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Characterization, expression profiling, and estradiol response analysis of *DMRT3* and *FOXL2* in clam *Cyclina sinensis*

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The clam *Cyclina sinensis* is one of the important economical aquaculture shellfish in China. However, the mechanisms of sex determination and differentiation in *C. sinensis* have not been fully studied. In this study, full-length cDNAs of *DMRT3* and *FOXL2* were cloned and functionally characterized. The ORF region of *CsDMRT3* consists of 1137 nucleotides, which encode 378 amino acids contains a conserved DM domain of *DMRT* family. The ORF region of *CsFOXL2* is 1245 bp, encodes 414 amino acids, and contains a conserved FH domain. Tissue-specific expression results showed that the higher expression level of *CsDMRT3* and *CsFOXL2* was found in the ovary and testis of *C. sinensis*. The expression levels of *CsDMRT3* and *CsFOXL2* also peaked at the maturation stage of male and female gonadal development, respectively. Moreover, the expression levels of *CsDMRT3* and *CsFOXL2* were significantly higher in the trochophore and D-larval stages than in other stages. The transcript levels of *CsDMRT3* reached the highest level at 11 months of age, while the *CsFOXL2* reached the highest level at 7 months of age. In estradiol-treated experiments, the expression levels of *CsDMRT3* and *CsFOXL2* in the gonads were highest at 5 μg/L estradiol treatment, and histologically, it was observed that the oocytes diameters became larger with increasing estradiol concentration. These results suggest that *CsDMRT3* and *CsFOXL2* play an important role in gonadal development and sex differentiation of *C. sinensis*.

KEYWORDS

***Cyclina sinensis*, *DMRT3*, *FOXL2*, expression analysis, estradiol response**

1 Introduction

Sex differences are essential characteristics of sexually reproducing organisms. In aquatic animals, species of both sexes grow at different rates (Liao, 2022). Therefore, understanding the sex development of aquatic animals is important. Key sex-related genes have been identified in bivalves, including *FOXL2*, *DMRT* family, *WNT4*, *FST*, *β-CATENIN*, and *SOXE*, which are involved in the development, gonad maintenance and germ cell formation of individual organisms (Christelle et al., 2014; Shi et al., 2014; Tong et al., 2015).

The *DMRT* (double-sex and mab-3-related transcription factor) gene family was first detected from fruit fly *Drosophila melanogaster*, where all genes in the family are transcription factors. The *DMRT* family is involved in the sex determination and differentiation of organisms, as well as in embryonic development and organ formation (Hildreth, 1965). Shinseog et al. (2003) found that *DMRT3*, *DMRT5*, and *DMRT7* exhibit sex specificity, suggesting their involvement in the gonadal development of mouse *Mus culus*. *DMRT3* transcripts in Japanese pufferfish *Takifugu rubripes* begin to express at the larval stage and are highly expressed in adult testes (Akihiko et al., 2006). Similar results were found in experiments with Atlantic cod (Hanne and Øivind, 2012).

FOXL2, forkhead transcription factor gene 2, is a member of the forkhead (FOX) family. The *FOXL2* coding region is highly conserved in *Homo*, *Capra hircus*, and *M. culus*. (Cocquet et al., 2002). *FOXL2* represses the male gene pathway during female gonadal differentiation and regulates and maintains ovarian development in mice (Loffler et al., 2003; Chris et al., 2005). Previous experiments have shown that *FOXL2* is expressed very differently in the testis and ovary of chicken *Gallus gallus domesticus*, and its testis expression is much lower than the ovary expression (Govoroun et al., 2004). Similar results were found during gonadal differentiation in frog *Rana rugosa* (Yuki et al., 2008). In invertebrates, *FOXL2* is involved in regulating embryonic development in sea urchin *Strongylocentrotus purpuratus* (Tu et al., 2006). Liu et al. (2013) found that *FOXL2* was expressed at a higher level in the D-larval stage of Zhikong scallop *Chlamys farreri*. The expression level of *FOXL2* in the freshwater mussel *Hyriopsis cumingii* gonad gradually decreases with age (Wang et al., 2020).

Estradiol (E2) is a sex steroid hormone produced by cholesterol metabolism and plays an important role in gonadal development and sex differentiation in animals. Specifically, Ni et al. (2012) found that the injection of estradiol into oysters induces sex reversal from male to female. Yan et al. (2011) treated razor clam *Sinonovacula constricta* with estradiol, causing an increase in oocyte diameter, suggesting that estradiol plays an endogenous regulatory role in the gonadal development of razor clam. Estradiol affects on the expression levels of sex-related genes. Estradiol exposure decreases the expression levels of *DMRT1* and *SOX9*, which are male key factors in *Pelodiscus sinensis* (Wang et al., 2014). The expression level of *FOXL2* was elevated in the ovaries of sea urchins *Mseocentrotus nudus* after estradiol exposure (Hu et al., 2021). The same result was also found in blue tilapia *Oreochromis aureus* (Cao et al., 2010).

To date, studies on genes related to sexual differentiation and the mechanisms of sexual differentiation among mollusks have mainly focused on oyster *C. gigas*, scallop *C. farreri*, and freshwater mussel *H. cumingii* (Liu, 2012; Tian et al., 2012; Christelle et al., 2014). However, detailed findings on the sex determination and differentiation of *C. sinensis* have not been reported. Understanding the sex determination and differentiation of *C. sinensis* is important for the reproduction and breeding of clams (Wei et al., 2020; Dong et al., 2021; Liao et al., 2022). In the present study, the full-length cDNAs of *DMRT3* and *FOXL2* were cloned first. The changes in the expression patterns of *DMRT3* and *FOXL2* were analyzed in different tissues, gonadal development stages, larval developmental stages, and different month-old clams. Finally, the expression levels of *DMRT3* and *FOXL2* of *C. sinensis* in response to estradiol exposure were investigated. This study will provide a reference for subsequent studies on gonad development and sex differentiation of *C. sinensis*.

2 Materials and methods

2.1 Ethics statement

All animal experiments were conducted in accordance with the guidelines and approval of the Animal Experiment Ethics Committee at Jiangsu Ocean University.

2.2 Animals and sampling

All clams of this study were obtained from Lianyungang Comprehensive Experimental Station, the national shellfish industry system of China. The clams were acclimated in a seawater tank (salinity: 25‰) for a week before experimental processing. During the acclimatization, the clams were fed with microalgae *Chaeroeeros moelleri* twice in the 8:30am and 18:00pm. The microalgae *Chaeroeeros moelleri* was purchased from Wudi Zaocheng Biotechnology company (Shandong, China).

