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ORIGINAL ARTICLE

Biology

Epigenetic variation of wild populations of the Pacific oyster *Crassostrea gigas* determined by methylation-sensitive amplified polymorphism analysis

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Abstract
Epigenetic variation can provide insights into ecological and evolutionary processes of eukaryote well-being, and can also be useful information for the conservation and management of wildlife. DNA methylation, a major epigenetic mechanism, plays a central role in gene regulation and phenotype variation in eukaryote genomes. However, in natural invertebrate populations, the potential role of epigenetic variation in DNA methylation polymorphisms and the association between this and genetic profiles remains largely unknown. In this work, we estimated epigenetic diversity and differentiation of the Pacific oyster *Crassostrea gigas*, from seven natural Chinese and Korean populations using the fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP) technique. In total, 636 MSAP loci revealed an unambiguous genome-wide methylation status (31.32–43.23% on average) as well as extensive epigenetic polymorphism (approximately 97%) at 5′-CCGG-3′ sites. The unweighted pair group method with arithmetic mean and principal coordinates analysis separated the seven *C. gigas* populations remarkably well, reflecting great epigenetic differentiation among populations, and in particular for the Korean population. Variation was higher in epigenetic structure than in genetic structure. Both multivariate analysis and a significantly different population molecular structure suggested that epigenetic variation might be determined by the combined effects of geographical barriers as well as current features and founder characteristics. A Mantel test showed a moderate but significant correlation between epigenetic and genetic profiles, indicating epigenetic variation coupled with genetic distribution in natural, wild *C. gigas* populations. This study provides some information on the role of DNA methylation in adaptive epigenetic divergence in wild marine bivalve populations.

Keywords Natural populations · Epigenetic differentiation · Conservation · DNA methylation · Genetic regulation

Introduction

Environmental certainty and uncertainty generally represent considerable selective forces on natural populations (Via et al. 1995; Jaenisch and Bird 2003). Organisms can employ a suite of regulatory strategies, and modify their phenotype, to cope with environmental fluctuations (Ghalambor et al. 2007). Recent work has indicated that epigenetic processes play crucial roles in phenotypic variation, which can lead to remarkable ecological and evolutionary success in natural plants and animals (Grant-Downton and Dickinson 2005; Bonasio et al. 2010; Feng et al. 2010; Faulk and Dolinoy 2011). Epigenetic status can provide insights into the ecological and evolutionary processes of eukaryote well-being, and is also of relevance to the conservation and management of wildlife.

Theoretically, epigenetic regulation is stably inheritable for gene products in the maintenance of genome integrity rather than in the modification of nucleotide sequences (Jablonka and Lamb 1998; Morris 2001). DNA methylation, a major epigenetic mechanism, consists mostly

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of 5′-cytosine methylation at CCGG sites (Klose and Bird 2006). Studies (Rando and Verstrepen 2007; Nicotra et al. 2015) have shown that DNA methylation responds to environmental change and signal integration in the eukaryote genome by a change in pattern, and plays a central role in gene regulation and phenotype variation. As a general rule, hypermethylation is routinely described as being correlated with the down-regulation of gene expression (Gonzalgo and Jones 1997; Rajasethupathy et al. 2012). The methylation-sensitive amplified polymorphism (MSAP) assay has proven to be a powerful integrator of genome-wide epigenetic status (Reyna-Lopez et al. 1997). Recent studies using MSAP have shown some meaningful results in methylation variation and epigenetic population structure at CCGG sites of natural plants (Ochogavía et al. 2009; Schulz et al. 2014; Avramidou et al. 2015; Venetsky et al. 2015), vertebrates (Morán and Pérez-Figueroa 2011; Liu et al. 2012) and invertebrates (Díaz-Freije et al. 2014; Jiang et al. 2016). There is increasing interest in the role of epigenetic processes in ecology and evolution. To date, there are few reports concerning genome-wide methylation shown by MSAP in wild bivalve populations.

Historically, the Pacific oyster *Crassostrea gigas* is of great importance as a representative domesticated bivalve species. *C. gigas* is distributed worldwide (Chaney and Gracey 2011) and is thus an important species in the global fishery and aquaculture industries (Ren et al. 2010). In the past few decades, wild *C. gigas* populations have faced considerable challenges from natural and anthropological stressors, and have shown unexpected mortality as a consequence (Aranishi 2006; Guo et al. 2015). As an eurythermal and euryhaline sessile filter-feeding species, *C. gigas* has a strong capability to cope with dynamic hostile environments, such as intertidal zones, and exposure to a variety of stressors (Miossec et al. 2009; Kawabe et al. 2010; Li et al. 2013). A species' capability to respond to environmental fluctuations can provide information on ecological and evolutionary processes. A growing number of reports exploring the genetic structure and differentiation of wild oyster populations have indicated that their genetic diversity is ecogeographically structured and might be partly determined by dispersal, gene flow, climatic factors and currents (Li et al. 2015; Zhong et al. 2016). However, their epigenetic profiles have scarcely been identified.

