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Characterization, expression, and functional analysis of testis-specific serine/threonine kinase 1 (*Tssk1*) in the pen shell *Atrina pectinata*

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ABSTRACT

Testis-specific serine/threonine kinase 1 (*Tssk1*) was named since its function was determined in mouse spermiogenesis. In our research, we cloned a homolog of the pen shell *Atrina pectinata Tssk1*, *ApTssk1*, and determined its expression characteristics at mRNA. The full length of *ApTssk1* cDNA was 1517 bp, with an open reading frame of 1089 bp (58–1146), which encodes a peptide including 362 amino acids. The homologous analysis indicated that the deduced amino acid sequences of *ApTssk1* shared a close identity with other reported *Tssk1*. In addition, the highly conserved fragment containing a serine/threonine protein kinase catalytic (S-TKc) domain was predicted to exist in *ApTssk1*. Results from the RT-qPCR display that *ApTssk1* was only transcribed in the male gonad of *A. pectinata*, and was highest in the mature testis. Therefore, this study indicated that *ApTssk1* might play a functional role during spermatogenesis in *A. pectinata*.

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Sequence analysis; S-TKc; spermatogenesis; maturation; bivalves

Introduction

Spermatogenesis is a complex process involving meiotic division of germ cells and their subsequent differentiation into highly specialized mature sperm (De Kretser et al. 1998). According to present knowledge, spermatogenesis can be divided into three phases: (i) proliferative or spermatogonial phase; (ii) meiotic phase; and (iii) spermiogenesis phase (Sharpe 1994). It is important that each round of spermatids differentiates into morphologically mature spermatozoa during spermatogenesis (Sharpe 1994). The molecular mechanisms of spermatogenesis are to a large extent unknown; however, there is increasing evidence that the testis-specific serine kinase (*Tssk*) family plays an important role in the control of spermatogenesis (Bielke et al. 1994; Shang et al. 2013).

The *Tssk* family consists of five members: *Tssk1* to *Tssk6*. Different members have been reported to be expressed at different stages of spermatogenesis (Li et al. 2011). *Tssk1* and *Tssk2* are expressed when spermatids differentiate into morphologically mature spermatozoa during spermiogenesis. This indicates the important roles of *Tssk1* and *Tssk2* in normal male reproduction (Xu et al. 2008). *Tssk3* is predominantly expressed in interstitial Leydig cells (Zuercher et al. 2000). *Tssk4*, also named *Tssk5*, is expressed exclusively in the testis (Chen et al. 2005; Li et al. 2011), and *Tssk4* has four alternative splicing variants at the transcriptional level in mouse testis (Wei et al. 2007). *Tssk6*, also called SSTK, has been reported to be expressed at the head of the elongated sperm (Spiridonov et al. 2005).

As a *Tssk* family member, mouse *Tssk1* was first cloned and characterized in 1994 using PCR with degenerate oligonucleotide primers targeting two highly conserved motifs within the protein kinase catalytic domain (Bielke et al. 1994). Prior research reports that *Tssk1* was found in mature spermatozoa, and many studies have shown that this gene plays an important role in mammalian spermatogenesis (Xu et al. 2008). *Tssk1* has been reported to be expressed in the testis of the bivalve mollusks, *Argopecten purpuratus* and *Mytilus edulis*, suggesting its role in spermatogenesis and/or sperm function in bivalves (Boutet et al. 2008; Ciocan et al. 2011). Some protein sequences of *Tssk1* are reported in bivalves, for example, *Crassostrea gigas* and *M. edulis* (Ciocan et al. 2011; Zhang et al. 2012). However, to date, no full-length cDNAs encoding *Tssk1* have been isolated from mollusks, and no information about *Tssk1* is available in the pen shell *Atrina pectinate* (Matsumoto et al. 2013).

Atrina pectinata is an important commercial shellfish in Asia and is a good experimental species to identify spermatogenesis-related genes in bivalves owing to its relatively stable sex composition (Tatsuya et al. 2005). In our study, we cloned the full-length cDNA of *Tssk1* from *A. pectinata*,

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and determined its expression pattern in various tissues during embryonic development and gametogenesis using a RT-qPCR method. Our research reveals the molecular characterization of *Tssk1* of *A. pectinata*, and identifies the expression characteristics of *Tssk1* during spermatogenesis. Our investigation indicates that bivalves and mammals use at least several similar mechanisms to control spermatogenesis. Therefore, this study provides some basic information for further research of its functional correlation with spermatogenesis and the breeding of bivalves.

