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RESEARCH PAPER

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Characterization of *nanos1* Homolog in the Olive Flounder, *Paralichthys olivaceus* (Temminck & Schlegel, 1846)

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Abstract

The *nanos* gene family plays a critical role during germline development in a wide array of organisms. The characteristics of the gene structure and function of *nanos1*, a *nanos* homolog, vary from species to species. Herein, we isolated the full-length cDNA of a *nanos1* homolog in the olive flounder, *Paralichthys olivaceus*, and analyzed its expression pattern. The full-length cDNA sequence of the olive flounder *nanos1* has 1215 base pairs (bp) and contains a 112 bp 5′-untranslated region, a 684 bp open reading frame that encodes a 228 AA peptide, and a 419 bp 3′- untranslated region. Phylogenetic analyses showed that the Nanos1 homolog of the olive flounder grouped with the nanos1 of teleost fish. We detected *nanos1* mRNA in all stages of embryogenesis using RT-PCR analyses. Our whole mount *in situ* hybridization results showed that *nanos1* was expressed in the diencephalon, midbrain, hindbrain, nose, medulla oblongata, retina, abdomen, and somatic gonadal cells. Our data indicate that the expression pattern of *nanos1* is not consistent from species to species, supporting that nanos1 had different functions in different organisms.

Introduction

The nanos protein is an RNA-binding protein containing two CCHC zinc-finger motifs (Hiroshi *et al*., 2009) that plays a critical role during germline development in a wide variety of organism. The function of the *nanos* gene family in primordial germ cell (PGC) migration and survival during early embryogenesis is evolutionarily conserved (Shen & Xie, 2010). However, the processes regulated by *nanos* vary among species and between different homologs (Ye *et al*., 2012). There is only one *Nanos* homolog in *Drosophila melanogaster*, while *Caenorhabditis elegans* and all the vertebrates have three *nanos* homologs. The *Nanos* homolog of *Drosophila* was shown to be involved in controlling germ cell migration, somatic cell fate suppression in the germline, and stem cell self-renewal maintenance (Asaoka-Taguchi, Yamada, Nakamura, Hanyu, & Kobayashi, 1999; Hayashi, Hayashi, & Kobayahsi, 2004; Wang & Lin, 2004). In *C. elegans*, *nanos* homologs (*nos-1* and *nos-2*) were required to regulate PGCs migrating into the somatic gonad and for the maintenance of germline cell viability during its larval development, while *nos-3* was involved in controlling the sperm-oocyte switch (Kraemer *et al*., 1999; Subramaniam & Seydoux, 1999). In mouse, *Nanos3* was responsible for maintaining PGCs, whereas *Nanos2* is involved in preventing germ cells from entering meiosis (Tsuda *et al*., 2003; Tsuda, Kiso, & Saga, 2006; Suzuki, Tsuda, & Saga, 2007).

Besides germline cells, *nanos* transcripts were also widely present in multipotent cells and somatic tissues. In invertebrates, such as the sea anemone, *nanos* was expressed in multiple somatic cell types during early

embryonic development (Extavour, Pang, Matus, & Martindale, 2005). In *Hydra magnipapillata*, *nanos* transcripts were found in multipotent interstitial cells, which developed into germ cells and a variety of somatic cell types (Mochizuki, Sano, Kobayashi, Nishimiya-Fujisawa, & Fujisawa, 2000). In vertebrates, such as humans, *NANOS1* was expressed in numerous tissues, like the heart, brain, liver, ovary, spleen, and testis (Julaton & Reijo Pera, 2011). In mouse, *Nanos1* transcripts were detected in the central nervous system and the seminiferous tubules of mature testes and (Haraguchi *et al*., 2003). The teleost fish medaka, has two *nanos1* paralogues, *nanos1a* and *nanos1b*. *nanos1a* was expressed in the cerebellum, diencephalon, hypothalamus, caudal wall of the mesencephalon, nose, peripheral ganglia, and the somatic cells surrounding the oocytes after the initiation of sexual differentiation. *nanos1b* was detected in the branchial arch, mesencephalon, nose, optic tectum, otic vesicle, retina, and parts of the telencephalon (Aoki, Nakamura, Ishikaw, & Tanaka, 2009). The adult Chinese sturgeon (*Acipenser sinensis*) expressed *nanos1* mRNA in the cerebellum, heart, hypothalamus, intestines, kidney, medulla oblongata, muscle, ovary, pituitary gland, spleen, and telencephalon midbrain (Ye *et al*., 2012). Despite the broad characterization of the expression and function of members of the *nanos* gene family, there is little information about the expression pattern of *nanos1* during embryonic development.

