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Molecular and expression characterization of a *nanos1* homologue in Chinese sturgeon, *Acipenser sinensis*

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ABSTRACT

The *nanos* gene family was essential for germ line development in diverse organisms. In the present study, the full-length cDNA of a *nanos1* homologue in *A. sinensis*, *Asnanos1*, was isolated and characterized. The cDNA sequence of *Asnanos1* was 1489 base pairs (bp) in length and encoded a peptide of 228 amino acid residues. Multiple sequence alignment showed that the zinc-finger motifs of Nanos1 were highly conserved in vertebrates. By RT-PCR analysis, *Asnanos1* mRNAs were ubiquitously detected in all tissues examined except for the fat, including liver, spleen, heart, ovary, kidney, muscle, intestines, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata. Moreover, a specific polyclonal antibody was prepared from the *in vitro* expressed partial *As*Nanos1 protein. Western blot analysis revealed that the tissue expression pattern of *As*Nanos1 was not completely coincided with that of its mRNAs, which was not found in fat, muscle and intestines. Additionally, by immunofluoresence localization, it was observed that *As*Nanos1 protein was in the cytoplasm of primary oocytes and spermatocytes. The presented results indicated that the expression pattern of *Asnanos1* was differential conservation and divergence among diverse species.

1. Introduction

The *nanos* gene encoding an RNA binding protein containing a zinc-finger motif was essential for germ line development in diverse organisms, although the regulated processes varied among species and between different homologs. The *nanos* gene was first identified as a maternal effect gene in *Drosophila* required for abdomen formation (Lehmann and Nusslein-Volhard, 1991). Further study indicated that the single *nanos* homolog was involved in germ cell migration, suppression of somatic cell fate in the germ line and maintenance of stem cell self-renewal (Asaoka-Taguchi et al., 1999; Hayashi et al., 2004; Wang and Lin, 2004). However, three *nanos* homologs (*nos-1*, *nos-2* and *nos-3*) had been identified in *C. elegans*. *Nos-1* and *nos-2* were required for the incorporation of PGCs into the somatic gonad and maintaining germ cell viability during larval development, while *nos-3* controlled the sperm–oocyte switch mechanism in hermaphrodites via its direct interaction with FBF, a Pumilio family protein (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). Similarly, in vertebrates, *nanos* played important roles in germ cell as well. In zebrafish, *nanos1* was required for the PGCs survival during embryogenesis, and to maintain oocyte production in adult (Draper et al., 2007; Koprunner et al., 2001). Three *nanos* homologs were identified in mouse. Among them, *nanos2* and *nanos3* were involved in germ cell function. *Nanos2* knockout mice were sterile, and resulted in reduce of size and weight of testis and a complete loss of spermatogonia, while female mice appeared developmentally normal and were fertile. Characterization of *nanos3* targeted disruption revealed the totally loss of germ cells in both sexes (Tsuda et al., 2003). Later studies showed that *nanos3* was implicated in the maintenance of PGCs during migration by repression of apoptosis

Abbreviations: bp, base pairs; h, hour; UTR, untranslated region; RT-PCR, reverse transcription-polymerase chain reaction; PGCs, primordial germ cells; MS-222, 3-aminobenzoic acid ethyl ester methanesulfonate-222; SMART, switching mechanism at 5-end of RNA transcript; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol tetraacetic acid; PVDF, polyvinylidene fluoride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; PBS, phosphate buffer solution; PI, propidium iodide.

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(Suzuki et al., 2007). As for human, there were also three *nanos* genes, and the function of *nanos2* and *nanos3* in the development of human germ cell had been particularly studied (Julaton and Reijo Pera, 2011; Kusz et al., 2009).

In addition to its germline functions, *nanos* was expressed widely in multipotent cells and somatic tissues. The homologue of *nanos* gene was identified in diverse invertebrates, such as *Nematostella vectensis* (Extavour et al., 2005), *Ilyanassa obsoleta* (Rabinowitz et al., 2008), *Hydra magnipapillata* (Mochizuki et al., 2000), *Platynereis dumerilii* (Rebscher et al., 2008), *Bombyx mori* (Zhao et al., 2008), *Hemicentrotus pulcherrimus* (Fujii et al., 2006), *Strongylocentrotus purpuratus* (Juliano et al., 2010) and so on. In sea anemone, *nanos* gene was expressed in multiple somatic types during early embryogenesis (Extavour et al., 2005), while *Hydra nanos* was expressed in multipotent interstitial cells, which produced both several somatic cell types and germ cells (Mochizuki et al., 2000). In vertebrates, such as human, *nanos1* was found in various tissues, such as brain, heart, liver, spleen, ovary, testis and so on (Julaton and Reijo Pera, 2011). By β-gal hischemical staining, mouse *nanos1* was predominantly expressed in the hippocampal formation of the adult brain, which was also observed in the cerebellum, lateral geniculate body in the thalamus, ventral tegmental area, piriform cortex, superior colliculus in the mesencephalon, and olfactory bulb (Haraguchi et al., 2003). In medaka, *nanos1* was duplicated, resulting in *nanos1a* and *nanos1b*. By in situ hybridization, *nanos1a* was detected in the nose, diencephalon, hypothalamus, caudal wall of the mesencephalon, cerebellum, and peripheral ganglia, while *nanos1b* was found in the parts of the telencephalon, nose, retina, optic tectum, mesencephalon, otic vesicle, and branchial arch; after the onset of sexual differentiation,*nanos1a* was expressed in the somatic cells surrounding the oocytes (Aoki et al., 2009). However, the expression pattern of *nanos1* in somatic tissues and its role in germ cell were still unknown.

