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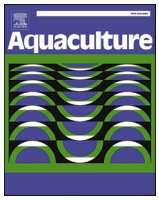

Examination of the role of *CgSox-like* in sex determination and gonadal development in the Pacific oyster *Crassostrea gigas*

Dongfang Sun [a], Hong Yu [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

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ABSTRACT

The Pacific oyster (*Crassostrea gigas*), as the most productive cultured shellfish in the world, has considerable economic importance and value. Yet research on its sex determination remains lacking. Here, a novel HMG-box-containing homolog of the sox gene was uncovered in *C. gigas* (named *CgSox-like*). Alignment with the HMG-box of sox genes from other animals revealed that *CgSox-like* had a high similarity to the *Sox* gene of scallops. Among different adult tissues, *CgSox-like* was mainly expressed in the gonads. qPCR analysis revealed that *CgSox-like* and *CgSox8*, a sox gene family gene mainly expressed in male gonads, shared similar expression patterns, with elevated expression as the male gonads developed. RNA interference and dual luciferase reporter assays demonstrated that *CgSox-like* was located upstream of *CgSox8* and promoted the expression of *CgSox8*. In addition, analysis of *CgSox-like* and *CgSox8* methylation patterns indicated that both genes were in hypomethylation state during the gonadal development. Finally, demethylase inhibitors resulted in elevated expression levels of *CgSox-like* and *CgSox8* genes, suggesting that epigenetics was engaged in modulating the expression of sex-related genes in the Pacific oyster. Taken together, the novel gene *CgSox-like* was implicated in the sex determination or gonadal development process in male oysters. These results will provide valuable information for the investigation of sex determination mechanism and sex control breeding.

1. Introduction

Sex determination is recognized as a complex regulatory mechanism capable of influencing many aspects of an organism's functional systems (Sarre et al., 2004). More than simply providing solutions for the reproductive survival and evolution of species, sex directly controls the phenotypic and physiological processes of organisms, such as reproductive development, biochemical metabolism, and differentiation (Angelopoulou et al., 2012). Sexual differentiation, on the other hand, is an extremely complex ontogenetic process and its developmental direction is regulated by sex determination mechanisms. In most species, sex selection and sex determination are determined by a combination of genetic and environmental factors, and the underlying regulatory mechanisms are traditionally referred to as environmental sex determination (ESD) and genetic sex determination (GSD) (Uller and Helantera, 2011). Several genes, including the members of sox gene family, were proved to be associated with sex determination. Sox genes are the family of SRY (sex determination region of the Y chromosome) related genes that encode a series of SOX (SRY-related HMG-box) family transcription factors, all members of which share a highly conserved HMG-box DNA binding domain homologous to the SRY-related HMG box (Bowles et al., 2000). Sox transcription factors are considered to be target-specific transcription factors and regulators of chromatin structure as they are participating in early developmental processes including sex determination (Jay et al., 1997). Currently, the *Sry* gene is the only mammalian sex-determining gene identified in the sox family. In addition, *Sox8* and *Sox9* are decisive for testis formation, and *Sox5* and *Sox6* are involved in mouse spermatocyte formation (Cohen-Barak et al., 2001; Schepers et al., 2003; Takada and Koopman, 2003). The first concern in exploring sex-determining genes in fish, given the unique position of the sox gene family in mammalian sex determination, was to identify whether sox genes such as *Sry* and *Sox9* were also present. However, *Sry* is not found in fish, presenting other sex-determined genes, depending on the species (Edgecombe et al., 2021). *Sox9* has been extensively studied in fish, having an important role in gametogenesis, specifically in supporting cells (Nakamura et al., 2008). In bivalves, sox genes are reported in *Chlamys farreri*, *Crassostrea gigas* and *Patinopecten yessoensis* (He et al., 2013; Santerre et al., 2014; Yu et al.,

* Corresponding author at: Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China.
E-mail address: qili66@ouc.edu.cn (Q. Li).

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2017). At present, ten sox genes are identified in *C. gigas*. *CgSoxE*, which is highly homologous to mammalian *Sox8* and *Sox9*, but they do not show unique expression. While *CgSox8* or *CgSry-like* shows sexually dimorphic expression and is considered to be participated in the oyster sex determination pathway (Zhang et al., 2014).

Molluscan sex determination mechanisms are diverse and complex in type. Therefore, investigations related to sex determination in mollusks not only enrich the knowledge base for understanding animal sex, but also provide a practical basis for reproduction and sex control in shellfish culture. *C. gigas* is a representative species for the examination of sex determination in bivalves, with respect to the exceptional phenomenon of sex reversal and essential economic and ecological value. In oysters, several progresses were made with the respect to the study of sex determination. The genes (*CgDMl*, *CgDsx/CgDmrt1*, *CgSox8*, *CgSoxE*, *Cg-Foxl2*, *Cg-catenin*, *Cg-Foxl2os*, etc.) associated with the sex determination signaling pathway in oysters were obtained by homologous gene cloning and high-throughput sequencing analysis (Yue et al., 2021; Zhang et al., 2014), and functional validation of some genes was performed (Sun et al., 2022b). Epigenetic investigations revealed remarkable differences in DNA methylation levels between sexes and between gonads at different stages of development (Riviere, 2014; Sun et al., 2022a). In fish, DNA methylation in the promoter region of sex-related genes is involved in negatively regulating gene expression (Navarro-Martin et al., 2011). Similarly, genes displaying a negative correlation between promoter methylation and expression are present in oysters (Riviere, 2014), but sex-related genes are not regulated by DNA methylation in mature gonads as they are in fish. This may be relevant to the reality that certain genes must be activated or inactivated depending on environmental inputs to follow different developmental pathways (Elango et al., 2009). In other words, regulation of gene expression may be involved in the effects of transient methylation in oysters (Roberts and Gavery, 2012). However, whether sex-related genes are transiently methylated and regulate gene expression in oysters remains unclear.

In this study, the uncharacterized LOC105322460 was found by BLAST comparison with the seed sequence of the sox family. Homologous sequence comparison revealed high similarity to the *Sox* gene of scallop *Mizuhopecten yessoensis*, hence the name *C. gigas Sox-like* (*CgSox-like*). Expression analysis showed that *CgSox-like* (GeneBank accession no. LOC105322460) was mainly expressed in the male gonads and shared similar expression patterns with *Sox8* (GeneBank accession no. LOC105319856), with elevated expression as the male gonads developed. Subsequently, the DNA methylation patterns on *Sox8* and *Sox-like* were analyzed with the aim of exploring the epigenetic mechanisms underlying sex determination in oysters.

