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# Gonad transcriptome analysis reveals the differences in gene expression related to sex-biased and reproduction of clam *Cyclina sinensis*

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Sexual differentiation and gonad development are important biological processes for bivalve species. The clam *Cyclina sinensis* is an important cultured marine bivalve widely distributed along with the coastal areas of China. However, the information related to sexual determination/differentiation and gonadal development of *C. sinensis* almost has no reported. To study the molecular mechanisms of its sexual determination/differentiation and gonadal development, transcriptome analysis was performed in the gonad of *C. sinensis*, and the potential biological functions of reproduction-related gene were also investigated in this study. The results showed that 1 013 and 427 genes were differentially expressed in the ovary and testis, respectively. KEGG enrichment analysis showed that the DEGs in the gonad were significantly clustered in progesterone-mediated oocyte maturation, cell cycle and oocyte meiosis. Further analysis showed that 23 genes were mainly involved in sex determination/differentiation, including *Dmrt1*, *Sox2/4/9*, *Foxl2*, *β-catenin* and *GATA-type zinc finger protein 1-like*. Twenty key genes were mainly involved in the process of spermatogenesis, and five genes encode steroid biosynthesis and metabolism. Fifteen genes related to ovarian development were also identified in this study, such as *Vitellogenin*, *MAM and LDL-receptor class A domain-containing protein 1* and *Cell division cycle protein 20 homolog*. Moreover, 50μg/L estradiol treatment significantly up-regulated the expression levels of *CsVg* in the ovary and hepatopancreas. These results highlight the genes involved in sexual determination/differentiation and gonadal development, which enhance our understanding for further studies of reproduction and breeding of *C. sinensis* and other marine bivalves.

KEYWORDS

**Cyclina sinensis, sex-differentially expressed genes, spermatogenesis, ovarian development, vitellogenin**

## Highlights

- 23 genes involved in sex determination/differentiation of *C. sinensis* were identified.
- Twenty key genes played important role in the process of spermatogenesis of *C. sinensis*.
- 15 key genes played important role in the ovarian development of *C. sinensis*.
- 50μg/L estradiol treatment significantly up-regulated the expression levels of *CsVg*.

## 1 Introduction

Sexual reproduction is one of the most universal phenomena in the animal kingdom, and it includes sexual determination/differentiation and gonadal development (Li et al., 2016a; Liu et al., 2016). Sexual determination/differentiation refers to the event that triggers the bipotential primordia to make the fate decision to become testes or ovaries, and it is very flexible in fish, with several species showing a chromosomal basis and others with influencing environmental factors (Devlin and Nagahama, 2002; Sandra and Norma, 2010). Previous studies showed that sex-determining/–differentiating pathways share common genes such as *SRY-related HMG box 9* (*SOX9*), *wingless-type MMTV integration site family member-4* (*WNT4*), *R-spondin1* (*R-SPO1*) and *transcription factor forkhead box L subfamily member 2* (*FOXL2*), among vertebrates and invertebrates, (Bertho et al., 2016; Major et al., 2019; Estermann et al., 2020; Broquard et al., 2021). Among those genes, Sox9 has been identified and proved its crucial roles in male sex determination, male gonad development, and Sertoli cell development and differentiation (Vidal et al., 2002; Chaboissier et al., 2004; Barrionuevo et al., 2009).

WNT4 is a key regulator of ovarian development in mammals, and R-spondin1 tips the balance in sex determination (Capel, 2006; Farhadi et al., 2021). Ning et al. (2021) reported that the transcription factor FoxL2 is an evolutionarily conserved gene playing pivotal roles in regulation of early ovarian differentiation and maintenance in animals. Similarly, as the primary reproductive organs, the ovary and testis play keys role in gametogenesis and steroid regulation of vertebrates and invertebrates (Nagaraju, 2011; Senthilkumaran and Kar, 2021).

As the second largest phylum of invertebrates, mollusks exhibit different reproductive strategies: they are dioecious, hermaphrodites or exhibit sex reversal (Li et al., 2018). Therefore, the studies on reproduction of mollusk, especially commercial mollusk species has received significant research attention in recent years (Wang et al., 2020; Broquard et al., 2021; Shangguan et al., 2022). For example, Santerre et al. (2014) reported that *SoxE* and *β-catenin* are involved in early gonadic differentiation of Pacific oyster *Crassostrea gigas*. Shangguan et al. (2022) reported that *17-alpha-hydroxylase/17,20 lyase* (*cyp17a1*) plays an important role in gonadal development of *Hyriopsis cumingii*. Meanwhile, several gonadal transcriptome analyses identified candidate genes (*SoxH*, *Sox9*, *Foxl2*, *Doublesex-and mab-3-related transcription factor 1* (*Dmrt1*), *sex determining protein Fem-1* (*Fem-1*), *Beta-catenin*, *wnt4*, *cyp17a1*, *Estradiol 17-beta-dehydrogenase 2* (*17β-hsd2*) and *Vitellogenin* (*Vg*)) that participate in gender determination/differentiation and gonadal maturation pathway of mollusks, including *Crassostrea hongkongensis*, *C. gigas*, *Patinopecten yessoensis*, *Sinonovacula constricta*, *Pinctada margaritifera*, *Tegillarca granosa*, *Chlamys nobilis* and *Hyriopsis schlegelii* (Teaniniuraitemoana et al., 2014; Zhang et al., 2014; Tong et al., 2015; Li et al., 2016a; Chen et al., 2017; Yue et al., 2018; Broquard et al., 2021; Yao et al., 2021; Zeng et al., 2022). Despites the identification of some candidate genes, studies on sex determination/differentiation and gonadal maturation genes in bivalves and other molluscs were still limited, and more sex and gonad related genes and their functions need to be studied.

The clam, *Cyclina sinensis*, is one of the important cultured marine bivalves found widely along the coastal areas of China, Korea, Japan, and Southeast Asia (Wei et al., 2020; Ge et al., 2021; Liao et al., 2022). To date, the research on *C. sinensis* mainly focuses on genetic evaluation, breeding techniques, nutritional value and physiological responses (Wei et al., 2020; Dong et al., 2021; Ge et al., 2021; Liao et al., 2022). However, the information related to sexual determination/differentiation and gonadal development of *C. sinensis* is limited. Transcriptome sequencing provides an effective way to obtain large amounts of sequence data, has been widely applied to model and non-model species (Qian et al., 2014). The availability of the *C. sinensis* genome and the reduced cost of next-generation sequencing provide the opportunity for enhance our understanding of the complex regulation mechanisms of reproduction (Wei et al., 2020; Dong et al., 2021; Ge et al., 2021; Liao et al., 2022). Therefore, the present study sequenced *C. sinensis* gonad transcriptomes and investigated the potential biological functions of reproduction-related genes. The results of this study will help us understand the general underlying molecular mechanisms of bivalve reproduction and provide a scientific basis for the sexual control and breeding of shellfish.

## 2 Materials and methods

### 2.1 Ethics statement

All the study design and animal experiments were conducted in accordance with guidelines of Jiangsu Ocean University's Animal Care and Use Committee.

### 2.2 Sample collection

The female and male *C. sinensis* used in this experiment were purchased from a commercial farmer in Qinzhou, Guangxi, China. After being transferred to the laboratory, the clams were acclimated in cement tanks (length×width× depth=100cm×100cm×80cm) filled with natural seawater (salinity 24 ± 1 ppt). All clams were fed daily

at 17:00 with a mixture of *Nannochloropsis oceanica* and *Chaetoceros mulleri* quantitatively. After acclimation for seven days, the clams were placed in an ice bath for anesthetisation, and the ovary or testis was dissected. The sampled ovary or testis was divided into two pieces: one piece was immediately frozen in liquid nitrogen for total RNA extraction, and the other piece was fixed in 4% paraformaldehyde for histological examination. Based on the histological features of previously reported by Yan (2009), the gonadal development was classified into five stages: proliferation stage (stage I), growing stage (stage II), maturation stage (stage III), spawning stage (stage IV), and spent stage (stage V). Based on the staging results, the ovary or testis tissues from three individuals at growing stage were selected for transcriptome analysis.