Past studies have shown that the gonadal development of *C. sinensis* can be divided into five stages: proliferation stage, growing stage, maturation stage, spawning stage, and spent stage (Racotta et al., 2003; Liao, 2022). Based on above gonadal staging system, eight male and eight female clams in the growing stage were selected and adductor, mantle, pipe, gill, foot, hepatopancreas and gonad were collected. Ovarian and testis samples were also collected from *C. sinensis* with different gonadal development stages, respectively. During the larval development of *C. sinensis*, the fertilized egg, trochophore, D-larvae, and umbo larvae were sampled in a 1.5 mL DNase/RNase-free centrifuge tube. In addition, the gonads of juvenile clams aged 3–12 months were also collected every month from October 2020 to September 2021. All tissues were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

2.3 RNA isolation, cDNA synthesis, and RACE PCR

Total RNA from the clam tissues was isolated using TRIzol reagent (TaKaRa, Japan), following the manufacturer's instruction, and then stored at −80°C. PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Tokyo, Japan) was used for first-strand cDNA synthesis. Ready-cDNA, which was used as the template for RACE PCR, was synthesized using the SMART™ RACE cDNA Amplification Kit (Clontech, USA) in accordance with the manufacturer's instructions.

The program of touchdown PCR was conducted in the 5′- and 3′-RACE PCR amplification as follows: 5 cycles of 95°C for 30 s, 72°C for 30 s, 72°C for 2 min; 5 cycles of 95°C for 30 s, 70°C for 30 s, 72°C for 2 min; 5 cycles of 95°C for 30 s, 68°C for 30 s, 72°C for 2 min; and 20 cycles of 95°C for 30 s, 66°C for 30 s, 72°C for 2 min, and then 72°C for 7 min for elongation. The PCR products were purified using a DNA Purification Kit (CWBiotech, Beijing, China), and the purified products were subcloned into a pEASY-T1 vector (Transgen, China) and sequenced by Ruibiotech Company (Beijing, China). The sequences of all the primers used are displayed in Table 1.

2.4 Sequence and phylogenetic analysis

All the sequence data were analyzed using DNAStar 11.1. The open reading frame (ORF) of genes was predicted by the ORF finder. The conserved domains and genomic structures were analyzed using the online software Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/) and Splign (https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi), respectively. Phylogenetic trees were constructed using MEGA 5.0 with the Neighbor-joining (NJ) method. Amino acid sequences from other species were downloaded from the GenBank database.

2.5 Quantitative real-time PCR

Primer Premier 5.0 was used to design specific primers for qPCR (Table 1). The qPCR was then conducted using the SYBR Premix Ex Taq™ kit (Takara, Japan) on a StepOnePlus Real-Time PCR system (Applied Biosystems) according to the instructions. *β-actin* was selected as the reference gene in the qPCR reaction system. The $2^{-\Delta\Delta CT}$ method was used for the analysis of the relative gene expression.

2.6 Estradiol exposure

Estradiol regent was obtained from Sigma (St. Louis, MO, USA). The stock solution (4 mg/mL) of estradiol was prepared in ethanol. Two-year-old *C. sinensis* samples in the growing stage were selected for the experiment. The average length and body weight of the clams were in the range of 3.2–3.4 cm and 13–15 g, respectively. First, 180 healthy clams were randomly divided into three groups of 60 with three duplicates in each group. Clams were exposed to estradiol (E2: control group, 5 μg/L, and 50 μg/L; Wu, 2019). The same feeding regime was kept during the exposure. Uneaten food and feces were removed before water renewal. The water was changed daily, and fresh hormones were

TABLE 1 Primers used in this study.

Primer	Sequence (5′–3′)	Purpose
DMRT3-5GSP	GCGTCGGAATACGTCTGTGTCCATCT	RACE
DMRT3-5NGSP	TTCTGTCGCCTGCTGTCTTTTC	RACE
DMRT3-3GSP	AATCGGCGTTCAAGCCATTACCAA	RACE
DMRT3-3NGSP	CAATCTTGGACTTCCGTTTCCGCAT	RACE
FOXL2-5GSP	TTTCCTTGTCGGCAACAGAGCG	RACE
FOXL2-NGSP	ACGGTGACAGGATTGGATTTAGG	RACE
Upm-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE
Upm-short	CTAATACGACTCACTATAGGGC	RACE
NUP	AAGCAGTGGT AACAACGCAGAGT	RACE
DMRT3-F	CTGAAAAGACAGCAGGCGACA	qPCR
DMRT3-R	GCTTGACTTGCGGGTATCGA	qPCR
FOXL2- F	ACTTGCTTCCTGTGGATACGG	qPCR
FOXL2- R	TAAATGGCTCGCTCTGTTGC	qPCR
β-actin-F	CCTGGTATTGCCGACCGTAT	qPCR
β-actin-R	TTGGAAGGTGGACAGTGAAGC	qPCR

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added. After 21 days of exposure, the gonad tissues of the clam were frozen in liquid nitrogen and stored at −80°C for RNA extraction. The remaining ovaries were fixed in 4% paraformaldehyde (PFA) overnight. Then, paraffin-embedded ovary samples were sliced on a microtome, and hematoxylin-eosin staining was carried out. Finally, ovary sections of 12 clams were observed under a Nikon 90i microscope, and the oocyte diameter was recorded.

2.7 Statistical analyses

All experimental data are expressed as mean ± SD and were analyzed using SPASS 23.0. The homogeneity of variance for all data was tested using Levene's method. When the homogeneity variance was unsatisfactory, the percentage data were processed by taking the arcsine or square root. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with Duncan's test on the gene expression levels, and statistical significance was defined at P value <0.05.

3 Results

3.1 Sequence analysis of *DMRT3* and *FOXL2* in *C. sinensis*

In the study, the cDNA sequences of *CsDMRT3* (GenBank accession No: OP970557) and *CsFOXL2* (GenBank accession No: OP970558) were obtained by partial cDNA cloning and RACE technique. The ORF region of *CsDMRT3* was 1137 bp, which encoded 378 amino acids containing the DM domain (16–69 aa) and DMA domain (189–228 aa; Figure 1A). The ORF sequence of *CsFOXL2* was 1245 bp. The deduced amino acid sequence was 414 aa and contains the FH domain (177–267 aa; Figure 1B).

