



A Novel Imprinted Gene (*Sp-Pol*) With Sex-Specific SNP Locus and Sex-Biased Expression Pattern Provides Insights Into the Gonad Development of Mud Crab (*Scylla paramamosain*)

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Identification and exploring the role of novel sex-related genes is a crucial step for understanding the regulation mechanism of sexual development. In the present study, we identified a novel sex-related gene (designated as *Sp-Pol*) upstream of a sex-specific single nucleotide polymorphism (SNP). SNP1888 together with *Sp-Pol* were mapped on LG32 (which is a sex-related linkage group) of a high-density genetic map. The full-length cDNA of *Sp-Pol* consists of 1703 bp with an open reading frame (ORF) encoding 204 amino acids, a 344 bp 5'-UTR and a 744 bp 3'-UTR. Phylogenetic analysis showed that *Sp-Pol* may need to be classified as a new gene family due to the very low sequence identity with other known genes (less than 25% identity). The sex-biased expression pattern of *Sp-Pol* starts from crablet stage V (C5) with approximately three- to four-fold higher in males than in females. *Sp-Pol* was expressed at a higher level in gonads compared to other tissues, with the highest expression level in the testis. In testis, a downward trend was observed in the expression level of *Sp-Pol* from the testis stage I (T1) to testis stage III (T3). After unilateral eyestalk ablation, the expression level of *Sp-Pol* significantly increased in testis and hepatopancreas in males, while it was downregulated in the hepatopancreas of females. Fluorescence *in situ* hybridization (FISH) assay revealed that *Sp-Pol* transcripts were strongly localized in the epithelia of seminiferous tubules of the testis, and in the ovary, it was detected in the oogonium cells. These findings showed that *Sp-Pol* may play crucial roles in the gonad development of *S. paramamosain*.

Keywords: eyestalk ablation, gonad development, sex-related gene, SNP, mud crab

INTRODUCTION

Understanding the sexual differentiation mechanism is a key point for sexual manipulation and improving the reproductive efficiency of crustaceans (Niksirat et al., 2014, 2016; Cui et al., 2021a). Female and male crustaceans display different growth rate (Shi X. et al., 2019). For instance, male crayfish and prawns grow larger and faster than females, and all-male individual rearing is desired. In contrast, female crab and shrimp have a better growth performance than male individuals (Ventura et al., 2012). Female crustaceans are considered more valuable in terms of egg production because they can mate with more males with no negative impact on the percentage of berried females (Niksirat et al., 2015a; Harlioğlu and Farhadi, 2017). In addition, males and females have different nutritional values and flesh quality (Niksirat et al., 2015b; Wu et al., 2019).

Crustacean female sex hormone (*CFSH*) and insulin-like androgenic gland hormone (*IAG*) are the two major hormones that regulate the sexual differentiation of female and male crustaceans (Ventura and Sagi, 2012; Zmora and Chung, 2014). Androgenic gland (AG) ablation and *IAG* silencing resulted in full sex reversal from male to female (feminization) in *Macrobrachium rosenbergii* (Rosen et al., 2010; Ventura and Sagi, 2012). However, the mechanisms underlying the regulation of sexual differentiation and most of the key factors upstream and downstream of the *IAG* in the “eyestalk-AG-testis” endocrine axis are still poorly understood (Guo et al., 2019). So far, the only functional sex reversal has been reported in *M. rosenbergii* by AG manipulation and *IAG* silencing (Aflalo et al., 2006; Ventura and Sagi, 2012). The *IAG* in *M. rosenbergii* is exclusively expressed only in AG rather than in the other tissues (Ventura et al., 2012; Ma et al., 2013). While in other crustaceans including shrimps, crayfish, and crabs (Mareddy et al., 2011; Li et al., 2012; Zhang et al., 2014; Shi L. et al., 2019; Jiang et al., 2020c) the *IAG* is specifically expressed in the AG, it also expressed in other tissues. Therefore, *IAG* may be not the only key regulator of sexual differentiation (Farhadi et al., 2021a).

Recently, several sex-related genes have been identified in crustaceans (Ma et al., 2012; Jin et al., 2013), suggesting that sexual differentiation in crustaceans is more complicated than previously expected. These sex-related genes include different types, namely *Sxl*, *Fru*, *Fem-1*, *Tra*, *Tra-2*, *Dsx*, *Mab-3*, *Sry*, *Sox9*, *Foxl2*, *Wnt4*, and *Dmrt1* (Chandler et al., 2018; Yang et al., 2018; Zhang et al., 2018; Farhadi et al., 2021b). For example, it has been documented that *CFSH*, *Sox*, *Dsx*, *Slx*, *Dmrt*, *GEM*, and *Fem-1* regulate the expression of *IAG*. *IAG* expression is negatively regulated by *GEM*, *Fem-1*, and *CFSH* (Jin et al., 2019; Shi L. et al., 2019; Zheng et al., 2019; Jiang et al., 2020b). In contrast, *Slx*, *Dsx*, and *Dmrt* silencing significantly decrease the expression of *IAG* indicating the existence of positive regulation (Abayed et al., 2019; Tan et al., 2020). Although most of these sex-related genes have been detected in both sexes, different expression patterns have been observed between the two sexes. *IAG*, *Sox*, *Dmrt*, *Dsx*, *Vasa*, and *Slx* exhibit male-biased expression patterns and play crucial roles in testis development and spermatogenesis. *CFSH*, *Fem-1*, *Wnt4*, *FAMeT*, *Slo*, *Tra-2*, *BMPs*, *UCHLs*, *Erk2*, *Cdc2*, *EGFR*, *Vg*, *VgR*, and *VIH* have higher expression levels in

females and play important roles during ovarian development (Xu et al., 2018; Abayed et al., 2019; Jin et al., 2019; Shi L. et al., 2019; Zheng et al., 2019; Jiang et al., 2020a; Zhou et al., 2020; Farhadi et al., 2021b). Therefore, identification of sex-related genes and characterization of their functions are crucial steps for understanding the sex determination and sexual differentiation mechanism in crustaceans.

Mud crab (*Scylla paramamosain*) is an important economic crustacean species and mainly distributed in the south-eastern coastal area of China (Wang et al., 2005; Wu et al., 2019). There is a great interest in the culture of *S. paramamosain* due to their high price, large size, and fast growth in captivity (Shi X. et al., 2019; Cui et al., 2021b). The annual yield of *S. paramamosain* reached 236,769 tons in 2019, including 160,616 tons for aquaculture production and 79,153 tons for capture (Bureau of Fisheries Management, 2020).

In the present study, we verified a female-specific locus named SNP1888 and a novel sex-related gene (*Sp-Pol*). Then we mapped both SNP1888 and the gene on LG32 of a previously constructed high-density genetic map. The expression pattern of *Sp-Pol* in different tissues and different sexual development stages was analyzed. Unilateral eyestalk ablation was carried out to find out the regulation mechanism of *Sp-Pol*. Fluorescence *in situ* hybridization (FISH) localized the *Sp-Pol* mRNA in the testis and ovary. The present study provides novel insights into the gonad development of *S. paramamosain*.

