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Molecular characterization, sexually dimorphic expression, and functional analysis of 3′-untranslated region of *vasa* gene in half-smooth tongue sole (*Cynoglossus semilaevis*)

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ABSTRACT

Vasa is a highly conserved ATP-dependent RNA helicase expressed mainly in germ cells. The *vasa* gene plays a crucial role in the development of germ cell lineage and has become an excellent molecular marker in identifying germ cells in teleosts. However, little is known about the structure and function of the *vasa* gene in flatfish. In this study, the *vasa* gene (*Csvasa*) was isolated and characterized in half-smooth tongue sole (*Cynoglossus semilaevis*), an economically important flatfish in China. In the obtained 6425-bp genomic sequence, 23 exons and 22 introns were identified. The *Csvasa* gene encodes a 663-amino acid protein, including highly conserved domains of the DEAD-box protein family. The amino acid sequence also shared a high homology with other teleosts. *Csvasa* expression was mainly restricted to the gonads, with little or no expression in other tissues. Real-time quantitative polymerase chain reaction analysis revealed that *Csvasa* expression levels decreased during embryonic and early developmental stages and increased with the primordial germ cell proliferation. A typical sexually dimorphic expression pattern of *Csvasa* was observed during early development and sex differentiation, suggesting that the *Csvasa* gene might play a differential role in the proliferation and differentiation of male and female primordial germ cells (PGCs). *Csvasa* mRNA expression levels in neomales were significantly lower than those in normal males and females, indicating that the *Csvasa* gene might be implicated in germ cell development after sex reversal by temperature treatment. In addition, medaka (*Oryzias latipes*) PGCs could be transiently labeled by microinjection of synthesized mRNA containing the green fluorescence protein gene and 3′-untranslated region of *Csvasa*, which confirmed that the *Csvasa* gene has the potential to be used as a visual molecular marker of germ cells and laid a foundation for manipulation of PGCs in tongue sole reproduction.

1. Introduction

In sexually reproducing organisms, germ cells, derived from primordial germ cells (PGCs), play important roles in gonadal differentiation and transmission of genetic information through the generations [1,2]. Primordial germ cells differentiate from blastomeres during the early stages of

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embryogenesis and migrate into the presumptive gonads, giving rise to sperm in males and eggs in females. Several molecular markers have recently been used to study PGCs in Metazoa, including *vasa*, *dnd*, and *nanos* [3]. Among these, the *vasa* gene was the first molecular marker for PGCs in teleosts, and researchers have developed a great interest in it [4].

The *vasa* gene encodes an ATP-dependent RNA helicase belonging to the DEAD (Asp-Glu-Ala-Asp)-box family, which was originally reported in fruit fly (*Drosophila melanogaster*) and considered as the maternal-effect gene required for abdominal segment formation and the proliferation and differentiation of germ cells [5]. Many studies revealed that the *vasa* gene was specifically expressed in germline cells and played important roles in germline development [6–17]. Sequence analysis of the *vasa* gene demonstrated a high conservation of gene structure during evolution. The *vasa* gene is therefore an excellent germ cell marker and has been used extensively for researching the formation, proliferation, and differentiation of PGCs in teleosts [12–17].

Recent years have seen the development of techniques to trace live PGCs in teleosts using microinjection of a fusion gene containing regulatory regions of *vasa* gene and green fluorescence protein (GFP) gene [17–22]. The 3′-untranslated region (3′UTR) of *vasa* has been verified to be sufficient for regulation of specific expression in the PGCs of zebrafish (*Danio rerio*) [18], medaka (*Oryzias latipes*) [21,22], Atlantic salmon (*Salmo salar*) [17], and a range of teleost species [20]. Migration patterns of PGCs in embryos and proliferation of PGCs during development can now be analyzed by tracing live PGCs visually, and labeled PGCs from some teleost species have been successfully isolated for cryopreservation and surrogate broodstock of endangered and commercial species [23,24].

Half-smooth tongue sole (*Cynoglossus semilaevis*) is one of the most valuable marine flatfish in China, which possesses a ZZ/ZW genetic sex determination system. Females of the species grow two to three times bigger than males [25], the phenomenon of natural sex reversal in genetic female tongue sole was observed [26], and it was also found that physiological sex of juvenile tongue sole was subject to reversal by an influence of temperature [27]. Artificial gynogenesis was carried out to obtain the super-females and some progress has been made [28]. Understanding the sex determination mechanism will accelerate the development of monosex female production. It was reported that germ cells played a crucial role in female sex determination in zebrafish [2], medaka [29], and three-spined stickleback (*Gasterosteus aculeatus*) [30], but in loach (*Misgurnus anguillicaudatus*) and goldfish (*Carassius auratus*), germ cells were not the primary factor for sex determination [31,32]. The role of germ cells in gonadal sex differentiation remains unclear, partly because of limited knowledge about the expression and functional analysis of germ cell makers in flatfish species. In addition, the tongue sole broodstocks cannot guarantee eggs production under artificial culture conditions. Primordial germ cell transplantation and surrogate broodstock might be employed to improve reproduction, yet no molecular marker for PGCs has been reported in this species. Therefore, information about the tongue sole *vasa* gene (*Csvasa*), a germ cell and PGC molecular marker, would not only broaden our understanding of sex determination but also provide the basic knowledge of PGC labeling, cryopreservation, transplantation, and surrogate broodstock in this species.

In this study, we cloned and characterized cDNA and genomic sequence of *Csvasa*, studied *Csvasa* mRNA expression levels in different tissues and embryonic stages by real-time quantitative PCR (qPCR), and sex-linked differential expression of *Csvasa* was also detected during early developmental and sex differentiation stages. The function of *Csvasa* 3′UTR was also analyzed by microinjecting synthesized mRNA into fertilized medaka eggs.

2. Materials and methods

2.1. Fish and samples

The half-smooth tongue sole used in this study were obtained from Haiyang High-Tech Experimental Base (Haiyang, Shandong Province, China) during the spawning season. Tissue samples (which included heart, liver, gill, skin, blood, kidney, intestine, brain, spleen, muscle, pituitary, and gonads) were collected from 1-year-old tongue sole, and immediately dipped into liquid nitrogen and stored at −80 °C until further analysis. Twenty body trunks (4–66 days post-hatching, dph) and ten gonads (80–150 dph) from different developmental stages of tongue soles were collected and stored in liquid nitrogen. To determine the genetic sex, the fins of these samples were placed in 100% ethanol for DNA extraction. The embryos that were cultured about 40 hours between fertilization and hatching under the temperature of 23 ± 0.5 °C and larvae of different developmental stages were placed in liquid nitrogen and stored at −80 °C until RNA extraction. The sex reversal induction of tongue sole fry by temperature treatment was carried out as previously described [27]. Briefly, the fry at 28 dph were cultured in tanks with a temperature of 28 °C until 100 days and then grown into adults under a natural water temperature.

The work described in this article have been carried out in compliance with the EU Directive 2010/63/EU for animal experiments; http://ec.Europa.eu/environment/chemicals/lab_animals/legislation_en.hem; Uniform Requirements for manuscripts submitted to Biomedical journals http://www.icmje.org.

2.2. Physiological sex and genetic sex identification

The physiological sex of each tongue sole was identified using gonad-specific expression genes (*dmrt1* and *cyp19a1a*) [27]. Genomic DNA of each sample was extracted using the traditional phenol chloroform method [28]. A pair of sex-linked SSR primers (CseF-SSR1 and SChen-1) was used for genetic sex identification [28]. The genetic male produced a 206-bp band and the genetic female produced two DNA bands of 206 and 218 bp. Fish with female genetic sex but male phenotypic sex were regarded as neomales.

