



Gonad Transcriptome Analysis of the Razor Clam (*Sinonovacula constricta*) Revealed Potential Sex-Related Genes

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The razor clam, *Sinonovacula constricta* is a commercially important bivalve in the western Pacific Ocean, yet little is known about the mechanisms of sex determination/differentiation and gametogenesis. In the present study, the comparative transcriptome analysis of adult gonads (female gonads and male gonads) was conducted to identify potential sex-related genes in *S. constricta*. The number of reads generated for each target library (three females and three males) ranged from 31,853,422 to 37,750,848, and 20,489,472 to 26,152,448 could be mapped to the reference genome of *S. constricta* (the map percentage ranging from 63.71 to 71.48%). A total of 8,497 genes were identified to be differentially expressed between the female and male gonads, of which 4,253 were female-biased (upregulated in females), and 4,244 were male-biased. Forty-five genes were identified as potential sex-related genes, including *DmrtA2*, *Sox9*, *Fem-1b*, and *Fem-1c* involved in sex determination/differentiation and *Vg*, *CYP17A1*, *SOHLH2*, and *TSSK* involved in gametogenesis. The expression profiles of 12 genes were validated by qRT-PCR, which further confirmed the reliability and accuracy of the RNA-Seq results. Our results provide basic information about the genes involved in sex determination/differentiation and gametogenesis, and pave the way for further studies on reproduction and breeding in *S. constricta* and other marine bivalves.

Keywords: *Sinonovacula constricta*, gonad, transcriptome analysis, sex determination/differentiation, gametogenesis

INTRODUCTION

The razor clam, *Sinonovacula constricta* is a marine bivalve, living in the lower to mid intertidal zones along the western Pacific Ocean coast (Morton, 1984; Wang and Xu, 1997). Razor clam farming began almost 500 years ago in southeastern China, particularly in the coastal areas of Fujian and Zhejiang Province (Wang et al., 1993). In China, *S. constricta* has become one of the most commercially important cultured clam species and has huge market potential. Therefore, it is urgent to further improve the technique in the field of artificial propagation and culture of this

species. For the *S. constricta* selection program, the aim is to construct inbred lines with a variety of traits, such as rapid growth, that underlie successful reproduction.

Compared with other economically important molluscan species, *S. constricta* is dieocious (i.e., with fixed gender; individuals do not change sex), and the gonad maturation period is from September to October (Wu and Xu, 2000). Furthermore, it is difficult to differentiate between female and male clams with external morphological characteristics, especially before sex maturity (Wang et al., 1993). Therefore, gonadal maturity and synchronicity cannot be estimated by appearance, yet they are important factors for successful artificial reproduction. In the artificial reproduction conditions of bivalves, the successful hatching of selected spat relies on production of gametes and embryos from synchronous breeders. Therefore, it is essential to achieve genetic improvement for controlled reproduction, which depends on understanding molecular mechanisms and factors that control reproduction (Vahirua-Lechat et al., 2008; Moullac et al., 2013). However, the mechanisms that underlie gonad development and sex determination/differentiation in *S. constricta* are poorly understood, and there is no gene yet identified as an actor involved in gonad development or sex determination.

Gender is the product of sex determination/differentiation. Genetic or environmental processes that establish the gender of an organism, lead to specific molecular cascades, which can change an undifferentiated gonad into an ovary or a testis (Penman and Piferrer, 2008; Piferrer and Guiguen, 2008). Previous genetic studies have shown that some genes expressed in a sexually dimorphic manner in the progress of activation of the ovarian or testis pathway or repression of the alternative pathway, and this process determines the resulting sex or gonad development in model organisms (Piferrer and Guiguen, 2008). In bivalves, studies have identified some genes related to sex determination/differentiation or gametogenesis, for example, *Dmrt* (Doublesex- and mab-3-related transcription factor) and *SoxE* involved in male determination in *Crassostrea gigas* (Naimi et al., 2009a; Santerre et al., 2014), and *Foxl2* and *Beta-catenin* involved in female determination in *Chlamys farreri* (Liu et al., 2012; Li et al., 2014). In recent years, transcriptome sequencing analysis has been widely used to screen for potentially sex-related genes in bivalves. In *C. gigas*, genes involved in sex-determining pathways, such as *SoxH* and *Foxl2* were identified by male and female gonad transcriptome analysis (Zhang et al., 2014). In *Pinctada margaritifera*, genes such as *Dmrt*, *Fem-1*, *Foxl2*, and *Vitellogenin* (*Vg*) were identified as potential sex differentiation/determining genes based on transcriptional change in male and female gonads at different development stages (Teaniniuraitemoana et al., 2014). In *Patinopecten yessoensis*, transcriptome sequencing revealed that the *Foxl2* gene was ovary-biased and *Dmrt1*, *SoxH*, *Fem-1*, and *Sox9* were testis-biased (Yang et al., 2016; Li et al., 2016; Zhou et al., 2019). In *Tegillarca granosa*, genes such as *Sox*, *Foxl2*, and *Beta-catenin* were identified as sex-related genes from the gonad transcriptome data (Chen et al., 2017). The *Vg* gene was identified to be expressed especially in female gonads in *C. gigas* (Matsumoto et al., 2003), *C. farreri*

(Qin et al., 2012), and *P. yessoensis* (Yang et al., 2016). Clearly, studies on sex-related genes not only contribute to improving our knowledge about molecular mechanisms of sex determination/differentiation and gonad development, but also may be applicable to reproductive control of cultured mollusks. However, little is known about the sex-related genes in *S. constricta*.

Recently, the genomic sequence and a large number of transcriptomic data of *S. constricta* have been available (Niu et al., 2013; Ran et al., 2019; Dong et al., 2020), however, the genes related with sex determination/differentiation or gametogenesis were still not identified. In this study, we analyzed the female and male gonadal transcriptomes of *S. constricta* using Illumina sequencing technology, compared expression patterns of differentially expressed genes between female and male gonads, and identified potential sex-related genes. This study aims to identify genes that potentially involved in sex determination/differentiation or gametogenesis. Our results will be useful for further studies on reproduction control and artificial propagation practice in *S. constricta*.

MATERIALS AND METHODS

Animal Sampling and RNA Extraction

Two-year-old mature clams were collected from the Technology Innovation Base of Marine and Fisheries of Ningbo, Zhejiang province, China and used for gonadal transcriptomic sequencing. During the reproduction season, the mature gonads of *S. constricta* are full of sperms or eggs. The gender of clams was confirmed by observing the presence of sperms or eggs under a microscope. Three female and three male clams were selected, and their gonad tissues were sampled and frozen in liquid nitrogen and then stored at -80°C until RNA extraction. The three females were designed as *ScF1*, *ScF2*, and *ScF3* and three males were designed as *ScM1*, *ScM2*, and *ScM3*. Total RNA from each gonad tissue was isolated with Trizol reagent (Invitrogen, United States) according to manufacturer's instructions. DNase I was used to remove DNA contamination from the isolated RNA, and the RNA quantity and quality were evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, United States).

cDNA Library Construction, Sequencing, and Reference-Based Assembly

Six cDNA libraries were constructed according to the standard protocol provided by Biomarker Technologies Co. (Beijing, China). Briefly, mRNA was purified from total RNA with oligo (dT) magnetic beads and fragmented with divalent cations buffer under elevated temperature. First-strand cDNA was generated using random hexamer-primed reverse transcription followed by the synthesis of second-strand cDNA using RNase H and DNA polymerase I. Then the double-strand cDNA was purified and washed with elution buffer followed by end repair, dA-tailing and ligated to sequencing adapters. Afterward, adaptor-ligated fragments were purified, and suitable fragments were chosen as templates for PCR amplification. The prepared cDNA libraries

were sequenced on the Illumina HiSeq 2500 platform (San Diego, United States) with 150 bp paired-end model.

