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ORIGINAL ARTICLE

Inheritance and Variation of Genomic DNA Methylation in Diploid and Triploid Pacific Oyster (*Crassostrea gigas*)

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Abstract DNA methylation is an important epigenetic mechanism that could be responsive to environmental changes indicating a potential role in natural selection and adaption. In order to evaluate an evolutionary role of DNA methylation, it is essential to first gain a better insight into inheritability. To address this question, this study investigated DNA methylation variation from parents to offspring in the Pacific oyster *Crassostrea gigas* using fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP) analysis. Most of parental methylated loci were stably transmitted to offspring segregating following Medelian expectation. However, methylated loci deviated more often than non-methylated loci and offspring showed a few de novo methylated loci indicating DNA methylation changes from parents to offspring. Interestingly, some male-specific methylated loci were found in this study which might help to explore sex determination in oyster. Despite environmental stimuli, genomic stresses such as polyploidization also can induce methylation changes. This study also compared global DNA methylation level and individual methylated loci between diploid and triploid oysters. Results showed no difference in global methylation state but a few ploidy-specific loci were detected. DNA methylation variation during polyploidization was less than autonomous methylation variation from parents to offspring.

Keywords DNA methylation · F-MSAP · Heritability · Polyploidy

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Introduction

Epigenetics refers to processes capable of inducing changes in genetic activity without altering the underlying DNA sequence (Jablonka and Lamb 1998). Environmental changes could trigger phenotypic variation in various organisms through epigenetic regulation including DNA methylation, histone modifications as well as non-coding RNA activity (Herrera and Bazaga 2010). Compared with genetic variation, epigenetic modification shows higher mutation rates to generate new phenotype (Finnegan 2002; Richards 2008) and a considerable proportion of methylation variation is stably transmitted across generations (Cervera et al. 2002; Riddle and Richards 2005; Vaughn et al. 2007; Verhoeven et al. 2010a). Considering the phenotypic changes and heritability, epigenetics is proposed to be involved in natural selection and adaption (Rapp and Wendel 2005; Richards 2006; Jablonka and Raz 2009). DNA methylation is the most well-studied epigenetic modification which varies significantly in distribution and context among phylogenetic groups (Colot and Rossignol 1999). Previous studies about DNA methylation usually focus on mammals and plants in which the function was well studied. For example, DNA methylation has been reported to play an important role in genome stabilization (Wolffe and Matzke 1999), gene expression regulation (Okano et al. 1999; Zhang et al. 2006) and reduction of transcriptional noise (Bird 1995).

Despite the biological and evolutionary significance of epigenetics, surprisingly, little is known about this mechanism in invertebrates. Recent research in a handful of invertebrates revealed that invertebrate genomes are far less methylated than vertebrate (Suzuki et al. 2007; Gavery and Roberts 2010, 2013; Lyko et al. 2010; Olson and Roberts 2014a; Riviere 2014). DNA methylation in invertebrate is predominantly found in gene bodies, but the function remains unclear. Recently, an emerging possible explanation is that the role of gene body DNA

methylation is dependent on gene function (Riviere 2014). Hypermethylation is supposed to be associated with highly expressed housekeeping genes while hypomethylation with regulated and/or inducible genes (Elango et al. 2009; Hunt et al. 2010; Sarda et al. 2012). Absence of methylation is supposed to facilitate a variety of transcriptional opportunities to increase phenotypic plasticity in invertebrate (Roberts and Gavery 2012). This phenomenon provides an adaptive potential especially for species living in highly fluctuating environment. In order to evaluate an evolutionary role of DNA methylation, it is important to first gain a better insight into the heritability. Transgenerational epigenetic inheritance has been investigated in mammal (Guerrero-Bosagna et al. 2010; Manikkam et al. 2012) and plants (Cubas et al. 1999; Manning et al. 2006), but in bivalves, it remains blank (Gavery and Roberts 2014).

