White Rose Research Online
eprints@whiterose.ac.uk
https://eprints.whiterose.ac.uk
Universities of Leeds, Sheffield and York

Deposited via The University of Sheffield.

White Rose Research Online URL for this paper:
https://eprints.whiterose.ac.uk/id/eprint/239228/

Version: Accepted Version

Article:

de Carvalho, C.F., Planidin, N.P., Villoutreix, R. et al. (2025) Linking DNA methylation to localised genetic differentiation in Timema cristinae stick insects. Molecular Ecology, 34 (22). e70049. ISSN: 0962-1083

https://doi.org/10.1111/mec.70049

© 2025 The Authors. Except as otherwise noted, this author-accepted version of a journal article published in Molecular Ecology is made available via the University of Sheffield Research Publications and Copyright Policy under the terms of the Creative Commons Attribution 4.0 International License (CC-BY 4.0), which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/

Reuse
This article is distributed under the terms of the Creative Commons Attribution (CC BY) licence. This licence allows you to distribute, remix, tweak, and build upon the work, even commercially, as long as you credit the authors for the original work. More information and the full terms of the licence here: https://creativecommons.org/licenses/

Takedown
If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

UNIVERSITY OF LEEDS
University of Sheffield
UNIVERSITY of York

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

Authors: Clarissa F. de Carvalho[1,†]*, Nicholas P. Planidin[2], Romain Villoutreix[2], Víctor Soria-Carrasco[3], Rüdiger Riesch[2,4], Jeffrey L. Feder[5], Jon Slate[1], Patrik Nosil[1,2,], Zachariah Gompert[6]

Affiliations:
[1]*School of Biosciences, University of Sheffield; Sheffield, S10 2TN, UK*
[2]*SETE, Univ Université Paul Sabatier, CNRS, UMR-5321, F-09200 Moulis, France*
[3]*John Innes Centre; Norwich, NR4 7UH, UK*
[4]*Department of Biological Sciences, Centre for Ecology, Evolution and Behaviour, Royal Holloway University of London; Egham, TW20 0EX, UK.*
[5]*Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana, 46556, USA*
[6]*Department of Biology, Utah State University; Logan, Utah, 84322, USA*
†*Present address: Departamento de Ecologia e Biologia Evolutiva, UNIFESP; Diadema, 09972-270, Brazil*
**Corresponding authors*

Email: clarissa.carvalho@unifesp.br

Abstract

Understanding speciation is a fundamental goal in evolutionary biology. Genome scans of genetic differentiation (F_{ST}) have become a cornerstone of speciation research, helping identify genomic regions likely involved in population divergence and speciation. While such studies have advanced our understanding, the relationship between epigenetic mechanisms and genetic differentiation 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 often show greater F_{ST} than expected by chance. Strikingly, the magnitude of this accentuation of F_{ST} in DMRs 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 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 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 (*i.e.,* genetic differentiation), which leads to population divergence and reproductive isolation (Coyne & Orr, 2004; Mayr, 1963; Schluter, 2001; Seehausen et al., 2014). In this context, a key metric for quantifying genetic differentiation is the fixation index (F_{ST}). Genome scans using F_{ST} have revealed that selection, recombination, and gene flow interact to produce heterogeneous landscapes of genetic differentiation across the genome (Feder et al., 2012; Wolf & Ellegren, 2017). This is because divergent selection on locally adapted traits can create ‘peaks’ of high F_{ST}, while gene flow typically homogenizes the genetic differences between populations, generating F_{ST} ‘valleys’ (Hohenlohe et al., 2012; Via & West, 2008). It is important to note that F_{ST} peaks can also arise from other processes unrelated to adaptive divergence, such as background selection – accentuated in regions of reduced recombination (Cruickshank & Hahn, 2014). Given its ability to capture genome-wide patterns of divergence, F_{ST} has become a foundational metric in speciation research.

Genome scans based on F_{ST} have been instrumental in identifying candidate regions driving population divergence across the speciation continuum (e.g., 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) linked F_{ST} peaks to plumage differences between crow sub-species, maintained despite their gene flow. While such studies have deepened our understanding of speciation, a focus solely on peaks of genetic differentiation may overlook regulatory mechanisms that can also drive divergence (Pfennig et al., 2010). In this context, epigenetic mechanisms, such as DNA methylation, may play a key role by modulating gene expression and thereby potentially contributing to phenotypic differentiation and population divergence (Pavey et al., 2010; Smith & Ritchie, 2013). In particular, it remains unclear to what extent and why regions of differential methylation between populations are associated with accentuated genetic differentiation relative to the rest of the genome (accentuated F_{ST}, hereafter). This question is the focus of the present study.

DNA methylation – the addition of a methyl group to the fifth carbon of a cytosine (5mC) – is the most extensively studied epigenetic mechanism in ecological and evolutionary studies. DNA methylation can influence gene expression by modifying the accessibility of DNA to transcriptional machinery, often leading to the repression or activation of specific genes (Bird, 2002; Law & Jacobsen, 2010; Suzuki & Bird, 2008). Although much of the variation in DNA methylation among individuals reflects their genetic background (Dubin et al., 2015; Richards, 2006; Sepers et al., 2023; Taudt et al., 2016), DNA methylation patterns can be influenced by a variety of processes. For example, DNA methylation patterns can change due to errors in maintenance pathways, and in response to environmental changes, thereby mediating phenotypic plasticity (Duncan et al., 2014; Hu & Barrett, 2023; Husby, 2022). Consequently, variation in DNA methylation and associated phenotypic variation can arise not only from genetic factors, but also through environmental variation and genotype-environment interactions (*i.e.,* G × E) (Adrian-Kalchhauser et al., 2020; Richards, 2006). Beyond its regulatory role, DNA methylation can influence mutation rates. This is because 5mCs are susceptible to deaminate into thymines (*i.e.,* C/T transitions) and mutate at rates that are higher than any other substitution – thus potentially contributing directly to genetic variation (Holliday & Grigg, 1993; Ossowski et al., 2010; Tomkova & Schuster-Böckler, 2018). Notably, DNA methylation has been shown to be transmitted across one or more generations in several 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), insects (Wang et al., 2016; Yagound et al., 2020), and yeast (Stajic et al., 2019).

These processes indicate that DNA methylation can both influence and be influenced by genetic variation, raising the possibility that it may be associated with *genetic differentiat*ion between populations. Indeed, several studies have reported genome-wide correlations between genetic differentiati*on* and differential methylation (Aagaard et al., 2022; Boman et al., 2024; de Carvalho et al., 2023; Foust et al., 2016; Herrera et al., 2016; Medrano et al., 2014; Platt et al., 2015; Venney et al., 2021). However, the extent to which methylation is associated with fine-scale patterns of genetic differentiation across different parts of the genome remains largely overlooked. That is, although genome-wide associations between DNA methylation variation and F_{ST} have been described, it remains unclear whether regions that differ in methylation also exhibit accentuated genetic differentiation – and, if so, what mechanisms might explain such a pattern (but see Heckwolf et al., 2020; Ord et al., 2023; Venney et al., 2024). In this study, we address this gap by investigating whether genomic regions that differ in DNA methylation between populations (specifically, insect populations adapted to different host-plant species) also tend to exhibit accentuated F_{ST}. Having identified such an association, we further explore the mechanisms that may underlie it (Table 1, Fig. 1).

There are several non-mutually exclusive explanations for why genomic regions that differ in DNA methylation between populations might exhibit accentuated F_{ST}. For example, selection on phenotypes influenced by DNA methylation could indirectly affect genetic variation through linkage disequilibrium (LD) 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 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 influence DNA methylation, either directly by controlling DNA methylation variation (Dubin et al., 2015), or via LD (Xu et al., 2020). In both cases, divergence in DNA methylation would exhibit higher F_{ST} in specific regions of the genome. Such an association between accentuated F_{ST} values in DMRs is expected to be even more pronounced with reduced gene flow and greater geographical isolation, which limits homogenization and allows selection to drive genetic and epigenetic differentiation (i.e, a selection-gene flow hypothesis).

Beyond the selection-gene flow hypothesis, genomic regions that differ in DNA methylation between populations may also exhibit accentuated F_{ST} if they fall within regions of low recombination (*e.g.*, as shown in plants; Melamed-Bessudo & Levy, 2012; Peñuela et al., 2022), where background selection is stronger. Background selection reduces local genetic diversity, which can inflate F_{ST} even in the absence of divergent selection (Booker et al., 2020; Cruickshank & Hahn, 2014; Noor & Bennett, 2009). Alternatively, the mutagenic potential of DNA methylation (*I.e,* 5mC→T transitions) could directly elevate F_{ST} in certain genomic regions by introducing directional (*i.e.,* biased) mutations in one population relative to another (Ord et al., 2023; Venney et al., 2024). Because mutation rates would also influence methylation levels (Xia et al., 2012), this process could generate co-occuring patterns of accentuated F_{ST} and differential methylation. In this work, we tested for a fine-scale relationship between population divergence in DNA methylation and accentuated F_{ST}, and examine potential explanations for such a pattern using genetically divergent populations of *Timema cristinae* stick insects (Vickery, 1993) – a model system for speciation studies.

Timema cristinae are wingless, plant-feeding insects distributed throughout the Santa Ynez mountains in California, USA. These insects primarily live and feed on two host-plant species: *Adenostoma fasciculatum* (Rosaceae), and *Ceanothus spinosus* (Rhamnaceae) (Nosil & Crespi, 2006; Sandoval, 1994). Divergent selection associated with these host plants contributes to partial reproductive isolation between the host-plant associated populations, resulting in distinct *Adenostoma* and *Ceanothus* ecotypes (Nosil, 2007). A previous study showed that genome-wide DNA methylation differences among *T. cristinae* populations are strongly positively correlated with genetic and geographical distance, while also exhibiting moderate host-plant effects (de Carvalho et al., 2023). Importantly, that study used methylome-environment association analyses to designate genomic regions that differ in DNA methylation between host-plant ecotypes – hereafter referred to as differentially methylated regions (DMRs). In the present study, we focus on these ecotype-associated DMRs, as these may reflect methylation responses to ecological differences between host-plant environments. This makes these DMRs plausible candidates for investigating patterns of accentuated genetic differentiation. Notably, previous work did not address the question of how locus-specific genetic differentiation relates to these ecotype-associated DMRs in *T. cristinae*, which forms our focus here.