Materials and methods

Experimental specimens

Healthy *A. pectinata* (n = 15), averaging 22 ± 5 cm in shell length, were obtained from July 2013 to June 2014 from the commercial market in Penglai, Shandong, China. The different developmental stages of eggs were obtained as previously described (Chen et al. 2015). The embryos were stored at -80 °C until RNA isolation was performed. The gonad, mantle, gill, and muscle were dissected and immediately frozen in liquid nitrogen and stored at -80 °C until use. For each *A. pectinata*, gonad tissues were sampled for RNA extraction and fixed in an aqueous Bouin's solution for histology.

Histological analysis of the gonad

Histological analysis was performed as previously described (Heude-Berthelin et al. 2001; Tatsuya et al. 2005). For the analysis of natural male gonadal differentiation, gonad development was classified into different stages (Figure 1): Stage 0, resting stage with undifferentiated gonad; Stage I, the gonad in early gametogenesis; Stage II, maturation stage with active spermatogenesis; and Stage III, mature stage with ripe gonads.

RNA extraction and cDNA synthesis

Total RNA was isolated from *A. pectinata* using TRIzol reagent (Invitrogen, USA). Total RNA was treated with DNase I (TaKaRa, Dalian, China) to remove the genomic DNA. Then, cDNA was synthesized using DNase-treated RNA with reverse transcriptase M-MLV (TaKaRa, Dalian, China) and OligodT as primers under reverse transcription PCR, performed as per the manufacturer's protocol.

