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Molecular and expression characterization of two somatostatin genes in the Chinese sturgeon, *Acipenser sinensis*

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ABSTRACT

Chinese sturgeon (*Acipenser sinensis*) is a rare and endangered species and also an important resource for the sturgeon aquaculture industry. SMART cDNA was synthesized from the hypothalamus of Chinese sturgeon, and the full-length cDNAs of two somatostatin (*SS*) genes were cloned and sequenced. The first cDNA (*AsSS1*) encodes a 116-amino acid protein that contains the SS_{14} sequence at its C-terminal extremity. *AsSS1* shows high identity to that of human and other vertebrates. The second cDNA (*AsSS2*) encodes a 111-amino acid protein that contains the somatostatin variant [Pro^2]-SS_{14} at its C-terminal extremity. Both the two *SS* mRNAs were expressed in brain and pituitary with different mRNA levels. But in peripheral tissues, *AsSS2* was more widely distributed than *AsSS1*. High mRNA levels of *AsSS2* were found in liver, kidney and heart, while low mRNA levels of *AsSS2* were also detected in ovary. Throughout embryogenesis and early larval development only *AsSS2* mRNAs were detected. Furthermore, in the hypothalamus of one to five year-old Chinese sturgeon, *AsSS2* but not *AsSS1* maintained stable expression. The mRNA distribution suggests that the Chinese sturgeon *AsSS2* products play important physiological functions in adult fish as well as in cell growth and organ differentiation in embryo and larva development.

1. Introduction

The neuropeptide somatostatin (SS) was originally isolated from the ovine hypothalamus due to its ability to inhibit growth hormone secretion from rat anterior pituitary cells (Brazeau et al., 1973). Since then, many somatostatins have been identified in vertebrates (Conlon et al., 1997). They have been believed to be multifunctional peptides widely distributed throughout the central nervous system (CNS) and peripheral tissues, to play a vast array of physiological roles in neuromodulation and osmoregulation, and to affect various aspects of growth, development and metabolism (Patel, 1999; Lin and Peter, 2001).

In vertebrates, most of the somatostatin peptides have been recommended to be from the products of at least four genes, and to be located at the C-terminal extremity of the precursors (Tostivint et al., 2008). The somatostatin 1 (*SS1*) gene has been cloned from all vertebrate classes from agnathans to mammals (Conlon et al., 1997; Lin et al., 1998; Tostivint et al., 2004). Somatostatin 2 (*SS2*), the duplicated gene of *SS1* by a segment/chromosome duplication, has been characterized in numerous vertebrates, including frog (Vaudry et al., 1992; Tostivint et al., 1996), rat (de Lecea et al., 1996), human (de Lecea et al., 1997; Fukusumi et al., 1997), mouse (de Lecea et al., 1997), chicken (Trabucchi et al., 2003), lungfish (Trabucchi et al., 1999), goldfish (Lin et al., 1999), white sturgeon (Trabucchi et al., 2002), grouper (Ye et al., 2005), zebrafish (GenBank accession No. BG307388), opossum ((DS)ENSMODG00000002663), and pufferfish (GenBank accession No. AL296478), shark (GenBank accession No. AAVX01232114.1). In mammals, SS2 was originally called as cortistatin (CST). Because SS2s and CSTs possess a proline residue at position 2, all the [Pro^2] somatostatin variants, that are orthologous to cortistatins (Tostivint et al., 2006), have been now termed SS2 (Tostivint et al., 2008). Moreover, two additional somatostatin genes *SS3* and *SS4* have been identified from teleost fish. The first cDNA sequence of *SS3* was cloned in anglerfish (Hobart et al., 1980), and then in rainbow trout (Moore et al., 1995, 1999), goldfish (Kittilson et al., 1999), zebrafish (GenBank accession No. XM_689051) and orange-spotted grouper (Ye et al., 2005). In comparison with SS1, SS3 contains at least two substitutions, $Phe^7 \rightarrow Tyr^7$ and $Thr^{10} \rightarrow Gly^{10}$. *SS4* gene encodes a peptide with [Tyr^6, Ser^{10}, Arg^{11}, Ala^{13}], and occurs only in species of the group of ostariophysi (Tostivint et al., 2008). The

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zebrafish is now the only vertebrate in which all four *SS* genes have been identified. It seems that the somatostatins belong to a large polygenic family that also includes urotensin II and urotensin II-related peptide (Tostivint et al., 2006).

Chinese sturgeon (*Acipenser sinensis*) is one of the Acipenseriformes, a group of cartilage ganoid with an evolutionary history of over 200 million years (Wei et al., 1997; Birstein et al., 1997). As compared with other sturgeon species, its stock has declined dramatically due to overfishing, loss of natural habitat for reproduction and interference by other human activities (Wei et al., 1997; Billard and Lecointre, 2001; Wei, 2003). To save this species more effectively and to develop its aquaculture industry for the future, artificial propagation has been attempted since 1983, but mature males and females have not been obtained from the cultured offspring. One of the major reasons is the lack of data regarding the first early years in the life of the sturgeon. Therefore, the understanding of its growth and reproduction regulation should start from the early stages. Recently, we have initiated a systematic molecular study in Chinese sturgeon, and identified some important genes relative to growth and reproduction, such as three gonadotropin subunits common α, FSHβ and LHβ (Cao et al., 2009), GH/PRL family and thyroid stimulating hormone subunit β (TSHβ) (in preparation). In the present study, two distinct somatostatin cDNAs were cloned from the hypothalamus of Chinese sturgeon. Moreover, their molecular and expression pattern were characterized in the central nervous system and peripheral tissues as well as in different stages of embryogenesis and hypothalamus from one to five year-old Chinese sturgeon.

