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RESEARCH ARTICLE

Analysis of DNA methylation in tissues and development stages of pearl oyster *Pinctada fucata*

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Abstract In this study, we developed a methylation-sensitive amplification polymorphism technique to investigate DNA methylation profiles in different tissues and in the early-development stages of the pearl oyster *Pinctada fucata* (*P. fucata*). Methylation levels in adductor muscle, digestive gland, axe foot, heart, and gill ranged from 11.71 to 14.71 %, and significant differences ($P < 0.05$) between methylation levels in different tissues were observed. The DNA methylation levels of sperm, egg cells, two-cell embryos, morula embryos, trochophore larvae and D-shaped larvae were 13.51, 11.80, 12.14, 12.60, 14.65 and 13.18 %, respectively. Development stages of two-cell embryos, morula embryos, trochophore larvae and D-shaped larvae indicated a higher number of identical DNA methylation status loci in the egg, compared to that in the sperm. It is probable that DNA methylation patterns of the progeny are mainly influenced by the egg, while the sperm may become increasingly important during the process of early embryo development. The observed differences in methylation levels in the tissues and the development stages of *P. fucata* suggest that DNA methylation may act as an epigenetic regulator during tissue differentiation, individual growth, and development.

Keywords DNA methylation · MSAP · Tissue · Development stage · *Pinctada fucata*

Introduction

Pinctada fucata (*P. fucata*), a species of pearl oyster, is extensively cultured along three coastal provinces of China (Guangdong, Guangxi, and Hainan). A number of threats to this well-established aquaculture industry have recently been identified: a decline of pearl quality in *P. fucata* within established breeding programs, genetic degeneration due to inbreeding (Wada and Komaru 1994), disease outbreaks (Miyazaki et al. 1999), and environmental threats (Liu et al. 2012). Most genetically-improved strains in the aquaculture industry were developed through conventional selective breeding techniques (Hulata 2001). In the case of *P. fucata*, encouraging results have recently been obtained from a selective breeding programmed, the purpose of which was to achieve high-quality pearl production, in terms of pearl color and weight (Wada and Komaru 1996) and other desirable shell traits (He et al. 2008). The application of DNA markers such as amplified fragment length polymorphism (Yu and Chu 2006), microsatellite markers (Wu et al. 2013), and expressed sequence tags (Wang et al. 2011), have changed the approach to genetic research in this field of aquaculture.

Many observed phenotypic changes are a consequence of DNA variation, but there is also increasing evidence that phenotypic changes occur in the absence of DNA sequence

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polymorphism (Jiang et al. 2013b). Besides causing changes to neucleotide base sequences (of A, T, C, and G), 5-methylcytosine is regarded as a major epigenetic modification, sometimes referred to as "the fifth base" in eukaryotic DNA (Field and Blackman 2003). In higher organisms, DNA is modified by enzymatic conversion of many cytosine residues to 5-methylcytosine (Razin and Riggs 1980). This occurs predominantly at the symmetrical dinucleotide CpG, or at other sites such as CpNpG and GpC (Clark et al. 1995; Pontecorvo et al. 2000). In mammals, cytosine methylation plays a role in deactivating non-coding DNA, including introns, repetitive elements, and active transposable elements (Jones and Takai 2001). DNA methylation also plays a role in genomic imprinting, X inactivation, and suppression of homologous recombination (Mohandas et al. 1981; Li et al. 1993; Colot and Rossignol 1999). Based on the methylation-sensitive isoschizomer and selective PCR, a methylation-sensitive amplification polymorphism (MSAP) technique was developed to evaluate 5-methylcytosine in the sequence 5′-CCGG-3′ (Reyna-Lopez et al. 1997). One of the isoschizomer pairs is *Hpa*II and *Msp*I. Both enzymes recognize the 5′-CCGG-3′ sequence and can cut unmethylated DNA. *Hpa*II does not cut the methylated sites, except the hemi-methylated site of external cytosine, while *Msp*I cuts the methylated sites at the internal cytosine of 5′-CCGG-3′ (Baurens et al. 2003). The MSAP technique has been used for surveying the extent and pattern of CpG methylation in the genome (Xiong et al. 1999), analyzing the relationship between DNA methylation and heterosis (Li et al. 2013), and studying DNA methylation related to bacterial resistance (Sha et al. 2005). In shellfish, associations have been found between genetic and DNA methylation profiles ($P < 0.01$) and the influence of DNA methylation on gene expression and early development of *Crassostrea gigas* (Gavery and Roberts 2010; Jiang et al. 2013b; Riviere et al. 2013). The methylated genes in *C. gigas* were also found to associate with high transcript abundance and low variation in expression between tissue types (Gavery and Roberts 2013). No studies of DNA methylation have however been reported for *P. fucata*.

