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RESEARCH ARTICLE

Expression and DNA methylation pattern of reproduction-related genes in partially fertile triploid Pacific oysters *Crassostrea gigas*

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Abstract Partial or complete sterility is an obvious feature in triploid Pacific oyster (*Crassostrea gigas*) which contributes to improving rearing performances. Despite the significance of sterility, the molecular mechanism behind it remains elusive and related research was limited. This study focused on six reproduction-related genes and compared their different behavior in gene expression and DNA methylation pattern between triploid and diploid oysters in order to provide more molecular information. The gonadal development of triploid oyster was examined by histology before molecular analysis. Gametogenesis disturbance was observed in triploid oysters at different development stages (stage II and III) with more serious impairment in females. QPCR showed significant gene expression difference between diploid and triploid in two genes: *putative Vg* and *cgER*. Gene expression of *putative Vg* was delayed in triploids while for *cgER* triploid oyster showed higher expression and the difference was significant at stage III. DNA methylation pattern of these two genes were further investigated by bisulfite sequencing. Between diploid and triploid oysters, no difference was observed in total methylation level but some individual loci showed different patterns: significantly high methylation rate of loci 2284 in *cgER* was observed in triploid oyster which has a higher expression of this gene. This study indicated that *putative Vg* and *cgER* might play a role in partial sterile in triploid *C. gigas*. Gene expression could be regulated by the methylation pattern at specific individual locus, which deserves equivalent attention as well as total DNA methylation level.

Keywords Triploid · Partial sterility · Gene expression · DNA methylation · *Crassostrea gigas*

Introduction

Triploidization is an efficient strategy used in the Pacific oyster (*Crassostrea gigas*) to improve rearing performances. The additional set of chromosomes causes reduced reproductive performance with partial or complete gonadal sterility. The reduction in gametogenesis might contribute to faster growth and better survival through reallocation of energy from gonadal development to somatic growth and resistance (Allen and Downing 1986; Garnier-Géré et al. 2002). Besides, it also improves marketability with better taste during the reproductive period (i.e. summer) and limits the propagation of these individuals, having little interference with natural genetic diversity (Normand et al. 2008). Considering all the advantages, triploid oyster was wildly cultured in different countries such as USA, Australia, China, France and Chile (Guo 2004; Hubert et al. 2009; Nell 2002).

Despite the advantages of gonadal sterility in triploids, the mechanism behind it remains elusive. Previous studies focusing on histological observations revealed that the additional set of chromosome in triploid oyster caused disorder in chromosome segregation and cell division and then resulted in locked gametogenesis (Jouaux et al. 2010; Que et al. 1997). But histological observation cannot unravel the essence and studies on molecular mechanism should be promoted. Dheilly et al. (2014) conducted the first comprehensive transcriptome profiling in diploid and triploid

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oysters, revealing numerous different expressed genes, which provided fundamental information in molecular mechanism. However, further study focusing on candidate genes are of greater importance which deserves our effort.

A few reproduction-related genes have been cloned in *C. gigas* which makes study on candidate genes feasible. Genes involved in gonadal development were first chosen as candidate genes. Besides, genes contributing to sex determination were also considered because Dheilly et al. (2014) implicated those genes responsible for the impaired gametogenesis. Finally, six cloned genes (*Cg-DM1, Cg-Foxl2, Cg-SoxE, Cg-β-catenin, putative Vg* and *cgER*) related to sex determination and gonadal development were chosen as candidate genes to investigate the molecular mechanism under sterile in *C. gigas*.

It is well known that the occurrence of sterile in triploid involves gene expression changes, then it brings another question, how are the differently expressed genes regulated? Except for the regulatory sequences, gene expression is also controlled by epigenetic modification such as DNA methylation, RNA interference and so on (Chen 2007; Strömqvist et al. 2010). An increasing number of studies have demonstrated that DNA methylation is associated with transcription level. In our previous study, we compared the DNA methylation level between diploid and triploid oysters and found some ploidy-specific methylated loci (Jiang et al. 2016). Considering the ploidy-specific methylated loci and the regulatory function of DNA methylation, it is reasonable to hypothesize that DNA methylation might regulate gene expression in the process of sterile in triploid oyster.

This study focused on six reproduction-related genes and compared their different behavior in gene expression and DNA methylation pattern between triploid and diploid oysters in order to provide more information unraveling the molecular mechanism under sterile.

Materials and methods

Oyster materials

The oyster materials were constructed in July 2013 with detail description in our previous study (Jiang et al. 2016). Triploid oysters were induced by cytochalasin B (CB, 0.5 mg/l) for 15 min starting when about 50% of the eggs released the first polar body. To keep the consistency of genetic background, both diploid and triploid oysters were sampled from the same full-sib family treated by CB. Two gonadal development stages were investigated with the age of 8 and 10 months respectively. Ploidy status was estimated by flow cytometry using DAPI staining of nuclear DNA. A 3-mm-thick gonad-visceral tissue block from each oyster was fixed in Bouin's fluid for histological analysis. The rest gonad tissue were frozen by liquid nitrogen and stored at −80 °C.