Chinese sturgeon (*Acipense sinensis*) is one of the most primitive Actinopterygii species and belongs to Acipenseriformes, with some characters between chondrichthyes and osteichthyes. To date, it is a rare and endangered species, due to over-fishing for meat and production of caviar, destruction of their spawning grounds and other anthropogenic interferences (Birstein et al., 2002; Wei et al., 1997). To save this species more efficiently and to be able to develop an aquaculture industry, artificial propagation has been attempted since 1983. A severe problem is that Chinese sturgeon is an extremely late and asynchronous sexual maturation species. Generally, sexual maturity in males is reached between 8 and 18 years and in females between 14 and 26 years, respectively (Chen et al., 2006). However, with the improving of biotechnology, germ cell transplantation is believed to be an available and fast method for protecting this species. In order to implement this plan, germ cells must be isolated firstly. Currently, it is widely acknowledged that *nanos* can be used as a marker for germ cells (Extavour and Akam, 2003). In present study, we identified a *nanos*-related gene (*nanos1*) in Chinese sturgeon and examined its mRNA and protein expression patterns in diverse somatic tissues. Additionally, the subcellular localization of *As*Nanos1 protein in the gonad was reported.

2. Materials and methods

2.1. Animals and samples

All Chinese sturgeons used in this study were cultured in Taihu station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Deep anesthesia was induced by a 0.05 % solution of MS-222 (Sigma, USA). The tissues samples from two and a half years old female Chinese sturgeons (about 1.11 m in length and 7.2 kg in weight) were collected within 30 min of exsanguinations by tailing and immediately dipped into liquid nitrogen and stored at −80 °C in May 2011. Additionally, surgical procedures were used to obtain the testis and ovary tissues from 4.5 years old Chinese sturgeons in May 2012. The experimental procedures were based on the standards of the Chinese Council on Animal Care.

2.2. RNA extraction and SMART cDNA synthesis

Total RNAs were extracted using SV total RNA isolation system (Promega, USA). The quality of RNAs was measured at A260 nm and the purity from the ratio A260:A280 nm (Eppendorf Biometer, Germany). Double strands cDNAs were synthesized and amplified according to the reports described previously (Li et al., 2005) using the Switching Mechanism at 5-end of RNA Transcript (SMART) cDNA Library synthesis Kit (Clontech, USA). Briefly, 100 ng of total RNAs were reverse-transcribed in 42 °C for 1 hour at the presence of both 3′ SMART CDS primer II A and SMART II oligonucleotide. And 2 μL of first-strand reaction products were used in each 100 μL long-distance PCR system containing 0.2 μM PCR primer II A. The LD-PCR parameters were 95 °C for 5 sec and 68 °C for 6 min on PTC-200 thermal cycler for 15 cycles. 5 μL of PCR products were separated and checked by electrophoresis on 1% agarose gels containing ethidium bromide.

2.3. Cloning of the full-length cDNA of Asnanos1

The full-length of *Asnanos1* cDNA was amplified by 5′- and 3′-RACE (rapid amplification of cDNA ends). Degenerate sense and antisense primers were designed and synthesized according to a nucleotide alignment of different *nanos* cDNAs. The 5′ end of the cDNA was amplified with a 5′PCR anchor primer (5′-AP, Table 1) and specific antisense primer (*Asnanos1*-5′RACE reverse). The 3′-end of *Asnanos1* cDNA was amplified using specific sense primers (*Asnanos1*-3′RACE forward1 and *Asnanos1*-3′RACE forward2) and a PCR anchor primer corresponding to the terminal anchor sequence of the cDNA (3′-AP, Table 1). All PCRs were performed on a PTC-200 thermal cycler (Bio-Rad, USA) by denaturation at 94 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min and an additional elongation at 72 °C for 10 min after the last cycle. The PCR mixture contained 1U Taq DNA polymerase (MBI, USA) together with 0.2 mM of each dNTPs, a suitable reaction buffer (MBI), 1.5 mM $MgCl_2$, 15 pmol of each primer and 2 μL diluted SMART cDNA. The amplified DNAs were visualized by electrophoresis of ethidium bromide stained

Table 1
Sequences of the primers used for PCR.

Primer name	Purpose	Sequence (5′ to 3′)
Degenerate_forward	Partial	TTYAAYTTYTGGAACGACTAC
Degenerate_reverse	Partial	TGRGCRTTGTCACCRTTRG
Asnanos1-3′RACE forward1	RACE	CTGCTGTTGCCCCTCTTC
Asnanos1-3′RACE forward2	RACE	TGTTACCCGATAACATCGGAGAA
Asnanos1-5′RACE reverse	RACE	ACTGCATAGGGGGCAAGTGTAA
Asnanos1-RT-F	RT-PCR	AACGACTACCTGGGACTTTCTACT
Asnanos1-RT-R	RT-PCR	ACTGCATAGGGGGCAAGTGTAA
Asβ-actin-F	Control	TGGACTTGGCTGGTCGTGAC
Asβ-actin-R	Control	CTGGCAGCTCATAGCTCTTC
Asnanos1-E-F	Express	GGATCCTTGAATGCTGGAAACTCT
Asnanos1-E-R	Express	CTCGAGTCTCTTACCCCCAACTG
3′ SMART CDS primer II A	SMART cDNA	AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$ N$_{-1}$ N
SMART II oligonucleotide	SMART cDNA	AAGCAGTGGTATCAACGCAGAGTACGCGGG
primer II A	SMART cDNA	AAGCAGTGGTATCAACGCAGAGT
3′-AP	RACE	GGTATCAACGCAGAGTACTT
5′-AP	RACE	ATCAACGCAGAGTACGCGGG