The olive flounder (*Paralichthys olivaceus*) is a native species to the Western Pacific, that is distributed from the Sea of Okhotsk to the southeastern Russian shores, and along the Japanese coast to the South China Sea. It is a commercially important marine fish in East Asia that has been cultured for more than 20 years. Li *et al*. (2015) detected *nanos3* transcripts in migrating PGCs and germ cells of the olive flounder. However, the function of these transcripts and the expression pattern of other *nanos* homologs in the olive flounder remains unknown. In this study, we aimed to characterize the *nanos1* olive flounder homolog and to elucidate whether the function of the *nanos* subfamily members is conserved among vertebrates.

Materials and Methods

Fish Culture and Sample Collection

Olive flounders were cultured under controlled conditions (a light/dark cycle of 14 h / 10 h, a temperature of 15 ± 1°C, and aerated seawater) at a fish farm in Rongcheng, China. The fish were fed with fresh fish diet twice daily. Fertilized eggs were obtained by artificial insemination (Jiao *et al.*, 2015), and cultured at 15 ± 1°C in a 1 m^3 tank with aerated seawater. The developmental stages were identified by monitoring the embryos with a stereoscope every 15 min and comparing the observations to those of a previous study (Tian, Chen, Yan, & Ji, 2004). To isolate the RNA, the samples (embryos or tissue) were rapidly frozen in liquid nitrogen after Trizol reagent (Invitrogen, Waltham, MA, USA) was added. The samples were stored at −80°C. For in situ hybridization, eggs at different stages were fixed in paraformaldehyde (PFA) (4%, 1× PBS) at 4°C overnight. Then, PFA was replaced by methanol (100%), and the samples were stored at −20°C (Li *et al.*, 2015).

RNA Isolation and Cdna Synthesis

Total RNA was extracted from samples (flounder gonads and different developmental stage embryos) using Trizol (Invitrogen, Waltham, MA, USA), following the manufacturer's instructions. The total RNA was digested with RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) (30 min at 37°C), to remove DNA. M-MLV reverse transcriptase (Promega Corporation) was used to synthesize the First-strand cDNA with oligo-dT from 1μg total RNA, following the enzyme instructions.

Sequence Alignment and Phylogenetic Analysis

Clustal Omega (http: //www.ebi.ac.uk/Tools) were used to do alignment with amino acid sequence of Nanos homologs from flounder and other species (Sievers *et al*., 2011). The evolutionary history was deduced by the neighbor-joining method (Saitou & Nei, 1987), and a phylogenetic tree was built using MEGA6 (https://www.megasoftware. net/, Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The phylogeny was tested by the bootstrap method (10000 replicates) (Felsenstein, 1985). The Poisson correction method was used to calculate evolutionary distances (Zuckerkandl & Pauling, 1965). There were 25 amino acid sequences in the analysis; all positions with gaps and missing data were completely deleted. There was a total of 86 positions in the final dataset.

Isolation of the Olive Flounder Nanos1

The coding region of the olive flounder *nanos1* gene was isolated by reverse transcription polymerase chain reaction (RT-PCR) from olive flounder ovary cDNA. RT-PCR was performed using *Taq* DNA polymerase (CoWin Biotech, Beijing, China) and the primers *nanos1*-Fw and *nanos1*-Rw (Table 1). After purification, the PCR products were ligated into the pEASY-T3 vector (TransGen Biotech Inc., Beijing, China) and sequenced.

The SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) was used to construct the SMARTer 5'RACE and 3'RACE cDNA libraries. The 5'UTR and 3'UTR of *nanos1* were isolated using the RACE program following the manufacturer's instructions, using specific primers (nanos1-5-1 and nanos1-5-2 for the 5'UTR; nanos1-3-1 and nanos1-3-2 for the 3'UTR; Table 1), and the universal primer mix (UPM) (Clontech, Mountain View, CA, USA). After the

purification, the PCR products were ligated into the pEASY-T3 vector and sequenced.

