Received: 9 March 2022 | Revised: 21 June 2022 | Accepted: 27 June 2022

DOI: 10.1111/mec.16586

ORIGINAL ARTICLE

Genome-wide methylation in the panmictic European eel (*Anguilla anguilla*)

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Funding information
Interreg Project Under the Øresund-Kattegat-Skagerrak Program; Danish Council for Independent Research, Natural Science, Grant/Award Number: 7014-00167B

Handling Editor: Katie Lotterhos

Abstract

The role of methylation in adaptive, developmental and speciation processes has attracted considerable interest, but interpretation of results is complicated by diffuse boundaries between genetic and non-genetic variation. We studied whole genome genetic and methylation variation in the European eel, distributed from subarctic to subtropical environments, but with panmixia precluding genetically based local adaptation beyond single-generation responses. Overall methylation was 70.9%, with hypomethylation predominantly found in promoters and first exons. Redundancy analyses involving juvenile glass eels showed 0.06% and 0.03% of the variance at SNPs to be explained by localities and environmental variables, respectively, with GO terms of genes associated with outliers primarily involving neural system functioning. For CpGs 2.98% and 1.36% of variance was explained by localities and environmental variables. Differentially methylated regions particularly included genes involved in developmental processes, with Hox clusters featuring prominently. Life stage (adult versus glass eels) was the most important source of inter-individual variation in methylation, probably reflecting both ageing and developmental processes. Demethylation of transposable elements relative to pure European eel was observed in European X American eel hybrids, possibly representing postzygotic barriers in this system characterized by prolonged speciation and ongoing gene flow. Whereas the genetic data are consistent with a role of single-generation selective responses, the methylation results underpin the importance of epigenetics in the life cycle of eels and suggest interactions between local environments, development and phenotypic variation mediated by methylation variation. Eels are remarkable by having retained eight Hox clusters, and the results suggest important roles of methylation at Hox genes for adaptive processes.

KEYWORDS
adaptive processes, *Anguilla anguilla*, epigenetics, Hox clusters, hybridization, methylation

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1 | INTRODUCTION

It is increasingly appreciated that epigenetics, defined as modifications of DNA that affect expression of genes but without changing the DNA sequence (Dupont et al., 2009), is highly important in developmental, adaptive and evolutionary processes (Adrian-Kalchhauser et al., 2020; Anastasiadi et al., 2021; Gore et al., 2018; Greenberg & Bourc'his, 2019; Jablonka, 2017; Jablonka & Raz, 2009; Lind & Spagopoulou, 2018; Stajic et al., 2019; Verhoeven et al., 2016). Epigenetics mechanisms include DNA methylation, histone modifications and small RNAs (Deans & Maggert, 2015; Feil & Fraga, 2012; Law & Jacobsen, 2010), where DNA methylation has so far attracted the most interest. This involves addition of a methyl group to a nucleotide, in most cases cytosin (C), which in animals primarily occurs at CpG sites (C followed by G in the genome sequence). Gain or loss of methylation at CpG sites in regulatory regions can lead to silencing or reactivation of genes, with hypomethylation of promoter regions generally leading to increased transcription in vertebrates (Christensen et al., 2021; Greenberg & Bourc'his, 2019; Jones, 2012; Law & Jacobsen, 2010; Moore et al., 2013).

Methylation plays key roles in developmental processes and cell differentiation (Greenberg & Bourc'his, 2019). In mammals this involves two events of reprogramming during embryogenesis, but general knowledge about major changes in methylation is scarce in other animals, including those that undergo extensive metamorphosis. Importantly, methylation can be environmentally induced and transferable across cell divisions (Feil & Fraga, 2012). There is furthermore some evidence, particularly in plants but less clear-cut in animals that epigenetic marks can be transferable across generations. This can ultimately lead to traits being inherited despite not being coded by the DNA sequence (Anastasiadi et al., 2021; Gapp et al., 2014; Richards, 2006; Schmitz et al., 2013; Skvortsova et al., 2018).

The environmental inducibility of methylation states raises the possibility that this could represent rapid adaptive mechanisms in response to spatial and temporal environmental variation (Angers et al., 2020; Feil & Fraga, 2012), and it is assumed to be a major component in phenotypic plasticity and may also be involved in knock-on effects, that is early perceived environmental cues leading to phenotypic change later in life (Jonsson et al., 2022). Indeed, some studies report significant methylation differences of functional importance associated with environmental variation (Artemov et al., 2017; Gugger et al., 2016; Heckwolf et al., 2020; Le Luyer et al., 2017; Merondun et al., 2019; Metzger & Schulte, 2018; Schmitz et al., 2013; Wogan et al., 2020). A distinction has usually been made between genetic adaptation (encoded by DNA) resulting from evolution across generations; and phenotypic plasticity, within-generation responses of individuals to environmental conditions, for example, by adjustments in physiology (Gienapp et al., 2008; Kawecki & Ebert, 2004). Whereas methylation from this perspective could be viewed as a source of non-genetic adaptation, it is increasingly realized that the boundaries between genetic and non-genetic factors are unclear and involve complex interactions, also in the case of epigenetics (Adrian-Kalchhauser et al., 2020; Taudt et al., 2016; Verhoeven et al., 2016). In particular, a proportion of methylation may be under genetic control (Richards, 2006), as for instance demonstrated by different non-recombining chromosome inversions showing different methylation (Sun et al., 2021). Differences in methylation patterns between populations could therefore ultimately reflect individual- and population-level genetic differences in genes controlling methylation (Anastasiadi et al., 2021; Dubin et al., 2015; Richards, 2006; Taudt et al., 2016). It would therefore be of significant interest to study epigenetic patterns and its association with geographical and environmental variation in species where genetically based adaptation can be ruled out.

Methylation also has the important role to repress transposable elements (TEs), thus preventing deleterious proliferation of TEs in the genome (Jones, 2012; Slotkin & Martienssen, 2007). Derepression of TEs by demethylation has been found to occur in some cases of hybridization, leading to harmful reactivation and proliferation of transposons (Laporte et al., 2019; Michalak, 2009; O'Neill et al., 1998; Ungerer et al., 2006). This could potentially represent postzygotic barriers, but the importance of TE de-repression relative to other postzygotic barriers remains unclear. It would therefore be of interest to study this in cases where environmental conditions experienced by species are similar and gene flow between species is still ongoing.

The European eel (*Anguilla anguilla*) represents an excellent model for further increasing our knowledge about the role of methylation in adaptive and developmental processes. It is distributed across environmental conditions spanning from Sub-arctic climates in Iceland to Sub-tropical environments in North Africa (Tesch, 2003). It spawns in the Southern Sargasso Sea in partial sympatry with its sister species American eel (*A. rostrata*) (Kleckner et al., 1983; Munk et al., 2010), which shows a very similar life history and is distributed along similar environmental gradients along the American Atlantic coast (Tesch, 2003). European eel larvae are transported by ocean currents towards the European and North African coastal regions. The recently arrived juveniles metamorphose into so-called glass eels, settle in freshwater and coastal marine habitats and go through an additional stage of metamorphosis until they mature as silver eels and undertake their >5000 km spawning migration back to the Sargasso Sea (Schmidt, 1923; Tesch, 2003). Previous results based on anonymous methylation markers suggest major differences between life stages (Trautner et al., 2017), but it is unknown if these differences represent functionally important methylation in relation to developmental stages or merely ageing effects (Anastasiadi & Piferrer, 2020; Horvath & Raj, 2018).

Both European and American eel are remarkable by being panmictic species, that is despite being distributed across a wide range of environmental conditions they mate randomly in the Sargasso Sea (Als et al., 2011; Côté et al., 2013; Enbody et al., 2021; Palm et al., 2009; Pujolar, 2014b); although see contrasting views by for example, Baltazar-Soares et al. (2014). Signals of spatially varying selection have been detected in both European and American eel (Babin et al., 2017; Gagnaire et al., 2012; Pavey et al., 2015; Pujolar, 2014b; Williams

et al., 1973), but this is expected to be a single generation effect as individuals may end up in environments that differ considerably from those of their parents (Gagnaire et al., 2012; Pujolar, 2014b). The resulting absence of genetically based local adaptation suggests that phenotypic plasticity could play an important role in the species' ability to persist in different environments, although there is also evidence for a role of weak, polygenic selection to occur (Côté et al., 2014; Pavey et al., 2015). For instance, American glass eels sampled at geographically and environmentally different localities showed different growth rates and transcriptomic reaction norms in common garden settings when exposed to different salinities (Côté et al., 2014, 2009). This suggests that phenotypic plasticity interacts with processes that have occurred at local scales at a very early stage coinciding with the arrival of glass eels at the sites; either genetic variation shaped by within-generation selection (as the species is panmictic) and/or epigenetic imprints. However, knowledge about differences in methylation across geography and environments is currently lacking.

Finally, European and American eel can hybridize (Albert et al., 2006; Avise et al., 1990; Jacobsen et al., 2017; Pujolar et al., 2014a), as is also the case for other Anguillid species (Barth et al., 2020). Genomic analyses suggest a protracted speciation process by involving episodes of isolation and secondary contact and with ongoing gene flow (Nikolic et al., 2020). Given the incomplete speciation process it is of interest to assess if TE derepression occurs in hybrids between European and American eel.

