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Complementary genomic and epigenomic adaptation to environmental heterogeneity

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Abstract

While adaptation is commonly thought to result from selection on DNA sequence-based variation, recent studies have highlighted an analogous epigenetic component as well. However, the relative roles of these mechanisms in facilitating population persistence under environmental heterogeneity remain unclear. To address the underlying genetic and epigenetic mechanisms and their relationship during environmental adaptation, we screened the genomes and epigenomes of nine global populations of a predominately sessile marine invasive tunicate, *Botryllus schlosseri*. We detected clear population differentiation at the genetic and epigenetic levels. Patterns of genetic and epigenetic structure were significantly influenced by local environmental variables. Among these variables, minimum annual sea surface temperature was identified as the top explanatory variable for both genetic and epigenetic variation. However, patterns of population structure driven by genetic and epigenetic variation were somewhat distinct, suggesting possible autonomy of epigenetic variation. We found both shared and specific genes and biological pathways among genetic and epigenetic loci associated with environmental factors, consistent with complementary and independent contributions of genetic and epigenetic variation to environmental adaptation in this system. Collectively, these mechanisms may facilitate population persistence under environmental change and sustain successful invasions across novel environments.

Yangchun Gao and Yiyong Chen contributed equally to this study

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adaptive evolution, biological invasion, epigenetic adaptation, genetic adaptation, natural selection

1 | INTRODUCTION

Marine organisms have experienced frequent and intensive environmental challenges associated with global climate change that notably affect their persistence, abundance, and distribution (Donelson et al., 2019; Eirin-Lopez & Putnam, 2019). Dissecting the strategies and associated molecular mechanisms that enable marine populations to respond to fluctuating environments is critical to better predict their evolutionary responses and geographical distributions under global climate change. The responses of wild populations exposed to environmental change are commonly characterized by 'move, acclimate/adapt or die' (Donelson et al., 2019). Among these responses, adaptation is expected to be key to avoid extinction, particularly for sessile organisms. In these taxa, contemporary processes alone, such as behavioral, physiological, or morphological changes, are usually insufficient to allow them to cope with environmental stress. The long-standing view on adaptation emphasizes that selection on DNA-based variation can result in genotypes with high fitness in the local environment, as has been shown in a range of invasive and native taxa (Bock et al., 2015; Pina-Martins et al., 2019; Thomas et al., 2017). However, when adaptation occurs from de novo as opposed to standing genetic variation, it may take longer for beneficial alleles to reach high frequencies in a population (Ni et al., 2019).

Recent advances have shown that epigenetic variation, particularly for the most commonly studied type in animals – DNA methylation – could not only change phenotypic outcomes but also contribute to evolutionary and adaptive potential of wild populations (Adrian-Kalchhauser et al., 2020; Hu et al., 2019; Hu & Barrett, 2017). Unlike genetic variation, epigenetic variants can be directly influenced by environmental conditions and provide a faster route to respond to environmental change (Fu et al., 2021; Heckwolf et al., 2020; Richards et al., 2017; Verhoeven et al., 2016). Epigenetic changes can also facilitate evolution when stably transmitted across generations or by increasing genetic mutation rate (Schmid et al., 2018). However, our knowledge of the relationship between genetic and epigenetic variation comes mainly from laboratory-reared model species, and the two sources of variation are often studied in isolation in most studies of wild populations (but see in Heckwolf et al., 2020; Kronholm et al., 2017; Hu et al., 2019). Thus, their contribution to and relationship with environmental adaptation in natural settings remains to be established (Donelson et al., 2019; Eirin-Lopez & Putnam, 2019).

In principle, genetic and epigenetic variation are not mutually exclusive, and may interactively contribute to adaptation (Adrian-Kalchhauser et al., 2020; Verhoeven et al., 2016). While most studies in humans and plants have revealed a strong genetic component of epigenetic variation (Taudt et al., 2016), comparable investigations for wild animal populations are yet to systematically explore their relationship and relative contribution in facilitating population-level adaptation to environmental variation (Hu & Barrett, 2017). An active debate centers on whether epigenetic variation contributes to environmental adaptation in wild populations either independently or as an intermediate step under genetic control (Dubin et al., 2015; Kronholm et al., 2017). Both genetic and epigenetic variation can affect functional genes and biological pathways, thus contributing to adaptation under changing environments (Heckwolf et al., 2020; Liew et al., 2020). More precisely, if candidate genetic and epigenetic variation act on the same genes or biological pathways, the same functions may be regulated at these two different levels. If, on the other hand, they influence different genes or biological pathways, then they probably have distinct roles with respect to responses to environmental challenges. Thus, examining shared and distinct genes and biological pathways harbouring candidate adaptive genetic and epigenetic variation (i.e. using a 'functional perspective') could facilitate a better understanding of their adaptive significance and relationship to environmental adaptation.

Botryllus schlosseri is a predominately sessile ascidian used as a model system in evolutionary biology and invasion science (Bock et al., 2012; Zhan et al., 2015). Previous phylogenetic and population genetic studies have revealed that *B. schlosseri* is a species complex, including five genetically distinct and morphologically cryptic species (A–E) (Bock et al., 2012, but see Brunetti et al., 2020 for morphological differences). Of these, only one (species A, hereafter '*B. schlosseri*') has invaded globally, although its origin and invasion routes are still controversial. Regarding the origin of this species, one hypothesis is that *B. schlosseri* is native to European waters (Bock et al., 2012; Zhan et al., 2015). Other hypotheses, however, propose that *B. schlosseri* is native to the Indo-Pacific (Carlton, 2005), the northeastern Pacific (Teske et al., 2014), the Pacific (Nydam et al., 2017), or the Mediterranean Sea (Ben-Shlomo et al., 2006). Regarding invasion routes, *Botryllus schlosseri* was reported as introduced to the northwestern Atlantic in the mid-1800s (Verrill, 1871) and the western Pacific in 1929 (Van Name, 1945). After that, these populations were subsequently introduced to the eastern Pacific in 1940s (Cohen & Carlton, 1995; Lejeusne et al., 2011), although the population in Seattle was first observed in the early 1970s (www.psp.wa.gov/downloads/ANS/MISM_Online.pdf). Its successful invasion and widespread geographical distribution were probably facilitated by its wide ecological niche and species-specific traits (Epelbaum et al., 2009). For example, this colonial tunicate can reproduce both sexually and asexually, which probably enables its high survival rate and rapid range expansion in invaded regions worldwide (Gasparini et al., 2015; Reem et al., 2017). The high invasion success, wide

geographical range, as well as the publication of a draft genome for species A (Voskoboynik et al., 2013), make *B. schlosseri* an attractive model to investigate the role of genetic and epigenetic variation in environmental adaptation in marine populations.

In this study, we investigate genetic and epigenetic mechanisms of adaptation to contrasting environments in *B. schlosseri*. We first generated genome-wide genetic and DNA methylation data from nine global *B. schlosseri* populations. We then examined the environmental factors that most probably influence genetic and methylation patterns. Finally, to analyse the relationship between genetic and epigenetic variation and their relative contributions to environmental adaptation, we explored the overlap of functional genes and biological pathways harbouring putatively adaptive genetic and epigenetic variation.

2 | MATERIALS AND METHODS

2.1 | Sample and environmental data collection

Botryllus schlosseri colonies were collected from nine locations (nine to 10 colonies per location) across the Northern Hemisphere during 2008–2012 (Figure 1a; Table S1). To maximize the DNA yield, a total of six to 10 genetically identical zooids were separated from each colony under a light dissecting microscope, and genomic DNA was extracted using the proteinase K method by following Waters et al. (2000). The mitochondrial *cytochrome c oxidase subunit I* (*COI*) fragment was amplified using the LCO1490/HCO2198 primer pair (Folmer et al., 1994) to verify species identity. This step confirmed that all colonies of the nine populations were part of the globally-invasive clade A.