The raw reads from the six samples were pre-processed by trimming the adaptor sequences and filtering the ambiguous or low-quality short reads with software (Grabherr et al., 2011). Then the resulting high-quality reads from all samples were mapped to the reference genome of *S. constricta* (WSYO00000000.1) using HISAT2 (Kim et al., 2015). String Tie software (Shen et al., 2014) were used to assemble mapped reads and compared with reference genome annotation to discover novel genes. All novel genes were subjected to annotation with different databases, including Swiss-Prot,¹ NR (NCBI non-redundant protein database),² GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes).³ BLASTX searches were conducted in the NR database and Swiss-Prot database with an *e*-value threshold of 1E-5.

Sample Relationship Analysis

The correlation coefficient between biological replicates was used to evaluate sample repeatability, and low correlation sample was removed for further analysis. In order to study the global transcriptomic differences and correlation among samples from the two sexes, Principal component analysis (PCA) was conducted and a heat map was generated. PCA was performed on the basis of the average fragments per kilobase of transcript per million mapped reads (FPKM) values for all expressed genes in each sample, and sample repeatability analysis was presented via heat map using R scripts.

Analysis of DEGs (Differentially Expressed Genes) and Function Enrichment

Gene expression level was generally estimated by mapping clean reads to the trinity transcript assembly by RSEM for each sample (Li and Dewey, 2011). Meanwhile, the gene expression level was presented in a normalized expressed value as FPKM. Afterward, reconstruction of transcript assemblies was performed by the reference genome annotation-based transcripts assembly program within the Cufflinks software package to obtain a comprehensive set of transcripts for further differential analysis. The DESeq2 R package was used to identify differentially expressed genes (DEGs) between the two sexes (Anders and Huber, 2010). DEGs were defined as $|\text{Log}_2\text{FoldChange}| > 1$ and a false-discovery rate < 0.01 . The transcriptional pattern variations between the two sexes were assessed by DEG union, and R scripts were used to generate a heat map of the DEGs.

For enrichment analysis, all DEGs were mapped to terms in the KEGG and GO databases using BLAST algorithm with an *E*-value of $\leq 1e-5$, and the significantly ($P < 0.05$) enriched KEGG and GO terms in DEGs were identified compared with the transcriptome background.

¹<http://www.ebi.ac.uk/swissprot/>

²<http://www.ncbi.nlm.nih.gov/>

³<http://www.genome.jp/kegg/>

Gene Expression Analysis by Quantitative Real-Time PCR (qRT-PCR)

To validate the transcriptome sequencing data and the genes that might be potential sex-related genes obtained from DESeq analysis, 12 DEGs were chosen and quantified using quantitative real-time PCR (qRT-PCR). Total RNA was first extracted from each sample by using Trizol Reagent (Invitrogen), and cDNA was then synthesized using the PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, Japan). Finally, qRT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, United States) according to manufacturer's instructions on an ABI 7500 Fast Real-time PCR System (Applied Biosystems, United States). The 18S rRNA gene was chosen as the reference gene to serve as an internal control. The 12 pairs of prime sequences used for qRT-PCR analysis were listed in **Table 1**. Each 20 μ L reaction system contained the following components: 10 μ L iTaq Universal SYBR Green Supermix, 1 μ L each primer, 0.8 μ L cDNA, and 7.2 μ L deionized water. The procedure of qRT-PCR was set as following: 94°C for 20 s and 40 cycles of 94°C for 3 s, 60°C for 15 s, and 72°C for 10 s. The gene relative expression level was calculated with the $2^{-\Delta\Delta\text{ct}}$ methods, and the results were compared using one-way analysis of variance in SPSS 20.0 (SPSS, United States). $P < 0.05$ was considered to be statistically significant. The fold changes of 12 genes in female samples vs. male samples were calculated via FPKM, and the $\log_2\text{FoldChange}$ values of these genes obtained by RNA-Seq and qRT-PCR were used for graphical presentation.

To detect tissue expression pattern of some DEGs, eight tissues, including hepatopancreas, gill, mantle, siphon, adductor muscle, foot, female gonad and male gonad, were collected from three female and three male individuals, respectively, at mature stage. And gene expressions levels were analyzed by qRT-PCR followed the aforementioned procedure.

Sequence Analysis of Sex-Related Genes

The amino acid sequences used for sequence analysis were all retrieved from GenBank. Protein domain structure analysis was performed with SMART software. Multiple sequence alignments were performed by the ClustalW2 program and phylogenetic trees with bootstrap values were constructed with MEGA 7.0 software using the neighbor-joining method. The numbers in the branches of phylogenetic trees represent the bootstrap values from 1,000 replicates, and all the sequence GenBank accession numbers were listed in **Supplementary Table 1**.

RESULTS

Sequence Analysis and Gonad Transcriptome Assembly

To better understand the mechanism of sex determination/differentiation and gametogenesis in *S. constricta*, a comparative transcriptomic analysis was conducted. Six cDNA libraries (Female: *ScF1*, *ScF2*, and *ScF3*; Male: *ScM1*, *ScM2*, and *ScM3*) were sequenced and assembled. The number of reads generated for each library ranged from 31,853,422 to 37,750,848,

with Q30 values ranging from 94.34 to 94.79% (**Table 2**). Among the clean reads, the number of reads that could be mapped to the reference genome ranged from 20,489,472 to 26,152,448, and the percentage of clean reads ranged from 63.71 to 71.48% in the different libraries (**Table 2**). After alignment with the reference genome, 30,651 genes (including 10,092 novel genes) were obtained from global gonad transcriptomes, and 24,198 genes (including 5,279 novel genes) were function-annotated in the GO, NR, KEGG and Swiss-Prot databases. We deposited the transcriptome data into the NCBI Sequence Read Archive (SRA) under accession numbers SRR9937008–SRR9937013.

DEGs Identification and Function Enrichment Analysis

PCA revealed strong clustering associated with sex, and there were obvious differences in transcript expression between ovaries and testis excluding the *ScM1* sample (**Figure 1A**). Meanwhile,

a heat map plot for sample relationship analysis also showed that sex identification of samples was exact, except for the *ScM1* sample (**Figure 1B**). The Pearson's correlation coefficient (r^2) were 0.102 and 0.264 between *ScM1* and *ScM2*, *ScM1*, and *ScM3*, respectively, with low coefficient, so the RNA-Seq data from the *ScM1* sample were excluded from further analysis.

For the differential expression analysis, 8,497 DEGs were identified between female gonad group and male gonad group. Compared to female gonad group, 4,244 genes were significantly up-regulated in male gonad group, accounting for 49.95% of all significant DEGs, and 4,253 genes were significantly down-regulated in male gonad group, accounting for 50.05% of all significant DEGs (**Figure 2**). Of all the significant DEGs, 6,823 genes were function-annotated in the GO, Swiss-Prot, KEGG and NR databases by BLASTX with a cut-off *E*-value of 1×10^{-5} .

