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Correlation between Genome Methylation Level and Growth Trait of Pearl Oyster, *Pinctada fucata*

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ABSTRACT

The function of genome methylation in invertebrates has only recently been characterized in-depth. In this study, we firstly investigated the associations of genome methylation level and growth traits in pearl oyster, *Pinctada fucata* (*P. fucata*). The incidence of type II locus (hemi-methylation locus) was significantly correlated with the shell height and the shell length, with Pearson correlation coefficients of 0.204 and 0.233, separately. The incidence of type IV locus (super methylated locus) on the other hand was significantly correlated with the shell length, with Pearson correlation coefficient of 0.217. The genome methylation levels were significantly positively correlated with shell height, shell length, shell width, total weight and shell weight ($P<0.05$). The incidence of type II locus (hemi-methylation locus) was also significantly positively associated with growth rates of the shell height and the shell length, and Pearson correlation coefficients were 0.268 and 0.298, respectively. The results indicated that genome methylation of *P. fucata* may play a pivotal role in the formation of growth trait, and could be considered for growth trait selection in breeding.

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Authors' Contributions
MXH conceived and designed the study and collected samples. YGL performed the experiments, analyzed the data and wrote the article.

Key words
***Pinctada fucata*, Growth trait, Growth rate, Genome methylation, Correlation analysis.**

INTRODUCTION

The pearl oyster, *Pinctada fucata*, is a bivalve that is cultured for pearl production in Guangdong, Guangxi and Hainan of China. The growth traits of *P. fucata*, such as shell length, shell width and shell weight, were associated with the pearl quality. Generally speaking, oysters with higher growth trait value can produce pearls of good quality. The genetic degeneration caused by inbreeding and environmental threats makes the pearl production of *P. fucata* no longer commercially sustainable (Wada and Komaru, 1994; He *et al*., 2008; Liu *et al*., 2012). The quantitative trait loci for growth of *P. fucata* have been identified which may contribute to the improvement of pearl quality (Li and He, 2014).

The phenotypic formations within a species are mainly dependent on both genetic and epigenetic heritable factors (Jiang *et al*., 2013). Chemical modification of DNA bases, 5-methylcytosine, plays a key role in epigenetic gene regulation (Breiling and Lyko, 2015). DNA methylation is one of the key processes that could affect individual development, genomic imprinting, sex chromosome inactivation and transposable element silencing (Zentner and Henikoff, 2014). Differentially methylated genes are organized in gene networks related to the cellular development, growth, and carbohydrate metabolism (Kwak *et al*., 2014). This implies that methylation of growth related networks might influence the hormone level, growth traits and mRNA expression (Zhao *et al*., 2015).

Many studies have shown that genome methylation may play critical role in the formation of heterosis. Genome methylation level of the *Larix kaempferi* heterotic hybrids may play critical role in the formation of heterosis. Genome(26.47%) was significantly lower than the midparent value (33.80 %), and increased gene expression in the heterotic hybrids was associated with its overall low genomic methylation level (Li *et al*., 2013). The genome methylation, demethylation and hypermethylation levels were positively correlated with heterosis in rice. Significant positive association was also observed for genome methylation and hypermethylation levels with plot yield, plot yield heterosis and grain yield. The incidence of demethylation was associated with all the traits studied except grain yield (Sakthivel *et al*., 2010). Methylation of quantitative trait loci across genome contributed to complex traits formation. For example, several methylation quantitative trait loci across the genome of *Arabidopsis* accounted for 60 to 90% of the flowering time and primary root length. These methylation

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quantitative trait loci are heritable and can be subjected to artificial selection (Cortijo *et al*., 2014). The DNA methylation markers are potentially linked to phenotypic variations, especially in closely related strains (Takata *et al*., 2005). The above studies highlight the need to integrate epigenetic information into population genetics studies (Johannes *et al*., 2009).

