

Study on the Heterosis of the First Generation of Hybrid between Chinese and Korean Populations of *Scapharca broughtonii* using Methylation-Sensitive Amplification Polymorphism (MSAP)

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Received: September 9, 2015 Accepted: September 20, 2015 Online Published: November 6, 2015

doi:10.5539/jmbr.v5n1p56 URL: <http://dx.doi.org/10.5539/jmbr.v5n1p56>

Abstract

DNA methylation is known to play an important role in the regulation of gene expression in eukaryotes. In this study, the author assessed the extent and pattern of cytosine methylation in the *Scapharca broughtonii* genome using the technique of methylation-sensitive amplified polymorphism (MSAP). The results showed that, DNA methylation rate 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; there was significantly different between the parents and the offspring: 31.6% of 5'-CCGG sites in the *Patinopecten yessoensis* of Korean populations genome were cytosine methylated, and in the *Patinopecten yessoensis* of Chinese populations were 33%, the methylation rates of F1 was 29.98%; four classes of patterns were identified in a comparative assay of cytosine methylation in the parents and hybrid, increased methylation was detected in the hybrid compared to the parents at some of the recognition sites, while decreased methylation in the hybrid was detected at other sites. It indicated that the alteration of methylation resulted from cross-breeding, and the inbreeding did not change the methylation ratio and patterns; The DNA cytosine methylation has a relationship with the heterosis.

Keywords: *Scapharca broughtonii*, DNA methylation, methylation-sensitive amplified polymorphism (MSAP), heterosis

1. Introduction

Scapharca broughtonii (Mollusca, Bivalve, Arcoida), one of the most important marine commercial bivalve species, mainly distributes in the coasts of BoHai Sea and North of Yellow Sea, China. Because of its large body, rapid growth rate, delicious tastes and high protein and vitamin contents, the export in the exchange rates of ark shell is higher in the aquatic products, and *Scapharca broughtonii* has become one of the most popular farming mollusks in North China due to its high economic value in recent years. However, with the deterioration of marine ecological environment resulted from the extended farming scale and frequency human activities in coastal waters, and over-fishing, the wild resources decreased. The mass mortality has become a major constraint for the development of the *Scapharca broughtonii* culture. It is imperative for us to actively manage the resource and turn to breed high adversity resistance, fast-growing variety using traditional and new breeding methods. The hybridization of different populations proved to be a good way of breeding.

The genetic basis of heterosis has been debated for decades, dominance, pseudo-overdominance, real overdominance, and epistasis are the major genetic models proposed to explain heterosis (Crow, 2000; Lamkey & Edwards, 1997; Lippman & Zamir, 2007; Reif et al., 2006), but there is still a striking discordance between an extensive use of heterosis in variety development and our understanding of the basis of heterosis (Birchler, Auger, & Riddle, 2003; Reif et al., 2006). In recent years, many research thought that the molecular basis of heterosis may be attributed to the increased gene expression level in the hybrid or to the altered regulation of gene expression in the hybrid either at the global level or for specific classes of genes (Leonardi, Damerval, Hebert, Gallais, & Vienne, 1991; Romagnoli, Maddaloni, Livini, & Motto, 1990; A. Tsiftaris, Kafka, Polidoros, & Tani, 1997; S. Tsiftaris, 2006). Two different alleles brought together in the hybrid may create a combined allelic expression pattern in the hybrids. Alternatively, at some loci, allelic interaction or a change in the spectrum of trans-acting

factors causes gene expression in the hybrid to deviate from simple additive allelic expression patterns of the parents (Birchler et al., 2003; Gibson & Weir, 2005). Considering effects of DNA methylation on gene expression, there may be a relationship between DNA methylation and the expression of heterosis (Finnegan, Peacock, & Dennis, 2000; Rangwala & Richards, 2004).

DNA cytosine methylation is the most common covalent modification of DNA in eukaryotes, in recent years, DNA methylation has received considerable attention in eukaryotic organisms (Xiong, Xu, Saghai Maroof, & Zhang, 1999), which plays an important role in many aspects of biology, including differential gene expression, cell differentiation, genomic imprinting, chromatin inactivation, transposable elements and gene silencing, and so on (Finnegan et al., 2000; Paszkowski & Whitham, 2001; Tariq & Paszkowski, 2004).