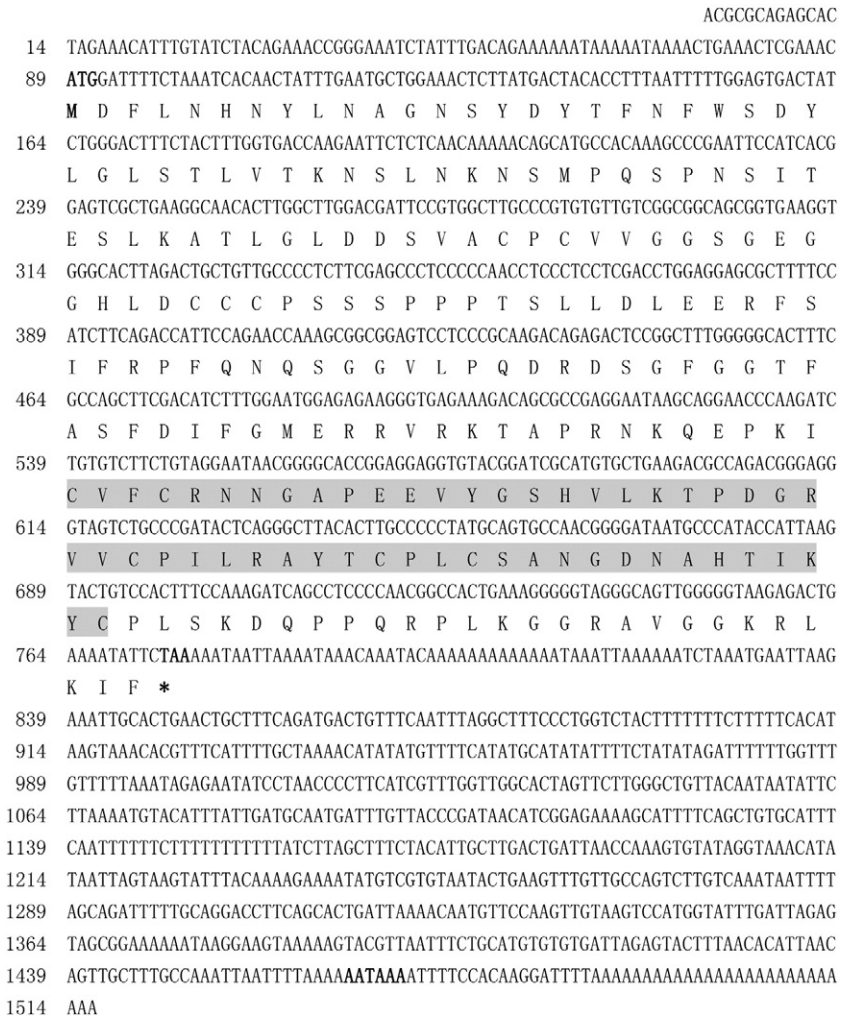

Fig. 1. Nucleotide sequence of the Chinese sturgeon *nanos1* and its deduced amino acids sequence. The nucleotide and amino acid residue number are shown on the left. The initiation codon, termination codon and consensus polyadenylation signal AATAAA are in bold. The zinc-finger motif is shaded in gray. This sequence has been deposited in the GenBank nucleotide database, under accession no. JQ410472.

agarose gel, cloned into pMD18-T vector (Takara, Japan), and sequenced (Sangon, China).

2.4. Database and sequence analysis

The cDNA and deduced amino acid sequences were analyzed by BLAST program (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequence identity was performed using CLUATAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2). Multiple alignments were performed with the MAP method at BCM Search Launcher web servers (http://searchlauncher.bcm.tmc.edu/) and the printing output was shaded by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed using Mega 4.1 molecular evolutionary genetic analysis software package by bootstrap analysis 1000 replicates using neighbor-joining.

2.5. RT-PCR analysis

For RT-PCR, total RNAs extracted from different tissues (including liver, spleen, heart, ovary, fat, kidney, muscle, intestines, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata) from two to two and a half years old cultured Chinese sturgeons were isolated using SV Total RNA Isolation System according to the manufacturer's instructions (Promega, USA). Four hundred nanogram total RNAs were respectively reverse-transcribed with PrimeScript RT reagent Kit With gDNA Eraser (Takara, Japan) as described by the manufacturer. All of the resultant cDNAs were respectively diluted 1:3, and then used as templates. The primer pairs, *Asnanos1*-RTF and *Asnanos1*-RTR (Table 1), were designed to detect the differentially expressed pattern of *Asnanos1*. Amplification reactions were performed in a volume of 25 μL containing 2 μL cDNA as template DNA, 0.5 μM each primer, 0.5 U Taq polymerase (MBI), 0.1 μM of each dNTP (Pharmacia, USA), 10× buffer for Taq polymerase (MBI). Each PCR cycle included denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 20 sec. As a positive control for the RT-PCR analysis, *Asβ-actin* was amplified by primers *Asβ-actin*-F/*Asβ-actin*-R (Table 1) to determine the template concentration and to provide an external control for PCR reaction efficiency under the same reaction conditions as *Asnanos1*.

2.6. Production of fusion proteins and the polyclonal antibody

A partial cDNA fragment encoding 216 aa of *As*Nanos1 was amplified used primers in Table 1, double-digested with *BamH* I and *Xho* I, subcloned into the pET32a (+) vector (Novagen, Germany), and transformed into the expression cell, *E. coli* BL21 (DE3). The fusion protein expression was induced with IPTG (final concentration 1 mM), and the bacterial cells were harvested respectively by centrifugation after culturing for 4 h in a medium containing 50 μg/mL of ampicillin at 37 °C. After detecting the fusion protein only in inclusion

body, the harvested bacteria were suspended thoroughly in bacterium lysis buffer (50 mM Tris–HCl, pH 7.9, 0.2 M NaCl, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM Benzamide), lysed on ice by lysozyme (0.2 mg/mL) with 1% Triton-100, violently ruptured on ice by ultrasonic wave. The inclusion body was purified by sucrose solution (40% sucrose, 10 mM Tris–HCl, pH 7.9, 0.2 M NaCl, 1 mM EDTA). The purified inclusion body was suspended by electrophoresis 2× loading buffer (0.1 M Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol Blue, 10% 2-mercaptoethanol), and about 4 mg fusion protein was isolated and purified by 12% SDS-PAGE electrophoresis as described previously(Dong et al., 2004). Then, the fusion protein was directly excised from the gel and grinded to cream (Dong et al., 2004), and used to prepare polyclonal antibody by immunizing rabbit as described previously (Xia et al., 2007). Briefly, white rabbits were first injected with complete Freund Adjuvant at the vola. A week later, 500 μg of the purified protein with the gel was injected into the swollen lymphoid node. After the first injection of the antigen, two times of booster injections were done with the same antigen amount for two weeks. The rabbits were bled 10 days after the last booster and serum samples were stored at −80 °C until use.