## 2.3 RNA extraction and transcriptomic sequencing

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, USA) according the manufacturer's instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). The integrity and purity of the total RNA quality were determined by 2100 Bioanalyser (Agilent Technologies) and quantified using the ND-2000 (NanoDrop Technologies).

RNA purification, reverse transcription, library construction and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China) according to the manufacturer's instructions (Illumina, San Diego, CA). The transcriptome library was prepared following TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA) using 1μg total RNA. Then, the synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition following Illumina's library construction protocol. After quantification by TBS380, paired-end RNA sequencing library was sequenced with the Illumina NovaSeq 6000 sequencer (2 × 150 bp read length).

## 2.4 Transcriptome assembly and differential expression analysis

The raw paired-end reads were trimmed and quality controlled by fastp with default parameters (Chen et al., 2018). Then, clean data from the samples were used to perform *de-novo* assembly with Trinity (Grabherr et al., 2011). The assembled transcripts were assessed and optimized with BUSCO (Manni et al., 2021), TransRate (Smith-Unna et al., 2016) and CD-HIT (Fu et al., 2012). All the assembled transcripts were searched against the National Center for Biotechnology Information (NCBI) protein non-redundant (Nr), Swiss-Prot, Pfam, Clusters of Orthologous Groups of proteins, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLASTX to identify proteins that had the highest sequence similarity with the given transcripts to retrieve their function annotations, and a typical cut-off E-values less than $1.0\times10^{-5}$ was set.

The expression level of each transcript was calculated using the fragments per kilobase of exon per million mapped reads (FPKM) method (Trapnell et al., 2010). To identify differentially expressed genes (DEGs) across samples, we considered DEGs with $|\log2(\text{foldchange})| \geq 1$ and P-value $\leq 0.05$ to be significantly different expressed genes. To identify DEGs that were significantly enriched in GO terms and metabolic pathways at P-value ≤0.05, we applied Goatools and KOBAS were applied for GO functional enrichment and KEGG pathway analysis, respectively (Xie et al., 2011).

## 2.5 Quantitative real-time PCR

Eight genes involved in gonadal development and sexual determination/differentiation were randomly selected for validation using qPCR. A reverse first-strand cDNA synthesis kit (RR036A, Takara Bio, Japan) was used to synthesize first-strand cDNA. Primer pairs were designed using Primer 6.0, and all the primer sequences are listed in Table 1. *β-actin* was used as the internal control to normalize

TABLE 1 Specific primers used to in qPCR.

| Primer name | Sequence (5′→3′) | Tm (°C) |
|---|---|---|
| *Testis-specific serine/threonine-protein kinase 1* | F: *TGAAATGCGAGAATCTGCTGT*<br>R: *CATGAAGGGGTAGATGGTAAGG* | 60.2 |
| *Sperm-associated antigen 6* | F: *TGGCAATGGCTGTCATAGTCT*<br>R: *TGAGGCATCTGCTCTGAGGTA* | 60.2 |
| *Doublesex and mab-3-related transcription factor 1 (Dmrt1)* | F: *TGTATCCGTCAAACCCTTCCT*<br>R: *CCCACTGCACCATAGCCAAA* | 60 |
| *MAM and LDL-receptor class A domain-containing protein 1* | F: *TCCAGCAGCCATGTTTGAGA*<br>R: *ACTTGTTGGTTGCCCCGTTA* | 60 |
| *Cell division cycle protein 20 homolog* | F: *GGTGTCATAGCAACGGGTGG*<br>R: *AGGGGCTCAAACTGAAACTTCT* | 60 |
| *Vitellogenin* | F: *TTGCTTGGAAGGATTATGGATG*<br>R: *GGCTTTACAGGATTTCTGGGTTT* | 60 |
| *Prostaglandin reductase 1* | F: *GACAATGTGGGAGGCGAGTT*<br>R: *CTGCCCAGCCTATGAACCCT* | 60 |

*(Continued)*

TABLE 1 Continued

| Primer name | Sequence (5′→3′) | Tm (°C) |
|---|---|---|
| *Forkhead box protein L2 (Foxl2)* | F: *ACTTGCTTCCTGTGGATACGG*<br>R: *TAAATGGCTCGCTCTGTTGC* | 60 |
| *β-actin* | F: *CCTGGTATTGCCGACCGTAT*<br>R: *TTGGAAGGTGGACAGTGAAGC* | 60 |

target gene expression. qPCR was carried out with a StepOnePlus™ real-time PCR system using SYBR1 Green I (TakaRa, Japan) according to the manufacturer's instructions. The volume of qPCR reaction system was 20 μl containing 2 μl of cDNA template, 0.4 μl of each primer (10 mM), 10 μl of SYBR premix, 0.4 μl of ROX Reference Dye (50×) and 6.8 μl of water. PCR conditions were as follows: 95°C for 30s; 40 cycles of 95°C for 5s, 60/60.2°C for 30 s, and 72°C for 30 s. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

## 2.6 Phylogenetic and sequence analysis of *C. sinensis* vitellogenin

Based on the results of transcriptome sequencing, the higher expression level of *CsVg* was found in the ovary of *C. sinensis*, the *CsVg* was selected for the further analysis in this study. The amino acid sequence and open reading frame (ORF) of *CsVg* were predicted by the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The conserved domains of *CsVg* and other species were analyzed using the online software Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1). Subsequently, ClustalX and MEGA 5.0 software were used to carry out multiple sequence alignment and phylogenetic tree construction of neighbor-joining (NJ) system. The amino acid sequences of other species used in these analyses were obtained from GenBank, including *Xenopus laevis* (AB092605.1), *Oryzias latipes* (AB092605.1), *Caenorhabditis elegans* (X56213.1), *Portunus trituberculatus* (DQ000638.1), *Scylla paramamosain* (KU987908.1), *Litopenaeus vannamei* (AY321153.2), *Haliotis discus hannai* (AB360714.1), *Scapharca broughtonii* (MG580782.1), *Tegillarca granosa* (JQ266265.2), *Crassostrea angulate* (JX218047.2), *Crassostrea gigas* (AB084783.1), *Saccostrea glomerata* (KU194475.1) and *Ostrea edulis* (XM_048903027.1).

## 2.7 Expression pattern analysis of *C. sinensis* vitellogenin

To assay the gene expression of *CsVg* in different tissues, several tissues (ovary, hepatopancreas, adductor, pipe, gill, foot, mantle) were dissected from the clams in the gonadal growing stage. In this experiment, different tissues from three individual *C. sinensis* were used for tissue-specific expression analysis. Meanwhile, the ovary from female clams at each gonadal developmental stage were also collected to determine the abundance of *CsVg* transcripts. A total of 6-8 clams were used for sampling in each stage. Total RNA isolation, cDNA synthesis and PCR reactions were all performed as described above.

## 2.8 Estradiol treatment experiment

To analyze the transcriptional response of *CsVg* to estradiol, we performed an *in vivo* exposure experiment using *C. sinensis* at the gonadal growing stage was performed. Specifically, 120 healthy clams were randomly divided into three groups of 30. The clams were exposed for 21 days to various nominal estradiol concentration (0+ethanol μg/L, 5 μg/L and 50 μg/L) in accordance with previous literature (Wu et al., 2019). During exposure, the clams were maintained in a 20 L plastic container, and uneaten food and feces were removed before water renewal. Throughout the experiment, the seawater with estradiol was changed daily to ensure that the concentration of estradiol in each group remained invariable during the experiment. At the end of the estradiol exposure, the ovary and hepatopancreas were dissected and stored at -80°C for RNA extraction.

