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Molecular cloning, expression analysis of insulin-like growth factor I (IGF-I) gene and IGF-I serum concentration in female and male Tongue sole (*Cynoglossus semilaevis*)

Qian Ma [a,b], Shu-Fang Liu [a,*], Zhi-Meng Zhuang [a], Zhong-Zhi Sun [a], Chang-Lin Liu [a], Yong-Quan Su [b], Qi-Sheng Tang [a]

[a] Key Laboratory for Fishery Resources and Eco-environment, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China
[b] College of Oceanography and Environmental Science, Xiamen University, Xiamen 361005, China

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ABSTRACT

Insulin-like growth factor I (IGF-I) is a polypeptide hormone that regulates growth during all stages of development in vertebrates. To examine the mechanisms of the sexual growth dimorphism in the Tongue sole (*Cynoglossus semilaevis*), molecular cloning, expression analysis of IGF-I gene and IGF-I serum concentration analysis were performed. As a result, the IGF-I cDNA sequence is 911 bp, which contains an open reading frame (ORF) of 564 bp encoding a protein of 187 amino acids. The sex-specific tissue expression was analyzed by using 14 tissues from females, normal males and extra-large male adults. The IGF-I mRNA was predominantly expressed in liver, and the IGF-I expression levels in females and extra-large males were 1.9 and 10.2 times as much as those in normal males, respectively. Sex differences in IGF-I mRNA expressions at early life stages were also examined by using a full-sib family of *C. semilaevis*, and the IGF-I mRNA was detected at all of the 27 sampling points from 10 to 410 days old. An increase in IGF-I mRNA was detected after 190 day old fish. The significantly higher levels of IGF-I mRNA in females were observed after 190 days old in comparison with males ($P<0.01$). The IGF-I concentrations in serum of mature individuals were detected by ELISA. The IGF-I level in the serum of females was approximately two times as much as that of males. Consequently, IGF-I may play an important role in the endocrine regulation of the sexually dimorphic growth of *C. semilaevis*.

1. Introduction

As in other vertebrates, many of the growth-promoting actions are regulated by the growth hormone/insulin-like growth factor (GH/IGF) axis in fish (Duan, 1997). In this axis, IGF-I plays a major physiological role in the growth and development of fish species (Moriyama et al., 2000). Due to its important function in regulating growth, the IGF-I gene becomes an important candidate gene and has been studied extensively in various aquaculture species. Its cDNA nucleotide sequences are available for many teleosts, including Pleuronectiformes species, such as the Southern flounder (*Paralichthys lethostigma*) (Luckenbach et al., 2007), the Senegalese sole (*Solea senegalensis*) (Funes et al., 2006) and the Chilean flounder (*Paralichthys adspersus*) (Escobar et al., 2011).

Dyer et al. (2004) demonstrated that plasma IGF-I concentrations were highly correlated with growth rates in some fish species. Moreover, the hepatic IGF-I mRNA expression levels of the coho salmon (*Oncorhynchus kisutch*) were also positively correlated with body growth rates (Duan et al., 1995). These reports suggest that measuring IGF-I concentration and mRNA expression levels may provide a reliable index of growth status. Besides, the importance of IGF-I in the development of larvae and juveniles has become apparent in many teleost species. For instance, recombinant tilapia IGF-I significantly increased the body weight and length of juvenile tilapia in the early life stages (Chen et al., 2000). Furthermore, IGF-I mRNA could be detected in *P. adspersus* larvae at eight days post-fertilization (Escobar et al., 2011). Similar results were observed in the rabbitfish (*Siganus guttatus*) that IGF-I mRNA were strongly expressed in the larvae (Ayson et al., 2002). These results imply that the IGF-I expression exists a developmental pattern starting from the early life stages to sex maturity period (Duan, 1998). Therefore, IGF-I may present as a candidate factor in detecting growth patterns of teleost species during the whole developmental stages.

Sexually dimorphic growth exists in many teleosts. In certain fish species, such as tilapia fishes, males grow faster and larger than females, while in the Tongue sole (*Cynoglossus semilaevis*), the Chinook salmon (*Oncorhynchus tshawytscha*), the Common carp (*Cyprinus carpio*) and the Atlantic halibut (*Hippoglossus hippoglossus*), females grow faster and larger than males. *C. semilaevis* is an increasingly important marine flatfish of potentially great aquacultural value in China (Deng et al., 1988; Liu et al., 2005). This species exhibits a typical sexual dimorphism

* Corresponding author at: Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Nanjing Road 106, Qingdao 266071, China. Tel./fax: +86 532 85811514.
E-mail address: liusf@ysfri.ac.cn (S.-F. Liu).

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in which females grow two to three times faster and larger than males. The physiologic mechanism of this sexual growth dimorphism in *C. semilaevis* is of great interest and remains to be examined. Owing to the important functions of IGF-I in regulating growth, determining the relative IGF-I concentration in serum and its mRNA expression levels between females and males during the different contrasting developmental stages may help elucidate the role of IGF-I in the sex-associated dimorphic growth of *C. semilaevis*. However, the expression pattern of the growth-related genes associated with fish sexual dimorphism has thus far only rarely been reported (Degani et al., 2003).

To gain a better understanding of this sex-associated dimorphic growth phenomenon, the full length IGF-I cDNA of *C. semilaevis* was cloned, and its sex-specific tissue expression was examined in this study. Moreover, to examine the differences of IGF-I between the mature female and male individuals at the protein level, the concentration of IGF-I in serum was detected by ELISA. Additionally, IGF-I mRNA expression levels between the sexes at different developmental stages were determined to gain insight into the mechanisms of sexual growth dimorphism.