For some complex traits of aquaculture species, methylation information of the genome is especially imperative for estimation of breeding values and for implementation of genomic selection (Moghadam *et al*., 2015). The cytosine methylation was found to play important role in the major morphological traits change of common octopus, *Octopus vulgaris* (Díaz-Freije *et al*., 2014). Genome methylation level was negatively related to the shell length, the gross weight and the weight of soft body, whilst positively related to the shell broadness and the shell height in the ark shell, *Scapharca broughtonii* (Sun *et al*., 2015). Genome methylation variation from parents to offspring of Pacific oyster *Crassostrea gigas* was investigated, and most of parental methylated loci were found to be stably transmitted to offspring following Medelian expectation (Jiang *et al*., 2016). No report of relationship genome methylation level with growth trait of *P. fucata* that affected the pearl quality were found.

In the present study, we used a methylation-sensitive amplification polymorphism (MSAP) technique to investigate the relationships of genome methylation levels, growth traits and growth rates of pearl oyster, *P. fucata*. The aim of this study was to enhance the fundamental understanding of role of DNA methylation in growth trait formation of *P. fucata*.

MATERIALS AND METHODS

Samples and DNA extraction

The breeding program of *P. fucata* was carried out at the Marine Biology Research Station, Daya Bay, Chinese Academy of Sciences, Shenzhen, China. A female and a male adult oysters were obtained from a wild population in Dapeng Bay, Shenzhen, China. The two parents were sacrificed for artificial breeding and used as founders of a family in Apirl 2012. Each oyster in the family (N=150) was numbered and shell height (SH), shell length (SL), shell width (SW), total weight (Wt) were measured on November 3, 2012, separately. The SH, SL, SW, Wt, soft tissue weight (Wf), and shell weight (Ws) of 98 oysters were then measured on June 2, 2013. Growth rates of different traits were calculated using the formula: increased growth trait value / time (months), from November 3, 2012 to June 2, 2013. The adductor muscle of 100 individuals, which included the two parents and 98 progeny, were sampled and preserved in 95% ethanol for DNA extraction. 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 100 mg adductor muscle tissue was placed in 350 µL lysis buffer; to which 25 µL of Proteinase K was added. The mixture was vortexed and incubated at 60 °C for 30 min until the sample solubilized. DNA was extracted with 350 µL chloroform: isoamyl alcohol (24:1), in MBL buffer and vortex-mixed for 15 s. The DNA-containing solutions were washed by buffers from the kit, diluted, and preserved in ultrapure water. The concentration of DNA was estimated with a spectrophotometer (Nanodrop), using $OD_{260}/_{280}$. The quality of DNA was analyzed using 1% agarose gel electrophoresis.

Methylation-sensitive amplified polymorphism (MSAP) procedure

The procedure of MSAP technique containing a four-step process: DNA digestion, adapter ligation, pre-amplification, and selective amplification. Genomic DNA (200 ng) of each adductor muscle sample was digested by two pairs of restriction enzymes: *Eco*RI and *Msp*I (or *Eco*RI and *Hpa*II). The digested fragments were ligated to the *Eco*RI adapter and the *Hpa*II–*Msp*I adapter (Li *et al*., 2015a). The digested-ligated DNA was diluted (1:10) and used as the template for pre-amplification reaction. The total volume of pre-amplification PCR was 20 µL, including 10.5 µL 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 digested-ligated product, and 6.5 µL ddH_2O. The PCR thermal cycle was as follows: 5 min at 94 °C, 20 cycles each of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, with a final elongation at 72 °C for 5 min. The pre-amplification products were diluted at 1:10 and used for selective PCR. Adapters, pre-amplification primers and eight primer pairs that could yield clear MSAP bands are listed in Table I. The selective PCR primers were designed by adding two bases to the 3′ end of the Eco+A primer or HM+T primer (Sun *et al*., 2015). The total volume of selective PCR was 20 µL, containing 10.5 µL Premix Ex Taq (Takara, Dalian, China), 0.5 µL 20 pmol/µL *Eco*RI primers, 2 µL 20 pmol/µL *Hpa*II-*Msp*I primers, 1 µL of the diluted pre-amplification product, and 6 µL ddH_2O. The PCR thermal cycle was as follows: 13 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min, with degradation temperate of 0.7 °C each cycle. This was followed by 23 cycles each of 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 PCR products were mixed with 10 µL formamide loading buffer, heated at 95 °C for 15 min and then chilled on ice. The denatured PCR products were separated on 8% denaturing

polyacrylamide gel and detected by silver staining.

Table I.- Primer combinations and adapters used for MSAP analysis.

