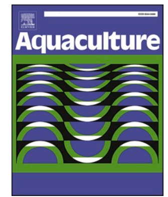
Aquaculture 547 (2022) 737421

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*

Hang Yang [a], Qi Li [a,b,*]

[a] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

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, overdominance, 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

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

https://doi.org/10.1016/j.aquaculture.2021.737421
Received 27 August 2021; Accepted 30 August 2021
Available online 8 September 2021
0044-8486/© 2021 Elsevier B.V. All rights reserved.

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 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 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 parents were collected, respectively. Then a 2 × 2 complete diallel cross was performed to produce two parental populations (HH and OO) and reciprocal hybrid populations (HO and OH).

30 individuals of 6-month-old juveniles were sampled from each population, and the body growth traits of each individual including shell height, shell length, shell width and total weight were measured. One-way ANOVA determining statistical significance were performed employing SPSS version 25.0 (SPSS Inc.) with the statistical significance set at $P < 0.05$. And the mid-parent heterosis (MPH) and the best-parent heterosis (BPH) of the F1 hybrids were calculated by the formula: $MPH = (F1 - MP)/MP \times 100\%$, and $BPH = (F1 - BP)/BP \times 100\%$, where MP represents the mid parent values, BP represents the mean value of best parent, and F1 indicates the mean performance of hybrids (Guo et al., 2017).

2.2. Genomic DNA extraction

The adductor muscle of each individual was dissected and saved at −80 °C for F-MSAP analysis. Genomic DNA of muscle was extracted by phenol-chloroform method as the previously described (Li et al., 2006). The quality of DNA samples was assessed by 1% polyacrylamide gel electrophoresis, and the concentration and purity of DNA were detected using NanoDrop-2000.

2.3. F-MSAP analysis

The detected DNA was diluted into 100 ng/μL of working fluid. The F-MSAP (fluorescence-labeled methylation-sensitive amplified polymorphism) analysis procedure was conducted as described previously (Zhang et al., 2017). Briefly, 100 ng of each genomic DNA was digested with 2 U *MspI* + 2 U *EcoRI* and 2 U *HpaII* + 2 U *EcoRI* (Thermo) by heating in 37 °C for 4 h, respectively.

Then, T4 DNA ligase (Thermo) was used to attach the linked adapters (I and II) to the digested DNA fragments at 16 °C, overnight. The restriction-ligation products were diluted 10 times as templates for the Pre-amplification that was performed by 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, followed by 60 °C incubation for 30 min. Then, selective amplification was carried out using 20 times diluted pre-amplification products and a total of twelve primer combinations with the following cycling profile: 2 min of denaturing at 94 °C, then 10 cycles of 20 s at 94 °C, 30 s at 66 °C, and 2 min at 72 °C, with a 1 °C decrease in the annealing temperature of each cycle, followed by 20 cycles of 20 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C, with 60 °C incubation for 30 min. *EcoRI* primers were 5′-labeled with different fluorescent molecular markers. All adapters, primers and primer markers are shown in Table 1.

The PCR products were run on an Applied Biosystems 3730xl DNA Analyzer using the GeneScan™-1200 LIZ™ Size Standard. Data analysis using GeneMapper 4.0 Software and then the data including DNA fragments range from 50 bp to 1200 bp were rendered in the form of an Excel table for further scoring. According to the differential sensitivity of isoschizomers to DNA methylation, the F-MSAP patterns were classified into four types: type I (1,1) refers to non-methylation loci that presents in both *EcoRI/HpaII* (H) and *EcoRI/MspI* (M) products the bands are detected; type II (0,1) refers to fully methylation loci that presents only in *EcoRI/MspI* products; type III (1,0) refers to hemimethylation loci that presents only in *EcoRI/ HpaII* products; type IV (0,0) is absent in both combined digestion products, which is considered to be an uninformative loci on account of genetic polymorphism or hyper-methylation and is discarded in the following analysis.

2.4. Expression analysis of Dnmt1 and Dnmt3

2.4.1. RNA extraction and cDNA synthesis

Adductor muscle of 10 juveniles from parental populations (HH and OO) and hybrid populations (HO and OH) were sampled for RNA extraction, which were part of the samples used for F-MSAP analysis. The total RNA of each sample was extracted with TRIzol reagent according to the manufacturer's instruction. Then, the quality of RNA was assessed by 1% polyacrylamide gel electrophoresis, and the concentration and purity of RNA were detected using NanoDrop-2000. After treated with gDNA wiper (Vazyme) to eliminate gDNA contamination, total RNA was reverse-transcribed into cDNA using HiScript III 1st Strand cDNA Synthesis (Vazyme) following the manufacture's instruction.