In this study, we assessed the epigenetic biodiversity and differentiation within and among natural, wild *C. gigas* populations from seven geographically isolated regions, using the fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP) assay. Our study aimed at exploring the amount and structure of cytosine methylation at CCGG sites in a genome-wide manner, and finding correlations by comparing epigenetic and genetic differentiation. The observed epigenetic variation in the wild Pacific oyster is of potential value for the study of invertebrates.

Materials and methods

Oyster material and DNA extraction

C. gigas from seven geographically isolated sites in eastern China and Inchon, Korea, were sampled under permit. Adult oysters from Zhoushan (ZS), Dalian (DL), Qingdao (QD), Weihai (WH), Weifang (WF), and Ganyu (GY) in China, and Inchon (KI) in Korea, were selected from 2013 to 2016 (Table 1; Fig. 1). Adductor tissue was harvested and frozen at − 80 °C for DNA extraction. Genomic DNA was extracted from adductor tissue using a modified phenol–chloroform protocol (Li et al. 2006).

F-MSAP analysis

As with MSAP, F-MSAP involves the use of two methylation-sensitive restriction enzymes, *Hpa*II and *Msp*I. *Hpa*II, which is inactive when either of the two cytosines is fully methylated, cleaves hemi-methylated DNA, whereas *Msp*I cleaves 5′-C5mCGG-3′. The isoschizomers *Hpa*II and *Msp*I both recognize the tetranucleotide sequence 5′-CCGG-3′, but display different sensitivity to cytosine methylation (Shaked et al. 2001; Jiang et al. 2016). The level of DNA methylation for each individual can be measured based on the number of polymorphic bands between the *Msp*I and *Hpa*II MSAP reactions. In this study, we followed the protocol provided by Jiang et al. (2016) that was established for the analysis

Table 1 Sampled populations of the Pacific oyster *Crassostrea gigas*

Population	Location	Sampling period	Geographical coordinates
ZS	Zhoushan, Zhejiang Province, China	2013	30.0°N, 122.3°E
DL	Dalian, Liaoning Province, China	October 2016	38.9°N, 121.4°E
QD	Qingdao, Shandong Province, China	November 2016	35.7°N, 120.4°E
WH	Weihai, Shandong Province, China	May 2016	36.4°N, 121.3°E
WF	Weifang, Shandong Province, China	May 2016	37.1°N, 119.5°E
GY	Ganyu, Jiangsu Province, China	August 2016	34.8°N, 119.2°E
KI	Inchon, Korea	2014	36.4°N, 126.6°E

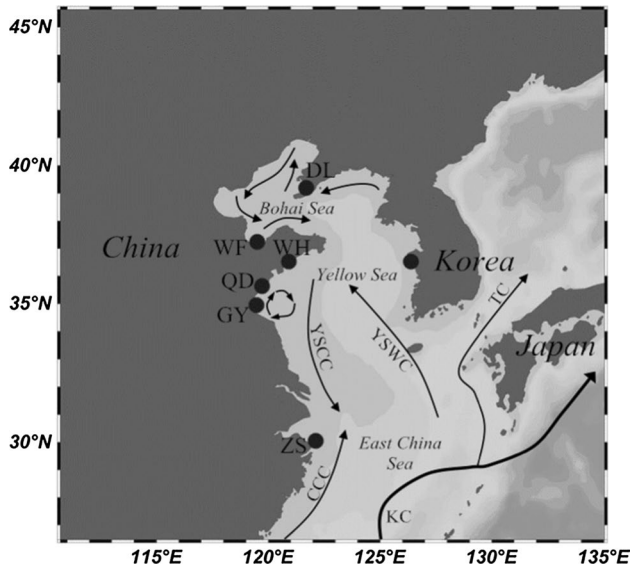

Fig. 1 Map of the sampling locations and ocean currents. *ZS* Zhoushan, Zhejiang Province, China; *DL* Dalian, Province, China; *QD* Qingdao, Shandong Province, China; *WH* Weihai, Shandong Province, China; *WF* Weifang, Shandong Province, China; *GY* Ganyu, Jiangsu Province, China; *KI* Inchon, Korea; *KC* Kuroshio Current; *TC* Tsushima Current; *YSWC* Yellow Sea Warm Current; *YSCC* Yellow Sea Coastal Current; *CCC* China Coastal Current

