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Growth differences and dimorphic expression of growth hormone (GH) in female and male *Cynoglossus semilaevis* after male sexual maturation

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ABSTRACT

Half-smooth tongue sole, *Cynoglossus semilaevis*, is an ideal model to investigate the regulatory mechanisms of sexual growth dimorphism in fish species. The aim of the study was to investigate the effect of differential age of sexual maturity for females and males on growth and GH mRNA expression in *C. semilaevis*. The body weight differences between the sexes were not significant in *C. semilaevis* at age 5 months when females and males were all immature. Significant differences in body weight between the sexes were found after early sexual maturation of males at the age of 9 months. The body weight of 21-month-old females (621.4 ± 86.4 g), still not immature, was even 3.28 times higher than that of the males (189.7 ± 14.4 g). The cDNAs encoding GH in *C. semilaevis* was cloned. The GH gene is 2924 bp long and consists of six exons and five introns. The results of qRT-PCR showed that GH mRNA levels of the immature females were not significantly different from that of immature males at age 5 months. However, GH mRNA levels of the immature females were significantly higher compared with those of the mature males at age 9 months ($P<0.05$). At age 11 months, GH mRNA levels of females were even 6.4-fold higher than that of males. In conclusion, for the first time we show that early sexual maturity of males is the main cause of sexual growth dimorphism in *C. semilaevis* and exert significant effect on GH mRNA expression.

1. Introduction

Cynoglossus semilaevis, is an important cultured marine fish species in China (Chen et al., 2008a) and showed significantly sexual dimorphism in growth. However, the physiologic mechanisms of sexual growth dimorphism in *C. semilaevis* remain unknown. Earlier studies in teleosts have shown that there is a significant correlation between age at first maturity and growth. For instance, it is reported that Atlantic halibut males exhibit relatively slow growth due to precocious sexual maturation comparing with females (Jákupsstovu and Haug 1988; Hagen et al., 2006). The body weights of cultured male turbot reached a plateau and leveled off around 1.0 kg after sexual maturation (Imsland et al., 1997). Recently, there were some reports concerning the histological analysis of ovary and testis development in *C. semilaevis* (Liu et al., 2009; Ma et al., 2006). However, no data is available about the relation between differential age of sexual maturity for females and males and the sexual growth dimorphism.

Growth hormone (GH), prolactin (PRL), placental lactogen (PL), and somatolactin (SL) belong to a family of polypeptide hormones with similar structure and overlapping biological characteristics (Li et al., 2005). GH is a single chain polypeptide having two intramolecular disulfide bonds. It, mediated by specific receptors, regulates somatic growth (Peter and Marchant 1995) and other biological actions of fish such as reproduction (Trudeau 1997), immunity (Yada et al., 1999), and osmoregulation (Sakamoto et al., 1997). As a result, GH is considered to be a central endocrine regulator of growth (Knobil and Hotchkiss 1964). Besides, a lot of studies have confirmed a role of GH in promotion of growth with varying degrees in many fish species (Schulte et al., 1989; Ben-Atia et al., 2000; Cavri et al., 1993; McLean et al., 1993; Acosta et al., 2008; Sekin et al., 1985). In seeking genetic factors affecting differential growth between the sexes, the growth hormone (GH) is naturally considered a primary candidate. In the past decades, GH cDNAs of many species have been cloned and a lot of general aspects of the structure, actions, expression, and signal transduction of GH are becoming clear (Sekin et al., 1985; Timmermans-Sprang et al., 2008). However, to date, GH gene has not been isolated and characterized in all *Cynoglossidae* species.

Recently, several studies have succeeded to screen many genes which showed sexual dimorphic expression in somatic tissues of adult mice or rats (Rinn et al., 2004; Ahluwalia et al., 2004; Vawter et al., 2004). Degani et al. (2003) examined a significantly higher expression of growth hormone in female European eels in comparison to the

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males. Davis et al. (2008) also found that hepatic mRNA expression of GHR1, GHR2, IGF-I and IGF-II as well as IGF-I levels were higher in tilapia males than in females, reflecting greater growth rate in males. But no data is available about the GH mRNA expression differences between the sexes in *C. semilaevis*.

The objectives of our present study were: (1) to compare the growth performance between female and male *C. semilaevis* at various stages; (2) to identify the relation between differential age of sexual maturity for females and males and the sexual growth dimorphism; (3) to clone the cDNAs encoding GH in *C. semilaevis* and examine the tissue expression of GH mRNA; (4) to investigate GH mRNA expression differences between the sexes, and relation of sexual growth dimorphism and GH mRNA expression differences between the sexes.