The Pacific oyster *Crassostrea gigas*, an important economic species, represents an excellent model for studying epigenetic modifications. Its dramatic morphophysiological changes, successive hermaphrodites, and highly stressful intertidal habitat require the implementation of transient transcriptomes. The control of these transcriptomes likely implicates epigenetic mechanisms. Several studies about epigenetic modification were demonstrated in oyster. Genome-wide DNA methylation profile was generated and the function in development, phenotypic plasticity, and regulation of genes was investigated (Gavery and Roberts 2010, 2013; Roberts and Gavery 2012; Riviere et al. 2013; Olson and Roberts 2014a). Recently, Olson and Roberts (2014b) characterized the genome-wide methylome of *C. gigas* sperm and larvae from two full-sib families nested within a maternal half-sib family across developmental stages and indicated the inheritance of DNA methylation. However, transgenerational inheritance investigating changes of DNA methylation pattern from parents to offspring directly has not been addressed not only in oyster but also in other bivalves. To what extent could methylated loci be passed on from parents to offspring in *C. gigas*? Besides diploid oyster existed in nature, triploids as an economically important organism also attract our attention. Polyploidization is confirmed to be able to trigger immediate methylation alterations during the first or first few generations in plants (Wang et al. 2004; Paun et al. 2007). How does polyploidization influence DNA methylation pattern in *C. gigas*? To address these two questions: DNA methylation inheritance pattern from parents to offspring and DNA methylation variation during polyploidization in *C. gigas*, we conducted this study.

Materials and Methods

Oyster Materials

One-year-old diploid oysters were sampled from Weihai Bay Shandong province and matured for 2 months indoors, using controlled temperature (20 °C) to induce gonadic maturation. Gametes were rinsed and placed into separate buckets by stripping the gonad from a single female and male and then fertilized. Two families were constructed. In one family, triploid oysters were induced by cytochalasin B (CB, 0.5 mg L^{-1}) for 15 min starting when about 50 % of the eggs released the first polar body. The other family was not treated and used as diploid control. The treated and control families were then reared separately in different tanks at the same condition. After a year, offspring were sampled and ploidy status of these samples was assessed individually by flow cytometry using DAPI staining of whole DNA content of the nucleus. Both diploids and triploids were obtained in treated group. Adductor muscle and gonad from offspring as well as adductor muscle from parents were frozen by liquid nitrogen and stored at −80 °C until further process.

DNA Isolation and F-MSAP Genotyping

Genomic DNA was extracted from the adductor muscle and gonad in 20 individuals from control group using a modified phenol–chloroform protocol (Li et al. 2006). For the treated group, 13 and 10 offspring were used to extract DNA from adductor muscle and gonad, respectively, in both diploid and triploid families. Parents' DNA was extracted only from the adductor muscle because the gonad was dissected in artificial fertilization.

The fluorescent-labeled methylation-sensitive amplified polymorphism (F-MSAP) analysis, an AFLP modified protocol, was used to detect methylation polymorphism as described by Xu et al. (2000). Compared with the highly informative standard bisulfate converted DNA sequencing, F-MSAP is a cheaper and less labor-intensive method to scan genome-wide DNA methylation pattern. Its effectivity and reliability have been verified by southern blot (Xiong et al. 1999; Marfil et al. 2009) and bisulfate sequence (Yang et al. 2011). Considering all these advantages, we applied F-MSAP in this study. F-MSAP substitutes the frequent cutter *Mse*I by *Hpa*II and *Msp*I, which recognize the same tetranucleotide sequence 5′-CCGG-3′, but has different sensitivities to methylation at cytosines. *Hpa*II is inactive when any of the two cytosines is fully methylated but cuts the hemimethylated 5′-CCGG-3′ at a lower rate compared to the unmethylated sequence, whereas *Msp*I cuts 5′-C^{5m}CGG-3′, but not 5′-^{5m}CCGG-3′. Genomic DNA (100 ng) was fragmented with *Eco*RI and *Hpa*II/*Msp*I (2 U each) in a restriction buffer in a total volume of 10 μL and subsequently ligated with adapters. Pre-selective amplification was performed on diluted restriction-ligation reaction products with pre-selective primers (*Eco*RI+A, *Hpa*II/*Msp*I+T). After an initial denaturation at 72 °C for 2 min, 20 PCR cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C were performed, followed by a final 30-min extension at 60 °C. Selective amplification was