of genome-wide oyster DNA methylation. Briefly, 100 ng of genomic DNA was digested with *Eco*RI + *Hpa*II/*Msp*I in a 10-μL volume containing 2 U *Eco*RI (Thermo), 2 U of either *Hpa*II or *Msp*I enzyme (Thermo), and 10× Tango buffer for 4 h at 37 °C. The *Eco*RI sticky ends and one of the *Hpa*II/*Msp*I sticky ends were ligated to the DNA after digestion by adding to each final digestion mixture 5 μL of a mix containing 5 μM of *Eco*RI adapter, 50 μM of *Hpa*II/*Msp*I adapter (Table 2), 1 U of T4 DNA ligase (Thermo) and 10× T4 DNA ligase buffer in a final volume of 20 μL. The mixture was then incubated at 16 °C overnight. The digested-ligated DNA was then diluted at 1:10 with $TE_{0.1}$ and used as the template for the pre-amplification reaction. The pre-amplification reaction cycling conditions were 20 cycles at 94 °C for 20 s, 56 °C for 30 s and 72 °C for 2 min, followed by 60 °C incubation for 30 min. After the pre-amplification, the polymerase chain reaction products were diluted 1:10 with $TE_{0.1}$. Selective amplification was performed for 2 min of denaturing at 94 °C, then ten cycles of 20 s at 94 °C, 30 s at 66 °C, and 2 min at 72 °C, with a 1 °C decrease in the annealing temperature of each cycle, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min 72 °C, with 60 °C incubation for 30 min. All adapters and primers are listed in Table 2. Eight primer combinations were employed during the selective amplification stage. *Eco*RI selective primers were labeled using a 6-carboxyfluorescein reporter molecule. The whole experiment was repeated twice in order to retain only the fully reproducible MSAP bands for further processing.

Data collection and statistical analysis

The F–MSAP reactions were loaded simultaneously onto an ABI 3130 Genetic Analyzer (Applied Biosystems) and the DNA methylation status of the loci was subsequently obtained with GeneMapper version 4.0 software. To reduce the potential impact of size homoplasy, only unambiguous and intense bands, ranging in size from 50 to 500 base pairs, were scored. We further converted the MSAP data into a binary matrix (Cervera et al. 2002).

To elucidate epigenetic structure among different geographically isolated regions, the epigenetic variation was calculated by the Dice (1945) and Jaccard (1908) coefficient algorithms, respectively. A group-clustering analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA). Furthermore, principal coordinate analysis (PCoA) was used to provide an indication of genome-wide variability and to summarize the data in a few synthetic variables. All the above analyses were conducted using the numerical taxonomy system of

Table 2 Adapters and primers used for methylation-sensitive amplified polymorphism

Adapter and primer	*Eco*RI (5′–3′)	*Hpa*II/*Msp*I (5′–3′)
Adapter I	CTCGTAGACTGCGTACC	GACGATGAGTCTAGAA
Adapter II	AATTGGTACGCAGTCTAC	CGTTCTAGACTCATC
Pre-amplification primer	GACTGCGTACCAATTCA	GATGAGTCTAGAACGGT
Selective primer	GACTGCGTACCAATTCATC	GATGAGTCTAGAACGGTGC
	GACTGCGTACCAATTCACG	GATGAGTCTAGAACGGTGT
	GACTGCGTACCAATTCACG	GATGAGTCTAGAACGGTAC
	GACTGCGTACCAATTCAGG	GATGAGTCTAGAACGGTAT
	GACTGCGTACCAATTCACT	GATGAGTCTAGAACGGTAG
	GACTGCGTACCAATTCACT	GATGAGTCTAGAACGGTGT
	GACTGCGTACCAATTCAAG	GATGAGTCTAGAACGGTAC
	GACTGCGTACCAATTCACA	GATGAGTCTAGAACGGTAT

multivariate programs–PC (NTSYS-PC) software package, version 2.10e (Applied Biostatistics, USA), and were conducted with 10^3 replicates. A Mantel test implemented in the isolation by distance (IBD) Web service program (Jensen et al. 2005) was performed to test the IBD model by correlating geographic distance to epigenetic distance and genetic distance.

In addition, to further explore the contribution of epigenetic and genetic profiles to the structure of the *C. gigas* populations, MSAP loci were then classified as either methylation-susceptible loci (MSL) or non-methylated loci (NML), depending on whether the observed proportion of methylated states across all samples exceeded a user-defined error rate-based threshold (5% by default) (Bonin et al. 2007; Pérez-Figueroa 2013; Díaz-Freije et al. 2014). Those fragments showing polymorphism of the same state at least twice were used for subsequent analyses (Herrera and Bazaga 2010; Díaz-Freije et al. 2014). Using the R package msap (http://msap.r-forge.r-project.org) (Pérez-Figueroa 2013), MSAP data were partitioned into the two distinct methylation types [MSL (DNA methylation pattern) and NML (genetic structure)] to study the variation of CCGG-methylation states and determine the correlation among all seven populations. Shown in Table 3 are: (1) the total number of loci, (2) the percentage of polymorphic loci (P_{MSL} and P_{NML}), and (3) the mean Shannon's information index (I_{MSL} and I_{NML}), (4) the multilocus analysis of molecular variance (AMOVA; φ*ST*) (, . Additionally, gene flow among populations was calculated based on the MSL and NML by POPGENE 1.32.

