Electronic Journal of Biotechnology 24 (2016) 49–55

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Expression analysis of *vasa* in Asian paddle crab (*Charybdis japonica*) exposed to Bisphenol A

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ARTICLE INFO

Article history:
Received 7 July 2016
Accepted 6 October 2016
Available online 19 October 2016

Keywords:
Aquatic environments
BPA
DEAD-box
Endocrine disrupting
Expression analysis
Expression patterns
Histological observation
vasa
vasa cDNA
vasa gene
vasa mRNA

ABSTRACT

Background: Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) with a weak estrogen-like activity in fish that is found ubiquitously in aquatic environments. However, there has been little study about BPA on the endocrine disrupting effects of crab. In the present study, cDNA of *vasa* was cloned and characterized in the *Charybdis japonica*. Histological structures of testis and expression patterns of *vasa* gene in the testis of *C. japonica* after treatment with BPA were investigated.
Results: The cDNA of *vasa* is composed of 3051 bp with a 2166 bp open reading frame encoding 721 AA. The deduced amino acid sequence contained eight conserved domains of the DEAD-box protein family. The tissue distribution showed that *vasa* mRNA was specifically expressed in ovary and testis. Histologically, the sperm cells were decreased in number and an acellular zone was seen in the testis. The transcript level of *vasa* gradually increased with a significant difference between the experimental and control groups. After BPA exposure with 0.50 and 1.00 mg/L for 1, 3, 6 and 9 d, the expression levels of *vasa* increased.
Conclusion: These findings suggest that BPA can increase the expression level of *vasa* mRNA and influence the development of the testis in *C. japonica*.

1. Introduction

The effects of endocrine disrupting chemicals (EDCs) on normal reproduction and development of animals have received much attention. Xenoestrogen is a kind of common EDCs, pesticides and synthetic chemicals that have been reported to show a feminization effect, including Diazinon [1], Bisphenol A (BPA) and 4-nonylphenol (4-NP) [2]. Bisphenol A (BPA), existing widely in industrial wastewater, domestic sewage and sludge, is considered to be an endocrine disrupting chemicals (EDCs) with estrogen-like activity. In recent years, more reports have been made about the effects of BPA on the gonadal differentiation and developmental genes of aquatic organisms. The expression levels of both *cyp19a* and *cyp19b* were increased in *Rivulus marmoratus* exposed to 600 μg/L BPA [2] and the male-specific gene *dmrt1* was down-regulated while the female-specific gene *dax1* was up-regulated following BPA exposure in the mangrove killifish (*Kryptolebias marmoratus*) [3].

DEAD-box protein is a family of ATP-dependent RNA helicase, which is widely found in species from bacteria to mammals [4]. The DEAD-box protein family shared eight characteristic sequence motifs and participated in several vital cell processes, including RNA splicing, editing and processing, initiation of mRNA expression, nuclear export and degradation [5].

Vasa is one of the important members of the DEAD-box family [6], and was originally identified in *Drosophila melanogaster*, where it was considered to be a germ cell marker genes gene [7]. Vasa plays an important role in the formation of germ cells, gametogenesis, establishment of polarity, and regulation of mRNA transcription in some developmental genes [8,9]. Previous studies have shown that the *vasa* mRNA is specifically expressed in the germ cells of most species. Therefore, *vasa* is widely used as a molecular marker for gametogenesis and primordial germ cells (PGCs) origin, migration and differentiation, etc. [10].

The differences of expression level of *vasa* mRNA was discovered in different stages of gametogenesis. In *Spinibarbus caldwelli*, the *vasa* mRNA was detected in each phase of the oogonia and oocytes, but the expression level of *vasa* was highest in the oogonia and was gradually reduced in all phases of ooctyes; during the spermatogenesis, *vasa* mRNA was detected in spermatogonia, but not in spermatocytes and

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.10.002

spermatid [11]. A similar phenomenon was observed in *Carassius auratus gibelio* [12].