2. Materials and methods

2.1. Experimental animals

Oysters (shell height: 74.1 ± 8.9 mm, weight: 37.3 ± 7.7 g) were collected from the sea area of Sanggou Bay, Rongcheng, China (37.11°N , 122.35°E), where the annual average water temperature is 13 °C and the salinity is about 31‰ (Deng et al., 2017). Thirty oyster gonad sampling was performed monthly during gonad development. In addition, six oysters were randomly selected to take whole tissues for gene expression distribution experiments. All gonadal tissues were taken in two copies, one fixed in Bouin's fixative and the other frozen in liquid nitrogen. As the oyster sex in the resting stage could not be identified by histological analysis, the following sampling strategy was adopted: in January, oysters were anesthetized with 5% magnesium chloride, small pieces of gonadal tissue were taken, then the oysters were tagged and released back to the sea area for transient rearing. Oysters were retrieved for sex identification after gonadal maturation so that the sex of oysters sampled during the resting stage could be confirmed. Considering the high mortality rate caused by anesthesia as well as in vivo sampling, 200 oysters were sampled during the resting period. Additional 20 oysters were sampled in January for histological analysis to determine that the gonads sampled were in the resting stage.

2.2. Bioinformatics analysis of sox protein sequences

The HMG-box consistent sequence (DHVKRPMNAFMVWSRGERRKIAQQNPDMHNSEISKRLGKRWKLLSESEKRPFIEEAERLRAQHMKDYPDYKYRP RRKKK) of the sox gene family was selected as the seed sequence, compiled into fasta format and matched in the local BLAST genome database (cgigas_uk_roslin_v1). The sox gene sequences were selected according to the amino acid homology matches in the oyster local BLAST genomic database. Meanwhile, the online software Pfam and SMART for conserved domain prediction were performed to ensure that the above candidate amino acid fragments contained HMG-box conserved domains. Construction of sox gene family NJ (Neighbor Joining) phylogenetic tree using MEGA7.0 software and amino acid sequences. Search for homologous genes was performed in the UniProt database utilizing the BLAST program with the amino acid sequence of *CgSox-like*. Homology analysis and NJ phylogenetic tree construction of the *CgSox-like* were performed on DNAMAN 9.0 and MEGA 7.0 software with HMG-box sequences.

2.3. RNA interference experiments

The template required for in vitro transcription was synthesized using primers with the T7 promoter sequence added and genomic DNA (See Table 1). Double-stranded RNA was synthesized by in vitro

Table 1
Primers used in this study.

Names	Primers (5′ - 3′)	Purpose
Sox-like F	GCTCGGAGCACCTGCGTCATGTTC	PCR
Sox-like R	GACCATGAGTCCACGGCAGTGCAC	
qPCR_Sox-like F	AGCCTCGTGTGTCTATCCAAACT	qPCR
qPCR_Sox-like R	TGTATCTCTATTGTCTGGCGTGG	
qPCR_Sox8 F	TCAAACACGGTAACACTTCAGGA	
qPCR_Sox8 R	ATAGATTCTTGAGAGGTGGACGG	
qPCR_Dmrt1 F	ACCTGTGGGTCCTTGCCTT	
qPCR_Dmrt1 R	GCTCTTGATTGGTGCTCTATGG	
qPCR_Foxl2 F	CACAGTGTGGTTACAACGCAATGC	
qPCR_Foxl2 R	CCTGTCAGTCCAGTACGAGTAATGC	
BS_Sox-like F	GAGAATATATATATATGTATTTTGT	BS-PCR
BS_Sox-like R	ACCATTTCTATATACATCCACTACAAT	
BS_Sox8 F	ATAGGGAAATTTAGAATTAATTA	
BS_Sox8 R	CTAAATTTCCTATAAAAACCTACTAT	
BS_Dmrt1 F	ATATTAGGAGATATATAAAGTTTGGA	
BS_Dmrt1 R	TCAATTTCAAATATATATTAAATTAC	
BS_Foxl2 F	GTGTTAAAATGATATGGAGAGAG	
BS_Foxl2 R	CTAATATTACATTCAATTCCCACAA	
pro_soxlike F + kpn1	ggggtacc*AGAGACTTTATTGATTGATGCTTGA	Double luciferase experiment
pro_soxlike R + xho1	ccctcgag*ATGGATGCATTCAGACTTCAGTC	
cds_soxlike F + kpn1	ggggtacc*ATGAGCTTTACCGACAGATCTTGCA	
cds_soxlike R + xho1	ccctcgag*TCACAGAAAATTTGCTCGCAGAGGC	
pro_sox8 F + kpn1	ggggtacc*TCGAGCAAGAATATAATGCTAAATG	
pro_sox8 R + xho1	ccctcgag*CGACGTCATTTAGCGCGATTCTAT	
cds_sox8 F + bamh1	cgcggatcc*ATGCAATCTGAAAGCAAAGAAAAAATG	
cds_sox8 R + xho1	ccctcgag*TCAATTCCTGAGTCGTTTTGATGCC	

* Lowercase letters indicate enzyme cut sites and conservation bases.

transcription utilizing the T7 RiboMAX™ Express RNAi System (Promega, USA) according to the instructions. Oysters were anesthetized with 5% magnesium chloride for 6 h, and then a few germ cells were aspirated with a fine needle to determine the sex. Since *Sox-like* and *Sox8* were expressed mainly in male oysters, interference experiments were conducted only on male oysters. Ten oysters in the experimental group and ten oysters in the control groups were injected with 40 μL of double-stranded RNA(1 μg/μL) and 40 μL of DEPC water through the closed-shell muscle, respectively. Sampling was performed after 24 h of RNA interference. The gonads were rapidly placed in liquid nitrogen and stored at −80 °C for RNA extraction.

2.4. DNA and RNA extraction

The male and female gonadal tissues were divided into resting, proliferative and mature stages according to histological analysis. Genomic DNA was extracted from the gonads at different developmental stages with the DNA extraction kit (Tiangen, China). Extraction of RNA with TRIzol reagent (Invitrogen, USA) was performed by taking 30 mg of tissue with 1 mL of TRIzol, followed by chloroform extraction, isopropanol sedimentation, 75% ethanol washing, and finally solubilization with DEPC water.

2.5. PCR amplification of CgSox-like in male and female genomic DNA

PCR amplification was performed with 5 female and 5 male oyster genomic DNA as templates employing *CgSox-like* specific primers and 2 × Master Mix (Vazyme, China) (See Table 1). According to the instructions for the PCR reaction, 50 ng of DNA template was used and the primer annealing temperature was set to 57 °C. Amplified fragments were detected using 1% gel electrophoresis and then photographed.

