High nucleotide diversity accompanies differential DNA methylation in naturally diverging populations

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Abstract

Epigenetic mechanisms such as DNA methylation are thought to comprise an invaluable adaptive toolkit in the early stages of local adaptation, especially when genetic diversity is constrained. However, the link between genetic diversity and methylation status has been scarcely examined in natural populations, despite its potential to shed light on the evolutionary forces acting on methylation state. Here, we analysed reduced-representation bisulfite sequencing and whole genome pool-seq data from marine and freshwater stickleback populations to examine the relationship between DNA methylation variation (between- and within-population), and nucleotide diversity in the context of freshwater adaptation. We find that sites that are differentially methylated between populations have higher underlying nucleotide diversity in both populations, with diversity higher among sites that gained methylation in freshwater than those that lost it. Strikingly, while nucleotide diversity is generally lower in the freshwater population as expected from a population bottleneck, this is not the case for sites which lost methylation which instead have elevated nucleotide diversity in freshwater compared to marine. Subsequently, we show that nucleotide diversity is higher among sites with ancestrally variable methylation and also positively correlates with the sensitivity to environmentally induced methylation change. Both suggest that as selection on the control of methylation state becomes relaxed, so too does selection against mutations at the sites themselves. Increased epigenetic variance in a population is therefore likely to precede genetic diversification.

Keywords: DNA methylation, stickleback, epigenetic, nucleotide diversity, pi, mutation

Abbreviations:

DM – Differential Methylation

DMC – Differentially Methylated Cytosine

DMR – Differentially Methylated Region

DNAme – DNA methylation

FW – Freshwater

FW-hyper – Hypermethylated in Freshwater population

FW-hypo – Hypomethylated in Freshwater population

Non-DMC – Non-Differentially Methylated Cytosine

RRBS – Reduced Representation Bisulfite Sequencing

SD – Standard Deviation

SNP – Single Nucleotide Polymorphism

Introduction

DNA methylation (DNAme) is an epigenetic mark whose roles in genome regulation, including gene expression regulation and transposable element suppression, have been well studied (He et al. 2011). Its role in local adaptation and long term evolution however, for example via plasticity, remains a topic of active debate. Methylome data support a potential role for DNAme in local adaptation in several species (Dubin et al. 2015; Sammarco et al. 2022), revealing that some genomic regions show differential methylation (DM) between different locally adapted populations. Populations (Johnson & Kelly, 2020) and species (Vernaz et al. 2021) with low genetic divergence from one another have been found to differ considerably in DNAme patterns at environmentally relevant loci, raising the possibility that flexibility of DNAme can be a source of phenotypic variation which increases adaptive potential of populations when genetic diversity is challenged. However, DNAme is far from independent of genetic diversity; it is determined by the presence of sites with the capacity to be methylated (typically a in a CpG context) and is subject to *trans*- and *cis*-regulation (Villicaña and Bell 2021). Therefore, the potential for methylation and corresponding plasticity is determined by the local genomic context, and thus the 'epigenetic potential' of a population evolves at the sequence level (Kilvitis et al. 2017). It is also established that the epigenetic conformation of the genome affects the propensity for sequence change. Notably, DNAme is well known to influence mutation rates due to the higher susceptibility of methylated Cs to spontaneous deamination to form thymine (Xia et al. 2012; Poulos et al. 2017; Zhou et al. 2020). Therefore, DNAme and sequence variation are intertwined (see also Adrian-Kalchhauser et al., 2020).

Despite the interdependence of DNAme and sequence variation, the potential importance of this link in local adaptation has been largely overlooked. For example, typical workflows for detection of differential methylation tend to exclude CpG sites that are not detected at a certain coverage in a certain proportion of individuals (e.g. Akalin et al., 2012), and therefore it could be assumed that genetic diversity of those sites is irrelevant. However, these sites may nevertheless harbour genetic variants in the population, the relative frequencies of which may be informative about evolutionary forces acting on methylation state, potentially allowing further dissection of the manner in which DM evolves in the context of local adaptation. Indeed, methylation sites within certain promoters have already been shown to exhibit selective sweep signatures in *Arabidopsis* (Shirai et al. 2021). Epigenetic diversification is one of many possible routes to local adaptation (e.g. Smith et al., 2016) but may occur in conjunction with others, such as selection on discrete new mutations (hard sweeps) or on standing genetic variation (soft sweeps) (Bernatchez 2016; Hermisson and Pennings 2017). For example, if epigenetic modifications at multiple loci could confer similar adaptive benefit, epigenetic diversification could occur in conjunction with a soft sweep. Furthermore, given that methylated cytosines

incur a heightened mutation risk, a gain in methylation in a divergent population may increase mutation rates at affected sites and *vice versa*. There is therefore a need to examine the relationships between differential methylation and nucleotide diversity in divergent populations.

The threespine stickleback fish (*Gasterosteus aculeatus*) has long been a popular model to study the genetics and, more recently, the epigenetics, of local adaptation. Ancestrally a marine fish, *G. aculeatus* has repeatedly and rapidly colonised freshwater habitats over the last few millennia, with large waves of colonisations having occurred since the formation of glacial lakes following the last ice age (Jones et al. 2012; Roberts Kingman et al. 2021). Freshwater-adapted morphs show numerous phenotypic adaptations including the loss of armour plating (Barrett et al. 2008) and changes to kidney morphology (Hasan et al. 2017). Phenotypic and genetic divergence has been observed over short time scales (Lescak et al. 2015), with large shifts in frequencies of particular alleles having been observed over just a few years in newly established lake populations (Roberts Kingman et al., 2021). Such rapid fixation of alleles on the basis of standing genetic variation is characteristic of a soft sweep (Bernatchez 2016). However, the rapid adaptability and high plasticity of sticklebacks (Day et al. 1994) also makes the contribution of epigenetic variation to freshwater adaptation compelling. Multiple studies have used bisulfite sequencing to reveal differentially methylated sites or regions between marine and freshwater populations, some of which are in the vicinity of genes relevant to freshwater adaptation (Smith et al. 2015; Artemov et al. 2017; Heckwolf et al. 2020; Hu et al. 2021).

A potential role for epigenetic variation in freshwater adaptation is especially pertinent given that the formation of freshwater populations has been characterised by population bottlenecks, constraining genetic diversity. Steep declines in the effective population size *Ne* have been observed in newly established freshwater populations both from time series experiments (Aguirre et al. 2022) and ancient DNA (Kirch et al. 2021). Interestingly, in their comparison of gill DNA methylomes between marine and freshwater fish in the White Sea region, Artemov et al. (2017) showed that freshwater fish had higher variance of DNAme compared to marine fish, in line with the idea that higher epigenetic variation could compensate for reduced genetic diversity, enhancing the adaptive potential of a population following a bottleneck.

While genome and / or epigenome data have been generated from multiple stickleback populations across the Northern hemisphere, the White Sea population complex is unique in that a variety of different data types have been generated from populations inhabiting the same region, including DNAme (Artemov et al., 2017), mRNA and small RNAseq (Rastorguev et al. 2016; Rastorguev et al. 2017), and whole genome pool-seq (Terekhanova et al., 2014; 2019).

Freshwater colonisation in this region has occurred relatively recently, with the oldest sampled lake estimated to have been formed approx. 700 years ago. Nucleotide diversity is likely to have decreased overall in this freshwater population due a past bottleneck (Terekhanova et al. 2014), but could be reduced further at specific sites due to purifying selection if the function of those sites becomes more important. However, diversity may increase due to lifting of selective constraints at sites whose function is less important in the new environment, or through locally elevated mutation rate due to methylation gain. Shifts in nucleotide diversity at DMCs may therefore be informative about the evolutionary forces acting on DNAme during local adaptation.

To address these intriguing hypotheses we combine epigenetic and genetic data from the White Sea stickleback population complex to study the interactions between methylation differences and nucleotide diversity during freshwater colonisation. We examined nucleotide diversity in relation to methylation divergence, variance, and the environmental inducibility of methylation state, considering both variance and inducibility as indicators of the relative stringency DNAme regulation.