Reverse Transcription-PCR (RT-PCR)

RT-PCR using *Taq* DNA polymerase was conducted on embryos of different developmental stages to test the expression of *nanos1* during embryogenesis. The final volume of the PCR reaction was 25µl and contained 4 µL of cDNA (1:50 dilution) as a template, and the primers *nanos1*-Fw and *nanos1*-Rw (Table 1). *Beta-actin* (*β-actin*) was used as a control. The primers for this reaction were *β-actin*-Fw and *β-actin*-Rw (Table 1). The PCR program was: denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 1 min, renaturation at 55°C for 30 s, and elongation at 72°C for 1 min; with a final elongation step of 72°C for 10 min.

Whole-Mount in Situ Hybridization

An antisense RNA probe was synthesized by *in vitro* transcription with T7 RNA polymerase (ThermoFisher Scientific, Waltham, MA, USA), the linearized plasmid containing a *nanos1* cDNA fragment and a DIG RNA labeling mix (Roche Applied Science, Mannheim, Germany). The probe was purified using SigmaSpin™ Sequencing Reaction Clean-Up (Sigma-Aldrich, St. Louis, MO, USA). Whole-mount *in situ* hybridization was conducted according to a previously described method (Zhang, Tan, Zhang, & Xu, 2006), with some modifications. The pre-hybridization and hybridization temperature was 70°C. Polyvinylalcohol (Final concentration 2%) was added to the alkaline buffer containing NBT/BCIP (Roche). After staining, the stained embryos were fixed in 4% PFA overnight at 4°C. After changing the storage solution to PBST buffer, the embryos were incubated in glycerol to take the photographs. We performed cross frozen sections of 10-15 µm with some frozen embryos (Jiao *et al*., 2015). All digital images were taken using a Leica DM LB2 microscope with a Leica DFC420C (Leica, Wetzlar, Germany).

Results

Isolation and Characterization of Olive Flounder Nanos1

The full-length olive flounder *nanos1* cDNA has 1215 base pairs (bp) and is composed by a 112 bp 5'-untranslated region (UTR), a 684 bp open reading frame (ORF), and a 419 bp 3'-UTR. The olive flounder *nanos1* homolog encodes a protein of 227 AA with an RNA-binding domain, and a conserved zinc-finger domain (Figure 1A). The deduced amino acid sequence of the olive flounder Nanos1 showed higher similarity to the Nanos1 from teleost fish than to those of other species. For example, flounder Nanos1 was 58% and 44% identical to medaka (*Oryzias latipes*) Nanos1a and Nanos1b, respectively; and 64% identical to the Chinese sturgeon (*Acipenser sinensis*) Nanos1. In contrast, the olive flounder Nanos1 showed only 31.8% and 29.4% identity to the NANOS1 of human and mouse, respectively (data not shown). The results from our phylogenetic analysis indicated that the olive flounder Nanos1 homolog grouped with Nanos1 homologs of other teleost fishes and its closest relative was the Chinese sturgeon Nanos1 (Figure 1B).

Spatiotemporal Pattern of Nanos1 Transcripts During Olive Flounder Embryo Development

The expression patterns of *nanos1* during embryogenesis were observed by whole mount *in situ* hybridization (Figure 2). *Nanos1* was expressed in the head at 75% of the epiboly stage (Figure 2A). From 90% of the epiboly stage, the *nanos1* transcript was detected in the diencephalon and hindbrain (data not shown). At the 24.25 hpf stage, the transcript was also detected in the trunk neural crest, which develops into the medulla oblongata (Figure 2C). At the 25.75 hpf stage, *nanos1* mRNA expression was also detected in the retina (Figure 2D). At the 28.75hpf stage, the transcript was detected in the nose and weakly in the olfactory bulb (Figure 2E). At the 35.75hpf stage, the expression was also observed in the branchial arch (Figure 2F). At the 50.75hpf stage, the transcript was detected in the abdomen (Figure 2H). Finally, at the hatching stage, the *nanos1* transcript was present in the somatic gonadal cells (Figure 2I).

The expression levels of *nanos1* during embryo development were analyzed by reverse transcription-

Table 1 Sequences of the primers used for PCR.