Environmental data spanning the interval of 2005–2012 were collected from World Ocean Atlas 2013 of the National Oceanic and Atmospheric Administration (https://www.nodc.noaa.gov/; Table S1). We included nine sea surface temperature-related, salinity-related and dissolved oxygen-related variables: the lowest monthly minimum temperature (T_min), the annual average temperature (T_ave), the highest monthly maximum temperature (T_max), the lowest monthly minimum salinity (S_min), the annual average salinity (S_ave), the highest monthly maximum salinity (S_max), the lowest monthly minimum dissolved oxygen (O_min), the annual average dissolved oxygen (O_ave) and the highest monthly maximum dissolved oxygen (O_max).

2.2 | Characterization of genomic variation

To characterize genomic variation, we constructed restriction site-associated DNA (2b-RAD) libraries using the *BcgI* restriction enzyme (Wang et al., 2012). The library construction, high-throughput sequencing with Illumina technology and single nucleotide polymorphism (SNP) genotyping followed Gao et al. (2018). Briefly, SNPs were first detected using the ref_map.pl program in STACKS version 2.1 (Catchen et al., 2013). The populations program in STACKS was used to filter SNPs with the following parameters: $-r = 0.7$, $H_O = 0.05$, –min_maf = 0.05, and --write_single_snp (see more details in Gao et al., 2018). Additionally, we retained those SNPs genotyped in at least seven populations to reduce false positives. Genetic differentiation index (F_{ST}) was calculated to estimate divergence among *B. schlosseri* populations using 1000 permutations in ARLEQUIN version 3.5 (Excoffier & Lischer, 2010), with significance adjusted based on sequential Bonferroni correction. To assess the effects of environment on genetic divergence among populations, we performed Pearson's correlation analyses between genetic distance (pairwise F_{ST}) and environmental distance. Environmental distance was calculated based on Euclidean distance between pairwise populations using PRIMER version 5.0 (Clarke & Gorley, 2001).

2.3 | Population genetic structure

We characterized patterns of relatedness among populations using principal component analysis (PCA), STRUCTURE, and discriminant analysis of principal components (DAPC). PCA was performed using the R package SNPRelate (Zheng et al., 2012). DAPC was calculated in the adegenet R package (Jombart et al., 2010). STRUCTURE was performed in STRUCTURE version 2.3 (Pritchard et al., 2000), the number of assumed genotypic clusters K were set from 1 to 9 with 10 replicates for each K value. Each analysis was performed with a period burnin length of 20,000 followed by 20,000 Markov chain Monte Carlo steps. We subsequently evaluated the model fit of the STRUCTURE analysis using EvalAdmix (Garcia-Erill & Albrechtsen, 2020). All of the required file format conversions were conducted using PGDSpider version 2.1 (Lischer & Excoffier, 2012), using VCF files as input.

2.4 | Identification of candidate adaptive genetic loci

To investigate genomic signatures of selection, we combined two population differentiation (PD)-based outlier detection methods (pcadapt and BayeScan) and two environmental association (EA) methods (LFMM and BayeScEnv). For PD methods, we first identified outlier SNPs potentially under selection using the pcadapt R package (Luu et al., 2017), which accounts for population genetic structure. We used a K value of five, in line with results of population genetic structure (PCA, DAPC and STRUCTURE analyses; Figure 1b, Figure S1a–d). However, the EvalAdmix analysis suggested nine clusters as the best fit for our data with minimum amount of nonzero residuals within clusters (Figure S1e). Even so, we consider the EvalAdmix result less robust due to the high sensitivity of this method to continuous genetic variation (Pečnerová et al., 2021), which is the case in our samples as shown by PCA result (Figure 1). The second PD analysis implements a Bayesian method to identify outliers with BayeScan version 2.1 (Foll & Gaggiotti, 2008).

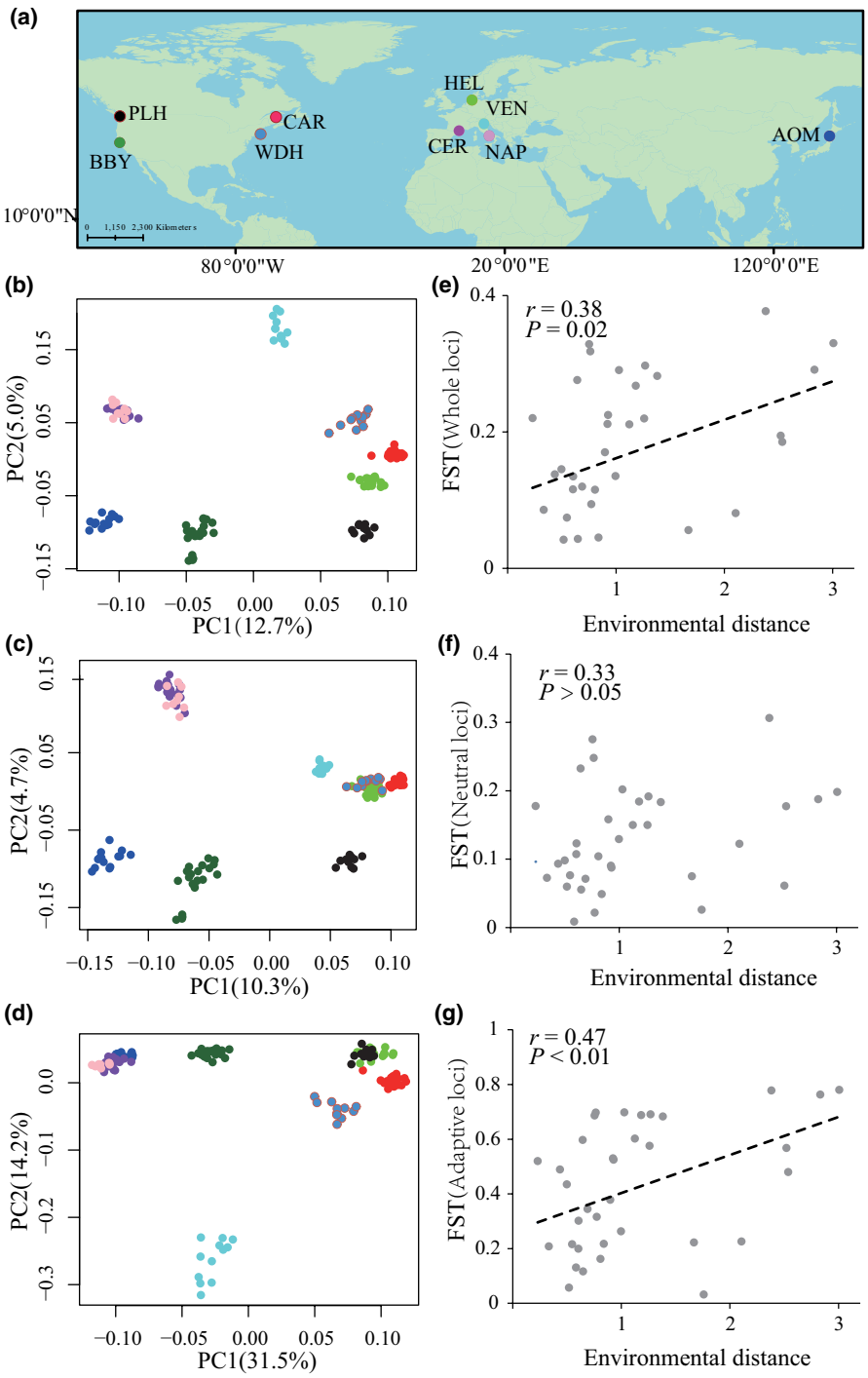

FIGURE 1 Population genetic structure of *Botryllus schlosseri*. (a) Sampling sites of nine *B. schlosseri* populations from the Northern Hemisphere. (b) Genetic structure based on the whole single nucleotide polymorphism (SNP) data set. (c) Neutral structure based on putatively neutral SNPs. (d) Adaptive structure based on candidate adaptive SNPs. (e) Pearson's correlation between environmental distance and genetic divergent index (F_{ST}) based on the whole SNPs. (f) Pearson's correlation between environmental distance and F_{ST} based on neutral SNPs. (g) Pearson's correlation between environmental distance and F_{ST} based on candidate adaptive SNPs; The map was made by ArcGIS version 10.0 (ESRI Company). Colours represent individual populations. Black dashed lines in e–g indicate the fitted regression lines with significant correlations ($p < .05$) between F_{ST} and environmental distance, grey dots indicate the F_{ST} and environmental distance value between pairwise populations [Colour figure can be viewed at wileyonlinelibrary.com]

