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Alternative splice variants and differential relative abundance patterns of *vasa* mRNAs during gonadal development in the Chinese mitten crab *Eriocheir sinensis*

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ABSTRACT

Gonadal development usually involves alternative splicing of sex-related genes. Vasa, a highly conserved ATP-dependent RNA helicase present mainly in germ cells, has an important function in gonadal development. As an important sex-related gene, recent evidence indicates that different splice variants of *vasa* exist in many species. In this study, there was identification of two types of *vasa* splice variants in the Chinese mitten crab *Eriocheir sinensis*, termed *Esvasa-l* and *Esvasa-s*, respectively. Furthermore, splice variants of *Esvasa-s* were sub-divided into *Esvasa-s1*, *Esvasa-s2*, *Esvasa-s3*, *Esvasa-s4,* and *Esvasa-s5*, based on differing numbers of TGG repeats. Results from genomic structure analyses indicated that these forms are alternatively spliced transcripts from a single *vasa* gene. Results from tissue distribution assessments indicate the *vasa* splice variants were exclusively expressed in the gonads of male and female adult crabs. *In situ* hybridization results indicate *Esvasa* mRNA was mainly present in the cytoplasm of previtellogenic oocytes. As oocyte size increased, relative abundance of *Esvasa* mRNA decreased and became distributed near the cellular membrane. The *Esvasa* mRNA was not detectable in mature oocytes. In testis, *Esvasa* mRNA was detected in spermatids and spermatozoa, but not in spermatogonia and spermatocytes. Notably, results from qPCR analysis of *Esvasa-l* and *Esvasa-s* indicate there are different relative proportions during gametogenesis, implying that splice variants of the *Esvasa* gene may have different biological functions during crab gonadal development.

1. Introduction

Alternative splicing or the capacity to produce different combinations of exons is a fundamental means of regulating protein structures (Bush et al., 2017). Results from sequence-based and microarray-based analyses indicate in *Drosophila*, the expression of the axon guidance receptor gene can result in production of approximately 38,016 different protein isoforms (Schmucker et al.,

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2000), and in most cases, the protein variants resulting from alternative splicing can have important and different functions, ultimately have profound effects on cell fate (Roy et al., 2013; Gamazon and Stranger, 2014).

The VASA protein is an ATP-dependent RNA helicase belonging to the DEAD (Asp-Glu-Ala-Ssp)-box family (Linder and Fuller-Pace, 2013), and is known to have essential functions in abdominal segment formation, the proliferation and differentiation of germ cells, and gonadal development (Shinomiya et al., 2000; Tsunekawa et al., 2000; Miyake et al., 2006; Wang et al., 2012a; Qiu et al., 2013). Therefore its homologs have been characterized in many organisms, including in the model fish zebrafish (*Danio rerio*) (Yoon et al., 1997) and medaka (*Oryzias latipes*) (Saito et al., 2006). Results of previous studies indicate multiple *vasa* genes exist with at least one expressed splice variant in many species, and the gene expression pattern and function of *vasa* alternative spliced mRNAs also differs among species (Luo et al., 2013; Kaushik et al., 2015). For example, there are five variants (1, 2, 4, 5 and 6) in testes of adult buffalo (Kaushik et al., 2015). The rate of transcription of VASA 1 gene was markedly greater than that of all except the VASA 6 gene. The relative ratio for variants was 100:1.0:1.6:0.9:48. To date, *vasa* homologs have also been identified in many crustaceans (Sagawa et al., 2005; Aflalo et al., 2007; Nakkrasae and Damrongphol, 2007; Sellars et al., 2007; Feng et al., 2011; Simeo et al., 2011; Wang et al., 2012a, b; Qiu et al., 2013). There, however, are no data regarding the identification of its splicing variants in crabs.

The Chinese mitten crab *Eriocheir sinensis* is one of the most important cultured crustacean species in China, with a commercial value of approximately US$ 5.7 billion in 2015 (Liu et al., 2019). Due to intensive propagation in aquaculture enterprises, there have been several problems arise including sexual precocity (Wu et al., 2011). Typically, with precocious sexual development of crabs, sexual maturation occurs in a 1-year period compared with the 2 years typical of crabs in their natural habitat, resulting in substantially smaller size and lesser commercial fiscal value to the aquaculture enterprises (Wu et al., 2011; Wang et al., 2012a; Liu et al., 2019). Hence characterization of the *vasa* gene, would contribute to understanding the regulation of germ cell differentiation and gonadal development in the Chinese mitten crab. In the present study, therefore, there was identification of two *vasa* splice variants in *E. sinensis*, analysis of protein sequence differences, and investigation of the expression of genes that encode these splice variants. To the best of our knowledge, this is the first report on the alternative splicing of the *vasa* gene in crabs and shrimps.

2. Materials and methods

2.1. Sample collection

Chinese mitten crabs were purchased from a local fisheries market and transported back to the laboratory where the research on the tissues was conducted. All of the crabs were maintained at 25 ± 2 °C in 30 L aerated aquaria for 3 days. Subsequently, different tissues, including brain, thoracic ganglion, ovary, testis, heart, gill, muscle, eyestalk, and hepatopancreas were collected, and immediately frozen in liquid nitrogen until use for gene and protein assessments. Additionally, the gonads prepared for *in situ* hybridization (ISH) were fixed directly in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C, dehydrated in a series of methanol solutions (25%, 50%, 75%, and 100%), and stored in 100% methanol at −20 °C.