2.6. Relative gene expression levels detection

Reverse transcription was performed by synthesizing 1 μg of RNA into cDNA with HiScript® II Q Select RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). Real-time PCR was conducted utilizing the ChamQ SYBR Color qPCR Master Mix (Vazyme, China) on a LightCycler 480 II detection system (Roche, Switzerland) according to the manufacturer's instructions. The total volume of the real-time PCR reaction was 20 μL, including 10 μL Master Mix, 0.4 μL primers, and 10 ng cDNA (See Table 1). Quantitative analysis of *CgSox-like* and *CgSox8* in different tissues and different periods of gonadal development was performed using three biological replicates and two technical replicates. qPCR related to RNA interference and injection of inhibitors experiments was performed using six biological replicates and two technical replicates. The reaction program was 95 °C for 5 min; and 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. Elongation factor I (*Ef1*) gene was similarly performed as an internal reference and relative expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7. Interaction between Sox-like and Sox8

To investigate the interaction relationship between *Sox-like* and *Sox8*, the binding sites of *Sox*-like and *Sox8* protein to the promoters of *Sox8* and *Sox-like*, respectively, were predicted through the online website https://jaspar.genereg.net/. Subsequently, the relationship was verified by the dual fluorescence reporter assay. The promoter sequences of *Sox-like* and *Sox8* upstream-2000 bp were synthesized and then introduced into the pGL3 vector by double digestion, named pGL3-Sox-like and pGL-Sox8, respectively. Similarly, the open reading frame sequences of *Sox-like* and *Sox8* were synthesized and introduced into the pCDNA vector, named pCDNA-Sox-like and pCDNA-Sox8. The plasmids were transfected into 500 μL human embryonic kidney 293 T (HEK-293 T) cells by transfection reagent according to the instructions. Transfection experiments to verify the binding of transcription factor *Sox8* to the *Sox-like* promoter were performed as follows: (1) 500 ng pGL3 (control); (2) 300 ng pGL3 and 300 ng pCDNA (control); (3) 300 ng pGL3-Sox-like, 300 ng pCDNA-Sox8. Transfection experiments to verify the binding of transcription factor *Sox-like* to the *Sox8* promoter were performed as follows: (4) 300 ng pGL3-Sox8, 300 ng pCDNA-Sox-like. Three wells were transfected for each group and two experimental replicates were performed. A further 50 ng of pRL-TK plasmid was added to each group to monitor transfection efficiency. The cell culture medium was renewed after 12 h and luciferase activity was detected after 48 h with the luciferase assay kit (Promega, US) and the Synergy NEO2 instrument.

2.8. Primers design for bisulfite sequencing PCR (BS-PCR)

Primers used for BS-PCR were designed for bisulfite-transformed DNA using the online software MethPrimer (http://www.urogene.org/methprimer2/). Due to DNA damage by sulfite, the amplified sequences were designed to be no longer than 600 bp and to contain as much CpG as possible within −2 kb located upstream of the translation start site (Li and Dahiya, 2002). In the *CgSox8* promoter region, the primers were designed to contain 12 CG sites from −1251 to −808 upstream of the TSS, and for the promoter region of the *CgSox-like*, the primers were designed to contain 12 CG sites from −1487 to −1124 upstream of the TSS (See Table 1).

2.9. Methylation analysis of Sox-like and Sox8 by BS-PCR

The BS-PCR template was transformed with 1 μg of DNA from different developmental stages of the gonads by the Heavy Sulfite Transformation Kit (Tiangen, China). The 2 × Taq Plus Master Mix II (Dye Plus) enzyme (Vazyme, China) was used for PCR amplification. BS-PCR of *Sox-like* and *Sox8* genes were performed to detect their DNA methylation levels at different periods of gonad development, with three biological replicates in each period. Amplifications of targeted fragments were performed with the 20 μL reaction system consisting of 10 μL Master Mix, 0.8 μL of each forward and reverse primer, and 30 ng of templates (See Table 1). The PCR program for amplifying was used: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 30 s; 72 °C for 5 min; and 4 °C for infinity. The target fragments were recovered by gel cutting, and the recovered products were ligated to pMD19-T (Takara, Japan) vectors for 5 h at 16 °C and then transformed into DH5α competent cells (Vazyme, China). Ten monoclonal clones were randomly selected for Sanger sequencing. DNA methylation levels, sulfite conversion efficiency calculations, and data visualization presentations were performed utilizing BiQ_Analyzer software.

2.10. DNA methyltransferase and demethylase inhibitor assay

To characterize the influence of DNA methylation on *CgSox-like* and *CgSox8*, oysters were treated with DNA methyltransferase inhibitors Decitabin (5-Aza-2′-deoxycytidine, MedChemExpress) and demethylase inhibitors Bobcat 339 hydrochloride (Bobcat 339, MedChemExpress) at gonadal developmental maturity. 5-Aza-2′-deoxycytidine and Bobcat 339 hydrochloride were dissolved with DMSO according to the instructions and diluted in sterile water to 10 mM and 4 mM, respectively. Subsequently, 20 μL of 1% DMSO (control), 10 mM 5-Aza-2′-deoxycytidine, and 4 mM Bobcat 339 were injected into the molluscum of the oyster utilizing a syringe. Injections were made every 48 h for a total of 7 injections. Finally, two copies of gonadal tissue were taken, one fixed using Bouin's fixative for histological observation to identify sex and the other for quantitative analysis of *CgSox-like* and *CgSox8*.

2.11. Statistical analysis

The mean and standard errors of the mean (SEM) were utilized to

represent the qRT-PCR data, and ANOVA was used to analyze the differences between the two groups. Methylation levels were expressed as the number of C-site points where methylation occurs divided by the total number of C-site points and presented as the mean and SEM. The relationship between DNA methylation and corresponding gene expression was assessed with Pearson's rank correlation coefficient. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Sox protein sequence characterization

Gene structure was the basis for gene function, gene organization, and evolution. To describe the structural variety of sox genes in oysters, the sox gene sequences and the corresponding HMG-box structural domain sequences were compared. The phylogenetic tree containing HMG-box structural domains screened indicated that *Sox-like* and *Sox5* clustered as a group, then cluster together with transcription factor 7-like, and other sox genes clustered into another group (Fig. 1A). The number of sox gene introns varied from zero to nine, indicating the structural diversity of sox genes in oysters. The number of exon-intron structures was relatively conserved in the same phylogenomic in oysters (Fig. 1B). Three motifs were identified in sox proteins, but motif types difference existed on different phylogenetic groups (Fig. 1C). HMG-box structural domains were included in all sox genes (Fig. 1D). Amplification of *Sox-like* in male and female genomic DNA revealed that the gene was present in both male and female genomes (Fig. 1E).

3.2. Structural and phylogenetic analysis of sox proteins

Comparison of the HMG conserved structural domain of *CgSox-like* with other animals in the database revealed high homology with LOC111134402 of *Crassostrea virginica* and with sox of *M. yessoensis*, with 91% and 68% similarity, respectively. (Fig. 2A). The phylogenetic analysis revealed that *CgSox-like* clusters with the sox of bivalve (*C. virginica* and *M. yessoensis*) and then with *Sox9* of *Diaphorina citri*. The *CgSox-like* homologs of other species were clustered into another group (Fig. 2B).