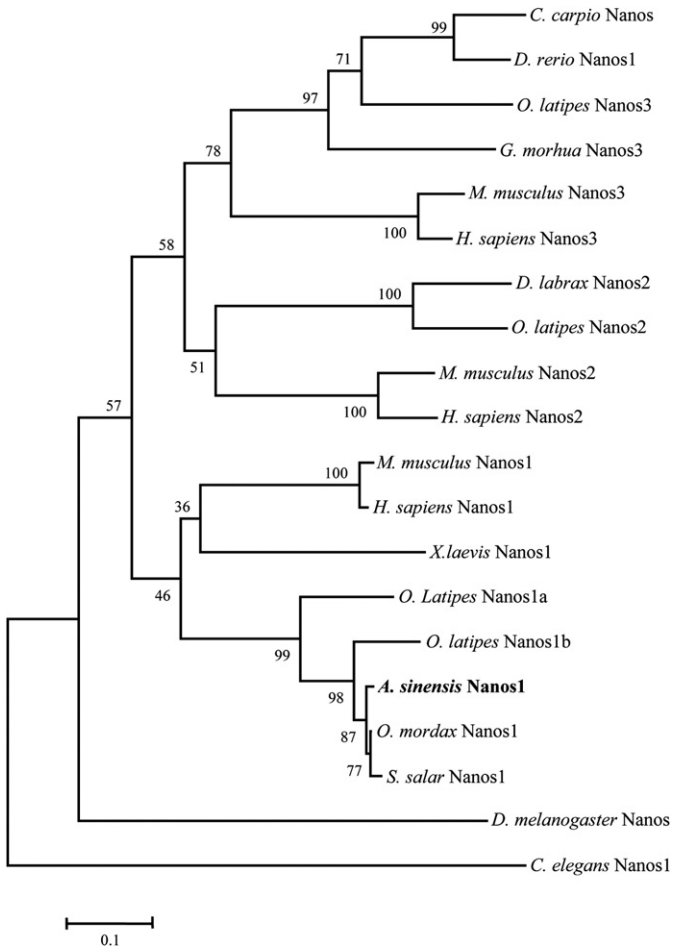

Fig. 2. Phylogenetic relationships of Chinese sturgeon Nanos1 gene and other Nanos proteins. Phylogenetic relationship of *nanos* gene family proteins is analyzed with Mega 4.1 program by bootstrap analysis using neighbor-joining (1000 replicates). The sequences were obtained from GenBank database. *C. carpio* (GenBank ID: BAJ76659), *D. rerio* (GenBank ID:AAL15474), *O. latipes* (GenBank ID: NP_001116300, NP_001153919, BAG84599 and NP_001153941), *G. morhua* (GenBank ID: ADV36251), *M. musculus* (GenBank ID: NP_918948, NP_918953 and NP_848508), *H. sapiens* (GenBank ID: NP_001092092, NP_001025032 and NP_955631), *D. labrax* (GenBank ID: CBN81978), *X. laevis* (GenBank ID: NP_001081503), *A. sinensis* (GenBank ID: JQ410472), *O. mordax* (GenBank ID: ACO09328), *S. salar* (GenBank ID: NP_001135057), *D. melanogaster* (GenBank ID: NP_476658), *C. elegans* (GenBank ID: NP_496358).

2.7. Western blot analysis

The protein extracts prepared from somatic tissues of 2.5 years old Chinese sturgeon were subjected to Western blot analysis. Equivalent (0.1 g) tissues were respectively sampled from liver, spleen, heart, ovary, fat, kidney, muscle, intestines, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata of immature fish. The tissue samples were homogenized respectively in 1 mL of chilled extract buffer (20 mM HEPES, 100 mM β-glycerol phosphate, 20 mM EGTA, 15 mM $MgCl_2$, 2 mM PMSF, 1 mM DTT, 2.5 μg/mL Leupeptin, 2.5 μg/mL Aprotinin, pH7.3). After they were separated on 15% SDS-PAGE gel, the proteins were electrophoretically blotted to PVDF membranes (Millipore, USA). The membranes were blocking with 5% dry milk in TBS buffer [100 mM NaCl, 100 mM Tris–HCl, pH 7.5]. The blocked membranes were incubated with rabbit *As*Nanos1 antisera at a dilution of 1:250 in TBS buffer containing 1.0% dry milk at 4 °C for 16 h. The membranes were washed three times for 10 min each in TBST buffer (TBS buffer containing 0.1% Tween-20) and then incubated with 1:5000 diluted alkaline phosphatase conjugated goat anti-rabbit IgG. After washing three times for 10 min each in TBST buffer, detection was performed using BCIP/NBT. Additionally, to test the specificity of *As*Nanos1 antibody, the antiserum was preabsorbed with the purified recombinant *As*Nanos1 protein for 16 h at 4 °C. As a negative control, the preimmune serum of the same rabbit was used for Western blot detection.

