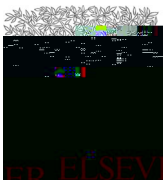
Aquaculture 547 (2022) 737421



Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

The DNA methylation level is associated with the superior growth of the hybrid crosses in the Pacific oyster *Crassostrea gigas*

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ARTICLE INFO

Keywords:
Crassostrea gigas
Heterosis
DNA methylation
F-MSAP
Dnmt3

ABSTRACT

Pacific oyster *Crassostrea gigas* is one of the most important global aquaculture bivalve species, and its growth traits have been improved by heterosis in the progeny produced by hybridization. However, comprehension of the molecular mechanisms implicat-ed in heterosis still remains elusive. In this study, a diallel cross between a selected line "Haida No. 1" and an orange-shell line of *C. gigas* was generated. The mid-parent heterosis and best-parent heterosis analysis on growth traits, including shell height, shell length, shell width and total weight, showed that the hybrid crosses exhibited a growth heterosis relative to the parental crosses. And the fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) revealed that the total DNA methylation level was significantly lower in hybrid crosses than in parental crosses, and the total methylation level was negatively associated with growth traits. Moreover, the mRNA expression level of DNA methyltransferases gene 3 (*Dnmt3*) of *C. gigas* was significantly different among four populations, which was also positively correlated to the total DNA methylation level. This work firstly provided clues for correlations between the heterosis of growth traits, total DNA methylation level and *Dnmt3* mRNA expression and will facilitate the understanding of heterosis formation in the oyster.

1. Introduction

Heterosis, or hybrid vigor, refers to the phenomenon that F1 hybrids produced by the hybridization of parents with different genetic bases are superior to one parent or both parents in terms of growth, survival and stress resistance (Birchler et al., 2010; Ma et al., 2019). Hybrid breeding based on heterosis is one of the most important breeding methods and has been widely used in the improvement and production of plant (Fujimoto et al., 2018) and animal germplasm (Song et al., 2013). There are many successful precedents in the genetic and breeding work of mollusks that use hybridization to improve seed quality (Cruz and Ibarra, 1997; Hedgecock et al., 1995; Rahman et al., 2000). Although heterosis contributes to increased yield, the underlying molecular mechanisms governing heterosis are poorly elucidated. Since the first discovery of heterosis by Charles Darwin (Fujimoto et al., 2018), many efforts including genomic and transcriptome analysis have been conducted on the DNA and mRNA levels to dissect the genetic mechanism of heterosis, and major classical models encompassing dominance, over-dominance, epistasis, and non-additive gene expression (Bruce, 1910; Chen, 2010; Shang et al., 2016; Yu et al., 1997) were expounded. The new nuclear-cytoplasmic relationship of hybrids is composed of the parental genomes and the cytoplasm mainly from the female parent (Ou et al., 2019). Therefore, heterosis can be regarded as an external manifestation of gene expression regulation.

Epigenetic mechanisms could alter gene expression and trigger phenotypic variation without entailing changes in the DNA sequence (Bonasio et al., 2010). Further demonstration that hybrids of epigenetic parental lines showed vigorous growth (Dapp et al., 2015), strongly supports the involvement of epigenetic regulation and interactions in heterosis in addition to genetic factors. DNA methylation, essentially the methylation of cytosine nucleotides, is the first identified epigenetic mechanism (Bird, 1986) and has been extensively studied. Accumulated studies suggested that DNA methylation could alter the chromatin structure (Naqvi et al., 2014; Sun et al., 2019), DNA stability and DNA conformation (Klose and Bird, 2006), also could interfere with the interaction mode between DNA molecule and its binding proteins (Banerjee et al., 2019), which ultimately regulates gene's expression and further induce changes in phenotype. Ou et al. (2019) found that the DNA methylation was closely correlated with the growth heterosis formation of hybrid fry in snakehead fish; Jiang et al. (2007) reported that

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https://doi.org/10.1016/j.aquaculture.2021.737421
Received 27 August 2021; Accepted 30 August 2021
Available online 8 September 2021

DNA methylation could be involved in the heterosis formation in pig hybrids. In the Pacific oyster *Crassostrea gigas*, a significant association between the DNA methylation and the genetic context was demonstrated (Jiang et al., 2013), and DNA methylation frequency in relation to the gametogenesis was verified (Zhang et al., 2018), suggesting that DNA methylation plays a role in oyster life.