DNA methylation analysis has been approached either by studying global levels of cytosines methylated or by analyzing specific gene sequences (Jacobsen, Sakai, Finnegan, Cao, & Meyerowitz, 2000; Luff, Pawlowski, & Bender, 1999; Riddle & Richards, 2002; Soppe et al., 2000). There are several methods used for detecting DNA methylation, such as bisulfite conversion, methylation-sensitive restriction enzymes, methyl-binding proteins, methylation-sensitive amplified polymorphism (MSAP), and anti-methylation cytosine antibodies (Zilberman & Henikoff, 2007). Among these, two methods are routinely used for the detection of DNA methylation in the tissues of eukaryotic organisms. These depend on the application of bisulfites or methylation-sensitive restriction enzymes. Bisulfites convert unmethylated cytosine into thymine, thus allowing the detection of cytosine methylation. Some restriction enzymes (isoschizomers) share the same recognition sites but show differential sensitivity to DNA methylation. Thus, polymorphic DNA fragments can be generated after digestion of methylated genomic DNA with isoschizomers (Xu, Li, & Korban, 2000). Methylation sensitive amplified polymorphism (MSAP) analysis is based on the use of isoschizomers for detection of DNA methylation. It is an adaptation of the amplified fragment length polymorphism (AFLP) technique (Reyna-Lopez, Simpson, & Ruiz-Herrera, 1997), in which the isoschizomers HpaII and MspI are employed as 'frequent-cutter' enzymes for AFLP, instead of the usual MseI. HpaII and MspI recognize the same tetranucleotide sequence (5'-CCGG-3'), but display differential sensitivity to DNA methylation. HpaII is inactive when either of the two cytosines is fully methylated, but cleaves hemi-methylated 5'-CCGG-3' at a lower rate than the unmethylated sequence. MspI cleaves 5'-C^{5m}CCGG-3', but not 5'-^{5m}CCGG-3'. MSAP allows for detection of genetic diversity throughout the genome without any prior knowledge of the nucleotide sequence (Vos et al., 1995) and has been successfully applied in various studies.

In this study, the MSAP technique was firstly used to analyze the *Scapharca broughtonii* genome DNA cytosine methylation. We discussed the differences in the level of cytosine methylation among the parents and the offspring, the differences of methylation patterns between the parents and offspring, the correlation between the methylation and phenotypic traits, and emphasized the discussion on the molecular basis of heterosis in terms of the DNA methylation.

2. Materials and Methods

2.1 Sampling

The *Scapharca broughtonii*, Chinese populations (6.9±0.53 cm of shell length), were collected randomly from Penglai sea area (Shandong province, China) in April, 2014. Korean populations (7.2±0.47 cm) were collected randomly from Incheon sea area in April, 2014. The heterozygous F1 (6.7±0.62 cm) was a cross between the *Scapharca broughtonii* of Chinese and Korean populations.

2.2 DNA Extraction

The sample DNA was extracted from adductor muscle by traditional phenol-chloroform method. Approximately 100 mg of adductor muscle was dissected out and transferred to an Eppendorf tube containing 500 µL of a lysis solution (50 mmol L⁻¹ Tris-Cl pH 8.0, 10 mmol L⁻¹ EDTA, 10% sodium dodecylsulfate [SDS] and 200 µg mL⁻¹ proteinase K) at 55 °C for 3 h. DNA was extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform, and then precipitated with two volumes of ice-chilled absolute ethanol and 1/10 volume of 3 mol L⁻¹ sodium chloride at -20 °C for 1 h. The rough extraction was washed with 70% ethanol for three times, natural dried and resuspended in 50 µL autoclaved ddH₂O. Extracted DNA was stored at -20 °C.

2.3 MSAP Analysis

The MSAP protocol was adapted from Xiong et al. (Xiong et al., 1999). Briefly, DNA was double-digested with one of the methylation sensitive enzymes HpaII or MspI, which cuts at the CCGG site, and then with the methylation insensitive EcoRI. Two digestion reactions were set up at the same time for each genomic DNA sample, each containing 400 ng of DNA with 3 Units of isoschizomers either HpaII or MspI (Fermentas) and 2 µL

10×Buffer Tango™ in a final volume of 20 µL for 6 h at 37°C, and then add in 3 Units of EcoRI (Fermentas) and 4µL 10×Buffer Tango™ in a final volume of 30 µL for 6 h at 37 °C.

