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**Linking DNA methylation to genetic differentiation in Timema cristinae stick insects**

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**Abstract**

Understanding speciation is a fundamental goal in evolutionary biology. Genomic regions of accentuated differentiation among populations often reveal patterns and mechanisms of species formation. While substantial progress has been achieved on this front for genetic variation, the contribution of epigenetic mechanisms to divergence patterns remains unclear. Here, we present evidence that DNA methylation is associated with regions exhibiting accentuated genetic differentiation between populations of *Timema cristinae* stick insects. We do so by integrating analyses of differentially methylated regions (DMRs) between individuals from different host-plant species with genomic sequencing. Our results reveal that DMRs exhibit accentuated genetic differentiation (*FST*) between populations. Strikingly, the strength of this association increases with the geographical distance between populations. We present results evaluating the contributions of mutation, reduced recombination, gene flow and selection to these divergence patterns. The overall results are consistent with a role for a balance between selection and gene flow, a finding further supported by evidence for selection in a previously-published survival field experiment. Nevertheless, details of our results suggest that selection on DMRs might be indirect and not strictly host-related. Our results establish associations between methylation and genetic change, but further work is required to clarify the exact causes of this association. Nonetheless, our results provide insight into how the interplay of epigenetic and genetic variation may influence population divergence and potentially contribute to speciation.

**1. Introduction**

Understanding the mechanisms underlying the origin of species is a central goal of evolutionary biology (Coyne & Orr, 2004; Darwin, 1859; Dobzhansky, 1937; Seehausen et al., 2014). The speciation process involves the accumulation of phenotypic and genetic differences that lead to population divergence alongside the development of reproductive isolation between populations (Mayr, 1963; Schluter, 2001). Researchers have thus often studied speciation through investigating the patterns and mechanisms that underpin the accumulation of genetic differences (*i.e.,* genetic differentiation) among populations (Coyne & Orr, 2004; Seehausen et al., 2014). The understanding of population divergence and the processes underlying it has greatly improved with the advent of next-generation sequencing technologies, enabling the use of genome scans across diverse taxa (Fraser & Whiting, 2020; Lotterhos & Whitlock, 2014; Wolf & Ellegren, 2017). In this context, genome scans have become a powerful tool to understanding population divergence and speciation.

Genome scan studies analyze patterns of genetic variation across the genome to identify regions with pronounced differentiation among populations, often measured by metrics like the fixation index (*FST*). These studies are based in the principle that differing levels of selection and gene flow can leave characteristic patterns across the genome. High gene flow typically homogenizes the genetic differences between populations, while selection on locally adapted traits is expected to increase genetic differentiation for loci associated with these traits. Consequently, the interplay of selection and gene flow between population pairs can produce a heterogeneous landscape of genetic differentiation across the genome, often visualized as ‘peaks’ and ‘valleys’ of *FST* (Hohenlohe et al., 2012; Via & West, 2008; Wolf & Ellegren, 2017). Thus, selection drives *FST* peaks by limiting introgression in specific genomic regions, while gene flow reduces genetic differentiation, and creates valleys. Nevertheless, it is important to note that *FST* peaks can also arise from other processes, such as background selection (Cruickshank & Hahn, 2014).

Numerous studies have identified genomic regions with accentuated *FST* between populations, illustrating how genetic differentiation can spread gradually or abruptly across the genome as populations diverge (Burri et al., 2015; Marques et al., 2018; Nadeau et al., 2012; Nosil et al., 2021; Poelstra et al., 2014; Turbek et al., 2021). For example, Poelstra et al., (2014) used genome scans to study divergence between two crow species: Corvus corone and Corvus cornix. By identifying genomic peaks of FST, they found regions under selection associated with plumage differences, which were maintained despite gene flow elsewhere in the genome. While these findings have helped to advance our understanding of speciation, a focus solely on genetic variation may overlook additional complexity in the evolutionary processes driving population divergence (Pfennig et al., 2010). In this context, investigating mechanisms of gene regulation alongside genetic variation could provide key insights into the patterns and processes underlying differentiation.

Gene regulation plays a pivotal role in driving phenotypic diversity and influencing traits that are key to ecological adaptation (King & Wilson, 1975; Lafuente & Beldade, 2019; Prager & Wilson, 1975; Prud’homme et al., 2007). Within this regulatory landscape, epigenetic mechanisms might add an additional layer to influence gene expression and thereby contribute to phenotypic and genetic differentiation, population divergence, and ultimately speciation (Pavey et al., 2010; Smith & Ritchie, 2013). However, the extent to which epigenetic mechanisms are associated with evolutionary divergence remains largely unexplored. In this study, we address this gap by investigating epigenetic variation in diverging populations of a stick insect, providing a complementary perspective on the role of genetic and epigenetic mechanisms in population divergence.

DNA methylation is the most extensively studied epigenetic mechanism in ecological and evolutionary studies, and involves the addition of a methyl group to the fifth carbon of a cytosine. Although DNA methylation patterns are typically determined by the genetic background (Dubin et al., 2015; Richards, 2006; Sepers et al., 2023; Taudt et al., 2016), they are also dynamic, frequently changing due to occasional errors in its several maintenance pathways, and in response to environmental changes. By responding to the environment, DNA methylation can serve as a molecular mechanism mediating phenotypic plasticity (Duncan et al., 2014; Hu & Barrett, 2023; Husby, 2022). Consequently, variation in DNA methylation and its phenotypic outcomes can arise not only from genetic factors, but also through environmental variation or genotype-environment interactions (*i.e.,* G × E) (Adrian-Kalchhauser et al., 2020; Richards, 2006). Moreover, DNA methylation can also be mutagenic and generate genetic polymorphisms. This is because methylated cytosines are more prone to transition into thymines (*e.g.,* C/T) than any other substitution (Holliday & Grigg, 1993; Ossowski et al., 2010; Tomkova & Schuster-Böckler, 2018), potentially contributing directly to genetic variation and differentiation. Notably, DNA methylation can also be transmitted across one or more generations in diverse taxa, including flowering plants (Schmid et al., 2018; van der Graaf et al., 2015), fish (Heckwolf et al., 2020; Kelley et al., 2021), mammals (Fitz-James & Cavalli, 2022; Skvortsova et al., 2018), and insects (Wang et al., 2016; Yagound et al., 2020). Taken together, DNA methylation can influence phenotypic and genetic variation and thus evolution on different timescales, spanning a continuum from transient acclimation effects to genetic evolution (Laine et al., 2022; Venney et al., 2023).