In the present study, we used a MSAP technique to investigate the level of cytosine methylation in the tissues, and in the early development stages, of *P. fucata*. We also sequenced the fragments that were differentially methylated among development stages, and compared the importance of the egg cell and the sperm in DNA methylation inheritance. The aim of the present study is to enhance the fundamental understanding of DNA methylation polymorphism in *P. fucata*, and to provide a basis for further studies on the potential role of DNA methylation in genetic breeding.

Materials and methods

Materials and DNA extraction

Three adult oysters were collected from a wild population of *P. fucata* (Daya Bay, Shenzhen, China). Tissue samples were taken from the adductor muscle, digestive gland, axe foot, heart, and gill of each individual, for the purpose of DNA extraction. In addition, separate collections were also made of sperm and egg cells, produced by three pairs of adult *P. fucata* individuals, for DNA extraction and artificial insemination. After fertilization, samples of the two-cell embryos, the morula embryo, the trochophore larvae and the D-shaped larvae were collected. Genomic DNA was extracted using an E.Z.N.A mollusk DNA Kit (Omega Bio-Tek, Inc, Georgia, USA) in accordance with the manufacturer's instructions. Approximately 50 mg of sample was placed in 350 µl ML1 lysis buffer to which 25 µl of Proteinase K was added. The sample was then vortex-mixed and incubated at 60 °C for 30 min until the entire sample was solubilized. DNA was extracted with 350 µl chloroform: isoamyl alcohol (24:1), in MBL buffer (in 0.5 volume of absolute ethanol) and vortex-mixed for 15 s. The DNA-containing solutions were washed by buffers (provided by the kit), diluted, and preserved in ultrapure water. The concentration of extracted DNA was estimated with a spectrophotometer (Nanodrop), using OD260/280. DNA quality was analyzed using agarose gel electrophoresis.

MSAP assay

MSAP is a modified protocol based on the AFLP method (Reyna-Lopez et al. 1997). The procedure can be described as a four-step process: DNA digestion, adapter ligation, pre-amplification, and selective amplification. The adapters and primers used in the experiment, as described by Xiong et al. (1999), with minor modifications, are listed in Table 1. Genomic DNA (400 ng) from each sample was digested by two pairs of restriction enzymes: *Eco*RI and *Msp*I (or *Eco*RI and *Hpa*II). Digestion reactions took place over 6 h at 37 °C and were terminated by exposure to warmer conditions (70 °C) for 10 min. The digested fragments were then ligated to the *Eco*RI adapter and the *Hpa*II–*Msp*I adapter. The *Eco*RI adapter and *Hpa*II–*Msp*I adapter are double-stranded fragments, designed for the isoschizomer digestions by annealing the corresponding adapter primers, as described in Table 1. The digested-ligated DNA was then diluted (1:10) and amplified, using pre-amplification primers Eco + A and HM + T (each with a selective nucleotide at the 3′ end). The total volume used in the PCR reaction was 20 µl, including 10.5 µl PCR

Table 1 Adapter and primer sequences used in MSAP analysis

Primers/adapters	Sequences (5′-3′)
*Eco*RI adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
*Hpa*II-*Msp*I adapter	GATCATGAGTCCTGCT
	CGAGCAGGACTCATGA
Eco + A	GACTGCGTACCAATTCA
HM + T	ATCATGAGTCCTGCTCGGT
Eco + ATA	GACTGCGTACCAATTCATA
Eco + AAT	GACTGCGTACCAATTCAAT
Eco + AAG	GACTGCGTACCAATTCAAG
Eco + ACT	GACTGCGTACCAATTCACT
Eco + ACA	GACTGCGTACCAATTCACA
Eco + ATG	GACTGCGTACCAATTCATG
Eco + ATC	GACTGCGTACCAATTCATC
HM + TGA	ATCATGAGTCCTGCTCGGTGA
HM + TTT	ATCATGAGTCCTGCTCGGTTT
HM + TTG	ATCATGAGTCCTGCTCGGTTG
HM + TTA	ATCATGAGTCCTGCTCGGTTA
HM + TAT	ATCATGAGTCCTGCTCGGTAT
HM + TCT	ATCATGAGTCCTGCTCGGTCT
HM + TCA	ATCATGAGTCCTGCTCGGTCA