In eukaryotes, the DNA methylation, mainly occurring on carbon 5 of cytosine (5mC), is orchestrated by DNA methyltransferases (DNMTs) that convert cytosine into 5-methylcytosine (5mC) using S-adenosyl methionine (SAM) as a methyl-donor (Salbaum and Kappen, 2012). Six *Dnmt* homolog genes have been reported in mammals to date: *Dnmt1*, *Dnmt2*, *Dnmt3A*, *Dnmt3B*, and *Dnmt3L*. DNMT1, as the maintenance DNMTs, preserves DNA methylation after DNA replication or cell division, which has a preference for methylation of hemimethylated sites and converts them into fully methylated sites (Robertson, 2002). Indeed, DNMT2 does not involve in DNA methylation, instead it transfers methyl groups to RNA (Schaefer and Lyko, 2010). DNMT3A and DNMT3B, as de novo methyltransferases, establish de novo DNA methylation, and Dnmt3L is a regulatory factor of DNMT3A and DNMT3B. Wang et al. (2014) identified only one ortholog each for DNMT1, DNMT2 and DNMT3 in *C. gigas* by performing homologous searches. However, it is still largely unknown how *Dnmts* genes functions in oysters.

C. gigas is a commercially important bivalve species with Mu eurythermal and euryhaline characteristics, and has contributed weightily to oyster aquaculture industry worldwide. With intensive cultivation in recent years, the cultivated oysters have begun to appear such phenomena as slow growth, increased mortality (Solomieu et al., 2015) and low meat yield due to inbreeding and successive breeding. Due to the heterosis or the combination of desired traits from parental species, hybrid breeding has become an important means of shellfish genetic improvement, which is widely used in the improvement of the oyster (Hedgecock et al., 1995).

In this study, a diallel cross between two culture lines of *C. gigas*, a fast-growing line "Haida No. 1" and an orange-shell inbred line, was carried out. Heterosis of reciprocal combinations in growth traits were analyzed. The fluorescent-labeled methylation-sensitive amplified-polymorphism (F-MSAP) technique was used to assess genome-wide DNA methylation differences and M explore the association between heterosis and DNA methylation levels. Simultaneously, the comparative analysis of *Dnmt1* and *Dnmt3* expression suggested *Dnmt3* probably be a regulator for heterosis. The objective of this study was to determine the modulation of DNA methylation on the formation of growth heterosis in oyster hybrids.

2. Materials and methods

2.1. Oyster and sampling

A fast-growing line "Haida No. 1" (H) and an orange-shell inbred line (O) of *C. gigas* were used in this study. The "H" line was produced by mass selection for fast growth annually since 2006 (Li et al., 2011), and the inbred line was established by four individuals (two males and two females) with orange shell color based on two generations of family selection and six generations of mass selection (Han et al., 2019). In May 2020, 100 oysters from two selected lines (H and O) as pa

Table 1
Primers and adapters uesd in this study.