Subsequently, the digested DNA fragments from the two reactions were ligated separately with an equal volume of the ligation solution containing 5µL digested fragments with 5 U of T4 DNA Ligase (Trans), 5 pmolL-1 EcoRI adapter, 50 pmolL-1 HpaII/MspI adapter, and 4µL 5×T4 DNA Ligase Buffer in a final volume of 20 µL at 16°C for overnight. The reactions were stopped by incubating at 65°C for 10 min and diluted to 200µL for PCR amplification.

Preamplification was conducted by using 5µL of the above ligation product with E0/HM0 primers in a volume of 20µL containing 2 µL PCR buffer, 0.1 mmolL-1 each dNTPs, 20 ng of each primer and 0.1 U Taq polymerase (TIANGEN). The reaction involved 27 cycles of 94°C for 30 s, 56°C for 1min, 72°C for 1 min, with a final extension at 72°C for 10 min. The preamplified products were then diluted to 600µL and stored at -20°C before use.

Ingredients of the selective amplification were the same as described above using 2.5µL of diluted preamplification mixture DNA. The selective amplification was performed by the touchdown program using amplification primers. The PCR conditions were as follows: 13 cycles at 94°C for 30 s, 0.7°C per cycle from 65 to 56°C for 30 s and 72°C for 1 min; and another 23 cycles of PCR amplification were used following the touchdown program. The denaturing step was done at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 10 min.

The final selective amplification products were denatured, separated on a 6% polyacrylamide sequencing gel, and visualized by silver staining.

All reactions were performed in triplicate to avoid false positive results. If results were reproducible, the sample was used for further analysis. Only clear and reproducible bands that appeared in four independent PCR amplifications were scored.

2.4 Restriction of Isolated and Re-Amplified Fragments

The special bands were excised directly from the polyacrylamide gels on the plate using a razor blade. The bands were rehydrated with 50µL of sterile distilled water, heated at 98°C for 5 min and let cool slowly to room temperature for the night. The tubes were centrifuged at 12,000 g for 10 min and the supernatant transferred into a fresh tube. Aliquots of 5µL were used as template for re-amplification in a total PCR reaction volume as the selective amplification with the same primer combinations. The products were checked on 1.5% agarose gel for the presence of the bands.

Then, two sets of digestion reactions were carried out simultaneously, in the first reaction, 5µL of re-amplification PCR product was added to 15µL of the digestion system above described, the reamplified fragment was excised from the H lane. The second digestion reaction was carried out in the same way, except that MspI was used in place of HpaII, and the reamplified fragment was excised from the M lane. The digestion products were checked on 1.5% agarose gel with the re-amplification PCR product.

3. Results

The isoschizomers HpaII and MspI recognize and digest 5'-CCGG-3 sites, but display differential sensitivity to DNA methylation. HpaII is inactive if one or both cytosines are fully methylated (both strands methylated), but cleaves hemimethylated sequences (only a single DNA strand is methylated) or no methylation sequences; whereas, MspI digests inner methylation of double-stranded DNA or no methylation. Hemimethylation of either of the two cytosines would lead to the appearance of a fragment in the amplification product from the EcoRI+HpaII digest but not the EcoRI+MspI digest; on the contrary, it is a full methylation site; and if the fragments appeared in the products of the two digestions, the cytosine were not methylated(Lu et al., 2006).

3.1 The Correlation between Methylation Rates and Phenotypic Traits

The methylation may affected *Scapharca broughtonii* phenotypic traits were studied with adductor muscle DNA samples of *Scapharca broughtonii* using 9 pairs of primers (Table 1). And Figure 1 show the correlation between methylation rates and phenotypic traits, results showed that DNA methylation rate 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, there was great correlativity between the DNA methylation and the gross weight (from Korean, the correlation coefficient is -0.59) (Figure 1). It indicated that DNA methylation affected on the shell length, the gross weight and the weight of soft body, DNA methylation may play an important part during the growth and development of the organisms, in the aspect of survival rate it has a different effect on Chinese and Korean populations.