3.3. Expression analysis of CgSox-like and CgSox8 in tissues and at different gonad developmental stages

The relative expression of *CgSox-like* and *CgSox8* genes in different tissues was detected. The qPCR results showed that *CgSox-like* and *CgSox8* were uniquely expressed in gonads, but mainly in the male gonads. The expression levels of *CgSox-like* and *CgSox8* in male gonads were 39 times and 162 times higher than those in female gonads, respectively (Fig. 3A). The expression patterns of *CgSox-like* and *CgSox8* (the only sox gene previously identified to be specifically expressed in the male gonads) were then analyzed during different periods of gonad development. In male oysters, *CgSox-like* expression levels were remarkably higher than that of *CgSox8* in resting and proliferative phases, but not in the mature phases. Furthermore, the expression levels of these two genes displayed a progressively increasing pattern and a strong positive correlation (Pearson = 0.8673, $P = 0.02525$) (Fig. 3B). Both *CgSox-like* and *CgSox8* were expressed at very low levels during gonadal development in females, except for *CgSox8* in the mature stage with no expression.

3.4. Interaction relationship between Sox-like and Sox8

The expression levels of *CgSox-like* and *CgSox8* showed a strong concordance, so we firstly verified their correlation utilizing RNA interference. Injection of *CgSox-like* double-stranded RNA decreased the

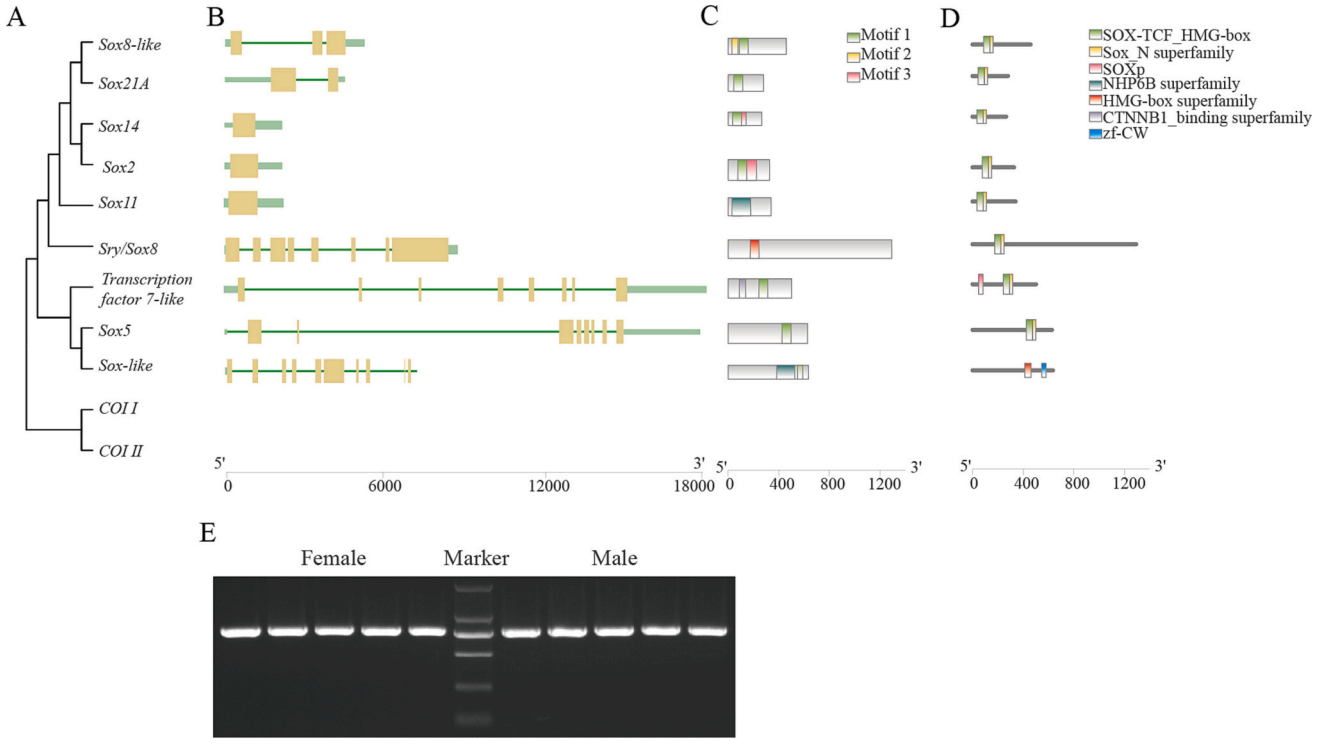

Fig. 1. Structural analysis of *CgSox-like* and PCR validation of *CgSox-like* in male and female genomes. (A) Phylogenetic tree of *C. gigas* sox proteins. *CoiI* and *CoiII* as out-group sequences. (B) Exon-intron structure of *C. gigas* sox genes. (C) Conserved motifs of *C. gigas* sox genes, which were indicated by different colored boxes. (D) Conserved HMG-box domain of *C. gigas* sox genes. (E) PCR amplification products of the 770 bp *CgSox-like* fragments in the genomes of female and male oysters. Amplification was performed in each of the 5 female and male genomic DNAs. Marker lane is 100 bp, 250 bp, 500 bp, 750 bp, 1000 bp and 2000 bp from bottom to top.