## 2.9 Statistical analysis

Data are presented as the mean ± standard error (SE). Statistical analyses were performed using SPSS Statistics V22.0. P value of $< 0.05$ was considered statistically significant. When normal distribution and/or homogeneity of variances were not achieved, data were subjected to the Kruskal-Wallis H nonparametric test, followed by the Games-Howell nonparametric multiple comparison test.

# 3 Result

## 3.1 Gonad developmental stages of *C. sinensis*

After dissection, the structure of the ovary and testis can be easily distinguished by microscopic observation of gonadal sections. Histological results showed that the ovaries and testes of *C. sinensis* used for RNA sequencing were at growing stage (stage II) of gonadal maturation (Figure 1).

## 3.2 Sequencing and *de novo* assembly

Two cDNA libraries were constructed from the ovary and testis of *C. sinensis*. After adaptor sequences and low-quality reads were

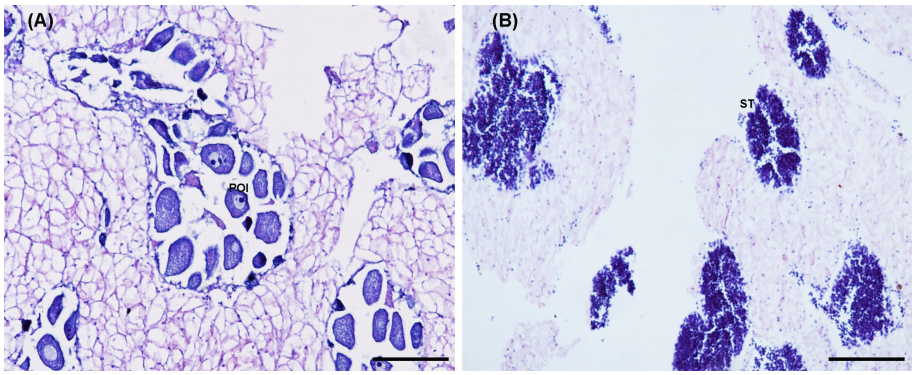


FIGURE 1
Histological characteristics of the ovary **(A)** and testis **(B)** of the *C sinensis* for transcriptome sequencing. POI, pre-vitellogenesis oocyte; ST: spermatid. Scale bars, 100 μm.

removed, 278 million clean reads, including 140 109 554 reads from the ovary libraries and 137 762 234 reads from the testis libraries, were obtained from the ovary and testis of the *C. sinensis* transcriptome (Table 2). Among these clean reads, the Q30 in each sample was above 90%. All reads were submitted to the website of NCBI (PRJNA906186).

All clean reads were assembled into 134 124 unigenes, and the minimum, longest, and average lengths of unigenes were 201, 29 212 and 1 095 bp, respectively. Among the unigenes, 77 305 unigenes (57.64%) were successfully annotated by searching against the Nr, Swissport, Pfam, COG, GO and KEGG databases. Especially, 22 095 (28.59%) unigenes were annotated to the Nr database, whereas 15 142 (19.59%) unigenes can be fully annotated to the SwissProt database (Figure 2). In addition, the species most represented in BLASTx searches included *Pecten maximus* (18.5%), *Mizuhopecten yessoensis* (17.23%) and *Crassostrea gigas* (4%) (Figure 3).

## 3.3 Analysis of differentially expressed genes

By comparing the databases of ovary and testis, 1 440 DEGs were detected in the transcriptome, of which 1 013 and 427 DEGs were upregulated and downregulated, respectively. To analyze the functions of these DGEs, we conducted GO and KEGG analyses. GO annotation analysis indicated that these DEGs were annotated to three functional ontologies, i.e. molecular function (399 transcripts), cellular component (504 transcripts), and biological process (493 transcripts) (Figure 4). In the biological process category, the metabolic process (GO:0008152) and cellular process (GO:0009987) level 2 terms were the most abundant terms. In the cellular component category, the membrane part (GO:0044425) and cell part (GO:0044464) level 2 terms were the most abundant terms. In the cellular component category, the binding (GO:0005488) and catalytic activity (GO:0003824) level 2 terms were the most abundant terms. KEGG enrichment analysis showed that these DEGs were enriched in 220 specific KEGG metabolic pathways. Several DEGs were mapped to several pathways related with reproduction, such as progesterone-mediated oocyte maturation, cell cycle, cAMP signaling pathway and oocyte meiosis. The top 20 most significantly enriched metabolic pathways are represented in Figure 5.

TABLE 2 Raw reads and quality control of reads for cDNA libraries of *C. sinensis* transcriptome.

| Type | Ovary | Testis |
|---|---|---|
| **Raw reads** | 140 937 740 | 138 575 290 |
| **Clean reads** | 140 109 554 | 137 762 234 |
| **Q20 (%)** | 97.87 | 97.91 |
| **Q30 (%)** | 93.57 | 93.71 |
| **GC content (%)** | 39.81 | 39.55 |

## 3.4 Key genes involved in sexual determination/differentiation and gonadal development

Through the analysis of the overall gene expression profiles of gonads, several candidate genes involved in various processes of sex determination/differentiation and gonadal development were identified (Table 3). Among the candidate genes, twenty-three key genes were mainly involved in sexual determination/differentiation. Specifically, the transcript levels of *Foxl2* and *GATA-type zinc finger protein 1-like* in the ovary were significantly higher than those in the testis. The expression levels of *Dmrt1*, *Sox9, Fem-1, Follistatin, Transformer-2 protein homolog alpha isoform X5, Zinc finger Y-chromosomal protein 1-like, E3 ubiquitin-protein ligase MARCHF3 isoform X1, Synaptonemal complex protein 1 isoform X2, Transcription factor Runt* and *Protein dpy-30* in the testis were higher than those in the ovary. Twenty key genes were mainly involved in the process of spermatogenesis, and the higher expression levels of *Spermatogenesis-associated protein 17-like isoform X1, Meiotic recombination protein SPO11-like isoform X1, Meiotic recombination protein REC8 homolog isoform X2, Ropporin-*

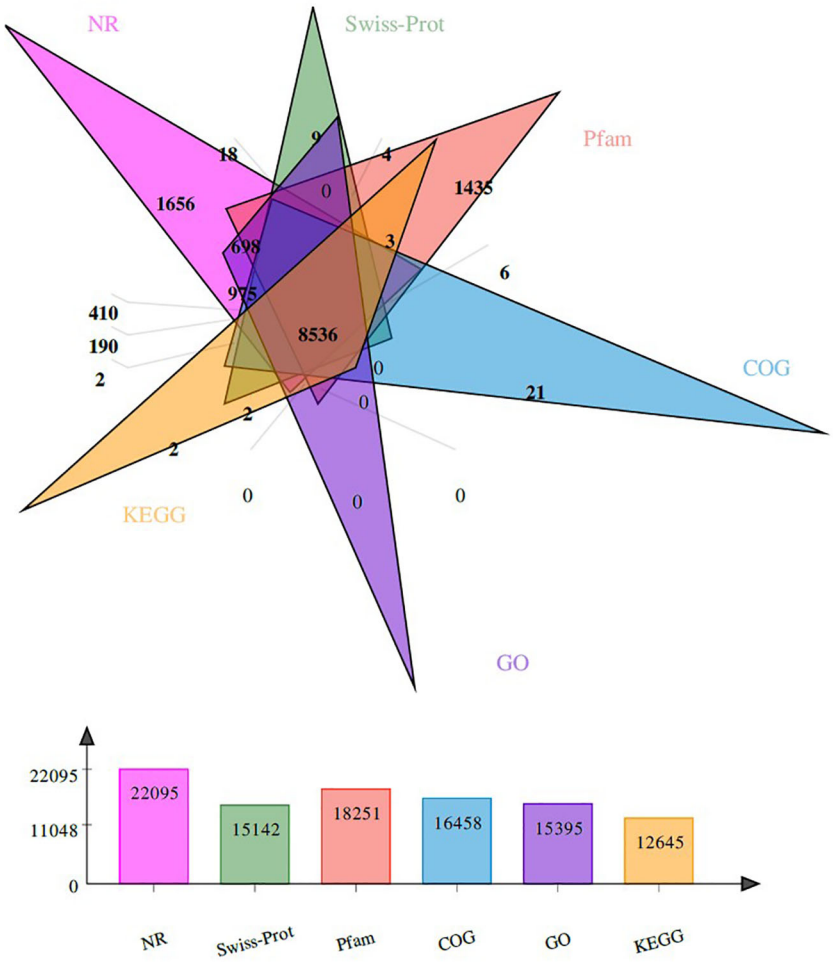


FIGURE 2
The distribution of unigenes in six databases. The colors show different databases. The number represents the total number of unigenes.