The marine swimming crab *Charybdis japonica* (family Portunidae; infraorder Brachyura), is a common cave dweller in coastal areas, and widely distributed in China, Japan, Korea and Malaysia [13]. It is one of the most important marine crab species and has been cultured in the coastal areas of the Yellow & Bohai seas of China. In this paper, we isolated the cDNA sequence of *vasa* and investigate the characteristics of *vasa* expression of *C. japonica* after BPA exposure.

2. Materials and methods

2.1. Animals

Crabs (*C. japonica*) with an average mass of (56 ± 2.5) g were obtained from Aquatic Products Market of Lianyungang, Jiangsu Province, China. The crabs were acclimated to laboratory conditions for 7 d in fiberglass tanks, water temperature ranging from 20 to 22°C, water salinity of 27 ± 1‰. Crabs were fed fresh clam meat in three times each day.

2.2. Cloning of vasa cDNA and rapid-amplification of cDNA ends (RACE)

Total RNA was isolated from gonads of crab using the TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Reverse transcriptions were performed using Reverse Transcriptase M-MLV (TaKaRa) with the oligo (dT)18 primers according to the manufacturer's protocol.

The partial sequence of *vasa* cDNA was amplified with a pair of degenerate primers (Table 1), which were designed based on highly conserved domains of the known *vasa* sequences from previously reported crustaceans. The PCR was executed in the following conditions: 94°C for 3 min: 35 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 1.30 min; and 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gel; the target DNA fragment was purified using a Gel Extraction kit (OMEGA) and then cloned into pMD18-T vectors (TaKaRa), and sequenced by Majorbio Company, Shanghai, China.

To obtain the full-length sequence of *vasa*, the rapid amplification of cDNA ends (RACE) was performed. A number of gene-specific primers (Table 1) were designed according to the *vasa* cDNA fragment obtained above. The 3′end of *vasa* was obtained in accordance with the manufacturer's instructions of 3′-Full RACE Core set ver. 2.0 (Takara, Japan). The extracting, purifying, and sequencing of the desired size fragments were performed as mentioned above.

The 5'end of *vasa* was obtained according to a protocol described by Dieffenbach and Dveksler [14], where the primers (Table 1) for 5′RACE were designed based on the partial sequence of *vasa* cDNA. In brief, the first strand cDNA was synthesized with 2 μg total RNA as a template and V-5-R was the reverse primer using M-ML V reverse transcriptase (Promega). The cDNA was reacted with RNase H (MBI) and then a poly (A) tail was added at the 5′ end using terminal deoxynucleotidyl transferase (MBI). The cDNA, containing the poly (A) tail was used as template for the first PCR. Amplification was performed with a universal forward primer Oligo $(dT)_{16}$ AP and a specific reverse primer V-5-R1. The nested PCR, which used the first PCR product as a template, was carried out with a forward primer AP and a specific reserve primer V-5-R2. The extracting, purifying, and sequencing of the desired size fragment were performed as mentioned above.

Table 1
Primers used for fragment cloning, 5′ and 3′ RACE and qRT-PCR of *vasa*.

Primer names	Sequence 5′-3′	use
V-F	ATGAAKGTRACWGGAGAKGAGCC	*vasa* fragment cloning
V-R	ACGAGCWGCMACDGMRGTAGCC	*vasa* fragment cloning
V-3-F1	5′ TACCAGGAGCAGCGTGAGGAAGC3′	*vasa* 3′ RACE
V-3-F2	5'GAGTGCTTGTGGCTACCTCCGTT 3'	*vasa* 3′ RACE
V-5-R	5'CCCTGTGCCTTTGTTGTCAGAATC3'	*vasa* 5′ RACE
V-5-R1	CCCCCTGTGCCTTTGTTGTCAGAATC	*vasa* 5′ RACE
V-5-R2	CTCCTCTCCCATACCCCCTGTGCCT	*vasa* 5′ RACE
Oligo$(dT)_{16}$AP	CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTT	*vasa* 5′ RACE
AP	CTGATCTAGAGGTACCGGATCC	*vasa* 5′ RACE
vasa-F1	GATAGCCAAGAGAAGGTTTTGGT	*vasa* RT-PCR
vasa-R1	TACTGGTTAGACCTGAAGGCAGA	*vasa* RT-PCR
q vasa-F	CAAGAGAAGGTTTTGGTGTTTG	*vasa* q RT-PCR
q vasa-R	GTATCCTACGCCTCGAATGTCT	*vasa* q RT-PCR
actin-F	GTCGACAATGGCTCCGGGATG	*beta-actin* RT-PCR
actin-R	GTGGTGCCAGATCTTCTCCAT	*beta-actin* RT-PCR
q actin-F	CGTCCCCATCTATGAAGGTTACT	*beta-actin* q RT-PCR
q actin-R	AAGTCAAGAGCGACATAGCAAAG	*beta-actin* q RT-PCR