The predicted molecular weight of the *CsDMRT3*-encoded protein was 41.38 kDa, whereas the theoretical isoelectric point was 8.05. The α helix of the CsDMRT3 protein was 25.93%, the extended strand was 2.65%, the β strand was 3.17%, and the disordered was 68.25% (Figures 2A, C). Among the protein encoded by *CsFOXL2*, the proportions of α-helix, extended chain, β-turn, and irregular coiling were 27.54%, 8.94%, 5.80%, and

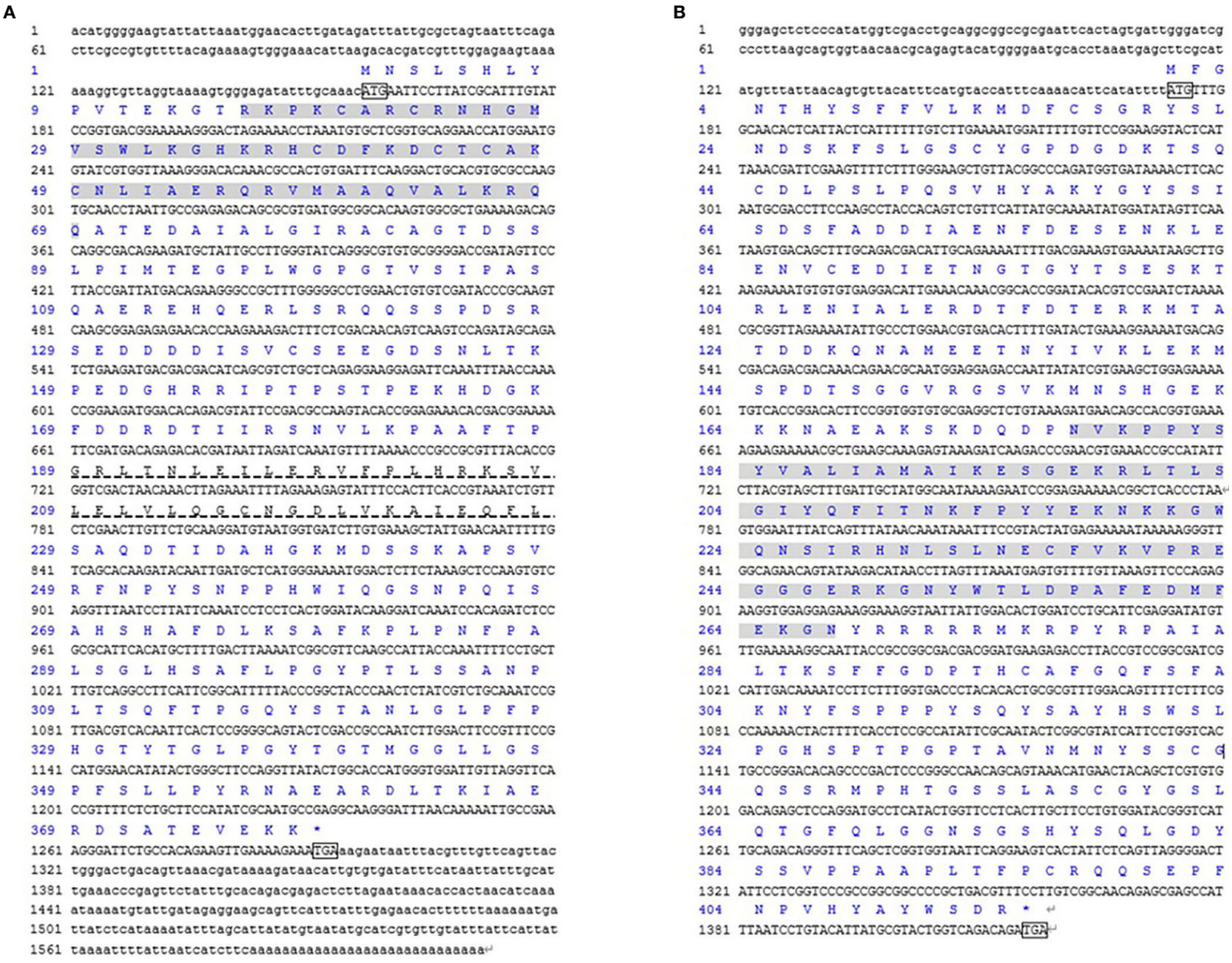

FIGURE 1
The *DMRT3* **(A)** and *FOXL2* **(B)** cDNA and deduced amino acid sequence of *C. sinensis*. The ATG/TGA indicated by the box starts codon/stop codon; shaded regions represent the DM domain and the FH domain, respectively. The DMA domain was indicated by virtual underline.

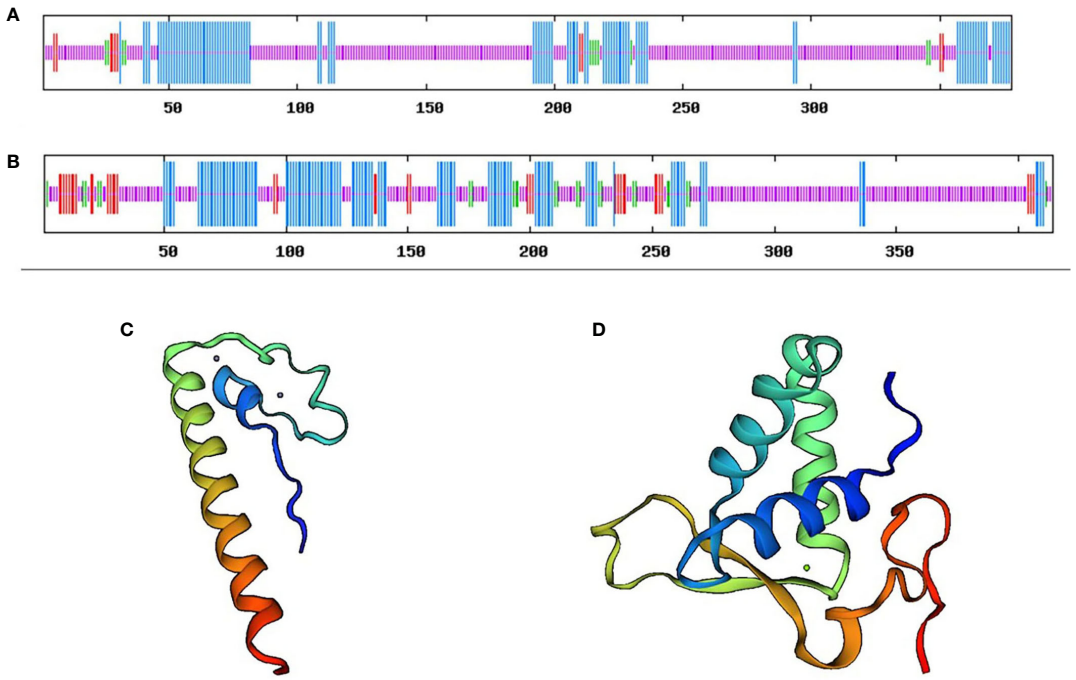

FIGURE 2
The secondary structure and tertiary structure prediction of *DMRT3* **(A)** and *FOXL2* **(B)** in *C. sinensis*. Blue represents the alpha helix, red represents the extended strand, green represents the beta-turn, and orange represents the random coil.

