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Analysis of DNA Methylation Level by Methylation-Sensitive Amplification Polymorphism in Half Smooth Tongue Sole (*Cynoglossus semilaevis*) Subjected to Salinity Stress

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Abstract Increasingly arisen environmental constraints may contribute to heritable phenotypic variation including methylation changes, which can help the animals with development, growth and survival. In this study, we assessed the DNA methylation levels in three tissues (gonad, kidney and gill) of half smooth tongue sole under the salinity stress. The methylation-sensitive amplification polymorphism (MSAP) technique was applied to illustrate the regulation of epigenetic mechanism in environmental stimuli. Fish were subjected to 15 salinity treatment for 7 and 60 days, respectively. A total of 11259 fragments were amplified with 8 pairs of selective primers. The levels of methylated DNA in different tissues of females and males without salinity stress were analyzed, which were 32.76% and 47.32% in gonad; 38.13% and 37.69% in kidney; 37.58% and 34.96% in gill, respectively. In addition, the significant difference was observed in gonad between females and males, indicating that discrepant regulation in gonadal development and differentiation may involve sex-related genes. Further analysis showed that total and hemi-methylation were significantly decreased under 15 salinity for 7 days, probably resulting in up-regulating salt-tolerance genes expression to adjust salt changing. With the adjustment for 60 days, total and hemi-methylation prominently went back to its normal levels to obtain equilibrium. Particularly, full methylation levels were steady along with salinity stress to maintain the stability of gene expression. Additionally, the data showed that gonads in females and gills in males were superior in adaptability. As a result, DNA methylation regulates tissue-specific epiloci, and may respond to salinity stress by regulating gene expression to maintain animal survival and activity.

Key words DNA methylation diversity; half smooth tongue sole; salinity stress; MSAP

1 Introduction

Nowadays the importance of epigenetic mechanism has brought growing attention in gene expression and regulation, characterized by histone post-translational modification and DNA methylation (Chinnusamy, 2009). In as early as year 1975, it was elucidated that cytosine methylation at CpGs can be considered as a genetic marker in vertebrate, and the genomic methylation occurs in pervasive CpGs (Ehrlich *et al*., 1982; Gama-Sosa *et al*., 1983). CpG methylation repressed gene transcription directly by inhibiting specific gene regulatory elements, or making methyl-binding proteins incorporated to the methylated DNA ultimately 'closing' the chromatin structure (Boerboom *et al*., 1999; Fitzpatrick and Richards, 1991; Ziller *et al*., 2013). In *Paralichthys olivaceus*, the differential CpG methylation levels in promoters could be the reason for various expression of *dmrt1* and *cyp19a* in the gonad (Wen *et al*., 2014). In addition, methylation of the *sb cyp19a* promoter caused the lower expression of *cyp19a* in temperature-dependent European Sea Bass (Laia *et al*., 2011). Therefore, associated with gene silence or super-activity, DNA methylation has been widely proposed to be a predominant epigenetic mechanism without changing the DNA sequences (Habu *et al*., 2001; Bird, 2002). In recent decades, epigenetic studies have already defined the crucial roles of DNA methylation in X chromosome inactivation (Allen *et al*., 1992), gene imprinting (McGrath and Solter, 1984; Tycko, 1997; Henckel and Arnaud, 2010), memory and ageing regulation (Tserel *et al*., 2014), and miRNA expression (Lujambio *et al*., 2008). Recent studies have made great advancements in the field of DNA methylation regulatory mechanism. Compared with normal somatic cell, promoter DNA methylation of specific-expression genes differs in mouse germline (Hackett *et al*., 2012). Similarly, Li *et al.* (2011) investigated the shoot regeneration of *Arabidopsis*, finding that DNA methylation and histone modifications are

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important in modulating auxin signaling and WUS expression. Overall, DNA methylation emphasizes its crucial role in tissue-specific and developmental stage-specific gene expressions.