2.3. Sequence analysis and alignment

The cDNA was analyzed using DNAstar7.0 and Vector NTI 10 (Invitrogen). The protein structures were predicted using online analysis tools such as ScanProsite (http://prosite.expasy.org/scanprosite), and the properties of the amino acid sequence was analyzed by the ExPASy ProtParam tool (http://www.expasy.ch/tools/protparam.html). Multiple alignments of the amino acid sequences were performed using the online tool in EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree was generated using MEGA 4.0 [15]. The data was re-sampled by 1000 bootstrap replications to determine the confidence indices within the phylogenetic tree.

2.4. Expression analysis of vasa mRNA in different tissues

To detect the *vasa* mRNA expression in different tissues, total RNA from heart, gills, intestine, hepatopancreas, muscle, testis and ovary was extracted using TRIZOL reagent (Invitrogen). The first strand of cDNA was synthesized with the oligo (dT)18. The primers (vasa-F1, vasa-R1) (Table 1) were used for analyzing *vasa* mRNA expression in different tissues. RT-PCR was run as follows: 94°C for 3 min; 30 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 30 s; and 10 min at 72°C for the ending extension. β-Actin was used as the preference gene (Table 1). The PCR was performed as follows: 94°C for 3 min; 22 cycles of 94°C for 45 s, 58°C for 45 s, 72°C for 30 s; followed by a final extension at 72°C for 10 min.

2.5. BPA exposure and sample collection

BPA (≥99.0%) and β-estradiol (≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of BPA was prepared by dissolving solid in ethanol (analytical grade). Subsequently, the desired concentrations of BPA were prepared by diluting the stock solution in sea water.

Crabs were randomly divided into six groups, with each containing 36 individuals. Based on the results of the acute toxicity test in previous study of *C. japonica* exposure to BPA [16], four groups were tested at different concentrations of BPA (0.125, 0.25, 0.50 and 1.00 mg/L), with one control group (only ethanol) and a β-estradiol group (0.01 mg/L) as a positive control. All experiments were conducted in triplicate using independent samples. After 1, 3, 6, 9 and 15 d of BPA exposure, the testis of crabs from each group were frozen in liquid nitrogen immediately and stored at -80°C prior to the test of gene expression. Samples of testis from 1.00 mg/L BPA concentration group were fixed in Bouin's solution for light microscopic histological examination at d 15.