2.3. Cloning full-length cDNA and genomic DNA sequences of Csvasa gene

Total RNA was isolated from the gonads of adult half-smooth tongue sole using TRIzol reagent (Invitrogen, USA)

according to the manufacture's instructions. First-strand cDNA was synthesized from 1 μg of total RNA, using transcript first-strand cDNA synthesis kit (Takara, Dalian, China). A 1097-bp cDNA fragment of the *Csvasa* gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using primers Vasa-mid-A and Vasa-mid-S (Table 1), which were designed according to the partial sequence of *Csvasa* from whole-genome and transcriptome sequencing [33]. Then rapid amplification of cDNA ends (RACE)-specific primers Vasa-5'GSP and Vasa-3'GSP (Table 1) were designed based on the partial cDNA fragment. Using a BD SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA), 5′- and 3′-RACE were employed to isolate a full-length cDNA sequence according to the manufacturer's protocol. The PCR conditions were as follows: five cycles at 94 °C for 30 seconds and 72 °C for 3 minutes, five cycles at 94 °C for 30 seconds, 70 °C for 30 seconds and 72 °C for 3 minutes, then 27 cycles at 94 °C for 30 seconds, 68 °C for 30 seconds, and 72 °C for 3 minutes. The amplification products were separated using 1.0% agarose gel using the DL2000 marker. Purified fragments were cloned into pMD18-T simple vector (Takara) for sequencing. Sequences were assembled using the Vector NTI software package (Invitrogen). The *Csvasa* cDNA sequence was analyzed by bioinformatics, and three pair of primers (Vasa-DNA-S1, Vasa-DNA-A1; Vasa-DNA-S2, Vasa-DNA-A2; and Vasa-DNA-S3, Vasa-DNA-A3) (Table 1) were designed to amplify the genomic sequence. Polymerase chain reactions were performed as follows: initial step at 94 °C for 3 minutes, followed by 35 cycles for 30 seconds at 94 °C, 30 seconds at 60 °C, and 3 minutes at 72 °C, and a final extension at 72 °C for 10 minutes. DNA fragments were ligated into pMD18-T vector and sequenced.

2.4. Sequence analysis and alignment

Homology searches were performed using an online Website (http://www.ncbi.nlm.nih.gov/BLAST/). Exon-intron boundaries were determined by comparing *Csvasa* cDNA and DNA sequence. A phylogenetic tree was constructed with Mega 5.2 software using the neighbor-joining method with a bootstrap analysis of 1000 replications [34]. The deduced amino acid sequences were aligned using the AlignX program in Vector NTI software. Sequences used for alignment were as follows: medaka (BAB61047), zebrafish (AAI29276), human (*Homo sapiens*) (AAF72705), mouse (*Mus musculus*) (EDL18409), fruit fly (AAF53438), frog (*Xenopus laevis*) (AAC03114), and chicken (*Gallus gallus*) (BAB12337).

2.5. Real-time quantitative PCR of Csvasa gene

Owing to the difficulty of dissecting the gonads from fish at 4 to 66 dph because of their small size, each body trunk was divided into several parts. The putative gonad parts (4–66 dph) and gonads were identified and confirmed using gonad-specific expression genes (*dmrt1* and *cyp19a1a*), respectively [27]. Thirty embryos or three to six gonads (gonad parts) of the same gender and age were pooled as one sample for RNA isolation. Three samples were collected from each stage for replicate experiments. Total RNA was extracted from each sample of different developmental stages and tissues of six healthy adults and reversely transcribed as described above. cDNA was used as the template for qPCRs. The expression of each sample was performed in triplicate using qPCR. Quantitative PCR was carried out on an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) in 20 μL reactions using SYBR Premix Ex Taq (Takara). Reactions were performed using specific primers (Vasa-RT-A, Vasa-RT-S; and Actin-RT-A, Actin-RT-S) (Table 1) based on the manufacturer's manual, and β-actin was used as the internal reference [35]. Cycling parameters were as follows: 95 °C for 10 seconds, 40 cycles at 95 °C for 5 seconds, 60 °C for 34 seconds, followed by disassociation curve analysis to check amplification specificity. *Csvasa* expression was analyzed using the relative quantification method and 7500 system SDS software (Applied Biosystems).

Table 1
Primers and their sequences used in this study.

Primer	Primer sequences (5′–3′)	Usage
CseF-SSR1	GAGGCCGACAGGATCGTAC	Genetic sex identification
SChen-1	TACGACGTACTCCGGTGGTTTT	Genetic sex identification
P450a-A	CAGGTAGGAGGTTGCTGGGTA	Gonad identification
P450a-S	CAGGAGGAAGAACTTGGGATTT	Gonad identification
Dmrt1-A	GGTGAGGATGTGACCCAGTGT	Gonad identification
Dmrt1-S	ACGGGCTGAAATCGCAAG	Gonad identification
Vasa-mid-A	TGACGACATACTGGTGAACG	Partial fragment clone
Vasa-mid-S	TGGAAATCAGAGGGCGAG	Partial fragment clone
Vasa-5′ GSP	GAGTCGCACAGGGCAGCCTCCTCAAAT	5′ RACE PCR
Vasa-3′ GSP	GCATTGATGAGTATGTTCACCGCATTGGG	3′ RACE PCR
Vasa-DNA-A1	GAATATGGATGACTGGGAAGAGACG	DNA fragment PCR
Vasa-DNA-S1	ACACGGCTCGTGACTGTGAGCTA	DNA fragment PCR
Vasa-DNA-A2	CGTCTCTTCCCAGTCATCCATATTC	DNA fragment PCR
Vasa-DNA-S2	GAATATGGATGACTGGGAAGAGACG	DNA fragment PCR
Vasa-DNA-A3	CATCCATTTATTGGTCCTTATCGTCA	DNA fragment PCR
Vasa-DNA-S3	CCAAAAATGTCAGCAGATCTGGATATG	DNA fragment PCR
Vasa-RT-A	AGGGCGAGACAGATGACCG	qPCR
Vasa-RT-S	CCCAGGCAGCATTGATGAGTA	qPCR
Actin-RT-A	GCTGTGCTGTCCCTGTA	qPCR
Actin-RT-S	GAGTAGCCACGCTCTGTC	qPCR
Vasa3U-A	GGGGTACCGCTTGGATTACATCCATTTATTGG	3′ UTR clone
Vasa3U-S	CCGCTCGAGGAGGAAGAGGAAGAATGGGAGTAG	3′ UTR clone

Each relative expression result was calculated using the $2^{-\triangle\triangle Ct}$ method.

2.6. Preparation of GFP-Csvasa 3′UTR mRNA and microinjection

The 3′UTR of *Csvasa* mRNA was amplified using primers (Vasa3U-A, Vasa3U-S) (Table 1) digested with *Xho*I and *Kpn*I, and cloned into the corresponding site of GFP-*Drnos* 3′UTR (supplied by Doctor Lin [22]), and then designed as the GFP-*Csvasa* 3′UTR vector. The resultant vector was linearized by *Kpn*I digestion for *in vitro* transcription. Chimeric mRNA containing the GFP gene fused to 3′UTR of the *Csvasa* gene was synthesized using a mMessage machine SP6 kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Japanese medaka (*Oryzias latipes*) were maintained and artificially induced for spawning as previously described [22]. GFP-*Csvasa* 3′UTR mRNA (200 ng/μL, 0.05% phenol red) was injected into fertilized medaka eggs at the one-cell stage. Injected embryos were cultured in Holtfreter's solution at 28 °C. To visualize GFP expression, embryos at different stages were observed and photographed under a fluorescence microscope (Nikon, Japan).

