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Characterization, Tissue-Specific and Developmental Stage Expression of Somatostatin in *Coilia nasus*

Siyu Yang[1], Fukuan Du[2] and Pao Xu[1,2]*

[1]*Fisheries College, Nanjing AgriculturalUniversity, Wuxi 214128, China*
[2]*Key Laboratory of Freshwater Fisheries and Germplasm Resources Utilization, Ministry of Agriculture, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, Jiangsu, China*

ABSTRACT

Estuarine tapertail anchovy (*Coilia nasus*) is a rare and endangered species and also an important resource with high economic value. Somatostatin (SS) is a neuropeptide family which effects growth, development and metabolism. In this study, full-length of one type of *SS* cDNA from *C. nasus* was synthesized, cloned and sequenced. This *SS* cDNA encodes a protein with 114 amino acids that contains the SS14 sequence at its C-terminus. This putative peptide is identical to that generated by the *SS1* gene in other vertebrates. Tissue distribution of *C. nasus SS1* mRNA was analyzed by real-time polymerase chain reaction (PCR), which demonstrated high expression level in the brain. During embryogenesis, *SS1* mRNA was detected during early-stage embryonic development, decreased during subsequent developmental stages then increased gradually from the stage of midgastrula onward. This study provides some basic evidence that SS1 may play a role in growth, development and metabolism in *C. nasus*, and provides a basis for further study of SS neuropeptide family in *C. nasus*.

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Authors' Contributions
PX conceived and designed the study. SY cloned the gene and detected the somatostatin gene expression. FD analyzed the data. SY and PX wrote the article.

Key words
Coilia nasus, Somatostatin, Molecular cloning, Growth hormone, Cortistatin, Embryonic development.

INTRODUCTION

The neuropeptide somatostatin (SS) was isolated from ovine hypothalamus firstly. It is a cyclic tetradecapeptide that was found to inhibit the release of growth hormone (GH) from rat pituitary (Brazeau *et al*., 1973). Many types of SS have been cloned with the same amino acid (aa) sequence in vertebrates subsequently, from agnathans to mammals (Conlon *et al*., 1997). SS effects growth, development and metabolism of vertebrates in various ways and plays important roles in neuromodulation and osmoregulation (Patel, 1999; Lin and Peter, 2001). As a multifunctional peptide, SS is widely distributed in the central nervous system and peripheral organs where it acts as both a neurotransmitter / neuromodulator and a hormone (Olias *et al*., 2004; Viollet *et al*., 2008).

Fish growth is generally considered to be associated with the control of the growth hormone-insulin-like growth factor-I (GH-IGF-I) axis, which relies on the secretion of GH. SS influences organismal growth at several levels of GH-IGF-I axis, it inhibits production and release of GH, reduces sensitivity of GH and inhibits IGF-I production and secretion as well as IGF-I sensitivity (Mofiyama *et al*., 2000; Sheridan *et al*., 2000). Some other related peptides have been characterized in various vertebrate species since the discovery of SS (Lin and Peter, 2001; Liu *et al*., 2010).

Somatostatin multigene family consists of six homologous genes: *SS1*, *SS2*, *SS3*, *SS4*, *SS5* and *SS6* (Liu *et al*., 2010). In zebra fish, all six distinct genes have been identified while in stickleback, medaka and Takifugu, only the *SS1–SS5* genes have been characterized (Liu *et al*., 2010). The different styles of SSs result from tissue-specific processing of SS precursor molecules (preprosomatostatin, PSS) and the multiple *PSSs* genes. Three distinct PSSs have been characterized in fish: PSS-I which contains SS-14 at its C-terminus, PSS-II which contains [Tyr7, Gly10]-SS-14 at its C-terminus, and PSS-III which contains [Pro2]-SS-14 at its C-terminus (Tostivint *et al*., 2004a; Tostivint *et al*., 2006; Tostivint *et al*., 2008). In mammals, SS2 was called cortistatin (CST) firstly. Because SS2 and CST have a proline residue at position 2 (Pro^2) and Pro^2 SS variants are orthologous to CSTs (Tostivint *et al*., 2004a).