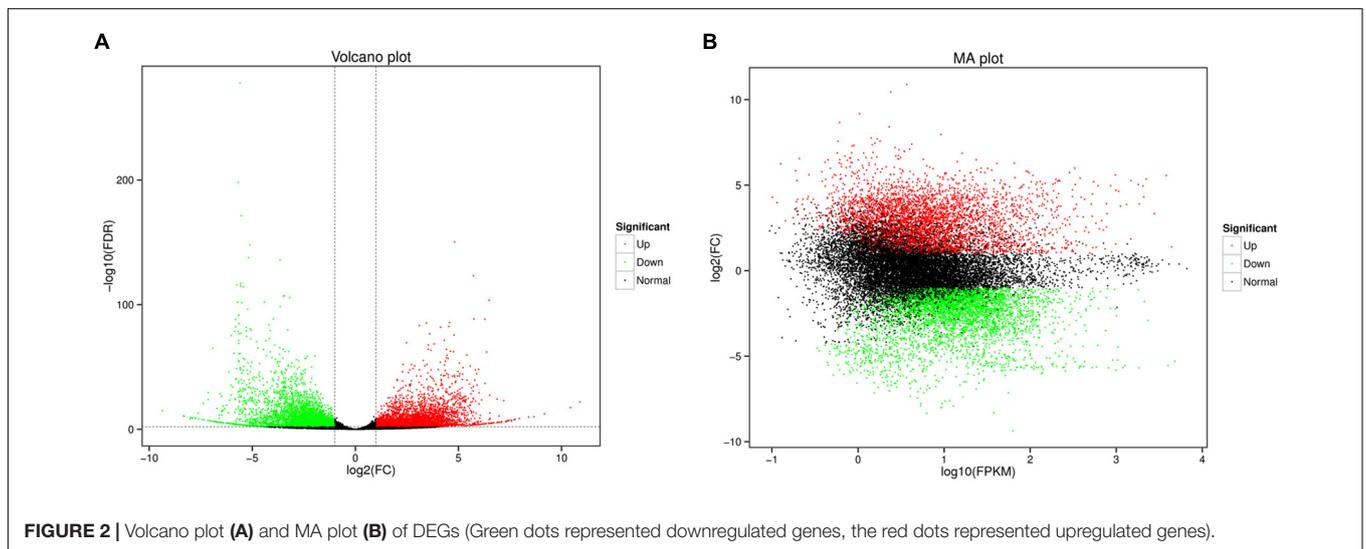
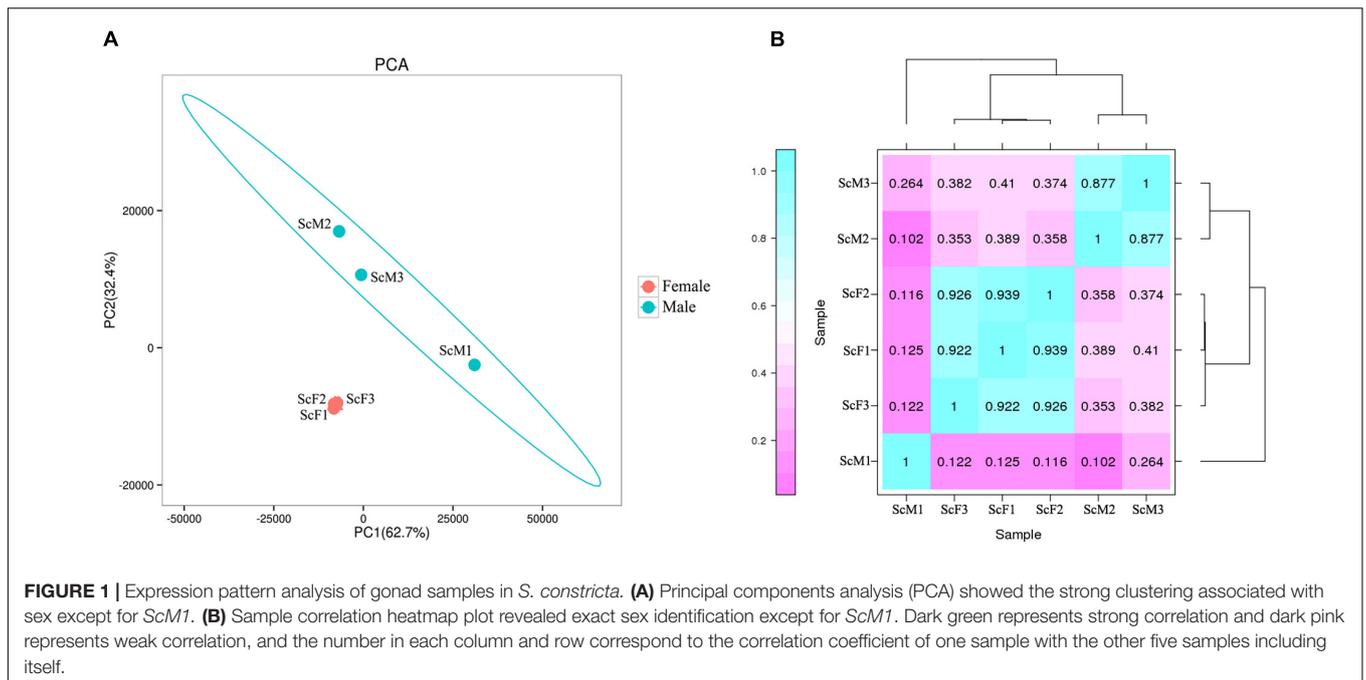
To explore the potential functions of these DEGs in sex determination/differentiation and gametogenesis, GO and KEGG pathway enrichment analyses were performed.

TABLE 1 | The information of genes validated by qRT-PCR.

Gene ID	Gene name	Annotation	Primer sequence(5'–3')
ctg423.9/MZ440691	<i>DmrtA2</i>	Doublesex and mab-3 related transcription factor A2-like protein	F: ACACCGAAATGTGCCAGA R: TCCTCCAGCGACAATAA
ctg57.8/MZ440689	<i>Fem-1b</i>	Feminization-1b	F: TTCTTGGCGGAATGGACG R: TCGGATGTCTTGGTGGGC
ctg186.37/MZ440688	<i>Fem-1c</i>	Protein fem-1 homolog C-like	F: CAGATTTGGAACAGGTCGGAT R: AGATGCTGCTCTCAGTGGTGTT
ctg163.27/MZ440690	<i>Sox9</i>	Transcription factor SOX-9-like	F: ACCACAAGCCAGCCTAT R: GCGAGGTTGCCTTGGAAA
novel gene_19266	<i>CYP17A1</i>	Steroid 17-alpha-hydroxylase/17,20 lyase isoform X1	F: GGTGGCTCTCATTTGTGGTCTT R: AAGGCAGTCGTGATGTCGTTG
ctg318.17	<i>Smaktcr</i>	Sperm motility kinase Tcr mutant form-like	F: TGACCCCGTGTCTGTAATG R: ATGGCACTGAACGGAATAGC
ctg1.91	<i>TSSK</i>	Testis-specific serine/threonine kinase	F: CCCGTTGATGTGAAGAAGGT R: ACTTTGAAAATCTCGTGGCG
ctg674.14	<i>GATA-7</i>	GATA zinc finger domain-containing protein 7-like	F: CGCACGCACCGCATTCAC R: ATGAGACGCTTCCGCCCG
novel gene_31922	<i>SOHLH2</i>	Spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2	F: AGGGAGCGTATCAAGGACAGC R: TTGCCCTTCCTGTTGAAAATG
novel gene_13121	<i>FoxA1</i>	Forkhead box protein A1-A-like	F: GGGGTGGATAAGGAGAGTGG R: TGGGTCCTGAAGCGATG
ctg1559.4	<i>Beta-catenin</i>	Beta-catenin	F: AGACCCTCTCCAGCGTTTC R: GGCTTACACAGGAACAGGT
novel gene_30040	<i>Vg</i>	Vitellogenin	F: CAGATGTGGTGTAGAGATGGAAT R: GGACATACGAGAATGAGGTAGGTT
	18S rRNA		F: TCGGTTCTATTGCGTTGGTTTT R: CAGTTGGCATCGTTTATGGTCA

