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EST dataset of pituitary and identification of somatolactin and novel genes in 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, however, a few genes have been identified in this species. We report here construction of a pituitary cDNA library from a 24 years old female Chinese sturgeon just after its spawning, and obtained 2,025 ESTs from the library. 885 unique sequences were identified, which were categorized into 12 functional groups. More than half of the unique sequences (57%) do not match with annotated sequences in the public databases. Three of these novel genes were further identified. Notably, a full-length of cDNA (1,143 bp) encoding somatolactin of 232 amino acids was identified. Phylogenetic analysis showed 97% amino acid identity with White sturgeon somatolactin. RT-PCR analysis indicated that the somatolactin mRNA was only detected in pituitary. Pituitary-specific expression of the somatolactin suggested that the protein may play important physiological functions in pituitary-endocrine system of the Chinese sturgeon.

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Introduction

Chinese sturgeon, a rare and endangered species, is one of the largest freshwater fishes, and found only in China. An adult Chinese Sturgeon ranges from 3 to 4 m in body length. It belongs to the Acipenseriformes, a group of Chondrichthyans with an evolutionary history of over 140–160 million years. Since 1980s, its stock has declined dramatically due to overfishing, loss of natural habitat for reproduction and interference by other human activities [1, 2]. Nowadays, the species was listed as a Grade I protected animal in China and Appendix II species in the CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora).

To protect this species more effectively and to develop its aquaculture industry, 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 research data regarding early years in the life of the sturgeon. Therefore, understanding of its growth and reproduction regulation should start from the early stages. Recently, we have initiated a systematic study in molecular biology of the Chinese sturgeon, and identified some important genes involved in growth and reproduction, such as three gonadotropin subunits common α, FSHβ and LHβ [3], and two somatostatins [4].

Somatolactin (SL) is a pituitary hormone of the growth hormone (GH)/prolactin (PRL) family. It is also fish-specific and the newest member of the family of hormones [5]. Since the isolation of SL from flounder (*Paralichthys olivaceus*) [5], it has been found in a variety of fish species

such as Atlantic cod (*Gadus morhua*) [6]; chum salmon (*Oncorhynchus keta*) [6]; Atlantic halibut (*Hippoglossus hippoglossus*) [7]; lumpfish (*Cyclopterus lumpus*) [7]; gilthead sea bream (*Sparus aurata*) [8]; European eel (*Anguilla anguilla*) [9]; goldfish (*Carassius auratus*) [10]; African lungfish (*Protopterus annectens*) [11]; European seabass (*Dicentrarchus labrax*) [12]; zebrafish (*Danio rerio*) [13]; Atlantic salmon (*Salmo salar*) [14]; Mozambique tilapia (*Oreochromis mossambicus*) [15]; and blackhead seabream (*Acanthopagrus schlegelii*) [16]. However, for the species in Acipenseriformes, SL was reported only in white sturgeon (*Acipenser transmontanus*) [11]. SL functions are still poorly understood.

In the present study, a pituitary cDNA library from the Chinese sturgeon was constructed and a set of ESTs were sequenced from the library. Full-length of cDNAs encoding somatolactin and several novel proteins were identified. Phylogenetic analysis of the somatolactin showed the highest amino acid identity with White sturgeon somatolactin. Expression analysis indicated that the somatolactin is pituitary-specific. The abundant sequence information, especially novel genes and SL data are an enriched resource for further studies in the Chinese sturgeon.

Materials and methods

Fish sampling

The pituitary sample used for the library construction was collected from a 24-year-old female Chinese sturgeon (*Acipenser sinensis*) with 192 kg, which was mature and just spawned. The individuals of 2-, 3-, and 4-year-old male Chinese sturgeon were the aquaculture offspring propagated from the wild sturgeons and cultured in Taihu Station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. All the tissue samples were collected within 30 min of exsanguination by tail-cut and immediately dipped into liquid nitrogen and stored at −80°C. The experimental procedures, approved by the Chinese government, are based on the standards of the China Council on Animal Care.