2.8. Immunofluorescence localization

The sampled Chinese sturgeon gonad tissues were immediately immersed into 4% paraformaldehyde 12 h at 4 °C, dehydrated in a graded methanol series, and then frozen at −20 °C. Before the samples were sectioned, they were first hydrated in a graded methanol series, and immersed into PBS buffer (containing 30% saccharose) for 12 h at 4 °C. Then, the samples were embedded in Optimal Cutting Temperature (O.C.T., Sakure), and quickly cooled in liquid nitrogen. The cooled samples were sectioned at 8 μm with frozen microtomy (Leica, Germany), and collected on TESPA-coated slides. The sections were incubated for 1 h with 5% dry milk in PBS at room temperature to prevent non-specific binding of antibodies. The sections were incubated for 12 h at 4 °C with rabbit *As*nanos1 antisera diluted 1:250. After five rinses in PBST, they were exposed to 10% goat serum for 1 h and finally to fluorescein-conjugated goat anti-rabbit IgG (H + L) for 1 h diluted 1:100. After five-time washes in PBS, the slides were stained with PI for 10 min at room temperature, washed three times in PBS, and subjected to confocal microscope (Zeiss, Germany). In the control, the specific *As*Nanos1 antisera were replaced with pre-immune serum.

3. Results

3.1. Molecular characterization of Asnanos1

Using degenerate primers and RACE strategy, we cloned *Asnanos1* from the ovary of Chinese sturgeon. The cDNA and deduced amino acid sequences of *Asnanos1* were shown in Fig. 1. The *Asnanos1* cDNA was 1,489 bp in total length (polyA tail excluded), which consisted of a 88 bp 5′-untranslated region (UTR), a coding sequence of 687 bp and a 714 bp 3′-untranslated region (UTR). A consensus polyadenylation signal AATAAA was located 18 bp upstream from the poly (A) tail. *Asnanos1* encoded a predicted propeptide of 228 amino acid residues which contained a zinc-finger motif conserved in *nanos* gene family (Fig. 1).

3.2. Phylogenetic analysis and comparison of Nanos1 sequence

Molecular phylogenetic tree of Nanos1 was constructed by the neighbour-joining method. As shown in Fig. 2, two distinct clusters were found in vertebrate: one cluster characterized by Nanos1 and

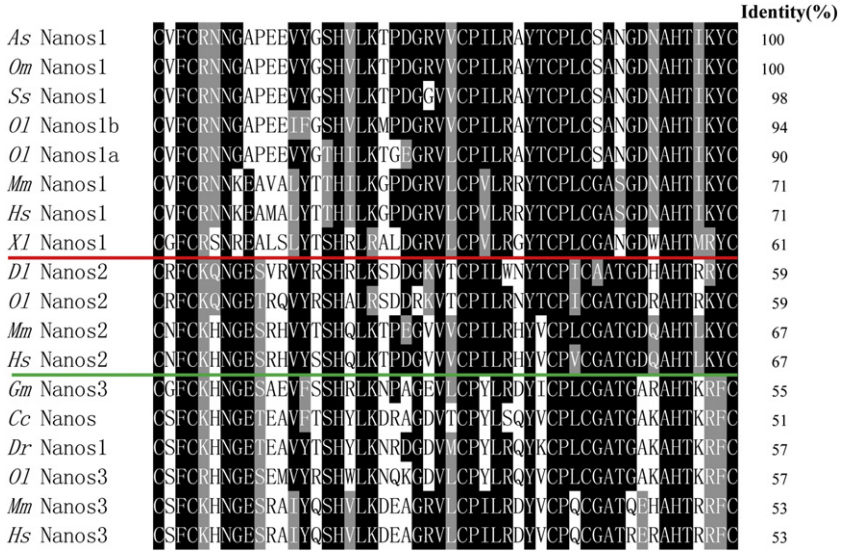

Fig. 3. Amino acid alignment of the zinc-finger domain of Nanos. Multiple alignments were performed with the ClustalX 2.0 and the printing output was shaded by BOXSHADE 3.21. Identical residues are in black, conservative substitutions are in gray. Homologues are arranged into three clusters: Nanos1 (above the red line), Nanos2 (between the red and green line) and Nanos3 (below the green line). The amino acid identities of *nanos* genes are shown on the right. *As* (*A. sinensis*), *Om* (*O. mordax*), *Ss* (*S. salar*), *Ol* (*O. latipes*), *Mm* (*M. musculus*), *Hs* (*H. sapiens*), *Xl* (*X. laevis*), *Dl* (*D. labrax*), *Gm* (*G. morhua*), *Cc* (*C. carpio*), *Dr* (*D. rerio*).

the other by Nanos2 and Nanos3. The sequence obtained in this study was clustered together with Nanos1. Surprisingly, zebrafish Nanos1 fell into the clade of Nanos3.

Alignments and identities of the deduced amino acid sequence of the zinc-finger motif of *As*Nanos1 were compared with that of other species including fish, amphibian and mammals. The zinc-finger motif of *As*Nanos1 had highly amino acid sequence identities to that of other vertebrates, which ranged from 53% to 100% (Fig. 3). In fish, Nanos1 showed extremely high similarity which was above 90%. However, the similarity of the putative amino acid of *As*Nanos1 with that of other vertebrates varied greatly. In the cluster of Nanos1, it shared 83% and 84% identity with that of *Osmerus mordax* and *Salmo salar*, respectively, and was 41%, 33% and 35% identical to the Nanos1 of *Xenopus laevis*, *Mus musculus* and *Homo sapiens*, respectively; in the cluster of Nanos2 and Nanos3, it displayed lower similarities, such as 28% to the Nanos2 of *Dicentrarchus labrax*, 30% to the Nanos3 of *Gadus morhua*, 29% to the Nanos2 of *Oryzias latipes*, 25% to the Nanos3 of *O. latipes*, 38% to the Nanos2 of *M. musculus*, 28% to the Nanos3 of *M. musculus*, 39% to the Nanos2 of *H. sapiens* and 28% to the Nanos3 of *H. sapiens* (data not shown).

3.3. Asnanos1 mRNA expression in somatic tissues

To determine how *Asnanos1* was distributed in somatic tissues, RT-PCR analysis was performed on fourteen tissues including liver, spleen, heart, ovary, fat, kidney, muscle, intestines, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata. As shown in Fig. 4, internal control *Asβ-actin* was found in all tissues examined, and *Asnanos1* mRNAs were ubiquitously detected in all tissues except for the fat. However, its expression level seemed to vary by tissues. In the central nervous system, higher mRNA levels of *Asnanos1* were observed in the pituitary, telencephalon, hypothalamus and cerebellum, whereas in the midbrain and medulla oblongata, *Asnanos1* mRNAs were hardly detected. In the peripheral tissues, lower levels of *Asnanos1* were found in the liver, heart, ovary, kidney, muscle and intestine, while the expression of *Asnanos1* was seemed to hardly observe in the spleen.