Here, we applied both whole genome sequencing and bisulphite sequencing of eels from geographical locations ranging from Iceland to Morocco. As the functional significance of methylation depends on the specific genomic categories (e.g., promoters and exons) being methylated, we first made use of whole genome information to characterize the general methylation landscape of European eel. Subsequently, we (1) tested the hypothesis that differences in methylation is present among glass eels from different localities and are furthermore associated with differences in environmental parameters. Given the panmictic nature of the species we further predicted that such methylation differences should vastly exceed genetic differences, even if within-generation selection occurs. (2) We assessed if methylation differences are present between glass and adult eels, and if so if this can be ascribed to the pronounced stages of metamorphosis or to mere ageing effects (Horvath & Raj, 2018). (3) By analysing methylation in European x American eel hybrids we tested the hypothesis that transposon methylation does not differ from pure European eel, reflecting the prolonged and incomplete speciation process.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 50 European eels were analysed, representing seven locations in Europe and Northwestern Africa, spanning 30 degrees of latitude (Figure 1, Table 1, Table S1). The samples were collected between 2001 and 2016 (Table 1). Icelandic samples were collected for the present study, whereas the remaining samples have previously been analysed using RAD sequencing (Pujolar, 2014b; Pujolar et al., 2015). Thirty-nine individuals were glass eels (juvenile eels recently arrived at the coasts), whereas 11 adult individuals (silver eels, i.e., about to undertake their spawning migration) were included from two locations (Burrishoole, Ireland; Valencia, Spain). Three individuals (two from Iceland and one from Ireland) were detected as hybrids with American eel (one F1 hybrid and two backcrosses in the direction of European eel), determined using species-diagnostic single nucleotide polymorphisms (SNPs; Pujolar et al., 2014a). For silver eels, tissues consisted of muscle, whereas for glass eels DNA was extracted from the tail end, composed primarily of muscle. DNA was extracted using the E.Z.N.A. Tissue DNA Kit (OMEGA, Bio-tek) following the manufacturer's recommendations. Whole-genome sequencing (WGS) and whole-genome-bisulphite sequencing (WGBS) was outsourced to Novogene Europe. Sequencing was conducted 150 base pair (bp) paired-end on the Illumina HiSeq platform and aimed for a minimum coverage of 10x.

2.2 | Mapping WGS reads and calling SNPs

The WGS reads were filtered using Trim Galore version 0.4.1 (https://github.com/FelixKrueger/TrimGalore) and mapped to a recent chromosome level European eel genome assembly (Rhie et al., 2021; GenBank accession: GCA_013347855.1) using the BWA-MEM algorithm of BWA version 0.7.17 (Li & Durbin, 2009). The resulting SAM files were sorted by coordinate and were converted to BAM format using samtools version 1.9 (Li et al., 2009). A VCF file of SNPs encompassing all 50 individuals was generated from the BAM files using bcftools version 1.9 (Li et al., 2009), constraining the minimum mapping quality to 20. Only biallelic SNPs with minimum variant quality of 20 and with combined coverage falling between 500 and 750 were kept. The coverage thresholds were decided upon inspecting the coverage distribution of the SNPs (Figure S1).

Genome-wide observed heterozygosity (H_O) of the individuals was calculated from the VCF file by dividing the number of heterozygous sites with adjusted genome lengths. We used this measure to corroborate the hybrid status of individuals and as a quality check of the data; in a panmictic species with expectedly almost no inbreeding, genome-wide H_O should be very similar across individuals. The genome length was adjusted per individual by correcting for the missing sites generated by the SNP calling process. A PCA aimed at analyzing genetic relationships among sampled individuals was conducted using the R function "prcomp" (R Core Team, 2018) on the genotype table of the individuals, where the genotypes were denoted as the number of alternative alleles.

2.3 | Mapping WGBS reads and calling methylation

A total of 36 individuals succeeded in WGBS (Table S1) with degradation and insufficient yields of DNA causing failure in sequencing

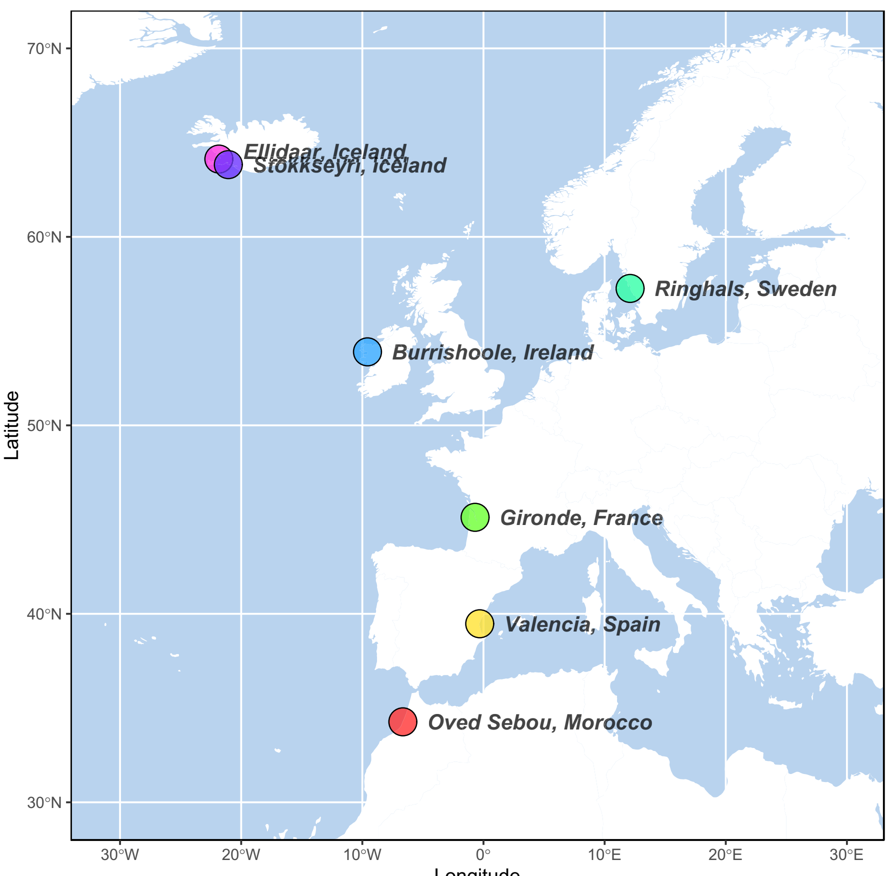


FIGURE 1 Map showing the sample localities of the analysed European eels. The colour coding for the localities applies to all the figures in this paper unless specified otherwise

TABLE 1 Overview of analysed individuals and environmental parameters

Location	Abbreviation	Time of sampling	Latitude	Longitude	SST30	MDL	Chlorophyll	*N* (WGS)	*N* (WGBS)
Glass eels									
Ellidaar, Iceland	Ell	2016-05-16	64.1268	−21.8419	6.26	1015.7	1.371	5	5
Stokkseyri, Iceland	Sto	2016-05-15	63.8324	−21.0603	7.13	1000.2	1.905	6	6
Burrishoole, Ireland	Bur	2008-03-15	53.8989	−9.5742	9.23	640.6	4.48	5	3
Ringhals, Sweden	Rin	2005-03-14	57.2633	12.1025	4.12	633.9	18.175	5	5
Gironde, France	Gir	2008-04-16	45.1193	−0.693	11.33	766.9	11.055	5	3
Valencia, Spain	Val	2010-01-15	39.4724	−0.3107	15.2	562.6	3.17	6	3
Oved Sebou, Morocco	Seb	2001-04-28	34.2698	−6.654	17.5	776.3	2.665	7	5
Adults									
Burrishoole, Ireland	Bur	2010	53.8989	−9.5742	NA	NA	NA	7	3
Valencia, Spain	Val	2012	39.4724	−0.3107	NA	NA	NA	4	3

Note: SST30 denotes mean sea surface temperature (°C), MDL mean day length (in minutes) and chlorophyll mean chlorophyll concentration (mg per m^3) across 30 days prior to sampling. N(WGS) denotes sample size for whole genome sequencing and N(WGBS) sample size for whole genome bisulphite sequencing.

of the remaining individuals. The WGBS reads were filtered using Trim Galore by allowing "--trim1" and were mapped to genomes using Bismark version 0.22.3 (Krueger & Andrews, 2011). Reads were mapped to the individual genomes obtained from the WGS data instead of the general reference genome. This was considered necessary due to the exceptionally high genetic diversity of the European eel (Pujolar et al., 2013), leading to lower mapping success when using the reference genome. Default parameters were used except for a relaxed gap penalty ("--rdg 2,1 --rfg 2,1").

We subsequently ran "bismark_methylation_extractor" and "bismark2bedGraph" (Krueger & Andrews, 2011) to extract all the sequenced CpG sites together with their methylation status. The information was stored in the COVERAGE files in the output. During the extraction process, the first two bps of all the Read 2 files were

removed based on the M-bias plots. CpG sites containing mutations were excluded. As CpG is palindromic and complementary CpGs are synchronized in methylation due to dnmt1 activity during DNA replication, complementary CpGs were merged. The COVERAGE files of all the individuals were merged using a custom script. This generated a file where the CpGs of all the individuals were aligned by coordinate. Within each individual, the CpGs with coverage lower than five were marked as missing. CpGs missing in more than half of the individuals were filtered out. CpGs whose combined coverage (across individuals) fell outside the range between 115 and 539 were removed. These coverage thresholds were decided from the coverage distribution (Figure S2).

To assess differences in global methylation among individuals, PCA was conducted using the R function "prcomp" on the methylation matrix. The data points were the individuals and the variables were the methylation level of the CpG sites.

2.4 | Methylation in genomic categories

We used the gene annotation file provided with the reference genome sequence, and for each transcript we defined two potential promoter regions, one is from 1 to 500 bp upstream from the TSS (transcription start site), referred to as "promoter_1", and the other from 501 bp to 1000 bp upstream, referred to as "promoter_2. We also identified CpG islands using cpgplot in the EMBOSS version 6.6.0.0 package (Madeira et al., 2019). We further predicted and annotated the repetitive sequences for the reference genome using RepeatModeler v1.0.11 and RepeatMasker v4.0.9-p2 (http://www.repeatmasker.org). Based on the annotation, we divided the reference genome of European eel into 17 non-exclusive categories (Table S2) and examined methylation patterns within each of these categories. The aligned CpGs were assigned into the genomic categories using the "intersect" command of bedtools version 2.29.0 (Quinlan & Hall, 2010).