2. Materials and methods

2.1. Fish culture and sampling

In August 2007, 120 four-year-old *C. semilaevis* cultured in Mingbo fisheries Co., Ltd., (Laizhou, Shandong province) were transported to a pool (60 m^3) of a constant photoperiod (16 h of light: 8 h of darkness), and a constant water temperature (23 °C) to stimulate natural maturation. The culture and reproductive manipulation of *C. semilaevis* were performed according to routine methods (Jiang and Wan 2005). In October 2007, about 500 ml eggs from four females and 1 ml semen from six males were collected by abdominal massage of natural mature *C. semilaevis*. After artificial fertilization, the fertilized eggs were hatched at a 2 m^3 tank with 1–2 daily changes of total water volume and gentle aeration. The hatched larvae were cultured in triplicates in 40 m^3 pool according to routine methods (Jiang and Wan 2005) till February 2009. At 80 days, 5 M (months), 9 M, 11 M, and 21 M of age, the fish were sacrificed by an overdose of MS-222, and the body weight of females (n = 9−15, 3–5 samples per pool) and males (n = 9−15, 3–5 samples per pool) was respectively determined. Gonads of 9 and 21-month-old *C. semilaevis* (n = 4) were collected for gonad histology. Correspondingly, the pituitary tissues of each sex from each pool were respectively collected and stored in liquid nitrogen for RNA extraction and qRT-PCR analysis. The fin clips were respectively collected from twelve 80-day-old fry, and then fixed in 100% ethanol for DNA extraction and genetic sex determination.

C. semilaevis (1.5 years old) were obtained from Haiyang High-Tech Experimental Base (Haiyang, Shandong province). Fourteen tissues including blood, brain, eye, gill, head kidney, intestine, liver, muscle, pituitary, skin, testis, spleen, stomach, and trunk kidney were collected, frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Gonad histology and assessment of sperm quality

The gonad from each individual was fixed in Bouin's fixative, dehydrated through series of graded ethanol (50–99.9%) and xylene, and embedded in paraffin. The paraffin containing the tissues was sectioned in 6 μm, and stained with hematoxylin and eosin (Chen et al., 2008a). In total three sections of the testes or ovaries were evaluated from each fish by microscopical observation.

Semen from 9-month-old males was collected by applying gentle abdominal pressure. Then, an aliquot (0.5 μl) of semen was placed on a glass slide and 50 μl of distilled water was added to activate the sperm. After homogenization, sperm motility was immediately observed under light microscope (magnification: 100×) and percentage of motile sperm was recorded (increments of 5%) (Ji et al., 2008).

2.3. Genetic sex identification of Cynoglossus semilaevis

Genetic sex identification of *C. semilaevis* was determined according to Chen et al. (2007; 2008a). The genomic DNA from each fry was extracted using a traditional proteinase-K digestion and phenol-chloroform protocol with RNase treatment (Blin and Stafford, 1976). The female-specific PCR primers were CseF305N1 and CseF305C1 (Table 1). The individuals producing a female-specific fragment of 160 bp were determined to be genotypic females, whereas males did not produce this fragment.

2.4. Clone of Cynoglossus semilaevis partial GH cDNA

Total RNA was isolated from pituitaries of *C. semilaevis* using Trizol reagent (Invitrogen) according to manufacturer's instruction. Reverse transcription (RT) was performed at 30 °C for 10 min and then 42 °C for 60 min in a total volume of 20 μl consisting of 1 μg total RNA, 1 × M-MLV buffer, 0.5 mM each dNTP, 1.25 μM random primers, 200 U M-MLV Reverse Transcriptase (Takara, Dalian, China).

Two pairs of degenerate primers, GH-f1, GH-r1, GH-f2, and GH-r2, were designed according to conserve sequences of GH gene in other teleosts (Table 1) and used for amplifying the GH cDNA fragment of *C. semilaevis*. In the first round PCR, 1 μl RT product as template, GH-f1 and GH-r1 as primers, 30 cycles of amplification were performed using a cycle profile of 94 °C for 40 s, 51 °C for 30 s, and 72 °C for 40 s. Then, 1 μl of 50-fold diluted first-round PCR product as a nested PCR template, GH-f2 and GH-r2 as primers, PCR were performed using the same cycle profile as that for the first-round PCR.

To isolate full-length GH cDNA, two specific primers (5′GSP and 3′ GSP) (Table 1) were designed according to the amplified partial GH cDNA sequences. 5′GSP primer was for amplification of 5′ end of *C. semilaevis* GH cDNA, and 3′GSP primer was for 3′ end. 10 × Universal Primer Mix (UPM) used for 5′-RACE and 3′-RACE are Long primer and Short primer (Clontech). To identify GH genomic organization, 10 primers (Table 1) were designed to amplify introns of GH gene. Exon-intron junctions were deduced according to the known GH sequences of the other vertebrates.