RNA extraction and SMART cDNA synthesis

Total RNAs were extracted using SV total RNA isolation system (Promega, Madison, USA). The RNA quantity was measured spectrophotometrically at A260 nm by biophotometer (Eppendorf, Hamburg, Germany). The RNA quality was assessed by the ratio of A260:A280 nm and gel-electrophoresis to ensure the integrality. The cDNAs were synthesized from 50 ng of total RNAs according to previous reports [17–19] using the Switching Mechanism at 5′ end of RNA Transcript (SMART) cDNA Library Construction Kit (Clontech, Madison, USA). Briefly, 50 ng of total RNA was reversely-transcribed at 42°C for 1 h at the presence of both 3′ BD SMART cDNA synthesis (CDS) primer II A (5′-AAGCAGTGGTATCAACGCAGAGTACTVN-3′) (N = A, C, G, or T; V = A, G, or C), and BD SMART II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′). Then 2 µl of first-strand reaction product was added into each 100 µl long-distance PCR system containing 0.2 µM PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′). The LD PCR parameters were 95°C for 15 s and 65°C for 30 s and 68°C for 6 min on Perkin–Elmer PCR System 2400 for 20 cycles. Five microliters of the amplified products were separated by electrophoresis on 1% agarose gels.

cDNA library construction and data analysis

The cDNAs were ligated to pMD-18T vector (Promega) and the plasmids were used to transform *E. coli* DH5α super competent cells (TransGen Biotech, Beijing, China). The colonies were randomly picked for single-pass sequencing of the 5′-termini from the above constructed SMART cDNA library. DNA sequencing was performed using dRhodamine terminator cycle sequencing Kit and ABI PRISMTM 310 Genetic Analyzer (Perkin Elmer, Waltham, USA).

In order to identify useful sequences, all ESTs > 200 bp in length after elimination of vector sequence were clustered using blastclust (ftp.ncbi.nih.gov/blast) and assembled using CAP3 (http://seq.cs.iastate.edu/download.html). Then they were compared to sequences in the protein and nucleotide databases (SwissProt, nt and nr) in GenBank using the BLAST algorithms [20]. Only matches with e-values of $\leq 10^{-5}$ were considered as significant, and accepted as homological sequences of the known genes. The known genes were categorized following the classifications of the Gene Ontogeny Consortium (GO) [21]. The webbased, ESTScan was used to scan those unmatched sequences with any of the public databases to identify whether they contained potential ORFs [22]. Protein domains were predicted using InterProScan at http://www.ebi.ac.uk.

Identification of somatolactin and novel cDNAs

EST analysis revealed a full-length cDNA of *AsSL*. Nucleotide sequence identity analysis was performed using the BLAST program (GenBank, NCBI). Amino acid sequence alignment and similarity analysis of *AsSL* with other fish somatolactin were carried out by Clustal multiple sequence alignment program. Phylogenetic analysis was performed using Neighbor-Joining (NJ) method. Novel genes were determined using the BLAST program, which showed no hits.

RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNAs were extracted from different tissues, including pituitary, hypothalamus, telencephalon, midbrain, cerebellum, medulla oblongata, spinal cord, heart, liver, spleen, kidney and gonad, from 2-, 3-, and 4-year-old male Chinese sturgeon, using SV Total RNA Isolation System according to the manufacturer's instructions (Promega).

The isolated RNAs were reverse-transcribed with M-MLV Reverse Transcriptase (Promega) and oligo(dT)15 (Promega) as described by the manufacturer. Total volume for each reaction is 25 μl containing 15 μl of the isolated RNAs, 10 mM of each dNTP, 200 units of M-MLV RT, and 40 units of rRNasin® Ribonuclease Inhibitor with 5× M-MLV buffer (Promega). The reaction mixture was incubated at 37°C for 1.5 h.

PCR reactions were performed in volume of 25 μl containing 1 μl cDNA as template, 0.2 μM each primer, 0.5 units Taq polymerase (MBI Fermentas, Burlington, Canada), 5 mM of each dNTP, 1× Buffer for Taq polymerase (MBI Fermentas). β-actin was amplified to determine the template concentration for PCR reaction efficiency under the same reaction conditions. The information of primer sequences were provided in Table 1.