In this context, we here integrate previously published whole-methylome and whole-genome data from natural and experimental *T. cristinae* populations. We used methylome data from de Carvalho et al. (2023), and we leveraged whole-genome sequencing data from populations with varying degrees of divergence (Soria-Carrasco et al., 2014). We first tested the hypothesis that DMRs exhibit accentuated genetic differentiation (e.g., higher F_{ST} relative to the genome-wide levels; Table 1, Fig. 1). Having found such a pattern, we then tested different non-mutually exclusive hypotheses to explain it. Specifically, we explored whether a balance between selection and gene flow, along with the roles of mutation and reduced recombination, could explain these divergence patterns. By investigating the fine-scale associations between DNA methylation and genetic variation, our study provides new insights into whether and how epigenetic mechanisms may be associated with genetic differentiation during speciation.

2. Material and Methods

2.1. Differentially methylated genomic 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*. That study conducted whole-genome bisulfite sequencing (WGBS) on individuals from 12 host-associated populations spanning nine geographic localities. These included four localities dominated by *Adenostoma,* two dominated by *Ceanothus*, and three ‘parapatric’ sites where the two host-plant species coexist in close proximity. Importantly, the sampled populations differed not only in host-plant habitat, but also in geographical proximity and climatic conditions (as summarized by WorldClim climatic variables; Harris et al., 2014). Although host-plant species and climatic variables are generally correlated, the sampling locations were deliberately selected to disentangle these factors. Consequently, there was little correlation between them in the sampled sites (Pearson correlation r=0.00, P=0.847; de Carvalho et al., 2023). From each population, two similarly-sized female specimens were selected for study (n = 24 samples; Fig. 2). 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 (WGS; 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 focused on DMRs between host-plant ecotypes identified by de Carvalho et al. (2023). These DMRs were delineated through a methylome-environment (i.e., host plant) association analysis using binomial mixed models (MACAU v1.0.0; Lea et al., 2015). This analysis was applied to 1kbp non-overlapping methylation tiling windows across the genome, requiring a minimum coverage of 10 reads per window (hereafter referred to as '1kbp methylation tiles'; see de Carvalho et al., 2023 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 cut-off thresholds. Following de Carvalho et al. (2023), we here focused on DMRs that were defined 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 focused mainly on DMRs delimited by the *p-value* < 0.0004, the most stringent cut-off.

Previously, de Carvalho et al. (2023) identified several patterns across the range of *p-value* cut-offs for defining DMRs that are relevant to this study. For example, DNA methylation distances in DMRs were more closely associated with host-plant ecotype than with genetic, geographical or climatic differences. This ecotype-specific signal contrasted with genome-wide methylation patterns, which were primarily explained by geographical distance (Fig. 2C). Additionally, DMRs were distributed genome-wide across all chromosomes, with a mean of 72% overlapping annotated genes across cut-offs (range 70.6% – 73.4%, 95% confidence interval, CI). This proportion closely matched the 73.5% genic overlap observed for the entire dataset used in the methylome-environment analyses. This indicates that genes did not harbor disproportionately more DMRs than expected by chance. This detailed characterization of DMRs provides a foundation for investigating the fine-scale association between DNA methylation and accentuated F_{ST}, the central focus of the present study.

2.2. F_{ST} *values in DMRs relative to the genomic background*

We began by testing the hypothesis that DMRs exhibit accentuated genetic differentiation. Specifically, we tested whether DMRs exhibited significantly higher F_{ST} values compared to the genomic background, indicating accentuated differentiation. To this end, we estimated F_{ST} using WGS data originally published by Soria-Carrasco et al. (2014), and reanalyzed by Lucek et al. (2019), which aligned all data to the same reference genome (v1.3c2) used by de Carvalho et al. (2023) to estimate DMRs. This data comprised 160 specimens from eight populations (Fig. 2). These populations represent a subset of those analyzed in the WGBS study, plus one additional, more geographically distant population (R12). Including these populations allowed us to capture varying stages of population divergence in our study (see Supporting Information for details).

We estimated pairwise genetic differences between each population pair (n=28 comparisons) within the same 1kbp methylation tiles used in the methylome-environment association analysis – but here based on SNPs. We used allele-frequency estimates for 3,252,350 SNPs from the WGS data (see Lucek et al., 2019 for details on DNA sequencing, alignment, variant calling and allele-frequency estimation for these SNPs). Using the SNPs located within the 1kbp methylation tiles, we estimated mean heterozygosity (H_S) and F_{ST} between each population pair. H_S was estimated as $H_S = \sum [p_1 1 - p_1) + p_2 (1 - p_2)] / L$; where p_1 and p_2 represent the non-reference allele frequencies in the populations 1 and 2, respectively. The sum was divided by the number of loci (*L*) within the methylation tiles.

Multilocus F_{ST} was calculated as $F_{ST} = \sum (H_T - H_S) / \sum (H_T)$ where H_S is the mean heterozygosity within populations, and H_T is the total heterozygosity across each population pair: $H_T = 2\bar{p}(1 - \bar{p})$ where $\bar{p} = (p_1 + p_2)/2$. The 95% confidence intervals for multilocus F_{ST} estimates were obtained from 1,000 bootstrap replicates where individual DMRs were sampled with replacement. We then evaluated how F_{ST} 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 whether DMRs exhibited significantly greater F_{ST} values compared to the genomic background (*i.e.,* accentuated F_{ST}), we analyzed the same 1kbp methylation tiles for both DMR identification and F_{ST} estimation. For each population pair, we calculated the mean F_{ST} across DMRs at multiple *p-value* cut-offs (ranging from 0.0004 to 0.0061). These observed means were compared against null distributions generated by 1,000 iterations of random sampling – with an equal number of 1kbp methylation tiles as defined for each *p-value* cut-off. To measure how elevated F_{ST} values were in DMRs relative to random expectations, we calculated the ratio of the observed mean F_{ST} to the mean of the null distribution (the 'x-fold difference'). Statistical significance was estimated using empirical *p-value* from the randomization tests. This analysis was performed separately for: (1) all genome-wide DMRs, and (2) only genic DMRs (i.e., overlapping with annotated gene regions), and (3) only non-genic DMRs.

Our analyses provided initial evidence for the predicted greater F_{ST} values in DMRs relative to the genomic background, indicating accentuated genetic differentiation (see Results for details). This pattern was further corroborated by significant overlap between DMRs and 1kbp methylation tiles falling within the top 5% of F_{ST} values across and between population comparisons (see Supporting Information). Notably, both approaches – the quantification of F_{ST} values in DMRs and the overlap tests with regions in the top 5% of F_{ST} values – revealed that DMRs exhibited accentuated F_{ST} in some population comparisons, but not in others. As such, we next explored potential processes that could be underlying such a pattern of heterogeneity among population pairs. In the following sections, we tested three non-mutually exclusive hypotheses regarding the potential contributions of (1) selection-gene flow balance, (2) reduced recombination, and (3) mutagenic effects of DNA methylation to the pattern of accentuated F_{ST} observed in DMRs.

2.3. The selection-gene flow balance hypothesis: genetic differentiation in DMRs across geographical distances

We first tested whether accentuated F_{ST} in DMRs reflects a balance between selection and gene flow. Under this hypothesis, the magnitude to which DMRs exhibit higher F_{ST} relative to the rest of the genome is predicted to increase with the geographical distance between populations (Fig. 1, Prediction 1). In other words, we tested whether populations separated by greater geographical distances show a stronger association between DMRs and accentuated F_{ST} than populations that are geographically closer – an enrichment of the DMR-F_{ST} association with geographical isolation. This expectation is based on evidence that populations separated by greater geographical isolation experience reduced gene flow, which weakens its homogenizing effect on genetic differentiation, a pattern previously documented in *T. cristinae* for morphological traits, cryptic coloration, and genome-wide differentiation (Lindtke et al., 2017; Nosil et al., 2012; Nosil & Sandoval, 2008;

Sandoval, 1994b). Because gene flow tends to constrain genetic differentiation, DMRs under direct or indirect selection – via LD with causal mutations or otherwise correlated with selected traits – should exhibit higher F_{ST} values relative to the background level in more geographically isolated populations. This effect likely requires moderate levels of gene flow, because, under very low gene flow rates, genome-wide divergence can obscure signatures of localized selection in DMRs.

To test our prediction, we estimated, for each population pair, the mean F_{ST} within DMRs (delimited by $P < 0.0004$) and divided it by the mean of a null distribution generated by permutation tests as described above (the x-fold difference). Subsequently, we assessed the relationship between the x-fold difference of F_{ST} values in DMRs and the logarithm of geodesic geographical distances between each population pair using Mantel tests (10,000 permutations, R package *vegan* v2.6-6.1; Oksanen et al., 2022). This analysis was repeated for three distinct DMR sets: (1) all DMRs, (2) genic DMRs only, and (3) non-genic DMRs only.

Having found support for the main prediction from the selection-gene flow balance hypothesis (see Results), we then evaluated whether accentuated F_{ST} in DMRs could be explained by their location in gene-dense genomic regions – where a higher number of sites may be subject to selection. This scenario would align with the selection-gene flow balance hypothesis, as DMRs near selected regions in gene-rich areas could exhibit significantly greater genetic differentiation through genetic hitchhiking. However, our analyses did not support this explanation (see Supporting Information).

2.4. The selection-gene flow balance hypothesis: potential sources of selection underlying the observed geographical pattern

We next investigated potential sources of selection that could underlie the observed geographical pattern under the selection-gene flow balance hypothesis. We did so by examining two key environmental factors: host-plant species and climatic variation. Host-plant species – the environmental factor associated with differential methylation in DMRs (de Carvalho et al., 2023) – may also be linked to genetic differentiation in these regions, either through direct selection on DMRs, or indirect selection via LD with genetic loci. In addition, climate-associated loci in *Timema* are known targets of selection (Chaturvedi et al., 2022), suggesting that climatic variables could also contribute to the observed patterns of genetic differentiation. Similar to DMRs, these climate-associated loci are distributed genome-wide. The eight populations from which we obtained F_{ST} data differ both in host-plant species (four *Adenostoma* and four *Ceanothus*, Fig. 2) climatic conditions (see Supporting Information).