57.73%, respectively (Figures 2B, D). The predicted molecular weight of the *CsFOXL2*-encoded protein was 46.55 kDa, whereas the theoretical isoelectric point was 7.55.

A comparison of the amino acid sequence encoded by the *CsDMRT3* with other species (Figure 3A) showed that *DMRT3* contains a conserved DM domain. The highest identity of *CsDMRT3* with *Mercenaria mercenaria* was 85.1%, and it shares 40.5%, 48.4%, 48.7%, 35.8%, and 36.2% identity with homologs in *Pomacea canaliculate*, *Aplysia californica*, *Crassostrea virginica*, *Acanthaster planci*, and *Limulus polyphemus*, respectively. Therefore, *DMRT3* is relatively conserved in shellfish, especially in bivalves. The comparison of the sequence of *FOXL2* (Figure 3B) showed that the FH domain is also present in other species. *CsFOXL2* shares 84.7%, 62.3%, 58.1%, 57.9%, 51.4%, and 49.0% identity with homologs in *Mercenaria mercenaria*, *Dreissena polymorpha*, *Haliotis rubra*, *Gigantopelta aegis*, *Octopus bimaculoides*, and *Owenia fusiformis*, respectively.

Phylogenetic tree was constructed based on the sequence of the *CsDMRT3* with 12 species by using the Neighbor-Joining method, and the results indicate that *CsDMRT3* cluster with the other bivalves and stay farther away from humans and mice (Figure 4A). Phylogenetic analysis showed that *CsFOXL2* formed a cluster with those of other bivalve shellfish species (Figure 4B).

3.2 Expression levels of *DMRT3* and *FOXL2* in different tissues and gonadal development stages of *C. sinensis*

Tissue-specific expression results showed that the expression level of *CsDMRT3* in the testes significantly higher than that of other tissues, including the hepatopancreas, ovary, and foot (Figure 5A). During the gonadal development of *C. sinensis*, the expression level of *CsDMRT3* in the testis increased significantly from the proliferation stage to maturation stage, and reached the peak level at the maturation stage (Figure 6A). Subsequently, the expression of *CsDMRT3* in the testis of *C. sinensis* continued to decline from spawning stage to spent stage (Figure 6A).

The present result showed that the highest expression level of *CsFOXL2* was found in the foot of *C. sinensis*. Moreover, the results showed that the levels of *CsFOXL2* in the ovaries of *C. sinensis* were higher than that in the testis (Figure 5B). Further analysis revealed that the expression level of *CsFOXL2* in the ovary increased significantly from the proliferation stage to maturation stage, and reached the peak level at the maturation stage throughout the reproductive cycle of *C. sinensis*. Then, the expression level of *CsFOXL2* in the ovary decreased significantly from spawning stage to spent stage. The lowest expression level of *CsFOXL2* was found in the spent stage (Figure 6B).

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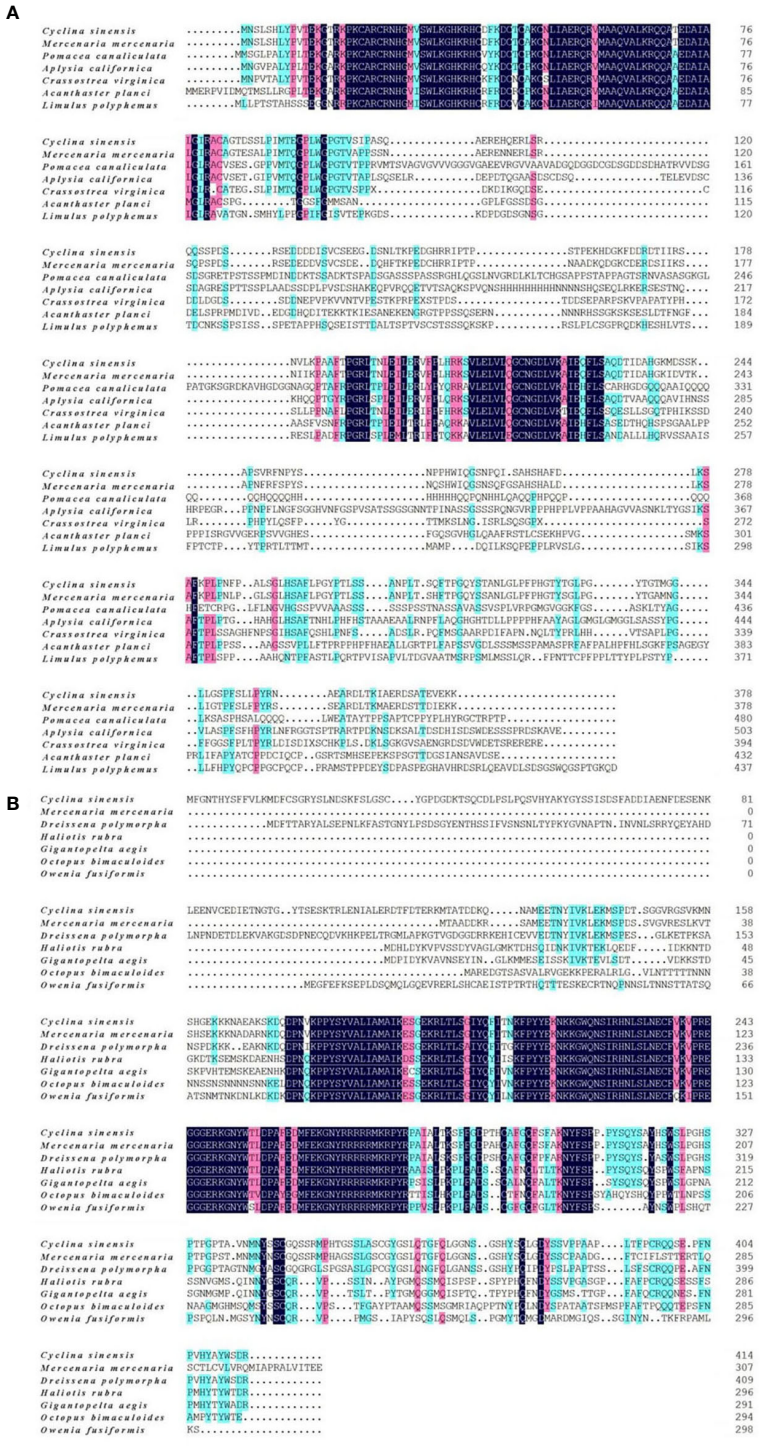

FIGURE 3
The amino acid alignment of *DMRT3* **(A)** and *FOXL2* **(B)** from *C. sinensis* and other species. Different colors represent residues sharing homology, cyan, pink and black regions indicate homology above 50%, above 75%, and equal to 100%, respectively.