Primer pairs/ adapters	Sequences (5'-3')
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
*Hpa*II-*Msp*I adapter	GATCATGAGTCCTGCT CGAGCAGGACTCATGA
Eco+A	GACTGCGTACCAATTCA
HM+T	ATCATGAGTCCTGCTCGGT
1	Eco+AGT: GACTGCGTACCAATTCAGT HM+TTT: ATCATGAGTCCTGCTCGGTTT
2	Eco+ACA: GACTGCGTACCAATTCACA HM+TCT: ATCATGAGTCCTGCTCGGTCT
3	Eco+AAG: GACTGCGTACCAATTCAAG HM+TTT: ATCATGAGTCCTGCTCGGTTT
4	Eco+ATA: GACTGCGTACCAATTCATA HM+TTT: ATCATGAGTCCTGCTCGGTTT
5	Eco+AAT: GACTGCGTACCAATTCAAT HM+TAT: ATCATGAGTCCTGCTCGGTAT
6	Eco+AAG: GACTGCGTACCAATTCAAG HM+TTG: ATCATGAGTCCTGCTCGGTTG
7	Eco+ATC: GACTGCGTACCAATTCATC HM+TCA: ATCATGAGTCCTGCTCGGTCA
8	Eco+ATG: GACTGCGTACCAATTCATG HM+TTA: ATCATGAGTCCTGCTCGGTTA

Statistical analysis

To calculate the genome methylation level of each adductor muscle sample, the DNA banding patterns from the amplification of genomic DNA (digested with *Eco*RI and *Hpa*II, or *Eco*RI and *Msp*I) were simultaneously analyzed. Four types of methylation patterns were revealed by MSAP technique: Type I locus with same length of amplified fragments in both *Msp*I and *Hpa*II lanes, indicating no methylation modification of a 5´-CCGG-3´ locus; Type II locus displaying an amplified band after restriction with *Hpa*II but not after restriction with *Msp*I, representing 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, indicating inner methylation of double-stranded DNA (fully methylated locus) (Lu *et al*., 2008) and Type IV locus with no bands in both enzyme combinations but other samples showing fragment at that position, indicating a fully-methylated mCmCGG site (super methylated locus) (Fulneček and Kovařík, 2014). Methylation levels of a sample was calculated by the following formulae: Fully methylation level (%) = sum of Types III and IV loci/sum of Types I, II, III and IV loci. Genome methylation level (%) = sum of types II, III and IV loci/ sum of Types I, II, III and IV loci. Pearson correlation analysis was performed to investigate the relationship between genome methylation level, growth trait and growth rate by SPSS version 19.0, a *p*-value of 0.05 was considered statistically significant.

RESULTS

Genome methylation levels of parents and progeny

The genome methylation status of two parents and 98 progeny in the *P. fucata* family were detected by MSAP technique. Eight pairs of PCR primers were used to detect cytosine methylation at 5´-CCGG-3´ sites within the genome of oysters. A total of 24277 5´-CCGG-3´ loci were detected, including 21095 Type I loci, 506 Type II (hemi-methylated) loci, 1068 Type III (fully methylated) loci and 1608 Type IV (super methylated) loci in the family. Among these loci, type III and type IV loci were both considered as fully methylated loci, with a total number of 2676. The incidences of Type II, Type III, Type IV and fully methylated loci were 2.08%, 4.39%, 6.62% and 11.01%, respectively. The genome methylation level of the progeny was 13.23±1.6%. The average methylation level of the parents was 12.88%, with a value of 11.13±4.7% for the female parent and 14.63±4.8% for the male parent. The genome methylation level of progeny was higher than the average methylation level of the two parents.

Correlation between growth traits of the progeny

Growth traits of 98 progeny from the *P. fucata* family were measured on June 2, 2013 and correlation analysis were performed. The mean value of SH, SL, SW, Wt, Wf, and Ws were 44.59±5.52 mm, 46.48±4.70 mm, 13.93±1.72 mm, 9.69±3.25 g, 3.39±1.24 g and 5.21±1.68 g, separately. Pearson correlation coefficients between the growth traits ranged from 0.809 to 0.959 ($P<0.01$) (Table II).