2.7. Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Differences in *Csvasa* expression during different development stages or between different tissues were analyzed by one-way ANOVA followed by Duncan multiple comparison tests. Differences in *Csvasa* expression between males and females during the same and different developmental stages were analyzed by two-way ANOVA followed by Duncan multiple comparison tests. Differences between different sexes of the same stage were detected using a Student's *t*-test. The data were tested for normality and log-transformed to ensure homogeneity of variances before performing an ANOVA. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. cDNA and genomic sequences of Csvasa gene

The expected 1097 bp fragment of *Csvasa* was obtained by RT-PCR, and the two remaining regions were produced by 3′- and 5′–RACE. Fragments were cloned and sequenced, overlapping segments were spliced *via* Vector NTI, and a full-length 2445 bp *vasa* cDNA sequence (GenBank ID: KF819358) was isolated. In the present study, the *Csvasa* sequence consisted of a 5′UTR of 100 bp, an open reading frame of 1992 bp, and a 3′UTR of 353 bp containing a polyadenylation signal. The sequence of the open reading frame encodes a 663-amino acid protein. A total of 23 exons and 22 introns were identified by comparing the *Csvasa* cDNA sequence with the 6425 bp genomic DNA fragments (Fig. 1). Exon lengths varied between 21 bp and 452 bp, and all introns followed the GT-AG splicing rule, except the intron 8, which was found to have a GC-AG splice site. In accordance with the bioinformatics analysis, the predicted protein contained eight conserved domains of the DEAD-box protein family, including GYRKPTPIQ (Q motif), AQTGSGKT, GG, TPGR, DEAD, SAT, RGLD, and HRIGRTGR. In addition, the N-terminus comprised 10 arginine-glycine repeats, five arginine-glycine-glycine repeats, and the sequence EARKF. Well conserved tryptophan (W), glutamic acid (E), and aspartic acid (D) residues were also present in N-terminal and C-terminal regions as typical characteristics of Vasa protein (Fig. 2).

3.2. Phylogenetic and alignment analysis

To support the homology of the putative amino acid sequences of Vasa and determine the phylogenetic relationship between tongue sole Vasa (*Cs*Vasa) and other species, a phylogenetic tree was constructed with full-length Vasa sequences from 29 species belonging to six phyla, including Chordata, Nematoda, Cnidaria, Arthropoda, Echinodermata, and Mollusca. Phylogenetic analysis revealed that there were eight main branches, five of them corresponding to invertebrates. The remaining three branches belonged to vertebrates including teleost fish, amphibians, birds, and mammals. *Cs*Vasa belonged to homologues of the teleosts and clustered with Senegalese sole (*Solea senegalensis*) (Fig. 3A). The *Cs*Vasa sequence was aligned with other typical Vasa sequences available in the GenBank by the BLASTP program. The result revealed that the putative *Cs*Vasa shared 43.3% to 71.3% identity with typical Vasa of other organisms (Fig. 3B).

3.3. Csvasa expression in different tissues and during embryogenesis

Real-time quantitative PCR was performed to study *Csvasa* mRNA expression levels in different tissues including heart, liver, gill, skin, blood, kidney, intestine, brain, spleen, muscle, pituitary, and gonads of 1-year-old tongue sole. The sampled fish had ovaries at stage III of development and testes at stage IV. The results showed that *Csvasa* expression was mainly restricted to the gonads, with higher levels in the ovaries. Expression was also observed at a very low level in gill, liver, and heart, but was undetectable in other tissues (Fig. 4).