BayeScan was run for 20 pilot runs with a length of 5000 iterations after a burnin of 50,000 steps with prior odds of 100. Those SNPs with false discovery rate (FDR) lower than 5% were considered as outliers for both methods. For environmental association methods, we considered population genetic structure and implemented a hierarchical Bayesian mixed model implemented in LFMM version 1.2 (Frichot et al., 2013) to screen for candidate loci significantly associated with the environment. The number of latent factors was also set to five as inferred from population genetic structure. SNPs with a z-score higher than five were considered significantly associated with environmental variables (Frichot et al., 2013). Second, we identified significant environment-associated loci using BayeScEnv (de Villemereuil et al., 2014), which assumes that all geographic populations are independent and incorporates environmental information in the form of 'environmental differentiation'. To maximize power while reducing the false discovery rate, our final data set of candidate adaptive loci consisted of overlapping loci identified by both PD and EA methods. We additionally built a neutral SNP data set by excluding all potential outliers detected by any of the four methods described above (pcadapt, BayeScan, LFMM and BayeScEnv).

2.5 | Characterization of epigenomic variation

A 2b-RAD-like protocol, MethylRAD (Wang et al., 2015) was selected to perform reduced methylome sequencing for genome-wide DNA methylation profiling. MethylRAD can specifically distinguish methylated cytosines between CG and non-CG by using methylation-dependent restriction enzymes. This is an efficient DNA methylation profiling approach, amendable to high-throughput sequencing (Wang et al., 2015). Genomic DNA from the same samples used in 2b-RAD was digested using the methylation-dependent restriction enzyme *FspEI* (NEB, USA), which can recognize 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine (5-hmC), cut on the 3'-side of the modified cytosine at a fixed distance (N_{12}/N_{16}), and finally produce 32-bp fragments with methylated restriction sites in the middle. Steps for DNA methylation sequencing library construction were according to Wang et al. (2015). The pooled libraries were sequenced using single-end sequencing (SE50) on the Illumina Hiseq 4000 sequencing platform (Illumina).

Raw methylRAD data were filtered using SOAPnuke version 1.1.1 (http://soap.genomics.org.cn) with default parameters to remove primer linker, contamination, and low-quality reads (average Phred quality score per read <30). Filtered reads were further demultiplexed based on barcodes and trimmed to remove an extra of 2 bp from the 3' end of each read to eliminate artefacts at ligation sites. Clean reads were used for subsequent analyses based on a reference-based method (Wang et al., 2015). Briefly, *FspEI* sites (CCGG and CCWGG) were first extracted from the genome of *B. schlosseri* to build a catalog of reference sites. Clean reads were then mapped against the reference sites using SOAP2 (Li et al., 2009) with no repeat hits ($r = 0$) and with two mismatches allowed (v = 2). DNA methylation level for each site was determined using the normalized read depth (reads per million, RPM = (read coverage per site/high-quality reads per library) × 1,000,000), which was extracted from the mapping results using soap.coverage implemented in the SOAP2 program.

2.6 | Population epigenetic structure

DNA methylation level for each site was determined using the RPM method as described above. We inferred population DNA methylation structure using the multidimensional scaling (MDS) method implemented in the R package edgeR (Robinson et al., 2010). Prior to MDS, DNA methylation sites were filtered to retain only loci detected in at least eight colonies for each population. Methylation distance was calculated on Bray-Curtis distance of methylation levels (RPM) between pairwise populations in PRIMER.

2.7 | Identification of candidate adaptive methylation loci

To identify candidate adaptive methylation loci, we used two methods that are conceptually similar to the PD and EA methods as described above for genetic analyses. For the PD-similar method, we identified differentially methylated loci (DML) between clusters inferred using all methylation loci based on the quantile-adjusted conditional maximum-likelihood (qCML) method implemented in the R package edgeR (Robinson et al., 2010). To acquire a reliable data set of DML, we retained only methylation loci that occurred in at least 40 colonies for each of the two clusters inferred using all methylation loci. Loci were considered to be DML based on fold change (FC) ≥2 and Benjamini-Hochberg corrected $p \leq .01$ (Wang et al., 2015). For the EA-similar method, we used a hierarchical Bayesian mixed model implemented in the R package lfmm to screen for candidate loci exhibiting significant association with the environmental data ($p < .05$), and set the number of latent factors to two as inferred by population epigenetic structure. Similar to the genetic level, our final data set of candidate adaptive epigenetic loci consisted of overlapping loci identified by both edgeR and lfmm. To acquire a neutral data set, we excluded all potentially adaptive methylation loci, which were identified in both edgeR and lfmm.

2.8 | Spatial structure and redundancy analysis (RDA)

Spatial structure can confound environmental effects on population variation if environmental variables are spatially autocorrelated (Benestan et al., 2016). We tested the effect of spatial distribution on population structure using the principal coordinates of neighbour matrices (PCNM) analysis as described in Chen et al. (2021). Spatial factors were represented with distance-based Moran's eigenvector map (dbMEMs) variables, which were transformed from latitudes and longitudes of sampling sites using the R package PCNM (Dray et al., 2006).

We performed redundancy analysis (RDA) to test and quantify the influence of environmental and spatial variables on genetic and epigenetic variation. Allele frequency of SNPs and methylation levels of CCGG loci were considered as response variables for the genetic and epigenetic levels, respectively. A total of 11 factors, including nine environmental factors and two spatial factors (V1 and V2) were considered as explanatory variables. We adopted the vif function in the R package vegan to reduce collinearity of the 11 factors. The SNP allele frequency and methylation level data were detrended using the decostand function with the hellinger method implemented in the R package vegan (Dixon, 2003). We used the forward.sel function in R to select significant explanatory variables to construct a parsimonious RDA model with the highest adjusted coefficient of determination (R_{adj}^2) (Dray et al., 2006). We performed partial RDA (pRDA) using the R package vegan and partitioned variance using the varpart function with 1000 permutations to examine the relative contribution of environmental factors and spatial distribution to genetic or epigenetic variation (Chen et al., 2021).

2.9 | Gene annotation

Sequences containing candidate adaptive genetic and DNA methylation sites were queried against the genome of *B. schlosseri* to search for putatively adaptive genes using BLAST 2.5.0+ with an e-value

threshold of 1.00E-05. Only genes that were within 20 kb upstream or downstream of candidate adaptive SNPs (considering the effect of linkage disequilibrium) and within 5 kb upstream or downstream of candidate adaptive methylation loci (considering effects in promoter and gene body regions) were considered as candidate adaptive genes. Blast results were further filtered with coverage = 100% and similarity = 100%. A systematic functional interpretation of candidate adaptive genes was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis in DAVID 6.7 (Huang et al., 2009). Parameter settings of gene count = 2, ease = 0.1 and $p < .05$ were used to filter significantly enriched KEGG pathways.