To quantify these effects, we fit Bayesian linear mixed models (BLMMs) using Markov Chain Monte Carlo (MCMC) Clarke et al., 2002; Gompert, Lucas, et al., 2014). Our models incorporated all combinations of geographical, host-plant species and climatic distances to explain the log ratio of the x-fold difference of F_{ST} 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). Briefly, climate PC1 summarizes variation in annual temperatures, temperatures during the coldest and wettest periods, and overall temperature constancy across seasons (e.g. isothermality, seasonality). Climate PC2 primarily captures temperature extremes during the warmest month, and the warmest and driest quarters (Fig. S1; Supporting Information). We treated the two PCs separately, calculating standardized Euclidean distances for each. 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. We used weakly informative priors for fixed effects (normal distributions with mean = 0 and precision = 0.001) and vague gamma priors (shape = 1, rate = 0.01) for random effect and residual precisions. 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 accentuated F_{ST} in DMRs reflects 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 specimens were collected in *Adenostoma*, tissue-sampled (allowing WGS), and then transplanted onto either *Adenostoma* or *Ceanothus* bushes. Surviving individuals were recaptured eight days later, providing an opportunity to compare the genetic change between the released and surviving individuals (see Gompert, Comeault, et al., 2014 for details). The results, together with more recent studies, indicate that selection affected some genomic regions – either directly or indirectly via LD with such regions – although genetic drift also 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 genomic regions (Fig. 1, Prediction 2). Such a pattern would be consistent with DMRs being influenced by selection, even if the specific targets and sources of selection remain unclear. 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 of 'balance' is supported by previous studies in *T. cristinae* (Nosil et al., 2018, 2021a, 2024; Riesch et al., 2017).

We estimated the allele-frequency changes from WGS 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). Given that most allele-frequency changes in the original experiment were not host-related (Gompert, Comeault et al., 2014), we initially focused on overall allele-frequency shifts, calculated as the difference between release and recapture allele frequencies (pooled across host plants). If DMRs exhibited elevated allele-frequency changes relative to the genomic background, we then tested whether there changes were particularly pronounced in the context of host-plant shift.

We used a standardized measure of change relative to initial genetic diversity, calculated as: $\Delta p = (|p_1 - p_0|) / \sqrt{[p_0(1 - p_0)]}$, where p_0 and p_1 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. We performed 1,000 randomizations to create this null distribution. We conducted these analyses in DMRs designated by the *p-value* cut-off $P < 0.0004$.

To evaluate the robustness of our findings under more stringent criteria, we repeated the analyses using a stricter cut-off of $P<0.0001$. We focused on DMRs located within genes, as gene bodies are the primary targets of DNA methylation in Timema *and* other insects (Bewick et al., 2017; Provataris et al., 2018) . Additionally, results for non-genic DMRs were not significant (1.08x, P=0.388). We further excluded chromosome 8 from this analysis because it harbors strong signals of selection associated with color and color-pattern variation – traits that were not our focus of this present study.

2.6. The reduced recombination hypothesis

Accentuated F_{ST} values in specific genomic regions could 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. 1). To explore this possibility as well, we tested whether patterns of accentuated F_{ST} in DMRs could be affected by reduced recombination in the genomic regions where DMRs are located. 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 overlapped with structural variants (SVs) mapped in *Timema* (*e.g.*, deletions, inversions, tandem duplications) (Lucek et al., 2019). Structural variants are known to suppress recombination rates by disrupting homologous chromosome pairing during meiosis, which interferes with crossover formation (Hoffmann & Rieseberg, 2008; Kirkpatrick, 2010; Noor et al., 2001). Thus, we predicted that DMRs would tend to overlap with SVs. To test this, we used SV data from (Lucek et al., 2019), which included 492 inversions, 223 tandem duplications and 194 deletions across *T. cristinae* genome, using the same WGS data as the one used here. We assessed whether DMRs at various *p-value* cut-offs at least partially overlapped with these SVs using R (R Core Team, 2023). All SVs located in un-mapped scaffolds were excluded.

Next, we examined whether DMRs tended to display lower mean heterozygosity indices (H_S) than the genomic background, given that 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 procedure described with F_{ST}, drawing 1kbp random samples from the methylation tiles 1,000 times at varying *p-value* cut-offs.

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 WGS data (Lucek et al., 2019; Soria-Carrasco et al., 2014) for F_{ST} and H_S 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 and then we estimated genotypes by applying an allele-frequency based prior to the genotype likelihoods (as in 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 1kbp 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 direct mutagenic effects of DNA methylation could contribute to the patterns of accentuated F_{ST} observed in DMRs. Methylated cytosines are significantly more prone to mutate into thymines than unmethylated cytosines, with mutation rates exceeding those of most other point mutations (Holliday & Grigg, 1993; Ossowski et al., 2010). Such biased mutation rates could elevate F_{ST} by generating directional mutations in one population relative to another, particularly in genomic regions with marked methylation differences between populations (*e.g.,* DMRs). This long-standing hypothesis posits a direct link between DNA methylation and genetic differentiation (Guerrero-Bosagna, 2017), yet it has been rarely tested in natural populations (but see Venney et al., 2024). We thus here tested the hypothesis that the observed accentuated F_{ST} in DMRs results from mutagenic effects of DNA methylation. If this was the case, we expected accentuated F_{ST} values in DMRs' to be associated with CpG context genetic polymorphisms, specifically C/T transitions (or from G/A in the complementary strand; Fig. 1). In other words, in this context, differences in methylation could directly cause biased genetic differences between populations.

To test our prediction, we used WGS data from Soria-Carrasco et al. (2014) and estimated F_{ST} for individual SNPs located within DMRs delimited by $P < 0.0004$ (n = 283 SNPs), rather than using 1 kbp windows as in previous analyses. For each SNP, we identified the type of genetic polymorphism (*i.e.*, C/T, A/T, etc.) and identified whether it occurred in CpG context – wher*e methylated cytosines are more likely prone to mutagenesis*. Note that SNPs in CpG context had been previously removed from the methylation tables to avoid spurious methylation calls (de Carvalho et al., 2023).

Because F_{ST} is strongly correlated with minor allele frequency (MAF) – with a mean Spearman correlation of 0.68 across all population pairs (all $P < 0.001$) – for each population pair, we binned the F_{ST} values based on MAF for each SNP within DMRs 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 MAF criterion for each comparison. Using permutation tests, we tested whether C/T or G/A SNPs in CpG contexts (*i.e.,* those most likely to result from 5mC → T deamination) exhibited higher F_{ST} values than these mutations in non-CpG contexts. Finally, we used Mantel tests to evaluate the relationship between F_{ST} in mutation-bias SNPs and geographical distance 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 predicted that DMRs would exhibit higher F_{ST} values than genome-wide levels (*i.e.,* accentuated F_{ST}). Consistent with this prediction, DMRs (genic and non-genic combined) and genic DMRs tended to show significantly higher F_{ST} relative to background levels across various *p-value* cut-offs, whereas non-genic DMRs did not (see Figs. S2-S4). For example, among DMRs delimited by $P<0.0004$, 24 out of 28 population pairs (86%) showed generally higher F_{ST} values for all DMRs (genic and non-genic combined) and genic DMRs compared to the null expectations ($P < 0.001$, binomial test; Figs. S2-S3). Within this group, 14 individual population comparisons also exhibited significantly higher F_{ST} in DMRs relative to the genomic background ($P<0.05$; permutation tests; Figs. S2-S3). In these 14 comparisons, DMRs exhibited an average of 2.3-fold higher F_{ST} than the background (95% CI: 2.2x – 2.5x), while the remaining comparisons showed a mean of 1.3-fold (95% CI: 1.0x – 1.6x). Taken together, these results indicate that DMRs are associated with accentuated F_{ST}, but more strongly so for some population comparisons than others.

Similar patterns were observed when analyzing the overlap between DMRs and genomic regions within the top 5% F_{ST} values cross population comparisons (Fig. 3B; Supporting Information). The collective findings provide initial evidence that DMRs are associated with regions of accentuated F_{ST}. We next examined three different hypotheses to explain such a pattern.

3.2. DMRs show increasing genetic differentiation with geographical distance

To explain the observed pattern of accentuated F_{ST} in DMRs – particularly its presence in some some population comparisons but not others – we tested the hypothesis of a balance between selection and gene flow. Specifically, we predicted that the magnitude of higher F_{ST} in DMRs relative to the genomic background would increase with geographical distance between populations. This would indicate an enrichment of the association between DMRs and accentuated F_{ST} at larger geographical distances (Fig. 1, Prediction 1). This expectation follows from the idea that reduced gene flow allows selection to drive greater genetic differentiation within localized regions of the genome.

Consistent with this prediction, the x-fold difference in F_{ST} between DMRs and the genomic background increased significantly with greater geographical distance between population pairs (r=0.76, P<0.001; Mantel test; Fig. 3). At the largest distances, DMRs exhibited threefold higher F_{ST} than the genomic background (~3.0x), compared to ~1.0x at the smallest distances. This geographic pattern remained consistent when only genic DMRs were analyzed (r = 0.75, P = 0.001; Mantel test; Fig. S5), with similar ~3.0-fold higher F_{ST} at the largest distances. In contrast, non-genic DMRs displayed no such geographical pattern (r= -0.38; P=0.963; Mantel test), instead showing lower F_{ST} values than the genomic background at the largest distances (~0.5x; Fig. S6).

