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Signatures of polygenic adaptation align with genome-wide methylation patterns in wild strawberry plants

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Summary

- Epigenetic inheritance can drive adaptive evolution independent of DNA sequence variation. However, to what extent epigenetic variation represents an autonomous evolutionary force remains largely elusive.
- Through gene ontology and comparative analyses of genomic and epigenomic variation of wild strawberry plants raised in distinct drought settings, we characterized genome-wide covariation between SNPs (single nucleotide polymorphisms) and DMCs (differentially methylated cytosines).
- Covariation between SNPs and DMCs was independent of genomic proximity, but instead associated with fitness-related processes such as stress responses, genome regulation and reproduction. We expected this functional SNP-DMC covariation to be driven by adaptive evolution canalizing SNP and DMC variation, but instead observed significantly lower covariation with DMCs for adaptive than for neutral SNPs. Drought-induced DMCs frequently co-varied with tens of SNPs, suggesting high genomic redundancy as a broad potential basis for polygenic adaptation of gene expression.
- Our findings suggest that stress-responsive DMCs initially co-vary with many SNPs under increased environmental stress, and that natural selection acting upon several of these SNPs subsequently reduce standing covariation with stress-responsive DMCs. Our study supports DNA methylation profiles representing complex quantitative traits rather than autonomous evolutionary forces. We provide a conceptual framework for polygenic regulation and adaptation shaping genome-wide methylation patterns in plants.

Keywords: adaptive evolution, adaptive potential, facilitated epigenetic variation, *Fragaria*, methylome, multi-locus adaptation, non-genetic inheritance, polygenic adaptation

Introduction

The ability of natural populations to adapt to novel environmental stressors is a key driver of biodiversity, particularly in an era of accelerated environmental change. Understanding the molecular mechanisms that allow populations to withstand environmental perturbations therefore is fundamental to biodiversity conservation. Quantitative genetics theory predicts that the probability of populations to keep pace with environmental change increases with molecular variation underlying phenotypic traits affecting fitness (Wright, 1948; Fisher, 1930; Payne & Wagner, 2019). While heritable genetic variation is thought to be the dominant driver of trait evolution, a rising number of studies suggests an important role for epigenetic variation and non-genetic inheritance in governing adaptive trait-environment covariation (Meröndun et al. 2019; De Kort et al. 2020a; Wogan et al. 2020). However, it remains elusive to what extent genetic variation dictates epigenetic responses to environmental changes (Burgess, 2019; Cavalli & Heard, 2019). If genetic variation drives all genome-wide epigenetic and regulatory processes in response to environmental stress, then epigenetic variation should be considered a consequential quantitative trait that is under genetic control, rather than an independent evolutionary force.

As a non-genetic inheritance mechanism, intergenerational transmission of epigenetic signatures is deeply interwoven with DNA sequence inheritance (Danchin *et al.*, 2019; Adrian-Kalchhauser *et al.*, 2020; Medrano *et al.*, 2021). Epigenetic marks can be established, erased, maintained and recognized by a large repertoire of DNA sequence motifs and proteins. As a consequence, allelic variants of these protein-coding genes can affect the genomic distribution, stability and inheritance of epigenetic variation. Epigenetic inheritance is also suggested to be highly context-dependent and probabilistic rather than linear and deterministic, also referred to as facilitated epigenetic inheritance (Richards, 2006). For example, epigenetic marks frequently integrate across larger genomic regions with dynamic epigenetic landscapes, and gene activity depends on the combined actions of many epigenetic marks that spatially and temporally co-occur (Jones & Liang, 2009; Marchal & Miotto, 2015; Adrian-Kalchhauser *et al.*, 2020).

Most adaptive epigenetic variation may thus, either directly or moderated by environmental influences, arise from adaptive genetic variation, questioning the importance of epigenetic adaptation as a *gene-independent* evolutionary mechanism. Uncoupling epigenetic from genetic evolution is, however, challenging, requiring experimental work on isogenic or inbred lines each raised under various environmental settings and preferably over multiple generations, or comparative studies integrating genomic, gene expression and epigenomic analysis of genotypes that underwent distinct evolutionary trajectories. Experimental inbred studies show that environmental stress generates

ample epigenetic variation (Verhoeven *et al.*, 2010; Herman & Sultan, 2016; Berbel-Filho *et al.*, 2019), but that epigenetic responses to treatments are genotype-specific (Herman and Sultan 2016; Berbel-Filho et al. 2019, but see Xu et al. 2019). The observation that each inbred line generates a specific epigenetic profile may suggest non-independence between DNA sequence polymorphisms and differential methylation (Herman & Sultan, 2016; Berbel-Filho *et al.*, 2019). Comparative genome-epigenome studies, on the other hand, frequently find more differentially methylated genomic regions than DNA sequence polymorphisms, and little evidence for causative mutations underlying epigenetic variation (but see Herrera and Bazaga 2010; van Moorsel et al. 2019; Yagound et al. 2019), suggesting that (i) part of the epigenetic variation can arise independent from genetic evolution (Schmid *et al.*, 2018; Stajic *et al.*, 2019), and/or (ii) single DNA sequence polymorphisms (e.g. in methyltransferase enzymes catalysing genome-wide DNA methylation) pleiotropically affect epigenetic variation across large genomic regions (e.g. Kankel et al. 2003). Molecular mechanisms such as polygenic and pleiotropic regulation of epigenetic variation likely are omnipresent throughout the genome of most species (Wagner & Zhang, 2011; Smith *et al.*, 2015; Frachon *et al.*, 2017; Csilléry *et al.*, 2018; Adrian-Kalchhauser *et al.*, 2020), posing a huge challenge to disentangling epigenetic from genetic adaptive evolution.

DNA methylation islands, i.e. genomic regions characterized by high-density methylation, can have large effects on gene expression and phenotypic fitness (Cortijo *et al.*, 2014; Jeziorska *et al.*, 2017; Jeong *et al.*, 2018). For example, up to 90% of heritability for flowering time could be explained by a small number of islands of differential methylation in isogenic *Arabidopsis* lines (Cortijo *et al.*, 2014). In addition, many DNA methylation islands are linked to DNA sequence variation in transposable elements, gene bodies and intergenic regulatory elements (Suzuki & Bird, 2008; Gent *et al.*, 2013; Zhang *et al.*, 2018; Adrian-Kalchhauser *et al.*, 2020). Genomic islands of differential methylation, i.e. DNA methylation islands with significantly different methylation levels between populations, may therefore especially arise near genetic polymorphisms that are under the influence of divergent natural selection (see also Kawakatsu et al. 2016). Identifying to what extent islands of differential methylation can arise independent of genetic variation may help elucidating the role of genome-wide methylation landscapes in adaptive evolution.

Epigenetic variation may contribute disproportionally to adaptive evolution where the amount of standing genetic diversity is low, for example in clonal species, following demographic bottlenecks, and/or where selection pressures are variable or extremely high (Dapp *et al.*, 2015; Latzel *et al.*, 2016; Ardura *et al.*, 2017; Artemov *et al.*, 2017; Thorson *et al.*, 2017; Wibowo *et al.*, 2018). A recent study comparing population epigenomic signatures between a steep altitudinal gradient and a much wider continental gradient in the clonal species *Fragaria vesca* (woodland strawberry), demonstrated that

genomic islands of epigenetic divergence arise even at fine spatial scale (<2km), and persist through the next generation (De Kort et al. 2020a). How these epigenetic patterns behave relative to genetic variation nevertheless remains an open question that is key to understanding the molecular basis of adaptive evolution.

Aiming to shed new light on the interplay between epigenetic and genetic adaptive evolution, we compare genome-wide methylation profiles with genome-wide SNP (single nucleotide polymorphism) data from 20 *F. vesca* individuals originating from three nearby populations and raised in a common garden to control for short-lived environment-driven epigenetic signatures. A genomic bisulphite treatment was used to convert unmethylated cytosines into thymines thereby allowing quantifying cytosine methylation across the genome. While whole genome bisulphite sequencing (WGBS) is the most preferred method for methylation profiling, it complicates single nucleotide polymorphism (SNP) calling, with an increased occurrence of false calls particularly at CT and AG variants (Lindner *et al.*, 2022). Various techniques are being developed to deal with bisulphite-induced ascertainment bias, allowing dual use of bisulphite-treated genomes (Xu *et al.*, 2019; Liew *et al.*, 2020; Lindner *et al.*, 2022). Here, we relied on stringent filtering and complementary downstream methods to corroborate our findings.

We used a hierarchical sampling design involving a steep altitudinal population gradient, which has been demonstrated to generate fine-scale adaptive trait divergence in *F. vesca* (De Kort et al. 2020b), and subjected five of the individuals of one population (N=10) to acute drought stress. Specifically, five individuals originated from the low and high-altitudinal population, and ten from the mid-altitudinal population. While our major goal was to elucidate the role of adaptive evolution in driving SNP-DMC associations along an environmental gradient, the drought treatment helped disentangling epigenetic DMC (differentially methylated cytosine) patterns that are expected to be gene-independent (stress-induced) from SNP-associated DMC patterns. Through distinguishing between neutral and adaptive SNPs (genetic outliers) we also tested whether differential methylation between natural populations is associated to adaptive evolution acting upon nearby SNPs. We then explored to what extent acute stress, adaptation evolution, genomic proximity, and the tendency of DMCs to co-occur in genomic islands of differential methylation together explain genome-wide epigenetic and genetic covariation. Gene ontology enrichment tests served to assess whether SNP-DMC covariation is associated with specific biological functions. To consolidate the role of adaptive evolution in shaping genome-wide SNP-DMC covariation, we performed multivariate trait association analyses. We finally tested to what extent genes associated to flowering time, an important fitness trait with high heritability and adaptive divergence in *F. vesca* (De Kort et al. 2020b), are under genetic and/or epigenetic control. Flowering genes, which regulate vernalisation and timing of flowering in response to various environmental

stimuli including light conditions and ambient temperature, are prime examples of adaptive DNA sequences that can be subject to evolutionary divergence (e.g. Andrés and Coupland 2012) and of which transcription is fine-tuned by epigenetic modifications (Cortijo *et al.*, 2014; Bratzel & Turck, 2015). As a results, flowering genes are representative for adaptive evolution governed by the joint and complementary actions of genetic and epigenetic processes.

Materials and Methods

Study system

Fragaria vesca is a self-compatible clonal species of deciduous forest edges and gaps, with a relatively small diploid genome (2n = 14; 219 Mbp, Edger *et al.*, 2018). Seeds were collected from *F. vesca* plants at three locations along a fine-scale altitudinal gradient in the French Pyrenees (<2kms between populations, Table S1), including five plants from a low and high altitudinal population (400 and 1200 masl, resp.), and ten plants from a mid-altitudinal population (900 masl). Clear adaptive trait divergence has previously been observed along the fine-scale gradient (covering 9 populations, with ca. 13 plants per population), coinciding with various topographical variables including elevation, aspect and slope (De Kort et al. 2020b). Among the most heritable and adaptive traits was flowering vigour per unit biomass, demonstrating that this fitness trait is under strong selection even at a very fine spatial scale.

