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1 # DNA methylation differences between stick insect ecotypes

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Abstract:

Epigenetic mechanisms, such as DNA methylation, can influence gene regulation and affect phenotypic variation, raising the possibility that they contribute to ecological adaptation. Beginning to address this issue requires high-resolution sequencing studies of natural populations to pinpoint epigenetic regions of potential ecological and evolutionary significance. However, such studies are still relatively uncommon, especially in insects, and are mainly restricted to a few model organisms. Here, we characterize patterns of DNA methylation for natural populations of *Timema cristinae* adapted to two host plant species (*i.e.,* ecotypes). By integrating results from sequencing of whole transcriptomes, genomes, and methylomes, we investigate whether environmental, host, and genetic differences of these stick insects are associated with methylation levels of cytosine nucleotides in the CpG context. We report an overall genome-wide methylation level for *T. cristinae* of ~14%, with methylation being enriched in gene bodies and impoverished in repetitive elements. Genome-wide DNA methylation variation was strongly positively correlated with genetic distance (relatedness), but also exhibited significant host-plant effects. Using methylome-environment association analysis, we pinpointed specific genomic regions that are differentially methylated between ecotypes, with these regions being enriched for genes with functions in membrane processes. The observed association between methylation variation and genetic relatedness, and with the ecologically-important variable of host plant suggest a potential role for epigenetic modification in *T. cristinae* adaptation. To substantiate such adaptive significance, future studies could test if methylation can be transmitted across generations and the extent to which it responds to experimental manipulation in field and laboratory studies.

Keywords: DNA methylation, epigenetics, natural populations, insects, host-plant adaptation

36 **Introduction**

37 Epigenetic mechanisms are receiving increasing attention as possible contributors to phenotypic
38 diversity, adaptation and evolution (Bossdorf *et al.*, 2008; Hu & Barrett, 2017; Richards *et al.*, 2017;
39 Verhoeven *et al.*, 2016). However, questions remain about the processes shaping epigenetic variation in
40 natural populations, and their ecological and evolutionary consequences (Husby, 2022; McGuigan *et al.*,
41 2021). Currently, the best-studied epigenetic mechanism is DNA methylation, a covalent addition of a methyl
42 group (CH_3) at the fifth carbon (C-5) of a cytosine (hereafter 'methylation'). Like other epigenetic
43 mechanisms, methylation markscan influence gene regulation without altering the DNA sequence (Law &
44 Jacobsen, 2010). For instance, methylation can affect the binding of transcription factors to promoters,
45 influence chromatin structure and modulate gene expression (Cedar & Bergman, 2009). As a consequence, it
46 can define and influence the phenotypes of cells and organisms (Duncan *et al.*, 2014). We, therefore, focus
47 on methylation here.

48 In comparison with DNA sequence, methylation has potentially complex and highly variable dynamics
49 (Fig. 1). For instance, changes in methylation marks can be induced by specific environmental conditions
50 (Le Luyer et al., 2017; Van Antro et al., 2023). Through the regulation of gene expression, methylation might
51 mediate phenotypic responses to environmental stimuli and thus influence phenotypic plasticity (Bogan et
52 al., 2023; Lee et al., 2022; Lloyd & Lister, 2022; Venney et al., 2021). In addition, methylation can also be
53 affected by the genetic background in different steps of its molecular pathway (Adrian-Kalchhauser *et al.*,
54 2020; Richards, 2006). For example, methylation could be completely dependent on the genotype (Dubin *et*
55 *al.*, 2015), loosely potentiated by the genetic background (Morgan *et al.*, 1999), or largely independent of it
56 (*i.e.* methylation marks that are entirely environmentally-induced or resultant from processing errors, both
57 often referred to as 'pure' epigenetic variation; (Cubas *et al.*, 1999; Yao *et al.*, 2021). Lastly, methylation
58 patterns may persist from one to several generations. Indeed, methylation marks have been shown to be
59 transmitted across generations in plants (van der Graaf *et al.*, 2015; Zhang *et al.*, 2018), vertebrates
60 (Heckwolf et al., 2020; Hu et al., 2021; Kelley et al., 2021; Vernaz et al., 2022), and insects (*i.e.,* bees,
61 Yagound *et al.*, 2020). Despite recent advances, the relative contributions of genetic variation, environmental
62 effects, and their interaction in shaping patterns of methylation and their persistence across generations

63 generally remains unclear, even in the best studied model organisms (Adrian-Kalchhauser *et al.*, 2020;
64 Anastasiadi *et al.*, 2021; Stajic & Jansen, 2021). Thus, understanding how methylation varies with
65 ecologically relevant factors and with genetic variation can be an important first step in the path to resolve
66 the role of methylation in ecological and evolutionary processes, including adaptation.

67 In this context, it is important to characterize patterns of methylation variation in natural populations
68 that are genetically heterogeneous and to test for possible ecological associations (Bossdorf *et al.*, 2008;
69 Richards *et al.*, 2017). This is because such variation may be missed in laboratory experiments and therefore
70 studies in nature complement laboratory approaches (Herrera *et al.*, 2014; Husby, 2022; Ledón-Rettig, 2013).
71 Several studies in nature have now shown that methylation can vary across environmental gradients (see
72 reviews: Hu & Barrett, 2017; Richards *et al.*, 2017; Verhoeven *et al.*, 2016), contribute to invasion potential
73 (Hawes *et al.*, 2018), and influence host-parasite interactions (*e.g.,* Hu *et al.*, 2018; Sagonas *et al.*, 2020).
74 However, the majority of studies of natural methylation were performed with anonymous markers or with
75 reduced representation sequencing (rather than whole-methylome sequencing), which can limit inferences
76 and conclusions (Lea *et al.*, 2017). Therefore, high-throughput and resolution sequencing data are required to
77 better identify and pinpoint differentially methylated genomic regions and genes associated with
78 environmental differences, reflecting a possible involvement with ecological adaptation. We provide such a
79 study here for the stick insect *Timema cristinae* (study system details below).

80 Notably, methylation patterns and mechanisms also vary phylogenetically (Feng *et al.*, 2010; Zemach
81 *et al.*, 2010), further complicating efforts to draw general conclusions based on comparisons from a few
82 restricted groups. For example, DNA methylation marks tend to be concentrated in actively expressed genes
83 in teleost fishes, but are found upstream of inactive genes in mammals (Adrian-Kalchhauser *et al.*, 2020;
84 McGaughey *et al.*, 2014) and birds (Lindner *et al.*, 2021). Moreover, insects and other invertebrates exhibit
85 lower levels of genome-wide methylation compared to vertebrates (Suzuki & Bird, 2008; but see de
86 Mendoza *et al.*, 2019), such that whole-methylome sequencing may be critical to detect significant
87 differences among insect populations (de Carvalho, 2023). In addition, many studies have investigated
88 insects' methylation only in laboratory settings (*e.g.*, (Bain et al., 2021; de Carvalho, 2023; Duncan et al.,
89 2022; Libbrecht et al., 2016; X. Wang et al., 2016; Yagound et al., 2020). As a consequence, how methylation

90 varies in insects' natural populations remains unclear. Therefore, studies that combine high resolution
91 epigenomic tools with relevant ecological variables in natural populations of different study systems, most
92 notably insects, are needed to help resolve the importance of methylation to ecological and evolutionary
93 processes, which could differ among organisms (Husby, 2022).