2.10 | Genes and KEGG pathways overlapping between genetic and epigenetic levels

To understand the functional relationship between candidate adaptive genetic and epigenetic variation, we calculated and compared the overlap in functional genes and significantly enriched KEGG pathways between the two types of variation. We retrieved genes and KEGG pathways at genetic and epigenetic levels from 3691 candidate adaptive SNPs and 6265 candidate adaptive methylation sites, respectively. MethylRAD follows the similar library construction and sequencing protocols as 2b-RAD, with the only difference being that methylRAD uses methylation-dependent restriction enzymes (Wang et al., 2015).

The enzymes used for 2b-RAD and methylRAD have different recognition sites and cut at different frequencies to produce fragments for sequencing, so it is not possible to analyse the proportion of specific versus nonspecific genes in nonshared fragments. Because when different genetic and epigenetic loci are found associated with environmental variables, this may be wrongly concluded as genetic and epigenetic variation independently contribute to adaptive landscape. Thus, to eliminate the influence of different enzymes, we additionally focused on 'overlaps', that is, fragments containing shared genes annotated by both SNPs from 2d-RAD and methylated loci from methylRAD, and analysed functional difference between adaptive genetic and epigenetic loci. The original data set of genes was composed of shared genes derived from all 8363 SNPs and all 18,629 methylation loci. The overlapping analyses of putatively adaptive genes and KEGG pathways between genetic and epigenetic levels were further assessed based on their shared genes.

3 | RESULTS

3.1 | Genomic footprints of adaptation to environmental heterogeneity

We sampled 135 *B. schlosseri* colonies from nine coastal locations exhibiting environmental heterogeneity across the Northern Hemisphere (Figure 1a, Figure S2 and Table S1). A total of 512.78 million clean reads with an average coverage of 13x per locus were produced by 2b-RAD sequencing (Table S2). After stringent filtering, a total of 8363 high quality single nucleotide polymorphisms (SNPs) were genotyped across all samples (Table S2). Based on this SNP data set, high and significant pairwise genetic differentiation (average $F_{ST} = 0.20$, ranging from 0.04 to 0.41, $p < .001$) was detected for all but one analysed population pair (Table S3). Clear population genetic divergence was also supported by STRUCTURE, PCA, and DAPC (Figure 1b, Figure S1).

To investigate genomic footprints of adaptation, we designated candidate adaptive SNPs using different methods. We identified 896 candidate adaptive SNPs (F_{ST} outliers) based on two population-differentiation (PD) analyses (the sum of pcadapt and BayeScan) and 3319 candidate adaptive SNPs based on two environment-association (EA) analyses (the sum of LFMM and BayeScEnv). Collectively, a total of 3691 candidate adaptive SNPs were identified by these four methods (Figure 2a, Table S4), among which 524 SNPs were shared between PD and EA analyses (Figure 2a). Subsequently, we measured population genetic structure with a strictly filtered neutral SNP data set (4672 SNPs) and the shared candidate adaptive SNP data set (524 SNPs), respectively. Patterns of population genetic structure were different between neutral and adaptive SNP data sets (Figure 1c,d). For example, in analyses based on the neutral SNP data set, the population in Aomori (Japan; AOM) was clearly separated from populations in Canet-en-Roussillon (France; CER) and Naples (Italy; NAP; Figure 1c). By contrast, all three of these populations clustered together in analyses based on the shared candidate adaptive SNPs (Figure 1d). Significant Pearson correlation between environmental distance and genetic distance was revealed with the whole SNP data set ($r = 0.38$, $p = .02$; Figure 1e) and shared candidate adaptive SNP data set ($r = 0.47$, $p < .01$; Figure 1g), suggesting a pattern of genetic isolation by environment (IBE). Moreover, seven out of nine separate Pearson correlation tests based on each environmental factor also exhibited significant IBE (Figure S3). For the neutral SNP data set, the Pearson tests indicated no evidence of IBE ($r = 0.33$, $p > .05$; Figure 1f). These results suggest that environmental conditions are of importance in influencing total and adaptive genetic variation in *B. schlosseri* populations.

To assess the effect of environmental variables on population genetic variation, we conducted RDA. After forward selection and collinearity analyses, two environmental factors (T_min: the lowest monthly minimum temperature, S_max: the highest monthly maximum salinity) and one spatial structure vector (V2) transformed from latitudes and longitudes of sampling sites were retained as the primary explanatory variables in the RDA model. The parsimonious RDA model was globally significant (P-model = 0.001) with an adjusted coefficient of determination (R_{adj}^2) of 0.330 (Figure 2b). Partial redundancy analysis (pRDA) revealed that T_min explained the largest proportion (20.70%) of the total variation, followed by S_max (10.16%) and V2 (8.42%), indicating T_min is probably the leading driver of population genetic variation (Figure 2c). Based on the 524 shared candidate adaptive SNPs, we obtained panels of environment-shared and environment-specific genomic loci. Specifically, 247 SNPs were shared among three environmental groups, and 109 were group-specific, including 72 temperature-specific, 14 salinity-specific and 23 oxygen-specific

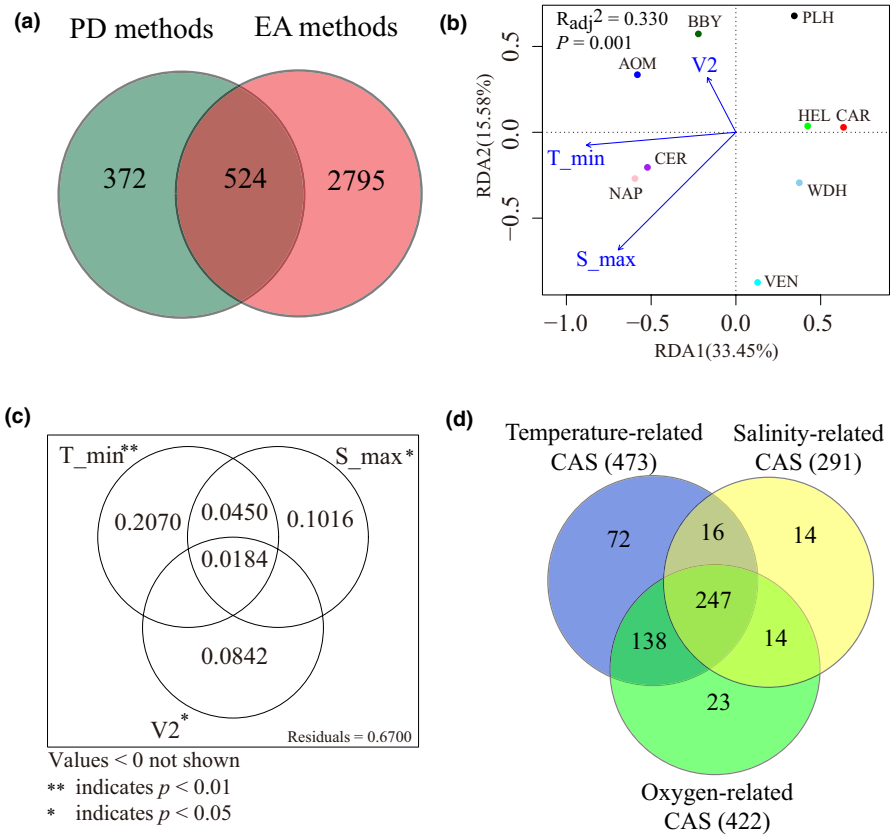

FIGURE 2 Candidate adaptive single nucleotide polymorphism (SNPs) and their correlations with environmental factors. (a) Venn diagram of candidate adaptive SNPs identified by both PD methods (pcadapt and BayeScan) and EA methods (LFMM and Bayescenv). (b) redundancy analysis plot based on the whole SNP data set. (c) Variance partitioning analysis (VPA) of T_min, S_max and V2. (d) Number of SNPs that significantly correlate with temperature, salinity and oxygen from 524 candidate adaptive SNPs (CAS). T_min indicates the lowest monthly minimum temperature, S_max indicates the highest monthly maximum salinity, and V2 indicates spatial factor [Colour figure can be viewed at wileyonlinelibrary.com]

SNPs (Figure 2d). The environmental factor-shared and -specific patterns were also verified by the overlapping analyses based on each environmental factor within their individual groups (Figures S4a–c, Table S5), indicating the importance of each environmental factor in affecting population genetic variation.