Different animal species resist to various kinds of biotic and abiotic stresses, depending on gene expression and reprogramming metabolism, to modify a physiology equilibrium for development, growth and survival. Growing evidences indicate that environmental and genetic stimuli may contribute to heritable phenotypic variation with methylation changes (Angers *et al*., 2010). Arsenic-exposion results in DNA hypermethylation in the promoters of gene p53 and p16 in human beings (Chanda *et al*., 2006) and peripheral blood leukocyte in Bangladeshi adults (Pilsner *et al*., 2007). When exposing adult zebrafish to estrogens, the methylation level in the 5'-flanking region of Vitellogenin was decreased, resulting in gene expression abduction (Stromqvist *et al*., 2010). In addition, Navarro-Martín *et al*. (2011) found that DNA methylation influenced by temperature may lead to sex ratio shifts in European sea bass. Varriale *et al*. (2006) also discovered Antarctic fishes are more likely than tropical fishes in DNA methylation, indicating that temperature is related with DNA methylation. Methylation-sensitive amplification polymorphism (MSAP) technology is a modified classic AFLP, with the isoschizomers *Msp*I and *Hpa*II which can recognize and cleave the same CCGG sites with different methylation sensitivity, allowing the detection of DNA methylation (Shan *et al*., 2013; Mastan *et al*., 2012; Sun *et al*., 2014; Zhao *et al*., 2015). *Msp*I is sensitive only to internal methylated cytosine, while it cleaves the methylated sites but not the external one. To the contrast, *Hpa*II is inactive if both strands cytosine are fully methylated, while it cuts the hemi-methylation sequence when the outer cytosine is methylated in a single strand. Methylated sites that at both external and internal cytosine or external cytosine in double-strand, or internal cytosine in single-strand, are unable to be detected by either enzyme. Consequently, the compositions of *EcoR*I/*Msp*I and *EcoR*I/*Hpa*II profiles allows detecting the methylation level at one site. MSAP technology has proven to be reliable, simple and highly efficient in detecting genomic cytosine methylation. But it should be considered that true values may be underestimated due to a limit of endonuclease enzyme unrecognized methylation in non-CCGG sequence (Yaish *et al*., 2014). MSAP has been made important advances in epigenetic variation. Using MSAP, the hypermethylation occurring along with the deep-aestivation in Adult sea cucumbers was observed, suggesting the importance of DNA methylation in regulating global transcriptional suppression during aestivation (Zhao *et al*., 2015). In addition, MSAP has been applied to detect the tissue-specific DNA methylation patterns in pigs (Ma *et al*., 2012) and Zhikong Scallop (Sun *et al*., 2014) to study the epigenetic regulatory mechanism in tissue differentiation and development.

As a major constrain for fish survival and development, salinity stress also induces physiological drought indirectly, which may contribute to heritable phenotypic variation with methylation changes. The ability of tolerating salinity stress varies in different fish species, depending on epigenetic modifications. The researches of salinity stress have increased significantly. However, most of them are predominantly focused on feed efficiency, energy equilibrium, oxygen consumption rate and ammonia excretion rate, as well as other physic-biochemical aspects (Alliot *et al*., 1983; Koshiishi *et al*., 1986; Watanabe *et al*., 1993). There is rarely study on epigenetic modification that regulates the gene expression in response to salinity stress in fish. As a commercially valuable euryhaline flatfish, half smooth tongue sole (*Cynoglossus semilaevis)* is widely distributed in the coastal water of China. It is gaining importance as one of the most potential marine species for Chinese aquaculture, considering its commercial value, appealing taste, lacking natural resource and easy domestication (Liao *et al*., 2009). In addition, female individuals grow faster than male ones, with two to three times larger in size. Moreover, in terms of genomic DNA, the studies of epigenetic modification on adaptability and tissues-specificity have poorly been explored. In summary, the present study was carried out to investigate the changes of DNA methylation and polymorphism of methylation patterns, especially for growth and osmotic regulation, in three tissues (gonad, kidney and gill) of *Cynoglossus semilaevis* when subjected to salinity stress. The MSAP technique revealed stimulatory effect on genome-wide DNA methylation by differential regulation of gene expression. Additionally, the epigenetic diversity and differentiation among tissues were also studied in this research.

2 Materials and Methods

2.1 Animal Treatment, Stress Treatments and Sampling

A cohort of 10 months old female and male half smooth tongue sole (*Cynoglossus semilaevis*) with an average body weight 101.58 ± 32.07 g and average body length 25.39 ± 3.52 cm were collected from a commercial fish farm (Qingdao, China). Prior to the experiment, by diluting the seawater with fresh water, salinity was decreased to the suitable range of 15 (Wang *et al*., 2003) at the rate of 5 daily. Fish were randomly distributed into 6 pools with forty fish in each one. The fish cultured in natural sea water (salinity 30) were considered as control group (NS). The fish cultured in salinity 15 for 7 (LS1) and 60 (LS2) days were regarded as treatment groups. The experiments were carried out at 22℃–24℃ with continuous aeration (dissolved oxygen $>6\,mg\,L^{-1}$). The fish were fed twice a day with compound feed (3%–5% of body weight) before half seawater was replaced daily. Fish (n=6, NS, LS1 and LS2) were harvested from each pool, anaesthetized with 0.15% MS-222 (Sigma, St. Louis, MO). Gonad, kidney and gill were rapidly isolated from the fish under sterile conditions. They were snap-frozen in liquid nitrogen and stored at −80℃ till further analysis. Three females and three males from each treatment were taken for MSAP analysis.