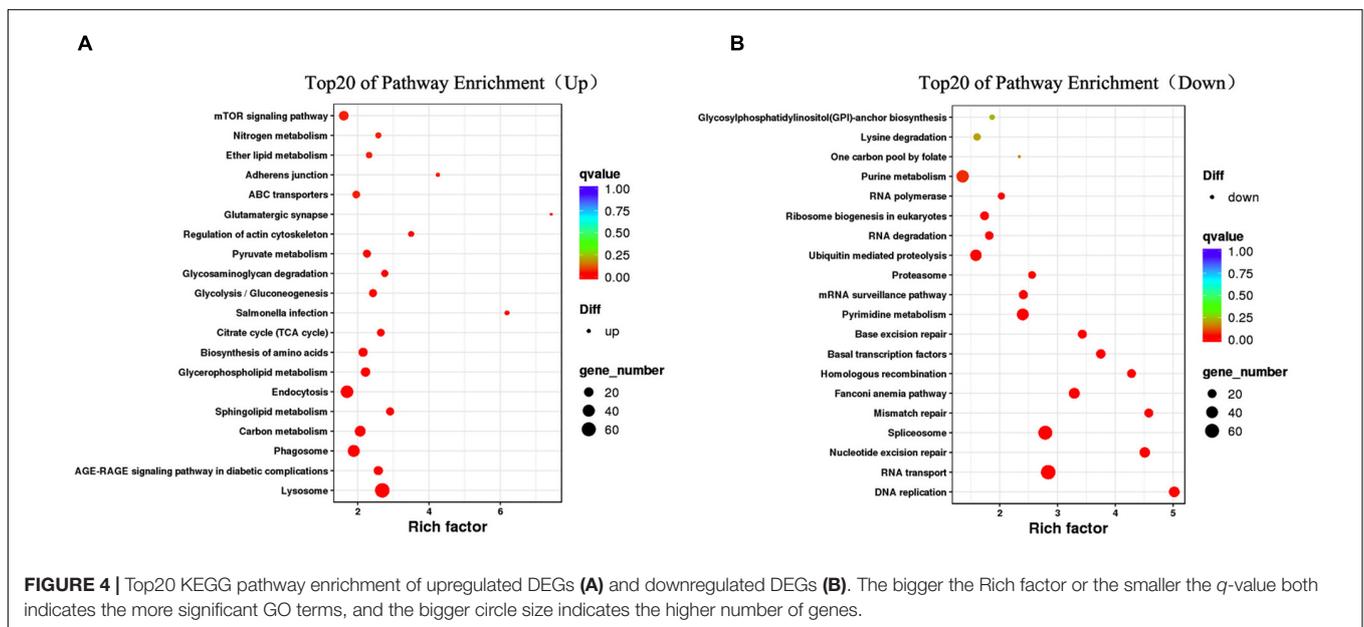
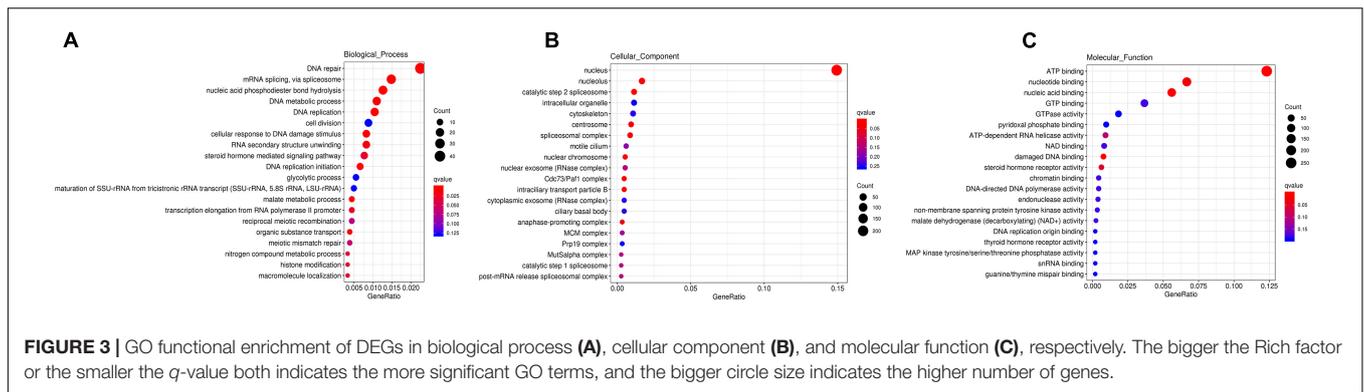
TABLE 2 | Summary statistics of gonad transcriptome sequencing for *S. constricta* from ovary and testis.

Sample ID	Female			Male		
	ScF1	ScF2	ScF3	ScM1	ScM2	ScM3
Clean reads	36,051,818	37,073,896	37,750,848	33,338,138	31,853,422	32,159,464
Base pairs	4,533,013,162	4,662,926,212	4,746,412,276	4,193,274,532	4,007,164,874	4,045,487,056
Mapped reads	25,768,615	25,789,370	26,152,448	22,453,357	20,553,483	20,489,473
Mapped percentage (%)	71.48%	69.56%	69.28%	67.35%	64.53%	63.71%



GO functional enrichment analysis showed that 2,746 DEGs had a GO ID and could be categorized into 1,965 functional groups in three main categories: biological process, cellular component, and molecular function. Additionally, 151 GO terms were significantly enriched ($P < 0.05$). Biological process GO terms related to DNA repair (GO:0006281), DNA replication (GO:0006260), and nucleic acid phosphodiester bond hydrolysis (GO:0090305) were significantly enriched ($P < 0.05$), as were molecular function GO terms related to ATP binding (GO:0005524) and nucleic acid binding (GO:0003676) and cell cellular component GO terms related to nucleus (GO:0005634), catalytic step 2 spliceosome (GO:0071013), and Cdc73/Paf1 complex (GO:0016593) (**Figure 3**).

In the KEGG analysis, 1,364 DEGs had KEGG assignments and were associated with 221 pathways. **Figure 4** shows the top 20 enriched pathways. DNA replication, nucleotide excision repair, spliceosome, Fanconi anemia pathway, lysosome, RNA transport, and carbon metabolism were significantly enriched in KEGG pathways ($P < 0.05$), which showed patterns similar to those of the GO terms. Specifically, lysosome, endocytosis, phagosome, sphingolipid metabolism, carbon metabolism, and glycerophospholipid metabolism were significantly enriched in male gonad group ($P < 0.05$) (**Figure 4A**), whereas RNA transport, spliceosome, pyrimidine metabolism, ubiquitin mediated proteolysis, Fanconi anemia pathway, DNA replication, and nucleotide excision repair were significantly enriched in female gonad group ($P < 0.05$) (**Figure 4B**). Some of the



DEGs were enriched in the progesterone-mediated oocyte maturation, estrogen signaling pathway, oocyte meiosis, oxytocin signaling pathway, ovarian steroidogenesis, mTOR signaling pathway, FoxO signaling pathway, Wnt signaling pathway, GnRH signaling pathway, ubiquitin-mediated proteolysis, and insulin secretion, which are known to be involved in male and female germ cell development. Some of these GO term and KEGG pathway enrichments were closely related to sex determination/differentiation or gametogenesis. At least 45 genes were identified as sex-related genes that might be involved in sex determination/differentiation or gametogenesis, and most of them were enriched in the GO function terms and KEGG pathways mentioned above (Supplementary Table 2).

