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Cloning and Expression Analysis of a Giant Gourami *Vasa*-Like cDNA

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Molecular marker is useful in the development of testicular cells transplantation for detecting donor-derived germ cells in the recipient gonad. In this study, a giant gourami (*Osphronemus goramy*) *vasa*-like gene (GgVLG) was cloned and characterized for use as a molecular marker for germ cells in this species. Nucleotide sequence analysis revealed that GgVLG comprises 2,340 bps with an open reading frame of 1,962 bps encoding 653 amino acids. The deduced amino acid sequence contained 17 arginine-glycine or arginine-glycine-glycine motifs and eight conserved motifs belonging to the DEAD-box protein family. The GgVLG sequence showed high similarity to *Drosophila vasa*, common carp *vasa* homolog and tilapia *vasa* homolog for 66.2, 85.9, and 90.7%, respectively. In adult tissues, the GgVLG transcripts were specifically detected in ovary and testis. *In situ* hybridization analysis showed that GgVLG mRNA was detected in oocytes of the ovary and spermatogonia of the testis. There was no signal detected in the spermatocytes, spermatids and other gonadal somatic cells. Thus, consensus sequences, specific localization of GgVLG mRNA in the germ cells, amino acid sequence similarity and phylogenic analysis all suggest that GgVLG is the giant gourami *vasa*-like gene. Further, GgVLG can be used as a molecular marker for giant gourami germ cells.

Key words: germ cell transplantation, ovary, spermatogonia, testis, giant gourami, *vasa*

INTRODUCTION

Giant gourami (*Osphronemus goramy*) is an important freshwater cultured fish species in Java and Sumatera. The Directorate General of Aquaculture has programmed to increase significantly production of this species (Department of Marine Affairs and Fisheries, 2005). One of the obstacles to meet the production target is seed availability from hatchery. Breeders cultivate fry for 2-3 years to acquire first sexual maturity broodstock. Further, induced maturation and artificial spawning to control seed production of giant gourami remain to be developed. Currently, fry is produced by natural spawning in pond. This seed production system involves maintenance of giant gourami broodstock, which requires considerable space, cost, and labor. Consequently, the need therefore exists to establish a novel method for seed production of giant gourami.

A technique for fish germ cell transplantation using primordial germ cells (PGCs) or spermatogonia (SG) as donor germ cells had recently been developed (Okutsu *et al*. 2006). Donor germ cells are microinjected into the peritoneal cavities of newly hatched embryos. They subsequently migrate toward and colonize the genital ridges of the recipient embryos. Furthermore, donor-derived germ cells proliferate and differentiate into mature eggs and sperm in the allogeneic (Takeuchi *et al*. 2003; Okutsu *et al*. 2006) and xenogeneic recipient gonads (Takeuchi *et al*. 2004; Okutsu *et al*. 2007); the resulting gametes produce live fry through fertilization. Thus, if the giant gourami germ cell could be transplanted into well-controlled reproduction and smaller fish species such as Nile tilapia, then giant gourami gametes might more easily and rapidly be produced in surrogate Nile tilapia kept in aquaria. Hence, the maintenance of giant gourami broodstock in pond would no longer be required.

Identification and isolation of SG containing spermatogonial stem cell (SGSC) population is necessary to prepare the germ cell of giant gourami for transplantation studies. However, little is known about the number and localization of SG in giant gourami testis. We noted that *vasa* gene homologs have been found to express specifically in the germ cell linage of taxa ranging from insects to mammals (Raz 2000). In the teleosts examined to date, the *vasa* gene was observed to express in the premeiotic germ cells of zebrafish (Olsen *et al*. 1997; Yoon *et al*. 1997), medaka (Shinomiya *et al*. 2000), tilapia (Kobayashi *et al*. 2000), rainbow trout (Yoshizaki *et al*. 2000), gibel carp (Xu *et al*. 2005), goldfish (Xu *et al*. 2005), gilthead sea bream (Cardinali *et al*. 2004), shiro-uo (Miyake *et al*. 2006), swamp eel (Ye *et al*. 2007), and Pacific bluefin tuna (Nagasawa *et al*. 2009). The *vasa* gene is thus an excellent candidate for use as a general molecular marker of PGC and SG. Therefore, we cloned a giant gourami *vasa*-like gene (GgVLG) complementary DNA (cDNA) and analyzed GgVLG mRNA expression using farmed giant gourami as the first step towards establishing germ cell transplantation in giant gourami.