2. Materials and methods

2.1. Experiment design and sample sources

The experiment was designed to characterize IGF-I cDNA sequence and examine differences of IGF-I concentrations in serum and mRNA expression levels between the female and male *C. semilaevis*. Mature female and male individuals of the same age were used to detect IGF-I levels in serum and the mRNA tissue expression distribution pattern. In addition, two extra-large male individuals were used to compare the IGF-I mRNA expression level with normal female and male fish. The information on the above-mentioned fish samples is listed in Table 1. After being rapidly dissected from the above-mentioned 14 live individuals, 14 tissues (blood, brain, gill, gonad, heart, intestine, kidney, liver, muscle, pituitary, spinal cord, skin, spleen and stomach) were immediately frozen in liquid nitrogen, and kept at −80 °C until use. The blood samples for ELISA analysis were allowed to clot for 30 min before centrifugation for 15 min at 1000 *g*, and serum was collected and stored at −20 °C.

To minimize factors influencing gene expression, such as genetic background, as well as ontogenetic and environmental influences, a full-sib family of *C. semilaevis* was constructed and grown to supply samples used in examining the difference in the ontogenetic expression pattern in IGF-I mRNA among siblings at the different developmental stages. Using a pair of wild parent fish caught in the Yellow Sea, a full-sib family was constructed in October 2008 at the Zhonghai Hatchery (Qingdao, Shandong Province) and grown in the same indoor concrete tank with the optimal conditions as described by Liu et al. (2004). Four to six individuals at each developmental stage (interval of 10 days between 10 to 150 days old, interval of 20 days between 150 to 350 days old, 380 and 410 days old) were randomly collected from the full-sib family. As a result, a total of 138 fish were sampled under the condition of an empty stomach in the early morning. After determining the body length and weight, the whole visceral mass section was dissected under anatomical microscope. All the viscera samples were frozen in liquid nitrogen for RNA and DNA extraction.

2.2. DNA isolation and cDNA synthesis

Genomic DNA was extracted using a standard phenol–chloroform extraction procedure (Sambrook et al., 1989) from the viscera and muscle tissues. The quality and concentration of DNA were assessed by agarose gel electrophoresis and measured with NanoVue™ (GE Healthcare). Finally, DNA was diluted to 100 ng/μL and stored at −20 °C for future use.

Total RNA was extracted from frozen tissues of adult fish and viscera samples of different developmental stages using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The isolated RNA samples were suspended in DEPC-treated water and quantified using NanoVue™ (GE Healthcare) at A_{260} nm and A_{280} nm, and then analyzed for its integrity on agarose gel. The first-strand cDNA was synthesized from total RNA using Reverse Transcriptase M-MLV (Takara Bio., China) following the manufacturer's instructions.

2.3. Genetic sex identification

Genetic sex identification for the samples from the full-sib family was determined using the female-specific SCAR marker developed by Chen et al (2007), with a pair of female-specific PCR primers (CseF382N1 and CseF382C1). A female-specific fragment of 350 bp was amplified from the genotypic female individuals. The results were further verified by another sex-specific marker which amplifies particular fragments from differently sexed individuals (unpublished data).

2.4. Cloning of IGF-I cDNA

According to the conserved IGF-I cDNA sequences from other teleost species, a pair of degenerate primers, IGF-F and IGF-R (Table 2), was designed to enable cloning of the corresponding partial fragment of IGF-I cDNA. PCR amplification was performed in a typical reaction, the condition was one initial denaturing step of 3 min at 94 °C, followed by 32 cycles of 30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C, and a final 10 min at 72 °C.

Based on the obtained partial fragment of IGF-I cDNA, four specific primers, IGF-5′-OUTER, IGF-5′-INNER, IGF-3′-OUTER and IGF-3′-INNER (Table 2) were designed for amplification of the cDNA ends of the IGF-I gene using the 5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0 (Takara Bio., China) following the manufacturer's instructions.

All the amplified fragments of the expected sizes were purified with a Tiangen gel extraction kit (Tiangen, China) and cloned into a pMD18-T vector (Takara Bio., China), then transformed into *Escherichia coli* DH5α and sequenced by the Beijing Genomics Institute (Beijing, China).

2.5. Sequence and phylogenetic analysis

The full length cDNA of IGF-I was assembled by aligning the overlapping fragments and the primer sequences. The signal peptides were predicted with Signalp 3.0 (http://genome.cbs.dtu.dk/services/SignalP). Putative IGF-I amino acid sequences of *C. semilaevis* and other known vertebrates were used to construct a phylogenetic tree using the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) with MEGA version 3.1. In the analysis, the gaps were deleted and a 1000 bootstrap procedure was used to test the robustness of the

Table 1
Mature *Cynoglossus semilaevis* samples.

Samples	Numbers of individuals	Age (years)	Mean body length (mm)	Mean body mass (g)	Sample source
Females	6	3	583.0 ± 61.3	1479.2 ± 280.8	Mingbo hatchery station (Laizhou, Shandong Province)
Males	6	3	333.3 ± 14.3	170.3 ± 16.9	
Extra-large males	2	2	555.0 ± 21.2	1107.5 ± 116.7	Xinyongfeng hatchery station (Tianjin, China)

All data are expressed as the mean + S.D.