2.2. Complementary DNA (cDNA) synthesis, sequencing, and alignment

Total RNA was extracted from the tissues using TRIzol reagent (Takara, Japan) according to the manufacturer's instructions. The $A_{260/280}$ and $A_{260/230}$ ratios of all the RNAs prepared were determined using a Nanodrop 2000c spectrophotometer (Thermo, USA), with the values being within the ranges of 1.9 to 2.1 and 2.0 to 2.5, respectively. The cDNA was synthesized using PrimeScript® 1 st Strand cDNA Synthesis Kit (Takara) after removing potential contaminating genomic DNA with RNase-free DNase I (Promega, USA). The crab ovary was used as template for PCR to synthesize cDNA, with primers (Table 1) which were designed based on the sequence deposited in GenBank (accession number HM459853) and an ovary transcriptomic library (data not shown). The PCR products were ligated into pGEM®-T vector (Promega), transformed into competent *Escherichia coli* DH5α cells, plated on LB-agar Petri dishes, and incubated overnight at 37 °C. Subsequently, positive clones were picked and sequenced on an ABI PRISM 3730 Automated Sequencer using BigDye terminator v3.1 (Applied Biosystems, USA). By comparison with genomic DNA of *Eriocheir sinensis vasa* (*Esvasa*) using the software Bioedit (Hall, 1999), the locations of exons and introns were confirmed.

Table 1
Primers used in study.

Gene	Primer	Sequence (5′–3′)	Purpose
Vasa	*Esvasa* F1	GACGAGGACTGGGATGATGAACCCGT	RT-PCR/qPCR
	Esvasa R1	CTTGACCTCTACCTCTACCGC	RT-PCR/qPCR
	Esvasa R2	AGCACTTCCCTTGTCCCTCCT	RT-PCR
	Esvasa F2	ACTTTTCAAAGGAGTGATTTTGGT	RT-PCR/qPCR/probe
	Esvasa R3	AATGGTACAAATAAAGACATCTGCT	RT-PCR/qPCR/probe
β-actin	*β-actin* F1	CGACGGTCAGGTCATCACCA	RT-PCR/qPCR
	β-actin R1	ACGTCGCACTTCATGATGGA	RT-PCR/qPCR

2.3. Tissue distribution of Esvasa mRNA

Total RNAs from various tissues were reverse transcribed (RT). The RT-PCR procedures were conducted using pairs of gene-specific primers (Table 1). The reaction conditions were: 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. Furthermore, *β-actin* was amplified as an internal control. The RT-PCR products were separated using electrophoresis on a 2.0% agarose gel and there was verification using sequencing procedures.

2.4. In situ hybridization (ISH)

A 273-bp DNA fragment amplified using primers *Esvasa* F2 and *Esvasa* R3 was sub-cloned into a pGEM®-T vector (Promega), and was used for generating the sense or anti-sense RNA probes. The ISH was conducted using a previously described method (Ma et al., 2016, 2018) with minor changes. Briefly, paraffin sections of ovary and testis were deparaffinized with xylene, and rehydrated in a series of graded alcohols for 5 min in each solvent. After rinsing, sections were treated with 5 μg/mL proteinase K for 30 min. Subsequently, slides were washed, covered with 4% paraformaldehyde, and pre-hybridized for about 1 h at 55 °C. Then, slides were incubated with 500 ng/mL of the antisense or sense probes in hybridization solution overnight at 55 °C. Slides were washed the next day and treated with anti-DIG alkaline phosphatase Fab fragments (Roche, Switzerland) in blocking solution for 2 h and washed with washing buffer, followed by detection buffer. After chromogenic reaction with NBT/BCIP solution (Thermo Fisher Scientific, USA), slides were counterstained with eosin at room temperature.

2.5. Quantitative real-time PCR (qPCR) of Esvasa mRNA

The expression profile of *Esvasa* mRNA in testes and ovaries at various developmental stages was measured using qPCR. About 200 ng of total RNA from each sample were reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the protocol provided by the manufacturer. qPCR was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad) in a 20 μL reaction mix containing 1 μL cDNA, 4 μL 5× iScript reaction mix, 0.3 μL each primer (10 μM), and 14.4 μL nuclease-free water. The qPCR cycling conditions were 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 10 s and 60 °C for 30 s, followed by dissociation curve analysis at 95 °C/15 s, 60 °C/1 min, and 95 °C/15 s to verify the amplification of a single product. The values for relative abundance of mRNA ($n = 3$) of *Esvasa* were normalized to *β-actin* (Table 1). The triplicate fluorescence intensities of each sample were compared and converted to fold differences. A one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison tests was performed using SPSS 20.0 to determine whether there were any statistically significant differences between groups (Ma et al., 2016). The qPCR data were expressed as the mean + standard error (SE). Significance was accepted at the level of $P < 0.05$.

