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The role of plastic and evolved DNA methylation in parallel adaptation of threespine stickleback (*Gasterosteus aculeatus*)

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Abstract

Repeated phenotypic patterns among populations undergoing parallel evolution in similar environments provide support for the deterministic role of natural selection. Epigenetic modifications can mediate plastic and evolved phenotypic responses to environmental change and might make important contributions to parallel adaptation. While many studies have explored the genetic basis of repeated phenotypic divergence, the role of epigenetic processes during parallel adaptation remains unclear. The parallel evolution of freshwater ecotypes of threespine stickleback fish (*Gasterosteus aculeatus*) following colonization of thousands of lakes and streams from the ocean is a classic example of parallel phenotypic and genotypic adaptation. To investigate epigenetic modifications during parallel adaptation of threespine stickleback, we re-analysed three independent data sets that investigated DNA methylation variation between marine and freshwater ecotypes. Although we found widespread methylation differentiation between ecotypes, there was no significant tendency for CpG sites associated with repeated methylation differentiation across studies to be parallel versus nonparallel. To next investigate the role of plastic versus evolved changes in methylation during freshwater adaptation, we explored if CpG sites exhibiting methylation plasticity during salinity change were more likely to also show evolutionary divergence in methylation between ecotypes. The directions of divergence between ecotypes were generally in the opposite direction to those observed for plasticity when ecotypes were challenged with non-native salinity conditions, suggesting that most plastic responses are likely to be maladaptive during colonization of new environments. Finally, we found a greater number of CpG sites showing evolved changes when ancestral marine ecotypes are acclimated to freshwater environments, whereas plastic changes predominate when derived freshwater ecotypes transition back to their ancestral marine environments. These findings provide evidence for an epigenetic contribution to parallel adaptation and demonstrate the contrasting roles of plastic and evolved methylation differences during adaptation to new environments.

KEYWORDS
DNA methylation, epigenetics, *Gasterosteus aculeatus*, parallel evolution, plasticity

1 | INTRODUCTION

The extent to which similar phenotypes evolve in independent populations inhabiting similar environments, that is, parallel (or convergent) evolution, is a major question in evolutionary biology (Colosimo et al., 2005; Fang et al., 2020; Losos, 2011; Wake et al., 2011). Uncovering the molecular basis of parallel evolution provides an opportunity for assessing the deterministic role of natural selection (Bolnick et al., 2018; Stern, 2013). While a large number of studies have explored the mechanisms of parallel evolution at genomic (Andrade et al., 2021; Fang et al., 2021; Magalhaes et al., 2021), transcriptomic (Fischer et al., 2021; Hanson et al., 2017; Jacobs & Elmer, 2021; Wang et al., 2020), and proteomic (Corbett-Detig et al., 2020) levels in a wide range of animal taxa, the degree of parallelism in epigenetic processes during repeated environmental transitions remains relatively unexplored. Recent genome-wide studies have demonstrated DNA methylation variation between populations that have adapted to ecologically divergent environments (Artemov et al., 2017; Caizergues et al., 2022; Heckwolf et al., 2020; Le Luyer et al., 2017; Leitwein et al., 2021; Smith et al., 2015). However, it has been difficult to evaluate the parallelism of epigenetic changes under natural conditions because these studies either have not included replicate populations in each environment type or have focused on epigenetic shifts occurring in response to anthropogenic changes. It has been suggested that epigenetic modifications, particularly DNA methylation in animals, can independently and directly regulate phenotypic plasticity by influencing gene expression abundance and transcription, and contribute to adaptive evolution by inducing inter- and trans-generational phenotypic change (Feil & Fraga, 2012; Heard & Martienssen, 2014; Heckwolf et al., 2020; Hu et al., 2021; Jones, 2012; Lim & Brunet, 2013; Neri et al., 2017). In addition, DNA methylation can indirectly affect plasticity and evolution as a function of its high spontaneous mutation rate and by regulating transposable elements and gene duplication (Dyson & Goodisman, 2020; Hu & Barrett, 2017; Keller & Yi, 2014; Richards et al., 2017; Verhoeven et al., 2016). Modification of the methylome might therefore be an important mechanism underlying parallel phenotypic differentiation driven by divergent natural selection.

Plastic changes can occur in the same or opposite direction as adaptive evolution in a novel environment, with concordance in the direction of these processes being indicative of adaptive plasticity (Conover et al., 2009; Ghalambor et al., 2007; Grether, 2005; Lande, 2009; Velotta & Cheviron, 2018). Theory suggests that this concordance (or lack thereof) can affect the trajectory subsequent evolution (Ghalambor et al., 2007; Grether, 2005; Lande, 2009), but few studies have empirically tested the role of environmentally induced plasticity within the context of parallel adaptation (Fischer et al., 2021; Hanson et al., 2017; Oke et al., 2016; Torres Dowdall et al., 2012). In the context of environmental change, the phenotypic plasticity retained in derived populations that have adapted to novel environments can facilitate their readaptation to ancestral environments, although the relative roles of plasticity and evolution in "forward" versus "reverse" adaptation is inconsistent between studies (Brennan et al., 2022; Ho et al., 2020). In general, the role of epigenetic mechanisms in regulating the interplay between plasticity and evolution within the context of parallel adaptation is unknown.