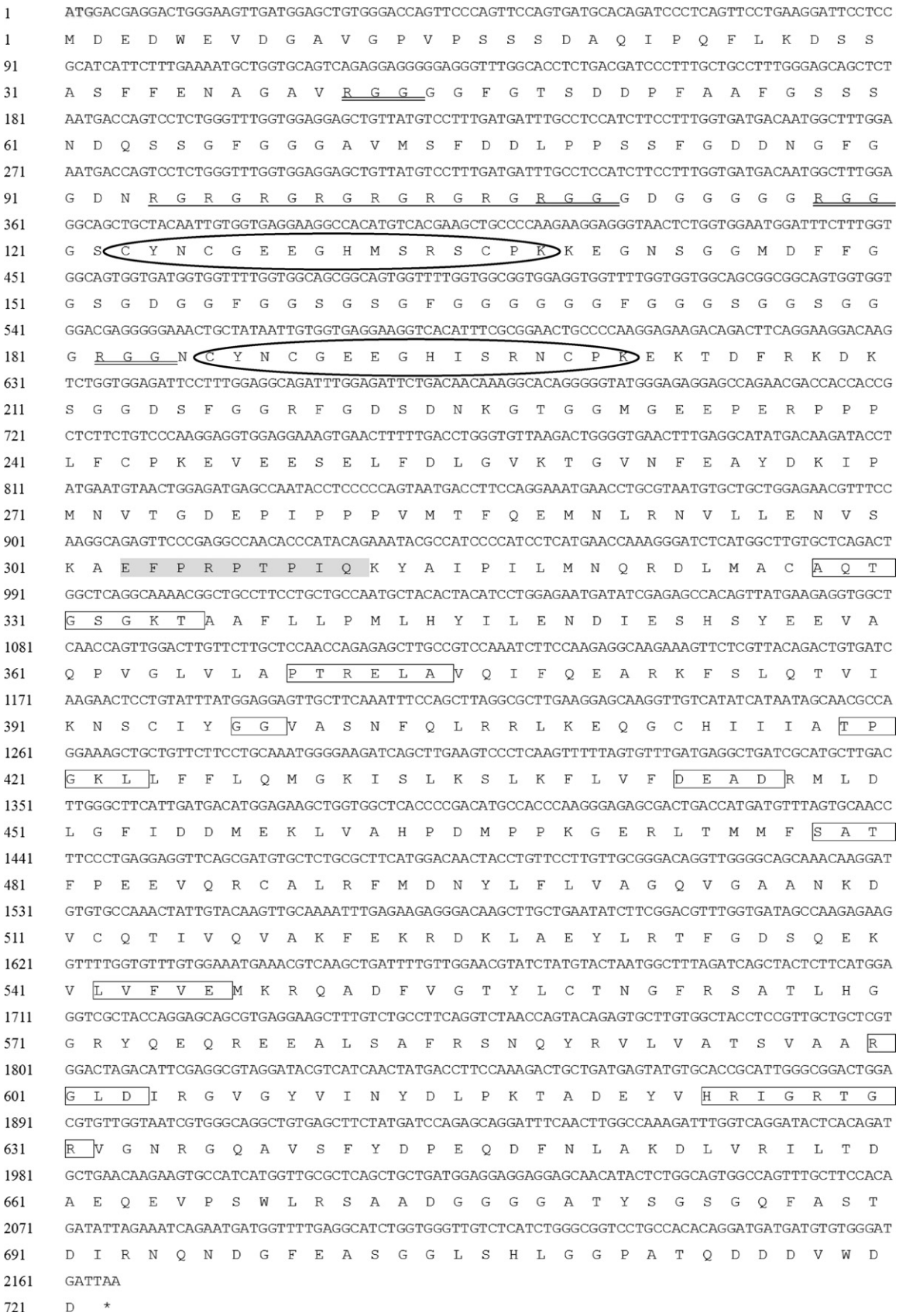

Fig. 1. Nucleotide and deduced amino acid sequence of *vasa* in *C. japonica*. The Q-motif is shadowed. The eight conserved regions of the DEAD-box protein family and GG doublet are boxed in black. RG and RGG repeats are underlined and double underlined, respectively. The two Zn-finger motifs (CCHC) are shown inside ovals.

2.6. Expression analysis of vasa after BPA treatment

Real-time quantitative PCR (qRT-PCR) was carried out to determine the *vasa* expression of *C. japonica* exposed to BPA using the SYBR Green I chimeric fluorescence method. The qRT-PCR primers of *vasa* are listed in Table 1. The *β-actin* gene was used as the endogenous control (Table 1). The cycling program for the qRT-PCR was 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s; followed by 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The efficiency of the reactions was checked by analysis serial dilutions of cDNA. The calculation of the relative expression level of genes used the $2^{-\Delta\Delta Ct}$ method [17].