performed on diluted pre-selective amplification products with the following cycling profile: 2 min of denaturing at 94 °C, then 10 cycle 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 each cycle, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min 72 °C, with a final extension of 30 min at 60 °C. Twenty-one selective amplification primer pairs were chosen for PCR (E-AAC+HM-TAC/TCA/TGA, E-ACA+HM-TAC/TCA, E-ACT+HM-TCT/TGA, E-ATC+HM-TAC/TCA/TTG/TGC, E-ACG+HM-TCA/TGA, E-ACC+HM-TGT/TAT/TCC, E-AGG+HM-TAC/TGT/TAT/TAA, E-AGC+HM-TAC). *Eco*RI primers were labeled using a 6-FAM reporter molecule. PCR products were loaded simultaneously with a GeneScan™-500 LIZ™ Size Standard into an ABI 3130 Genetic Analyzer (Applied Biosystems). Fragment analysis and F-MSAP scoring was subsequently obtained using GeneMapper v4.0 software.

Data Analysis: DNA Methylation Changes from Parents to Offspring

Considering possible methylation variations induced by CB, DNA methylation changes from parents to offspring were detected in both control and treated groups (including diploid and triploid oysters). Comparison of DNA methylation in adductor muscle between control group (CA) and diploid oyster in treated group (DA) displays influence of CB on methylation changes. DNA methylation variation induced by polyploidy states is detected by assessment of adductor muscle between DA and triploids (TA). Four types of DNA methylation status were identified (Table 1): non-methylated loci (1–1; type I fragments) if it occurred in both the *Msp*I and *Hpa*II lanes, fully methylated loci (0–1; type II fragments) if present in the *Msp*I lane but not in the *Hpa*II lane, hemimethylated loci (1–0; type III fragments) if present in *Hpa*II but not in *Msp*I, and uninformative loci (0–0; type IV fragments) if absent in both *Hpa*II and *Msp*I which is discarded in the following analysis (Blouin et al. 2010). According to the parents' methylation status, F-MSAP bands were divided into two groups (Table 1): both parents are unmethylated locus (PUL); one or both parents are methylated locus (PML).

Analysis of DNA methylation changes from parents to offspring was performed following Verhoeven et al. (2010b). If a locus segregates following Mendelian expectation, it could be regarded as heritable. Thus, we investigate segregation of all methylated loci to see to what extent the methylated loci could be inherited. Considering the fact that some inheritable loci might deviate from Mendelian expectation because of various factors, unmethylated loci were used as genetic information. DNA methylation variation between parents and offspring was investigated through comparing segregation of polymorphic PUL and PML. Based on presence/absence scores, polymorphic markers were tested for deviation from Mendelian segregation using exact chi square tests for goodness of fit. Mendelian expectations are worked out in six marker classes for PML based on parental genotype and offspring's ploidy: $M_0P_1/M_1P_0,CA$ (one of two parental loci (paternal or maternal) is methylated (including fully methylated and hemimethylated), control group), M_1P_1,CA (both parental loci are methylated, control group), $M_0P_1/M_1P_0,DA$ (one of two parental loci (paternal or maternal) is methylated, diploid offspring), M_1P_1,DA (both parental loci are methylated, diploid offspring), $M_0P_1/M_1P_0,TA$ (one of two parental loci (paternal or maternal) is methylated, triploid offspring), and M_1P_1,TA (both parental loci are methylated, triploid offspring). This classification goes on with PUL (Fig. 1). Fragments that are present in one parent (marker class "M_0P_1/M_1P_0") either do not segregate (when parents are homozygous at the marker locus) or segregate with an expectation of 1/2 (when one parent is heterozygous, in which case, half of the progeny inherit the fragment). Fragments that are present in both parents ("M_1P_1") either do not segregate (when one or both parents are homozygous) or segregate with an expectation of 3/4 (when both parents are heterozygous).