3.3. Host-plant ecotype and climatic differences are not strong predictors of the DMR-F_{ST} association

We subsequently evaluated the potential sources of selection acting on DMRs (either directly or indirectly). Specifically, we assessed whether environmental factors (*e.g.,* host-plant ecotype and climate) were associated with patterns of accentuated F_{ST} in DMRs, beyond their association with geographical distance. Our BLMM results indicated that the model combining geographical and climatic PC1 distances best explained variation in x-fold F_{ST} difference in DMRs across population pairs. Among predictors, geographical distance had the strongest and most credible effects (β_{GEOG}=0.93 [0.61, 1.24 95% equal-tail probability interval, ETPI]); while climatic distances showed a more moderate and negative effect (β_{PC1}= -0.30 [-0.58, -0.02, 95% ETPI]; Table S1). In other words, whereas the association between accentuated F_{ST} and DMRs increased with geographical distance, it actually declines with climatic distance. The second best model combined geographical, PC1 and PC2 distances, with PC2 showing a weak effect, but in the same positive direction as geographical distance and with credible intervals crossing zero (β_{GEOG}=0.85 [0.38, 1.32]; β_{PC1}= -0.30 [-0.59, -0.02]; β_{PC2}= 0.10 [-0.33, 0.50], 95% ETPI). Contrary to our expectations, these analyses further suggest that accentuated F_{ST} 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 complementary prediction: that allele-frequency changes within DMRs would be more extreme than genome-wide expectations in a field experiment. Consistent with this prediction, although only weakly, allele-frequency changes in DMRs (designated by a $P<0.0004$) were marginally higher than expected by chance (1.1x, $P=0.091$, randomization test). Applying a more stringent cut-off ($P<0.0001$) yielded a slightly stronger and statistically significant genetic response relative to null expectations (1.2x, $P=0.045$, randomization test; Fig. 4). Although modest, these effects are are noteworthy, given that this short-term experiment at a single site likely under-represents the broader selective landscape experienced by these organisms. In contrast, we observed no significant allele-frequency change in DMRs when analyzing transplants between different host-plant species were analyzed separately (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 F_{ST} in DMRs is affected by reduced recombination, we employed three different proxies. 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. Across all population comparisons and *p-value c*ut-offs, mean heterozygosity within DMRs did not significantly differ from genome-wide expectations (all $P > 0.10$, randomization tests). Across the 28 population pairs and 10 p-value cut-offs, mean heterozygosity in DMRs ranged from 0.92x to 1.04x relative to the genomic background, with an overall mean of 0.98x (Fig. S12). Third, we estimated LD among the genetic variants within DMRs for each population. In seven of eight populations, LD within DMRs was comparable to null expectations (Table S2). Together, these findings do not support the hypothesis that reduced recombination strongly underlies the observed pattern of accentuated F_{ST} in DMRs.

3.6. No significant evidence of methylation mutagenic effects explaining accentuated F_{ST} *in DMRs*

Lastly, we tested the hypothesis that the mutagenic effects of methylation contribute to the association between accentuated F_{ST} and 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 marginally higher median F_{ST} than comparable mutations outside CpG context at MAF ≤ 0.05, though this difference was not statistically significant (1.28x, $P=0.256$, permutation tests; Fig. 5). Additionally, the F_{ST} values for these SNPs were not significantly associated with geographical distance (Table S3). One SNP (lg1_scaf1290:791133) showed a significant association between F_{ST} and geographical distance (Mantel test $r = 0.85$, $P = 0.022$), but its F_{ST} values remained below 0.04, even at the largest geographical distances.

When MAF was not controlled for, median F_{ST} of SNPs putatively affected by methylation’s mutagenic effect did not significantly differ from other C/T and G/A SNPs (1.0x, $P=0.643$, permutation tests). Notably, some C/T and G/A SNPs showed elevated F_{ST} values in some population comparisons, whereas SNPs subject to methylation’s mutagenic effects did not (Fig. 5). Overall, our results do not support the hypothesis that methylation’s mutagenic effects strongly explains the accentuated F_{ST} observed in DMRs.

4. Discussion

Genetic differentiation underlies population divergence and speciation. Yet, genetic differentiation – often quantified by the F_{ST} metric – 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), F_{ST} 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 on population genetic drivers of genetic differentiation, the association between DNA methylation and fine-scale genetic differentiation remains poorly studied. Our study bridges this gap by investigating associations between DNA methylation patterns and genomic regions of accentuated F_{ST} between populations of *T. cristinae* stick insects. Our findings demonstrate an association between genetic and epigenetic divergence, particularly for geographically distant population pairs. The results suggest a potential role for DNA methylation in speciation, though further research is needed to determine the underlying mechanisms. The results are summarized in Table 2, and discussion of our main findings follows.

4.1. DMRs tend to exhibit accentuated F_{ST}

We began our investigation by testing the hypothesis that genomic regions that differ in DNA methylation between populations are associated with regions of accentuated genetic differentiation. Our findings support this hypothesis, by showing that DMRs tended to show significantly higher F_{ST} values compared to the genomic background, but with effects that vary among populations spanning different degrees of geographical and baseline genetic divergence. This result suggests that regions exhibiting differential methylation could be more likely to diverge genetically than other regions of the genome. Our results are consistent with the few 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 significant association between DNA methylation differences and accentuated F_{ST} 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 environmentally-induced regulation of gene activity (Pavey et al., 2010; Smith & Ritchie, 2013).

4.2. Patterns of accentuated F_{ST} *in DMRs is best explained by selection-gene flow balance*

We found that the magnitude of the association between DMRs and accentuated F_{ST} increased with greater geographical distance between population pairs – a pattern driven primarily by genic DMRs, but not observed in non-genic DMRs. This trend suggests that reduced gene flow and / or increased divergent selection in geographically isolated populations may facilitate genetic divergence in DMR-associated loci, consistent with the selection-gene flow balance hypothesis. Under this model, the effects of selection on differentially methylated genomic regions intensifies as gene flow declines or as divergent selection increases with greater geographical isolation. Past work has shown that gene flow declines with increased geographical distance, whereas the possibility of increased divergent selection with increased distance remains more speculative (Nosil et al., 2012). Consistent with this interpretation, we found that DMRs exhibited marked allele-frequency changes in a single-generation selection experiment. Together, these findings suggest that reduced gene flow and selection may contribute to elevate F_{ST} in DMRs.

While genetic drift could theoretically elevate F_{ST}, it is unlikely to entirely explain the observed patterns of accentuated F_{ST} in DMRs because: (1) drift typically produces genome-wide differentiation, not localized patterns of accentuated F_{ST}; (2) the notable patterns of accentuated F_{ST} in genic DMRs (*i.e.,* functional regions) with geographical distance is unlikely under neutral processes; and (3) that non-genic DMRs did not show greater DMR-F_{ST} association with geography, further supporting selective mechanisms. Therefore, while drift may contribute to some background signal, the observed patterns can be better explained by a selection-gene flow balance.

4.3. DMR-F_{ST} *association is likely influenced by environmental factors beyond host plant and climate*

To identify potential sources of selection, we evaluated the contributions of host-plant and climatic variation in explaining the observed pattern of progressively higher F_{ST} in DMRs with increasing geographical distance. This pattern was best explained by a combination of geographical distance and climatic differences summarized by PC1. However, climate differences along PC1 were moderately but negatively associated with such a pattern (Table S1), suggesting that populations in divergent climates actually exhibit reduced genetic differentiation in DMRs. Thus, although climate variation may contribute to patterns of accentuated F_{ST} in DMRs, it does so in a direction inconsistent with expectations under a selection-gene flow balance. Additionally, while climate PC2 showed a weak positive association with accentuated F_{ST} in DMRs, there was considerable uncertainty in its contribution as estimated by the BLMM.

Given that the DMRs are associated with host-plant ecotype, we expected host-plant species to contribute to the geographical pattern of genetic differentiation in DMRs. Unexpectedly, we found no significant association between that host-plant ecotype and this geographical pattern. One possibility is that methylation patterns in DMR are primarily induced by host-plant environments (*e.g.,* the nutritional content or the stress imposed by each habitat), independent of geography or gene flow. Alternatively, methylation differences in DMR could 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). In summary, while our results support a role of a selection-gene flow balance in shaping genetic differentiation in DMRs, the specific sources of selection remain unclear.

It is also important to note that selection may operate through multiple mechanisms. For example, it may act on methylation-influenced phenotypes (*e.g.,* Schmid et al., 2018) in LD with genetic variants. Alternatively, selection may 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 genotype-methylation interactions – patterns that may not be detected through genetic data alone (Adrian-Kalchhauser et al., 2020; Morgan et al., 1999). These scenarios 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). As such, future studies should focus in not only identifying the sources of selection in DMRs, but also the target of selection (see discussion below). Regardless of the underlying mechanisms, the processes affecting DMRs appear to amplify – or at least better coincide with – genetic differentiation in the context of reduced gene flow.

4.3. Little support for the reduced recombination and mutagenic effects hypotheses

We evaluated two alternative (but not mutually-exclusive) hypotheses to the selection-gene flow balance, but we did not find support for either. First, we found no significant association between DMRs and any proxy used to estimate reduced recombination. This suggests that the patterns of accentuated F_{ST} in DMRs are unlikely to result from background selection in reduced recombination regions. 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 high-resolution recombination map for *T. cristinae* would allow directly testing of how DNA methylation, genetic differentiation, and reduced recombination interact.

Second, we found no evidence that potential mutagenic effects of methylation at CpG sites could underlie the observed accentuated F_{ST} in DMRs. If this hypothesis held, as shown in sticklebacks and *Coregonus* whitefish (Ord et al., 2023; Venney et al., 2024), we would expect C/T and G/A SNPs in CpG contexts (prone to deamination of methylated cytosines) to show elevated F_{ST}. However, F_{ST} in these SNPs did not differ significantly from other contexts, potentially due their low MAF. This suggests that while methylated CpGs may have a higher mutation rate (Holliday & Grigg, 1993; Ossowski et al., 2010), such mutations are likely rare and do not commonly reach high frequencies that would contribute significantly to genetic differentiation within DMRs. Thus, the resolution of our analysis was possibly constrained by the nature of the data in our study system (*i.e.,* low MAF). Future studies should pair WGS and WGBS from the same individuals to more precisely evaluate the association between DNA methylation and genetic mutations. Taken together, these results suggest that neither reduced recombination nor methylation-induced mutation sufficiently explain the patterns of accentuated F_{ST} in DMRs.