Building on these DNA methylation properties, we here investigated if DNA methylation is associated with the accumulation of genetic differences during population divergence. For example, selection on phenotypes influenced by DNA methylation could indirectly affect genetic variation through linkage disequilibrium between epigenetic and genetic loci. As a result, when advantageous traits associated with DNA methylation are selected, genetic variants in LD with these methylation loci (e.g., within the same chromosomal region) are more likely to be passed on to the next generation (Planidin et al., 2022; Platt et al., 2015). Conversely, selection acting on genetic variants could exert direct or indirect effects on DNA methylation patterns (*i.e.,* by controlling DNA methylation variation or via LD, respectively). Both pathways could lead to accentuated genetic differentiation in genomic regions where DNA methylation patterns differ between populations. Other processes could also cause such a pattern. For example, the mutagenic effects of DNA methylation could itself lead to the accentuated genetic differentiation among populations by increasing genetic polymorphism. In each of these cases, we might expect an association between DNA methylation patterns and accentuated genetic differentiation (*FST*) among populations, a pattern we tested for here using *Timema cristinae* stick insects (Vickery, 1993).

*Timema cristinae* are wingless, plant-feeding insects primarily found in two host-plant species: *Adenostoma fasciculatum* (Rosaceae), and *Ceanothus spinosus* (Rhamnaceae) (Nosil & Crespi, 2006; Sandoval, 1994). Divergent selection associated with the two host-plant species contributes to partial reproductive isolation between the populations associated with them, resulting in distinct *Adenostoma* and *Ceanothus* ecotypes (Nosil, 2007). A previous study has shown that genome-wide differences in *T. cristinae* DNA methylation are strongly positively correlated with genetic distances among populations, but also exhibit moderate host-plant effects (de Carvalho et al., 2023). Importantly, that study has used methylome-environment association studies to designate genomic regions that are differentially methylated between host-plant ecotypes (*i.e.,* differentially methylated regions, DMRs). However, such previous work has primarily focused on genome-wide comparisons between genetic and epigenetic variation, leaving unexplored how genetic differentiation relates to population-level differences in DNA methylation in *T. cristinae*.

In this context, we here combined previously published methylome and whole-genome data from natural and experimental *T. cristinae* populations*.* First, we tested the hypothesis that differential DNA methylation is more pronounced in genomic regions with accentuated *FST* among populations. To this end, we leveraged whole-genome sequencing data from populations with varying degrees of divergence (Soria-Carrasco et al., 2014)⁠ to test if DMRs are enriched in regions of accentuated *FST*. Having found such a pattern, we subsequently tested different hypotheses of processes to explain it. We explored how a balance between selection and gene flow, along with the roles of mutation and reduced recombination, can explain these divergence patterns. Our approach of investigating the relationship between DNA methylation and genetic variation thus explores less-studied (*i.e.,* epigenetic) mechanisms associated with genetic differentiation during speciation.

**2. Material and Methods**

*2.1. Differentially methylated regions between host-plant ecotypes*

Here, we used data from de Carvalho et al. (2023), which examined DNA methylation differences between host-plant ecotypes of *T. cristinae*. This previous study conducted whole-genome bisulfite sequencing on samples from 12 host-associated populations across nine geographic localities. The sampled localities were all within the patchy and heterogeneous chaparral landscape, the natural habitat of *T. cristinae*. The sampling included four localities dominated by *Adenostoma,* two by *Ceanothus*, and three ‘parapatric’ sites where the two host-plant species coexist in close proximity. The populations varied not only by the host-plant ecotype, but also by geographical distances and climatic variables estimated using the WorldClim database (Harris et al., 2014). Two similarly-sized female specimens were used from each population, resulting in a total of 24 samples. The analysis pipeline involved mapping the methylation data to the reference genome v.1.3c2 (Nosil et al., 2018), and using whole-genome sequencing data (Soria-Carrasco et al., 2014) to minimize confounding single nucleotide polymorphisms (SNP) that could affect methylation calls. Specifically, C/T and G/A SNPs were removed to improve data accuracy (see de Carvalho et al., 2023 for details).

In the present study, we used differentially methylated genomic regions (DMRs) between host-plant ecotypes identified by de Carvalho et al. (2023). These DMRs were estimated through a methylome-environment (host plant) association analysis using binomial mixed models (MACAU v1.0.0; Lea et al., 2015)⁠. This analysis was applied to 1kbp non-overlapping tiling windows on the methylation data across the genome, requiring a minimum coverage of 10 reads per window (see de Carvalho et al. for details on filtering criteria). The DMRs were designated by examining the tail of the empirical *p-value* distribution generated by MACAU, allowing the properties of DMRs to be assessed across various threshold cut-offs. Following de Carvalho et al. (2023), we here reported results based on empirical *p-value* percentiles, specifically from the 0.04th to the 0.4th percentile (corresponding *p-values* from 0.0004 to 0.0061), resulting in DMR counts ranging from 25 to 258 DMRs. In more downstream analyses, we focus mainly on DMRs delimited by the *p-value* < 0.0004, which balances stringency and pattern robustness.

In their study, de Carvalho et al. (2023) reported three main patterns across the range of *p-value* thresholds for DMR designation. First, that DMRs were distributed genome-wide across different linkage groups, with the majority (~ two-thirds) located within genes. This trend is consistent with the genome-wide distribution of methylation tiles, which also had around two-thirds located within genes. Second, that methylation distances in DMRs were more closely associated with host-plant ecotype than with genetic, geographical or climatic differences. Such an ecotype-specific pattern for DMRs contrasted with genome-wide trends in methylation differences, which were mostly explained by geographical distance. Third, DMRs were functionally enriched for processes related to protein metabolism and membrane processes, particularly signal transduction and transmembrane transport. This characterization of DMRs provides a foundation for understanding the association between genetic and epigenetic differentiation, bridging previous findings with the present study's focus.

*2.2. Overlap between DMRs and genetic regions with accentuated* FST *values*

We began our investigation by testing the hypothesis that DNA methylation is linked to the accumulation of genetic differences during population divergence. Specifically, we predicted that DMRs would co-occur with genomic regions showing accentuated *FST* between diverging populations. To test this, we quantified the *FST* values across the genome and focused on the extreme tails of the *FST* distribution. We defined accentuated *FST* as the 5% values at the upper tail of the distribution. We then examined whether these regions of extreme *FST* values overlapped (*i.e.,* co-occurred) with DMRs more than expected by chance – which would indicate an enrichment of accentuated *FST* in DMRs.

For this analysis, we used whole-genome sequencing data previously published by Soria-Carrasco et al. (2014), comprising 160 specimens from eight populations (Fig. 1). We first estimated pairwise genetic differences between each population pair (n=28 comparisons) within the same 1kbp methylation tiling windows used in the methylome-environment association analysis. We relied on maximum-likelihood allele-frequency estimates for 3,252,350 SNPs from the whole-genome data (see (Lucek et al., 2019)⁠ for details on DNA sequencing, alignment, variant calling and allele-frequency estimation). Using the SNPs located within the methylation tiles, we estimated mean heterozygosity (*HS*) and *FST* between each population pair. *Hs* was estimated as follows:

where *p1* and *p2* represent the non-reference allele frequencies in the populations 1 and 2, respectively, with the sum divided by the number of loci (*L*) within the methylation tiles. Multilocus *FST* was calculated as:

where *HS*is the mean heterozygosity within populations, and *HT*  is the total heterozygosity across each population pair. The 95% confidence intervals for multilocus *FST* estimates were obtained from 1,000 bootstrap replicates where individual DMRs were sampled with replacement. We then evaluated how *FST* varied according to different *p-value* thresholds for DMR designation, using cut-offs ranging from the 0.4th to the 0.04th percentile of *p-values* (0.0004 to 0.0061).