MATERIALS AND METHODS

Animals and Sampling

Samples including embryos, zoeae, megalopa, and crablets were obtained from a crab farm (named Raoping West Coast Biotechnology Co., Ltd.) and aquatic product market in Chaozhou City, Guangdong Province of China. After being transported to the laboratory, they were kept for adaption at room temperature. Then tissues including testis, ovary, heart, hepatopancreas, muscle, gill, thoracic ganglion, intestine, and stomach were quickly dissected and stored in RNA keeper solution (Vazyme Biotech Co., Ltd., Nanjing, China) at -80°C for RNA and DNA extraction. The total RNA was extracted using Trizol Reagent (Invitrogen, CA, United States) following the manufacturer's protocol. First-strand cDNA was synthesized using ReverTra Ace[®] qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Genomic DNA was extracted using TIANamp Marine Animals DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) and treated with RNase A to remove residual RNA.

Verification of Sex-Specific SNP1888 and Discovery of *Sp-Pol* Gene

A potential female-specific SNP locus named SNP1888 was identified from Cluster_39896 by RAD-seq in our previous study (Shi et al., 2018). In order to verify SNP1888 is a real sex-specific locus, we first designed a pair of primers for PCR amplification, and then the PCR products of a total of 195 crabs (106 females and 89 males) were sequenced by bidirectional

sequencing. The genotypes of SNP1888 were defined by the peak types according to the sequencing chromatograms: double peaks indicate heterozygous and single peak indicates homozygous. Based on gene clone technology and bioinformatics analysis, we identified a novel gene (designated as *Sp-Pol*) at the upstream of SNP1888 (SNP1888 is located at the 3'-UTR of *Sp-Pol*).

Cloning and Sequence Analysis of *Sp-Pol*

The *Sp-Pol* cDNA sequence was achieved by the rapid amplification of cDNA ends (RACE) technique using SMARTer RACE cDNA Amplification Kit (Clontech, United States). Nested PCR amplifications were carried out using a universal primer mix (UPM outer primer) and a nested universal primer (NUP inner primer), in conjunction with two gene-specific primers (GSPs) designed based on the cDNA sequence. PCR was performed on an Eppendorf Mastercycler[®] ep realplex (Eppendorf, Germany) with specific primers (Table 1) using the following conditions: denaturation at 94°C for 5 min; 35 cycles of amplification at 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and final elongation at 72°C for 5 min. The PCR product was ligated into Simple T1 vector (Transgen, Beijing). The recombination was then transformed into *Escherichia coli* strain K12, and positive clones were sequenced. Then, positive clones were sequenced using an automatic DNA sequencer in Sangon Biotech Co., Ltd. Later, the gene sequence was blasted with the NCBI database to find its homologs.

Localization of SNP1888 and *Sp-Pol*

The sex-specific SNP1888 and *Sp-Pol* were localized in the high-density genetic linkage map of *S. paramamosain*. The obtained genotypes of SNP1888 in the mapping family with a total of 129 individuals by PCR amplification and Sanger sequencing (Shi et al., 2018) were integrated with all SNP genotypes obtained in our previous study (Waiho et al., 2019) to reconstruct the genetic map by Joinmap 5.0 software.

Bioinformatic Analysis

The sequences of *Sp-Pol* were analyzed based on the sequences in GenBank using BLASTN and BLASTX (available at¹). The ORF was identified using Editseq program (Lasergene, United States). Compute pi/Mw tool² was used to predict the molecular weight and isoelectric point of the *Sp-Pol* protein. NetPhos 3.1 Server³, NetOGlyc 4.0 Server⁴, and NetNGlyc 1.0 Server⁴ were used to predict the potential phosphorylation, O-glycosylation, and N-glycosylation sites, respectively. The three-dimensional (3D) protein structure and secondary structure of a protein were predicted using SWISS-MODE⁵ and self-optimized prediction method with alignment (SOPMA), respectively. The sequences were aligned using Bioedit software version 7.2⁶. The phylogenetic diagram was generated by the neighbor-joining method (bootstrap analysis of 1,000 replicates) using Mega X software (Kumar et al., 2018).

Quantitative Real-Time PCR (qRT-PCR) and Expression Models Analysis

The qRT-PCR was used to investigate the *Sp-Pol* mRNA expression levels at different developmental stages and different tissues. The testis and ovary developmental stages were identified based on the morphology of gonads (Waiho et al., 2017; Wu et al., 2020). A LightCycler[®] 480 Instrument II (Roche, Switzerland) was used for the qRT-PCR analysis. The qRT-PCR using SYBR Green method (TIANGEN Biotech, Beijing, China) was performed under the following conditions: denaturation at 95°C for 3 min; 40 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 15 s. Specific primers for *Sp-Pol* and housekeeping genes (EF1 α and 18S rRNA) are listed in Table 1.

¹www.ncbi.nlm.nih.gov/blast

²https://web.expasy.org/compute_pi/

³http://www.cbs.dtu.dk/services/NetPhos/

⁴http://www.cbs.dtu.dk/services/NetOGlyc/

⁵https://www.swissmodel.expasy.org/

⁶http://bioedit.software.informer.com/7.2

TABLE 1 | Primers used for fragment verification, 3'/5' – RACE, qRT-PCR, FISH, and RNAi of *Sp-Pol*.

Primer name	Sequence (5' – 3')	Description	Tm (°C)	Amplification Efficiency (%)
Sp-Pol-F1	CTACAGCACCCGCACCATCCCT	cDNA fragment verification	63.2	–
Sp-Pol-R1	TCCATGTCGGCGCAAACCTTGCT	cDNA fragment verification	62.5	–
Sp-Pol-F2	GTCATTACCCGTTTTATCAACTCAC	DNA fragment verification	54.9	–
Sp-Pol-R2	GCCTTATGTCCTTATTCAATCCGT	DNA fragment verification	55.4	–
3' RACE-outer	ACAGCACCCGCACCATCCCTATCC	For 3' - RACE	64.8	–
3' RACE-inner	CCCACCGCCAACACACGGAAATCT	For 3' - RACE	64.1	–
5' RACE-outer	TGATGAGTGGACAGGCTGCTGATGG	For 5' - RACE	62.8	–
5' RACE-inner	AACAAGCAATCCATAATGACTCTG	For 5' - RACE	54.1	–
RT- <i>Sp-Pol</i> -F	ATGTAAGTGAAGCCATCCCTGAGAC	For qRT-PCR	60.7	93.6
RT- <i>Sp-Pol</i> -R	CTCCCAGAGGTGTTAGACGAAGATTTAG	For qRT-PCR	58.3	–
18S-F	GGGGTTTGCAATTGCTCTCCC	For qRT-PCR	57.1	98.8
18S-R	GGTGTGTACAAAGGGCAGGG	For qRT-PCR	59.2	–
EF-1 α -F	CTACAAGTGTGGTGGCATCG	For qRT-PCR	56.4	94.8
EF-1 α -R	CGTCGATGATGGTACCGTAG	For qRT-PCR	55.5	–
Sp-Pol- FISH	GCTAGAGCTTGCATCTCATTCATGCTGCA	For FISH	–	–

Gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). In addition, the expression models analysis in mRNA of ovaries and testis were determined by Sanger sequencing through the peaks and their colors in the chromatogram.

