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A potential germ cell-specific marker in Japanese flounder, *Paralichthys olivaceus*: identification and characterization of lymphocyte antigen 75 (Ly75/CD205)*

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Abstract Some germ cell marker genes, such as *vasa*, *nanos*, and *dead end* (*dnd*), have been identified in fish. Recently, lymphocyte antigen 75 (Ly75/CD205) has been identified as a mitotic germ cell-specific cell-surface marker in several fish species. In this study, the Japanese flounder *Paralichthys olivaceus* ly75 homolog (*ly75*) was cloned and its expression pattern in gonads was analyzed. The full-length cDNA of *ly75* was 7 346 bp, with an open reading frame (ORF) of 5 229 bp. The ORF encoded a protein containing 1 742 amino acids with a predicted molecular mass of 196.89 kDa. In adult tissues, *ly75* transcripts were detected in all analyzed tissues but abundantly in the testis. In in-situ hybridization analyses, *ly75* mRNA was predominantly localized in oocytes in the ovary and spermatogonia in the testis, but *ly75* mRNA was not detected in oogonia, spermatocytes, spermatids, or spermatozoa. These results indicated that *ly75* could be a potential germ cell-specific marker in *P. olivaceus*, as in other fishes.

Keyword: Ly75/CD205; Japanese flounder; *Paralichthys olivaceus*; gonads; germ cell-specific marker

1 INTRODUCTION

Germ cells are highly specialized cells that play an extremely important role in fish reproduction (Kurokawa et al., 2007). In teleost fish, mature gametes are acquired from primordial germ cells (PGCs) through a series of basic biological processes. Among them, the PGCs, the diploid oogonia, and spermatogonia are known as special stem cells that provide the foundation for gametogenesis through self-renewal and differentiation (Schulz et al., 2010; Nakamura et al., 2011; Lacerda et al., 2012; Kagawa, 2013). However, the molecular and cellular mechanisms underlying the continuous production of mature gametes in adult fish are poorly understand. Therefore, for the molecular, cellular, and functional characterization of germ cells, germ cell marker genes such as *vasa*, *nanos*, and *dead end* (*dnd*) have been identified in recent decades (Nagasawa et al., 2010; Li et al., 2015; Wang et al., 2015b). Among them, Nanos 2, a type of *nanos*, has been extensively studied and is regarded as a specific marker of germline stem cells in fish (Suzuki et al., 2007). Lymphocyte antigen 75 (*ly75*, also called *CD205*) was first identified as a germ cell surface marker in rainbow trout (Nagasawa et al., 2010). Subsequently, *ly75*homologs have been cloned from several other fish species (Nagasawa et

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al., 2012, 2013; Presslauer et al., 2014). In mammals, Ly75 plays a role in the immune system and functions as an antigen-uptake receptor in dendritic cells (Jiang et al., 1995; East and Isacke, 2002). Although the role of Ly75 in fish germ cells is unknown, its amino acid sequence has a highly conserved region, and its expression is restricted specifically to spermatogonia (SG). Therefore, it is considered to be a practical mitotic germ cell-specific cell-surface marker and it has been used in SG transplantation in fish (Nagasawa et al., 2010, 2012).

Japanese flounder (*Paralichthys olivaceus*), a cold water benthic flatfish, is one of the most important aquaculture species in China, Korea, and Japan (Radonic and Macchi, 2009; Si et al., 2016). Due to its high commercial value, it has been intensively studied, and most research on its reproductive biology has focused on gametogenesis (Fan et al., 2014; Wang et al., 2015a). Although some germ cell markers have been identified in *P. olivaceus*, there is still much to learn about its germline cells, including their molecular and cellular makeup and functions. Therefore, in this study, we identified a potential germ cell-specific marker, lymphocyte antigen 75 (*ly75*), in *P. olivaceus*, and investigated its gene expression pattern using real-time quantitative PCR (RT-PCR) and in-situ hybridization (ISH) analyses.