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).

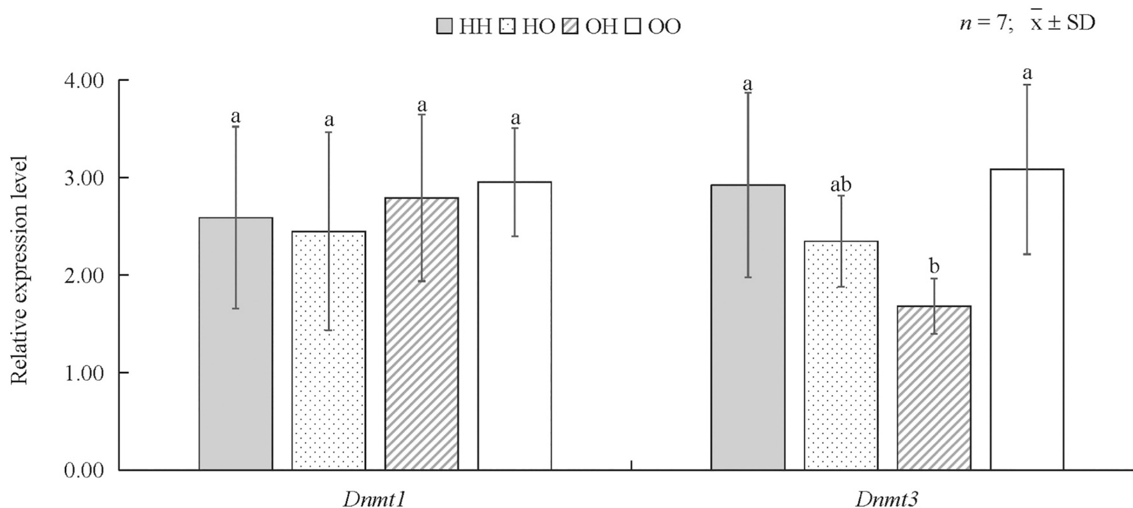


Fig. 1. The mRNA expression levels of *Dnmt1* and *Dnmt3* in adductor muscles of four populations of *C. gigas*.

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 width, total weight and shell weight were 14.9%, 12.9%, 18.2%, 33.2% and 17.3%, respectively. Likewise, Hedgecock and Davis (2007) used three inbreeding lines of *C. gigas* to conduct complete diallel cross, and the

