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Molecular characterization and differential expression of the myostatin gene in *Coilia nasus*

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ABSTRACT

Estuarine tapertail anchovy (*Coilia nasus*, junior synonym *C. ectenes*) is a widely distributed and commercially important aquaculture species, although its growth in aquaculture settings is so slow as to pose a serious practical problem. In order to understand the molecular mechanisms of growth, we cloned the myostatin gene in *C. nasus* (*CnMSTN*) by homologous cloning methods. Its full-length cDNA is 2252 bp, with a 1125-bp open reading frame (ORF) that encodes a 374-amino acid protein. The CnMSTN protein is predicted to contain domains typical of MSTN, including a TGFb-propeptide domain and a TGFB domain. Gene expression patterns were detected by RT-qPCR. *CnMSTN* is expressed strongly in the muscle and brain, and comparatively lower in the gills, liver, spleen, intestine, trunk kidney and head kidney. The effects of stress on the muscle and brain MSTN levels were evaluated by RT-qPCR. *CnMSTN* in the muscle was positively regulated by loading and transport stress, but brain *CnMSTN* expression was not affected. We found NaCl could reduce the death rate caused by loading and transporting stress, and in this group, *CnMSTN* mRNA expression in the muscle revealed increased, but decreased in the brain. Further, in the fasting experiment, the *CnMSTN* mRNA revealed decrease in the muscle, on the contrary, it showed increase in the brain. Selection upon variants of the MSTN gene has shown great potential in breeding work for mammals, and our results provide the basic knowledge for breeding of *C. nasus*.

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1. Introduction

Estuarine tapertail anchovy (*Coilia nasus*, junior synonym *C. ectenes*) is widely distributed in the Yangtze River, the coastal waters of China, Korea, and the Ariake Sound of Japan (Jiang et al.). It is a commercially important species due to its nutritive value and delicacy. However, excessive fishing and changes in aquatic ecology have almost caused extinction of the species in the middle reaches of the Yangtze River (Zhang et al., 2005). In recent years, a number of research projects have been done by many scientists, breeding in ponds, artificial propagation, and larval rearing techniques have been established (Xu et al., 2011; Zhang and Zheng, 2006). As a result, the threat to *C. nasus* resources has been alleviated. However, the body weight of farmed *C. nasus* is only up to 100 g through two or more years, hence, the growth rate should be improved. Therefore, the growth rate of *C. nasus* is a serious practical problem facing aquaculture.

Myostatin (MSTN), a member of the transforming growth factor-β (TGFβ) superfamily, is a negative regulator of muscle deposition (Lee and McPherron, 1999). Individual muscle fibers of homozygous MSTN knockout mice are double or triple in mass compared with those in heterozygous and wild-type litter-mates (McPherron et al., 1997a). Moreover, the natural mutations of the myostatin gene in the two breeds of cattle, the Belgian Blue and the Piedmontese, both of which possess a double muscling phenotype (Bass et al., 1999; Casas et al., 1998; Grobet et al., 1998; McPherron and Lee, 1997), supported this discovery. The finding that variation at a single gene showed such a major effect on processing yields provided a potential channel for improving processing yields of animals using knockout technology. Therefore, molecular characterization of the myostatin locus of important domesticated species such as pigs, chickens, cows, sheep and dogs has been completed (Boman and Vage, 2009; Boman et al., 2009; Clop et al., 2006; Grobet et al., 1997; Mosher et al., 2007; Stratil and Kopecny, 1999; Yang et al., 2001). This work is likewise important for improving *C. nasus* yields. However, the functions of myostatin are quite different in fishes from those in mammals, where multiple gene copies are differentially expressed in many tissues such as the gill, liver, muscle, intestine, spleen, heart, brain, eye, stomach, skin, testis, ovary, and kidney (Pan et al., 2007; Swofford, 2000; Wise et al., 2007; Ye et al., 2007).