	Hpa II/*Msp I*(5′-3′)	*EcoR* I(5′-3′)	Fluorescence
Adapters I	CGTTCTAGACTCATC	CTCGTAGACTGCGTACC	
Adapters II	GACGATGAGTCTAGA A	AATTGGTACGCAGTCTAC	
Preamplification primers	GATGAGTCTAGAACGGT	GACTGCGTACCAATTCA	
Selective amplification primers	GATGAGTCTAGAACGGTCA	GACTGCGTACCAATTCACA	ROX
	GATGAGTCTAGAACGGTGT	GACTGCGTACCAATTCACG	NED
	GATGAGTCTAGAACGGTAG	GACTGCGTACCAATTCACT	FAM
	GATGAGTCTAGAACGGTAT	GACTGCGTACCAATTCAAG	HEX
	GATGAGTCTAGAACGGTGT	GACTGCGTACCAATTCACA	ROX
	GATGAGTCTAGAACGGTAC	GACTGCGTACCAATTCACG	NED
	GATGAGTCTAGAACGGTGT	GACTGCGTACCAATTCACT	FAM
	GATGAGTCTAGAACGGTAT	GACTGCGTACCAATTCAAC	HEX
	GATGAGTCTAGAACGGTAT	GACTGCGTACCAATTCACA	ROX
	GATGAGTCTAGAACGGTGC	GACTGCGTACCAATTCATC	NED
	GATGAGTCTAGAACGGTAT	GACTGCGTACCAATTCAGG	FAM
	GATGAGTCTAGAACGGTAC	GACTGCGTACCAATTCAAG	HEX
Dnmt1 primers	CTCGCTCATCGCCTCATA	TGCGGGACTCCGTAACTC	
Dnmt3 primers	TTGCCGCCAAGCATAGGAA	AAGTCACACAGACGACATAAGGAG	
EF I primers	AGTCACCAAGGCTGCACAGAAAG	TCCGACGTATTTCTTTGCGATGT	

2.4.2. Quantitative real-time PCR

Based on the sequence of *Dnmt1* (GenBank access no. LOC105330054) and *Dnmt3* (GenBank access no. LOC105334030) in *C. gigas* obtained from NCBI databases (NCBI, Bethesda, MD, USA), the *Dnmt1* and *Dnmt3* primers used in qRT-PCR were designed by Primer Premier 5.0 (Table 1). Elongation factor I (*EF I*) gene was used as the reference gene to normalize gene expression by real-time PCR (Kozera and Rapacz, 2013). qPCR-PCR was performed using ChamQ SYBR Color qPCR Master Mix (Vazyme) via the LightCycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, UK). Expression levels of *Dnmt1* and *Dnmt3* were calculated by using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and data were analyzed by SPSS Statistics Software (version 25.0; IBM) using *t*-test. *P*-value less than 0.05 was considered as statistical significance.

3. Results

3.1. Superior performance of hybrid juveniles

Various growth traits including shell height, shell length, shell width and total weight of oysters were compared between the parental and hybrid populations (Table 2). Apparently, the hybrid juveniles showed significant growth advantage to the parental juveniles in terms of shell height and total weight. The mean shell length of HH, HO, OH and OO oysters were 35.61, 35.78, 37.08 and 34.67 mm, respectively, and there was no statistically significant difference in shell length among four populations. Likewise, the same situation was observed concerning shell width. And all of the growth traits had positive MPH and BPH values, the highest value of MPH was total weight, which was up to 30.43%. Generally, the growth superiority of hybrids was observed.

Table 2
Comparison of growth traits and heterosis among different populations at juvenile stage of *Crassostrea gigas*.

	Shell height (mm)	Shell length (mm)	Shell width (mm)	Total weight (g)
HH	70.94 ± 8.72^{b}	35.61 ± 3.91^{a}	19.28 ± 3.29^{a}	27.37 ± 7.54^{b}
HO	77.32 ± 5.72^{a}	35.78 ± 4.25^{a}	20.98 ± 2.96^{a}	34.80 ± 6.93^{a}
OH	74.12 ± 7.40^{ab}	37.08 ± 4.68^{a}	20.10 ± 4.68^{a}	31.28 ± 5.24^{a}
OO	65.70 ± 7.98^{c}	34.67 ± 4.21^{a}	19.63 ± 4.38^{a}	23.29 ± 5.49^{c}
MPH (%)	10.84	3.67	5.38	30.43
BPH (%)	6.75	2.32	6.33	20.70

3.2. Genome-wide DNA methylation levels

Considering the tissue specificity of methylation, only adductor muscle was employed in methylation study. Twelve primer-pair combinations that could produce a good amplification were used and DNA methylation profiles in juveniles of four populations were analyzed (Table 3). In general, the non-methylated loci accounted for the majority of the total loci, and the full methylated loci was the main type methylation in oyster. There were significant differences in total methylation, total methylation loci and hemimethylation loci between hybrid populations (HH and OO) and self-crossing populations (OH and HO) ($P < 0.05$). And the total DNA methylation extent ranged from 28.75% (HO) to 37.12% (OO) of adductor muscle samples.