3. Results

3.1. Splice variants of Esvasa gene

There was a search conducted of the crab ovarian transcriptome dataset previously established in the research laboratory where the present study was conducted (unpublished data). An *Esvasa* transcript was identified with great similarity to the published *vasa* homologs of shrimp and crabs. To confirm the sequence of this mRNA transcript, RT-PCR was conducted using the primers *Esvasa* F1 and *Esvasa* R2, followed by direct sequencing. Interestingly, two amplified bands were detected and there were six variant sequences detected after sequencing of 60 clones. The longest variant, defined as *Esvasa-l* (NCBI accession number MK408432), is 3747 bp in length. Nevertheless, shorter transcripts contain five variants with different numbers of TGG repeats, defined as *Esvasa-s1*, *Esvasa-s2*, *Esvasa-s3*, *Esvasa-s4*, and *Esvasa-s5* (NCBI accession numbers MK408433 - MK408437) (Fig. 1A), respectively. From results of genomic sequence analysis, there was detection of only a single copy of the *Esvasa* gene in the crab genome (unpublished data), indicating that the variants of *Esvasa* are derived from alternative mRNA splicing. Compared with the genomic DNA sequence of *Esvasa*, both *Esvasa-l* and *Esvasa-s* which contain 14 exons, separated by 13 introns (Fig. 1B). The region absent from *Esvasa-s* included Exons 4 and 5, while most of the nucleotides of Exon 5 were absent in the *Esvasa-l* variant.

3.2. Characterization of the Esvasa gene

The *Esvasa-l* gene encodes a deduced protein containing 743 amino acids, with an estimated molecular weight of 79.5 kDa and an isoelectric point of 5.08. Further analysis indicated that there were nine conserved motifs of the DEAD-box family in ESVASA-l, including motif Q (A_{323}KYSKPTPIQ$_{332}$), motif I (A_{349}QTGSGKT$_{356}$), motif Ia (P_{390}TRELA$_{395}$), motif Ib (T_{440}PGKF$_{444}$), motif II (D_{464}EAD$_{467}$), motif III (S_{499}AT$_{501}$), motif IV (L_{562}VFVE$_{566}$), motif V (R_{620}GLD$_{623}$), and motif VI (H_{644}RIGRTGR$_{651}$) (Fig. 2). Additionally, other motifs, such as glycine (G)-rich regions, RGG repeats, and a zinc finger, were also present in the N-terminus of ESVASA-l, and are associated with binding to nucleic acids. Compared with *Esvasa-l*, *Esvasa-s* variants encode 697, 696, 695, 694, and 693 amino acids, respectively. The splice site differences between *Esvasa-s* variants and *Esvasa-l* were outside the conserved domain of the DEAD-box family. Thus, these *Esvasa-s* variants still contain the conserved domains and functional motifs of the DEAD-box family.