Data Analysis: DNA Methylation Changes Between Diploid and Triploid Offspring

Individual fragments were classified as either "methylated locus (ML)" or "unmethylated locus (UL)." Markers with proportion of discordant *Hpa*II-*Msp*I bands (i.e., number of

Table 1 Illustration of different types of loci used in this study

*Hpa*II	*Msp*I	Types	Paternal	Maternal	Types
1	1	I	+	+	PML
0	1	II	+	−	PML
1	0	III	−	+	PML
0	0	IV	−	−	PUL

1 and 0 refer to the presence and absence, respectively, of a fragment; plus and minus signs refer to methylated (including fully methylated and hemimethylated) and unmethylated locus, respectively
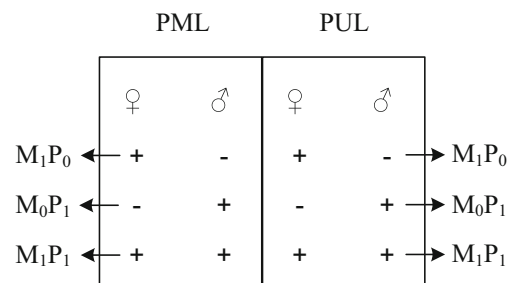

Fig. 1 Illustration of three marker classes for PML and PUL based on parental genotype. ♀ refers to maternal loci and ♂ refers to paternal loci. For PML, "+" refers to bands 01 and 10 while "−" refers to bands 11 and 00. For PUL, "+" refers to bands 11 while "−" refers to bands 00

individuals with contrasting *Hpa*II-*Msp*I scores for the fragment divided by total number of individuals assayed) exceeded a 5 % threshold are regarded as ML. This analysis only focus on the polymorphic ML in treated group which was scored as 1 (0–1 or 1–0), 0 (1–1), and missing (0–0) in the following analyses. We divided the samples into four groups considering the ploidy and tissue: adductor muscle of diploids (DA), gonad of diploids (DG), adductor muscle of triploids (TA), and gonad of triploids (TG). Principal component analysis (PCA) and between-group Eigen analysis (BPCA-PCA among groups based on PCA among individuals, (Parisod and Christin 2008)) were computed on F-MSAP data using ADE-4 software.

Results

DNA Methylation Changes from Parents to Offspring

Considering tissue-specific methylation, only adductor muscle was analyzed in this part. The 21 primer combinations produced a total of 252~470 markers with loci segregating among offspring (polymorphic between parents and offspring) to be 231~336 in control and treated groups (Table 2). *De novo* methylation and demethylation were found in both control and treated groups with a consistent pattern. For the control group, 30 loci showed de novo methylation out of 470 unmethylated parental loci, while in the 308 methylated parental loci, 22 loci showed demethylation in offspring with significant decrease on methylation level. This mode of DNA methylation variation was further verified in treated diploid and triploid offspring with 18/16 de novo methylation fragments and 25/27 demethylation loci (Table 2).

Polymorphic PUL and PML were further used to analyze genetic and epigenetic segregation ratio in offspring, and results were present in Fig. 2 and Table 3. Of the methylated loci, 90.6 % were stably transmitted to offspring segregating following Medelian expectation in control group, while the

Table 2 Epigenetic variation from parents to offsprings in adductor muscle of the control group (CA) and diploids (DA) and triploids (TA) in the treated group

Loci	CA		DA		TA	
	PML	PUL	PML	PUL	PML	PUL
Total Num.	308	470	252	376	252	383
Polymorphic Num.	264	336	239	305	231	267
Monomorphic Num.	44	134	13	71	21	116
De novo methylation	–	30	–	18	–	16
Demethylation	22	–	25	–	17	–