2.5 | Correlation between methylation and gene expression

We anticipated that distinct methylation patterns of the first exons and the promoters of the transcripts would indicate a functional role in regulating gene expression. We furthermore also considered first introns, as Anastasiadi et al. (2018) reported inverse relationships between methylation of this genomic category and gene expression. Gene expression profiles were not generated in the present study, but we used a published transcriptome data set of the European eel (Bracamonte, Johnston, Monaghan, et al., 2019; NCBI BioProject: PRJNA419718 and PRJNA547691) as an approximate measure. This means that we could assess patterns of general association between gene expression and methylation in genomic categories, but not specific changes in gene expression as a result of differential methylation. The data set is comprised of Illumina paired-end reads from 30 experiments (20 individuals). The reads were filtered using Trim Galore version 0.4.1 and were mapped to the reference genome with the guidance of the gene annotation using HISAT2 version 2.1.0 (Kim et al., 2019). The expression profile of all the transcripts was called for each experiment using StringTie version 2.0 (Kovaka et al., 2019). Upon inspecting the expression profile and visually examining the transcripts in IGV version 2.7.2 (Robinson et al., 2011), five experiments were removed due to low numbers of expressed transcripts.

The expression levels (measured using fragments per kilobase of transcript per million [FPKM] mapped reads) of the transcripts were averaged across the experiments, and the average values were used to correlate with the methylation levels of the first exons, first introns and the promoters. The methylation level for each first exon, first intron or promoter was represented with the average methylation level across all the individuals.

2.6 | Identification of lowly-methylated sites (LMSs)

Due to the importance of lowly-methylated sites (LMSs) in activating genes in an otherwise globally methylated genome (Nakamura et al., 2014), we inspected the genome-wide distribution pattern of LMSs. In order to include all meaningful LMSs across all individuals, a CpG with a methylation level lower than 0.05 in at least two individuals was defined as an LMS. This threshold was chosen because the CpGs of this methylation level showed the highest tendency of clustering together (Figure S3). The criterion of a minimum of two individuals aimed to decrease the possibility of false positives caused by modest sequencing coverage.

2.7 | Association of SNPs and methylation with localities and environmental parameters

We used redundancy analysis (RDA; Forester et al., 2018; Legendre & Legendre, 2012) to study association of SNPs and methylation, respectively, with local environments. The analysis was conducted in the vegan package (version 2.5–6: Oksanen et al., 2008) in R. Only non-hybrid glass eels were included in the analyses, encompassing 32 individuals for the SNP and 25 for the methylation data. Two rounds of RDA were implemented. The first round had sampling locations (dummy variables) as explanatory variables. This analysis aimed to examine the genetic or methylation response to the sampling locations regardless of their environmental composition. The second round had sea surface temperature (SST30), chlorophyll concentration and mean day length (MDL) as explanatory variables (Table 1), all encompassing means of 30 days prior to the date of sampling. This analysis can be regarded as representing a targeted subset of the (unknown) environmental composition represented by the sampling locations. Remotely sensed sea surface temperatures encompassing a resolution of 0.25 degree latitude × 0.25 degree longitude on a global grid and measured for each day, were provided by the NOAA/OAR/ESRL

PSD, Boulder, Colorado, USA, (http://www.esrl.noaa.gov/psd/) and retrieved using the function extractOISSTdaily from the R script NOAA_OISST_ncdf4.R (http://lukemiller.org/index.php/2014/11/extracting-noaa-sea-surface-temperatures-with-ncdf4/). Data on chlorophyll concentration were extracted from the CCI-OC Data Portal (https://www.oceancolour.org/; Sathyendranath et al., 2019). Mean day length data were obtained from the photoperiod calculator at https://www.ou.edu/research/electron/internet/solarjav.html. SST30 and chlorophyll concentration were included to reflect basic abiotic and biotic properties of the environments, whereas MDL was included to represent diurnal and seasonal variation among localities, possibly associated with for example, genetic or methylation variation at circadian genes. By including mean values for the 30 days preceding sampling, we aimed to capture as much as possible the environmental conditions the glass eels were exposed to either at the site or close to the site during the last stages of oceanic transport.

RDA was conducted for the SNP and methylation data separately. For the SNP data, we filtered out loci with overall minor allele count lower than four and removed SNPs containing missing values, thus retaining 18,337,468 SNPs. For the methylation data, we filtered out CpGs with more than five missing values across individuals, retaining 1,934,985 CpGs. Missing values in the methylation data were replaced with cross-individual mean values of the corresponding CpGs. The *p*-value of each RDA was calculated through 5000 permutations and *p*-values of the RDA axes were calculated using 2000 permutations each.

For each significant RDA axis, we extracted the loadings of the SNPs or the CpGs. SNPs or CpGs with extreme loadings were defined as outliers. For SNPs, we used four times the standard deviation away from the mean as the threshold. For CpGs, we used three times the standard deviation away from the mean as the threshold. This difference in thresholds was used to obtain comparable number of outliers between the two data sets.

2.8 | Methylation related to developmental stage and hybridity

We used P_{ST} combined with methylation difference to search for methylation functionally related to developmental stage and hybridity. P_{ST} is a measure of phenotypic differentiation between groups (Leinonen et al., 2013; Pujol et al., 2008), here accommodated to evaluate methylation divergence between groups in developmental stage or hybridity and calculated using a custom script in R. We adopted criteria of P_{ST} higher than 0.8 and methylation difference higher than 0.35 to define outliers. For the developmental stage, we compared adults and glass eels. For each group, each CpG had to be scored for at least two individuals.

The outliers were assigned to the 17 genomic categories defined above to check for enrichment. The outlier enrichment in a category was calculated as:

$$\text{outlier enrichment} = \log_2 \frac{O(\text{frq})}{E(\text{frq})}$$

where *O*(frq) was the observed frequency of the outliers in the category, and *E*(frq) was the expected frequency by random chance. *E*(frq) was obtained by assigning all the CpGs to the genomic categories. A positive enrichment implies overrepresentation of the outliers in the category, and a negative enrichment indicates underrepresentation. The significance of the enrichment was tested by comparison to the confidence interval of null hypothesis, that is, no enrichment. The confidence interval was defined using binomial distribution.

2.9 | GO term enrichment analysis for the outliers

For each set of outliers (both SNPs and methylation data), we extracted all genes that overlapped with the outliers within a 3000 bp range upstream and downstream. The 3000-bp threshold was decided according to the median length of the intergenic regions (Table S2, Figure S4). The resulting gene list was tested for gene ontology (GO) term enrichment using the “weight01” algorithm of the “topGO” package (Alexa et al., 2006; Schulz et al., 2007) in R. The *p*-values of the GO terms were adjusted following Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995). The GO IDs of the genes were retrieved by blasting the genes against the Swiss-Prot database (release 2021_03, The UniProt Consortium, 2019).

2.10 | Defining DMRs from the outliers

We developed a method for identifying DMRs (differentially methylated regions) from a set of methylation outliers based on the distribution of the neighbouring distances among the outliers. Such distributions show two peaks. The peak with a higher mean is a geometric distribution, representing neighbouring distances of randomly distributed outliers with uncertain functional value and not easily separable from noise. In contrast, the peak with lower mean represents regions with multiple closely located outliers, hence strongly indicative of functional roles. We identified these regions as DMRs and obtained them by grouping the outliers in the small-mean peak according to distance. A threshold, *K*, was set for the minimum number of outliers required in a DMR, in order to filter out the noise generated by the large-mean peak. *K* was obtained from the following inequality.

$$\frac{\sum_{X=K}^{\infty} E(a_X)}{\sum_{X=K}^{\infty} O(a_X)} < \text{FDR}$$

here a_X represents the number of DMRs containing *X* outliers. FDR is the false discovery rate, which we set as 0.01 in this study. $O(a_X)$ is the observed series of a_X, and $E(a_X)$ is the expected series of a_X. $E(a_X)$ was calculated as:

$$E(a_X) = N \cdot p^{X-1} \cdot (1-p)^2$$

where N is the number of neighbouring distances in the large-mean peak assuming a geometric distribution, and p is the expected ratio of neighbouring distances in the small-mean peak under this distribution.

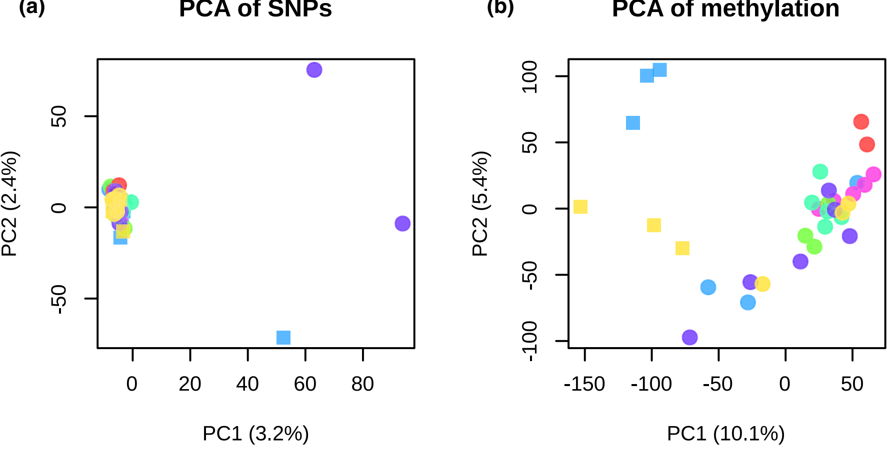
3 | RESULTS

3.1 | Genetic variation

An overview of whole genome sequencing and mapping statistics for each individual is provided in Table S3. A total of 74,040,803 SNPs were obtained from the WGS data. The majority of individuals showed highly similar levels of heterozygosity (Figure S5), ranging from 0.00981 to 0.01084 (mean: 0.01028), whereas the three admixed individuals exhibited higher levels (0.01156–0.01232). Four individuals from Morocco showed higher heterozygosity (0.01161–0.01229), almost similar to the hybrids. However, species-diagnostic SNPs (Pujolar et al., 2014a) confirmed them not to be hybrids. Such variation in heterozygosity would not be expected in a panmictic species with high effective population size, and we found that the elevated heterozygosity was most likely due to cross-sample contamination (see Supporting Information, Note S1), with contamination rate ranging from 3.4% to 6.0%. We therefore excluded these individuals from all subsequent analyses, although we note that we observed no noticeable impacts on the methylation-based analyses. A principal component analysis (PCA) based on SNPs (Figure 2a) showed virtually no divergence between the majority of individuals, consistent with the assumption of panmixia of the species (Als et al., 2011; Enbody et al., 2021; Palm et al., 2009; Pujolar, 2014b), whereas the three hybrids showed separation along PC1 and PC2.

