

# Characterization and Expression Analysis of Myogenin Gene in White Muscle of Chinese Mandarin Fish, *Siniperca chuatsi*

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## Abstract

Myogenin plays a crucial role in myoblast differentiation and maturation. In the present study, the myogenin gene structure and expressional patterns in *Siniperca chuatsi* were characterized. Sequence analysis indicated that the myogenin shared a similar structure and the conserved bHLH domain with other vertebrate myogenin genes. Sequence alignment and phylogenetic analysis showed that *Siniperca chuatsi* myogenin shared homologous with *Epinephelus coioides*, *Sparus aurata*, *Takifugu rubripes* and *Salmo salar*. Whole-mount *in situ* hybridization revealed that myogenin expression was first detected in the gastrula stage embryos and high levels of expression at the 24 somite stage. After the 24 somite stage, myogenin expression began to decrease in the anterior somites where somatic cells were differentiated. Further, the muscle structural gene, MyHC, and myogenin, are concomitantly expressed during *S. chuatsi* embryonic development as assayed with whole-mount *in situ* hybridization. In the adult fish, the myogenin showed the highest levels of expression in the brain compared with the kidney, spleen, liver, heart and white muscles. Our work on the myogenin gene from the mandarin fish has provided useful information for fish molecular biology and fish genomics.

**Keywords:** *Siniperca chuatsi*; Myogenin; White muscle; Developmental expression

## Introduction

Fish skeletal muscle is composed of two spatially separated fibers, white, fast-twitch muscle make up the bulk of fish body, whereas red, slow-twitch fibers are found in a narrow mid-lateral band immediately under skin [1] and it provides an excellent model for the study of muscle development and the mechanisms underlying muscle cellularity [2]. Similar to other vertebrates, fish skeletal muscle development involves a series of events in which cells in the paraxial mesoderm are firstly induced to form myoblasts and subsequently differentiate into myotubes, and the myotubes then mature into myofibers and grow by recruiting other myoblasts [3]. This process, termed as myogenesis, is controlled by many extracellular signaling molecules together with a family of basic helix-loop-helix (bHLH) intracellular transcription factors known as the myogenic regulatory factors (MRFs) [4-6]. Four members of MRFs have been identified in fish including MyoD, Myf5, myogenin and MRF4 which exert a pivotal role in muscle cell specification, proliferation and differentiation during muscle development [7,8]. Several earlier studies confirmed that MyoD and Myf5 are required for the determination of myoblasts, while myogenin and MRF4 function in myofiber differentiation by recruiting structural proteins, such as myosin proteins [9-11]. In view of spatial and temporal expression during embryonic development, MyoD and Myf5 are restricted to adaxial and posterior cells and their expression are also appeared earlier than Myogenin and MRF4 [5]. These results demonstrated that the MRF members act in a concert manner during myoblast specification and myofiber differentiation.

Like other MRF factors, the myogenin cDNAs have been isolated from several teleost species, including but not limit to, zebra fish (*Danio rerio*) [12,13], striped bass (*Morone saxatilis*) [14], electric fish (*Sternopygus macrurus*) [15], channel catfish (*Ictalurus punctatus*) [16], blue catfish (*I. furcatus*) [16], white catfish (*Ameiurus catus*) [16], flounder (*Paralichthys olivaceus*) [17] and sea bream (*Sparus aurata*) [3]. Comparison of those earlier reports on the myogenin gene structure, expression patterns and regulatory pathways among different fish species, it shows certain similarity with some species-

specific characterization [9]. Therefore, further characterization of the myogene, its expression pattern and regulatory mechanism in other fish species, especially some commercially important aquaculture species could provide useful information in fish muscle development biology and potential application in fish aquaculture.

Improving fish production is a continuous goal of the aquaculture industry, and thus a better understanding of the molecular control of muscle development of the fish species could be beneficial for the fish culture [18]. The Chinese mandarin fish, *Siniperca chuatsi* is one of the most commercially important species in aquaculture in China as well as in eastern Asia [19,20]. High nutritional value and the appealing taste of the mandarin fish stimulate a large-scale aquaculture [21,22]. Therefore, in the present study, we choose the important muscle regulatory factor, myogenin, to investigate its gene structure, spatial and temporal expression, as well as its correlated expression with myosin heavy chain gene during muscle development in *S. chuatsi*. Our studies provide important insights into the structure and expressional characterization of myogenin in fish.