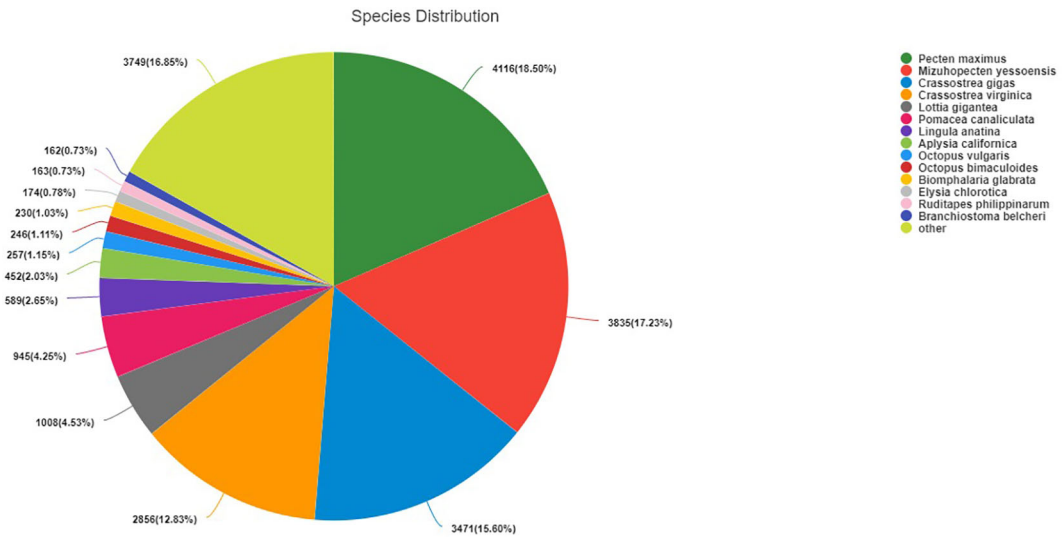


FIGURE 3
Species distribution of BLASTx hits.

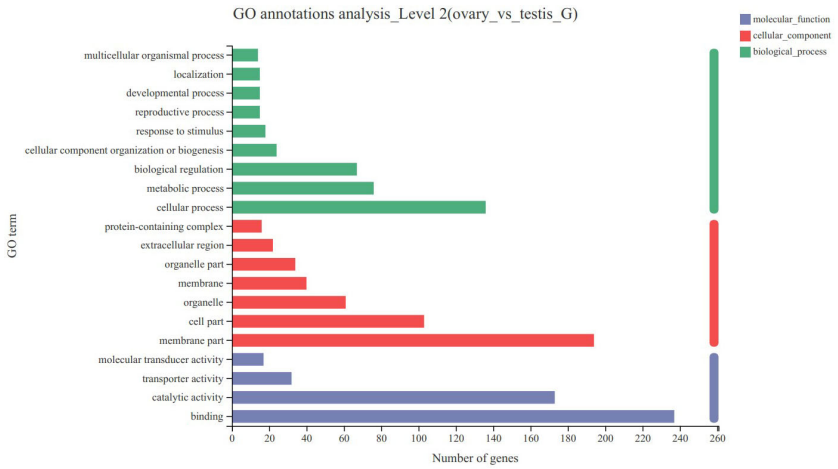


FIGURE 4
GO annotation analyses of differentially expressed genes (DEGs) in *C. sinensis* transcriptome. The left vertical coordinate indicates the second level of GO classification. The horizontal coordinate indicates the number of unigene/transcript included in this secondary classification, and the three colors on the right indicate the three major branches of GO.

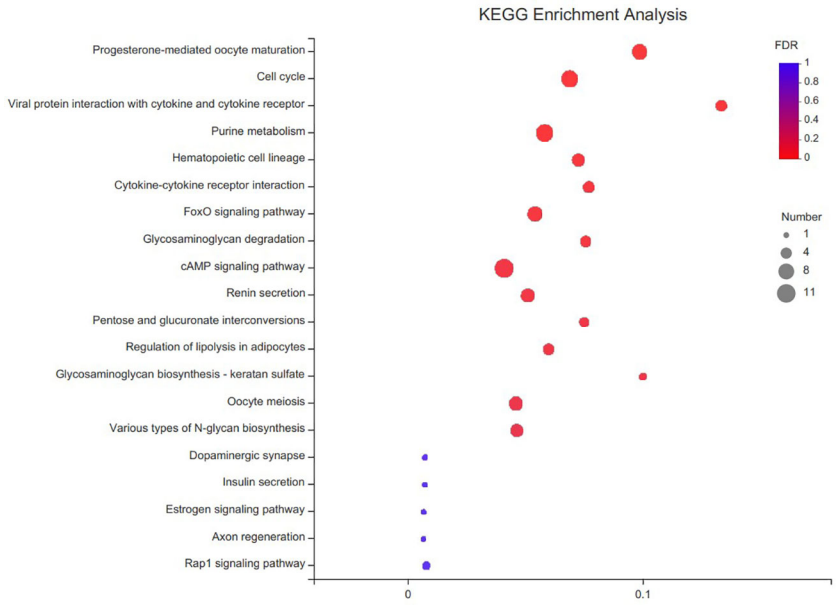


FIGURE 5
KEGG enrichment analyses of differentially expressed genes (DEGs) in *C. sinensis* transcriptome. The vertical coordinate is the name of the KEGG metabolic pathway and the horizontal coordinate is the number of transcript/unigene annotated to the pathway.

*1-like protein, Sperm-tail PG-rich repeat-containing protein 2-like* (*STPG1*), *Testis-specific serine/threonine-protein kinase 1, Testis-specific serine/threonine-protein kinase 3-like, Sperm-associated antigen 6, Kelch-like protein 10, Armadillo repeat-containing protein 4-like isoform X1* and *G2/mitotic-specific cyclin-B3-like* were found in the testis. Moreover, fifteen genes related to ovarian development and five genes encoding steroid biosynthesis/metabolism were identified in the ovary and testis.

## 3.5 Validation of gene expression by qPCR

Eight selected important genes related to sexual determination/differentiation and gonadal development of *C. sinensis* were determined using qPCR to validate the RNA-seq results. qPCR results showed that *Dmrt1*, *Testis-specific serine/threonine-protein kinase 1* and *Sperm-associated antigen 6* had significantly higher expression levels in male gonads (Figure 6), whereas *Foxl2*, *Vg*,

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TABLE 3 Summary of differential and non-differentially expressed genes related to sex determination/differentiation and gonadal development in the transcriptome of *C. sinensis*.