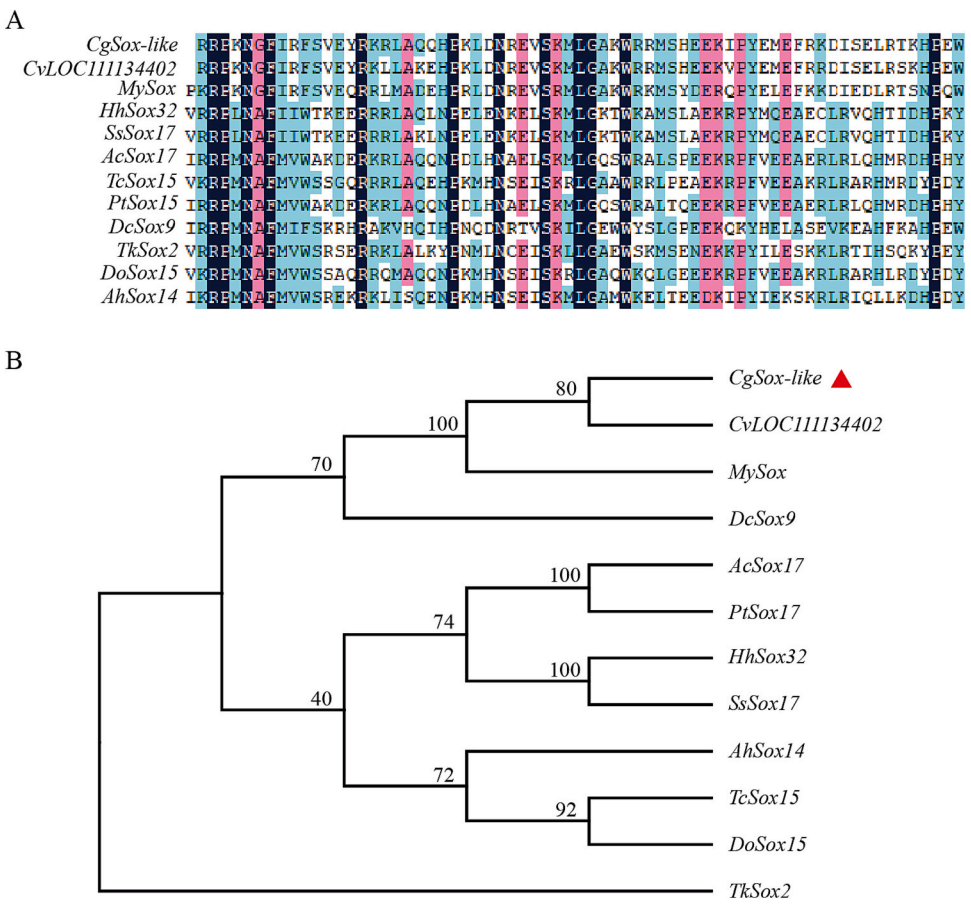

Fig. 2. Alignment and phylogenetic analysis of amino acid sequences for the sox genes of oysters and other species. (A) Alignment of HMG-box domain of sox and selected HMG-box domain genes. (B) Phylogenetic tree of protein sequences of sox and selected HMG-box domain genes. Species names are abbreviated as Ac for *Anolis carolinensis*, Ah for *Acromyrmex heyeri*, Cg for *Crassostrea gigas*, Cv for *Crassostrea virginica*, Dc for *Diaphorina citri*, Do for *Dipodomys ordii*, Hh for *Hucho hucho*, My for *Mizuhopecten yessoensis*, Pt for *Pseudonaja textilis*, Ss for *Salmo salar*, Tc for *Terrapene carolina triunguis*, Tk for *Thelohanellus kitauei*. Accession numbers: *AcSox17*, A0A803TA82; *AhSox14*, A0A836FGS8; *CvLOC111134402*, A0A8B8EHA4; *DcSox9*, A0A3Q0JLX7; *DoSox15*, A0A1S3EYB5; *HhSox32*, A0A4W5R256; *MySox*, A0A210QXS4; *PtSox17*, A0A670XQ95; *SsSox17*, A0A1S3NCZ2; *TcSox15*, A0A674KC23; *TkSox2*, A0A0C2N6F9.

expression levels of *CgSox-like* by 85.6% and that of *CgSox8* by 40% (Fig. 4A). Similarly, after injection of *CgSox8* double-stranded RNA, the expression levels of *CgSox8* decreased by 75.9% and that of *CgSox-like* decreased by 39% (Fig. 4A). These results indicated that the injected double-stranded RNA exerts a significant interference effect on the target genes and demonstrated the interrelationship between *CgSox-like* and *CgSox8*. Meanwhile, the expression levels of *CgDmrt1* and *CgFoxl2* genes were detected after interference. The results showed that the expression levels of *CgDmrt1* and *CgFoxl2* were not dramatically changed after interference with *CgSox-like* and *CgSox8* (Fig. 4A).

Next, the online software prediction results showed the presence of multiple sox transcription factor binding sites upstream of *CgSox-like* (Fig. 4B) and multiple HMG-box binding sites upstream of *CgSox8* (Fig. 4C). Finally, the dual luciferase reporter results showed that the transcription factor SOX-like was able to activate the transcription of the *CgSox8* promoter (Fig. 4D).

3.5. DNA methylation patterns of CgSox-like and CgSox8 during gonadal development

To clarify the methylation profiles of *Sox-like* and the regulation of transient methylation, the methylation levels of the *Sox-like* gene at different developmental periods of the gonads were investigated by BS-PCR. The results of Sanger sequencing displayed that the conversion efficiency of DNA was >99% in all cases. The 12 CpG sites in the promoter region of *Sox-like* gene presented an overall hypomethylation status during different developmental periods of gonads, with methylation levels varying from 2.51% to 5.05% (Fig. 5A). Meanwhile, the methylation levels of *CgSox8* were also examined to elucidate DNA methylation patterns of sex-related genes. The results showed that *CgSox8* promoter exhibited hypomethylation in different periods of gonad development, with the methylation levels ranging from 0 to 1.65% (Fig. 5B). Furthermore, the methylation levels of the *CgSox-like/CgSox8* promoter region did not differ significantly between female and male gonads, and likewise between different periods of gonad development.

DNA methylation was generally considered to be associated with gene expression. Therefore, we subsequently performed Pearson correlation analysis on the methylation levels and expression levels of genes to detect the regulatory role of methylation on gene expression. Given that *CgSox8* hardly underwent methylation, only *CgSox-like* correlation analysis was performed. The results showed that the methylation levels of *Sox-like* and gene expression levels presented a positive correlation with a Pearson correlation coefficient of 0.6845 ($P = 0.1336$) (Fig. 5C).

3.6. Expression levels of Sox-like and Sox8 after inhibitor treatment

BS-PCR results demonstrated that these two genes were demethylated, but it was uncertain whether their expression levels were indirectly controlled by DNA methylation. Therefore, oysters were treated with DNA methyltransferase and demethylase inhibitors to observe the expression levels of the two genes. In females, gene expression levels did not show significant differences after inhibitor treatment. In males, *Sox8* and *Sox-like* expression levels were not altered after 5-Aza-2′-deoxycytidine injection. However, after Bobcat 339 injection, the expression levels of *Sox-like* and *Sox8* were significantly increased, which were 1.93 and 1.47 times higher than the control group, respectively (Fig. 6). These suggested that inhibition of DNA demethylases, i.e., elevated DNA methylation, resulted in the activation of *Sox-like* and *Sox8* expression in males.