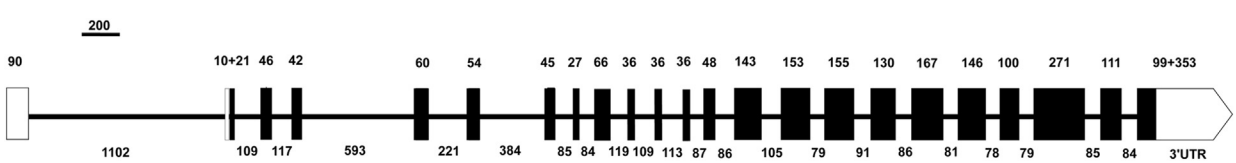

Fig. 1. Schematic representation of the *Csvasa* gene. Exons are represented by boxes (coding region by black box; UTR by blank box) and introns are denoted by black lines. Exon and intron lengths are indicated above and below the schematics, respectively. Scale bar = 200 bp.

```
   1 GCAGAAACGCTCTGCACACGGCTCGTGACTGTGAGCTAGGACAGTGCGGCGAAATCAAGCTGAAGTTTACCTCAGCTTACACTTCACCAG
  91 gtatgatttcgaattaattatatacatgtttaagctttagattattttatttttattatgaaatgttgataccttaatcgtttgttgctcg
 181 agtaaggttaagttcatgtgacatcgtttgctgctaatttttccgccgcttcgaaagaatgttctcgattcttttcttttttttttttagc
 271 gaatgcaccttggtttttttatttcgttttaacttaatgtttgaaagatacatttggtgtttattggtatttgcagctcgttgatttttct
 361 gatctccattgtacgtcaatgtagttacgaagtgaggccaaaagtcttgaccctggttaactacgtaaaaagttaggagttaaagttaat
 451 ttttgaaataatgcactaaataacaacgggtaggttcaaagattgagtgtattgcttttcaaacgtcagtgaaacgctgtaaattagga
 541 gtcgttcgcaagagcacgaggctggatgtgttaaattgcacaaaacttaatttacgtctcgcgatgtttccaatttcccggtatttgttg
 631 cgccctttttgtgaagactacacaaaccagatccccataacggggttatacgatccttttttttaaaccacgtttttttcttttcttttatcc
 721 aagaatccgagacctgtctatgtaggaaaactattcgtgacctgtaatattataaaaattgctcactttcactcagtttgtttacattca
 811 atattcccatttttatctcaatcatatatgtcaaaatcatacttttgaataagcagcttttcattgctgcctttatatgctggttgcacat
 901 acaaattaatcattctttagttttttgtgacctatggaataatttaaattacactactgcctctcactgggtctgctttcttttggcaaa
 991 ctacacctttcagagatgtcctggctgattatacaaaaaaggggggaaatgggcagataaggagaccacatgcagaatagaaaagcagag
1081 tttggcctgtacatgttctgtgtgtttatcaagataactgtaccagaataagtaagggtcatggctgcactgtgtcaaatgtggatgtgt
   1                                   M  D  D  W  E  E  T
1171 ctcattttaatttgcatttcagGCTGTTGAATATGGATGACTGGGAAGAGACGgtatgtttactgtaccaaccagtatcatagaaaacct
   8                                                                                 E  N  A  E  V  V
1261 ttactatgctaattgtgtccaactgcagttctttctaactgtcaacacctgatcaatgacctttgattatagGAAAATGCAGAAGTGGTG
  14 S  V  I  S  C  A  S  T  E  G
1351 TCTGTAATCAGCTGTGCCTCAACAGAAGgtgattttactttccctttctggtttctgtcatttctgggaaaaatattttatgatacatca
  24                                                        S  R  E  H  F  W  K  D  D  S  S
1441 tggtgtttactgctaaaattaaactatgtataactgtaagataacactgtttcagGTTCAAGGGAACACTTCTGGAAAGATGACAGTTCT
  35 G  K  R
1531 GGAAAGAgtaagaaaaaaagagtgaacctgtttgccctttgtgaaccctgcgtaagatttttgtcttaatttgttcaggcagtggaggtt
1621 ggcgaggcaggtggggaagatttacagattctctttttctcaagtaattcatttcaatctatgttgttgaacattttggaaattgctagat
1711 gacttgaaatgggacatagcagtcgctagatggcattttatgtcctggtttggaaaagaagggttggtgttcactagtataagacctgca
1801 tggacagtgcagacaaggagtccggtgataaatacaactgttagaaacattgatttttctctccaaatgttaaataaaactagaatctct
1891 gagatgatgatgtgctttaatctaaaatctggcaaaactactatactaccattctctaaacaattgccacagttgttaatgcttctgcct
1981 aatctgaacagtagagacacaatgaagctatagaagagagatgagtcccacaaacaaaacttaatgaattaatgaaatattaggggtttg
  38                                                                        A  D  E  K  S  N  G  T  G  T
2071 tggctgttgaggcacaaaactaactgggctgttttctctgtcattatgtgccttctgcagGAGCTGATGAGAAAAGTAACGGCACTGGAA
  48  I  T  G  T  S  S  F  N  Q  D
2161 CCATTACAGGAACCTCAAGCTTCAACCAAGgttgagtttgttaatgataatatgttggattttgatttacttaattaagttttgatgttc
2251 tgccacacagtacatgtctggtttgttttccatatgttagaactagttgcaaactatcatatcataactatcgtgtcccttaatccgttt
  58                                                                                 W  E  F  F  K  N
2341 aacgatgacacaatgaaggcttaaatgtaaaaagtcattaccccactgaccctctgatttgtcacaagcagATTGGGAATTCTTCAAGAA
  64  T  E  G  Q  Q  G  G  I  R  G  R  G
2431 CACAGAAGGACAACAAGGTGGTATTAGAGGAAGAGgtgagaataatccgtcacctgcttcaagggacatttaaggcgaactatgtagctc
2521 cctttcctgaaatccgtactgagctcattgttggaccctgtcaataattcacacaatcggcacactcaccattaaagacacaattcaagg
2611 atacaccacaccagtgtttacaatgacgtccctttagctttcaacaaaaaggaatacaagaaacaagacaaattgtgtgaagctagaaac
2701 cttattcaaaatgtttcactctcaaatatgctgtgtgactccctgtgaaattgaaattattcagtgttatttttttaagaattccctgtgt
  76                                                                        R  G  R  G  F  G  R  E  N  R
2791 agtaaatcctcaagattgtgtcattcattcatttcttcaattctatgtttatttgtcagGACGCGGCAGAGGATTTGGGAGAGAGAACAG
  86  S  D  F  N  G
2881 AAGTGATTTCAATGgtatgttcatggttcctacttccttaatgtacattcaaatagcctgctgcactgaacttattctactctttatttt
  91           D  S  N  G  F  C  E  S  E
2971 ttttcacagGAGACAGCAATGGATTTTGTGAAAGCGgcaagtgttatggactatctttatattaatatattctgtttgatattgctaagt
 100                                 F  R  G  V  S  R  G  G  H  G  G  R  G  G  R  G  R  G  F  R
3061 gataataacagtgctggatgatgtgaacagAGTTCAGAGGAGTAAGCCGAGGAGGTCATGGTGGCCGAGGAGGAAGAGGAAGAGGCTTCA
 120  R  G
3151 GACGTGgtatgaactaccatttttttttcactttgtgtaatgatatgtgttcataataggaatatgtcagaaataaccacatccctcatt
 122                                    T  E  G  G  S  R  G  G  F  R  E  D
3241 ggtgccagtcagtgatgttgaccattttgctgcagGCACTGAAGGAGGCTCCAGGGGAGGCTTCAGAGAAGgtaataagatataataaca
3331 tgtttttttaaaaaatattttgtcaatgtgcaaagatatttatttttgtgttgttcagcctattatgattaatgggtaataatttattgcag
 134  G  E  Q  R  G  R  G  G  F  R  G  G
3421 ACGGAGAACAAAGAGGTAGAGGAGGCTTTAGAGGAGgtacaaaaacacttttttttgcccctgtgatccatataaaagtgacgatgataac
 146                                                                        Y  R  G  K  G  E  N  N  F  F
3511 cagtgatgtttcaggagacaagtagcttgtattaatccatggcatttgtgtaacggcagGTTACCGTGGAAAGGGCGAAAATAACTTTTT
 156  Q  E