## Materials and Methods

### Fish and embryo preparation

The mandarin fish (*Siniperca chuatsi*) were raised at Hunan Aquatic Research Institute, Changsha, Hunan, China. The white muscle, liver, kidney, spleen, heart and brain were collected from 5 adult fishes at an

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average body weight about 0.5 kg. The isolated tissues were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until use. Embryos were obtained by artificial fertilization and cultured in freshwater aquaria at approximately  $20^{\circ}\text{C}$ . Embryos of different developmental stages were collected from fertilization to hatching and they were then fixed in 4% paraformaldehyde for whole mount *in situ* hybridization.

### Isolation of the myogenin gene of *S. chuatsi*

The genomic DNA was extracted from the white muscle tissues of the fishes as described by Chu et al. [23]. The extracted DNA was used as a template for PCR amplification. A 1.7 kb DNA fragment containing the first exon and the part of the first intron was generated using the MG1 and MG2 primers and the 5'-flanking region of the myogenin gene sequence was cloned using the MG3 and MG4 primers based on the corresponding region of the published teleost myogenin genes. The remaining part of myogenin genomic sequences was cloned by another round of PCR using the MG5 and MG6 primers. All of the fragments were sequenced on a model 377XL DNA sequencer (Applied Biosystems, Foster City, CA, USA).

To determine the intron-exon junctions in myogenin genomic sequence, total RNAs were extracted from the obtained fish tissues using TRIzol methods. Briefly, 5 g of frozen the tissue was chopped into fine pieces and homogenized with a hand mortar and then extracted with TRIzol<sup>®</sup> Reagent (Life Technologies, P/N15596-018, China), according to the manufacturer's standard protocol. The RNA was then purified on an RNeasy Min Elute Cleanup kit (Qiagen 7402, China Branch) and RNA purity was ensured by obtaining a 260/280 nm ratio equal to 2.0. All of the resulted RNAs were stored at  $-80^{\circ}\text{C}$  until use. Synthesis of the first strand cDNA was performed with oligo (dT)<sub>20</sub> and Superscript II reverse transcriptase (Invitrogen, USA) as described by Chu et al. [24]. The cDNAs were used as a template for amplification. The myogenin cDNA was isolated by PCR using the MG1 and MG7 primers. The PCR fragment was cloned into the pGM-T vector and it was transformed by electroporation into the Escherichia coli strain DH10B. The sequencing was performed on a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA).

### Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out essentially as reported using a digoxigenin (DIG)-labeled riboprobes [25]. The *S. chuatsi* myogenin exon 1 sequence was amplified with PCR using the MG8 and MG9 primers and cloned into pMD18-T vector (TaKaRa, Japan). The purified plasmid DNA was linearized by *Sph*I, followed by *in vitro* transcription with SP6 RNA polymerase to generate the antisense RNA probe. The reverse-transcription reaction and antisense digoxigenin labeling was done in a 20  $\mu\text{L}$  reaction mixture including 5  $\mu\text{L}$  ddH<sub>2</sub>O, 2  $\mu\text{L}$  6.5  $\times$  NTP, 2  $\mu\text{L}$  DTT, 4  $\mu\text{L}$  5 $\times$ buffer, 2  $\mu\text{L}$  Digoxigenin-UTP, 1  $\mu\text{L}$  RNase inhibitor and 1  $\mu\text{L}$  SP6 enzyme. Embryos of various stages were hybridized with the digoxigenin-labeled antisense RNA probe in 50% formamide, 20 $\times$ SSC, heparin 50 mg/ml, tRNA 50 mg/ml, and 0.1% Tween 20 at  $65^{\circ}\text{C}$ , followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and by staining with substrates Nitro blue tetrazolium (NBT) and 5-bromo, 4-chloro, 3-indolil phosphate (BCIP) to produce purple insoluble precipitates. The typical stained embryos were photographed in glycerol under the microscope (DM LB2, Leica) with a Nikon 4500 digital camera.