For a previous study (De Kort et al. 2020a), one random seedling per plant (N=20) was grown in a growth chamber with standardized soil moisture and light conditions after germination in petri-dishes (two week germination period). The plants were potted in potting soil (LP2D, Peltracom). Two times a week, the plants were watered and reshuffled to make sure they all experienced similar environmental variation. To help disentangling genetic from epigenetic variation, five samples from the mid-altitudinal Pyrenees were raised under drought stress through applying three consecutive drought treatments starting two months after germination (Table S1). At the start of each treatment, watering stopped until leaves went limp (6-10 days), after which plants were watered for three days to allow partial recovery of the soil. The plants were allowed to rehydrate for one week after the last drought treatment to remove the most unstable drought-induced epigenetic effects. The plants were kept in a growth chamber with regular growth lamps and a day/night regime of 16/8 hours and 22/18°C. No fertilizer was added in the course of the experiment. Ca. three months after germination, one leaf per plant was collected in liquid nitrogen prior to DNA extraction (see De Kort et al. 2020a).

Whole-genome bisulphite sequencing, and methylation and SNP profiling

Library preparation, bisulphite conversion and whole-genome sequencing was performed by De Kort et al. (2020a), and rendered 76 417 704 ± 13 837 490 high quality reads (mean ± standard deviation) per sample (See Supporting Methods and Fig. S1-S3 for more details). Methylkit v1.10.0 (Akalin *et al.*, 2012) was used by De Kort et al. (2020a) to call significant differentially methylated cytosines (DMCs), which were deemed significant where (i) at least 3 samples per population had a minimum cytosine coverage of 5x, (ii) at least a 25% difference in methylation between at least two populations was observed, and (iii) q-values <0.01 (see Supporting Methods for more details). Because DMCs often tend to cluster together, we assessed the tendency of DMCs to co-occur (< 100 bp between DMCs) along genomic blocks of 1 kb using the genomic R package "bumphunter v1.12". We defined DMC islands where at least five DMCs were found within these 1 kb blocks. Because we identified DMCs in offspring, significant methylation differences between the parental conditions are assumed to be inherited from parents to offspring (intergenerational inheritance). Therefore, cytosines with significantly differentiated methylation levels between the three populations and between the drought stress treatments are referred to as inherited and drought-induced DMCs, respectively. Note that the DMCs inherited from the parental generation (i) may in part reflect the environmental conditions experienced by the parents, and (ii) are insensitive to our drought treatment (no inherited DMC was also a drought-induced DMC). SNP calling was conducted with the ANGSD-software (Korneliussen *et al.*, 2014) from high coverage (30x and 13x genome coverage before and after bisulphite treatment, respectively) 30 bam files that were generated previously on a Hiseq (PE75, 8 lanes) by De Kort et al. 2020a. Stringent filtering (see Supporting Methods) rendered a total of 7,192 SNPs (Table S3).

PCadapt was used to identify SNPs with exceptional genetic patterns that reflect adaptive genetic differentiation (Luu *et al.*, 2017). A total of four principal components captured most background genetic variation (K=4, Fig. S4 and S5; See Supporting Methods). The SNPs that deviated significantly from neutral background structure along the principal components (expressed as the Mahalanobis distance and with q<0.05) were identified as putatively adaptive loci. Because no *a priori* definition of populations is required for this analysis, PCadapt is (i) not restricted to signatures of selection at the population level, and (ii) insensitive to unbalanced sampling sizes. However, outlier detection methods generally perform poorly for low sample sizes, rendering high false discovery rates particularly for rare alleles. We thus implemented a leave-one-out strategy, by removing every sample once for a range of MAF thresholds (0, 0.05, 0.075 and 0.1), and applied PCadapt on each of these subsets (20 subsets x 4 MAF thresholds = 80 analyses, see table S4 and Supporting Methods). We observed a strong impact of MAF threshold, and the highest number of consistent outliers was found for MAF and q-value thresholds of 0.1 (Fig. S6). Outliers with consistent signatures of selection were thus defined under these threshold conditions for all downstream analyses.

DMC-SNP covariation

To assess covariation between epigenetic and genetic population structure, we first performed a Principal Component Analysis (PCA) on the methylation frequency and allele matrix, respectively. Individual genotypes were coded as 0, 1 or 2 representing allele counts (homozygous for reference allele, heterozygous, and homozygous for alternative allele). The methylation matrix is composed of methylation frequencies along the individual x DMC matrix, i.e. the transposed DMC matrix as provided in Table S2. Then, to identify the general degree of covariation between genetic and epigenetic population structure, we performed a coinertia analysis between the principal components of the SNP matrix and the principal components of the DMC methylation percentages (R package "ade4") (Dray & Dufour, 2007). This rendered 19 co-inertia axes delineating the multidimensional molecular space, of which the first two axes covered most covariation between allele and methylation frequencies (76.8; Fig. S7). The overall strength of the covariation between SNPs and DMCs was measured through the RV-coefficient, and its significance was determined using 1000 Monte Carlo permutations. Mahalanobis distances, representing the dissimilarity between SNPs and DMCs in the two-dimensional coinertia space, were calculated for each SNP-DMC pair, using the R package "*StatMatch*". The degree of covariation between each SNP-DMC pair was calculated as [maximum Mahalanobis distance between SNP and DMC scores - pairwise SNP-DMC Mahalanobis distance], divided by the maximum Mahalanobis distance to obtain a covariation index between 0 (corresponding to the maximum Mahalanobis distance) and 1 (corresponding to maximum covariation). The SNP-DMC pairs with a covariation index > 0.9 were considered as co-varying SNP-DMCs (n=3,902 ~ 0.56% of all SNP-DMC pairs).

A mixed model was generated to test whether the degree of SNP-DMC covariation depended upon (i) the logarithmic distance between each SNP and all DMC islands, (ii) natural selection (consistent outliers vs. background SNPs), (iii) DMC clustering (number of linked DMCs in the DMC island), and (iv) acute drought stress (DMC type: drought-induced DMCs vs. inherited DMCs), while controlling for SNP and DMC cluster ID (two random factors):

Covariation ~ Log10(Distance) + Natural selection + DMC clustering + DMC type + (1 | SNP) + (1 | DMC Cluster ID)

This model served to address the following hypotheses in respective order: (i) SNP-associated DMCs are predominantly cis-acting (i.e. close to co-varying SNP) rather than trans-acting, (ii) SNP-associated methylation differentiation is linked to natural selection acting upon SNPs rather than to neutral processes, (iii) SNP-DMC covariation is more likely where more dense DMC islands are involved, and (iv) SNP-DMC covariation is more pronounced for DMCs inherited from parental environmental

conditions than for drought stress-induced DMCs. The hypothesis that adaptive evolution facilitates SNP-DMC covariation stems from the increasingly recognized importance and abundance of genetic variants governing non-genetic inheritance (Danchin *et al.*, 2019; Adrian-Kalchhauser *et al.*, 2020; Medrano *et al.*, 2021). Therefore, natural selection-driven canalization of allelic variants may result in canalization of associated epigenetic variants, resulting in increased SNP-DMC covariation.

The covariation between SNPs and DMCs may not be reflected by specific alleles directly resulting into higher or lower methylation frequencies, but may rather reflect alleles altering the methylation sensitivity or *variability* of co-varying DMCs. To test how the allelic composition of SNPs alters methylation profiles of co-varying DMCs, we first calculated methylation variability as the coefficient of variation (SD/mean) in methylation frequencies across all samples. A higher coefficient of variation thus corresponds to higher methylation variability. We then tested the amount of SNP-DMC covariation explained by (i) the absolute difference in average methylation *frequencies* between SNPs that were homozygous vs. heterozygous for the reference allele (Δ methylation frequency, normalized to obtain values between 0 and 100), and (ii) the absolute difference in methylation *variability* between SNPs that were homozygous vs. heterozygous for the reference allele (Δ methylation variability). In addition, we hypothesize that the relationship between SNP-DMC covariation and methylation patterns differed between DMCs arising from drought stress than for inherited DMCs that were not affected by drought stress (fixed effect "DMC type"), because acute environmental stress may reduce variation in methylation frequencies. Finally, because only a tiny fraction of the SNPs was homozygous for the alternative alleles (0.25%), these were excluded from the mixed model:

Covariation ~ (Δ methylation frequency × DMC type) + (Δ methylation variability × DMC type) + (1 | SNP) + (1 | DMC Cluster ID)

Functional enrichment analysis

For each SNP and DMC, the nearest gene within 1 kb distance was determined, as well as their putative involvement in flowering (Table S5 and S6). We retrieved gene ontology terms (GOs; Table S7) from the Genome Database for Rosaceae (GDR, Jung *et al.* 2019) for *F. vesca* v4.0.a2. Using Fisher's exact tests (R package "TopGO"), we then assessed which biological processes and molecular functions were particularly overrepresented in (i) outlier SNPs, (ii) drought DMCs, (iii) inherited DMCs, (iv) DMCs co-varying with SNPs (covariation > 0.9) and (v) SNPs co-varying with DMCs, as compared to the full *F. vesca* genome. We hypothesized an enrichment of (i) stress-related GO terms in each of these groups (and drought DMCs in particular), (ii) fitness-related GOs in outlier SNPs and in inherited DMCs, and (iii) genome regulatory GOs in all DMCs.

To test to what extent the covariation between SNPs and DMCs is linked to functional enrichment or functional similarity, we first grouped all GO terms in broad GO categories, including "stress" (e.g. response to water and immune response), "reproduction" (e.g. flowering and seed development), "growth" (e.g. photosynthesis and mitosis), "genome regulation" (e.g. regulation of transcription and nucleotide binding), "translation" (e.g. protein processing and ribosome biogenesis), "metabolism" (e.g. metabolic process and catalytic activity) and "signalling" (e.g. signal transduction and transferase activity) (Table S7). When a gene was involved in multiple GO categories, we prioritized reproduction, followed by stress, growth, genome regulation, translation, signalling and metabolism (e.g. a gene involved in reproduction, genome regulation and metabolism was assigned the GO category reproduction). This order was chosen to emphasize any potential association with fitness, as stress responsiveness and reproductive traits are known to be frequent targets of natural selection across fine scale gradients. We then tested whether SNP-DMC covariation could be explained by "SNP GO category" and/or "DMC GO category" rather than by "genomic distance" (i.e. three fixed effect variables), using a mixed model with "SNP ID" and "DMC cluster ID" as random effects. In this model, we additionally included the binary fixed effect variable "functional similarity" indicating whether or not a SNP-DMC pair shared the same GO category:

Covariation ~ Log10(Distance) + SNP GO Categories + DMC GO Categories + functional similarity + (1 | SNP) + (1 | DMC Cluster ID)

To assess the strength of the relationship between genomic vs. functional variation and SNP-DMC covariation, we calculated the variance uniquely explained by each variable while partialling out random effects (R package "partR2").

Phenotypic association analysis

We used phenotypic data of sister plants that were raised in similar conditions (moist vs. dry) to evaluate whether the adaptive SNP-DMC covariation signals observed in this study can be linked to adaptive trait divergence. While the use of sister plants may cause some noise in the hypothesized relationship between adaptive trait variation and SNP-DMC covariation, their shared parental and environmental origin is expected to render very similar evolutionary signals. Specifically, a total of 83 sister plants (on average 4.3 sister plants per genotype) were monitored for specific leaf area (SLA), flower density, runner density, growth and stomatal density as part of a large common garden trial detailed in De Kort et al. 2020b, and for which significant adaptive divergence was observed (De Kort et al. 2020b, Table S10). The phenotypic data were averaged per genotype (Table S1) and then used in redundancy analyses (RDA, R package "*vegan*") to detect associations between adaptive trait variation and (i) methylation frequencies of DMCs co-varying with at least five SNPs vs. (ii) methylation

frequencies of DMCs not co-varying with any SNP. The methylation frequencies of DMCs represent the multivariate response matrix, with the five traits along with altitude and treatment as explanatory variables. Because adaptive evolution along the altitudinal gradient is hypothesized to drive SNP-DMC covariation, we expected that DMCs co-varying with SNPs manifest more pronounced associations with adaptive traits and altitude than DMCs that do not co-vary with SNPs. Treatment was included to account for methylation variation resulting from the drought treatment. An additional RDA model was ran to corroborate the adaptive role of outlier SNPs, using the individual alleles of the consistent outliers as response matrix and the adaptive traits as response variables.