2.2 Total Genomic DNA Isolation and MSAP Analysis

Genomic DNA from each tissue was isolated using Marine Animal Genomic DNA Kit (TransGen, Beijing, China) according to the manufacturer's instruction. Finally, the concentration of total DNA was quantified by the nucleic acid analyzer Biodropsis BD-1000 (OSTC, China) and the integrity of purified DNA templates was detected with 1.5% agarose gel.

In the MSAP system, DNA samples were digested with methylation sensitive isoschizomers (*Msp*I and *Hpa*II) (TaKaRa, Dalian, China) combined with an internal control restriction enzyme (*EcoR*I) (TaKaRa, Dalian, China) separately. The reaction was conducted as following: to the genomic DNA (600 ng), 0.1% BSA 0.2 μL, 10×T Buffer 3.5 μL, *Msp*I (or *Hpa*II) 3 U, *EcoR*I 4.5 U, and sterile Milli Q water were added to a total volume of 20 μL. The reaction mixture was incubated at 37℃ for 4 h, and then was inactivated at 65℃ for 10 min. The ligation reaction solution included digested products 10 μL, 10×T4 DNA Ligase Buffer 2 μL, 50 μmol L^{-1} *Msp*I/*Hpa*II adapter (HM-A: 5'-CGAGCAGGACTCATGA-3', Table 1) 5 μL, 5 μmol L^{-1} *EcoR*I adapter (E-A:5'-CTCGTAGACTGC GTACC-3', Table 1) 2 μL, 350 U μL^{-1} T4 DNA Ligase (TaKaRa, Dalian, China) 1 μL. The ligation reaction was performed at 16℃ overnight. A 1 μL of the above ligated DNA was pre-amplified in 20 μL volume with 10×EasyTaq@ Buffer 2 μL, 2.5 mmol L^{-1} dNTPs 2 μL, 10 μM of each pre-amplification primer (E0: 5'-GACTGCGTAC CAATTCA-3' and HM0: 5'-ATCCATGAGTCCTGCTC GGT-3', Table 1) 2 μL, 10 U μL^{-1} EasyTaq@ DNA Polymerase 0.3 μL, sterile Milli Q water 10.7 μL. The PCR program was as follows: 94℃ degeneration for 5 min, 25 cycles of 94℃ for 30 s, 56℃ for 1 min and 72℃ for 1.5 min. Then 72℃ extension for 10 min and 60℃ incubation for 30 min. Selective amplification was conducted in 20 μL volume with 10-fold diluted pre-amplification product 5 μL, 10×EasyTaq@ Buffer 2 μL, 2.5 mmol L^{-1} dNTPs 2 μL, 10 μmol L^{-1} of each selective-amplification primer (E000 and HM000, Table 1) 2 μL, 10 U μL^{-1} EasyTaq@ DNA Polymerase 0.3 μL, sterile Milli Q water 6.7 μL. The selective amplification program were run under the conditions: 94℃ degeneration for 5 min, subsequent 13 cycles of 94℃ for 30 s, 64℃ for 30 s (successively reduced each cycle by 0.7℃) and 72℃ for 1.5 min; following 20 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 2 min. Finally 72℃ extension for 10 min and 60℃ incubation for 30 min. The 5 μL of selective amplification products were loaded onto 8% non-denaturing polyacrylamide gel in 1×TBE buffer to separate by electrophoresis at 200 V for 2.5 h. Stained the gels with EB solution for 30 min, finally, the fragments were scanned and visualized by gel imaging and analysis system (ChampGel, China) for further data analysis.

Table 1 Sequences of adapters, pre- and selective-primers for MSAP

Name	No.	Sequence (5'-3')
Adapter	E-A	5'-CTCGTAGACTGCGTACC-3'
		5'-AATTGGTACGCAGTCTAC-3'
	HM-A	5'-CGAGCAGGACTCATGA-3'
		5'-GATCATGAGTCCTGCT-3'
Pre-primers	E0	5'-GACTGCGTACCAATTCA-3'
	HM0	5'-ATCCATGAGTCCTGCTCGGT-3'
Selective-primers	E1	5'-GACTGCGTACCAATTCAGT-3'
	E2	5'-GACTGCGTACCAATTCAGA-3'
	E3	5'-GACTGCGTACCAATTCAAG-3'
	E4	5'-GACTGCGTACCAATTCACT-3'
	E5	5'-GACTGCGTACCAATTCAAC-3'
	E6	5'-GACTGCGTACCAATTCACA-3'
	E7	5'-GACTGCGTACCAATTCACT-3'
	HM1	5'-ATCCATGAGTCCTGCTCGGTAC-3'
	HM2	5'-ATCCATGAGTCCTGCTCGGTAG-3'
	HM3	5'-ATCCATGAGTCCTGCTCGGTTC-3'