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 anaesthesia was induced by a 0.05% solution of MS-222 (Sigma). The tissue samples from a 2-year-old female Chinese sturgeon (1.05 m in length and 4.5 kg in mass) were collected within 30 min of exsanguinations by tailing and immediately dipped into liquid nitrogen and stored at −80 °C. To study the ontogenetic expression profiles, fertilized embryos and larvae from different stages were identified, collected in liquid nitrogen and stored at −80 °C. 10 individuals of 1-, 2-, 3-, 4- and 5-year-old Chinese sturgeon (two individuals per age group) were used for temporal transcriptional expression analysis. One year-old individuals were a female and a male; two and three year-old were male; four and five year-old were female (data not shown). The average length and mass of individuals in each age group were 0.60 m and 0.92 kg, 1.02 m and 5.25 kg, 1.22 m and 8.4 kg, 1.26 m and 8.3 kg, 1.30 m and 11.9 kg, respectively. The experimental procedures, approved by the Chinese government, are based on the standards of the China Council on Animal Care.

2.2. RNA extraction and SMART cDNA synthesis

Total RNA was extracted using SV total RNA isolation system (Promega, USA). The quality of RNA was measured at A260 nm and the purity from the ratio A260:A280 nm (Eppendorf Biometer). Double strand 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).

2.3. Cloning and sequencing

AsSS cDNAs were amplified by 3′- and 5′-RACE (rapid amplification of cDNA ends) as described previously (Li et al., 2005). Degenerate sense and antisense primers (*AsSS1*-F and *AsSS1*-R, *AsSS2*-F and *AsSS2*-R, Table 1) were designed and synthesized according to a nucleotide alignment of different *SS1* and *SS2* cDNAs including nearly all the animals available in the GenBank at NCBI website. The 3′-end of *AsSS1* and *AsSS2* cDNAs was amplified using sense primers (*AsSS1*-F2 and *AsSS2*-F2, Table 1) and a PCR anchor primer corresponding to the terminal anchor sequence of the cDNA (3′-AP, Table 1). The 5′-ends of the two cDNAs were amplified with a 5′-PCR anchor primer (5′-AP, Table 1) and specific antisense primer (*AsSS1*-R2 and *AsSS2*-R2, Table 1). All PCRs were performed on a PTC-100 thermal cycler (Bio-Rad) by denaturation at 94 °C for 4 min, followed by 35 cycles of amplification at 94 °C for 30 s, 56 °C for 40 s and 72 °C for 2 min and an additional elongation at 72 °C for 7 min after the last cycle. The PCR mixture contained 1 U Taq DNA polymerase (MBI, Fermentas) together with 0.2 mM of each dNTPs (Pharmacia), a suitable reaction buffer (MBI, Fermentas), 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 agarose gel, cloned into pMD18-T vector (Takara), and sequenced.

2.4. Database and sequence analysis

Nucleotide sequence identity was performed using the BLAST program (GenBank, NCBI). The glycosylation sites and the cleavage site for the putative signal peptide were predicted using software at ExPASy Molecular Biology Server (*http://www.expasy.pku.edu.cn*). 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 mega3.1 molecular evolutionary genetic analysis software package by bootstrap analysis 1000 replicates using neighbour-joining. The following *SS* genes (acronym, GenBank accession number/references) were included in the analysis: *Homo sapiens* (*Hs*SS1, BC032625; *Hs*CST/*HsSS2*, AF013252), *Rattus norvegicus* (*Rn*SS1, V01271; *Rn*CST/*RnSS2*, NM012835), *Gallus gallus* (*Gg*SS1, X60691; *Gg*SS2, DQ279789), *Rana ridibunda* (*Rr*SS1, U68136; *Rr*SS2, U68137), *Epinephelus coioides* (*Ec*SS1, AY677120; *Ec*SS2, AY677122; *Ec*SS3, AY677121); *Lophius americanus* (*La*SS1, V00640; *La*SS3, V00641); *Carassius auratus* (*Ca*SS1, U40754; *Ca*SS2, U72656; *Ca*SS3, U60262); *Protopterus annectens* (*Pa*SS1, AF126243; *Pa*SS2, AF126244); *Acipenser transmontanus* (*At*SS1, AF395849; *At*SS2, AF395850); *Danio rerio* (*Dr*SS1, AF435965; *Dr*SS2, BG307388; *Dr*SS3, XM_689051; *Dr*SS4, AJ238017); *Ictalurus punctatus* (*Ip*SS1, M25903; *Ip*SS-22/*Ip*SS4, J00945); *Ctenopharyngodon idella* (*Ci*SS1, EU571475; *Ci*SS2, EU571477; *Ci*SS3, EU571476); *Ornithorhynchus anatinus* (*Oa*CST/SS2, XM_001509770); *Monodelphis domestica* (*Md*CST/SS2, (DB)ENSMODG00000002663); *Callorhinchus millis* (*Cm*SS2, AAVX01232114.1); *Oncorhynchus mykiss* (*Om*SS1, Kittilson et al., 1999; *Om*SS3, OMU32471); *A. sinensis* (*AsSS1*, FJ792687; *AsSS2*, FJ792688).

Table 1
Primers used for PCR amplification of *AsSS1*, *AsSS2* and *Asβ*-actin.