To explore plastic and evolved DNA methylation patterns in parallel adaptation, we used threespine stickleback fish (*Gasterosteus aculeatus*), an important model for studies of ecological adaptation. Worldwide, thousands of populations of threespine stickleback have repeatedly colonized freshwater habitats from ancestral marine habitats, where they have subsequently undergone a suite of characteristic morphological and physiological adaptations to produce distinct marine and freshwater "ecotypes" (Bell & Foster, 1994). Importantly, prior studies have demonstrated epigenetic responses to changes in salinity conditions (Artemov et al., 2017; Heckwolf et al., 2020; Hu et al., 2021; Metzger & Schulte, 2018; Smith et al., 2015). By comparing DNA methylation patterns between three independent pairs of marine and freshwater stickleback populations, including one pair in which marine and freshwater populations were reciprocally challenged with opposing salinity environments, we ask two questions: (1) To what extent does methylation level show parallel changes between ecotypes? (2) What is the relative role of methylation plasticity versus evolution during adaptation to novel and ancestral environments? Answering these questions will help to understand what role epigenetics might play during parallel adaptation, and how epigenetic modifications might regulate the interplay between plasticity and evolution.

2 | MATERIALS AND METHODS

2.1 | Experimental design

To explore the parallel methylation patterns between ecotypes, we reanalysed publicly available data sets including three independent pairs of marine and freshwater stickleback populations, with methylation levels measured by reduced representation bisulphite sequencing (RRBS) on fish tissue collected from Scotland (fillet; Smith et al., 2015), Canada (caudal fin; Hu et al., 2021), and Russia (gill; Artemov et al., 2017) (hereafter referred to as the "SC study", "CA study", and "RU study", respectively; Table S1). To explore the roles of plastic and evolved methylation variation during marine and freshwater adaptation, we analysed methylation levels measured in the RU study, sampled from marine and freshwater fish acclimated to either their native salinity environment or the opposite salinity environment. Following Artemov et al. (2017), we hereafter refer to these groups of fish as "MM" (marine fish acclimated to saltwater), "MF" (marine fish acclimated to freshwater), "FF" (freshwater fish acclimated to freshwater), and "FM" (freshwater fish acclimated to saltwater), respectively.

2.2 | Data preparation

To remove adapter contamination, low-quality reads, and bases artificially introduced during library preparation, we trimmed reads

using Trim Galore! version 0.6.6 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), with the rrbs option.

We then used the program Bowtie2 version 2.3.4.3 (Langmead & Salzberg, 2012), implemented in Bismark version 0.22.3 (Krueger & Andrews, 2011) to align trimmed reads for each sample to the stickleback reference genome (ENSEMBL version 103) with default settings, except for tolerating one nonbisulphite mismatch per read. We only included reads that mapped uniquely to the reference genome, and cytosines that had at least 5× coverage in downstream analyses. Only CpG context cytosine methylation was analysed because CpG methylation is the most common functional methylation in vertebrates (Suzuki & Bird, 2008).

2.3 | Parallel methylation patterns

To identify parallel methylation patterns, we first identified cytosines that were present in samples across all three studies, using the R package methylKit version 1.4.1 (Akalin et al., 2012). Read coverage was then normalized between samples, using the median read coverage as the scaling factor. A minimum of five reads in all samples was required at a CpG site for that site to be analysed (Hu et al., 2018; Walker et al., 2015; Wan et al., 2016). We removed CpG sites that were in the 99.9th percentile of coverage from the analysis to account for potential PCR bias. We removed all CpGs located on the sex chromosome to reduce potential sex bias, following Heckwolf et al. (2020). This approach will not remove all sex-biased CpGs because they are not all located on the sex chromosome, but it has been suggested that less than 0.1% of CpG sites on autosomal chromosomes are affected by sex specific methylation in threespine stickleback (Metzger & Schulte, 2018). To improve methylation estimates, we corrected for SNPs, which could have resulted in an incorrect methylation call if C-to-T and G-to-A SNPs were falsely interpreted as unmethylated cytosines (Heckwolf et al., 2020; Le Luyer et al., 2017). SNPs were identified and filtered using the methylation value of each CpG site of all samples for input to Bis-SNP version 0.82.2 (Liu et al., 2012) with the default parameters, and following steps in Hu et al. (2021). To perform PCA, we further filtered SNPs that (1) had more than 10% missing data across all samples, (2) were located on the sex chromosome, and (3) had high linkage disequilibrium (pairwise $r^2 > 0.8$ within a window of 1 Mb). In total, we retained 239 SNPs and 13,184 CpGs that passed these filtering steps. Principal component analysis (PCA) on the filtered CpGs and SNPs showed similar patterns, with marine and freshwater population pairs from the same country clustered together and separated from other population pairs (Figure S1). This clustering is probably due to the genetic similarity of populations sampled from the same geographic area (i.e., the pairs from each study) and tissue-specific effects on methylation (Anastasiadi et al., 2021; Horvath, 2013; Vernaz et al., 2021; Watson et al., 2021; Weyrich et al., 2016).

Based on the 13,184 CpG sites that passed the filtering step above, we first identified differential methylation cytosines (DMCs) between marine and freshwater fish within each study (hereafter referred to as "ecotype-DMCs"), using the R package methylKit. CpG sites were considered to be DMCs with a false discovery rate correction Q-value <0.01. To control for tissue-specific effects (and other factors exclusive to single studies), we then performed pairwise comparisons between studies, and retained any ecotype-DMCs that were not exclusive to a single study. We designated ecotype-DMCs as parallel when they showed the same direction of methylation difference between ecotypes in two (or three) studies.