2.3 MSAP Data Scoring

In the electrophoresis profiles (Fig.1), only unambigu-

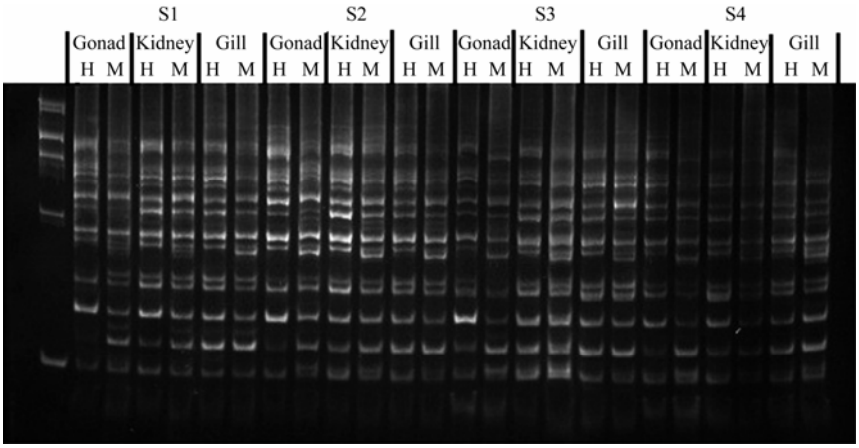

Fig.1 An example of MSAP profiles amplified by a pair of selective-primer (HM2 and E3) in half smooth tongue sole, showing the different types of DNA methylation. S1, S2 were male fish under salinity 15 for 7 days; S3, S4 were female fish under salinity 30. H stands *Hpa*II/*EcoR*I lane; M stands *Msp*I/*EcoR*I lane. Hemi-methylation, viz. TypeI, was defined while only in H lane not in M lane detected the presence of bands. Full methylation, viz. TypeII, was defined while only in M lane not in H was detected the presence of bands. Total methylation, viz. Type III, was defined while both in H and M lanes detected the presence of bands.

ous and reproducible fragments were scored for data statistics. The different types of bands were counted three times to minimize artificial error. According to the electrophoresis profiles, '1' and '0' show the presence and absence of bands in the lanes, respectively. Hemi-methyllation, viz. TypeI, was defined while only in *Hpa*II/ *EcoR*I (H) lane not in *Msp*I/*EcoR*I (M) lane the presence of bands was detected. Full methylation, viz. TypeII, was defined while only in *Msp*I/*EcoR*I lane not in *Hpa*II/ *EcoR*I lane the presence of bands was detected. Total methylation, viz. Type III, was defined while both in *Hpa*II/*EcoR*I and *Msp*I/*EcoR*I lanes the bands were detected. The absence of bands in both *Hpa*II/*EcoR*I and *Msp*I/*EcoR*I lanes were considered to be genetic polymorphism or hyper-methylation (Keyte *et al*., 2006). The formula was calculated for methylation ratio:

$$\text{Hemi-methylation ratio (\%)} = \text{TypeI bands}/(\text{TypeI bands} + \text{TypeII bands} + \text{TypeIII bands}) \times 100 ,$$

$$\text{Full methylation ratio (\%)} = \text{TypeII bands}/(\text{TypeI bands} + \text{TypeII bands} + \text{TypeIII bands}) \times 100 ,$$

$$\text{Total methylation ratio (\%)} = (\text{TypeI bands} + \text{TypeII bands}) \times 100/(\text{TypeI bands} + \text{TypeII bands} + \text{TypeIII bands}) \times 100 .$$

2.4 Statistics Analysis

The significant difference of methylation levels between control and treatment groups in different tissues were performed using the SPSS 19.0, followed by one-way ANOVA within Duncan's multiple range tests. Independent Samples t-test was conducted to determine differences of DNA methylation between female and male fish in each tissue. Statistical significance was accepted as $P< 0.05$.