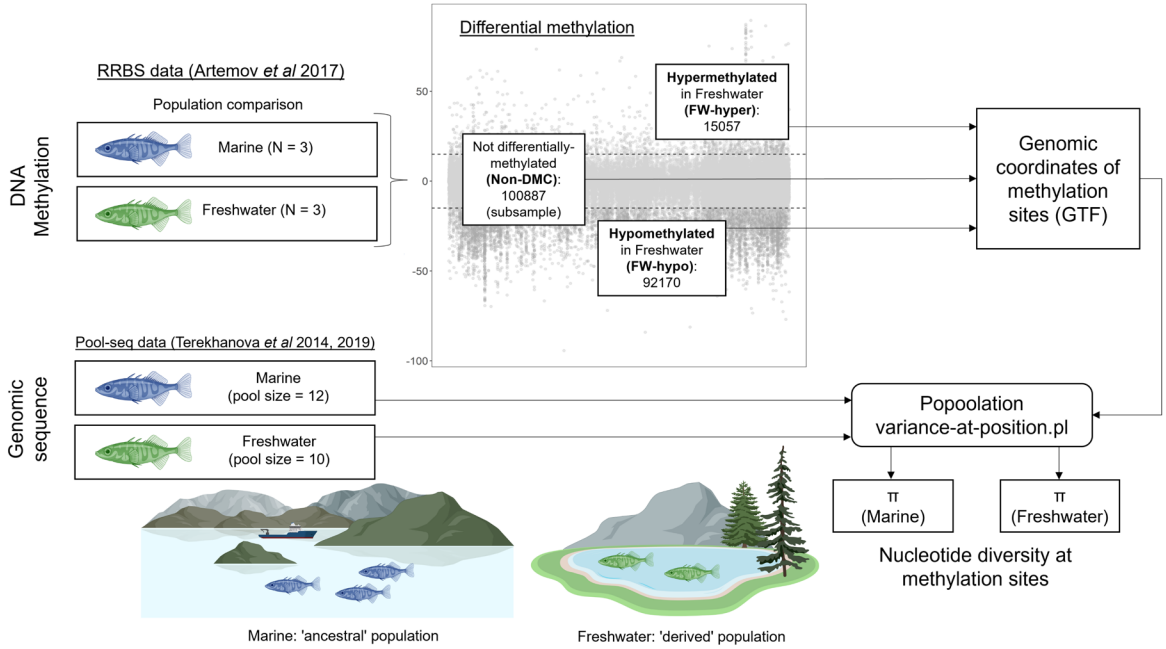


Figure 1. Analysis workflow for obtaining nucleotide diversity estimates for differentially methylated sites. Site-level differential methylation analysis was performed to compare marine (considered as 'ancestral' population) vs freshwater sticklebacks (considered as 'derived' population) using RRBS data previously published by Artemov *et al* (2017). Taking the marine population as the reference methylation state, sites were classified as FW-hyper (significantly higher % of methylated copies in freshwater compared to marine), not differentially-methylated (Non-DMC; no significant difference in % methylated copies between populations), or FW-hypo (significantly lower % of methylated copies in freshwater compared to marine). For non-DMCs, a subset of the total was used, comprising ~11% of the total non-DMCs (see methods). Coordinates of sites belonging to the three site classes (Non-DMC, FW-Hypo, and FW-hyper) were compiled in a GTF file for use with the variance-at-position.pl script from the Popoolation toolkit. The nucleotide diversity (π) of each site class on each chromosome was estimated from whole-genome pool-seq data (Terekhanova *et al*, 2014 & 2019) of marine and freshwater fish derived from the same or similar geographic locations as those taken for the RRBS data.

Results

Elevated nucleotide diversity accompanies differential methylation but level depends on the direction of methylation change

For generation of DNAme and genome sequence data, respectively, both Artemov et al. and Terekhanova et al. sampled freshwater fish from the same lake (Lake Mashinnoye) and marine fish from nearby coastal locations in the Kandalaksha gulf. A combined analysis of samples from these two datasets therefore allowed us to identify differentially methylated cytosines in CpG context (DMCs) between marine and freshwater populations and examine the nucleotide diversity of these sites in separate samples of those populations (**Fig. 1**).

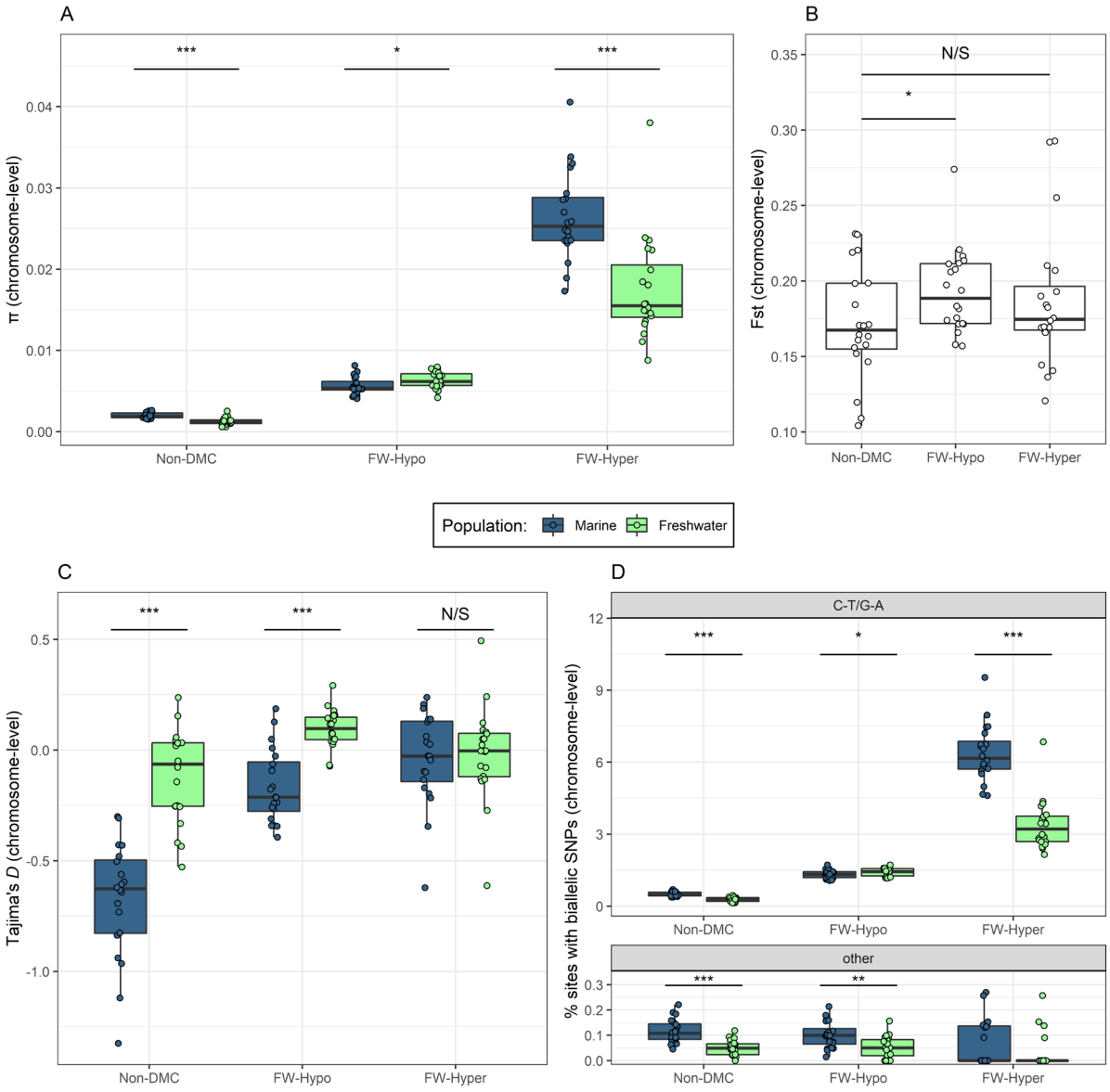
We first derived measures of nucleotide diversity of differentially methylated (DMCs) and non-differentially methylated DNAme sites (Non-DMCs) in the form of π (average number of pairwise differences within population) (Nei and Li 1979), Watterson's θ (Watterson, 1975), Tajima's D (Tajima 1989), and pairwise Fst (Weir and Cockerham 1984). All nucleotide diversity measures were derived from pool-seq data at methylation sites detected from RRBS data (**Fig. 1**). In a site-level differential methylation analysis, we used a standard workflow to detect DMCs in RRBS individuals which retained sites for which a methylation call was obtained in all of the analysed individuals. These included six individuals as part of the main population comparison and a further five which were used for a subsequent analysis of site inducibility. Therefore, our analysis relied on the assumption that even sites that are conserved across RRBS individuals would show some level of genetic diversity in the slightly larger subsets of the populations profiled by pool-seq. Considering Non-DMCs as representing a baseline level of nucleotide diversity at cytosines in CpG context, we compared the π of Non-DMCs with that of DMCs that either lost methylation (significantly fewer methylated copies, FW-hypo) or gained methylation in freshwater compared to marine (significantly more methylated copies, FW-hyper). The Non-DMCs we considered here represented a subsample of the total number of Non-DMCs which was extracted by taking one random Non-DMC within 2Mb of each DMC.