2.7. Statistical analysis

Experimental data is shown as mean ± SEM. The data was analyzed with a one-way ANOVA, followed by Duncan's multiple comparison test, with $p < 0.05$ indicating a significant difference, using SPSS 16.0 for Windows.

3. Results

3.1. Molecular characterization of vasa and sequence analysis

The full length cDNA of *vasa* was successfully acquired from *C. japonica* using RT-PCR and RACE methods. The size of the full cDNA of *vasa* (GenBank accession no. KF995354) was 3051 bp, which contained a 5′-untranslated region (UTR) of 100 bp, an open reading frame (ORF) of 2166 bp encoding a putative 721 AA (amino acid), and a 3′-UTR of 785 bp. The predicted *vasa* protein has a calculated molecular weight of 77.14 kDa and a theoretical isoelectric point of 5.04. The deduced amino acid sequence contained eight conserved domains of the DEAD-box protein family, including AQTGSGKT (I), PTRELA (I a), TPGKL (I b), DEAD (II), SAT (III), LVFVE (IV), RGLD (V), HRIGRTGR (VI), and a GG doublet. In addition, EFPRPTPIQ (Q-motif) was present in 17 amino acids upstream of AQTGSGKT (I). Moreover, seven arginine–glycine repeats (RG) and, four arginine–glycine–glycine triad repeats (RGG) were present, with two zinc finger motifs (CCHC) in the N-terminus (Fig. 1).