3 Results

3.1 Epigenetic Diversity Between Tissues in Natural Sea Water

To assess DNA methylation status in three tissues of female and male half smooth tongue sole that were subjected to salinity stress for 7 d and 60 d, a total of 21 selective primer pairs were used to amplify the MSAP fragments, while only high repeatability and unambiguous primers (E1/HM1, E3/HM1, E4/HM1, E5/HM1, E7/HM1, E3/HM2, E4/HM2, E5/HM2, E7/HM2, E7/HM3) were utilized in large-scale amplification. In total, the results indicated that 3764, 3704, 3791 fragments were amplified in gonad, kidney and gill, respectively. Furthermore, 378/450, 325/393, 397/293 were detected as TypeI/TypeII methylation sites for each of the above three tissues, respectively. Most of the fragments were amassed from 100 bp to 700 bp, while nearly no fragments that are above 1000 bp were observed.

For half smooth tongue sole without salinity stress, different DNA methylation degree in each tissue of females and males are shown in Table 2. In female fish, total methylation levels were close to identical in gonad, kidney and gill, which were 32.98%, 38.13% and 37.58%, respectively. The hemi-methylation levels were 16.79%, 20.63% and 20.90%, which accounted for the main dominant type of the above three tissues, respectively. As for full methylation, the substantial fraction, it made nearly no difference across gonad, kidney and gill, taking percentage about 15.97%, 17.50%, 16.68%, respectively. Furthermore, total and full methylation levels have showed that there were no significant differences between the tissues, but significant differences of hemi-methylation levels were observed between gonad and kidney.

Table 2 Levels of DNA methylation across surveyed tissues of female and male half smooth tongue sole under non-salinity stress

Sex	Methylation type	Gonad	Kidney	Gill
Female	Hemi-methylation	16.79±0.66^{a}	20.63±0.88^{b}	20.90±3.38^{ab}
	Full methylation	15.97±1.22^{a}	17.50±4.11^{a}	16.68±5.34^{a}
	Total methylation	32.98±1.68^{a}	38.13±4.60^{a}	37.58±2.08^{a}
Male	Hemi-methylation	29.86±5.72^{a}	19.05±3.23^{a}	22.90±8.58^{a}
	Full methylation	17.47±7.04^{a}	18.64±5.60^{a}	12.06±7.21^{a}
	Total methylation	47.32±1.41^{a}	37.70±5.01^{b}	34.96±6.47^{b}

Notes: Data represents mean ± SD; Different lowercases on the data are showing significant difference from each other ($P<0.05$, Duncan's test).

In male fish, total methylation was as high as 47.32% in gonad, followed by 37.70% in kidney and 34.96% in gill. Hemi-methylation turned out 29.86%, 19.05% and 22.90% for these tissues, respectively. As for full methylation, the results indicated that it was quite similar across gonad, kidney and gill, with 17.47%, 18.64% and 12.06%, respectively. It should be noted that total methylation in gonad was the highest which significantly exceeded those in the other tissues.

3.2 Total Methylation Levels of Various Tissues Subjected to Different Salinity Stress

For salinity stress, different DNA methylation levels in each tissues for female and male half smooth tongue soles are shown in Fig.2A, Fig.2B and Fig.2C, respectively. In female fish, total methylation (including hemi and full methylation) levels of genomic DNA in gonad under NS, LS1 and LS2 were 32.98%, 28.06% and 41.57%, respectively, and the level under LS2 was significantly higher as compared to NS (by 8.59%, P value 0.005) and LS1 (by 13.51%, P value 0.001). In kidney and gill, as subjected to different salinity stresses, total methylation levels under NS was dropping under LS1 (by 11.04%, P value 0.005 and 18.23%, P value 0.001, respectively) and rising in kidney and gill under LS2, changing obviously.

In the gonad tissue of male fish cultured under salinity stress, total methylation was as high as 47.32% with NS, falling significantly to 24.87% with LS1 (P value 0.001), then rising significantly to 42.86% with LS2 (P value 0.02). In kidney, total methylation under NS was 37.70%, contrasted to LS1, significantly dropping to 22.60% (P

value 0.031) and later rising to 34.87% under LS2. In gill, total methylation was declined from 34.96% to 23.14% firstly (*P* value 0.011), then surged to 38.86% under above salinity stress (*P* value 0.003).