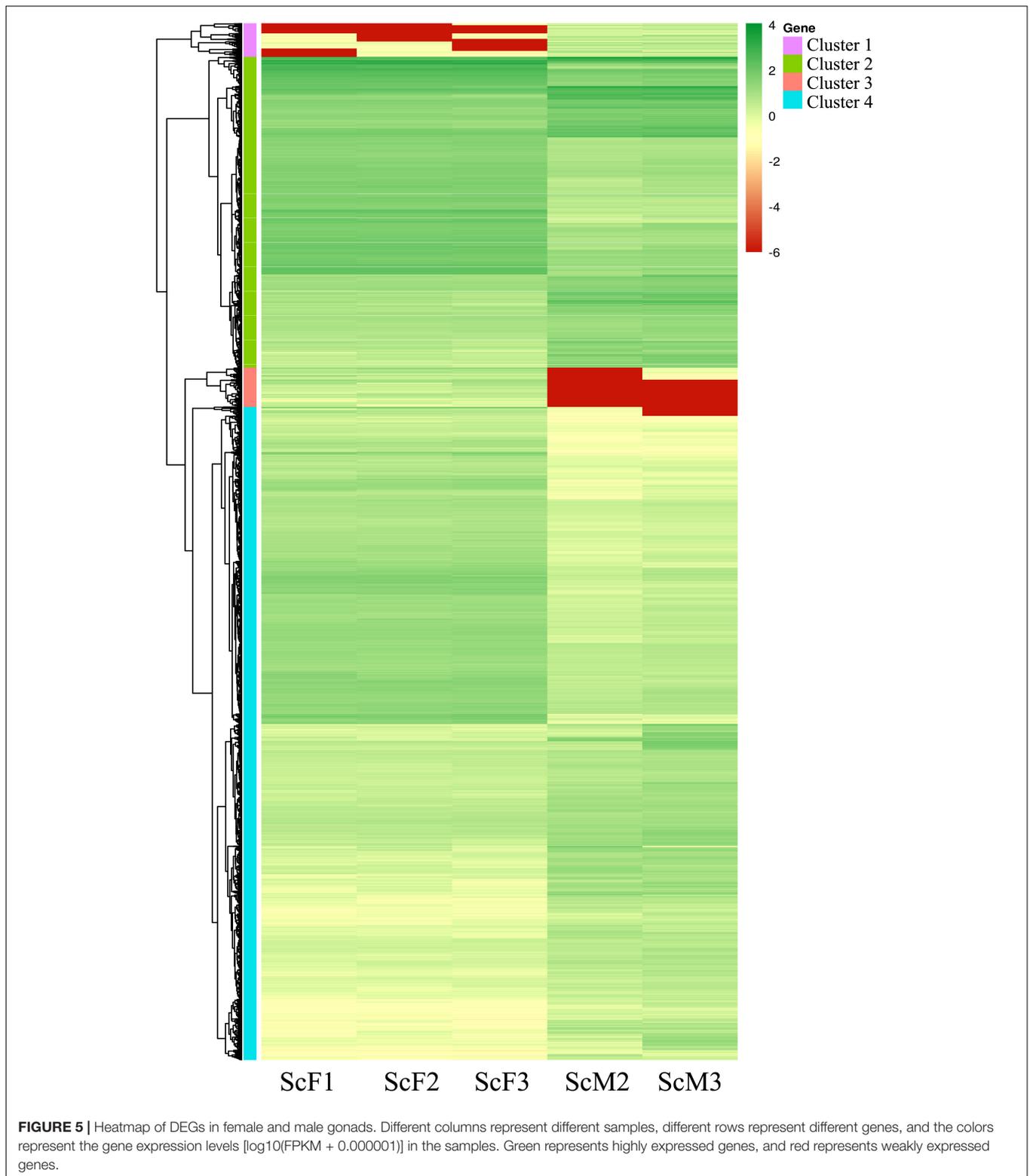
Identification of DEGs Related to Sex Determination/Differentiation and Gonad Development

A heat map illustrating the hierarchical clustering of the genes differentially expressed between female and male gonads was conducted to visualize the overall gene expression pattern,

and four clusters with similar gene expression patterns were generated (Figure 5). From the clusters, a catalog of 45 genes potentially involved in sex-determination/differentiation or gametogenesis was generated (Supplementary Table 1). The roles of these genes are unknown in *S. constricta*, but most of them have been identified as playing important roles in sex-determination/differentiation or gametogenesis in other organisms.

Cluster 1 included 277 genes, of which 41 genes showed significant similarities to known proteins, and 64 genes possessed GO assignments. And several genes known to be involved in male sex determination/differentiation were identified, including *Fem-1b* (*Fem-1b*, ctg57.8) and *Fem-1c* (*Fem-1c*, ctg186.37). Genes such as *E3 ubiquitin-protein ligase R2-like* (*R2*, novel gene_12556) and *sperm motility kinase Tcr mutant form-like* (*Smoktcr*, ctg318.17 and ctg318.18) were also found, and they have been described as playing roles in spermatogenesis.

In cluster 2(including 2,545 genes), *Beta-catenin* (*Beta-catenin*, ctg1559.4, and ctg1559.5) and *3-oxo-5-alpha-steroid 4-dehydrogenase 1* (*SRD5A1*, ctg350.13) were detected, and they are known to be involved in sex determination/differentiation. Genes



involved in oogenesis and oocyte maturation were also found, including *mitotic apparatus protein p62-like isoform X2* (MP62, ctg197.11) and *Lis1* (*Lis1*, ctg955.10). Some genes involved in catalyzing steroid biosynthesis and metabolism were also found,

such as *17beta-HSD14* gene, which is of great importance to gametogenesis.

In cluster 3 (including 323 genes), we identified genes encoding proteins involved in spermatogenesis and oogenesis,

such as *spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 2* (*SOHLH2*, novel gene _31922) and *spermatogenesis-associated protein 24-like* (*SPATA24*, ctg9357.1). A few genes implicated in glycoprotein biosynthesis and metabolism were also found, such as *vitellogenin* (*Vg*, novel gene _30040) and *vitellogenin-6* (*Vg-6*, novel gene _30041), which are important for female gonad development.

Of the 5352 genes in cluster 4, a large number of genes were found to be differentially expressed between females and males and involved in oogenesis or spermatogenesis. Genes potentially involved in male sex determination included *double sex and mab-3 related transcription factor A2-like protein* (*DmrtA2*, ctg423.9) and *transcription factor Sox-9-like* (*Sox9*, ctg163.27). Genes implicated in spermatid differentiation and development, including *meiotic recombination protein SPO11-like* (*SPO11*, ctg915.3) and *testis-specific serine/threonine-protein* (*TSSK*, ctg1.91; *TSSK1*, ctg1.92; *TSSK4*, ctg207.28), were found, as were genes associated with oogenesis, including *cytochrome P450 26A1* (*CYP26A1*, ctg146.9) and *steroid 17-alpha-hydroxylase/17,20 lyase-like* (*CYP17A1*, ctg67.144). *Serine-protein kinase ATM-like isoform X2* (*ATM*, ctg637.23), which is involved in female gamete generation and oocyte development, was also identified.

Gene Expression and Sequence Analysis of Sex-Related Genes

Twelve genes were chosen for qRT-PCR analysis to validate the differential expression results identified by RNA-Seq. qRT-PCR results showed that the expression levels of *DmrtA2*, *Sox9*, *Fem-1b*, *Fem-1c*, *CYP17A1*, *Smoktcr*, *TSSK*, and *GATA-7* in male gonads were significantly higher than those in female gonads ($P < 0.05$, **Figure 6A**), whereas *SOHLH2*, *FoxA1*, *Beta-catenin*, and *Vg* were significantly highly expressed in female gonads than in male gonads ($P < 0.05$, **Figure 6B**). The expression patterns of these DEGs validated by qRT-PCR were generally consistent

with the RNA-Seq results (**Figure 7**), which further confirmed the reliability and accuracy of the RNA-Seq data.