4.3. Absence of host-plant effects on the selection-gene flow patterns

The selection-gene flow balance hypothesis emerged as the most consistent explanation for the patterns of accentuated F_{ST} observed in DMRs. However, despite the fact that DMRs reflect methylation differences between host-plant ecotypes, we found no evidence that host-plant species explained the pattern of increased DMR-F_{ST} association with greater geographical distance. Similarly, allele-frequency changes in DMRs during the transplant experiment were also unrelated to host-plant species. In this section, we explore potential explanations for these unexpected findings.

One possible explanation is that DMRs overlap with pleiotropic genes involved in responding to multiple environmental cues. Host plants habitat may induce methylation changes in these pleiotropic genes via differences in chemical composition, nutrient availability, or stress signals (Chen et al., 2019; Gupta & Nair, 2022), leading to consistent methylation differences across ecotypes – regardless of geographical proximity or gene flow. While such 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). However, if these same genes are also targets selection from broader environmental pressures (*e.g.,* aspects of the climate or climatic impacts on host plants not captured by our climate variables), this could lead to genetic differentiation at DMR-associated loci. This way, the co-occurrence of methylation differences and accentuated F_{ST} may reflect a scenario in which pleiotropic genes integrate methylation responses to host plants with genetic adaptation to other environmental drivers. explaining the co-occurrence of methylation differences and accentuated.

An alternative explanation is that DMR-associated loci are in LD with regions under selection from environmental gradients unrelated to host-plant use. In other words, selective pressures acting on the loci unrelated to host plants could indirectly drive genetic divergence at the DMR-associated loci, creating a pattern where DMRs exhibit accentuated $F_{ST.}$. While this mechanism could explain the patterns of accentuated F_{ST} in DMRs, it remains unclear why genetic variants associated with DMRs would consistently exhibit strong LD with selected loci unrelated to host-plant habitat.

Although these scenarios are plausible, it is also possible that the absence of host-plant effects in our results reflects limitations of the available data (de Carvalho et al., 2023). Despite capturing biologically meaningful methylation differences, the DMR dataset was based on small sample sizes, pooling different tissues, and had restricted genomic coverage, likely limiting the number of DMRs detected. Additionally, our analyses integrated data from three different sources and different geographical scales (i.e., DMRs, SNPs from WGS, and transplant experiment), which potentially complicates the identification of specific mechanisms. Future studies should address these limitations by using larger sample sizes and paired WGS and WGBS data from the same individuals to enable a clearer understanding of the mechanisms underlying the selection-gene flow balance and its role in linking DNA methylation to accentuated $F_{ST.}$.

4.4. Future directions for understanding the relationship between DNA methylation and accentuated genetic differences in speciation

While our findings demonstrate that differences in DNA methylation are associated regions of pronounced genetic differentiation – and that a selection-gene flow balance best explains these patterns – several key questions remain. In particular, future studies in *Timema* should aim to (1) directly test the extent to which host-plant habitats induce methylation changes, and (2) determine how such changes influence phenotype and fitness. Even if these methylation differences reflect generalized stress responses (*e.g.*, Gupta & Nair, 2025), they may still have ecological and evolutionary relevance, especially if they co-occur with loci under selection. To further clarify the relationship between DNA methylation and genetic differentiation, it is also essential to distinguish genetically controlled methylation (*e.g.*, via cis-regulatory variants or local genetic context) and environmentally induced changes (Husby, 2022). Integrating methylation data with allele-frequency shifts and selection signals in the same individuals would provide a powerful framework for understanding causality and the role of epigenetic mechanisms in genetic differentiation and population divergence.

Moreover, future studies should evaluate whether the higher-level phenotypes associated with methylation variation are subject to selection, and clarify the form of such selection (*e.g.*, stabilizing, directional or divergent selection). The extent to which DNA methylation is stably inherited across generations will also influence its potential role in driving or reinforcing genetic differentiation. Depending on this stability, methylation may act as a short-term plastic response or become integrated into long-term evolutionary trajectories via genetic assimilation or epigenetic selection (Gopalan-Nair et al., 2024; Schmid et al., 2018; Stajic et al., 2019). Pursuing these directions will deepen our understanding of the complex interplay between genetic and epigenetic variation and offer new perspectives on the roles of DNA methylation in population divergence, adaptation and speciation.

4.5. Conclusion

DNA methylation can respond to environmental cues and modulate gene expression, potentially influencing traits under selection. This makes methylation a candidate mechanism contributing to population-level genetic differentiation. In this study, we establish an association between DNA methylation and accentuated F_{ST} in populations pairs with varying levels of divergence, showing evidence consistent with a balance between selection and gene flow. However, the specific targets of selection and underlying mechanisms remain unclear. While our results support that epigenetic variation contributes to genetic differentiation, the full understanding of how DNA methylation contributes to population divergence is still in its early stages, representing a key frontier in population epigenetics research. Overall, our results suggest that epigenetic variation may not only provide insights on regions of accentuated genetic differentiation, but also add new layers of complexity to our understanding of population divergence. Beyond serving as a marker of pronounced 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.

Acknowledgments: PN and CFdC were supported by Royal Society of London (RG140369) and FAPESP 2020/07556-8. The support and resources from the Center for High Performance Computing at the University of Utah are gratefully acknowledged.

Author contributions: CFdC, PN and ZG conceived the idea and wrote the manuscript. CFdC and ZG conducted analyses. All authors contributed to writing and revision.

Competing Interest Statement: The authors declare no competing interests.

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). The scripts can be accessed in the link: https://zenodo.org/records/14566136?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjhjMGY0NzU4LWQ1NzctNDYwZi1hMDRmLTAwMDRkOTFhZTA4NyIsImRhdGEiOnt9LCJyYW5kb20iOiI5OGEyY2QzYmJhMmFkYzNiYTUzZjExMGJjNzYyYjJlYiJ9.Z5GBl5UYSyQ0iXCwCuw5XYzUGnb_ZuB1ctynxZaouzvtMQu90JeGk4tiDKYFy9aC6rxdqyZu6cUJIwyjDKS23w

References

Aagaard, A., Liu, S., Tregenza, T., Braad Lund, M., Schramm, A., Verhoeven, K. J. F., Bechsgaard, J., & Bilde, T. (2022). Adapting to climate with limited genetic diversity: Nucleotide, DNA methylation and microbiome variation among populations of the social spider Stegodyphus dumicola. *Molecular Ecology*, *31*(22), 5765–5783. https://doi.org/10.1111/mec.16696

Adrian-Kalchhauser, I., Sultan, S. E., Shama, L. N. S., Spence-Jones, H., Tiso, S., Keller Valsecchi, C. I., & Weissing, F. J. (2020). Understanding “Non-genetic” Inheritance: Insights from Molecular-Evolutionary Crosstalk. *Trends in Ecology and Evolution*, *35*(12), 1078–1089. https://doi.org/10.1016/j.tree.2020.08.011

Begun, D. J., & Aquadro, C. F. (1992). Levels of naturally occurring DNA polymorphism correlate with recombination rates in D. melanogaster. *Nature*, *356*(6369), 519–520. https://doi.org/10.1038/356519a0

Bewick, A. J., Vogel, K. J., Moore, A. J., & Schmitz, R. J. (2017). Evolution of DNA methylation across insects. *Molecular Biology and Evolution*, *34*(3), 654–665. https://doi.org/10.1093/molbev/msw264

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes & Development*, *16*(1), 6–21. https://doi.org/10.1101/gad.947102

Boideau, F., Richard, G., Coriton, O., Huteau, V., Belser, C., Deniot, G., Eber, F., Falentin, C., Ferreira de Carvalho, J., Gilet, M., Lodé-Taburel, M., Maillet, L., Morice, J., Trotoux, G., Aury, J.-M., Chèvre, A.-M., & Rousseau-Gueutin, M. (2022). Epigenomic and structural events preclude recombination in Brassica napus. *New Phytologist*, *234*(2), 545–559. https://doi.org/10.1111/nph.18004

Boman, J., Qvarnström, A., & Mugal, C. F. (2024). Regulatory and evolutionary impact of DNA methylation in two songbird species and their naturally occurring F1 hybrids. *BMC Biology*, *22*(1), 124. https://doi.org/10.1186/s12915-024-01920-2

Booker, T. R., Yeaman, S., & Whitlock, M. C. (2020). Variation in recombination rate affects detection of outliers in genome scans under neutrality. *Molecular Ecology*, *29*(22), 4274–4279. https://doi.org/10.1111/mec.15501

Burri, R., Nater, A., Kawakami, T., Mugal, C. F., Olason, P. I., Smeds, L., Suh, A., Dutoit, L., Bureš, S., Garamszegi, L. Z., Hogner, S., Moreno, J., Qvarnström, A., Ružić, M., Sæther, S. A., Sætre, G. P., Török, J., & Ellegren, H. (2015). Linked selection and recombination rate variation drive the evolution of the genomic landscape of differentiation across the speciation continuum of Ficedula flycatchers. *Genome Research*, *25*(11), 1656–1665. https://doi.org/10.1101/gr.196485.115

Chaturvedi, S., Gompert, Z., Feder, J. L., Osborne, O. G., Muschick, M., Riesch, R., Soria-Carrasco, V., & Nosil, P. (2022). Climatic similarity and genomic background shape the extent of parallel adaptation in Timema stick insects. *Nature Ecology and Evolution*, *6*, Article 6. https://doi.org/10.1038/s41559-022-01909-6

Chen, W., Dong, Y., Lin, L., Saqib, H. S. A., Ma, X., Xu, X., Zhang, L., Jing, X., Peng, L., Wang, Y., Vasseur, L., He, W., & You, M. (2019). Implication for DNA methylation involved in the host transfer of diamondback moth, Plutella xylostella (L.). *Archives of Insect Biochemistry and Physiology*, *102*(2), e21600. https://doi.org/10.1002/arch.21600

Clarke, R. T., Rothery, P., & Raybould, A. F. (2002). Confidence limits for regression relationships between distance matrices: Estimating gene flow with distance. *Journal of Agricultural, Biological, and Environmental Statistics*, *7*(3), 361–372. https://doi.org/10.1198/108571102320

Comeault, A. A., Soria-Carrasco, V., Gompert, Z., Farkas, T. E., Buerkle, C. A., Parchman, T. L., & Nosil, P. (2014). Genome-wide association mapping of phenotypic traits subject to a range of intensities of natural selection in Timema cristinae. *The American Naturalist*, *183*(5), 711–727. https://doi.org/10.1086/675497

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sinauer Associates.