2.5. Rapid amplification of the cDNA ends (RACE)

Both 5′-RACE and 3′-RACE were carried out using BD SMART™ RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instruction. PCR was carried out with 5 cycles of 94 °C 30 s and 72 °C 3 min, 5 cycles of 94 °C 30 s, 70 °C 30 s and 72 °C 3 min, followed by 25 cycles of 94 °C 30 s, 68 °C 30 s and 72 °C 3 min. All amplified products were run on a 1.5% agarose gel using D2000 as marker (Tiangen, Beijing). Bands of expected sizes were excised from the gel and purified by Tiangen gel extraction kit (Tiangen, Beijing). The purified fragment was cloned into pBS-T vector, propagated in E. coli DH5α, and sequenced.

2.6. Sequence analysis

DNA and deduced amino acid sequences were analyzed with EditSeq and DNAMAN software. The signal peptides were predicted with Signalp3.0 (http://genome.cbs.dtu.dk/services/SignalP). Multiple alignments of amino acid sequences were determined using ClustalW. Mega 3.1 was used to construct phylogenetic trees using the neighbor-joining method.

2.7. Tissue expression of GH mRNA transcripts

Total RNA of fourteen tissues was respectively extracted and the reverse-transcription of mRNA was performed as described above. GH-RT-f and GH-RT-r was designed for PCR analysis for *C. semilaevis* GH mRNA, and β-Actin-f and β-Actin-r for β-Actin mRNA. PCR was run as follows: initial incubation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s; 55 °C for 30 s, and 72 °C for 40 s, with a final extension of 7 min at 72 °C. 10 μl amplification products were electrophoresed on 1.2% agarose gel with a DL2000 DNA marker.

Table 1
Primers and their sequences used in this study.

Primers	Primer sequences (5′ to 3′)	Utilizations
CseF305N1	CTCCCCTGACCTTCCTTT	Genetic sex identification
CseF305C1	CGGCAGCACAATTATTACA	Genetic sex identification
GH-f1	CCTTCACCTGVTBGCTCA	Partial fragment clone
GH-r1	GAABCCHYCTGCTCCATC	Partial fragment clone
GH-f2	YCTTCACCTGSTBGCTMA	Partial fragment clone
GH-r2	GAABMCHYCTGCTCCRTC	Partial fragment clone
5′GSP	CAGCAGTTCGTAGTTCCGCTTTTGTGAT	5′ region clone
3′GSP	CTATGAGAACCTGGGCGGCAACGAAT	3′ region clone
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	5′ or 3′ RACE
UPM-short	CTAATACGACTCACTATAGGGC	5′ or 3′ RACE
GH-intron-1f	GTGAACTGAAGAAAACGTCAGAATC	Intron 1 of GH
GH-intron-1r	AGTATTTCTGAGCAAGCAGGTGA	
GH-intron-2f	TCACCTGCTTGCTCAGAAATACT	Intron 2 of GH
GH-intron-2r	TCAGGGTTGCAGAAGTCTTGATG	
GH-intron-3f	CAAGACTTCTGCAACCCTGATAAC	Intron 3 of GH
GH-intron-3r	GAATTCCCAGGAGTCAACCAGTC	
GH-intron-4f	ACTGGTTGACTCCTGGGAATTCT	Intron 4 of GH
GH-intron-4r	CCAGGTTCTCATAGAAGTTTCCAA	
GH-intron-5f	ACAGCATCACTCTCCCAGTAACTC	Intron 5 of GH
GH-intron-5r	GGCTACTGTGAGATATGTTTCCACC	
GH-RT-f	CCATCAAGACTTCTGCAACCCTGA	RT-PCR or quantitative RT-PCR
GH-RT-r	GGCTACTGTGAGATATGTTTCCACC	RT-PCR or quantitative RT-PCR
β-Actin-f	CCAACAGGGAAAAGATGACC	Internal control
β-Actin-r	TTCTCCTTGATGTCACGCAC	Internal control

2.8. Quantitative RT-PCR (qRT-PCR)

The RNA was respectively extracted from pituitaries of females and males in various growth stages according to the methods as described above. Quantitative real time PCR (qPCR) analysis was conducted in 7500 ABI real time PCR system (Applied Biosystems, Foster city, CA). Quantitative real time PCR reaction systems (20 μl) consists of 10 μl SYBR Premix Ex Taq (2×), 0.4 μl of GH-RT-f and GH-RT-r, 1 μl cDNA, 0.4 μl ROX reference dye II. The PCR amplification procedure was initial denaturation at 95 °C for 10 s, 40 cycles of 95 °C for 5 s, 60 °C for 34 s, followed by disassociation curve analysis to determine target specificity. The expression of β-Actin was used as internal control. The triplicate fluorescence intensities for GH gene in females and males, as measured by crossing point (Ct) values, were compared by the relative quantification method using 7500 system SDS software (Applied Biosystems). PCR specificity was assessed by melting curve analysis.