94 Here, we investigate natural methylation variation in *Timema cristinae* stick insects, a model for
95 adaptation and speciation. *Timema cristinae* are wingless, plant-feeding insects native to the Santa Ynez
96 Mountains in California (USA, Vickery, 1993, see *Supplementary Materials* for details about *n*atural history
97 and population genetic patterns). Previous studies have identified important ecological variables affecting the
98 evolution of this species, most notably the host plant species the insect lives and feeds on, which defines two
99 ecotypes (Nosil, 2007; Nosil & Crespi, 2006; Sandoval, 1994a, 1994b). *Timema cristinae* are primarily found
100 on the host plants *Ceanothus spinosus* (Rhamnaceae) and *Adenostoma fasciculatum* (Roseaceae; Fig. 2).
101 Divergent selection between these hosts contributes to ecological reproductive isolation between ecotypes,
102 most markedly via selection on a color-pattern trait that confers crypsis against visual predators (Nosil &
103 Crespi, 2006). Specifically, the presence versus absence of a white dorsal stripe is highly heritable and is
104 encoded by a major locus on linkage group 8 (LG8), named *Mel-Stripe* (Nosil *et al.*, 2018). The ecotypes
105 also differ in traits other than color-pattern, such as body size (Nosil & Crespi, 2006), host-plant preference
106 (Nosil *et al.*, 2006), mate choice (Nosil *et al.*, 2002), and cuticular hydrocarbons (CHCs) (Riesch *et al.*,
107 2017). Thus, host plant is a key ecological variable that affects adaptation in this species. In addition, it has
108 been shown that *Timema* can be locally adapted to different climatic conditions (Chaturvedi *et al.*, 2022),
109 making climate another important ecological variable to investigate in this group. These ecological details
110 make *Timema* an interesting candidate to investigate patterns of DNA methylation in the context of possible
111 adaptive significance.

112 To this end, we integrated whole-methylome, genome, and transcriptomic data from natural
113 populations of *T. cristinae*. Our strategy was three-pronged. Our first objective centered on describing the
114 methylome of *T. cristinae*. This involved characterizing general patterns of methylation across the *T.*
115 *cristinae* genome, assessing how levels of methylation in *T. cristinae* compare to what has been published on
116 other insect species (*e.g.* (Bewick et al., 2017; Glastad *et al.*, 2019; Provataris *et al.*, 2018), and determining

117 whether and how methylation covaries with gene expression levels in the stick insect. Second, we tested for
118 associations of genome-wide methylation with factors indicative of its possible role in adaptation, namely:
119 genetic variation, geographical distance, and two environmental factors: climatic variation and host-plant
120 use. Third, we used methylome-environment analyses to investigate the association between methylation
121 variation and environmental differences for specific genetic regions. We did so by examining whether DNA
122 methylation in *Timema* is associated with the use of different host-plant species, and if any such differentially
123 methylated regions (DMRs) consistently displayed particular molecular functions. To our knowledge, our
124 results are one of the first (or the first) population-level studies to investigate whole-methylome variation in a
125 wild species of insect.
126

127 **Methods**

128 ***Sampling***

129 Our sampling strategy was designed to capture methylation variation across the patchy and
130 heterogeneous chaparral landscape that *T. cristinae* inhabits. Here, a 'population' is defined as all *T. cristinae*
131 collected within a continuous patch of one of the host plant species at a given geographic locality, following
132 previous work (Nosil, 2007). *Timema cristinae* is found along an elevational range that varies from 300m up
133 to 1200m of altitude, representing climatic differences along its distribution (Comeault *et al.*, 2015; Nosil *et*
134 *al.*, 2018). The key factors dictating the choice of populations for this study were: species and abundance of
135 host plants, elevation, and the climatic and geographical distance between populations (see the
136 *Supplementary Materials* for more details).

137 We selected 12 populations spanning 9 geographic localities in the Santa Ynez Mountains (Fig. 2).
138 This comprised four localities with only *Adenostoma,* two with only *Ceanothus*, and three localities where
139 patches of the two different host species grow side-by-side (*i.e.,* 'parapatric' populations; Fig. 2B.; Table S2).
140 In this design, the geographic distance between the populations varied from a parapatric setting (*i.e.,* no clear
141 geographic separation) to patches that were geographically separated by up to 11km (*i.e.* 'allopatric'
142 populations; Fig. 2B). Individuals from the 12 populations were sampled on the same date (25th April 2017).

143 Specimens were collected using sweep nets and flash frozen using liquid nitrogen before being store at -
144 80°C to minimize the effects of sampling on methylation.
145 Two similarly-sized females were selected from each population (n=24 samples in total) to perform
146 whole-genome bisulfite sequencing (Table S3). The sample size per population used in this work is
147 comparable to other studies using whole-genome bisulfite sequencing (*e.g.,* Gore *et al.*, 2018; Metzger &
148 Schulte, 2018), and is further increased in downstream analyzes as the samples are grouped per ecotype.
149
150 ***Whole-genome bisulfite sequencing (WGBS)***
151 Half of each specimen's body (cut longitudinally) was used to isolate its genomic DNA using
152 DNeasy Blood and Tissue Kits (Qiagen). Our method of DNA isolation therefore generated a mix of DNA
153 from different tissues, and, as a result, our measures of methylation should be considered as whole body
154 estimates. This procedure has been used in a number of other studies in insects (*e.g.,* Bain *et al.*, 2021;
155 Glastad *et al.*, 2016; Lewis *et al.*, 2020; Yu *et al.*, 2023), and is a reasonable starting point for the first
156 epigenetic study within a system. The samples were treated with sodium-bisulfite before being sequenced,
157 which converts non-methylated cytosine residues into uracil, but leaves 5-methyl-cytosines unaffected
158 (Cokus *et al.*, 2008). Thus, only the cytosines that are methylated are retained after this treatment, and the
159 unmethylated ones are output as uracil, later amplified as thymine following polymerase chain reaction. We
160 included a 1% lambda phage (cl857 Sam7 Lambda phage DNA, Promega Corporation) spike in each library
161 as an unmethylated control in the WGBS. One *T. cristinae* sample (individual *17_0015*) was also sequenced
162 without sodium-bisulfite treatment as an additional control (*i.e.,* the non-methylated cytosines in the sample
163 were not affected). The sodium-bisulfite treatment and high-throughput sequencing were performed by the
164 Biomedicum Functional Genomics Unit (FuGU, Helsinki). The libraries were sequenced using the Illumina
165 NextSeq 500 platform, with High Output 2 x 150 bp runs. In total, three flow cells with four lanes were run.
166 See *Supplementary Materials* for details regarding the bisulfite sequencing steps.
167
168 ***Filtering and mapping reads and initial quality control steps***