Table 1. List of MSAP primers and adapters used

	EcoRI	MspI/HpaII
Adapters	EA ₁ :5'-CTC GTA GAC TGC GTA CC-3' EA ₂ :5'-AAT TGG TAC GCA GTC TAC-3'	HMA ₁ :5'-GAT CAT GAG TCC TGC T-3' HMA ₂ :5'-CGA GCA GGA CTC AGA A-3'
Primers for Preamplification	E ₀ :5'-GAC TGC GTA CCA ATT C-3'	HM ₀ :5'-ATC ATG AGT CCT GCT CGG G-3' HM ₁ : 5'-ATC ATG AGT CCT GCT CGG GC TGA-3'
Primers for Selective Amplification	E ₁ : 5'-GAC TGC GTA CCA ATT C ACA-3'	HM ₂ : 5'-ATC ATG AGT CCT GCT CGG GC TGT-3'
	E ₂ : 5'-GAC TGC GTA CCA ATT C AGT-3'	HM ₃ : 5'-ATC ATG AGT CCT GCT CGG GC TAT-3'
	E ₃ : 5'-GAC TGC GTA CCA ATT C AAC-3'	HM ₄ : 5'-ATC ATG AGT CCT GCT CGG GC TAC-3'
	E ₄ : 5'-GAC TGC GTA CCA ATT C GTC-3'	HM ₅ : 5'-ATC ATG AGT CCT GCT CGG GC TCA-3'
	E ₅ : 5'-GAC TGC GTA CCA ATT C GCT-3'	HM ₆ : 5'-ATC ATG AGT CCT GCT CGG GC TCT-3' HM ₇ : 5'-ATC ATG AGT CCT GCT CGG GC TTC-3' HM ₈ : 5'-ATC ATG AGT CCT GCT CGG GC TTA-3'

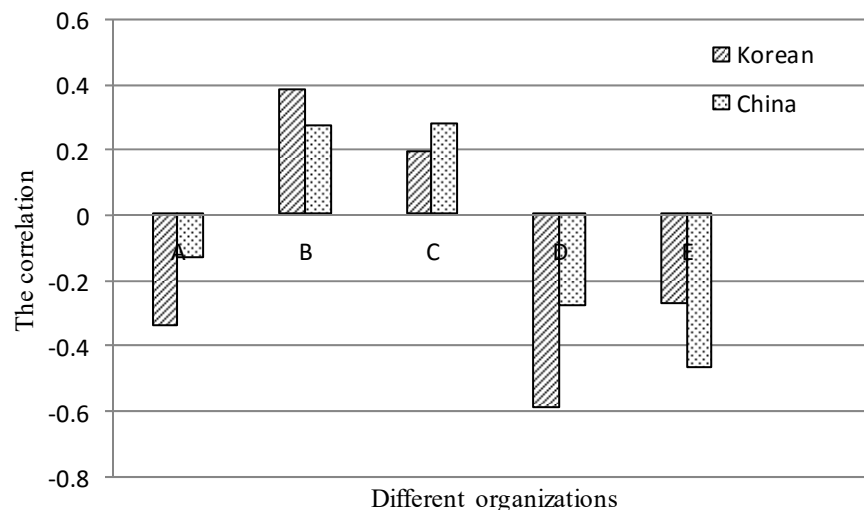


Figure 1. The correlation between methylation rates and phenotypic traits

A: The shell length; B: the shell broadness; C: the shell height; D: the gross weight; E: the weight of soft body

3.2 The Methylation Rates of Parental Lines and Hybrid

The two parental lines, their F1 hybrid were compared using tissue from adductor muscle with the same primers (Table 1). A total of 732 fragments were amplified, each of the fragments represented a recognition site cleaved by one or both of the isoschizomers. Firstly, the two parents showed significantly different degree of methylation (Figure 2): 197 differentially amplified fragments were detected in *Scapharca broughtonii* from Korean and 183 were observed in *Scapharca broughtonii* from China. Thus, approximately 31.6% of 5'-CCGG sites in *Scapharca broughtonii* (from Korean) genome were cytosine-methylated, in *Scapharca broughtonii* from China were 33%. In *Scapharca broughtonii* from Korean, the full methylation rate was 26.58%, the hemimethylation rate was 4.82%, in *Scapharca broughtonii* from China, the full methylation rate was 28.68%, and the hemimethylation rate was 5.39%. The methylation rates of F1 was less than the parents (Figure 2), a total of 231 fragments were amplified, and the rates of the methylation were 29.98%. In F1, full methylation of internal cytosines accounted for 79.94% of the methylated sites, and the remaining 20.06% were due to hemimethylation. The F1 inclined to the female parent on the traits, so the methylation rate was close to the *Scapharca broughtonii* from Korean, it was 31.6%.