Premix Ex Taq (Takara, Dalian, China), 1 μl 20 pmol/μl primer Eco + A, 1 μl 20 pmol/μl primer HM + T, 1 μl diluted ligation product, and 6.5 μl ddH_2O. The PCR cycling protocol was as follows: 5 min at 94 °C, 20 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final elongation at 72° C for 10 min. Following the PCR reaction, the pre-amplification products were diluted at 1:10 and used as a template for selective PCR. Only the primer pairs obtained from *Eco*RI primers (Eco + ATA, AAT, AAG, ACT, ACA, ATG or ATC) in combination with *Hpa*II-*Msp*I primers (HM + TGA, TTT, TTG, TTA, TAT, TCT or TCA), that could yield clear bands and could detect the methylation loci, were used for selective amplification. The total volume of selective PCR was 20 μl, including 10.5 μl Premix Ex Taq (Takara, Dalian, China), 0.5 μl 20 pmol/μl *Eco*RI primer, 2 μl 20 pmol/μl *Hpa*II–*Msp*I primer, 1 μl of the diluted pre-amplification product, and 6 μl ddH_2O. The PCR cycling conditions were as follows: 13 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, with a degradation temperate of 0.7 °C each cycle. This was followed by 27 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final elongation at 72 °C for 5 min. The selective PCR products, mixed with 10 μl formamide loading buffer, were heated at 95 °C for 10 min and rapidly chilled on ice. The denatured PCR products were separated on an 8 % denaturing polyacrylamide gel at 200 V for 3.5 h and detected by silver staining.

Cloning and sequencing of development stage methylation polymorphic fragments

Fragments of different methylation status among development stages were randomly removed from denaturing polyacrylamide gels on the plate, using a razor blade. The gel slices were rehydrated in 100 μl sterile water, heated at 95 °C for 10 min and slowly cooled to room temperature. The samples were placed into tubes and centrifuged at 12,000*g* for 5 min. Aliquots of 1 μl of supernatant were used as templates for reamplification. PCR reactions were performed with the same primer combinations and reaction conditions as those used in pre-amplification. The PCR products were separated on a 1.3 % agarose gel and the bands were recovered with a gel extraction kit (Promega, Madison, USA) in accordance with the manufacturer's instructions. The purified DNA samples were subsequently sent for direct bidirectional sequencing.

Data analysis

Only clearable fragments were scored, in terms of presence or absence in the denaturing polyacrylamide gel. To estimate the DNA methylation status of the 5′-CCGG-3′ loci, the DNA banding patterns from the amplification of genomic DNA (digested with *Eco*RI and *Hpa*II, or *Eco*RI and *Msp*I) had to be simultaneously analyzed. In this study, the MSAP bands that revealed methylation patterns could be divided into four types: Type I: locus with same length of amplified fragments in both *Msp*I and *Hpa*II lanes, indicating inner methylation of single-stranded DNA, or no methylation; Type II: locus displaying an amplified band after restriction with *Hpa*II but not after restriction with *Msp*I, indicating outer methylation of single-stranded DNA (hemi-methylation locus); Type III: locus showing an amplified band after restriction with *Msp*I (but not after restriction with *Hpa*II) were considered for inner methylation of double-stranded DNA (Lu et al. 2008); Type IV: locus with no bands in both enzyme combinations but another treated sample showing the presence of a fragment at that position, indicating a fully-methylated mCmCGG site (Fulneček and Kovařík 2014). The DNA methylation level was calculated using the following formula:

$$\text{DNA methylation level } (\%) = \text{Total methylated loci} / (\text{sum of Types I, II, III and IV loci}).$$

The sum of types III and IV loci represents the fully methylated loci. The sum of types II, III and IV loci represents the total methylated loci.