Histological analysis

The tissue blocks were fixed in Bouin's fluid for 24–36 h, dehydrated and then embedded in paraffin wax. Sections of 6 µm thickness were prepared, stained with haematoxylin and counter-stained with eosin following routine histological techniques. The specimens were examined microscopically to determine the sex and stages of gametogenesis on the basis of criterion described by Jouaux et al. (2010) using an optical microscope (Olympus BX50). According to histological analysis, 12 diploid oysters (six females and six males) and 12 triploid oysters (six females and six males) at different gonadal development stages were chosen in the following study.

RNA extraction and reverse transcription (RT)

Total RNA was extracted from ovary/testicle tissue using TRIzol reagent (Invitrogen) following manufacturer's instructions. RNA concentration of each specimens was quantified on NanoDrop2000 (Thermo Fisher Scientific) and the integrity was check by 1.5% agarose gel. The first-strand cDNA was synthesized using PrimeScript® RT reagent Kit (TaKaRa). Total RNA (500 ng) was reverse transcribed in a total volume of 10 µl (4 µl 5×PrimeScript Buffer 2, 1 µl RT Primer Mix, 1 µl PrimerScript RT Enzyme Mix and 4 µl RNase free dH_2O). Reaction conditions were at 37 °C for 15 min, then at 85 °C for 5 s.

Quantitative PCR (qPCR)

Expression of reproduction-related genes was estimated by qPCR in triplicate on LightCycler 480 real-time PCR instrument (Roche Diagnostics, Burgess Hill, UK) using SYBR1 Premix Ex TaqTM (TaKaRa). Enlongation factor (EF) was used as an endogenous control (Renault et al. 2011). QPCR primers for *putative Vg* were designed using PRIMER 5 (http://www.premierbiosoft.com/) software, and the primers for other genes were obtained from (Matsumoto et al. 2007; Naimi et al. 2009a, b; Santerre et al. 2014; Table 1). PCR amplification was conducted in 20 µl volumes containing 1×SYBR Premix Ex TaqTM, 10 µM PCR Forward Primer, 10 µM PCR Reverse Primer and 200 ng DNA. Reaction conditions were as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s and finally melting curve analyses were performed to verify specific amplification. The comparative threshold cycle (CT) method was used to

Table 1 Primer sequences used for real time quantitative PCR

Gene	Primer	Sequence (5′-3′)
EF	qfEF	ACCACCCTGGTGAGATCAAG
	qrEF	ACGACGATCGCATTTCTCTT
Cg-DM1	DMS	CCATGGAGTGGTGTCCGC
	DMASB	GTTGCCTTTCTGCTATCAGGG
Cg-Foxl2	qFoxl2S2	AATATCAGGGATGGGCACAA
	qFoxl2AS1	GTCCTTGGGTGCAGGAACTA
Cg-SoxE	SoxEqPCRS1	GCCATTCAAGGAGAAAACGA
	SoxEqPCRAS1	TGTAAGAGCTGGTCCAGGATG
Cg-β-catenin	βCatqPCRS1	CCATGATGGTTCACCAGTTG
	βCatqPCRAS1	ACAGCGAGTGGTCTCCAAGT
putative Vg	vgF	CCGATGTCAATGGCTTATGG
	vgR	GAGGTTGGTTGTTGTTTGGAT
cgER	Primer 6	CCTACTCGACCCCTCCCTATC
	Primer 4	CACCCCTCACATGACCACAC

Table 2 Primer sequences used for Bisulfite-PCR

Primer	Sequence (5′-3′)
Vg-CG1-F	TAATTGTGTTGATATATTATTTAGTTAGTA
Vg-CG1-R	TTTTAAACTCTTTATTTTAAAAATC
Vg-CG2-F	GGATAATTAAATATTATTAAAAATATTAAG
Vg-CG2-R	CCATAACTATAAACATTAAACCTTC
Vg-CG3-F	TGTGTTTGAATTGGTAGTTTAGTAT
Vg-CG3-R	ACAAATACCAAAACCAAAACCTTAATA
Vg-CG4-F	GGATATTTGATGAAGATATAATAGTATAGT
Vg-CG4-R	AACATTTAAACACCTACTTAAAATAAATAA
Vg-CG5-F	ATTGATTTAATTAAGATTGTTAAGTTTATT
Vg-CG5-R	TAAAAATTCCTCTCCCTATAAAAA
Vg-CG6-F	TAAGATGGGAGTAGTAGTC
Vg-CG6-R	CAACAATAATCTTACGATAA
ER-CG1-F	TAGTTGATAAATATGTTATTATTAAAGAAG
ER-CG1-R	TCTAACATAAACTTCTACCACTAAA
ER-CG2-1F	TTATGTTTTAGTGGTAGAAGTTTATGTTAG
ER-CG2-1R	AAAAACCACCATACTATTAATCAAAC
ER-CG2-2F	ATTATGTTTTAGTGGTAGAAGTTTATGTTA
ER-CG2-2R	AAAAACCACCATACTATTAATCAAAC
ER-CG3-F	TTTGTATGGAGGTGTTATG
ER-CG3-R	CGCCTAAAAATACCAATAC
ER-CG4-F	GATTTGTATTTTTAGTAATGTTTGT
ER-CG4-R	CCTTTACTAAATTAAAACTACCACTC
ER-CG5-F	TTTAGAGGTGCGACGTTT
ER-CG5-R	TTTCAATCTCCCTCGTTT

Two primers were used to amplify the ER-CG2 islands

analyze gene expression (Livak and Schmittgen 2001). The relative mRNA levels were normalized to EF transcripts using the following formula, $N = 100 * 2^{(CtEF–CtCg\text{-}gene)}$.