3.2 | Epigenomic footprints of adaptation to environmental heterogeneity

For analyses focusing on patterns of DNA methylation, we obtained a total of 20.46 million clean reads with an average coverage of 11× per locus for CCGG and 25× for CCWGG (W = A or T; Table S6). After stringent filtering, a total of 12.44 million CCGG reads and 1.75 million CCWGG reads were retained and successfully mapped to 18,629 and 972 loci in the reference genome, respectively (Table S6). Since the number of CCWGG loci was relatively small, and since all of these loci were located in intergenic regions, which are less likely to provide clear functional insights (Figure S5a), we focused on CCGG loci for downstream analyses (Figure S5b). Based on the DNA methylation data set of CCGG loci, the nine populations were assigned to two groups - AOM, BBY, CER, VEN, and NAP were clustered into group 1, while PLH, WDH, CAR, and HEL were included in group 2 (Figure 3a).

To investigate the epigenomic footprint of adaptation to environmental heterogeneity, we adopted population-differentiation (edgeR) and environment-association (LFMM) methods. We identified 2845 differentially methylated loci based on the edgeR analysis and 4277 environment-associated methylation loci based on the LFMM analysis (Figure 4a, Table S4). Collectively, a total of 6265 candidate adaptive methylation loci were identified by these two analyses with 857 of the loci identified by both analyses (Figure 4a). Similar to population structure analyses at the genetic level, we also assessed population epigenetic structure with putatively neutral and candidate adaptive methylation loci, respectively. Consistent with epigenetic structure inferred based on all loci, two groups were also observed based on the data sets of putatively neutral methylation loci (Figure 3b) and adaptive methylation loci (Figure 3c). Based on the data set of putatively adaptive methylation loci, group 1 is distinguished from group 2 by contrasting environmental conditions (Figure 3d). Similar to the genetic IBE patterns, significant correlations between methylation divergence and environmental distance were revealed based on the whole loci and the putatively adaptive methylation loci (Figure 3e, g). No signal of significant IBE was detected based on putatively neutral methylation loci (Figure 3f).

To further identify key drivers of population epigenetic variation, we also performed RDA. Consistent with results at the genetic level, two environmental factors (T_min and S_max) and one spatial structure vector (V2) were retained, and the RDA model was globally significant (p-model.001) with an R_{adj}^2 of 0.192 (Figure 4b). After accounting for the influence of other factors, T_min still explained 8.23% of the population epigenetic variation (Figure 4c), followed by V2 (4.40%) and S_max (4.22%), indicating T_min is also likely to be the leading driver for population epigenetic variation. Moreover, we observed both environment-shared and environment-specific

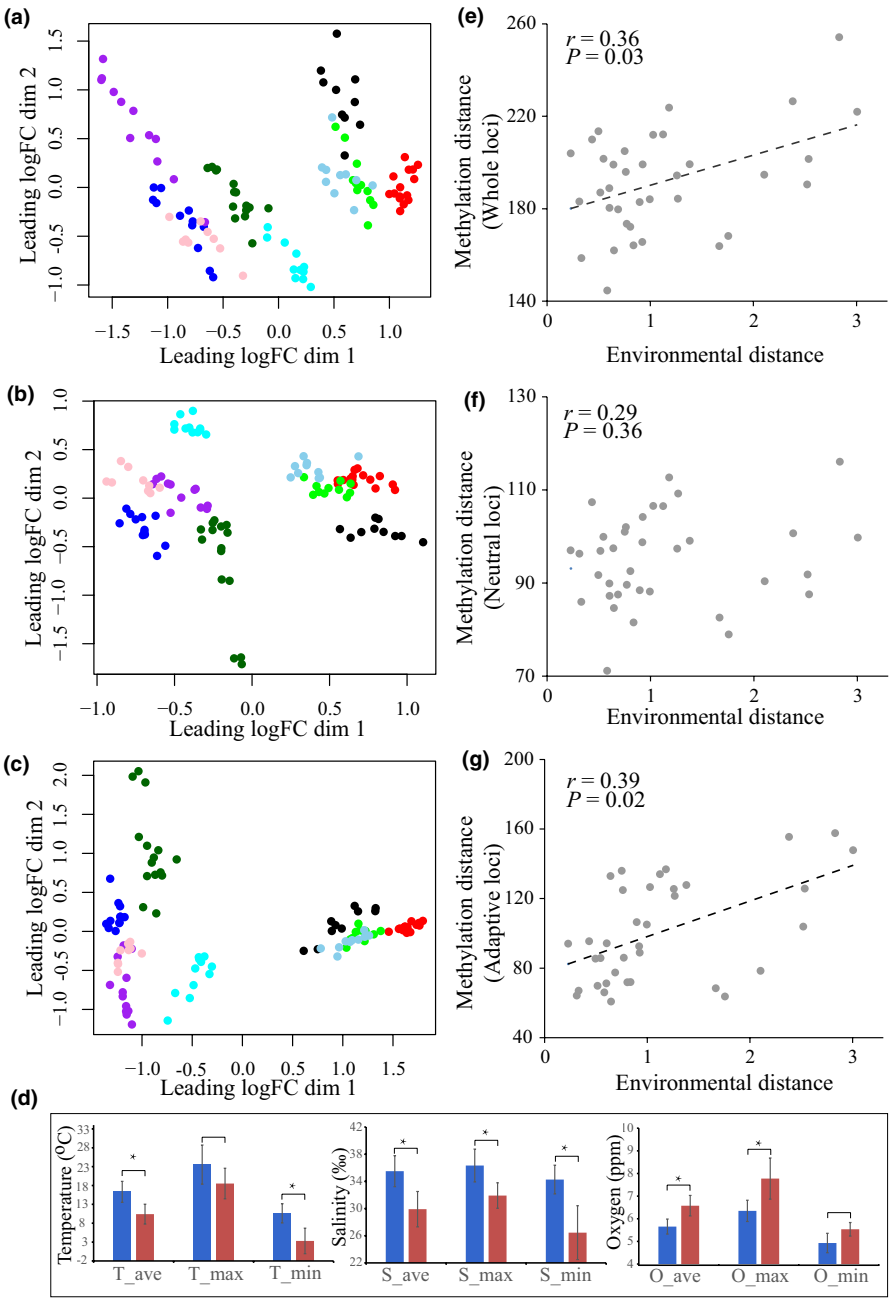

FIGURE 3 Population epigenetic structure of *Botryllus schlosseri*. (a) Epigenetic structure based on all methylation loci. (b) Neutral epigenetic structure based on putatively neutral methylation loci. (c) Adaptive epigenetic structure based on putatively adaptive methylation loci. (d) Comparisons of average values of nine environmental factors between two epigenetic clusters. Blue indicates cluster I, including AOM, BBY, CER, NAP and VEN. Red indicates cluster II, including PLH, WDH, CAR and HEL. '*' indicates significance at $p < .05$. (e) Pearson's correlation between environmental distance and methylation distance based on all methylation loci. (f) Pearson's correlation between environmental distance and methylation distance based on putatively neutral methylation loci. (g) Pearson's correlation between environmental distance and methylation distance based on putatively adaptive methylation loci. Black dashed lines in e–g indicate the fitted regression lines with significant correlations ($p < .05$) between methylation distance and environmental distance, grey dots indicate the methylation distance and environmental distance value between pairwise populations [Colour figure can be viewed at wileyonlinelibrary.com]

candidate adaptive methylation loci. Specifically, 138 loci were shared among the three environmental variables, while 458 were group-specific, including 103 temperature-specific, 71 salinity-specific and 284 oxygen-specific candidate adaptive methylation loci (Figure 4d). Similar results were obtained in analyses based on each environmental factor within each group (Figures S6a–c).