Results

Gene identification and functional categorization of pituitary

After elimination of some short and vector sequences, a total of 2,025 EST sequences larger than 200 bp were obtained from the Chinese sturgeon pituitary cDNA library. The ESTs generated in this study have been submitted to GenBank (accession no. EV823676-EV825700). The average EST length was 578 bp. To determine whether open reading frames (ORFs) were present in the unannotated ESTs, we further used the web-based program, ESTScan, to scan these ESTs. 885 unique sequences were used for gene functional category analysis. As shown in Fig. 1, by comparison with sequences in the public databases, more than half of the ESTs (58%) have no hits. The second group contains of some binding activity-related genes, which accounts for 17%. The following groups are the genes involved in catalytic activity (8.1%), the genes encoded structural molecules (5.4%), the genes of unknown functions (5.4%), and others including the transcription regulators, enzyme regulations, the electron carriers, the molecular transducers, the translation regulators and the antioxidants (Fig. 1).

Molecular characterization of AsSL cDNA

A full-length of cDNA encoding somatolactin was identified (Fig. 2a). The cDNA of 1143 bp length was predicted to encode a mature protein of 232 amino acids. Sequence of *AsSL* has been deposited in GenBank database under accession number EU656141. The *AsSL* cDNA consisted of 34 bp 5′-untranslated region (UTR) and a 410 bp 3′-UTR. A consensus poly adenylation signal ATTAAA was located 11 bp upstream from the polyA tail. It contains a putative N-linked glycosylation site at residue Asn145 (Fig. 2a).

Phylogenetic relationship of AsSL and other fish SLs

Sequence analysis showed that *AsSL* shares 97% amino acid identity with SL of White sturgeon (Table 2). Alignment of the amino acid sequences of the SL*s* between the Chinese sturgeon and white sturgeon revealed that there were only six amino acid residues different from each other (Fig. 2a). The identities with other fishes ranged from 47 to 71%.

Table 1 The primer sequences and PCR condition used in RT-PCR

Gene	cDNA length (bp)	ORF length (bp)	Primer sequence (5′–3′)	Annealing temperature (°C)
Somatolactin	1,143	699	F: GCAGAAGGTGAAAGTATTG	50
			R: TGTAGCAGCTCATCTTGTC	
EV824874	652	252	F: CAACCACGGGATGGCTCT	55
			R: TCACTCGGGTGTTAGAAT	
EV823739	1,145	330	F: ACATCCGGTTTGCGTCCT	54
			R: CCAGCTTCCTCTTTTACA	
EV824455	865	306	F: CAGTATAATACATGGGGC	55
			R: ACTTTCTGTGTGGAGCGC	
β-actin	1,180	738	F: TTATGCCCTGCCCCACGCTATC	60
			R: CGTGTGAAGTGGTAAGTCCGT	

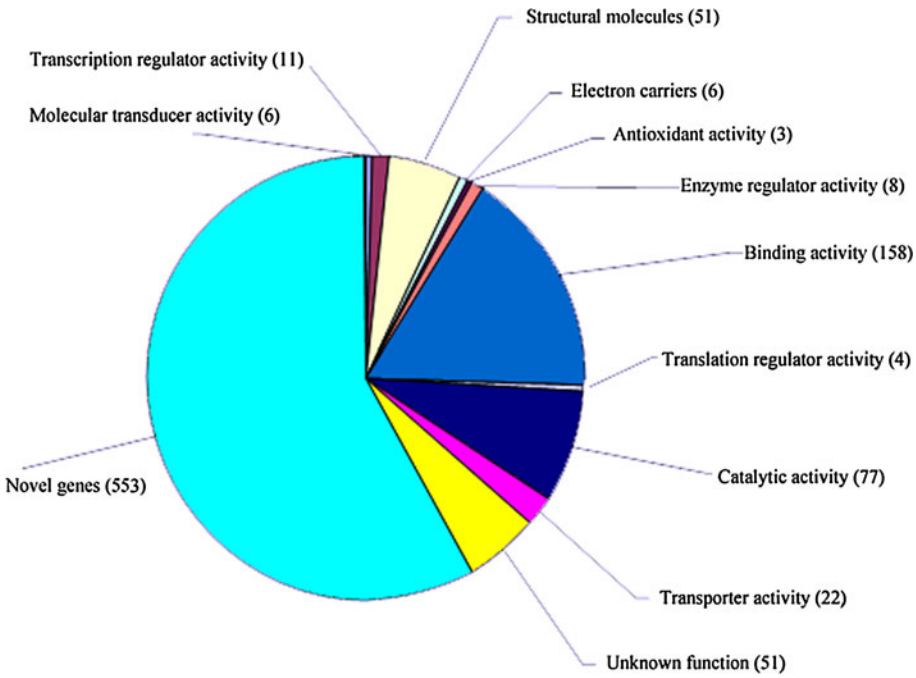

Fig. 1 Gene functional categories of pituitary cDNAs of a Chinese sturgeon. 2,025 expressed sequence tags (ESTs) were obtained and 885 unique sequences were included and classified into 12 functional categories. More than half of the ESTs (57%) have no hits in the public databases. GenBank accession no. EV823676-EV825700