Bisulfite modification and sequencing analysis

Genomic DNA was extracted from the same tissues for RNA isolation using modified phenol–chloroform protocol (Li et al. 2006). DNA concentration was quantified on NanoDrop (Thermo Fisher Scientific) and the integrity was check by agarose gel electrophoresis. Two hundred nanogram genomic DNA was bisulfite-modified by the Methylamp™ DNA Modification Kit (EPIGENTEK), according to the protocol recommended by manufacturer. All the six candidate genes were blast with the genome data to get the full length and upstream noncoding region of genes. CpG islands of the six candidate genes were searched in putative promoter region (the upstream noncoding regions and the following 100 base pair in coding region, −2000~100 bp) and all the exons using online MethPrimer design software (http://www.urogene.org/methprimer/index1.html).

Bisulfite PCR (BS-PCR) primers were designed based on the bisulfite-modified DNA to amplify CpG islands (Table 2). All the PCR products were separated on 1.5% agarose gel, purified using the SanPrep Colum DNA Gel Extraction Kit (Sangon, China) and then cloned. Ten positive recombinant colonies of each sample were sequenced using Big Dye Terminator Cycle Sequencing Kit (ver. 3.1, Applied Biosystems) on an ABI PRISM 3730 (Applied Biosystems) automatic sequencer. After bisulfite treatment, unmethylated cytosine deaminate to uracil while the 5-methylcytosine remains unchanged. Based on this criteria, the methylation status could be determined. Considering the fact that cytosine out of the context of a CpG dinucleotide is always unmethylated, the efficiency of the bisulfite modification was calculated by the number of cytosine converted to uracil divided by the total number of cytosine.

Results

Histological analysis of triploid and diploid oysters

In *C. gigas*, the gonad is a diffuse organ with numerous tubules invaginated in a connective tissue, surrounded the digestive system. Expansion of the gonadal tubules in the gonadal area varies broadly during the cyclic development at the expense of the storage tissue. Gonad condition was graded by histological analysis according to Berthelin et al. (2001). Samples with the age of 8 months were at stage II gonadal development (Fig. 1), while the others with 10 months were at stage III (Fig. 2). Differences in gametogenesis could be discovered between diploid and triploid oyster with triploid exhibiting disturbed gametogenesis. All the 34 triploid samples we collected showed significant