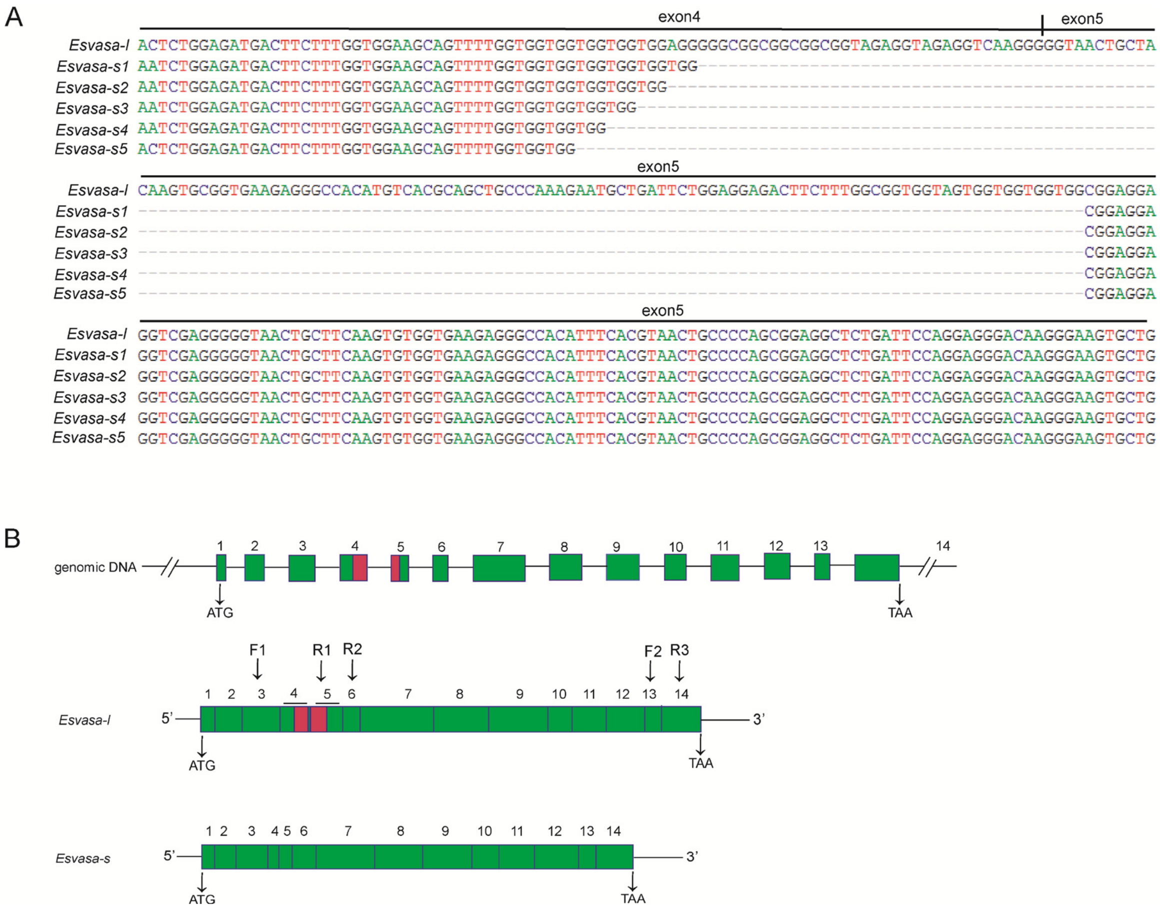

Fig. 1. Sequence analysis of splice variants of the *Esvasa* gene; A, genomic structure of *Esvasa* and its transcriptional mRNAs, *Esvasa-l* and *Esvasa-s*; Numbered boxes indicate exons of the *Esvasa* gene; Translation initiation and stop codons are located in Exon 2 and 15, respectively; Red boxes labeled in Exon 5 and 6 indicate alternative splice variants of *Esvasa-l* and *Esvasa-s*; Relative location of *Esvasa* F1, R1, R2, F2, and R3 primers are indicated; B, sequence difference between *Esvasa-l* and *Esvasa-s*; In general, *Esvasa-s* variants comprised five different sequences localized in Exon 5.

3.3. Tissue distribution of Esvasa mRNA

Due to sequence limitations, there could not be the designing of primers exclusive to *Esvasa-s,* therefore, the variant *Esvasa-s* could not be distinguished from *Esvasa-l.* Tissue distribution of *Esvasa* was, therefore, evaluated using the primer sets *Esvasa* F1/*Esvasa* R1 (to amplify *Esvasa-l* only), *Esvasa* F1/*Esvasa* R2 (to amplify *Esvasa-l* and *Esvasa-s*), and *Esvasa* F2/*Esvasa* R3 (to amplify a mixture of *Esvasa* variants) (Fig. 1B and Table 1), respectively. As depicted in Fig. 3, RT-PCR products were exclusively detected in the testis and ovary. As expected, the use of *Esvasa* F1/*Esvasa* R1 primer set resulted in amplification of only one fragment approximately 300 bp in size, and the *Esvasa* F2/*Esvasa* R3 primer set a fragment about 550 bp in size. Two bands (about 500 bp and 650 bp in size) were detected using the primer set *Esvasa* F1/*Esvasa* R2 as well. There were no bands detected in any of the somatic tissues when using these three primer sets.

3.4. Relative abundance mRNA transcript patterns of Esvasa in the ovary and testis

The relative abundances of mRNA transcripts of *Esvasa*, *Esvasa-l,* and *Esvasa-s* at different stages of ovary and testis development were evaluated by qPCR using the primer pairs *Esvasa* F1/*Esvasa* R1 and *Esvasa* F2/*Esvasa* R3. There were large relative abundances of *Esvasa* mRNA in previtellogenic oocytes and vitellogenic oocytes (Fig. 4A). The relative abundances of Esvasa mRNA relatively less in the later compared to earlier developmental stages of vitellogenic oocytes and after germinal vesicle breakdown (GVBD). For *Esvasa-l*, the relative abundances profiles were similar to that of total relative abundance of *Esvasa* mRNA transcripts. In contrast, for *Esvasa-s* the relative smaller abundances of mRNA transcript in previtellogenic oocytes, while relative abundances' of *Esvasa-s* were markedly greater in vitellogenic oocytes and late vitellogenic oocytes as compared with the previtellogenic oocytes. In GVBD developmental stage, there was a marked relative decrease compared with earlier developmental stages in abundance of *Esvasa-s* mRNA. The relative abundances of *Esvasa-l* and *Esvasa-s* mRNA transcripts during different stages of oogenesis were approximately 12:1, 2:1, 1:1, and 12:1, respectively (Fig. 4A).