Primers	Sequence (5′-3′)
AsSS1-F	GTACTCSAGYTTGGAARGAHG
AsSS1-R	CTGGWGAKAASATGARGAWC
AsSS2-F	CCTGKWGAGGACAGACTGTCTGT
AsSS2-R	CAGGGKGCTTTRCGAKCTCTCWG
AsSS1-F2	ATGCTCTCCTCTCGTCTCCAG
AsSS2-F2	GAGCAAAGAGAGGAAGGAGGG
3′-AP	CGAGGAGGACGACATGTTTTTTTTTTTT
5′-AP	AGTCAATGTAGGATGGACAGG
AsSS1-R2	AGAACAGGGAGGGTGGGAGAG
AsSS2-R2	GTTATAGACGGCCCTCTCCT
AsSS2-RTF	CAGGCTTGTTGGATGGAGTG
AsSS2-RTR	CTGGGTGTTAGCAGGATGTG
Asβ-actin-F	TCCGTGACATCAAGGAGAAGC
Asβ-actin-R	TACCGCAAGATTCCATACCC

2.5. Spatial and temporal expression analyses of the AsSS1 and AsSS2

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA extracted from different tissues (including liver, kidney, spleen, fat, heart, ovary, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, medulla oblongata and spinal cord) from a 2-year-old female Chinese sturgeon was isolated using SV Total RNA Isolation System according to the manufacturer's instructions (Promega, USA). Total RNAs of embryos at the first cleavage, multicellular stage, blastula stage, gastrula stage, blastopore stage, tail bud stage, rudiment of heart stage, muscle contract stage, heartbeat stage, head to tail stage, pre-hatching stage and 1-day larvae were isolated. Total RNAs of hypothalamus isolated from 10 individuals of 1-, 2-, 3-, 4- and 5-year-old Chinese sturgeon were used for temporal transcriptional expression analysis. The quality and purity of the RNAs were checked by electrophoresis of the samples in a 1% agarose gel with ethidium bromide staining, and quantified by the ratio A260:A280 nm (Eppendorf Biometer).

Total RNAs were reverse-transcribed with M-MLV Reverse Transcriptase and oligo d $(T)_{15}$ (Promega, USA) as described by the manufacturer. All of the resultant cDNAs were respectively diluted 1:10, and then used as templates. The primer pairs, *AsSS1*-F2/*AsSS1*-R2 and *AsSS2*-RTF/*AsSS2*-RTR (Table 1), were designed to detect the differentially expressed pattern of *AsSS1* and *AsSS2* respectively. Amplification reactions were performed in a volume of 25 μL containing 1 μL cDNA as template DNA, 0.5 μM each primer, 0.5 U Taq polymerase (MBI, Fermentas), 0.1 μM of each dNTP (Pharmacia), and 1× buffer for Taq polymerase (MBI, Fermentas). Each PCR cycle included denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 50 s. The RT-PCR was carried out as described previously (Zhang and Gui, 2004). Briefly, 10 duplicate reactions were performed by alternate cycle numbers from 15 to 33 to ensure that the semi-quantitative RT-PCR