169 The BS-converted raw reads were filtered using Trimmomatic 0.36 (Bolger *et al.*, 2014), and read
170 quality assessed using FASTQC v0.11.5 (Andrews *et al.*, 2015; see *Supplementary Materials*). High quality
171 reads were initially mapped to the BS-transformed phage DNA (GenBank-EMBL: J02459) using Bismark
172 0.16.1 (Krueger & Andrews, 2011), yielding a mean of 737,086 reads uniquely mapped across samples
173 [626,125 – 848,047; 95% confidence interval, CI]. These estimates were used as a proxy to calculate the BS-
174 conversion efficiency in each sample. We obtained a mean of 0.3% of methylated cytosines followed by
175 guanines (*i.e.,* C-phosphate-G dinucleotide; CpG hereafter) across samples, representing 99.7% conversion
176 efficiency (Table S4). We focused on CpGs because it is the main context in which DNA methylation occurs
177 in animals, including insects (Feng *et al.*, 2010; Zemach *et al.*, 2010).
178 Non-phage reads (mean 23,262,914 [23,151,953 – 23,373,875, 95% CI]) were aligned to the *T.*
179 *cristinae* reference genome 1.3c2 (Nosil *et al.*, 2018), which was first BS-converted *in silico* (following
180 Krueger & Andrews, 2011). The mapping yielded a mean of 10,232,740 [9,803,341 – 10,662,139] reads
181 uniquely mapped (mapping efficiency of 44.0% [43.3% – 44.7%]; Table S5). In the BS-control sample (*i.e.,*
182 individual *17_0015*), we expected an elevated percentage of cytosines being called as methylated by
183 Bismark, because the unmethylated ones were not affected by the treatment (*i.e.,* not transformed into
184 thymines). Indeed, Bismark called 98.0% methylation for CpG context in this sample, implying the software
185 was sensitive to detect cytosines in the methylated state. Methylation calling for every cytosine was
186 performed using Bismark (Krueger & Andrews, 2011), yielding a total of 37,622,963 cytosines. The
187 downstream analyses reported below do not involve a de-duplication step. Our results are likely robust to
188 PCR duplicates given the extremely high correlation of methylation status between datasets that do versus do
189 not remove PCR duplicaes (r ~ 1.0, see *Supplementary Materials* for details).
190
191 ***Genetic sequencing (genotyping-by-sequencing)***
192 We obtained new genotyping-by-sequencing data (GBS) from the 24 individuals with sequenced
193 methylomes to estimate genome-wide variation among these specific individuals. To ensure adequate sample
194 sizes for calling single nucleotide polymorphisms (SNPs) and estimating genome-wide variation for the
195 study populations, we also included previously published GBS accessions of other *T. cristinae* specimens

196 from the same populations studied here, when available (Comeault *et al.*, 2015; Lindtke *et al.*, 2017; Riesch
197 *et al.*, 2017; Table S6). If not available (*i.e.,* BT-A, OUT-A, OUT-C, SC-C, and SCN-A populations; Table
198 S2), we obtained new GBS data from individuals of those populations (Table S7).

199 To acquire new GBS data, genomic DNA was isolated using DNeasy Blood and Tissue Kits (Qiagen),
200 and genomic libraries were prepared following Parchman *et al.* (2012), as previously implemented in
201 *Timema* (Comeault *et al.*, 2015; Nosil *et al.*, 2012, 2018), and Peterson *et al.* (2012). These samples were
202 sequenced using an Illumina HiSeq2000 platform at the National Center for Genome Research (Santa Fe,
203 New Mexico, USA). Reads from all data sets were processed together (following Comeault *et al.*, 2015;
204 Nosil *et al.*, 2018; Riesch *et al.*, 2017), and mapped to the *T. cristinae* reference genome 1.3c2 (Nosil *et al.*,
205 2018). SNPs were called by a custom *Perl* script (Comeault *et al.*, 2014) using SAMTOOLS (Li *et al.*, 2009)
206 *mpileup* and BCFTOOLS. We used the consensus caller, excluding all alignments with a phred-scale
207 mapping quality score below 20, and requiring the probability of the data to be less than 0.05 under the null
208 hypothesis that all samples were homozygous for the reference allele to call a variant. We filtered out
209 variants that had reads for fewer than 50% of the individuals, a quality score below 20, a depth greater than
210 10 times the number of individuals, more than two alleles, or a minor allele frequency lower than 1%
211 (yielding 533,420 SNPs). Custom *Perl* scripts were used along with a C++ program (alleleEst 0.1b) to
212 estimate the genotypes based on a hierarchical Bayesian model (Gompert *et al.*, 2013). Following previous
213 studies in *Timema* (*e.g.,* Comeault *et al.*, 2015; Riesch *et al.*, 2017), we calculated the empirical Bayesian
214 posterior probabilities for the genotypes of each locus using the genotype likelihoods estimated by
215 BCFTOOLS along with Hardy–Weinberg priors from our estimated allele frequencies. For we ran three
216 independent MCMC chains of 10,000 steps, saving samples every 10th step, and removed the first 5,000
217 steps as burn-in. We then computed the posterior mean genotype as a point estimate of the genotype for each
218 individual, at each locus.

219

220 ***Controlling for genetic polymorphisms (single nucleotide polymorphisms, SNPs) in methylation calls***

221 BS-seq data can lead to erroneous methylation calls when bisulfite transformed data (from C-to-T or
222 G-to-A in the reverse strand) is subsequently aligned to a reference genome. This is because a natural SNP

223 could be assigned as a confounding differently methylated position. Therefore, controlling for SNP variation
224 is an important quality control step to confidently assess methylation levels (Lea *et al.*, 2017). Notably, this
225 step is rarely conducted (exceptions aside, *e.g.,* (Heckwolf *et al.*, 2020; Hu *et al.*, 2021; Schmid *et al.*, 2018)
226 and is a technical strength of our study.

227 To this end, we first used the BS-control sample (*17_0015*, Table S3) to estimate potential C/T and
228 G/A SNPs and compare these to its BS-treated equivalent. Based on this comaparison, we estimated 0.5% of
229 the CpGs in the methylation data were in fact SNPs (*Supplementary Materials*). We then used the GBS data
230 along with 98 previously published whole-genome sequences (Riesch *et al.*, 2017; Soria-Carrasco *et al.*,
231 2014) to make a list of C/T and G/A SNPs that could confound the methylation calling. From this list, 10.5%
232 [10.4%–10.6%] of SNPs overlapped with CpG sites in the BS-treated samples (Table S8, *Supplementary*
233 *Materials*). These values are considerably higher than the proportion of SNPs called in the BS-control
234 sample with its BS-treated equivalent (only 0.5%). Thus, with this latter approach, there was likely an
235 overestimation of confounding SNPs in the methylation tables, but the approach is conservative and ensures
236 most methylation polymorphisms (SMPs) we study are unlikely to be genetic polymorphisms (SNPs). Thus,
237 we removed all the CpG sites in the BS-treated data that overlapped with SNPs to reduce these confounding
238 effects.

239

240 ***Description of* T. cristinae *methylome***

241 For each sequenced individual, methylation levels were calculated for each site as the total number of
242 methylated cytosines (*i.e.* unconverted C) divided by the total number of reads mapped to the site. We used
243 the annotated *T. cristinae* genome 1.3c2 from Villoutreix *et al.* (2020) to obtain information about DNA
244 methylation patterns in genes, in both exons and introns. Only the genes with InterPro (EMBL-EBI) or Gene
245 Ontology accessions (GO, UniProt) were selected, resulting in a total of 19,383 retained genes. Information
246 about repetitive elements and transposons were extracted from the *T. cristinae* RepeatMasker database
247 (which includes short and long tandem and interspersed repeats; Villoutreix *et al.*, 2020). Results are
248 represented in Table S10. We used enrichment analyses to estimate the probability that CpGs in different
249 regions of genes and flanking sequences were more methylated than expected by chance (*Supplementary*