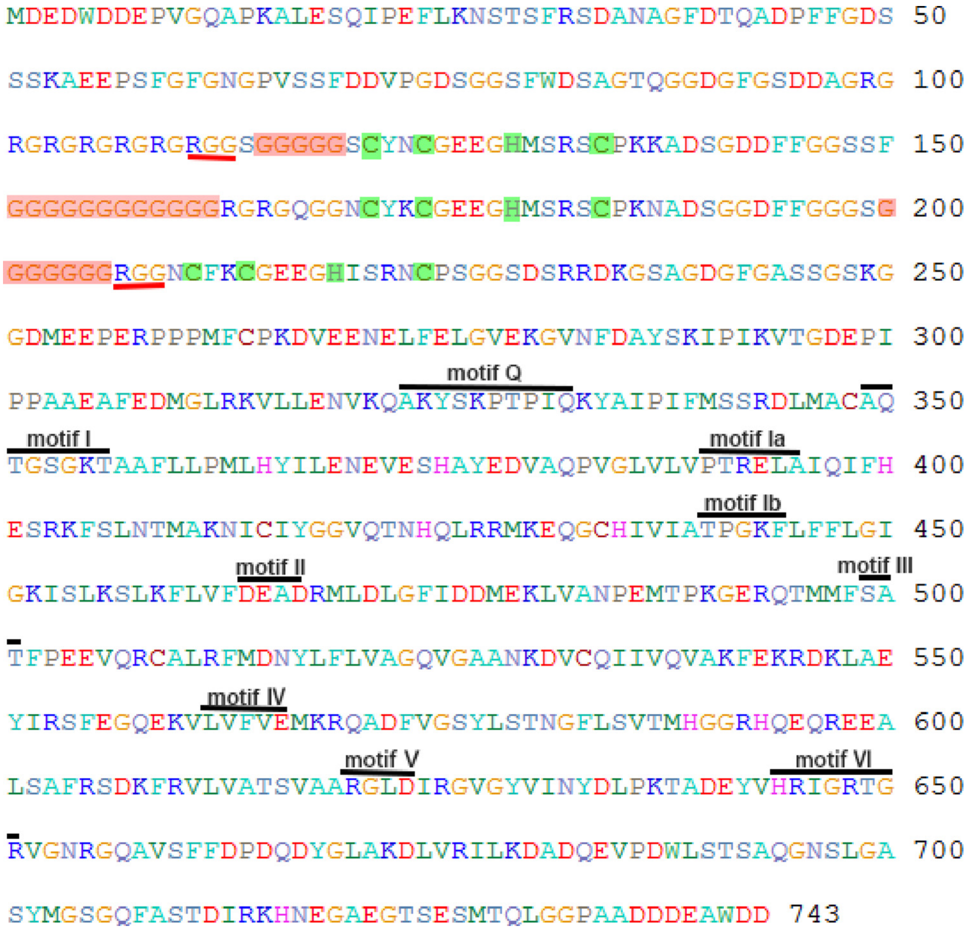

Fig. 2. Deduced amino acid sequence of *Esvasa-l*; Conserved motifs of the DEAD-box protein family are marked with black lines; Conserved amino acid residues CCHC of the Zn-finger are indicated with green background; Glycine (G)-rich regions in the N-terminus are highlighted using red background; RGG repeats are underlined in red.

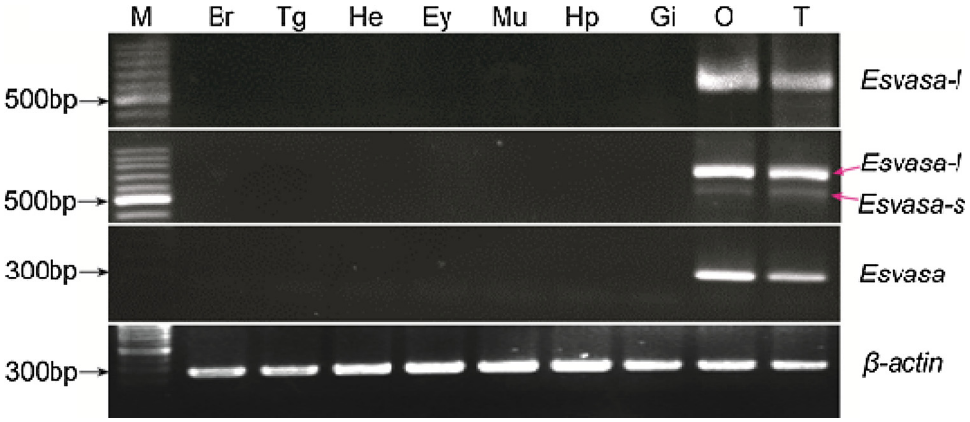

Fig. 3. Tissue distribution of *E. sinensis Esvasa*; *Esvasa* F1 and *Esvasa* R1 primers were used to obtain *Esvasa-l* only; *Esvasa* F1 and *Esvasa* R2 primers were used to obtain a mixture of *Esvasa-l* and *Esvasa-s* with different lengths; and *Esvasa* F2 and *Esvasa* R3 primers were used to obtain a mixture of *Esvasa-l* and *Esvasa-s* with the same length; M, DNA marker; Br, brain; Tg, thoracic ganglion; He, heart; Ey, eyestalk; Mu, muscle; Hp, hepatopancreas; Gi, gill; O, ovary; and T, testis.