Many studies have shown that *SS1*, *SS2* and *SS5* genes almost appeared from two rounds of whole genome duplication (2R) at the early stages of vertebrate evolution (Tostivint *et al*., 2006; Liu *et al*., 2010). However, the *SS5* gene is thought to have disappeared in the tetrapod lineage over time. The *SS4* gene has been verified in all teleost species while been viewed as particular in the ostariophysi previously (Liu *et al*., 2010). *SS4* is deemed to a paralog of *SS1* which arose during the teleost-specific third round of tetraploidization (3R) (Liu *et al*., 2010). *SS3* and *SS6*

* Corresponding author: xup13806190669@163.com
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arose from tandem duplication of the *SS1* and *SS2* genes (Tostivint *et al*., 2004). *SS3* has been found in all teleost species investigated so far (Tostivint *et al*., 2006), while *SS6* is only known to be found in zebrafish (Quan *et al*., 2013).

The estuarine tapertail anchovy *(Coilia nasus,* junior synonym *C. ectenes)* is an important fishery resource and famous for its nutritive value and food delicacy (Xu *et al*., 2011). It is an anadromous fish species belonging to the order Clupeiformes and family Engraulidae. This species is widely distributed in the Yangtze River and the coastal areas of China (Jiang *et al*., 2012). *C. nasus* reaches sexual maturity at 2-3 years old and migrates up river for spawning in fresh water from April to October each year (Liu *et al*., 2014). *C. nasus* is a popular fish due to its high nutritional value and market position as a food delicacy. However, excessive fishing and degradation of aquatic ecology has caused a sharp decline in populations of *C. nasus* in the middle reaches of the Yangtze River (He *et al*., 2008). To combat this issue, a host of research projects including artificial spawning and larval rearing techniques have been carried out to help alleviate threat to this fish population (Liu *et al*., 2014). However, further research is required to better address population threats to this important resource.

In this study, we obtained the full-length sequence of *C. nasus SS1* cDNA and characterized its tissue-specific expression patterns for the first time. We also investigated the expression of *SS1* mRNA during embryonic development. The research about *C. nasus* growth and development is necessary on account of the reduced sharply population. An improved understanding of *SS1* gene regulation and its control of growth and development in *C. nasus* will be beneficial for solving problems associated with feeding, growth and reproduction of this species in culture.

MATERIALS AND METHODS

Experimental animals

Healthy *C. nasus* were cultured in Yixing Fisheries (Jiangsu Province, China). Before experimental use, the fish were maintained at Wuxi Agricultural University in a 28.26×1.7 m^3 aquarium supplied with a continuous flow of fresh water at 11–13°C under natural photoperiod. Fish were fed to satiety twice a day at 08:00 and 16:00 for 40 minutes with a commercial pellet diet (TECH-BANK, Ningbo, China).

Tissue collection and RNA extraction

For cDNA cloning and tissue expression analysis, three individuals with an average body weight of 11.9±0.62g were deeply narcotized with an overdose of tricaine methanesulfonate (MS-222) (Sigma) after 24-hour food deprivation. The tissue (heart, brain, liver, intestines, kidney, head kidney, spleen and muscle) were rapidly dissected, snap-frozen in liquid nitrogen and stored at -80°C until RNA isolation was conducted. To study the ontogenetic expression profiles, fertilized embryos and larvae from different developmental stages (fertilized, 2-cell, multicellular, midgastrula, neurula, muscular contraction, pre-hatching, post-hatching, seven days post-hatch) were collected following natural spawning of the brood stock. A total of 30 embryos or 20-25 larvae were pooled at each stage and immediately dipped into liquid nitrogen and stored at -80°C until RNA isolation.

Total RNA was extracted from the samples using RNAiso Plus (TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's protocol. The quantification and purity of RNA was measured by spectrophotometer (Thermo Scientific, Waltham, MA, USA).The samples with an absorption ratio (260 nm / 280 nm) between 1.8 and 2.1 were used for cDNA synthesis.