To test the prediction that DMRs co-occur with genomic regions exhibiting accentuated *FST* among populations, we used two analytical approaches, one focused on individual pairs of populations and one on average patterns across pairs of populations. First, for each population pair, we conducted randomization tests to assess whether DMRs were significantly enriched in accentuated *FST* (the top 5% values for each pair) compared to a null model. Observed *FST* values were compared to the null distributions generated by randomly sampling 1kbp methylation tiling windows 1,000 times, with the number of sampled tiles varying according to *p-value* stringency used to designate DMRs.

The second approach consisted in calculating the average *FST* across the 28 population pairs, and assessing whether the proportion of DMRs associated with accentuated *FST* was higher than expected by chance. For this, we focused on DMRs delimited by the *P* <0.0004 (the 0.04th percentile of the *p-value* empirical distribution). Accentuated *FST* was defined as the top 5% values of mean *FST* across population pairs. We estimated the proportion of DMRs displaying these accentuated *FST* values and tested the significance of the overlap between high *FST* regions and DMRs through randomization, permuting *FST* values across loci 1,000 times. These two approaches provided some initial evidence supporting the prediction that DMRs are associated with regions of accentuated *FST* between populations. As such, we subsequently explored the processes that could be underlying such a pattern.

*2.3.Geographical patterns of* FST *enrichment in DMRs*

Building on the initial evidence showing DMRs are enriched in regions with accentuated *FST*, we examined whether this association follows a geographical pattern. Specifically, we investigated whether the enrichment for accentuated *FST* in DMRs increases with greater geographical distance between populations. Populations experiencing varying levels of geographical isolation tend to show differing gene flow effects on genetic differentiation (Fig. 2), a pattern documented in *T. cristinae* (Lindtke et al., 2017; Nosil et al., 2012; Nosil & Sandoval, 2008)⁠. Since high gene flow tends to constrain genetic differentiation, DMRs could be expected to show more accentuated *FST* values with greater geographical isolation. This analysis would offer further insight into the patterns underlying enrichment of accentuated *FST* in DMRs.

To evaluate this, we estimated the degree of *FST* enrichment in DMRs (delimited by *P <* 0.0004). Specifically, for each population pair, we estimated the mean *FST* within DMRs and divided by the mean of a null distribution, calculated using permutation tests as described above (*i.e.,* ‘x-fold difference’). Subsequently, we assessed the regression between the degree of *FST* enrichment in DMRs and geographical distances between each population pair. The geographical distances were estimated based on the geodesic distance between each population pair then logarithmically transformed. We then estiamted the association between the degree of *FST* enrichment in DMRs and geographical distances using a Mantel test with 10,000 permutations using the R package *vegan* v2.6-6.1 (Oksanen et al., 2022). We performed this step three times: across all DMRs, using only genic DMRs, and using only non-genic DMRs.

Our analyses revealed that the degree of *FST* enrichment in DMRs gets more accentuated with increased geographical distances, representing a key finding of our study (see Results). This pattern suggests a balance between selection and gene flow influencing DMRs, either directly or indirectly. To explore this, in the following sections we tested the hypothesis that a selection-gene flow balance drives *FST* enrichment in DMRs. Additionally, we evaluated two alternative but non-mutually exclusive hypotheses: reduced recombination and the potential mutational effects of methylation. Another possibility is that the enrichment reflects the proximity of DMRs to gene-dense regions, where natural selection and LD could influence *FST*. However, our analyses showed no significant differences in gene counts between DMRs and non-DMR methylation tiles in 20 kbp and 100 kbp genomic windows. The median difference in gene counts was 0 in both cases (*P =*0.999 and *P*=0.981, permutation tests). These results suggest that gene density cannot explain *FST*  enrichment in DMRs. Consequently, we excluded gene density as a factor and focused on the three primary hypotheses (Fig. 2).

*2.4. The selection-gene flow balance hypothesis: association with environmental factors*

The increasing enrichment of extreme *FST* in DMRs with greater geographical isolation may result from a drift-gene flow or a selection-gene-flow balance. Under a selection-gene flow balance, selection would counteract gene flow in specific regions, leading to accentuated *FST*  values in DMRs that become more pronounced with increasing isolation. Based on this, we hypothesized that the observed pattern is due to a selection-gene flow balance. To test this hypothesis, we first predicted that environmental factors, in addition to geographical distance, would be associated with the enrichment of *FST* within DMRs. Specifically, we examined the contributions of host-plant ecotype and climatic variables, both of which are potential drivers of selection. Host-plant ecotype, which is associated with DMRs, may also drive genetic differences within these regions through selective pressures on either epigenetic or genetic variation. Moreover, climate-associated loci in Timema are known to be under selection(Chaturvedi et al., 2022), suggesting that climatic variables could also play a role. Similar to DMRs, these climate-associated loci are distributed across the genome.

The eight populations from which we obtained *FST* data differ in host-plant species (four *Adenostoma* and four *Ceanothus* populations, Fig. 1). These populations are also exposed to different climatic conditions, as shown by principal component analyses of climatic data summarized across *T. cristnaie* populations in Nosil et al. (2018). The first two principal components explained around 92% of the variance (PC1=66.4%, PC2=25.5%). PC1 reflects annual temperatures, as well as temperatures during the coldest and wettest periods,and also captures temperature constancy across the seasons (e.g. isothermality, seasonality). PC2 primarily represents temperatures in the warmest month, and in the warmest and driest quarters (see also de Carvalho et al., 2023). Among the populations studied, LA, MR1A and MR1C are found in regions with highest precipitation indices, while R12A and R12C are located in drier regions and with higher isothermality. HVA, HVC and PRC are located in regions with highest temperatures during the warmest and driest quarters (Fig. S1). These climatic and host-plant variations were used to test the hypothesis that *FST* enrichment within DMRs is associated with these factors.

To test this prediction, we employed a Bayesian framework using Markov Chain Monte Carlo (MCMC) based analyses (Bayesian linear mixed models, BLMM; Clarke et al., 2002; Gompert, Lucas, et al., 2014)⁠. This model incorporated geographical, host-plant species and climatic distances to explain the log ratio of observed over expected *FST* values for loci within DMRs. Host-plant distances were coded as 0 (same host plant) or 1 (different host-plant species), while climatic variables were derived from WorldClim data (Harris et al., 2014), as summarized in (Nosil et al., 2018). We calculated standardized Euclidean distances using the PC1 and PC2 summarizing these climatic variables. Model fit was assessed using the deviance information criterion (DIC), implemented in the *rjags* R package (Plummer, 2018)⁠. The model was run in three parallel chains with 10,000 iterations, a burn-in of 2,000 iterations, and a thinning interval of 5. All analyses were performed using R v4.3.2 (R Core Team, 2023).