3601 TCAAGgttgtatgtcaagtatttgtacaaaaatgttttgatggtttaaccttagtaccatttatttttactgaagagactttgtttgatgc
 158     K  E  N  K  E  L  K  Q  R  D  E  G  S  A  D  K
3691 agAGAAAGAAAACAAAGAATTAAAACAGAGGGATGAAGGCAGTGCTGACAgtaagtagatctgttcactgtactgtaaccagttctgcag

 174                                                  P  K  V  S  Y  I  P  P  A  L  A  E  D  E
3781 tgggattgttcctcagcttactctttcctcctgctctgacccacagAGCCAAAAGTCAGTTATATCCCTCCAGCCCTCGCTGAGGATGAA
 188 N  S  V  F  A  H  Y  E  T  G  I  N  F  D  K  Y  D  D  I  L  V  N  V  S  G  T  N  P  P  Q
```

```
3871 AACTCAGTTTTTGCTCACTATGAGACTGGAATCAATTTTGACAAGTATGACGACATACTGGTGAACGTCAGTGGAACCAACCCGCCACAG
 218 A  I  M
3961 GCAATAATGgtatgatcaaatgcagtatgtctagactgaaattatgcataagagctacaaatctgccccccctgcccccaccctcctgacg
 221                        T  F  E  E  A  A  L  C  D  S  L  S  K  N  V  S  R  S  G  Y  V  K
4051 ccactgcatgtttactgtgtttagACATTTGAGGAGGCTGCCCTGTGCGACTCTTTATCCAAAAATGTCAGCAGATCTGGATATGTCAAG
 243 P  T  P  V  Q  K  H  G  I  P  I  I  S  A  G  R  D  L  M  A  C  A  Q  T  G  S  G  K  T
4141 CCGACTCCTGTACAGAAGCACGGCATTCCTATCATTTCTGCTGGCAGAGATCTCATGGCCTGTGCACAGACGGGGGTCTGGAAAAACGgtg
 272                                                                                    A  A  F  L  L
4231 agatgagagcagagttgaatttaacatgagcagtaaatgtgattagatcgaaatgaaattcctcgtccttctgcagGCTGCATTCCTGCT
 277  P  I  L  H  Q  L  M  I  G  G  A  S  S  S  F  F  S  E  L  Q  E  P  K  A  I  I  V  A  P  T
4321 GCCCATCCTGCATCAGCTGATGATAGGCGGAGCATCATCATCTTTCTTCAGTGAGCTGCAGGAGCCTAAGGCCATTATTGTGGCTCCAAC
 307  R  E  L  I  N  Q  I  F  L  E  A  R  K  F  A  Y  G
4411 CAGGGAGCTTATAAACCAGATTTTTCTGGAGGCCAGGAAATTTGCCTATGGgtataatttttataacaaactgtggctatgattaaaaaaa
 324                                                    T  V  V  R  P  V  V  V  Y  G  G  V  S
4501 caaacaacattgaggtaataaaagagctgcttctgcttcttctttctcccagAACTGTTGTGCGTCCAGTGGTTGTTTACGGTGGAGTCA
 337   I  R  H  Q  V  S  D  L  N  K  G  C  N  V  L  C  G  T  P  G  R  L  M  D  M  I  E  R  G  K
4591 GTATTAGACACCAGGTTAGTGACCTTAACAAGGGATGTAATGTCCTTTGTGGAACACCAGGGCGGCTGATGGACATGATAGAGAGAGGAA
 367                                                                                            V
4681 AGgtaaagataaacaaaaggatttgcaactgcttgagtttgttgtcattataaagggtctgaatacggatgctgtgtgatcatcttagGT
 368  G  L  S  K  V  Q  Y  F  V  L  D  E  A  D  R  M  L  D  M  G  F  E  P  D  M  R  R  L  V  A
4771 TGGGTTGAGCAAGGTGCAATACTTTGTTCTGGATGAGGCTGATCGAATGTTGGACATGGGCTTCGAGCCAGATATGCGCCGCCTGGTTGC
 398  S  P  G  M  P  S  R  E  G  R  Q  T  L  M  F  S  A  T  F  P  E  E  I  Q  K
4861 CTCTCCTGGAATGCCATCTAGGGAGGGCCGTCAGACACTGATGTTTAGCGCTACCTTCCCTGAGGAAATCCAGAAgtatgagcctttatc
 423                                                                    L  A  A  D  F  L  K  P
4951 tgctgcgattattttaactgttaaggagaaacatatgtttcacacttttttctttttttctttttaaagACTTGCAGCGGACTTCCTCAAGCC
 431  D  Y  L  F  L  A  V  G  I  V  G  G  A  C  S  D  V  E  Q  T  F  I  E  V  G  K  F  S  K  R
5041 AGACTATCTCTTCTTGGCTGTCGGAATAGTGGGTGGAGCTTGCAGTGACGTAGAGCAGACGTTTATCGAAGTTGGCAAGTTCTCAAAGAG
 461  E  Q  L  L  D  L  V  K  T  C  G
5131 GGAACAGCTTCTTGATCTTGTGAAGACATGTGgtacttttttattttagttttttttttagcttattcctgccacagacagatttctcc
 472                       N  E  R  T  I  V  F  V  E  K  K  R  Q  A  D  F  I  A  T  F  L  C  Q
5221 aatacagtctaatgttgcagGAAATGAGCGCACCATTGTGTTTGTGGAGAAGAAGAGACAGGCGGATTTTATCGCCACATTCCTGTGTCA
 495  E  N  V  P  T  T  S  I  H  G
5311 GGAGAATGTTCCAACCACCAGCATTCATGGgtacgttacatgttcaacacttgtacagcaactactctggacttgtataggtgtaataat
 505                     D  R  E  Q  W  Q  R  E  Q  A  L  A  D  F  K  Y  G  K  C  P  V  L  V  A
5401 attttatttttgtactgcagGGACCGTGAGCAGTGGCAGCGAGAGCAGGCCTTAGCAGACTTTAAATACGGCAAATGTCCAGTCCTGGTTG
 529   T  S  V  A  A  R  G  L  D  I  P  D  V  Q  H  V  V  N  F  D  L  P  G  S  I  D  E  Y  V  H
5491 CAACCTCTGTGGCAGCCCGCGGTCTGGATATTCCTGATGTTCAGCATGTGGTCAACTTCGACCTCCCAGGCAGCATTGATGAGTATGTTC
 559   R  I  G  R  T  G  R  C  G  N  V  G  R  A  V  S  F  F  D  T  D  V  D  G  H  L  S  R  P  L
5581 ACCGCATTGGGAGAACTGGCCGCTGTGGGAACGTGGGTAGAGCGGTGTCTTTCTTTGACACAGATGTTGACGGTCATCTGTCTCGCCCTC
 589   I  S  I  L  S  K
5671 TGATTTCCATCCTGTCGAAGgtgagagcttggaactgggctatttttttccaaaagtgatttaaaaggaaaaactccagtggtcatgtttg
 595                A  Q  Q  E  V  P  P  W  L  A  Q  L  A  F  S  S  A  S  S  G  G  N  F  N  A
5761 agttgtcattgacagGCTCAGCAGGAGGTGCCTCCTTGGTTGGCGCAGTTGGCTTTTAGCAGTGCCAGCTCTGGAGGAAACTTCAACGCC
 620 N  K  N  N  F  A  F  T  D  S  R  K
5851 AACAAGAACAACTTTGCTTTTACAGATTCCAGGAAGgtaacagtaccaagatcagccttttttattttcagttctctgtgcttttctctc
 632                              G  Q  Q  R  G  S  S  F  Q  G  N  T  V  Q  S  Q  V  T  A  V
5941 agtgtttttttctatttccttttgtttagGGTCAGCAAAGAGGATCATCTTTTCAGGGCAACACTGTGCAGAGCCAGGTGACAGCTGTG
 652 L  Q  P  P  P  E  E  E  E  E  W  E  *
6031 CTCCAGCCTCCACCTGAGGAAGAGGAAGAATGGGAGTAGAGAAATTACATGGAGCAGCAGTGCCACACATTGACCAGTTTAGTTGTTGTT
6121 TTTCATTTGTTGCAGTGTGTGGAGTTTTATCACAGTATTTTTGCTACTGTGAACAAAAAAGACAAAAATGTTTATAATGTCATGTAAGAT
6211 TCTGAGATTCAATCTGTTGTGGTTATACCAAAGGTTTTTTTTCCCCGCAAAAGTAGAAGTGAAAATGTGAATTGACGACAAAAGGGGTAA
6301 AACCACTTCATGTCAGAGGAGACAACCATGGGATTTCTTTTATTCTTCTGTTTTTTTGATAAATAATTGCATTAAAATGGTTAATGTCTT
6391 GACGATAAGGACC AATAAA TGGATGTAATCCAAGC AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. (*continued*).