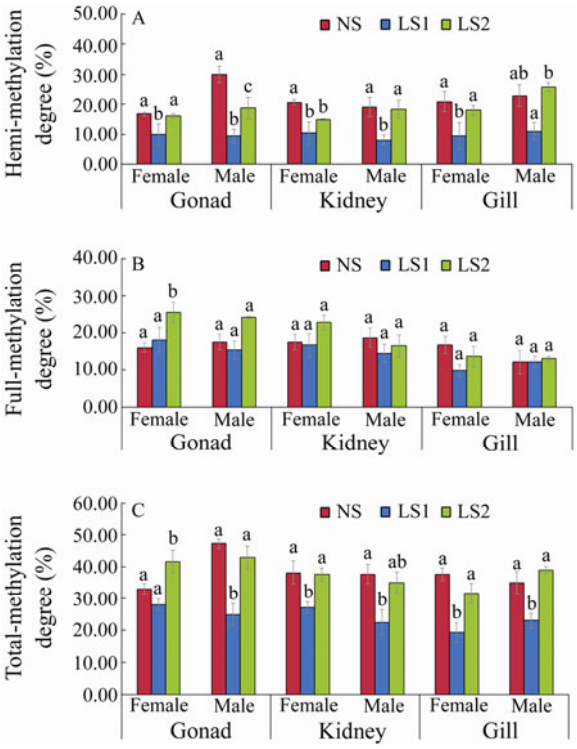

Fig.2 Three types of methylation levels (%) in three tissues of half smooth tongue sole subjected to salinity stress. Normal salinity (NS), 15 salinity for 7 days (LS1) and 15 salinity for 60 days (LS2). Data represents mean ± SD, different lowercase letters represents significances subjected to salinity stress ($P < 0.05$, Duncan's test).

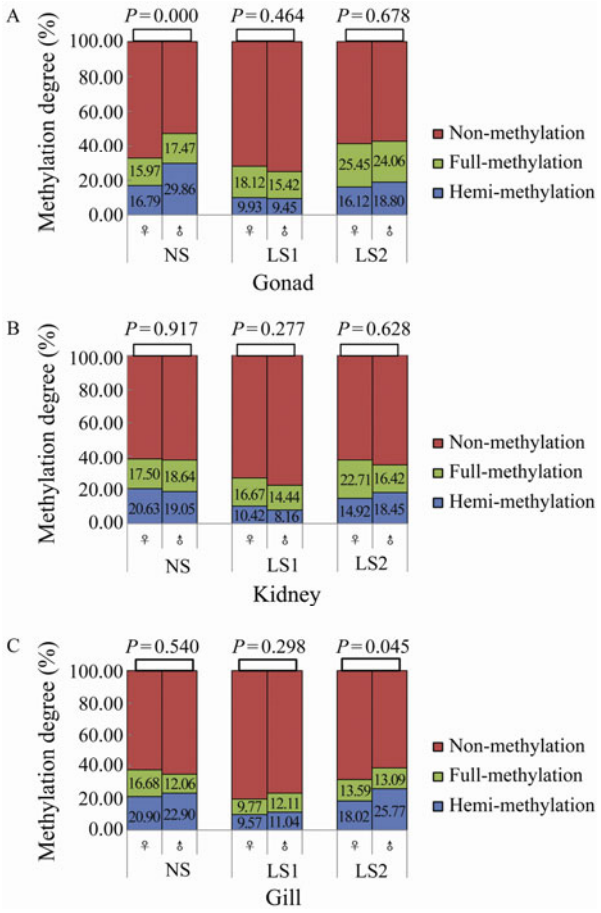

Fig.3 Variations in levels of DNA methylation across females and males for gonad (A), kidney (B) and gill (C) subjected to salinity stress. The independent samples t-test was conducted to compare the total methylation between females and males. $P < 0.05$ represents statistically significant. Normal salinity (NS), 15 salinity for 7 days (LS1) and 15 salinity for 60 days (LS2). Data represents mean ± SD, different lowercase letters represents significances subjected to salinity stress ($P < 0.05$, Duncan's test).

In almost all the three tissues with salinity stress in both female and male fish, full methylation was observed nearly with no significant changes. Meanwhile, the consistent regularity that first down then up in two types of methylation was displayed. The changes of total methylation could be traced to significant variation in hemi-methylation levels, along with small, slightly or no significant change in full-methylation levels.

3.3 The Gender Bias in Tissues with Different Salinity Stresses

To estimate gender bias among tissues with different salinity stresses, total methylation levels were analyzed with Independent Samples *t*-test ($P < 0.05$). As shown in Fig.3A, 3B and 3C. The male fish were significantly higher than in female fish in gonad (by 14.57%, *P* value 0.00) under NS, and in gill (by 7.25%, *P* value 0.045) under LS2. There was no significant difference in kidney and gill with NS. The total methylation in females was higher than in males, while the difference was not significant.