Cruickshank, T. E., & Hahn, M. W. (2014). Reanalysis suggests that genomic islands of speciation are due to reduced diversity, not reduced gene flow. *Molecular Ecology*, *23*(13), 3133–3157. https://doi.org/10.1111/mec.12796

Darwin, C. (1859). *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. London: Murray.

de Carvalho, C. F., Slate, J., Villoutreix, R., Soria-Carrasco, V., Riesch, R., Feder, J. L., Gompert, Z., & Nosil, P. (2023). DNA methylation differences between stick insect ecotypes. *Molecular Ecology*, *32*(24), 6809–6823. https://doi.org/10.1111/mec.17165

Dobzhansky, T. (1937). *Genetics and the origin of species* (1st ed.). Columbia University Press.

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M. S., Osborne, E. J., Casale, F. P., Drewe, P., Kahles, A., Jean, G., Vilhjálmsson, B., Jagoda, J., Irez, S., Voronin, V., Song, Q., Long, Q., Rätsch, G., Stegle, O., Clark, R. M., & Nordborg, M. (2015). DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. *eLife*, *4*(MAY), e05255. https://doi.org/10.7554/eLife.05255

Duncan, E. J., Gluckman, P. D., & Dearden, P. K. (2014). Epigenetics, plasticity, and evolution: How do we link epigenetic change to phenotype? *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *322*(4), 208–220. https://doi.org/10.1002/jez.b.22571

Ellegren, H., & Galtier, N. (2016). Determinants of genetic diversity. *Nature Reviews Genetics*, *17*(7), 422–433. https://doi.org/10.1038/nrg.2016.58

Fargeot, L., Loot, G., Prunier, J. G., Rey, O., Veyssière, C., & Blanchet, S. (2021). Patterns of Epigenetic Diversity in Two Sympatric Fish Species: Genetic vs. Environmental Determinants. *Genes*, *12*(1), Article 1. https://doi.org/10.3390/genes12010107

Feder, J. L., Egan, S. P., & Nosil, P. (2012). The genomics of speciation-with-gene-flow. *Trends in Genetics*, *28*(7), 342–350. https://doi.org/10.1016/j.tig.2012.03.009

Fitz-James, M. H., & Cavalli, G. (2022). Molecular mechanisms of transgenerational epigenetic inheritance. *Nature Reviews Genetics*, *23*(6), Article 6. https://doi.org/10.1038/s41576-021-00438-5

Foust, C. M., Preite, V., Schrey, A. W., Alvarez, M., Robertson, M. H., Verhoeven, K. J. F., & Richards, C. L. (2016). Genetic and epigenetic differences associated with environmental gradients in replicate populations of two salt marsh perennials. *Molecular Ecology*, *25*(8), 1639–1652. https://doi.org/10.1111/mec.13522

Gompert, Z., Comeault, A. A., Farkas, T. E., Feder, J. L., Parchman, T. L., Buerkle, C. A., & Nosil, P. (2014). Experimental evidence for ecological selection on genome variation in the wild. *Ecology Letters*, *17*(3), 369–379. https://doi.org/10.1111/ele.12238

Gompert, Z., Feder, J. L., & Nosil, P. (2022). Natural selection drives genome-wide evolution via chance genetic associations. *Molecular Ecology*, *31*(2), 467–481. https://doi.org/10.1111/mec.16247

Gompert, Z., Lucas, L. K., Buerkle, C. A., Forister, M. L., Fordyce, J. A., & Nice, C. C. (2014). Admixture and the organization of genetic diversity in a butterfly species complex revealed through common and rare genetic variants. *Molecular Ecology*, *23*(18), 4555–4573. https://doi.org/10.1111/mec.12811

Gopalan-Nair, R., Coissac, A., Legrand, L., Lopez-Roques, C., Pécrix, Y., Vandecasteele, C., Bouchez, O., Barlet, X., Lanois, A., Givaudan, A., Brillard, J., Genin, S., & Guidot, A. (2024). Changes in DNA methylation contribute to rapid adaptation in bacterial plant pathogen evolution. *PLOS Biology*, *22*(9), e3002792. https://doi.org/10.1371/journal.pbio.3002792

Guerrero-Bosagna, C. (2017). Evolution with no reason: A Neutral view on epigenetic changes, genomic variability, and evolutionary novelty. *BioScience*, *67*(5), 469–476. https://doi.org/10.1093/biosci/bix021

Gupta, A., & Nair, S. (2022). Heritable Epigenomic Modifications Influence Stress Resilience and Rapid Adaptations in the Brown Planthopper (Nilaparvata lugens). *International Journal of Molecular Sciences*, *23*(15), Article 15. https://doi.org/10.3390/ijms23158728

Gupta, A., & Nair, S. (2025). Epigenetic processes in insect adaptation to environmental stress. *Current Opinion in Insect Science*, *67*, 101294. https://doi.org/10.1016/j.cois.2024.101294

Harris, I., Jones, P. D., Osborn, T. J., & Lister, D. H. (2014). Updated high-resolution grids of monthly climatic observations—The CRU TS3.10 Dataset. *International Journal of Climatology*, *34*(3), 623–642. https://doi.org/10.1002/joc.3711

Heckwolf, M. J., Meyer, B. S., Häsler, R., Höppner, M. P., Eizaguirre, C., & Reusch, T. B. H. (2020). Two different epigenetic information channels in wild three-spined sticklebacks are involved in salinity adaptation. *Science Advances*, *6*(12). https://doi.org/10.1126/sciadv.aaz1138

Herrera, C. M., Medrano, M., & Bazaga, P. (2016). Comparative spatial genetics and epigenetics of plant populations: Heuristic value and a proof of concept. *Molecular Ecology*, *25*(8), 1653–1664. https://doi.org/10.1111/mec.13576

Hoffmann, A. A., & Rieseberg, L. H. (2008). Revisiting the Impact of Inversions in Evolution: From Population Genetic Markers to Drivers of Adaptive Shifts and Speciation? *Annual Review of Ecology, Evolution, and Systematics*, *39*(Volume 39, 2008), 21–42. https://doi.org/10.1146/annurev.ecolsys.39.110707.173532

Hohenlohe, P. A., Bassham, S., Currey, M., & Cresko, W. A. (2012). Extensive linkage disequilibrium and parallel adaptive divergence across threespine stickleback genomes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *367*(1587), 395–408. https://doi.org/10.1098/rstb.2011.0245

Holliday, R., & Grigg, G. W. (1993). DNA methylation and mutation. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *285*(1), 61–67. https://doi.org/10.1016/0027-5107(93)90052-H

Hu, J., & Barrett, R. D. H. (2023). The role of plastic and evolved DNA methylation in parallel adaptation of threespine stickleback (Gasterosteus aculeatus). *Molecular Ecology*, *32*(7), 1581–1591. https://doi.org/10.1111/mec.16832

Husby, A. (2022). Wild epigenetics: Insights from epigenetic studies on natural populations. *Proceedings of the Royal Society B: Biological Sciences*, *289*(1968). https://doi.org/10.1098/rspb.2021.1633

Kelley, J. L., Tobler, M., Beck, D., Sadler-Riggleman, I., Quackenbush, C. R., Arias Rodriguez, L., & Skinner, M. K. (2021). Epigenetic inheritance of DNA methylation changes in fish living in hydrogen sulfide–rich springs. *Proceedings of the National Academy of Sciences*, *118*(26), e2014929118. https://doi.org/10.1073/pnas.2014929118

Kirkpatrick, M. (2010). How and why chromosome inversions evolve. *PLoS Biology*, *8*(9). https://doi.org/10.1371/journal.pbio.1000501

Law, J. A., & Jacobsen, S. E. (2010). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews Genetics*, *11*(3), 204–220. https://doi.org/10.1038/nrg2719

Lea, A. J., Tung, J., & Zhou, X. (2015). A Flexible, Efficient Binomial Mixed Model for Identifying Differential DNA Methylation in Bisulfite Sequencing Data. *PLoS Genetics*, *11*(11), 1–31. https://doi.org/10.1371/journal.pgen.1005650

Lindtke, D., Lucek, K., Soria-Carrasco, V., Villoutreix, R., Farkas, T. E., Riesch, R., Dennis, S. R., Gompert, Z., & Nosil, P. (2017). Long-term balancing selection on chromosomal variants associated with crypsis in a stick insect. *Molecular Ecology*, *26*(22), Article 22. https://doi.org/10.1111/mec.14280

Liu, Y., Sarkar, A., Kheradpour, P., Ernst, J., & Kellis, M. (2017). Evidence of reduced recombination rate in human regulatory domains. *Genome Biology*, *18*(1), 193. https://doi.org/10.1186/s13059-017-1308-x

Lucek, K., Gompert, Z., & Nosil, P. (2019). The role of structural genomic variants in population differentiation and ecotype formation in *Timema cristinae* walking sticks. *Molecular Ecology*, *28*(6), 1224–1237. https://doi.org/10.1111/mec.15016

Marques, D. A., Jones, F. C., Di Palma, F., Kingsley, D. M., & Reimchen, T. E. (2018). Experimental evidence for rapid genomic adaptation to a new niche in an adaptive radiation. *Nature Ecology & Evolution*, *2*(7), 1128–1138. https://doi.org/10.1038/s41559-018-0581-8

Mayr, E. (1963). *Animal species and evolution*. Harvard University Press.