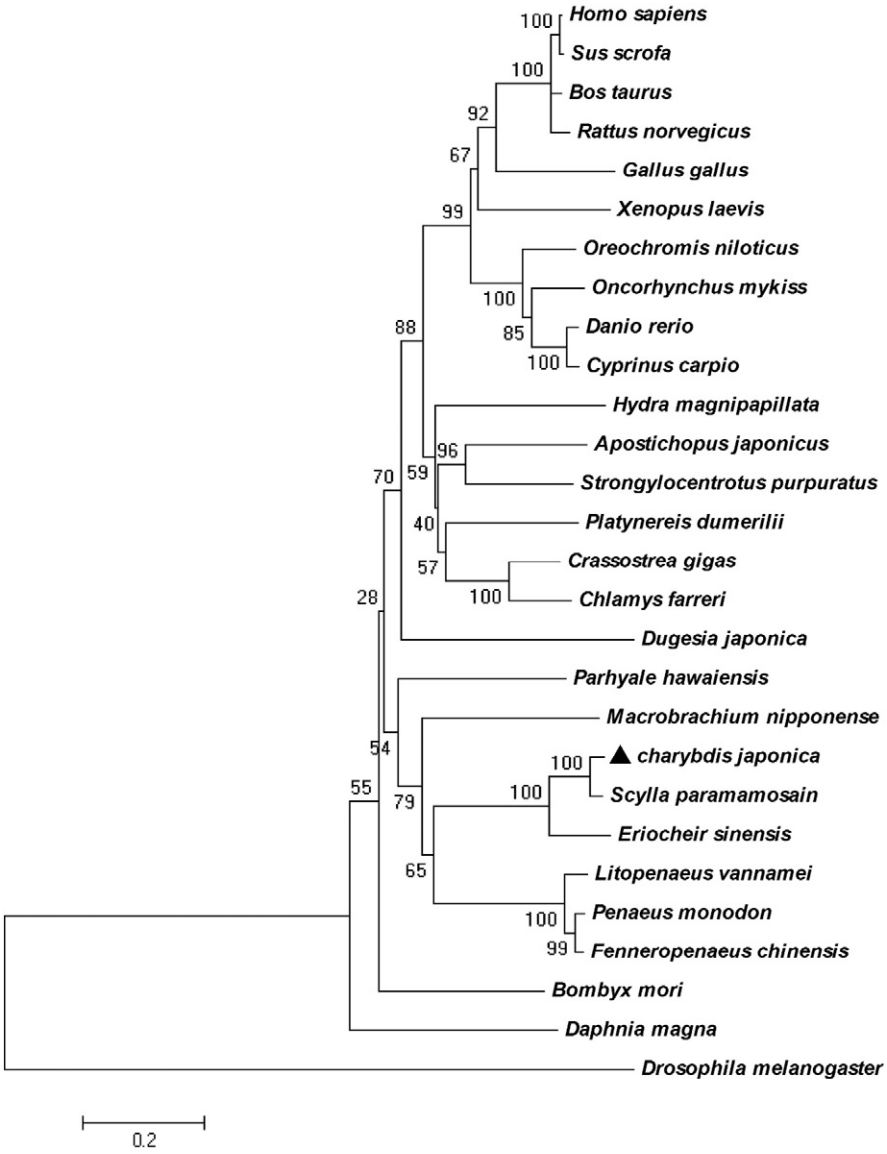

Fig. 2. Phylogenetic tree based on a deduced amino acid alignment using the neighbor-joining method for VASA sequences in *C. japonica* and other vertebrates. *C. japonica* VASA are marked with a black triangular form. Numbers on nodes indicate bootstrap vales from 10,000 replicates.

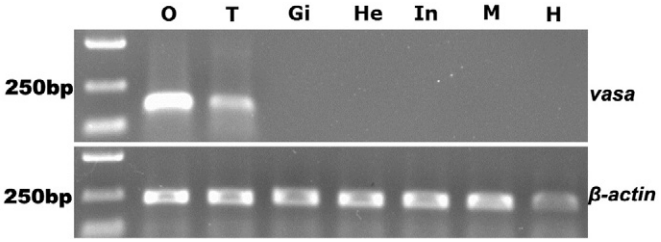

Fig. 3. Tissue distribution of *vasa* expression in adult *C. japonica* by RT-PCR, O: ovary; T: testis; Gi: gill; He: hepatopancreas; In: intestines; M: muscle; H: heart.

3.2. Vasa sequence alignment and phylogenetic analysis

A comparison of the deduced amino acid sequence with those of other species ranging from invertebrates to vertebrates, revealed that the highest homology was shared with the green mud crab *Scylla paramamosain* (ACZ92304.1) and the Chinese mitten crab *Eriocheir sinensis* (ADM64419.1) (91.6–72.1% similarity). Low similarities were found with other species (<50%), such as *Litopenaeus vannamei* (AAY89069.2), *Macrobrachium nipponense* (AEQ19569.1), *Apostichopus japonicas* (ACA05234.1), *Sus scrofa* (AY626785.1), *Homo sapiens* (AY004154.1), *Rattus norvegicus* (AAB33364.1), *Oncorhynchus mykiss* (NP_001117665.1), and *Danio rerio* (AAI29276.1). Phylogenetic analysis showed that *C. japonica* VASA was more closely related to VASA homologs in other crabs, such as *S. paramamosain* and *E. sinensis*, compared to vertebrates, such as *H. sapiens*, *Mus musculus* (AFC17964.1), *D. rerio*, among others from the evolutionary process (Fig. 2).

3.3. Tissue-specific expression of vasa mRNA

The distribution patterns of *vasa* in different tissues including ovary, testis, muscle, heart, intestine, gill and hepatopancreas of adult *C. japonica* were analyzed by RT-PCR. As shown in Fig. 3, *vasa* mRNA was specifically expressed in the gonads and the expression, level of *vasa* in the ovary was higher than that in the testis. The expression of *vasa* mRNA was not detected in five other tissues that were tested.

3.4. Effect of BPA on histological structure of the C. japonica testis

Light microscopic sections of testis from *C. japonica* exposed to BPA are shown in Fig. 4. Compared to the control group, the amount of sperm cells was reduced and the acellular zone was observed in testis of *C. japonica* exposed to 1 mg/L BPA.