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MATERIALS AND METHODS

Fish. Ten immature giant gourami fish, *Osphronemus goramy* were obtained from National Center for Development of Freshwater Aquaculture, Sukabumi. Body weight of fish was 1.03 ± 0.17 kg (mean ± standard deviation).

RNA Isolation and Synthesis of cDNA. Testes were excised from male giant gourami with gonadosomatic indexes (GSIs) of 0.0089%. The testes were homogenized and used for total RNA extraction using Isogen reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized using the Ready-To-Go You-Prime First-Strand Beads Kit (GE Healthcare UK Ltd., England) with an oligo (dT) primer (5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT TTT TTT TTT TTT TTT TTT-3') according to the manufacturer's instructions.

Cloning of the *Vasa* cDNA Fragment by Reverse Transcription Polymerase Chain Reaction (RT-PCR). RT-PCR was performed with degenerate primers as reported by Nagasawa *et al*. (2009). Primer mix-*vasa*-Fw: 5'-TYCTDCARCAGYTGATGG-3' and mix-*vasa*-Rv: 5'-TCAAACTTSCK-GGCYTCMA-3' were designed using the highly conserved regions of *vasa* homologs from eight fish species with the following GenBank accession numbers: butterfly fish (*Pantodon buchholzi*): AF479823, gilthead sea bream (*Sparus aurata*): AF520608, medaka (*Oryzias latipes*): AB063484, Nile tilapia (*Oreochromis niloticus*): AB032467, rainbow fish (*Melanotaenia fluviatilis*): AF479824, rainbow trout (*Oncorhynchus mykiss*): AB032566, shiro-uo (*Leucopsarion petersii*): AB098252, tetra (*Hyphessobryon ecuadoriensis*): AF479821, and zebrafish (*Danio rerio*): NM_131057).

The PCR reaction was conducted at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 20 s at 56 °C and 30 s at 72 °C, followed by a final extension step of 72 °C for 3 min. The cDNA was amplified using Takara Ex *Taq* (Takara Bio Inc., Shiga, Japan). PCR products were electrophoresed on a 2.0% agarose gel, and the cDNA fragments that showed the predicted molecular weight were isolated using an UltraClean-15 DNA Purification Kit (MO BIO Laboratories, Inc., CA, USA). The purified cDNA fragments were subcloned into a pGEM T-Easy plasmid vector (Promega, WI, USA), and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). Sequence determination was performed on ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Cloning of Full-length *Vasa* cDNA. The 3' and 5' rapid amplification of cDNA ends (RACE) were performed to isolate a full-length cDNA sequence. After determining the DNA sequence of a partial *vasa* cDNA fragment, two *vasa*-specific primers (Fw-3'-RACE1: 5'- TGA GAC TGT TGG ATG TGA TCG GAA GA -3', Fw-3'-RACE2: 5'- TAA GCT GAG GTA CCT GGT GCT AGA -3') was synthesized for use as the forward primer for 3'-RACE, and adapter primers (AP1: 5'-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3', AP2: 5'-CTA TAG GGC ACG CGT GGT-3') were used as the reverse primers for 3'-RACE. PCR reactions were performed according to the method described previously (Yoshizaki *et al*. 2000). 5'-RACE was carried out using a GeneRacer Kit with SuperScript III RT (Invitrogen, CA, USA) according to the manufacturer's instructions. Two primers for giant gourami *vasa* cDNA (Rv-5'-RACE1: 5'-GCT GCC ACT CCG TCT GCC ATC A-3', Rv-5'-RACE2: 5'-GCA GCC GTT TTG CCC GAT CC-3') were synthesized for use as reverse primers for 5'-RACE. The cDNA was amplified using Takara LA *Taq* (Takara Bio Inc., Shiga, Japan). We estimated the molecular weight and *pI* of giant gourami *vasa* homolog using a Compute *pI*/Mw tool (http://au.expasy.org/tools/ pi_tool.html). Moreover, its similarity and identity were calculated by LALIGN (http://www.ch.embnet.org/softwareLALIGN_form.html) using *vasa* homolog sequences from other species. The LALIGN programs compare two sequences and look for local sequence similarities.