4 Discussion

In the recent years, MSAP has been widely applied in revealing epigenetic modifications under adverseness, which is modified the classic AFLP (Shan *et al*., 2013; Mastan *et al*., 2012; Sun *et al*., 2014; Zhao *et al*., 2015). This method depends on the function of *Hpa*II and *Msp*I, the isoschizomers which can recognize and cleave the same CCGG sites with different methylation sensitivity. DNA methylation has been well-researched in marine vertebrate, mammals and plants. In addition, regulating gene expression and tissue differentiation and development (Wu *et al*., 2001), DNA methylation plays an important role in resisting the adversity stress (Bird, 1986; Wada *et al*., 2004; Hashida *et al*., 2006; Pilsner *et al*., 2007). Salinity is a vital environment factor that has been widely studied in fish growth and physiological ecology (Likongwe *et al*., 1996; Tian *et al*., 2010). It has been reported some salt-tolerant plants adjust themselves in molecular levels, with changing gene expression or stare a new gene expression, to defense salinity stress (Xie *et al*., 2005). Notably, some salt tolerance-related genes had already comprehensively explored, such as aquaporins gene PtAQP, chloride intracellular channel gene PtCRT in

Portunus trituberculatus (Wang *et al*., 2014), and TRPV4, PRLI, Na^+-K^+-ATPase alpha1 (NKAα1) genes in *Oreochromis mossambicus*, *O. hornorum* and their hybrids (Liu, 2014). These genes played important roles in mediating the osmotic pressure. Being sensible to salinity variation, some hormones that can act in somototrophic axis such as cortisol, prolactin, GH and IGF group, were also involved in osmoregulation process (Boeuf and Payan, 2001). Furthermore, salinity notably acts as an environmental factor on physiology and biochemistry, histology and digestive enzyme activity, causing adverse conditions to the growth, development and survival of animals (Chen *et al*., 1998; Wang and Zhu, 2002; Liu *et al*., 2010). Currently, a great deal of studies have been carried out focusing on the reproductive biology, early development and physio-biochemical changes in response to abiotic stress in half smooth tongue sole, whereas poor attentions have been given to molecular regulatory mechanism of salinity stress. In the present study, we use MSAP to evaluate genomic DNA methylation levels in various tissues of half smooth tongue sole subjected to salinity stress. The results showed that there was a significant difference in methylation levels with salinity stress among and within different tissues. Remarkably, the methylation levels significantly differed in female and male fish.

DNA methylation is essential to tissue development and differentiation (Choe, 2008). For instance, Finnegan *et al*. (2000) had reported that DNA methylation level in plants ranged from 4.6% to 30%, while 40% to 50% in birds and mammal, and marine animals mainly amassed 20% to 50% (Leutwiler *et al*., 1984). In this study, the methylation levels of half smooth tongue sole were calculated to be 34.96%–47.32% for above tissues, which was slightly higher than medium. Non-methylation levels had taken the vast majority, as in *Chlamys farreri* (Sun *et al*., 2014) and pigs (Ma *et al*., 2012). Meanwhile, hemi-methylation levels exceeded full methylation levels, indicating that methylation of single-stranded DNA is superiority in CCGG sites in half smooth tongue sole, inferring the function of DNA methylation is important, to some extent, in gene regulation and function. The result was consistent with those reported in pigs (Ma *et al*., 2012) and chicken (Xu *et al*., 2011), and was different to those in *Crassostrea gigas* (Jiang *et al*., 2014) and *Apostichopus Japonicus* (Guo *et al*., 2013). The difference overall revealed that dominant position for full or hemi-methylation differs in different species. In addition, full methylation levels steadily ranged from 12.06% to 18.64% in three tissues, with no significant difference along with salinity stress, suggesting that it is necessary in maintaining the stability of gene expression, which was vital important in animal survival. In adversity stress, DNA methylation had an essential role in tolerability with differential fragment increased expression (Shan *et al*., 2011). As a result of changes in salinity, hemi and total methylation in three tissues of both female and male half smooth tongue soles presented a complete consistent tendency, with significant dropping in LS1 then striking rising in LS2. The result suggested that salinity stress, to some extent (15), could result in gene expression changing in half smooth tongue sole, to ensure its survival and activity. In response to 15 salinity for 60 days, half smooth tongue sole acclimatize themselves with low-salt-tolerance and regulation ability. Consequently, DNA methylation returned to its normal levels. Kovalchuk *et al*. (2003) had provided that hypomethylation, a defense mechanism, was a simple and indirect protection way to adversity stress, with accurately regulating gene expression. Recently, Li *et al.* (2011) had shown the identical changing tendency of DNA methylation in *Carthamus tinctorius* under salt stress, which also supports our results. In female fish, the total methylation in gonad under LS1 decreased not significantly ($P > 0.05$) compared to kidney and gill, indicating that gonad was superior in adaptability. Furthermore, in male fish, the total methylation in kidney had no significant rebound under LS2, showing the long reaction time in kidney. As a euryhaline species, half smooth tongue sole can survive with both high and low salinities. Gill is considered to be the main regulatory organ in adjusting different salt degrees (Pisam *et al*., 1991). Correspondingly, total methylation in gill was the lowest and conspicuously declined under LS1, revealing gill was more adaptable than gonad and kidney in male fish.