2 MATERIAL AND METHOD

2.1 Experimental animals

Nine Japanese flounders (three 8-month-old males, three adult males, and three adult females) were obtained from Oriental Ocean Sci-Tech Co., Ltd. (Shandong Province, China). The samples were collected and excised after being anesthetized with a 0.05% (w/v) solution of ethyl 3-aminobenzoate methane sulfonate (Sigma-Aldrich, Shanghai, China). To explore the function of *ly75* in subsequent transplantation in marine fish, tissues including both types of testes, adult ovaries, and the gill, intestine, liver, kidney, spleen, stomach, heart, and muscle from 8-month-old males were rapidly excised and frozen in liquid nitrogen. Half of each testis and ovary sample was fixed in Bouin's fluid and 4% paraformaldehyde in Sorensen's phosphate buffer (0.1 mol/L, pH 7.2) for at least 24 h and then preserved in 70% ethanol. All animal work was conducted according to relevant national and international guidelines and was approved by the Institute of Oceanology, Chinese Academy of Sciences.

Table 1 Primers used in this study

Purpose	Primer name	Sequence
Mid	*ly75*F1	ccaaacagcaggtcccagatgtggaagtg
	*ly75*R1	cggtgtgactgtggtcggtccagcggtag
	*ly75*F2	agagcccttccacatcggcaaccacacc
	*ly75*R2	tctgttatgatgctggtggcgtccgact
3′RACE	3′*ly75*-1	gaagaaatcgtccccccactgtgccgtc
	3′*ly75*-2	tggcactggttctcttcatcatcatcct
5′RACE	5′*ly75*-1	ggacaatccaggggaatctgcgtcgggg
	5′*ly75*-2	ctctcacccaagaatcattagtgtcccg
RT-PCR	*β-actin*-F	ccttcaccaccacagccgagag
	β-actin-R	attccacaggactccataccga
	RT-*ly75*-F	gcctgccaaataccccgaggaaagacgc
	RT-*ly75*-R	gacatccgacgaccagcgattgaacacg
Probe	T-*ly75*-F	gcctgccaaataccccgaggaaagacgc
	T-*ly75*-R	tgagacaaggagccagtttcccccaatg

2.2 Cloning of full-length *ly75* cDNA

Total RNA was extracted from the testes of 8-month-old Japanese flounder using an RNA fast 200 kit (Fastagen Biotech, Shanghai, China). First-strand cDNA was synthesized using a Transcript First-strand cDNA synthesis kit (TransStart, TransGen, Beijing, China), according to the manufacturer's instructions. The cDNA fragment of the *ly75* gene was amplified by PCR with primers (Table 1) designed according to the highly conserved regions of *ly75*/*cd205* homologs from other fish species (Table 1). The PCRs were performed using KOD-Plus-Neo (Toyobo, Tokyo, Japan) in a PTC-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Subsequently, 5′- and 3′-rapid amplification of cDNA ends (RACE) were carried out using a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) using the gene-specific primers shown in Table 1. The reaction mixture contained 2 μL 10×buffer, 2 μL 2 mmol/L dNTPs, 1.4 μL 25 mmol/L $MgSO_4$, 0.6 μL forward and reverse primers (10 μmol/L), 11 μL ddH_2O, 0.4 μL KOD-Plus-Neo (1 U/μL), and 2 μL cDNA. The thermal cycling conditions were as follows: 2 min at 94°C; 10 s at 98°C, 30 s at *T*m °C, and 1 min at 68°C for 40 cycles; and finally 10 min at 72°C. The molecular mass and p*I* of predicted poly75 polypeptide were estimated using the compute pI/Mw tool (http://web.expasy.org/compute_pi/).

2.3 Phylogenetic and bioinformatics analyses

Homology searches of the nucleotide and deduced amino acid sequences of *ly75* were conducted at the

National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The deduced amino acid sequences were aligned using AlignX in Vector NTI 11.5 Suite (Life Technologies, Carlsbad, CA, USA). The percentages of similarity and identity among fish Ly75 homologs were calculated using LALIGN (http://www.ch.embnet.org/software/LALIGN-form.html). A phylogenetic tree was constructed using Mega 4.1 software with bootstrap analysis of 1 000 replicates, by the neighbor-joining method. The signal peptide sequences were determined by SignalP (http://www.cbs.dtu.dk/services/SignalP/). A domain structure analysis was carried out using SMART (http://smart.embl-heidelberg.de/). Transmembrane helices in proteins were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).