Phylogenetic Analysis. A homology search of the deduced amino acid sequence of the obtained cDNA was carried out using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLASTP). The deduced amino acid sequences were aligned using Genetyx version 7, and phylogenic tree were constructed by the unweighted pair-group method with arithmetic mean (UPGMA) method.

RT-PCR Analysis. Total RNA extraction and cDNA synthesis were performed using various organs (gill, fin, muscle, liver, intestine, testis, and ovary) of immature giant gourami as described above. The PCR reaction was conducted with giant gourami *vasa*-specific primers. The forward primer was located between nucleotides 912 and 937 (Fw-PCR: 5'- GTT CCT GCT CCC AAT TCT GCA GCA G -3'), while the reverse primer was located between nucleotides 2,296 and 2,319 (Rv-PCR: 5'-ACG TTC TGT CTG TCA GAC ACA TTG-3'). The PCR reaction was performed at 94 °C for 3 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 1.30 min at 72 °C, followed by a final extension step of 72 °C for 3 min. The cDNA was amplified using Takara Ex *Taq* (Takara Bio Inc., Shiga, Japan). β-actin gene expression also was analyzed as an internal control for equal loading of RNA with a set of primers; forward (5'-GAC AAC GGM TCY GGY-3') and reverse (5'-TAG AAG GTG TGR TGC-3'). PCR products were electrophoresed on a 0.7% agarose gel.

***In Situ* Hybridization Analysis with Digoxigenin (DIG)-labeled RNA Probes.** *In situ* hybridization was performed using a method developed by Yoshizaki's laboratory. A 1.4 kb cDNA *vasa* fragment (nucleotides 912–2,319 of *vasa*) was subcloned into the pGEM T-easy vector. Sense and antisense RNA probes were transcribed *in vitro* using DIG-labeled uridine triphosphate (UTP) (Roche, Mannheim, Germany) and T7 RNA polymerase (Promega). For the *in situ* hybridization (ISH) of tissue sections, tissue samples from the central region of the gonads were fixed at 4 °C for 16 h in Bouin's solution. After dehydration in increasing concentrations of ethanol, a portion of each sample was embedded in paraffin wax and cut into 5-µm serial sections using a microtome. The paraffin sections

were then mounted on Matsunami Adhesive Slides (MAS; Matsunami Glass Ind., Ltd., Osaka, Japan), dewaxed, and dehydrated by immersion in a xylene-ethanol series. The sections were stained with hematoxylin-eosin (HE) or processed for ISH with DIG-labeled RNA probes. The sections were then permeabilized, acetylated, and incubated with a hybridization mixture of 1 µg/ml RNA probe, 50% formamide, 29 saline-sodium citrate (SSC) (pH 4.5), 50 µg/ml transfer RNA (tRNA), 50 µg/ml heparin, 1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate. After hybridization at 65 °C for 16 h, the sections were washed as follows: twice in 59 SSC/50% formamide at 65 °C for 30 min, twice in 29 SSC/50% formamide at 65 °C for 30 min, and once in 19 SSC/25% formamide: 19 Tris buffered saline containing 0.1% Tween-20 (TBST) at room temperature (RT) for 30 min. The sections were then placed in NTE buffer [500 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM ethylenediamine tetraacetic acid (EDTA)] at 37 °C for 5 min before being washed twice in 0.59 SSC at 65 °C for 20 min and then twice 19 TBST at RT for 5 min. Hybridized DIG-labeled probes were visualized using a tyramide signal amplification (TSA) Plus 2,4-dinitrophenyl (DNP) alkaline phosphatase (AP)-System (PerkinElmer, CA, USA) as the indirect immunodetection method. Nonspecific binding was blocked in freshly prepared TNB buffer (100 mM Tris–HCl pH 7.5, 0.5% blocking reagent) for 30 min at RT in moist chambers. The sections were incubated for 1 h at RT with anti-DIG-POD Fab fragments (diluted 1:500 in the TNB buffer) (Roche), before being washed twice in TNT wash buffer (100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 5 min. DNP-tyramide was diluted (1:50 in the diluent provided) and the sections were incubated for 10 min at RT. The DNP-tyramide signal amplification procedure was performed twice in order to increase the signal-to-noise ratio in ISH. After two washes of 5 min each, anti-DNP-AP (diluted 1:100 in the TNB buffer) was applied to the sections for 1 h at RT. Finally, each of the sections was rinsed twice in wash buffer for 5 min. The sections were then incubated in a NTMT solution (100 mM NaCl, 100 mM Tris–HCl pH 9.5, 50 mM MgCl2, 0.1% Tween-20, mM, 1 mM Levamisole) containing 0.0035% of nitroblue tetrazolium (NBT; Roche) and 0.0018% of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) at RT in the dark. After the color reaction had occurred, the slides were mounted using Entellan neu (Merck KGaA, Darmstadt, Germany) and sections were counterstained using Nuclear Fast Red (NFR) (Vector Laboratories, CA, USA) for 16 h. The resulting sections were observed under a BX-50 microscope (Olympus, Tokyo, Japan).