250 *Materials*). All these enrichment analyses were performed using R (v3.6.3) (R Core Team, 2020)
251 (*Supplementary Materials*).
252
253 ***RNA-seq analyses***
254 We generated RNA-seq data from 18 of the same 24 individuals that were used for methylation
255 analysis (Table S3), with the goal of testing for a general association between gene expression and
256 methylation. The RNA extractions, library preparations and sequencing were performed by Genome Quebec.
257 Total RNA for each individual was extracted from the remaining half of the specimens' bodies using the
258 Quiacube animal tissue kit and protocol (18 individuals were used because these were the only ones with
259 sufficient remaining material after WGBS to perform RNA-seq). Details about library preparation and about
260 filtering reads are in the *Supplementary Materials.* The reads were aligned to the *T. cristinae* reference
261 genome 1.3c2 (Nosil *et al.*, 2018) using STAR v2.7.3a (Dobin *et al.*, 2013), and sequenced read pairs were
262 assigned to genes in the *T. cristinae* reference genome using *featureCounts* (Liao *et al.*, 2014). In this work,
263 we focused on testing for general correlation between methylation levels and gene expression. This is
264 because the RNA-seq did not yield sufficient data to overlap strongly enough with the differentially
265 methylated regions to test for an association with differential methylation of specific regions (an opportunity
266 to be pursued by furture studies). To estimate the relationship between methylation levels and gene
267 expression, we performed variance-stabilized transformation of these data using the R package *DESeq2*
268 v1.28.1 (Love *et al.*, 2014). We ranked genes according to their mean expression across the 18 samples
269 before being divided into 100 bins (see *Supplementary Materials* for details on *STAR* and *DESeq2* steps). For
270 each bin, we plotted the mean ± standard error of methylated CpGs per gene and performed a Spearman
271 correlation test using R.
272
273 ***Association between genome-wide methylation and genetic, geographical and environmental distances***
274 We tested for a relationship between genome-wide methylation and factors that could suggest its
275 possible role in adaptation: genetic variation, geographical distance (based on Herrera et al., 2016), host plant
276 use and climatic variation. To this end, we estimated pairwise distances between the 24 individuals for each

277 examined factor (see *Supplementary Materials* for further context). We used methylKit v1.0.0 (Akalin *et al.*,
278 2012) to generate a single table with methylation calls at each site for all 24 individuals (*i.e.,* SMPs; see
279 *Supplementary Materials*). Euclidean distances in methylation levels were then estimated between each pair
280 of the 24 sequenced individuals. Genetic distances using GBS data from the same 24 individuals were
281 estimated using RapidNJ 2.3.0.2 (Simonsen *et al.*, 2008). This software calculates pairwise distances
282 between individuals based on the Kimura two-parameter model (Elias & Lagergren, 2007; Kimura, 1980;
283 Simonsen & Pedersen, 2011). We first estimated the association between the pairwise genome-wide
284 methylation distances and the pairwise genome-wide genetic distances using Mantel tests, performed in the
285 *vegan* R package (Oksanen *et al.*, 2022), based on 10,000 permutations.

286 We then also fit Bayesian linear mixed models to determine the factor or combination of factors best
287 explaining genome-wide methylation distances between individuals (Clarke *et al.*, 2002; Gompert *et al.*,
288 2014), including random effects accounting for the pairwise nature of the predictor and response distance
289 matrices. The Bayesian approach uses a Markov chain Monte Carlo framework to estimate the regression
290 coefficients and deviance information criterion (DIC) for model selection. The model was fit via the *rjags* R
291 package (Plummer, 2018), including genetic, geographical, host-plant and climatic distances to explain the
292 methylation distances between individuals. Geographical distances were calculated using the geodesic
293 distance between coordinate points and then logarithmically transformed (Rousset, 1997). Host was coded by
294 whether a pair of insects were collected from the same (0) or different plant species (1). We obtained climate
295 information from WorldClim database (Harris *et al.*, 2014) at each locality. We used the two main axes from
296 a principal component analysis (PCA, PC1=66.4% and PC2=25.5%, Fig. S1, Table S1; S*upplementary*
297 *Materials*) to summarize 19 bioclimatic variables. We estimated the climatic distances between individuals
298 using Euclidean distances. We ran three chains of the model, with 10,000 iterations and a burn-in of 2,000
299 iterations, thinning interval of 5. All statistics were performed using R (R Core Team, 2020).

300

301 ***Methylome-environment association analyses using MACAU: designating DMRs***

302 To further investigate the association between host plant and methylation, we delimited subsets of the
303 genome that were most strongly differentially methylated between host ecotypes (*i.e.,* differentially

304 methylated regions, DMRs, hereafter). We did so using an approach analogous to genotype-environment
305 association methods (GEA), which search for correlations between genetic variation and environmental
306 variables (Forester *et al.*, 2018). Specifically, we performed a methylome-environment association analysis
307 using binomial mixed models (MACAU: Mixed-model association for count data via data augmentation)
308 (Lea *et al.*, 2015). Briefly, this model estimates whether a variable (predictor) is associated with methylation
309 levels at a specific locus or region. Its binomial component handles methylation count data, estimating the
310 level of methylation for each CpG locus based on the number of reads with methylated cytosines and the
311 total coverage. In addition, MACAU controls for population structure by incorporating a matrix of pairwise
312 genetic kinship (in our case, estimated from GBS genetic data) – treated as the variance-covariance matrix
313 for the heritable component of the random effects. The kinship matrix contributes to the value of the
314 response variable, but does not affect its non-heritable part (Lea *et al.*, 2015). This method can enhance the
315 power to detect a potentially true association between methylation variation and the environment (Lea *et al.*,
316 2017). The non-heritable component consists of the variation that is due to independent environmental noise
317 (see Lea *et al.* 2015 for details concerning MACAU).
318 For the MACAU analysis, we summarized the methylation data into 1 kilobasepair (kbp) non-
319 overlapping tiling windows to increase the per region sequencing coverage, and to enhance the model's
320 statistical power. Coverage outliers above the 99.9th percentile, identified with methylKit v1.0.0 (Akalin *et*
321 *al.*, 2012) were removed from the analysis along with SNP variants (see S*upplementary Materials* for
322 details). We retained the regions with a minimum coverage of 10 reads per tile comparable to other studies
323 (*e.g.,* Heckwolf *et al.*, 2020), yielding 428,092 methylation tiles. Following Lea *et al.*, 2016, we excluded all
324 methylation tiles that were consistently hypo-methylated (mean DNA methylation level < 10%) and hyper-
325 methylated (mean DNA methylation level > 90%). We further excluded tiles in which the standard deviation
326 of methylation among the samples was below 5% (*i.e.,* least variable regions). These steps yielded 82,696
327 1kbp methylation tiles (64,713 in assembled linkage groups of the reference genome, of which 73.5% of the
328 tiles were located within genes).
329 We used the 1kbp methylation tiles to model the association between host plant and methylation
330 levels. We additionally used the first two principal component axes of climatic variation as covariates along

331 with the BS-conversion efficiency estimated for each sample (using the non-methylated Lambda phage), and
332 flow cell batch to remove possible variation due to technical artifacts. The matrix of pairwise kinship was
333 inferred using GBS data (Zhou *et al.*, 2013), see *Genetic sequencing* section). We used the tail of empirical
334 distribution of *p-values* output from MACAU to designate DMRs differing between hosts as regions of
335 interest, examining the robustness of the properties of the DMRs to different cut-off thresholds. Here, we
336 report results based on empirical *p-value* percentiles, varying from the 0.04th to the 0.4th most significant
337 percentile of *p-value*s (the corresponding *p-values* ranged from 0.0004 to 0.0061) to designate DMRs. We
338 found qualitatively similar results across different cut-offs (see *Discussion* for further elaboration). The
339 MACAU analyses were performed with 100,000 sampling steps and burn-in of 50,000 iterations, with the
340 filtering ratio threshold equal 1.