2.9. Statistical analysis

The transcript level of each gene was described with its relative concentration ($RC_{gene}/RC_{ß-actin}$). All data were expressed as mean ± SE and analyzed by one-way ANOVA followed by LSD multiple comparison tests using SPSS 10.0 (SPSS Co. Ltd, Chicago). A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Growth differences between the sexes in Cynoglossus semilaevis and gonad histological observation

The body weight of females and males was not significantly different at both 80 days and 5 months of age (Fig. 1A). Significant differences in body weight between the sexes were found following 9 months of growth ($P<0.05$), whereby the females reached 59.6 ± 9.4 g and the males 36.6 ± 5.6 g. Histological examinations showed that the ovaries of 9-month-old females were immature (Stage II) (Fig. 1B and C). However, the testes from four 9-month-old males had all been mature and contained a lot of spermatocytes and spermatozoa (Fig. 1B and A). Semen could be obtained by applying gentle abdominal pressure of 9-month-old males and sperm showed high motility (>60%) after activation. At age 21 months, the body weight of females (621.4 ± 86.4 g) was even 3.28 times higher than that of the males (189.7 ± 14.4 g). Moreover, the oocytes of 21-month-old females did not become polarized and were not immature (Stage III) (Fig. 1B and D).

3.2. cDNA and genomic sequence of Cynoglossus semilaevis GH

In the first round PCR, one poor band of about 323 bp was obtained using the degenerate primer GH-f1 and GH-r1. And the subsequent nested PCR produced a clear expected DNA fragment of 250 bp. On the basis of the sequence of the obtained 250 bp fragment, two specific primers 5′GSP and 3′GSP were designed and used for 5′-RACE and 3′-RACE, respectively. Distinct bands of predicted size approximately 580 bp and 291 bp, respectively, were produced in 5′-RACE and 3′-RACE. After subcloning, sequencing, and assembling analysis with DNAMAN software, the full-length GH cDNA sequences of 818 bp in *C. semilaevis* was obtained (Genbank accession No. FJ608663), including a 66 bp 5′ terminal UTR, 603 bp encoding region and 149 bp 3′ terminal UTR including a polyadenylation signal (AATAAA) (Fig. 2A). The 603 bp encoding region encodes a protein with 200 amino acid residues, which represented the precursor of *C. semilaevis* GH composed of a signal peptide with 17 amino acids and a mature GH polypeptide with 183 amino acids. A full length of 2924 bp GH genomic DNA was obtained (Fig. 2A) and six exons (exon I, 10 bp; exon II, 132 bp; exon III, 114 bp; exon IV, 144 bp; exon V, 141 bp; exon VI, 59 bp) and five introns (intron I, 113 bp; intron II, 348 bp; intron III, 542 bp; intron IV, 415 bp; intron V, 194 bp) were identified in *C. semilaevis* GH, which was similar to the GH genes of Japanese flounder, yellowtail, and channel catfish (Fig. 2A). However, there were five exons and four introns in channel catfish (Fig. 2B). The second intron of *C. semilaevis* GH, which is 348 bp long, is larger than its counterparts in other teleosts because of the presence of microsatellite repeats: $(TAGA)_{13}$, $(AGAT)_{19}$, and $(TG)_{10}$.

3.3. Sequence alignment and phylogenetic analysis

The deduced *C. semilaevis* GH amino acid sequences had 71.5%, 65.5%, 65%, 64%, 58.4%, 53%, 46.5%, 44%, 35.5%, and 31% identity with