*2.5. The selection-gene flow balance hypothesis: allele-frequency changes in DMRs*

To further test the hypothesis that the enrichment of accentuated *FST* in DMRs results from a selection-gene flow balance, we used a previously published release-recapture survival field experiment that compared the genetic composition of surviving versus non-surviving individuals (Gompert, Comeault, et al., 2014)*.* In that study, 491 specimenswerecollected, tissue-sampled (allowing whole-genome sequencing), and then transplanted onto *Adenostoma* or *Ceanothus* bushes. Surviving individuals were recaptured eight days later, providing an opportunity to compare the genetic change of the released and surviving individuals (see (Gompert, Comeault, et al., 2014) for details). Prior work with this experiment observed changes in the phenotypic frequencies of heritable color-pattern morphs, along with allele-frequency shifts between release and recapture. These results along with more recent studies indicated that selection affected some genomic regions, either directly or indirectly via LD with such regions, although genetic drift might also have contributed to observed changes (Gompert, Comeault, et al., 2014; Gompert et al., 2022).

Using this data, we predicted that allele-frequency changes within DMRs would be more extreme than that observed for other regions across the genome (Fig. 2), consistent with DMRs being influenced by selection even if the specific targets of selection remain unknown. Although the experiment took place in a different year from the de Carvalho et al. (2023) methylation survey, we assumed that genetic variation remained relatively stable across years, due to a balance between selection, gene flow, and other evolutionary processes. This assumption is supported by previous studies in *T. cristinae* (Nosil et al., 2018, 2024; Riesch et al., 2017)⁠*.*

We estimated the allele-frequency changes from whole-genome sequencing data based on estimates from (Nosil et al., 2018). This data set comprised 6,175,495 SNPs sequenced across 491 individuals (Nosil et al., 2018). Allele-frequency changes were calculated as the difference between release and recapture allele frequencies, independent of host plant. A standardized measure of change, relative to initial genetic diversity, was calculated as:

(*1*)

where *p0* and *p1* represent allele frequencies before and after the selection event. We calculated the mean *Δp* for all SNPs within DMRs, and compared this to a null distribution generated from *Δp* values in SNPs within an equivalent number of random 1kbp methylation tiles (non-DMRs). We performed 1,000 randomizations to create this null distribution. We conducted these analyzes in DMRs designated by the *p-value* cut-off *P* < 0.0004. To evaluate the robustness of the observed patterns under more stringent criteria, we repeated the analyses using a stricter threshold of *P<*0.0001. We focused our attention on DMRs located within genes because they are the main target of methylation in *Timema,* but also in other insects (Bewick et al., 2017; Provataris et al., 2018), and because the results were not significant for non-genic DMRs (1.08x, *P=*0.388). We further excluded LG8 because it contains the strongest selection signals related to the genomic regions that affect color and color-pattern, which was not our focus in this study.

*2.6. The reduced recombination hypothesis*

Accentuated *FST* values in specific genomic regions can arise from processes beyond the selection-gene flow balance, such as background selection in regions of reduced recombination (Booker et al., 2020; Cruickshank & Hahn, 2014; Noor & Bennett, 2009)⁠ (Fig. 2). To explore this, we hypothesized that the enrichment of accentuated *FST* in DMRs is due to reduced recombination in these genomic regions. We tested this hypothesis using three approaches based on different proxies for reduced recombination: (1) overlap with structural variants, (2) low heterozygosity, and (3) high linkage disequilibrium.

First, we investigated whether DMRs overlap with structural variants (SVs) mapped in *Timema* (*i.e.*, gains or losses of DNA segments as well as chromosomal rearrangements) (Lucek et al., 2019)⁠. Structural variants are known to often suppress recombination rates significantly (Hoffmann & Rieseberg, 2008; Kirkpatrick, 2010; Noor et al., 2001)⁠. As such, our prediction was that DMRs would tend to co-occur with SVs. To test this prediction, we used the SV data from (Lucek et al., 2019)⁠, checking whether DMRs at various *p-value* cut-offs at least partially overlapped with these SVs using R (R Core Team, 2023). We repeated this operation across the different *p-value* cut-offs designating DMRs. We excluded all SVs that were located within unmapped scaffolds.

Next, we examined whether the DMRs with accentuated *FST* values displayed lower mean heterozygosity indices (*HS*) than the genomic background, as reduced recombination regions often exhibit low heterozygostity (Begun & Aquadro, 1992)⁠. As such, we predicted that DMRs would show lower heterozygosity than the genomic average. To test this prediction, we used the same proceedings applied with *FST*, drawing 1kbp random samples from the methylation tiles 1,000 times at varying *p-value* cutoffs.

Finally, we evaluated linkage disequilibrium (LD) levels within DMRs, as high LD levels relative to background may indicate reduced recombination. Thus, we predicted that LD within DMRs would be higher than the genome-wide average. To test this prediction, we used the whole-genome sequencing data (Lucek et al., 2019; Soria-Carrasco et al., 2014) for *FST* and *HS* estimation to compute LD among SNPs within the DMRs, defined by a P<0.0004 cut-off for each population. We used an empirical Bayesian approach to estimate genotypes for each individual and locus. Specifically, we extracted the genotype likelihoods from the *VCF file*, then we estimated genotypes based on a custom *Perl* script from Comeault et al. (2014)⁠. For each population, we calculated a correlation matrix of SNP genotypes within DMRs, which was then squared and averaged. To ensure robust estimates of LD (the average squared correlation in a tile), we included only methylation tiles with more than five SNPs. The LD distribution for DMRs was compared to a null distribution generated from random 1kbp windows within each population across 10,000 iterations to obtain *p-values*.

*2.7. The methylation mutagenic effects hypothesis*

Finally, we evaluated whether the mutagenic effects of DNA methylation is associated with the accentuated *FST v*alues in DMRs. These effects stem from the fact that methylated cytosines have a significantly higher likelihood of mutating into thymines compared to unmethylated cytosines, and this mutation occurs at rates much higher than other types of point mutations (Holliday & Grigg, 1993; Ossowski et al., 2010)⁠. These higher mutation rates could lead to accumulated genetic mutations (*i.e.,* thus accentuated *FST*) in regions with marked methylation differences between populations (*e.g.,* DMRs). This is a standing hypothesis that directly links DNA methylation levels with genetic variability (Guerrero-Bosagna, 2017)⁠, but it has been tested less often in natural populations (but see (Venney et al., 2024)). We thus here tested the prediction that the observed enrichment in accentuated *FST*in DMRs results from mutagenic effects of DNA methylation. If this was the case, we expected accentuated *FST* values in DMRs’ to be associated with CpG context genetic polymorphisms, specifically C/T transitions (or from G/A in the complementary strand; Fig. 2). In other words, in this context, differences in methylation could directly cause genetic differences between populations.