Results

Based on a PCadapt outlier analysis, a total of 127 SNPs (1.77% of 7,192 SNPs) deviated significantly from background genetic structure of 20 *F. vesca* individuals originating from three mountainous populations and raised under two distinct soil moisture settings (Fig. **1a**, Table S1 and S3). These outlier SNPs were enriched for several biological processes, including "regulation of a biological trait" and "flowering" (Table S8), corroborating the adaptive nature of the outliers. Of these potentially adaptive SNPs, 30 (23.62%) were highly consistent outliers across all leave-one-out outlier analyses (Fig. **1a**). These outliers showed a strong and significant association with adaptive traits (Fig. S8, R^2adj = 16.40), as compared to neutral SNPs (R^2adj = 2.77, Table S12 and S13). Because only 13 of these consistent outliers could be annotated, enrichment analysis on this small sample did not provide additional insights (Table S8). A total of nine SNPs were within or near a total of eight flowering genes (Table S6), two of them (22.22% of flowering genes) showing signatures of fine-scale adaptation (Fig. **1a**).

Across the genome, a total of 619 DMCs were found along the sampling gradient, most of which were found in isolation or loosely linked (79.16%) while 20.84% was clustered in 13 genomic islands of differential methylation (Fig. **1b**). Most of the DMCs were in CG context (71.6%), followed by CHG (18.7%) and CHH (9.7%) context (see De Kort et al. 2020 for detailed analysis of methylation across the DMC contexts). None of the DMC islands arose from the drought treatment. Instead, they were inherited from the parental plants growing along a steep altitudinal gradient, and contained on average 10 DMCs (up to 29 DMCs) within 1kb linkage blocks. Drought stress gave rise to 19 solitary DMCs (Fig. **1b**). The drought-induced DMCs were enriched for stress-related GO terms including response to oxidative stress and sulfate transport, which are key plant responses to drought stress (Chan *et al.*, 2013) (Table S8). The inherited DMCs were overrepresented by responses to environmental stimuli (e.g. light detection and response to osmotic stress), in addition to reproduction (e.g. pollination and seed dormancy). This overrepresentation of fitness-related processes suggests the involvement of

natural selection in the maintenance of inherited DMCs. Islands of DNA methylation were not enriched for notable ecological processes, although a transcription factor (FvH4_2g01260, MCM1) with DNA replication-related GO terms was differentially methylated at six cytosines (Table S5, Table S8). We finally found two significant DMCs within or near a putative flowering time gene encoding red/far red light photoreceptor FvPhyB (Fig. **1b**, Fig. **2a**), suggesting their involvement in constitutive, altitude-dependent expression of flowering.

A multivariate co-inertia analysis aiming to elucidate the degree of covariation between SNP allele frequencies and DMC methylation levels across the 20 samples revealed significant covariation between genome-wide SNP and DMC signatures (RV-coefficient = 41.97%, $p<0.001$, Fig. **2**), and most covariation aligned with the altitudinal gradient (first axis in Fig. **2c**). Although genotypes raised under drought stress clustered together with genotypes that did not receive a drought treatment (light blue genotypes in Fig. **2c**), drought-induced DMCs showed markedly high covariation with SNPs (i.e. they clustered together with the SNPs, Fig. **2a** and **2b**). A total of 15 drought-induced DMCs (78.95%) and 148 inherited DMCs (24.67%) co-varied with a total of 2550 SNPs (35.46%), indicating that DMCs typically have a broad genetic basis (on average 16 SNPs co-vary with a DMC). The proportion of drought-induced DMCs co-varying with SNPs is notably high (Fig. **2d**), and linear mixed models confirmed that SNPs were significantly more likely to co-vary with drought-induced DMCs than with inherited DMCs (Fig. **3a**, Table 1, Table S8).

Contrary to the expectation that adaptive evolution facilitates SNP-DMC covariation, significantly higher covariation with DMCs was found for background SNPs as opposed to outlier SNPs (Fig. **3b** and **3c**), and covariation of DMCs with SNPs was higher for solitary DMCs than for DMC islands (Fig. **3c**, Table 1, Table S9). In addition, covariation between a DMC and a SNP did not translate into obvious associations between allele frequencies and methylation frequencies. Instead, co-varying SNPs were associated with differential methylation variability, but only for inherited DMCs (Fig. **4**, Table 1, Table S9). Drought-induced DMCs did not display altered methylation variability, likely because drought stress canalized methylation levels. In all co-varying SNP-DMC pairs involving inherited DMCs (n=35 with a covariation index > 0.9; Table S11), SNPs resulted in higher vs. lower methylation variability when they were homozygous vs. heterozygous for the reference allele. We did not consider the homozygous state of the alternative allele because it was underrepresented (<1% of all SNPs).

Interestingly, the amount of covariation between SNPs and DMCs did not depend on their pairwise genomic distance (Fig. **5a**, Table 1, Table S9), suggesting that the degree of SNP-DMC covariation has a functional basis. We thus tested whether SNPs and DMCs sharing the same GO category (e.g. both involved in reproduction) were featured by increased covariation, and whether covariation depended

on SNP or DMC GO category. We found that shared GOs were not responsible for high SNP-DMC covariation (partial R^2 <0.1%), and SNP-DMC covariation did not vary according to SNP GO category (partial R^2 = 1.3%, Fig. **5a**, Table S9). However, there was a marked difference in SNP-DMC covariation depending on DMC GO category (partial R^2 = 9.5%). Reproduction DMCs in particular manifested consistently low SNP-DMCs covariation (Fig. **5c** and **5d**). SNPs and DMCs involved in growth and genome regulation on the other hand had the highest SNP-DMC covariation (Fig. **5d**). DMCs co-varying with SNPs were particularly enriched for GOs related to stress responses (e.g. response to abiotic stimulus, response to oxidative stress, light detection) and to genome regulation (e.g. regulation of histone modification) (Table S8). Interestingly, DMCs involved in stress responses typically only rendered average SNP-DMC covariation unless the associated SNP was involved in genome regulation and reproductive processes (Fig. **5d**). Likewise, reproductive SNPs only rendered high SNP-DMC covariation where the associated DMC was involved in stress or in genome regulation (Fig. **5d**). Our GO analysis on co-varying SNPs and DMCs furthermore allowed us to detect a functional link for a specific co-varying SNP-DMC pair, where the DMC is involved in responses to oxidative stress while the co-varying SNP is embedded in a cellular (aerobic) respiration gene (Table S8). Aerobic respiration has been shown to cause oxidative stress (Anand et al. 2019). It is thus most likely that SNPs in genes affecting aerobic respiration cause changes in oxidative stress and associated methylation patterns, particularly under stress.

Both DMCs co-varying with SNPs and DMCs that do not co-vary with SNPs were significantly associated with adaptive trait variation along the altitudinal gradient (Fig. 6). However, the association was stronger for the DMCs not co-varying with SNPs (R^2adj = 61.77% vs. 42.05%), which corresponds to the decreased SNP-DMC covariation for loci with signatures of adaptation (Fig. 3C). The tendency of genotypes to form runners (asexual reproduction) and SLA had the most pronounced association with these DMCs (Fig. 6B). Altitude and drought treatment also contributed considerably to the methylation patterns, which is in line with the observation that drought DMCs in particular co-varied with SNPs along the sampling gradient (Fig. 2).

Discussion

Epigenetic variation is tightly linked to environmental and fitness differences, implying its involvement in adaptive evolution. Understanding the genetic and environmental mechanisms driving adaptive epigenetic variation therefore is key to evolutionary and molecular biology. It nevertheless remains unclear to what extent epigenetic evolution can occur independent of genetic polymorphisms. However, demonstrating the degree of interdependence between DNA sequence and DNA

methylation signatures is challenging, particularly because DNA methylation marks can be triggered by multiple genetic polymorphisms each affecting to various extent environment-dependent methylation signatures. Here, we used *Fragaria vesca* as a model species and applied multivariate and comparative genomics-epigenomics analyses to show that (i) many SNPs are linked to one or several DMC(s), with higher covariation when SNPs are not currently targeted by selection, (ii) inherited DMCs frequently co-vary with many SNPs, and (iii) this covariation is functional, and mostly linked to fitness-related processes, genome regulation and stress responses. All results point to differential DNA methylation marks representing quantitative traits that can be the target of selection rather than sources for gene-independent evolution. Based on our findings, we propose a framework of polygenic adaptation shaping genome-wide methylation levels.

Along a steep environmental gradient, 127 SNPs were found to significantly deviate from background genetic structure while methylation levels of ca. 600 cytosines were significantly differentiated. The adaptive nature of these genetic and epigenetic polymorphisms was corroborated by enrichment of ecological processes such as responses to environmental stimuli and flowering (Table S8). Inherited epigenetic signatures differed markedly from those arising from drought stress. Specifically, drought only induced 19 DMCs (as opposed to 600 inherited DMCs) that were still detectable after one week of rehydration, suggesting that most environmentally induced epigenetic marks, as detected in studies without rehydration (e.g. Kuromori et al. 2021; Zhao et al. 2022), are short-lived and non-heritable. Inherited DMCs, on the other hand, may be stable enough to persist through meiosis (Calarco *et al.*, 2012; Ragunathan *et al.*, 2015; Gehring, 2019). However, they can also re-establish across generations under genetic influence following meiotic erasure (Richards, 2006; Wang & Moazed, 2017; Walker *et al.*, 2018). While it is challenging to disentangle these competing processes, it becomes increasingly clear that epigenetic signatures are under strong genetic control (Lemire *et al.*, 2015; Tikhodeyev, 2018; Danchin *et al.*, 2019; Adrian-Kalchhauser *et al.*, 2020; Medrano *et al.*, 2021). Here, many DMCs co-varied with multiple SNPs, supporting the notion that non-genetic inheritance is at least partly genetically determined.

Theoretical and empirical data show that adaptive evolution typically is a polygenic process, whereby many SNPs can have the same impact on gene expression and fitness (Jain & Stephan, 2017; Barghi *et al.*, 2020; Fagny & Austerlitz, 2021). This genomic redundancy allows individuals with distinct genetic backgrounds to evolve to similar environmental stressors. In our study, many putatively adaptive DMCs (N=51, 8.5%) each co-varied with over 15 SNPs - predominantly in different genes (Table S11) and not currently targeted by natural selection (PCadapt) - indicating that multiple genes can alter the susceptibility of a cytosine to environmental stress. This altered susceptibility to stress was reflected by changes in methylation variability (Fig. **4**), and corresponds to earlier studies proposing that most

epigenetic inheritance is probabilistic rather than linear and deterministic (Jones & Liang, 2009; Marchal & Miotto, 2015; Adrian-Kalchhauser *et al.*, 2020). A polygenic basis underlying this environmental susceptibility allows for natural selection targeting different SNPs associated with the same DMC in different populations, consequently reducing SNP-DMC covariation across populations, and is in line with studies demonstrating polygenic adaptation of gene expression (He *et al.*, 2016; Hämälä *et al.*, 2020). Thus, low SNP-DMC covariation does not necessarily imply SNP-independent evolution of DMCs, but may indicate polygenic selection on a broad genetic basis shaping genome-wide methylation levels. Although many of these SNPs may coincidentally rather than causally co-vary with the same DMC due to similar allele frequency distributions, additional findings suggest that at least part of the SNP-DMC associations are functional and involved in polygenic adaptation.