341 In addition to MACAU approach, we used Bayesian regressions performed in R (R Core Team, 2020)
342 to estimate the combination of factors best explaining the methylation distances between individuals for the
343 DMRs, following the same approach detailed above (Clarke *et al.*, 2002; Gompert *et al.*, 2014). We
344 estimated Euclidean distances on methylation levels calculated as the total number of methylated cytosines
345 divided by the total number of reads mapped to the 1kbp tile. We fit the model using genetic, geographical,
346 host plant and climatic distances to explain the methylation distances in DMRs delimited by the different
347 quantiles of the *p-value* distribution, and also genome-wide (*i.e.,* all methylation tiles used at MACAU).
348

349 ***Gene ontology (GO) enrichment in DMRs***

350 To test for GO enrichment in DMRs, we used the R package TopGO v2.52.0 (Alexa & Rahnenfuhrer,
351 2023). For this analysis, we used the tilling windows within genes (n=47,696) and the *T. cristinae* genome
352 annotation (v1.3c2; Villoutreix et al., 2020). The analysis was performed comparing methylated regions that
353 were significantly different according to host plants (*i.e.,* DMRs according to different quantiles of the
354 empirical *p-value* distribution) versus the remaining tilling windows. Fisher's Exact Test was used to
355 calculate the significance of the enrichment.
356

357 **Results**

358 ***DNA methylation is enriched in the gene body and correlated with gene expression levels***

359 We first quantified general patterns of DNA methylation in *T. cristinae*. To begin with, we observed 360 that the *T. cristinae* genome only possesses DNA methyltransferase 1, which maintains methylation patterns 361 after cell division (Goll & Bestor, 2005), but does not contain DNA methyltransferase 3, that adds 362 methylation in a d*e novo* manner (Table S9; see *Supplementary Materials* for further discussion). We next 363 estimated that the overall methylation level of cytosine nucleotides in CpG context was around 14% in *T.* 364 *cristinae*. Methylation marks mainly targeted gene bodies, with enriched levels in both exons and introns 365 compared to the genomic background levels (2.4x, P<0.001 for both genomic features, permutation test; Fig. 366 3A). Methylation was not elevated for short repeats or transposable elements (TEs). If anything, TEs 367 appeared slightly impoverished in methylation (enrichment 0.9x, P<0.001, permutation test).

368 Gene body methylation levels tended to increase from 5’→3’ in genes (Fig. 3B), and were higher in 369 genes with more exons (t=32.6, P<0.001; unpaired t-test; Table S11). Gene ontology enrichment analyses 370 indicated that highly-methylated genes generally had housekeeping functions (*i.e.,* involved in basic cellular 371 functions), while those with lower methylation levels tended to have more dynamic functions (*e.g.,* cell 372 signaling and membrane receptors; Tables S12-S13). Finally, we found that methylation was positively 373 correlated with levels of gene expression (Spearman rho=0.57, P<0.001, Fig. S2).

374

375 ***Genome-wide DNA methylation varies with genetic distance and host-plant ecotype***

376 We next tested for associations of genome-wide methylation with genetic, geographic, host plant and 377 climatic differences among individuals and collection sites. We found that pairwise methylation distances 378 were mainly correlated with genetic distance between populations (r=0.65, P<0.001, Mantel test; Fig. 4). 379 Methylation also significantly varied with geographical distance (r=0.33, P<0.001, Mantel test), which could 380 result from the association between genetic and geographical distances (*i.e.,* isolation-by-distance, IBD, 381 r=0.50, P<0.001, Mantel test). In contrast, genome-wide methylation distances did not vary with host-plant 382 considered alone (r=0.00, P=0.78, Mantel test), or with climate (r=0.00, P=0.40, Mantel test).

383 Multivariate analyses using Bayesian linear mixed model regression indicated that genetic and host 384 differences together represented the best fit model explaining methylation distances, according to the lowest

385 distance information criterion (DIC=324.8; Table S14). In this model, genetic distances had a major
386 explanatory effect on genome-wide methylation (standardized regression coefficient: β_{GEN}= 1.00, [0.92,
387 1.08; 95% equal tail probability interval, ETPI]), while host plant had a smaller but nonetheless credible
388 negative effect (β_{HOST}= -0.08, [-0.18, -0.02; 95% ETPI]).

389

390 ***Differentially methylated regions across the genome associated with host plant ecotype***

391 To further assess the relationship between methylation and host, we conducted a second, more fine-
392 grained analysis focused on differentially methylated gene regions (DMRs) displaying ecotype associated
393 differences to augment the broad genome-level results presented above. Specifically, we used 1kbp tilling
394 windows to identify and evaluate regions distinguishing the ecotypes by a methylome-environment
395 association analysis (*i.e.,* MACAU). This model accounts for the genetic covariance among individuals (see
396 *Methods;* Lea *et al.*, 2015) and, thus, is similar in this regard to methods commonly employed in genotype-
397 environment association methods and genome-wide association mapping studies.

398 Three general patterns emerged across the range of *p-value* cutoffs used to designate the DMRs (Table
399 S15). First, we found that the DMRs were distributed genome-wide, and, thus, were not restricted to one or a
400 few specific linkage groups or gene regions (Fig. 5; Fig. S3). More than two-thirds of the DMRs were
401 located within gene bodies (mean 72.0% [70.6% – 73.4%, 95% CI], Table S15). This proportion matches that
402 of the full set of methylation tiles analyzed with MACAU (*i.e.,* 73.5%), therefore, it does not indicate a
403 particular enrichment of DMRs within genes. Overall, a significantly greater proportion of the DMRs (mean
404 61.3% [59.6% – 62.9%, 95% CI]) showed higher levels of methylation for stick insects collected from
405 *Adenostoma* than *Ceanothus* (1.2x, P<0.001, permutation test, *Supplementary Materials*; Table S15). The
406 absolute difference in the mean methylation levels for DMRs between individuals of different ecotypes was
407 on average 13.1% [12.8% – 13.4%, 95% CI].

408 Second, our results suggest that methylation distances in DMRs are better explained by host-plant
409 ecotype than by genetic, geographical or climatic differences. Specifically, using Bayesian linear mixed
410 models, we found that the best supported model to explain methylation variation in the different quantiles
411 designating DMRs was a combination of geographical distance and host ecotype (Table S16). The results

412 revealed that DMRs clearly distinguished ecotypes, showing more elevated standardized regression
413 coefficients in all quantiles (*e.g.,* 0.04th quantile: β_{HOST}=0.29 [0.27 – 0.31; 95% ETPI]; 0.4th quantile:
414 β_{HOST}=0.73 [0.69 – 0.78]; Fig. 5B-C; Fig S4). In contrast, the DMRs were only weakly associated with
415 geographic distance (*e.g.,* 0.04th quantile: β_{GEOG}=0.01 [0.00 – 0.02]; 0.4th quantile: β_{GEOG}=0.02 [0.00 – 0.05]),
416 and the models including genetic distances were not among the best fit (Table S16). These patterns for
417 DMRs contrasted markedly with genome-wide trends outside DMRs, where the best model that explained
418 methylation differences only involved genetic distance (Table S17), and not host plant (*i.e.,* consistent with
419 the results from the previous section indicating an isolation-by-distance pattern, IBD; β_{GEN}=0.80 [0.67 –
420 0.93; 95% ETPI]; Fig. 5D).
421 Third, we found that gene ontology (GO) enrichment analyses support an element of non-randomness
422 in the function of the DMRs. Specifically, DMRs were generally statistically enriched for functions related to
423 protein metabolism and membrane processes, especially signal transduction and trans-membrane transport
424 activity (Tables S18-S21). Interestingly, we did not find any DMR located within *Mel-Stripe*. Considering
425 the genetic regions surrounding of *Mel-Stripe, o*ne DMR was found located 6Mb from one extremity of the
426 locus and a few at ~3Mb from the other extremity (Table S22), but these regions were not enriched in DMRs
427 (*e.g.,* it was expected by chance, Table S23).
428
429 **Discussion**
430 In this work, we provide an integrative approach that combines whole methylomes, genomes and
431 transcriptomes to characterize patterns of methylation within and among natural populations of *T. cristinae.*
432 This integrated and fine-grained strategy allowed us to detect potentially important and different patterns of
433 methylation in nature for stick insects, which might have not been observed (*i.e.*, missed) with a less
434 integrative and coarser approach. We elaborate on this issue below.
435 We began by describing general patterns of methylation at species level. We found *T. cristinae* lacks
436 the *de novo* methyltransferase (DNMT3), which is responsible for transferring methyl groups to DNA. It has
437 been previously shown that many other insect groups lack this enzyme (Bewick et al., 2017; Provataris et al.,
438 2018), and that the maintenance methyltransferase (DNMT1) might be capable of adding methyl tags to