3.5. Expression analysis of vasa mRNA in response to BPA

The expression level of *vasa* in the testis of *C. japonica* exhibited significant changes with exposure to BPA (Fig. 5). The *vasa* mRNA level in the group with a low concentration of BPA (0.125 mg/L) was maintained at a lower level without any significant difference from the control group. When crabs were exposed to 0.25 mg/L BPA for 9 and 15 d, the transcript level of *vasa* gradually increased with a significant difference from the control group. BPA exposures of 0.50 and 1.00 mg/L for 1, 3, 6 and 9 d led to gradually increased, expression levels of *vasa*, though a relative declined occurred after day 15. In the positive control group (E_2), the *vasa* mRNA level was significantly higher than that of the control group.

4. Discussion

4.1. Characterization of vasa

The DEAD box protein family is ATP-dependent RNA helicases, which has eight conserved domains [18]. In the present study, the full-length *vasa* cDNA of *C. japonica* was acquired, and like other species, the deduced amino acid sequence of *C. japonica* had eight conserved domains. In addition, a glycine-rich region in the N-terminus and multiple RGG repeats were observed in *C. japonica* Vasa, which is similar to the Vasa structure described in previous studies [19,20]. The RGG/RG motifs in the N-terminus are considered to be associated with RNA binding [21]. The zinc finger motifs (CCHC) were also observed in the N-terminus of *C. japonica* Vasa, which may have an effect on binding with nucleic acid [22]. These results suggest that the *vasa* gene is relatively conserved for the protein structure, implying that it has a unique biological function for the DEAD-box family proteins.

4.2. Tissue distribution of vasa mRNA

In previous studies, *vasa* mRNA was shown to be highly expressed in ovary and testis, whereas its expression was very weak in other tissues [23,24]. In crustaceans, the *vasa* of the mud crab *S. paramamosain* was expressed only in the ovary and testis [25]. Similar results were observed in *M. nipponense* [26]. In the present study, the *vasa* were specifically expressed only in ovary and testis of *C. japonica*, which is

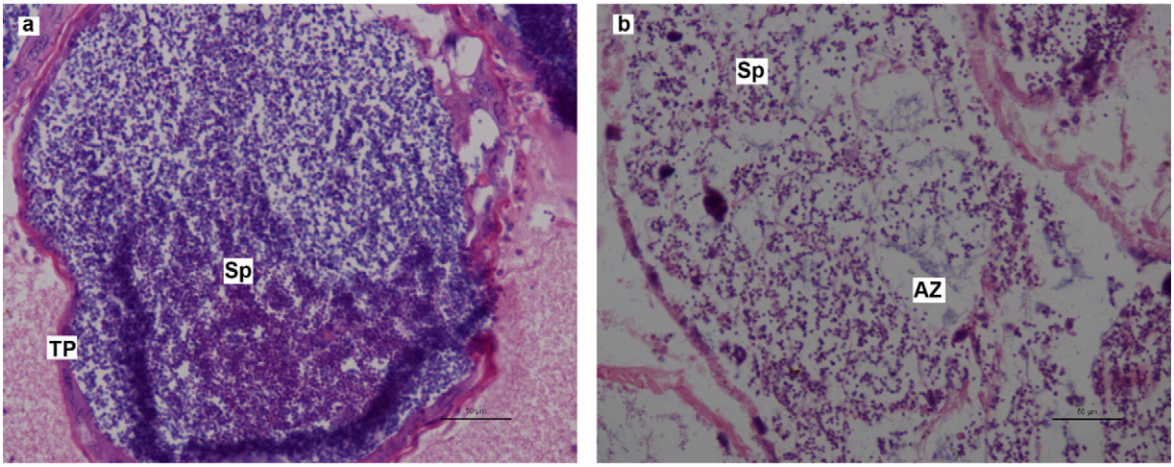

Fig. 4. Images of the testis of *C. japonica* exposed to BPA. (a): control group; (b): BPA treatment group. TP: Tunica Propria, Sp: Spermatocyte, AZ: Acellular Zone.