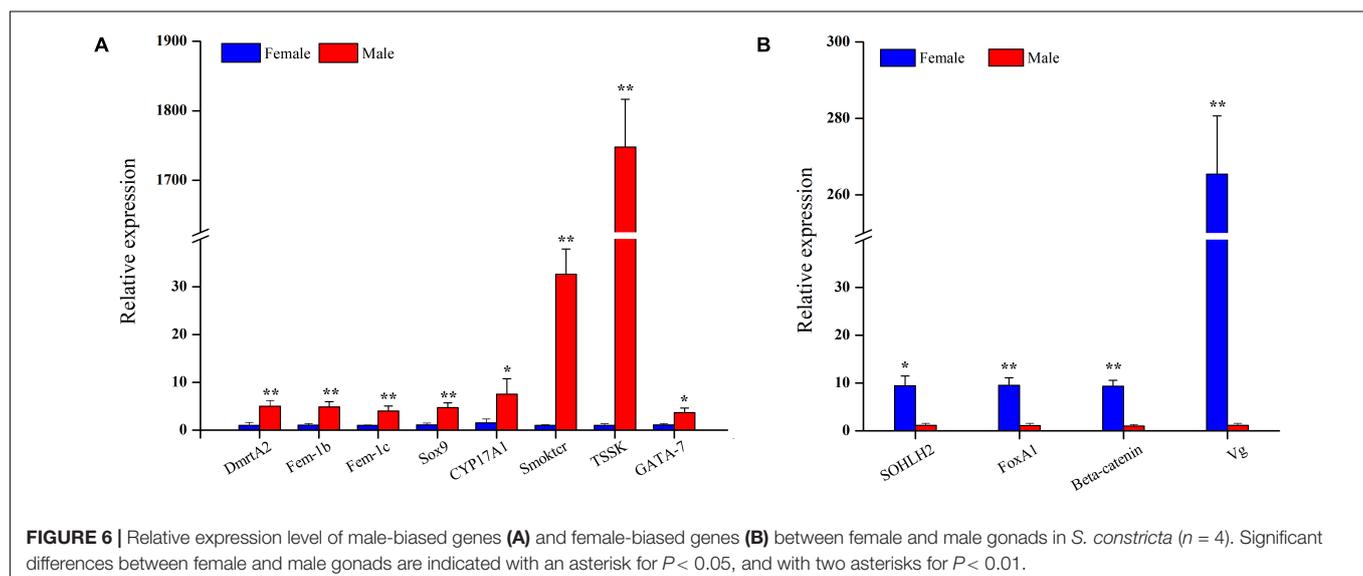
To investigate potential sex-related genes of *S. constricta*, four crucial genes *DmrtA2*, *Sox9*, *Fem-1b*, and *Fem-1c* were further examined and analyzed, and named *ScDmrtA2*, *ScSox9*, *ScFem-1b*, and *ScFem-1c* in *S. constricta*, respectively.

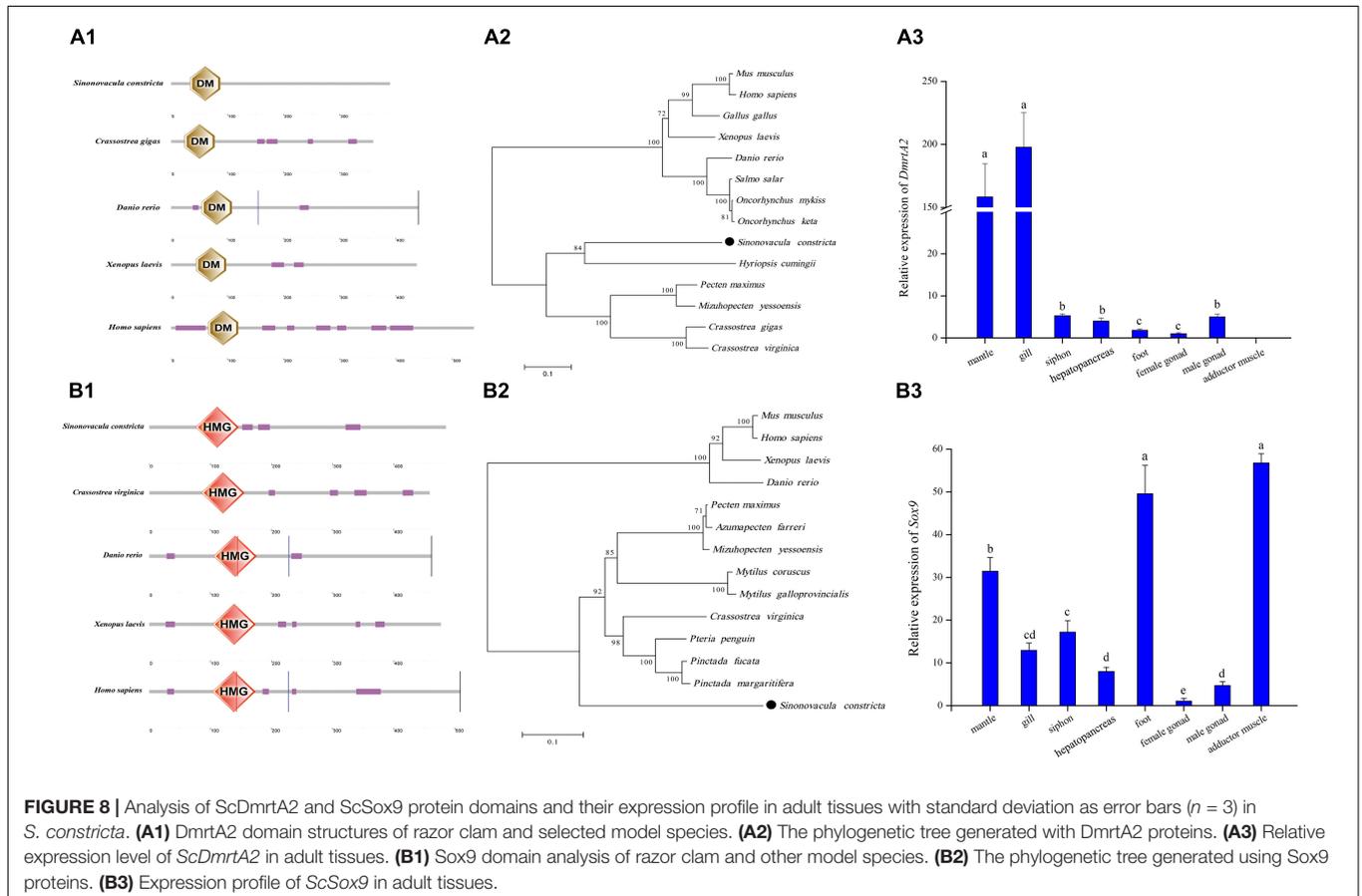
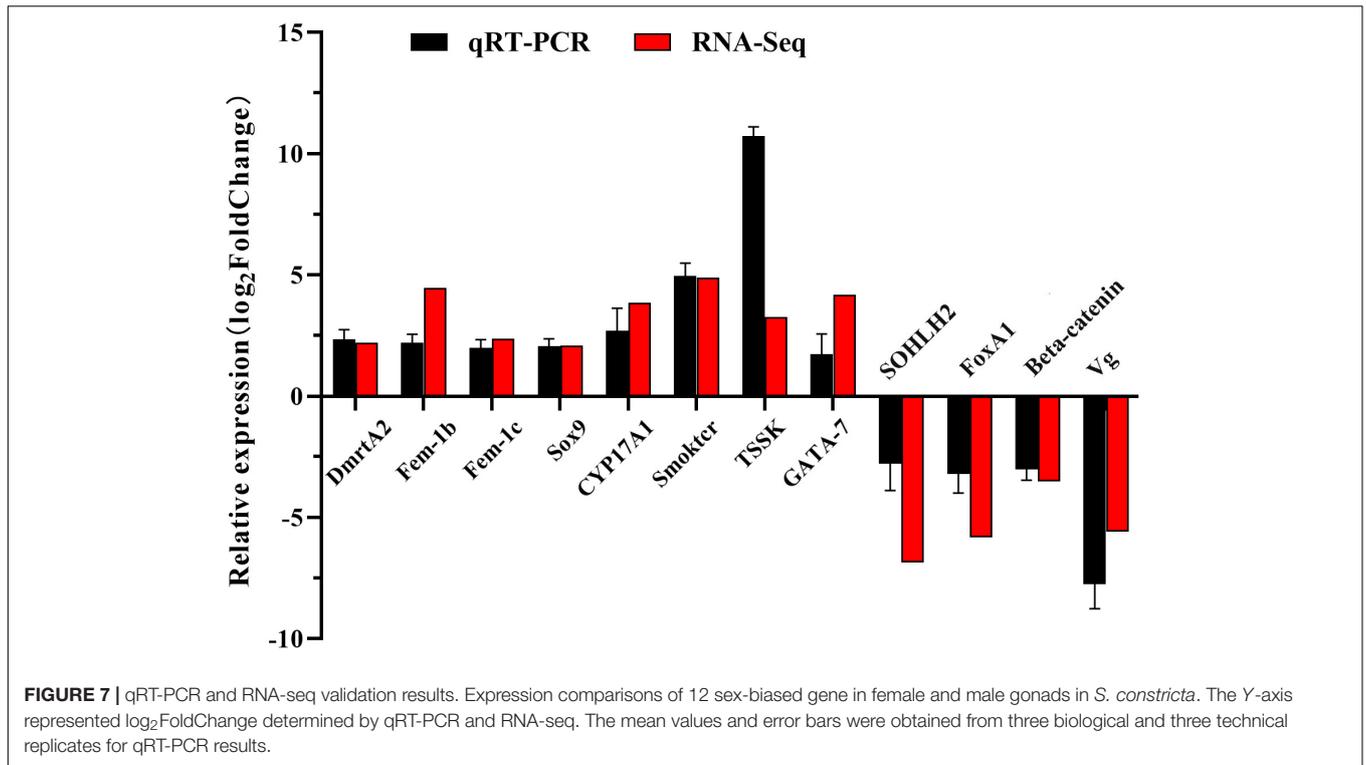
ScDmrtA2