First, we found significantly reduced SNP-DMC covariation for SNPs with signatures of divergent selection, indicating that adaptive evolution is involved in the maintenance of relatively long-lived (inherited) methylation signatures. In the framework of polygenic adaptation shaping genome-wide methylation levels, DMCs that are key to fitness arise from divergent selection on multiple SNPs, consequently reducing SNP-DMC covariation. We found markedly low SNP-DMC covariation where DMCs are embedded in sequences associated with reproduction (Fig. **5**). Considering that fitness is shaped by highly complex traits (e.g. sexual and asexual reproduction) that have been found to be under strong selection along the fine-scale gradient studied here (De Kort et al. 2020b), the lack of SNP-DMC covariation across reproduction genes suggests a role for adaptation in uncoupling epigenetic from genetic variation. Moreover, we found that methylation profiles associated with low SNP-DMC covariation were markedly similar to adaptive trait patterns (Fig. **6b**), further corroborating a role for adaptive evolution underpinning reduced SNP-DMC covariation. Methylation patterns associated with high SNP-DMC covariation, on the other hand, were significantly but less markedly linked to adaptive trait patterns. This may point to a broad epigenetic basis underpinning adaptive traits upon which natural selection can act, through their association with genetic variants, when environmental conditions change.

Second, high SNP-DMC covariation was found where DMCs were involved in responses to drought (Fig. **2d, 2f**) and to environmental stress (response to stimuli, pH regulation, response to oxidative stress; Inzé and Montagu 1995; Isaksson 2015; Zhu 2016; Table S8), particularly when the co-varying SNPs were associated with genome regulation and reproduction (Fig. **5c**). Such SNP-DMC associations may confer stress-responsiveness to fitness traits, thereby allowing a species to adjust key fitness traits while allowing appropriate stress responses under changing environmental conditions (Agarwal *et al.*, 2010; Lasky *et al.*, 2014; Le *et al.*, 2014). Stress-responsive elements have frequently been identified near flowering genes (Kim *et al.*, 2004; Andrés & Coupland, 2012), and genetic polymorphisms in

transcription factor binding sites of fitness genes have been shown to affect responsiveness of these genes to various environmental stressors (Lasky et al. 2014). Previously observed drought stress-dependent flowering vigour along the same altitudinal gradient reinforces a putative role for SNP-DMC associations in conferring stress-responsiveness to fitness traits (De Kort et al. 2020b). One particular SNP-DMC pair exemplifies such functional covariation. The respective drought-induced DMC is embedded in a gene responding to oxidative stress, and co-varied with a SNP in a gene involved in aerobic respiration (Table S8). This SNP did not manifest any signature of adaptation along our sampling gradient, but is embedded in a gene involved in a key fitness trait (aerobic respiration, Anand et al. 2019) governing responses to drought and oxidative stress more than 300 kbp away. Taken together, SNP-DMC covariation most likely is functional because (i) it is independent of genomic proximity but instead associated with many SNPs with diffused *trans*-effects, (ii) it is significantly associated with drought stress-induced epigenetic signatures (Fig. **2d, 3a**), (iii) it is significantly affected by the involvement of DMCs in reproductive genes, (iv) co-varying DMCs are enriched for processes related to stress responses and genome regulation, (v) co-varying SNPS are enriched for processes related to respiration and hormone regulation, and (vi) these findings align with patterns of adaptive divergence of stress-responsive fitness-related traits observed previously along the same environmental gradient (Fig. 6, De Kort et al. 2020b).

Our findings suggest that stress-responsive cytosines can be targeted by many fitness genes across the genome, allowing the same DMC to orchestrate a versatile stress response that depends on the genomic and environmental context. For example, drought-responsive DMCs each co-varied with an average of 113 SNPs as compared to inherited DMCs, which had on average three co-varying SNPs. Two of these drought-induced DMCs were involved in oxidative stress and oxidation-reduction processes and co-varied with 217 and 381 SNPs, respectively (Table S8). Genome-wide trans-acting epigenetic properties have correspondingly been proposed to provide populations with the flexibility of allowing adaptive responses to various stresses (Hou *et al.*, 2014; Liu *et al.*, 2019).

We expected part of the SNPs to be associated with many DMCs through affecting genome-wide methylation levels (e.g. SNPs in methyltransferase genes). Only 22 SNPs (0.31%), however, co-varied with more than 10 DMCs (on average less than 1 DMC), and none of these SNPs were associated with genome regulation (Table S8). It has been suggested that mutations with pleiotropic effects on gene expression are often deleterious due to their genome-wide impact (Denver *et al.*, 2005; Wittkopp *et al.*, 2008), possibly explaining the lack of SNPs co-varying with large numbers of DMCs. Alternatively, we did not capture the SNPs with genome-wide impact due to the removal of genetic variants by the bisulfite treatment and/or overly stringent SNP filtering parameters. In a model where most DMCs arise from a few *trans*-acting SNPs with multiple and interactive downstream effects (Denver *et al.*,

2005; Lyko, 2017), it becomes extremely challenging to detect SNPs with genome-wide impact. Importantly, our finding that one SNP typically co-varies with only few DMCs suggests that the fitness effects inferred by such SNPs work through a limited number of epigenetic marks. This would imply that individual DMCs can have important fitness effects. While experimentally testing the phenotypic impact of individual epigenetic marks is particularly challenging, we here show that (i) the identification of DMCs co-varying with SNPs can elucidate important candidate epi-variants that are expected to have detectable fitness impacts, and (ii) our DMCs collectively manifest strong associations with adaptive traits (Fig. 6). Our findings thus suggest that solitary DMCs are frequently inherited (through inheritance of co-varying SNPs) and associated with putatively adaptive functions, and should therefore not be ignored when studying epigenetic evolution.

Islands of differential methylation are thought to be more likely than solitary DMCs to affect gene expression and biological functions (Paun *et al.*, 2019; Teschendorf & Relton, 2019). Although we found more divergent methylation patterns for DMCs in genomic islands than for solitary DMCs (De Kort et al. 2020a), we found little indication for DMC islands to be involved in adaptive evolution (e.g. no enrichment of adaptive functions, Table S8). However, a total of seven out of 12 islands of differential methylation, including the top three largest islands, were associated with annotated transposable elements (Table S5), indicating that transposable elements contribute to the divergence of methylation islands along our steep environmental gradient. Adopted transposable elements have been demonstrated to exert important phenotypic effects (e.g. Quadrana et al. 2019), which could explain why the seven transposable elements identified here have distinct methylation levels across the samples.

Our SNP-DMC covariation estimates probably are highly conservative. First, the bisulphite treatment of our genomes eliminated many genetic variants across the genome (76% missing data), consequently decreasing the number of SNPs that could contribute disproportionally to the SNP-DMC covariation matrix. Second, false positive SNP calls that may have arisen from the bisulphite treatment likely have caused additional genomic noise, to some extent blurring the evolutionary signals present in our samples. However, this effect has been shown to be limited as compared to the loss of variants due to bisulphite treatment (Lindner et al. 2022). We nevertheless acknowledge that SNP calling methods specifically designed for bisulphite treated genomes (see e.g. Xu et al. 2019; Wiener et al. 2020) could have rendered stronger results, particularly when comparison with untreated genomes is possible. Third, while multi-variate tools have high potential for detecting subtle and polygenic signals (Forester *et al.*, 2018), they do not capture interactive effects between markers that likely contribute considerably to the SNP-DMC interdependence. Dense panels of genetic and epigenetic variants could explore the evolutionary basis of SNP-DMC covariation in more depth. Fourth, our methylomes were

restricted by tissue (leaves) and treatment (one environmental stressor), most likely causing underestimated SNP-DMC covariation estimates. Fifth, genetic variants other than SNPs, such as copy number variations (CNVs), insertions and deletions can render differential methylation and thus contribute to the interdependence between methylation status and genomic variation (Huang & Chain, 2021). Finally, much of the gene-dependent epigenetic variation may be linked to genetic elements other than SNPs. Transposable elements, for example, are among the most abundantly methylated regions in the genome and may thus explain part of the "missing" SNP-DMC covariation. In our study, the assignment of transposable elements to most of our DMC islands confirms the epigenomic importance of transposons. Transposable elements, rather than SNPs, being the driving force of genomic islands of differential methylation likely explains why SNP-DMC covariation decreases with increasing DMC clustering (Fig. **3**). While DMCs in transposable elements generally had low covariation with SNPs, we did observe two transposable elements with high SNP-DMC covariation (>0.9; Tables S5 and S11). This could result from methylation-directed TE mobilization causing genetic variation across the genome (Baduel & Colot 2021). Considering that at least 22% of *the F. vesca* genome sequence consists of transposable elements (Shulaev *et al*. 2011; Edger *et al*. 2018), studying the role of transposable elements as drivers of SNP-DMC covariation and adaptive evolution represents a promising research avenue.

Conclusion and research opportunities

The interdependence between genetic and epigenetic variation and its evolutionary significance remains largely unexplored. Based on our findings, we proposed a framework of polygenic adaptation shaping genome-wide methylation levels. This framework assumes that inherited and stress-responsive epigenetic signatures are inseparably from organismal fitness and play a key role in adaptive evolution. While validation of our framework, e.g. through expression analyses, functional validation and/or experimental evolution, is required for making general statements regarding the role of genetic variation and polygenic selection in driving genome-wide methylation patterns, the framework paves the way for addressing key questions in ecology and evolution. For example, do genetic variants in presumably pleiotropic genes such as methyltransferase genes drive adaptive evolution in response to environmental stressors through affecting methylation levels across specific genomic regions? And does polygenic adaptation towards increased methylation at DMCs facilitate genetic assimilation through increased mutation rates at these DMCs? Through our framework, we also stress that experimentally testing hypotheses on the phenotypic impact of individual DMCs, while considering their dependence upon *trans*-acting genetic polymorphisms, represents an important research avenue (see e.g. Taudt et al. 2016). Finally, our study is based on methylation differentiation between pre-defined conditions (populations and drought). While this is the most commonly used

approach to study ecologically relevant epigenetic signatures, outlier detection methods applied to single methylation polymorphisms without *a priori* definition of population structure (similar to PCadapt) could reveal a more complete set of potentially adaptive patterns. Together, we provide important exploratory insights into the evolutionary interplay between genetic and epigenetic variants, but further research is required to make conclusive statements with respect to the genetic and evolutionary basis of genome-wide methylation.

We further propose that our framework can inform on evolutionary potential in the context of global change. Specifically, we found that stress-induced DMCs co-varied more readily with SNPs than inherited DMCs, suggesting that environmental stress exposes the genetic basis of stress responsiveness. Stress-induced DMCs may thus reflect the potential of a population to evolve stress responses and, as such, maintain fitness under changing stress levels. The number of DMCs identified following a specific environmental stressor may correspondingly approximate the potential of a population to evolve in response to environmental change. Although we only subjected one population to drought stress in our study, comparison of stress-induced methylomes between populations could inform on their adaptive potential.

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Author contributions

HDK designed the study, performed the data-analyses, and wrote the manuscript. TT and FVN performed the bio-informatics analyses (read assembly, SNP calling, DMC calling). JA provided the collection of flowering genes. TPH commented on the manuscript. OH supervised the study and commented on the manuscript.

Data Availability

All data needed to perform the data-analyses are provided in the supporting information. Sequence reads are stored in Genbank under accession number PRJNA635047. Filtered DMCs used for all analyses are stored in Dryad (doi.org/10.5061/dryad.zs7h44j6r).