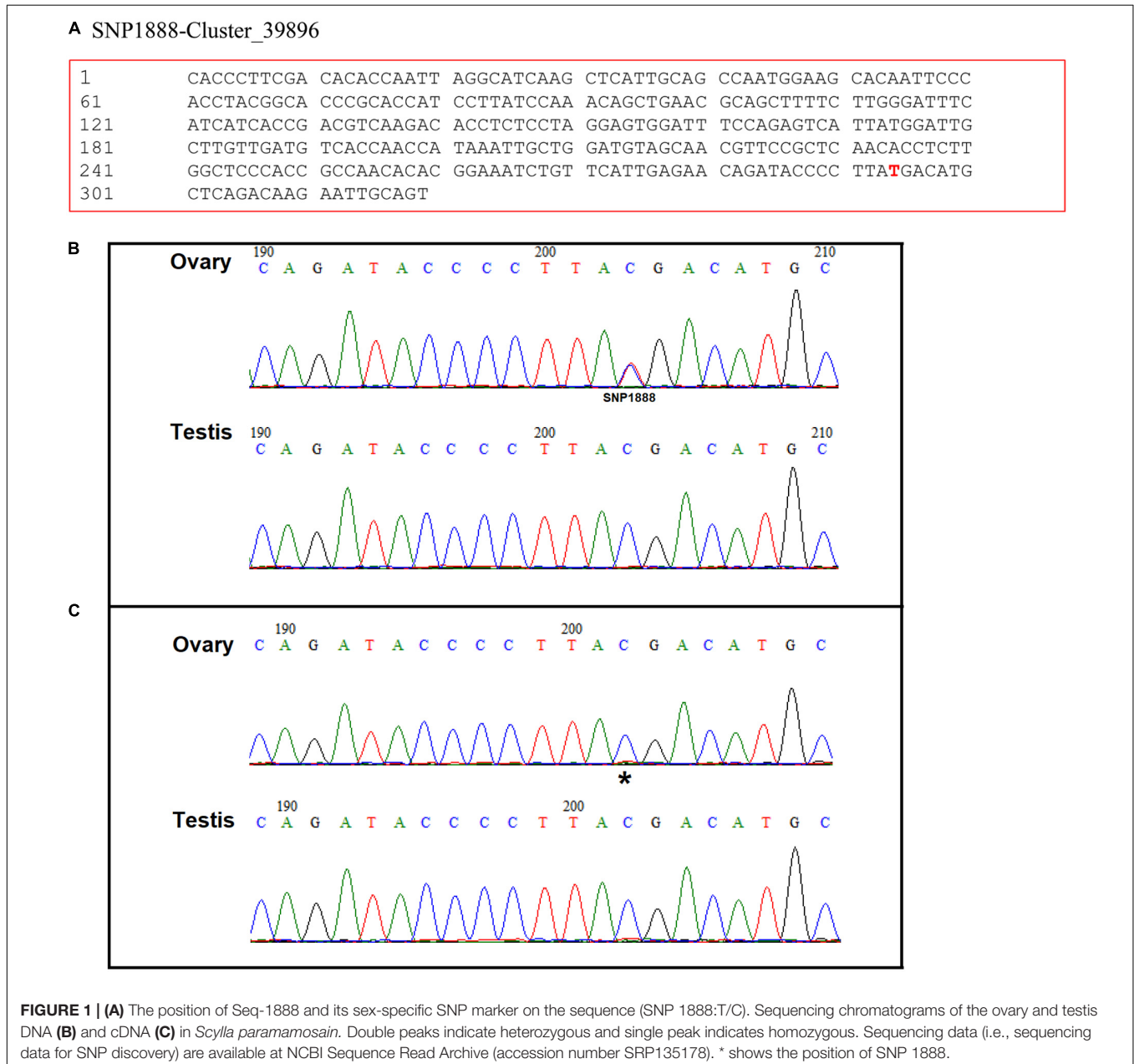
Unilateral Eyestalk Ablation

Males (221.2 ± 23.3 g; $N = 20$) and females (211.4 ± 19.8 g; $N = 30$) *S. paramamosain* were randomly divided into two groups. Before the ablation of the eyestalk, animals were adapted for 7 days. Hot tweezers were used to remove the eyestalks. No eyestalk was removed in the crabs from the control group.

Seven days later, gill, muscle, hepatopancreas, and gonads were dissected to detect the mRNA level of *Sp-Pol*.

Fluorescence *in situ* Hybridization (FISH)

After dissection, gonads samples (mean weight of male crabs: 107.4 ± 23.1 g; mean weight of female crabs: 124.8 ± 21.9 g) were used for the preparation of frozen sections. The OCT-embedding tissue was cut into $3 \mu\text{m}$ sections by a frozen section machine (Thermo). The fluorescein-labeled DNA probe (5'-FAM-GCT AGAGCTTGCATCTCATTCCATGCTGCA-3') was synthesized based on the cDNA sequence of *Sp-Pol* by the Sangon Biotech Company (Shanghai, China) (Table 1). Before hybridization, slides were digested using proteinase K for 5 min. Then,



sections were immersed in 75, 85, and 100% ethanol for 1 min, respectively. Hybridization was carried out at 65°C overnight with FAM-labeled *Sp-Pol* probe (10 µl for each slide). The sections were subjected to nuclear staining by DAPI and observed using Axio Observer 5 fluorescence microscopy (ZEISS, Germany).

Statistical Analysis

The experimental data were analyzed using SPSS version 21.0 (Chicago, IL, United States). The normality and homoscedasticity of the data were investigated using Kolmogorov-Smirnov and Levene's test. The proportional data were normalized using square root arcsin transformation. To compare the expression level of *Sp-Pol* in different tissues and different developmental stages a one-way ANOVA was utilized. Significant differences between groups were determined using the Tukey's HSD *post hoc* test in each case. The mRNA level of *Sp-Pol* between the eyestalk ablated and the control group was analyzed using an independent samples *t*-test. Significances were accepted at $P < 0.05$.

RESULTS

Verification of Sex-Specific SNP1888

The position of SNP1888 in Cluster_39896 is shown in **Figure 1A**. With a pair of primers (F: CTACGGCA CCCGCACCATCCTT, R: AGCAAGTTTGGCCGACATGGA), a specific product was obtained by PCR. According to the

sequencing chromatograms, SNP1888 showed heterozygous (T/C) in all 106 females and homozygous (C/C) in all 89 males (**Figures 1B,C**).

Characterization of *Sp-Pol*

The full-length cDNA of *Sp-Pol* was 1,703 bp and contains a 5'-UTR of 344 bp, a 3'-UTR of 744 bp, and an ORF of 615 bp encoding a total of 204 amino acid residues (aa) (**Figure 2A**). The deduced protein has a molecular mass of 22.74 kDa, with an estimated isoelectric point (PI) being 8.50. Analysis by SignalP 5.0 software revealed that the deduced peptide does not contain a putative signal peptide. Twenty-eight potential phosphorylation sites were identified at 14 serine residues (aa 6, 14, 25, 26, 29, 30, 31, 33, 40, 79, 100, 101, 131, and 173) and 14 threonine residues (aa 19, 20, 22, 27, 28, 54, 59, 73, 80, 81, 127, 136, 164, and 195). A potential N-glycosylation site (162NQTCT165) and six potential O-glycosylation sites (Ser6, Ser14, Thr19, Ser26, Thr27, Thr148) were also identified. The SOPMA program showed that the *Sp-Pol* protein contained 33.82% α helix, 3.43% β turn, 55.39% random coil, and 7.35% extended chain (**Figure 2B**).

