevalence of natural epigenetic variation along
312 a steep environmental gradient, and put these findings into a much wider geographical context.
313 Collectively, our results indicate that epigenetic memories develop both at small and large spatial
314 scales, each associated with distinct epigenetic signatures. Specifically, methylation in non-CG
315 context gains in importance as the spatial scale increases, and this translates into more methylation
316 differentiation of regulatory sequences and transposable elements. Conversely, divergence of CG
317 methylation was more pronounced at the fine-scale altitudinal gradient, where it may guide
318 adaptive gene expression in response to environmental variability.

319 We found that epigenetic memories, predominantly in CG context, can diverge even at very small
320 spatial scale (< 2 km), indicative of epigenetic isolation-by-ecology. This finding is in strong contrast
321 to an earlier study that was unable to detect significant epigenetic differences between alpine herb
322 populations originating from three elevations and grown in a common garden using 150
323 methylation-sensitive AFLPs (MS-AFLPs) (Nicotra *et al.* 2015). In our study, methylation in CG-
324 context increased from low to high altitude, suggesting that high altitudinal environments trigger
325 activation of genes predominantly underpinning amino acid metabolism, intracellular transport,
326 responses to light conditions and cellulose catabolism (Supp. Table 6). Furthermore, we observed a
327 pronounced increase in epigenetic diversity for CG DMCs from low to high altitude, suggesting that
328 epigenetic diversity for ecologically adaptive methylation sites may be favorable where the
329 environment is more heterogeneous. While evidence supporting DNA methylation differentiation in
330 response to environmental heterogeneity is still lacking, several lines of evidence have
331 demonstrated increased up-regulation of genes involved in environmental responses to novel
332 and/or stressful conditions through gene body methylation (Artemov *et al.* 2017; Dixon *et al.* 2018).

333 Fine-scale epigenetic patterns differed substantially from large-scale epigenetic patterns. As
334 compared to the fine-scale altitudinal gradient, the large spatial gradient was featured by (i) an
335 increase in the number of DMCs with two orders of magnitude, (ii) more intense methylation
336 differentiation (on average 70% vs. 40%), (iii) a more prominent role for non-CG differentiation and
337 differential transposon suppression, and (iv) more pronounced population structure, particularly for
338 ecologically adaptive DMCs. Although the CG-context constituted the most divergent methylation
339 patterns irrespective of spatial scale (Fig. 2), the proportion of non-CG DMCs increased considerably
340 from small to large spatial scale (Fig. 2D). This finding is in agreement with a large-scale study on *A.*
341 *thaliana* showing that non-CG demethylation associated with transposon activity was abundant
342 where temperature reached extreme levels (Keller *et al.* 2016). It is unclear, however, whether
343 transposon activity was more related to temperature extremes than to demographic history and
344 range dynamics, given that highest transposon activity was observed at *A. thaliana*'s range edges
345 where its distribution becomes more scattered (Beck *et al.* 2008; Alonso-Blanco *et al.* 2016). Here,
346 non-CG DMCs lost methylation and were less diverse (Fig. 4, 6) from east to west, which,
347 hypothetically, results from ecology-driven activation of transposons towards the edge of the
348 distribution range of *F. vesca* (defined by the Pyrenees in the southwest of its distribution), where
349 transposon activation through demethylation may provide opportunities for genetically
350 impoverished populations to boost genetic change. Although this would be in line with earlier
351 studies pointing to an evolutionary rescue mechanism for transposons during range expansions
352 (Stapley *et al.* 2015; Rey *et al.* 2016b), more research on the spatial distribution of transposon
353 activity and its role in evolution is required to validate this assumption. Nevertheless, the
354 observation that transposons are differentially suppressed at large spatial scale and not along a fine-
355 scale steep gradient, suggests that differential suppression of transposon activity follows
356 biogeographic storylines rather than fine-scale environmental clines.

357 The more pronounced epigenetic clustering for neutrally behaving cytosines along the spatial
358 gradient than along the altitudinal gradient (Fig. 3) may suggest epigenetic isolation-by-distance

359 (Whipple & Holeski 2016). However, most clustering occurred at putatively adaptive DMCs indicating
360 a more dominant role for isolation-by-ecology over isolation-by-distance. Given that roughly 30% of
361 altitudinal DMCs were shared with the spatial DMCs, part of the adaptive epigenetic divergence
362 along both spatial scales may be driven by parallel ecological processes. This spatial ecological
363 parallelism underlying methylation patterns is corroborated by the strong overlap in enriched gene
364 ontology processes between both spatial scales (Fig. 5, Supp. Table 5, 6).

365 While the origin-dependent epigenetic memories (i.e. altitudinal and spatial DMCs) were stably
366 transmitted to the F1 generation, acute drought stress-induced epigenetic signatures were weak
367 (Fig. 1,2). This suggests that repeated exposure to stressful conditions is required for acquiring a
368 detectable epigenetic memory, and emphasizes the importance of historical stress experience for
369 the generation of an epigenetic memory. Vice versa, our results suggest that the loss of an
370 epigenetic memory requires long-term release of stressful conditions, and that multiple generations
371 without stress exposure are required for completely resetting the epigenetic machinery. Multi-
372 generational persistence of epigenetic signatures (i.e. epigenetic carryover) and thus slow trans-
373 generational loss of epigenetic memory is a typical epigenetic mechanism observed in common
374 gardens quantifying epigenetic variation across generations (Paszkowski & Grossniklaus 2011; Miska
375 & Ferguson-Smith 2016; Proulx *et al.* 2019). Given the natural ubiquity of transgenerational
376 epigenetic inheritance, at least part of the epigenetic patterns observed in our F1 generation is
377 expected to reflect such epigenetic carryover. Nevertheless, the precise extent of multi-generational
378 methylation inheritance requires additional generations of epigenetic profiling.

379 Collectively, our findings provide novel insights into the natural prevalence of adaptive epigenetic
380 divergence and the processes driving epigenetic memories at distinct spatial scales. We showed that
381 significantly different epigenetic memories, presumably concentrated in or near gene bodies, arise
382 at fine spatial scales. At large spatial scale, epigenetic memories also diverge at the level of
383 regulatory sequences and transposons. We hypothesize that genetic and epigenetic responses

384 complementary support fitness in heterogeneous environments, and that non-CG demethylation
385 increases in importance as genetic variation gets depleted. Further research involving higher
386 resolution sampling and a multi-generational common garden is required to shed more light on the
387 role of epigenetic variation at distinct spatial scales. This would particularly increase our
388 understanding of epigenetic memory acquisition and divergence as an adaptive strategy of natural
389 populations that could enhance their ability to cope with global change stressors.

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394

395 **AUTHOR CONTRIBUTIONS**

396 HDK coordinated the research, performed data-analyses of DMCs and wrote the manuscript. FVN
397 performed all bio-informatics analyses. MD assisted with field sampling. All co-authors provided
398 comments and suggestions to the first version of the manuscript.

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