3.3 Expression levels of *DMRT3* and *FOXL2* in larvae development and different months of *C. sinensis*

During the period of larval development of *C. sinensis*, *CsDMRT3* had the lower expression level in fertilized egg and umbo larval stages. However, the expression level of *CsDMRT3* in the trochophore and D-larval stages was significantly higher than that of other two stages. (Figure 7A). For the expression levels of *CsDMRT3* in *C. sinensis* at different months of age, the results showed that the transcript level of *CsDMRT3* in the gonads at different months of age showed a sharp increase from 5 months of

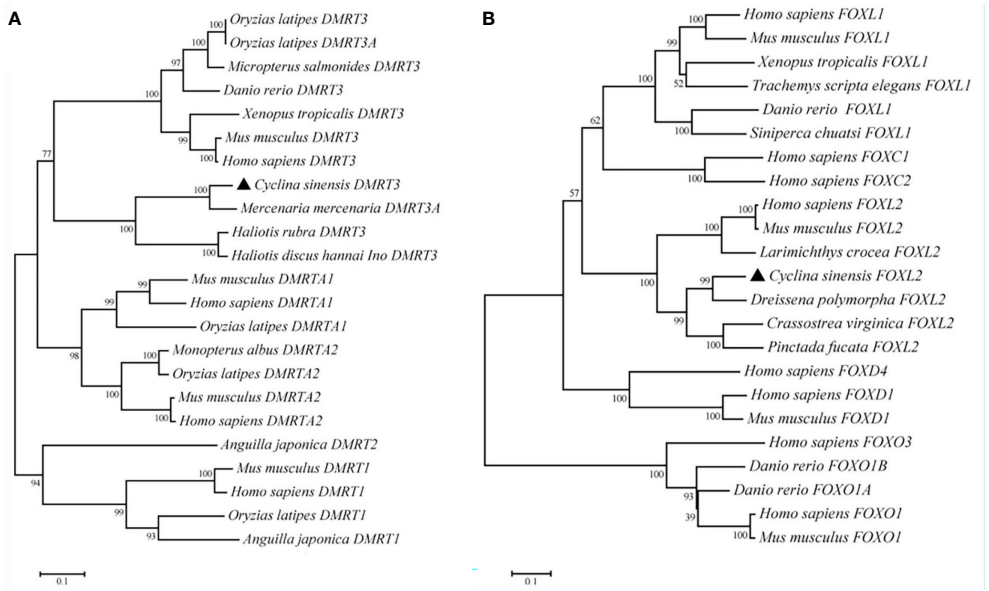

FIGURE 4
The phylogenetic tree of *DMRT3* **(A)** and *FOXL2* **(B)** from *C. sinensis* and other species. *Note: C. sinensis* were marked by a black triangle.

age to 11 months of age. Moreover, the expression level of *CsDMRT3* reached the lowest level in the gonads of 12-month-old *C. sinensis* (Figure 8A).

The expression level of *CsFOXL2* increased significantly from the fertilized egg to D-larval stage, while the expression level of *CsFOXL2* declined sharply in the umbo larval stage (Figure 7B). For the expression levels of *CsFOXL2* in *C. sinensis* at different months of age, the results showed that the low transcript levels of *CsFOXL2* were found in 3 to 5 months of age, increased at 6 months of age, and then dropped at 9 months of age (Figure 8B).

3.4 Expression levels of *CsDMRT3* and *CsFOXL2* in gonads after estradiol exposure

Results of estradiol exposure experiments showed that 5 μg/L estradiol treatment caused a significant increase in *CsDMRT3* transcript level of male gonads. However, no significant changes in *CsDMRT3* expression levels were observed at 50 μg/L estradiol treatment compared to the control group (Figure 9A). Moreover, estradiol treatment up-regulated the expression level of *CsFOXL2* in female gonads compared to the control group (Figure 9B).

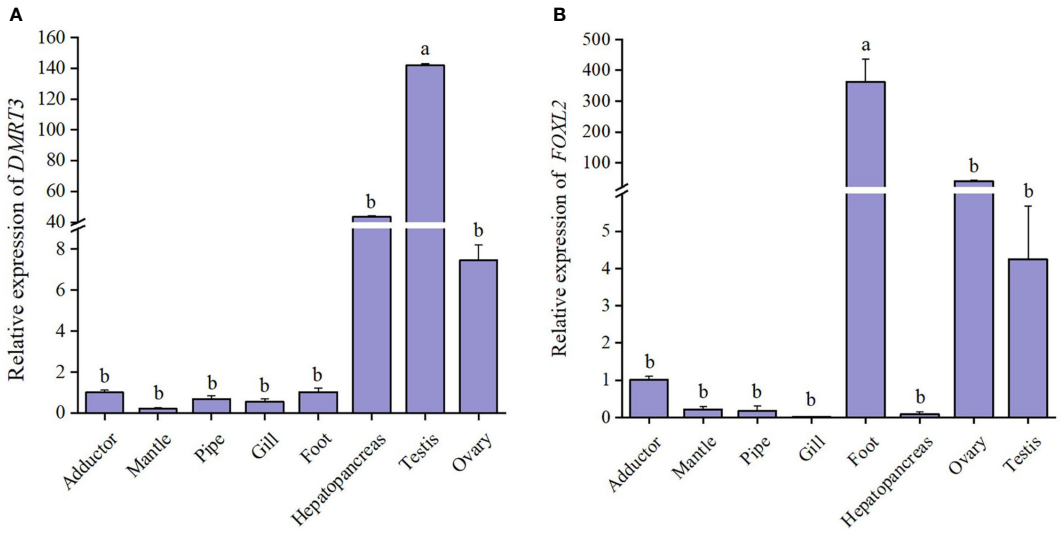

FIGURE 5
The relative expression levels of *DMRT3* **(A)** and *FOXL2* **(B)** in different tissues of *C. sinensis*. Different letters represent significant differences (*P<0.05*).