Cloning of SS1 cDNA in C. nasus

Two micrograms of total RNA was used for first strand cDNA synthesis using a PrimeScript™ RT Reagent Kit (TaKaRa). Primers (22S and 22A; Table I) for partial SS fragment amplification were designed according to a nucleotide alignment of different SS cDNAs from nearly all animal sequences available in GenBank (http://www.ncbi.nlm.nih.gov/). The cDNA fragment was cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), transformed into DH5α *Escherichia coli* cells (TaKaRa) and subsequently cycle-sequenced (Boshang, Shanghai, China).The full-length *C. nasus SS* cDNA sequence was amplified from brain sample by 5′- and 3′- rapid-amplification of cDNA ends PCR (Zhuandao, Wuhan, China).

Structural analysis

Nucleotide and deduced amino acid sequences were analyzed using basic local alignment search tools (BLAST) BLASTn and BLASTp, respectively (http://www.ncbi.nlm.nih.gov). The open reading frame (ORF) was predicted using Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple sequence alignments were generated using ClustalX 1.83 (Thompson *et al*., 1997). The structure analysis was estimated using SMART (http://smart.embl-heidelberg.de). A phylogenetic tree based on the amino acid sequences was constructed using the neighbor-joining method of ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) (Thompson *et al*., 1994) and MEGA 5.1 programs (http://www.megasoftware.net/index.html) (Tamura *et al*., 2011). The analysis reliability was assessed by 1000 bootstrap replicates.

Table I.- Sequences and function of primers used in this study.

Primer name	Primer sequence (5-3')	Applications
22S	5'ATGCTATCCTTGCGGCTCCA3'	SS1 cloning
22A	5'ACGACGTGAAGGTTTTCCAGAAG3'	SS1 cloning
24S	5'CTGGCAGAACTGTTGTCCGAG3'	SS1 qPCR
24A	5'TCACGAGGTGCGAGCATAGAG3'	SS1 qPCR
B1	5'GAATCATTTCCAAAGAGCAGGT3'	β-actin qPCR
B2	5'GGGTCAGGATACCTCTCTTGCTCTG3'	β-actin qPCR

Real-time PCR analysis of SS1 mRNA

The tissue distribution and ontogenetic expression of SS mRNA in *C. nasus* was examined by real-time quantitative PCR. First strand cDNA was synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa). The primers for real-time quantitative PCR were designed using nucleotide sequences obtained for *C. nasus* SS cDNA (primers 24S and 24A; Table I). *C. nasus β-actin* cDNA was amplified as an internal standard (primers B1 and B2; Table I). Real-time quantitative PCR was performed on the Bio-Rad CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA) using Ssofast Eva Green Supermix (Bio-Rad). Amplifications were performed in quadruplicate, using the following cycling parameters: 95°C for 30s, followed by 40 cycles of 5s at 95°C and 5s at 60°C. The expression of target genes was calculated relative to control gene expression using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

One-way analysis of variance followed by the Duncan test (SPSS Inc., Chicago, IL, USA) was performed in order to identify significant differences between samples. All data were expressed as mean±SEM. Differences were considered significant when P-values less than 0.05 were obtained.

RESULTS

Full-length SS1 cDNA in C. nasus

The full-length cDNA sequence of *C. nasus SS1* was obtained by rapid-amplification of cDNA ends PCR (Fig. 1). The cDNA (GenBank accession No.KX013373) nucleotide sequence was 714bp in length, comprising a 345bp ORF and a 369bp 3′-untranslated region (3′-UTR).