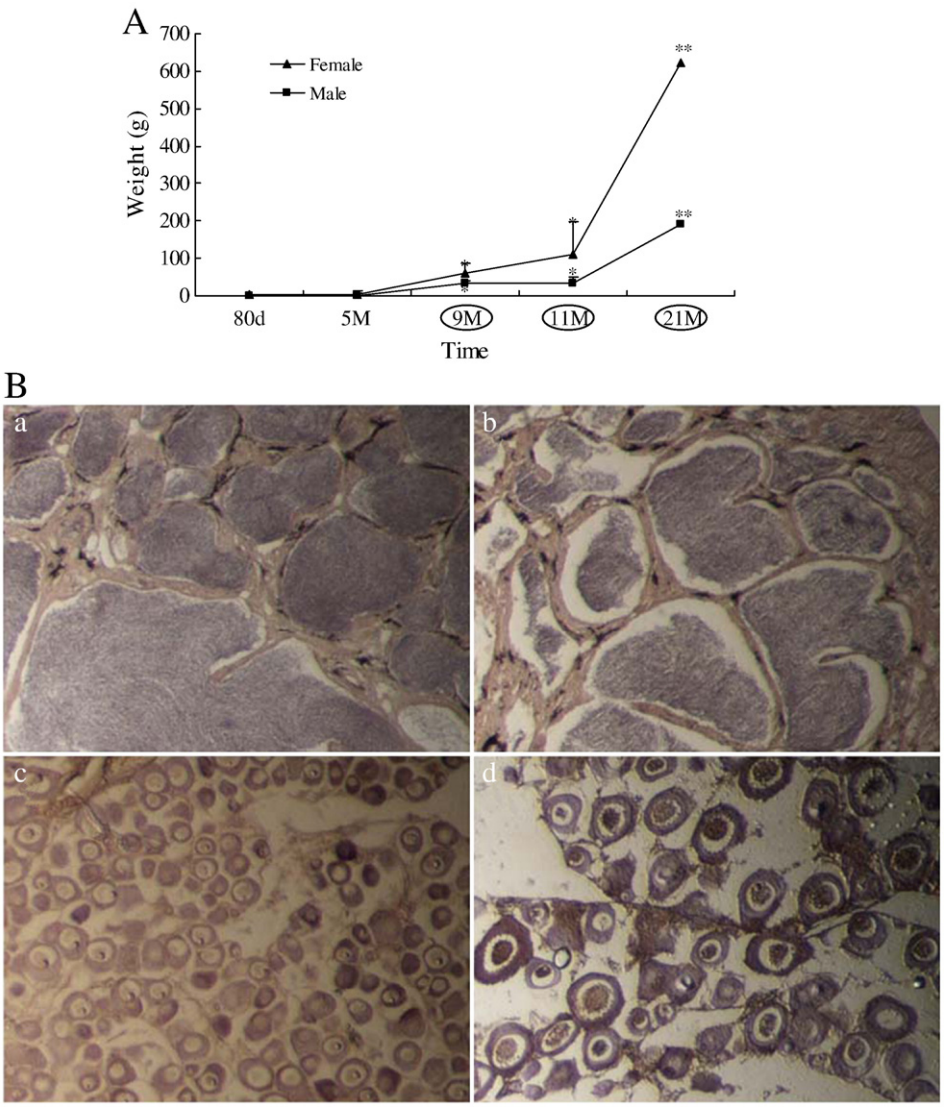

Fig. 1. Comparison of the growth performance of *Cynoglossus semilaevis* between the sexes in various growth stages (A). Histological sections of the *C. semilaevis* gonads (B). a: testis of 9-month-old males; b: testis of 21-month-old males; c: ovary of 9-month-old females; d: ovary of 21-month-old females. ⬭ indicated that the males were mature and the females immature at these stages. * showed the significant differences in body weight between female and males at this stage. D: day, M: month.

that of Senegalese sole, gilthead sea bream, orange-spotted grouper, red sea bream, Japanese flounder, rainbow trout, common carp, grass carp, African clawed frog, and porcine. A comparison of the deduced amino acid sequences of *C. semilaevis* and *Solea senegalensis* revealed that there was much divergence of amino acid residues of the signal peptide and the conserved region located at the ′3'-end of GH (Fig. 3A). The *C. semilaevis* GH amino acid sequences and several published GH sequences in other organisms were used to infer phylogenetic relationships. The results of analysis showed that *C. semilaevis* GH was evolutionary more related to the Pleuronectiform (Senegalese sole and Japanese flounder) and Perciform species (gilthead sea bream, orange-spotted grouper, and etc.), but less related to Cyprinid (common carp and grass carp) and Salmonid (rainbow trout and coho salmon) fish species, and distally related to the amphibian, avian, and mammalian species (Fig. 3B).

3.4. GH mRNA tissue distribution and expression differences between the sexes

GH mRNA expression in different tissues were analyzed. GH transcripts were highly abundant in *C. semilaevis* pituitary, but not present in blood, brain, eye, gill, head kidney, intestine, liver, muscle, ovary, skin, spleen, stomach, and trunk kidney (Fig. 4).

The results of qRT-PCR showed that GH mRNA levels of the immature females were not significantly different from that of immature males at age 5 months, which is consistent with their growth performance (Fig. 5). However, GH mRNA levels of the immature females were significantly higher compared with those of the mature males at age 9 or 11 months ($P<0.05$) (Fig. 5). At age 11 months, GH mRNA levels of females were even 6.4-fold higher than that of males. It showed the effect of sexual maturity of males on its GH mRNA expression. Additionally, at age 9 or 11 months, the difference of GH mRNA levels between the sexes also reflects the difference in growth between the females and males. At age 21 months, although the body weight of females was significantly higher than that of the males, the ovary of females was in stage III and there was not significantly different of GH mRNA levels between the sexes.