Finally, the similarity between epigenetic and genetic profiles was calculated with the standardized Mantel coefficient using 10^3 permutations for significance (Mantel 1967), using the NTSYS-PC software package. According to Cervera et al. (2002), Dice similarity coefficients (Sneath and Sokal 1973) were compared independently from both epigenetic and genetic profiles in the NTSYS-PC software package. Additionally, we calculated pairwise Nei's unbiased measures of genetic identity and genetic distance (Nei 1978) of both profiles by POPGENE 1.32 (Yeh et al. 1999; Zhong et al. 2016) and calculated pairwise AMOVAs. We then compared these data in the NTSYS-PC.

Results

Genome-wide analysis of methylation profiles

The level of methylation for each individual can be measured by the number of sites with polymorphic bands between the *Msp*I and *Hpa*II MSAP reactions out of the total number of MSAP sites. Examples of radioactively labeled and fluorescently labeled MSAP patterns are shown in Fig. 2. To this end, 636 reproducible methylation sites were analyzed in all populations. Namely, for each site, only variations which had originated from cytosine methylation (polymorphism between the *Msp*I and *Hpa*II MSAP reactions) were considered.

The average level of methylation varied statistically between all seven populations (Fig. 3). Notably, the level

Table 3 Collection sites of wild *C. gigas* populations, total methylation-susceptible loci (*MSL*) diversity and comparison with non-methylated loci (*NML*) diversity indexes

Population	MSL				NML			
	Sites	P_{MSL} (%)	I_{MSL}	AMOVA	Sites	P_{NML} (%)	I_{NML}	AMOVA
ZS	328	67.38	0.5743 ± 0.1059		308	33.77	0.5335 ± 0.1242	
DL	347	70.89	0.5835 ± 0.0993		289	42.91	0.5407 ± 0.1269	
QD	391	74.17	0.5749 ± 0.1147		245	34.29	0.5404 ± 0.1393	
WH	347	70.03	0.5863 ± 0.1055		289	50.52	0.5562 ± 0.1244	
WF	371	71.16	0.5797 ± 0.1062		265	36.98	0.5366 ± 0.1326	
GY	377	74.54	0.5729 ± 0.1099		259	48.06	0.5369 ± 0.1309	
KI	352	49.72	0.5859 ± 0.1011		284	26.41	0.5171 ± 0.1338	
Mean	359	68.25			277	38.99		
Total	395	96.71	0.5972 ± 0.1126		241	91.29	0.4452 ± 0.1607	
Among groups				17.12				6.361
Within groups				41.94				18.26
				φ*ST* = 0.2899, *P* < 0.0001				φ*ST* = 0.2584, *P* < 0.0001

Analysis of molecular variance (*AMOVA*) among groups and within groups

P_{MSL} (%) Percentage of polymorphic MSL loci, I_{MSL} Shannon's information index based on MSL loci, P_{NML} (%) percentage of polymorphic NML bands, I_{NML} Shannon's information index based on NML loci; for other abbreviations, see Table 1

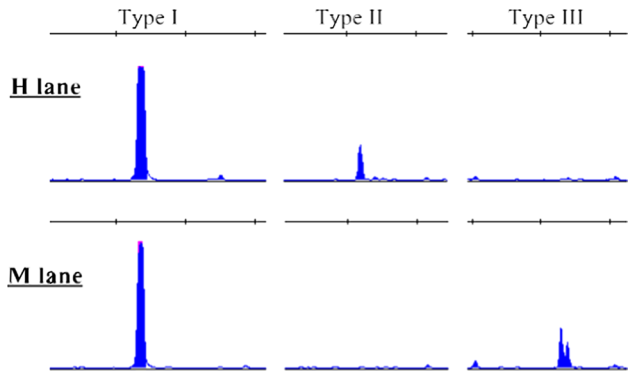

Fig. 2 Examples of methylation-sensitive amplified polymorphism (MSAP) banding patterns in *Crassostrea gigas*. *H lane H* Digested by *Eco*RI/*Hpa*II, *M lane* digested by *Eco*RI/*Msp*I, *Type I* absence of methylation due to the presence of bands in both the *EcoR* I/*Hpa* II and *Eco*RI/*Msp*I digestion, *Type II* bands appeared in the *Eco*RI/*Hpa*II digestion but not in the *Eco*RI/*Msp*I digestion, *Type III* generated bands obtained in the *Eco*RI/*Msp*I digestion but not in the *EcoR*I/*Hpa*II digestion