The 3′-UTR contains two polyadenylylation signal motifs (AATAAA) located 41 and 241bp upstream of the poly (A) tail. The ORF encodes a precursor of 114aa and

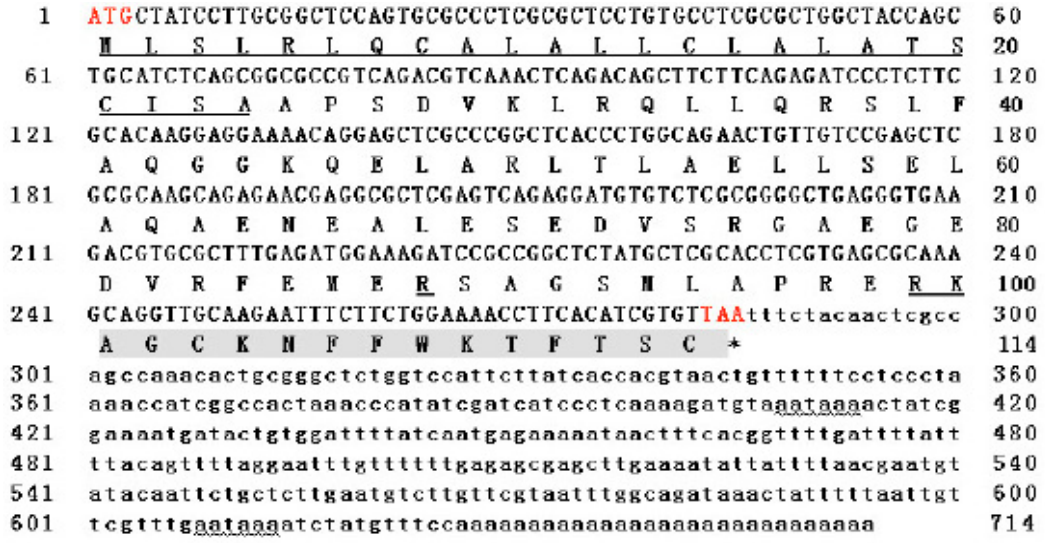

Fig. 1. Nucleotide and predicted amino acid sequences of *C. nasus SS1*. Signal peptides are underlined. SS sequence is shaded in gray. Double underline indicates the potential cleavage sites (Arg87 and Arg98–Lys99). Wave underlining represents polyadenylylation signals.