4. Discussion

In the present study, we found the differential age at first maturity for males and females in *C. semilaevis*. It is reported that the weight or volume of mature testis from *C. semilaevis* is only 1/200–1/900 of

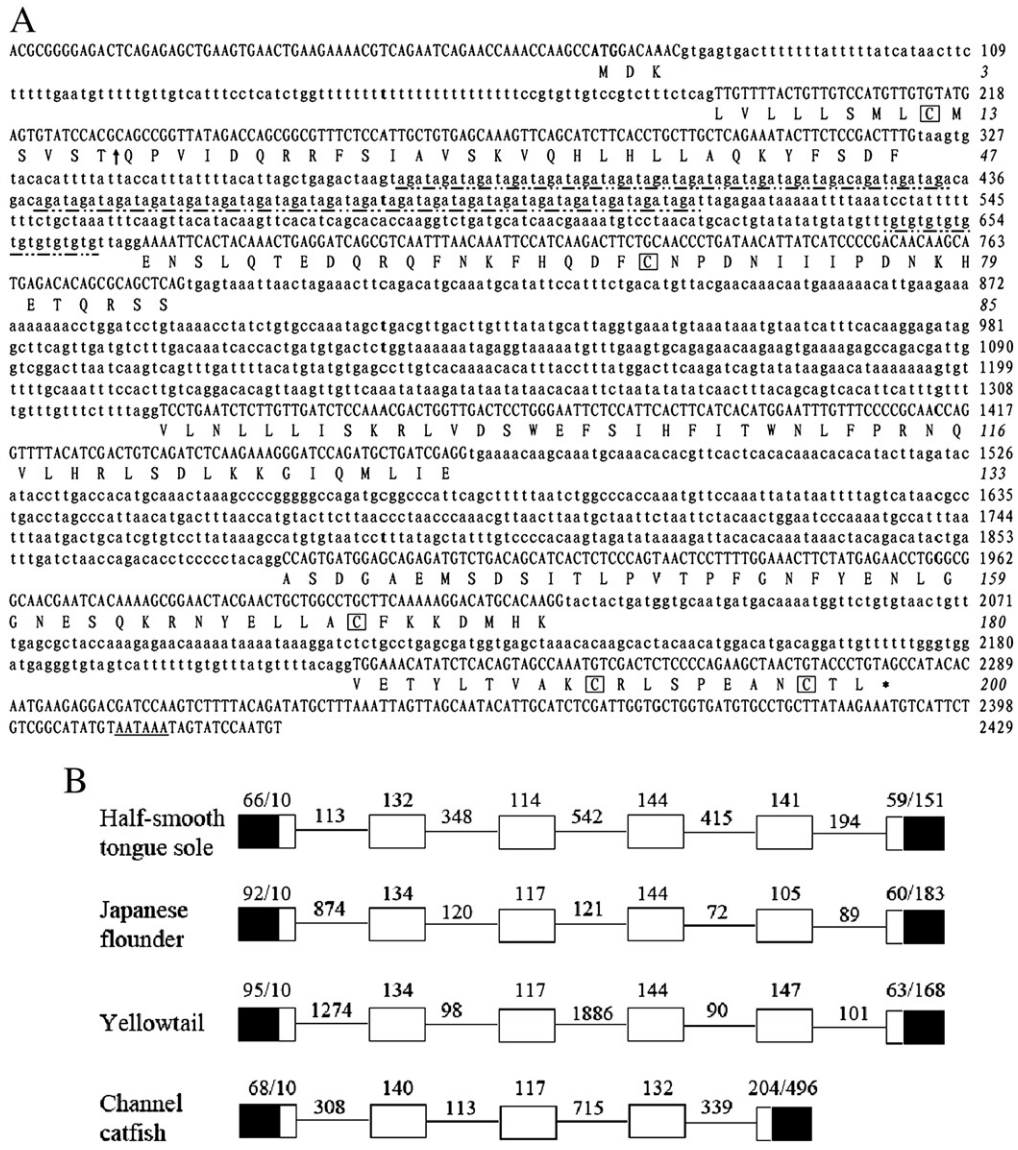

Fig. 2. Genomic sequences (GenBank Accession No. FJ608663) (A) and schematic illustration (B) of *Cynoglossus semilaevis* GH. Exons are in uppercase and introns are in lowercase. The arrow between the signal peptide (aa 1–17) and the mature peptide (aa 18–200) indicates the signal peptide cleavage site. The asterisk indicates the stop codon (TAG). The nucleotides corresponding to the polyadenylation signals in the 3′-untranslated region (AATAAA) are underlined. The five cysteine residues are boxed. Microsatellite motifs are underlined with ▬ ▪ ▪ .

mature ovary (Jiang and Wan, 1988). The differential age at maturity for males and females can influence adult population sex ratio and offset the lower reproductive capacity of males. However, the precocity is an undesirable trait in many fish species because very often the early sexual maturation is correlated with negative aspects such as reduced growth and feed conversion (Bromage et al., 2001). It is reported that Atlantic halibut males exhibit relatively slow growth due to precocious sexual maturation comparing with females (Jákupsstovu and Haug, 1988; Hagen et al., 2006). Felip et al. (2006) found that precocious European sea bass grew up to 18% less in weight and 5% less in fork length than their counterparts during their second annual cycle of life. In this study, we also found that body weight between the sexes was not significantly different at both 80 days and 5 months of age, when females and males were all immature (Liu et al., 2009; Ma et al., 2006), and significant difference of body weight between sexes was found after early sexual maturation of males at the age of 9 months. As a result, early sexual maturation of males is the main cause of sexual growth dimorphism in *C. semilaevis*.