A full-length transcript of *ScDmrtA2* (GenBank no.: MZ440691) was identified with an opening reading frame (ORF) of 1,170 bp encoding 389 amino acids. The deduced amino acids contain the DM domain (34–87 aa) consensus sequences (**Figure 8A1**). The phylogenetic tree showed that *ScDmrtA2* was first clustered with *DmrtA2* of *Hyriopsis cumingii*, and then grouped with the clade including scallops (*Pecten maximus* and *Mizuhopecten yessoensis*) and oysters (*C. gigas* and *Crassostrea virginica*). Finally, the molluscan *DmrtA2* clade was clustered with the vertebrate *DmrtA2* clade (**Figure 8A2**). Expression profile of *ScDmrtA2* in different adult tissues revealed that *ScDmrtA2* expressed significantly higher in mantle and gill ($P < 0.05$) than other tissues, and the expression level in male gonads is also significantly higher than that in female gonads ($P < 0.05$) (**Figure 8A3**).

ScSox9

ScSox9 (GenBank no.: MZ440690) was identified with ORF of 1,458 bp encoding 485 aa. An HMG domain consensus sequences were found from 76 to 146 aa in the deduced amino acids (**Figure 8B1**). The phylogenetic tree showed that *ScSox9* was clustered with *Sox9* of mollusks with the high bootstrap support (92), and then clustered with *Sox9* of vertebrate species (bootstrap support 100) (**Figure 8B2**). Tissue expression analysis showed that *ScSox9* was expressed in all nine examined tissues, and expressed significantly higher in foot and adductor muscle than in other tissues ($P < 0.05$) (**Figure 8B3**). Meanwhile, the relative expression level of *ScSox9* in male gonads is about 4.25 folds higher than in female gonads.





ScFem-1b and ScFem-1c

Two Fem-1 family genes, Fem-1b (GenBank no.: MZ440689) and Fem-1c (GenBank no.: MZ440688), were identified with complete ORF sequences of 1,911 and 1,869 bp, respectively. The deduced amino acid sequences of Fem-1b and Fem-1c were 636 and 622 aa, respectively, both of which contain seven ankyrin (ANK) repeats at N-terminus and two ankyrin repeats at C-terminus. The ANK repeats are characteristics of the Fem family proteins (Figures 9A1,A2). The phylogenetic tree showed that ScFem-1b and ScFem-1c had closer relationships to other mollusks, and were both grouped with mollusks with high bootstrap support (Fem1b 92 and Fem-1c 100), respectively (Figure 9B). Both *ScFem-1b* and *ScFem-1c* were expressed in all tissues, and both of them expressed at highest level in male gonads among all detected tissues ($P < 0.05$) (Figures 9C1,C2).

DISCUSSION

By analyzing the female and male gonad transcriptome of *S. constricta*, we identified at least 45 sex-related genes, including *DmrtA2*, *Sox9*, *Fem-1b*, *Fem-1c*, and *Beta-catenin* potentially involved in sex determination or differentiation, and *Vg*, *CYP17A1*, *TSSK*, and *SOHLH2* potentially involved in gametogenesis.

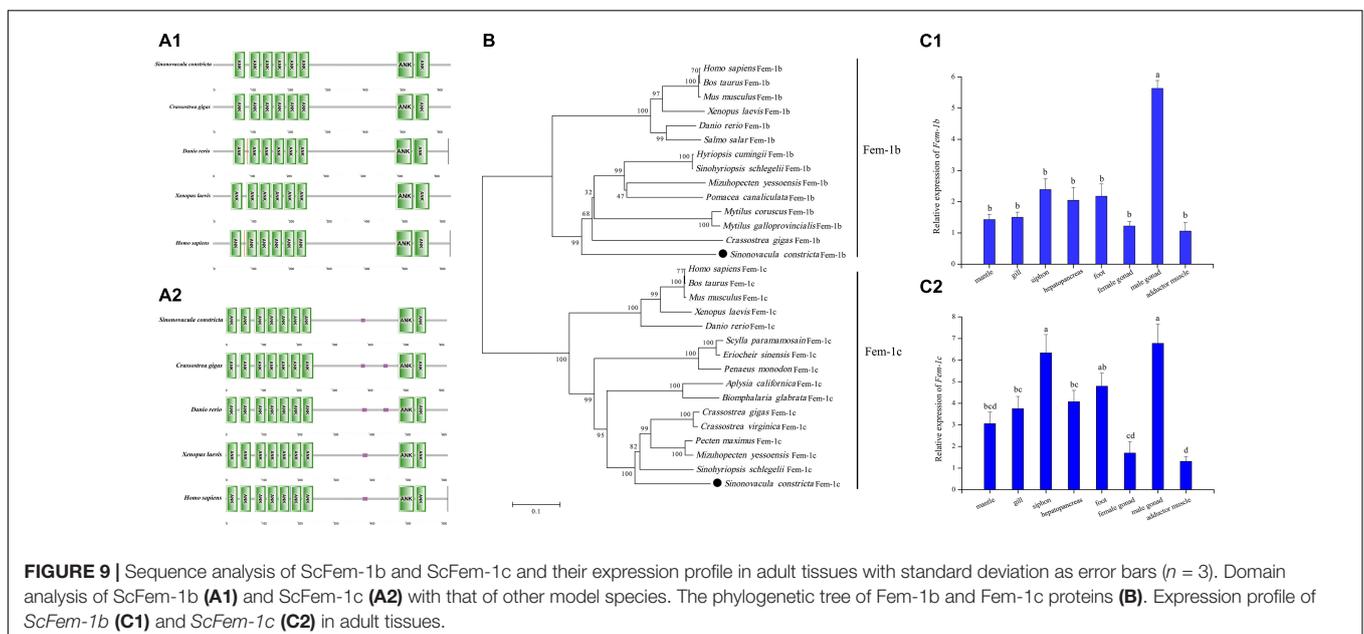
Potential Sex Determination/Differentiation Genes in *S. constricta*