DNA methylation is essential to tissue development and differentiation (Choe, 2008). In female fish, hemi-methylation levels in gonad was significantly ($P < 0.05$) lower than those in kidney and gill. To the contrast, total methylation in male gonad was significantly higher ($P < 0.05$). The different DNA methylation levels show that there are tissue specificity in epigenetic regulation during differentiation process. For example, DNA in DLY pig adipose tissue was more methylated than in muscle for energy storage and endocrine (Liu, 2009). Likewise, when studying the effects of promoter methylation on the regulation of human blood T cells, Yano *et al*. (2003) found cells regulate the genes through tissue-specific DNA methylation levels. For many animals, like chicken, pig, aquatic animals and plants, DNA methylation levels had been identified to be significantly different (Tang *et al*., 2006; Xu *et al*., 2011; Guo *et al*., 2011). As far as the establishment of intact genomic methylation patterns in higher animal development (Grunau *et al*., 2000; Song *et al*., 2005), two sets of full demethylated-DNA were remethylated during embryonic development (Walsh *et al*., 1999), involving in embryo normal development and differentiation. As a result, tissue-specific methylation patterns of individual fish were formed. Although gene expression in different tissues are not the same, DNA methylation in kidney and gill are very similar. The phenomenon is common in many species (Cao *et al*., 2009; Xu *et al*., 2011; Guo *et al*., 2013) because the regulation mechanism of gene expression are various, including not only DNA methylation, but also histone modification, RNA interference, and so on. Moreover, activation and inhibition of various genes are common in different tissues, resulting the increased or decreased methylation

levels which contributed to the no significant difference in methylation levels. Particularly, DNA methylation in gonad (under NS) and gill (under LS2) in male is obviously higher than in female ($P<0.05$). As we all know females-half smooth tongue soles grow two to three times faster than males in size. Moreover, the natural sex reversal of female half smooth tongue sole is universal. Currently, the researches on the sex-determining genes in animals are basically carried out in *dmrtl*, *Amy*, *sox9*, *SRY*, *foxl2*, *sfl*, *tra2* and others (Fukada *et al*., 1995; Ito *et al*., 1995; Raymond *et al*., 1998; Smith *et al*., 1998; Matsuda *et al*., 2002; Nakamoto *et al*., 2005; Tannour *et al*., 2010; Dong *et al*., 2011; Chen *et al*., 2014). Having investigated the sex determination mechanism in half smooth tongue sole, *dmrt1* (Deng and Chen, 2008), *wt1a* (Zhang *et al*., 2013) and *gadd45g3* (Liu *et al*., 2014) were proved to be gender-related genes which were necessary for testis maturation. Additionally, *cyp19a* (Deng *et al*., 2009) and *foxl2* (Dong *et al*., 2011) were higher expression genes in ovary, playing a major role in embryonic development and sex determination. Considering the function of DNA methylation in tissue development and differentiation, the different levels are probably relevant to the difference between female and male fish. Overall, the underlying molecular mechanism of individual difference needs further study.

Based on MASP technique, we preliminarily studied the DNA methylation levels in three tissues of *Cynoglossus semilaevis* subjected to salinity stress. The results show that DNA methylation is different between tissues, presenting 34.97% to 47.32%, suggesting the important role of DNA methylation in tissue-specific epiloci. Hemi- and total DNA methylation levels strikingly dropped when salinity decreased to 15. With acclimation for 60 days, the methylation levels significantly rose to the normal levels. Further analysis speculated that DNA methylation may respond to salinity stress with stimulating or inhibiting the gene expression. In addition, the methylation level in gonad of male fish is prominently higher compared to female fish under normal salinity. Our study takes the first look at tissue-specific DNA methylation patterns of the whole genomes in *Cynoglossus semilaevis* when subjected to salinity stress, which is an initial step towards epigenetic regulatory mechanism in low-salinity tolerance. However, there is still much work to be done. Firstly, if DNA methylation occurs on key genes which can contribute to the difference in size of female and male fish, which gene needs to be determined. Secondly, more attention should be given to study the key salt-resisting genes and the mechanism of DNA methylation on resisting the salinity stress. Finally, the relationship of DNA methylation and gene expression needs further study.

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