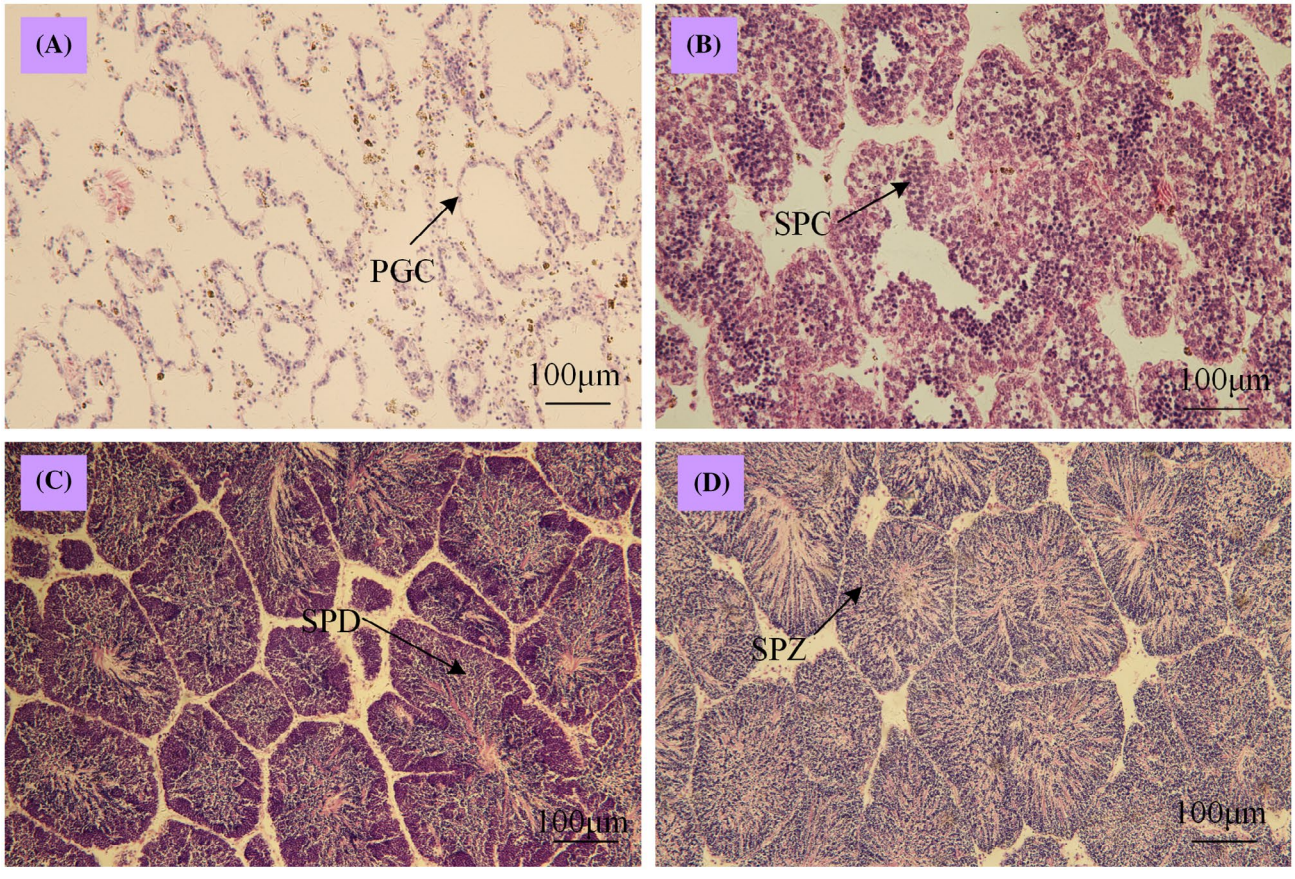

Figure 1. Histological features of *A. pectinata* male gonads.
Notes: A, stage 0; B, stage I; C, stage II; and D, stage III. PGC, primordial germ cell; SPC, spermatocytes; SPD, spermatids; and SPZ, spermatozoa.

Table 1. Primers used in this study.

Primers	Sequences(5′→3′)	Application
ApTssk1-F1	TTGCAGTCAAAATCATCGATA-GAAMNAARGVNCC	Degenerate primers
ApTssk1-R1	GTCGTCATCCAAAAGTGTAAA-GTHYAGNGMYAC	
ApTssk1-F1	TTGCAGTCAAAATCATCGATA-GAAMNAARGVNCC	
ApTssk1-R2	GTCGTCATCCAAAAGTGTAAA-GTHYAGNGMYAC	
ApTssk1-F1	TTGCAGTCAAAATCATCGATA-GAAMNAARGVNCC	
ApTssk1-R3	GTCGTCATCCAAAAGTGTAAA-GTHYAGNGMYAC	
ApTssk1-3′-F1	GGCCAAGGCTACGACATCCA-CACAT	3′RACE
ApTssk1-3′-F2	TTGGTGAAAATGGCGATGTTC-TACG	
ApTssk1-5′-R1	CATACCTGACGTGTCCAGTTCCG-GGCC	5′RACE
ApTssk1-5′-R2	TCGTAGAACATCGCCATTTTCACCA	
dTAP	GGCCACGCGTCGACTAGTAC$(T)_{16}$	3′RACE adaptor
dGAP	GGCCACGCGTCGACTAGTAC$(G)_{10}$	5′RACE adaptor
AP	GGCCACGCGTCGACTAGTAC	RACE adaptor
ApTssk1-rF	AAGTTGGCAATGGAGGAAG	RT-qPCR
ApTssk1-rR	CACAGCACGATACGTTGGT	
Ap28S-rF	AAGCGGGAAGAGCCCAGCAC	cDNA synthesis
Ap28S-rR	AGAGGCGGTCGCCAGTAAA	
Oligo(dT)-adaptor	GGCCACGCGTCGACTAG-TAC$(T)_{16}$VN	
M13–47	CGCCAGGGTTTTCCCAGTCACGAC	Vector primer
RV-M	GAGCGGATAACAATTTCACACAGG	

Cloning the internal fragment of ApTssk1

Four degenerated primers, *ApTssk1*-F1, *ApTssk1*-R1, *ApTssk1*-F2, and *ApTssk1*-R2 (Table 1), were designed based on the conserved regions of amino acid sequences from the sequence of *Homo sapiens*, *Mus musculus*, *Crassostrea gigas*, and *Aplysia californica*. An internal fragment of *Tssk1* was amplified by polymerase chain reaction (PCR). The cDNA synthesized using the total RNA from the gonad of *A. pectinata* was used as a PCR template. First-round PCR was performed using the primers *ApTssk1*-F1 and *ApTssk1*-R1. The PCR program included an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 49 °C for 30 s, and elongation at 72 °C for 45 s, followed by extension at 72 °C for 7 min. Then, nested PCR was carried out with *ApTssk1*-F2 and *ApTssk1*-R2. The amplification PCR products were analyzed by electrophoresis on 1.5% agarose gel. The target PCR products were purified using an agarose gel DNA purification kit and ligated with the pMD-19T vector (TaKaRa, Dalian, China). Vectors containing the target sequences were transformed into *E.coli* DH5α. The recombinants were identified through blue-white color selection and PCR screening with M13–47 and RV-M primers (Table 1). The positive clones were sequenced by BGI (Beijing, China).