We observed consistent associations between differential methylation and nucleotide diversity. DMCs showed elevated π compared to Non-DMCs regardless of population, with the highest π observed amongst sites which gained methylation in freshwater (FW-hyper) (**Fig. 2A**). When comparing π between populations for each category of sites, π was slightly though significantly reduced in freshwater compared to marine at Non-DMCs, reflecting the expected reduction in nucleotide diversity in the derived population. π calculated for sliding windows across chromosome 1 suggested that the pattern of reduced diversity in freshwater is a genome-wide feature (**Fig. S1A**) which likely resulted from a past bottleneck. Freshwater

178 π was similarly lower at FW-hyper sites, but not at FW-hypo sites which rather showed
179 elevated π in freshwater compared to marine. Watterson's *θ* showed a similar pattern to that
180 of π (**Fig. S2**). Patterns of between-population diversity (Fst) were not so pronounced: When
181 compared to Non-DMCs, FW-hypo sites had slightly increased Fst, but FW-hyper sites did not
182 (**Fig. 2B**). Tajima's *D* was mostly negative in both populations as indicated by sliding windows
183 across chromosome 1 (**Fig. S1B**), but was relatively higher in freshwater, consistent with the
184 scenario of a population bottleneck following freshwater colonisation. Tajima's *D* of
185 methylation sites largely reflected this chromosome-wide pattern of higher values in
186 freshwater, with the exception of FW-hyper sites which did not differ in Tajima's *D* between
187 the two populations (**Fig. 2C**). Within populations, Tajima's *D* tended to be higher among
188 DMCs than non-DMCs, with the exception that in freshwater, FW-hyper sites showed no
189 difference in Tajima's *D* compared to Non-DMCs.

190 These patterns of elevated nucleotide diversity were not driven by enrichment for DMCs in
191 regions of high diversity. Rather, elevated π of DMCs was found to be strongly localised
192 around individual DMCs (**Fig. S3**). The pattern was largely consistent across different genomic
193 features including CpG islands, gene bodies, promoters, and intergenic regions, with the
194 exception of DMCs which fell within DMRs (**Fig. S3**).

195 Next, we determined which mutation type(s) were most likely to be driving the elevated π of
196 DMCs, and specifically whether this was driven by an over-abundance of C-T transitions. To
197 this end, the percentages of sites in each category harbouring biallelic SNPs of different types
198 (C-T/G-A, C-A/G-T, or C-G/G-C) were calculated from the pool-seq data. The majority of SNPs
199 were C-T/G-A, comprising 92% of SNPs across all categories in marine and 95% in
200 freshwater. The proportion of sites harbouring biallelic C-T/G-A SNPs across the three
201 categories of methylation site and two populations showed essentially an identical pattern to
202 that of π, with FW-hyper sites harbouring the highest proportion of C-T/G-A SNPs in both
203 populations (**Fig. 2D**). Marine had more C-T/G-A SNPs than freshwater in the Non-DMC and
204 FW-hyper categories but not the FW-hypo category, in which the freshwater population had
205 more C-T/G-A SNPs, also consistent with the pattern observed in π. Meanwhile, the % of
206 other SNP types showed no clear differences between the site categories and was not
207 elevated in the freshwater population amongst FW-hypo sites. Therefore, both the general
208 increased nucleotide diversity amongst DMCs and the heightened diversity of FW-hypo sites
209 in freshwater seemed to be driven by a greater occurrence of C-T mutations.



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211 **Figure 2. Nucleotide diversity of differentially methylated sites. (A)** π (average number of pairwise
212 differences), **(B)** Fst (marine vs. freshwater), and **(C)** Tajima's *D* estimated from pool-seq of marine and
213 freshwater sticklebacks for three classes of methylation site identified from RRBS individuals and
214 classified according to the direction of methylation difference in freshwater fish compared to marine
215 (Not differentially-methylated (Non-DMC), hypomethylated in freshwater (FW-Hypo), or
216 hypermethylated in freshwater (FW-Hyper)). Each measure was estimated for each chromosome
217 separately, such that one point represents an estimate from one chromosome. Significance stars derive
218 from paired Wilcoxon tests (comparison of chromosome pairs). **(D)** % of sites in each site class
219 harbouring biallelic SNPs of the type C-T/G-A (upper panel) or other types (C-A/G-T or C-G/G-T, lower
220 panel), estimated separately for each chromosome. Significance stars derive from paired *T*-tests.

221 *Higher diversity of infrequently-methylated DMCs*

222 The finding that sites which gained methylation in freshwater (FW-hyper) had the highest π
223 and highest proportion of C-T/G-A mutations (**Fig. 2**) was contrary to expectations, as these
224 sites would be expected to be infrequently methylated in the ancestral population and
225 therefore not at high risk of mutation via deamination. We therefore tested the relationship
226 between π and the distributions of mean % methylation across the three site categories. Here,
227 mean % methylation refers to the average % of copies on which the C is methylated, or in
228 other words the average frequency of the methylation mark among copies of given CpG site.

229 Because pool-seq data are not appropriate for estimating π at the level of a single site, we
230 used a ranking procedure to examine the relationship between methylation frequency and π.
231 Sites were divided into ranks according to their mean per-site % methylation, with higher ranks
232 containing sites with higher % methylation. This ranking was performed separately for each
233 population and each site category, and a measure of π obtained for each rank. We found that
234 Non-DMCs displayed a bimodal density distribution, with most sites either very frequently or
235 very infrequently methylated (**Fig. 3A**, left). The relationship between rank mean %
236 methylation and π was clearly non-monotonic for non-DMCs, with the highest values
237 appearing at low to intermediate methylation of around 25% (**Fig. 3B**, left). Meanwhile, the
238 distributions of % methylation of DMCs were markedly different to those of Non-DMCs. FW-
239 hypo sites were not characterised by a complete loss of methylation in the FW population.
240 Instead, they were characterised by a shift from mostly high % methylation in marine to mostly
241 intermediate % methylation in FW (**Fig. 3A**, middle). The higher π of FW-hypo sites in
242 Freshwater appeared to be driven largely by sites in the low-intermediate range (**Fig. 3B**,
243 middle). Meanwhile, FW-hyper sites were not characterised by a gain of methylation at
244 previously bare sites, but instead were characterised by a shift from low-intermediate %
245 methylation in marine to high % methylation in FW (**Fig. 3A**, right). Amongst FW-hyper sites,
246 those with the highest methylation clearly contributed to the lower π of these sites in the
247 freshwater population (**Fig. 3B**, right). In addition to absolute rank in % methylation, we also
248 examined the relationship of π with the mean change in % methylation (i.e. the extent of hypo-
249 or hypermethylation). We observed that among FW-hypo sites, the freshwater population had
250 the largest increases in the π where the hypomethylation was strongest (**Fig. 3C** and **Fig. 3D**).
251 FW-hyper sites also increased in π with the extent of hypermethylation (**Fig. 3C**), but this also
252 corresponded with greater loss of π in FW (**Fig. 3D**). Meanwhile, sites with stronger
253 methylation change in either direction (methylation loss or gain) had higher Fst (**Fig. 3E**).