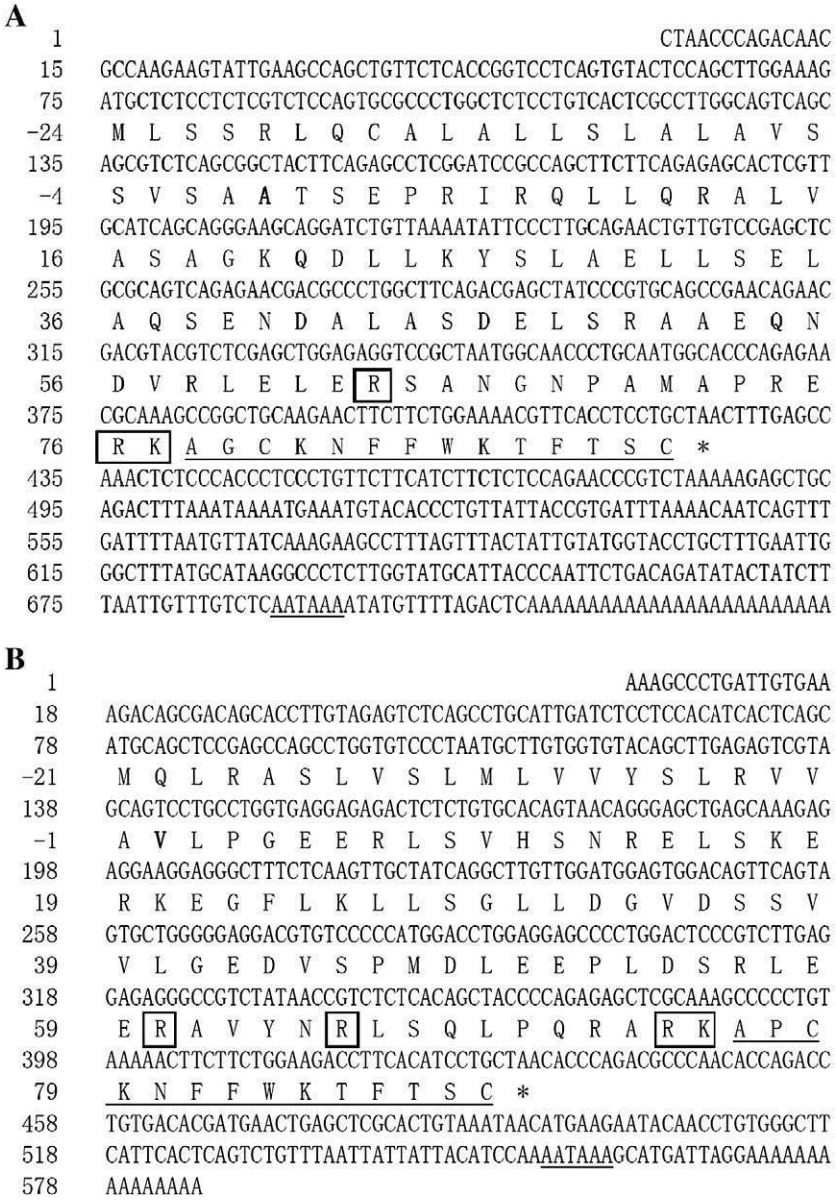

Fig. 1. Nucleotide and deduced amino acids of the *AsSS1* (A) and *AsSS2* (B). The first amino acid of the mature peptide is in bold and numbered as +1 and amino acids of the signal peptide are given negative numbers. Consensus polyadenylation signals AATAAA are underlined. Potential enzymatic cleavage recognition sites are boxed. The amino acid sequences for somatostatin-14 (SS_{14}) and somatostatin-14 variant [Pro^2]-SS_{14} are underlined at the C-terminus of each precursor. *AsSS1* and *AsSS2* have been deposited in the GenBank nucleotide database, under accession No. FJ792687 and No. FJ792688 respectively.

products were in a linear range of accumulation. After the cycle number was optimized, temporal and spatial expression analysis of *AsSSs* were completed by RT-PCR from the different stage samples. Thirty-four cycles were performed, followed by a final extension at 72 °C for 7 min. 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 a semi-quantitative external control for PCR reaction efficiency under the same reaction conditions as *AsSS1* and