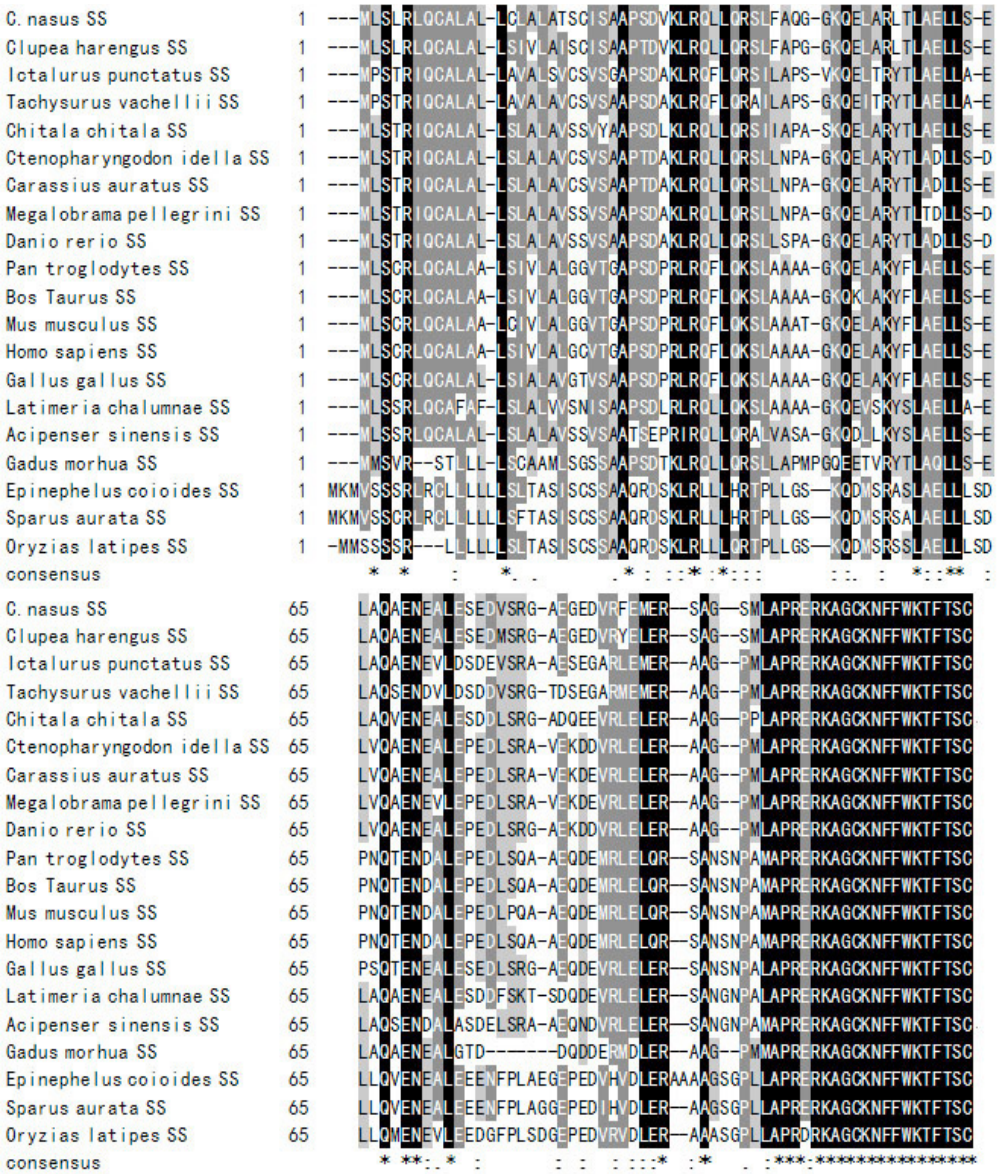

Fig. 2. Amino acid sequence comparison of *C. nasus* SS1 with the sequences of SS1 precursors from several other species. Residues that are conserved in more than half of the listed peptides are shaded. Identical amino acids are indicated by an asterisk.

contains a 24-residue potential signal sequence. The *C. nasus* SS1 aa sequence includes a dibasic processing site at position 98-99 (Arg-Lys), potentially processing a mature 14-aa peptide whose sequence (AGCKNFFWKTFTSC) is identical to SS. There is also a processing site at position 87 (Arg), potentially yielding a somatostatin-26 isoform.

The mature *C. nasus* SS1 peptide shares high homology with other species (Fig. 2), including *Clupea harengus* SS1 (92%), *Ictalurus punctatus* SS1 (72%), *Tachysurus vachellii* SS1 (71%) and *Latimeria chalumnae* SS1 (70%). The phylogenetic tree was constructed based on the deduced precursor sequence of SS1, SS2 and SS3, the *C. nasus* SS1 was grouped within the fish SS1 subfamily (Fig. 3).

Tissue distribution of SS1 mRNA

C. nasus SS1 mRNA expression levels in different tissue were analyzed by real-time quantitative PCR. As shown in Figure 4, *C. nasus SS1* is distinctly expressed in the brain where mRNA was quantified at its highest level. In some peripheral tissue including spleen, intestine, head kidney and muscle, *SS1* mRNA expression was weak.

However, in heart, liver and kidney the *C. nasus SS1* mRNA expression level was higher comparatively.