3.2 | Global methylation

An overview of whole genome bisulphite sequencing, mapping statistics and methylation for each individual is provided in Table S4. Methylation was analysed in 33 individuals (after removal of contaminated individuals). A total of 7,484,974 CpG sites were identified (out of 24,369,391 sites in the reference genome). The overall methylation of 70.9% is within the range observed in vertebrates (Head, 2014) and of similar magnitude as observed in fishes like three-spined stickleback (*Gasterosteus aculeatus*) (70.3%; Metzger & Schulte, 2018) and zebrafish (*Danio rerio*) (80%; Feng et al., 2010). The methylation level distribution exhibited a U-shape with the two peaks at the ends of the range of methylation (corresponding to hyper- and hypomethylation; Figure 2c), and this pattern was highly



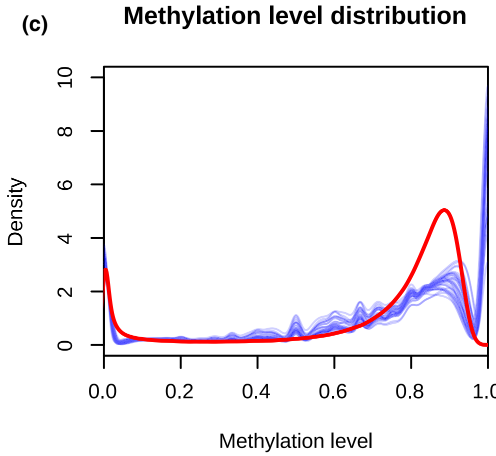


FIGURE 2 Principal component analysis (PCA) based on (a) single nucleotide polymorphisms (SNPs) and (b) methylation, respectively, showing the relationships among individuals, with numbers in parentheses indicating the percentage of variance explained by each PC. The colours indicate the sampling localities, as shown in Figure 1. Squares denote adult eels and circles denote glass (juvenile) eels. (c) Distribution of methylation level across CpG sites. The blue lines represent single individuals. The red line represents the average across all individuals

consistent across individuals. A PCA based on methylation of CpG sites separated adult and glass eels along PC1 and PC2 (Figure 2b).

3.3 | Methylation in genomic categories

We divided the reference genome into 17 non-exclusive functional categories according to genomic annotation (Table S2) and found methylation in most categories, including first introns to be high (Figure 3). However, promoters and the first exons were notable exceptions and showed the lowest methylation levels. Visual inspection of the methylation level across the chromosomes also indicated a high frequency of methylation valleys at the beginning of genes (see Figure S6 for an example from Chr_01). The methylation level of each CpG was highly consistent across individuals for the promoters and the first exons (Figure 3).

Comparison of methylation and gene expression using the transcriptome data set by Bracamonte, Johnston, Knopf, et al. (2019) showed that genes with hypomethylated first exons were overall highly expressed, and those with hypermethylated first exons generally showed lower expression levels (Figure 4a). This contrasted with first introns (Figure 4b), where only weak association was found between hypo- and hypermethylation and gene expression. For promoter 1 and 2 regions there was association between hypo- and hypermethylation and gene expression, although the patterns were weaker than for first exons (Figures 4c,d).

We also found that repetitive regions, especially transposable elements (TEs), showed very low numbers of hypomethylated CpGs (Figure S7), probably related to TE silencing. This was further supported by the observation that the CpGs in TEs exhibited the lowest methylation variation (Figure 3).

We found that the CpG islands were overall highly methylated (Figure 3), in accordance with other studies (Deaton & Bird, 2011) and in contrast to previous notions that the hypomethylated CpGs are mainly confined to the CpG islands in promoter regions (Saxonov et al., 2006). The CpG islands were not enriched in promoters (Figure S8), but were enriched in CDS and LTR, both being highly methylated.

PCA performed for each of the genomic categories generally separated adults and glass eels (Figure S9), similar to genome-wide methylation patterns (Figure 2b). This suggests that the methylation differences between developmental stages exist in all categories and is the dominant source of variance.

3.4 | Characteristics of lowly-methylated sites (LMSs)

We identified 1,099,209 lowly-methylated CpG sites (LMSs), which tended to cluster into local groups (Figure S3). They were highly enriched in the promoters and the first exons and were underrepresented in the repetitive sequences (Figure S10). Some genomic regions exhibited particularly high density of LMSs (Figure S11). The gene clusters and genes identified in these regions included: all eight Hox clusters of the European eel (see Figure S11 and Table S5 for genomic coordinates), the two largest protocadherin clusters (12 copies on Chr_03 and 6 copies on Chr_09), the two largest olfactory receptor clusters (110 copies on Chr_09 and 125 copies on Chr_12) and a zscan2 cluster (5 copies on Chr_08). The genes included: zic gene pairs, tbx, tfap2 and homeobox genes other than Hox. The Hox and protocadherin clusters, homeobox genes, zic pairs and tbx have previously been reported in medaka fish (*Oryzias latipes*) and/or threespine stickleback to reside in large hypomethylated domains (Metzger & Schulte, 2018; Nakamura et al., 2014), hence consistent with their high density of LMSs found in this study. We provide an extended description of the distribution of LMSs in Supporting Information, Note S2.

3.5 | Genetic and methylation response to local environments

Redundancy analysis (RDA: Forester et al., 2018; Legendre & Legendre, 2012) was used to study association of genetic or methylation variation with local environments experienced by glass eels. That left 32 individuals for the genetic data and 25 for the methylation data. Two rounds of RDA were implemented, with the first having the sampling locations (dummy variables) as explanatory variables, thus examining the genetic or methylation response to the sampling locations encompassing a range of (undefined) environmental parameters. For the genetic data (SNPs), 0.06% of the variance was explained by the location variables, and for the methylation data (CpG sites), 2.98% was explained (Figure 5a). The results from both data sets were significant (p-value: .0028 and .0004, respectively). We further extracted outliers from the loading scores of the first axes, since for both data sets only the first axes were significant (p-value: .0075 and .0170, respectively). For the SNP data, 11,949 outliers were obtained. GO term enrichment analysis for the surrounding genes revealed high abundance of genes involved in nervous system development and functioning (Table S6). We inspected the genome-wide distribution of the outliers and found regions with high outlier density (Figure 5b). However, the genes in these regions do not show much functional overlap with the major GO terms.

For the methylation data, 23,912 outliers were found. After grouping the outliers into regions according to their neighbouring distances, this led to the identification of 1523 DMRs (Table S7). Genes in these regions were enriched with functions related to developmental processes (Table S8). The genome-wide distribution of the outliers displayed multiple high-density regions (Figure 5b). Genes in these regions exhibited high correlation with the major GO terms. In particular, seven of the eight Hox clusters turned out to be high-density regions.

The second round of RDA had sea surface temperature (SST30), mean day length (MDL) and chlorophyll concentration as explanatory variables, all representing means over the last 30 days prior to

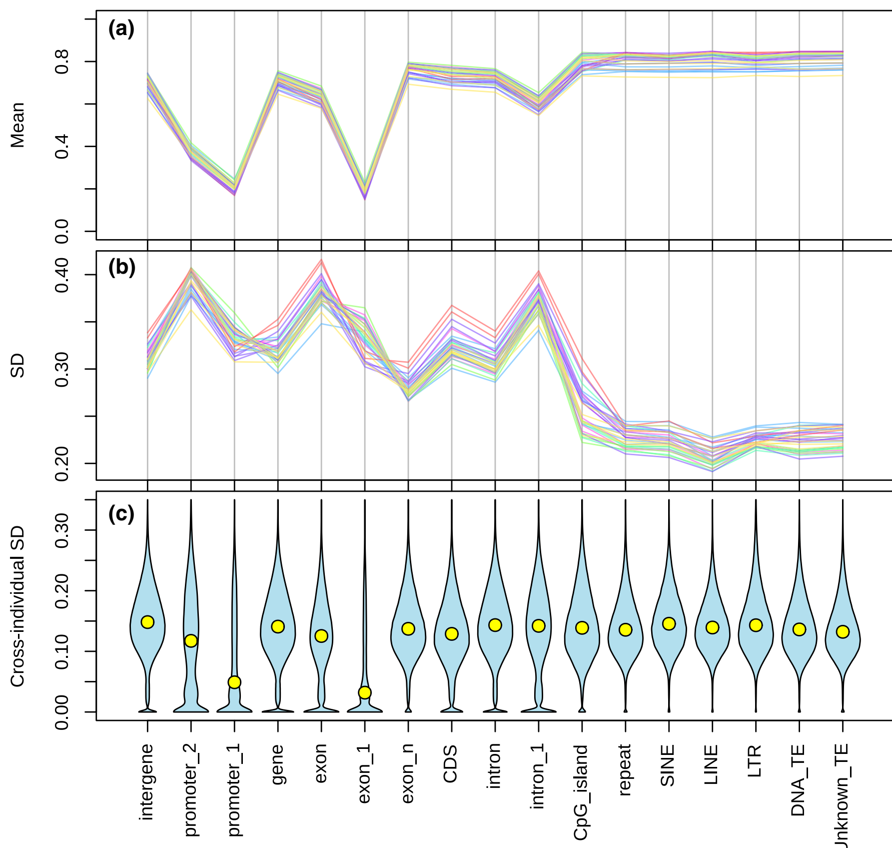


FIGURE 3 Methylation in genomic categories. Each line represents one individual, and the colour indicates sampling location as specified in Figure 1. (a) Average methylation level of all the sequenced CpG sites in each category, and (b) the standard deviation. (c) Interindividual variation of methylation in genomic categories are shown in the lower panel. Each violin represents the distribution of standard deviation of methylation level calculated across individuals per CpG site. Yellow dots indicate medians