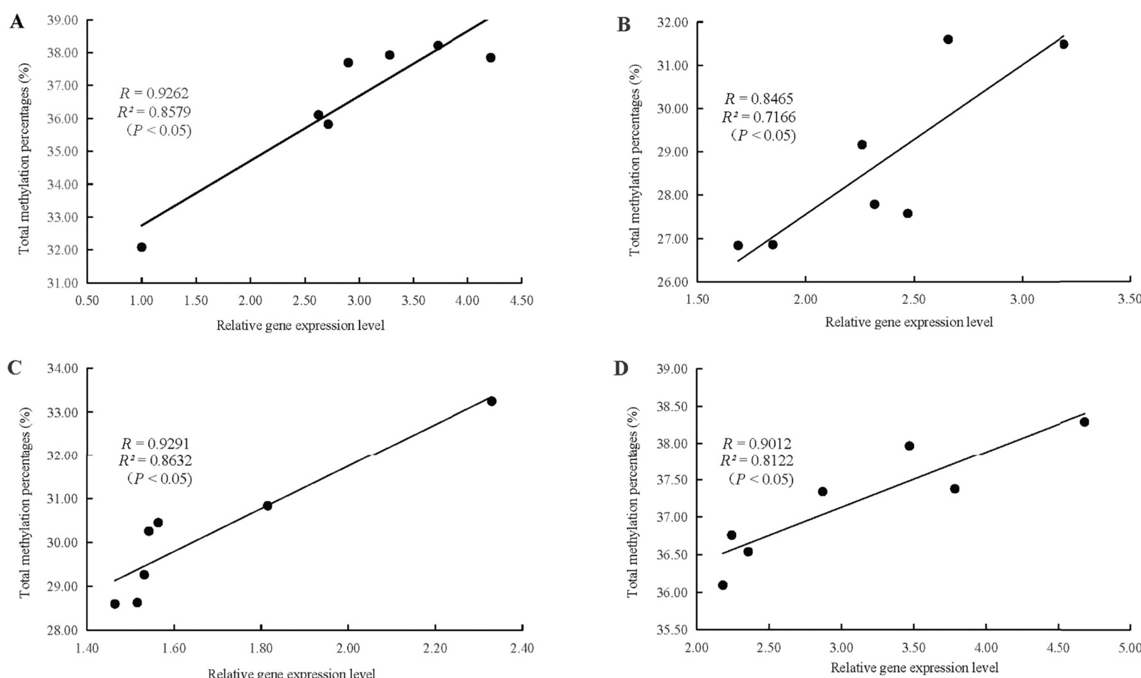


Fig. 2. Correlation of the total DNA methylation level and *Dnmt3* mRNA expression in juvenile stage of *C. gigas*. (A) HH. (B) HO. (C) OH. (D) OO.

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 lines mating.

4.2. The DNA methylation associated with heterosis formation in hybrid populations

As we all know, growth traits are the key parameters that can be directly observed and easily optimized in shellfish, and also the main measure of heterosis. Although the genetic basis of heterosis is complicated (Shahzad et al., 2020), there is growing evidence that the heterosis, referring to the phenotypic differences between hybrid progenies and parental progenies is the result of gene regulation, environmental factors and so on. Moreover, several studies have demonstrated that the methylation change exerts an influence on genes' expression (Li et al., 2021) and contributes to heterosis formation on hybrid performance (Jiang et al., 2007; Ou et al., 2019). For instance, it was found that differences in hybrid methylation compared with parents may contribute to the heterosis, as it provides an explanation for heterosis (Jiang et al., 2007). In fish, the total DNA methylation levels of hybrid offspring were lower than those of parental offspring, and the negative correlation between the DNA methylation levels of fry and its growth performance including body length and body height was also discovered (Ou et al., 2019). In addition, Jiang et al. (2013) investigated the epigenetic variation of *C. gigas*, and a few bands between the two populations showed significantly different frequencies in DNA methylation, which possibly could contribute to phenotypic changes in selective breeding. Similarly, the methylation levels of hybrid offspring were significantly lower than those of parental offspring in this study, and the methylation levels of HO populations that had optimal growth traits were the lowest, which indicated the total methylation level was negatively associated with growth traits. Usually, in heterozygotes, the methylation patterns from the parents are sufficiently modulated, with complex methylation and demethylation changes while maintaining most stable transmission (Grimm et al., 2019). This result indicated that the DNA methylation would involve in the potential mechanism of the growth heterosis formation in hybrids, but it still requires extensive investigations to illuminate the process how the DNA methylation contributes to the superior growth traits forming in the oyster hybrids.

4.3. The association between DNA methylation and Dnmt genes

In eukaryotes, DNA methylation is classified into two categories: de novo DNA methylation and DNA maintenance methylation (Yu et al., 2021), controlled by DNMTs (Iguchi et al., 2020). Because only one ortholog each for *Dnmt1* and *Dnmt3* were identified in *C. gigas* (Wang et al., 2014), *Dnmt1* and *Dnmt3* in *C. gigas* were amplified and its mRNA expression were analyzed by RT-qPCR in this study. *Dnmt1* is involved in the maintenance of post-replication methylation patterns, promoting the transfer of parental DNA methylation state to the newly synthesized DNA of the progeny (Goll and Bestor, 2005). Wang et al. (2014) found that the relatively high expression level of *Dnmt1* at the early developmental stages was concerned with requirement of maintaining methylation during the particularly active phase of cell division. In present study, *Dnmt1* had a relatively higher expression level than *Dnmt3* in the hybrids. But we also observed that the expression of *Dnmt1* among four populations was no significant difference, which couldn't indicate that it contributes to inter-population differences in DNA methylation levels. On the contrary, the mRNA expression level of *Dnmt3* in hybrids was significantly lower than that in the parental populations. As de novo DNA methylation methyltransferase, *Dnmt3* establishes methylation on unmethylated DNA and contributes to metabolism and development (Kaneda et al., 2004; Okano et al., 1999). It has been documented that *Dnmt3* had a regulatory effect on the early developmental stage of aquatic animals (Firmino et al., 2017; Smith et al., 2011). And it was deduced that *Dnmt3* could be a regulator of heterosis formation in oyster.