Primer name	Sequence (5' to 3')
nanos1-5-1	GGAGTCGTCTTCCAAACCCAGGGTGG
nanos1-5-2	GGTGGCTTTAAGAGACTCGGTGATGG
nanos1-3-1	CGGCAGAGTCCTGTGTCCCATCCTCC
nanos1-3-2	CCTCCGTGCGTACACCTGCCCCCTCTG
nanos1-Fw	ATGGATTTCTTGGATCACAG
nanos1-Rw	TCAGAAGCTTTTCAGCCTTTT
β-actin-Fw	ACTACCTCATGAAGATCCTG
β-actin-Rw	TTGCTGATCCACATCTGCTG

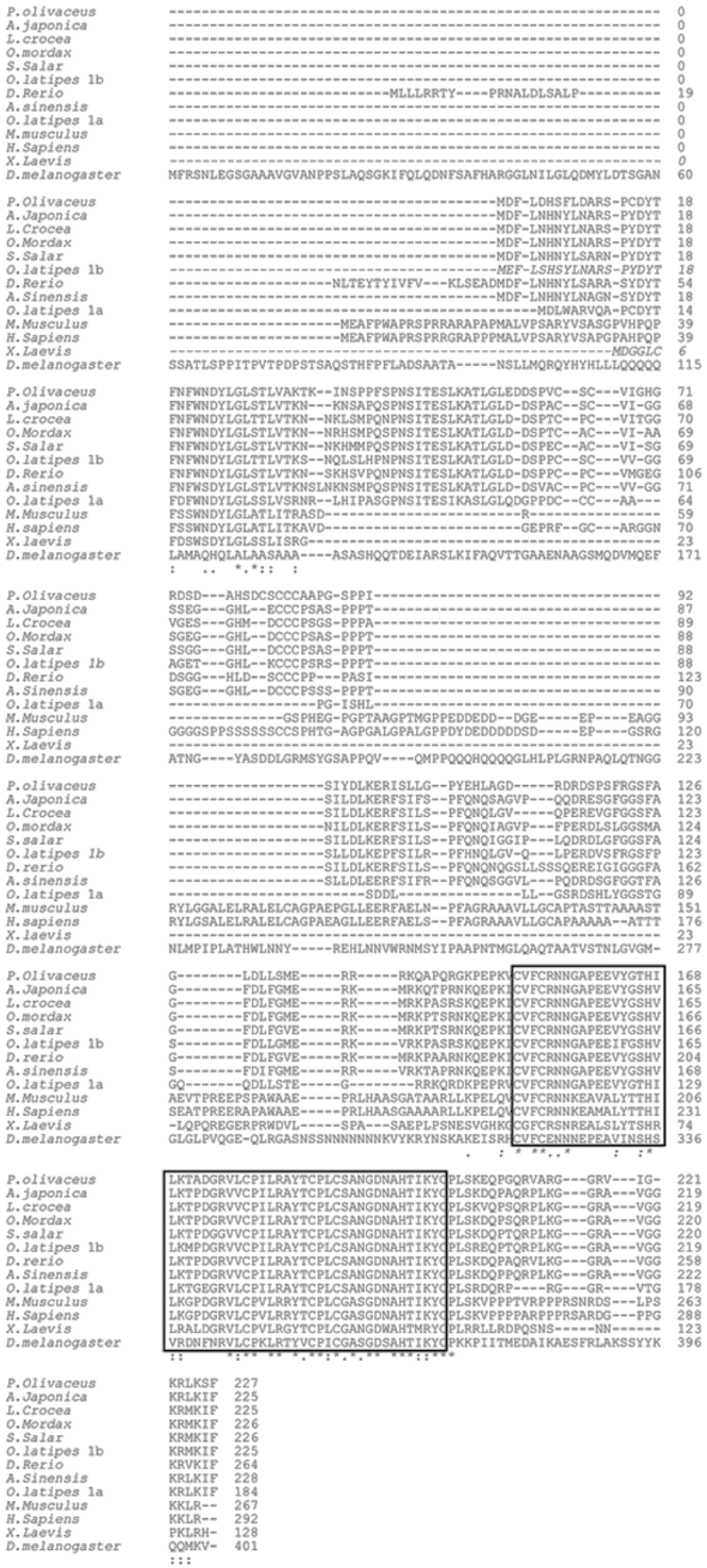

Figure 1A. Comparison of olive flounder (*Paralichthys olivaceus*) nanos1 protein and nanos homologs in other species.

An asterisk (*) indicates positions that have a single, fully conserved residue. A colon (:) indicates conservation between groups with strongly similar properties; A period (.) indicates conservation between groups with weakly similar properties. An open rectangle (□) indicates the presence of a zinc finger domain.