2.4 | The roles of plastic and evolved methylation changes during adaptation

To explore if methylation plasticity facilitates or hinders divergence, we compared the association between directions of plastic versus evolved methylation differences of fish experiencing either marine or freshwater conditions in the RU study. We filtered CpGs and SNPs using the aligned reads of MM, MF, FM, and FF fish in the RU study as described above, and retained 1159 SNPs and 1,054,873 CpGs that passed these filtering steps. We define plastic differences as CpGs with methylation levels that are significantly different between fish of the same ecotype that are tested in different environments (i.e., DMCs between MM and MF or between FF and FM). We define evolved differences as CpGs with methylation levels that are significantly different between different ecotypes tested in the same environment (i.e., DMCs between MF and FF or between FM and MM). We used the directions of methylation change to determine whether plastic and evolved methylation differences were in concordant or nonconcordant directions in scenarios that mimic either freshwater or marine adaptation. During freshwater adaptation we investigate plastic differences in the "nonadapted" marine ecotype (MM-MF) and then test for concordance with the evolved differences by comparing ecotypes in freshwater conditions (MF-FF). Here, we are assessing whether the plastic change occurring in the nonadapted ecotype when moving from its native environment to the novel environment mirrors the evolved difference that we see between ecotypes in the novel environment. During freshwater adaptation, concordance between plastic and evolved differences would be reflected by the same DMCs showing hyper- (or hypo-) methylation in MM relative to MF (plastic), and in MF relative to FF (evolved). Nonconcordance would be reflected by hypermethylation in MM relative to MF (plastic), and hypomethylation in MF relative to FF (evolved) (or vice versa). Conversely, during marine adaptation we investigate plastic differences in the "nonadapted" freshwater ecotype (FF-FM) and then test for concordance with evolved differences by comparing ecotypes in marine conditions (FM-MM).

To further understand the relative roles of plastic and evolved methylation during adaptation, we distinguished between two categories of DMCs, using an approach similar to that described in Ho et al. (2020): (1) plastic-only (PO), which are CpGs with showing plastic differences but not evolved differences (i.e., DMCs between MM and MF but not between MF and FF for freshwater adaptation and DMCs between FF and FM but not between FM and MM for marine

adaptation), and (2) evolved-only (EO), which are CpGs with evolved differences but not plastic differences (i.e., DMCs between MF and FF but not between MM and MF for freshwater adaptation, and DMCs between FM and MM but not FF and FM for marine adaptation). Based on the same 1,054,873 CpGs that passed the filtering step above, we identified 210,633 DMCs categorized as PO or EO in freshwater and marine adaptation.

2.5 | The genetic basis of methylation

We annotated genes associated with parallel ecotype-DMCs using the stickleback reference genome from Ensembl 103 database and the R packages biomaRt version 2.34.2 (Durinck et al., 2005, 2009) and ChIPpeakAnno version 3.12.7 (Zhu, 2013; Zhu et al., 2010). Gene names were assigned to DMCs only when DMCs were located within promoters (defined as upstream 1 kb and downstream 1 kb from the transcription starting site [TSS] [Akalin et al., 2015]) or genes. We gave the precedence to promoters over genes when DMCs were located inside both features. We performed gene ontology (GO) analysis on genes associated with parallel DMCs using the R package topGO version 2.28.0 (Alexa et al., 2006). The gene pools against which we compared genes associated with parallel DMCs were the genes associated with the 13,184 CpGs that passed the filtering step.

Plasticity itself can be a target of natural selection and can be controlled by genetic variation or autonomous epigenetic variation (Arnold et al., 2019). Thus, to explore the relative contribution of genetically controlled vs. autonomous methylation variation to methylation plasticity, we examined the association between the 1159 filtered SNPs and the 210,633 DMCs identified above, using the R package MatrixEQTL version 2.3 (Shabalin, 2012). We fit an additive linear model to test if the number of alleles (coded as 0, 1, 2) predicted percentage methylation level (value ranging from 0 to 1) at each CpG identified as a DMC, including ecotype (marine or freshwater) and acclimation environment (native or opposite salinity environment) as covariates. We used a Bonferroni-corrected multiple-test corrected threshold, set it to genome-wide significance for GWAS and divided by the number of DMCs tested (i.e., $5\times10^{-8}/210{,}633 = 2.37\times10^{-13}$) to minimize false positives (Orozco et al., 2015). We calculated the distance between a SNP and a DMC within a significant SNP-DMC pair and defined a SNP as *cis*-acting if the SNP was located within 1 Mb from its associated DMC or *trans*-acting if the SNP was located more than 1 Mb from its associated DMC (Zhang et al., 2014).