To test our prediction, we used whole-genome sequencing data from Soria-Carrasco et al. (2014) and estimated *FST* for each SNP, rather than for 1 kbp methylation windows as done previously. We focused on SNPs that were located within the DMRs delimited by *P* < 0.0004 (n = 283 SNPs). We then identified the type of genetic polymorphism (*i.e.*, C/T, A/T, etc.) of each SNP, and whether or not they were located in CpG context (*i.e.* likely to have emerged due to the methylation mutagenic effects). Note that SNPs in CpG context had been previously removed from the methylation tables to avoid spurious methylation calls (de Carvalho et al., 2023)*.*

To address the strong correlation observed between *FST* in SNPs within DMRs and their minor allele frequencies (MAF)(mean across 28 Spearman correlations: 0.68, all *P<* 0.001), we binned the *FST* values based on MAF for each SNPs within DMRs for each population pair using *VCFtools* v0.1.15. Across the 28 population pairs, at least 90% of the SNPs showed MAF ≤ 0.05 (mean 95.2% [92.3% – 98.1%; standard deviation]). Therefore, we focused on SNPs meeting this criterion for each comparison. Using permutation tests, we tested whether C/T or G/A SNPs in CpG context (‘mutation-bias SNPs’) exhibited higher *FST* values than these mutations in other sequence contexts. Finally, we evaluated the relationship between *FST* in mutation-bias SNPs and geographical distance using Mantel tests across 28 population comparisons, implemented in the R package *vegan* v2.6-6.1 (Oksanen et al., 2022).

**3. Results**

*3.1. Accentuated genetic differentiation between populations in DMRs*

We hypothesized that DNA methylation is associated with the accumulation of genetic differences during population divergence, predicting that DMRs would overlap with regions of accentuated *FST*. We found support for this prediction, as DMRs tended to show an enrichment in *FST* values relative to genome background levels (Fig. S2). Specifically, 15% of the DMRs exhibited accentuated *FST*, a proportion that is marginally higher than expected by chance when considering all populations together as equal (expected=5%; *P=*0.077; randomization test using mean *FST* across all comparisons; Fig. 3). When analyzed by individual population pairs, 4 out of 28 pairs (14% of the comparisons) exhibited an enrichment of accentuated *FST* in DMRs (expected=5%, *P<*0.050 for each of the four pairs; probability of 14% of the comparisons being significant: *P=*0.012). Additionally, in 24 out of 28 population pairs (86%), the DMRs exhibited *FST* values that exceeded the mean expectation under the null model (*P* < 0.001, binomial test; Fig. S2). Together, these findings provide some initial evidence that DMRs overlap with regions of accentuated *FST* among populations. We next examined whether the enrichment of pronounced *FST* values in DMRs followed a geographical pattern. As shown below, the accentuation of genetic differentiation in DMRs becomes stronger and more significant once spatial variation is accounted for.

*3.2.*  *DMRs exhibit more strongly accentuated* FST *with increasing geographical distance*

We observed a geographical pattern of *FST*enrichment in DMRs. Specifically, we found that the enrichment of accentuated *FST* values in DMRs increases significantly with greater geographical distance between population pairs (*R2*=0.58, *P*<0.001; Mantel test; Fig. 3). At the largest distances, *FST* values in DMRs are nearly three times higher (~3.0x) than those observed in the genome background. This geographic pattern remains consistent when only genic DMRs are analyzed (*R2*=0.57, *P=*0.001; Mantel test; Figs. S3,S4), showing an enrichment of ~3.0x compared to the background levels at largest distances. In contrast, analyses restricted to non-genic DMRs reveal no such geographical pattern of increased *FST* enrichment with greater geographical distance (*R2*=0.14; *P*=0.963; Mantel test), instead showing an enrichment of ~0.5x at the largest distances (Figs. S5,S6).

*3.3.*  *Geographical and climatic distances drive* FST *enrichment in DMRs, independent of host-plant ecotype*

We subsequently tested the hypothesis that the enrichment of accentuated *FST* in DMRs results from a selection-gene flow balance. To do so, we first tested the prediction that environmental factors (*e.g.,* host-plant ecotype and climate) would be associated with the enrichment of *FST* within DMRs, beyond its association with geographical distances. Our results with BLMM indicated that the model combining geographical and climatic distances was the one that best explained accentuated *FST* in DMRs. Geographical distances showed the largest and most credible effects, while climatic distances showed a more moderate effect (βGEOG=0.97 [0.64, 1.28; 95% ETPI] and βCLIM= -0.31 [-0.61, -0.02, 95% ETPI], the standardized regression coefficients for geographical and climatic distances, respectively; Table S1). These analyses further suggest that accentuated *FST* in DMRs is not associated with host-plant ecotype (Table S1; Figs. S7-S11).

*3.4.*  *DMRs exhibit marked allele-frequency changes in a survival field experiment*

To further test the selection-gene flow hypothesis, we examined a different prediction: that allele-frequency changes within DMRs in a field experiment would be more extreme than for other regions across the genome. Weakly consistent with this prediction, we found that the allele-frequency changes in DMRs (designated by a *P<*0.0004 cut-off, *i.e.*, the 0.04th quantile of the *p-value* distribution) were marginally higher than expected by chance (1.1x, *P=*0.091, randomization test). Applying a more stringent cut-off (*P<*0.0001) to designate DMRs revealed a slightly stronger and statistically significant genetic response relative to null expectations (1.2x, *P=*0.045, randomization test; Fig. 4). Although these effects are modest, they are noteworthy, given that this short-term experiment at a single site is unlikely to capture the full spectrum of selection pressures these organisms face. Additionally, we observed no significant allele-frequency change in DMRs when analyzing transplants between different host plant species alone (1.1x, *P=*0.212 in DMRs delimited by *P<*0.0004; 1.1x, *P=*0.303 in DMRs delimited by *P*<0.0001; randomization test).

*3.5.*  *DMRs do not exhibit significant evidence of reduced recombination*

To test the hypothesis that accentuated *FST* in DMRs is driven by reduced recombination, we employed three different approaches. First, we examined whether DMRs overlapped with SVs mapped in *Timema,* but found no overlap with any structural variants. Next, we tested if DMRs exhibited lower heterozygosity than expected by chance. Mean heterozygosity within DMRs did not significantly differ from genome-wide expectations in any comparison (all *P* > 0.05, randomization tests). Across the 28 population pairs and 10 p-value cut-offs, mean enrichment of heterozygosity in DMRs ranged from 0.92x to 1.04x, with an overall mean of 0.98x (Fig. S12). Finally, we estimated LD among the genetic variants within DMRs for each population, and found that seven out of eight populations exhibited LD levels within DMRs comparable to null expectations (Table S2). Together, these findings do not support the hypothesis that reduced recombination explains the accentuated *FST* observed in DMRs.