Cortisol is secreted from the interrenal gland in teleosts (Mommsen et al., 1999), and its production is enhanced by stress treatment in interrenal cells (Young, 1988). Several studies have addressed the regulatory interaction between cortisol and *MSTN* gene expression (Lang et al., 2001; Ma et al., 2001, 2003), but the results are still controversial,

Abbreviations: MSTN, myostatin; CnMSTN, *Coilia nasus* myostatin; ORF, open reading frame.

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moreover, no efforts has yet been committed to understanding the relationship of stress, cortisol and *MSTN* expression in fish. Here, we report molecular cloning, sequencing and differential expression of the myostatin gene from *C. nasus*, evaluate the effects of stress treatment on the muscle and brain MSTN levels in *C. nasus*, and investigate possible relationships between stress, cortisol, and *MSTN* levels following treatment.

2. Materials and methods

2.1. Experimental animals

C. nasus (average weight, 9.6 g) were adapted to a $7.0 \times 5.0 \times 1.0\ m^3$ aquarium with a water temperature of 24.5 ± 1.0 °C, pH 7.8, and dissolved oxygen concentration of 9.2 ± 0.5 mg O_2/L dechlorinated and aerated water. Fish were fed twice daily, at 7:00 AM and 5:00 PM. At the onset of the experiments, all fish appeared healthy.

2.2. Cloning and sequencing of C. nasus MSTN (CnMSTN)

Total RNA of *C. nasus* muscle was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and treated with RNase-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesized from RNA using Moloney murine leukemia (M-MLV) reverse transcriptase (Takara, Osaka, Japan). Sequence alignment of *MSTN* nucleotide sequences from a variety of species was performed with the ClustalX 1.83 multiple-alignment software. The primers were designed based on the conserved nucleotides of the sequences of thirteen species reported previously: *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Canis familiaris*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, *Danio rerio*, *Takifugu rubripes*, *Oryzias latipes*, *Takifugu rubripes* and *Oncorhynchus mykiss* (Table 2). A 276-bp fragment of the *MSTN* gene was amplified from *C. nasus* muscle cDNA using the primer pair R796-1/R796-2 (Table 1). The resulting fragments were separated on 1.0% agarose gel and purified using the Axygen DNA gel extraction kit (Axygen, Union City, CA, USA). The purified fragments were cloned into the pMD-18T vector (Takara, Dalian, China) by the TA cloning strategy and sequenced (BGI, Shenzhen, China). The 276-bp fragment was confirmed to be a homologous sequence of the *MSTN* gene from *D. rerio* by using BlastX. The 5′ and 3′ ends of the *CnMSTN* cDNA were obtained using the rapid amplification of cDNA ends (RACE) approach (Zhuan Dao, Wuhan, China).

2.3. Analysis of nucleotide and amino acid sequences

The nucleotide and predicted amino acid sequences of *CnMSTN* were analyzed using DNA figures software (http://www.bio-soft.net/sms/index.html). The similarity of *MSTN* from *C. nasus* with *MSTN* from other organisms was analyzed using the BLASTP search program (http://www.ncbi.nlm.nih.gov/blast). The domain structures were predicted using the SMART program (http://smart.embl-heidelberg.de/). The amino acid sequence was compared with those of other species' MSTNs using ClustalX 1.83 (http://www.ebi.ac.uk/clustalW/) and GeneDoc (http://www.nrbsc.org/gfx/genedoc/).

2.4. RT-qPCR analysis of CnMSTN mRNA expression profiles

For tissue distribution analysis of *CnMSTN*, total RNA was extracted from the gill, liver, spleen, trunk kidney, head kidney, brain and intestine from healthy *C. nasus* using TRIzol Reagent (Invitrogen, USA). Each of the samples was obtained from five individuals and triplicated to control for inter-individual differences.