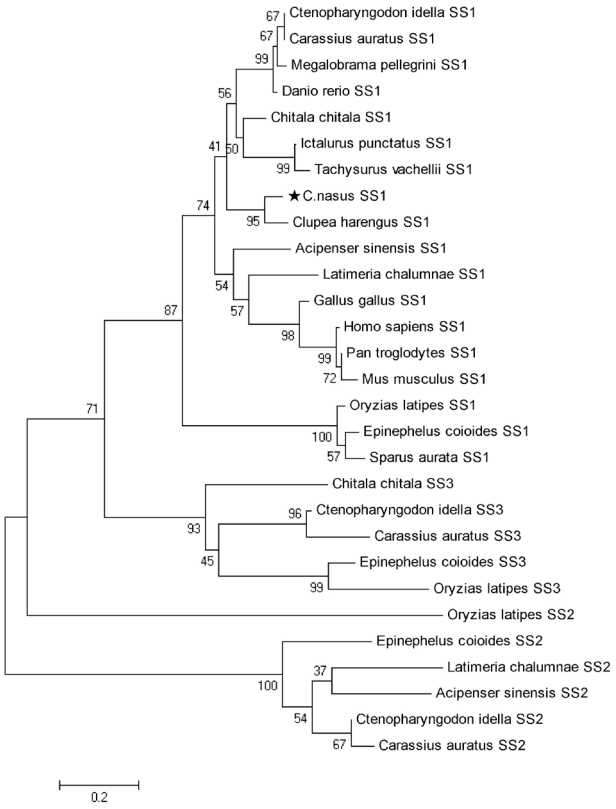

Fig. 3. Phylogenetic analysis of *C. nasus* SS1 amino acid sequences. Scale bar indicates the substitution rate per residue. Numbers at nodes indicate the bootstrap value as percentages obtained for1000 replicates. GenBank accession numbers: *Clupea harengus* SS1 (XP_012673158.1); *Ictalurus punctatus* SS1 (NP_001187259.1); *Tachysurus vachellii* SS1 (ADX32485.1); *Danio rerio* SS1 (AAH76254.1); *Latimeria chalumnae* SS1 (XP_005992512.1); *Ctenopharyngodon idella* SS1 (ACB69423.1); *Carassius auratus* SS1 (AAD09359.1); *Megalobrama pellegrini* SS1 (AAO92644.1); *Chitala chitala* SS1 (AAK97070.2); *Acipenser sinensis* SS1 (ACN88148.1); *Epinephelus coioides* SS1 (AAU93565.1); *Oryzias latipes* SS1 (XP_004084505.1); *Sparus aurata* SS1 (AFO52507.1); *Homo sapiens* SS1 (AAH32625.1); *Pan troglodytes* SS1 (JAA20675.1); *Mus musculus* SS1 (EDK97673.1); *Gallus gallus* SS1 (CAA42747.1); *Latimeria chalumnae* SS2 (XP_005994064.1); *Ctenopharyngodon idella* SS2 (ACB69425.1); *Carassius auratus* SS2 (AAD09631.1); *Acipenser sinensis* SS2 (ACN88149.1); *Epinephelus coioides* SS2 (AAU93567.1); *Oryzias latipes* SS2 (XP_004084506.1); *Ctenopharyngodon idella* SS3 (ACB69424.1); *Carassius auratus* SS3 (AAD09626.1); *Chitala chitala* SS3 (AAV48555.1); *Epinephelus coioides* SS3 (AAU93566.1); *Oryzias latipes* SS3 (ALD51535.1).

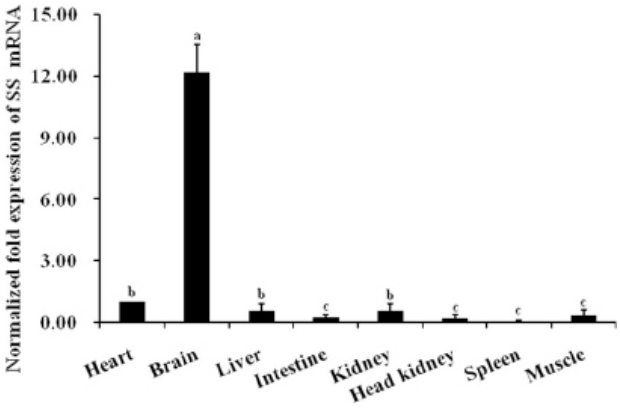

Fig. 4. Profile of *C. nasus SS1* mRNA expression determined by real-time quantitative PCR and normalized against *β-actin* as an internal standard. Error bars represent standard error of the mean. Different letters indicate groups that differ significantly.