The analysis on growth traits and methylation extent showed that the shell height, shell width and total weight is closely correlated with the methylation. In comparison with other populations, the hybrid population HO has the optimal growth traits and the lowest total methylation level. On the contrary, the growth traits of the parental population OO was the worst and meanwhile its total methylation level is the highest.

3.3. Expression profiles of Dnmt1 and Dnmt3

The relative expression level of *Dnmt1* and *Dnmt3* were assessed by qRT-PCR using *EF I* as the reference genes. C_t values of genes were < 35 in all samples, illustrating that the mRNA expression of *Dnmt1* and *Dnmt3* could be detected (Fig. 1). The mRNA expression level of *Dnmt1* in HH population was set as baseline (1.0), and the relative expression of other populations were determined as the ratio of expression relative to that in HH population. The relative mRNA expression level of *Dnmt1* in OO population was higher but not of significant differences ($P > 0.05$) than that in other populations. However, the mRNA expression level of *Dnmt3* was significantly different among the four populations, embodied

Table 3
DNA methylation state in different populations of *Crassostrea gigas*.

DNA methylation patterns	HH	HO	OH	OO
I	396	457	448	351
II	89	67	92	83
III	135	118	105	124
Total methylated bands	224	185	197	207
Fully methylated bands (%)	14.38	10.44	14.26	14.87
Hemi-methylated level (%)	21.81	18.31	16.27	22.25
Total methylated level (%)	36.19	28.75	30.53	37.12

Note: Total methylated bands = II + III; Fully methylated level (%) = II/(I + II + III); Hemi-methylated level (%) = III/(I + II + III); Total methylation level (%) = (II + III)/(I + II + III).

in that the expression levels of *Dnmt3* mRNA in hybrids was significantly lower than that in the parental populations.

3.4. The correlation of Dnmt genes and methylation level

The linear regression analysis between *Dnmt3* expression and total DNA methylation level were implemented, and the results showed there was a significant linear relationship between them (Fig. 2). The decision coefficient in HH, HO, OH and OO were 0.9262, 0.8465, 0.9291 and 0.9012 ($P < 0.05$), respectively. Also, the correlation coefficients indicated that the total methylation level was positively related to the *Dnmt3* mRNA expression level.

4. Discussion

4.1. The heterosis of hybrids

The heterosis in intraspecific hybridization between families, populations and lines of marine mollusks was extensively applied to improve growth performance and its substantial evidence for the pervasiveness were accumulated, such as pearl oyster *Pinctada fucata martensii* (Yang et al., 2018), Pacific abalone *Haliotis discus hannai* (Boamah et al., 2020), Pacific oyster *C. gigas* (Hedgecock et al., 1995; Kong et al., 2017) and boring giant clam *Tridacna crocea* (Zhang et al., 2020). For example, Yang et al. (2018) utilized two full-sib families of pearl oyster to develop a 2 × 2 complete diallel cross, and found that the mid-parent heterosis values of shell height, shell length, shell

shell heights of six hybrid crosses all showed positively heterosis in the larval stage. Similarly, in the present study, two lines of *C. gigas* which had been successively selected were used for diallel crossing, and the hybrid populations had strong heterosis. The MPH values of shell height, shell length, shell width and total weight were 10.84%, 3.67%, 5.38% and 30.43%, respectively. The results reflected that the shell height and body weight of hybrid populations were significantly larger than those of parental populations, which was in accord with that of previous studies on marine shellfish species, demonstrating that the heterosis effects in *C. gigas* could be applied by z

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