The *C. semilaevis* females grow faster than males. The low growth rate of males weakens the quality of the fish, and thus leads to an overall reduction in production. Recently, to solve the problem, artificial gynogenesis (Chen et al., 2008b) and molecular marker-assisted sex control techniques (Chen et al., 2008a) were developed in order to generate all-female stocks in *C. semilaevis*. However, *C. semilaevis* has a ZZ/ZW sex determination mechanisms and it is very difficult to obtain WW superfemales or distinguish WW superfemales from the progeny of ZW female × ZW sex-reversed male crosses. Many studies showed that the manipulation of environmental parameters, such as temperature (Adams and Thorpe, 1989), photoperiod (Norberg et al., 2001), and feed availability (Rowe and Thorpe, 1990) at seasonally critical times, can result in reduced maturation. In the future, we can postpone the sexual maturation of *C. semilaevis* males using environmental manipulation to improve the production.

In the present study, the GH cDNA in *C. semilaevis* was cloned and it was a first report of GH cDNA sequences in Cynoglossidae species. The *C. semilaevis* pre-GH amino acid sequences had relatively higher identity with Senegalese sole (71.5%) (Pendón et al., 1994). Five cysteine residues were found in *C. semilaevis* GH as those for other fish species (Peyush et al., 2000; Law et al., 1996). These cysteine residues are involved in the formation of two disulfide bonds, which plays an

Fig. 3. (A) Comparison of the amino acid sequences of GH from half-smooth tongue sole, *Cynoglossus semilaevis* (FJ608663) and Senegalese sole, *Solea senegalensis* (P45643). The different amino acid residues between them were boxed. (B) Dendrogram graphically showing the relations for various fish GH. The scale bar was bootstrap values obtained after 1000 resampling and refers to percentage divergence. The relative genetic distances are indicated by the scale bar and the branch lengths. Gilthead sea bream, *Sparus aurata* (P08591), Silver sea bream, *Sparus sarba* (AAT48995), Red sea bream, *Pagrus major* (P08591), Yellowtail, *Seriola quinqueradiata* (M35622), Yellowfin porgy, *Acanthopagrus latus houttuyn* (P45654), Orange-spotted grouper, *Epinephelus coioides* (AAK57697), Japanese flounder, *Paralichthys olivaceus* (D29737), Rainbow trout, *Oncorhynchus mykiss* (M24684), Coho salmon, *Oncorhynchus kisutch* (M19999), Channel catfish, *Ictalurus punctatus* (AF267989), Grass carp, *Ctenopharyngodon idella* (M27094), Common carp, *Cyprinus carpio* (M27000), African clawed frog, *Xenopus laevis* (X14602), Chicken, *Gallus gallus* (M35609), Monkey, *Macaca mulatta* (L16556), Porcine, *Sus scrofa* (U19787), Bovine, *Bos taurus* (M27325).

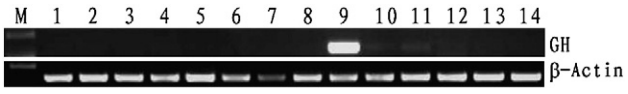

Fig. 4. RT-PCR expression analysis of GH mRNA from various tissues of *Cynoglossus semilaevis*. M: DL2000 marker. 1. blood; 2. brain; 3. eye; 4. gill; 5. head kidney; 6. intestine; 7. liver; 8. muscle; 9. pituitary; 10. skin; 11. gonad; 12. spleen; 13. stomach; and 14. trunk kidney.

important role in determining its biological activity (Chang et al., 1992). The putative signal peptide of *C. semilaevis* GH is 17 amino acids long as orange-spotted grouper and pejerrey (Li et al., 2005; Sciara et al., 2006) and shorter than carp (Chao et al., 1989), goldfish (Law et al., 1996), and rohu (Venugopal et al., 2002), which has 22 amino acids residues.