| Functional category | Gene id | Gene name | log2 Fold changes (testis/ovary) | *P-value* |
|---|---|---|---|---|
| Sex determination/differentiation | | | | |
| | TRINITY_DN20554_c0_g1 | *Doublesex and mab-3-related transcription factor 1* (*Dmrt1*) | 2.10 | 3.77E-03 |
| | TRINITY_DN32439_c0_g1 | *Transcription factor SOXC*(*Sox4*) | -0.20 | 7.24E-01 |
| | TRINITY_DN12181_c0_g1 | *Transcription factor SOXB1*(*Sox2*) | -0.75 | 3.33E-01 |
| | TRINITY_DN5766_c0_g1 | *Transcription factor SOXE*(*Sox9*) | 0.63 | 2.84E-01 |
| | TRINITY_DN17013_c0_g1 | *Forkhead box protein L2* (*Foxl2*) | -4.47 | 1.27E-05 |
| | TRINITY_DN18205_c0_g2 | *Wnt-4a* | -0.79 | 4.91E-01 |
| | TRINITY_DN7453_c0_g2 | *GATA-type zinc finger protein 1-like* | -4.21 | 8.97E-04 |
| | TRINITY_DN1084_c0_g1 | *Growth arrest and DNA damage-inducible protein GADD45 alpha* | -0.43 | 5.04E-01 |
| | TRINITY_DN4959_c0_g1 | *β-catenin* | 0.03 | 9.60E-01 |
| | TRINITY_DN3871_c0_g1 | *Fem-1* | 1.32 | 1.05E-01 |
| | TRINITY_DN26641_c0_g1 | *Follistatin* | 1.19 | 8.01E-01 |
| | TRINITY_DN14753_c0_g1 | *Transformer-2 protein homolog alpha isoform X5* | 0.64 | 2.90E-01 |
| | TRINITY_DN7640_c0_g1 | *Structural maintenance of chromosomes protein 5-like* | 1.25 | 8.71E-02 |
| | TRINITY_DN4319_c0_g1 | *Serine/threonine-protein kinase PLK1-like* | 1.17 | 8.71E-02 |
| | TRINITY_DN11738_c2_g2 | *Zinc finger Y-chromosomal protein 1-like* | 2.45 | 5.13E-02 |
| | TRINITY_DN11833_c0_g2 | *E3 ubiquitin-protein ligase MARCHF3 isoform X1* | 11.8 | 1.24E-08 |
| | TRINITY_DN12272_c0_g2 | *Synaptonemal complex protein 1 isoform X2* | 4.48 | 1.40E-05 |
| | TRINITY_DN5752_c0_g1 | *Core histone macro-H2A.1-like isoform X1* | 0.47 | 4.76E-01 |
| | TRINITY_DN11990_c0_g1 | *Platelet-derived growth factor subunit A-like* | 0.30 | 6.89E-01 |
| | TRINITY_DN5555_c0_g1 | *Transcription factor Runt* | 1.09 | 5.33E-02 |
| | TRINITY_DN27415_c0_g1 | *Protein dpy-30* | 0.68 | 2.11E-01 |
| | TRINITY_DN16278_c0_g1 | *Elongation factor 1 alpha* | -0.90 | 1.08E-01 |
| | TRINITY_DN28419_c0_g1 | *Paramyosin* | -0.78 | 1.86E-01 |
| Spermatogenesis | | | | |
| | TRINITY_DN8046_c2_g1 | *Spermatogenesis-associated protein 17-like isoform X1* | 2.57 | 1.44E-04 |
| | TRINITY_DN122_c0_g1 | *Meiotic recombination protein SPO11-like isoform X1* | 4.52 | 1.37E-05 |
| | TRINITY_DN7476_c0_g1 | *Meiotic recombination protein REC8 homolog isoform X2* | 5.11 | 7.41E-05 |
| | TRINITY_DN3408_c0_g1 | *Ropporin-1-like protein* | 3.84 | 4.27E-06 |
| | TRINITY_DN16340_c0_g1 | *Sperm flagellar protein 2-like isoform X4* | 0.29 | 6.28E-01 |
| | TRINITY_DN7095_c0_g1 | *Sperm-tail PG-rich repeat-containing protein 2-like* | 5.35 | 1.73E-07 |
| | TRINITY_DN46387_c0_g1 | *Testis-specific serine/threonine-protein kinase 1(Tssk1)* | 10.8 | 5.73E-10 |
| | TRINITY_DN1043_c0_g1 | *Testis-specific serine/threonine-protein kinase 3-like (Tssk3)* | 7.78 | 1.19E-08 |
| | TRINITY_DN2241_c0_g1 | *Armadillo repeat-containing protein 4-like isoform X1* | 3.53 | 1.28E-05 |
| | TRINITY_DN5793_c0_g1 | *Motile sperm domain-containing protein 1 isoform X1* | 1.17 | 5.66E-02 |
| | TRINITY_DN33601_c0_g1 | *Nucleoside diphosphate kinase homolog 5-like* | 1.41 | 2.03E-02 |
| | TRINITY_DN7758_c0_g1 | *SH3 domain-containing YSC84-like protein 1* | 0.15 | 7.81E-01 |
| | TRINITY_DN6087_c0_g1 | *Axonemal dynein light chain p33* | 1.24 | 4.24E-02 |
| | TRINITY_DN10017_c0_g1 | *Sperm-associated antigen 6* | 2.96 | 4.56E-05 |

*(Continued)*

TABLE 3 Continued

| Functional category | Gene id | Gene name | log2 Fold changes (testis/ovary) | *P-value* |
|---|---|---|---|---|
| | TRINITY_DN10999_c0_g1 | *Kelch-like protein 10* | 10.9 | 1.87E-10 |
| | TRINITY_DN6562_c0_g1 | *LIM homeobox 9* | 0.27 | 6.77E-01 |
| | TRINITY_DN5365_c0_g1 | *Small ubiquitin-related modifier 2-like* | 0.35 | 5.10E-01 |
| | TRINITY_DN386_c0_g1 | *Ubiquitin conjugating enzyme isoform 1* | 0.26 | 6.56E-01 |
| | TRINITY_DN2167_c0_g1 | *Mitotic checkpoint protein BUB3* | 0.16 | 8.11E-01 |
| | TRINITY_DN2008_c0_g1 | *G2/mitotic-specific cyclin-B3-like* | 3.49 | 1.73E-04 |
| Ovarian development | | | | |
| | TRINITY_DN1182_c0_g1 | *Vitellogenin* | -6.60 | 5.42E-09 |
| | TRINITY_DN5981_c0_g2 | *Estrogen receptor gamma-like isoform X1* | -0.59 | 4.11E-01 |
| | TRINITY_DN1678_c0_g1 | *Estrogen-related receptor* | 0.06 | 9.23E-01 |
| | TRINITY_DN3450_c0_g1 | *Vitellogenin receptor-like isoform X2* | 0.52 | 5.21E-01 |
| | TRINITY_DN38987_c0_g4 | *MAM and LDL-receptor class A domain-containing protein 1* | -6.00 | 7.38E-04 |
| | TRINITY_DN9377_c0_g1 | *Melatonin receptor type 1B-B-like* | -1.40 | 3.48E-02 |
| | TRINITY_DN744_c0_g1 | *E3 ubiquitin-protein ligase HUWE1-like isoform X4* | -0.40 | 4.58E-01 |
| | TRINITY_DN2483_c0_g1 | *Ubiquitin thioesterase OTUB1-like* | 1.05 | 8.96E-02 |
| | TRINITY_DN2249_c0_g1 | *26S proteasome regulatory subunit 7* | 0.63 | 2.37E-01 |
| | TRINITY_DN8661_c0_g1 | *Cell division cycle protein 20 homolog* | -9.43 | 4.41E-12 |
| | TRINITY_DN42248_c0_g1 | *Receptor-type guanylate cyclase Gyc76C-like* | -2.53 | 2.50E-01 |
| | TRINITY_DN5631_c0_g1 | *Prostaglandin reductase 1* | -1.58 | 3.95E-02 |
| | TRINITY_DN26216_c0_g1 | *Transcription factor GATA-4 isoform X1* | -1.22 | 3.18E-02 |
| | TRINITY_DN3852_c0_g2 | *Wilms tumor protein 1-interacting protein homolog* | -0.11 | 8.60E-01 |
| | TRINITY_DN19696_c0_g1 | *Fascin-like* | -0.92 | 1.08E-01 |
| Steroid biosynthesis/metabolism | | | | |
| | TRINITY_DN982_c0_g1 | *Steroid 17-alpha-hydroxylase/17,20 lyase-like (CYP17A)* | -0.73 | 2.02E-01 |
| | TRINITY_DN25278_c0_g2 | *17-beta-hydroxysteroid dehydrogenase 14-like* | -0.12 | 8.40E-01 |
| | TRINITY_DN18332_c0_g3 | *Estradiol 17-beta-dehydrogenase 2-like* | -0.20 | 8.97E-01 |
| | TRINITY_DN13675_c0_g1 | *Hydroxysteroid dehydrogenase-like protein 2* | 0.25 | 6.94E-01 |
| | TRINITY_DN27506_c0_g1 | *Estrogen sulfotransferase-like isoform X2* | -1.73 | 2.61E-01 |

Fold changes (Log2 ratio) in gene expression.