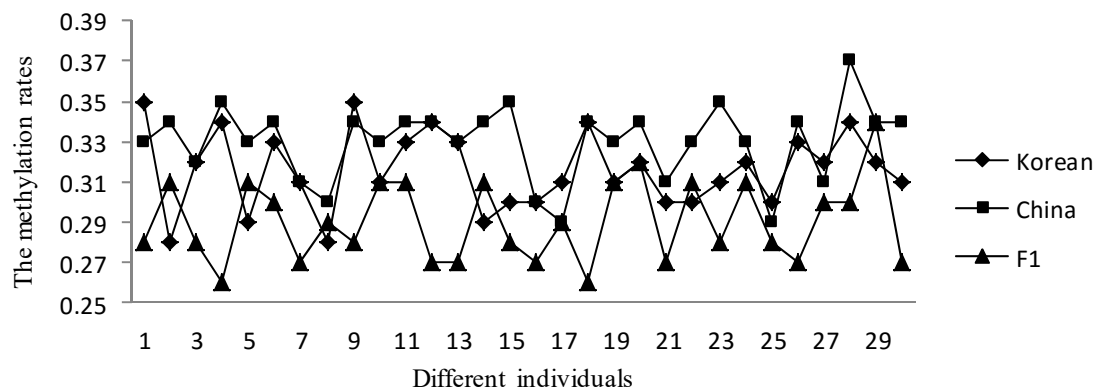


Figure 2. The methylation rates of parental lines and hybrid

Each group chose 30 individuals; each site stands for the average methylation rate of 9 pairs of primes.

3.3 Differential Methylation Patterns among Parental Lines and Hybrid

Four major classes of banding patterns were identified among the differentially amplified fragments (Table 3). In the first class (class A), the same methylation sites were detected in both parents and in the hybrid; these are referred to as monomorphic with respect to cytosine methylation, within the resolving power of this technique. In the four groups, 19 sites detected by 9 primer pairs reflected full methylation of the internal cytosine, and 6 sites were the result of hemimethylation. The second class (class B) showed simple Mendelian inheritance of the methylated bands, irrespective of the enzyme digest: A band that was detected in either parent was also detected in the hybrids. Class B could be divided into four subclasses, B1, B2, B3 and B4 (Table 2). B1 averagedly accounted for 3.1% methylated sites, B2 did 16.7%, B3 did 2.1% and B4 did 26.0% methylated sites. This class accounted for totally 46.9% methylated sites. Class C represents an increase in the level of methylation in the hybrid compared to the parental lines: a site detected in one or both parental lines was not observed in the hybrid (Table 2). Class C could be divided into 3 subclasses, C1 (a band was only revealed in *P. yessoensis*), C2 (a band was only revealed in *C. farreri*), C3 (a band was revealed in both of *C. farreri* and *P. yessoensis*). On the contrary, class D indicated demethylation in the hybrid genome. That is, a band was observed in the hybrids, but not in the parental lines (Table 2). The two classes occupied 26% methylated sites; with 18.7% belong to C and 7.3% to D.

Table 3. Patterns of cytosine methylation in parental lines and their F1 hybrid

Patterns	<i>Scapharca broughtonii</i> from Korean		<i>Scapharca broughtonii</i> from China		F1 hybrid		Number of sites
	HpaII	MspI	HpaII	MspI	HpaII	MspI	
A1	-	+	-	+	-	+	19
A2	+	-	+	-	+	-	6
B1	-	+	-	-	-	+	3
B2	-	-	-	+	-	+	16
B3	+	+	-	+	+	+	2
B4	-	+	+	+	+	+	25
C1	-	+	-	-	-	-	10
C2	-	-	-	+	-	-	6
C3	-	+	-	+	-	-	2
D1	-	+	-	-	+	+	0
D2	-	-	-	+	+	+	7

“-” stands for no band was found at the site; “+” stands for a band was found at the site.

4. Discussion

In recent years, there has been an increased interest in understanding the role of DNA methylation in regulating gene expression during the growth and development of the organisms (Chen, Ma, Chen, Song, & Zhang, 2009). The methylation sensitive amplified polymorphism (MSAP) technique has been used in various studies on cytosine methylation, and has proven to be a powerful tool for investigating DNA methylation. In this study, we have adapted the MSAP technique for the detection of cytosine methylation in the *Scapharca broughtonii* genome, the results showed that this technique is highly efficient for investigating the cytosine methylation of *Scapharca broughtonii*; all bands detected displayed good stability, reproducibility, and consistency.