3.4. Characterization of AsNanos1 protein expression pattern

To confirm the specificity of *As*Nanos1 antibody, preimmune serum and preabsorbed antiserum of the same rabbit were also subjected to Western blot detection. As shown in Fig. 5, the specific *As*Nanos1 protein band was found only by the antiserum.

To examine the expression of the *As*nanos1 protein in diverse somatic tissues, Western blot was introduced. As shown in Fig. 6, internal control actins were observed in all tissues examined. A single band of expected size (about 25 kDa), *As*Nanos1, was found widely in the liver, spleen, heart, ovary, kidney, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, and medulla oblongata, whereas

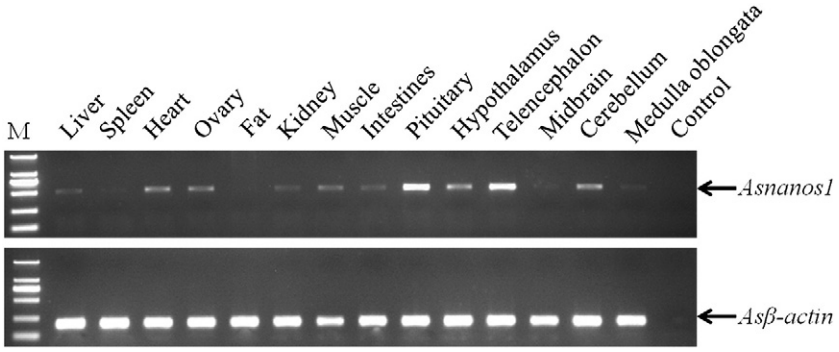

Fig. 4. *Asnanos1* tissue distribution detected by RT-PCR. *Asβ-actin* was used as RT-PCR control. M is the 2 kb DNA Ladder marker.

in the fat, muscle and intestines, it was not detected. We noted that the expression pattern of the *As*Nanos1 protein was not completely coincident with its mRNA.

3.5. Immunofluoresence localization and cellular distribution of AsNanos1in the gonad

In aspect of histology, sex differentiation of the gonads of Chinese sturgeon was completed by the age of 9 months, and the gonads entered into stageI, during which mitosis of oogonia occurred. With the primary oocytes appearing, the ovaries reached stage II when fishes were 2.5-3.0 years old (Chen et al., 2006). In other words, Chinese sturgeons examined in this study did not enter in meiosis, and their gonads corresponded to early stages of oogonial proliferation. At this stage, primary oocytes had formed and grown continuously.

To investigate the relationship of *As*Nanos1 protein with gonad development, we further analyzed its subcellular localization in the gonad by immunofluorescence staining. In the ovary of 2.5 years old Chinese sturgeons, *As*Nanos1 was restricted to primary oocytes in which *As*Nanos1 was cytosolic (Fig. 7B). Under high magnification, it was obviously observed that *As*Nanos1 protein enriched around the cell membrane (Fig. 7F). Meanwhile, the preimmune rabbit serum did not generate reproducible staining (Figs. 7A and E). On the other hand, in the ovary and testis of 4.5 years old Chinese sturgeons, *As*Nanos1 was only detected in the cytoplasm of primary oocytes and spermatocytes, respectively (Figs. 7D, H, J and L).

4. Discussion

In the present study, we identified the *nanos1* gene in the Chinese sturgeon, and analyzed its expression patterns on mRNA and protein level in somatic tissues of immature individuals. Additionally, the cellular distribution of *As*Nanos1 in the gonad was investigated. This is the first time to systematically reveal the expression pattern of the Nanos1 protein in vertebrates.

Several lines of evidence, including protein sequence and structure and phylogenetic sequence comparisons, supported the view that *Asnanos1* was homologous to the *nanos1* gene. In general, the 3′UTR was necessary for the location of *nanos* gene. As for the localization, the *Xcat-2* 3′UTR was found to be both required and sufficient for mitochondrial cloud localization (Zhou and King, 1996), while the 3′UTR of zebrafish *nos1* was essential for directing specific expression of the protein in the PGCs (Koprunner et al., 2001). Therefore, the function of the 3′UTR of *nanos1* was important and conserved.

Based on the phylogenetic analysis of the *nanos* genes, two cohorts (Nanos1, Nanos2 and Nanos3) were identified in vertebrates, and

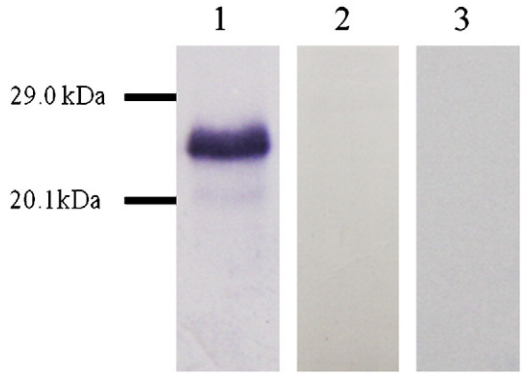

Fig. 5. Specificity confirmation of *As*Nanos1 antibody by Western blot analysis. Lanes 1, 2, 3 are Western blot against endogenous *As*Nanos1 protein in ovary extracts by using anti-*As*Nanos1 serum, anti-*As*Nanos1 serum that pre-absorbed with the purified recombinant *As*Nanos1 protein and pre-immune rabbit serum, respectively.