PML one or both of the parents are methylated locus; *PUL* both of the parents are unmethylated locus
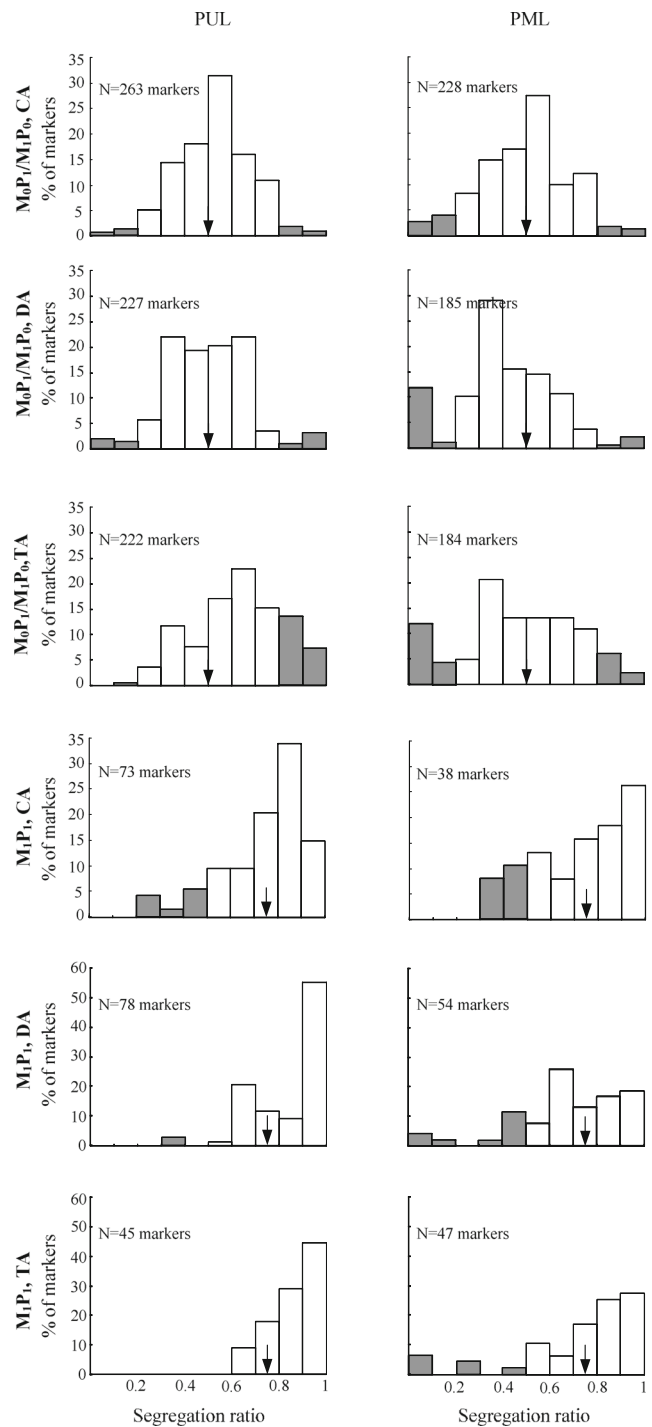

Fig. 2 Segregation ratios of all PUL and PML markers in adductor muscle of control and treated groups (including diploid and triploid oysters). Individuals are divided into six groups depending on parental genotype and offspring's ploidy: M_0P_1/M_1P_0,CA; M_1P_1,CA; M_0P_1/M_1P_0,DA; M_1P_1,DA; M_0P_1/M_1P_0,TA; M_1P_1,TA. *Arrows* indicate Mendelian segregation expectation. *Gray bars* indicate markers deviating significantly from Mendelian segregation expectation

treated diploid and triploid offspring showed a greater percentage of variation. Deviating PUL markers showed similar proportion in CA (4.0 %) and DA (4.8 %) which is much less than

Table 3 Proportion of PUL and PML markers that deviate significantly from Mendelian segregation in adductor muscle of the control group (CA) and diploids (DA) and triploids (TA) in the treated group

Type	Expected ratio	Total markers			Deviating markers*		
		CA	DA	TA	CA	DA	TA
Poly-PUL							
M_0P_1/M_1P_0	1:1	263	227	222	11 (4.2 %)	16 (7.0 %)	48 (21.6 %)
M_1P_1	3:1	73	78	45	8 (11.0 %)	2 (2.6 %)	0 (0.0 %)
Mono-PUL		134	71	116	0	0	0
Sum		470	376	383	19 (4.0 %)	18 (4.8 %)	48 (12.5 %)
Poly-PML							
M_0P_1/M_1P_0	1:1	228	185	184	22 (9.6 %)	29 (15.7 %)	45 (24.5 %)
M_1P_1	3:1	38	54	47	7 (18.4 %)	10 (18.5 %)	6 (12.8 %)
Mono-PML		44	13	21	0	0	0
Sum		308	252	252	29 (9.4 %)	39 (15.5 %)	51 (20.2 %)