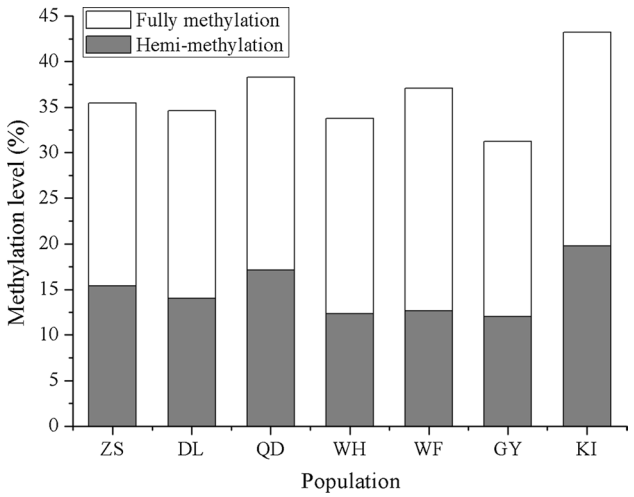

Fig. 3 Average level of cytosine methylation in CCGG sites as assessed by MSAP in natural *C. gigas* populations. For abbreviations, see Figs. 1 and 2

of the total methylation in Inchon, Korea was higher than that of populations from other areas ($P < 0.01$). The total methylation level ranged from 31.32% (GY) to 43.23% (KI), with a mean of 36.24% at the species level. The average level of full methylation was somewhat similar, except for WF and KI populations ($P < 0.01$). For hemi-methylation, the level of ZS, QD and KI was higher than that of the rest of the populations ($P < 0.05$). There was a greater difference between hemi-methylation as compared with full methylation across the populations.

Clustering analysis

A phylogenetic tree was built based on the methylation patterns of total 636 CCGG sites from F-MSAP for all seven populations (Fig. 4; sample number—KI 1–8, ZS 9–16, DL 17–24, QD 25–32, WH 33–40, WF 41–48, and GY 49–56), in which the phylogenetic tree significantly clustered the accessions (matrix correlation $r = 0.8031$). In the phylogenetic tree (Fig. 4), individuals from KI were significantly clustered in one group based on their methylation patterns, as were those from ZS, DL and WH. The phylogenetic tree showed a clear separation between the Chinese and Korean populations: KI, originating from Korea, belonged to one cluster, while the other cluster included the six Chinese *C. gigas* populations. In addition, the latter cluster was further divided into two subgroups: the first subgroup containing ZS and DL, and the second subgroup containing QD, WH, WF and GY. Both QD and WF populations were clustered in two main groups: the first group contained five accessions, while the second group, which is far from the first group, contained three accessions. This might indicate a high level of epigenetic variation in populations QD and WF.

A principal coordinate analysis (PCoA) of epigenetic variation was performed in multivariate space to determine the genome-wide variability of the populations based on different methylation patterns (Fig. 5; samples KI 1–8, ZS 9–16, DL 17–24, QD 25–32, WH 33–40, WF 41–48, and GY 49–56). The seven populations showed obvious clusterings on a three-dimensional plot (summarizing 78% of the total inertia). Additionally, the methylation status of *C. gigas* showed an obvious regional correlation in most areas (Fig. 5). The WH, WF and GY populations were interwined, while the ZS, DL, QD populations were interwoven. However, KI showed more variation than the other populations, especially than DL. In general, the results of the PCoA were consistent with those of the UPGMA.

Epigenetic and genetic structure

Using the R package msap, all seven populations were then treated as one group for the next analysis. Approximately 97% (382 of 395) were polymorphic in the full sample, but only 49.72–74.54% were polymorphic within samples of MSL (Table 3). Similarly, about 91% (220 of 241) could be regarded as polymorphic in the full sample, but only 26.41–50.52% were polymorphic within samples of NML (Table 3). For the among-population analysis, the first axis of the PCoA (data not shown) based on the covariance matrix (MSL) summarized only 11.5% of the total inertia, and the first 47 axes, together representing 98.1% of the information contained in the epigenetic data set, were used for further analyses. For NML, the first axis summarized only 10.6% of the total inertia, and the first 55 principal