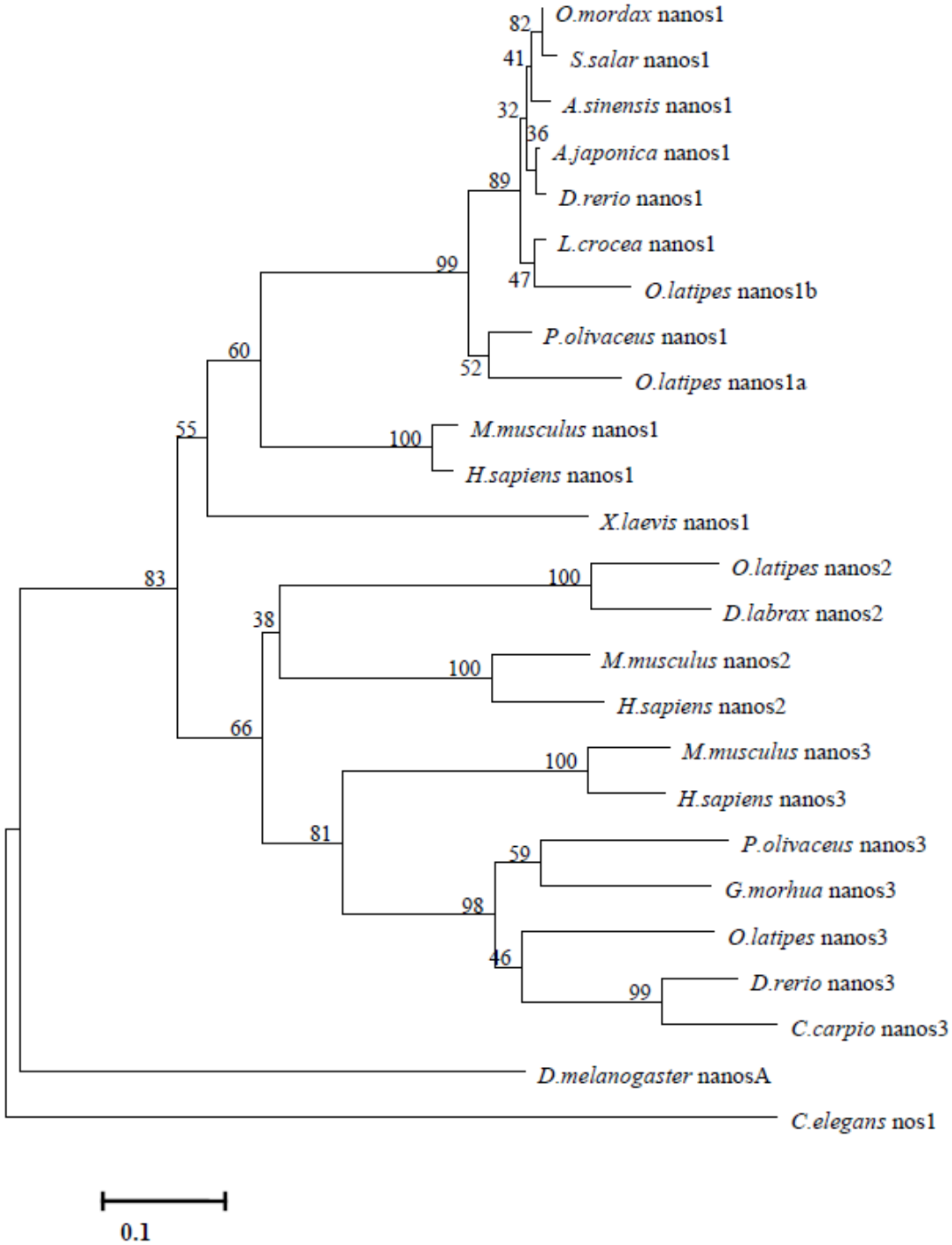

Figure 1B. Phylogenetic relationships of the olive flounder Nanos1 protein and nanos homologs in other species**.**