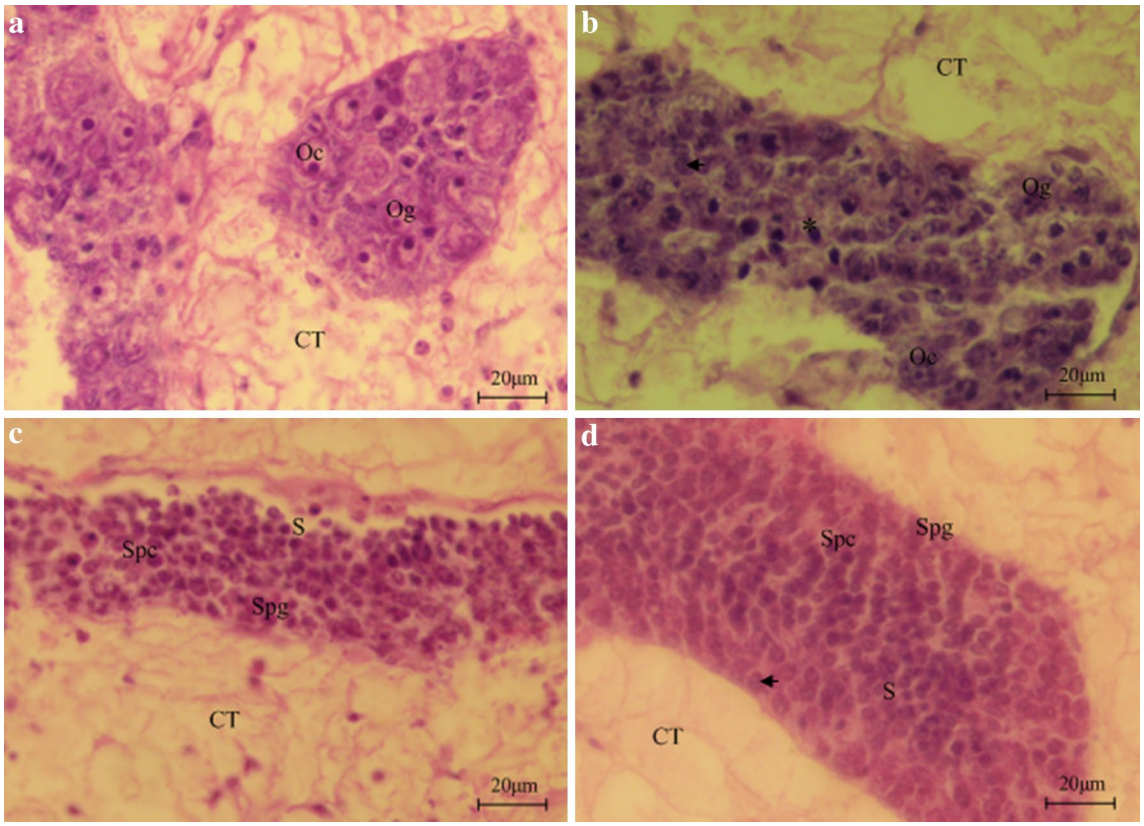

Fig. 1 Male and female gametogenesis at stage II in diploid and triploid *C. gigas*. Female showing germinal lineage from oogonia (Og) to previtellogenic oocytes (Oc) without (**a**) or with (**b**) locked (*arrowhead*)/retracted (*asterisk*) nuclei for diploid and triploid oysters, respectively. Males exhibiting the whole germinal lineage including spermatogonia (*Spg*), spermatocytes (*Spc*) and spermatids (*S*) without (**c**) or with (**d**) locking events for diploid and triploid oysters, respectively. *CT* conjunctive tissue

reduction in gonadal development, which were determined to be β-pattern according to Jouaux et al. (2010).

Stage II is associated with vitellogenesis in females and presence of the whole germinal lineage in males. At this stage, sex determination was possible for both diploid and triploid oysters. Diploid females (Fig. 1a) presented oogonia (Og) and previtellogenic oocytes (Oc) with an eosinophilic nucleolus while the triploids showed germinal lineage with locked or retracted nuclei (Fig. 1b). Both diploid (Fig. 1c) and triploid (Fig. 1d) males in stage II presented a germinal cell lineage including spermatogonia (Spg), spermatocytes (Spc) and spermatids (S) while some of germinal cell in triploid showed a clear demonstration of locking events.

Stage III corresponds to a ripe gonad containing mainly mature oocytes or spermatozoa. Diploid females' oocytes (Fig. 2a) appeared polygonal in shape, filling the gonadal tubules. In triploid ovarian (Fig. 2b), mature gametes are always present but less numerous with locking events in the nuclei of germinal cells. Ovarian in triploid oysters presented a continuum of germinal cell lineage with some oogonia and previtellogenic oocytes which is determined as β2 pattern according to Jouaux et al. (2010). In male oysters, a continuum of germinal cell lineage with a large majority of spermatids and spermatozoa were observed in both diploid and triploid. In diploid males (Fig. 2c), expanded gonadal tubules full of mature gametes were observed, whereas the triploid (Fig. 2d) presented reduced number of mature spermatozoa and the gonadal area contained a larger part of conjunctive storage tissue than diploid males.

Expression of six candidate genes in triploid and diploid oysters

Six genes related to sex determination and gonadal development (*Cg-DM1, Cg-Foxl2, Cg-SoxE, Cg--catenin, putative Vg* and *cgER*) were chosen as candidate genes. Based on gender and gonadal development, all the samples are classified into four groups: ovarian at stage II (Ov.StII), ovarian at stage III (Ov.StIII), testicle at stage II (Te.StII) and testicle at stage III (Te.StIII). The variation of gene expression at two gonadal development stages in triploid and diploid oysters was shown in Fig. 3.