*As*Nanos1 clustered with the clade of Nanos1. However, zebrafish Nanos1 was found in the group of Nanos3. In fact, according to its sequence, expression characteristics and function (Draper et al., 2007; Koprunner et al., 2001), it should belong to the cohort of Nanos2 and Nanos3. Alignment of the zinc-finger motif of *nanos* genes among different vertebrate species showed that it was highly conserved (Fig. 3). Nevertheless, the identities of various deduced amino acid of *nanos* genes varied greatly, especially among different clusters. Collectively, these results indicated that zinc-finger domain had evolutionary conservation and functional significance.

In vertebrates, previous studies had identified three kinds of *nanos* (*nanos1*, *nanos2* and *nanos3*), and *nanos1* might function in various somatic tissues, while *nanos2* and *nanos3* were both specially implicated in germ line (Aoki et al., 2009; Draper et al., 2007; Haraguchi et al., 2003; Jaruzelska et al., 2003; Julaton and Reijo Pera, 2011; Koprunner et al., 2001; Kusz et al., 2009; MacArthur et al., 1999; Sada et al., 2009; Tsuda et al., 2003; Wang and Lin, 2004). However, *nanos1* was also expressed in germ cells of some vertebrate species, including zebrafish, medaka, frog, mouse and human. In frog, *nanos1* was detected in PGCs, oocyte and testis (Lai et al., 2011; Mosquera et al., 1993). Additionally, mouse *nanos1* was observed in seminiferous tubules of testis (Haraguchi et al., 2003), while human *nanos1* was found in both ovary and testis (Jaruzelska et al., 2003; Julaton and Reijo Pera, 2011). Similarly, in the present study, both *Asnanos1* mRNAs and proteins were expressed in the gonad. In the ovary of *B. mori* larvae, Nanos proteins were primarily localized in oocytes. Moreover, co-localization of Vasa and Nanos further revealed that Nanos was a germline marker in silkworm (Zhao et al., 2008). Besides, in *Botryllus primigenus*, *nanos* was a strong marker of spermatogonia and spermatocytes, although it was also detected in the somatic tissues (Sunanaga et al., 2008). Nevertheless, mice targeted knockout of *nanos1* was fertile (Haraguchi et al., 2003). Consequently, it was difficult to determine whether *nanos1* function in germ cells or not.

In vertebrates, the expression of *nanos1* in nervous system has been found in zebrafish, medaka, frog, mouse and human (Aoki et al., 2009; Haraguchi et al., 2003; Julaton and Reijo Pera, 2011; Koprunner et al., 2001; Lai et al., 2011). In this study, besides ovary and testis, *Asnanos1* was expressed in central nervous system as well (Figs. 4 and 6). Hence, the expression of *nanos1* in brain may conserve in vertebrates. The *nanos1*-deficient mouse developed without any detectable abnormality; meanwhile, no significant neural defect was found (Haraguchi et al., 2003). Whereas the development of peripheral nervous system in *Drosophila* required the translational repressor of *nanos*, suggesting its function in dendrite morphogenesis (Ye et al., 2004). Additionally, localization and translation control of *nanos* mRNAs were essential for the neuron morphogenesis (Brechbiel and Gavis, 2008). Therefore, further studied were needed to determine the function of *nanos1* in nervous system.

The mRNAs and proteins of *Asnanos1* were detected in peripheral tissues as well, such as liver, spleen, heart and kidney. Presently, the similar expression pattern of mRNAs was not found in other vertebrates except human. However, *nanos* genes in invertebrates were likely to have similar expression pattern. In *B. mori*, RT-PCR and Western Blot analysis confirmed that *nanos* was expressed in diverse somatic tissue, such as fat body, blood, midgut and so on (Zhao et al., 2008). Similarly, during early cleavage stages of polychaete *Capitella* sp. I, *nanos* transcripts were found in the presumptive brain, mesodermal bands and developing foregut (Dill and Seaver, 2008), while in snail, loss of *nanos* function led to defects of heart, muscle and intestine (Rabinowitz et al., 2008), indicating *nanos* function in peripheral tissues. In diverse sea urchin species, *nanos* was identified in the descendants of micromeres and subsequently became confined to the coelomic pouches, from which the adult rudiment would form, suggesting that the conserved molecular factor was involved in the formation of multipotent progenitor cells which were responsible for the generation of the entire adult body, including somatic cells

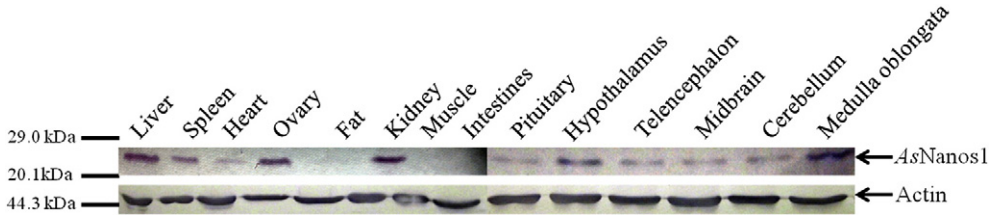

Fig. 6. Western blot detection of *As*Nanos1 protein in immature individuals' tissues. Ubiquitous expression of β-actin is used as the internal control.

and germ cells (Fujii et al., 2006; Juliano et al., 2010). In a word, the expression of *nanos* in various somatic tissues might be conserved in invertebrates.

By immunofluoresence localization, it was observed that *As*Nanos1 proteins were in the cytoplasm of primary oocytes of 2.5 years old Chinese sturgeon, which were around the cell membrane (Fig. 7B and F); however, with the growth of the primary oocytes, it has spread in the total cytoplasm (Figs. 7D and H); meanwhile, *As*Nanos1 was detected in the cytoplasm of primary spermatocytes of 4.5 years old Chinese sturgeon (Figs. 7J and L). Therefore, it was reasonably inferred that *As*Nanos1 could be considered as a candidate marker of primary oocytes and spermatocytes. Nevertheless, immunohistochemical analysis revealed that *Bp*Nanos was detected in the spermatogonia and spermatocytes, but not in the young oocytes (Sunanaga et al., 2008). In frog, from gastrula stage on in frog, Nanos1 was observed mainly in a perinuclear location (Lai et al., 2011). Likewise, the presence of human Nanos1 proteins was mainly in a perinuclear subregion of the cytoplasm of spermatogonia and spermatocytes, while it was not observed in the postmeiotic oocytes (Jaruzelska et al., 2003). Taken together, the expression characterization of Nanos1 protein varied in different species.