439 DNA (Kausar et al., 2021; Wang et al., 2006). However, to which extent DNMT1 in insects can lead to large
440 *de novo* methylation changes, such as modifying global methylation levels in response to environmental
441 stimuli or re-establishing methylation after erasure between generations, is unclear. Considering some lines
442 of evidence suggesting that there might not be reprogramming of methylation between generations in some
443 insect species (Wang et al., 2016; Yagound et al., 2020), it is possible that DNMT3 may not be needed for
444 that step (Glastad et al., 2019). In any case, additional studies focusing on the molecular mechanisms and
445 functions of DNMTs are needed in insects to understand how these different enzymes could contribute to
446 methylation variability and transmission across generations – important processes that could affect the
447 adaptive potential of methylation.

448 We reported overall genome-wide methylation level of this stick insect to be around 14%. Considering
449 the relatively low methylation levels (compared to organisms more commonly used in ecological studies;
450 Laine et al., 2022). This illustrates how having whole methylomes was likely important for having the
451 requisite variation here to discover the patterns reported. At the same time, such levels of methylation are
452 higher than those reported for most insects studied to date (Bewick *et al.*, 2017), but characteristic of those
453 with incomplete metamorphosis ("Hemimetabola" insects; Glastad et al., 2019; Glastad, Gokhale, et al.,
454 2016; Provataris et al., 2018; Wang et al., 2014). Also characteristic of this group of insects (Provataris *et al.*,
455 2018), methylation in *T. cristinae* mainly targeted the gene body (*i.e.* both exons and introns), and was
456 impoverished in repeat elements (Fig. 3A). Gene body methylation is thought to stabilize and up-regulate
457 gene expression and to reduce transcriptional noise by preventing transcription initiation outside start sites
458 (with some suggestion that gene body methylation might be shaped by natural selection; (Muyle *et al.*, 2022;
459 Neri *et al.*, 2017). In our results, we showed methylation levels increased from 5'→3' in gene bodies of *T.*
460 *cristinae* (Fig. 3B). As longer genes are likely more prone to transcription noise (Muyle *et al.*, 2022),
461 elevated methylation levels could act to suppress spurious transcription in *T. cristinae*, assuring the integrity
462 of the genes' function.

463 We found enrichment of different cellular functions depending on methylation level: genes involved in
464 housekeeping functions were associated with increased methylation, while genes involved in signal
465 transduction pathways showed decreased methylation levels. We detected a positive correlation between

466 methylation and gene expression levels, which may reflect the general trend for housekeeping genes to be
467 constitutively expressed at higher levels. However, recent studies have shown that gene body methylation
468 has a strong dose-dependent effect on gene expression in *Arabidopsis thaliana* (He *et al.*, 2022; Shahzad *et*
469 *al.*, 2021). While plants and insects are distantly related, our results suggest that gene body methylation may
470 directly influence gene expression in other organisms, including *Timema*. Our findings therefore reveal
471 patterns implying that methylation is associated with gene regulation in a manner that can affect higher-level
472 phenotypes via gene expression itself that is often viewed as a 'molecular phenotype' (Ranz & Machado,
473 2006).

474 From our survey of methylation variation in *T. cristinae* natural populations, two different and largely
475 contrasting patterns emerged. One is at the genome-wide scale, where overall methylation differences were
476 observed among individuals that largely reflect their genetic relatedness and degree of geographic separation
477 from one another in nature. Our results suggest that variation in methylation could be affected by gene flow,
478 which decreases as geographical distance increases – following an isolation-by-distance pattern (Herrera *et*
479 *al.*, 2016). In addition, such a pattern suggest a significant amount of methylation variation is due to
480 differences in the genetic background (Adrian-Kalchhauser *et al.,* 2020; Richards, 2006; Taudt *et al.*, 2016).
481 The relationship between methylation and genetic variation is complex. Methylation may be sequence-
482 dependent, with certain genomic regions being more prone to methylation than others (*e.g.,* transcription
483 factor binding sites; Onuchic et al., 2018) or be regulated by specific genic variations (*e.g.,* variants in genes
484 encoding DNA methyltransferase; Dubin et al., 2015). On the other hand, methylation can also affect genetic
485 variation, either directly via methylated cytosines transitioning to thymines at relatively elevated rates (*i.e.,*
486 C-to-T mutation bias; Holliday & Grigg, 1993; Ossowski et al., 2010) or via the process of genetic
487 accommodation (Danchin et al., 2019; Klironomos et al., 2013). Therefore, future studies should further
488 investigate the association between methylation and genetic variation to establish their effects on this
489 interplay. Finally, it is important to note that methylation marks that are transmitted between generations
490 independently from genetic variation (*e.g.,* van der Graaf *et al.*, 2015) could also contribute to the isolation-
491 by-distance trend observed in this study, a topic that should also be subject to further studies.

492 The second pattern is that specific gene regions within the genome were differentially methylated
493 between ecotypes related to the host plant that the insect uses. In contrast to genome-wide patterns, the
494 MACAU analysis identified multiple DMRs that varied with ecotype in the wild, but not with geographic
495 distance. Thus, different ecotypes of *T. cristinae* tend to show considerable methylation differences in DMRs
496 regardless of whether their natal host populations are located side-by-side (*i.e.,* parapatric) or allopatric and
497 separated by larger physical distances. Thus, these host DMRs may be viewed in some respect as
498 methylation QTL outliers associated primarily with host plant use that were identified by 'GWA-like'
499 methodology implemented in MACAU. Such a pattern could result from environmental induction and
500 plasticity, if the methylation marks are modified directly by host-plant use. Alternatively, it could reflect
501 divergent selection between ecotypes, if the methylation differences are transmissible between generations
502 and affect insect fitness in a host-dependent manner. Resolving these possibilities will require future work to
503 determine the degree to which methylation of DMRs is transmitted across generations in combination with
504 manipulative selection experiments testing for host-associated fitness differences. It is possible that some
505 combination of all of these processes is involved, particularly as they are not mutually exclusive.

506 Our results further showed that the DMRs were widely distributed across the genome. This result
507 resembles the genome-wide differences between ecotypes found in previous studies (Soria-Carrasco et al.,
508 2014). In contrast, no DMRs were found within a major locus controlling for color and pattern (*Mel-Stripe*),
509 with only a few DMRs located in the proximity of this locus. Our results thus do not argue for particular
510 epigenetic regulation of genes located within *Mel-Stripe*. Therefore, it seems very likely that the association
511 between methylation and host plants involves traits other than cryptic color-pattern and genetic regions
512 beyond the *Mel-Stripe* locus. In this respect, our results suggested that DMRs were mostly related to protein
513 metabolism and especially to membrane processes, including putative functions in signal transduction and
514 ion channel activity. Among the DMRs designated by more extreme quantiles, we also detected GO terms
515 related to synaptic processes, but the statistical power is too low to test for enrichment (*i.e.,* Tables S18-S19).
516 Some of the genes with ion-channel activity functions could be linked to the transmission of nerve impulse
517 (Gasque *et al.*, 2006). DNA methylation changes in genes with these functions have been previously
518 implicated in synaptic transmission and memory formation in mammals (Campbell & Wood, 2019; Halder et

519 al., 2015), but the relationship between these processes and host-plant use in insects is less clear. The links
520 between these functions, differential methylation, and host-associated fitness differences is an area ripe for
521 future study.