the date of sampling. Similar to the first round of RDA, less variance (0.03%) was explained by environmental variables for the genetic data as compared to the methylation data (1.36%; Figure 6a). The results were significant for both data sets (p-value: .0396 and .0056, respectively), but only the first axes were significant or marginally significant (p-value: .0915 and .0325, respectively). A total of 12,124 outliers were obtained from the genetic data, with surrounding genes enriched with the same major GO terms as in the RDA involving localities (Table S9). Similar to the first round of RDA, the genome-wide distribution of the outliers showed some high-density regions (Figure 6b), but with no functional overlap of major GO terms observed for the genes in these regions. For the methylation data, 19,311 outliers were found, from which 803 DMRs were identified (Table S10). Genes in these DMRs were enriched with functions related to developmental processes (Table S11), and the outliers showed several high-density regions (Figure 6b), encompassing among others six of the Hox clusters. Inspection of outliers associated with the individual environmental parameters did not reveal obvious differences (Table S12); the major GO terms were primarily associated with development regardless of the environmental parameter. A total of 9% of the SNP outliers (1092 SNPs) and 39.5% (7632 CpGs) of the methylation outliers overlapped with those identified in the first round of RDA, reflecting the fact that the three environmental parameters represent a subset of the total environmental variation among sites.

A total of 30 GO terms overlapped between outlier SNPs and DMRs for the RDA involving locality as explanatory variable (6.9% of DMR GO terms), and seven GO terms overlapped for SNPs and DMRs identified using environmental variables as explanatory variables (2.3% of DMR GO terms). The overlapping GO terms are highlighted in Tables S6 and S9.

3.6 | Methylation related to developmental stage and hybridization

For developmental stage, the P_{ST} distribution between glass eels and adults indicated that the two groups were divergent in methylation throughout most of the genome, and the methylation difference distribution showed adults to be overall hypomethylated compared to glass eels (Figure 7a). Using criteria of P_{ST}>0.8 and methylation difference>0.35, we identified 10,767 hypomethylated and 3411 hypermethylated outliers in adults relative to glass eels. According to the neighbouring distance distribution of the outliers, hypomethylated outliers seemed to be more randomly distributed across the genome whereas hypermethylated outliers were highly targeted (Figure 7b). We examined the enrichment of the outliers in the 17 genomic categories defined above. The hypomethylated outliers were significantly enriched in intergenic regions, and hypermethylated outliers were significantly enriched in the intergenic

FIGURE 4 Correlation between methylation and gene expression, the latter based on gene expression data from Bracamonte, Johnston, Knopf, et al. (2019) and Bracamonte, Johnston, Monaghan, et al. (2019). Methylation was divided into three categories, that is, overall, hypomethylated (methylation level ≤ 0.2) and hypermethylated (>0.2). We added a value 1e-7 to the expression level to visualize the unexpressed genes in the logarithmic scale. (a) Correlation between exon_1 methylation and gene expression. (b) Correlation between intron_1 methylation and gene expression. (c) Correlation between promoter_1 methylation and gene expression. (d) Correlation between promoter_2 methylation and gene expression

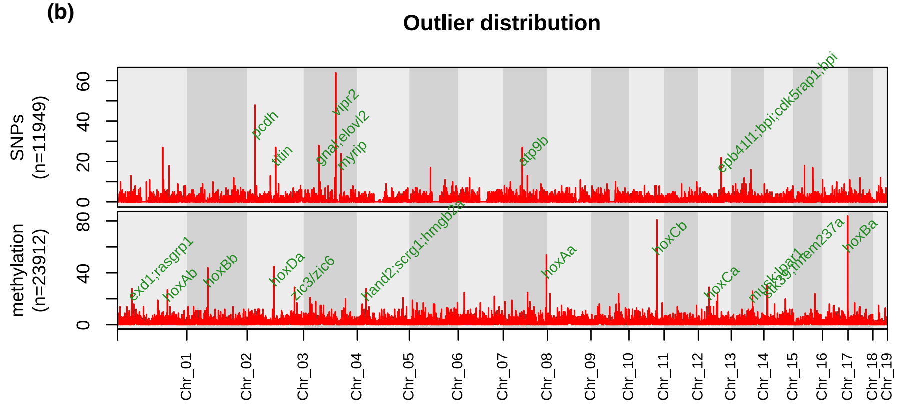
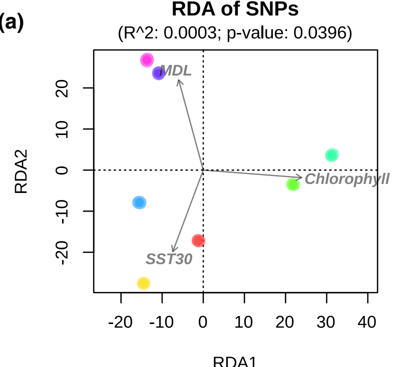
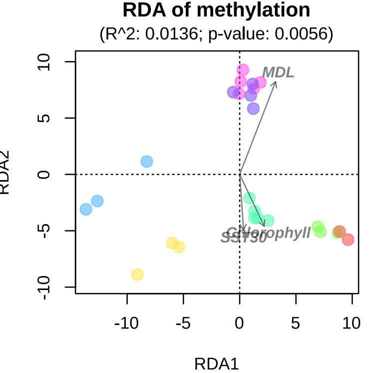


FIGURE 5 (a) Redundancy analyses (RDA) of SNPs and methylation (CpGs), respectively, using location as explanatory variable. (b) Genomic distribution of RDA outliers for SNPs and methylation (CpGs), respectively. Genes associated with high density regions (peaks) are indicated

(a)





(b)

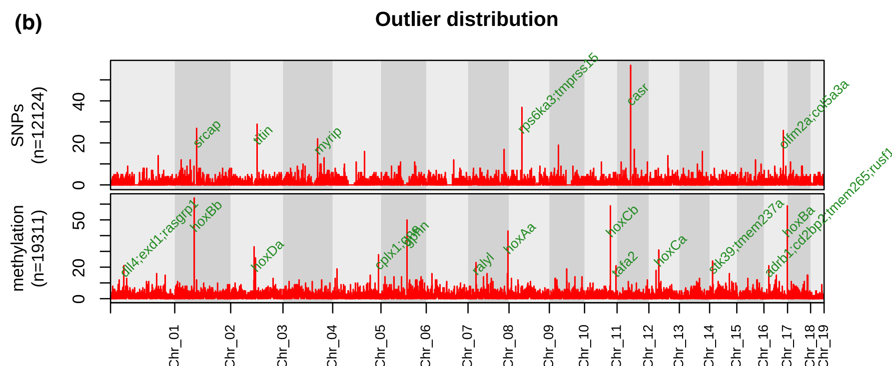


FIGURE 6 (a) Redundancy analyses (RDA) of SNPs and methylation (CpGs), respectively, using environmental parameters (mean day length [MDL], sea surface temperature [SST30] and chlorophyll concentration [chlorophyll]) as explanatory variables. b) Genomic distribution of RDA outliers for SNPs and methylation (CpGs), respectively. Genes associated with high density regions (peaks) are indicated

regions, the promoters and the first introns (Figure 7c). From the hypomethylated outliers, 389 DMRs were identified (Table S13). No significantly enriched GO terms were found from the genes residing in these DMRs (Table S14). From the hypermethylated outliers, 577 DMRs were found (Table S13), enriched with transcription regulators and development-related genes (Table S14).

The genome-wide distribution of the outliers corroborated the neighbouring distance distribution (Figure 7d). The outliers hypomethylated in adults were primarily randomly distributed. Hence, despite their abundance being three times higher than hypermethylated outliers, only six high-density regions were identified, encompassing four genes that were all related to development and cell differentiation. In comparison, the hypermethylated outliers encompassed 25 high-density regions. The three highest peaks correspond to two copies of nfix and one pura. They are involved in the initiation of DNA replication and transcription (Gronostajski, 2000). Two copies of nfic and one nfia were also among the high-density regions. They have similar functions as nfix, and together they cover five of the six nuclear factor I genes in the European eel genome. Interestingly, the two copies of nfix and nfic were found in both hypomethylated and hypermethylated DMRs (Table S13). This may be related to alternative splicing of these genes, as the isoforms of these genes tend to have different or even opposite effects (Gronostajski, 2000). The genome contains two copies of zic1/zic4 gene pair, and both copies were found within the high-density regions. These genes are crucial for nervous system development in embryos. Three copies of igf2bp genes were found, and there are in total five copies in the genome. However, the hypermethylated DMRs represented all five copies (Table S13). Igf2bp genes are RNA-binding factors. They play direct roles in transport and translation of mRNAs and protect them from endonuclease and miRNA attacks. Igf2bp plays important role in nervous system development. Two copies of bmi1 were found in the high-density regions, out of the three copies in the genome. They are components of PRC1 complex, which induce gene repression through histone modification. PRC1 regulate many genes during development, including the Hox clusters. For the rest of the genes in the high-density regions, most are related to developmental processes. We also note that in contrast to outliers associated with local environments, none of the Hox clusters were found in high-density regions, and among DMRs only hoxDa on Chr_03 was visibly hypermethylated (Table S13).