*MAM and LDL-receptor class A domain-containing protein 1*, *Cell division cycle protein 20 homolog* and *Prostaglandin reductase 1* had significantly higher expression levels in female gonads (Figure 6). The qPCR results showed that the relative expression patterns of these eight genes were consistent with the RNA-seq results (Figure 6).

## 3.6 Sequences analysis of *CsVg*

The *CsVg* sequence contains a 5584 bp open reading frame encoding 1860 amino acids. Comparison of the amino acid sequence encoded by the *CsVg* with the homologues from other species showed that *CsVg* contains a conserved DUF1943 and VWD domains (Figure 7A). However, the typical lipoprotein N-terminal domain of known lipid transport proteins was not found in the amino acid sequence of *CsVg*. Finally, the phylogenetic tree obtained by NJ method revealed that the *CsVg* from bivalves was clustered in a separate clade from the *CsVg* from vertebrates and invertebrates (Figure 7B). Evolutionarily, the protein sequence of CsVg is more similar to that of the Pacific abalone *Haliotis discus hannai*.

## 3.7 Tissue distribution and temporal expression profiles during gonadal development of *CsVg*

Tissue distribution analysis demonstrated that *CsVg* was expressed in various tissues of female *C. sinensis* (Figure 7C).

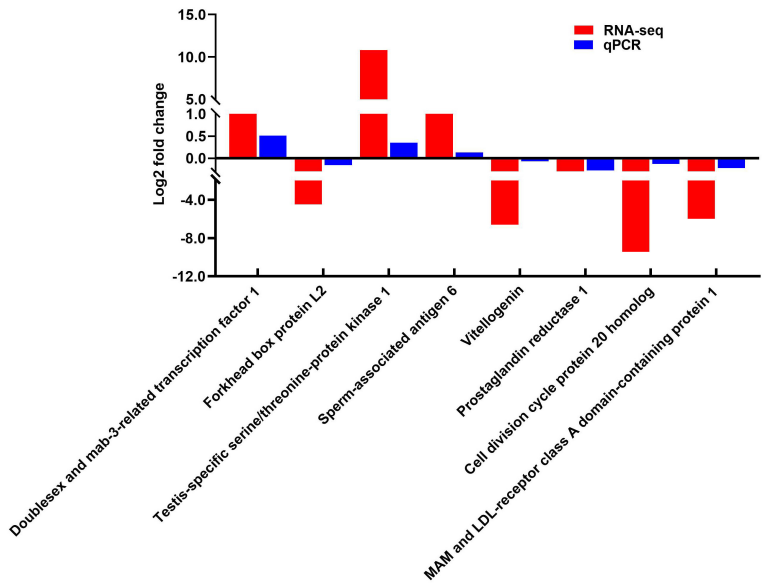


FIGURE 6
Expression levels of eight selected DEGs normalized to β-actin of *C. sinensis* was determined by qPCR.

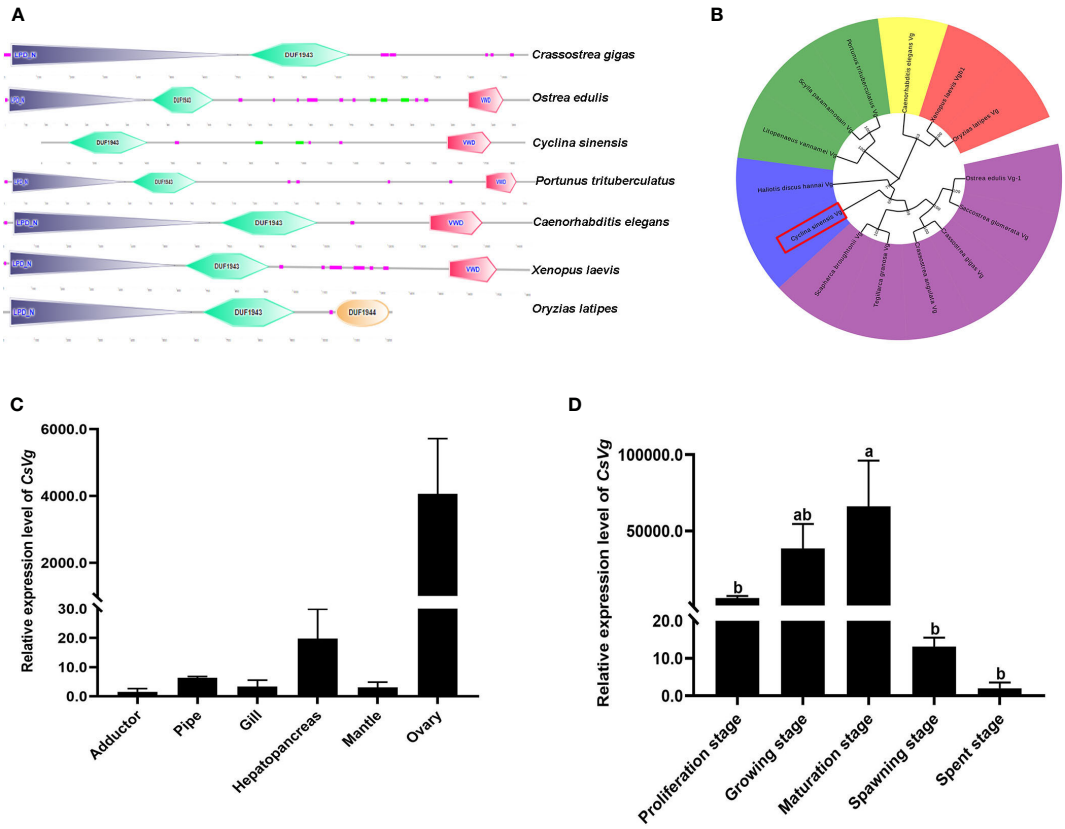


FIGURE 7
Sequence analysis of *CsVg* and its expression profile in tissues of *C sinensis*. **(A)** Domain structures of *CsVg* and other selected species. **(B)** Phylogenetic tree based on amino acid sequences of *Vg* gene **(C)** Relative expression of *CsVg* in different tissues **(D)** Relative expression of *CsVg* in different ovarian development stages. The columns with different letter indicate significant difference ($P < 0.05$).

Remarkably, *CsVg* had significantly high expression level in the ovary and hepatopancreas. To test the correlation of *CsVg* expression level with the gonadal development stages, the relative abundance of *CsVg* transcripts was detected in different stages of ovarian development by qPCR. As shown in Figure 7D, the expression level of *CsVg* in the ovary increased continuously from proliferation stage to maturation stage of *C. sinensis*, and it reached the peak level at maturation stage. However, the expression level of *CsVg* in the ovary decreased from spawning stage to spent stage.

### 3.8 *In vivo* effect of estradiol on *CsVg* expression

The expression level of *CsVg* in the ovary of *C. sinensis* exposure with estradiol is shown in Figure 8A. Compared with the control treatment, the expression level of *CsVg* in the ovary increased in a dose-dependent manner by estradiol treatments. Moreover, the expression levels of *CsVg* in the ovary of *C. sinensis* significantly increased in the 50μg/L estradiol treatment. In the hepatopancreas, estradiol treatments (5μg/L, 50μg/L) also significantly up-regulated the expression levels of *CsVg* compared with the control (Figure 8B).