The GenBank accession numbers for the *nanos* homologs used in this analysis are as follows: *P. olivaceus nanos1* (XXXXXX), *P. olivaceus nanos3* (KR855714), *Anguilla japonica nanos1* (AB674328.1), *Larimichthys crocea nanos1* (KF690631.1), *Osmerus mordax nanos1* (BT074904.1), *Salmo salar nanos1* (NM_001141585.1), *Oryzias latipes nanos1a* (AB437935.1), *O. latipes nanos1b* (NM_001160469.1), *O. latipes nanos2* (NM_001160447.1), *O. latipes nanos3* (NM_001122828.1), *Danio rerio nanos1* (XM_003199836.3), *D. rerio nanos3* (AY052376.1), *Homo sapiens NANOS1* (NM_199461.2), *H. sapiens NANOS2* (NM_001029861.2), *H. sapiens NANOS3* (NM_001098622.2), *Mus musculus Nanos1* (NM_178421.3), *M. musculus Nanos2* (NM_194064.2), *M. musculus Nanos3* (NM_194059.2), *Acipenser sinensis nanos1* (JQ410472.2), *Cyprinus carpio nanos3* (AB576134.1), *Gadus morhua nanos3* (HM451457.1), *Dicentrarchus labrax nanos2* (FQ310508.3), *Xenopus laevis nanos1* (NM_001088034.1), *Drosophila melanogaster NanosA* (NM_057310.4), and *Caenorhabditis elegans nos1* (NM_063957.1). The sum of the optimal tree's branch length = 4.78793245. The percentage of replicate trees in which the associated taxa clustered together are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, and the units of branch lengths are the same as those of the evolutionary distances used to infer the phylogenetic tree. The units of evolutionary distances represent the number of amino acid substitutions per site (Zuckerkandl & Pauling, 1965).

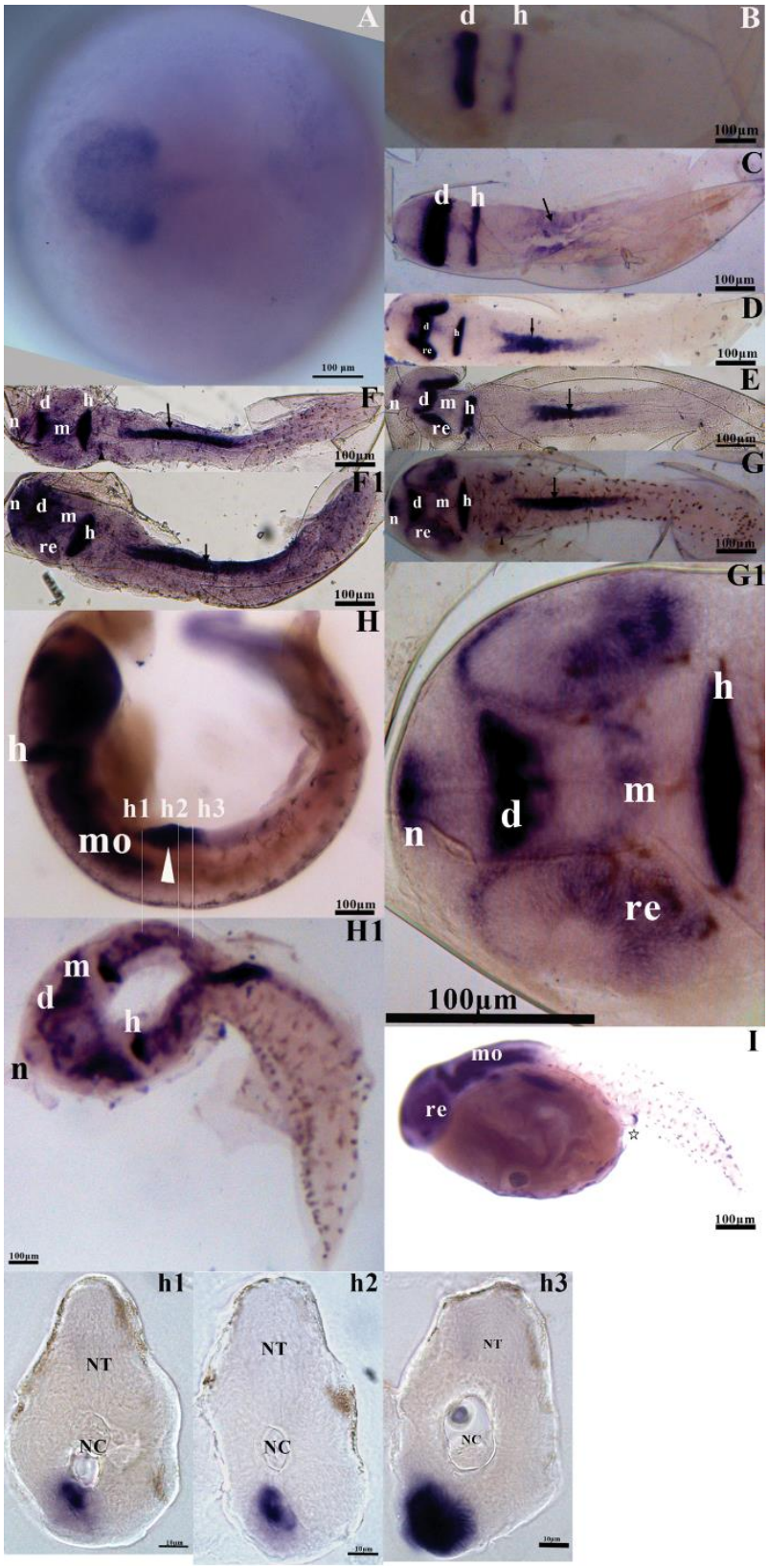

Figure 2. Spatial distribution of olive flounder *nanos1* transcripts during embryogenesis.