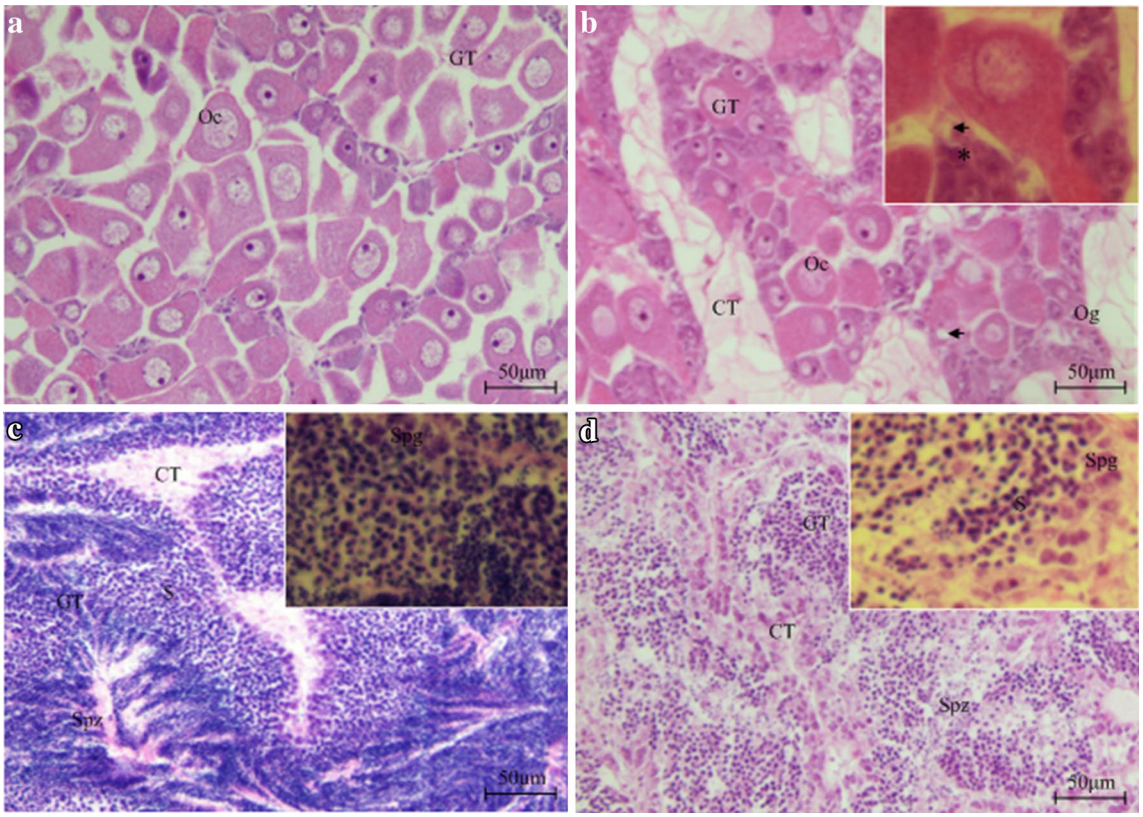

Fig. 2 Male and female mature stages of gametogenesis in diploid and triploid *C. gigas*. **a** Diploid females present gonadal tubules (GT) full of mature gametes, whereas triploids **b** exhibit locking events (locked nuclei: *asterisk*; clear cytoplasmic area: *arrow*). **c** Diploid males present gonadal tubules full of mature gametes whereas triploids exhibit reduced tubules (**d**). *CT* conjunctive tissue; *Og* Oogonia; *Oc* Oocyte; *Spg* Spermatogonia; *S* Spermatid; *Spz* Spermatozoa

In all the genes, only two genes display significant variation between diploid and triploid oysters: *putative Vg* at ovarian development stage II and *cgER* at ovarian development stage III. For the different expressed genes, three more individuals are added in each group to quantify gene expression and the results of all six samples (including the previous three) are given in Table 3 which further confirmed the accuracy. To explore the regulatory mechanism in gene expression, the DNA methylation status in these two genes were analyzed in the following.

Efficiency of bisulfite modification

In order to evaluate the efficiency of the bisulfite modification step, the cytosine conversion rate out of the context of CpG dinucleotide was examined. Sequencing results showed that almost all the cytosine was chemically converted to uracil with efficiency above 99%. The high conversion efficiency indicated the validity of bisulfite modification and ensured the accuracy of the following results.

DNA methylation analysis of differently expressed genes

DNA methylation analysis was conducted on two genes (*putative Vg* at ovarian development stage II and *cgER* at ovarian development stage III) which were differently expressed in diploid and triploid oysters. Six CpG islands were found in *putative Vg* with one in putative promoter region (−213 to −97 bp) and five in exons (exon 5, 7, 8, 9, 13). In *cgER*, only five CpG islands were found in exons (two island in exon 1 and one in exon 2, 6, 10 each) and none was found in putative promoter region.

Table 2 showed the primer sequences to amplify CpG islands. Since the ER-CpG2 islands is too long to successfully amplify and clone, it was amplified using two primers. Using bisulfite modification and sequencing, the DNA methylation pattern of all the CpG islands were characterized and the results were given in Table 4. For *putative Vg*, DNA methylation was barely observed in the whole six CpG islands with almost all the cytosine conversed to uracil. The lack of methylation was consistent in diploid and triploid oysters in spite of the different gene expression. For *cgER*, two CpG islands (ER-CG4 and ER-CG5) located in