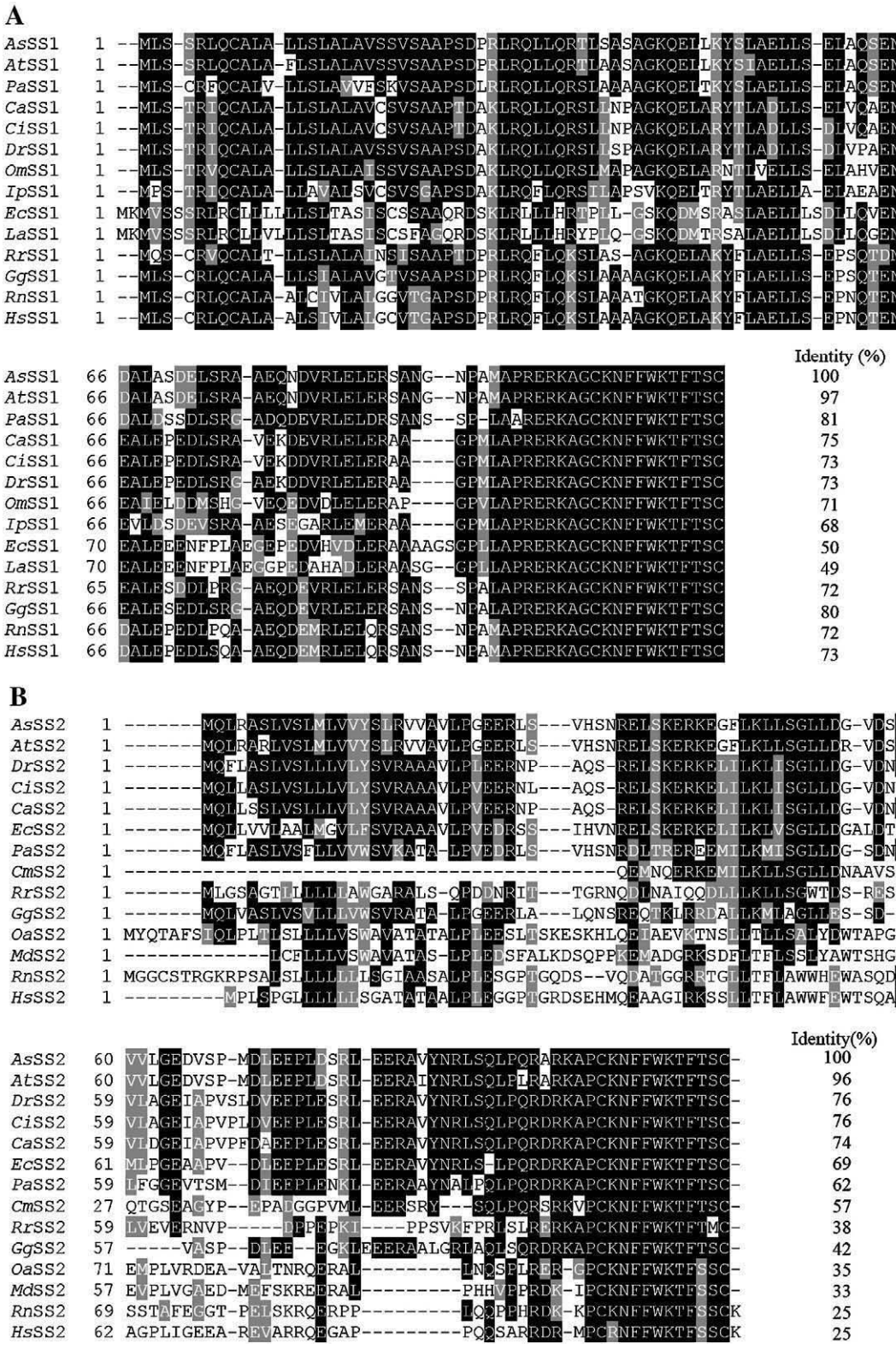

Fig. 2. Alignment of the predicted amino acid sequences of the Chinese sturgeon *AsSS1* (A) and *AsSS2* (B) with its homologues from other species of vertebrates. Multiple alignments were performed with the MAP method at BCM Search Launcher web servers and the printing output was shaded by BOXSHADE 3.21. Unrooted neighbour-joining phylogenetic tree (C) was based on the alignment of amino acid sequences of *AsSSs* with those of other vertebrates. The horizontal branch lengths are proportional to the estimated divergence of the sequence from the branch point. The sequences were extracted from GenBank databases as listed in Materials and methods.

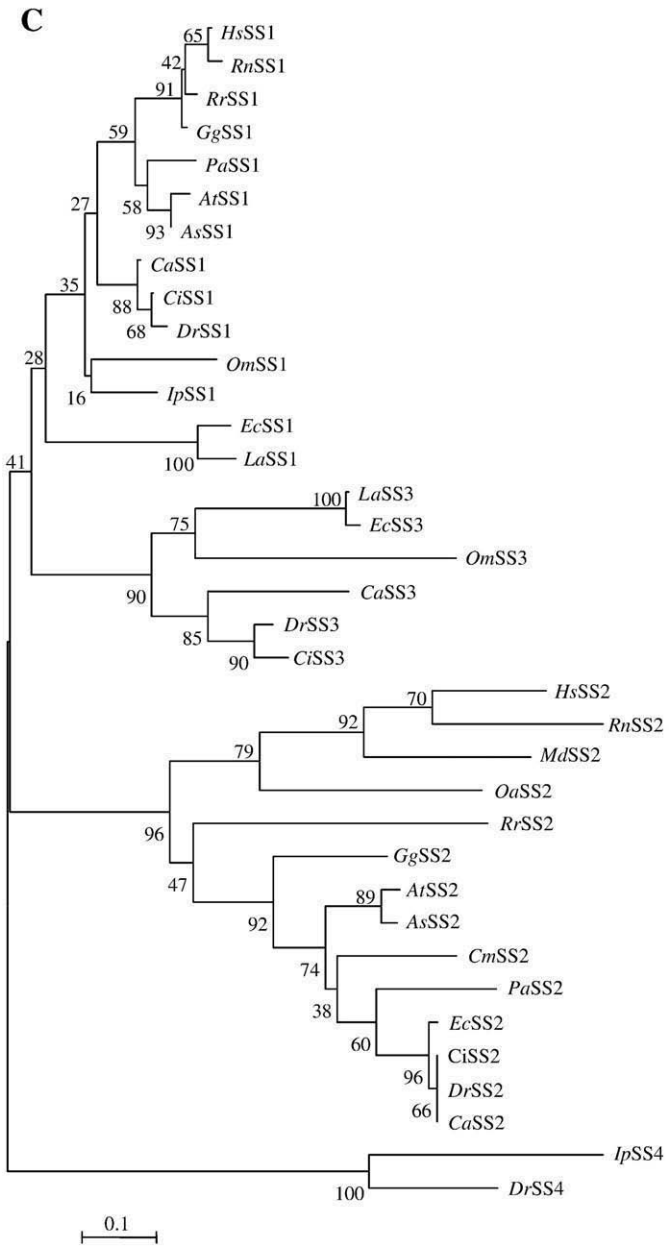

Fig. 2 (*continued*).

AsSS2. About 25% of each PCR product was separated by electrophoresis on 1.5% agarose gel with 0.5 μg/mL ethidium bromide in Tris-borate ethylenediaminetetraacetic (TBE) acid buffer, and the separated PCR products were visualized under ultraviolet (UV) light. Each experiment was repeated three times.

3. Results

3.1. Cloning and molecular characterization of Chinese sturgeon AsSS1 and AsSS2

To isolate *SS* homologues from the Chinese sturgeon, we designed two pairs of degenerate primers against the conserved regions of different *SSs*. Two fragments were firstly produced from the SMART cDNA of Chinese sturgeon hypothalamus. Following the fragment sequence, we cloned and sequenced the full-length cDNA of *AsSS1* and *AsSS2* using RACE strategy. The cDNA sequences and deduced amino acid sequences are shown in Fig. 1. The *AsSS1* cDNA is 709 bp in total length (poly (A) tail excluded), and consists of 74 bp 5′-untranslated region (UTR), a coding sequence of 351 bp, and a 284 bp 3′-UTR. A consensus polyadenylation signal (AATAAA) was found at 15 bp upstream from the poly (A) tail. The cleavage site for the putative signal peptide was predicted by means of Signal P and was located between amino acid position 24 and 25 (Fig. 1A).

The *AsSS2* cDNA is 550 bp in total length (poly (A) tail excluded) and has an open reading frame of 336 bp, starting with the first ATG codon at position 78 and ending with a stop TGA codon at position 413. A consensus polyadenylation signal (AATAAA) is located 11 bp upstream from the poly (A) tail. When a signal peptide of 21 aa is removed, the proposed mature *AsSS2* consists of 90 aa residues (Fig. 1B). The identity of the cDNA sequence between *AsSS1* and *AsSS2* was 57%, but the identity of amino acids was only about 33.6%.