Representative images of various developments stages. (**A**) 75% epiboly, top view, head to the left. (**B**) 22.75hpf. (**C**) 24.25hpf. (**D**) 25.75hpf. (**E**) 28.75hpf. (**F**) 37.75hpf. (**G**) 40.75 hpf, head to the left, dorsal view, **F1**, 37.75 hpf, head to the left, ventral view. **G1**, magnification of **G**. **H**, **H1**, 50.75 hpf. **H**, side view. **H1**, dorsal view of head. h1, h2, h3, cross-section of **H**, dorsal to top. I, hatching stage, head to the left, dorsal view. m, midbrain; d, diencephalon; h, hindbrain; re, retina; n, nose; mo, medulla oblongata. NT, neural tube; NC, notochord. **(D-G**) The black arrow indicates the trunk neural (which will later form the medulla), (**G**) the black arrowhead indicates the branchial arch, (**H**) the white arrowhead indicates the abdomen, (**I**) the white star indicates the somatic gonadal cells.

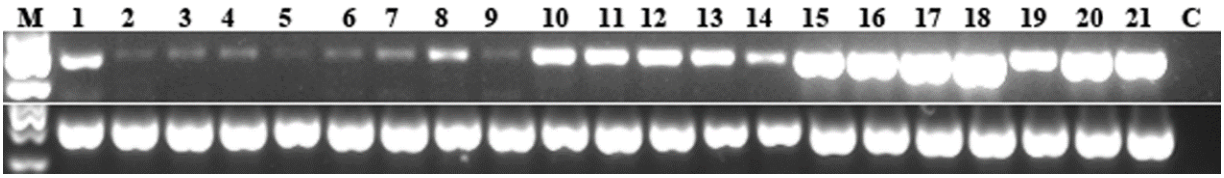

Figure 3. Reverse transcription-PCR analysis of *nanos1* expression during olive flounder embryogenesis.

Up, *nanos1*; Down *β-actin*. M, Marker; 1, unfertilized egg; 2, 1-cell; 3, 2-cell; 4, 4-cell; 5, 8-cell; 6, 16-cell, 7, 32-cell; 8, multicellular stage; 9, blastula; 10, 50% epiboly; 11, 70% epiboly; 12, 90% epiboly,13, 22.75hpf; 14, 24.75hpf, 15, 26.hpf, 16, b31.hpf, 17, 37hpf, 18, 43.45hpf, 19, 51.45hpf, 20, 56.45hpf. 21, hatching stage; C, Blank control.

PCR (Figure 3). *nanos1* was expressed at all the stages analyzed, including in unfertilized embryos, which suggested that *nanos1* was maternally inherited. Prior to the blastula stage, expression was very low.

Discussion

In this study, we isolated the olive flounder *nanos1* homolog and characterized its expression patterns during the process of embryonic development by *in situ* hybridization and RT-PCR.

It has been proposed that an additional fish-specific genome duplication (FSGD) event occurred during teleost evolution (Amores *et al*., 1998; Venkatesh, 2003). In medaka, there are two *nanos1* homologs, *nanos1a* and *nanos1b*, which are believed to have originated from a gene duplication event (Aoki *et al*., 2009). In our study, we isolated one *nanos1* homolog from the olive flounder. Our phylogenetic analysis showed that the olive flounder nanos1 homolog was most closely related to Chinese sturgeon nanos1 that to any other species, and clustered into a group that included most nanos1 of teleost fish, including medaka Nanos1b, whereas medaka nanos1a was in another cluster. Additionally, there were two Nanos1 homologs in other fish: the stickleback and the tetraboron (Aoki *et al*., 2009). Therefore, considering that the teleosts are believed to have undergone a genome-wide duplication event and the discovery of multiple *nanos* paralogues in other teleost fish, it is likely that there is another *nanos1* in the olive flounder.