Cloning the full-length cDNA of ApTssk1

Based on the obtained internal fragment of *ApTssk1*, the 3′ and 5′ ends were cloned using the RACE method with adapter primers and gene-specific primers (Table 1). For the 5′-end, the male gonad cDNA was purified using a DNA purification kit (TaKaRa, Dalian, China) and tailed by poly C and terminal deoxynucleotidy1 transferase (TdT) (Fermentas, USA). PCR was initially carried out with the primers *ApTssk1-5′*-R1 and dGAP with an annealing temperature of 60 °C; the semi-nested PCR was performed with the specific reverse primer *ApTssk1-5′*-R2 and the adapter primer AP with an annealing temperature of 60 °C. The 3′-end RACE PCR was performed with the specific primer *ApTssk1-3′*-F1 and the adapter primer dTAP with an annealing temperature of 60 °C; the semi-nested PCR was carried out with the primer *ApTssk1-3′*-F2 and an adaptor primer AP with an annealing temperature of 60 °C. The PCR products were gel-purified, cloned, and sequenced as described above.

Sequence analysis and phylogenetic analysis

The *ApTssk1* amino acid sequences, protein molecular weight, and isoelectric point (pI) were predicted using lasergene 5.1 (DNASTAR Inc., USA). The amino acid sequence of *ApTssk1* was analyzed by ExPASy (http://www.expasy.org). The three-dimensional modeling was predicted by the Swiss-model (http://swissmodel.expasy.org). The homology searches of nucleotide and protein sequences of *ApTssk1* were conducted with the BLAST program (http://www.ncbi.nlm.gov/blast). Multiple alignments of the *ApTssk1* sequence were performed with ClustalW multiple alignment programs (http://www.ebi.ac.uk/clustalw). A phylogenetic tree was constructed using the neighbor-joining method by MEGA 5.0 (Arizona State University, USA) based on the sequences of *Tssk1* in a public database (Tamura et al. 2007).

Gene expression analysis by quantitative real-time PCR

The mRNA levels of *ApTssk1* from various tissues, different developmental stages of embryos, and gonads of *A. pectinata* were analyzed by quantitative real-time PCR. The RT-qPCR was performed on a Roche LightCycler 480 real-time PCR system using SYBR Green Real time PCR Master Mix (TaKaRa, Dalian, China) according to the manufacturer's protocols. Conditions for RT-qPCR were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 20 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After amplification, fluorescence data were converted to threshold cycle values. The transcript levels of *ApTssk1* were normalized against *28S* transcript levels.

Results

Cloning and sequence analysis of Tssk1 from A. pectinata

An internal fragment of *Tssk1* from *A. pectinata* was amplified using degenerate primers; then, based on the partial length of *ApTssk1*, the 5′ and 3′ ends were cloned using the RACE method with gene-specific primers and adapter primers, so we obtained the full length of *ApTssk1*. The full-length *ApTssk1* cDNA sequence of *A. pectinata* obtained in the present study was submitted to the NCBI database and an accession ID (KT736043) was acquired.

The complete sequence of the *ApTssk1* cDNA was 1517 bp, containing a 5′ untranslated region (UTR) of 57 bp, a 3′ UTR of 371 bp with a canonical polyadenylation signal-sequence AATAAA and a poly (A) tail, and an open reading frame (ORF) of 1089 bp (Figure 2(A)). The ORF encoded a polypeptide of 362 amino acids including 20 types of amino acids. The calculated molecular mass of *ApTssk1* protein was 42.39 kDa and the theoretical isoelectric point (pI) was around 9.14 (Figure 2(B)). The tertiary structure of the *ApTssk1* protein was based on template 4d28.1.A (3.30Å), which shared 36.90% identity with the *ApTssk1* protein (Figure 2(C)). The domain analysis of *ApTssk1* showed that the protein contains a serine/threonine protein kinases catalytic (S-TKc) domain between 72 and 329 aa, which revealed ATP binding and a protein kinase activity molecular function of *ApTssk1* (Figure 2(A)) (Hanks et al. 1988).