The DNA methylation levels are varied in different species: 16.3% of the methylation has been reported to occur in the rice genome (Xiong et al., 1999), 35–43% in *Arabidopsis* of different ecological type (Cervera, Ruiz-Garcia, & Martinez-Zapater, 2002), 33% in wheat (Horváth et al., 2002), 15.7% in the developing seeds from *Brassica napus* (Lu et al., 2006). In animals, the pigs' methylation ratio is about 10%; the grass carp's methylation is very high, about 75.9%. Our research found that 31.6% of 5'-CCGG sites in *Scapharca broughtonii* from Korean genome were cytosine methylated, in *Scapharca broughtonii* from China were 33%, the DNA methylation level is different significantly to other organism, the differences may come from the detection method (such as the number of primes, the reaction conditions and time), the experiment material (different tissues: adductor muscle, mantle, gill filaments, gut, and gonad; or different collection time), and the genetic factors (the genetic factors play a more important part). Riddle (Riddle & Richards, 2002) found that natural variation in NOR methylation results from a combination of genetic and epigenetic mechanisms.

The purpose of this study was to develop an approach to investigating the possible role of methylation in the expression of heterosis. The genetic basis of heterosis has been debated for decades, but the mechanism underlying heterosis remains mysterious. Before the 1990s, two major hypotheses have been promulgated to explain this phenomenon: the dominance hypothesis and the overdominance hypothesis, but they are ideal and very limited, because they all thought that one trait was controlled by one allele. The fact is that more and more studies show that the appearance of quantitative character was the result of many alleles acting together. Many researchers used QTL method to study the heterosis, and put forth the hypothesis of epistasis, but the results were also not satisfactory (Z. Li et al., 2001; Luo et al., 2001; Xiao, Li, Yuan, & Tanksley, 1995; Yu et al., 1997).

Now, many researchers believe that the molecular basis of heterosis may be attributed to the increased gene expression level in the hybrid (Leonardi et al., 1991; Romagnoli et al., 1990; A. Tsafaris et al., 1997; S. Tsafaris, 2006) or to the altered regulation of gene expression in the hybrid either at the global level or for specific classes of genes.

The phenotypic traits that the organism expressed are the results of gene expression, an individual take on an advantage in a trait, it should be the result of over-expression of some genes or under-expression of some genes, that is to say, the over-expression and under-expression of some genes lead to the expression of heterosis together. Romagnoli et al. (Romagnoli et al., 1990) found that heterosis may derive from simple dominant or codominant gene effects in addition to the increased expression of certain loci. Tsafaris et al. (A. Tsafaris et al., 1997) found that the transcriptional level of 35 gene were higher than the parents lines. Sun et al. (Sun et al., 2004) found that 30% genes were differentially expressed between hybrids and their parents, which play an important role for hybrids to demonstrate heterosis. Li et al. (X. Li, Wei, Nettleton, & Brummer, 2009) found that nonadditive expression of transcript levels may contribute to heterosis for biomass yield in alfalfa. Zhao (Zhao, Chai, & Liu, 2007), Meng (Meng, Ni, Wu, & Sun, 2005), Uzarowska et al. (Uzarowska et al., 2007) received the similar results.

Considering effects of DNA methylation on gene expression (Finnegan et al., 2000; Jacobsen et al., 2000; Rangwala & Richards, 2004), Hyper-methylation can lead to the gene silence, and demethylation can lead to the over-expression (Neves, Heslop-Harrison, & Viegas, 1995; Sardana, O'Dell, & Flavell, 1993), there may be a correlation between the DNA methylation and heterosis.

A negative correlation was found between DNA methylation and the economic characters of shell length, shell height, soft body weight and adductor muscle weight, Wan (Wan, 2008) found that, in the Chongqing mountainous cattle and their hybrids, methylation affected heart girth and body weight highly significantly, methylation and body slanting length significantly. Methylation content and heterosis rates of heart girth, body weight showed significant correlation, it just indicated indirectly that DNA methylation have a correlation with the gene expression. Zhang, Shiu, Cal, and Borevitz (2008) found that cytosine methylation alterations immediately upstream or downstream of the gene were inversely correlated with the degree of expression variation for that gene. Jin found a direct relationship between cytosine methylation alteration and gene expression variation. So we can say DNA cytosine methylation alteration lead to the expression of heterosis in some extent.