All adult samples were from Bur and Val, with each locality represented by three adults and three glass eels. We repeated the analysis above for each locality in order to assess parallelism in methylation differences between adults and glass eels. For Bur, 37,727 outliers were found, and 6007 of them overlapped with the outliers found above (42.37%; the percentages are relative to the number from the analysis encompassing all adult versus all glass eels). For Val, 36,739 outliers were found of which 5411 (38.16%) overlapped. A total of 1860 (13.12%) outliers were shared by all three sets. The percentages remained similar when only hypo- or hypermethylated outliers were considered. Under a null hypothesis that the three sets of outliers are uncorrelated, the expected percentages would be 1.95%, 1.90%, 0.037%.

For hybridity, we defined hybrids and the non-hybrids as two groups. The methylation profiles of the two groups based on P_{ST} were only mildly divergent (Figure 8a). We obtained 8577 hypomethylated and 667 hypermethylated outliers in hybrids relative to non-hybrids. Both sets of outliers were largely randomly distributed in

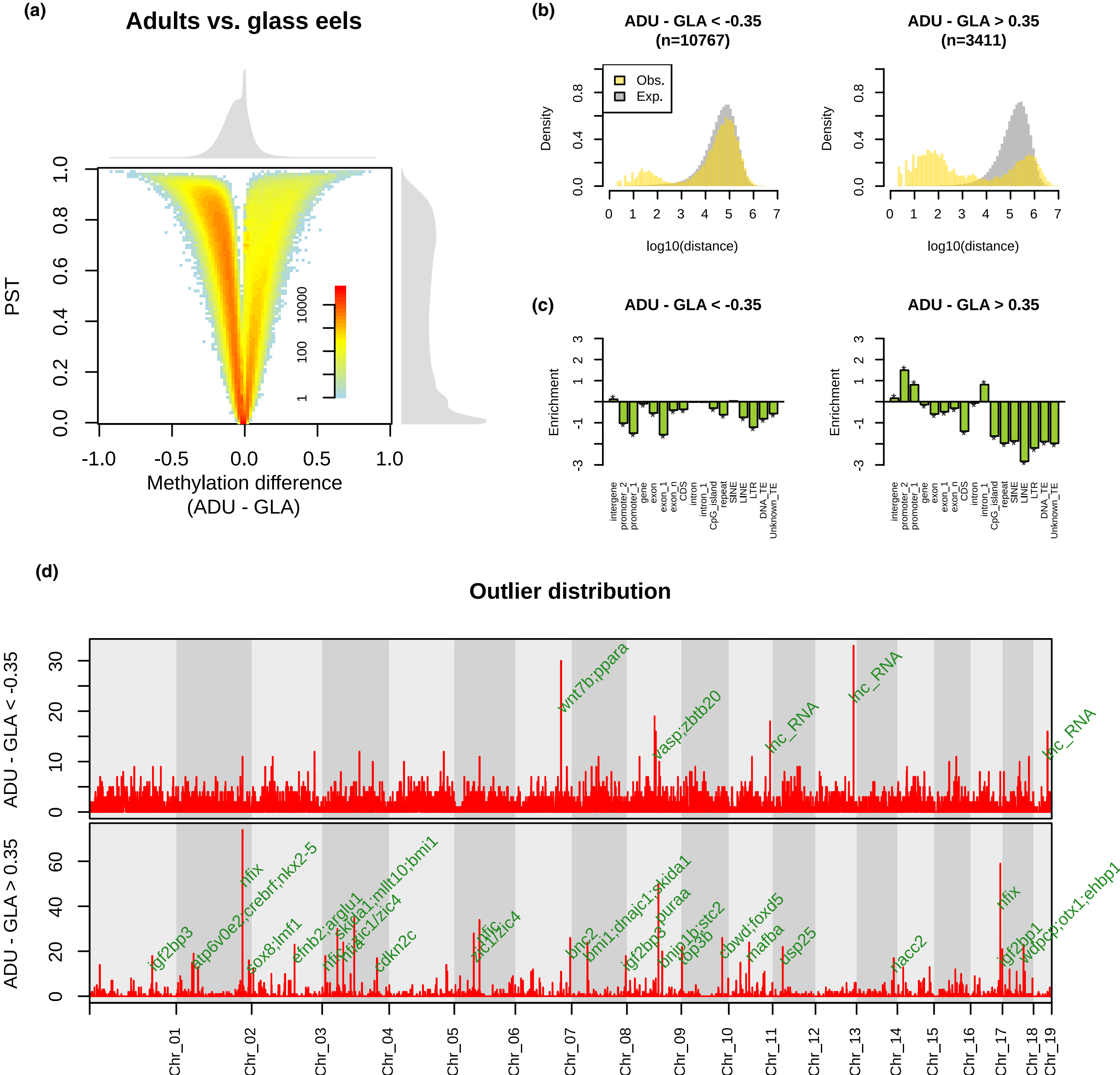


FIGURE 7 Analysis of methylation differences between life stages. (a) Joint distribution of P_{ST} and methylation difference between adults and glass eels. The methylation difference was calculated as the average methylation level of adults minus the average methylation level of glass eels. (b) Distribution of neighbouring distance (logarithmically scaled) among CpG outliers related to the developmental stage. The left panel represents outliers hypomethylated in adults, and the right panel outliers hypermethylated in adults. (c) Outlier enrichment in genomic categories. Positive values indicate overrepresentation of the outliers in the category and negative values implicate underrepresentation. Asterisks indicate significance (alpha = 0.05). (d) Genome-wide distributions of hypo- and hypermethylated outliers, respectively. Genes associated with high density regions (peaks) are indicated

the genome (Figure 8b). The hypomethylated outliers were significantly enriched in intergenic regions, repetitive regions and DNA TEs (Figure 8c), with patterns in the last category suggesting demethylation of transposons in hybrids. The hypermethylated outliers were significantly enriched in intergenic regions and promoters (Figure 8c). A total of 129 hypomethylated and 90 hypermethylated DMRs were found (Figure 8d, Table S15), with no significant enrichment of any GO terms found for either set of DMRs (Table S16).

4 | DISCUSSION

The results of this study, along with other recent papers (Christensen et al., 2021; Leitwein et al., 2021; Wellband et al., 2021), represent some of the first data on methylation at the whole genome level in fishes, and we observed a complex methylation landscape that is associated with the general functional roles of methylation. In relation to our specific research objectives we found (1) outlier SNPs in glass

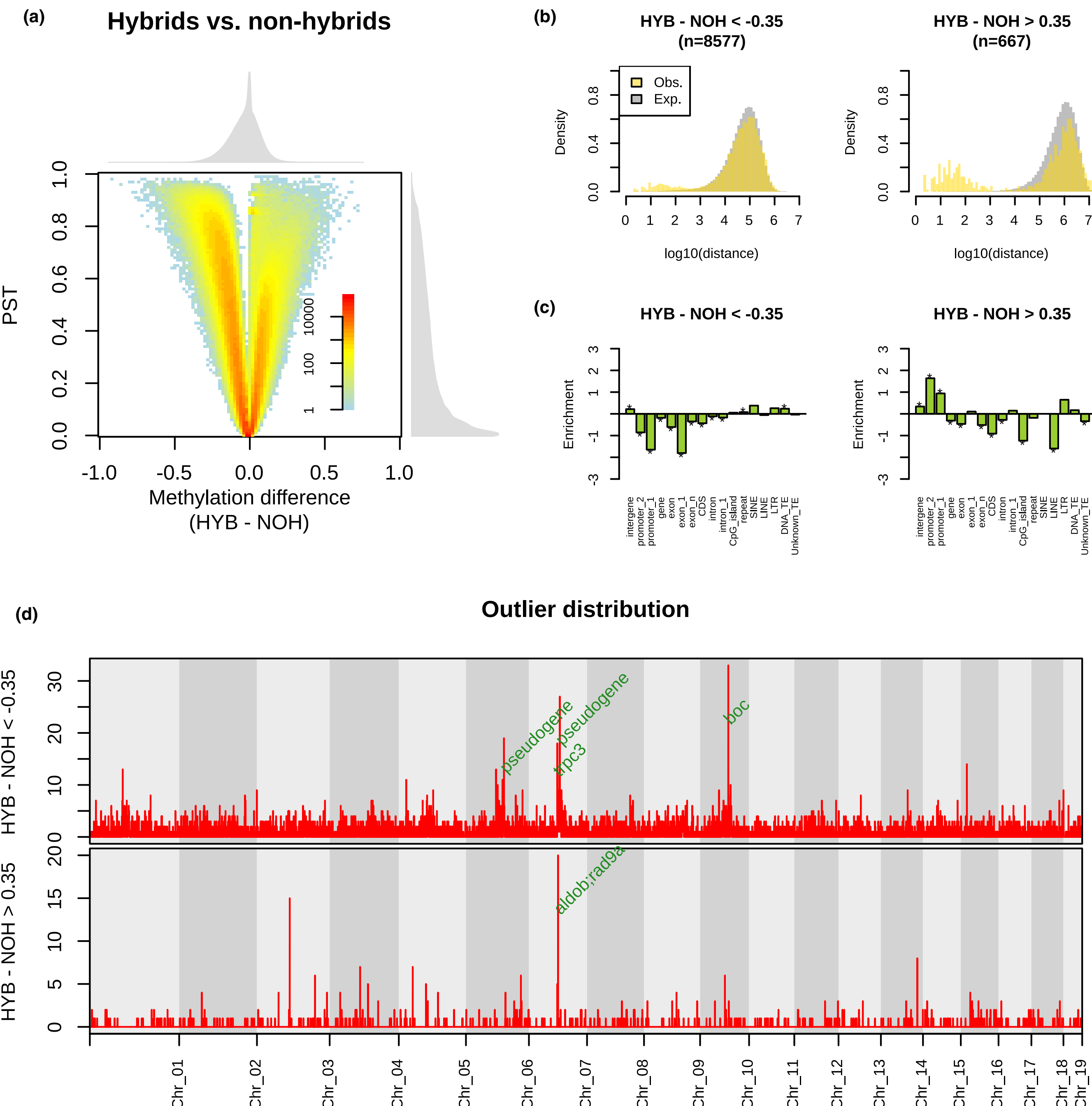


FIGURE 8 Analysis of methylation differences between hybrids and non-hybrids. (a) Joint distribution of P_{ST} and methylation difference between hybrids and nonhybrids eels. The methylation difference was calculated as the average methylation level of hybrids minus the average methylation level of non-hybrids. (b) Distribution of neighbouring distance (logarithmically scaled) among CpG outliers related to hybrid or non-hybrid status. The left panel represents outliers hypomethylated in hybrids, and the right panel outliers hypermethylated in hybrids. (c) Outlier enrichment in genomic categories. Positive values indicate overrepresentation of the outliers in the category and negative values implicate underrepresentation. Asterisks indicate significance (alpha = 0.05). (d) Genome-wide distributions of hypo- and hypermethylated outliers, respectively. Genes associated with high density regions (peaks) are indicated