The RT-PCR detected that the olive flounder *nanos1* transcript in unfertilized embroys but the expression remained low until the blastula stage. This result showed that *nanos1* mRNA is inherited maternally, as is the case in *Drosophila*, mouse, and *Xenopus* (Bergsten & Gavis, 1999; Haraguchi *et al*., 2003; Lai, Zhou, Luo, Fox, & King, 2011). The *nanos1* mRNA of the olive flounder was not analyzed in the whole-mount *in situ* hybridization experiments until 50% epiboly, which coincided with the low expression levels observed by RT-PCR.

nanos1 transcripts have been detected in the nervous system of vertebrates such as frog, medaka, zebrafish, mouse, and human (Haraguchi *et al*., 2003; Aoki *et al*., 2009; Julaton & Reijo Pera, 2011; Lai *et al*., 2011), with the function of *nanos1* varying from species to species. For example, no significant neural defects were observed in *Nanos1*-deficient mice (Haraguchi *et al*., 2003), while *Nanos* was required for the formation of the peripheral nervous system in *Drosophila* (Ye *et al*., 2004; Brechbiel & Gavis, 2008). In medaka, *nanos1a* and *nanos1b* showed different expression patterns in the nervous system, *nanos1a* was expressed in the caudal wall of the mesencephalon, part of the cerebellum, part of the diencephalon, the habenula, the rostral hypothalamus, part of the hypothalamus, and the peripheral ganglia. *nanos1b* was presented in the telencephalon, the proliferation zone of the retina, the optic tectum, and the optic vesicle.

In this study, the expression of *nanos1* in the olive flounder exhibited a mixture of the *nanos1a* and *nanos1b* expression patterns previously observed in medaka (the expression was detected in the diencephalon, the proliferation zone of the retina, and the nose). The olive flounder *nanos1* was also expressed in the medulla oblongata similar to the expression patterns of mouse *nanos1* (Haraguchi *et al*., 2003). Additionally, the mouse *nanos1* showed a different expression pattern from that of medaka *nanos1a* or medaka *nanos1b*, but similar to that of both medaka *nanos1* (Aoki *et al*., 2009). Therefore, our data suggested that the function of *nanos1* might be species-specific. Further investigation is required to establish the role of the olive flounder *nanos1* in the development of the nervous system.

The olive flounder *nanos1* was also expressed in the abdomen, which could represent the progenitor cells of the gut, similar to *Drosophila*, where *nanos A* was expressed in the abdomen (Lehmann & Nusslein-Volhard, 1991). *nanos* was expressed the midgut in *Bombyx mori* (Zhao *et al*., 2008), in the developing foregut of the polychaete *Capitella* spp. (Dill & Seaver, 2008), and in the intestine of a snail (*Ilyanassa obsoleta*) (Rabinowitz, Chan, Kingsley, Duan & Lambert, 2008). In adult Chinese sturgeons (*A. sinensis*), *nanos1* was also expressed in the intestine (Ye *et al*., 2012). Thus, the expression of *nanos* in peripheral tissues may not only be conserved in invertebrates (Ye *et a*l., 2012) but also in vertebrates.

nanos1 was mainly expressed in various somatic tissues of vertebrates (Jaruzelska *et al*., 2003; Haraguchi *et al*., 2003; Aoki *et al*., 2009;). However, its expression was also detected in germ cells of *Xenopus* (Mosquera,

Forristall, Zhou, & King, 1993; Lai *et al*., 2011;) and in the gonads of species such as *Xenopus*, mouse, and human (Haraguchi *et al*., 2003; Jaruzelska *et al*., 2003; Julaton & Reijo Pera, 2011). In teleost, *nanos1* transcripts were also detected in the gonads or germ cells. In medaka, *nanos1a* expression has been observed in the somatic cells that surround the meiotic germ cells (Aoki *et al*., 2009), while *nanos1* transcripts were found in the gonads of adult Chinese sturgeons (*A. sinensis*) (Ye *et al*., 2012). In the olive flounder, it was more likely that *nanos1* was expressed in the somatic gonadal cells during the hatching stage. Thus, the function of *nanos1* in the germ cells or gonads of the olive flounder will require further elucidation.

Conclusion

In conclusion, the sequence of the olive flounder *nanos1* homologue was conserved with those of other species. *Nanos1* expression patterns have been shown to be species-specific, implying that *nanos1* function could be both divergent and convergent depending on the species.

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