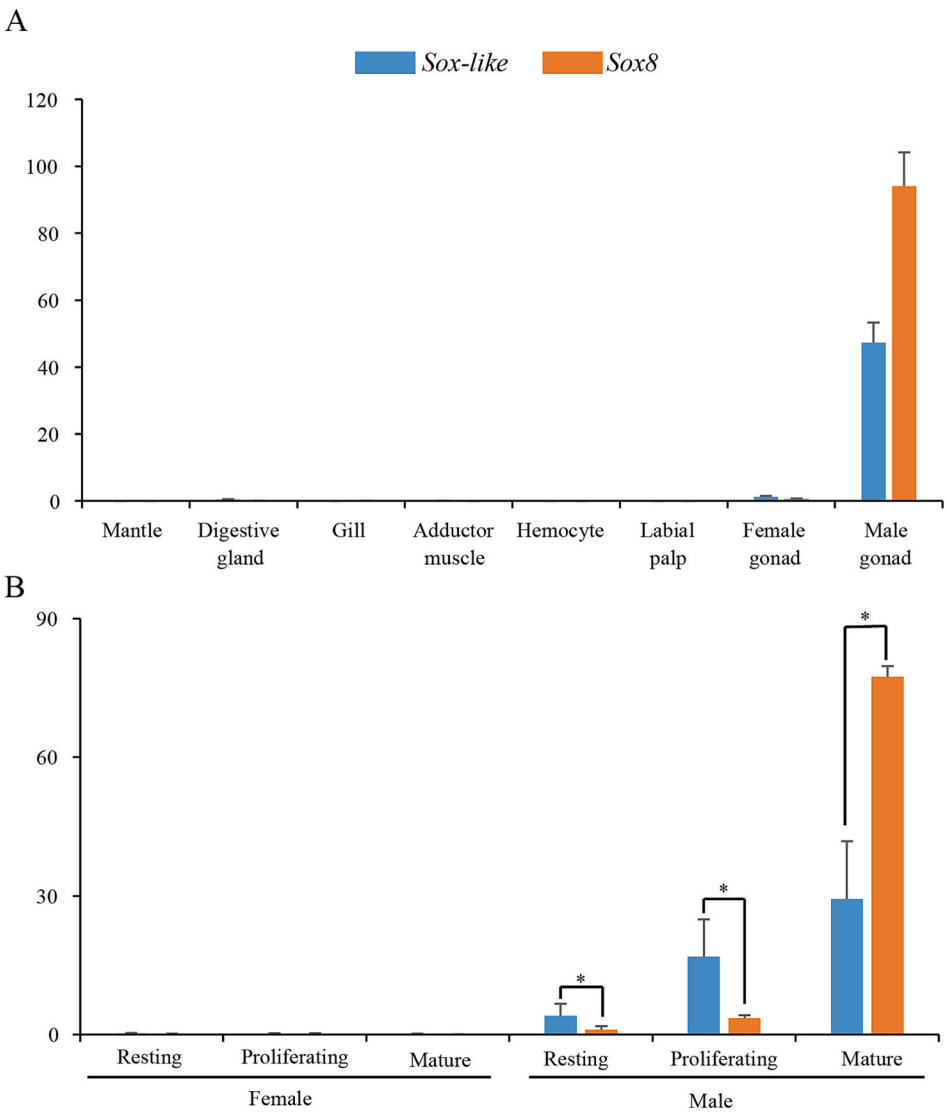

Fig. 3. Expression patterns of *Sox-like* and *Sox8* in different tissues and different periods of gonadal development. (A) Average gene expression of *CgSox-like* and *CgSox8* in different tissues. (B) Mean expression levels of *CgSox-like* and *CgSox8* during different gonadal categories. Bars represent standard error and asterisks indicate significant differences.

4. Discussion

In this study, a novel gene uniquely expressed in the gonads was identified as well as explored its expression patterns during the gonad development. Its gene expression patterns and DNA methylation patterns in gonad development shed new light on the mechanisms of DNA methylation-mediated regulation during bivalves' sex determination/gonad development.

4.1. Homology analysis of Sox-like

The HMG structural domain superfamily consists of the TCF/SOX/MATA group, which typically contains a single sequence-specific HMG structural domain, and the HMG/UBF group, which contains multiple HMG structural domains. HMG structural domain motifs are highly conserved, while outside the HMG structural domain, sox sequences are heterogeneous (Bowles et al., 2000). In fish, the major subfamilies involved in sex determination or gonadal differentiation are *Sox—B1*, *SoxE*, *SoxF*, and *Sox8* (Hu et al., 2021). A similar identification of *Sox* family genes participating in sex determination or gonadal development, such as *SoxE*, *Sox8*, and *Sox9*, has also been observed in bivalves (Teaniniuraitemoana et al., 2014; Zhang et al., 2014). However, only the *Sox8* (*Sox30/Sry*) was recognized to be involved in sex determination or gonadal development in bivalves (Yu et al., 2017; Zhang et al., 2014). In addition, *Sox5* plays a key regulatory role in sex determination in the medaka (Schartl et al., 2018). Phylogenetic analysis of Sox genes in *C. gigas* showed that the new genes *CgSox-like* and *CgSox5* belong to the same group, suggesting that they may play the same functions in sex determination/differentiation.

4.2. Sox-like is associated with sex determination or gonadal development

Although *CgSox-like* was present in both sexes, *CgSox-like* was only expressed in gonads with high expression in male gonads and very low expression in female gonads, indicating a critical role of *CgSox-like* in male gonadal differentiation. The expression patterns of *Sox-like* and *Sox8* were highly consistent in male gonads at different developmental stages, demonstrating that *Sox-like* and *Sox8* together promote the development of male gonads. Interestingly, the expression level of *CgSox-like* was higher than that of *CgSox8* in the resting and proliferative phases, while the opposite was true in the maturation phase. With the same expression pattern of *Dmrt1* (Yue et al., 2021), we could determine