The first-strand cDNA was synthesized with the ReverTra Ace® qPCR RT kit (Toyobo, Japan), and RT-qPCR was employed to detect the *CnMSTN* expression profiles using *β-actin* and *18sRNA* as a reference gene. The RT-qPCR primers 23S/23A for *CnMSTN*, B1/B2 for *β-actin* and 42S/42A for 18sRNA (Table 1) shared similar Tm values and were designed to amplify 180-bp 136-bp and 114-bp fragments, respectively. RT-qPCR was performed on the ABI 7500 real-time PCR system (ABI, USA) using 2× SYBR green real-time PCR mix (Takara, Japan). PCR amplification was performed in triplicate, using the following cycling parameters: 94 °C for 2 min; followed by 40 cycles of 15 s at 94 °C, 15 s at 60 °C, and 45 s at 72 °C. All samples were analyzed in triplicate and the expression of target genes was calculated as relative folds with the $2^{-\triangle\triangle CT}$ method.

CnMSTN protein expression in tissue was analyzed using an enzyme-linked immunosorbent assay kit (Zhaorui, Shanghai, China), as described by the manufacturer.

2.5. Stress experiment

In March 2012, three $7.0 \times 5.0 \times 1.0\ m^3\ m^2$ ponds were stocked with 120 juvenile *C. nasus* each. The fish were acclimated to the ponds for approximately 14 months before the experiment, at which time the fish were 15 months old. Excess fish were stocked in the ponds in order to ensure subsequent access to the intended numbers. Five fish were removed from each pond and euthanized with 70 mg/L buffered tricaine methanesulfonate (MS-222). These 15 fish were the non-stressed controls and were processed immediately (see tissue sampling below). Then, another 75 fish were netted from each pond and 15 were loaded into each of the 15 tanks with a volume of $75 \times 55 \times 33\ mm^3$. These 15 tanks were divided into 5 groups which were used for sampling at different time-points; each group contained 3 tanks. Shaking once every 5 min to simulate the transport process, at 0 h, 2 h, 4 h, 6 h, 8 h after

Table 1
Sequences of primers used in this study.

Primer	Sequence	Usage
R796-1	GGAGTTCTCGTCGCAGTCTA	5′RACE
R796-2	AGACCTTTTGGGGCCTTCGG	5′RACE
457-1	GACGAGAACTCCCCAGAATC CCGCT	3′RACE
457-2	CGCTACAAGGCCAACTACTG CTCCG	3′RACE
MSTN-F1	CTGCAACCYTTCATGGAGGTGA	Homologous cloning forward primer
MSTN-R2	AGCATGTTGATGGSSGACATCTT	Homologous cloning reverse primer
23S	GCTTTGTTCACCAGGTGGGTG	Forward primer for *MSTN* RT-qPCR
23A	CCGGACTAGACTGCGACGAGA′	Reverse primer for *MSTN* RT-qPCR
B1	AACGGATCCGGTATGTGCAA AGC′	Forward primer for Beta-actin RT-qPCR
B2	GGGTCAGGATACCTCTCTTG CTCTG	Reverse primer for Beta-actin RT-qPCR
42S	TGATTGGGACTGGGGATTGAA	Forward primer for 18sRNA RT-qPCR
42A	TAGCGACGGGCGGTGTGT	Reverse primer for 18sRNA RT-qPCR

Table 2
GenBank accession numbers of MSTN used in this study.

Protein	Accession no.
Homo sapiens MSTN	NP_058642.1
Pan troglodytes MSTN	NP_001073388.1
Macaca mulatta	NP_001073588.1
Canis familiaris MSTN	NP_001002959.1
Gallus gallus MSTN	NP_001001461.1
Mus musculus MSTN	NP_034964.1
Rattus norvegicus MSTN	NP_062024.1
Takifugu rubripes MSTN2	NP_001027844.1
Salmo salar MSTN1a	NP_001117021.1
Oncorhynchus mykiss MSTN2	NP_001117755.1
Oncorhynchus mykiss MSTN1	NP_001117754.1
Salmo salar MSTN1b	NP_001117106.1
Takifugu rubripes MSTN1	NP_001027843.1
Oreochromis niloticus MSTN	XP_003458880.1
Oryzias latipes MSTN	NP_001188428.1
Oncorhynchus mykiss MSTN2a	NP_001118048.1
Drosophila melanogaster MSTN	NP_726606.1
Danio rerio MSTN	NP_571094.1

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