Mapping of Sex-Specific SNP1888 and *Sp-Pol*

The mapping results showed that the sex-specific SNP1888 with the *Sp-Pol* gene were mapped on LG32 of the sex-averaged high-density genetic linkage map (**Figure 3**). Hence, LG32 is suggested as a sex-related linkage group due to it already has 13 sex-specific

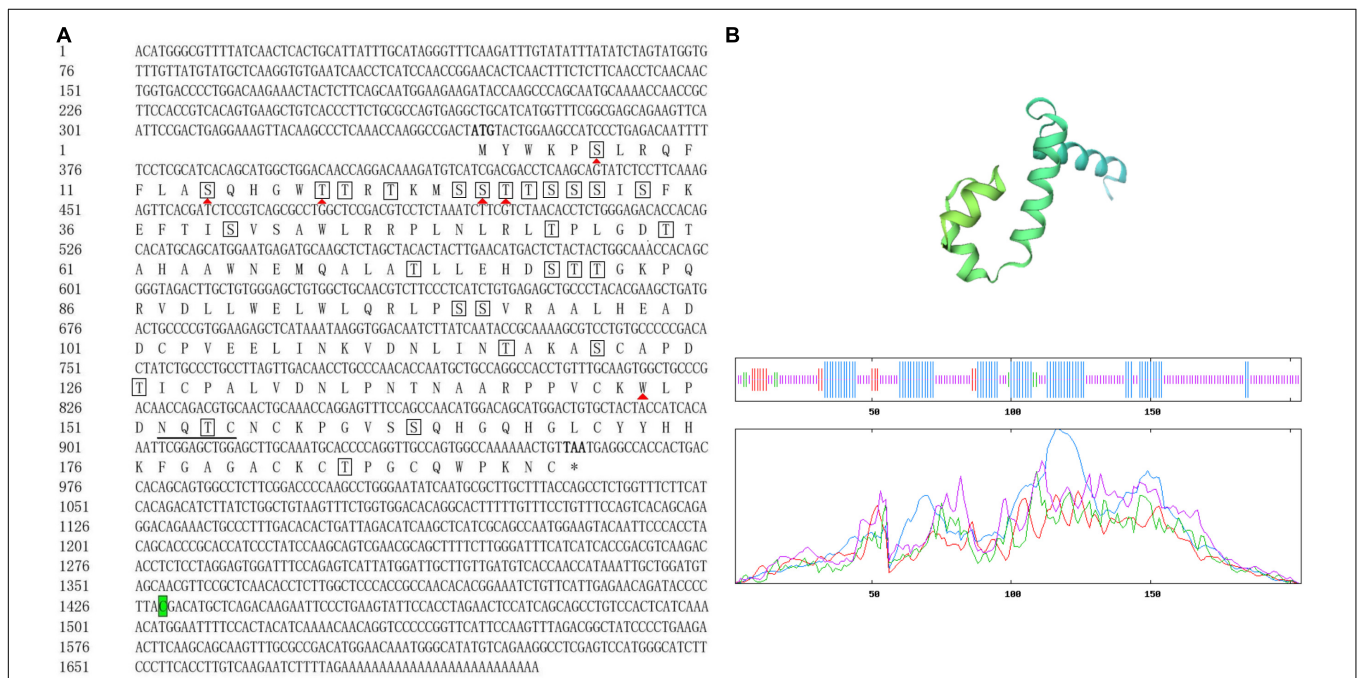


FIGURE 2 | Sequences of full-length complementary DNA and deduced amino acids (aa) of *Sp-Pol*. The number of nucleotide and amino acid are listed on the left side. Start (ATG) and stop codon (TAA) of the open reading frame are both indicated in bold. Termination of aa is denoted by asterisks. Predicted O-glycosylation sites are indicated by red solid triangles (▲). The potential N-glycosylation site (162NQTCT165) is underlined. Potential phosphorylation sites are in boxes. Letter with green background indicates the location of sex-specific SNP (SNP1888: C/T) (A). The three-dimensional (3D) protein structure of *Sp-Pol* and analysis of the secondary structure. The α -helix (blue), extended strand (red), random coil (purple), and β -turn (green) (B).

SNPs (SNP1888, SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP8, SNP9, SNP10, SNP11, SNP12) and exclusive sex QTLs of *S. paramamosain* (Waiho et al., 2019).

Multiple Alignments and Phylogenetic Analysis of Sp-Pol Protein

Bioinformatics analysis of the deduced amino acid sequence of Sp-Pol protein indicated its structural similarity to the Gag-Pol polyprotein family of peptides. Sp-Pol protein shared the highest identity with the Retrovirus-related Pol polyprotein from transposon opus (*Penaeus vannamei*) (ROT64510.1, 60.47% identity, bit score: 209, expectation value: $3.73e^{-61}$) (Figure 4). A phylogenetic tree was constructed by the neighbor-joining method using multiple alignments of 19 homologous proteins from Decapoda, Insecta, Echinodermata, and fish species (Figure 5). In this tree, the homologous proteins are divided into four separate clades: Decapoda, Insecta, Echinodermata, and fishes.

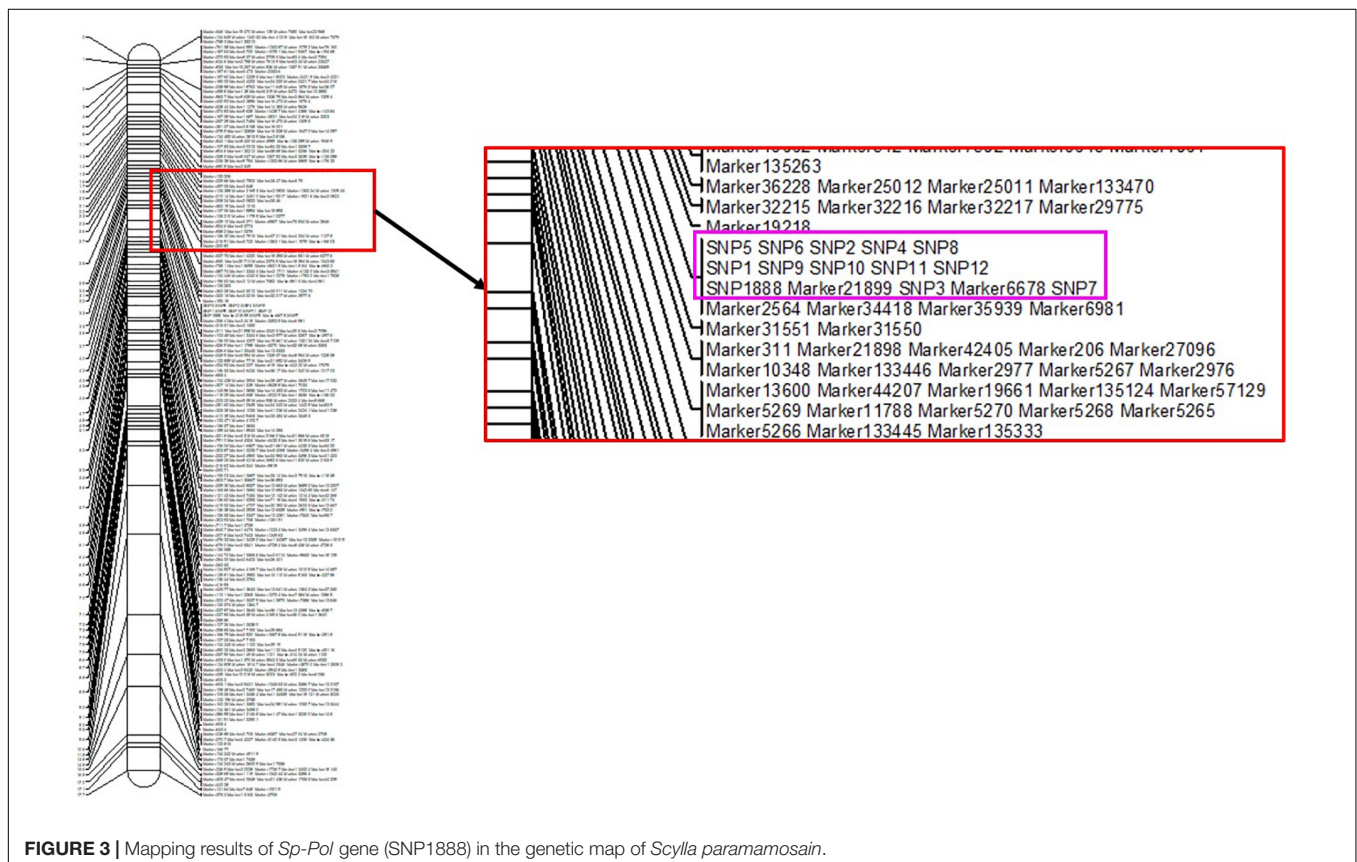
Expression of Sp-Pol in Different Tissues and Different Developmental Stages of Gonads