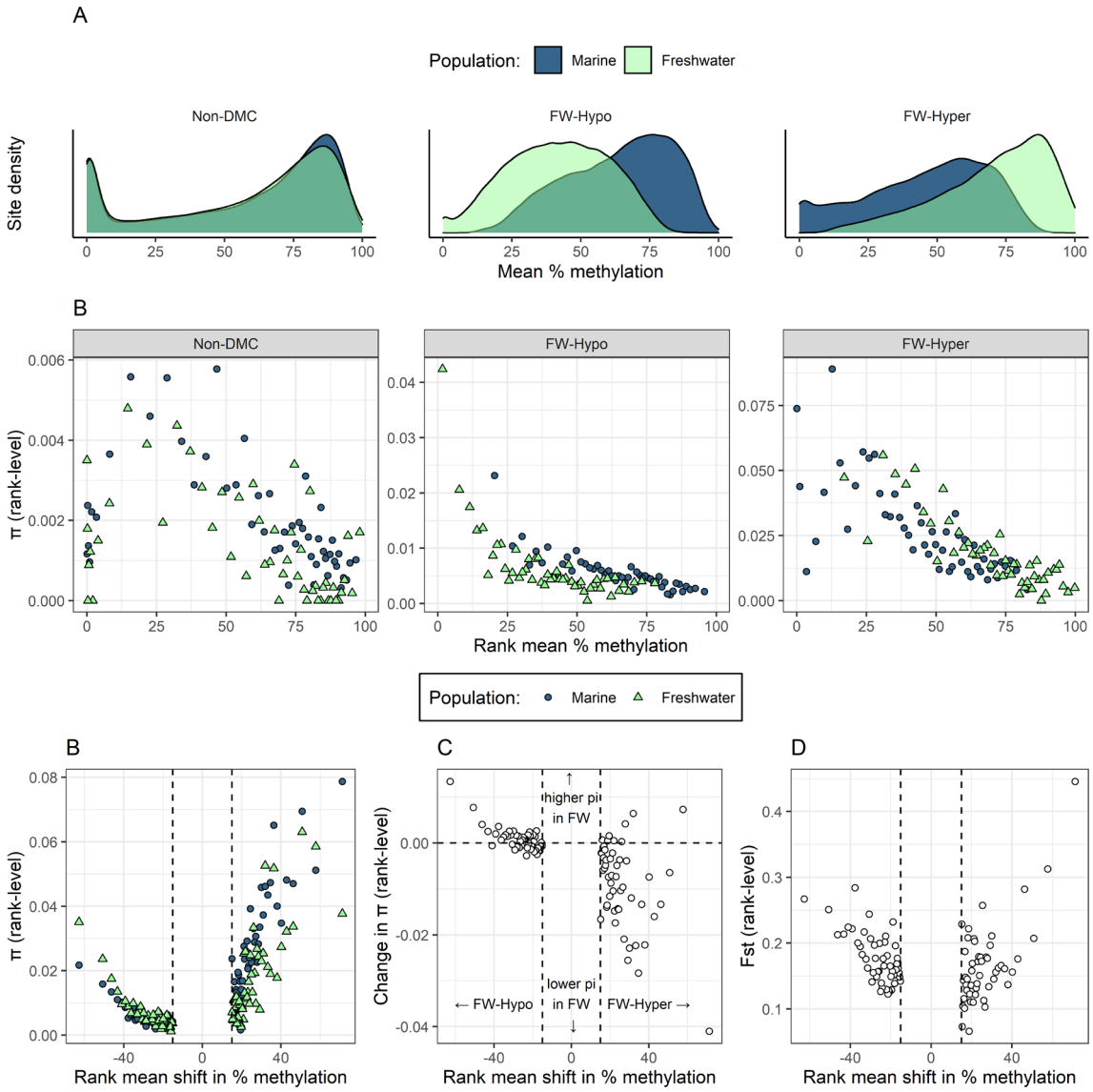

Figure 3. Distribution of mean % methylation (% of methylated copies at a given CpG site) in three site categories and their relationship with nucleotide diversity in marine and freshwater populations. (A) The relative distribution of sites along the mean % methylation in each population and site category. **(B)** π of sites that were ranked according to their mean % methylation in three site categories. A single π estimate was derived for all the sites in a given rank and π is plotted against the mean % methylation of sites in the rank. Sites were ranked separately for marine (blue circles) and freshwater (green triangles) populations and for Non-DMC (left panel), FW-hypo (middle panel), and FW-hyper sites (right panel). Each rank contains an average of 2038, 1843, and 301 sites for Non-DMC, FW-hypo, and FW-hyper, respectively. **(C-E)** Nucleotide diversity **(C)**, difference in nucleotide diversity (π of freshwater – π of marine) **(D)**, and pairwise FST (marine vs. freshwater) **(E)** of sites that were ranked according to the degree of hypo- or hypermethylation in freshwater. For each population, a single π estimate was derived for all the sites in a given rank. Each rank contains an average of 1843 and 301 sites for FW-hypo and FW-hyper sites, respectively.

High nucleotide diversity accompanies high variability in ancestral methylation

Considering that sites with intermediate mean methylation frequency are liable to have more variable methylation levels than those with very low or very high methylation frequency, we also considered the relationship between π and the standard deviation (SD) of % methylation. We predicted that sites with more variable methylation would have higher nucleotide diversity, reasoning that the methylation state of these sites is not stringently controlled and therefore mutations at these sites may have little impact on function. We first examined the distributions of standard deviations of % methylation for sites in the Non-DMC, FW-hypo and FW-hyper categories. Non-DMCs were largely invariable, with slightly more variable methylation in freshwater compared to marine (**Fig. 4A**, left), consistent with the observations of Artemov *et al* (2017). FW-hypo sites were characterised by a pronounced increase in SD from ancestral to derived population, shifting from a left-skewed distribution in marine to a Gaussian-like distribution in freshwater (**Fig. 4A**, middle). Meanwhile, FW-hyper sites, showed a skewed distribution in marine with an elongated plateau to the right, while methylation was less variable in freshwater (**Fig. 4A**, right).

To examine the relationship between π and the variability in methylation, sites were ranked according to the SD of % methylation in each population and site category. We observed that π increased steeply at an SD above ~15 in all three site categories in the marine population and two of the three site categories in freshwater (**Fig. 4B**). For the π of FW-hypo sites in FW however, there was no obvious relationship with the exception of two ranks showing highly elevated π at opposite ends of the range of SD values (**Fig. 4B**, middle). The high π of the lowest rank, which contradicted the trend observed in the other categories, is likely attributable to high ancestral diversity of sites that have almost completely lost methylation in freshwater (and therefore attain very low variance) but retain high ancestral nucleotide diversity. The relationship between π and the shift in SD of % methylation would appear to support this notion because sites with the largest decrease in SD in freshwater also have the highest π in marine (**Fig. 4C**). Shifts in the SD of methylation were also associated with shifts in π (**Fig. 4D**). Both amongst FW-hypo and FW-hyper sites, there was a trend towards reduced π with decreased SD and increased π with increased SD. No clear relationships were observed between the shift in mean % methylation and Fst for either FW-hypo or FW-hyper categories (**Fig. 4E**).