RESULTS

Cloning of *Vasa* cDNA and Phylogenetic Analysis. The full-length giant gourami *vasa* cDNA had an open reading frame of 1,962 bp that began with the first start codon, ATG, at position 100, and ended with a stop codon, TAG, at position 2061 (GenBank accession number: GQ422440). The open reading frame encoded 653 amino acids and the predicted sequence had a molecular mass of 70.9 kDa and a *pI* of 5.55. The deduced amino acid sequence showed 66.2% similarity and 48.0% identity with the *Drosophila vasa* (Hay *et al*. 1988; Lasko & Ashburner 1988), 85.9% similarity and 69.2% identity with the common carp *vasa* homolog (GenBank accession no.: AF479820), and 90.7% similarity and 77.0% identity with the tilapia *vasa* homolog (Kobayashi *et al*. 2000). The obtained amino acid sequence contained eight consensus sequences for the DEAD protein family (Figure 1, boxed) (Linder *et al*. 1989). The amino acid region between the N-terminus and amino acid position 150 contained 17 arginine-glycine repeats (Figure 1, single underline), and there were eight arginine-glycine-glycine repeats (Figure 1, double

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MDEWEEEETTTISTIALTSQSTNEGTQGDFWKPDSGESGRGRGGGGRGRRGGFKSSFSSG  60

GEERRDDGNNWNSTAAERGGFRGRGGRGRGRGFGRMDQSEFNGDDSGVCESGFRGGSRGG 120

RGSRGRGGFREAGDQGGRGGYGGGYRGKDEEIFAQGENKDPGKKDAIDGDRPKVTYVPPT 180

LPEDEDSIFAHYKTGINFDKYDDIMVDVSGTNPPQAILTFDEAALCETLRKNVSKSGYVK 240

PTPVQKHGIPIISAGRDLMACAQTGSGKTAAFLLPILQQLMADGVAASRFSELQEPEALI 300
                          ATP-A
VAPTRELINQIYLEARKFSFGTCVRPVVVYGGVSTAHQIREISRGCNVLCGTPGRLLDVI 360

GRGKVGLSKLRYLVLDEADRMLDMGFEPDMRRLVGSPGMPSKENRQTLMFSATYPEDIQR 420
                    ATP-B
MAADFLKTDYLFLAVGVVGGACSDVEQTFVQVTKFSKREQLLDLLKTTGTERTMVFVETK 480

RQADFIATFLCQEKVPTTSIHGDREQREREQALADFRSGKCPVLVATSVAARGLDIPDVQ 540

HVVNFDLPSNIDEYVHRIGRTGRCGNTGRAVSFYDPEADGHLARSLVGVLSKAQQEVPSW 600

LEEAAFSGPSSTGFNPPRKNFASTDTRQRGLLQDTSVMSQPAAQPAADEEEWE*       660
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Figure 1. Deduced amino acid sequences of a cloned giant gourami cDNA encoding a *vasa*-like gene. Amino acid residue numbers are shown on the right. Arginine-glycine repeats and arginine-glycine-glycine repeats in the N-terminal region are underlined and double underlined, respectively. Eight consensus sequences for the DEAD protein family, including an adenosine triphosphate (ATP)-A motif and an ATP-B motif are boxed. These sequence data are available from GenBank (accession no. GQ422440).