3 | RESULTS

3.1 | Parallel evolution of ecotypes was not associated with predominantly parallel methylation differentiation

To identify parallel methylation patterns, we first performed differential methylation analysis within the three pairs of marine and freshwater populations. Based on the 13,184 CpGs that passed the filtering step, we identified 717, 1422, and 2219 ecotype-DMCs in the CA, SC, and RU studies, respectively, with significantly more ecotype-DMCs displaying hypermethylation in marine fish relative to freshwater fish in the RU study (1814 hypermethylated and 405 hypomethylated DMCs; $G = 967.32$, df = 1, $p < 2.20\times10^{-16}$) and CA study (389 hypermethylated and 328 hypomethylated DMCs; $G = 5.20$, df = 1, $p = .02$) but not in the SC study (726 hypermethylated and 696 hypomethylated DMCs; $G = 0.63$, df = 1, $p = .43$) (Figure S2a). Individual fish clustered mainly by their ecotypes based on Euclidean distance of methylation levels in all three population pairs (Figure S2b–d). We found 108, 187, and 309 ecotype-DMCs shared between the CA and SC studies, between the CA and RU studies, and between the SC and RU studies, respectively, with 28 ecotype-DMCs shared between all three studies (Figure 1a).

When analysing the directions of methylation change in shared ecotype-DMCs, we found no tendency for parallel differences to predominate in any of the study comparisons (CA vs. SC: 51 parallel vs. 57 nonparallel, $G = 0.33$, df = 1, $p = .56$; CA vs. RU: 94 parallel vs. 93 nonparallel, $G = 5.34\times10^{-3}$, df = 1, $p = .94$; RU vs. SC: 164 parallel vs. 145 nonparallel, $G = 1.17$, df = 1, $p = .28$), with no significant correlations between the methylation change in CA versus SC ($p = .56$) and RU versus SC ($p = .63$), and a marginally significantly negative correlation between the methylation change in CA vs. RU ($\rho = -0.14$, $p = 4.79\times10^{-2}$) (Figures 1b–d). When comparing the locations of shared ecotype-DMCs that showed parallel methylation change to previously documented regions of parallel genomic divergence between marine and freshwater sticklebacks (Hohenlohe et al., 2010; Jones et al., 2012; Terekhanova et al., 2014), we found that only two (between CA and SC), one (between CA and RU), and three (between RU and SC) parallel ecotype-DMCs overlapped with previously identified regions.

3.2 | The roles of plastic and evolved differences in methylation during adaptation

Consistent with the Artemov et al. study from which we obtained the primary data (Artemov et al., 2017), when performing PCA on methylation levels of filtered CpG sites, we found that freshwater fish showed a higher degree of methylation plasticity than marine fish when exposed to salinity change, with marine fish clustering together regardless of salinity environment but freshwater fish exposed to saltwater clustering with marine fish (Figure S3). We found fewer DMCs with plastic differences among marine ecotypes challenged with freshwater (35,166 in MM–MF) than freshwater ecotypes challenged with saltwater (140,940 in FF–FM). To explore if methylation plasticity facilitates or hinders evolution, we analysed the concordance in directions of plastic and evolved differences during scenarios that mimic the osmoregulatory challenges of freshwater versus marine adaptation. For both freshwater and marine adaptation scenarios, we