*3.6.*  *No significant evidence of methylation mutagenic effects explaining accentuated* FST *in DMRs*

Lastly, we tested the hypothesis that the mutagenic effects of methylation could explain the accentuated *FST* values observed in DMRs. Among 283 SNPs within DMRs, 28 were identified as C/T or G/A mutations in a CpG context (24 of these had complete data across all population comparisons). These SNPs showed a marginal difference in median *FST* values compared to C/T or G/A SNPs in other sequence contexts at MAF ≤ 0.05, though this result was not statistically significant (1.28x, *P=*0.256, permutation tests; Fig. 5). Additionally, the *FST*values for these SNPs were not significantly associated with geographical distance (Table S3). One SNP (lg1\_scaf1290:791133) showed a marginal association between *FST* and geographical distance (Mantel test *r* =0.85, *P* = 0.022), but its *FST* values remained below 0.04, even at the largest geographical distances.

When MAF was not standardized, we observed that the median *FST* of SNPs likely arisen from methylation mutagenic effect did not significantly differ from other C/T and G/A SNPs (1.0x, *P*=0.643, permutation tests). However, we found that some C/T and G/A SNPs showed pronounced *FST*  values in some population comparisons, while ‘mutation-bias’ SNPs did not show any pronounced *FST* value (Fig. 5). Our results collectively indicate that the mutagenic effects of methylation does not appear to explain the accentuated *FST* observed in DMRs.

**4. Discussion**

The accumulation of phenotypic and genetic differences (*i.e.,* genetic differentiation) is pivotal to population divergence and species formation. Yet, this differentiation can widely vary across the genome. Influenced by factors such as the genetic architecture of adaptive traits, regional recombination rates, and mutation hotspots (Ellegren & Galtier, 2016), these patterns are further shaped by extrinsic processes like selection and gene flow, creating a heterogeneous landscape of genetic differentiation (Ravinet et al., 2017; Wolf & Ellegren, 2017). While much research has focused purely on population genetic drivers of genetic differentiation, our study takes a novel approach by examining the association between DNA methylation and genetic differentiation in diverging populations. Specifically, we investigated associations between DNA methylation patterns and genetic differentiation across the genome of T. cristinae stick insects populations. Our findings reveal new insights into the association between genetic and epigenetic variation in population divergence and suggest a potential role for DNA methylation in speciation, though the actual role of methylation in speciation remains uncertain and warrants further investigation. The results are summarized in Table 1, and discussion of our main findings follows.

*4.1. DMRs co-occur with regions of pronounced genetic differentiation*

We began our investigation by testing the hypothesis that DNA methylation is associated with the accumulation of genetic differences in diverging populations. Our findings support this hypothesis, by showing that DMRs tend to co-occur with regions of elevated genetic differentiation. Specifically, DMRs tended to be enriched for elevated *FST* values among populations spanning different stages of divergence. This result suggests that genomic regions exhibiting differential methylation could be more likely to diverge genetically than other regions of the genome. Our results are consistent with earlier studies that highlight a link between DNA methylation and genetic differentiation, potentially contributing to evolution (Heckwolf et al., 2020; Ord et al., 2023; Venney et al., 2024).

The co-occurrence of epigenetic differences and accentuated *FST* values in *T. cristinae* populations may suggest a role for epigenetic variation in the process of genetic differentiation and population divergence (Platt et al., 2015). For example, DNA methylation could act as a precursor or modulator of genetic differentiation by influencing gene expression levels and contributing to phenotypic variation, complementing the effects of genetic variation (Ord et al., 2023; Smith et al., 2016; Vernaz et al., 2022). This raises the possibility of an additional pathway for divergence: not only at the level of DNA sequences but also in the regulation of gene activity (Pavey et al., 2010; G. Smith & Ritchie, 2013).

Notably, we found that the enrichment of elevated *FST* values in DMRs increases with geographical distance between populations, particularly within genic DMRs. This pattern underscores the role of reduced gene flow among distant populations in facilitating genetic divergence, especially at DMR-associated loci. At the same time, it is important to note that the DMRs are associated with host-plant ecotype, displaying distinct methylation patterns that are independent of geographical proximity or levels of gene flow (de Carvalho et al., 2023). This could indicate that their methylation patterns are strongly induced by host-plant environments. Alternatively, these methylation differences may be driven by locally-adapted genetic variants (Dubin et al., 2015; Fargeot et al., 2021), or shaped by a combination of genetic and environmental factors (Adrian-Kalchhauser et al., 2020). Regardless of the underlying mechanisms shaping DNA methylation patterns, the processes affecting DMRs appear to amplify or better coincide with genetic differentiation in the context of reduced gene flow (see discussion below).

*4.2. Enrichment of accentuated* FST *in DMRs best explained by selection-gene flow balance*

To explain the enrichment of DMRs for accentuated *FST* with increasing geographical distance, we first hypothesized that this pattern could be explained by a balance between selection and gene flow. In this scenario, the effects of selection on differentially methylated genomic regions would intensify as gene flow decreased with greater geographical isolation. Consistent with this interpretation, we observed a moderate effect of climatic differences together with effects of geographical distance on *FST* enrichment in DMRs. This way, differences in climate such as temperature and precipitation could be a source of selection shaping genetic differentiation in DMRs. This result is consistent with previous studies showing some genomic regions in different Timema species are under selection driven from climatic differences (Chaturvedi et al., 2022). Furthermore, DMRs exhibited marked allele-frequency changes in a single-generation experiment. This result provides additional evidence for the role of selection in shaping genetic differentiation in differentially methylated regions, although the sources of selection remain unknown.

In addition to the selection-gene flow hypothesis, we tested for two alternative hypotheses. However, we found no support for either. First, we did not find a significant association between the proxies we used to estimate reduced recombination and DMRs. This suggests that *FST* enrichment in DMRs is unlikely due to background selection in regions of reduced recombination. Studies in plants and vertebrates have established a link between DNA methylation and reduced recombination, providing a potential molecular mechanism to interpret the interplay between genetic and epigenetic variation (Boideau et al., 2022; Liu et al., 2017; Melamed-Bessudo & Levy, 2012). However, this relationship is unclear in invertebrates, in general, which can show very different genomic functions for DNA methylation. A more refined analysis could use a recombination map in *T. cristinae* to directly understand the relationship between DNA methylation, genetic differentiation, and reduced recombination.

Second, we found no evidence that biased mutation in methylated CpGs could explain the observed pattern. Had this hypothesis been supported, it would have directly linked DNA methylation differences to genetic variation, as demonstrated in other sticklebacks (Ord et al., 2023; Venney et al., 2024). However, our analysis revealed that *FST* values of C/T and G/A SNPs in CpG contexts (*i.e.,* mutation-bias SNPs) did not significantly differ from other contexts. Mutation-bias SNPs did not show elevated *FST* values, possibly due to low MAF across the population comparisons. Although this suggests that while methylated CpGs may have a higher mutation rate (Holliday & Grigg, 1993; Ossowski et al., 2010), these mutations likely remain rare and do not reach fixation levels that contribute significantly to genetic differentiation within DMRs. Thus, the resolution of our analysis was possibly constrained by the available data, limiting our ability to provide the most powerful test of the mutation-bias hypothesis. Future studies on Timema should utilize whole-genome sequencing data for each bisulfite sequencing sample to achieve higher resolution and better evaluate biased mutation patterns.