Compared to the relative abundances of mRNA for *Esvasa* during oogenesis, the relative abundance of total *Esvasa* mRNA transcript increased during testicular development. For *Esvasa-l*, there were no significant differences in relative abundance of mRNA transcript among spermatogonia, spermatocytes, and spermatid stages. The relative abundance of mRNA transcript markedly

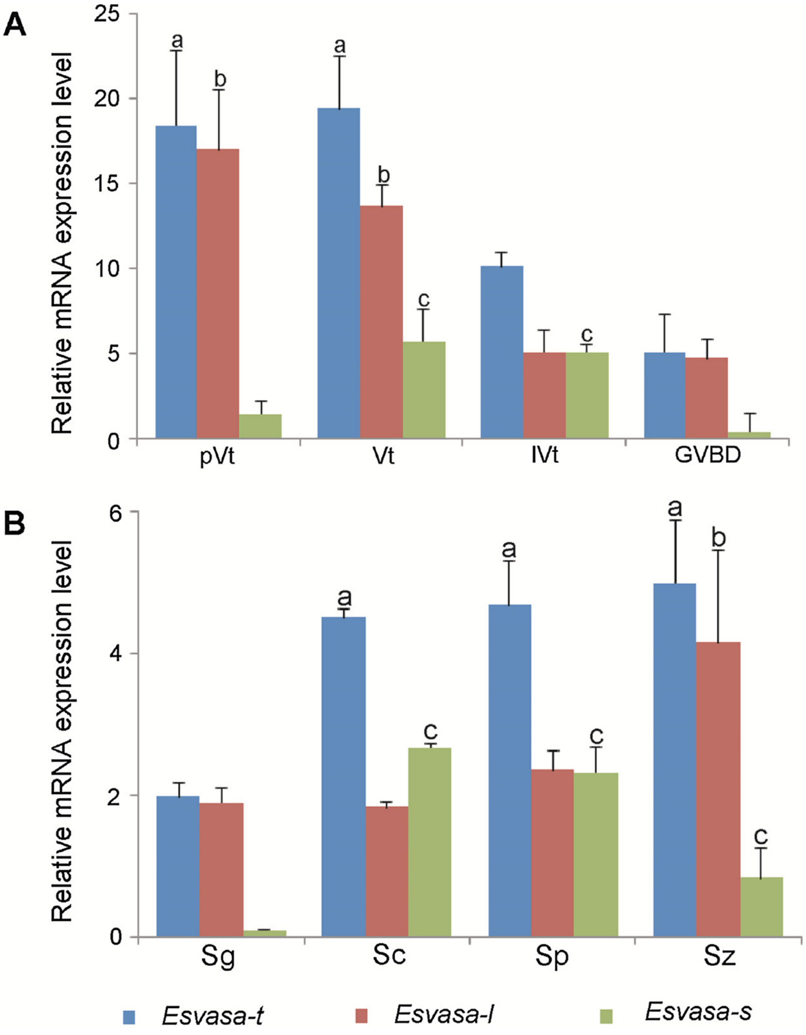

Fig. 4. Relative abundances of mRNA transcripts of *Esvasa* in different developing stages of ovary (A) and testis (B). pVt, previtellogenic oocyte stage; Vt, vitellogenic oocyte stage; lVt, later vitellogenic oocyte stage; GVBD, germinal vesicle breakdown; Sg, spermatogonia stage; Sc, spermatocyte stage; Sp, spermatid stage; and Sz, spermatozoa stage; *Esvasa-t* indicates total *abundance* of *Esvasa* and *Esvasa-l* mRNA transcripts; Relative abundance of *Esvasa-s* was obtained through analyzing the difference between *Esvasa* and *Esvasa-l*; qPCR data are expressed as the means + SE; Superscript letters indicate differences $P < 0.05$.

increased for *Esvasa-l* at the spermatozoa developmental stage. In contrast, the relative abundance of *Esvasa-s* mRNA transcript was small at the spermatogonia developmental stage, and marked increased during later stages of spermatogenesis. The relative mRNA abundances of *Esvasa-l* and *Esvasa-s* during different stages of testes development were approximately 20:1, 7:10, 1:1, and 5:1, respectively (Fig. 4B).

3.5. *Localization of the Esvasa mRNA in the gonads*

Consistent with qPCR results, there was a large amount of antisense *Esvasa* hybridization signal in previtellogenic oocytes, especially in the cytoplasm (Fig. 5A, D). With increasing cytoplasmic volume of the yolk, *Esvasa* mRNA decreases and is primarily localized near the cellular membrane in the vitellogenic oocytes (Fig. 5B, E). The *Esvasa* mRNA signals could not be detected in maturate oocytes (Fig. 5C, F). In testis, *in situ* hybridization signal for *Esvasa* was undetectable in spermatogonia and spermatocytes (Fig. 5H, I, K, L). The *Esvasa* mRNA was present in spermatids and spermatozoa until these cells were at a mature stage (Fig. 5J, M, N), while there was no signal detected in the negative control using the sense probe (Supplemental Fig. 1)

4. Discussion

To date, numerous *vasa* homologs have been identified, and the homologous DEAD-box and other motifs are conserved among