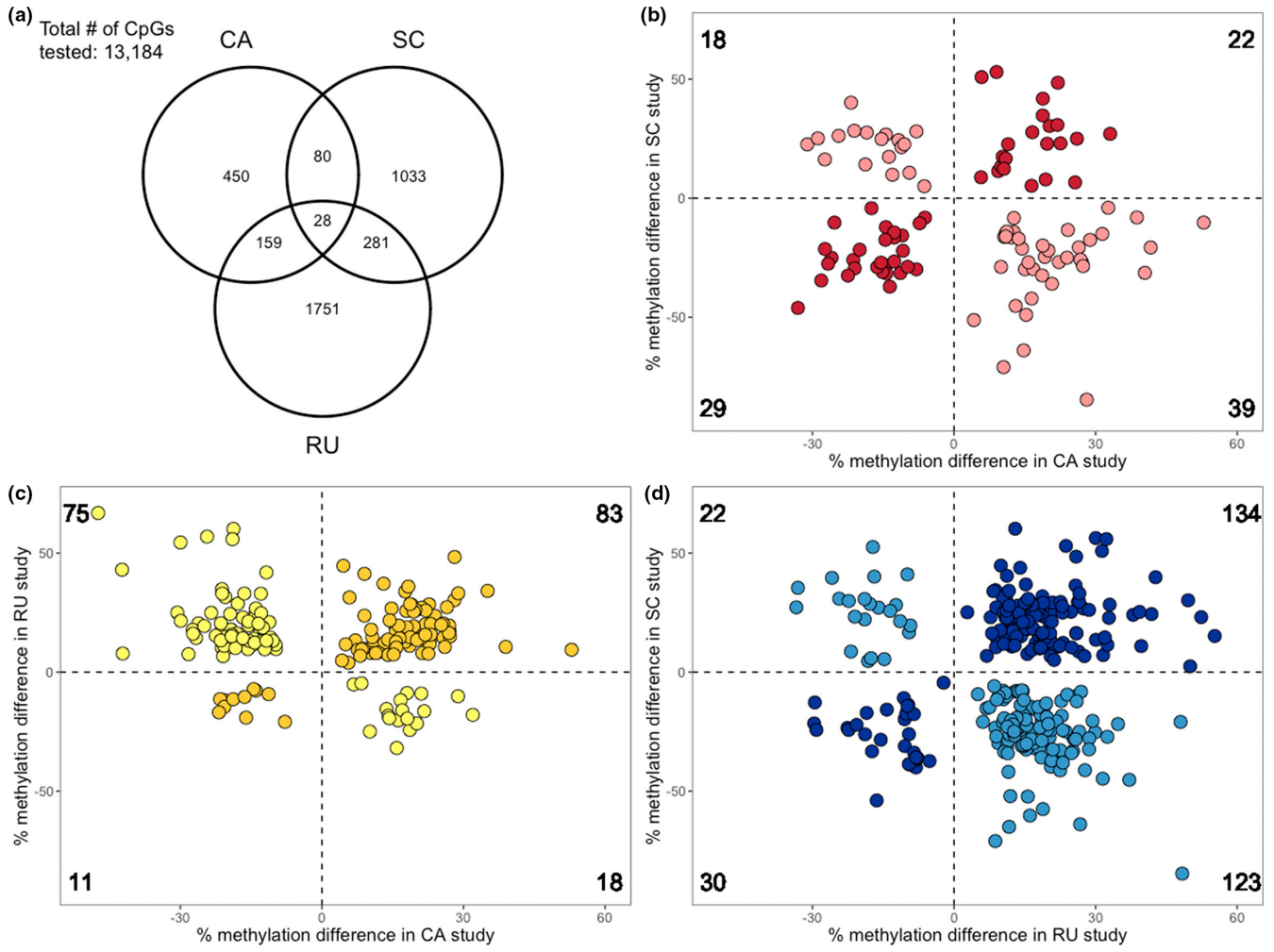

FIGURE 1 (a) Venn diagram showing differentially methylated cytosines (DMCs) between marine and freshwater ecotypes (ecotype-DMCs) shared between different studies. (b–d) Direction of methylation change of ecotype-DMCs shared between (b) CA and SC, (c) CA and RU, and (d) RU and SC. Each dot represents an ecotype-DMC shared between populations indicated on x-and y-axes. Shared DMCs with parallel and nonparallel direction of change are shown in darker and lighter colours in each panel, respectively. The number of ecotype-DMCs in each quadrant is indicated on the panels. The x- and y-axes indicate the difference in percentage methylation for ecotype-DMCs in marine fish relative to freshwater fish. Positive and negative values on x-axes and y-axes represent hyper- and hypomethylated ecotype-DMCs, respectively.

found significantly greater numbers of DMCs showing nonconcordant directions of plastic and evolved methylation differences (freshwater adaptation: 1523 concordant vs. 19,269 nonconcordant, $G = 17{,}930$, df = 1, $p < 2.20 \times 10^{-16}$; marine adaptation: 1302 concordant vs. 14,074 nonconcordant, $G = 12{,}396$, df = 1, $p < 2.20 \times 10^{-16}$; Figure 2). When analysing the patterns of methylation plasticity versus evolution in freshwater and marine adaptation, we found fewer PO-DMCs than EO-DMCs during freshwater adaptation (14,374 PO-DMCs vs. 148,392 EO-DMCs). In contrast, we found the opposite pattern during marine adaptation, with PO playing a more prominent role (125,564 PO-DMCs vs. 17,939 EO-DMCs) (Figure 3). The ratio of the number of PO-DMCs to that of EO-DMCs during marine adaptation was approximately 72 times the ratio during freshwater adaptation ($G = 8.81$, df = 1, $p = 3.00 \times 10^{-3}$).

3.3 | The genetic basis of methylation responses

Our functional analysis identified 91 genes annotated with the 309 parallel ecotype-DMCs, representing a list of core genes exhibiting repeatable responses upon colonization of freshwater environments (Table S2). While GO analysis showed no significant GO term enrichment, multiple genes annotated with parallel ecotype-DMCs were relevant to changes in the osmoregulatory environment, for example, ion channel (*cdkal1*, *cyp1b1*, *gria3a*, *VAV1*), development (*FOXC1*, *tenm1*, *nrp2b*), and signalling pathways (*gria3a*, *gucy2c*, *notch2*). Previous studies of the genetic architecture underlying parallel divergence in morphology, physiology and behaviour between marine and freshwater ecotypes have also identified functionally similar genes (Hohenlohe et al., 2010; Jones et al., 2012; Terekhanova et al., 2014); however, the set of specific