The difference in methylation levels among tissues was tested by one-way analysis of variance using SPSS Version 19.0, and the significance level was set to 0.05. The 5′-CCGG-3′ genome position, which represents different

Table 2 Methylation locus number and methylation level of tissues in three *P. fucata* individuals

Sample	Methylation type	Adductor muscle	Digestive gland	Axe foot	Heart	Gill
1	I	222	230	224	231	232
	II	5	9	7	4	7
	III	18	16	18	15	15
	IV	17	7	13	12	8
	Fully methylated loci	35	23	31	27	23
	Total methylated loci	40	32	38	31	30
	Methylation level (%)	15.26	12.21	14.50	11.83	11.45
2	I	228	229	231	233	234
	II	10	5	5	4	5
	III	12	17	15	15	18
	IV	16	15	15	14	9
	Fully methylated loci	28	32	30	29	27
	Total methylated loci	38	37	35	33	32
	Methylation level (%)	14.28	13.90	13.15	12.40	12.03
3	I	234	238	240	241	242
	II	10	10	8	9	10
	III	17	16	15	17	14
	IV	13	10	11	7	8
	Fully methylated loci	30	26	26	24	22
	Total methylated loci	40	36	34	33	32
	Methylation level (%)	14.59	13.13	12.40	12.04	11.67
	Mean ± SD[e] (%)	$14.71^{a} \pm 0.50$	$13.08^{bc} \pm 0.84$	$13.35^{b} \pm 1.06$	$12.09^{cd} \pm 0.28$	$11.71^{d} \pm 0.29$

Data with different superscript letters (a, b, c, d) are significantly different at $P < 0.05$

[e] SD: standard deviation; Type I locus: inner methylation of single-stranded DNA or no methylation; Type II locus: outer methylation of single-stranded DNA (hemi-methylation loci); Type III locus: inner methylation of double-stranded DNA; Type IV locus: fully methylated mCmCGG site. Fully methylated loci = III + IV. Total methylated loci = II + III + IV; Methylation level (%) = (II + III + IV)/I + II + III + IV

DNA methylation status between the sperm and the egg cell, was defined as the inheritance differential methylation position. Based on inheritance differential methylation positions, the methylated status of the two-cell embryo, the morula embryo, the trochophore larvae and the D-shaped larvae were compared with the egg cell and the sperm, in order to separately determine the number of identical methylation status loci.

Results

DNA methylation levels in different tissues of *P. fucata*

Seven pairs of PCR primers were used to detect cytosine methylation at 5′-CCGG-3′ sites within the genome of the adductor muscle, the digestive gland, the axe foot, the heart and the gill in each individual. For each MSAP primer combination, there were ten lanes, corresponding to five different tissues, each of which was digested with one of the two enzyme combinations: *Eco*RI and *Hpa*II, or *Eco*RI and *Msp*I. A total of 4,010 5′-CCGG-3′ loci were detected for tissue DNA methylation analysis. The number of loci counted for each primer combination varied from 27 to 48, with the average being 38. A total of 521 loci were found to be methylated, including 413 full-methylated loci and 108 hemi-methylated loci. MSAP screening indicated that the levels of DNA methylation in the adductor muscle, the digestive gland, the axe foot, the heart and the gill were 14.71 ± 0.50, 13.08 ± 0.84, 13.35 ± 1.06, 12.09 ± 0.28 and 11.71 ± 0.29 %, respectively. Among the tissues, the DNA methylation level was highest in the adductor muscle and lowest in the gill. The methylation level of the adductor muscle was significantly higher than that of all the other tissues ($P < 0.05$) (Table 2). The average DNA methylation level of the five tissues was 12.98 %.