3.3 | The relationship between putatively adaptive genetic and epigenetic variation

In order to assess the relationship between genetic and epigenetic variation potentially involved in adaptation, we examined the overlap in functional genes and KEGG pathways harbouring both putatively adaptive genetic and epigenetic loci. We detected a significant difference ($p < 2.2 \times 10^{-16}$, Fisher's exact test) between adaptive genes (2569) based on 3691 putatively adaptive SNPs (Table S7) and adaptive genes (2239) based on 6265 putatively adaptive methylation loci (Table S8) while using the total number of genes (3608 genes based on the whole SNPs and 4523 genes based on the whole methylation loci) as background. Among those adaptive genes, we identified 670 overlapping genes, 1899 genetic-specific genes, and 1569 epigenetic-specific genes (Figure 5a). As for KEGG analyses, we detected seven shared pathways between the two levels, with 12 genetic-specific and 18 epigenetic-specific KEGG pathways (Figure 5b, Tables S9, S10). In addition, the most significantly enriched KEGG pathway at the two levels was different (Figure 5c,d). For example, the metabolic pathway was identified

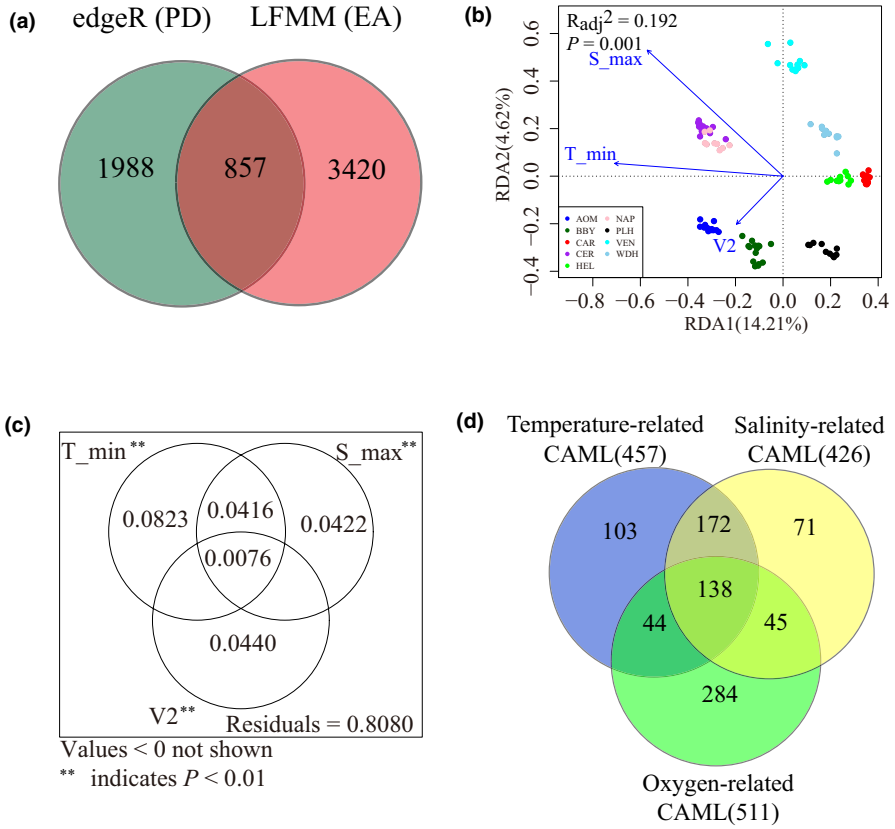

FIGURE 4 Putatively adaptive methylation loci and their correlations with environmental factors. (a) Venn diagram of putatively adaptive methylation loci identified by both edgeR and LFMM. (b) redundancy analysis plot based on the whole methylation loci. (c) Variance partitioning analysis of T_min, S_max and V2. (d) Number of methylation loci that significantly correlated with temperature, salinity and oxygen from 857 candidate adaptive methylation loci [Colour figure can be viewed at wileyonlinelibrary.com]

with the highest enrichment score at the genetic level (Figure 5c) in comparison to the biosynthesis of antibiotics pathway at the epigenetic level (Figure 5d). Moreover, within the seven overlapping KEGG pathways, we also detected both shared and genetic- or epigenetic-specific genes (Figure 5b). For example, in the RNA transport pathway, 20 genes were shared, whereas 16 genes and 15 genes were unique at the genetic and epigenetic levels, respectively (Figure 5b, Figure S7). Altogether, we detected not only overlapping genes and KEGG pathways, but also different genetic or epigenetic genes harbouring putatively adaptive loci, or even different genes in the same KEGG pathway, which also suggest that genetic and epigenetic variation may simultaneously play conjoint and distinct (i.e. complementary) roles during adaptation to different marine environments.

To eliminate potential bias introduced by our use of different enzymes in 2b-RAD (*BcgI*) and methylRAD (*FspEI*), we additionally focused on the shared genes, which were simultaneously identified by all SNPs and DNA methylation sites (Figure S8). We obtained a total of 3608 genes from all 8363 genetic loci and 4523 genes from all 18,629 methylation loci, and 2078 genes were shared between them (Figure S8). Among all shared genes, 1295 and 967 candidate adaptive genes were identified at the genetic and epigenetic levels, respectively. We found 621 shared genes, 674 genetic-specific and 346 epigenetic-specific genes (Figure S8). Furthermore, a total of 19 and 15 KEGG pathways were significantly enriched at the genetic and epigenetic levels, respectively (Figure S9a-b). We identified 12 overlapping KEGG pathways between the two levels, with seven genetic-specific and three epigenetic-specific KEGG pathways. For the 12 overlapping KEGG pathways, there were also overlapping genes and genetic or epigenetic level-specific genes (Figure S9c). Therefore, our results highlight the possible complementary function between genetic and epigenetic variation potentially involved in environmental adaptation.

4 | DISCUSSION

4.1 | Genomic signature of environmental adaptation

Elucidating the role of selective pressure in influencing genetic divergence between locally adapted populations is crucial for predicting future distributions in response to global climate change (Chen et al., 2021). In this study, we uncovered distinctive population genetic patterns associated with oceanographic sites that were discriminated by environmental conditions (Figure S1). We also identified a number of genetic loci significantly associated with environmental factors. The signal at these putatively adaptive loci is unlikely to be the result of hitchhiking effects. This is because SNPs retained for these analyses were evenly distributed across chromosomes, and were separated by an average distance of 69.35 kb (=580 Mb/8363 loci; Figure S10). We observed significant environment-associated genetic differentiation (IBE; Figure 1, Figure S3). This pattern has been documented in other marine populations in seascape genomic studies (e.g. Benestan et al., 2016; Layton et al., 2021; Sandoval-Castillo et al., 2018). These findings highlight the crucial role of natural selection imposed in different environments in affecting population genetic patterns and adaptation (Rellstab et al., 2015).