## 4 Discussion

The reproduction of marine invertebrates demonstrates a wide range of sexual reproduction traits, which often manifests through free spawning (Ostrovsky, 2021; Picard et al., 2021). As a typical buried shellfish, the research on the molecular mechanism of *C. sinensis* reproduction is a key basis work for its genetic selection. Therefore, the present study firstly sequenced *C. sinensis* gonad transcriptomes, screened out several candidate genes involved in the process of sexual determination/differentiation and gonadal development, and then analyzed the responses of several genes to estradiol. The results of this study will help us understand the general underlying molecular mechanisms of bivalve reproduction and provide a scientific basis for the sexual control and breeding of shellfish.

In this study, serveral DEGs were mapped to several pathways related with to reproduction, including progesterone-mediated oocyte maturation, cell cycle, cAMP signaling pathway, oocyte meiosis and estrogen signaling pathway, which suggests the significance of signal transduction systems and endocrine regulation in gonadal development of *C. sinensis*. Importantly, twenty-three potential sexual determination/differentiation genes were identified by analysis of the female and male gonad transcriptomes of *C. sinensis*, these genes included *Dmrt1*, *Sox9*, *Wnt-4a*, *Foxl2*, *GATA-type zinc finger protein 1-like*, *Elongation factor 1 alpha* (*EF-1-alpha*), *Transformer-2 protein homolog alpha isoform X5*, *Follistatin* and *Fem-1*. The discovery of these reported genes suggests that, similar to other bivalves and mammalian, these genes also play an important role in sexual determination/differentiation of *C. sinensis* (Li et al., 2016a; Yang et al., 2016; Yao et al., 2021). Robinson et al. (2022) reported that *Dmrt* are key genes for male and female development of mollusk by the analysis of RNAseq data from eight phylogenetically diverse bivalve species. Adzigbli et al. (2019) reported that *Sox9* and *Gata-type zinc finger protein 1* are involved in genetic sex determination of pearl oysters. The signalling molecule Wnt-4 is not only crucial for female sexual development, but its signalling is also well implicated in mammalian testis development (Vainio et al., 1999; Naillat et al., 2015). Foxl2 plays a major role in initiating ovarian differentiation in fish and bivalves (Jin et al., 2022; Sun et al., 2022). Follistatin1 not only acts as an inhibitory binding protein of activin in the regulation of oocyte maturation in adult females but also plays a potential role in the masculinization of juveniles (Jiang et al., 2012). *EF-1-alpha* has been reported to be expressed in male and female germ cells, and it may contribute to the massive protein synthesis required for egg production (Kinoshita et al., 2000; Zhou et al., 2002). Wang et al. (2021a) reported that Transformer-2 plays a potential regulatory role in embryonic sex determination and early gonadal development of Freshwater Pearl Mussel *Hyriopsis cumingii*. Differently to what reported in *H. cumingii*, the present study showed the higher expression level of *Fem-1* in the testis than in the ovary of *C. sinensis* (Wang et al., 2021b). The opposite result can potentially be explained by the different functions of various *Fem-1* isoforms in distinct species. Tan et al. (2001) reported that Fem-1 plays pivotal roles in sex determination of *Caenorhabditis elegans*, whereas Fem-2 has a role in apoptosis signaling. Therefore, further research is required in *C. sinensis* regarding what these genes target and how they function to determine sex.

Spermatogenesis is a complicated process of proliferation and division and involves numerous genes (Yu et al., 2009; Yue et al., 2018). Previous study reported that *spermatogenesis-associated*

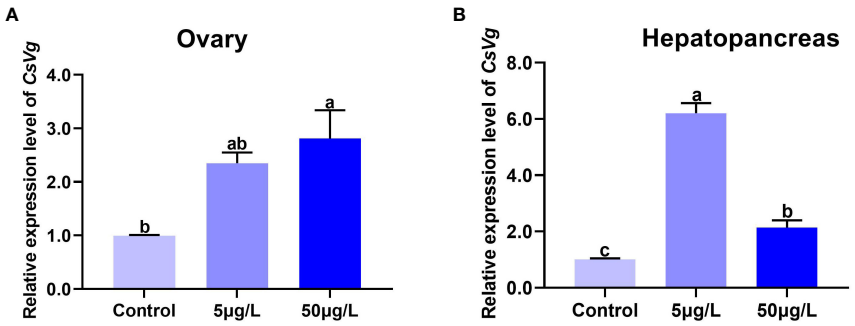


FIGURE 8
The expression level of *CsVg* in the ovary **(A)** and hepatopancreas **(B)** of *C sinensis* by different concentrations of estradiol. The columns with different letter indicate significant difference ($P < 0.05$).

*protein 17* is a testis-specific apoptosis genes and plays important roles in the gonadogenesis and testis development (Nie et al., 2011). *Meiotic recombination protein REC8* is a prominent component of the meiotic prophase chromosome axis, whereas *Meiotic recombination protein SPO11* initiates meiotic recombination by generating DNA double-strand breaks (Yoon et al., 2016; Paiano et al., 2020). Ropporin is a spermatogenic cell-specific protein and may be involved in sperm maturation (Chen et al., 2009). In this study, the *spermatogenesis-associated protein 17-like isoform X1*, *meiotic recombination protein SPO11-like isoform X1*, *meiotic recombination protein REC8 homolog isoform X2* and *Ropporin-1-like protein* were also identified in the testis, where all of them had higher transcript levels in the testis, demonstrating that the *spermatogenesis-associated protein 17-like isoform X1*, *meiotic recombination protein SPO11-like isoform X1 meiotic recombination protein REC8 homolog isoform X2* and *Ropporin-1-like protein* are involved in the spermatogenesis of *C. sinensis*. Notably, members of testis-specific serine/threonine kinases (Tssk) family are required for male fertility in mammals, and targeted deletion of *Tssk1* and *Tssk2* results in dysregulation of spermiogenesis (Shang et al., 2013; Wang et al., 2022). Several studies reported that *Tssk1/2*, *Tssk3*, *Tssk4* and *Tssk5* play a functional role during spermatogenesis of several mollusks, including the pen shell *Atrina pectinate*, abalone *Haliotis discus hannai* and Bay Scallop *Argopecten irradians* (Li et al., 2016b; Kim et al., 2019; Xue et al., 2021). The present study showed the higher expression level of *Tssk1* and *Tssk3* in the testis of *C. sinensis*, indicating that Tssk family may have a role in sperm differentiation in the testis and/or fertilization. Moreover, Murray and Hobbs (2022) reported that *Kelch-like protein homolog 10* and *Armadillo repeat-containing protein 4 isoform X2* are involved in spermatogenesis and spermatid development/maturation of male blue mussel *Mytilus edulis*, respectively. Similarly, the *Kelch-like protein homolog 10* and *Armadillo repeat-containing protein 4 isoform X2* have previously been identified in serveral bivalve species including *Mytilus galloprovincialis*, *Nodipecten subnodosus*, *Pinctada margaritifera* and *C. gigas* (Craft et al., 2010; Llera-Herrera et al., 2013; Teaniniuraitemoana et al., 2014; Gallardi et al., 2021). In agreement with previous results, the higher expression level of *Kelch-like protein 10* and *Armadillo repeat-containing protein 4-like isoform X1* was found in the testis, illustrating that these genes play a key role in the sperm development of *C. sinensis*. Sperm-associated antigen 6 (SPAG6) is an important flagellar protein required for normal flagellar and cilia motility (Jarrell et al., 2020). De Sousa et al. (2014) reported that the mRNA levels of SPAG6 showed a significant correlation with the gonad area of European Clam *Ruditapes decussatus*. The present study revealed the higher transcript levels of *SPAG6* in the testis, suggesting that *SPAG6* is essential for the spermatogenesis of *C. sinensis*.