Medrano, M., Herrera, C. M., & Bazaga, P. (2014). Epigenetic variation predicts regional and local intraspecific functional diversity in a perennial herb. *Molecular Ecology*, *23*(20), 4926–4938. https://doi.org/10.1111/mec.12911

Melamed-Bessudo, C., & Levy, A. A. (2012). Deficiency in DNA methylation increases meiotic crossover rates in euchromatic but not in heterochromatic regions in Arabidopsis. *Proceedings of the National Academy of Sciences*, *109*(16), E981–E988. https://doi.org/10.1073/pnas.1120742109

Morgan, H. D., Sutherland, H. G. E., Whitelaw, E., & Martin, D. I. K. (1999). Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics*, *23*(3), 314–318. https://doi.org/10.1038/15490

Nadeau, N. J., Whibley, A., Jones, R. T., Davey, J. W., Dasmahapatra, K. K., Baxter, S. W., Quail, M. A., Joron, M., ffrench-Constant, R. H., Blaxter, M. L., Mallet, J., & Jiggins, C. D. (2012). Genomic islands of divergence in hybridizing Heliconius butterflies identified by large-scale targeted sequencing. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *367*(1587), 343–353. https://doi.org/10.1098/rstb.2011.0198

Noor, M. A. F., & Bennett, S. M. (2009). Islands of speciation or mirages in the desert? Examining the role of restricted recombination in maintaining species. *Heredity*, *103*(6), 439–444. https://doi.org/10.1038/hdy.2009.151

Noor, M. A. F., Gratos, K. L., Bertucci, L. A., & Reiland, J. (2001). Chromosomal inversions and the reproductive isolation of species. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(21), 12084–12088. https://doi.org/10.1073/pnas.221274498

Nosil, P. (2007). Divergent host plant adaptation and reproductive isolation between ecotypes of Timema cristinae walking sticks. *The American Naturalist*, *169*(2), 151–162. https://doi.org/10.1086/510634

Nosil, P., & Crespi, B. J. (2006). Experimental evidence that predation promotes divergence in adaptive radiation. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(24), 9090–9095. https://doi.org/10.1073/pnas.0601575103

Nosil, P., Feder, J. L., & Gompert, Z. (2021a). Biodiversity, resilience and the stability of evolutionary systems. *Current Biology*, *31*(19), Article 19. https://doi.org/10.1016/j.cub.2021.01.022

Nosil, P., Feder, J. L., & Gompert, Z. (2021b). How many genetic changes create new species? *Science*, *371*(6531), 777–779. https://doi.org/10.1126/science.abf6671

Nosil, P., Gompert, Z., De Carvalho, C., Villoutreix, R., Zamorano, L., Sinclair-Waters, M., Planidin, N., Parchman, T., & Feder, J. (2024). Evolution repeats itself in replicate long-term studies in the wild. *Science Advances*, *10*(21). https://doi.org/10.1126/sciadv.adl3149

Nosil, P., Gompert, Z., Farkas, T. E., Comeault, A. A., Feder, J. L., Buerkle, C. a., & Parchman, T. L. (2012). Genomic consequences of multiple speciation processes in a stick insect. *Proceedings of the Royal Society B: Biological Sciences*, *June*, Article June. https://doi.org/10.1098/rspb.2012.0813

Nosil, P., & Sandoval, C. P. (2008). Ecological niche dimensionality and the evolutionary diversification of stick insects. *PLoS ONE*, *3*(4). https://doi.org/10.1371/journal.pone.0001907

Nosil, P., Villoutreix, R., de Carvalho, C. F., Farkas, T. E., Soria-Carrasco, V., Feder, J. L., Crespi, B. J., & Gompert, Z. (2018). Natural selection and the predictability of evolution in Timema stick insects. *Science (New York, N.Y.)*, *359*(6377), Article 6377. https://doi.org/10.1126/science.aap9125

Oksanen, J., Simpson, G., Blanchet, F., Kindt, R., Legendre, P., Minchin, P., & et al. (2022). *The vegan package* [Computer software]. https://cran.r-project.org/package=vegan

Ord, J., Gossmann, T. I., & Adrian-Kalchhauser, I. (2023). High Nucleotide Diversity Accompanies Differential DNA Methylation in Naturally Diverging Populations. *Molecular Biology and Evolution*, *40*(4), msad068. https://doi.org/10.1093/molbev/msad068

Ossowski, S., Schneeberger, K., Lucas-Lledó, J. I., Warthmann, N., Clark, R. M., Shaw, R. G., Weigel, D., & Lynch, M. (2010). The rate and molecular spectrum of spontaneous mutations in arabidopsis thaliana. *Science*, *327*(5961), 92–94. https://doi.org/10.1126/science.1180677

Pavey, S. A., Collin, H., Nosil, P., & Rogers, S. M. (2010). The role of gene expression in ecological speciation. *Annals of the New York Academy of Sciences*, *1206*(1), 110–129. https://doi.org/10.1111/j.1749-6632.2010.05765.x

Peñuela, M., Gallo-Franco, J. J., Finke, J., Rocha, C., Gkanogiannis, A., Ghneim-Herrera, T., & Lorieux, M. (2022). Methylation in the CHH Context Allows to Predict Recombination in Rice. *International Journal of Molecular Sciences*, *23*(20), Article 20. https://doi.org/10.3390/ijms232012505

Pfennig, D. W., Wund, M. A., Snell-Rood, E. C., Cruickshank, T., Schlichting, C. D., & Moczek, A. P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends in Ecology and Evolution*, *25*(8), 459–467. https://doi.org/10.1016/j.tree.2010.05.006

Planidin, N. P., de Carvalho, C. F., Feder, J. L., Gompert, Z., & Nosil, P. (2022). Epigenetics and reproductive isolation: A commentary on Westram et al., 2022. *Journal of Evolutionary Biology*, *35*(9), 1188–1194. https://doi.org/10.1111/jeb.14033

Platt, A., Gugger, P. F., Pellegrini, M., & Sork, V. L. (2015). Genome-wide signature of local adaptation linked to variable CpG methylation in oak populations. *Molecular Ecology*, *24*(15), 3823–3830. https://doi.org/10.1111/mec.13230

Plummer, Martyn. (2018). rjags: Bayesian Graphical Models using MCMC. *R Package Version 4-8.* https://cran.r-project.org/package=rjags

Poelstra, J. W., Vijay, N., Bossu, C. M., Lantz, H., Ryll, B., Baglione, V., Unneberg, P., Wikelski, M., Grabherr, M. G., & Wolf, J. B. W. (2014). The genomic landscape underlying phenotypic integrity in face of gene flow in crows. *Science*, *344*(6190), 1410–1414.

Provataris, P., Meusemann, K., Niehuis, O., Grath, S., & Misof, B. (2018). Signatures of DNA methylation across insects suggest reduced DNA methylation levels in Holometabola.

Genome Biology and Evolution, *10*(March), 1185–1197. https://doi.org/10.1093/gbe/evy066/4943971

R Core Team. (2023). *R: A language and environment for statistical computing.* [Computer software]. https://www.r-project.org/

Ravinet, M., Faria, R., Butlin, R. K., Galindo, J., Bierne, N., Rafajlović, M., Noor, M. A. F., Mehlig, B., & Westram, A. M. (2017). Interpreting the genomic landscape of speciation: A road map for finding barriers to gene flow. *Journal of Evolutionary Biology*, *30*(8), 1450–1477. https://doi.org/10.1111/jeb.13047

Richards, E. J. (2006). Inherited epigenetic variation—Revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401. https://doi.org/10.1038/nrg1834

Riesch, R., Muschick, M., Lindtke, D., Villoutreix, R., Comeault, A. A., Farkas, T. E., Lucek, K., Hellen, E., Soria-Carrasco, V., Dennis, S. R., De Carvalho, C. F., Safran, R. J., Sandoval, C. P., Feder, J., Gries, R., Crespi, B. J., Gries, G., Gompert, Z., & Nosil, P. (2017). Transitions between phases of genomic differentiation during stick-insect speciation. *Nature Ecology and Evolution*, *1*(4), Article 4. https://doi.org/10.1038/s41559-017-0082

Roberts, S. B., & Gavery, M. R. (2012). Is there a relationship between DNA methylation and phenotypic plasticity in invertebrates? *Frontiers in Physiology*, *2 JAN*(January), 1–5. https://doi.org/10.3389/fphys.2011.00116

Sandoval, C. P. (1994). The effects of the relative geographic scales of gene flow and selection on morph frequencies in the walking-stick Timema cristinae. *Evolution*, *48*(6), Article 6. https://doi.org/10.1111/j.1558-5646.1994.tb02220.x

Schluter, D. (2001). Ecology and the origin of species. *Trends in Ecology & Evolution*, *16*(7), 372–380. https://doi.org/10.1016/S0169-5347(01)02198-X

Schmid, M. W., Heichinger, C., Coman Schmid, D., Guthörl, D., Gagliardini, V., Bruggmann, R., Aluri, S., Aquino, C., Schmid, B., Turnbull, L. A., & Grossniklaus, U. (2018). Contribution of epigenetic variation to adaptation in Arabidopsis. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-06932-5

Seehausen, O., Butlin, R. K., Keller, I., Wagner, C. E., Boughman, J. W., Hohenlohe, P. a., Peichel, C. L., Saetre, G.-P., Bank, C., Brännström, Å., Brelsford, A., Clarkson, C. S., Eroukhmanoff, F., Feder, J. L., Fischer, M. C., Foote, A. D., Franchini, P., Jiggins, C. D., Jones, F. C., … Widmer, A. (2014). Genomics and the origin of species. *Nature Reviews Genetics*, *15*(3), 176–192. https://doi.org/10.1038/nrg3644

Sepers, B., Chen, R. S., Memelink, M., Verhoeven, K. J. F., & van Oers, K. (2023). Variation in DNA Methylation in Avian Nestlings Is Largely Determined by Genetic Effects. *Molecular Biology and Evolution*, *40*(4), msad086. https://doi.org/10.1093/molbev/msad086

Skvortsova, K., Iovino, N., & Bogdanović, O. (2018). Functions and mechanisms of epigenetic inheritance in animals. *Nature Reviews Molecular Cell Biology*, *19*(12), 774–790. https://doi.org/10.1038/s41580-018-0074-2

Smith, G., & Ritchie, M. G. (2013). How might epigenetics contribute to ecological speciation? *Current Zoology*, *59*(5), 686–696. https://doi.org/10.1016/j.cognition.2008.05.007

Smith, T. A., Martin, M. D., Nguyen, M., & Mendelson, T. C. (2016). Epigenetic divergence as a potential first step in darter speciation. *Molecular Ecology*, *25*(8), 1883–1894. https://doi.org/10.1111/mec.13561