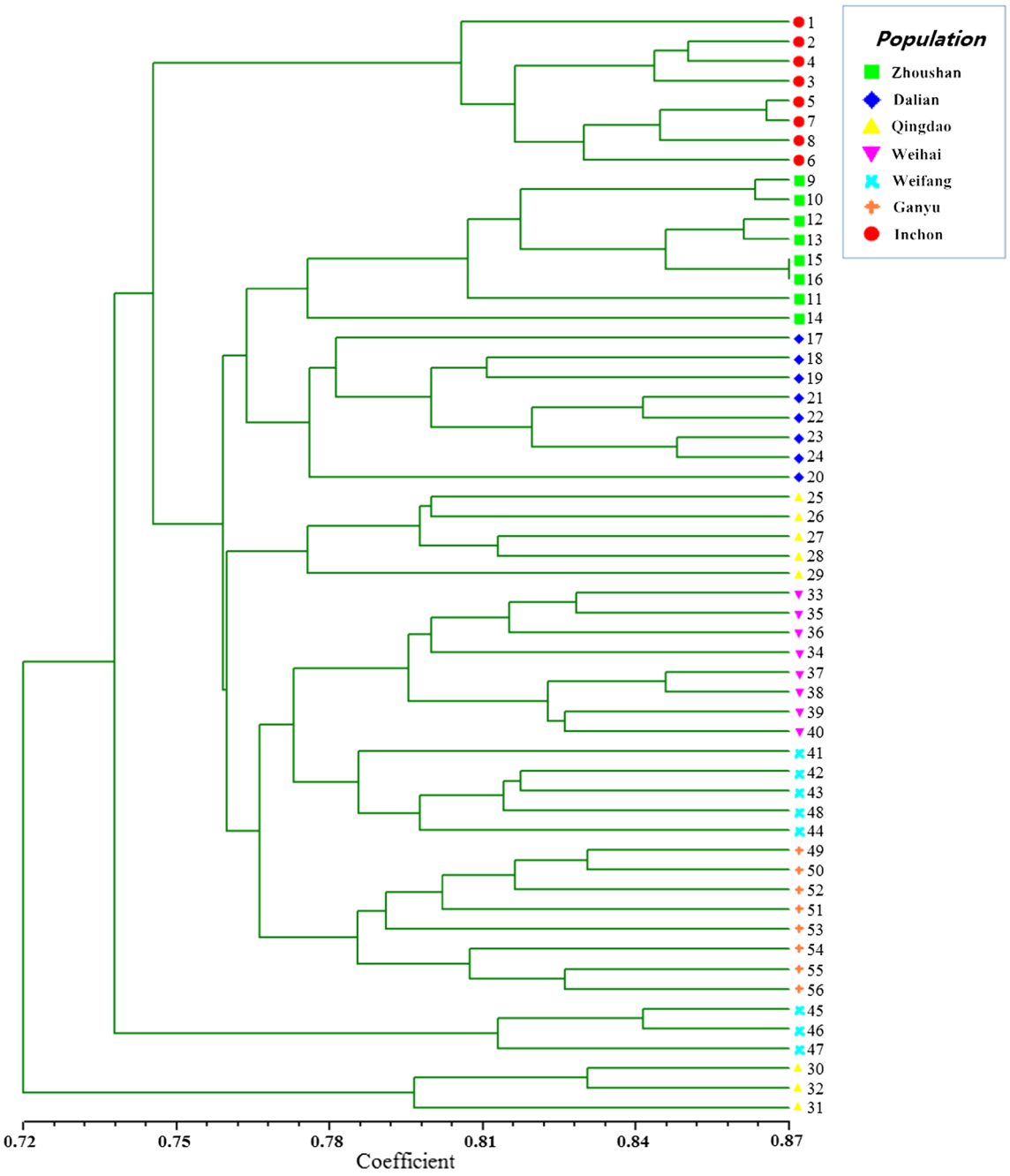

Fig. 4 Unweighted pair group method with arithmetic mean analysis by MSAP in natural *C. gigas* populations. Phylogenetic tree generated by 636 MSAP bands from accessions of seven populations: ZS, DL, QD, WH, WF, GY, and KI. The index indicates the collection site of each of the 56 populations. *Green lines* indicate separation. The level of epigenetic similarity is indicated on the axis. For abbreviations, see Figs. 1 and 2

component analysis axes represented 99.9% of the information contained in the genetic data set.

The AMOVA analyses (MSL $\varphi ST = 0.2899$, $P < 0.0001$, and NML $\varphi ST = 0.2584$, $P < 0.0001$) revealed significant differences among all populations in the epigenetic (MSL) and genetic (NML) profiles. Pairwise AMOVAs (Table 4) showed that all seven *C. gigas* populations were significantly different from one another in terms of both MSL and NML profiles ($P < 0.01$). Pairwise φST of MSL values (Table 4) ranged from 0.1357 (between WF and GY) to 0.4230 (between KI and DL), while that of NML values (Table 4) ranged from 0.1539 (between WH and DL) to 0.3664 (between WF and ZS). Statistical significance was assessed for the all pairwise AMOVA results ($P < 0.01$).

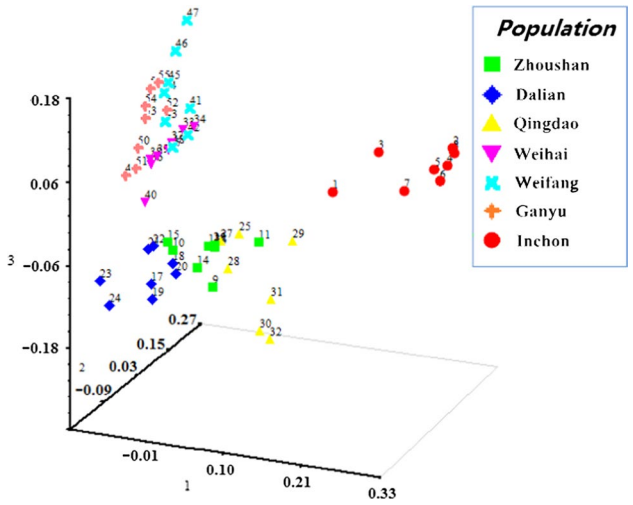

Fig. 5 Principal coordinate analysis (PCoA) by MSAP in natural *C. gigas* populations. PCoA analysis used the Dice similarity measure in the numerical taxonomy system of multivariate program-PC software for the MSAP analysis. PCoA produces an ordination based on a distance or dissimilarity matrix where similar groups are clustered on a three-dimensional plot. The index indicates the different populations