Moreover, correlation analysis showed the total methylation levels were significantly related to *Dnmt3* mRNA expression levels from four populations, which is consistent with the report in snakehead fish (Ou et al., 2019). Given the above, *Dnmt3*, involved in de novo methylation in oyster, probably modulates the heterosis formation in the hybrid. However, this hypothesis merits further investigation on the regulatory mechanisms of DNA methylation in excellent growth traits of oyster hybrid breeding.

5. Conclusion

In this study, the hybrids of *C. gigas* produced by intraspecific hybridization exhibited higher growth than the parental progeny. The F-MSAP analysis showed that the methylation levels in the hybrid offspring were significantly lower than that in the parental offspring, and the total weight and shell height was negatively associated with the total methylation level. Additionally, the *Dnmt1* mRNA and *Dnmt3* mRNA were detected, but only the mRNA expression level of *Dnmt3* was positively correlated to the total DNA methylation level, which deduced that *Dnmt3* was involved in heterosis formation of the hybrids. The study provides foundational information on heterosis formation in Pacific oyster *C. gigas*.

Credit author statement

Hang Yang: Investigation, Methodology, Data curation, and Writing - original draft. Qi Li: Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the grants from Earmarked Fund for Agriculture Seed Improvement Project of Shandong Province (2020LZGC016), Industrial Development Project of Qingdao City (20-3-4-16-nsh), and Science and Technology Development Project of Weihai City (2018NS01).

References

Banerjee, S., Wei, X.R., Xie, H.H., 2019. Recursive motif analyses identify brain epigenetic transcription regulatory modules. Comput. Struct. Biotechnol. J. 17, 507–515.
Birchler, J.A., Yao, H., Chudalayandi, S., Vaiman, D., Veitia, R.A., 2010. Heterosis. Plant Cell. 22, 2105–2112.
Bird, A.P., 1986. CpG-rich islands and the function of DNA methylation. Nature 321, 209–213.
Boamah, G.A., Wang, T., Chowdhury, I.A., Luo, X., Huang, M.Q., Xu, C.G., Ke, C.H., You, W.W., 2020. Sub-low salinity impact on survival, growth and meat quality of the Pacific abalone (*Haliotis discus hannai*) and hybrids. Aqua. Res. 51, 5184–5193.
Bonasio, R., Tu, S.J., Reinberg, D., 2010. Molecular signals of epigenetic states. Science 330, 612–616.
Bruce, A.B., 1910. The mendelian theory of heredity and the augmentation of vigor. Science 32, 627–628.
Chen, Z.J., 2010. Molecular mechanisms of polyploidy and hybrid vigor. Trends Plant Sci. 15, 57–71.