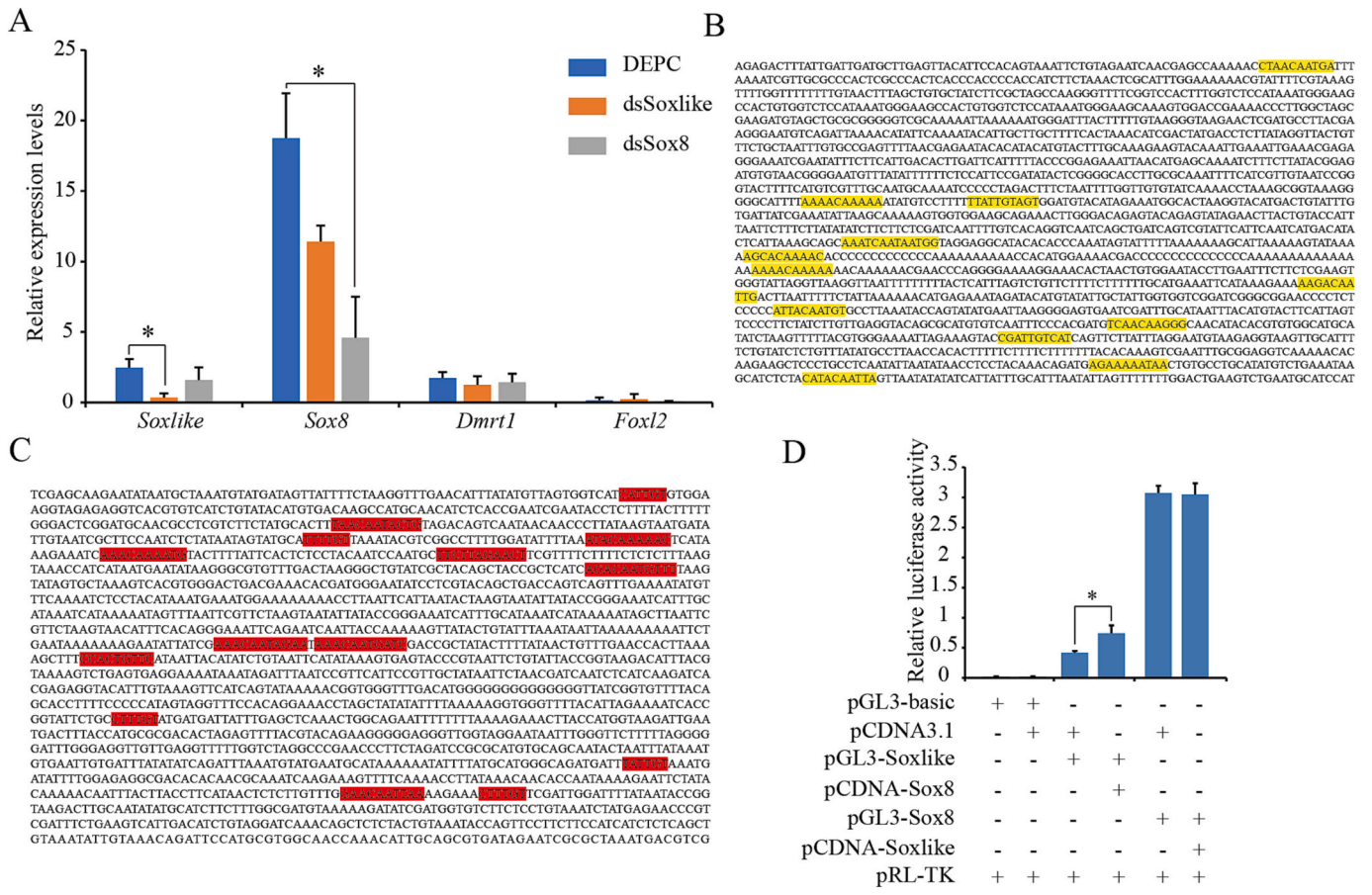

Fig. 4. Interaction of *CgSox-like* and *CgSox8*. (A) Expression levels of sex-related genes after RNA interference. Prediction of transcription factor binding sites 2000 bp upstream of *CgSox-like* (B) and *CgSox8* (C) Yellow boxes indicate *CgSox8* transcription factor binding sites and red boxes indicate HMG-box transcription factor binding sites. (D) Dual luciferase reporter assay demonstrating the activation of the *Sox8* promoter by the transcription factor *Sox-like*. "+" and "-" denote whether the plasmid was transfected into the HEK-293 T or not. Data are mean ± S·D and asterisks indicate significant differences ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

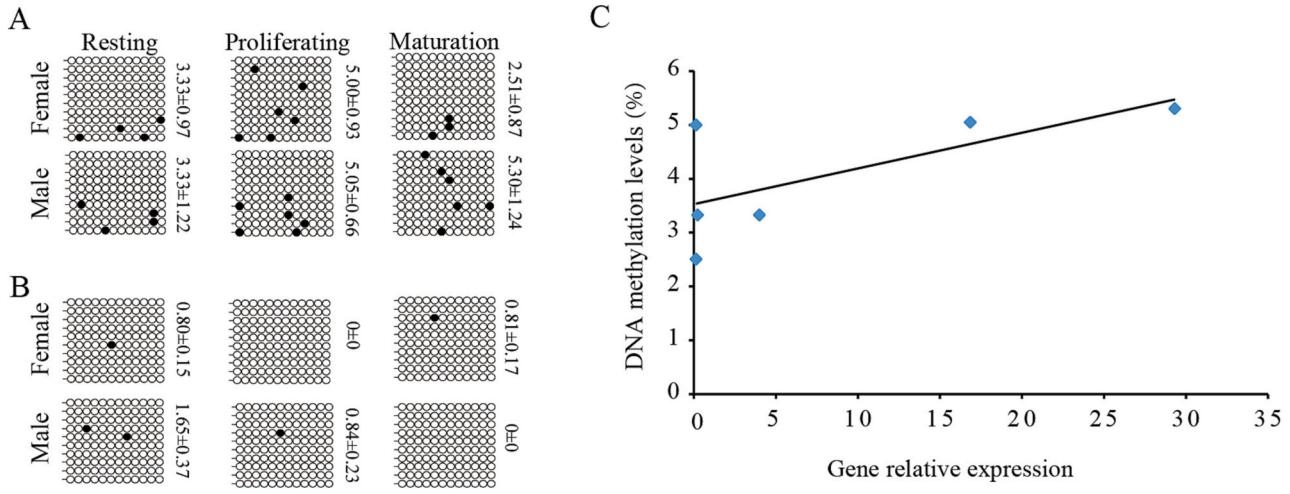

Fig. 5. Correlation analysis of DNA methylation and gene expression. DNA methylation of the *CgSox-like* (A) and *CgSox8* (B) in different periods of gonadal development. Open and filled circles indicate unmethylated or methylated positions, respectively. Three oysters per sample and ten clones per oyster were analyzed. Results are mean ± SEM. Correlation of methylation levels of *Sox-like* promoter and gene expression.

that a cascade of interactions existed between *CgSox-like*, *CgSox8* and *Dmrt1* in the sex determination pathway. Zhang et al. (2014) showed that *Sox8* was located the upstream of *Dmrt1*, but the expression level of *Dmrt1* was not altered after interfering with *Sox-like* and *Sox8*, implying that other pathways may exist to regulate the expression of *Dmrt1*. RNA interference and dual luciferase indicated that *Sox-like* was located the upstream of *Sox8* and was able to activate *Sox8* gene expression, which was consistent with *Sox-like* being expressed earlier than *Sox8*. These