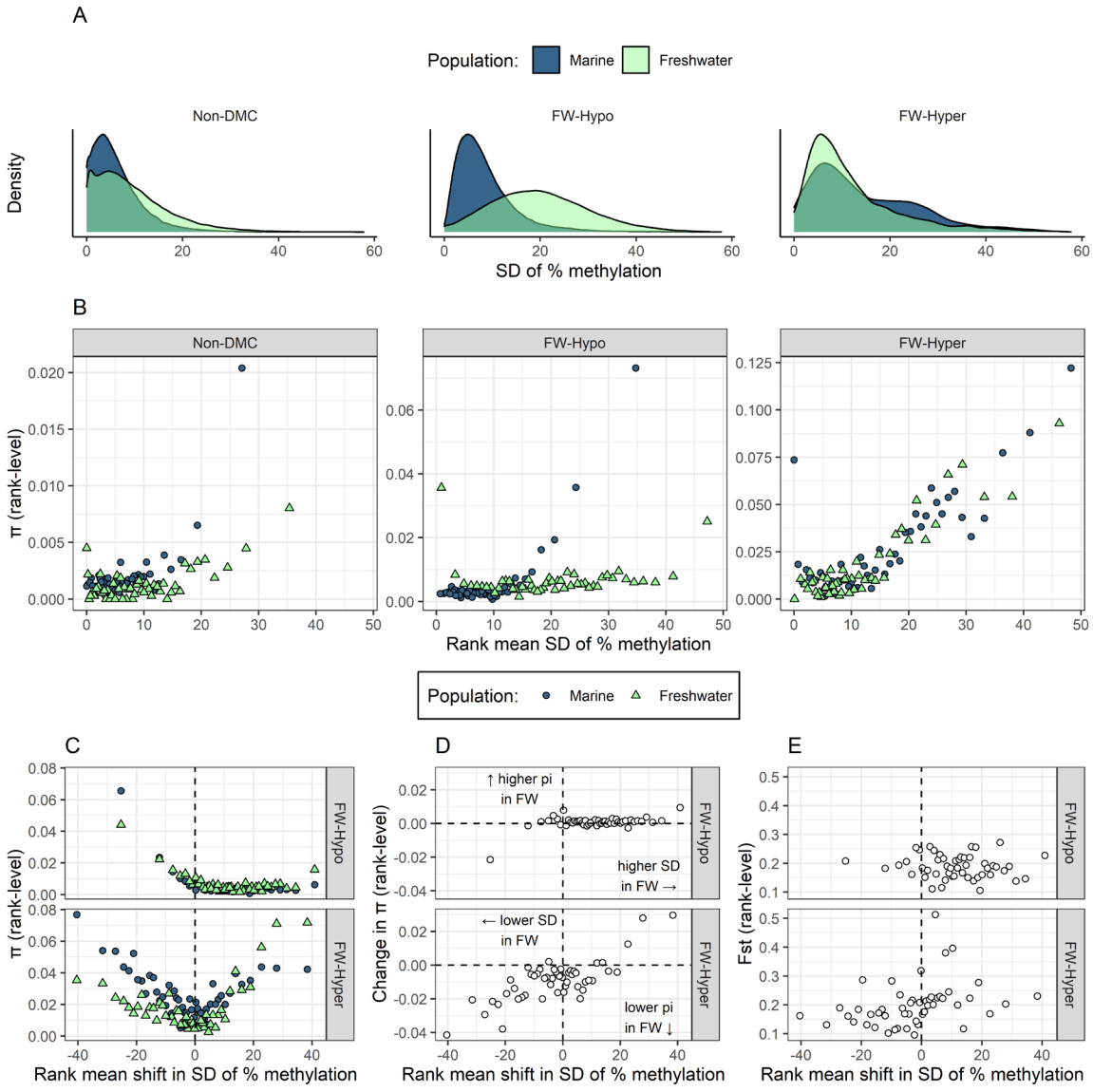

Figure 4. Distribution of standard deviation (SD) of % methylation levels in three site categories and two populations and their relationship with nucleotide diversity. (A) The relative distributions of SD of % methylation in each population and category. **(B)** π of sites that were ranked according to their SD of % methylation level. A single π estimate was derived for all the sites in a given rank and this rank-level π is plotted against the mean SD of sites in the rank. Sites were ranked separately for marine (blue circles) and freshwater (green triangles) populations and for Non-DMC (left panel), FW-hypo (middle panel), and FW-hyper sites (right panel). Each rank contains an average of 2038, 1843, and 301 sites for Non-DMC, FW-hypo, and FW-hyper sites, respectively. **(C-E)** π **(C)**, difference in π (π of freshwater – π of marine) **(D)**, and pairwise Fst (marine vs. freshwater) **(E)** of sites that were ranked according to difference in SD between marine and freshwater (SD of freshwater – SD of marine), where higher values represent higher SD in freshwater and lower values represent lower SD in freshwater. For each population, a single π estimate was derived for all the sites in a given rank. Each rank contains an average of 1843 and 301 sites for FW-hypo, and FW-hyper sites, respectively.

313 *Environmentally inducible DNA methylation is linked with higher nucleotide diversity*

314 So far, we have considered differential methylation in regard to losses or gains of methylation
315 that have been detected in a population ~700 years after its colonisation of a new environment.
316 While such changes may result from long-term processes, changes in methylation state can
317 also be directly induced by the environment. Such inducibility may be important for adaptation
318 but also subject to genetic variation. We therefore analysed additional samples from the
319 dataset by Artemov et al. (2017), as in addition to the main population comparison, the authors
320 also quantified gill methylation changes in fish from each population in response to a change
321 in environmental salinity. Fish from each population were exposed to the opposite conditions,
322 with marine fish exposed to reduced salinity and freshwater fish exposed to increased salinity
323 (**Fig. 5A**). Here, we reasoned that environmental inducibility of methylation state could also
324 reflect the degree of genetic versus environmental control of methylation state, similar to
325 increased SD of methylation possibly reflecting relaxed control of methylation state. Sites
326 whose methylation state is more responsive to the environment could be assumed to be under
327 looser genetic control and possibly relaxed selection. In total, sites that were induced in either
328 population constituted 3.9% of Non-DMCs, 12% of FW-hypo and 40% of FW-hyper sites, thus
329 representing a substantial enrichment of induced sites among DMCs and FW-hyper sites
330 especially. When considering the proportions of induced sites in each population separately
331 (**Fig. 5B**), freshwater had a higher proportion of induced sites than marine among FW-hypo
332 sites and a lower proportion among FW-hyper sites, a pattern that almost perfectly mirrored
333 that which was observed for π (**Fig. 2A**) and C-T/G-A mutations (**Fig. 2C**).

334 We find that sites that were induced in either population, or that were induced only in one of
335 the two populations, had elevated π compared to sites that were not induced in either
336 population (pairwise *T*-tests, all $P < 0.01$) (**Fig. 5C**), while the π of non-induced DMCs was
337 closer to that of Non-DMCs. Furthermore, the gain in π in freshwater among FW-hypo sites
338 appeared to be driven by sites that had gained inducibility (i.e. which were not previously
339 inducible by environment), as this elevated π was observed among sites that were induced
340 only in freshwater, but not sites that were induced only in marine. Meanwhile, amongst FW-
341 hyper sites, π was consistently lower in freshwater compared to marine, regardless of the
342 induced methylation category. When induced sites in each population were assigned to ranks
343 according to the mean absolute induced methylation change (i.e. regardless of whether salinity
344 change induced lower or higher methylation), π increased linearly with the mean induced
345 methylation change in both marine (linear model, $R^2 = 0.87$, $P < 0.001$) and FW populations
346 ($R^2 = 0.76$, $P < 0.001$) (**Fig. 5D**), suggesting that environmental inducibility can reliably predict
347 nucleotide diversity and vice-versa. A linear model of π as a function of inducibility and

population revealed a significant interaction between the two predictors (F(1) = 19.4, $P < 0.001$), reflecting the steeper relationship observed in the marine population.

Compared to non-induced sites, sites that were induced had significantly elevated pairwise Fst in both FW-hypo and FW-hyper categories (**Fig. 5E**). This elevated Fst was driven by sites that were induced in freshwater fish, as sites induced only in marine fish did not show elevated Fst compared to non-induced sites. The elevated Fst of FW-hypo sites (as seen in **Fig. 2B**) was therefore likely driven by sites that are inducible in the freshwater population. Finally, when induced sites in each population were ranked according to the degree of induced methylation change in that population, sites that were induced in the freshwater population showed a weak positive correlation between inducibility and Fst (linear model, $R^2 = 0.39$, $P < 0.001$), while sites that were induced in the marine population did not show such a correlation ($R^2 = -0.02$, $P = 0.63$) (**Fig. 5F**).