The φ*ST* values between KI and the other populations were up to > 0.33 and those of NML were up to > 0.27 (Table 4), indicating large differentiation and variation between KI and the other populations. To some degree, the MSL results are in agreement with those of the UPGMA and PCoA. Additionally, AMOVA analysis for both MSL and NML showed that the vast majority of variation existed within populations; however, the differentiation level among populations was relatively low (Table 3). There was relatively limited calculated gene flow among the populations (MSL Nm = 1.6837, NML Nm = 0.9196). IBD analyses showed that epigenetic and genetic distances were not significantly correlated with geographic distances ($P_{epi} = 0.9370$, $r_{epi} = 0.5165$; $P = 0.8780$, $r = 0.5896$), indicating that there were no significant phylogeographic structures of *C. gigas* in these areas. AMOVA for each population and among all populations revealed a higher variation in MSL than in NML. The Shannon index showed results similar to those in Table 3. The total Shannon index for methylation-susceptible polymorphic loci (MSL) ($I_{epi} = 0.5971$) was statistically higher than the corresponding value for NML ($I = 0.4452$) (Wilcoxon rank sum test with continuity correction $W = 37283$, $df = 6$, $P < 0.0001$). The among-population Shannon index for MSL showed more abundance than that of the within-population index; the results for NML were opposite (Table 3).

Correction between epigenetic and genetic profiles

The Mantel test was used to estimate the contribution of both epigenetic and genetic structure to natural *C. gigas* populations. Due to the deletion of MSL loci, we could not see any association between MSL and NML, as described above. So we calculated a similarity matrix using the transforming binary matrix described by Cervera et al. (2002) and the NML matrix as primitive data; the correlation between epigenetic and genetic profiles was evaluated based on Dice coefficients. Finally, the Mantel test showed some association between the transforming matrix and MSL profiles ($r = 0.4234$, $P = 0.0002$). A moderate but significant correlation was revealed between epigenetic and nucleotide sequence variation.

Discussion

The genome-wide methylation of a total of 636 CCGG sites of 56 wild *C. gigas* individuals from seven geographically isolated populations was analyzed. Based on an unbiased F-MSAP analysis, the cytosine methylation variation was extensive and population specific, indicating a high level of epigenetic variation in all populations. Compared with domesticated *C. gigas* (~ 26% methylation in mass selection populations) (Jiang et al. 2013), the DNA methylation frequency of natural populations was relatively high, suggesting that *C. gigas* populations in natural environments might exhibit more epigenetic diversity. Similar results in other species (Shaked et al. 2001; He et al. 2015; Venetsky et al. 2015) are in agreement with ours. More differences were shown in hemi-methylation patterns than in fully methylated patterns, perhaps because isoschizomer *Msp*I is insensitive to the methylation status of the internal cytosine

Table 4 Pairwise φ*ST* values of MSL (*below diagonal*) and NML (*above diagonal*) among seven natural populations of Pacific oyster; for abbreviations, see Table 1

	ZS	DL	QD	WH	WF	GY	KI
ZS	*	0.2256	0.3373	0.2806	0.3664	0.3387	0.2852
DL	0.2896	*	0.2659	0.1539	0.2233	0.2469	0.2704
QD	0.2488	0.2328	*	0.2493	0.2883	0.2549	0.3468
WH	0.2252	0.1931	0.1991	*	0.2058	0.1982	0.275
WF	0.1892	0.175	0.1485	0.1928	*	0.2360	0.2924
GY	0.2586	0.2111	0.1832	0.1965	0.1357	*	0.3138
KI	0.4018	0.4230	0.3355	0.3486	0.3508	0.4069	*

(Blouin et al. 2010; Liu et al. 2012). The UPGMA and PCoA analyses showed significant clustering for most individuals. Considerable epigenetic differences between natural populations have been reported in previous studies, such as for wild female great roundleaf bat (Liu et al. 2012), wild barley (Li et al. 2008), etc. The specific methylation patterns might have been the result of a founder effect in different geographical regions, and might have an evolutionary role in the adaptation of natural populations.

Due to the lack of effective geographical barriers in the sea, geographic distance has been suggested to have an effect on molecular differentiation in marine shellfish populations (Palumbi 1994). Due to their geographical location, individuals of each *C. gigas* population could have unique patterns of inherited cytosine methylation through adaptation. Isolation by geographical distance is considered the most likely hypothesis to explain the differentiation among populations of different localities on the basis of a significant variation between molecular mechanisms and geographical distance (Arias et al. 2010). Analysis of the methylation status and epigenetic structure showed that KI was clearly separate from the Chinese oyster populations, indicating that geographical isolation might play a role in population epigenetic differentiation. However, no significant correlation was found between the epigenetic profile and geographic distance, suggesting a weak pattern of isolation by distance.