Four potential male sex determining genes (*DmrtA2*, *Sox9*, *Fem-1b*, and *Fem-1c*) were all expressed significantly higher in male gonads than in female gonads ($P < 0.05$). In vertebrates, *Dmrt* is an important gene involved in sex determination/differentiation, which is a transcription factor and contains a characteristic zinc finger DM domain (Guo et al., 2005; Kopp, 2012). Mammals have at least seven DM domain genes, most of them

showed obvious gonadal expression (Xu et al., 2013). *DmrtA2* belongs to *Dmrt* family, and was expressed specially in testis in human, which suggested *DmrtA2* might be involved in sexual development (Ottolenghi et al., 2002). In adult zebrafish, *DmrtA2* was specially expressed in testis, ovary and brain, and the expression level is higher in testis than in ovary (Guo et al., 2004). Furthermore, zebrafish *DmrtA2* expression was restricted in developing germ cells and brain, especially in spermatogonia, spermatocytes, spermatids, and sperm cells, which suggested that *DmrtA2* was potentially involved in gonadal development and spermatogenesis (Guo et al., 2004; Xu et al., 2013). In this study, compared to female gonads, we detected significant elevation of *DmrtA2* gene expression in the testis of *S. constricta*, which might be involved in male gonad development and spermatogenesis, and this would be consistent with the patterns in zebrafish. *ScDmrtA2* were also detected to express significantly higher in mantle and gill than in other tissues ($P < 0.05$), and Yu et al. (2009) also found high expression of *DmrtA2* in mantle, gill, digestive diverticulum and adductor muscle besides male and female gonads in *Pinctada matensii*, suggesting that *DmrtA2* might exert other functions in mollusks.

We also found that the *Sox9* gene, a homolog of the *SoxE* gene, was male-biased and therefore might be a potential sex determination gene in *S. constricta*. The transcription factor *Sox9* containing an HMG domain, is a key gene in the mammalian testis determining pathway, and plays important roles in male gonadic differentiation and maintenance (Wagner et al., 1994; Knower et al., 2011). In *P. margaritifera*, the *Sox9* gene was also identified as a potential sex determination gene (Teaniuraitemoana et al., 2014). The expression profiles of *SoxE* in *C. gigas* suggested its important roles in early sex differentiation (Santerre et al., 2014).

Fem-1 gene, as a sex-determining gene for male body development and spermatogenesis, was first discovered in



Caenorhabditis elegans (Ventura-Holman et al., 1998). In vertebrates, the *Fem-1* family contains three conservative members: *Fem-1a*, *Fem-1b*, and *Fem-1c*, which are able to feminize nematode sex differentiation (Ventura-Holman and Maher, 2000; Krakow et al., 2001; Ventura-Holman et al., 2003). The *Fem-1* gene identified in *P. margaritifera* was thought to be related to sex determination (Teaniniuraitemoana et al., 2014), and the *Fem-1c* gene in *P. yessoensis* showed upregulated expression in females (Yang et al., 2016). Using both qRT-PCR and RNA-Seq, we observed significant increases in mRNA levels of the *Fem-1b* and *Fem-1c* genes in male gonads of *S. constricta*, which suggested their functions in sex determination/differentiation.

FoxA1, *FoxD2*, and *Beta-catenin* were also identified to be involved in the female sex determination/differentiation in *S. constricta*. Fox gene family members are transcription factors and some of which play important roles in ovarian development (Shimeld et al., 2010). For example, the *Foxl2* gene has been found to be involved in ovary differentiation and maintenance in vertebrates (Ottolenghi, 2005; Uhlenhaut et al., 2009). In mollusks, the *Foxl2* gene has been identified in *C. farreri* (Liu et al., 2012), *C. gigas* (Naimi et al., 2009b; Zhang et al., 2014), *P. yessoensis* (Li et al., 2016; Yang et al., 2016), and *P. margaritifera* (Teaniniuraitemoana et al., 2014), and it is thought to be a master regulator of female sex determination or ovarian maintenance. However, we did not find the *Foxl2* gene in our RNA-Seq results of *S. constricta*, although *FoxA1* and *FoxD2* were present and ovary-biased. In the canonical Wnt signaling pathway, *Beta-catenin* is a key transcriptional regulator and is essential for ovarian development and maintenance in mammals (Liu et al., 2009). *Beta-catenin* was found to be localized in the oocytes of the ovary, and its expression was significantly high in the ovaries in *C. farreri* (Li et al., 2014). Here, *Beta-catenin* was expressed in both male and female gonads, however, its expression level was significantly higher in the ovaries than in the testis, which suggested *Beta-catenin* might play important roles in ovarian differentiation and maintenance in *S. constricta*.

Identification of Genes Involved in Gametogenesis

In our study, some genes putatively involved in oogenesis and spermatogenesis were also identified. For example, *Vg*, *Vg-6*, and *SOHLH2* were female-biased, and *CYP17A1*, *TSSK*, *TSSK1*, and *TSSK4* were male-biased in *S. constricta*. *Vg* or *Vg-6* as the storage protein and non-polar molecular carrier has been speculated to be involved in combining and transferring proteins, lipids, carotenoids and vitamin to oocytes during oogenesis (Wallace, 1985). In *C. farreri* and *C. gigas*, *Vg* was found to be specifically expressed in ovaries (Matsumoto et al., 2003; Qin et al., 2012). In *S. constricta*, the expression levels of *Vg* and *Vg-6* were significantly higher in ovaries than in testis, suggesting their important roles in oocyte development. *SOHLH2* is a universal transcription regulator of both male and female germline differentiation, with distinct and sex-specific downstream pathways that induce genes important to spermatogonia differentiation and coordinate

oocyte differentiation without affecting meiosis I (Suzuki et al., 2011; Shin et al., 2017). In *S. constricta*, *SOHLH2* was expressed in both male and female gonads, with significantly higher expression in female gonads.

CYP17A1, a cytochrome P450 monooxygenase, is involved in the biosynthesis of steroid hormones, such as corticoid and androgen, which occurred in adrenal glands and gonads. Therefore, it indicates that *CYP17A1* is involved in gonad development (Yoshimoto et al., 2016). *TSSK*, *TSSK1*, and *TSSK4* exhibited high expression levels in male gonads of *S. constricta*. *TSSK* family members play important roles in cytoplasm reconstruction at the late stage of spermatogenesis. Knockout of *TSSK1* would result in spermatogonial or spermatocytic apoptosis deficiencies in mouse (Shang et al., 2010; Jha et al., 2013). Therefore, *TSSK*, *TSSK1*, and *TSSK4* were characterized as male-specific genes in adult mature gonads, indicating their important roles in spermatogenesis.

CONCLUSION

In conclusion, we identified 45 sex-related genes that might be potentially involved in sex determination/differentiation or gametogenesis in *S. constricta*. These genes included *DmrtA2*, *Sox9*, *Fem-1b*, and *Fem-1c* for sex determination/differentiation and *Vg*, *CYP17A1*, *SOHLH2*, and *TSSK* for gametogenesis. Identification of sex-related genes not only contributes to our understanding of reproduction in *S. constricta*, but also may be applicable to developing methods to control reproduction to support sustainable development of the clam farming.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The Illumina transcriptome raw data are available in the Sequence Read Archive (SRA) database at NCBI under accession number SRR9937008-SRR9937010 for female gonad and SRR9937011-SRR9937013 for male gonad of *S. constricta*. The names of the repository/repositories and accession number(s) can be found below: NCBI Genbank, accession numbers: MZ440688, MZ440689, MZ440690, and MZ440691.

AUTHOR CONTRIBUTIONS

YD and ZL conceived the project. XK collected the samples. HY, YD, and LH conducted the gonad transcriptome assembly, annotation, and transcriptome analysis. HY wrote the manuscript. LX revised the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.725430/full#supplementary-material>

Supplementary Table 1 | Forty-five sex-related genes identified in *S. constricta*.

Supplementary Table 2 | The information sequences used in this study.

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