Cruz, P., Ibarra, A.M., 1997. Larval growth and survival of two catarina scallop (*Argopecten circularis*, Sowerby, 1835) populations and their reciprocal crosses. J. Exp. Mar. Biol. Ecol. 212, 95–110.
Dapp, M., Reinders, J., Bediee, A., Balsera, C., Bucher, E., Theiler, G., Granier, C., Paszkowski, J., 2015. Heterosis and inbreeding depression of epigenetic *Arabidopsis* hybrids. Nat. Plants. 1, 15092.
Firmino, J., Carballo, C., Armesto, P., Campinho, M.A., Power, D.M., Manchado, M., 2017. Phylogeny, expression patterns and regulation of DNA methyltransferases in early development of the flatfish, *Solea senegalensis*. BMC Dev. Biol. 17, 11.
Fujimoto, R., Uezone, K., Ishikura, S., Osabe, K., Peacoc, W.J., Dennis, E.S., 2018. Recent research on the mechanism of heterosis is important for crop and vegetable breeding systems. Breed. Sci. 68, 145–158.
Goll, M.G., Bestor, T.H., 2005. Eukaryotic cytosine methyltransferases. Annu. Rev. Biochem. 74, 481–514.
Grimm, S.A., Shimbo, T., Takaku, M., Thomas, J.W., Auerbach, S., Bennett, B.D., Bucher, J.R., Burkholder, A.B., Day, F., Du, Y., Duncan, C.G., French, J.E., Foley, J.F., Li, J., Merrick, B.A., Tice, R.R., Wang, T.Y., Xu, X.J., Bushel, P.R., Fargo, D.C., Mullikin, J.C., Wade, P.A., 2019. DNA methylation in mice is influenced by genetics as well as sex and life experience. Nat. Commun. 10, 305.
Guo, H.B., Mendrikahy, J.N., Xie, L., Deng, J.F., Lu, Z.J., Wu, J.W., Li, X., Shahid, M.Q., Liu, X.D., 2017. Transcriptome analysis of neo-tetraploid rice reveals specific differential gene expressions associated with fertility and heterosis. Sci. Rep. 7, 40139.
Han, Z.Q., Li, Q., Liu, S.K., Yu, H., Kong, L.F., 2019. Genetic variability of an orange-shell line of the Pacific oyster *Crassostrea gigas* during artificial selection inferred from microsatellites and mitochondrial COI sequences. Aquaculture 508, 159–166.
Hedgecock, D., Davis, J.P., 2007. Heterosis for yield and crossbreeding of the Pacific oyster *Crassostrea gigas*. Aquaculture 272, 17–29.
Hedgecock, D., McGoldrick, D.J., Bayne, B.L., 1995. Hybrid vigor in Pacific oysters: an experimental approach using crosses among inbred lines. Aquaculture 137, 285–298.
Iguchi, E., Takai, A., Takeda, H., Kumagai, K., Arasawa, S., Eso, Y., Shimizu, T., Ueda, Y., Marusawa, H., Seno, H., 2020. DNA methyltransferase 3B plays a protective role against hepatocarcinogenesis caused by chronic inflammation via maintaining mitochondrial homeostasis. Sci. Rep. 10, 21268.
Jiang, C.D., Deng, C.Y., Xiong, Y.Z., 2007. Differences of cytosine methylation in parental lines and F1 hybrids of Large White × Meishan crosses and their effects on F1 performance. J. Agric. Biotechnol. 2, 195–200.
Jiang, Q., Li, Q., Yu, H., Kong, L.F., 2013. Genetic and epigenetic variation in mass selection populations of Pacific oyster *Crassostrea gigas*. Genes Genom. 35, 641–647.
Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., Sasaki, H., 2004. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature 429, 900–903.
Klose, R.J., Bird, A.P., 2006. Genomic DNA methylation: the mark and its mediators. Trends Biochem. Sci. 31, 89–97.
Kong, L.F., Song, S.L., Li, Q., 2017. The effect of interstrain hybridization on the production performance in the Pacific oyster *Crassostrea gigas*. Aquaculture 472, 44–49.
Kozera, B., Rapacz, M., 2013. Reference genes in real-time PCR. J. Appl. Genet. 54, 391–406.
Li, Q., Yu, H., Yu, R.H., 2006. Genetic variability assessed by microsatellites in cultured populations of the Pacific oyster (*Crassostrea gigas*) in China. Aquaculture 259, 95–102.
Li, Q., Wang, Q.Z., Liu, S.K., Kong, L.F., 2011. Selection response and realized heritability for growth in three stocks of the Pacific oyster *Crassostrea gigas*. Fish. Sci. 77, 643–648.
Li, D., Yang, Y., Li, Y.P., Zhu, X.H., Li, Z.Q., 2021. Epigenetic regulation of gene expression in response to environmental exposures: from bench to model. Sci. Total Environ. 776, 145998.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402–408.
Ma, L.L., Wang, Y.M., Ijaz, B., Hua, J.P., 2019. Cumulative and different genetic effects contributed to yield heterosis using maternal and paternal backcross populations in Upland cotton. Sci. Rep. 9, 3984.