Sp-Pol was expressed at a higher level in gonads compared to other tissues ($P < 0.05$). The highest expression of *Sp-Pol* was detected in testis with approximately four-fold higher than in the ovary ($P < 0.05$) (Figure 6A). Meanwhile, we found that *Sp-Pol*

had mono-allelic expression in the ovary, whereas in the testis both alleles were expressed. In the ovary, the mono-allele which owing nucleotide acid C at the SNP1888 locus was expressed, and the other allele which owing T was not expressed (Figures 1B,C). The expression level of *Sp-Pol* in different development stages of the ovary and testis is presented in Figure 6B. The highest expression level of *Sp-Pol* was observed at the first stage of testis development (T-1), and then the expression level decreased significantly from T-1 to T-2, and T-3 ($P < 0.05$). In the ovary, the expression of *Sp-Pol* was low during all five stages of ovary development (O-1 to O-5). In addition, the expression profile of *Sp-Pol* during embryo (E), zoeae (Z-1 to Z-5), megalopa (M), and crablets (C-1 to C-6) was assessed (Figures 6C,D). An upward trend was observed for males at the crablet stage V (C-5) and the crablet stage VI (C-6) ($P < 0.05$) (Figure 6C). Among zoea stages, *Sp-Pol* had the highest expression at the zoea I stage (Figure 6D).

The Effects of Unilateral Eyestalk Ablation on the mRNA Level of Sp-Pol

The eyestalk ablation significantly increased the expression level of *Sp-Pol* in the testis (4.7-fold) and hepatopancreas (2.1-fold) of males ($P < 0.05$) (Figure 7A). While in females, a downward trend was recorded in the *Sp-Pol* expression in hepatopancreas (2.2-fold) ($P < 0.05$) (Figure 7B). However, unilateral eyestalk ablation slightly increased the expression level of *Sp-Pol* in the ovary (1.5-fold), however, it was not statically significant



($P = 0.23$). Based on these results, *Sp-Pol* might be negatively regulated by eyestalk in testis and male hepatopancreas. Down-regulation of *Sp-Pol* in the female hepatopancreas showed the possible existence of positive regulation.

The Localization of *Sp-Pol*

The FISH assay showed that *Sp-Pol* transcripts were detected in both ovary and testis (Figure 8). In the ovary, *Sp-Pol* was

mainly expressed in oogonia cells (Figure 8A). In testis, *Sp-Pol* transcripts were strongly localized in epithelial cells of seminiferous tubules (Figure 8B).

DISCUSSION

Limited information is available on the sex-determination system of brachyuran crabs. For decades, scientists believed that the XY/XX sex-determination system is the only sex-determination system for crabs (Legrand et al., 1987). Recently, besides the XY/XX (Fang et al., 2020), the WZ/ZZ sex-determination system has been found in some crab species such as *Eriocheir sinensis* (Cui et al., 2015), *S. paramamosain*, *S. tranquebarica*, and *S. serrata* (Shi et al., 2018). Sex-specific SNP marker is considered a powerful tool for understanding the sex-determination system (Pan et al., 2015). Detection of female-specific SNP markers provides insights into a WZ/ZZ sex determination system in *S. paramamosain* (Shi et al., 2018). In the present study, SNP1888 was mapped on the LG32 of the genetic map, which is a sex-related linkage group, and was located very close to the positions of other sex-specific SNPs (SNP1, SNP2, SNP3, SNP4, SNP5, SNP6, SNP7, SNP8, SNP9, SNP10, SNP11, SNP12) (Shi et al., 2018; Waiho et al., 2019). The presence of female-specific SNP markers and their locations on the female linkage map supports the existence of a WZ/ZZ sex determination system in *S. paramamosain*.

Phosphorylation plays important roles in metabolism, proliferation, apoptosis, differentiation, and cell division. Protein phosphorylation is catalyzed by a group of enzymes called kinases, which add a phosphate group to serine, threonine, tyrosine, or, to a lesser degree, histidine residues (Song et al., 2017). Twenty-eight putative phosphorylation sites (14 sites on serine and 14 sites on threonine residues) in *Sp-Pol* protein showed that *Sp-Pol* protein may be activated by some kinases enzymes. The prediction of six potential O-glycosylation and one N-glycosylation site indicates that glycosylation may also affect the function of *Sp-Pol* protein. Glycosylation, like phosphorylation, is important due to the various roles in protein folding, protein trafficking and localization, cell-cell interactions, and epitope recognition (Caragea et al., 2007). Glycosylation can be classified into four types: N-linked, O-linked, Glypiation, and C-linked. In N-linked glycosylation, the oligosaccharide chain is attached to the amide nitrogen of asparagine. In O-linked glycosylation, the glycan is attached to the hydroxyl oxygen of serine or threonine (Julenius et al., 2005).

Sp-Pol protein had the highest similarity to a Retrovirus-related Pol polyprotein gene from *P. vannamei* (60.47% identity) and was also slightly homologous (20–35% identity, bit score: 50–55, expectation value: 10^{-3} – 10^{-6}) to a group of uncharacterized and hypothetical proteins from crustaceans, insects, echinoderms and fish species. Although *Sp-Pol* protein was closely related to a Retrovirus-related Pol polyprotein from *P. vannamei* and a hypothetical protein from swimming crab (*Portunustri tuberculatus*), it was distinct from other identified proteins. The very low similarity of *Sp-Pol* protein to other known homologous proteins and failure to predict functional domains indicates that