*Exact chi square goodness-of-fit tests, $\alpha=0.05$

that in TA (12.5 %). Relatively high deviating proportion in TA was probably caused by lethal of some genotype after polyploidization. In order to investigate methylation changes from parent to offspring, we compared segregation of PML and PUL in each groups. In four out of six marker classes (M_0P_1/M_1P_0,CA; M_0P_1/M_1P_0,DA; M_1P_1,DA; M_1P_1,TA), PML markers deviated more often from Mendelian expectation than PUL fragments ($P=0.016$, 0.005, 0.004, 0.026, respectively; Fisher's exact test for independence) showing de novo methylation and demethylation from parents to offspring. With similar proportion of deviation markers in PUL, CA and DA showed difference of deviation in PML indicating DNA methylation variation induced by CB. To sum up, a considerable autonomous methylation variation could build up from parents to offspring and abiological stimulation such as CB can induce DNA methylation variation.

Noticeably, 20 male-specific methylated loci were found with methylated loci exclusively found in male individuals. Ten out of these male-specific methylated loci were found in gonad of offspring with both of parents unmethylated. The other ten fragments came from paternal methylated loci and this methylation pattern exists in both gonad and adductor muscle. In contrast, none female-specific locus was found which makes the male-specific methylated loci more interesting.

DNA Methylation Changes Between Diploid and Triploid Offspring

Genomic DNA methylation state of diploid and triploid oysters in adductor muscle and gonad is present in Table 4. No significant difference was observed in diploid and triploid oysters in both tissues with full methylation and hemimethylation to be ~29 and 0.6 %, respectively. Comparison of deviating markers between diploids and triploids revealed more often deviation from Mendelian

expectation in triploids both in PUL and PML. However, the difference was significant only in PUL, but not in PML, indicating more genetic than epigenetic variation occurred in polyploidization. Considering the situation that the two families could differ in methylation at many sites, but the direction might not be consistently towards hypo- or hypermethylation leading to weakening the significance of total methylation level, we tested the difference of each individual band. Thirteen/eight out of 706 loci in adductor muscle/gonad exclusively existed in diploids or triploids (Table 4). Ploidy-specific loci were also observed in de novo methylated loci where three out of 31 were exclusively found in triploids. Noticeably, ploidy-specific loci observed in this study were less than de novo methylation loci which occurred in nature. An intriguing coincidence was found in the ploidy-specific bands: the diploid-specific bands are paternal methylated while the triploid-specific ones are maternal methylated only with an exception in the three de novo methylated loci. Furthermore, comparison of deviating markers between triploids and diploids in four marker classes revealed more often deviation from Mendelian expectation in triploids than in diploids in M_1P_1 groups (both in PUL and PML; $P=0$, 0.035 respectively, Fisher's exact test) indicating DNA methylation variation in different ploidy states.

PCA analysis of MSL was conducted in four groups divided by different ploidy states and tissues: adductor muscle of triploids (TA), gonad of triploids (TG), adductor muscle of diploids (DA), and gonad of diploids (DG). Different levels of DNA methylation variation between ploidy and tissues were observed. The output plots of the two first principal coordinates are shown in Fig. 3a. The first coordinate separates different tissues (9.1 % of variance explained) and the second coordinate separates triploids and diploids (7.9 % of variance explained). Furthermore, considering the male-specific methylated loci present in gonad, methylation patterns between male and female in gonad were also

Table 4 Genomic DNA methylation level and ploidy-specific methylated loci of diploid and triploid oysters in adductor muscle and gonad

Ploidy status and ploidy-specific loci	Full methylation		Hemimethylation	
	Adductor muscle	Gonad	Adductor muscle	Gonad
Diploid (%)	29.38	29.50	0.56	0.72
Triploid (%)	28.41	29.62	0.48	0.62
Diploid-specific Num.	5	4	2	0
Triploid-specific Num.	5	3	1	1

investigated (Fig. 3b). Difference between male and female was much greater than that in different ploidy states. Male oysters were clearly separated from female ones along the first coordinate (18.1 % of variance explained) while different ploidy states in the male oysters cannot be completely divided into two groups.

Discussion

DNA methylation pattern can be influenced by various factors such as environmental change, development stages, different tissues, and so on. To ensure the accuracy of this study, interfering factors were eliminated as much as possible: parents and offspring were raised in the same sea area, samples were collected in the same development stage, and data from different tissues were compared separately. However, it is impossible to eliminate all the interfering factors especially the environmental parameter which is an inevitable limitation.