3.2. Phylogenetic relationships of Chinese sturgeon and other vertebrate SSs

Multiple amino acid alignment of the deduced *AsSS1* and other known SS1 precursor proteins revealed interestingly evolutionary implication among Chinese sturgeon and other vertebrates (Fig. 2A). The highest identity of 97% was found between Chinese sturgeon *AsSS1* and white sturgeon *At*SS1, but extensive identities from 81% to 49 were observed between the *AsSS1* and other fish SS1. Intriguingly, the relatively high identities, ranging from 80% to 72%, were revealed between the *AsSS1* and SS1 of tetrapods including birds and mammals (Fig. 2A).

In comparison with SS1s, multiple amino acid alignment of the deduced *AsSS2* and other known SS2/CST precursor proteins showed a common evolutionary relationship. As shown in Fig. 2B, the identities between the *AsSS2* and other fish SS2 range from 96% to 57%, and the identities between the *AsSS2* and SS2/CST of other vertebrates range only from 42% to 25%.

Phylogenetic tree of vertebrate SSs was constructed by neighbour-joining. As shown in Fig. 2C, the all vertebrate SSs are divided into three clusters, the SS1 and SS3 cluster, the SS2 cluster, and the SS4 cluster. In the SS1 and SS3 cluster, SS1 and SS3 are obviously divided into two separate branches. Interestingly, three primitive and ancient teleost SS1, including Chinese sturgeon *AsSS1*, white sturgeon *At*SS1, and lungfish *Pa*SS1, are clustered, and then grouped together with SS1 of amphibians, birds and mammals. In the SS2 cluster, Chinese sturgeon *AsSS2* and white sturgeon *At*SS2 are grouped into the fish SS2 branch. The SS4 cluster is only composed of zebrafish *Dr*SS4 and catfish *Ip*SS4.

3.3. Distribution of two AsSS mRNAs in CNS and peripheral tissues

The distribution of *AsSS1* and *AsSS2* mRNA in CNS and peripheral tissues was examined by RT-PCR. As shown in Fig. 3, *AsSS1* is expressed only in CNS. Its high level of transcript is observed in the telencephalon, hypothalamus, pituitary and medulla oblongata, and the highest mRNA level is revealed in the telencephalon. A faint band is also detected in the cerebellum, midbrain and spinal cord but not in peripheral tissues such as liver, kidney, spleen, heart and ovary (Fig. 3A). *AsSS2* mRNA displays a wide distribution of peripheral tissues including liver, kidney, spleen and heart, and the highest mRNA level is observed in kidney (Fig. 3B). In CNS, *AsSS2* transcript is expressed in all subdivisions of brain and the spinal cord, and the highest mRNA level is detected in the hypothalamus (Fig. 3B).

3.4. Temporal expression analysis of AsSS1 and AsSS2

According to the cDNA sequence, we employed RT-PCR to investigate the temporal expression patterns of *AsSS1* and *AsSS2* during embryogenesis. The results showed that *AsSS1* mRNA was not detected from embryos at the first cleavage to one-day larvae (data

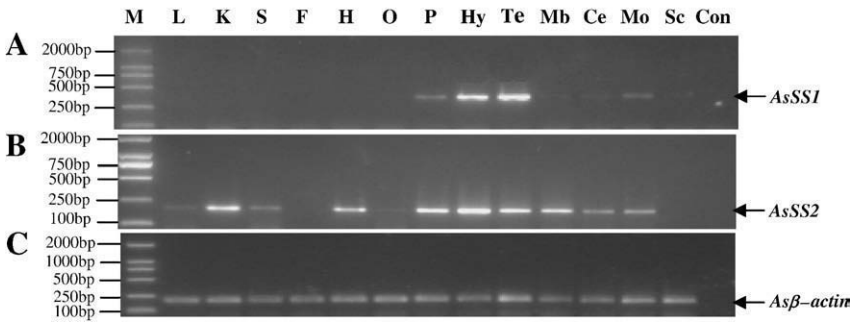

Fig. 3. *AsSS1* (A) and *AsSS2* (B) tissue distribution detected by RT-PCR. *Asβ*-actin (C) was used as RT-PCR control. M is the 2 kb DNA Ladder marker. L, liver; K, kidney; S, spleen; F, fat; H, heart; O, ovary; P, pituitary; Hy, hypothalamus; Te, telencephalon; Mb, midbrain; Ce, cerebellum; Mo, medulla oblongata; Sc, spinal cord; Con, blank control.

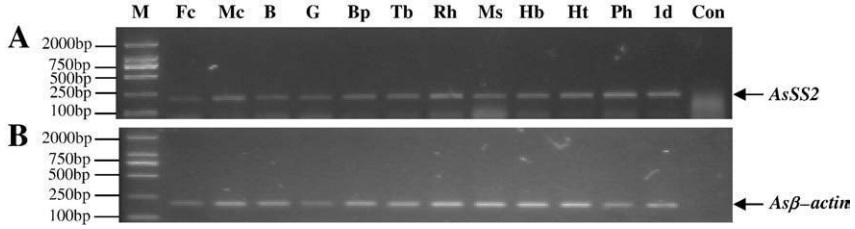

Fig. 4. Analysis of *AsSS2* (A) *mRNAs* during embryogenesis and one-day larvae by RT-PCR. *Asβ*-actin (B) was used as RT-PCR control. M is the 2 kb DNA Ladder marker. Fc, the first cleavage; Mc, multicellular stage; B, blastula; G, gastrula; Bp, blastopore; Tb, tail bud; Rh, rudiment of heart; Ms, muscle contract; Hb, heartbeat; Ht, head to tail; Ph, pre-hatching; 1d, 1-day larvae; Con, blank control.