2.4 Semi-quantitative RT-PCR analysis

Total RNA was extracted from tissue samples (including testis, ovary, gill, intestine, liver, kidney, spleen, stomach, heart and muscle) as described above. Specific primers (Table 1) were used to amplify *ly75* and *β-actin* by RT-PCR, and the primer specificity for each gene was verified by sequencing. The cDNA was synthesized from 1 μg total RNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China) following the manufacturer's instructions. The reaction mixture contained 12.5 μL 2×buffer, 9.5 μL ddH_2O, 0.5 μL forward and reverse primers (10 μmol/L), and 2 μL cDNA. All samples were run in triplicate. The thermal cycling conditions were as follows: 3 min at 95°C; 5 s at 95°C and 30 s at 62°C for 35 cycles; and 30 s at 95°C, 20 s at 60°C; and 30 s at 72°C, followed by a final elongation step at 72°C for 3 min. The PCR products were electrophoresed on 2% (w/v) agarose gels.

2.5 Histology

The testes and ovaries fixed in Bouin's fluid were dehydrated with an ethanol gradient, embedded in paraffin, and cut into a series of sagittal and cross sections (5 μm thick). The tissue sections were stained with hematoxylin and eosin (HE) for histological observations.

2.6 In-situ hybridization

The localization of *ly75* transcripts was analyzed by section in-situ hybridization (SISH), as described by Wang et al. (2015b). Samples were dehydrated using a methanol gradient, embedded in paraffin wax, and cut into 8-μm thick sections. Probes for *ly75* (Table 1) were individually synthesized using a DIG RNA Labeling Kit (SP6/T7) (Roche, Mannheim, Germany) following the manufacturer's instructions.

2.7 Immunohistochemistry

The localization of Nanos 2 was analyzed using immunohistochemical analyses. Samples were dehydrated with a methanol gradient, embedded in paraffin wax, and cut into 8-μm thick sections. The tissue sections were treated with lead citrate (BBI, Shanghai, China) for antigen retrieval and 3% H_2O_2 to block endogenous peroxidase. The primary antibody against Nanos 2 protein (BBI, China) was diluted 1:200 and incubated with the samples at 4°C overnight. The secondary antibody was goat anti-rabbit IgG (ABclone, China), which was diluted 1:800 and incubated with the samples for 30 min at room temperature.

3 RESULT

3.1 Cloning of *ly75* from Japanese flounder

The full-length cDNA of *ly75* (GenBank accession No. MF983803) was 7 346 bp, with an open reading frame (ORF) of 5 229 bp. The ORF encoded a protein consisting of 1 742 amino acids with a predicted molecular mass of 196.89 kDa. The deduced amino acid sequence of ly75 showed 70.7% identity and 90.1% similarity with that of *Cynoglossus semilaevis*, 49.3% identity and 74.9% similarity with that of zebrafish, 61.4% identity and 83.9% similarity with that of medaka, and 35.8% identity and 67.1% similarity with that of mouse. A phylogenetic tree analysis (Fig.1) revealed that *P. olivaceus* Ly75 was associated with teleost Ly75s and belonged to the Ly75 protein subfamily. In addition, analyses of the protein sequence of ly75 (Fig.2) revealed a predicted N-terminal signal peptide cleavage site between amino acid positions 28 and 29. And the sequence contained a cysteine-rich domain (RICIN), a fibronectin type II (FN2), 10 C-type lectin-like domains (CLECT), and a transmembrane domain (TM).

3.2 Analyses of *ly75* mRNA transcript levels in different tissues by RT-PCR

Transcripts of *ly*75 were abundantly detected in the testis of 8-month-old fish, and in the gill, kidney, spleen, stomach, and heart. Transcripts of *ly*75 were detected at low levels in the adult testis and ovary, intestine, liver, and muscle (Fig.3).