References

Adrian-Kalchhauser I, Sultan SE, Shama LNS, Spence-Jones H, Tiso S, Keller Valsecchi CI, Weissing FJ. **2020**. Understanding 'Non-genetic' Inheritance: Insights from Molecular-Evolutionary Crosstalk. *Trends in Ecology and Evolution* **35**: 1078–1089.

Agarwal P, Agarwal PK, Joshi AJ, Sopory SK, Reddy MK. **2010**. Overexpression of PgDREB2A transcription factor enhances abiotic stress tolerance and activates downstream stress-responsive genes. *Molecular Biology Reports* **37**: 1125–1135.

Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, Mason CE. **2012**. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. *Genome Biology* **13**: R87.

Anand A, Chen K, Yang L, Sastry A V., Olson CA, Poudel S, Seif Y, Hefner Y, Phaneuf P V., Xu S, *et al.* 2019. Adaptive evolution reveals a tradeoff between growth rate and oxidative stress during naphthoquinone-based aerobic respiration. *Proceedings of the National Academy of Sciences of the United States of America* **116**: 25287–25292.

Andrés F, Coupland G. **2012**. The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics* **13**: 627–639.

Ardura A, Zaiko A, Morán P, Planes S, Garcia-Vazquez E. **2017**. Epigenetic signatures of invasive status in populations of marine invertebrates. *Scientific Reports* **7**: 42193.

Artemov A V., Mugue NS, Rastorguev SM, Zhenilo S, Mazur AM, Tsygankova S V., Boulygina ES, Kaplun D, Nedoluzhko A V., Medvedeva YA, *et al.* 2017. Genome-Wide DNA Methylation Profiling Reveals Epigenetic Adaptation of Stickleback to Marine and Freshwater Conditions. *Molecular Biology and Evolution* **34**: 2203–2213.

Baduel P, Colot V. 2021. The epi-allelic potential of transposable elements and its evolutionary significance in plants. *Phil. Trans. R. Soc*. **B376**: 20200123

Barghi N, Hermisson J, Schlötterer C. **2020**. Polygenic adaptation: a unifying framework to understand positive selection. *Nature Reviews Genetics* **21**: 769–781.

Berbel-Filho WM, Rodríguez-Barreto D, Berry N, Garcia De Leaniz C, Consuegra S. **2019**. Contrasting DNA methylation responses of inbred fish lines to different rearing environments. *Epigenetics* **14**: 939–948.

Bratzel F, Turck F. **2015**. Molecular memories in the regulation of seasonal flowering: From

competence to cessation. *Genome Biology* **16**: 1–14.

Burgess DJ. **2019**. Testing the generality of non-genetic inheritance. *Nature Reviews Genetics* **20**: 3.

Calarco JP, Borges F, Donoghue MTA, Van Ex F, Jullien PE, Lopes T, Gardner R, Berger F, Feijó JA, Becker JD, *et al.* 2012. Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. *Cell* **151**: 194–205.

Cavalli G, Heard E. **2019**. Advances in epigenetics link genetics to the environment and disease. *Nature* **571**: 489–499.

Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ. **2013**. Balancing metabolites in drought: The sulfur assimilation conundrum. *Trends in Plant Science* **18**: 18–29.

Cortijo S, Wardenaar R, Colomé-Tatché M, Gilly A, Etcheverry M, Labadie K, Caillieux E, Hospital F, Aury J-M, Wincker P, *et al.* 2014. Mapping the epigenetic basis of complex traits. *Science* **343**: 1145–8.

Csilléry K, Rodríguez-Verdugo A, Rellstab C, Guillaume F. **2018**. Detecting the genomic signal of polygenic adaptation and the role of epistasis in evolution. *Molecular Ecology* **27**: 606–612.

Danchin E, Pocheville A, Rey O, Pujol B, Blanchet S. **2019**. Epigenetically facilitated mutational assimilation: epigenetics as a hub within the inclusive evolutionary synthesis. *Biological Reviews* **94**: 259–282.

Dapp M, Reinders J, Bédiée A, Balsera C, Bucher E, Theiler G, Granier C, Paszkowski J. **2015**. Heterosis and inbreeding depression of epigenetic *Arabidopsis* hybrids. *Nature Plants* **1**: 15092.

Denver DR, Morris K, Streelman JT, Kim SK, Lynch M, Thomas WK. **2005**. The transcriptional consequences of mutation and natural selection in *Caenorhabditis elegans*. *Nature Genetics* **37**: 544–548.

Dray S, Dufour A. **2007**. The ade4 Package: Implementing the Duality Diagram for Ecologists. *Journal of Statistical Software*, **22**(4), 1–20. doi: 10.18637/jss.v022.i04.

Edger PP, VanBuren R, Colle M, Poorten TJ, Wai CM, Niederhuth CE, Alger EI, Ou S, Acharya CB, Wang J, *et al.* 2018. Single-molecule sequencing and optical mapping yields an improved genome of woodland strawberry (*Fragaria vesca*) with chromosome-scale contiguity. *GigaScience* **7**: 1-7.

Fagny M, Austerlitz F. **2021**. Polygenic Adaptation: Integrating Population Genetics and Gene Regulatory Networks. *Trends in Genetics* **37**: 631–638.

Fisher RA. **1930**. The genetical theory of natural selection. Clarendon Press, Oxford.

Forester BR, Lasky JR, Wagner HH, Urban DL. **2018**. Comparing methods for detecting multilocus adaptation with multivariate genotype-environment associations. *Molecular Ecology* **27**: 2215–2233.

Frachon L, Libourel C, Villoutreix R, Carrère S, Glorieux C, Huard-Chauveau C, Navascués M, Gay L, Vitalis R, Baron E, *et al.* 2017. Intermediate degrees of synergistic pleiotropy drive adaptive evolution in ecological time. *Nature Ecology and Evolution* **1**: 1551–1561.

Gehring M. **2019**. Epigenetic dynamics during flowering plant reproduction: evidence for reprogramming? *New Phytologist* **224**: 91–96.

Gent JI, Ellis NA, Guo L, Harkess AE, Yao Y, Zhang X, Dawe RK. **2013**. CHH islands: *De novo* DNA methylation in near-gene chromatin regulation in maize. *Genome Research* **23**: 628–637.

Hämälä T, Guiltinan MJ, Marden JH, Maximova SN, dePamphilis CW, Tiffin P. **2020**. Gene Expression Modularity Reveals Footprints of Polygenic Adaptation in *Theobroma cacao*. *Molecular Biology and Evolution* **37**: 110–123.

He F, Arce AL, Schmitz G, Koornneef M, Novikova P, Beyer A, de Meaux J. **2016**. The Footprint of Polygenic Adaptation on Stress-Responsive *Cis* -Regulatory Divergence in the *Arabidopsis Genus*. *Molecular Biology and Evolution* **33**: 2088–2101.

Herman JJ, Sultan SE. **2016**. DNA methylation mediates genetic variation for adaptive transgenerational plasticity. *Proceedings of the Royal Society B: Biological Sciences* **283**: 20160988.

Herrera CM, Bazaga P. **2010**. Epigenetic differentiation and relationship to adaptive genetic divergence in discrete populations of the violet *Viola cazorlensis*. *New phytologist* **187**: 867–76.

Hou X, Zhou J, Liu C, Liu L, Shen L, Yu H. **2014**. Nuclear factor Y-mediated H3K27me3 demethylation of the SOC1 locus orchestrates flowering responses of *Arabidopsis*. *Nature Communications* **5**: 1–14.

Huang KM, Chain FJJ. **2021**. Copy number variations and young duplicate genes have high methylation levels in sticklebacks. *Evolution* **75**: 706–718.

Inzé D, Montagu M Van. **1995**. Oxidative stress in plants. *Current Opinion in Biotechnology* **6**: 153–158.

Isaksson C. **2015**. Urbanization, oxidative stress and inflammation: a question of evolving, acclimatizing or coping with urban environmental stress. *Functional Ecology* **29**: 913–923.

Jain K, Stephan W. **2017**. Modes of Rapid Polygenic Adaptation. *Molecular Biology and Evolution* **34**:

3169–3175.

Jeong H, Wu X, Smith B, Yi S V. 2018. Genomic landscape of methylation islands in hymenopteran insects. *Genome Biology and Evolution* **10**: 2766–2776.

Jeziorska DM, Murray RJS, De Gobbi M, Gaentzsch R, Garrick D, Ayyub H, Chen T, Li E, Telenius J, Lynch M, *et al.* 2017. DNA methylation of intragenic CpG islands depends on their transcriptional activity during differentiation and disease. *Proceedings of the National Academy of Sciences of the United States of America* **114**: E7526–E7535.

Jones PA, Liang G. **2009**. Rethinking how DNA methylation patterns are maintained. *Nature Reviews Genetics* **10**: 805–811.

Jung S, Lee T, Cheng C-H, Buble K, Zheng P, Yu J, Humann J, Ficklin SP, Gasic K, Scott K, *et al.* 2019. 15 years of GDR: New data and functionality in the Genome Database for Rosaceae. *Nucleic Acids Research* **47**: D1137–D1145.

Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddeloh JA, Riddle NC, Verbsky ML, Richards EJ. **2003**. *Arabidopsis* MET1 cytosine methyltransferase mutants. *Genetics* **163**: 1109–1122.

Kawakatsu T, Stuart T, Valdes M, Breakfield N, Schmitz RJ, Nery JR, Urich MA, Han X, Lister R, Benfey PN, *et al.* 2016. Unique cell-type-specific patterns of DNA methylation in the root meristem. *Nature Plants* **2**: 16058.

Kim HJ, Hyun Y, Park JY, Park MJ, Park MK, Kim MD, Kim HJ, Lee MH, Moon J, Lee I, *et al.* 2004. A genetic link between cold responses and flowering time through FVE in *Arabidopsis thaliana*. *Nature Genetics* **36**: 167–171.

Korneliussen TS, Albrechtsen A, Nielsen R. 2014. ANGSD: Analysis of Next Generation Sequencing Data. *BMC Bioinformatics* **15**: 356.

Kuromori T, Fujita M, Takahashi F, Yamaguchi-Shinozaki K, Shinozaki K. 2021. Inter-tissue and inter-organ signaling in drought stress response and phenotyping of drought tolerance. The Plant Journal **109**: 342-358.

De Kort H, Panis B, Deforce D, Van Nieuwerburgh F, Honnay O. **2020a**. Ecological divergence of wild strawberry DNA methylation patterns at distinct spatial scales. *Molecular Ecology* **29**: 4871–4881.

De Kort H, Panis B, Helsen K, Douzet R, Janssens SB, Honnay O. **2020b**. Pre-adaptation to climate change through topography-driven phenotypic plasticity. *Journal of Ecology* **108**: 1465–1474.

Lasky JR, Des Marais DL, Lowry DB, Povolotskaya I, McKay JK, Richards JH, Keitt TH, Juenger TE.

2014. Natural Variation in Abiotic Stress Responsive Gene Expression and Local Adaptation to Climate in *Arabidopsis thaliana*. *Molecular Biology and Evolution* **31**: 2283–2296.

Latzel V, Rendina González AP, Rosenthal J. **2016**. Epigenetic Memory as a Basis for Intelligent Behavior in Clonal Plants. *Frontiers in Plant Science* **7**: 1354.

Le TN, Schumann U, Smith NA, Tiwari S, Khang Au PC, Zhu QH, Taylor JM, Kazan K, Llewellyn DJ, Zhang R, *et al.* 2014. DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in *Arabidopsis*. *Genome Biology* **15**: 1–18.