Naqvi, H., Ilagan, Y., Krikun, G., Taylor, H.S., 2014. Altered genome-wide methylation in endometriosis. Reprod. Sci. 21, 1237–1243.
Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.
Ou, M., Mao, H.H., Luo, Q., Zhao, J., Liu, H.Y., Zhu, X.P., Chen, K.C., Xu, H.Y., 2019. The DNA methylation level is associated with the superior growth of the hybrid fry in snakehead fish (*Channa argus* × *Channa maculata*). Gene 703, 125–133.
Rahman, M.A., Uehara, T., Aslan, L.M., 2000. Comparative viability and growth of hybrids between two sympatric species of sea urchins (Genus *Echinometra*) in Okinawa. Aquaculture 183, 45–56.
Robertson, K.D., 2002. DNA methylation and chromatin - unraveling the tangled web. Oncogene 21, 5361–5379.
Salbaum, J.M., Kappen, C., 2012. Genetic and epigenomic footprints of folate. Prog. Mol. Biol. Transl. Sci. 108, 129–158.
Schaefer, M., Lyko, F., 2010. Solving the Dnmt2 enigma. Chromosoma 119, 35–40.
Shahzad, K., Zhang, X.X., Guo, L.P., Qi, T.X., Bao, L.S., Zhang, M., Zhang, B.B., Wang, H. L., Tang, H.N., Qiao, X.Q., Feng, J.J., Wu, J.Y., Xing, C.Z., 2020. Comparative transcriptome analysis between inbred and hybrids reveals molecular insights into yield heterosis of upland cotton. BMC Plant Biol. 20, 239.
Shang, L.G., Liang, Q.Z., Wang, Y.M., Zhao, Y.P., Wang, K.B., Hua, J.P., 2016. Epistasis together with partial dominance, over-dominance and QTL by environment interactions contribute to yield heterosis in upland cotton. Theor. Appl. Genet. 129, 1429–1446.
Smith, T.H., Collins, T.M., McGowan, R.A., 2011. Expression of the dnmt3 genes in zebrafish development: similarity to Dnmt3a and Dnmt3b. Dev. Genes Evol. 220, 347–353.
Solomieu, V.B., Renault, T., Travers, M.A., 2015. Mass mortality in bivalves and the intricate case of the Pacific oyster, *Crassostrea gigas*. J. Invertebr. Pathol. 131, 2–10.
Song, G.Y., Guo, Z.B., Liu, Z.W., Cheng, Q., Qu, X.F., Chen, R., Jiang, D.M., Liu, C., Wang, W., Sun, Y.F., Zhang, L.P., Zhu, Y.G., Yang, D.C., 2013. Global RNA sequencing reveals that genotype-dependent allele-specific expression contributes to differential expression in rice F1 hybrids. BMC Plant Biol. 13, 221.
Sun, Y.Y., Fan, M., He, Y.J., 2019. DNA methylation analysis of the *Citrullus lanatus* response to *Cucumber green mottle mosaic virus* infection by whole-genome bisulfite sequencing. Genes (Basel) 10, 344.
Wang, X.T., Li, Q.Y., Lian, J.M., Li, L., Jin, L.J., Cai, H.M., Xu, F., Qi, H.G., Zhang, L.L., Wu, F.C., Meng, J., Que, H.Y., Fang, X.D., Guo, X.M., Zhang, G.F., 2014. Genome-wide and single-base resolution DNA methylomes of the Pacific oyster *Crassostrea gigas* provide insight into the evolution of invertebrate CpG methylation. BMC Genomics 15, 1119.
Yang, J.M., Luo, S.J., Li, J.H., Zheng, Z., Du, X.D., Deng, Y.W., 2018. Transcriptome analysis of growth heterosis in pearl oyster *Pinctada fucata martensii*. FEBS Open Bio. 8, 1794–1803.
Yu, S.B., Li, J.X., Xu, C.G., Tan, Y.F., Gao, Y.J., Li, X.H., Zhang, Q.F., Saghai Maroof, M. A., 1997. Importance of epistasis as the genetic basis of heterosis in an elite rice hybrid. Proc. Natl. Acad. Sci. U. S. A. 94, 9226–9231.
Yu, Z.M., Zhang, G.H., Teixeira da Silva, J.A., Li, M.Z., Zhao, C.H., He, C.M., Si, C., Zhang, M.Z., Duan, J., 2021. Genome-wide identification and analysis of DNA methyltransferase and demethylase gene families in *Dendrobium officinale* reveal their potential functions in polysaccharide accumulation. BMC Plant Biol. 21, 21.
Zhang, X., Li, Q., Kong, L.F., Yu, H., 2017. DNA methylation changes detected by methylation-sensitive amplified polymorphism in the Pacific oyster (*Crassostrea gigas*) in response to salinity stress. Genes Genom. 39, 1173–1181.
Zhang, X., Li, Q., Kong, L.F., Yu, H., 2018. DNA methylation frequency and epigenetic variability of the Pacific oyster *Crassostrea gigas* in relation to the gametogenesis. Fish. Sci. 84, 789–797.
Zhang, Y.H., Zhou, Z.H., Qin, Y.P., Li, X.Y., Ma, H.T., Wei, J.K., Zhou, Y.Y., Xiao, S., Xiang, Z.M., Noor, Z., Li, J., Yu, Z.N., 2020. Phenotypic traits of two boring giant clam (*Tridacna crocea*) populations and their reciprocal hybrids in the South China Sea. Aquaculture 519, 734890.