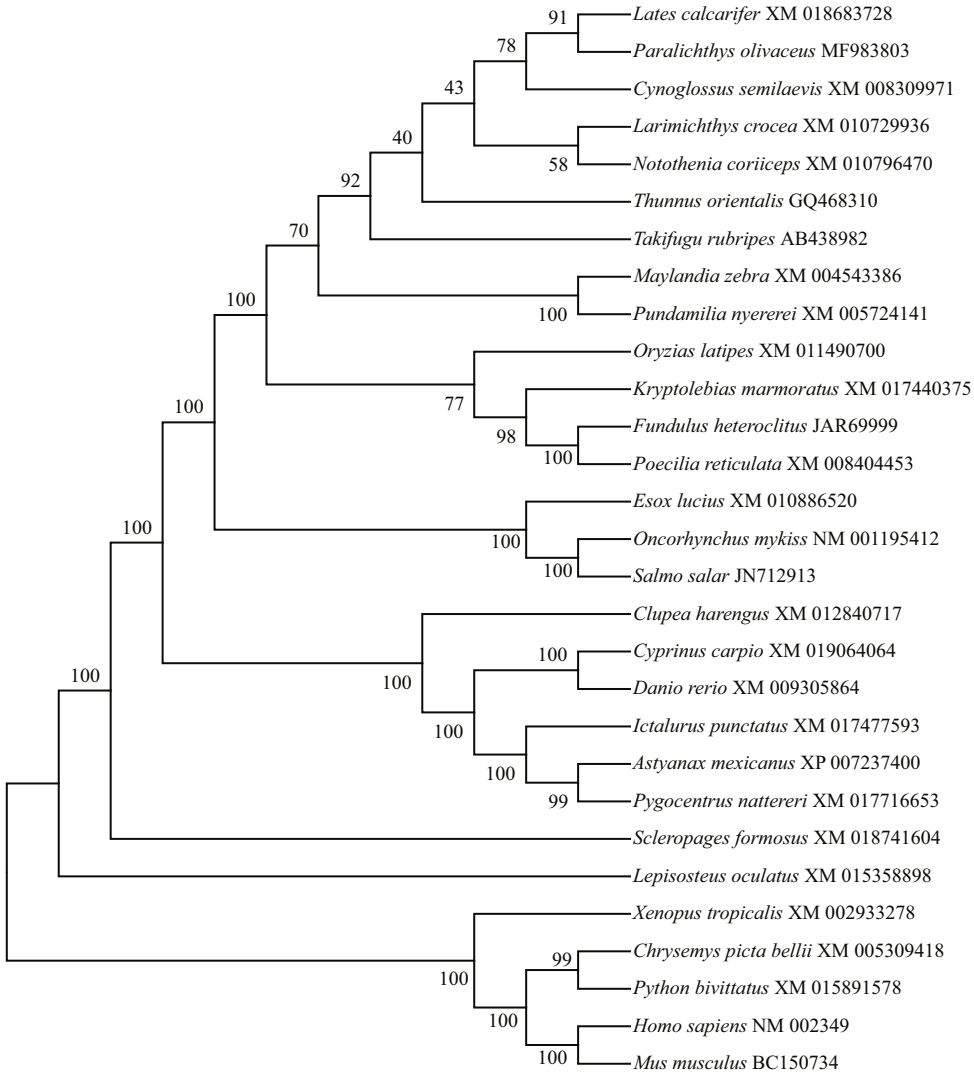

Fig.1 Phylogenetic tree of deduced amino acid sequences for Ly75 from Japanese flounder and other vertebrates constructed by MEGA 4.1 using neighbor-joining method

Numbers adjacent to nodes indicate bootstrap percentage value for 1 000 replicates (>80%). GenBank accession numbers of sequences are given after species name.

3.3 Detection of *ly75* mRNA in gonads by ISH analysis

The mRNA of *ly75* was detected in the testis and ovary by ISH. The localization of the Nanos 2 protein was also determined using immunohistochemical analyses. In adult ovaries, *ly75* mRNA was predominantly located in the cytoplasm of oocytes at stage III and IV, and could not be detected in oogonia and oocytes at stage II (Fig.4a, b). In the testis, *ly75* mRNA was predominantly detected in spermatogonia, and could not be detected in spermatocytes, spermatids, or spermatozoa (Fig.4f, g). The Nanos 2 protein was expressed in germline stem cells: spermatogonia in the testis (Fig.4j) and oogonia in the ovary (Fig.4d).