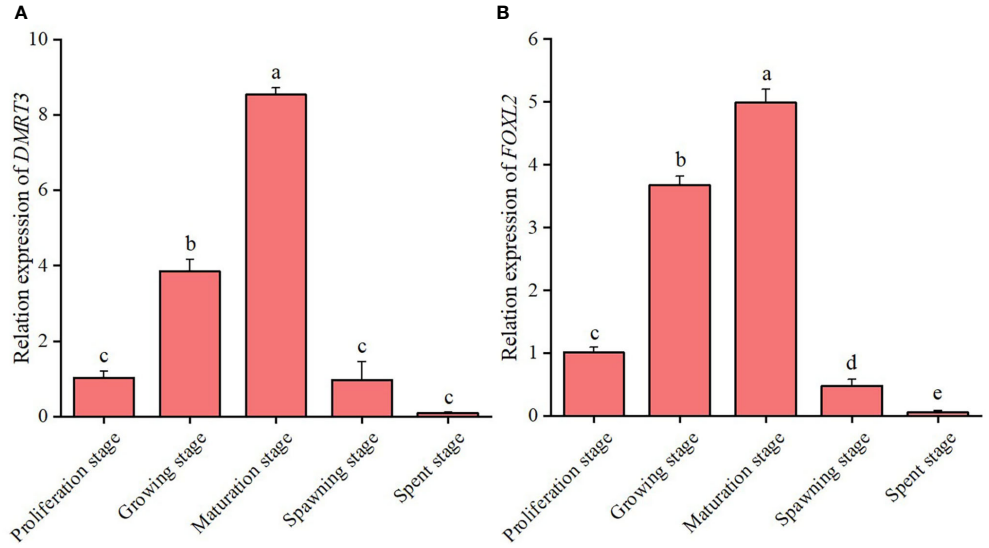

FIGURE 6
The relative expression levels of *DMRT3* **(A)** and *FOXL2* **(B)** in the different gonadal developmental stages of *C. sinensis*. The expression levels of *DMRT3* and *FOXL2* was only detected in testis and ovary, respectively. Different letters represent significant differences (*P<0.05*).

3.5 Ovaries histological analysis of estradiol exposure

Histological examination showed that estradiol exposure promoted the ovarian development of *C. sinensis* (Figure 10). Specifically, the main type of germ cells of *C. sinensis* was pre-vitellogenesis oocyte (POI) and the cytoplasm of POI was basophilic in the control group (Figure 10A). In the estradiol group (5 μg/L estradiol, 50 μg/L estradiol), the main type of germ cells of *C. sinensis* was post-vitellogenesis oocytes (POL) and the cytoplasm of POL is eosinophilic (Figures 10B, C). Further analysis revealed that the mean long diameters of the oocytes in control group, 5μg/L group and 50 μg/L group were 42.62 ± 1.50 μm, 59.56 ± 2.22 μm, and 69.05 ± 1.99 μm, respectively. The short diameters of oocytes after different estradiol treatments were 28.23 ± 1.01 μm, 38.30 ± 1.29 μm, and 46.82 ± 1.52 μm (Table 2).

4 Discussion

The *DMRT* family is a class of sex-determination and differentiation-related genes that are commonly found in organisms, and the genes in this family are highly conserved in invertebrates and vertebrates (Zhang et al., 2015; Craig et al., 2002).

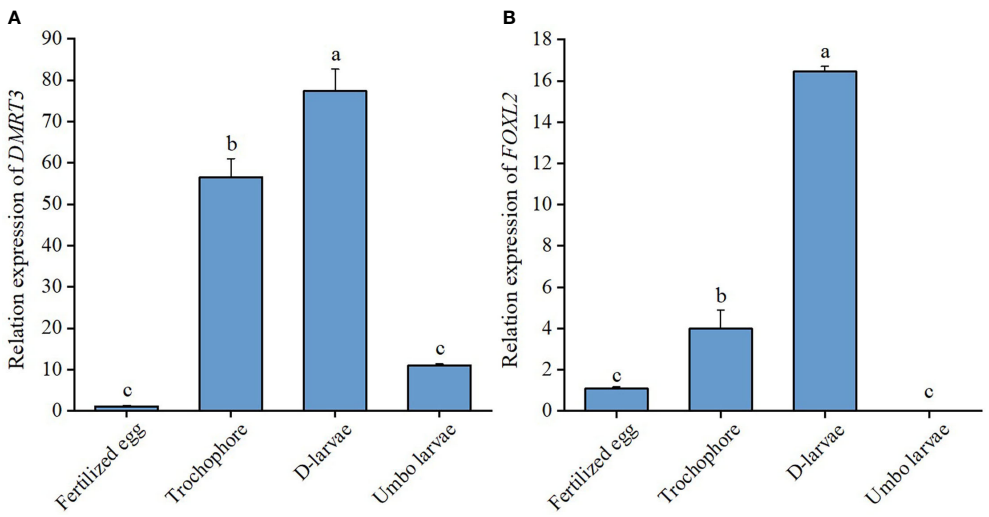

FIGURE 7
The relative expression levels of *DMRT3* **(A)** and *FOXL2* **(B)** in larval development stages of *C. sinensis*. Different letters represent significant differences (*P<0.05*).

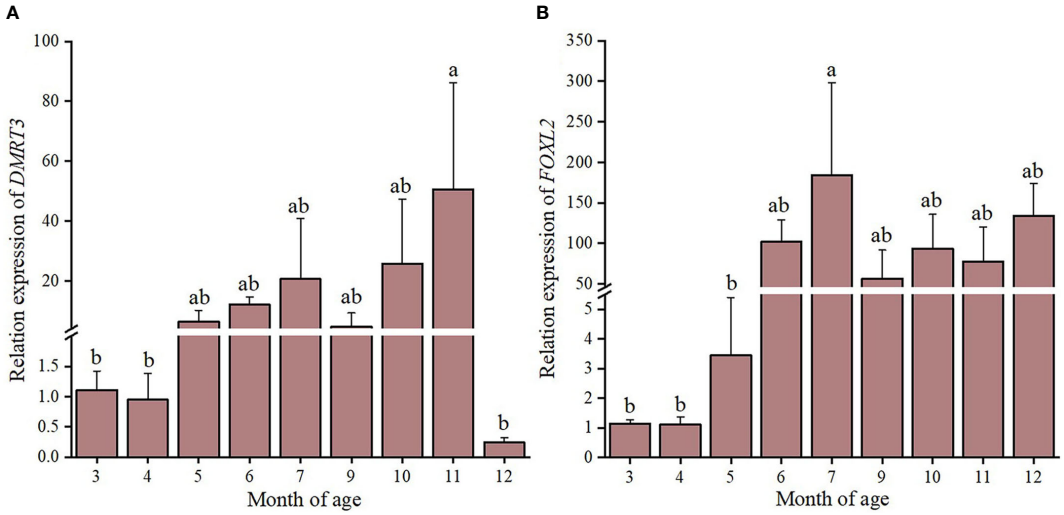

FIGURE 8
The relative expression levels of *DMRT3* **(A)** and *FOXL2* **(B)** in different months ages of *C. sinensis*. Different letters represent significant differences (*P<0.05*).