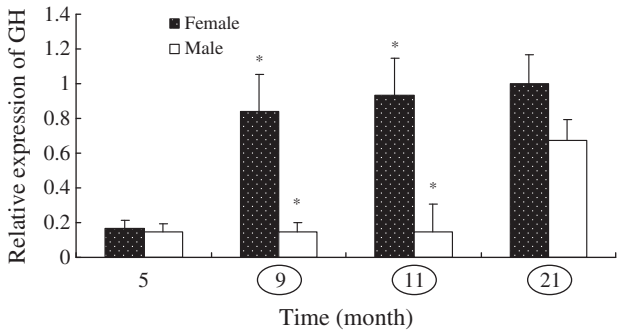

Fig. 5. Quantitative RT-PCR analysis of *Cynoglossus semilaevis* GH mRNA expression between females and males at various growth stages. ⬭ indicated that the males were mature and the females immature at these stages.

The *C. semilaevis* GH gene consists of six exons and five introns similar to the GH genes from teleosts belonging to orders Pleuronectiformes (Peyush et al. 2000), Salmoniformes (Sekin et al., 1985), and Perciformes (Law et al., 1996). However, there were five exons and four introns in channel catfish (Fig. 2B) (Sekin et al., 1985) or other Ostariophysi GHs (Venkatesh and Brenner, 1997). It is possible that the fifth intron of GH genes was either introduced into the GH gene of a common ancestor of orders Pleuronectiformes, Salmoniformes, and Perciformes after it branched off from the order Ostariophysi or was lost in the branch that gave rise to the Ostariophysi (Venkatesh and Brenner, 1997). The second intron of *C. semilaevis* GH, which is 348 bp long, is larger than its counterparts in other teleosts because of the presence of microsatellite repeats: $(TAGA)_{13}$, $(AGAT)_{19}$, and $(TG)_{10}$. However, the second introns of Pleuronectoidei species GH do not contain microsatellite repeats, which show that Soleoidei possibly branched off from their ancestor early. Moreover, there are significant differences in GH introns length among species (Fig. 2B) and the dispersion of microsatellite repeats in introns among species is somewhat different. To elucidate the evolutionary trails of teleost GH genes, further investigations in the genomic structures of other teleost GH are necessary. The microsatellite loci in second intron of *C. semilaevis* GH might be used for genotyping GH gene in the future.

The *C. semilaevis* GH transcripts were highly abundant in pituitary, but not present in other tissues. In rainbow trout, though high levels of GH transcripts were primarily detected in the pituitary tissue, low levels of GH transcripts were also found in ovary (Yang et al., 1999). In orange-spotted grouper, besides the high level of GH mRNA in the pituitary, the faint bands for PCR product were also found in the brain (free from pituitary), spleen, liver, gill, and ovary (Li et al., 2005). These data suggest that GH have differential expression pattern in various fish species.

An interesting finding was that GH mRNA levels of the females were not significantly different from that of males when females and males were all immature, and significant difference of GH mRNA levels between the sexes was observed after early sexual maturation of males. It indicated that sex steroids play an important role in regulating the GH mRNA expression. Degani et al. (2003) found that the GH mRNA levels in the European eel females treated by estradiol (E_2) were significantly higher than that in the males. In masu salmon, sGnRH at 1.0 nM elevated the amounts of GH mRNA examined in the pituitary cells of pre-spawning females (Onuma et al., 2005). Several studies in mammals also showed that many of physical dimorphisms in the brain were directly caused by sex hormone treatments (Gorski et al., 1978; Hutchison 1997). As a result, further studies will be needed for elucidating the effect of sex steroids on the GH mRNA expression in *C. semilaevis*.

GH mRNA levels of the females were not significantly different from that of males when the body weight of females and males was not different at age 5 months. However, GH mRNA levels of the females were also significantly higher than that of males when body weight of females was higher than that of males at age 9 or 11 months. GH plays an important role in regulating mRNA expression of many genes, some of which are known to be growth related genes. Gil et al. (2008) found that GH modified the expression of 224 genes in the tibial growth plate of young rats, 195 being up-regulated and 29 down-regulated. Ponce et al. (2008) discovered that both hepatic IGF-I and IGF-II mRNA levels in redbanded sea bream *Pagrus auriga* increased sharply (3.1- and 19-fold higher than control, respectively) 3 h after injection of porcine GH. Jarukamjorn et al. (2006) found that GH could significantly modify the expression profile of sexually dimorphic P450 genes. As a result, it is possible that sexually dimorphic expression of GH mRNA exerts the effects on the differential growth between the sexes in *C. semilaevis* by regulating the expression of other genes, especially the growth-related genes. But this hypothesis requires more detailed study.

In conclusion, our data show for the first time that significant difference of growth and GH mRNA levels between the sexes was observed only after early sexual maturation of males. The differential level of GH mRNA between the females and males was also consistent with growth difference between the sexes in *C. semilaevis*. These findings suggest that early sexual maturation of males play a role in regulating growth and GH gene expression, and GH gene is in turn responsible for growth differences between the sexes in *C. semilaevis*.

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