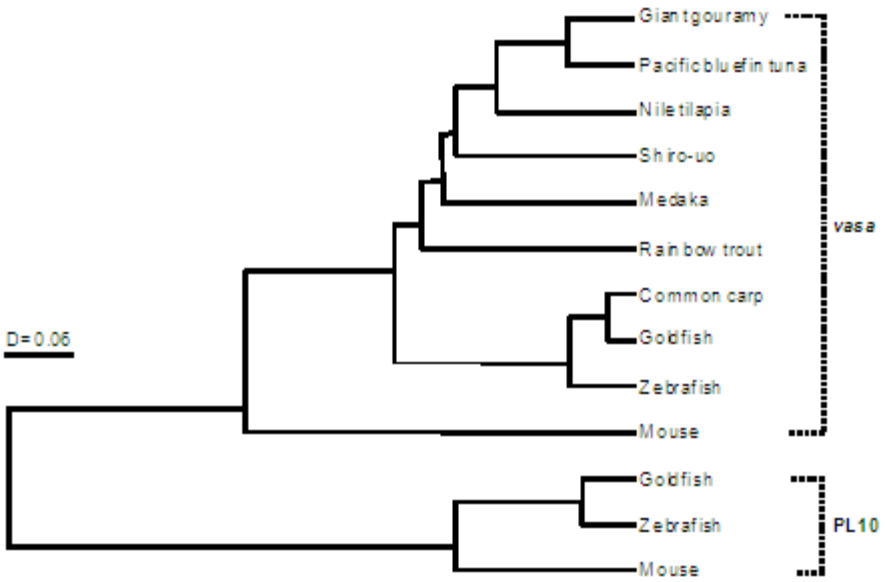

Figure 2. Phylogenic tree of the amino acid sequences of *vasa* and PL10 constructed using the UPGMA method. The length of horizontal lines indicates genetic distances. The GenBank accession numbers of the aligned amino acid and nucleic acid sequences were as follows: *vasa* (Pacific bluefin tuna: EU253482, common carp: AF479820, goldfish: AY773078, medaka: AB063484, mouse: AK014844, rainbow trout: AB032566, shiro-uo: AB098252, tilapia: AB032467, zebrafish: NM_131057) and PL10 (goldfish: AY842133, mouse: J04847, zebrafish: BC059794).

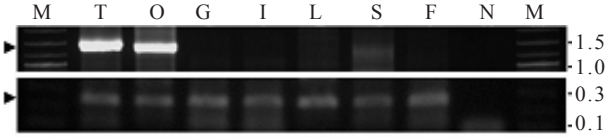

Figure 3. RT-PCR analysis of various tissues using *vasa*-specific primers. cDNAs from various tissues (testis, ovary, gill, intestine, liver, muscle, fin) were used for RT-PCR. β-actin was used as an internal control for RT-PCR amplification. Lane NC was a negative control containing no cDNA template. M represents molecular weight marker (2-log ladder DNA marker, BioLabs, Inc., New England).

underline). The phylogenic trees of the *vasa* genes and PL10 amino acid sequences belonging to the DEAD protein family constructed using UPGMA method are shown in Figure 2. The sequence obtained in this study belonged to the *vasa* family and showed a strong association with the other vertebrate *vasa* homolog examined.

Tissue Distribution of *Vasa* mRNA by RT-PCR. Tissue distribution patterns of *vasa* mRNA was analyzed in 2-year-old male (*pubertal testes*) and female (previtellogenic ovary) tissues (GSI was 0.0089 and 0.23%, respectively). While high levels of transcripts were detected in the gonads of both males and females, none was detected in other tissues (Figure 3).