Lemire M, Zaidi SHE, Ban M, Ge B, Aïssi D, Germain M, Kassam I, Wang M, Zanke BW, Gagnon F, *et al.* 2015. Long-range epigenetic regulation is conferred by genetic variation located at thousands of independent loci. *Nature Communications* **6**: 1–12.

Liew YJ, Howells EJ, Wang X, Michell CT, Burt JA, Idaghdour Y, Aranda M. **2020**. Intergenerational epigenetic inheritance in reef-building corals. *Nature Climate Change* **10**: 254–259.

Lindner M, Gawehns F, te Molder S, Visser ME, Oers K, Laine VN. **2022**. Performance of methods to detect genetic variants from bisulphite sequencing data in a non-model species. *Molecular Ecology Resources* **22**: 834–846.

Liu X, Li YI, Pritchard JK. **2019**. Trans Effects on Gene Expression Can Drive Omnigenic Inheritance. *Cell* **177**: 1022-1034.e6.

Luu K, Bazin E, Blum MGB. **2017**. pcadapt: an R package to perform genome scans for selection based on principal component analysis. *Molecular Ecology Resources* **17:** 67-77.

Lyko F. **2017**. The DNA methyltransferase family: a versatile toolkit for epigenetic regulation. *Nature Reviews Genetics 2017 19:2* **19**: 81–92.

Marchal C, Miotto B. **2015**. Emerging Concept in DNA Methylation: Role of Transcription Factors in Shaping DNA Methylation Patterns. *Journal of Cellular Physiology* **230**: 743–751.

Medrano M, Alonso C, Bazaga P, López E, Herrera CM. **2021**. Comparative genetic and epigenetic diversity in pairs of sympatric, closely related plants with contrasting distribution ranges in south-eastern Iberian mountains. *AoB PLANTS* **12**: plaa013.

Meröndun J, Murray DL, Shafer ABA. **2019**. Genome-scale sampling suggests cryptic epigenetic structuring and insular divergence in Canada lynx. *Molecular Ecology* **28**: mec.15131.

van Moorsel SJ, Schmid MW, Wagemaker NCAM, Gurp T, Schmid B, Vergeer P. **2019**. Evidence for rapid evolution in a grassland biodiversity experiment. *Molecular Ecology* **28**: 4097–4117.

Paun O, Verhoeven KJF, Richards CL. **2019**. Opportunities and limitations of reduced representation bisulfite sequencing in plant ecological epigenomics. *New Phytologist* **221**: 738–742.

Payne JL, Wagner A. **2019**. The causes of evolvability and their evolution. *Nature Reviews Genetics* **20**: 24–38.

Quadrana L, Etcheverry M, Gilly A, Caillieux E, Madoui MA, Guy J, Bortolini Silveira A, Engelen S, Baillet V, Wincker P, *et al.* 2019. Transposition favors the generation of large effect mutations that may facilitate rapid adaption. *Nature Communications* **10**: 1–10.

Ragunathan K, Jih G, Moazed D. **2015**. Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science* **348**: 1258699.

Richards EJ. **2006**. Inherited epigenetic variation - Revisiting soft inheritance. *Nature Reviews Genetics* **7**: 395–401.

Schmid MW, Heichinger C, Coman Schmid D, Guthörl D, Gagliardini V, Bruggmann R, Aluri S, Aquino C, Schmid B, Turnbull LA, *et al.* 2018. Contribution of epigenetic variation to adaptation in *Arabidopsis*. *Nature Communications* **9**: 4446.

Shulaev V, Sargent DJ, Crowhurst RN, Mockler TC, Folkerts O, Delcher AL, Jaiswal P, Mockaitis K, Liston A, Mane SP, *et al.* 2011. The genome of woodland strawberry (*Fragaria vesca*). *Nature Genetics* **43**: 109–116.

Smith G, Smith C, Kenny JG, Chaudhuri RR, Ritchie MG. **2015**. Genome-Wide DNA Methylation Patterns in Wild Samples of Two Morphotypes of Threespine Stickleback (*Gasterosteus aculeatus*). *Molecular biology and evolution* **32**: 888–95.

Stajic D, Perfeito L, Jansen LET. **2019**. Epigenetic gene silencing alters the mechanisms and rate of evolutionary adaptation. *Nature Ecology and Evolution* **3**: 491–498.

Suzuki MM, Bird A. **2008**. DNA methylation landscapes: provocative insights from epigenomics. *Nature reviews. Genetics* **9**: 465–76.

Taudt A, Colomé-Tatché M, Johannes F. 2016. Genetic sources of population epigenomic variation. Nature *Reviews Genetics* **17**: 319-332.

Teschendorf AE, Relton CL. **2019**. Statistical and integrative system-level analysis of DNA methylation data. *Nature Reviews Genetics* **19**: 129–147.

Thorson JLM, Smithson M, Beck D, Sadler-Riggleman I, Nilsson E, Dybdahl M, Skinner MK. **2017**. Epigenetics and adaptive phenotypic variation between habitats in an asexual snail. *Scientific Reports*

7: 14139.

Tikhodeyev ON. **2018**. The mechanisms of epigenetic inheritance: how diverse are they? *Biological Reviews* **93**: 1987–2005.

Verhoeven KJF, Jansen JJ, van Dijk PJ, Biere A. **2010**. Stress-induced DNA methylation changes and their heritability in asexual dandelions. *New phytologist* **185**: 1108–18.

Wagner GP, Zhang J. **2011**. The pleiotropic structure of the genotype-phenotype map: the evolvability of complex organisms. *Nature reviews. Genetics* **12**: 204–13.

Walker J, Gao H, Zhang J, Aldridge B, Vickers M, Higgins JD, Feng X. **2018**. Sexual-lineage-specific DNA methylation regulates meiosis in *Arabidopsis*. *Nature Genetics* **50**: 130–137.

Wang X, Moazed D. **2017**. DNA sequence-dependent epigenetic inheritance of gene silencing and histone H3K9 methylation. *Science* **356**: 88–91.

Wibowo A, Becker C, Durr J, Price J, Spaepen S, Hilton S, Putra H, Papareddy R, Saintain Q, Harvey S, *et al.* 2018. Partial maintenance of organ-specific epigenetic marks during plant asexual reproduction leads to heritable phenotypic variation. *Proceedings of the National Academy of Sciences* **115**: E9145–E9152.

Wittkopp PJ, Haerum BK, Clark AG. **2008**. Regulatory changes underlying expression differences within and between *Drosophila* species. *Nature Genetics* **40**: 346–350.

Wogan GOU, Yuan ML, Mahler DL, Wang IJ. **2020**. Genome-wide epigenetic isolation by environment in a widespread Anolis lizard. *Molecular Ecology* **29**: 40–55.

Wright S. **1948**. On the Roles of Directed and Random Changes in Gene Frequency in the Genetics of Populations. *Evolution* **2**: 279.

Xu J, Chen G, Hermanson PJ, Xu Q, Sun C, Chen W, Kan Q, Li M, Crisp PA, Yan J, *et al.* 2019. Population-level analysis reveals the widespread occurrence and phenotypic consequence of DNA methylation variation not tagged by genetic variation in maize. *Genome Biology* **20**: 1–16.

Yagound B, Smith NMA, Buchmann G, Oldroyd BP, Remnant EJ. **2019**. Unique DNA Methylation Profiles Are Associated with cis-Variation in Honey Bees. *Genome Biology and Evolution* **11**: 2517–2530.

Zhang H, Lang Z, Zhu JK. **2018**. Dynamics and function of DNA methylation in plants. *Nature Reviews Molecular Cell Biology* **19**: 489–506.

Zhao P, Ma B, Cai C, Xu J. 2022. Transcriptome and methylome changes in two contrasting mungbean genotypes in response to drought stress. *BMC Genomics* **23**: 80.

Zhu JK. **2016**. Abiotic Stress Signaling and Responses in Plants. *Cell* **167**: 313–324.

Supporting files

Fig S1. Number of reads sequenced and aligned against the *F. vesca* genome in each sample.

Fig. S2. Sequence duplication levels for each of the individuals subjected to whole genome bisulfite sequencing.

Fig. S3. Proportion of cytosines that were methylated across the genome, within each sample, and for each sequence context.

Fig. S4. Proportion genetic variance explained by each of 17 principal components.

Fig. S5. Background genetic structure represented by the two first principal components generated by PCadapt.

Fig. S6. Proportion of PCadapt outliers (from full dataset) that were detected consistently across 20 leave-one-out subsets.

Fig. S7. Proportion genetic-epigenetic covariance explained by each of 19 coinertia axes.

Fig. S8. Biplot of consistent outlier SNPs (red dots) along the two major RDA axes representing adaptive genomic variation explained by adaptive traits.

Fig. S9. Relationships between pairwise SNP-DMC covariation and differential DMC methylation associated with the allelic composition of the respective SNP.

Methods S1. Bio-informatics and molecular data generation.

Table S1. Geographical and treatment information of the 20 samples.

Table S2. Methylation percentage for all differentially methylated cytosines (DMCs) in each of 20 samples.

Table S3. All single nucleotide polymorphisms (SNPs) for each of 20 samples.

Table S4. Leave-one-out (LOO) SNP datasets and associated Pcadapt outlier Q-values.

Table S5. DMC characteristics.

Table S6. SNP characteristics.

Table S7. Gene ontology (GO) information associated with annotated SNPs and DMCs.

Table S8. Gene ontology enrichment results.

Table S9. Mixed model outcomes and residual distributions.

Table S10. Trait values for sister plants of the 20 samples subjected to WGBS.

Table S11. SNP-DMC pairs with covariation coefficients > 0.9.

Table S12. Results of redundancy analysis on traits.

Table S13. Allele frequency and heterozygosity of each consistent outlier in each population.

Table 1. Mixed model results.

SNP-DMC covariation variables (**Fig. 3**)			DMC signature associated with SNP-DMC covariation (**Fig. 4**)		
	F	R^2		F	R^2
Genomic distance (Log10)	5.70*	18.55/ 73.18	Δ methylation frequency [1]	0.58	10.07/ 82.76
Selection (outlier vs. backgr. SNPs)	21.25***		**Δ methylation variability [2]**	851***	
DMC (drought vs inherited)	56.61***		DMC (drought vs inherited)	60.53***	
DMC clustering (sqrt)	19.12***		[1] × DMC type	41.80***	
Selection × DMC clustering	65.91***		**[2] × DMC type**	523***	

The left model tested which SNP (single nucleotide polymorphism) and DMC (differentially methylated cytosine) features explain SNP-DMC covariation in *Fragaria vesca*. The right model tested whether high SNP-DMC covariation results in differential methylation frequency rather than differential methylation variability. The table provides ANOVA F-values with asterisks representing significance (* for $p<0.05$ and *** for $p<0.001$), and R^2 showing marginal (fixed effects only) and conditional (full model) explained variance (%), respectively. The strongest effects are in bold. See **Table S9** for model details (e.g. degrees of freedom), and **Figs. 3** and **4** for visualization of patterns.

Fig. 1. Genome-wide distribution of genetic (A) and methylation (B) polymorphisms along a fine spatial gradient in *Fragaria vesca*. Differential methylation represents the absolute difference in methylation between the two populations with the highest methylation difference. The blue SNPs are outliers with consistent signatures of selection along a series of "leave-one-out" analyses. Alternate grey-colors represent the seven chromosomes. The two most extreme flowering SNPs are located in FvH4_3g08390 (ATVOZ1,VOZ1: positive regulation of long-day photoperiodism), and FvH4_6g07870 (VIP5: vernalization, DNA binding), but do not show consistent signatures of selection along the series of "leave-one-out" analyses. The two flowering DMCs are located in FvH4_4g19750 (PhyB: red/far-red light photoreceptor).