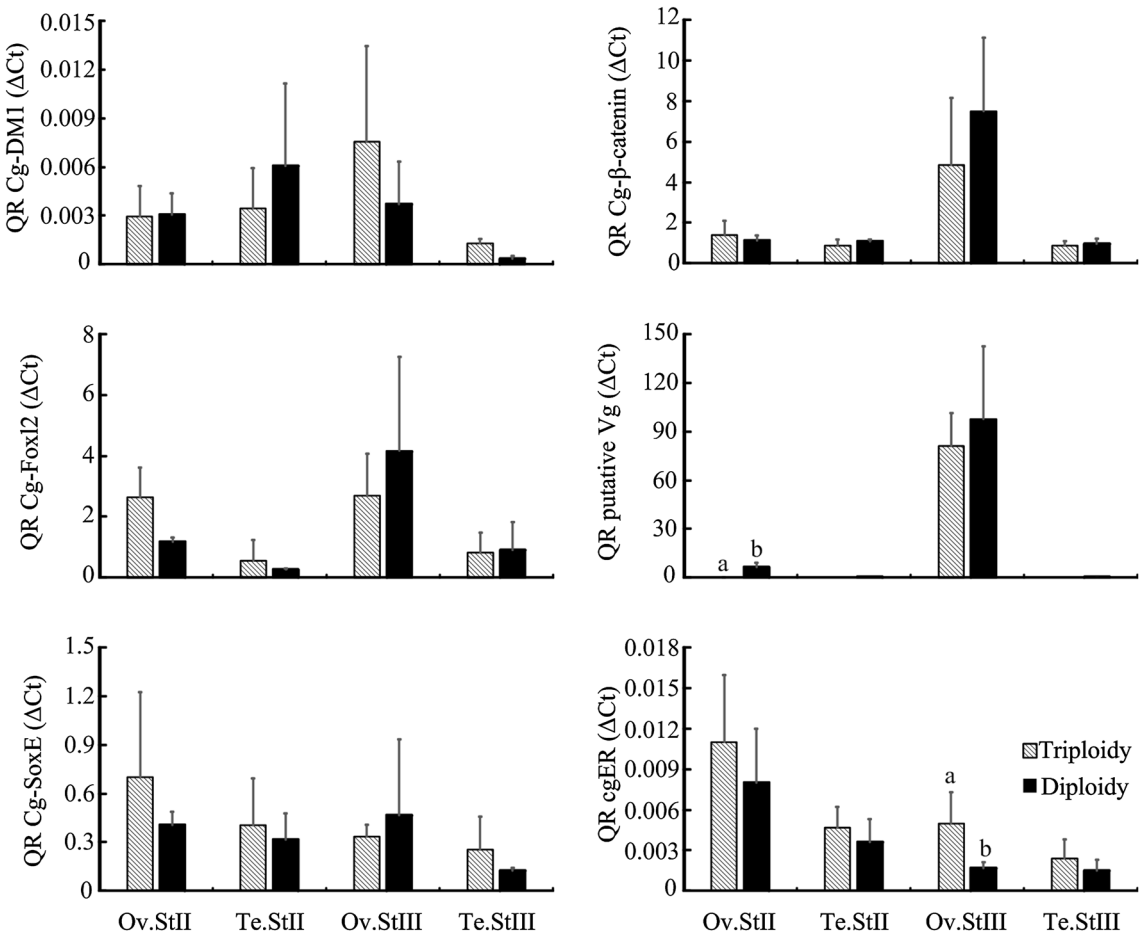

Fig. 3 Real time RT-PCR transcripts quantification relative to elongation factor in two developmental stages of triploid and diploid oysters. *Ov.StII* ovarian at stage II, *Ov.StIII* ovarian at stage III, *Te.StII* testicle at stage II, *Te.StIII* testicle at stage III, *QR* relative quantity, arbitrary units. Values are mean ± SEM of triplicates

Table 3 Real time RT-PCR transcripts quantification relative to elongation factor in *putative Vg* and *caER*

Sample ID	*Putative Vg*		*caER*	
	Tri	Di	Tri	Di
1	0.0850	11.3446	0.0069	0.0014
2	0.0380	6.3813	0.0034	0.0016
3	0.0531	3.9370	0.0036	0.0024
4	0.0236	3.6503	0.0085	0.0011
5	0.0130	7.0858	0.0050	0.0017
6	0.0642	6.7123	0.0071	0.0009
Avg	0.0461 ± 0.0225	6.5186 ± 2.3470	0.0057 ± 0.0017	0.0015 ± 0.0004

$N = 100 * 2^{(CtEF-CtCg\text{-}gene)}$

exon 6 and 10 were highly methylated, while the other three islands were hardly methylated. The methylation pattern of ER-CG4 was similar between diploid and triploid oysters with an average methylation level to be 96.67% (Fig. 4). Similar with ER-CG4, the cytosine in CpG dinucleotide was almost methylated in ER-CG5 (Fig. 5). The methylation level in triploids (96.90%) was a little higher than that in diploids (95.47%), but the difference was not significant. Despite the similarity of average methylation level between diploid and triploid oysters, the location of DNA methylation was quite different: the first eight CpG loci was all methylated in triploid while in the diploid, some loci were unmethylated, and the opposite situation was observed in the last two CpG loci; at the loci 2284, methylation rate in

Table 4 DNA methylation level of *putative Vg* and *cgER* in triploid and dipoloid oysters

CpG islands ID	Tri (%)	Di (%)
Vg-CG1	1.11	2.22
Vg-CG2	0.91	0.91
Vg-CG3	3.00	2.00
Vg-CG4	2.86	3.57
Vg-CG5	0.00	0.91
Vg-CG6	1.81	2.73
ER-CG1	0.00	0.00
ER-CG2	3.08	3.85
ER-CG3	3.33	1.67
ER-CG4	96.67	96.67
ER-CG5	96.90	95.47

triploid was significantly higher than that in diploid which is in consistence with gene expression between diploids and triploids.

Discussion

Triploid animals are generally considered to be sterile with the absence of fertile gametes. In *C. gigas*, two types of gametogenic pattern were described by Jouaux et al. (2010): α-pattern, producing abundant gametes with almost the same fecundity as diploids and β-pattern, which is associated with locked gametogenesis and only few mature gametes could be observed at sexual maturity. All of the triploid oysters obtained in this study were detected to be β-pattern which is a common phenomenon since the percentage of α-pattern is much lower in nature. Histological observation in this study showed that gametogenesis damage was more serious in females than males which is in accordance with Jouaux et al. (2010). However, in the crossing experiment between triploid and diploid oysters, triploid females produced more progeny than triploid males (Guo 2004). Considering these two literally paradox phenomenon, we could infer that oocyte produced by triploid females might be more fertile than spermatozoa produced by triploid males.