4 DISCUSSION

In this study, we isolated and characterized a *ly75* homolog from *P. olivaceus*. The deduced amino acid sequence of *ly75* was similar to that of *ly75s* of other teleosts, showing 90.1% similarity to that of *C. semilaevis*, 74.9% similarity to that of zebrafish (Presslauer et al., 2014) and 67.1% similarity to that of mouse (Jiang et al., 1995). The Ly75 amino acid sequence contained typical conserved domain structures including one RICIN, one FN2, 10 CLECT, and one TM, with high identities to those of other Ly75 proteins (Nagasawa et al., 2012). The phylogenetic tree constructed using the neighbor-joining method showed that the Ly75 protein was closely related to the Ly75 clade. Overall, these

1 Signal peptide RICIN
MLTLSGATLRLCLLTVFWATWSPADVQASEEDAFTIQHLGTGKCLGTGASANLSLITCNADSRSQMWKWGSGHRLFHVASSTCL

85 FN2
ALEVLSKRLSLVDCGANIPLSWHCLDGAVYTVYQMGLAVSESKVATKRDTNDSWVRGGSQDNICEKPYRVVHTINGNSAGDPC

168 CLECT-1
DFPFKFNGSWHHGCLPDADSPGLSWCATSSDYDQDRKKGNCLTREEGCQTLFAGPEGGFCYEFVSSATVTWQEALDSCRSQGA

251
DLFSLTGPNDLHSKTLLDGLGGMPERMWIGLHQLDMSQGWQWSDGSPLSFLHWEEGMPSTVAIMESDCGVLNSKQNFESEAC

333 CLECT-2
NKHLPYICKKSISASPTATTESIVYNETVCAVGWVPWNGWCYKLVKDEPKNFKDAQQHCAQTEGGGEGSLASLHSIDCKEMIST

417
NFHADGKFLDVWIGLVGILMNQTTVFKWNDQAPVTFTFWGPNEPVQPTQDPSCVFYSGESHGWRVGNCTERLPFMCQTKGEV

499 CLECT-3
RKSVSPAGCRFEDGWRRHGNSCYQVNTKKVSFKDRCNITIRNRFEQTFINRLLGEYINKETQYFWIGLQEIKNTGEYQWLSQDGS

584 CLECT-4
PHLLTYTNWGWSEPDQHGGCAVISTAQPLGKWELKNCTNFTAGTICRTDLSPPPTTEPELNSNASCPNGWMSRENMKYCYKVF

667
HEERLSRKRSWEEAERFCQALGANLPSFSNIADMKDLHSIMRDTISDNRFFWVGLNRRNPSDRSWVWSDGQPVSLKVIYDDFHE

751 CLECT-5
DDSYRRDCTAFKTTKSSLKHILVFLPPPLPFYAIPFHCDARLEWACQIPRGKTPKNPDWYNPVGHHETSIFVDGAEFWFVQEPKLA

837
FEEAKLFCNSNGSKVAAPSSSTAIIKIHQYLKNISSSTQQNWWVDLTEPGRIFPMTFTQMFFYHSVFLGRCISISPENLFPNHELSCQ

925 CLECT-6
QPLSFVCERHNITSVEIKPLEPQPAGQPCGNDSQSFRNKCYTLMSSTKPLSFKYANEQCQSARGTLVTISDQVEQDFITTLLPGMR

1011
NMERIWIGLKIKHNDPEWLDESPVNYLNFNPLLLGMHKAIQISRWDPESIDLCVYLINNPNSAMLGTWDYSSCTQFQNVAVCQH

1095 CLECT-7
YADKIEEPHINTKPFTIGNHTIQLLANNLTWFEALEQCRRNNMDLASVADTFFQSTLTVHVSRARTPMWIGLFSEDDGIHYRWTD

1180 CLECT-8
HSHTVFNRWSSDVTSGSCVYLDTDGFWKATECEEELGGAICHKPHKETITTPEDVAVKCPHKIHGPNWVPFKNNCYSFQLAASR

1264
WEQYDQGLIHDTCKNLYPNAEILTIRNAEENEFIKQQLLPFQTLAQFVWLGMFKDNKDNQVKWYDGTNVQYSNWANGRPDVD

1346 CLECT-9
RPFLAGITIGGNWLLVSNQGLFSEFKQRAVVTCKLDNEPKQDYNTTALDLLHYGNLIYSVVTRKLNWYQALEECGERGGHLASV

1430
HDIQHNEHVKLIAKTDGFPLWIGLSDQEVDGSAYEWSDGTRFDYKDNLSDSKERFISNKQEAVCVVVTPAGAWVKTSCNAMVD

1513 CLECT-10
GAICYTTHVTTSSQRARLRTSPEANHCPQSNDTSKGMSMWVQHQDHCYAFDMSFYNYSVYSMEQASKICQRMDAKLLTIKTKE

1596
ENDFVSKYITDNPLITGRVWLGMDMDTQGKPASWQDGSTLAYSNWKSGALGTGKKSSPHCAVMMAGDEGIWNFVSCQASYSR

1678 TM Cytoplasmic domain
VVCKTEAKSGGTPVALVLFIIILLALIAVIGFIVYRKKRAHFSSTVRYERTLDEFDTNSIITDAD

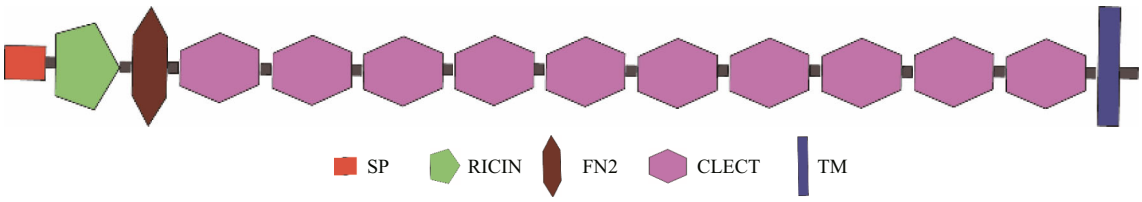

Fig.2 Molecular characterization of Japanese flounder ly75