DNA methylation level of early development stages

DNA methylation levels of the sperm, the egg cell, the two-cell embryo, the morula embryo, the trochophore larvae and the D-shaped larva were detected using 10 pairs of

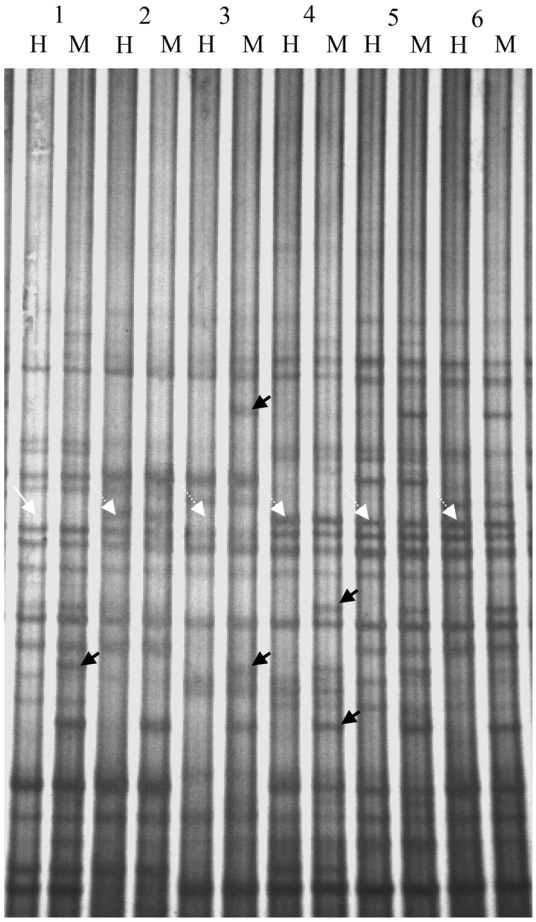

Fig. 1 MSAP fingerprints of sperm, egg and four development stages of *P.fucata.* MSAP fingerprints of egg cell, sperm and four development stages with primers Eco + ATC and HM + TCA. MSAP: methylation sensitive amplification polymorphism. *1* egg cell; *2* sperm; *3* two-cell embryo; *4* morula embryo; *5* trochophore larvae; *6* D-shaped larvae. H lanes were cut with *Eco*RI and *Hpa*II; M lanes were cut with *Eco*RI and *Msp*I. The *black arrows* indicate methylated loci, and the *white arrows* indicate the inheritance differential methylation position

selective PCR primers, obtained from *Eco*RI primers in combination with *Hpa*II-*Msp*I primers. For each MSAP primer combination there were 12 lanes, corresponding to six developmental-stage samples, each of which was digested with one of the two enzyme combinations: *Eco*RI and *Hpa*II, or *Eco*RI and *Msp*I (Fig. 1). The number of loci counted for each primer combination varied from 12 to 55, with an average of 29. A total of 5,238 5′-CCGG-3′ loci were detected for DNA methylation analysis. Among these loci, 680 were found to be methylated, including 427 full methylated loci and 253 hemi-methylated loci. The DNA methylation levels, of the sperm, the egg cell, the two-cell embryo, the morula embryo, the trochophore larvae and the D-shaped larvae were 13.51 ± 0.10, 11.80 ± 0.35, 12.14 ± 0.05, 12.60 ± 0.25, 14.65 ± 0.37 and 13.18 ± 0.41 %, respectively. Among the development stages, the DNA methylation level was highest in the trochophore larvae and lowest in the egg cell. During the early development of *P. fucata*, the DNA methylation levels showed an increasing trend. After fertilization, the DNA methylation level increased from 12.14 % (in the two-cell stage) to 14.65 % (in the trochophore larvae). The DNA methylation level of the trochophore larvae (14.65 %) was higher than that of the D-shaped larvae (13.18 %) (Table 3).

DNA methylation inherited from the egg cell and the sperm

The DNA methylation similarity between four early developmental stages (the two-cell embryo, the morula embryo, the trochophore larvae and the D-shaped larvae) and the egg cell or the sperm, were analyzed separately (Table 4). One hundred and thirty-eight inheritance differential methylation positions were detected and used for methylation similarity rate analysis. In Fig. 1, the white arrows showed one of the inheritance differential methylation positions. In the 5′-CCGG-3′ position, the egg cell was fully methylated (Type IV locus), while the sperm was inner-methylated (of single-stranded DNA), or not methylated (Type I locus). The methylation status of the two-cell embryo was the same as that of the egg cell, and those of the morula embryo, the trochophore larvae and the D-shaped larvae were the same as that of the sperm. In each of the four development stages there are a higher number of identical DNA methylation-status loci in the egg than in the sperm. For example, in the two-cell stage, 112 loci were detected to have an identical DNA methylation status with that of the egg (81.14 %), while only 10 loci had an identical methylation status as that of the sperm (7.22 %). During the process of early development, the incidence rate of identical methylation status loci between each of the four development stages and the egg cell decreased from 81.14 to 58.82 %, while the incidence rate of identical methylation-status loci with the sperm increased from 7.22 to 28.26 %.