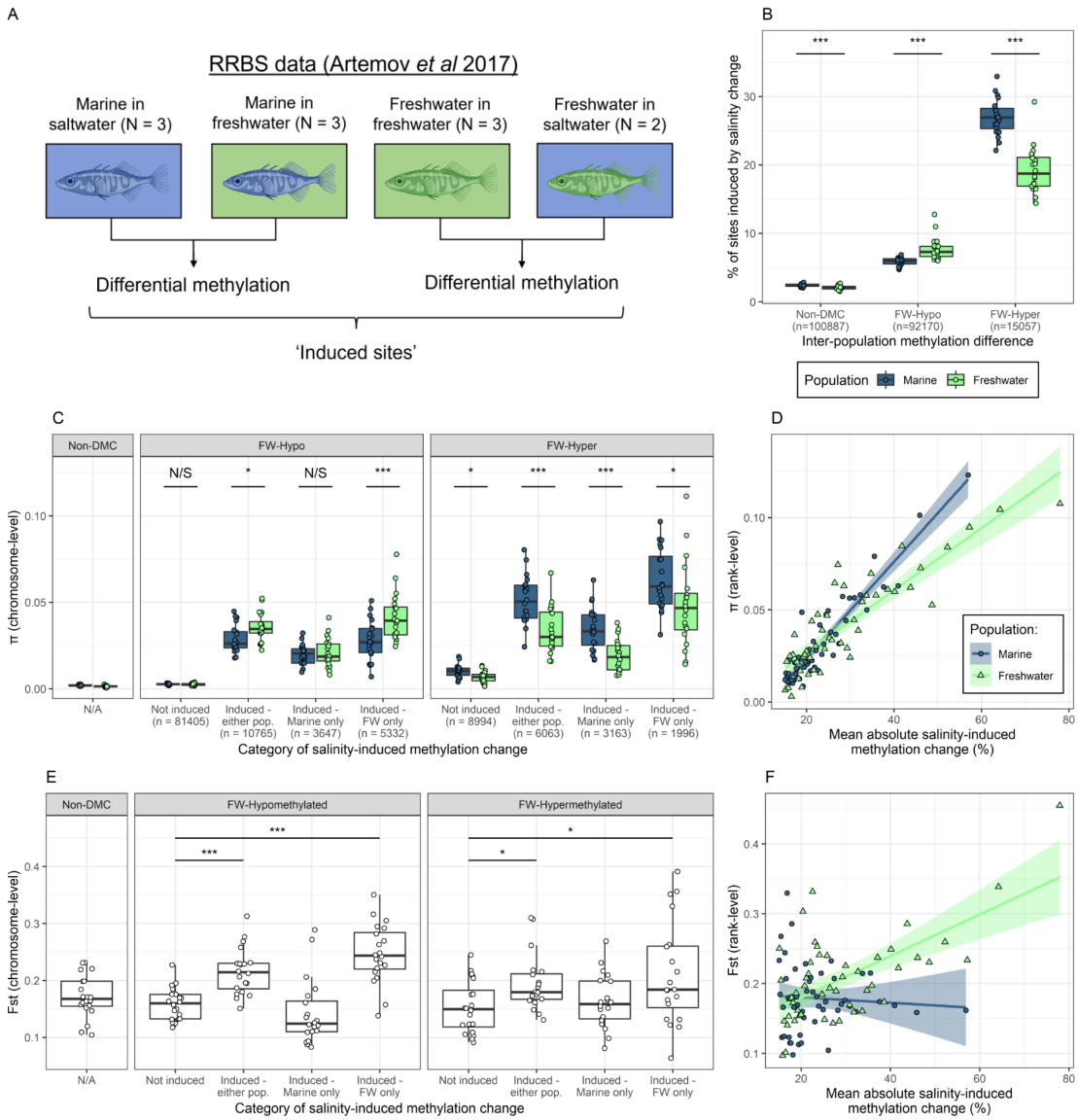

Fig. 5. Nucleotide diversity of differentially methylated sites in relation to their capacity for induced methylation change in response to environmental salinity. (A) Additional RRBS data deriving from experimental salinity treatments performed by Artemov et al (2017) (marine fish placed in freshwater and freshwater fish placed in saltwater) were used to identify sites that were inducible in response to salinity change in either of the two populations. **(B)** % of sites in the Non-DMC, FW-hypo, and FW-hyper categories that were induced in response to salinity change in the marine (blue) and freshwater (green) populations. **(C)** Per-chromosome estimates of π for FW-hypo and FW-hyper sites divided according their capacity for induced gill methylation change in response to a change in environmental salinity, considering sites that were induced in neither of the populations, either of the two populations, or only in one of the two populations (marine or freshwater). π of Non-DMCs is shown in separate panel for comparison. **(D)** π of inducible DMCs that were ranked according to their mean absolute induced change in % methylation (i.e. regardless of the direction of the change). Within each population, only sites that were significantly differentially methylated in response to salinity change (mean change in % methylation >/= 15) were considered. Separate ranks were obtained for marine and freshwater populations and a single π estimate obtained for each rank. Each rank contains an average of 238 sites for Marine and 242 sites for Freshwater. **(E)** Per-chromosome estimates of pairwise Fst (freshwater vs. marine) of FW-hypo and FW-hyper sites divided according to their capacity for induced methylation change. Fst of Non-DMCs is shown in separate panel for comparison. **(F)** Pairwise Fst of inducible DMCs that were ranked according to their mean absolute induced change in % methylation (i.e. regardless of the direction of the change).

Discussion

Here, we examined the relationship between DNAme changes and nucleotide diversity in an ancestral and a derived population of wild threespine stickleback. For this purpose, CpG sites with different methylation status in freshwater compared to the marine environment were considered as changes in DNAme that occurred in the course of freshwater colonisation. Our analyses show that genetic diversity is intimately linked to variable and inducible DNAme in these populations. This link between DNAme and nucleotide diversity is useful for inferring the evolutionary forces acting on methylation state and could also hint at the extent to which epigenetic changes precede sequence evolution.

Hypermutability of 5mC drives high nucleotide diversity at sites prone to methylation divergence

Despite applying stringent filtering to retain only Cs in CpG context that were detected in all RRBS individuals (requiring all individuals to be either C/C or heterozygous at reference CpG loci), we found that not only did DMCs harbour SNPs among the individuals represented in the pooled sequencing dataset, but were even enriched for them. Methylation changes occurred at sites which already had high standing genetic variation. In general, reduced nucleotide diversity was expected in the freshwater population given that colonisation of the freshwater environment likely entailed a population bottleneck (Terekhanova et al. 2014). This lower diversity was observed even for FW-hyper sites, despite the expectation that sites gaining methylation should incur higher mutation rates (Xia et al. 2012). However, FW-hypo sites – those that had lost methylation in freshwater – exhibited an increase in π in freshwater compared to marine, implying relaxed selection among these sites in the derived population.

Different measures of genetic diversity can provide further insight into possible evolutionary processes at a fine scale. As well as higher π, DMCs also had higher Tajima's *D* (higher proportion of intermediate-frequency alleles) than non-DMCs within the marine population, suggesting that those sites with a tendency to diverge in methylation state are those that were already under weaker selective constraint (Jackson et al. 2015). The generally higher Tajima's *D* in FW was expected following a recent population bottleneck (Stajich and Hahn 2005). However, this increase was not observed at FW-hyper sites, whose Tajima's *D* was comparable to that of Non-DMCs in the same population. This shows that the accumulation of intermediate-frequency alleles was impeded among these sites, possibly suggesting increased selective constraint. Fst was slightly elevated among FW-hypo sites, suggesting that sites that lost methylation are subject to differential selection.

DNA methylation and mutations rates are instrinsically linked by the hypermutability of 5mC. We subsequently showed that the patterns of nucleotide diversity are driven by higher

413 abundance of C-T/G-A SNPs amongst DMCs, but not other SNP types, suggesting that they
414 are driven by higher rates of spontaneous deamination of methylated CpGs. (Xia et al. 2012).
415 The hypermutability of 5mC may further explain why FW-hypo sites were >6x more common
416 than FW-hyper sites, as new methylations are more likely to be overridden by mutation than
417 new demethylations. Thus, stable gains in methylation are more difficult to attain than stable
418 losses.

419 *Relationships between nucleotide diversity and methylation frequencies further imply*
420 *differential selection of DMCs*

421 The relative frequencies at which sites are methylated (expressed as % methylation) captures
422 both intra- and interspecific variation and we therefore considered it a useful measure in our
423 inference of evolutionary forces acting on methylation state. Although the RRBS data derived
424 from a specific tissue – gill, such a tissue is nevertheless heterogeneous, comprising of
425 different specialised cell types (Pan et al. 2022). Very high or very low methylation frequencies
426 are therefore likely to comprise sites where methylation state is not cell type-specific, i.e. the
427 same state is consistently maintained across a majority of cell types. Although cell type-
428 specific methylation is likely to be important in some contexts (Loyfer et al. 2022), it could
429 nevertheless be inferred that sites with consistent methylation state are more likely to be
430 selectively constrained. We observed that among Non-DMCs, most sites had either very high
431 or very low % methylation, suggesting most sites have a methylation state that is consistently
432 maintained across cell types (i.e. consistently methylated or non-methylated). Those Non-
433 DMCs with intermediate methylation frequencies had higher π than those with very high or
434 very low frequencies, again consistent with stronger selective constraint on sites which are
435 consistently either methylated or non-methylated. The observation is consistent with previous
436 observations that sites in the human genome with low to intermediate methylation frequency
437 *in vitro* have higher mutation rates in human populations (Xia et al. 2012). We found that
438 differential methylation in the freshwater sticklebacks was characterised by shifts either
439 towards (FW-hypo) or away from (FW-hyper) intermediate methylation frequency. π increased
440 in freshwater with the degree of hypomethylation and decreased with the degree of
441 hypermethylation, while Fst tended to increase with the degree of change in either direction.
442 Combined, these patterns imply that differential methylation occurs alongside differential
443 selection, in that (stable) gains in methylation tend to be selectively constrained while sites
444 that lose methylation are released from selective constraint. This would make sense given that
445 a loss of methylation relinquishes the requirement of the locus to remain as a CpG
446 dinucleotide.

447 *Genetic variation reflects (ancestral) epigenetic variation*

448 Interspecific variability of DNA methylation remains understudied in natural populations, and
449 yet it may hint at the processes by which the methylome evolves. Using the same RRBS
450 dataset, Artemov et al. (2017) previously showed that methylation was more variable in the
451 freshwater population. Here, we show that this effect depends on differential methylation:
452 While higher variability was noticeable among the distributions of Non-DMCs, it appeared to
453 be driven largely by FW-hypo sites which showed a Gaussian-like distribution of SD values in
454 the FW population, in stark contrast to the left-skewed distribution of the same sites in marine.
455 FW-hyper sites meanwhile showed the opposite pattern, becoming less variable in FW. Loss
456 of methylation in FW therefore appears to be characterised by relaxed control of methylation
457 state, while gain of methylation characterised by tighter control. Indeed, heightened variability
458 of FW-hypo sites suggests that loosening of regulation is itself the cause of methylation loss.