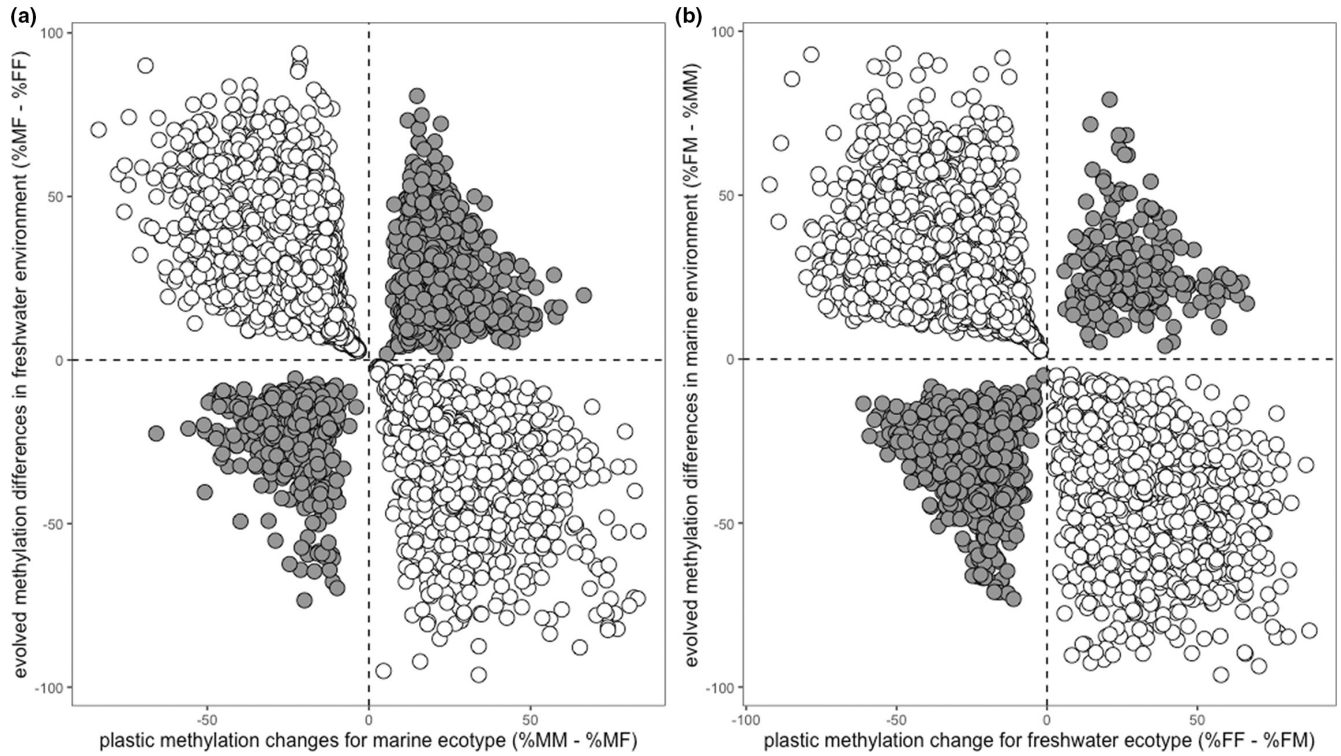

FIGURE 2 Relationship between ancestral methylation plasticity and evolved methylation difference in (a) freshwater and (b) marine adaptation scenarios. Each circle represents the methylation difference at a single cytosine with statistically significant plastic and evolved methylation differences between marine and freshwater ecotypes (see methods). Plastic methylation differences were more likely to be in a nonconcordant (white circles) than concordant (grey circles) direction to evolved methylation differences.

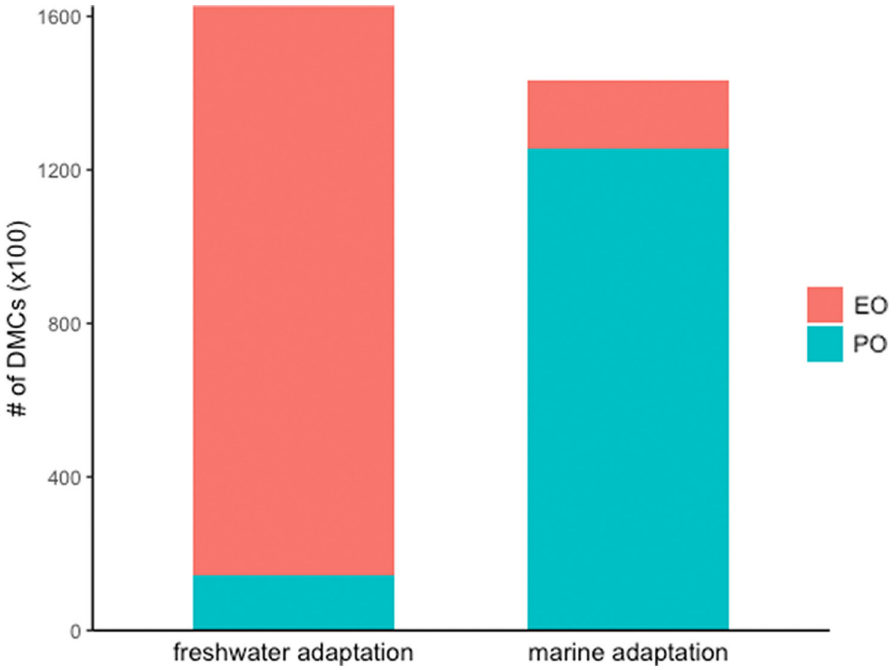

FIGURE 3 Number of differentially methylated cytosines (DMCs) that were plastic-only (PO, blue) or evolved-only (EO, red) in freshwater and marine adaptation scenarios.

genes implicated in genetic versus epigenetic parallel changes were largely nonoverlapping.

To explore the genetic basis of plastic versus evolved methylation variation, we performed an association analysis between SNPs and DMCs categorized as PO or EO. In total, we identified 8600 significant SNP-DMC pairs, corresponding to 559 unique SNPs and 215 unique DMCs, with the *p*-value distribution showing no evidence of test statistic inflation (Figure S4), suggesting that our analysis provides high power for testing for associations between epigenetic and genetic variation (Hu et al., 2021). Among the 215

unique DMCs, 165 and 171 were identified as PO-DMCs and EO-DMCs, respectively. Finally, we analysed the genetic regulatory landscape underlying PO- and EO-DMCs associated with SNPs. We found that all PO- and EO-DMCs were associated with at least one *trans*-acting SNP, whereas only 12 PO- and 18 EO-DMCs were associated with *cis*-acting SNPs. However, there was no significant enrichment for *trans*-acting SNPs associated with PO-DMCs (9 *cis*- and 507 *trans*-acting SNPs; $G = 1.17$, df = 1, $p = .28$) or EO-DMCs (12 *cis*- and 487 *trans*-acting SNPs; $G = 3.11 \times 10^{-3}$, df = 1, $p = .96$) when compared to the null distribution built on all *cis*- ($n = 14$) and *trans*-acting ($n = 559$) SNPs.