Fig. 2. SNP-DMC covariation patterns along two coinertia axes (A-E) and between inherited vs. drought-induced DMCs (F) in *Fragaria vesca*. The coinertia patterns (panels C-E) are based on a multivariate analysis aiming to reveal covariation between two multivariate matrices, including a DMC methylation frequency matrix (epigenetic population structure depicted in panel A), and a SNP allele composition matrix (genetic population structure depicted in panel B). Population genetic structure is higher than population epigenetic structure, pointing to strong genetic relatedness and the influence of environmental heterogeneity on epigenetic signatures. Panel C shows population structure based on SNP-DMC covariation. Here, the transparent dots represent DMC patterns, while the darker dots represent SNP patterns. The red dots in panels D and E represent drought stress-induced DMCs, and frequently co-vary with many SNPs (close to grey dots along both coinertia axes). The orange and green dots represent inherited DMCs and tend to cluster together as expected for DMCs defined along the same elevation gradient (i.e. part will have increased methylation at high elevation, while part will have decreased at high elevation, and vice versa for low elevation). Boxplots

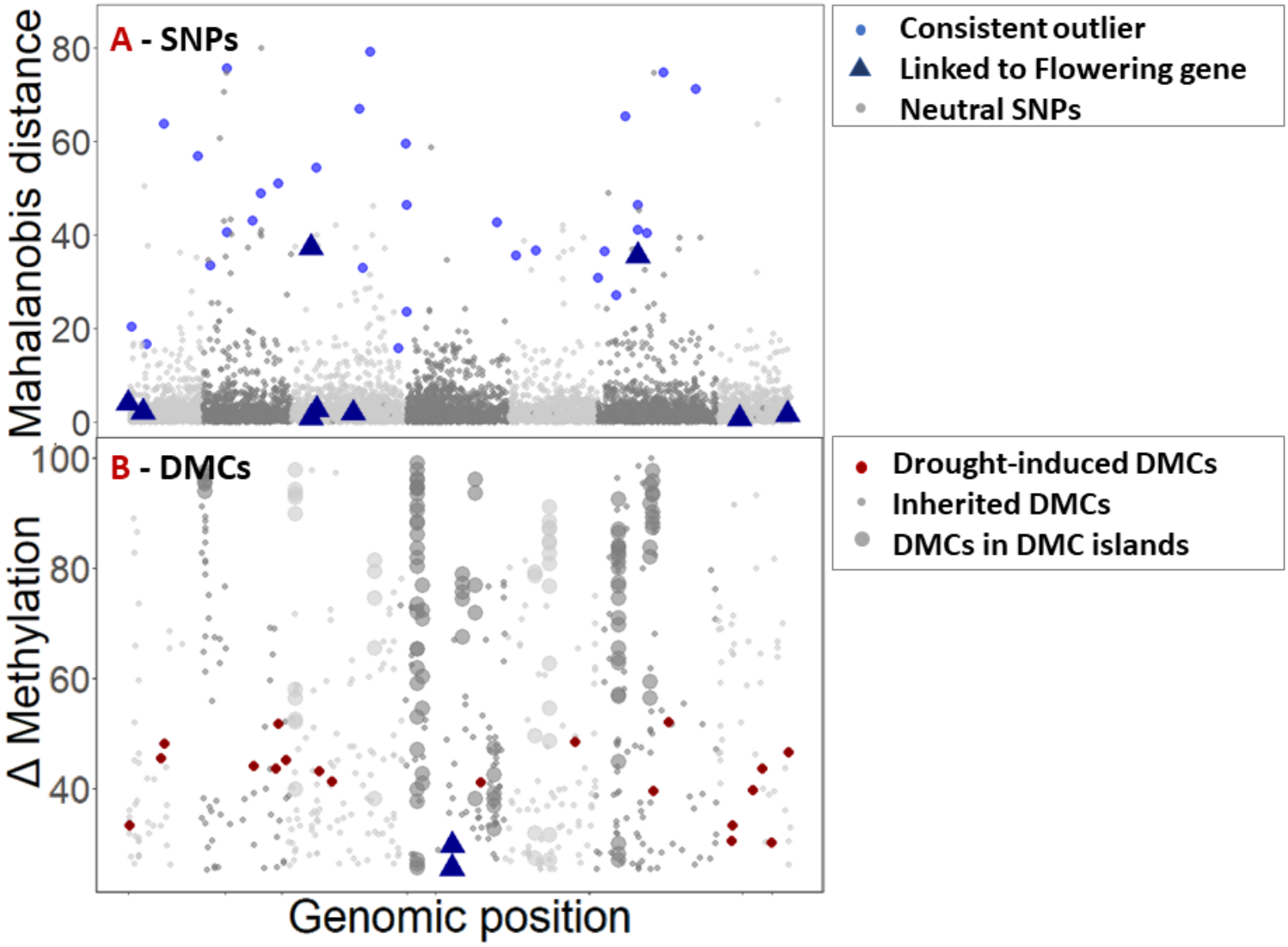
(panel F) show medians with interquartile ranges (boxes), data ranges (whiskers) and outliers (small grey dots).

Fig. 3. The amount and distribution of covariation between pairs of SNPs and DMCs across the *Fragaria vesca* genome, depending on DMC features (drought-induced vs. inherited: A, DMC clustering: B), and on SNP features (background vs. outlier SNPs: B and C). Marginal covariation was used to exclude pseudoreplication of shared SNPs and DMC islands across SNP-DMC pairs. DMC clustering (panel B) varied from 1 to 29 DMCs per cluster (Table S5); clusters of at least five DMCs within blocks of 1 kb were considered DMC islands. DMC islands typically have lower co-variation with SNPs than solitary DMCs (panel B), and outlier SNPs typically have lower co-variation with DMCs than neutral SNPs (panels B and C). Boxplots (panel B) show medians with interquartile ranges (boxes), data ranges (whiskers) and outliers (small grey dots).

Fig. 4. Relationships between pairwise SNP-DMC covariation and differential DMC methylation associated with the allelic composition of the respective SNP in *Fragaria vesca.* Differential methylation is represented by methylation variability (panel A) and frequency (B). SNP-DMC covariation is positively correlated with methylation patterns only for methylation variability at inherited DMCs (see Fig. S9 for relationships for drought vs. inherited DMCs separately). The full and dashed line represent a significant ($p<0.05$) and insignificant ($p>0.05$) correlation, respectively.

Fig. 5. The physical and functional aspects of SNP-DMC covariation in *Fragaria vesca,* including the genomic distance between co-varying SNPs and DMCs (A), the difference in SNP-DMC covariation between SNP (B) and DMC (C) gene ontology categories, and the Pearson correlation between SNP vs. DMC GO categories in terms of SNP-DMC covariation (D). The dashed red line in (B) and (C) represents the average SNP-DMC covariation across all possible SNP-DMC pairs. Boxplots (panel B) show medians with interquartile ranges (boxes), data ranges (whiskers) and outliers (small grey dots). DMCs in reproductive genes are featured by particularly low covariation with SNPs (panels C and D).

Fig. 6. Relationship between DMCs (differentially methylated cytosines, red dots) and predictors (arrows: traits, elevation and treatment) for each of 19 *Fragaria vesca* genotypes with trait information (Table S1). Length of arrows represent strength of relationship with predictors. Small red dots depict DMCs. Redundancy axes represent the epigenetic variation explained by the predictor variables (e.g. RDA1 contains most of the DMC variation explained by the predictors). DMCs that do not co-vary with any SNP (panel B) are associated with traits to a larger extent than DMCs co-varying with at least five SNPs (panel A).



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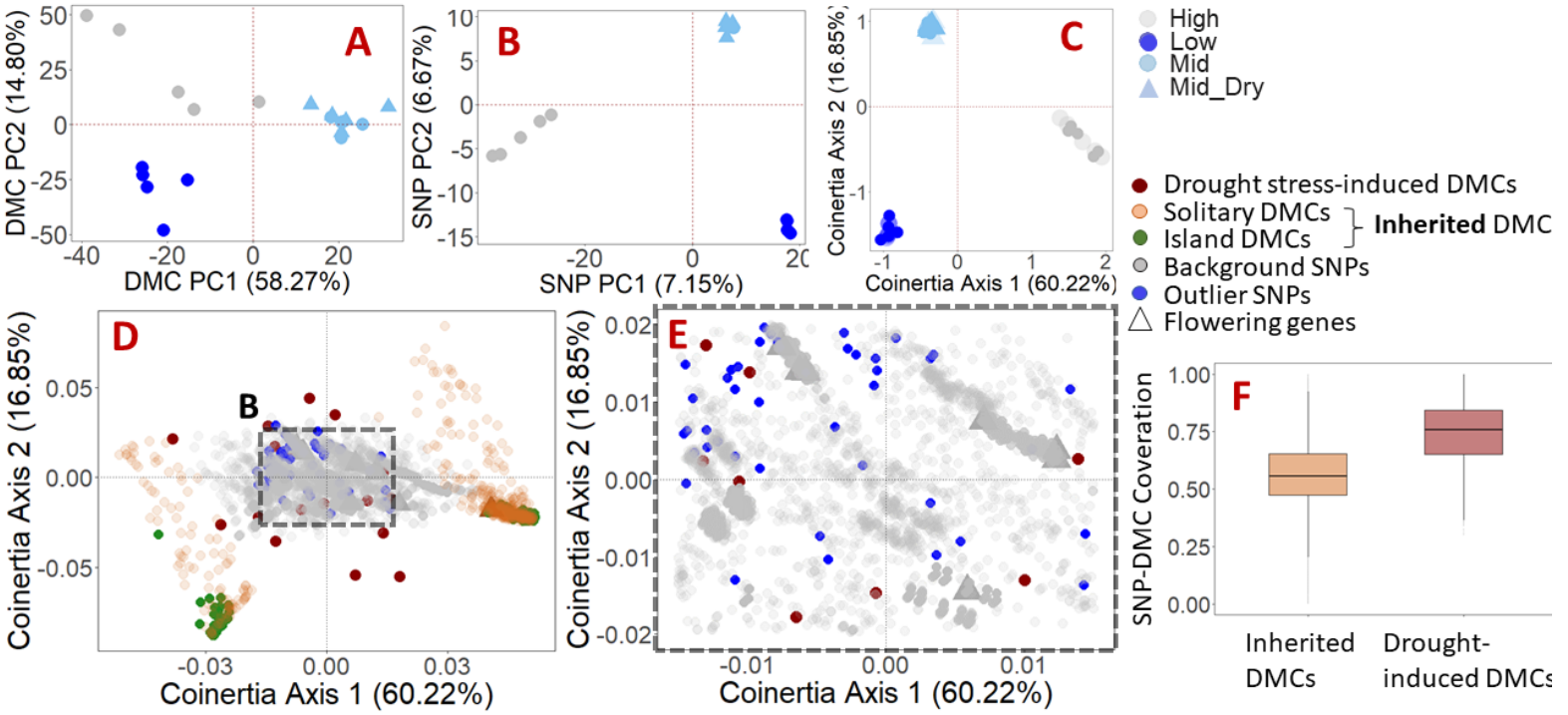