eels with respect to geographical location and environmental parameters, possibly reflecting within-generation selection. (2) In parallel with outlier SNPs we found differentially methylated regions in glass eels associated with geographical locations and local environments, indicating a role in local adaptive responses. Neighbouring genes particularly represented functions related to development, and especially Hox genes were prominent. (3) Life stage (glass and adult eels) was the overall strongest determinant of methylation differences among individuals, and a considerable portion of methylation differences was associated with genes of importance to developmental processes. (4) TEs were highly represented among methylation outliers between hybrids and non-hybrid European eel,

and were hypomethylated in hybrids, indicating that TE derepression also occurs in this system of incomplete speciation. We discuss these findings in more detail in the following.

4.1 | Methylation landscape in European eel

The general methylation landscape was in accordance with findings in other vertebrates (Brenet et al., 2011; Feng et al., 2010; Head, 2014; Metzger & Schulte, 2018), including overall high levels of genome-wide methylation but with promoters and first exons showing distinctly lower methylation and also higher variance in methylation. Moreover, the association between gene expression and methylation at first exons and promoters suggested a functional role of methylation in these genomic categories (Brenet et al., 2011; Jones, 2012). We found only weak association between methylation at first introns and gene expression, hence somewhat contrasting with the results by Anastasiadi et al. (2018). On the other hand, however, first introns showed enrichment among hypermethylated outliers in adult as compared to glass eels, suggesting a functional role of this genomic category. Some notable patterns were found in the analysis of lowly methylated sites (LMS), where specific gene clusters and genes, particularly Hox, protocadherin and olfactory receptor clusters coincided with large lowly methylated domains, in parallel to findings in medaka and three-spine stickleback (Metzger & Schulte, 2018; Nakamura et al., 2014). It has been previously suggested that such large hypomethylated domains act to suppress transcription of genes while at the same time retaining flexibility for transcription during development (Nakamura et al., 2014). This illustrates the complexity of patterns of methylation, as hypomethylation of promoter regions per se is otherwise positively associated with increased transcription (Moore et al., 2013).

4.2 | Genetic and methylation response to local environments

The unique life history of European eel causes it at the same time to be panmictic and yet to be distributed across geographically and environmentally highly divergent localities (Als et al., 2011; Enbody et al., 2021; Pujolar, 2014b), hence providing opportunities to assess epigenomic responses to environmental variables independent of population-specific genetic variation. However, despite panmixia, redundancy analyses nevertheless showed a low but significant proportion of genetic variance at the level of SNPs that was explained by locality and environmental variables (0.06% and 0.03%, respectively), and >10,000 outlier SNPs were identified. The finding of genomic regions with high densities of outlier SNPs and enrichment of GO terms associated with nervous system development and functioning further lends credibility to these findings representing genuine biological signals rather than false positives. In that sense, the findings are in accordance with previous findings in both European and American eel (Babin et al., 2017; Gagnaire et al., 2012; Pavey et al., 2015; Pujolar, 2014b; Williams et al., 1973), ascribed to within-generation selection and involving polygenic selection. The finding of specific genomic outlier regions as opposed to more even distribution of outliers is puzzling, as this would not be likely to occur as a result of selection within a single generation; panmixia would imply that offspring may end up in very different geographic localities as compared to their parents. Pavey et al. (2015) found evidence for polygenic divergence between freshwater and brackish/saltwater ecotypes of American eel and suggested genotype-dependent habitat choice as one possible explanation. It is possible that genotype-dependent habitat choice could also explain our results, but addressing this issue would require other studies and sampling designs.

A significant proportion of the variance in methylation was explained by locality and environmental parameters (2.96% and 1.36%, respectively). The fact that the specific environmental variables mean day length, sea surface temperature and chlorophyll concentration accounted for considerably less of the variance, as compared to locality, suggests that other environmental factors that vary across localities (and/or across years) may have important effects on methylation. One such factor could be local population density; for instance, sex determination in Anguillid eels is principally or exclusively environmentally determined (Geffroy & Bardonnet, 2016). It has been suggested that high population density of glass eels leads to predominance of initially fast-growing males and low density predominance of initially slow-growing females (Davey & Jellyman, 2005), although the mechanisms and factors involved may be considerably more complex (Côté et al., 2015; Geffroy & Bardonnet, 2016). Although specific information about density of glass eels at the sampling localities was not available, recruitment is known to vary considerably across the distributional range of the species (Bornarel et al., 2018; Dekker, 2003; Tesch, 2003), with the Bay of Biscay and the Iberian Coast (here represented by the Gironde sample) accounting for a major part of total recruitment (Bornarel et al., 2018). Hence, we find it plausible that methylation differences across samples could at least partly reflect differences in population density, also considering the high representation of developmental processes among significant GO-terms for DMRs. Indeed, it has previously been found in a bird species that changes in a social environment leading to increased competition also led to altered patterns of methylation (Rodriguez-Martinez & Galvan, 2019).

The finding of DMRs associated with chlorophyll-α concentration and sea surface temperature is not surprising, given the biological importance of these factors. The former of these can be considered a proxy of productivity, thereby affecting feeding and growth of glass eels. It is well established in humans and other vertebrates that diet can affect methylation (Lea et al., 2016; Zhang & Kutateladze, 2018). It is therefore biologically meaningful that most DMRs were associated with GO terms related to growth and developmental processes.

The life cycle and behaviour of European eel, including feeding and locomotory activity, is strongly affected by light regimes (Lopez-Olmeda et al., 2012; Tesch, 2003). Given the variation in mean day

length across sampling sites and dates, we anticipated a certain representation of circadian-related genes in DMRs associated with this environmental parameter. In fact, a previous population genomics study found a significant correlation between latitude and the circadian clock gene period (*Per*) indicative of within-generation selection (Pujolar, 2014b). At the methylation level in the present study, however, no DMRs were associated with circadian genes, although we note that this could be a result of analysing muscle tissue, whereas circadian genes would be expected to be functionally most important in brain tissue (Baras et al., 1998). Similar to the other environmental parameters, significant GO-terms were instead dominated by developmental processes. It is possible that different activity schemes associated with different light regimes could influence development, and mean day length could also be correlated with other environmental factors affecting development.

Remarkably, Hox clusters featured prominently among high-density regions of outliers, both in relation to localities and specific environmental parameters. Hence, Hox clusters are not only generally situated in large hypomethylated domains, but also represent some of the genomic regions showing most pronounced differential methylation among individuals from different localities and environments. Hox genes are of fundamental importance in developmental processes, notably with respect to determining body plans (Carroll, 2008; Duboule, 2007). It has previously been found that eels have retained a surprisingly large repertoire of duplicated Hox clusters and this has been suggested to underlie the two different body plans of the leaf-shaped larval stage (leptocephalus) and the glass and adult eel stages (Henkel et al., 2012). Our results suggest that variation in methylation of Hox genes (and by inference their regulation) could also be associated with phenotypic variation that develops in response to local environmental conditions. Our data do not allow for specifically associating methylation with phenotypic traits. However, examples of morphological variation exist in adult eels such as distinct narrow-headed and broad-headed types that exhibit different feeding preferences; these morphs are associated with different transcriptomic profiles already at the glass eel stage (De Meyer et al., 2017).

Other explanations should, however, also be considered, as glass eels arriving at different localities could show different ages and development, for instance involving different methylation of Hox clusters. Hence, if recruitment of glass eels exclusively occurs via the Gulf Stream, then glass eels would be expected to be younger in northern as opposed to southern localities. However, other ocean currents than the Gulf Stream are assumed to be involved in transport of larvae (Munk et al., 2010), age determination of glass eels is generally considered controversial (Bonhommeau et al., 2010), and it has been suggested that distance from inshore regions to the Continental Shelf could be the primary factor affecting age of newly recruited glass eel (Lecomte-Finiger, 1992). Hence, this scenario merits consideration, but is not possible to assess with the data and knowledge of recruitment patterns at hand.