a. deduced amino acid sequence from Japanese flounder *ly75* cDNA sequence. Amino acid residue numbers are shown on left. Signal peptide, cysteine-rich domain (RICIN), fibronectin type II (FN2), C-type lectin-like domains (CLECT), and transmembrane domain (TM) are indicated. Sequence data have been deposited in GenBank (accession number MF983803); b. domain structure analyses of Japanese flounder Ly75 by SMART.

results revealed that the clone identified from *P. olivaceus* was a Ly75 homolog.

The RT-PCR analyses of *ly75* transcript levels in different tissues revealed that *ly*75 mRNA was present in the testis, gill, and heart. Similar results were also reported for rainbow trout (Nagasawa et al., 2010), pacific bluefin tuna (Nagasawa et al., 2012), and Atlantic salmon (Nagasawa et al., 2013). This wide expression pattern was possibly related to the function of this protein in the immune system, as previously found in mammalian species (Jiang et al., 1995). Similar to the case in rainbow trout (Nagasawa et al., 2010) and Bluefin tuna (Nagasawa et al., 2012), *ly75* transcripts were abundant in the testis at the early development stage. The strong expression in the gonads indicated that *ly75* may play a key role in early gametogenesis in *P. olivaceus*.

The ISH analyses showed that *ly75* mRNA was present in oocytes at stage III and IV, but not in oogonia and oocytes at stage II and V in ovaries. In contrast, *ly75* transcripts were detected in oogonia and nucleolus-stage oocytes in rainbow trout (Nagasawa et al., 2010), and in oocytes at stages I, II, and III in zebrafish (Presslauer et al., 2014). Most fishes release eggs, and fish embryos rely upon the maternal provision of immunity molecules for protection against invading pathogens before their own immune system fully develops (Zhang et al., 2013). Therefore, in oogenesis, maternally transferred immunity molecules including lysozyme, lgM, and the egg yolk protein phosvitin are accumulated and stored in oocytes (Wang et al., 2017). Based on the present results, Ly75 as an immunity-related protein expressed in oocytes, may be related to maternally derived immunity molecules. In the adult testis, *ly75* mRNA was predominantly detected in SG, just its homologs were only detected in type A SG in rainbow trout (Nagasawa et al., 2010), zebrafish (Presslauer et al., 2014), and *Thunnus orientalis* (Nagasawa et al., 2012).

Immunohistochemical analyses revealed that the Nanos 2 protein was predominantly expressed in germline stem cells: spermatogonia in the testis and oogonia in the ovary, as in other fish (Suzuki et al., 2007; Huang et al., 2017). Several other genes have been reported as germ cell-specific markers in fish. In our previous study, *dnd* was identified as a gene essential for PGCs migration and oocytes and spermatocytes (Wang et al., 2015b). The results of the present study indicate that *ly75* could be a potential germ cell-specific marker in *P. olivaceus*, since it is specifically expressed in germ cells. In future research, we intend to functionally characterize this protein.