Sequence analysis of development stage methylation polymorphic fragments

Eleven DNA fragments (M6, M9, M13, M15, M23, M24, M25, M29, M30, M31 and M32) with differing methylation status among early development stages, were recovered, cloned and sequenced. The results showed that all of the 11 fragments were relatively short in length (96 to 285 bp). The *Eco*RI cleavage site "GAATTC" and the *Hpa*II-*Msp*I cleavage site "CCGG" were noted to be present in sequenced fragments. Further details relating to these observations can be found in Online Resource 1.

Table 3 Methylation locus number and levels of early development stages in *P. fucata*

Sample	Methylation type	Sperm	Egg cell	Two-cell embryo	Morula embryo	Trochophore larvae	D-shaped larvae
1	I	265	271	269	268	261	267
	II	16	11	12	14	20	16
	III	15	15	15	16	17	18
	IV	10	9	10	8	8	5
	Fully methylated loci	25	24	25	24	25	23
	Total methylated loci	41	35	37	38	45	39
	Methylation level (%)	13.39	11.43	12.09	12.41	14.70	12.74
2	I	248	253	252	250	245	249
	II	15	9	12	13	17	16
	III	16	17	16	18	17	18
	IV	8	8	7	6	8	4
	Fully methylated loci	24	25	23	24	25	22
	Total methylated loci	39	34	35	37	42	38
	Methylation level (%)	13.58	11.84	12.19	12.89	14.63	13.24
3	I	242	246	246	245	239	242
	II	14	12	9	13	17	17
	III	16	15	18	17	17	16
	IV	8	7	7	5	7	5
	Fully methylated loci	24	22	25	22	24	21
	Total methylated loci	38	34	34	35	41	38
	Methylation level (%)	13.57	12.14	12.14	12.50	14.64	13.57
	Mean ± SD[e] (%)	13.51 ± 0.10	11.80 ± 0.35	12.14 ± 0.05	12.60 ± 0.25	14.65 ± 0.37	13.18 ± 0.41

[e] SD: standard deviation; *Type I locus* inner methylation of single-stranded DNA or no methylation; *Type II locus* outer methylation of single-stranded DNA (hemi-methylation loci); *Type III locus* inner methylation of double-stranded DNA; *Type IV locus* fully methylated mCmCGG site. Fully methylated loci = III + IV; Total methylated loci = II + III + IV; Methylation level (%) = (II + III + IV)/I + II + III + IV

Table 4 Loci number and similarity rate of development stages with egg cell or sperm in inheritance differential methylation positions

Loci type	Two-cell stage	Morula stage	Trochophore larvae	D-shaped larvae
Identical methylation status loci with egg	(40, 38, 34)	(39, 37, 33)	(31,32,29)	(28,27,26)
	81.14 % ± 0.42	78.96 % ± 0.55	66.79 % ± 3.09	58.82 % ± 2.66
Identical methylation status loci with sperm	(4, 3, 3)	(7, 6, 5)	(11,9,7)	(14,13,12)
	7.22 % ± 0.89	12.98 % ± 1.20	19.41 % ± 2.89	28.26 % ± 0.53
Different methylation status loci with both egg and sperm	(5, 6, 5)	(3, 4, 4)	(7, 6, 6)	(7, 7, 4)
	11.62 % ± 1.30	8.05 % ± 1.74	13.77 % ± 0.87	12.89 % ± 2.94

Numbers in parenthesis, from left to right, represent the number of methylated loci, detected separately from three of the same development-stage samples

Discussion

The present research represents the first attempt to investigate DNA methylation in the pearl oyster *P. fucata*. Although the MSAP technique can only detect some cytosine methylation types at 5′-CCGG-3′ sites (Cervera et al. 2002), and the actual levels of methylation in the genome will be underestimated, this approach is much more cost effective than that of current practices in most laboratories (Lu et al. 2008). The sequence analysis of eleven development-stage differential methylated fragments indicated that these sequences contained *Eco*RI and *Hpa*II–*Msp*I cleavage sites. The detection of differential methylated fragments, and the correct enzyme digestion sites in the sequenced fragments, provides evidence that the MSAP technique is appropriate for DNA methylation analysis in *P. fucata*.