Multiple sequence alignment and phylogenetic analysis

The homologous analysis indicated that the deduced amino acid sequences of *ApTssk1* shared a close identity with other reported *Tssk1* (Figure 3(A)). *ApTssk1* had a 77%

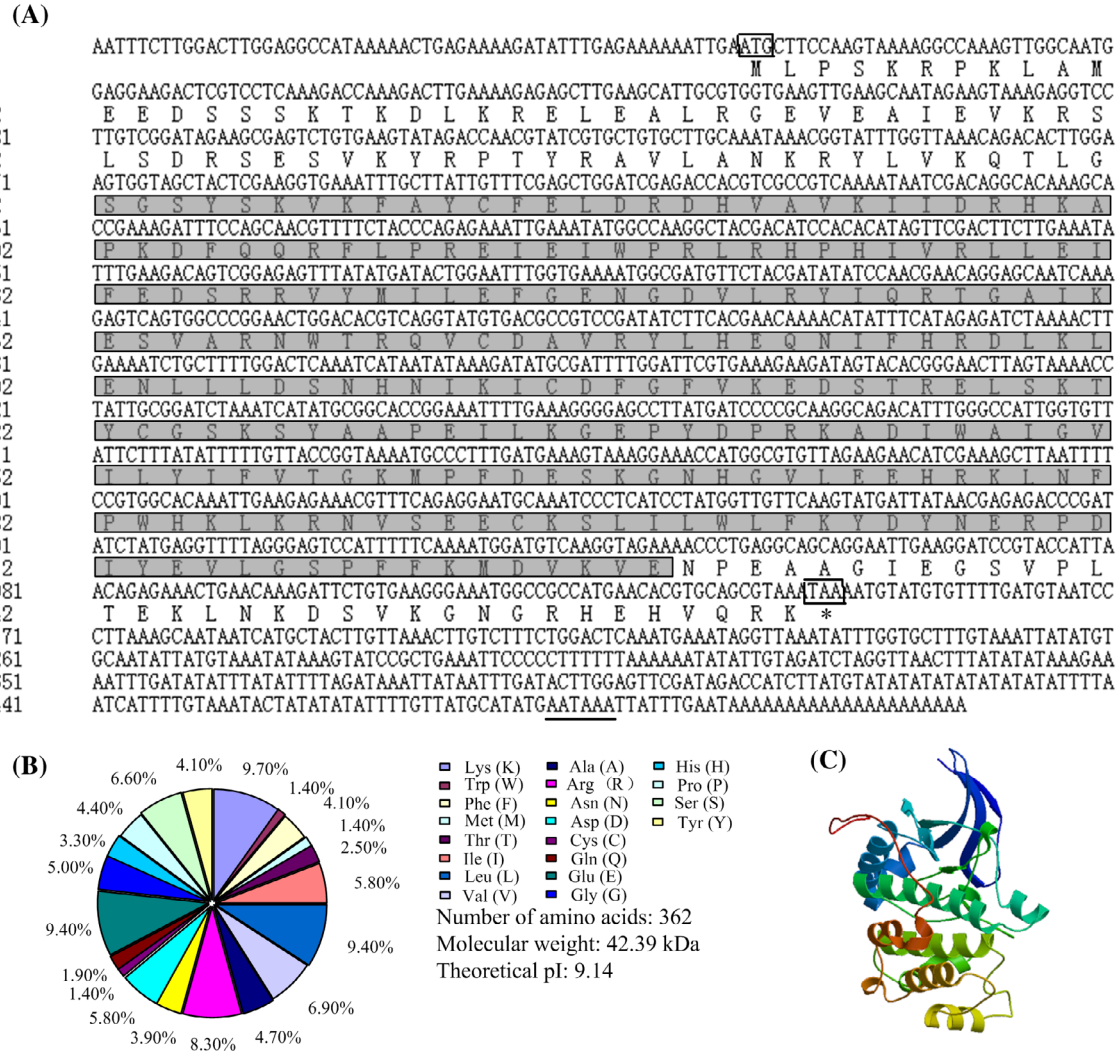

Figure 2. Sequence analysis of *Tssk1* from *A. pectinata.* A: Full-length cDNA sequence and deduced amino acid sequence of *ApTssk1*. Notes: The deduced amino acid sequence is shown upon the nucleotide sequence. The start (ATG) and stop (TAA) codons are marked by boxes. The S-TKc domain is shaded in light gray. The polyadenylation signal (AATAAA) is underlined. B: Amino acid composition of the *ApTssk1*. C: The predicted three-dimensional structure of the *ApTssk1* protein.