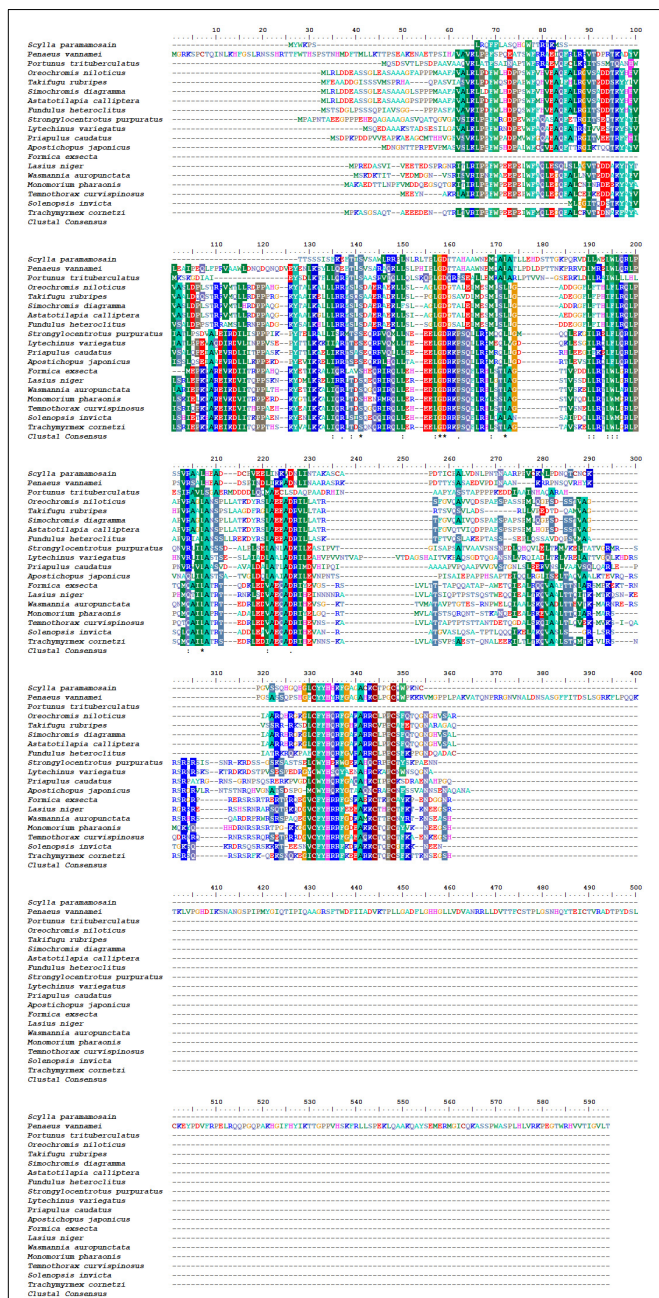
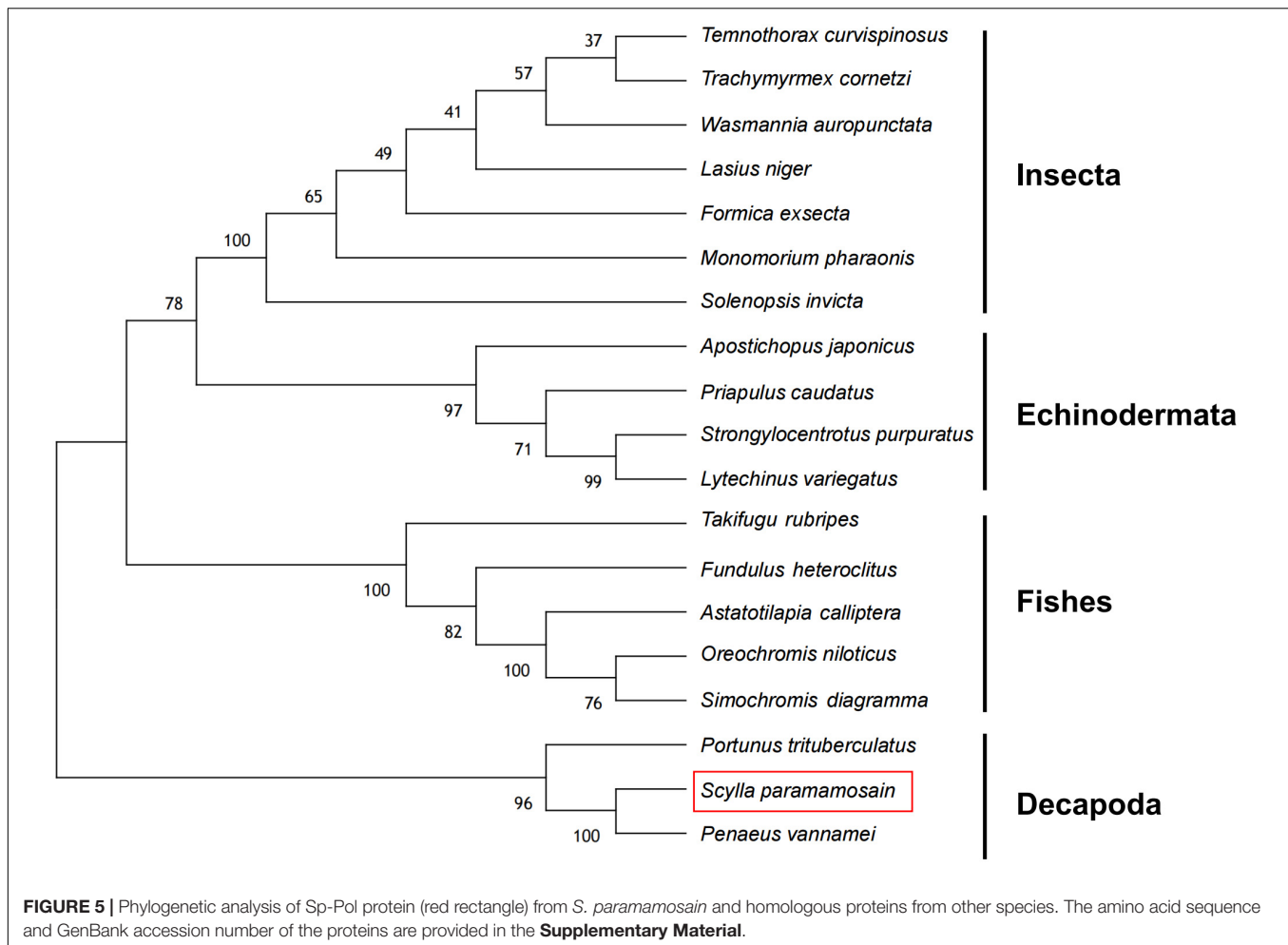


FIGURE 4 | Multiple alignments of deduced amino acids of *Sp-Pol* protein and its homologs. GenBank accession numbers are as follows: *Penaeus vannamei* (ROT64510.1); *Portunustri tuberculatus* (MPC10902.1).