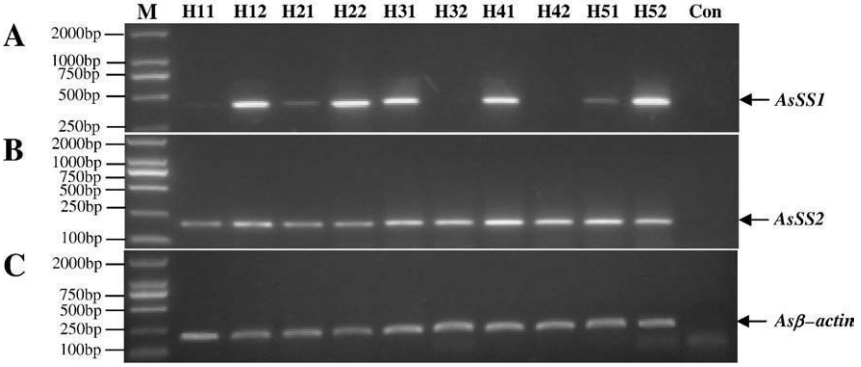

Fig. 5. RT-PCR detection of hypothalamus *AsSS1* (A) and *AsSS2* (B) mRNAs in immature individuals at 1— (lane H11, H12), 2— (lane H21, H22), 3— (lane H31, H32), 4— (lane H41, H42) and 5— (lane H51, H52) year age Chinese sturgeon. *Asβ*-actin (C) was amplified at the same conditions as a positive control in each sample. M is 2 kb DNA ladder markers and Con is blank control.

not shown); while *AsSS2* was found throughout the embryogenesis and the highest mRNA level appeared at the prophase of one-day larvae (Fig. 4).

Expression of *AsSS1* and *AsSS2* in the hypothalamus of immature Chinese sturgeon at five different ages (artificial propagated offspring) was examined. As shown in Fig. 5A, significant expression differences of *AsSS1* were revealed between the two individuals at the same age and among different ages of individuals. *AsSS1* mRNA was detected abundantly in one individual at each age, while another one just slightly detected or not detected (Fig. 5A). *AsSS2* mRNAs have similar expression between two individuals at the same age. Furthermore, expression appeared to be higher in the hypothalamus of 3-, 4- and 5-year-old Chinese sturgeon than those of 1- and 2-year-old (Fig. 5B).

4. Discussion

In the present study, two distinct *SS* genes were isolated in the hypothalamus of the Chinese sturgeon, which provides new evidence for the occurrence of two *SS* genes in Acipenseriformes. The deduced *AsSS1* protein consists of 116 aa, including a putative signal peptide of 24 aa, a conserved pair of basic residues and the SS1 sequence at its C-terminal extremity. Comparative analysis of the sequence of *AsSS1* and that of other vertebrates revealed that the structure of SS1 has been highly conserved during evolution (Fig. 2A). The aa sequence of *AsSS1* shows high degree of sequence identity with white sturgeon (97%) and lungfish (81%) and low degree of sequence identity with grouper (50%) and anglerfish (49%). In particular, the sequence of SS1 and the R–K (Arg^{76}–Lys^{77}) cleavage motif located upstream of SS1 are identical in all species studied to date. The monobasic cleavage site at Arg^{64} which can be used to generate SS-28 has been also fully preserved in all Osteichthyii (Tostivint et al., 2004). Although it is thought that this monobasic cleavage site is generally not used in teleosts (Conlon et al., 1997), the primary structure of the N-terminal segment of *As*SS-28 is conserved to that of the white sturgeon (Trabucchi et al., 2002) and with only one substitution ($Gly^4 \rightarrow Ser^4$) between Chinese sturgeon and human SS-28 (Fig. 2A).

For the *AsSS2*, cleavage at R–K (Arg^{74}–Lys^{75}) dibasic site would potentially yield a [Pro^2]-SS_{14} peptide and processing at monobasic

site +65 would generate a SS_{24} peptide (Fig. 2B). The monobasic site is also present in teleosts and lungfish. The strong conservation of SS2-derived SS_{24} suggests that it may represent a functionally active peptide. The [Pro^2]-SS_{14} product of *AsSS2* was identical with that of SS2 from white sturgeon and lungfish, and only differs in the Thr^{13} residue compared with [Pro^2, Met^{13}]-SS_{14} of frog (Lin et al., 1999; Trabucchi et al., 2002). About ten aa in the CST peptide are homologous to those of fish [Pro^2]-SS_{14}, especially with the same $Gly^2 \rightarrow Pro^2$ substitution. In the opossum and platypus, the SS2 peptides do not possess a Lys moiety in its C-terminus, suggesting that the acquisition of the residue happened late during evolution of the placental mammalian lineage (Tostivint et al., 2008). CST_{14} presented as a regulator of neuronal activity and sleep (de Lecea et al., 1996) and showed activity in depression of neuronal electrical activity (Fukusumi et al., 1997). The discovery of [Pro^2, Met^{13}]-SS_{14} in frog and CST/SS2 in mammals suggests that polygenic SS exists in all vertebrates from fish to human.