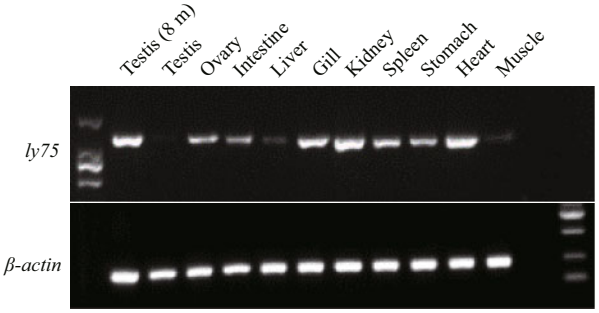

Fig.3 Tissue-specific expression of *ly75* mRNA as determined by RT-PCR (internal control, *β-actin*)

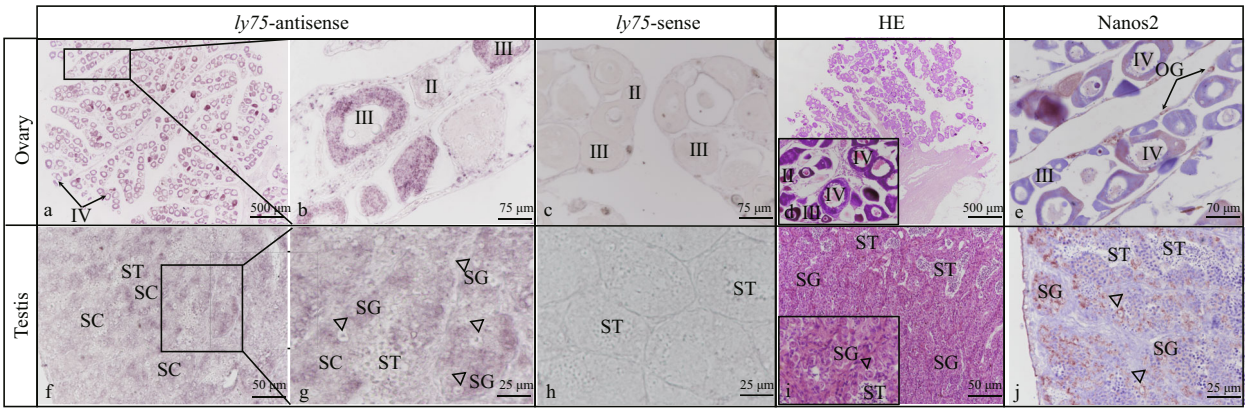

Fig.4 Distribution of *ly75* transcripts in adult *P. olivaceus* testis and ovary

In adult ovary (a–b), *ly75* mRNA was predominantly located in cytoplasm of oocytes at stage III and IV, and could not be detected in oogonia and oocytes at stage II. In adult testis (f–g), *ly75* mRNA was located in spermatogonia and could not be detected in spermatocytes and spermatids. b and g are magnifications of a and f, respectively; c and h are negative controls (sense probes). Hematoxylin- and eosin-stained sections of ovary (d) and testis (i). Nanos 2 protein in oogonia in ovary (e) and spermatogonia in testis (j). II: primary growth oocytes; III: perinucleolar or pre-vitellogenic ooctyes; IV: late vitellogenic oocytes; SC: spermatocytes; ST: spermatids.

5 CONCLUSION

We cloned Japanese flounder *ly75* and analyzed its expression pattern. In RT-PCR analyses, *ly75* transcripts were detected in all analyzed tissues but abundantly in the testis. The ISH analyses showed that *ly75* mRNA was predominantly localized in oocytes in the ovary and spermatogonia in the testis. We did not detect *ly75* mRNAs in the oogonia, spermatids, spermatocytes, or spermatozoa. Therefore, our results indicate that *ly75*, like *dnd*, could be a potential germ cell-specific marker in *P. olivaceus*.

7 DATA AVAILABILITY STATEMENT

The datasets generated and analyzed during the current study are not publicly available from NCBI (https://www.ncbi.nlm.nih.gov/) until Sep. 3, 2018 due to author request, but are available from the corresponding author on reasonable request.

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