The *DMRT* family-specific DM domain, which binds specifically in a zinc finger-like manner, is the main functional domain in the gene sequence Guo et al. (2020). Sinclair et al. (1990) found that zinc finger proteins can bind to other regulated genes and then regulate downstream genes, ultimately affecting testis development. The present results showed that no transmembrane structure was detected for the CsDMRT3 protein, suggesting that the DMRT3 protein in *C. sinensis* is an intracellular protein. DMRT3 protein has also been reported in studies on largemouth bass *Micropterus salmoides*, mandarin fish *Siniperca chuatsi*, and swamp eel *Monopterus albu*. Furthermore, the DM and DMA domains were present in the protein sequence encoded by *CsDMRT3*, indicating that the structure of *DMRT3* was conserved between vertebrate and invertebrates. Yu et al. (2007) found that 68% of the amino acid sequences in DM domains of *DMRT* were identical in 16 different evolutionary positions species.

The *DMRT* family is involved in sex determination and differentiation, gonadal development, and maintenance of organ function in organisms, and plays an important role in the development of individuals (Grandi et al., 2000; Loffler et al., 2003; Li et al., 2018). Previous experiments showed that *DMRT3* was expressed only in the testes of *Carassius auratus* (Wang et al., 2014). However, *DMRT3* transcripts have been detected in both testes and ovaries of zebrafish and Mandarin fish (Li et al., 2008; Han et al., 2021). Similar results were found in scallops and ctenophores (Feng et al., 2010; Zhao, 2021). Tissue distribution

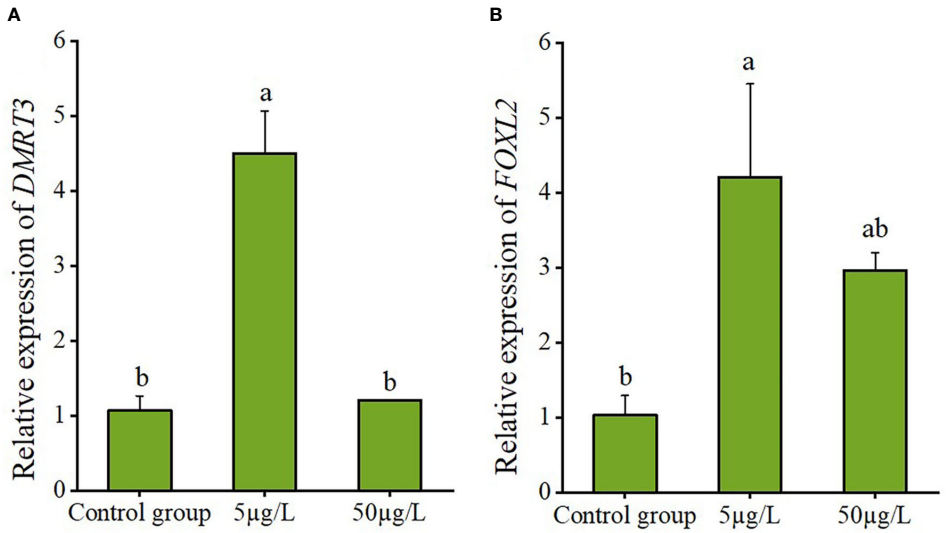

FIGURE 9
The relative expression levels of *DMRT3* in the testis **(A)** and *FOXL2* in the ovary **(B)** of *C. sinensis* under estradiol exposure treatments. Different letters represent significant differences (*P<0.05*).

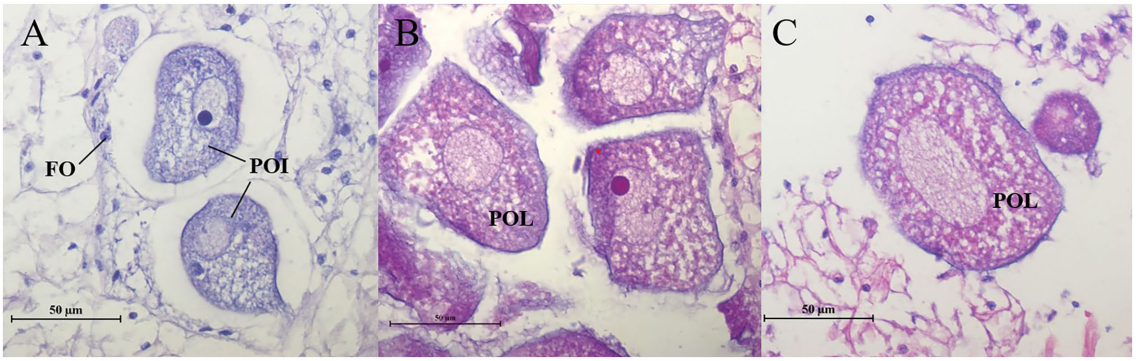

FIGURE 10
Ovaries histological characteristic of *C. sinensis* under estradiol exposure treatments. **(A)** control group, **(B)** 5 μg/L group, **(C)** 50 μg/L group. POI, pre-vitellogenesis oocyte; POL, post-vitellogenesis oocyte; FO, follicle cells.

analysis showed that the expression level of *CsDMRT3* in the testes significantly higher than in the ovary. The findings clarify that the expression level of *CsDMRT3* shows sexual dimorphism. The expression level of *CsDMRT3* in the testis reached the peak level at the maturation stage. The lowest expression level of *CsDMRT3* was found in the spent stage. Studies on the oyster *C. gigas* and scallops *Patinopecten yessoensis* yielded similar results (Amine et al., 2009; Zhao, 2021). Therefore, it is hypothesized that *DMRT3* is involved in the regulation of testis development and germ cell formation. The expression levels of *CsDMRT3* in larvae of different developmental stages were examined by qPCR. Results show that the expression level of *CsDMRT3* was the highest in the D-larval stage and low in the umbo larval stage, indicating that *CsDMRT3* was mainly synthesized in the D-larval stage. Similar features were found in the embryonic development of zebrafish and mice, and this finding is presumably related to the formation of the nervous system (Shinseog et al., 2003; Li et al., 2008; Wang and Luo, 2014). The expression level of *CsDMRT3* increased at 4 months of age and was highest at 11 months of age. Therefore, *CsDMRT3* is involved in the growth and development of the testis.