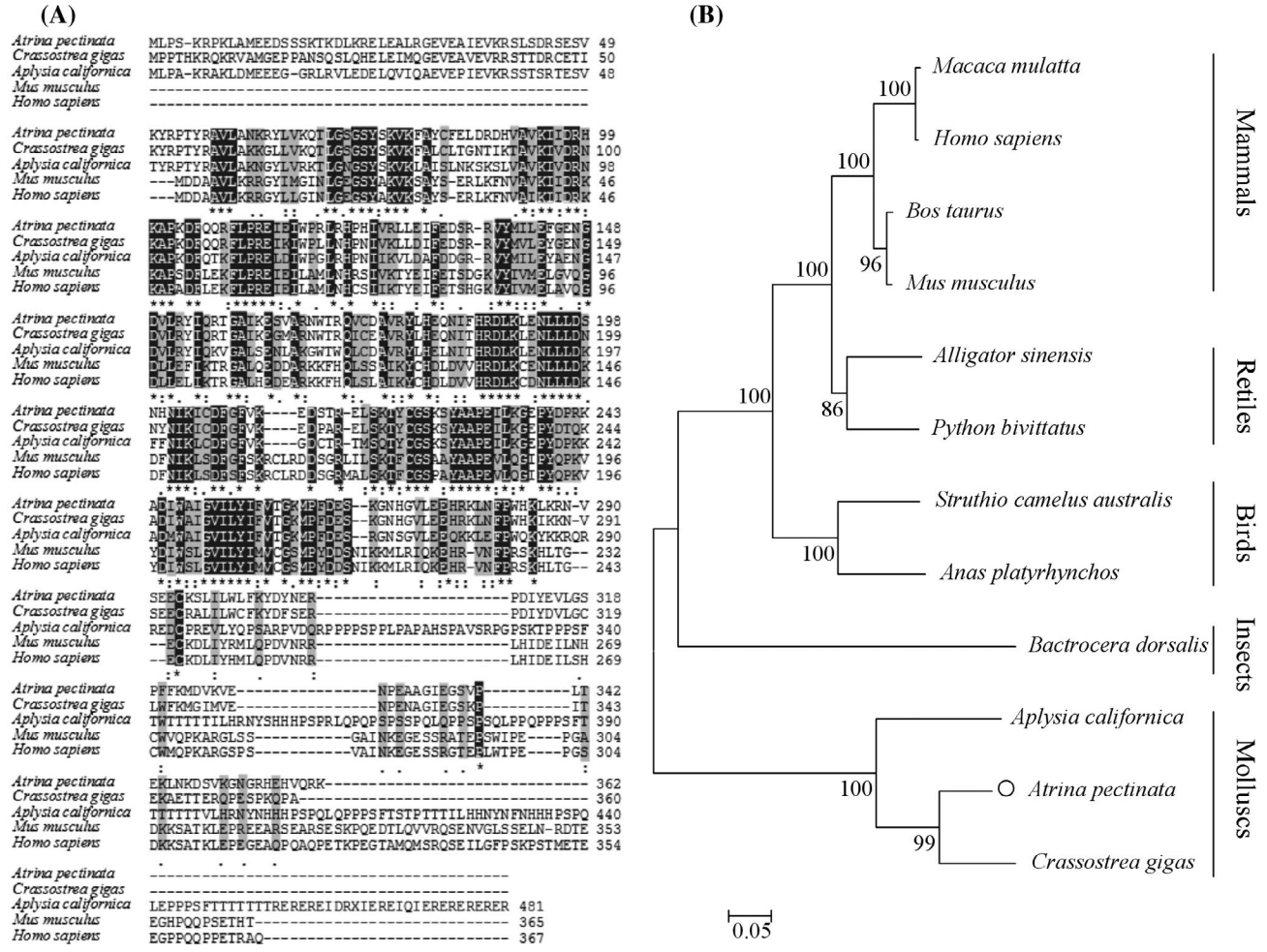

Figure 3. Multiple sequence alignment and phylogenetic analysis.
Notes: A: Multiple sequence alignment of the *ApTssk1* amino acid sequence among *Crassostrea gigas*, *Aplysia californica*, *Mus musculus*, and *Homo sapiens*. Completely conserved residues across all the aligned sequences are shaded in black and marked with an asterisk (*) below. Highly conserved residues are indicated by dots (.) and shaded in gray. Absent amino acids are indicated by dashes (-). B: Neighbor-joining phylogenetic tree of *ApTssk1* protein sequences and other selected species. Aligned sequences were bootstrapped 1000 times and the number associated with each internal branch was the local bootstrap value, which was an indicator of bootstrap confidence. The GenBank accession numbers are as follows: *Crassostrea gigas* (EKC40940.1), *Aplysia californica* (XP_005108426.1), *Mus musculus* (NP_033461.2), *Homo sapiens* (NP_114417.1), *Bos taurus* (DAA20397.1), *Macaca mulatta* (NP_001180397.1), *Python bivittatus* (XP_007435528.1), *Alligator sinensis* (XP_006021615.1), *Anas platyrhynchos* (EOB00127.1), *Struthio camelus australis* (XP_009665068.1), and *Bactrocera dorsalis* (JAC54086.1).