Soria-Carrasco, V., Gompert, Z., Comeault, A. a., Farkas, T. E., Parchman, T. L., Johnston, J. S., Buerkle, C. A., Feder, J. L., Bast, J., Schwander, T., Egan, S. P., Crespi, B. J., & Nosil, P. (2014). Stick insect genomes reveal natural selection's role in parallel speciation. *Science (New York, N.Y.)*, *344*(6185), Article 6185. https://doi.org/10.1126/science.1252136

Stajic, D., Perfeito, L., & Jansen, L. E. T. (2019). Epigenetic gene silencing alters the mechanisms and rate of evolutionary adaptation. *Nature Ecology & Evolution*, *3*(3), Article 3. https://doi.org/10.1038/s41559-018-0781-2

Suzuki, M. M., & Bird, A. (2008). DNA methylation landscapes: Provocative insights from epigenomics. *Nature Reviews Genetics*, *9*(6), 465–476. https://doi.org/10.1038/nrg2341

Taudt, A., Colomé-Tatché, M., & Johannes, F. (2016). Genetic sources of population epigenomic variation. *Nature Reviews Genetics*, *17*(6), 319–332. https://doi.org/10.1038/nrg.2016.45

Tomkova, M., & Schuster-Böckler, B. (2018). DNA Modifications: Naturally More Error Prone? *Trends in Genetics*, *34*(8), 627–638. https://doi.org/10.1016/j.tig.2018.04.005

Turbek, S. P., Browne, M., Di Giacomo, A. S., Kopuchian, C., Hochachka, W. M., Estalles, C., Lijtmaer, D. A., Tubaro, P. L., Silveira, L. F., Lovette, I. J., Safran, R. J., Taylor, S. A., & Campagna, L. (2021). Rapid speciation via the evolution of pre-mating isolation in the Iberá Seedeater. *Science*, *371*(6536), eabc0256. https://doi.org/10.1126/science.abc0256

van der Graaf, A., Wardenaar, R., Neumann, D. A., Taudt, A., Shaw, R. G., Jansen, R. C., Schmitz, R. J., Colomé-Tatché, M., & Johannes, F. (2015). Rate, spectrum, and evolutionary dynamics of spontaneous epimutations. *Proceedings of the National Academy of Sciences*, *112*(21), 6676–6681. https://doi.org/10.1073/pnas.1424254112

Venney, C. J., Mérot, C., Normandeau, E., Rougeux, C., Laporte, M., & Bernatchez, L. (2024). Epigenetic and Genetic Differentiation Between *Coregonus* Species Pairs. *Genome Biology and Evolution*, *16*(2), evae013. https://doi.org/10.1093/gbe/evae013

Venney, C. J., Sutherland, B. J. G., Beacham, T. D., & Heath, D. D. (2021). Population differences in Chinook salmon (Oncorhynchus tshawytscha) DNA methylation: Genetic drift and environmental factors. *Ecology and Evolution*, *11*(11), 6846–6861. https://doi.org/10.1002/ece3.7531

Vernaz, G., Hudson, A. G., Santos, M. E., Fischer, B., Carruthers, M., Shechonge, A. H., Gabagambi, N. P., Tyers, A. M., Ngatunga, B. P., Malinsky, M., Durbin, R., Turner, G. F., Genner, M. J., & Miska, E. A. (2022). Epigenetic divergence during early stages of speciation in an African crater lake cichlid fish. *Nature Ecology & Evolution*, *6*(12), Article 12. https://doi.org/10.1038/s41559-022-01894-w

Via, S., & West, J. (2008). The genetic mosaic suggests a new role for hitchhiking in ecological speciation. *Molecular Ecology*, *17*(19), 4334–4345. https://doi.org/10.1111/j.1365-294X.2008.03921.x

Vickery, V. R. (1993). Revision of Timema Scudder (Phasmatoptera: Timematodea) including three new species. *The Canadian Entomolgist*, *125*(4), 657–692.

Wang, X., Werren, J. H., & Clark, A. G. (2016). Allele-Specific Transcriptome and Methylome Analysis Reveals Stable Inheritance and Cis-Regulation of DNA Methylation in Nasonia. *PLoS Biology*, *14*(7), 1–21. https://doi.org/10.1371/journal.pbio.1002500

Wolf, J. B. W., & Ellegren, H. (2017). Making sense of genomic islands of differentiation in light of speciation. *Nature Reviews Genetics*, *18*(2), 87–100. https://doi.org/10.1038/nrg.2016.133

Xia, J., Han, L., & Zhao, Z. (2012). Investigating the relationship of DNA methylation with mutation rate and allele frequency in the human genome. *BMC Genomics*, *13*(8), S7. https://doi.org/10.1186/1471-2164-13-S8-S7

Xu, G., Lyu, J., Li, Q., Liu, H., Wang, D., Zhang, M., Springer, N. M., Ross-Ibarra, J., & Yang, J. (2020). Evolutionary and functional genomics of DNA methylation in maize domestication and improvement. *Nature Communications*, *11*(1), Article 1. https://doi.org/10.1038/s41467-020-19333-4

Yagound, B., Remnant, E. J., Buchmann, G., & Oldroyd, B. P. (2020). Intergenerational transfer of DNA methylation marks in the honey bee. *Proceedings of the National Academy of*

Sciences of the United States of America, *117*(51), 32519–32527. https://doi.org/10.1073/pnas.2017094117

Table 1. Hypotheses and predictions.

Hypothesis	**Predictions**	**Why to expect it**
Selection-gene flow balance	1. DMRs exhibit progressively higher F_{ST} compared to the genomic background with increasing geographical distance between populations	Gene flow homogenizes genetic variation; reduced gene flow in isolated populations allows selection to increase genetic differentiation in DMRs
	2. Allele-frequency changes within DMRs are more extreme than other genomic regions	Extreme allele-frequency changes following environmental shift suggest natural selection on DNA methylation and/or genetic variation (these results speak to the selection component of selection-gene flow balance).
Reduced recombination (i.e., higher background selection)	1. DMRs overlap with structural variants (SV)	Reduced recombination is associated with higher background selection and restists homogenization by gene flow – which leads to greater genetic differentiation. SVs suppress recombination; low heterozygosity and/or high LD indicates reduced recombination
	2. DMRs occurs with regions of low heterozygosity	
	3. DMRs occurs in regions of high linkage disequilibrium (LD)	
Direct mutagenic effects	4. Accentuated F_{ST} in DMRs' are associated with C/T polymorphisms (in CpG context)	Methylated cytosines deaminate to thymine at high rates and changes methylation levels, increasing genetic differentiation in differentially methylated regions

Table 2. Summary of findings on the association between DNA methylation and genetic differentiation. This table summarizes the evidence supporting each prediction from the hypotheses tested in this study (Table 1), categorized as none, weak, moderate, or strong based on statistical significance and pattern consistency.

Predictions	**Degree of evidence**
DMRs exhibit accentuated F_{ST} between populations relative to genome-wide levels	moderate
The magnitude to which DMRs exhibit higher F_{ST} relative to the genomic background increases with geographical distance between populations	strong
Patterns of accentauted F_{ST} in DMRs are associated with host-plant ecotype	none
Patterns of accentauted F_{ST} in DMRs are associated with climatic variables	weak
Allele-frequency changes within DMRs are more extreme than other genomic regions	weak
Patterns of accentuated F_{ST} in DMRs are associated with reduced recombination	none
Patternsof accentuated F_{ST} in DMRs are associated with C/T polymorphisms (in CpG context)	none

Figure Legends

Figure 1. Patterns, hypotheses and predictions for investigating the association between accentuated F_{ST} in differentially methylated regions (DMRs). (**A**) The selection-gene flow balance hypothesis posits that different levels of selection and gene flow on DMRs and associated loci could generate an association between accentuated F_{ST} in DMRs. Two predictions can be raised with this hypothesis. First, that the magnitude of the association between F_{ST} and DMRs increases with geographical isolation, reflecting stronger selection effects as gene flow decreases. Second, that DMRs will show greater allele-frequency (freq.) shifts in transplant experiments between environments compared to other methylation tiles. (**B**) The reduced recombination hypothesis posits that the patterns of accentuated F_{ST} in DMRs result from background selection in regions of reduced recombination. This predicts DMRs are concentrated in low-recombination regions. (**C**) The mutatagenic DNA methylation hypothesis posits that DNA methylation elevates F_{ST} 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 F_{ST} 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.

Figure 2. The *T. cristinae* study system and ecotype-associated differentially methylated genomic regionse (DMRs). (**A**) *T. cristinae* and its two host-plant ecotypes: *Adenostoma fasciculatum* and *Ceanothus spinosus*. Illustrations by Rosa Ribas. (**B**) Sampling loactions of study populations. The main map shows populations analyzed for differentially methylated regions (DMRs) using whole-genome bisulfite sequencing (WGBS; de Carvalho et al., 2023). The inset depicts populations with whole-genome sequencing (WGS) data from Soria-Carrasco et al. (2014), used to assess genetic differentiation (FST). WGS data covers a broader geographical range, including additional populations (R12; see Supplementary Information). (**C**) Methylation distance patterns across ecotypes.Pairwise Euclidean methylation distances reveal that DMRs (0.04th and 0.4th p-value quantiles) are strongly associated with host ecotype, whereas genome-wide methylation follows an isolation-by-distance pattern (Figure from de Carvalho et al., 2023). Abbreviation: geog. = geographic

Figure 3. Genetic differentiation (F_{ST}) 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 genic DMRs overlapping with top 5% F_{ST} values, a magnitude that is marginally more elevated than expected by chance (expected values: 5%, P=0.077). (**C**) Mean F_{ST} relative to the genomic background gets progressively higher with geographical distance between populations (logarithmically transformed). X-fold difference expresses relative F_{ST} values in DMRs compared to the background levels. (**D**) F_{ST} values in DMRs between some population pairs separated by different degrees of geographical isolation. The black bar represents the mean F_{ST} across methylation tiles (null expectation) and the red dots represent the F_{ST} in DMRs. DMRs tend to exhibit particularly higher F_{ST} values with increasing levels of geographic isolation.

Figure 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 $\Delta p=0.086$; null $\Delta p=0.071$; $P=0.045$). 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 F_{ST} 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.