To investigate the expression differences of *Csvasa* mRNA in gonads of males, females, and neomales after temperature treatment, expression of *Csvasa* mRNA was detected by qPCR in fish 2 years old. The ovaries of 2-year-old sampled fish were at stage IV to V of development and testes were at stage V. The number of sperms in the neomale testis was less than that in the normal testis. Lower expression levels of *Csvasa* mRNA were detected in the neomales, and relative higher levels were detected in males and females (Fig. 5).

Fig. 2. Genomic and deduced amino acid sequence (GenBank accession number:KJ664813 and KF819358) of *Cs*Vasa. Exons and introns are indicated in uppercase and lowercase, respectively. Shaded boxes represent the eight conserved motifs of DEAD-box protein family. Arginine-glycine (RG) and arginine-glycine-glycine (RGG) repeats in the N-terminus are marked by underline and double underline, respectively. The tryptophan (W), aspartic acid (D), and glutamic acid (E) residues in the C-terminus and N-terminus are shown inside boxes. The stop codon is indicated by an asterisk. The polyadenylation signal and poly-A tail are in bold.

To understand the expression patterns of *Csvasa* mRNA during embryonic development, expression levels were detected by qPCR. The results showed that *Csvasa* expression persisted throughout all embryonic stages, and expression levels gradually decreased from four-cell to hatching stage. Expression levels were high in embryos before the late gastrula stage and dropped abruptly from the late gastrula stage to the neurula stage. Expression levels decreased from the somites stage to the hatching stage, and much lower expression levels were detected at 1 dph (Fig. 6).

3.4. Sexually dimorphic expression of Csvasa during early development and sex differentiation

Sexually dimorphic expression of *Csvasa* was observed by qPCR from 4 to 150 dph. *Csvasa* expression levels gradually decreased from 4 to 16 dph in males and females, the lowest expression level in females was detected at 16 dph, and in males was detected at 46 dph. Expression levels increased continuously in both sexes until 150 dph. An interesting sexually dimorphic expression of *Csvasa* was observed during early development and sex differentiation, the expression levels in males were higher than that in females from 4 to 26 dph, and then females exhibited higher expression levels than males from 36 to 115 dph, but males showed higher expression levels again at 150 dph (Fig. 7).

3.5. Visualization of medaka PGCs by microinjection of GFP-Csvasa 3′UTR mRNA

In medaka fertilized eggs injected with GFP-*Csvasa* 3′UTR mRNA, GFP fluorescence in PGCs was observed over the whole blastodisc at stage 20 and was difficult to differentiate from that of somatic cells in the early segmentation period (Fig. 8A). Relatively strong GFP fluorescence in PGCs was observed (Fig. 8B, C) in presumptive gonad region aligned bilaterally along the trunk as the embryo developed, and the fluorescence signal gradually diminished after stage 29 (Fig. 8D). Primordial germ cells in medaka can be visualized by microinjection of GFP-*Csvasa* 3′UTR mRNA during embryogenesis.

4. Discussion

In the present study, a full-length 2445 bp cDNA and 6425 bp genomic DNA sequences of the *Csvasa* gene were isolated and analyzed. The length of *Csvasa* cDNA sequence in the N-terminus is shorter than another cDNA sequence (2602 bp) from GenBank (GenBank ID: HQ727561). The *Cs*Vasa sequence contained eight characteristic domains of the DEAD-box protein family, which are essential for ATPase-dependent RNA binding and unwinding activity [36,37]. Rich arginine-glycine and arginine-glycine-glycine repeats, G residues, and the EARKF sequence at the N-terminal region were observed. In the C-terminal region, six of the last eight amino acids were glutamic acid (E) residues, as observed in several single-stranded nucleic acid binding proteins [36]. These typical features indicated that the *Cs*Vasa belonged to a member of DEAD-box family. Phylogenetic analysis indicated that the *Cs*Vasa was closely aligned to the teleosts. Multiple sequence alignments showed that *Cs*Vasa had a considerable identity with other typical homologues. These results together provide further evidence that the *Cs*Vasa is a member of the Vasa homologues with an ATP-dependent RNA helicase function and suggests high conservation during evolution.

Tissue distribution analysis of *Csvasa* was performed by qPCR, and the gonadal stages of adult fish were determined as previously described [38,39]. The results showed that the *Csvasa* mRNA was mainly detected in gonads. Specific expression of the *vasa* gene in germ cells was verified to play a prominent role in germline development [40]. Except for the gonads, very low expression was observed in the gill, liver, and heart of tongue sole. *Vasa* mRNA expression has been reported in the heart and brain of rainbow trout (*Oncorhynchus mykiss*) [13] and the kidney and brain of an adult frog species [41]. This could be because Vasa is implicated in translational regulation of mRNAs involved in the specification and differentiation of tissue-specific cell types through its helicase activity [42]. It is possible that the role of *vasa* might be multipotent rather than confined to the germline. Tongue sole subject to high temperatures during early development and sex differentiation showed a high male phenotypic sex ratio (about 70%). Lower levels of *Csvasa* mRNA in neomales were detected and compared with normal fishes, and the results suggested that *Csvasa* expression might be restrained and the *Csvasa* gene might be implicated in germ cell development after sex reversal. We inferred that the *Csvasa* expression pattern might be more related to the genetic sex than to the physiological sex, and lower expression in neomales is also probably due to worse development of gonads under high temperature treatment. The exact reason will need to be further studied.