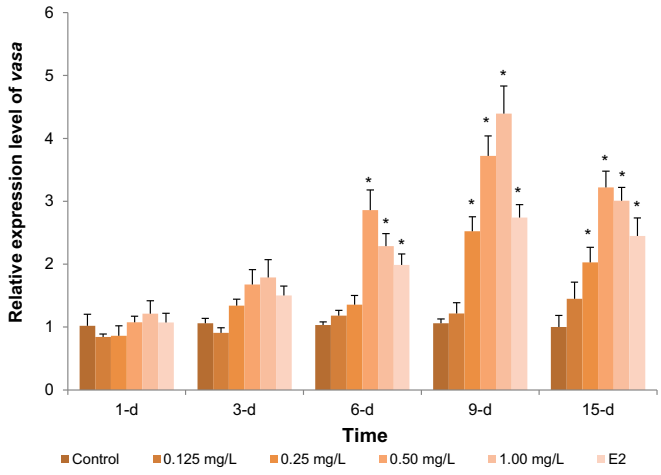

Fig. 5. Relative expression level of *vasa* in testis of *C. japonica* after exposure to BPA using quantitative real-time RT-PCR and *beta-actin* as an internal standard. Data are presented as mean ± SEM (n = 6). Note: * in column diagram indicates significant difference ($p < 0.05$) among the groups at the same test time.

consistent with previous studies. *vasa* gene likely plays an important role in oogenesis and spermatogenesis of *C. japonica*.

4.3. Effect of BPA on the histological structure of C. japonica

Previous studies have shown that some EDCs, such as nonylphenol (NP), BPA and estradiol (E2), can cause changes in the structure of testis from the zebrafish [27,28]. In the present study, a large number of acellular zones were noted in testis tissue of *C. japonica* after exposure to 1 mg/L BPA and the number of spermatocytes was also reduced. These observations are consistent with those of previous studies by Cao et al. [27] and Liu et al. [29] in the zebrafish exposed to BPA and NP, respectively.

4.4. Expression characteristics of vasa after exposure to BPA

The *vasa* gene is a specific marker for primordial germ cells (PGCs) in fish and plays a vital role in generation, migration, differentiation, and maturation of PGCs. Numerous experiments have demonstrated that some EDCs in the environment have feminizating effects on fish and effects on PGCs and the *vasa* gene [30,31]. Cardinali et al. [30] reported that the expression level of *vasa* was increased in *Sparus autata* exposed to E2. Moreover, the expression level of *vasa* was reported to be significantly up-regulated in *D. rerio* exposed to monocrotophos (unpublished data). Regarding the increasing expression of *vasa* induced by EDCs, two views have been put forward as explanations: 1) EDCs enhance the expression of *vasa* in the existing germ cells, and 2) the number of germ cells-specifically expressing *vasa* increases. Moreover, Hoshi et al. [32] reported that the number of germ cells was significantly increased in *Caenorhabditis elegans* exposed to E2 and BPA. In the present study, the *vasa* level was increased in the testis of *C. japonica* after exposure to BPA. This is consistent with the findings for *S. autata* after exposure to E2 [30] and *D. rerio* after exposure to monocrotophos, which was considered to be due to the increase in number of germ cells (unpublished data). Nevertheless, from observations of microstructure, the number of germ cells appeared to be decreased in *C. japonica* after exposure to BPA, and thus, the explanation would be that the increase in *vasa* expression due to the rise in *vasa* expression in the existing germ cells.

5. Concluding remarks

In the present study, cDNA of *vasa* from *C. japonica* was isolated and the expression level of *vasa* was identified after exposure to BPA. The change in the histological structure was also investigated. After exposure to BPA, the number of sperm cells was reduced along with testis degeneration. The *vasa* mRNA was specifically expressed in ovary and testis, and the expression level of *vasa* was increased after BPA exposure. These findings suggest that BPA can increase the expression level of *vasa* mRNA and influence the development of the testis in *C. japonica*.

Financial support

This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) (5511201401X), Open Foundation of key laboratory of East China Sea & Oceanic Fishery Resources Exploitation and Utilization, Ministry of Agriculture, P.R. China (Grant No. K201210), and Science and Technology Planning Project of Lianyungang City (CN1410). We also thank anonymous reviews and the Assistant Editor for providing valuable comments on the manuscript.

Conflict of interest

We declare that we have no conflict of interest.

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