Ontogeny of SS1 mRNA expression

During embryonic development of *C. nasus*, abundant *SS1* mRNA was first detected in fertilized eggs. As shown in Figure 5, the expression level of *SS1* mRNA drops gradually after fertilization as eggs progress though subsequent 2-cell stage, multicellular stage and midgastrula. However, from midgastrula to seven days post-hatch, a continuous rise of *C. nasus SS1* mRNA expression was observed. In comparison to mRNA levels at the fertilized stage, expression levels of *SS1* during pre-hatch, post-hatch and seven days post-hatch rose significantly.

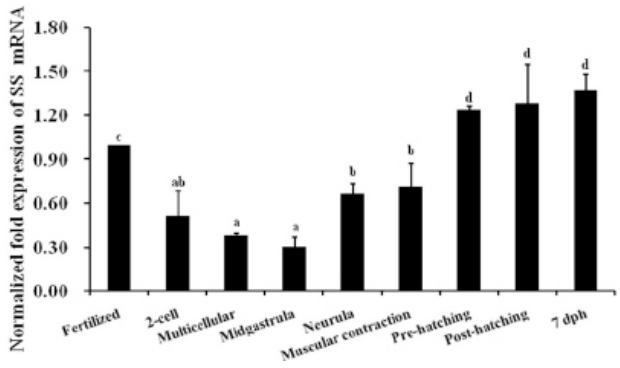

Fig. 5. Expression of *C. nasus SS1* mRNA at different developmental stages. The results are expressed as relative expression levels after standardization to *β-actin* gene expression. Bars represent the mean expression of three pooled samples of 30 embryos or 20–25 larvae each ±standard error. Different letters indicate groups that differ significantly. 7 dph; seven days post-hatch.

DISCUSSION

In vertebrates, most neuropeptides belong to multigene families that arise by successive gene duplications (Conlon and Larhammar, 2005). It is thought that the multiple *SS* genes arose by duplication of an ancient *SS1* gene which had already existed more than 500 million years ago in the ancestor of all extant vertebrates (Tostivint *et al*., 2004b; Tostivint *et al*., 2006). In mammals SS has two biologically active forms, SS-14 and SS-28 while there is an NH_2-terminal extension of 14-aa on SS-28 compared to SS-14 (Pradayrol *et al*., 1980). SS-14 and SS-28 are encoded by the same gene and processed from PSS-I in a tissue-specific way (Siehler *et al*., 2008). The SS hormone variants can bind to multiple SS receptors (SSTRs) subtypes with differing ligand affinities (Sheridan *et al*., 2000). The characteristics of ligand-selective binding have been reported for some SSTRs in trout and goldfish (Siehler *et al*., 2008). The SSTR5 has been found to preferentially bind SS-28 rather than SS-14 (Patel *et al*., 1994).

The present study describes the sequence characteristic of *C. nasus* SS1 mRNA. The deduced SS1 protein consists of 114-aa, including a putative signal peptide of 24-aa, and the conserved SS sequence at its C-terminal extremity. According to our phylogenetic analysis, the sequence and structures of SS1 are highly conserved in vertebrates. The amino acid sequence of *C. nasus* SS1 shows a high degree of identity with *Clupea harengus* SS1 (92%) and *Ictalurus punctatus* SS1 (72%). In addition, the sequence of SS and the R-K (Arg98-Lys99) cleavage motif located upstream are identical in all species studied to date. In *Acipenser sinensis*, the primary structure of the N-terminal segment of SS-28 is completely conserved with white sturgeon and has only one substitution (Gly4→Ser4) with human SS-28 (Li *et al*., 2009). In *Scylorhinus canicula*, two SS genes encode the same C-terminal 14 amino acid peptide sequence (AGCKNFFWKTFTSC) while outside of this region, their sequences are quite divergent. This strongly suggests that the tetradecapeptide form of SS is not the mature peptide (Quan *et al*., 2013).The PSSs of *Scylorhinus canicula* exhibit extra prohormone convertase consensus cleavage sites, possibly yielding N-terminally-extended forms. These longer peptides may bind their receptors more efficiently and thereby exert distinct functions (Seidah and Chrétien, 1994). Although the relationship between the molecular structure and function of SS needs further study, the conserved 14-aa sequence of the mature SS peptide suggests that they at least share some similar physiological functions across species (Xing *et al*., 2005).