The inconsistency between specific epigenetic differentiation and geographical distance might indicate the influence of oceanographic and/or other environmental factors (Liu et al. 2012; Venetsky et al. 2015) which may have limited the degree of epigenetic divergence of *C. gigas*. GY is located at the junction of Jiangsu and Shandong Provinces, and the mesoscale oceanic eddy and vast area of coastal soil around this particular island could block gene flow among populations. This has also been reported for the genetic differentiation of populations of *Mactra chinensis* (Ni et al. 2011) and *Octopus minor* (Gao et al. 2016). WF is located in the Bohai Sea, a semi-enclosed bay with a mildly sloping mud beach and shallow water, which means it is likely to have less water exchange and capacity for self-purification. Therefore, while the geographic distance from GY to WF is not small, the epigenetic variation between these locations is the lowest. On the other hand, there is relatively low epigenetic differentiation between WF and the other populations. As sessile organisms thriving in coastal oceans, the oysters may adapt to extensive and highly stressful environmental fluctuations (Miossec et al. 2009). Physical or biological factors may limit migration or free movement in benthic sessile organisms, and increase population differentiation of adult *C. gigas.* In this study, QD and WF showed more variation than the other populations. We inferred that other factors (sampling areas and human activities, etc.) could contribute to their extensive differentiation. Similar results have been shown in previous studies (Li et al. 2015; Venetsky et al. 2015), and suggest that sampling area might be important for epigenetic population variation. The above suggested that geographic isolation could not, in isolation, explain epigenetic heterogeneity. In conclusion, it appears that there is no single factor that can explain *C. gigas* epigenetic variation, which is why the interactions between geographical isolation, oceanographic and life history characteristics have been the focus of this study.

It is interesting that epigenetic variation was structured into distinct among- and within-population components in *C. gigas*. This result was consistent with studies on plants (Li et al. 2008) and mammals (Liu et al. 2012). Previous studies have shown that epigenetic variance may not only be independent of (Cervera et al. 2002; Vaughn et al. 2007; Avramidou et al. 2015), but also dependent on, genetic variation (Li et al. 2008; Herrera and Bazaga 2010; Jiang et al. 2013; Schulz et al. 2014; Liu et al. 2015) in natural and cultivated populations. According to Richards (2006), there are two possible extreme cases for the interaction between epigenetic and genetic profiles: epigenetic variation can be strongly associated with genetic variation, or be completely uncoupled from genetic variation. Changes in epigenetic phenomena might occur much more frequently than in genetic factors, therefore, any changes in methylation within species might shed light on how they evolved (Liu et al. 2012; Avramidou et al. 2015; Venetsky et al. 2015). In the present study, epigenetic characteristics showed higher polymorphism than genomic structures, which is in line with previous results on plants, mammals, marine fish and mollusks (Herrera and Bazaga 2010; Morán and Pérez-Figueroa 2011; Schulz et al. 2014; Wenzel and Piertney 2014; Liu et al. 2015). Nevertheless, Rico et al. (2014) and Avramidou et al. (2015) found that this was not the case in *Quercus ilex* trees and wild cherry *Prunus avium*.

In our study, the correction coefficient of epigenetic and genetic distribution was low but significant, indicating the existence of an association between epigenetic and genetic profiles. Likewise, a significant association was previously found by co-inertia analysis between genetic and epigenetic variation following mass selection of *C. gigas* populations (Jiang et al. 2013), which is in agreement with our result. In *C. gigas*, MSAP has been used to show that epigenetic effectors can induce changes in methylation in mass-selection and diploid and triploid populations (Jiang et al. 2013, 2016). A significant correlation suggested that methylation based on epigenetic variance might be associated with the control of genetic instability. Neutral drift could also account for the significant correlation between epigenetic and genetic variations, and high gene flow might minimize the population differences caused by this drift (Liu et al. 2012). In our study, we detected limited gene flow among *C. gigas* populations. Both above results indicated that stochastic processes

of drift could make epigenetic variations develop in parallel with genetic differences (Liu et al. 2012; Richards 2006) and lead to a moderate but significant correlation without a functional link between the two profiles. Besides the fact that both the epigenetic and genetic information was extracted from the same MSAP data set, different transformation probably leads to some biased estimates. Generally, our study supports the thesis that epigenetic variation is coupled with genetic variation. This study gives a first insight into the partitioning of epigenetic diversity in natural, wild Pacific oyster populations.

In conclusion, investigating DNA methylation polymorphisms in natural populations and evaluating the relevance of their epigenetic and genetic profiles are critical steps for assessing the potential role of epigenetic variation in natural invertebrate evolution. Using F-MSAP and multivariate analysis, we found that extensive epigenetic polymorphisms and distinct epigenetic structures might be partly determined by combined effects. Additionally, a moderate but significant association between epigenetic and genetic structure was verified in these natural oyster populations. This study provides some useful information on the role of DNA methylation in the adaptive epigenetic divergence of wild invertebrate populations.

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