Localization of *Vasa* mRNA-positive Cells in Gonads by ISH. In pubertal testes (GSI 0.0089%), *vasa*-positive signals were detected in SG (Figure 4, middle); both at SG types A and B (Figure, bottom). *Vasa*-positive signals in previtellogenic ovaries (GSI 0.022%) were detected in the oocytes (Figure, top-center). Conversely, no hybridization signals were observed in any of the cells when the sense probes were applied (Figure, top- and middle-right). We did not observe *vasa* positive signals in the gonadal somatic cells of both males and females. No *vasa* mRNA was also detected in spermatocytes and spermatids (Figure, bottom). Thus, *vasa* mRNA was predominantly localized in meiotic cells such as SG.

DISCUSSION

It has been demonstrated that *vasa* is a member of the DEAD protein family that possesses ATP-dependent RNA helicase activity (Hay *et al*. 1988). The deduced amino acid sequence of the clone isolated in this study contained eight consensus sequences for the DEAD protein family (Linder *et al*. 1989), including the ATP-A motif (AXXXXGKT) and the ATP-B motif (DEAD) (Pause & Sonenberg 1992). In addition, a glycine-rich region in the N-terminal region of giant gourami *vasa* was observed to contain 17 arginine-glycine repeats and 8 arginine-glycineglycine repeats, which is similar to that observed in the *vasa* orthologs of other species (Raz 2000). This glycine-rich region with several repeated motifs is believed to be a characteristic of single-stranded nucleic acid binding proteins, such as RNA helicase (Liang *et al*. 1994), and these findings strongly suggest that the cDNA clone isolated in this study encoded a DEAD protein possessing ATP-dependent RNA helicase activity. The phylogenic tree of *vasa* and the PL10 family revealed that the sequence obtained in this study belongs to a clade containing *vasa* homologs.

Next, we performed expression analysis of *vasa* mRNA by RT-PCR and ISH. Tissue distribution patterns of *vasa* mRNA was analyzed in adult male and female tissues by RT-PCR. These studies revealed that specific and abundant expression of *vasa* was detected in the testes and ovaries of adult giant gourami. Using section ISH, *vasa*-positive