Correlation analysis between growth traits and incidence of methylation loci

Pearson correlation analysis between growth traits (measured on June 2, 2013) and incidence of different methylation loci in 98 progeny were performed. The incidence of Type II loci was significantly correlated with SH and SL ($P<0.05$), with Pearson correlation coefficient of 0.204 and 0.223, respectively. The incidence of Type IV was significantly correlated with the SL, with Pearson correlation coefficients of 0.217. The genome methylation levels were all significantly correlated with the SH, SL, SW, Wt and Ws. More details are shown in Table III.

Table II.- Pearson correlation analysis between growth traits of *P. fucata*.

	SH	SL	SW	Wt	Wf	Ws
SH	1	0.92**	0.825**	0.896**	0.859**	0.844**
SL		1	0.809**	0.871**	0.824**	0.824**
SW			1	0.862**	0.858**	0.815**
Wt				1	0.933**	0.959**
Wf					1	0.873**
Ws						1

All correlations are statistically significant at the 1% level (data with double asterisk). SH, shell height; SL, shell length; SW, shell width; Wt, total weight; Wf, soft tissue weight; Ws, shell weight.

Table III.- Correlation analysis between growth traits and incidences of methylation loci.

	SH	SL	SW	Wt	Wf	Ws
Type II %	0.204*	0.223*	0.198	0.172	0.187	0.145
Type III %	0.120	0.097	0.039	0.118	0.032	0.125
Type IV %	0.198	0.217*	0.125	0.140	0.070	0.133
Genome methylation level	0.322*	0.336*	0.225*	0.262*	0.153	0.248*

For abbreviations see Table II. Data with an asterisk are statistically significant at the 5% level.

Table IV.- Correlations between growth rates and incidences of methylation loci.

Incidence of methylaiton loci	Growth rate of SH	Growth rate of SL	Growth rate of SW	Growth rate of Wt
Type II %	0.268*	0.298*	0.186	0.189
Type III %	-0.045	-0.120	-0.122	0.025
Type IV %	0.068	0.062	0.031	0.073
Genome methylation level	0.134	0.099	0.022	0.153

For abbreviations and statistical details, see Table II.

Correlation analysis between growth rates and incidences of methylation loci

The growth rates of traits in progeny from November 3, 2012 to June 2, 2013 were calculated. Correlation analysis between growth rates of SH, SL, SW, Wt and incidences of methylation loci were performed (Table IV). The incidence of Type II loci was significantly correlated with the growth rates of the SH and the SL ($P<0.05$), with Pearson correlation coefficients of 0.268 and 0.298, respectively. The incidence of other methylation loci showed no significant correlation with growth rates.

DISCUSSION

In our previous study, genome methylation levels of sperm, egg cells in *P. fucata* were 13.51 ± 0.10% and 11.80 ± 0.35%, respectively (Li *et al*., 2015a). In this study, genome methylation level of male parent was 14.63±4.8%, and the female parent with a methylation level of 11.13±4.7%. The results showed that genome methylation level of male parent (sperm) is higher than that of female parent (egg cell) in *P. fucata*. Sex differences in genome methylation levels may correspond to differentially expressed genes between females and males, which can have functional consequences on traits (Orozco *et al*., 2014). In human blood, a slightly higher genome methylation level in males than females were found, the methylation difference may play role in the process of X chromosome inactivation or sex determination (El-Maarri *et al*., 2007). DNA methylation difference between the male and the female *P. fucata* oysters may function in gene expression regulation, gene imprinting and sex determination. From the results we also know genome methylation level of progeny in *P. fucata* (13.23±1.6%) is lower than that of many other oysters, such as the Zhikong *Chlamys farreri* (14.9%-16.5%) (Sun *et al*., 2014), *Crassostrea gigas* (26.4%) (Jiang *et al*., 2013), and sea cucumber, *Apostichopus japonicus* (33.79%) (Zhao *et al*., 2015). The genome methylation level of progeny was higher than the average methylation level of the two parents of *P. fucata* (12.88%), and the methylation level was closer to male parent (14.63±4.8%) than that in female parent (11.13±4.7%). The DNA methylation modification of the progeny was adjusted to a higher level, and was the result of the cross of the parents. The function of increased genome methylation level in the progeny remains to be elucidated.