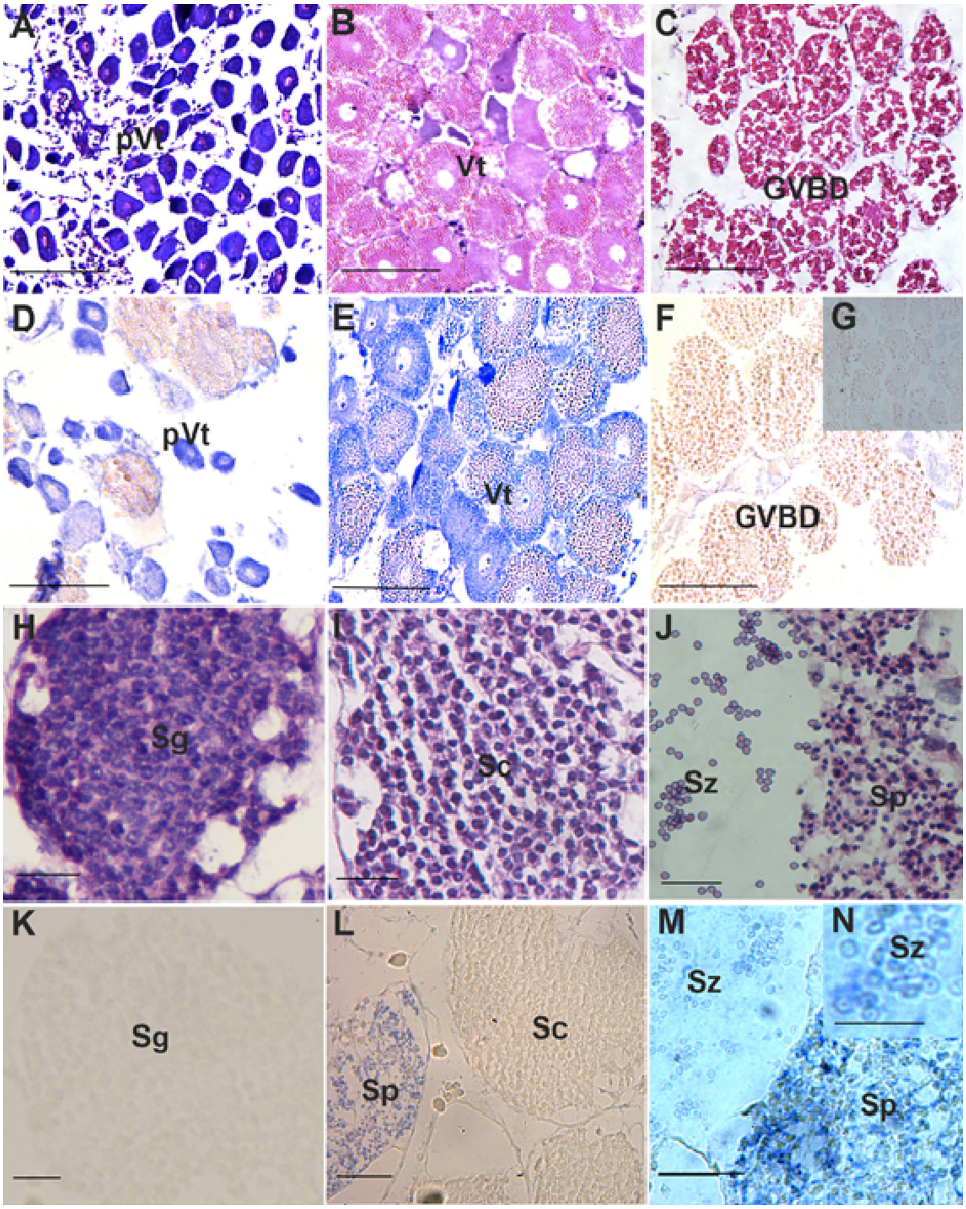

Fig. 5. Distribution of *Esvasa* mRNA during gametogenesis, detected by *in situ* hybridization with antisense probe (D–F and K–N) and sense probe (G), and hematoxylin-eosin staining (A–C and H–J). pVt, previtellogenic oocyte; Vt, vitellogenic oocyte; GVBD, germinal vesicle breakdown; Sg, spermatogonia; Sc, spermatocytes; Sp, spermatids; and Sz, spermatozoa; Scale bars = 100 µm.

different species, indicating functional consistency of these homologs among species. Like other *vasa* genes, both *Esvasa-l* and *Esvasa-s* contain motif Q, motif I, motif Ia, motif Ib, motif II, motif III, motif IV, motif V, and motif VI. Although one homolog of the *E. sinensis vasa* gene has been reported previously (Wang et al., 2012a), the sequences of *Esvasa-l* and *Esvasa-s* that was cloned in the present study differed by several bases. Furthermore, the cloning of *Esvasa-l* and *Esvasa-s* verified the existence of *vasa* splice variants, implying that these variants in *Esvas*a may have different functions in the Chinese mitten crab.

It is well-known that alternative splicing is a primary mechanism for the enhancement of transcriptome and proteome diversity, and is important in tissue development and physiology of animals (Chen and Manley, 2009). The *vasa* gene has splice variants in many species. For example, there are three *vasa* splice variants in humans, lacking 60 bp from Exon 7 and 8 in splice variant 1, 102 bp from Exon 7 and 9 in splice variant 2, and 447 bp from Exons 2 to 6 and 9 in splice variant 3 (Gamazon and Stranger, 2014). In the present study, the full-length open reading frame (ORF) of the *Esvasa* gene encoded for 743 amino acid residues, containing 14 exons. Although shorter splice variants were composed of the same number of exons, these lacked approximately 238 bp from Exon 4 and 5. The protein variants resulting from alternative mRNA splicing may differ in the presence or absence of structural or functional domains. The splice variants of *Esvasa-s* and *Esvasa-l* contain the conserved domains and functional motifs of the DEAD-box family, indicating that alternative splicing of *Esvasa* may not induce functional changes or differences in protein primary structure.