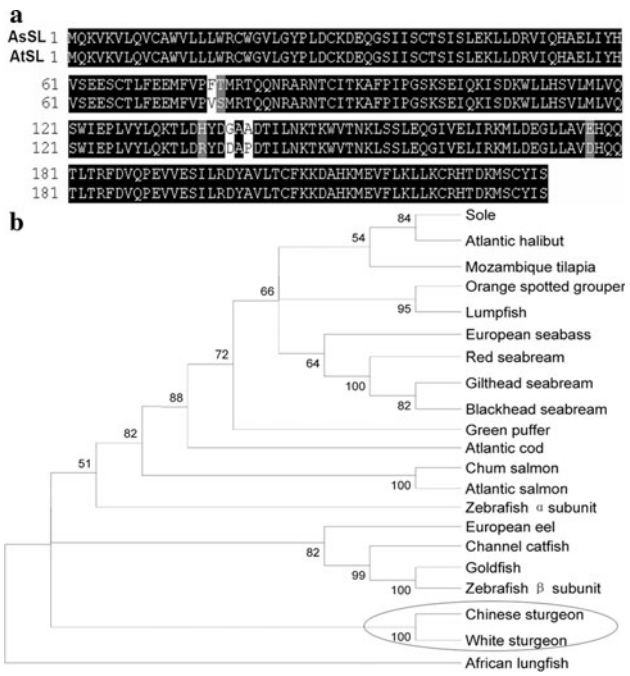

Fig. 2 **a** Alignment of the amino acid sequences of the somatolactin between the Chinese sturgeon (*As*) and white sturgeon (*Acipenser transmontanus*, *At*). A consensus sequence for polyadenylation signal is *boxed*. AsSL, the Chinese sturgeon somatolactin (Accession No. EU656141). The AtSL sequence was retrieved from the GenBank (Accession No. BAA32608). **b** Phylogenetic tree (Neighbour-Joining) of the amino acid sequences of AsSL and Somatolactin of the other fishes. Lengths of horizontal lines indicated genetic distance. Numbers in the branches represent the bootstrap values (%) from 100 replicates. The sequences were extracted from the GenBank databases and their accession numbers are listed in Table 2

Phylogenetic tree of fish SL*s* were constructed by Neighbor-Joining (NJ) method. As shown in Fig. 2b, the NJ tree of all fish SLs were divided into three main clusters, three ancient species including the Chinese sturgeon, white sturgeon and African lungfish was clustered together. SL of European eel, goldfish, Channel catfish (*Ictalurus punctatus*) and β subtype of zebrafish formed another branch. Other SLs were grouped as the third cluster (Fig. 2b).

Tissue distribution analysis of AsSL mRNA

RT-PCR was used to investigate the distribution of *AsSL* mRNA in central nervous system (CNS) and other tissues. As shown in Fig. 3, *AsSL* was expressed only in pituitary. The temporal expression patterns of *AsSL* mRNA in 2- to 4-year-old Chinese sturgeon individuals were also examined by RT-PCR. The result showed that *AsSL* mRNA was not detected from 2-year-old individual. However, in the 3- and 4-year-old individual samples, *AsSL* mRNA was detected only in pituitary (Fig. 3).