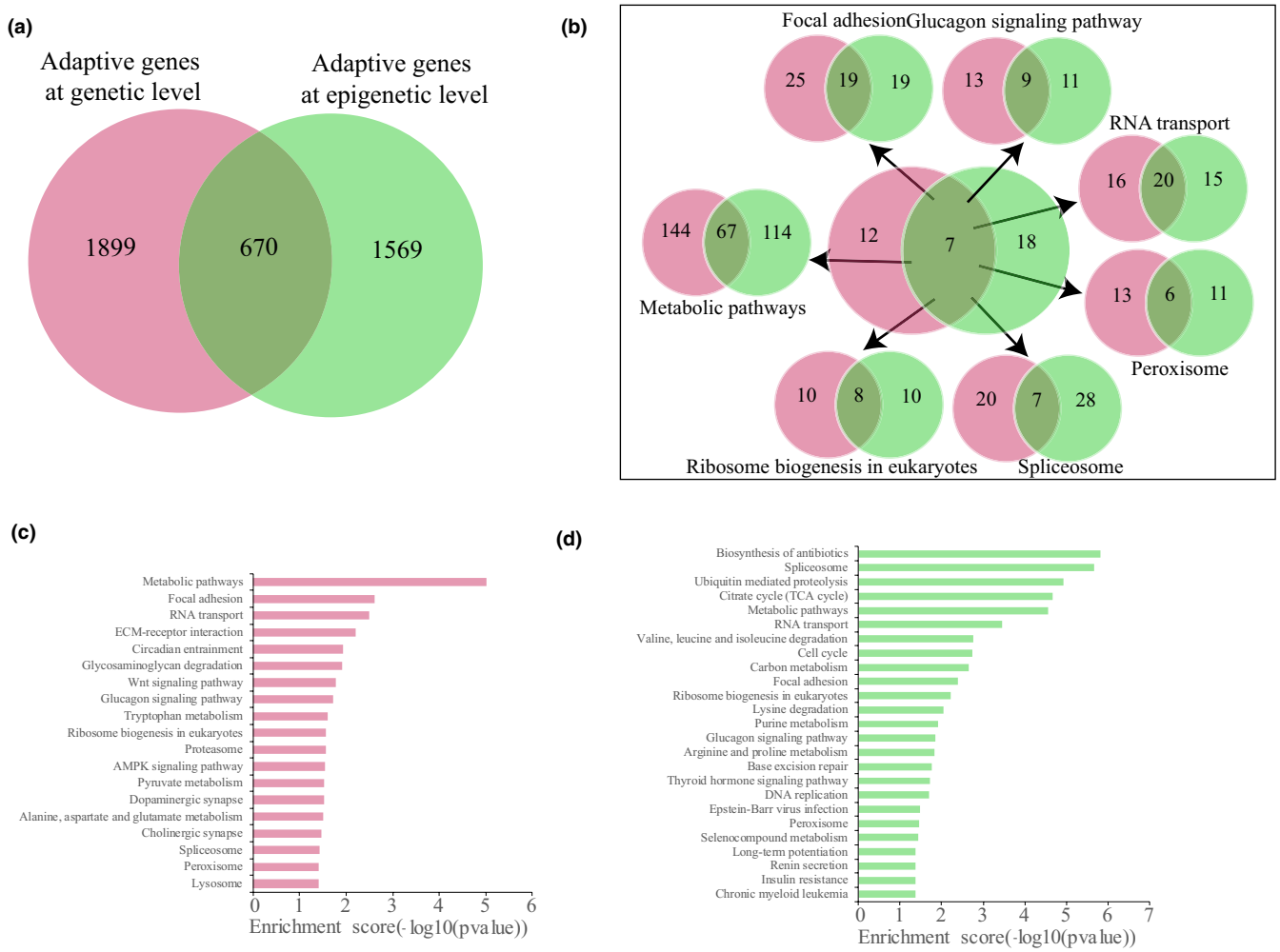

FIGURE 5 The functional relationship of putatively adaptive genetic and epigenetic genes. (a) Venn diagram of putatively adaptive genes at two levels. Putatively adaptive genes at the genetic level and epigenetic level were retrieved based on 3691 candidate adaptive single nucleotide polymorphisms and 6265 candidate adaptive methylation loci, respectively. (b) Overlapping rates of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and putatively adaptive genes in the same pathways between genetic and epigenetic levels. (c) KEGG pathways of putatively adaptive genes at the genetic level. (d) KEGG pathways of putatively adaptive genes at the epigenetic level. Light red and light green indicate genetic and epigenetic levels, respectively [Colour figure can be viewed at wileyonlinelibrary.com]

In the case of invasive species, associations between genotypes and environment can be driven by parallel introductions, when source populations colonize similar environments. For example, when adaptation occurs in human-altered habitats, this situation is known as the 'anthropogenically-induced adaptation to invade' (Hufbauer et al., 2012). In the case of parallel introductions, genetic differentiation between source and introduced populations adapted to the invasive range would be low. In contrast, we found high and significant genetic differentiation among populations (average $F_{ST} = 0.20$, Table S3). Thus, the significant genotype-environment associations detected here are most probably the result of selection in local environments in the invasive range, rather than parallel introductions. More importantly, we detected strong adaptive genetic divergence between some populations and their potential source populations. For example, two northwestern Atlantic populations (WDH and CAR) exhibited low genetic divergence and clustered together with one European population VEN based on neutral genetic structure (Figure 1b), supporting the records that northwestern Atlantic populations were introduced from Europe by shipping (Nydam et al., 2017). However, these populations exhibited high divergence based adaptive genetic loci (Figure 1d), indicating the importance of post-introduction adaptation among populations in their invaded regions. Post-introduction adaptation is known to be crucial to invasion success in a number of species (Bock et al., 2015), including in tunicates. For instance, rapid adaptive evolution after introduction to harsh environments in the Red Sea has been documented in the invasive ascidian *Ciona robusta* (Chen et al., 2018).

4.2 | Epigenomic signature of environmental adaptation

Investigating population epigenetic patterns in relation to the environment is one way of examining how epigenetics may contribute to environmental adaptation (Hu & Barrett, 2017; Ni et al., 2019). In our

study, all nine populations were grouped into two separate clusters based on putatively adaptive methylation loci, and significant environmental differences were detected between these two clusters (Figure 3c,d). Moreover, we uncovered high population epigenetic divergence and significant epigenetic IBE (Figure 3g). These results demonstrate high interpopulation DNA methylation differentiation at global geographical scales and further provide evidence of profound environmental effects on DNA methylation divergence. This finding is consistent with both laboratory experiments and studies in natural populations (Gugger et al., 2016; Huang et al., 2017).

The evolutionary potential of epigenetic variation largely depends on its transgenerational stability and the degree of autonomy from genetic variation (Hu & Barrett, 2017). In the case of *B. schlosseri*, reproductive modes, and in particular the ability to also reproduce asexually, should facilitate transgenerational epigenetic effects (Anastasiadi et al., 2021; Castonguay & Angers, 2012). Although a number of environment-induced DNA methylation changes are transient and reversible, some can still persist over multiple generations and contribute to transgenerational plastic phenotypes and adaptive evolution (Verhoeven et al., 2010). The inheritance of stress-induced variants in DNA methylation has been documented in plants and animals (Heckwolf et al., 2020; Liew et al., 2020; Weyrich et al., 2016). For instance, inheritable DNA methylation patterns were detected in a reef-building coral *Platygyra daedalea*, and most of intergenerational epigenetic changes were environment-induced other than genotype-dependent (Liew et al., 2020). These results indicate that DNA methylation is likely to play a crucial role in accommodation or adaptation to environmental heterogeneity in wild populations. In addition to environmental effects, genetic control and epimutations can also influence DNA methylation variation (Hu & Barrett, 2017; Taudt et al., 2016), and these factors may also interact, rendering it complicated to discriminate their respective effects in wild populations (Richards et al., 2017; Verhoeven et al., 2016).

4.3 | The relationship between genetic and epigenetic variation in environmental adaptation

Clarifying the relationship between genetic and epigenetic variation during local adaptation is crucial to understand how populations evolve in heterogeneous environments, particularly with respect to global climate change (Eirin-Lopez & Putnam, 2019). We first uncovered different patterns of epigenetic and genetic population structure, suggesting a certain level of autonomy in epigenetic variation. For example, while the VEN population was assigned into a separate group based on candidate adaptive genetic SNPs (Figure 1d), it was grouped together with four other populations (AOM, BBY, CER, NAP) based on candidate adaptive methylation loci (Figure 3c). Differences in putatively adaptive genetic and epigenetic population structure suggest that local environments may affect different loci or regions in the genome and in the epigenome. A similar pattern was reported for the Eastern oyster *Crassostrea virginica*, based on genome-wide comparisons of methylation and genetic structure, where the authors found a higher level of DNA methylation differentiation than genetic differentiation between populations (Johnson & Kelly, 2020). While testing the heritability of epigenetic variation was outside the scope of our study, future studies may include such a test to improve our understanding of the autonomy of epigenetic variation in this system (Hu et al., 2021).