459 π tended to increase with variability of methylation, which would support the hypothesis that
460 sites with less tightly maintained methylation state are under weaker selective constraint. This
461 pattern was consistent across all three site categories in the marine population but was absent
462 among FW-hypo sites in FW. Genetic variation therefore reflects ancestral, but not recently
463 acquired methylation variance. Relaxed selection on sites which lose methylation would lead
464 to an accumulation of C-T mutations, while stronger selective constraint would reduce the
465 nucleotide diversity of sites with stable methylation gain. Concordantly, sites that became less
466 variable in freshwater tended to lose π, while sites that became more variable tended to gain
467 pi. The pattern was more prominent however among FW-hyper sites which predominantly had
468 both decreased SD of methylation and decreased π. Among FW-hypo sites therefore, the lack
469 of correlation between SD and π in freshwater (or change in SD and change in π) can be
470 explained by the relatively young age of the freshwater population (~700 years) and
471 subsequent lack of time for mutations to accumulate.

472 While it is plausible that the increased π and SD of FW-hypo sites reflects relaxed selection
473 on the regulation of methylation state, an alternative hypothesis is that variable loss of
474 methylation reflects epigenetic responses that have occurred only in a fraction of individuals
475 in the population. This scenario would also be consistent with elevated π of the FW-hypo sites;
476 if these differential epigenetic responses were genotype-specific, the elevated π would reflect
477 standing genetic variation as opposed to new mutations. Such a scenario would be consistent
478 with a soft sweep (Hermisson and Pennings 2017), in which selection could act on many
479 different genetic and epigenetic loci, thus maintaining diversity at both levels.

480 The reduction in both π and SD at FW-hyper sites would suggest heightened selection for
481 new methylations at a subset of sites. While Fst values did not support the idea that these

482 sites were targets of a selective sweep, this may be difficult to detect due to the young age of
483 the population and high standing genetic variation of FW-hyper sites. We also note that, as
484 sites with newly gained methylation must escape deamination in several individuals in order
485 to be detected in the differential methylation analysis (due to stringent site filtering), FW-hyper
486 sites are likely to be enriched for the subset of new methylations that are under selective
487 constraint.

488 *Environmental inducibility of DNA methylation may predict sequence evolution*

489 The potential importance of epigenetic responses in mediating plastic responses has long
490 been discussed (Johnson & Tricker, 2010). Indeed, experimental evolution studies have
491 revealed the importance of chromatin silencing for mediating plasticity and population
492 expansion in rapidly evolving model organisms such as yeast (Stajic et al. 2019). Here we
493 considered environmental inducibility in a different context, in that the degree of environmental
494 inducibility of methylation state is (inversely) indicative of the degree of intrinsic regulation. We
495 found that elevated π of DMCs in general, as well as the elevated Fst of FW-hypo sites, was
496 driven by sites that were environmentally inducible, further supporting a hypothesis of relaxed
497 regulation and relaxed selective constraint of these sites. Furthermore, the gain in π among
498 FW-hypo sites was associated with increased environmental inducibility of these sites,
499 suggesting that nucleotide diversity is more likely to accumulate at sites where genetic control
500 of methylation is relaxed (and therefore more sensitive to the environment). Indeed, the
501 positive correlation between π and the degree of inducibility suggests that the more sensitive
502 the methylation state is to the environment, the more likely mutations are to be selectively
503 neutral. Therefore, shifts in inducibility may precede shifts in nucleotide diversity, as we also
504 interpreted for shifts in methylation variance. Our results suggest that the majority of
505 environmentally inducible sites are simply 'blowing in the wind' and do not have important
506 functions for plasticity which would constrain nucleotide diversity. Nevertheless, in their
507 analysis of Baltic Sea sticklebacks, Heckwolf et al. (2020) observed that the Fst of induced
508 sites (marine fish responsive to lower salinity) depended on whether the induced methylation
509 change occurred in the same direction as the 'evolved' methylation state observed in a
510 freshwater population; sites that were induced in the opposite direction (further from expected
511 state) showed higher Fst than those that were induced in the expected direction. This suggests
512 that some environmentally inducible sites are indeed constrained by selection due to the
513 importance of site plasticity. Here, we did not consider the direction of inducible change,
514 merely considering inducibility as a proxy for the relative weakness of intrinsic regulation.

515 Our observations would not be incompatible with the scenario of a soft sweep, as it is also
516 possible that plasticity of only some methylation sites is necessary to confer adaptation, while

517 others can mutate without detrimentally affecting the organism's capacity for adaptive
518 plasticity.

519 *Limitations and Future directions*

520 Here, we have revealed striking associations between genetic and epigenetic variation in
521 divergent stickleback populations. However, as we have examined only one population pair,
522 it is not known whether such patterns occur more broadly across different local adaptations
523 (in stickleback and other species) or whether they are idiosyncratic to the relatively recent
524 colonisation event considered in this study (~700 years). The existence of far older
525 populations, such as those in the Japanese archipelago which are estimated to have colonised
526 freshwater ~170,000 years ago (Kakioka et al. 2020), raises the question as to the fate of
527 differential methylation over longer periods. Over time, for example, the initially heightened
528 methylation variance may return to a less variable state due to refinement of methylation states
529 via selection or the removal of the CpG sites via accumulation of C-T transitions. Alternatively,
530 no accumulation of mutations over time would suggest that the heightened diversity of FW-
531 hypo sites merely reflects standing genetic variation.

532 If, indeed, heightened methylation variance arises due to relaxed control of methylation state,
533 the mechanisms by which this could occur are not known. Artemov et al (2017) suggested that
534 mutations in genes encoding epigenetic regulators may underlie increased methylation
535 variance, but did not identify any known epigenetic regulators in the vicinity of genomic regions
536 differentiating marine and freshwater populations in the White Sea region. *Trans*- and cis-
537 meQTL have however been identified in stickleback (Hu et al. 2021), some of which are indeed
538 in the vicinity of genomic regions of high Fst between marine and freshwater populations.
539 Differential selection on *trans*-meQTL in particular could have knock on effects on methylation
540 sites across the genome.

541 Another limitation is that the RRBS data we used came from only a single tissue type (gill),
542 and therefore we were unable to determine which methylation differences between
543 populations are tissue-specific and which are organism-wide. However, many divergent
544 methylation changes are not tissue specific, as a recent analysis of divergent cichlid ecotypes
545 showed that a high proportion of DMRs were shared across tissue types (Vernaz et al. 2021).

546 Finally, the detection of differential methylation is highly sensitive to the analytical methods
547 applied. Namely, the retention of individual-specific CpG sites can lead to the detection of
548 differential methylation simply due to differences in the abundance of CpGs available to be
549 methylated – i.e. directly due to SNPs (Wulfridge et al. 2019). We suggest that whether or not
550 individual-specific CpGs are retained in an analysis, and by extension the definition of
551 'differential methylation' should depend on the goals of the study. Here, by excluding

individual-specific sites we could be confident that the detected differential methylation arose through the differential action of the methylation machinery and not due directly to nucleotide variation at the sites themselves. The finding that these sites are nevertheless enriched for SNPs in the population is therefore all the more striking. We acknowledge that excluding individual-specific CpGs risks ignoring a potentially high proportion of methylation variation and, while it was not the goal of this study to extensively characterise this variation, the results should be interpreted with this in mind.

Conclusions

Relationships between genetic and epigenetic variation show that these different levels of variation are intertwined in natural populations. Nucleotide diversity in different methylation contexts is therefore informative of the evolutionary forces acting on methylation state and the origin of differential methylation itself. Subsequently, we identify evidence of differential selection on methylation sites which, combined with patterns of methylation variance and environmental inducibility, support a hypothesis that differential methylation is driven by shifts in the degree of intrinsic control of methylation state. Shifts in this control, it seems, precede increases in nucleotide diversity, and may therefore indicate parts of the genome where diversification is imminent. Heightened diversity of DMCs may also reflect a soft sweep which retains diversity at both genetic and epigenetic levels, a scenario compatible with previous genetic studies of local adaptation in stickleback (Terekhanova et al. 2014; Roberts Kingman et al. 2021). Indeed, our results support the idea that epigenetic variation should be incorporated alongside genetic mechanisms of adaptation in models of species adaptation and evolutionary potential (Bernatchez 2016). Our analyses further demonstrate the exciting potential held in published datasets for exploring combined patterns of genome and epigenome evolution.