4 | DISCUSSION

The role of epigenetic variation in regulating phenotypic plasticity and evolution has received increased attention in recent years (Hu & Barrett, 2017; Richards et al., 2017; Verhoeven et al., 2016; Vogt, 2021). However, the extent of parallelism in methylation divergence between independently evolved pairs of populations experiencing divergent natural selection remains unclear, as does the relative contribution of methylation plasticity versus evolution during adaptation in ancestral versus novel environments. We compared methylation variation between three independent pairs of marine and freshwater threespine stickleback populations. We found no tendency for shared ecotype-DMCs to show parallel directions of methylation differentiation. This finding might be surprising given the parallel phenotypic, genomic, and transcriptomic divergence that has been documented between marine and freshwater ecotypes (Barrett et al., 2008; Fang et al., 2020, 2021; Garcia-Elfring et al., 2021; Hohenlohe et al., 2010; Jones et al., 2012; Verta & Jones, 2019). However, there are also many counter-examples of morphological traits and genomic regions that do not show consistent divergence between ecotypes in stickleback and other fish species (Fang et al., 2020; Fitzpatrick et al., 2014; Oke et al., 2017; Pujolar et al., 2017; Stuart et al., 2017). Recent studies of parallel evolution at genomic and transcriptomic levels (Fang et al., 2020; Fischer et al., 2021; Hanson et al., 2017) have attributed these nonparallel patterns to geographic heterogeneity in access to standing genetic variation (Fang et al., 2020; Fischer et al., 2021; Fitzpatrick et al., 2014; Thompson et al., 2019), or low ecological similarity between replicates of the "same" ecotype (Morales et al., 2019; Rennison et al., 2019; Stuart et al., 2017).

A limitation of this study is that nonparallel patterns could potentially be ascribed to the heterogenous nature of the samples (e.g., tissue type and age at collection) that were used across the only three currently available data sets that report methylation differences between marine and freshwater sticklebacks. Although recent work has found a positive correlation between methylation changes at CpG sites across tissues and developmental stages (Lindner et al., 2021), the number of correlated CpG sites was small compared to all analysed CpG sites, consistent with the commonly observed tissue- and age-specific methylation patterns in animals (Anastasiadi et al., 2021; Bors et al., 2021; Contractor et al., 2004; Fairfield et al., 2021; Feil & Fraga, 2012; Horvath, 2013; Navarro-Martín et al., 2011; Rodriguez Barreto et al., 2019; Vernaz et al., 2021; Watson et al., 2021; Weyrich et al., 2016, 2020). In addition, while we attempted to minimize sex bias by excluding CpGs on the sex chromosome, some sex-specific methylation is known to occur on autosomal chromosomes (Metzger & Schulte, 2018) and thus the methylation patterns identified in our study might still be partially influenced by sex. By restricting our analyses of parallelism to only those DMCs shared between studies, we are using a conservative analytical approach to account for sample heterogeneity, in that all of the analysed CpG sites show a significant difference in methylation between ecotypes regardless of the tissue and sample preparation processes that were used. As such, the parallel ecotype-DMCs that we identified help to reveal a core set of CpGs associated with parallel marine-freshwater methylation divergence that hold despite being observed in heterogeneous samples from geographically distant populations. However, it is possible that the direction of this methylation differentiation between ecotypes is dependent on some difference between samples (e.g., tissue type), and thus that nonparallel DMCs could have been parallel if the samples had been more consistent across studies. Future research that investigates the parallelism of methylation patterns using homogenous sample types and standardized preparation processes would represent an important advance over the present study.

We found little overlap between the annotated genes implicated in genetic versus epigenetic marine-freshwater divergence, suggesting that these two mechanisms might act on different genes and complementarily regulate parallel adaptation. This would be consistent with previous methylation quantitative loci analysis showing that additive genetic variance explains a limited proportion of methylation variance between marine and freshwater stickleback (Hu et al., 2021). Yet, many of the genes implicated in parallel epigenetic divergence in this study were associated with osmoregulatory change, a pattern that has also been found in many studies of parallelism in stickleback at genomic and transcriptomic levels (Barrett et al., 2008; Fang et al., 2020, 2021; Garcia-Elfring et al., 2021; Hohenlohe et al., 2010; Jones et al., 2012; Verta & Jones, 2019). This suggests that autonomous epigenetic variation could provide an alternative (and potentially faster; Klironomos et al., 2013) route to achieve the same functional solutions.