The methylation of the *Scapharca broughtonii* from Korean and China are different, the cytosine methylation ratio of the filial generation will between the parents in theory, but the result was that the filial generation cytosine methylation ratio was lower than any parents. And four classes of patterns of cytosine methylation characterized by differences in degree of methylation between the hybrids and parental lines: (1) the same level of methylation in both parental lines and the hybrids, (2) the same level of methylation in either parent or hybrid, (3) an increased level of methylation in the hybrids compared to the parents, (4) a decreased level of methylation in the hybrids, and the number of demethylation sites were more than the number of the hypermethylation sites. Hepburn found the same result, Tsiftaris (A. Tsiftaris et al., 1997) found that hybrids were less methylated than inbreeds. It was further proposed that differential DNA methylation patterns in hybrids may play an important role in materializing heterosis

It has been widely recognized that, in animals, the inheritance of the epigenetic state through mitotic rounds of cell division is relatively faithful, in development (embryogenesis and gametogenesis) the epigenetic state is reset, that is, erased and reestablished; and parental epigenetic state in plants is often stably inherited to sexual progenies (Cubas, Vincent, & Coen, 1999; Monk, Boubelik, & Lehnert, 1987; Riddle & Richards, 2002). Penterman thought that demethylation processes may be the result of a deficiency in enzymatic maintenance after DNA replication or an active enzymatic process involving plant glycosylases with specific functions in genomic imprinting and to protect genes from potentially deleterious methylation (Grewal & Elgin, 2002; Grewal & Klar, 1996; Penterman et al., 2007). Grewal and Klar (Grewal & Klar, 1996) showed that the epigenetic modification of a reporter gene placed in the mating-type region of *Schizosaccharomyces pombe* could be inherited through mitosis and meiosis. Furthermore, they showed that loci influencing this process were, either directly or indirectly, involved with the organization of heterochromatin (Grewal & Klar, 1996). More recent work has shown that these modifiers include histone deacetylases, histone methyltransferases and other structural proteins associated with telomeres and centromeres (Grewal & Elgin, 2002).

Anyway, the change of the DNA cytosine methylation took place, though the mechanism is still unknown. Two different alleles brought together in the hybrid may create a combined allelic expression pattern in the hybrids. Alternatively, at some loci, allelic interaction or a change in the spectrum of trans-acting factors causes gene expression in the hybrid to deviate from simple additive allelic expression patterns of the parents (Birchler et al., 2003; Gibson & Weir, 2005). The patterns of the F1 DNA methylation experienced change and adjustments in order to coordinate the expression of the gene from the parents, most of the cytosine sites' methylation patterns could be inherited to next generation stably, some sites' methylation patterns experience hypermethylated and demethylated, the demethylation cause some gene expressing largely, the hypermethylation restrain the expression of some gene, with the participation of the environment, a new methylation patterns was formed which added together the parents' characters and can adapted to the circumstances very well, so the heterosis is the result of the differential expression of some gene, the DNA methylation also have to do with the expression of heterosis.

Generally speaking, after investigating the difference of methylation between the parents and the offspring, we can find out the special methylation sites which may play an important part in the expression of heterosis, and then using the methods of sequences analysis and genetic analysis to find out the inherent factor of heterosis. The genomes of the animals and plants contain a great quantity of CG dinucleotides, and the methylation sequences which control the expression of some functional gene also contain a great quantity of CG dinucleotides, so it is easy to find out the change regularity of the methylation. The advantages of MSAP include simplicity, rapidness, low cost, higher sensitivity, and high polymorphism, however, this technique also has some limitations associated with resolving power.

In some organisms, methylation also occurred in CAGs and CTGs. Furthermore, the types of non-methylation and inner-methylation of a single strand can not be distinguished as both HpaII and MspI are capable of recognizing the sites of non-methylation and inner-methylation of a single strand, thus revealing similar patterns of methylation following PCR amplification. Moreover, outer methylation of double strands can not be detected through MSAP analysis. For these reasons, actual levels of the cytosines methylation are likely to be significantly higher than those detected in this study. Notwithstanding some limits, the results here reported show this technique to be highly efficient for large-scale detection of cytosine methylation in *Scapharca broughtonii*. The ability to isolate and amplify these MSAP fragments may thus make possible direct identification of sequences which play a role during the expression of heterosis.

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