Our results, as well as those from previous studies, have revealed that both genetic and epigenetic variation may play an important role in environmental adaptation (Artemov et al., 2017; Richards et al., 2017). In our study, we conclude that these mechanisms contribute to local adaptation in contrasting marine environments in a complementary manner. For example, one candidate adaptive genetic locus 143229_26 on chromosome 9 is located in the *receptor-type tyrosine-protein phosphatase alpha gene* (*PTPRA*), which is a member of the *tyrosine-protein phosphatase* family. It plays a key role via the insulin receptor signalling pathway in coping with environmental stress (Chen et al., 2015). On the other hand, the *receptor-type tyrosine-protein phosphatase delta gene* (*PTPRD*) was annotated with one key DNA methylation locus chrUn_306504937. The *PTPRD* gene is also a member of *tyrosine-protein phosphatase* family and plays a similar role with the *PTPRA* gene at the genetic level involved in the insulin receptor signalling pathway (Tsai et al., 2010). Increasing methylation level of the *PTPRD* gene may inhibit gene expression, decrease the production of insulin, and increase glycogen storage, which is expected to provide essential energy to cope with environmental stress (Chen et al., 2015). The *PTPN1* gene, also a member of the *tyrosine-protein phosphatase* family, has been confirmed to contribute to low temperature adaptation by inhibiting the insulin receptor signalling pathway in migratory locusts (Ding et al., 2018). Thus, it is reasonable to assume that these functionally similar genes, for example, the *PTPRA* gene at genetic level and the *PTPRD* gene at epigenetic level, could collectively contribute to environmental adaptation. From the perspective of biological pathways, the metabolic pathway category was simultaneously enriched at both levels and harboured the largest number of putatively adaptive genes (Figure 5, Figure S9). The metabolic pathway has been reported in multiple invertebrate studies as being related to environmental adaptation, such as hypoxia adaptation in Tibetan migratory locusts *Locusta migratoria* (Ding et al., 2018), and seawater temperature and salinity adaptation in the Pacific oyster *Crassostrea gigas* (Li et al., 2018). Thus, the patterns of shared functional genes and biological pathways harbouring candidate adaptive loci indicate a conjoint contribution of genetic and epigenetic variation in regulating functions crucial to adaptation in heterogeneous marine environments.

Previous studies in model species such as mice and *Arabidopsis* have typically emphasized a strong influence of genetic variation on DNA methylation variation due to the close genomic locations between SNPs and methylation loci (Dubin et al., 2015; Orozco Luz et al., 2015). However, such results from model species are likely to be species-specific. In our study, we found some shared genes contained both adaptive genetic and epigenetic loci (nonspecific genes),

while other shared genes only contained adaptive genetic or epigenetic loci (specific genes), which is consistent with previous studies that also found nonoverlapped genomic locations but complementary functional roles of genetic and epigenetic variation in shaping adaptive landscape (Artemov et al., 2017). More importantly, distinct genes or biological pathways between genetic and epigenetic levels indicate the independent role of epigenetic variation from genetic variation. Some studies comparing DNA methylation variation between multiple laboratory inbred lines have shown the independent effect of epigenetic variation on phenotype and fitness. For example, the autonomy of epigenetic variation has been revealed through experimental evolution via manipulation of the genome and epigenome in the unicellular green alga *Chlamydomonas reinhardtii*, which uncovered an independent role of epigenetic variation in contributing to evolutionary adaptation (Kronholm et al., 2017). In addition, researchers have uncovered different patterns between DNA methylation sites (largely associated with climate variables) and SNPs (largely associated with spatial variables) in nonmodel species valley oak *Quercus lobata* (Gugger et al., 2016). In this case, climate-associated DNA methylation sites were not adjacent to putatively adaptive SNPs, indicating a partly independent effect of epigenetic variants from genetic variation in terms of the 'positional perspective'. An independent role of epigenetic variation from genetic variation has also been found in three-spine stickleback, for which only a moderate proportion (~30%) of epigenetic variation was explained by additive genetic variation (Hu et al., 2021). Because epigenetic variation is more sensitive to environmental change than genetic variation, and has a higher mutation rate than genetic variation, the relationship between genetic and epigenetic variation can be complex, and the regulatory role of the two sources of variation cannot be determined without further confirmatory experiments, such as gene editing techniques. Moreover, we acknowledge that distinct genes harbouring genetic and epigenetic variation may be due to limited ability to detect genetic variation underlying epigenetic variation, such as *trans*-acting genetic variants on epigenetic variation (Taudt et al., 2016). The reduced-representation genome sequencing methods used in our study, that is, 2b-RAD versus methylRAD, use different enzymes and produce RAD tags with a small number of overlaps, and therefore it is almost impossible for us to analyse the positional relationship between genetic and epigenetic variation. Whole genome-resolution sequencing approaches that simultaneously profile the whole genome and methylome would provide more comprehensive insights into genetic-epigenetic interaction from both perspectives under climate change in marine organisms.

Globally distributed, introduced populations of *B. schlosseri* are exposed to highly variable marine environmental conditions, which could impose strong selection pressure, triggering a variety of adaptive responses based on multiple mechanisms. Evolutionary adaptation through genetic mutation probably allows *B. schlosseri* populations to cope with persistent environmental stress and become a globally successful invader despite of intensive environmental heterogeneity over the long-term, as is known to occur in a range of other invasive species (Bock et al., 2015). In addition, this ascidian can rapidly adapt to novel or changing environments through microevolution from its ample standing genetic variation and a high mutation rate (Reem et al., 2017). On the other hand, introduction to novel environments at the early stage of a biological invasion would clearly benefit from epigenetic modifications that facilitate rapid response through phenotypic plasticity. Many empirical studies have provided evidence of epigenetic mechanisms facilitating rapid and flexible responses to environmental changes or stresses, which in turn contribute to invasion success when the founding populations harbour limited genetic variation (Gutekunst et al., 2018; Liebl et al., 2013; Schrey et al., 2012). Recent studies have revealed that DNA methylation diversity is greater than genetic diversity in wild populations, supporting the possible role of epigenetic changes in relaxing the negative consequences of genotype-environment mismatches in diverse metazoans, including molluscs, fishes, and cnidarians (Heckwolf et al., 2020; Hofmann, 2017; Johnson & Kelly, 2020). Collectively, our findings highlight that both distinct and complementary genetic and epigenetic mechanisms are likely to expand the adaptive capacity and invasion potential of *B. schlosseri* across distinct marine environments.

AUTHOR CONTRIBUTIONS

Aibin Zhan conceived the study and designed major scientific objectives. Aibin Zhan, G.D. and Juntao Huprepared ascidian materials for genome and epigenome sequencing. Yangchun Gao and Yiyong Chen conducted DNA extraction, genomic and epigenomic library construction. Shiguo Li, G.D. and Xuena Huang participated initial genome analysis. Yangchun Gao and Yiyong Chen conducted genomic and epigenomic analyses. Juntao Hu and G.D. participated in final data analysis and interpretation. Aibin Zhan, Yangchun Gao, and Yiyong Chen wrote the manuscript with input from all coauthors.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

RAD data and MethylRAD data have been uploaded to NCBI SRA with BioProject number: PRJNA810516. SNPs, methylation loci, environmental variables, codes and Supporting Information tables are available from the Dryad Digital Repository. https://doi.org/10.5061/dryad.44j0zpcgp.

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