Vitellogenin (Vg) is the precursor of yolk protein which is a source of nutrients during early development of embryonic development. *Putative Vg* gene was only expressed in female oysters and not in males. In the triploid oysters, the expression of this gene was delayed. At stage II, *Putative Vg* hardly expressed in triploid which is significantly lower than that in diploid females, whereas, at stage III, the expression increased rapidly and reached the same expression level with diploids. The delayed expression of *Putative*

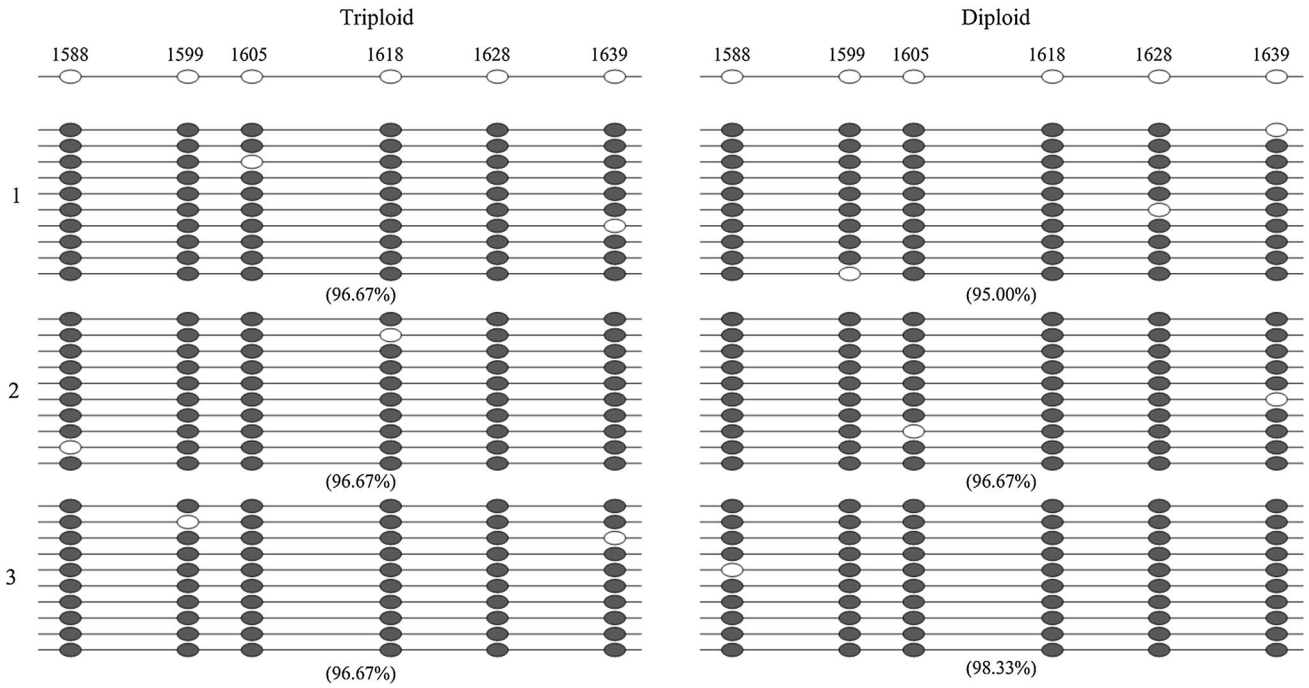

Fig. 4 DNA methylation patterns of ER-CG4 (exon 6) in three diploid and triploid oysters' ovarian at stage III. The *first line* indicates the localizations of studied CpG sites related to the sequence of *caER* coding region. *Numbers on the left* refer to three different oysters. *Filled* and *open circles* denote methylated and unmethylated sites, respectively. *Each line* represents one sequenced clone. The percentage indicates the methylation level (*under the line*), calculated as the number of methylated C divided by total number of C in all CpG site