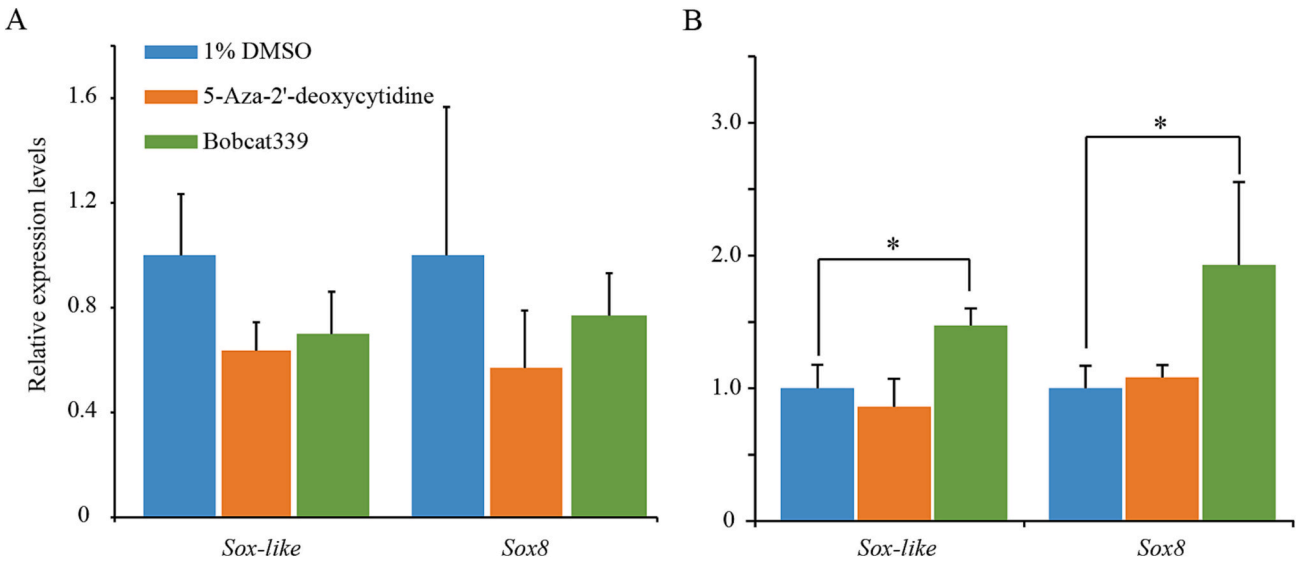

Fig. 6. DNA methyltransferase and DNA demethylase inhibitor assay. The detection of *CgSox-like* and *CgSox8* expression in female (A) and male (B) oysters. All data were normalized according to the control group (1% DMSO), and asterisks indicate significant differences ($P < 0.05$).

results implied the critical function of *CgSox-like* in sex determination or gonadal development in oysters.

4.3. Transient methylation in promoter regions does not participate in sex-related genes expression regulation

In ESD fish, external factors, like temperature, affect the expression of sex determination-related genes through DNA methylation (Anastasiadi et al., 2018; Navarro-Martin et al., 2011; Shao et al., 2014). Similarly, the phenomenon of sex reversal in oysters is strongly related to external environmental factors (Santerre et al., 2013), while differences in DNA methylation between males and females suggest that DNA methylation is also involved in the sex determination process in the oyster (Sun et al., 2022a). Analysis of DNA methylation profiles revealed that *CgSox-like* and *CgSox8* genes were demethylated at maturity, which may be related to the low methylation levels in promoter regions in invertebrates. In fish, methylation of the *Cyp19a* and *Dmrt1* promoters is able to respond to changes in temperature and is subsequently directly involved in their expression (Navarro-Martin et al., 2011; Oshima et al., 2008). In the oyster, promoter methylation possesses important functional outcomes, from cell differentiation during early development to ongoing environmental adaptation (Riviere, 2014). However, *CgSox-like* and *CgSox8* genes were demethylated in both female and male gonads, implying that they remained hypomethylated throughout gonadal development and did not undergo transient methylation. In invertebrates, hypermethylated genes are mainly housekeeping genes that maintain life activities, while luxury genes tend to be demethylated (Riviere et al., 2017; Roberts and Gavery, 2012). The mechanism for this is the protection of housekeeping genes and the adaptation of invertebrates to the complex and variable survival environment. In addition, transient methylation may play a role in the adaptation of oysters to external environmental influences (Roberts and Gavery, 2012). In the study, only the DNA methylation levels of the gene promoter region were examined, given that the methylation of the promoter is most directly related to gene expression. While, gene expression and methylation of the gene body region are positively correlated in invertebrates, which is attributed to the ability of gene body region methylation to increase transcriptional opportunities. Therefore, the regulation of genes by transient methylation through the gene body region cannot be denied, but at least in these two key genes the regulation of transient methylation does not occur in the promoter region.

4.4. The role of DNA methylation in sex determination or gonadal development

The DNA methylation pattern in oysters shows temporal variation, not only during different developmental periods, but also during gonad development (Riviere, 2014; Riviere et al., 2017). Meanwhile, DNA methylation is maintained in dynamic equilibrium by the action of DNA methyltransferases and demethylases (Bestor, 2000; Melamed et al., 2018). Changes in DNA methylation levels caused by variations in methylase-related genes lead to altered gene expression, resulting in fruit ripening or gonad maturation (Lang et al., 2017; Todd et al., 2019). The gonad development of the oyster is also accompanied by a large number of alterations in gene expression that may also be related to DNA methylation (Yue et al., 2018). A high percentage of sexual reversals were present in oysters, and the expression of sex-related genes was altered during sexual reversals (Broquard et al., 2020; Yue et al., 2020). To this end, we used inhibitors to suppress key enzymes associated with DNA methylation. In female oysters, gene expression was not significantly altered after inhibitor treatment, which may be related to the inhibitory effect of female determinants on the male-determined pathway (Shao et al., 2014; Yue et al., 2018). Decitabine, also known as 5-Aza-2′-deoxycytidine is a DNA methyltransferase inhibitor that inhibits both the initial methylation enzyme and the maintenance of methyltransferase activity, resulting in reduced levels of DNA methylation. Early embryonic development in oysters is inhibited through the utilization of decitabine (Riviere et al., 2013). However, the expression levels of *Sox-like* and *Sox8* did not change significantly in oysters. This may be related to the more pronounced expression of DNA methyltransferase during early embryonic development and the reduced expression in adult somatic tissues (Wang et al., 2014). In contrast, the treatment of oysters with Bobcat 339, a demethylase inhibitor, exhibited different results. In female mice, defects in the demethylase gene resulted in reduced expression of meiosis-related genes (Yamaguchi et al., 2012). Thus, reduced demethylase activity also affects gene expression during meiotic spermatozoa in oysters. Token together, DNA methylation is involved in sex determination and gonadal development, but not in the form of transient methylation of sex-associated gene promoters.

Author statement

Dongfang Sun: Investigation, Formal analysis and Writing - original

draft.

Hong Yu: Data Curation and Writing - review & editing.

Qi Li: Conceptualization, Supervision, Project administration and 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.

Data availability

Data will be made available on request.

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