Ovary are the primary reproductive organs, and their normal development is crucial for bivalve reproduction (Yang et al., 2016; Zhao et al., 2022). Notably, vitellogenesis is involved in the accumulation of the major yolk protein vitellin (Vn), which is important in the development and maturation of oocytes (Saavedra et al., 2012; Kang et al., 2014). As a synthetic precursor of Vn, vitellogenin (Vg) is considered a biological marker of ovarian development in vertebrates and invertebrates (Marin and Matozzo, 2004; Porte et al., 2006). In this study, sequence analysis results showed that *CsVg* has no the typical lipoprotein N-terminal domain region compared with other known species, which may be attributed to the involvement of different mechanisms of Vg receptor binding during endocytosis (Xie et al., 2009). Previous studies showed that the lipoprotein N-terminal domain is responsible for lipid binding (Morandin et al., 2014). Tissue distribution results showed that *CsVg* was expressed in various tissues of female *C. sinensis*, which is consistent with the results on Fujian oyster *Crassostrea angulate* and *C. gigas* (Matsumoto et al., 2003; Ni et al., 2014). Remarkably, qPCR data showed that *CsVg* was abundantly expressed in the ovary, indicating that *CsVg* plays crucial roles in the ovarian development of *C. sinensis*. To search for more clues regarding the interaction between *CsVg* and ovarian development, the temporal expression patterns of *CsVg* in the ovary was analyzed during the ovarian development of *C. sinensis*. A relatively abundant expression of *CsVg* in proliferation stage to maturation stage and a significantly low expression in spent stage further demonstrated that *CsVg* is involved in the ovarian development of *C. sinensis*. These results coincide with those of previous studies on bivalve species (Ni et al., 2014; Yang et al., 2016). Vitellogenesis in bivalves is under the control of sex steroid hormone as well as other oviparous animals, and the estradiol levels of bivalve exhibit a seasonal change associated with the reproductive cycle (Osada et al., 2003; Wang and Croll, 2003; Osada et al., 2004). Therefore, to obtain further gain insights into the endocrine regulatory mechanism of *C. sinensis* reproduction, the regulation of *CsVg* mRNA expression by estradiol were analyzed in the ovary and hepatopancreas. The present results showed that estradiol treatments significantly upregulated the mRNA expression of *CsVg* in the ovary, suggesting that estradiol is a primary promoter of *Vg* mRNA transcription in *C. sinensis*. Similar estradiol inducibility of *Vg* was demonstrated in other bivalve species (Osada et al., 2003; Andrew et al., 2010; Qin et al., 2012; Ni et al., 2014). Moreover, the Cs*Vg* mRNA transcription in the hepatopancreas was induced by estradiol, which implies that hepatopancreas may also be involved in the vitellogenesis of *C. sinensis*. Hepatopancreas is another important site of vitellogenesis in crustaceans (Feng et al., 2022). Nonetheless, the regulation mechanism of estradiol in the vitellogenesis of bivalve is largely unknown. In vertebrate, it is well known that estrogen regulates *Vg* gene transcription mainly through binding to estrogen receptors (ER) on the target organ (Nelson and Habibi, 2013). In this study, the *Estrogen receptor gamma-like isoform X1* (*ERγ*) and *Estrogen-related receptor* (*ERR*) were identified in the ovary, indicating that the estradiol-*ERγ*/*ERR*-*Vg* signalling pathway may play an important role the regulation mechanism of estradiol on the vitellogenesis in bivalve. The above hypothesis was also proposed for scallop, mussels and oyster (Wang and Croll, 2003; Ciocan et al., 2010; Ni et al., 2014).

Except for *Vg*, the present study also discovered other genes associated with ovarian development and steroid biosynthesis/metabolism in the transcriptome data. Specifically, the higher expression levels of *MAM and LDL-receptor class A domain-containing protein 1*, *Melatonin receptor type 1B-B-like*, *Cell division cycle protein 20 homolog*, *Prostaglandin reductase 1* and *Transcription factor GATA-4 isoform X1* were detected in the ovary, indicating that

those genes participate in the ovarian development, steroidogenesis, folliculogenesis and oocyte maturation of *C. sinensis*. A previous study showed that *MAM and LDL-receptor class A domain-containing protein 1* may serve as oogonia or oocyte membrane protein mediating diverse signal transduction to regulate oocyte development (Yue et al., 2018). Takahashi and Ogiwara (2021) reported that melatonin can directly regulate ovarian physiology, including steroidogenesis, folliculogenesis, oocyte maturation and ovulation, by binding to melatonin receptors. *Cell division cycle protein 20* is required for spindle assembly and chromosomal segregation during oocyte maturation (Yang et al., 2014). *Prostaglandin reductase 1* plays a negative physiological role in the development of oocytes and ovaries (Prasertlux et al., 2011). *Transcription factor GATA-4* can be a downstream effector of cAMP/PKA pathway in the regulation of *CYP19* gene during folliculogenesis and luteinization (Monga et al., 2012). In addition, five genes related to steroid biosynthesis/metabolism were identified in the transcriptome data. *CYP17A*has been viewed as a critical enzyme for the biosynthesis of sexual steroid, and it performs the 17-alpha-hydroxylation of progesterone and pregnenolone to 17-hydroxyprogesterone (17OHP) and 17-hydroxypreg nenolone, respectively (Athanasoulia et al., 2013). *17 beta-Hydroxysteroid dehydrogenases* (*17 beta-HSDs*) plays a key role in estrogen and androgen steroid metabolism by catalyzing the final steps of steroid biosynthesis (Marchais-Oberwinkler et al., 2011). The present study showed that the *CYP17A* and three genes related to beta hydroxysteroid dehydrogenase enzymes (17-beta-hydroxysteroid dehydrogenase 14-like, Estradiol 17-beta-dehydrogenase 2-like, Hydroxysteroid dehydrogenase-like protein 2) were identified in the gonad, suggesting that these genes are involved in the biosynthesis of sexual steroids in the *C. sinensis*. Moreover, the *Estrogen sulfotransferase-like isoform X2* was also found in the gonad, which indicates the presence ofa balance system of steroid hormone synthesis in clam gonads. A previous study reported that Estrogen sulfotransferase is a cytosolic enzyme that sulphatesestrogens to inactivate them and regulate their homeostasis (Yi et al., 2021).

## 5 Conclusion

In this study, transcriptome sequencing was used to explore the expression level of genes associated with sexual determination/differentiation and gonadal development of *C. sinensis*. The present study identified 23 genes (*Dmrt1*, *Sox2/4/9*, *Foxl2*, *β-catenin* and *GATA-type zinc finger protein 1-like*) involved in sexual determination/differentiation, 20 genes (*Spermatogenesis-associated protein 17-like isoform X1, Meiotic recombination protein SPO11-like isoform X1, Meiotic recombination protein REC8 homolog isoform X2, Testis-specific serine/threonine-protein kinase 1, Testis-specific serine/threonine-protein kinase 3-like, Sperm-associated antigen 6* and *Kelch-like protein 10*) related to spermatogenesis, 15 genes (*Vitellogenin, MAM and LDL-receptor class A domain-containing protein 1* and *Cell division cycle protein 20 homolog*) associated with the ovarian development and 5 genes involved in steroid biosynthesis/metabolism, respectively. Theresults will provide new insights into the mechanisms of sexual determination/differentiation and gonadal development in marine bivalves. In the future, the roles of these genes need to be elucidated to provide a scientific basis for the sexual control and breeding of shellfish.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI - PRJNA906186.

## Author contributions

ML: Experimental design, Writing - Original Draft, Data Curation. HN: Formal analysis, Data Curation. ZR: Data Curation, Validation. ZW: Formal analysis, Visualization. SY: Data Curation, Visualization. XL: Experimental design, Formal analysis. ZD: Writing-Editing, 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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