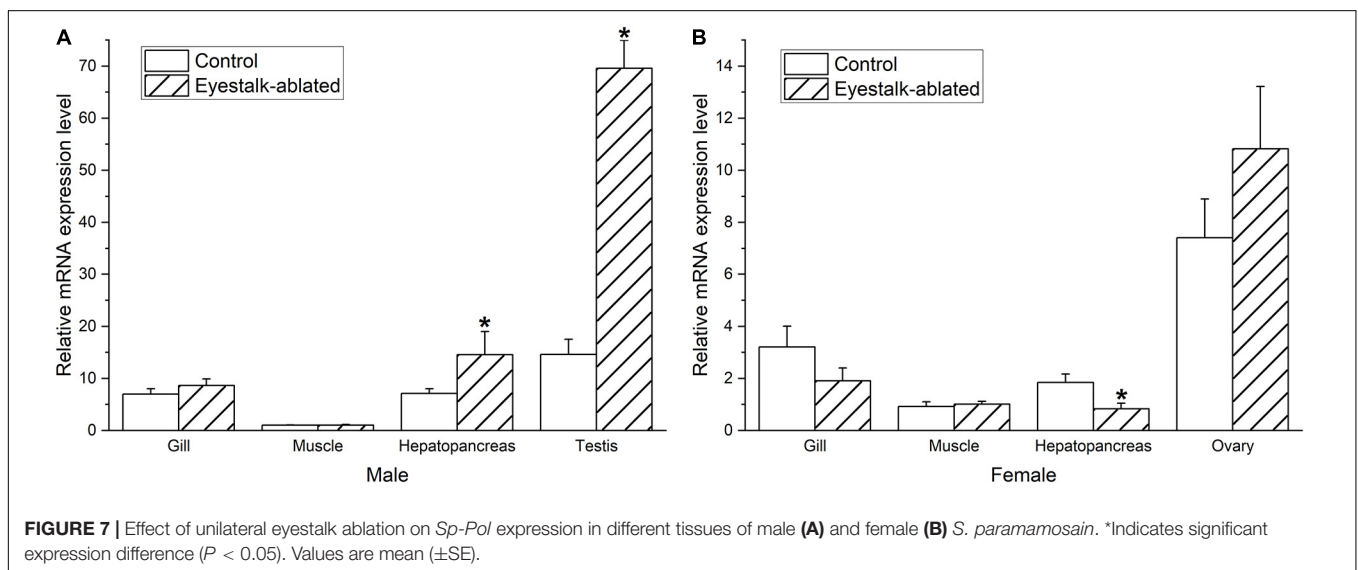
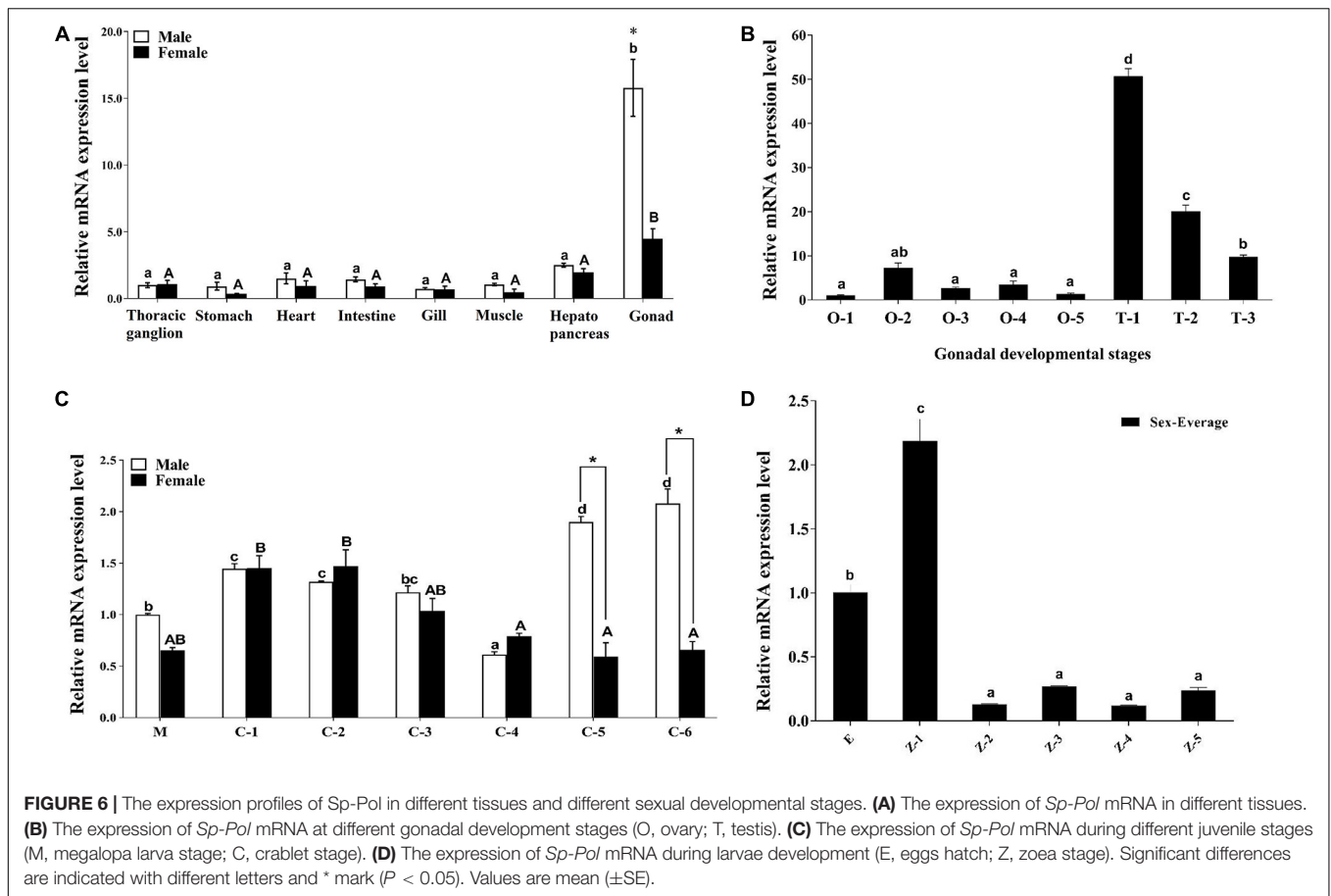
this gene may not belong to the Gag-Pol polyprotein family and can be reclassified as a new family. Notably, the protein encoded by the *Sp-Pol* gene (204 aa) was quite smaller than Retrovirus-related Pol polyprotein from *P. vannamei* (541 aa) and other homologous proteins.

Spatio-temporal expression analysis showed that *Sp-Pol* was expressed in all the examined tissues and during all life stages, suggesting that it has a wide range of functions in different tissues and developmental stages. Expression of *Sp-Pol* in gonads was higher than other tissues including heart, hepatopancreas, muscle, gill, thoracic ganglion, intestine, and stomach. Comparing the expression of *Sp-Pol* between females and males, male crabs displayed a higher level of *Sp-Pol* expression compared to females. The higher expression of *Sp-Pol* in testis revealed that it is likely to play more important roles in the sexual development of males compared to females. The lower expression of *Sp-Pol* in the ovary compared to testis may be due to the mono-allelically expression pattern of this gene in the ovary. In the ovary, the mono-allele which owing nucleotide acid C at the SNP1888 locus was expressed, and the other allele which owing T was not expressed. The mono-allele expression could result in several different outcomes at the transcriptional

level. There is a general trend for mono-allelically expressing cells to have fewer transcript levels than biallelically expressing cells (Eckersley Maslin and Spector, 2014).