While our results generally suggest that a selection-gene flow balance is the best explanation for the observed patterns, natural selection could be operating in several ways. For example, selection may act on methylation-influenced phenotypes independently of its genomic background (*e.g.,*  Schmid et al., 2018), but in LD with genetic variants. Alternatively, selection could primarily target genetic variation, with methylation changes arising secondarily (*e.g.*, Dubin et al., 2015). A third possibility involves selection acting on traits that emerge from interactions between the methylome and the genotype (a genotype-methylation interaction scenario), which might not be evident from genetic data alone (Adrian-Kalchhauser et al., 2020; Morgan et al., 1999). These potential mechanisms underscore the complexity of linking selection to DNA methylation patterns, a challenge even in the best-studied model organisms (Dubin et al., 2015; Heckwolf et al., 2020; Schmid et al., 2018)⁠.

*4.3. Absence of host-plant effects on enrichment of* FST *in DMRs and in the transplant experiment*

The selection-gene flow hypothesis was the most consistent explanation for the enrichment of accentuated *FST*observed in DMRs, particularly as geographical distance increases. Since DMRs represent pronounced methylation differences between *T. cristinae* from different host-plant ecotypes, we expected the enrichment of elevated *FST* in DMRs to align with host-plant ecotype. However, our results showed no detectable host-plant effect on this enrichment. Instead, we observed that the enrichment of elevated *FST* values in DMRs becomes more accentuated not only with increasing geographical distance, but were also moderately associated with climatic differences. Additionally, allele-frequency changes in DMRs from the transplant experiment were also unrelated to host-plant species. Below, we discuss two potential scenarios to explain these findings.

The first scenario involves pleiotropic effects at genes associated with DMRs, where a single gene may influence traits responding not only to host-plant habitat, but also to other environmental factors, such as climate. Notably, the DMRs are functionally enriched in pathways related to protein metabolism, signal transduction, and transmembrane transport (de Carvalho et al., 2023). Genes involved in these processes likely have pleiotropic effects and are critical for detecting environmental cues, regulating stress responses, and maintaining cellular homeostasis (King & MacRae, 2015; Zizzari & Ellers, 2011). For instance, signal transduction pathways could mediate responses of herbivores to both host-plant defense and abiotic stressors, while transmembrane transport systems might regulate detoxification of plant toxins (Amezian et al., 2021) and ion balance under varying climatic conditions (Chown et al., 2011).

It is possible that different host-plants species induce methylation changes in these pleiotropic genes through variations in chemical composition, nutrient availability, or stress signals. Such induction would result in consistent methylation differences between populations of different ecotypes, independent of geographical proximity or gene flow levels, as we observe in our results. While these changes might modulate gene expression and contribute to phenotypic plasticity, their effects may be transient or insufficient to drive significant allele-frequency changes (Roberts & Gavery, 2012). Simultaneously, these genes may encode traits subject to selection from broader environmental factors, such as climatic gradients, driving genetic differentiation at DMR-associated loci. This scenario involving pleiotropic genes, methylation responses to host plants, and genetic adaptation to other environmental pressures could lead to the co-occurrence of DNA methylation differences and accentuated genetic differentiation, as we observed.

The second scenario considers LD between loci associated with host-plant and non-host-plant second scenario considers LD between loci associated with host-plant and non-host-plant environmental variables. In this context, loci associated with DMRs may primarily reflect host-plant habitats. However, if these loci are in strong LD with other genetic regions under selection from broader environmental factors, such as climatic gradients, genetic variation at DMR-associated loci could appear differentiated as a by-product of this linkage. In other words, selective pressures acting on the non-host-plant loci could indirectly drive genetic divergence at the DMR-associated loci, creating a pattern where regions with DNA methylation differences would be enriched in accentuated *FST.*. While this mechanism could explain the enrichment of *FST* in DMRs, it remains unclear why genetic variants associated with DMRs would consistently exhibit strong LD with loci under selection from environmental factors unrelated to host-plant habitat.

While the proposed scenarios provide plausible explanations for the enrichment of accentuated *FST* in DMRs, further research is needed to validate these hypotheses. For example, future studies in *Timema* should test to which degree DNA methylation changes can be induced by host-plant habitat, and its effects on phenotype and fitness. Additionally, our results were derived from three separate datasets: one for DMRs, one for genetic differentiation, and one for allele-frequency change. Future studies should conduct a population survey such as the one developed for DMRs, but with larger sample sizes, and incorporating whole-genome data. This would enable direct, one-to-one associations between DMRs and genetic differentiation, providing higher resolution for disentangling the factors driving the co-occurrence of DMRs and accentuated *FST.* values.

*4.4. Future directions*

While our findings show that differences in DNA methylation are enriched in regions of pronounced genetic differentiation, and that a selection-gene flow balance best explains the observed patterns, several key questions remain. To fully understand the role of selection on DNA methylation, it is essential to disentangle the extent to which methylation variation is shaped by the genetic background and its mechanistic contribution to phenotypic variation and fitness (Husby, 2022). Future studies should further explore whether the phenotypes affected by DNA methylation variation are under selection, and the different forms of selection acting on it such as purifying, positive, or divergent selection. Identifying the targets and the type of selection at work could provide deeper insights into the evolutionary consequences of DNA methylation.

Much of our understanding of the potential role of DNA methylation in speciation remains uncertain. Therefore, future research should investigate whether methylation changes can contribute directly or indirectly to adaptation and reproductive isolation (McGuigan et al., 2021; Planidin et al., 2022). A clearer understanding of the extent to which DNA methylation is transgenerationally stable or erased between generations will also be essential, as this could significantly affect its potential for evolutionary change. Depending on the capacity of transgenerational transmission, the evolutionary consequences could thus range from enabling phenotypic plasticity, which could allow persistence in a different environment if the plasticity is adaptive (Duncan et al., 2014; Hu & Barrett, 2023; Smith & Ritchie, 2013), to positioning methylation as a direct target of selection, thereby channeling evolutionary change (Gopalan-Nair et al., 2024; Schmid et al., 2018; Stajic et al., 2019). These directions will deepen our understanding of the complex interplay between genetic and epigenetic variation, offering new perspectives on the role of DNA methylation in adaptation and population divergence.