Both theoretical and empirical studies have suggested a close relationship between the direction of plasticity and the trajectory of evolution (Baldwin, 1896; Ghalambor et al., 2007; Fischer et al., 2021), but whether plasticity typically facilitates or hinders evolution remains inconclusive (Fox et al., 2019; Kelly, 2019). We found that most plastic and evolved methylation differences were in nonconcordant directions. Assuming that evolved methylation differences reflect adaptive differentiation shaped by divergent natural selection (Brennan et al., 2022; Fischer et al., 2021; Ghalambor et al., 2015; Ho & Zhang, 2018), this suggests that a significant proportion of methylation plasticity is nonadaptive. However, we emphasize that testing the concordance between plastic and evolved

methylation differences does not provide conclusive evidence that a plastic change is adaptive (or nonadaptive). This would require fitness information that we do not have from these populations. As such, for any individual DMC it is possible that our assumption is flawed (e.g., if the methylation state of a freshwater fish in freshwater is less adaptive than that of a marine fish in freshwater). However, based on existing literature, we believe our assumption should be reasonable for the majority of DMCs in the data set. The predominance of putatively nonadaptive plasticity was consistent in both marine and freshwater adaptation scenarios, with 91.53% and 92.68% of CpGs showing nonconcordance, respectively. These results support models predicting that nonadaptive plasticity could facilitate adaptation by increasing the strength of directional selection in new environments (Ancel, 2000; Paenke et al., 2007; Price et al., 2003)—a prediction that has also been borne out in empirical studies focused on phenotypic and transcriptional plasticity (Dayan et al., 2015; Fischer et al., 2021; Ghalambor et al., 2015; Ho & Zhang, 2018; Schaum et al., 2013).

The reciprocal transplant experiment in RU study also allows us to test the relative roles of plastic and evolved methylation changes during adaptation to distinct environment types. We found a striking difference in the contributions of plastic versus evolved methylation changes in marine versus freshwater adaptation scenarios, with the ratio of plastic to evolved changes 72 times greater during the scenario mimicking marine adaptation. These findings are consistent with recent studies investigating transcriptomic changes between animals reciprocally transplanted to ancestral and derived environments (Brennan et al., 2022; Ho et al., 2020), and suggest that epigenetic plasticity might act as a long-term "memory" that facilitates readaptation to ancestral environments. However, an important caveat is that fish in the RU study were acclimated to salinity environments for only four days before testing, so it is possible that methylation changes in some CpGs are due to short-term acute stress that could differ from the methylation responses that would be observed under long-term rearing conditions. Short versus long-term exposure to environmental change has been shown to result in distinct transcriptomic patterns in fish and other aquatic species (Downey et al., 2022; Logan & Buckley, 2015). Further studies that compare short and long-term methylation responses in ancestral versus novel environments will be helpful for a more comprehensive understanding of the role of epigenetic plasticity in adaptation.

Finally, we found that both *cis*- and *trans*-acting genetic variants were associated with PO- and EO-DMCs. While a plastic change can occur without genetic variation, plasticity can also have a genetic basis (Kelly, 2019; Murren et al., 2015). Two main alternatives have been proposed for the genetic basis of plasticity: (1) the plasticity is influenced by environmentally sensitive genes (*cis*-acting) that have evolved as a byproduct of divergent natural selection (Via, 1993; Via et al., 1995) and (2) the mean and plasticity of a phenotype are influenced by separate (*trans*-acting) genes (Scheiner, 1993; Signor & Nuzhdin, 2018). It has been suggested that both *cis*- and *trans*-acting SNPs can play important roles in morphological (e.g., gill pigmentation, dorsal spine length and numbers, tooth numbers), gene expression, and methylation divergence between marine and freshwater ecotypes, but the relative contribution of *cis*- versus *trans*-acting SNPs to ecotype divergence has been inconsistent between studies (Hart et al., 2018; Hu et al., 2021; Ishikawa et al., 2017; Reid et al., 2021; Verta & Jones, 2019; Wucherpfennig et al., 2022). In this study we found that although DMCs were much more likely to be associated with *trans*-acting SNPs than *cis*-acting SNPs, the relative proportion of *trans*- vs. *cis*- associations was in-line with neutral expectations given the much greater number of *trans*-acting SNPs in the data set. We therefore do not have support for a greater contribution of either regulatory mechanism over the other.

In this study we identify a core set of CpGs associated with parallel methylation divergence between marine and freshwater stickleback ecotypes under divergent natural selection. In addition, we explored the roles of plastic and evolved differences in methylation during adaptation and demonstrate that nonadaptive plasticity could potentially facilitate evolution during both freshwater and marine adaptation. Notably, our data indicates starkly different contributions of plastic versus evolved methylation changes to freshwater versus marine adaptation, with evolved methylation changes contributing most to freshwater adaptation whereas plasticity predominates during marine adaptation. Finally, we explored the genetic basis of plastic and evolved methylation differences and found a predominantly *trans*-regulatory landscape underlying methylation variation. Our study adds to the few studies exploring parallel evolution from an epigenetic perspective and reveals an important role for nonadaptive epigenetic plasticity in adaptation.

AUTHOR CONTRIBUTIONS

Juntao Hu and Rowan D. H. Barrett conceived the study. Juntao Hu collected and analysed the data. Juntao Hu wrote the manuscript with input from Rowan D. H. Barrett. The authors have no conflicts of interest.

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CONFLICT OF INTEREST

The authors declared no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

Raw Illumina sequencing reads for all the analysed individuals can be downloaded from the NCBI database under accession number GSE82310, PRJNA587332, and PRJEB7912.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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