Methods

Datasets

We obtained a reduced-representation bisulfite sequencing (RRBS) dataset published by Artemov et al. (2017) (SRA project accession: PRJNA324599) comprising a total of six marine sticklebacks (of which three were exposed to lower salinity) and six freshwater sticklebacks (of which three were exposed to higher salinity; however, one of these three samples was excluded due to incomplete bisulfite conversion). The freshwater fish used for RRBS were sampled from Lake Mashinnoye, while the marine fish were sampled from the Kandalaksha gulf. Freshwater fish were also collected for pool-seq by Terekhanova et al. (2014) from Lake Mashinnoye (SRA run accession: SRR869609), while marine fish were collected from the Kandalaksha gulf as part of the 2014 study and a subsequent 2019 study (Terekhanova et al.

587 2019). We selected the 'White Sea, WSBS' sample from Terekhanova et al. (2019)
588 (SRR7470095) as the marine sample for our comparison, given that it has a similar pool size
589 to the Mashinnoye sample (12 vs 10) and a similar number of reads (64,176,648 vs
590 62,016,859 after quality trimming). Sequence files were obtained in FASTQ format from the
591 Sequence Read Archive (SRA) and European Nucleotide Archive (ENA).

592 *Data processing: RRBS*

593 Raw RRBS reads were trimmed using TrimGalore v0.6.6 using default settings. Alignment to
594 the Threespine stickleback v.5 assembly (Nath et al. 2021) and subsequent methylation calling
595 were carried out using Bismark v0.22.3 (Krueger and Andrews 2011) with Bowtie2 v2.3.4.1 as
596 the aligner (Langmead and Salzberg 2012). Methylation calls were not strand-specific. To
597 remove sites harbouring C-T/G-A SNPs which otherwise contribute erroneous counts of non-
598 methylated Cs, we ran BS-SNPer v1.1 (Gao et al. 2015) on each sample using a minimum
599 coverage of 5 and otherwise default settings, compiled the coordinates of all sites harbouring
600 C-T/G-A SNPs in any of the individuals (either homo- or heterozygous), and removed these
601 sites from the Bismark coverage files containing the methylation counts (counts of Cs and Ts
602 at each position).

603 *Data processing: Pool-seq*

604 Raw reads were trimmed with Trimmomatic v0.36 (Bolger et al. 2014) with the option
605 SLIDINGWINDOW:4:20 and otherwise default parameters. Only reads which remained paired
606 after trimming were kept. Reads were mapped to the Threespine stickleback v.5 assembly
607 with Bowtie2 v2.3.4.1 with default parameters. Sambamba v0.7.1 (Tarasov et al. 2015) was
608 used to filter the alignments to retain those with MAPQ >/=20 and to remove PCR duplicates.
609 Samtools v0.1.18 (Danecek et al. 2021) was used to generate a pileup file from each BAM
610 file, as required for the Popoolation and Popoolation2 toolkits.

611 *Identification of differentially methylated CpG sites (DMCs) and subsampling of non-DMCs*

612 Site-level differential methylation analysis was carried out using the methylKit R package
613 v1.22.0 (Akalin et al. 2012), inputting the SNP-filtered Bismark coverage files. We omitted
614 sites which did not have at least 5x coverage in each of the 11 samples as well as sites located
615 on the mitochondrial chromosome and two sex chromosomes (chromosomes XIX and Y). We
616 then filtered out sites that had either 0% or 100% methylation (i.e. no variation) in all samples
617 from the main population comparison (3x marine and 3x freshwater). Three differential
618 methylation analyses were then performed separately, comprising the comparisons also
619 described in Artemov et al (2017): (1) marine fish in saltwater vs freshwater fish in freshwater
620 (main population comparison), (2) marine fish in saltwater vs marine fish in freshwater (marine

low salinity treatment), and (3) freshwater fish in freshwater vs freshwater fish in saltwater (freshwater high salinity treatment). All groups comprised N=3 with the exception of freshwater fish in saltwater (N=2, due to low bisulfite conversion efficiency of sample SRR3632642). Regardless, we considered sites to be differentially methylated given a change in % methylation of >/= 15 and a FDR-corrected p-value of </= 0.05. The purpose of the experimental comparisons was to identify which population-DMCs were also induced by salinity change, and so we did not consider sites that were induced by salinity change but not differentially methylated between populations. We also did not consider the direction of induced change (hypo- or hypermethylated in response to salinity change). Subsequently, we detected 92,710 sites that were hypomethylated in freshwater compared to marine (of which 10,765 were induced in either population) and 15,057 sites that were hypermethylated in freshwater compared to marine (of which 6063 were induced in either population). 897,733 sites were not differentially methylated between populations, which we would use as reference sites when examining nucleotide diversity. Due to the possibility that DMCs could be distributed non-randomly across a chromosome and given that nucleotide diversity can vary across a chromosome (e.g. lower diversity in centromeric regions), we used a sampling procedure which randomly selected one non-differentially methylated site that was within 2kb upstream or 2kb downstream of each DMC. After removing duplicate samples, this resulted in a subsample of 100,887 non-differentially methylated CpG sites (non-DMCs).

Nucleotide diversity of site categories

We used the variance-at-position.pl script from the Popoolation toolkit (Kofler, Orozco-terWengel, et al. 2011) to calculate within-population nucleotide diversity statistics (π, Watterson's θ, and Tajima's D) for different categories of site (e.g. non-DMC, FW-hypo, FW-hyper) on each chromosome for each population. The decision to obtain estimates of π on a per-chromosome basis was because Popoolation's estimates of π are accurate over large numbers of sites, but not at the single site level (Kofler, Orozco-terWengel, et al. 2011). Each site was labelled with its chromosome and its category within the analysis (e.g. chrI FW-hypo) and the labelled category was entered as the 'gene ID' in a GTF file, such that variance-at-position.pl, which was developed to calculate diversity statistics per-gene, was instructed to calculate π for each combination of chromosome and site category. A similar procedure was used to obtain π for sites ranked according to (change in) mean % methylation, (change in) SD of % methylation, and absolute inducibility, whereby ranks were assigned using the bin() function from the OneR package and sites were labelled in the GTF according to their rank (regardless of chromosome), such that a single value of π was obtained for each rank. Variance-at-position.pl from Popoolation was run with the parameters --min-qual 20 --min-coverage 3 --min-count 2.

657 Fst for different categories of methylation sites (including ranked sites) were obtained using
658 the Popoolation2 toolkit (Kofler, Pandey, et al. 2011). 'Gene-wise' .sync files were obtained
659 from the pileup files using coordinates in the abovementioned gtf files and were used as input
660 for the 'fst-sliding.pl' script which was run with parameters --min-count 2 --min-coverage 3 --
661 pool-size 22 --min-covered-fraction 0 --max-coverage 1000 --window-size 1000000 --step-size
662 1000000.

663 *Percentage of sites with SNPs*

664 To obtain the % of sites within each result category (non-DMC, FW-hypo, and FW-hyper)
665 harbouring SNPs of different types (C-T/G-A and non-C-T/G-A) in the pool-seq samples, we
666 filtered the BAM files of each population to retain alignments corresponding with the positions
667 of interest. We then ran GATK HaplotypeCaller (McKenna et al. 2010) with the –sample-ploidy
668 set to the pool size x 2 (24 for marine and 20 for freshwater), and otherwise default settings.
669 The subsequent VCF file was then filtered using bcftools v1.10 to retain only biallelic SNPs at
670 the sites of interest. We subsequently extracted from the VCF a list of reference and alternate
671 alleles at sites of interest harbouring biallelic SNPs. We were therefore able to assign SNPs
672 as either 'C-T/G-A' or 'other', and calculate the % of sites in each result category harbouring
673 biallelic SNPs of one of those two classes.

674 *Statistical comparisons*

675 All plotting and statistical analyses were carried out in R version 4.2.0 (R Development Core
676 Team 2011), with plots generated using the ggplot2 package (Wickham 2011). For comparing
677 nucleotide diversity between populations, we first used Shapiro-Wilkes tests to determine
678 whether the distribution of pairwise differences (paired chromosomes) differed significantly
679 from a normal distribution. Paired *T*-tests were used in the case of normally-distributed
680 pairwise differences and paired Wilcoxon tests were used in the case of non-normally-
681 distributed pairwise differences. Linear models of nucleotide diversity parameters as a function
682 of inducibility were fit using the lm() function from the stats package.

683 **Supplementary material**

684 Supplementary figures and methods are available in the online supplementary material.

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687 Calculations were performed on UBELIX (http://www.id.unibe.ch/hpc), the HPC cluster at the
688 University of Bern. Elements of Figures 1 and 5 were created with BioRender.com.

Data availability

This work used entirely pre-existing datasets available under SRA project accessions PRJNA324599 (RRBS data), PRJNA204958, and PRJNA479509 (whole genome pool-seq). Code for performing analyses has been deposited on GitHub in the repository: https://github.com/jamesord/StickMethDiv.

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