522 In conclusion, understanding the role of methylation in adaptation has been proven challenging, even
523 in the best-studied model organisms (Heckwolf *et al.*, 2020; Hu & Barrett, 2023; Husby, 2022; Kelley *et al.*,
524 2021; Schmid *et al.*, 2018). Our findings therefore highlight the need for additional studies in other
525 ecological model organisms to fully elucidate the role methylation plays in adaptation. In this regard, our
526 current results for *T. cristinae* represent only a first step in gaining such an understanding, but an important
527 one that lays the groundwork for exciting future research. Our identification and characterization of the
528 pattern of DMRs within the genome and in nature positions us to target these regions to assess if the
529 methylation differences they display are genetically based versus environmentally induced by host use, or
530 some combination of the two. It also now sets the stage for manipulative transplant experiments in the field
531 and lab to assess their fitness consequences and therefore effectively test for the adaptive potential of
532 methylation changes.

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Data availability. Data, including custom code written for analyzes, have been archived in Dryad Digital Repository https://datadryad.org/stash/share/HCM4iWpKoYXSccwB-CGmxzHIVb-QHz74a-OgZWdGxb8 (temporarily for reviewers' access only). The accessions have been deposited in GenBank under the BioProject PRJNA1010130.

Benefit-Sharing. Not applicable

547 **Figure legends**

548 **Fig. 1. Factors affecting methylation variation and its consequences for phenotypic variation.** The
549 genetic background can influence methylation patterns. Additionally, environmental factors can affect
550 methylation variation independently of or via an interaction with the genetic background (G x E). Knowing
551 how methylation varies with these factors is required to understand if and how methylation might contribute
552 to variation in traits affecting fitness.

553

554 **Fig. 2. The *T. cristinae* study system.** (**A**) *T. cristinae* and their host plants: *Adenostoma fasciculatum* and
555 *Ceanothus spinosus*. The ecotypes not only differ by the frequency of the dorsal white stripe, but also by
556 differences in host-preference, body size, mate choice and cuticular hydrocarbons. (**B**) Map of the
557 populations used in this study, selected based on host-plant species and their abundance, as well as elevation,
558 climatic and geographic distance between populations (*Supplementary Materials*; Table S2). The general
559 study area is situated in the Santa Ynez Moutains, in California, USA.

560

561 **Fig. 3. Comparison of methylation levels for different components of the *T. cristinae* genome.** (**A**)
562 Methylation levels are enriched in both exons and introns (*i.e.*, the gene body) compared to genome-wide
563 levels, and genetic repeats tend to be impoverished in methylation. (**B**) DNA methylation levels in genes and
564 their flanking regions. The graph represents 1kbp in the 5' downstream flanking region, multiple exons and
565 introns in the depicted genetic region, and 1kbp in the 3' downstream region. The graph shows mean
566 methylation levels estimated at CpG sites found in at least 12 samples (n=14,656 genes). The x-axis
567 represents nucleotide position from the beginning or from the end of the genomic feature. To compare exons
568 and introns of different genes, we used the mean methylation in the first 100bp at 5' and the last 100bp 3' of
569 each exon and each intron, following Glastad *et al.* (2016) and Hunt *et al.* (2013).

570

571 **Fig. 4. Genome-wide methylation differences are correlated with genetic distance.** Pairwise methylation
572 distances were estimated using Euclidean distances between individuals, and genetic distance using the

573 Kimura two-parameter model using GBS alignments. Regression was evaluated for significance using a
574 Mantel test, with more complex multivariate analyses using Bayesian regression reported in the main text.
575
576 **Fig. 5. Evidence for association between methylation patterns of specific genetic regions and host plant**
577 **use (i.e., differentially methylated regions, DMRs, between ecotypes).** (**A**) Manhattan plot showing
578 association between methylation variation and ecotype across all 24 samples, for 1 kilobase-pair (kbp) tiling
579 windows using MACAU. (Lea *et al.*, 2015). Red points represent DMRs delimited by the 0.04th quantile of
580 the empirical *p-value* distribution ($P < 0.0004$, see Table S15 for details on other quantiles). Pairwise
581 methylation distances vary mostly according to ecotype in DMRs in different quantiles of the empirical *p-*
582 *value* distribution, here, represented by the (**B**) 0.04th quantile; and (**C**) 0.4th quantile (also see Fig. S4,
583 Table S16). (**D**) Genome-wide trends vary according to the geographical distances in an isolation-by-distance
584 pattern, whereas DMRs show more of a host association. Methylation distances were obtained with
585 Euclidean distances using the 1kbp windows in MACAU (also see Table S17).

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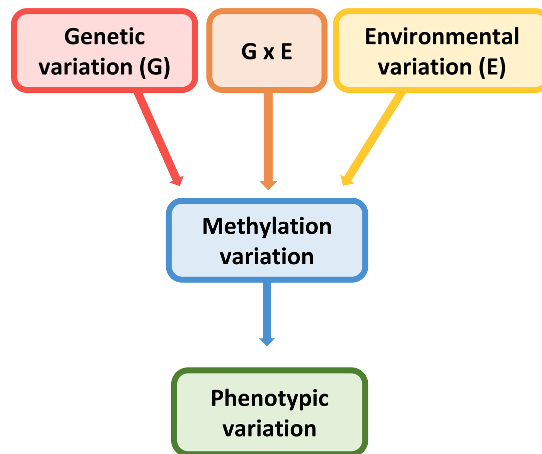


Fig. 1. Factors affecting methylation variation and its consequences for phenotypic variation. The genetic background can influence methylation patterns. Additionally, environmental factors can affect methylation variation independently of or via an interaction with the genetic background (G x E). Knowing how methylation varies with these factors is required to understand if and how methylation might contribute to variation in traits affecting fitness.

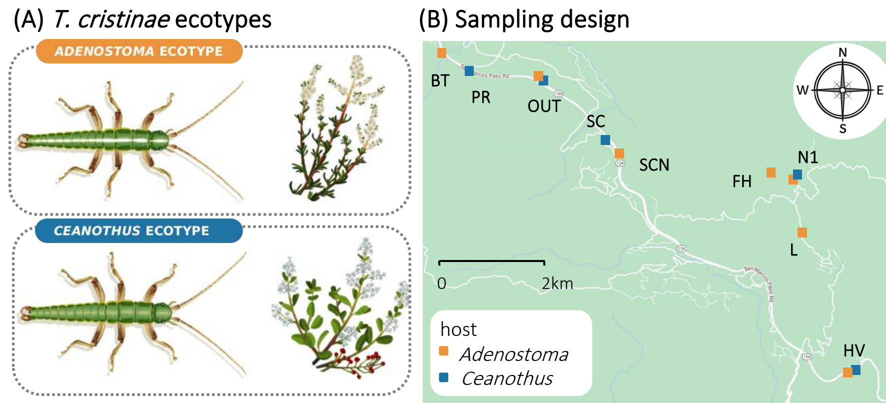


Fig. 2. The T. cristinae study system. (**A**) *T. cristinae* and their host plants: *Adenostoma fasciculatum* and *Ceanothus spinosus*. The ecotypes not only differ by the frequency of the dorsal white stripe, but also by differences in host-preference, body size, mate choice and cuticular hydrocarbons. (**B**) Map of the populations used in this study, selected based on host-plant species and their abundance, as well as elevation, climatic and geographic distance between populations (*Supplementary Materials*; Table S2). The general study area is situated in the Santa Ynez Moutains, in California, USA.