identity with *Crassostrea gigas*, 68% with *Aplysia californica*, 50% with *Mus musculus*, and 46% with *Homo sapiens*. The pairwise alignment showed many conserved amino acid residues from the S-TKc domain of *ApTssk1* with the other *Tssk1* sequences (Figure 3(A)).

To determine the evolutionary position of the *ApTssk1* protein, a phylogenetic tree was constructed by the neighbor-joining method after alignment (Figure 3B). In our phylogenetic tree, Tssk1 of *A. pectinata* and *C. gigas* was clustered together, and was closely related to other identified mollusk *Tssk1* orthologs. The *Tssk1* of these mollusks was more closely related to insects than that of vertebrates including birds, reptilians, and mammals (Figure 3(B)). This grouping was well-supported by bootstrapping.

Expression patterns of ApTssk1

The *ApTssk1* expression patterns among the various tissues, embryonic developmental stages and gametogenic stages, were analyzed by RT-qPCR. As shown in Figure 4(A), *ApTssk1* mRNA was only found in testis. *ApTssk1* was not expressed among the embryonic developmental stages (Figure 4(B)). According to the histological characteristic of *A. pectinata* testis, sperm development was divided into four stages (Figure 1). *ApTssk1* mRNA level increased from gonadal stage 0 to gonadal stage III. The highest levels were in gonadal stage III (Figure 4(C)). The results showed that *ApTssk1* transcripts were mostly localized in the mature spermatozoa of male gonads (Figures 1(D) and 4(C)).

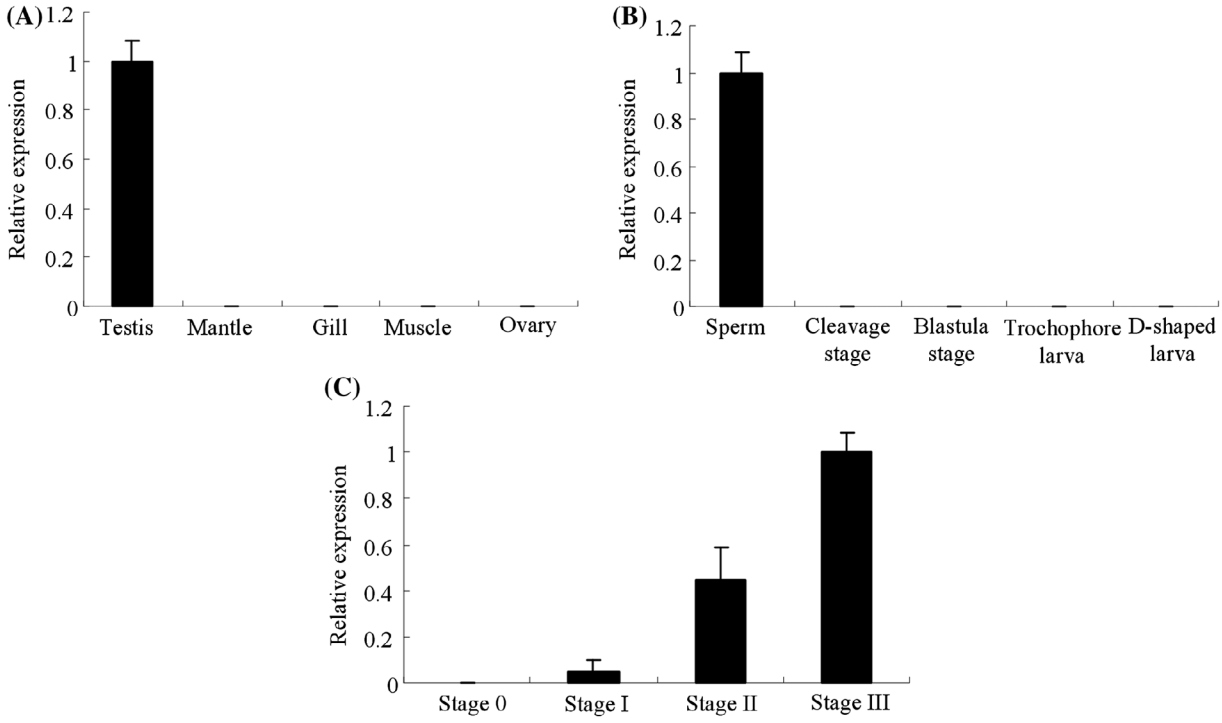

Figure 4. Expression patterns of *ApTssk1*.
Notes: A: Expression characterization of *ApTssk1* in various adult tissues. B: The temporal expression of *ApTssk1* during embryonic developmental stages. C: *ApTssk1* temporal expression during the adult male gametogenic cycle.