Identification of novel genes

Sequence analysis showed that 532 ESTs (57%) have no hits in the public databases, which indicated that they are novel genes. As an example of these genes, we further analyzed potential domains and expression of three novel genes (Fig. 4). Two genes (EV824874 and EV823739) encode proteins with signal peptides in their N-terminal region. These signal peptides will direct the proteins to

Table 2 Amino acid identities between *As*SL and other fish SL

Genus	Species	Identity (%)	GenBank no.
Dipnoi/lepidosireniformes	African lungfish (*Protopterus annectens*)	59	BAA33422
Chondrostei/acipenseriformes	White sturgeon (*Acipenser gueldenstaedtii*)	97	AAX36064
Teleostei/anguilliformes	European eel (*Anguilla anguilla*)	57	U63884
Cypriniformes	Zebrafish (*Danio rerio*) (α subunit)	64	NP_001032795
	Zebrafish (*Danio rerio*) (*β* subunit)	49	NP_001032763
	Goldfish (*Carassius auratus*)	47	CAU72940
Siluriformes	Channel catfish (*Ictalurus punctatus*)	52	AAF78945
Salmoniformes	Atlantic salmon (*Salmo salar*)	71	NP_001117076
	Chum salmon (*Oncorhynchus keta*)	71	BAA01487
Scorpaeniformes	Lumpfish (*Cyclopterus lumpus*)	62	AAC38004
Gadiformes	Atlantic cod (*Gadus morhua*)	64	BAA01486
Tetraodontiformes	Green puffer (*Tetraodon nigroviridis*)	58	AAR25694
Pleuronectiformes	Atlantic halibut (*Hippoglossus hippoglossus*)	69	AAC38003
	Sole (*Solea senegalensis*)	65	AAA61873
Perciformes	Orange spotted grouper (*Epinephelus coioides*)	66	AAN87889
	Red seabream (*Pagrus major*)	65	BAE43855
	Mozambique tilapia (*Oreochromis mossambicus*)	64	BAG50585
	Blackhead seabream (*Acanthopagrus schlegelii*)	63	ABZ88143
	Gilthead seabream (*Sparus aurata*)	63	AAA98734
	European seabass (*Dicentrarchus labrax*)	67	CAC16116

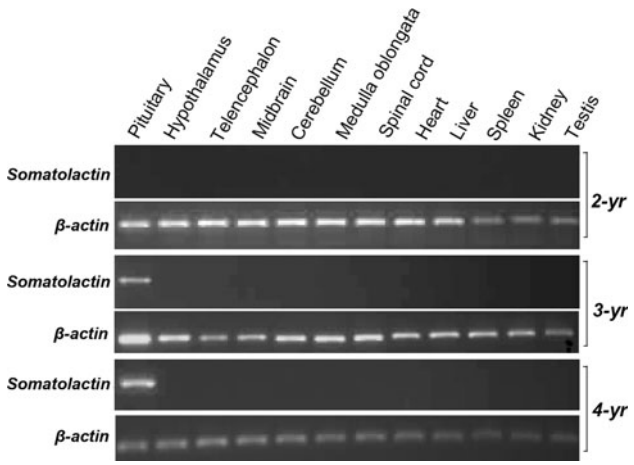

Fig. 3 Expression of *AsSL* gene in tissues of 2-, 3- and 4-year-old Chinese sturgeon detected by RT-PCR. *AsSL* mRNA was detected in pituitary at 3 and 4 year old. *β*-actin was used as a control. *AsSL*, 699 bp

certain organelles. Another one (EV824455) has a transmembrane domain in the middle region of the protein, which indicated its role as a transmembrane protein.

To analyze expression of these novel genes, RT-PCR was used from sample of 4-year-old Chinese sturgeon, which revealed three different categories of expression patterns (Fig. 5). The first category was constitutively expressed in nearly all tissues, for example, EV824874. The second category (EV823739) was abundantly expressed in pituitary and some tissues, whereas slightly in other tissues. The third category was presented abundantly in spleen, slightly in cerebellum and medulla oblongata, and nearly no signal was detected in pituitary.

Discussion

Sturgeon is an important resource for fish production. However, a few data are available for artificial manipulation in growth regulation, reproduction and genetic improvement. In this study, we have cloned and sequenced a set of 2,025 ESTs from the pituitary of the Chinese sturgeon. These sequence data are useful resource for further studies in the endangerous fish species, especially in species conservation, reproduction, growth, molecular biology and genetics. Expressed sequence tags (ESTs) analysis is an efficient approach to identify new genes and to profile gene expression in tissues or cells [23–26]. EST information can provide significant functional, structural and evolutionary information [27], and can also be used in many other applications, such as the discovery of molecular markers [28–30] and the detection of gene loci that influence a quantitative trait locus (QTL) of growth and reproduction [31–33] Recently, fish EST sequence resources are rapidly growing in molecular databases, but no EST data have been reported from any sturgeon species. Furthermore, because the sturgeons in Acipenseriformes arose