Expression patterns of *vasa* mRNA during embryogenesis have been studied using RT-PCR and whole-mount *in situ* hybridization for many teleosts, such as zebrafish [4], grass carp (*Ctenopharyngodon idella*) [8], Japanese flounder (*Paralichthys olivaceus*) [10], Atlantic cod (*Gadus morhua*) [15], turbot (*Scophthalmus maximus*) [16], and catfish (*Clarias gariepinus*) [43], and the consistent conclusion was that *vasa* mRNA was a maternally supplied transcript. For example, the expression levels in the grass carp during the early embryonic stages were high, even in fertilized eggs, and these high levels decreased gradually during cleavage and blastula stages and continued to decrease to lower levels until the gastrula stage. Four *vasa* transcripts have been isolated from Senegalese sole, *Ssvasa*1 and *Ssvasa*2 were maternally supplied, *Ssvasa*3 and *Ssvasa*4 were dependent on the *de novo* expression of the growing juveniles, which suggests that expression patterns of *vasa* mRNA could vary [11]. In the present study, the results suggest that the *Csvasa* transcript was also maternally supplied, as it was abundant in embryos before the late gastrula stage and decreased gradually from the late gastrula stage to the hatching stage, and expression levels from somites to the hatching stage were much lower than those of cleavage stages. Decreased expression levels of *Csvasa* might be from degradation of maternal *vasa* mRNA or a reduced PGC ratio caused by a rise in the total number of embryonic cells. Further studies are needed to determine the reasons.

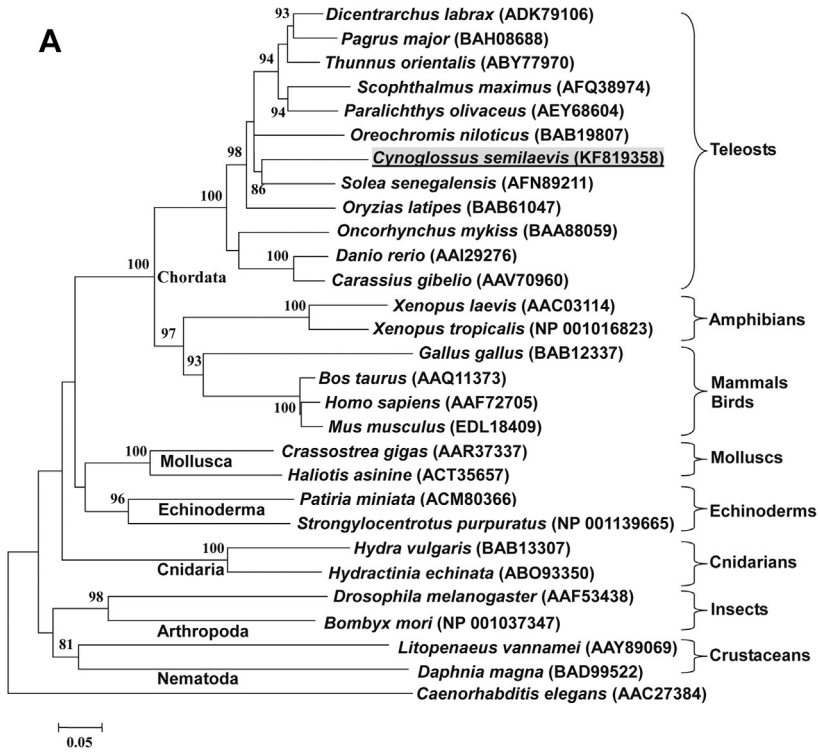

Fig. 3. Multiple alignment and phylogenetic tree of *Cs*Vasa and other DEAD-box family proteins. (A) Phylogenetic tree of *Cs*Vasa constructed by MEGA 5.1 using the neighbor-joining method. Bootstrap values of 1000 replicates (>80%) are indicated next to the branches. The sequences used for alignment are as follows: *Drosophila melanogaster* (*DmVasa*, AAF53438); *Homo sapiens* (*HsVasa*, AAF72705); *Mus musculus* (*MmVasa*, BAA03584); *Gallus gallus* (*GgVasa*, BAB12337); *Xenopus laevis* (*XlVasa*, AAC03114); *Danio rerio* (*DrVasa*, AAI29276); and *Oryzias latipes* (*OlVasa*, BAB61047). (B) Multiple alignment was generated with the AlignX program. Eight conserved motifs are framed in block. The identities of *Cs*Vasa to other Vasa proteins are indicated following the sequence alignment.

To understand the expression patterns of *Csvasa* in males and females, respectively, the sex linkage microsatellite marker and gonad-specific expression genes were used to distinguish sex and gonad parts. This method was used because sex could not be distinguished by the morphological characteristics and gonads could not be isolated during early development. In addition, the PGC proliferation and gonad sex differentiation have been previously described [44]. Expression of *Csvasa* mRNA gradually decreased from 4 to 16 dph in both sexes, except for the degradation of maternal *vasa* mRNA, a possible reason for the expression is that the amount of the maternally inherited *vasa* transcript became diluted with individual growth. Interestingly, the lowest expression level in females was observed at 16 dph, and males at 46 dph. We inferred that prolonged degradation of maternal *Csvasa* mRNA might be caused by higher expression in male larvae, and the *Csvasa* might play a more significant role in males before sex differentiation. Expression levels increased gradually in both sexes, and the primary gonads enlarged concomitant with the start of PGC division and mass proliferation at this stage. Similar expression trends have been reported in tilapia (*Oreochromis niloticus*) [45], zebrafish [46], and European sea bass (*Dicentrarchus labrax*) [9], suggesting a switch from maternal to *de novo vasa* expression. Expression levels in females were higher than those in males from 36 to 115 dph, which was probably because histological differentiation of the gonads in females was faster and earlier than that in males [47]. In European sea bass and catfish, it was reported that *vasa* expression levels in females were always higher than those in males during early development and sex differentiation [9,43]. It is, however, exceptional that the expression levels in male tongue sole were higher than the expression levels in females at 150 dph. The histological results suggest that histological differentiation in females happened earlier than in males, but cytological differentiation was faster in males than in females [47]. Although males were immature at 150 dph, a small population of sperm was observed by chance during sampling. This is consistent with the phenomenon that sexual maturity happens earlier in male than in female tongue sole.