In vertebrates, most neuropeptides are members of multigene families that extended by successive gene duplications (Conlon and Larhammar, 2005). The low identity (57%) between the two *As*SSs suggested that SSs might be phylogenetically ancient, multigene family of peptides and all *SS* genes come from an ancient gene (Fig. 2C). It was suggested that the multiple *SS* genes arose from the duplication of a very ancient *SS1* gene which was likely already present more than 500 million years ago in the ancestor of all extant vertebrates (Tostivint et al., 2004, 2008). The *SS2/CST* gene that appeared before the radiation of the gnathostomes arose from the *SS1* gene by segment/chromosome duplication; the *SS3* gene which existed only in teleost species resulted from a local duplication of the *SS1* gene, but the origin of zebrafish SS4 and catfish SS4 remained unknown (Tostivint et al., 2008). In fact, the zebrafish *SS4* and *SS1* genes are localized on two distinct chromosomes while the *SS3* gene maps very closely to *SS1* gene, which strongly suggested that zebrafish *SS3* and the *SS4* genes were paralogous (Tostivint et al., 2004, 2008). The *SS4* gene may arose from the fish-specific genome duplication that occurred relatively early in ray-finned fish lineage or may have appeared much later during teleost evolution (Tostivint et al., 2008).

The localization of the SSs has been studied in some species of teleost fish and other vertebrates by various methods, such as RT-PCR, in situ hybridization and immunohistochemistry. *SS* genes displayed different expression levels in the brain of lungfish (Trabucchi et al., 1999), white sturgeon (Trabucchi et al., 2002), goldfish (Yunker et al., 2003), grouper (Ye et al., 2005) and Siberian sturgeon (Adrio et al., 2008). In this study, the two *AsSS* genes expressed in all subdivisions of brain and pituitary with different expression levels (Fig. 3A, B). Both *AsSS1* and *AsSS2* were actively expressed in the telencephalon and hypothalamus, and slightly in the medulla oblongata. It seems that *SS* mRNAs were expressed abundantly in the hypothalamus of all vertebrates so far studied, suggesting that the *SS* gene products might play hypophysiotropic functions. But in the pituitary, expression of *SS* genes differs among species. *SS1* and *SS3s* were expressed in the pituitary of rainbow trout (Alexander et al., 2001). In white sturgeon, *SS2* but not *SS1* mRNA was found in the *pars intermedia* of the pituitary (Trabucchi et al., 2002). Goldfish *SS1* and *SS3*, but not *SS2* were detected in cultured pituitary cells (Yunker et al., 2003). Although both *AsSS1* and *AsSS2* were abundantly expressed in the pituitary of Chinese sturgeon, the paracrine and autocrine effects of *AsSSs* need to be examined. Different mRNA levels of the two *AsSSs* in brain and pituitary implicate that there are distinct roles among them.

It has been reported in mammals that SSs regulate some physiological processes of peripheral tissues by paracrine or autocrine actions, in addition to growth regulation at the level of pituitary through the inhibition of GH secretion. For instance, endogenous SS suppresses gastric acid secretion (Martinez et al., 1998) and seems to predominantly exert an inhibitory action on immune functions (Ferone et al., 2004). In fish, SSs also have effects on peripheral tissues and participate in a variety of growth, reproduction and metabolic processes (Macini et al., 1999; Sheridan et al., 2000; Very and Sheridan, 2002). In the present study, *AsSS1* was not detected in any peripheral tissues, while *AsSS2* was found in liver, kidney, spleen, heart and ovary (Fig. 3). The *AsSS2* mRNA levels in kidney and heart seemed to be higher than those of other peripheral tissues, suggesting the regulation function of *AsSS2* in growth, reproduction and immune reaction processes, but whether *AsSS2* acts in an endocrine, paracrine or autocrine way remains unknown.

Wang et al. (1995) suggested that SS might participate in the differentiation and development of tissues. In the pancreatic primordium of zebrafish, the first *SS3*-expressing cells were detected by in situ hybridization at the 16-somite stage (17 hours post fertilization (hpf)), whereas *SS1* expression was first detected in few cells at 24 hpf (Devos et al., 2002). Elevated expression of three grouper *SS* genes was detected through developing embryos to hatch-out larvae (Ye et al., 2005). All three of forms of *SS* genes were also detected in embryos of rainbow trout (Malkuch et al., 2008). In Atlantic cod, *SS1* was first detected in the pre-hatching stage and appeared to gradually increase with the development of cod embryos and larvae (Xu and Volkoff, 2009). In this study, *AsSS1* mRNA was not detected during embryogenesis while *AsSS2* maintained a relatively stable expression level from embryos at the first cleavage to pre-hatching stage, and the highest levels appeared to be present in one-day larvae (Fig. 4A). Furthermore, the expression level of *AsSS2* appeared to elevate in the hypothalamus of 3-, 4- and 5-year-old Chinese sturgeon compared to that of 1- and 2-years old (Fig. 5B). In contrast to *AsSS2*, the expression of *AsSS1* showed wide variation between individuals of the same age. *AsSS2* may play an important role not only in the process of organogenesis but also in the fully differentiated organism.

In summary, the present study characterized two distinct *AsSS* genes from the Chinese sturgeon, providing new evidences of the multiple *SS* genes in fish. Expression patterns of *AsSS1* and *AsSS2* in tissues, embryos and the hypothalamus of immature individuals suggest that SSs play important biological functions in Chinese sturgeon. Further studies about expression characterization of SS receptor and interaction between SSs and its receptors are needed to be explored.

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