The appearance of quantitative character is the result of many alleles acting together, and QTLs for growth traits have been identified for *P. fucata* (Shi *et al*., 2014; Li and He, 2014). Now, many researchers believe that the trait formation may be attributed to gene expression regulation, including the epigenetic mechanism of methylation (Calicchio *et al*., 2014). Growth traits of *P. fucata* are important in pearl production, however, little is known for its regulatory mechanism at epigenetic level. The methylation levels of genome or growth related genes were significantly associated with breeding traits or growth rate in aquaculture species. For example, comparison of genetic differences and genome methylation differences between mature and immature Atlantic salmon showed that early maturation may be mostly mediated by methylation process (Moran and Perez-Figueroa, 2011). Genome

methylation level was negatively related to the shell length, the gross weight and the weight of soft body, but positively related to the shell broadness and the shell height of the ark shell *Scapharca broughtonii* (Sun *et al*., 2015). The methylation of growth hormone promoter was negatively correlated with growth rate of Nile tilapia (*Oreochromis niloticus*) (Zhong *et al*., 2014). The present results show that genome methylation levels are significantly correlated with most of the growth traits in *P. fucata,* including SH, SL, SW, Wt and Ws ($P<0.01$). Shell traits of *P. fucata* are of particular interest to researchers due to their correlations with pearl production. Why the genome methylation levels are significantly correlated with nearly all of the measured growth traits? The possible reasons were as follows: the growth traits of *P. fucata* are significantly correlated at the 1% level (Table II), and the formation of traits may also involved in same growth-related metabolic pathway (Li and He, 2014). The differences of genome methylation in oysters may lead to large scale gene expression level difference, which may affect the formation of growth traits.

Methylation modifications are a possible source of heritable complex traits variation in the absence of change in DNA sequence (Johannes *et al*., 2009). DNA methylation differences of growth related genes are correlated with birth weight and could explain 70-87% of variance in birth weight (St-Pierre *et al*., 2012; Turan *et al*., 2012). Genome methylation levels of *P. fucata* were significantly positively associated with growth traits, indicating presence of a larger number of methylation loci in the oyster with higher growth trait value. There are many kinds of methylation loci in the genome, and it would be interesting to locate type of methylation loci which are significantly correlated with the growth rate of *P. fucata*. To answer this question, the relationship between methylation level and growth rate of *P. fucata* was investigated. The incidence of Type II loci (hemi-methylation loci) was significantly positively correlated with the growth rate of SH and SL. In the breeding process of *P. fucata*, the hemi-methylation loci levels of the two parents should be considered, which may be considered as standard reference for growth trait selection. A higher incidence of Type II loci (hemi-methylated loci) indicates that more loci were demethylated from fully methylated loci. Generally speaking, gene expression can be controlled by DNA methylation in the promoter region (Bell and Felsenfeld, 2000). For example, targeted DNA demethylation in human cells can lead to substantial increase in the expression of endogenous human genes (Maeder *et al*., 2013). In *P. fucata*, individuals with higher incidence of hemi-methylated loci indicated that more demethylation events had occurred with less hemi-methylated loci, which led to activation of more growth-related genes compared with correspondingly higher speed growth. We also found negative correlation of the incidence of Type III loci with growth rates of SH, SL and SW (Table IV). The results indicated that Type III loci may have negative regulatory effect on the growth. The effects of different methylation loci on growth trait formation need to be further elucidated, for which investigation on the methylation of growth related genes may be a feasible way. In *P. fucata*, methylation modification on promoter regions of *galectin* may have effects on mRNA expression regulation and immune reaction of mantle injury (Li *et al*., 2015b). In the future, the methylation modification of growth related genes involved in trait formation should be analyzed. DNA methylation markers correlated with growth traits may be identified for molecular marker-assisted breeding in *P. fucata*.

CONCLUSIONS

To conclude, this study is the first to analyze the relationship between methylation level and growth traits in *P. fucata*. Genome methylation levels were significantly related to the SH, SL, SW, Wt and Ws, and incidence of Type II loci was significantly correlated with the growth rates of SH and SL. DNA methylation may play an important role in growth traits formation. The genome methylation status should be considered in the breeding process, and further studies need to be undertaken for understanding the role of DNA methylation in growth regulation.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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