Localization of *SS1* gene expression has been investigated in some teleost fish and other vertebrates by various methods (Li *et al*., 2009). According to our real-time quantitative PCR results, *C. nasus SS1* mRNA was expressed abundantly in the brain. In fish, SS modulates growth mainly by inhibiting GH synthesis and secretion through the pituitary in the area of brain (Very and Sheridan, 2002; Sheridan and Hagemeister, 2010). Gene expression studies of *SS1* revealed different pattern in the brain of goldfish (Yunker *et al*., 2003), grouper (Xing *et al*., 2005) and Siberian sturgeon (Adrio *et al*., 2008) suggesting that *SS1* gene expression differs among species. In dogfish the *SS1* mRNA was detected in the brain specifically (Quan *et al*., 2013). Analogously, the expression level of Chinese sturgeon *SS1* was high particularly in the central nervous system (Li *et al*., 2009). These similar results with the present study suggested that the *SS1* gene corresponds to the hypophysiotropic form. *SS1* also has other effects such as reduces GH binding capacity in tissues as well as impinge on a variety of reproductive and metabolic processes (Very and Sheridan, 2002). The expression of *C. nasus SS1* in heart, liver and kidney is higher relatively compared to other peripheral tissues detected. Nutritional restriction results in increased plasma level of SS and reduced plasma levels of insulin (INS) and IGF-I in fasting fish (Sheridan and Hagemeister, 2010). As an anadromous fish, *C. nasus* generally stops feeding during the period of spawning migration, the main source of energy is stored energy (Nie *et al*., 2012). In *C. nasus*, the pancreas is mixed in with liver while *SS1* gene is expressed in pancreas in some species (Kittilson *et al*., 1999). These observations may be due to the effects of biological rhythms suggesting *SS1* may play role in metabolism and circulatory systems in *C. nasus*.

It has been reported that SS1 might participate in the differentiation and development of tissue (Wang *et al*., 1995). There is strong evidence showing that SS1 modulates cellular proliferation in humans (£awnicka *et al*., 2000). In this study, *SS1* mRNA was first detected in fertilized eggs of *C. nasus* and rose gradually from the stage of midgastrula onward. The highest expression level appeared at seven days post-hatch. Research showed that SS1 had effect on cell proliferation (Nelson and Sheridan, 2005). The *SS1* gene was first detected in a few cells at 24 h post fertilization in the pancreatic primordium of zebrafish (Devos *et al*., 2002). Three forms of *SS* genes were detected in rainbow trout embryos (Malkuch *et al*., 2008). While elevated expression of three *SS* genes was detected in developing embryos through to hatch-out larvae in grouper fish (Xing *et al*., 2005). Embryonic developed always along with the reassignment of energy while SS1 may modulate this energy allocation process. In Atlantic cod, *SS1* mRNA was first detected in the pre-hatch stage and increased gradually during development of embryos and larvae (Xu and Volkoff, 2009). The change

of *C. nasus SS1* mRNA expression provides new evidence that SS1 may participate in the process of embryonic development and organogenesis.

CONCLUSION

In conclusion, we characterized the cDNA encoding *SS1* in *C. nasus* firstly. Expression patterns in tissue and embryonic development suggest that SS1 has biological functions in *C. nasus*. Further studies should explore the interactions between SS1 and its receptors, the molecular character and biological function of other members in this multigene family as well as the physiological functions of this family in embryonic and larvae development.

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Conflict of interest statement

We declare that we have no conflict of interest.

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