On a whole, the functions of *nanos* genes were divergent and convergent in different species from sea anemone to human. In invertebrates, *nanos* family genes were detected broadly in multipotent cells and somatic tissues or somatic stem cells, while in vertebrates

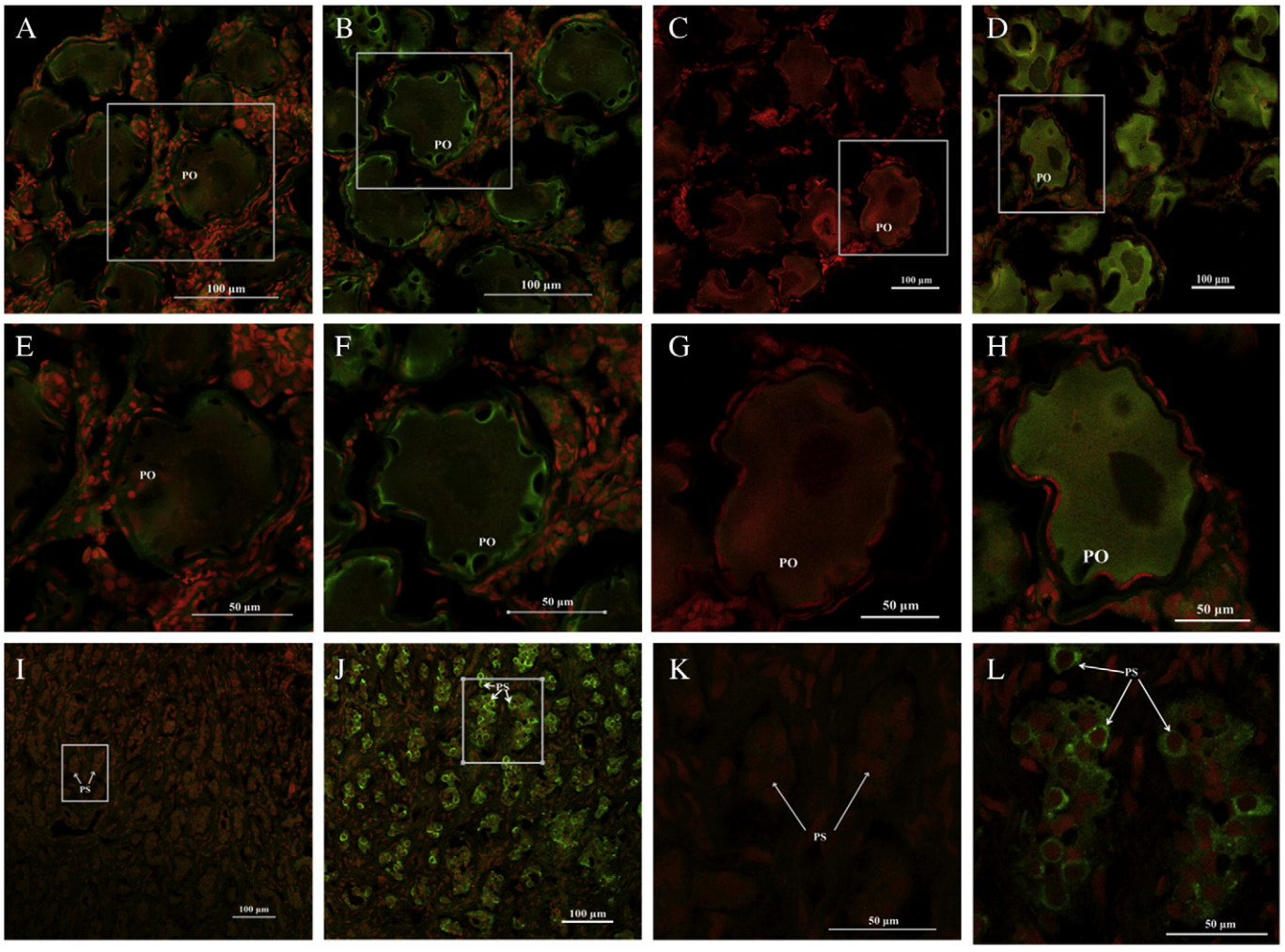

Fig. 7. Immunofluorescence localization of *As*Nanos1 in the gonad. Gonad sections were stained green for *As*Nanos protein and red for PI. (A) and (B), immunofluorescence staining of the ovary of 2.5 years old Chinese sturgeon by the pre-immune rabbit serum and anti-*As*Nanos1 serum, respectively. (C) and (D), immunofluorescence staining of the ovary of 4.5 years old Chinese sturgeon by the pre-immune rabbit serum and anti-*As*Nanos1 serum, respectively. (E), (F), (G), and (H) were high magnification of the boxed area in (A), (B), (C) and (D), respectively. (I) and (J), immunofluorescence staining of the testis of 4.5 years old Chinese sturgeon by the pre-immune rabbit serum and anti-*As*Nanos1 serum, respectively. (K) and (L) were high magnification of the boxed area in (I) and (J), respectively. PO: primary oocytes, PS: primary spermatocytes.

they were observed in germ cells and diverse somatic tissues. Recently, it was said that germ cells and somatic stem cells shared a common origin (Juliano and Wessel, 2010; Rebscher et al., 2008; Wu et al., 2011), which suggested that the ancestral function of *nanos* may be in totipotent stem cells or multipotent stem cells. Then, during the evolution, its function differentiated into germ cells and somatic stem cells, which were implicated in germ line and somatic tissues.

Acknowledgments

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