Our results show that already at the early life stage of glass eel, where individuals settle in their future nursery and foraging areas, differential methylation is present that is associated with geographical locations and/or environmental parameters. These differences have the potential to affect gene expression and phenotypes also later in life, and the results raise the possibility that epigenetics could in fact underlie differences in growth rates and transcriptomic reaction norms as observed in American eels from different localities (Côté et al., 2014, 2009). Nevertheless, it is a complex question if the methylation differences lead to phenotypic plasticity of adaptive value. This would require that environmental factors affecting methylation should also be predictive of the environmental conditions encountered later in life (Bateson et al., 2014). Analysis of methylation in older (yellow) eels from different localities and environments could shed further light on the role of methylation in adaptive processes in eels, and if the same cohorts could be followed from the glass eel stage this could allow for assessing the temporal stability and adaptive significance of methylation differences induced in early life. Finally, although our focus on a panmictic species should minimize interactions between genetic variation and methylation, the results also show some genetic variation associated with environmental factors. A genetic influence on methylation patterns can therefore not be ruled out entirely, although we note that the functional overlap between outlier SNPs and DMRs was limited. Richards (2006) distinguished between different categories of epigenetic variation, where genetic variation controls (*obligatory*) or influences (*facilitated*) epigenetic variation, whereas in the *pure* category epigenetic variation is independent of genetic variation. Indeed, empirical evidence now exists from a range of organisms showing that at least a part of methylation variation interacts with or is controlled by genetic variation (i.e., *obligatory* or *pure* epigenetic variation) that may furthermore interact with environmental conditions (Berbel-Filho et al., 2019; Dubin et al., 2015; Teh et al., 2014). It would be an interesting future research question if a higher proportion of epigenetic variation associated with environmental factors is *pure* in panmictic eels as compared to other species showing genetic differentiation and local adaptation across populations. However, this would obviously require a deeper understanding of interactions at the genetic and epigenetic levels, along with comparable data from relevant species.

4.3 | Differences in methylation between life stages

Patterns of global methylation clearly separated juveniles (glass eels) from adults (silver eels). Samples from juvenile eels could encompass other tissues than muscle, but muscle would nevertheless constitute the bulk of tissue analysed. We therefore find it less likely that the patterns of methylation should reflect different tissues as opposed to different life stages. Hence, with this caveat in mind life stage was the most important source of inter-individual variation in methylation, and it is noteworthy that this pattern showed high parallelism across the two environments from which adult eels were

sampled. The European eel life cycle is characterized by several metamorphoses; from larvae to glass eel, from glass eel to yellow (adult) eel, and from yellow eel to mature silver eel, all involving distinct morphological and physiological changes (Tesch, 2003). The extensive methylation differences observed could reflect extensive change of methylation associated with metamorphosis, as previously found in both vertebrate and invertebrate species (Covelo-Soto et al., 2015; Gegner et al., 2021; Kyono et al., 2020), but could also represent more gradual age-related changes in methylation (Horvath, 2013; Horvath & Raj, 2018; Issa, 2014). In that sense it was interesting that outliers that were hypomethylated in adult eels showed a relatively random genomic distribution, whereas hypermethylated regions showed a more targeted genomic distribution with enrichment of promoter regions and first introns. Genome-wide hypomethylation and hypermethylation of promoters is in fact a general pattern of methylation associated with ageing (Johnson et al., 2012).

On the other hand, the strong representation of developmental processes among GO terms for hypermethylated outliers supports links to metamorphic processes. Moreover, whereas our study analysed methylation in muscle tissue, a previous study of European eel using methylation-sensitive amplified polymorphisms (MSAP) and comparing life stages found little divergence in liver tissue but larger differences in gill and brain tissues (Trautner et al., 2017). These tissue-specific differences argue against merely age-related effects and support methylation differences being due to specific traits and environmental conditions encountered by the life stages, for example, fresh or brackish water in yellow eels and oceanic salinities to be encountered during the spawning migration of silver eels. In the case of muscle tissue, important differences in metabolic capacity and power output also develop between the yellow and silver eel stages (Egginton, 1986; Ellerby et al., 2001), ascribed to their long spawning migration. However, since our sampling included glass and silver eels, but not yellow eels, it remains uncertain exactly at which life stages the observed methylation differences have occurred. In total, it is possible that the distinct differences in methylation between glass and silver eels could reflect both ageing and metamorphosis, and it would require more extensive analysis of individuals at different age stages to fully resolve this.

Interestingly, whereas Hox clusters represented some of the most distinctive methylation outlier regions between glass eels from different localities and environments, they were not represented among methylation outlier regions between juvenile and adult eels, despite their importance in developmental processes. We do not rule out that differential methylation could exist between earlier life stages, notably leptocephali (larvae) and glass eels (as implicitly suggested by Henkel et al. (2012)). However, for the life stages covered in this study, differential methylation of Hox genes appears almost exclusively associated with environments. This decreases the possibility that the results obtained from glass eels could represent artefacts such as subtle differences in ages and developmental stages among individuals from different localities, as discussed previously.

4.4 | Methylation in European × American eel hybrids

Transposable elements (TEs) can be considered genomic parasites, and free proliferation of TEs in the genome is harmful. Hence, TEs are inactivated in particular by methylation mediated by small piRNA interacting with PIWI proteins (Goodier, 2016). Evolutionary "arms races" between TEs and genes in the PIWI-piRNA pathway have led to rapid evolution and divergence between species, that again results in incompatibilities in hybrids leading to de-repression of TEs (Aravin et al., 2007; Simkin et al., 2013). The resulting re-mobilization of TEs has been suggested as an important postzygotic reproductive isolation mechanism, even in cases of recent speciation (Laporte et al., 2019; Michalak, 2009; O'Neill et al., 1998; Ungerer et al., 2006).

The speciation history of European and American eel is complex and prolonged involving an initial period of reproductive isolation, presumably due to vicariance, followed by secondary contact and ongoing gene flow (Nikolic et al., 2020). Genomic outlier regions separating the species primarily represent genes related to energy and development, consistent with differences in length of spawning migration and larval phase duration of the two species (Jacobsen et al., 2014). Our results suggest, however, that postzygotic isolation does not only involve selection at ecologically important genes, but could also encompass intrinsic incompatibilities leading to demethylation of TEs, even despite ongoing gene flow. Even though we reject the hypothesis that transposon methylation does not differ between pure European eel and hybrids, we stress that the results presented here are preliminary and do not involve a comparison with the epigenome of pure American eel. Analysis of higher numbers of F1 hybrids and backcrosses could shed further light on TE demethylation and the extent to which it decays with each generation of backcrossing.

5 | CONCLUSIONS

Our study of a panmictic species where genetically based local adaptation cannot occur yielded important insights into the much debated issue of the ecological and adaptive role of methylation (Bossdorf et al., 2008; Flores et al., 2013; Rey et al., 2020; Verhoeven et al., 2016). In the absence of genetic differentiation and at most a limited degree of within-generation selection, variance in methylation between life stages, between hybrids and nonhybrids and between glass eels from different localities and environments was pronounced. Whereas the variance associated with life stages and hybridization concerns innate properties of the species, the association of methylation with localities and environmental variables does suggest that the genomes of eels can respond epigenetically to local conditions. We cannot entirely rule out the possibility that the genetic variation found to be associated with local environments could also interact with methylation. It can therefore not be concluded directly that methylation "substitutes" genetically based local adaptation, and this would also

require demonstration of the phenotypic effects of methylation and its adaptive value. However, there is certainly the possibility that at least some environmentally induced methylation at the glass eel stage is of adaptive value later in life. There are as yet few comparable studies of wild species quantifying variation of methylation in response to environmental factors. However, the 2%–3% of methylation variation associated with localities and environments in the early life stage of glass eel is considerably higher than the c. 0.01% of methylation associated with different salinities in experiments with three-spined sticklebacks (Metzger & Schulte, 2018), but lower than the c. 16% associated with river and hatchery environments in Coho salmon (*Oncorhynchus kisutch*) (Le Luyer et al., 2017).

In total, our study of a panmictic species shows that despite no genetic differentiation a portion of epigenetic variation is associated with local conditions and may contribute to adaptation of individuals. Along with other studies focusing on asexual species or species almost devoid of genetic variation (Angers et al., 2010; Berbel-Filho et al., 2019; Leung et al., 2016; Liu et al., 2019; Verhoeven & Preite, 2014), or analysing methylation-environment association while controlling for genetic structure (Gugger et al., 2016; Wogan et al., 2020), this provides evidence for the biological significance of epigenetic variation while controlling for aspects of genetic variation.

AUTHOR CONTRIBUTIONS

Michael M. Hansen, Shenglin Liu and Louis Bernatchez conceived and designed the study, Shenglin Liu conducted bioinformatics and statistical analyses, Aja Noersgaard Buur Tengstedt and Magnus W. Jacobsen identified and validated hybrid individuals, Jose Martin Pujolar, Bjarni Jónsson and Javier Lobón-Cervià provided samples and information, Shenglin Liu and Michael M. Hansen wrote the manuscript with input from all other authors. All authors read and approved the final version of the manuscript.

ACKNOWLEDGEMENTS

We thank Annie Brandstrup for technical assistance, Russel Poole, Eric Feunteun, Françoise Daverat, Gregory Maes and Håkan Wickström for providing samples and two anonymous reviewers for constructive comments and suggestions. This study was funded by the Danish Council for Independent Research, Natural Science (grant no. 7014-00167B to MMH) and MarGen II, an Interreg Project Under the Øresund-Kattegat-Skagerrak Programme.

CONFLICT OF INTEREST

The authors have declared no conflict of interest for this article.

DATA AVAILABILITY STATEMENT

Raw data files for both WGS and WGBS are available at NCBI (The National Center for Biotechnology Information) with accession number PRJNA812038. Data files with SNPs (VCF) and methylated sites and scripts used for analyzing the data are available through DRYAD (https://doi.org/10.5061/dryad.q2bvq83nm) (Liu et al., 2022).

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SUPPORTING INFORMATION

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

How to cite this article: Liu, S., Tengstedt, A. N. B., Jacobsen, M. W., Pujolar, J. M., Jónsson, B., Lobón-Cervià, J., Bernatchez, L., & Hansen, M. M. (2022). Genome-wide methylation in the panmictic European eel (*Anguilla anguilla*). *Molecular Ecology*, *00*, 1–21. https://doi.org/10.1111/mec.16586