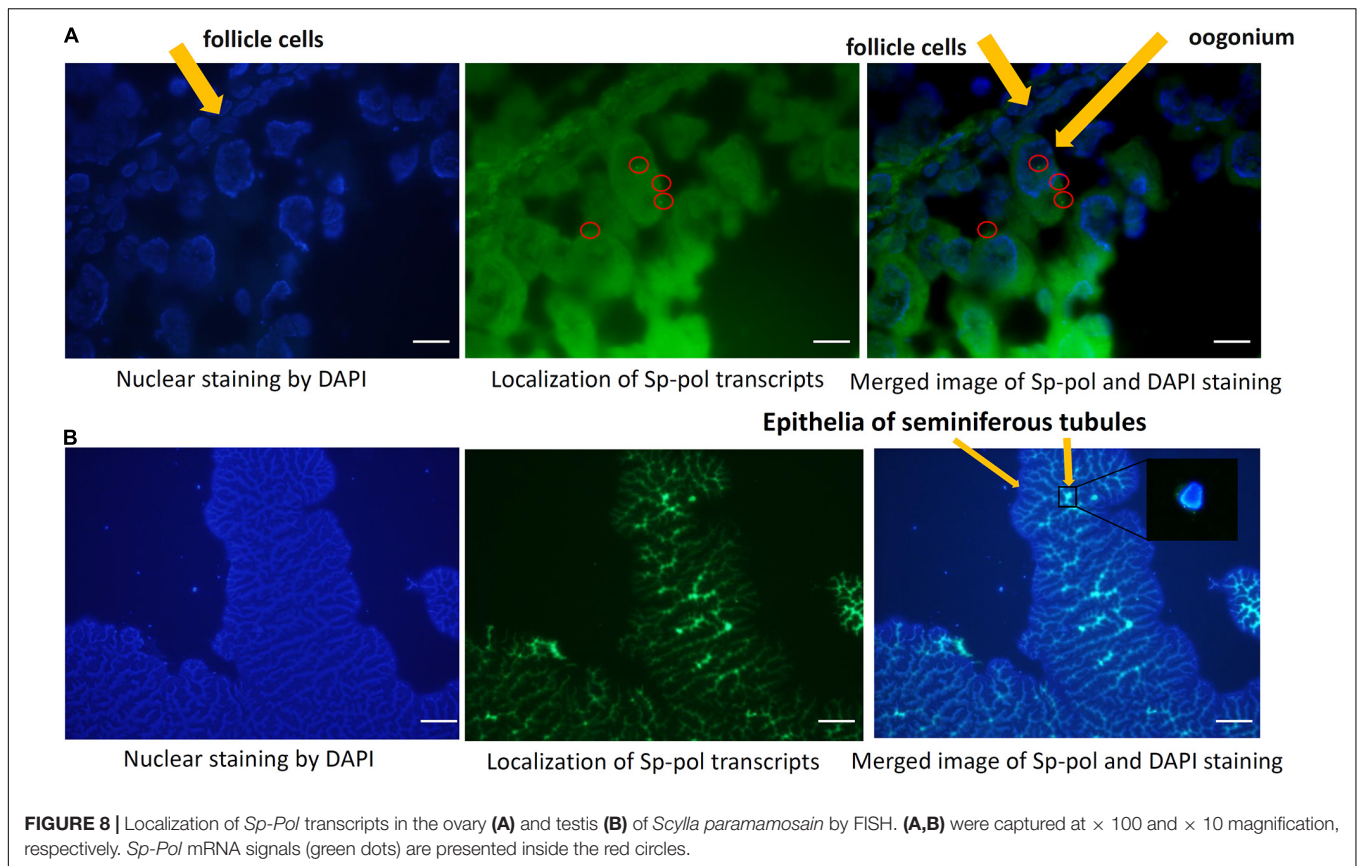
It has been revealed that several sex-related genes such as *CFSH*, *Sox*, *Dsx*, *Slx*, *Dmrt*, *GEM*, and *Fem-1* are involved in the “eyestalk-AG-testis” endocrine axis (Guo et al., 2019). These sex-related genes are mainly regulated by the neurohormones from eyestalk and play important roles in the IAG regulation (Shen et al., 2014; Ma et al., 2016; Shi L. et al., 2019; Zheng et al., 2019; Zhong et al., 2019).

Therefore, we hypothesized that *Sp-Pol* may be regulated by the eyestalk. Thus, we investigated the expression level of *Sp-Pol* after unilateral eyestalk ablation. Eyestalk ablation significantly up-regulated the expression level of *Sp-Pol* in the testis and male hepatopancreas and downregulated in female hepatopancreas. A similar expression pattern has been reported for IAG in the male blue crab, *Callinectes sapidus*. The expression of IAG was found to be up-regulated after eyestalk ablation in the testis and hepatopancreas (Chung et al., 2011). The significant changes of *Sp-Pol* mRNA in the hepatopancreas suggest *Sp-Pol* may be related to nutrient metabolism in the hepatopancreas. In decapods, the hepatopancreas is the major



source for providing energy to gonad development (Sokolowicz et al., 2006). The increase of hepatopancreas weight during the reproductive season has been documented in several studies (i.e., material and energy storage for oogenesis) (Farhadi and Harhoğlu, 2019). Vitellin (Vn) produced in the hepatopancreas is

the main component of yolk proteins, which is stored in oocytes and is a nutritional source for animal embryo growth (Tseng et al., 2001). Vn is processed from its precursor, vitellogenin (Vg). In *S. paramamosain*, both the hepatopancreas and ovary are sites of Vg synthesis, but the hepatopancreas is the primary



synthesis site (Huang et al., 2017). Therefore, further studies are required to evaluate the role of *Sp-Pol* in vitellogenesis and hepatic metabolism. Eyestalk ablation revealed that *Sp-Pol* expression may be regulated by inhibitory factors present in the eyestalk ganglia.

To investigate the potential role of *Sp-Pol* in gametogenesis, we performed FISH to locate *Sp-Pol* mRNA in the ovary and testis. In the ovary, *Sp-Pol* transcripts were detected in the oogonium cells but no signal was found in the follicle cells. In the testis, strong *Sp-Pol* signals were detected in the epithelia of seminiferous tubules. Signals decreased toward the center of the tubules of the testis, which means the expression level of *Sp-Pol* may progressively decrease from spermatogonia to spermatids. Spermatogenesis starts with the mitotic division of spermatogonia cells that locate on one side of the seminiferous tubules. Then, spermatogonia cells differentiate into primary spermatocytes. Spermatocytes divide into two equal haploid spermatids by Meiosis II. Later, spermatids differentiate into spermatozoa in the central region of the tubule (Zhang and Qiu, 2010). The role of the epithelium seminiferous tubules is not well studied in crustaceans. In fish, the cellular interactions between Sertoli cells and germ cells in the seminiferous epithelium play an important role during the spermatogenesis process (Batlouni et al., 2009). The structure of seminiferous epithelium changes from a Sertoli cell monolayer (containing spermatogonia cells during the non-reproductive season) to an active spermatogenic arrangement composed of spermatocytes and spermatozoa that

fill the tubular lumen (reproductive season) (Batlouni et al., 2006). Further studies are required to investigate the roles (i.e., long-term and short-term RNA interference) and regulation mechanism of *Sp-Pol* in *S. paramamosain*. It would be interesting to study the relationship of *Sp-Pol* with other sexual genes such as *IAG*, *CFSH*, *Sox* gene family, *Dmrt* gene family, etc. Based on the observation of mono-allelic expression of *Sp-Pol* in the ovary, further studies are required to find whether *Sp-Pol* is an imprinted gene or regulate by DNA methylation mechanism.

CONCLUSION

In the present study, we first verified SNP1888 being a female-specific SNP marker and then we identified a novel sex-related gene (*Sp-Pol*) upstream of SNP1888. Furthermore, we mapped SNP1888 and *Sp-Pol* on a high-density genetic map. Despite the fact that *Sp-Pol* protein has low homology to other homologous proteins in other species, *Sp-Pol* protein may not belong to the Gag-Pol polyprotein family and can be classified as a new family. *Sp-Pol* exhibited different expression patterns in different tissues, different early development stages. *Sp-Pol* was expressed much higher in the testis than in the ovary, which might be related to the mono-allelically expression pattern of this gene in the ovary. The changes of *Sp-Pol* expression in testis and hepatopancreas after eyestalk ablation showed that *Sp-Pol* is regulated by eyestalk neurohormones. These results

demonstrated that *Sp-Pol* may play important roles in the sexual development of *S. paramamosain*.

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 in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Ethics Committee of Shantou University.

AUTHOR CONTRIBUTIONS

AF: conceptualization, data curation, formal analysis, investigation, methodology, visualization, and writing – original draft. XS: conceptualization, data curation, formal analysis, investigation, and methodology. YiZ: formal analysis

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and investigation. YuZ and HZ: methodology and editing. SL: methodology and review. MI: review and editing. HM: conceptualization, methodology, funding acquisition, project administration, resources, supervision, validation, review, and editing. All authors contributed to the article and approved the submitted version.

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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.2021.727607/full#supplementary-material>

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