Vasa mRNA expression levels during gonad differentiation have been reported for several fish species [9,43–46]. However, accurate quantification of *vasa* mRNA levels in males and females during early development and sex differentiation using the sex-linked microsatellite marker and gonad-specific expression genes has been carried out in very few fish species [48]. Results of the present study suggest that *vasa* expression presented a sexually dimorphic pattern during this stage and that *vasa* gene might play a differential role in the proliferation and differentiation of male and female PGCs. Therefore, this information on *Csvasa* expression during the early development and sex

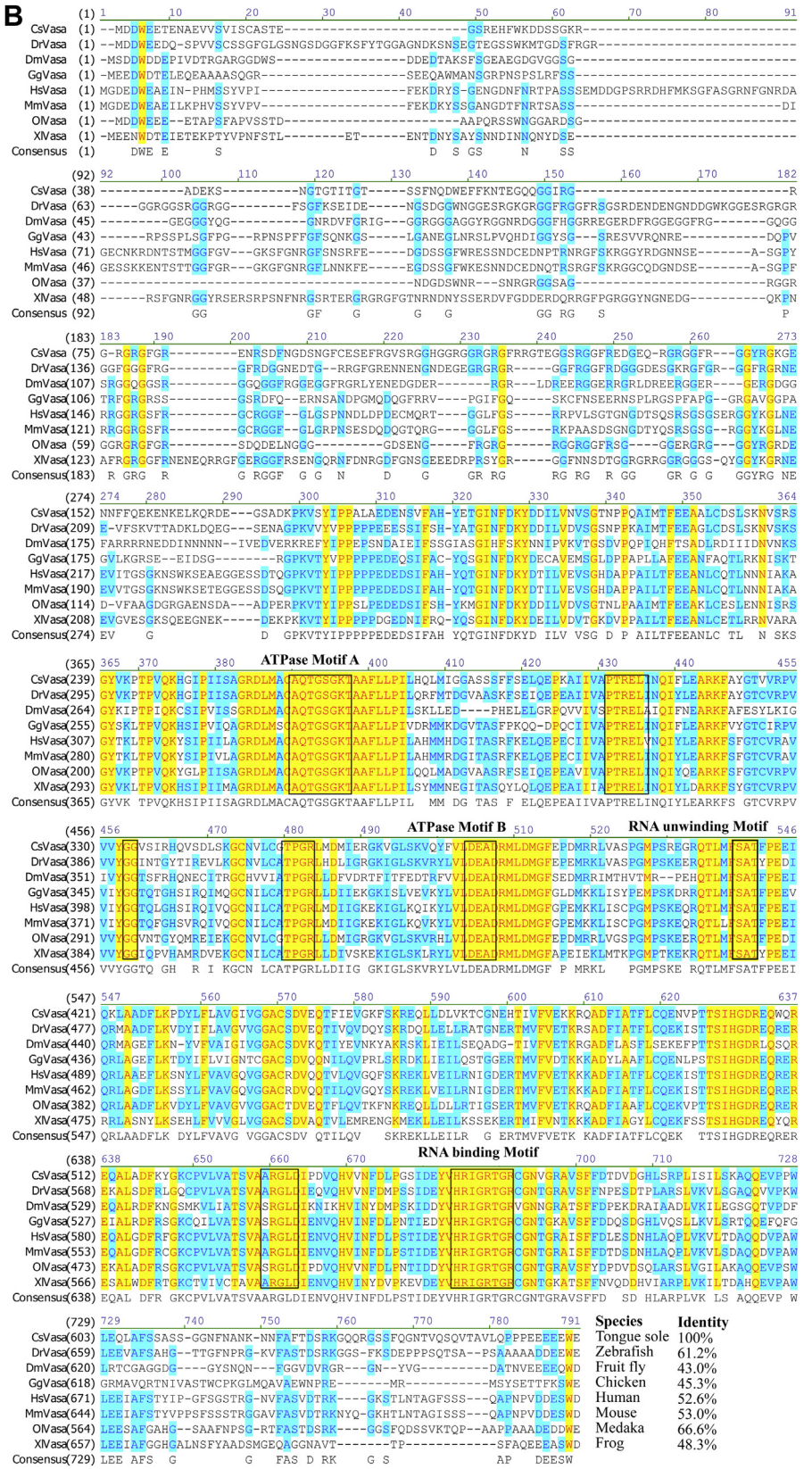

Fig. 3. (*continued*).

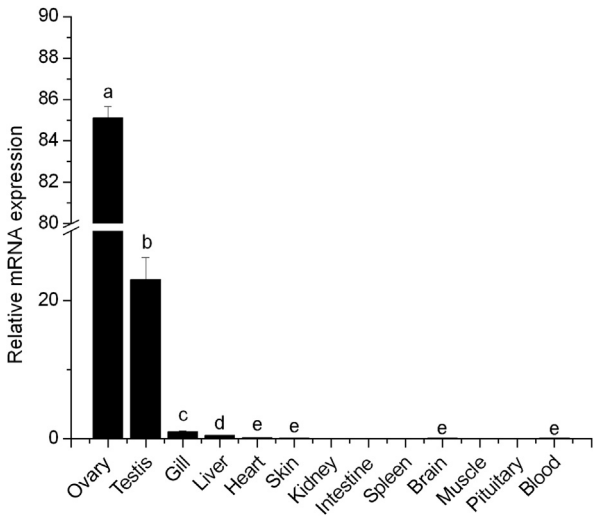

Fig. 4. The relative expression of *Csvasa* in various adult tissues. The data represent the mean normalized gene expression levels against the levels of β-actin mRNA ± the standard error of mean (SEM) from three separate individuals performed in triplicate (three biological replicates for gonads and six replicates for other tissues). Values with different superscripts differed with statistical significance ($P < 0.05$), calculated by one-way ANOVA followed by Duncan's multiple range tests using SPSS 17.0.

differentiation collected from this study may aid in the understanding of sex determination in half-smooth tongue sole.

Recently, PGC visualization and labeling in fish embryo has attracted considerable attention. However, a marker to identify PGCs of economically important flatfish species (e.g., tongue sole) is still lacking. Analysis of *vasa* transcriptional regulatory region contributes to the study of germline cells. The 3′UTR of *vasa* has an important function in the stabilization of mRNA; this being that it can eliminate mRNA from somatic cell lines by microRNA activity and stabilize mRNA in PGCs of several fish species. Embryonic injection of mRNA containing 3′UTR and the GFP gene have been used to

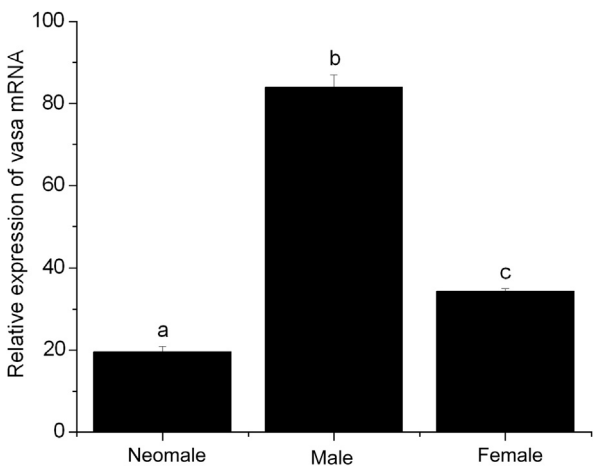

Fig. 5. The relative expression of *Csvasa* mRNA in the gonads of neomales, normal males, and females. The data represent the mean ± SEM from three separate individuals ($n = 3$). Values with different superscripts differed at $P < 0.05$.

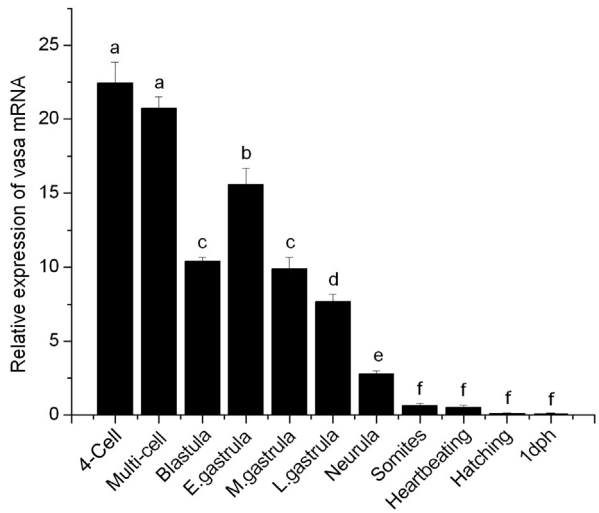

Fig. 6. The expression profile of *Csvasa* at different developmental stages of the embryos. Each sample consisted of a pool of 30 embryos, a pool of 30 larvae (1 dph). The data represent mean ± SEM from three separate samples ($n = 3$). Values with different superscripts differed at $P < 0.05$.

visualize and label PGCs in zebrafish [49], rainbow trout [20], medaka [50], and Atlantic salmon [17]. In the present study, GFP-*Csvasa* 3′UTR mRNA was injected into fertilized embryos of medaka. The results indicated that the GFP signal intensity gradually decreased in somatic cells because of the degradation of injected chimeric RNA; however, the intensity became clearer and larger in the presumptive gonad region. This indicated that GFP-*Csvasa* 3′UTR mRNA was specifically stabilized and expressed in PGCs of medaka during embryogenesis. These data confirmed the function of *Csvasa*

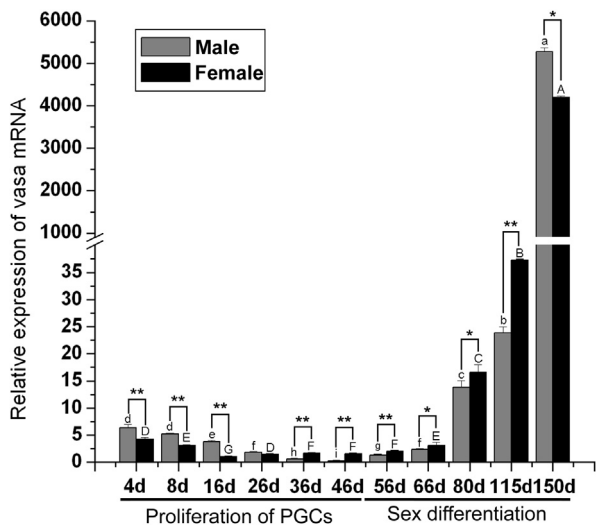

Fig. 7. The expression profile of *Csvasa* at different developmental stages of the gonads. The data represent mean ± SEM from three separate samples ($n = 3$). Each sample consisted of a pool of six body trunks (4–66 dph) and a pool of three gonads (80–150 dph). Values with different superscripts differed at $P < 0.05$, calculated by two-way ANOVA followed by Duncan's multiple range tests using SPSS 17.0. Asterisks show statistical significance between sexes (* $P < 0.05$; ** $P < 0.01$) at each sampling time after a Student's *t*-test.

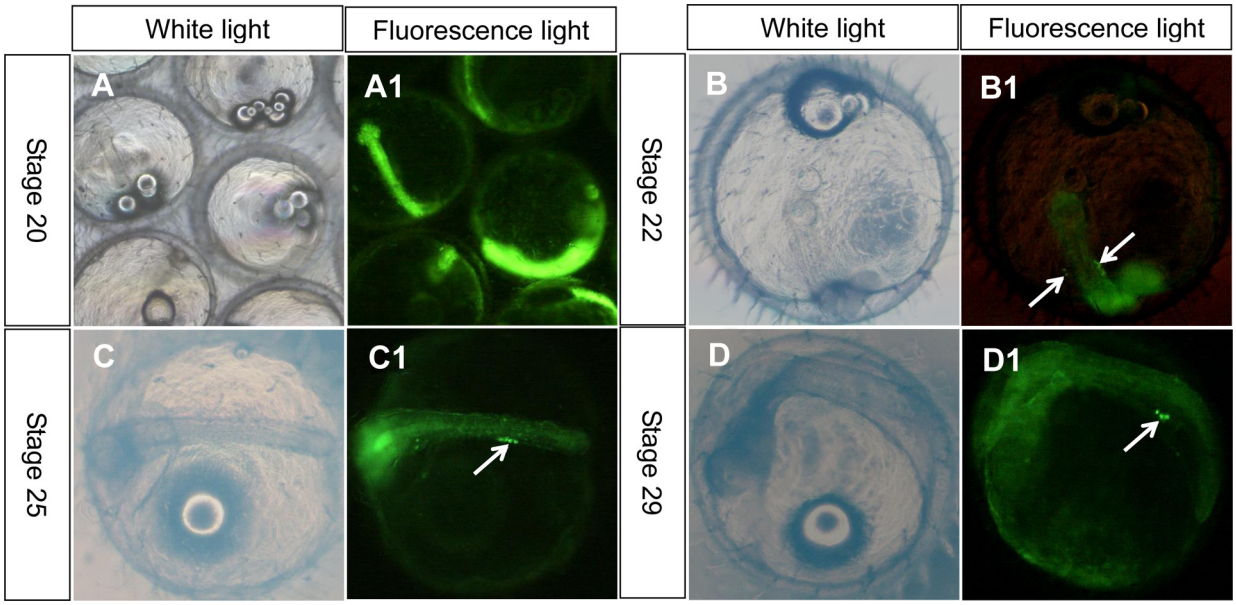

Fig. 8. Green fluorescence protein–labeled PGCs observed by microinjection of GFP-*Csvasa* 3′UTR mRNA in medaka embryos. PGCs are denoted by white arrows. (A–D) embryos of stage 20, stage 22, stage 25, and stage 29 were observed under light and fluorescence microscopy.

3′UTR, and will be helpful in the study of PGC visualization, cryopreservation, and transplantation for tongue sole.

In conclusion, the *Csvasa* gene was cloned and characterized in the present study. *Csvasa* mRNA was a maternally supplied transcript during the embryonic stage, and it was mainly observed in gonads of adult fish. Based on the accurate distinction of sex and gonads, typical sexually dimorphic expression of *Csvasa* was detected by qPCR during early development and sex differentiation. The PGC labeling function of *Csvasa* 3′UTR was verified in medaka embryos. Our results confirmed that the *Csvasa* gene may possibly be appropriate as a germ cell marker. In future studies, the *Csvasa* gene will be used as a molecular marker to illuminate the processes of PGC formation, migration and differentiation, and investigate PGC biotechnology for tongue sole reproduction.

Acknowledgments

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Competing interests

The authors declare that there are no conflicts of interest.

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