Inheritance of epigenetic modification is an important avenue in exploring its evolutionary significance. Previous studies about transgenerational inheritance usually focus on mammals and plants; little was known about bivalves. In mammal, clearing and re-establishment of DNA methylation with each generation is thought to be necessary to induce pluripotency of cells (Santos and Dean 2004). In contrast, plant displays a different pattern with a considerable proportion of the methylation marks being stably transmitted across generations

(Cervera et al. 2002; Riddle and Richards 2005; Vaughn et al. 2007; Verhoeven et al. 2010a). *C. gigas*, as a sessile living species, cannot directly interact with their offspring. Gavery and Roberts (2014) hypothesized that oyster may "inform" their offspring about recent environmental conditions through the transmission of epigenetic marks such as DNA methylation. This study showed that 90.6 % of methylated loci were stably transmitted to offspring segregating following Medelian expectation while a few loci showed de novo methylation and demethylation indicating methylation variation in transgenerational inheritance. This result is consistent with that in maize in which 6.59~11.92 % of methylated sites showed altered patterns from partens to intraspecific hybrids. Previous investigation of total amount of DNA methylation during embryonic development implied an epigenetic resetting event in oyster since it revealed lower methylation in the two to four cell stages and increasing in the morula and blastula (Riviere et al. 2013). However, an opposite point was speculated by Olson and Roberts (2014b) through comparing DNA methylation pattern of two full-sib families nested within a maternal half-sib family across developmental stages. More similarity between the two sires and their offspring compared to methylation pattern differences among developmental stages indicated inheritance of DNA methylation. Synthesizing these two studies and our results, we can infer that DNA methylation does not exactly inherit parental epigenetic allele like genetic allele does, allowing relatively frequent variation. Whether there exist a re-establishment of

Fig. 3 Principle component analysis (PCA) results for epigenetic differentiation between ploidy and tissues. F1 and F2 values show the contribution of the two principal components. *Triangles* represent triploids and *squares* for diploids. **a** *Open symbols* represent gonad individuals and *filled symbols* represent adductor. **b** *Open symbols* represent gonad of female individuals and *filled symbols* represent gonad of male
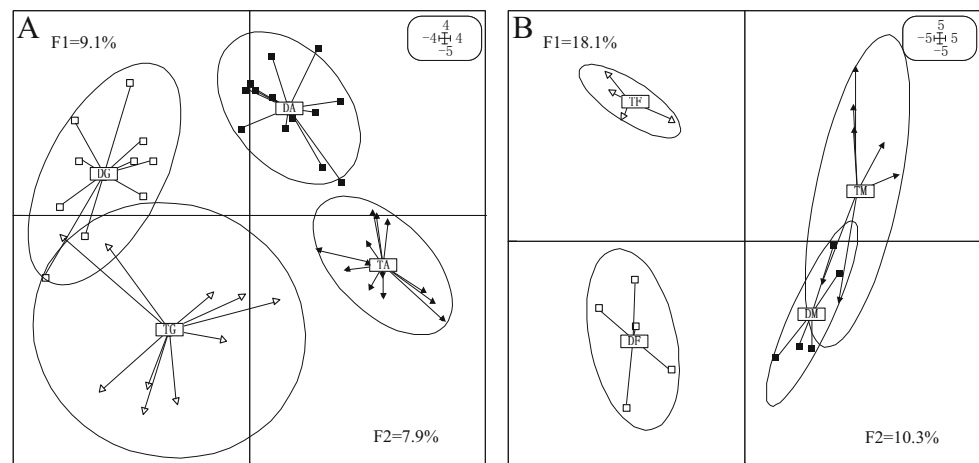
DNA methylation or not requires characterization of DNA methylation changes at finer temporal intervals.