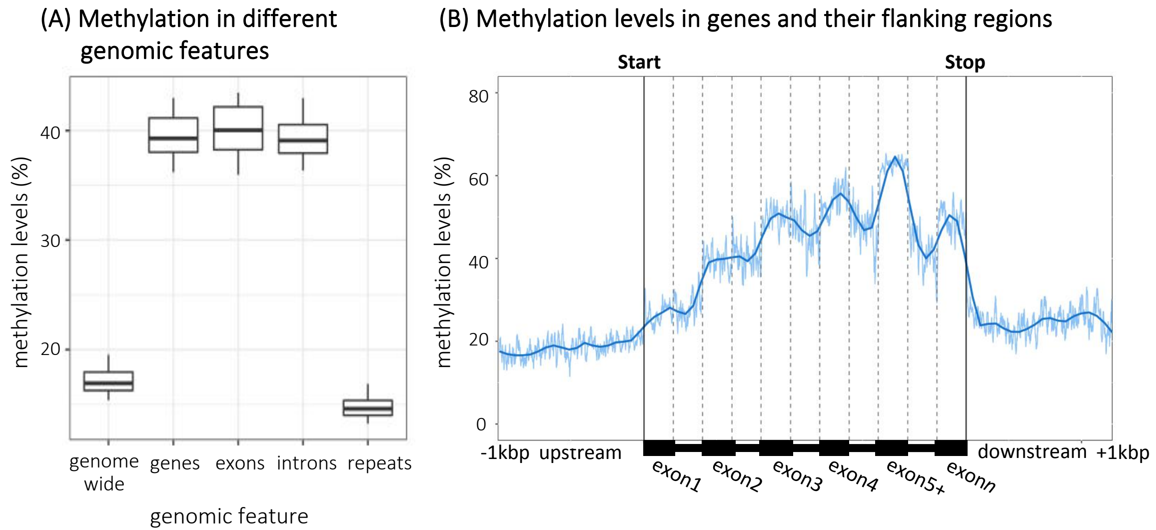


Fig. 3. Comparison of methylation levels for different components of the *T. cristinae* genome. (**A**) Methylation levels are enriched in both exons and introns (i.e., the gene body) compared to genome-wide levels, and genetic repeats tend to be impoverished in methylation. (**B**) DNA methylation levels in genes and their flanking regions. The graph represents 1kbp in the 5' downstream flanking region, multiple exons and introns in the depicted genetic region, and 1kbp in the 3' downstream region. The graph shows mean methylation levels estimated at CpG sites found in at least 12 samples (n=14,656 genes). The x-axis represents nucleotide position from the beginning or from the end of the genomic feature. To compare exons and introns of different genes, we used the mean methylation in the first 100bp at 5' and the last 100bp 3' of each exon and each intron, followingGlastad et al. (2016) and Hunt et al. (2013).

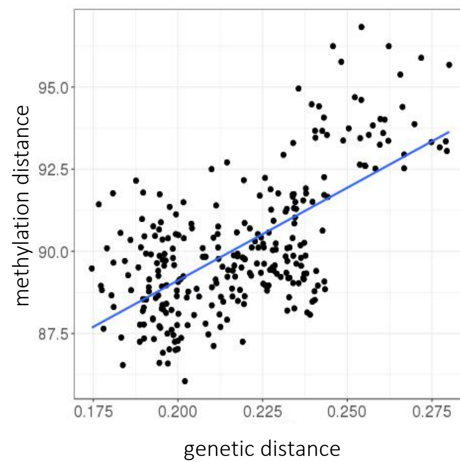
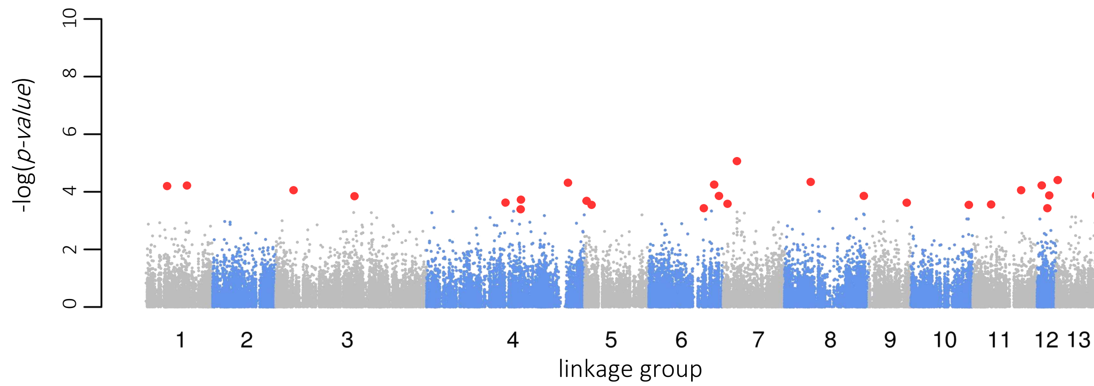
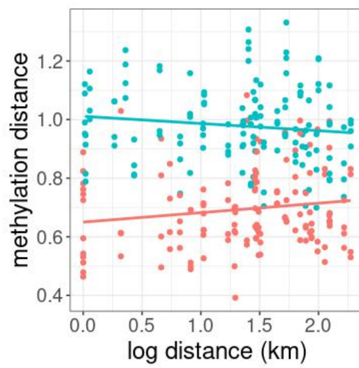
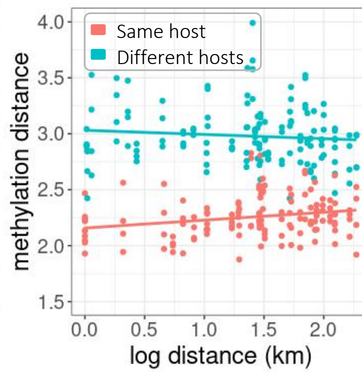


Fig. 4. Genome-wide methylation differences are correlated with genetic distance. Pairwise methylation distances were estimated using Euclidean distances between individuals, and genetic distance using the Kimura two-parameter model using GBS alignments. Regression was evaluated for significance using a Mantel test, with more complex multivariate analyses using Bayesian regression reported in the main text.







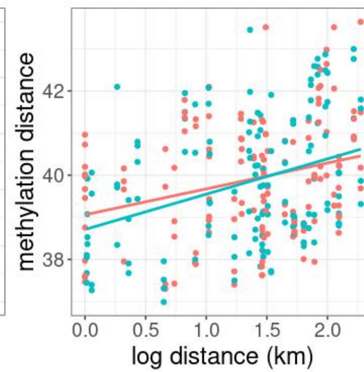


Fig. 5. Evidence for association between methylation patterns of specific genetic regions and host plant use (*i.e.*, differentially methylated regions, DMRs, between ecotypes). (**A**) Manhattan plot showing association between methylation variation and ecotype across all 24 samples, for 1 kilobase-pair (kbp) tiling windows using MACAU. (Lea *et al.*, 2015). Red points represent DMRs delimited by the 0.04th quantile of the empirical p-value distribution (P < 0.0004, see Table S15 for details on other quantiles). Pairwise methylation distances vary mostly according to ecotype in DMRs in different quantiles of the empirical *p-value* distribution, here, represented by the (**B**) 0.04th quantile; and (**C**) 0.4th quantile (also see Fig. S4, Table S16). (**D**) Genome-wide trends vary according to the geographical distances in an isolation-by-distance pattern, whereas DMRs show more of a host association. Methylation distances were obtained with Euclidean distances using the 1kbp windows in MACAU (also see Table S17).