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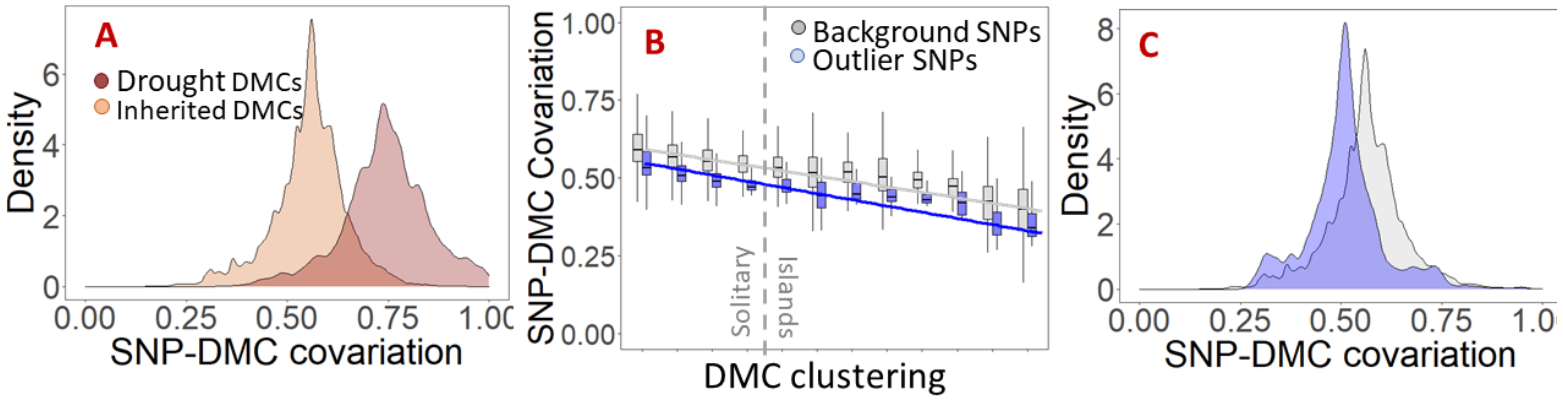

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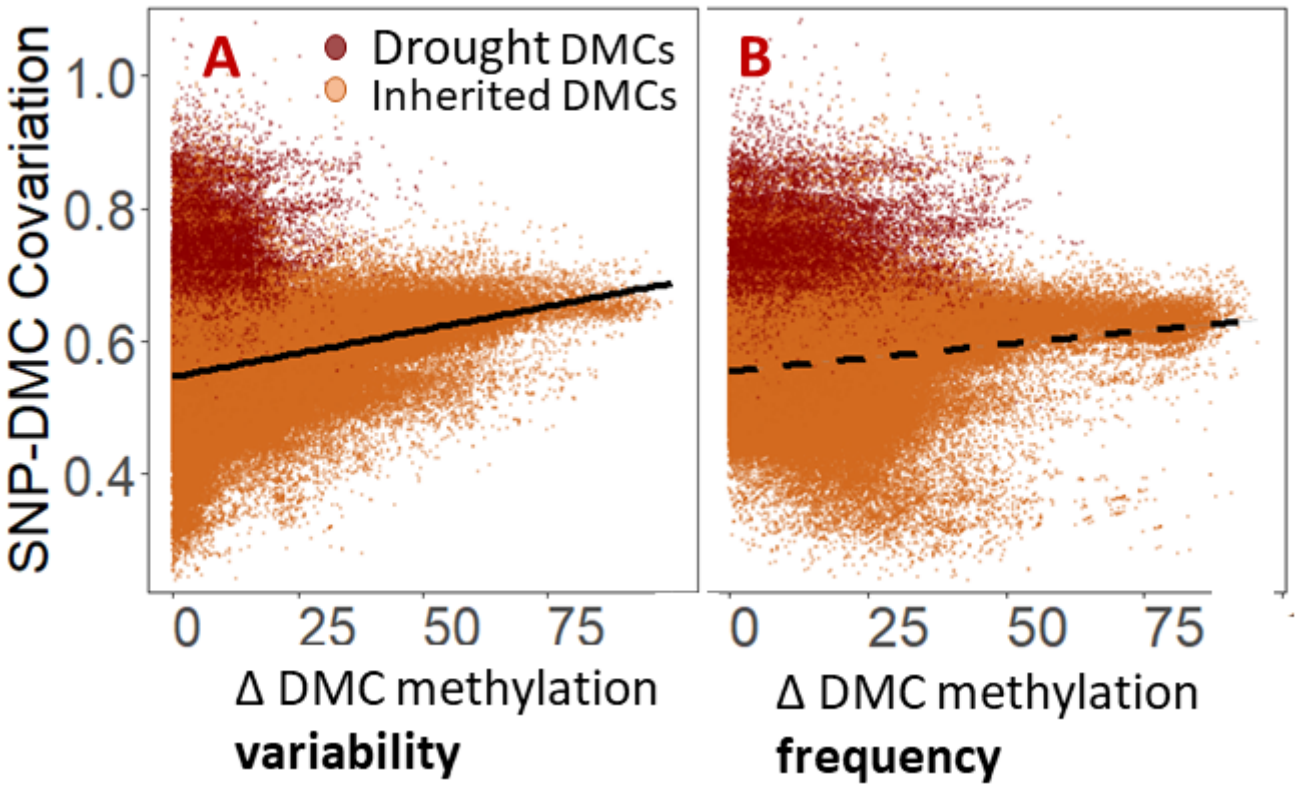

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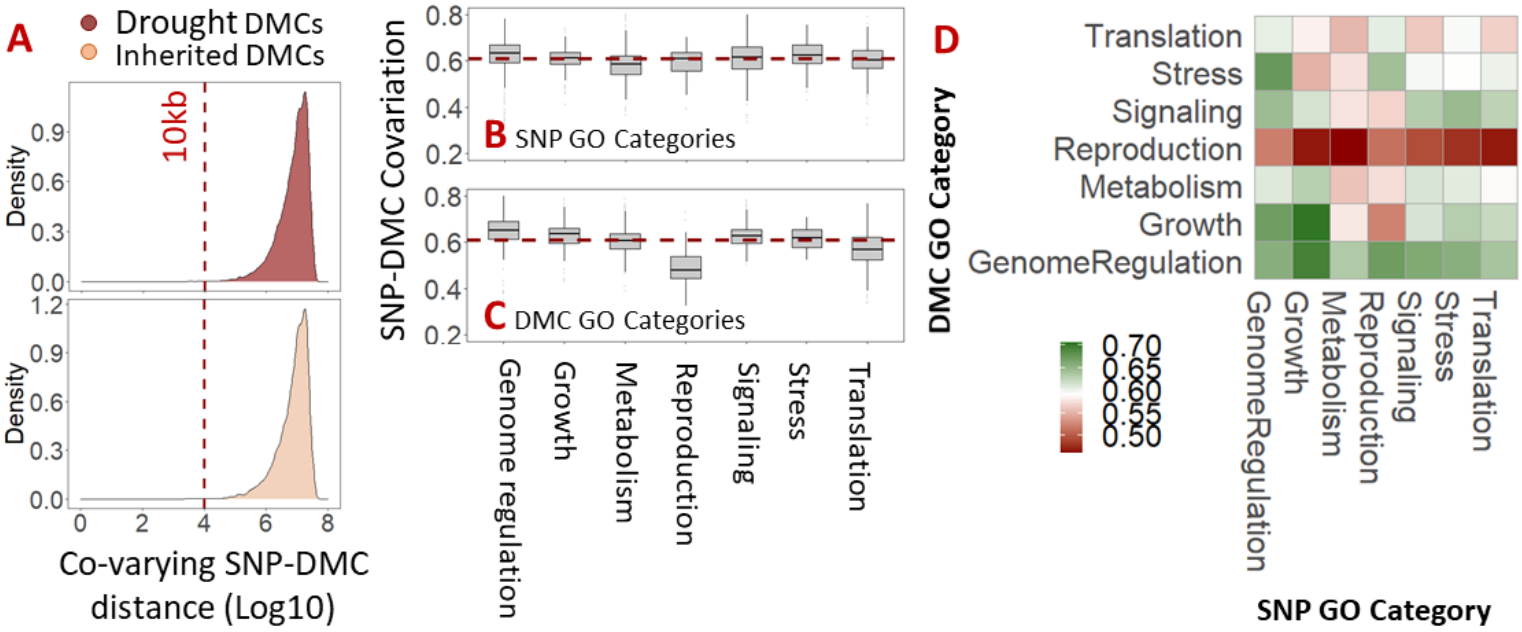

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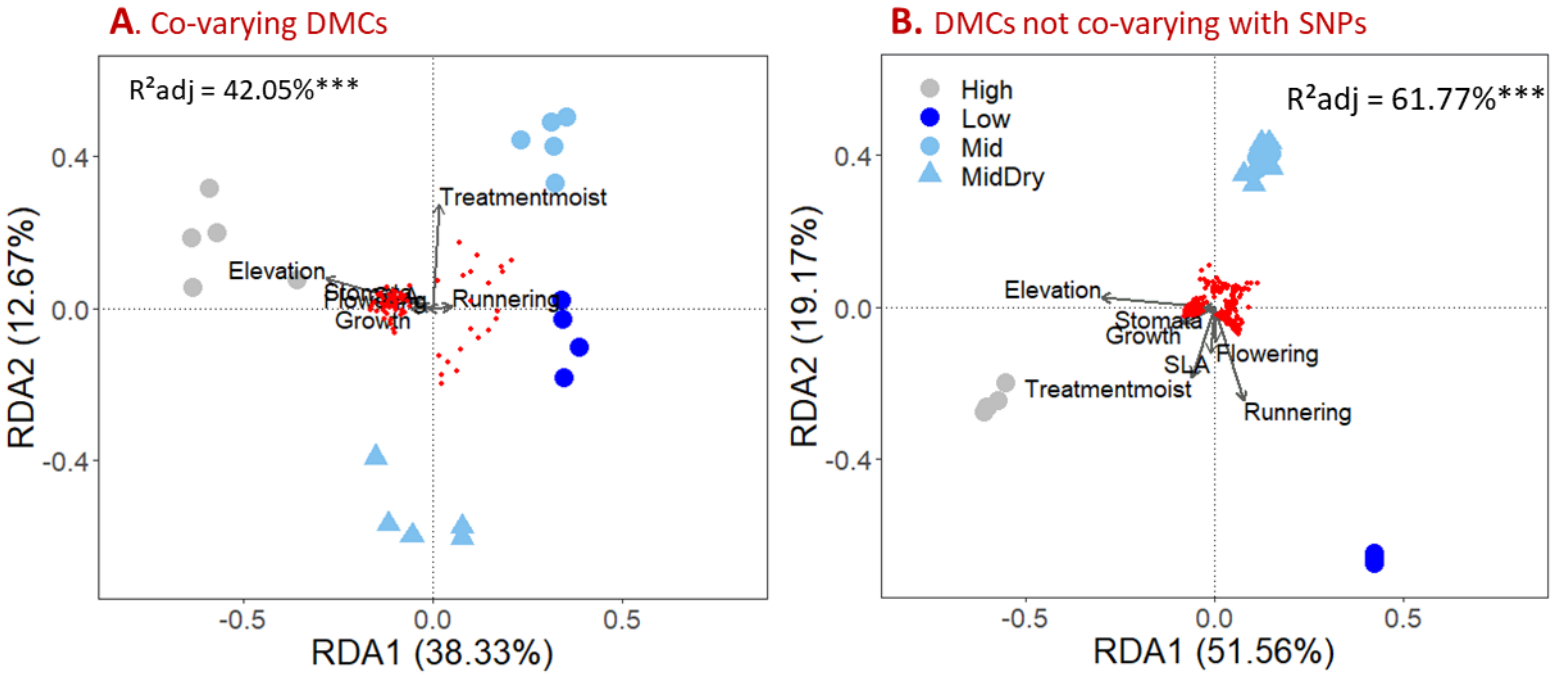

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