C. gigas is an irregular successive hermaphrodite due to a yearly gonad renewal from stem cells. The sex determination is thought to be controlled by a major gene and influenced by the environment (Hedrick and Hedgecock 2010; Guo et al. 2012). Environmental temperature is confirmed to influence sex ratio in *C. gigas* (Fabioux et al. 2005; Santerre et al. 2013). Occurrence of sex inversion without changes in DNA sequence indicates the importance of epigenetic mechanisms in sex determination. Gorelick (2003) hypothesized that sex differences are initially determined by different patterns of methylation on nuclear DNA of females and males. In species with temperature-dependent sex determination, small environmental changes could induce alteration in methylation patterns of virtually identical sex chromosomes, hence influencing determination of the sex. In sea bass, a species undergoing temperature-dependent sex determination, DNA methylation contributes to temperature-induced masculinization through regulating gene expression of aromatase (Navarro-Martin et al. 2011). Furthermore, studies about DNA methylation and steroidogenic genes indicate that epigenetics are the missing link between genetics, the environment, and endocrine functions (Zhang and Ho 2011). Function of DNA methylation in sex determination was often investigated in aromatase which irreversibly converts androgens into estrogens. In *C. gigas*, regardless of the cloned genes involved in the gonadic development or differentiation, none of them was clearly demonstrated to determine sex. Hence, genome-wide analysis at random marker loci provides a useful tool to investigate the relationship between DNA methylation and sex determination. This study revealed 20 male-specific alleles in which ten loci were exclusively found in gonad. Considering its exclusive presence in male gonad as well as the correlation between DNA methylation and sex determination, it is reasonable to hypothesize that these ten loci might be involved in sex determination in oyster. These fragments might provide candidates for future experiments aimed at understanding sex determination. Of course, the correlation of these male-specific loci with sex determination is only a hypothesis; these fragments might simply be related with other male-specific gene expression than sex determination especially for another ten male-specific loci present in both adductor muscle and gonad. The function of DNA methylation in regulating gene expression in *C. gigas* is still under debate. Riviere et al. (2013) discovered a negative relevance between DNA methylation and homeobox gene expression. But genome-wide profiling of DNA methylation in male gametes showed a positive association between methylation status with expression (Olson and Roberts 2014a). Considering the paradox, we cannot simply infer the function of DNA methylation arbitrarily, and issues should be discussed depending on situation.

Polyploidy is an important genomic feature for all eukaryotes which suggests an advantage for adaptive evolution since genome duplications create many opportunities for function divergence between duplicated genes. However, increased gene and genome dosages often cause some problems such as genome instabilities, chromosome imbalances, regulatory incompatibilities, and so on. Therefore, new mechanisms should be established to reconcile incompatibilities among duplicated genes. Epigenetic mechanisms are supposed to regulate the expression of duplicate copies of genes with similar or redundant functions in the early process of polyplidization (Chen 2007). In *Arabidopsis thaliana*, polyplidization is demonstrated to trigger immediate methylation alterations during the first or first few generations after the polyploidization event (Wang et al. 2004). The methylation effects are usually more pronounced in allopolyploidization than genome doubling per se (Salmon et al. 2005; Wang et al. 2006). This study showed methylation changes in oyster with different ploidies which is the first description in bivalves to our knowledge. Not only in oyster, methylation re-patterning is also observed in other species during autopolyploid (Scheid et al. 1996, 2003; Verhoeven et al. 2010b). Without global increasing or decreasing methylation changes, oyster showed altered methylation pattern only at a few loci which is less than that in allopolyploids in *Spartina* (Salmon et al. 2005). Ploidy-specific loci were even less than de novo methylation from parents to offspring indicating that DNA methylation might not be the major mechanism reconciling duplicated genome in oyster. While in insects, intragenic DNA methylation levels differ among individuals of distinct ploidy and are positively associated with levels of gene expression (Glastad et al. 2014). Epigenetic information was suggested to be important to maintain appropriate patterns of gene regulation in biological systems that differ in genome copy number. Function of epigenetic modification in polyploidization should be analyzed separately in different species. Another intriguing coincidence is the correspondence between ploidy-specific loci and paternal methylation as well as triploid-specific and maternal loci methylation. In triploids, demethylation occurs in paternal methylated loci, while maternal methylated loci which would be discarded in normal diploids are maintained. The correspondence between ploidy-specific loci and parents' methylation pattern was also observed in dandelion where most methylation changes in triploids involved methylation of paternally inherited marker alleles and not methylation of maternal alleles. Whether this phenomenon is just a coincidence or has a relationship with the important mechanism needs further study.

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