*4.5. Conclusion*

DNA methylation is affected by environmental change and regulates gene expression, which in turn affects phenotypic traits that may be subject to selection and thereby contribute to genetic differentiation among populations. This dynamic interplay suggests that regions with differential methylation may harbor genes that are crucial for adaptive traits, thereby influencing genetic differentiation and population divergence. In this study, we establish an association between DNA methylation and genetic differentiation in populations pairs with various levels of genetic divergence, with evidence consistent with a balance between selection and gene flow. However, much remains unknown regarding the specific targets of selection and the mechanisms at play. While our results provide evidence that epigenetic variation contributes to genetic differentiation, the full understanding of how methylation contributes to population divergence is still in its early stages, representing a key frontier in epigenetics research. Overall, our results suggest that epigenetic variation mayb not only provide insights on genetic differentiation, but also add new layers of complexity to our understanding of population divergence. Beyond serving as a marker of genetic differentiation, DNA methylation may facilitate or reinforce divergence between populations. Understanding the interaction between genetic and epigenetic variation can thus shed light on the mechanisms driving the accumulation of phenotypic and genetic differences across different stages of population divergence and speciation.

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**Data availability**: This manuscript does not contain new data, all of which is publicly archived as described in the Methods section. All scripts and code used for analysis has been archived at Zenodo (<https://doi.org/10.5281/zenodo.14566136>).

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**Table 1.** Summary of findings on the association between DNA methylation and genetic differentiation. This table summarizes the evidence supporting each hypotheses tested in this study, categorized as none, weak, or strong based onstatistical significance and pattern consistency. "Pronounced *FST* across DMRs and populations" refers to *FST* enrichment in DMRs across all population pairs (*Section 3.1*). " *FST* enrichment with geographical distance" examines whether *FST* in DMRs increases with distance between populations (*Section 3.2*). "Host effects" and "Climate effects" assess the relationship between *FST* enrichment in DMRs and host-plant and climatic factors (*Section 3.3*). "Allele frequency changes in DMRs" reflects the enrichment of allele-frequency changes in DMRs (*Section 3.4*), while "Reduced recombination effects" and "Mutagenic effects of DNA methylation" explore the role of recombination rates and methylation-induced mutation (*Sections 3.4* and *3.5*).

|  |  |
| --- | --- |
| **Hypothesis** | **Degree of evidence** |
| Pronounced *FST* across DMRs and populations | weak |
| *FST* enrichment with geographical distance | strong |
| Host-plant effects | none |
| Climate effects | weak |
| Allele frequency changes in DMRs | weak |
| Reduced recombination effects | none |
| Mutagenic effects of DNA methylation | none |

**Figure Legends**

**Figure 1. The T. cristinae study system.** (**A**) *T. cristinae* and their host-plant ecotypes: *Adenostoma fasciculatum* and *Ceanothus spinosus*. Drawings from Rosa Ribas. (**B**) Map of study populations. The main map shows populations analyzed for differentially methylated regions (DMRs) from de Carvalho et al. (2023). The smaller scheme depicts the map of populations with whole-genome sequencing data from Soria-Carrasco et al. (2014), used to investigate genetic differentiation (*i.e.*, *FST*).

**Fig. 2. Patterns, hypotheses and predictions for investigating the enrichment of accentuated *FST* in differentially methylated regions (DMRs).** (**A**) Expected geographical pattern of *FST* enrichment in DMRs. Populations with greater geographical isolation (*e.g.*, A x C) are expected to show stronger *FST* enrichment in DMRs compared to geographically closer populations (*e.g.*, A x B), due to differential gene flow effects on genetic differentiation. (**B**) The selection-gene flow balance hypothesis to explain the geographical patterns of *FST* enrichment in DMRs. This hypothesis posits that *FST* enrichment in DMRs increases with geographical isolation, reflecting stronger selection effects as gene flow decreases. One of the predictions of this hypothesis is that DMRs will show greater allele-frequency (freq.) shifts in transplant experiments between environments compared to other methylation tiles. (**C**) The reduced recombination hypothesis posits that the enrichment of accentuated *FST* in DMRs results from background selection in regions of reduced recombination. This predicts DMRs are concentrated in low-recombination regions. (**D**) The mutatagenic DNA methylation hypothesis posits that DNA methylation increases *FST* enrichment by promoting specific mutations. Methylated cytosines (with the red dot) tend to mutate into thymines in higher rates than any other point mutation (Holliday & Grigg, 1993; Ossowski et al., 2010). This hypothesis predicts higher *FST* T values for SNPs cytosine-to-thymine (C/T) SNPs in CpG contexts compared to other sequence contexts. Abbreviations: C=cytosine, T=thymine, G=guanine, A=adenine.

**Fig. 3. Genetic differentiation (*FST*) in DMRs across populations.** (**A**) Map of the populations (n=8 populations) used in this study, of both Adenostoma and Ceanothus ecotypes (orange and blue points, respectively). This includes parapatric populations (side by side), and with different levels of geographical isolation (Soria-Carrasco et al., 2014). (**B**) Percentage of DMRs with accentuated *FST* (‘high *FST*’ in the figure), a magnitude that is marginally more elevated than expected by chance (expected values: 5%, P=0.077). (**C**) Accentuation of *FST* in DMRs according to the pairwise geographical distance between populations (logarithmically transformed). X-fold enrichment expresses relative *FST* values in DMRs compared to the background levels. (**D**) *FST* values in DMRs between some population pairs separated by different degrees of geographical isolation. The black bar represents the mean *FST* across methylation tiles (null expectation) and the red dots represent the *FST* in DMRs. DMRs tend to exhibit particularly higher *FST* values with increasing levels of geographic isolation.

**Fig. 4. Field experiment testing for allele-frequency changes in DMRs.** (**A**) Design of field experiment, where 491 *T. cristinae* specimens to experimental bushes of *Adenostoma* or *Ceanothus*. Individuals were collected, tissue-sampled, and then transplanted. Survivors were recaptured after eight days, allowing comparison of genetic changes between the released and surviving individuals (Gompert, Comeault, et al., 2014). The analysis in this study focused on whether genetic changes were more pronounced in DMRs than expected by chance. Drawings from Rosa Ribas. (**B**) DMRs show a weak yet statistically significant enrichment in allele-frequency changes between release and recapture in a field survival experiment, compared to a null expectation obtained by random sampling (observed Δp=0.086; null Δp=0.071; *P*=0.045). The analysis shown here was performed on 1kbp windows located within genes, and DMRs were delimited using the cut-off of *P* < 0.0001.

**Figure 5. Mutagenic effects of DNA methylation on SNPs within DMRs.** (**A**) C/T or G/A SNPs within DMRs and their corresponding *FST* between all pairwise comparisons, separated at different levels of geographic isolation. Geographical distance was logarithmically transformed. SNPs that could be caused by mutagenic effects of DNA methylation in cytosines (thus in cytosines followed by guanines, CpG, context) are represented in red. (**B**) Same graph as in (A), but controlling for minor-allele frequencies (MAF) below or equal to 0.05, since a mean of 95.2% of SNPs across all 28 population pairs show MAF ≤ 0.05. Abbreviations: C=cytosine, T=thymine, G=guanine, A=adenine.