DNA methylation within animals ranges from undetectable levels to high levels of global methylation (Suzuki et al. 2007). The average DNA methylation level of tissues

in *P. fucata* was 12.98 %, which is lower than those of 26.4 and 53.99 %, determined in the tissues of *C. gigas* (Jiang et al. 2013b) and in swine (Yang et al. 2011), respectively. Differences in the methylation levels of these species may be attributed to the different materials used in the experiment. Significant differences in DNA methylation levels, observed among tissues of *P. fucata* ($P < 0.05$), may be related to specific gene expression during tissue differentiation. Normal DNA methylation is essential to the development of animals, including methylation pattern reprogramming in germ cells and early embryos that generate cells with a broad developmental potential (Reik et al. 2001). DNA methylation also plays a role in gene expression, regulating developmental pathways in plants (Finnegan et al. 1998). The epigenetic information, inherited from parents to offspring, is crucial for diverse biological processing (Daxinger and Whitelaw 2012). In the present study, we focused on DNA methylation changes during early development and on the inheritance of DNA methylation information in *P. fucata*. By means of MSAP analysis, we followed the DNA methylation status, at methylation-sensitive restriction loci, throughout the early development of *P. fucata*. The methylation level of early development stages were different and a progressive DNA methylation trend, from the two-cell stage to the D-shaped-larvae stage, was observed. Changes to DNA methylation levels in *P. fucata* followed a similar trend to that recorded in the oyster *C. gigas*: oocytes were heavily methylated and became significantly more methylated up to the morula stage, while the DNA appeared to be more methylated within the trochophore larva than within the D-shaped larva (Riviere et al. 2013). Changes in the methylation level during the early development of *P. fucata* are also consistent with trends observed in plants, with DNA methylation levels increasing with development, as observed in *Arabidopsis* (Ruiz-García et al. 2005). The DNA methylation level in mature leaves has been found to be higher than that of seedlings (Finnegan et al. 1996). Increased DNA methylation levels may also be required for the control of differential expression of imprinted genes and may function as possible epigenetic regulators during development (Lei et al. 1996; Finnegan et al. 2000).

In our experiments we found that the DNA methylation level of the sperm (13.51 %) was higher than that of the egg cell (11.80 %), while a higher number of identical methylation-status loci were observed in various development stages and in the egg cell, compared with those observed in the sperm. The question arises as to why DNA methylation inheritance is mainly influenced by the egg cell, whereas the sperm has a higher methylation level. The probable explanation is that the egg cell has a larger cellular volume, with more RNA-containing cytoplasm, and a greater degree of protein information, than is the case for the sperm. Conditions in the egg cell would thus be more adequate for the functioning of DNA methyltransferase, and thus for DNA methylation inheritance. In zebrafish, the oocyte methylome is also hypomethylated compared to that in the sperm. Nevertheless, the DNA methylome in zebrafish early embryos was mainly under the influence of the sperm. The paternal DNA methylation pattern is maintained throughout early embryogenesis, while oocyte methylome is gradually discarded and reprogrammed to attain a pattern that is similar to that of sperm methylome (Jiang et al. 2013a). We also found that the rate of identical methylation-status loci between different development stages and sperm increased from 7.22 to 28.26 % during the early development processes in *P. fucata*. This may indicate that the sperm become increasingly important, in terms of DNA methylation inheritance, during development. The underlying molecular mechanism of DNA methylation inheritance in *P. fucata* is unclear and needs to be further elucidated.

In summary, we have demonstrated that MSAP is an appropriate technique for DNA methylation polymorphism analysis in the genome of *P. fucata*. The different DNA methylation levels in tissues and development stages may be related to specific gene expression during tissue differentiation and individual development. While DNA methylation patterns in the offspring may be mainly under the influence of the egg, the sperm may becomes increasingly important in DNA methylation inheritance during development. In this study we provided further insights into the understanding of DNA methylation in *P. fucata* which will be useful for future study into the potential role of DNA methylation in molecular-selection breeding.

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Conflict of interest The authors declare no conflict of interest.

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