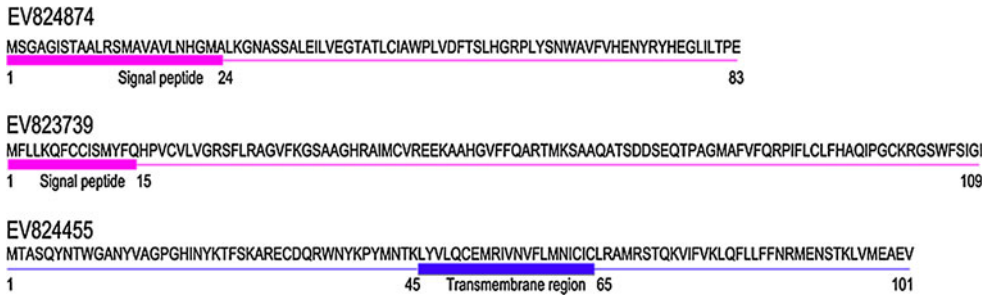

Fig. 4 Schematic view of amino acid sequences of three novel genes of the Chinese sturgeon. Two genes encode proteins with signal peptides (GenBank Accession Nos. EV824874 and EV823739). Signal peptides are in the N-terminal region. Another one (GenBank Accession No. EV824455) encodes a transmembrane protein. Transmembrane domain is located in the middle region of the protein

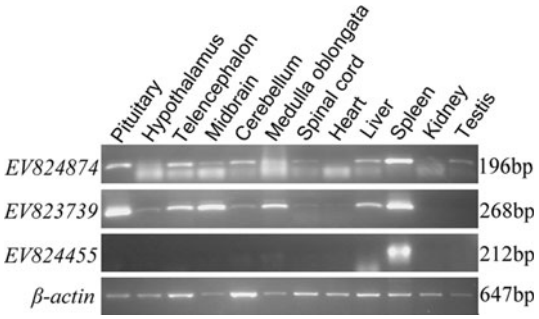

Fig. 5 RT-PCR detection of expression of three novel genes (EV824874, EV823739, and EV824455) in different tissues of 4-year-old Chinese sturgeon. β-actin was used as a control. Amplified sizes are showed in the right

from the close base of the actinopterygian radiation, the study on the pituitary EST analysis can provide us important information about the evolution of reproduction-related genes in the line leading to the teleosts.

We further analyzed full-length cDNAs of Somatolactin and three novel genes and characterized their expression patterns in the Chinese sturgeon. Somatolactin was first found in the flouder (*Paralichthys olivaceus*) [5]. Since then, it has been reported in a lot of fish species and was suggested to be involved in a lot of physiology processes such as gonadal steroid biosynthesis [34], gonadal maturation [35], stress response [36], Ca^{2+} regulation [37], energy mobilization [38], smoltification [39], and acid–base regulation [40]. Recently, studies revealed that Somatolactin deficient mutant (ci) medaka not only had aberrant white body color [41], but also had significantly higher hepato-somatic index (HSI), muscle triglycerides, liver triglycerides and lower plasma cortisol levels than the wild type medaka, indicated its important role in lipid metabolism, pigment cell development, and cortisol secretion [42, 43]. In this study, because of the limitation of the experimental samples, we can only use 2-, 3- and 4-year-old male individuals. Our previous studies revealed that in male Chinese stuegeon, spermatogonia and primary spermatocytes had formed in the testicular tissues of 2-year-old males, while in 4-year males, the testis had highly differentiated, and numerous primary spermatocytes existed in differentiated lobules [3]. Expression of *As*SL could be detected in 3- and 4-year-old male samples instead of 2-year-old, indicating the possible function of the *As*SL in the testis differentiation.

A large group of novel genes were identified in the present study, which showed that the Chinese stuegeon has special information in evolution. Further studies in the Chinese stuegeon will provide evolution clue of the rare and endangered species with an evolutionary history of over 140–160 million years. Furthermore, function studies in Somatolactin and novel genes of the Chinese sturgeon are also needed for efficient applications in the fish production.

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