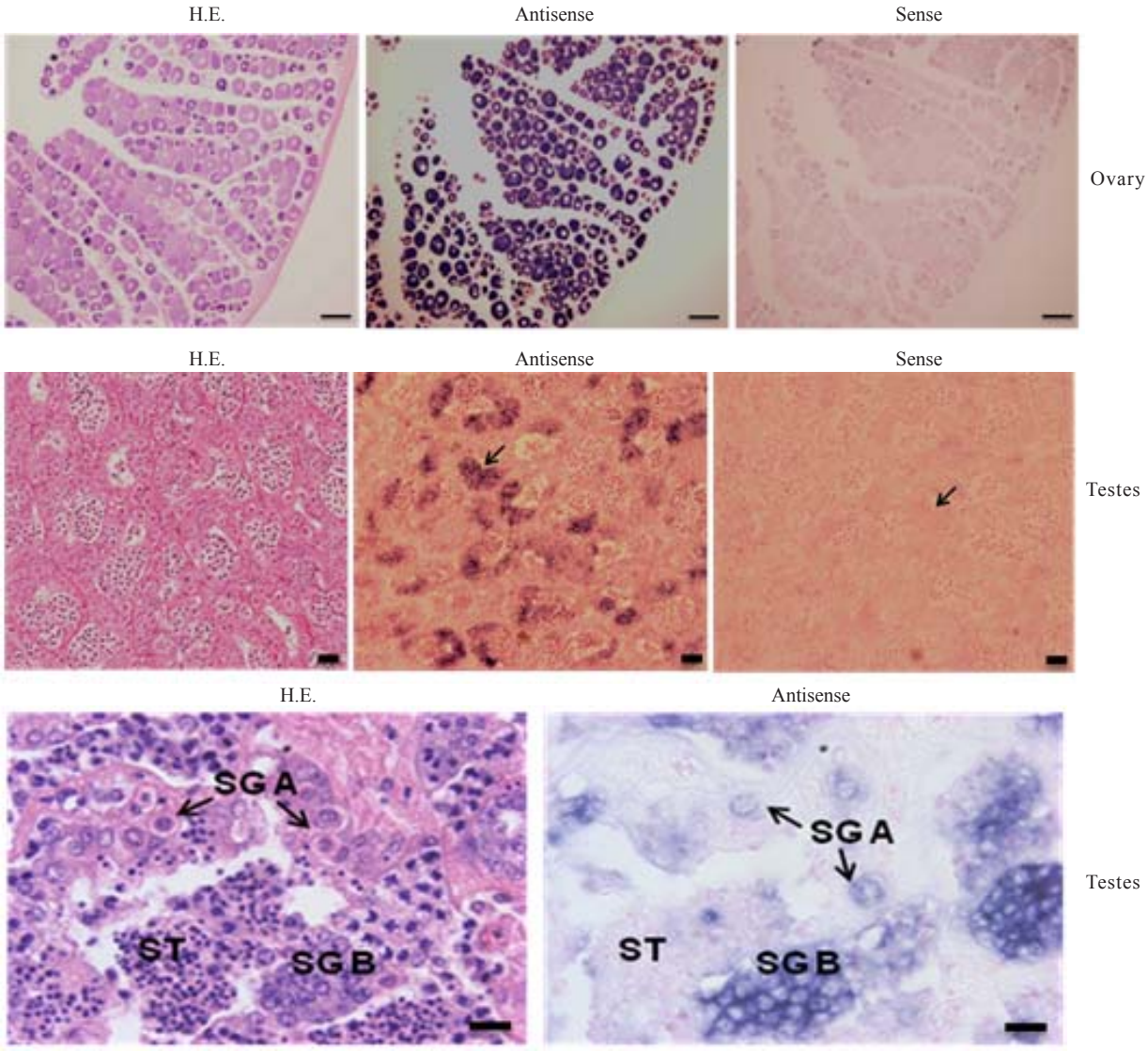

Figure 4. *Vasa* expression in ovary and testis. Sequential sections stained with hematoxylin-eosin (H.E.) (left) and hybridized with an antisense (middle) and sense (right) *vasa* probes. No unspecific staining is observed with the sense probe. The signal of *vasa* mRNA is specifically expressed in oocytes (top-center) and spermatogonia types A (SG A) and B (SG B). The scale bars represent 100 µm (ovary) and 20 µm (testis).

cells were detected in the testes and ovaries of adult giant gourami. In pubertal male fish, which contained mainly type SG, *vasa*-positive signals were specifically detected in SG. In previtellogenic ovaries, *vasa* mRNA was specifically localized in OC. Furthermore, no *vasa* mRNA was observed in SC, ST or any of the gonadal somatic cells. Taken together with nucleotide sequence and the spatial expression patterns, we concluded that the clone identified in this study is the giant gourami ortholog of the *Drosophila vasa* gene and designated it the giant gourami *vasa*-like gene (GgVLG). Similar expression pattern of *vasa* gene has also been reported in fish species including rainbow trout (Yoshizaki *et al*. 2000), tilapia (Kobayashi *et al*. 2000), and Pacific bluefin tuna (Nagasawa *et al*. 2009).

Development of SG transplantation technology to generate a surrogate parent fish capable of producing giant gourami gametes is ongoing in our laboratory. The use of highly concentrated SG population, especially SG type A as donor cells is expected to facilitate high colonization efficiency (Nagasawa *et al*. 2009). The results from the section HE and ISH showed that, the number of SG was lower in the testes of 2-year-old giant gourami. Thus, in order to increase the successful rate of transplantation in giant gourami using SG, it is desirable to determine the size of giant gourami containing high number of SG. In such observation, the GgVLG cDNA sequence will be a valuable tool for SG quantification. Further, the GgVLG sequence can also be used as a marker to identify donor cells colonized in recipient gonad using ISH and PCR methods.

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