FOXL2 has an important role in sex determination and differentiation, ovarian development and maintenance, embryonic development, and immune regulation in animals (He et al., 2020; Craig et al., 2005). Sequence analysis of the *CsFOXL2* in the present study showed that *CsFOXL2* contains an FH domain. The phylogenetic tree of the *FOX* family shows that *C. gigas FOXL2* is clustered together with *FOXL2*. Therefore, *FOXL2* has a highly conserved FH domain, which is typical of the fox family (Amine et al., 2009). The expression level of *CsFOXL2* was highest in the foot. *FOXL2* was highly expressed in the foot in the study of *H. cumingii*, indicating the possible existence of a mechanism in the foot that regulates sex determination and differentiation in clams (Wang et al., 2020). The transcript levels of *CsFOXL2* were higher in the ovary than in the testis. The same result was observed in *C. gigas*, where the expression level of *CsFOXL2* does not show sexual dimorphism (Amine et al., 2009). The expression level of *CsFOXL2* during gonad development initially increased and then decreased from the proliferation stage to the spent stage. This finding is consistent with the expression characteristics of *FOXL2* during scallop gonad development (Ning et al., 2021; Zhao, 2021). The involvement of *CsFOXL2* in early ovarian development and oocyte differentiation can be hypothesized based on the trend of *CsFOXL2* expression during ovarian development. In the present study, the expression level of *CsFOXL2* during larval development was analyzed. The results showed that *CsFOXL2* was transcribed in fertilized eggs, and Liu (2012) concluded that this phenomenon occurred, because *FOXL2* was expressed maternally. *CsFOXL2* was significantly highly expressed in the D-larval stage, but barely expressed in the umbo larvae stage. Similar results were observed for *C. farreri* and the bay scallop *Argopecten irradians irradians* (Liu et al., 2013). For the result, Ning et al. (2021) suggested that primordial germ cells (PGCs) may first appear in the D-larval stage. The D-larval stage is a period of rapid construction of larvae tissue in *C. sinensis*, including the initial formation of larval shells and visceral masses. Therefore, it is hypothesized that *CsFOXL2* is involved in the formation of certain organs of the larvae. The expression level of *CsFOXL2* reached its peak at 7 months of age. Therefore, *CsFOXL2* is involved in ovarian growth and development.

Fluctuations in sex hormone levels in the organism as the sexual maturation cycle changes, suggest that sex hormones may participate in

TABLE 2 The oocytes diameters of *C. sinensis* in different estradiol treatment.

Indices	Control	5 μg/L	50 μg/L
long diameters of oocytes/μm	42.62 ± 1.50^{c}	59.56 ± 2.22^{b}	69.05 ± 1.99^{a}
short diameters of oocytes/μm	28.23 ± 1.01^{c}	38.30 ± 1.29^{b}	46.82 ± 1.52^{a}

Data are meant ± SE. Different superscript letters indicate that means are significantly different (P<0.05).

gonadal development (Reis-Henriques and Coimbra, 1990; Matsumoto et al., 1997; Shangguan et al., 2022). Estradiol injection promoted the growth of sea scallop oocytes, demonstrating that estrogen is involved in the reproductive process in invertebrates (Wang and Croll, 2004). The expression level of *CsDMRT3* was significantly higher at estradiol: 5 μg/L compared with the control group. Combined with the specific expression of *DMRT3* in the male gonads, it is hypothesized that *DMRT3* is involved in the development and the maintenance of gonadal function in *C. sinensis* (Wu et al., 2019). After estradiol treatment, the expression level of *CsFOXL2* peaked at estradiol concentration of 5 μg/L, which was significantly higher compared with the control group. The experimental results suggest that estradiol treatment induced the upregulation of *CsFOXL2*. It indicates that estradiol may play a role in ovarian development by stimulating *FOXL2* to induce estrogenic feedback mechanism (Narisato et al., 2006; Li et al., 2014; Banh Quyen et al., 2021). However, the expression level of *CsFOXL2* was decreased at estradiol concentration of 50 μg/L compared with estradiol concentration of 5 μg/L. Similar results were observed in *C. elegans*. Zhu et al. (2019) concluded that the uptake of exogenous estrogen by fry leads to high estradiol concentrations in the body, resulting in a negative feedback mechanism that inhibits the production of endogenous estrogens. In the present experiment, the diameter of oocytes in the ovaries of *C. sinensis* remarkably increased with increasing estradiol concentration. The findings of this study are consistent with the study of Wang and Croll, 2004.

The results showed that *DMRT3* and *FOXL2* were involved in the development and maintenance of gonad in different tissues and gonad stages of *C. sinensis*. *CsDMRT3* may be a candidate regulator of male development. In addition, *FOXL2* may interact with *ESR* in oyster gonads to inhibit the expression of genes related to male development (Sun et al., 2022). *CsFOXL2* was barely expressed in the umbo larvae stage, while *CsDMRT3* was expressed at different stages of larval development, suggesting that the broader function of *CsDMRT3* in early development.

5 Conclusion

The present study showed that the expression level of *DMRT3* in *C. sinensis* was sexually dimorphic, indicating that *DMRT3* is likely to be involved in male gonad development and germ cell formation. *FOXL2* is closely associated with early organogenesis and ovarian development in *C. sinensis*. In addition, estradiol exposure stimulated ovarian development and oocyte growth. The results of this study provide valuable information for the subsequent study of sex determination and gonadal development of clam *C. sinensis*.

Data availability statement

The datasets presented in this study can be found in online repositories. The the cDNA sequences of CsDMRT3 can be found at GenBank, https://www.ncbi.nlm.nih.gov/genbank/, accession No: OP970557 and the sequences of CsFOXL2 can be found at GenBank, accession No: OP970558. Further inquiries should be directed to the corresponding author

Ethics statement

The animal study was reviewed and approved by the Animal Experiment Ethics Committee at Jiangsu Ocean University.

Author contributions

SY: experimental design, formal analysis, and writing - original draft. MX: data curation, and validation. JX: data curation. XL: experimental design and data curation. ML: experimental design and formal analysis. SW: formal analysis. SF: experimental design. ZD: writing-editing and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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