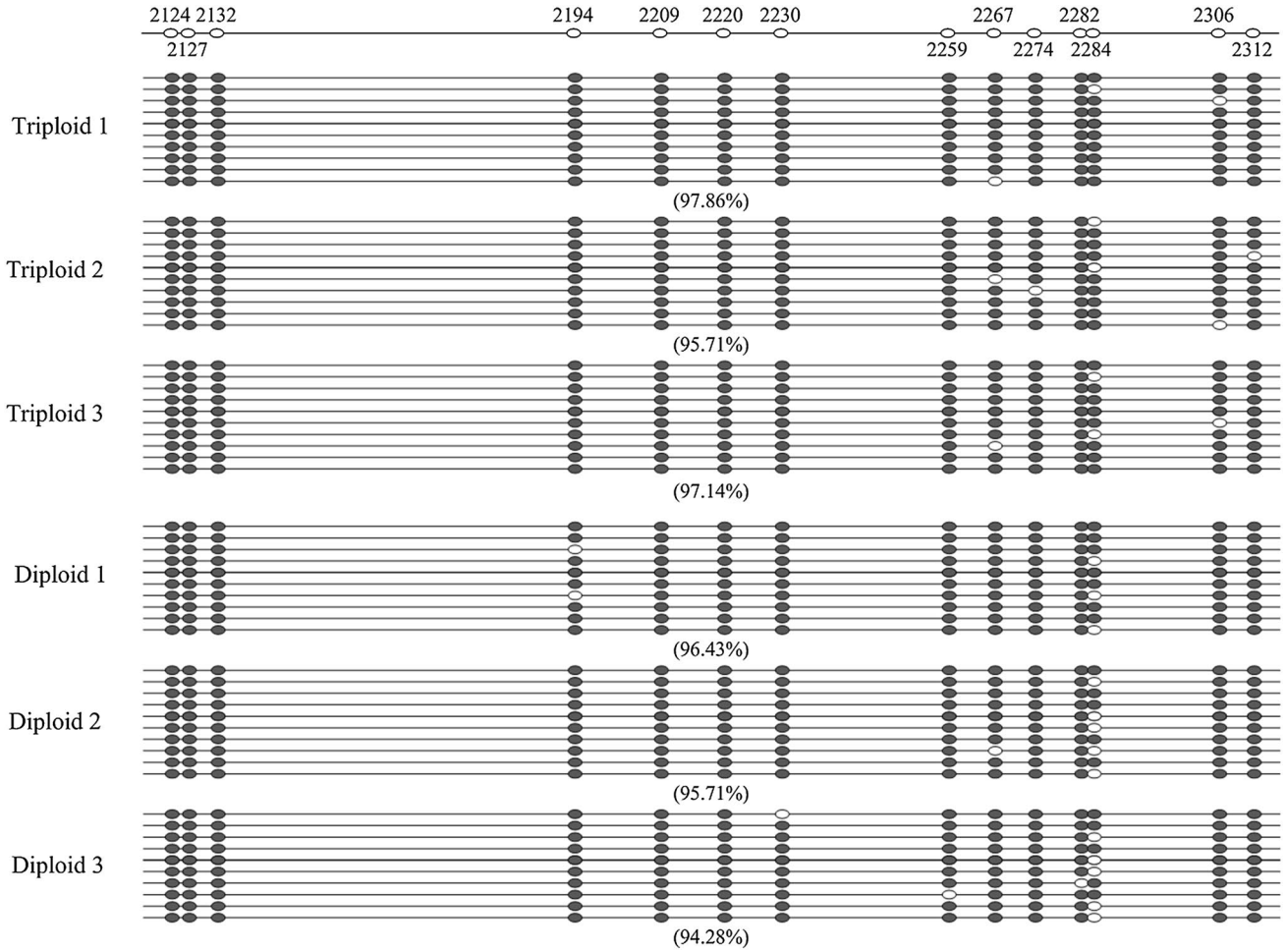

Fig. 5 DNA methylation patterns of ER-CG5 (exon 10) in three diploid and triploid oysters' ovarian at stage III. The *first line* indicates the localizations of studied CpG sites related to the sequence of caER coding region. *Numbers on the left* refer to three different oysters. *Filled* and *open circles* denote methylated and unmethylated sites, respectively. *Each line* represents one sequenced clone. The percentage indicates the methylation level (*under the line*), calculated as the number of methylated C divided by total number of C in all CpG sites

Vg might induce delayed synthesis of yolk protein and then influence gametogenesis.

The estrogen receptor (ER) is a member of the nuclear receptor superfamily, which could bind to estrogen and regulate reproduction through promoting or repressing target genes. Function of ER is clearly established in vertebrate (Pakdel et al. 1991). However, little was known in invertebrate, especially in the mollusk. In recent years, an increasing number of "vertebrate type" sex steroids has been discovered, but the signaling pathways and function of these steroids have not been elucidated which makes the significance of ER in reproduction under debate. Studies in scallop suggested that effects of the steroids in scallop may be achieved through activation of ER in the process of sexual maturation (Wang and Croll 2003, 2007). Nevertheless, the binding incidence and regulation in gene expression between estrogen and ER were not found in *Aplysia californica* (Thornton et al. 2003), *Octopus vulgaris* (Keay et al. 2006), *Mytilus edulis* (Puinean et al. 2006) and *C. gigas* (Matsumoto et al. 2007). Our study displayed that the expression of ER decreased from stage II to stage III and the expression was lower in diploid oyster compared with the triploids. This result showed that the expression of ER was not in a simple positive correlation with gonad maturation.

In mammal and plant, high methylation in gene promoter and coding regions is usually associated with gene silence while demethylation of these regions could active gene expression (Okano et al. 1999; Zhang et al. 2006). Similar regulatory mechanism was also discovered in aquatic animals such as fish. For example, in *Paralichthys olivaceus*, a negative relationship between gene expression and DNA methylation was found in *cyp17-II, dmrt* and *cyp19a* genes (Ding et al. 2013; Wen et al. 2014). In aquatic invertebrate, however, the regulation between DNA methylation and gene expression was complicated.

Previous studies in *C. gigas* observed both positive and negative regulation (Olson and Roberts 2014; Riviere et al. 2013). In our study, the differently expressed gene (*putative Vg*) between diploid and triploid have an approximate DNA methylation level, which indicate that DNA methylation might not regulate expression in some genes. In *cgER*, however, despite the similar total methylation level, methylation pattern in individual locus have a consistent trend with gene expression. This situation reminds us to care about not only total methylation level but also the individual locus in future studies.

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Compliance with ethical standards

Conflict of interest Qun Jiang declares that she does not have conflict of interest. Qi Li declares that he does not have conflict of interest. Hong Yu declares that she does not have conflict of interest. Lingfeng Kong declares that he does not have conflict of interest.

Ethical approval The research was conducted in the absence of any ethical issue on aquatic animal research.

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