The *Esvasa* mRNA distribution analysis was performed in various tissues in the present study. The results indicate the *Esvasa* mRNA was exclusively present in gonads. There has been verification that the specific presence of the mRNA of *vasa* in germ cells is related to important functions of the encoded proteins in germline development (Braat et al., 1999; Huang et al., 2014). Homologs of the *vasa* gene have been cloned in many decapods (Aflalo et al., 2007; Nakkrasae and Damrongphol, 2007; Wang et al., 2012a, b; Qiu et al., 2013), and the distribution patterns of these *vasa* homologs were also limited to the testis and ovary. The gonadal-specific distribution of *Esvasa-l* and *Esvasa-s* mRNAs further indicate that *vasa* gene function is conserved among species, and *vasa* mRNA or protein could represent an important molecular marker to study the origin and migration of germ cells in *E. sinensis* as in other organisms.

Results of previous studies indicate that there are marked differences in relative abundances of mRNA transcripts among *vasa* splice variants (Krovel and Olsen, 2004; Luo et al., 2013). This conclusion in previous studies was confirmed by results from the qPCR analysis in the present study where there was varying relative abundances of *Esvasa-1* and *Esvasa-s* mRNA transcripts during *E. sinensis* gametogenesis. Additionally, in the testis there was a marked relative abundance of *Esvasa-l* mRNA the mature developmental stage, while there was a greater relative abundance of *Esvasa-s* in spermatocytes and spermatids. It is speculated that *Esvasa-l* protein is involved in the formation of spermatozoa and the *Esvasa-s* protein is essential in the spermatocyte and spermatid stages. In the ovary, the *Esvasa-l* protein may regulate the growth and development of oocytes at an early stage, because there is a greater relative abundance of *Esvasa-l* mRNA at previtellogenic and vitellogenic developmental stages. In contrast, there was the greatest relative abundance of *Esvasa-s* mRNA at the vitellogenic developmental stage, implying that *Esvasa-l* and *Esvasa-s* proteins may have different functions during gonadal development. Further investigation of the splice variants *Esvasa-l* and *Esvasa-s*, however, are needed to elucidate functional differences of the splice variants.

The *Esvasa* mRNA is distributed in the cytoplasm of small early-stage oocytes at the beginning of oogenesis. As oocyte development progresses, the there is a lesser relative abundance of *Esvasa* mRNA, and there was no detection of this mRNA in mature oocytes. Similar mRNA relative abundance patterns for *vasa* have also been reported in the fruit fly (Tanaka et al., 2000), oyster (Fabioux et al., 2004)[30], and tilapia (Kobayashi et al., 2000). Although no ISH signal was detected in maturate oocytes, *vasa* mRNA was still detectable at this stage suing qPCR. This finding is consistent with results from studies with the freshwater prawns (Nakkrasae and Damrongphol, 2007) and mud crabs (Wang et al., 2012b). Owing to the large amount of yolk globules present, a weaker hybridization signal may be the result of masked signaling in mature oocytes. In *E. sinensis*, qPCR results indicate *Esvasa* mRNA was present in the early spermatogenesis stage, with increasing abundances in early testis development and greatest abundances in the mature testis. The relative mRNA abundances for *Esvasa* are similar and in large amounts in the testis at spermatocytes, spermatids, and spermatozoa stages. These findings imply that there is an essential function for *Esvasa* through the entire period of crab testis development. *In situ* hybridization results, however indicate there was only a positive signal in spermatids and spermatozoa, but not in spermatocytes. To investigate the reason for this, there was examination of the ISH sections and it was discovered that in testicular sections at the spermatocyte developmental stage there are also a few spermatids present due to asynchronous spermatogenesis, so the relative amounts of mRNA abundance in the spermatocyte developmental stage could result from the signal coming from spermatids instead of spermatocytes as indicated by *in situ* hybridization results.

In summary, for the first time there was isolation of two types of *Esvasa* splice variants in the Chinese mitten crab *E. sinensis*, and the different relative abundance patterns of mRNA for these two splice variants indicate these proteins may be involved in different biological processes during gonadal development. Specific gene expression profiles for *Esvasa-l* and *Esvasa-s* in the gonad indicate that the *Esvasa* mRNA or its protein could serve as an important molecular marker to study the origin and migration of germ cells in *E. sinensis*.

Author contributions

Gaofeng Qiu designed the experiments; Guocui Yang and Zhiqiang Liu performed the experiments; Jianbin Feng and Guocui Yang processed and analyzed the data; Ruirui Wang, Zhiqiang Liu, and Keyi Ma wrote the paper; Gaofeng Qiu and Keyi Ma revised the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2019.106131.

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