Discussion

In recent years, an increasing number of genes encoding protein kinases have been isolated by cDNA cloning (Lindberg & Hunter 1990; Richards et al. 2013). Some of these newly identified protein kinases contain catalytic and noncatalytic domains (Hunter & Manning 2015). In the central part of the catalytic domain, there is a conserved aspartic acid residue, which is involved in the catalytic activity of the enzyme (Hanks et al. 1988). In addition, there is a glycine-rich stretch of residues in the N-terminal extremity of the catalytic domain, which is important for ATP binding (Hanks et al. 1988). We have identified a 1517-bp full-length cDNA sequence in *A. pectinata*, which encodes a 362 amino acid sequence containing a serine/threonine protein kinases catalytic (S-TKc) domain between 72 and 329 aa (Figure 2(A)). This structural characteristic of the S-TKc domain implies important functions of *ApTssk1*, ATP binding and protein kinase activity.

Atrina pectinata homologs of *Tssk1* amino acid sequences in this study have a high percentage identity with *C. gigas Tssk1* (77%), suggesting that they are the authentic orthologs. Furthermore, a phylogenetic tree was constructed using the complete *Tssk1* genes of various species. *Tssk1* of *A. pectinata* and *C. gigas* was clustered together, and the *Tssk1* of mollusks was more closely related to insects than that of vertebrates including birds, reptiles, and mammals, like other genes that have been reported in mollusks (Santerre et al. 2014). *ApTssk1* is grouped together with all mollusk *Tssk1* genes of referred species, indicating they have a close genetic distance and may have evolved from the same ancestor (Shang et al. 2013). These results strongly suggest that *ApTssk1* is a member of the *Tssk1* subfamily. All *Tssk1* genes of referred species shared close amino acid sequences, which suggests a functional conservation of *Tssk1* (Shang et al. 2010).

Tssk1, a member of the highly conserved *Tssk* gene family widely reported to participate in spermatogenesis (Tanaka & Baba 2005), plays an important role in spermatid differentiation into morphologically mature spermatozoa in mammals (Shang et al. 2009, 2013). Some studies have shown that *Tssk1* is only expressed in the testis of humans and mice (Visconti et al. 2001; Li et al. 2011; Yang et al. 2012). The *ApTssk1* expression patterns were analyzed by RT-qPCR among the various tissues and the gonads in different phases of *A. pectinata*. Similar to *Tssk1* of mammals, the *ApTssk1* gene is mostly expressed in the male gonads, not in other tissues. The genes that are specifically transcribed during spermatogenesis are often those that are necessary for the maturation of sperm (Eddy 1998). *Tssk1* has been reported to be expressed at the testis of *A. purpuratus* and *M. edulis*; however, the homolog from *M. edulis* is upregulated in immature/early developing gonads, whereas the homolog from *A. purpuratus* is upregulated at the mature stage of gonads (Boutet et al. 2008; Ciocan et al.

2011). Similar to *Tssk1* of *A. purpuratus*, *ApTssk1* mRNA level increased from the resting to mature stages, and had the highest level in the mature stage (Figure 4(C)). This expression pattern suggests that *ApTssk1* might play functional roles during spermatogenic cell differentiation from spermatocytes and spermatids into sperm, like mammals. The function of *Tssk1* in spermatogenesis is complex (Gan et al. 2013; Silva et al. 2014). A method needs to be developed to elucidate the mechanisms of *Tssk1* in spermatogenesis.

In conclusion, we have described the molecular characterization and expression of *Tssk1* from *A. pectinata*. Our results strongly support the *ApTssk1* protein playing a functional role during spermatogenesis. As far as we know, it is the first full-length *Tssk* family gene isolated and characterized from *A. pectinata*, which provides some basic information for further researching of its functional correlation with spermatogenesis and the breeding of bivalves.

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