### Quantitative real-time PCR assays for the myogenin gene expression

Primers MG10 and MG11 were designed with Primer 5.0 for the real time PCR analysis of myogenin expression.  $\beta$ -actin was used as an internal reference gene to normalize target gene transcript levels. Real-time PCR was performed using SYBR Green PCR Mix, containing MgCl<sub>2</sub>, dNTP, and Hot start *Taq* polymerase. 2  $\mu\text{L}$  cDNA templates was added to a total volume of 25  $\mu\text{L}$  containing 12.5  $\mu\text{L}$  SYBR Green mix, and 1  $\mu\text{mol/L}$  each of forward and reverse primers. The following protocol was used: (i) pre-denaturation program (10 s at  $95^{\circ}\text{C}$ ); (ii) amplification and quantification program, 40 cycles (5 s at  $95^{\circ}\text{C}$ , 20 s at  $60^{\circ}\text{C}$ ); (iii) melting curve program (60-99 $^{\circ}\text{C}$  with heating rate of  $0.1^{\circ}\text{C s}^{-1}$  and fluorescence measurement). The identity of each product was confirmed by di-deoxy mediated chain termination sequencing at Sangon Biotechnology, Inc. (Shanghai, China). We calculated the relative expression ratio (R) of the mRNA by  $R = 2^{(\text{CT}(\beta\text{-actin}) - \text{CT}(\text{test}))}$  [26]. Negative controls were performed in which cDNA was substituted with water. The myogenin mRNA expression was analyzed by one-way ANOVA procedure and regression procedure of SPSS software (SPSS Inc., USA). Duncan's multiple range tests were used to compare the difference among different tissues. The differences were considered statistically significant and very significant when  $P < 0.05$  and  $P < 0.01$  respectively. Data are shown as means  $\pm$  SE.

### Bioinformatics analysis

The nucleotide sequences and inferred amino acid sequences were analyzed using DNA star. The BLAST program was used to identify homologous sequences in the Gene Bank database. The sequences were aligned with multiple alignment program CLUSTAL W.

## Results

### Molecular characterization of *S. chuatsi* myogenin gene

The *S. chuatsi* myogenin genomic gene was isolated from the white skeletal muscles. The complete genomic sequence of the myogenin spanned approximately 3.3 kb (Gene bank accession no. HQ724299), including 0.35 kb 5'-flanking sequence and 2.5 kb transcriptional unit followed by 0.45 kb 3' flanking sequence (Figure 1). The myogenin gene sequence contains three exons and two introns. The three exons were named as exon I, exon II and exon III with nucleotides of 537 bp, 96 bp and 120 bp, respectively, and the two introns, named intron 1 and intron 2, are of 803 bp and 95 bp each. The 5' promoter region analyzed with DNA star revealed that it contains two putative E-box sites (CAnn TG), a MEF-2 (myocyte enhancer factor-2) binding site (TAAATTTA) and a MEF3 (myocyte enhancer factor-3) binding site (TCAGGTTT). The two E boxes were located at 184 and 170 bp, while MEF2 and MEF3 at 238 and 359 bp upstream of the ATG starting codon, respectively (Figure 2). Comparison of the *S. chuatsi* myogenin promoter sequences with other two fish species, *Sparus aurata* (EF462192) and *Epinephelus coioides* (HM190251). The nucleotide sequence and location of the regulatory elements are highly conserved among the three fish species (Figure 2).

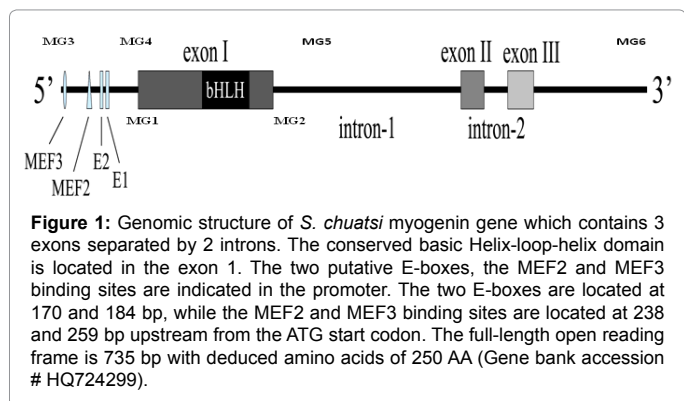
### Comparison of *S. chuatsi* myogenin protein sequence and phylogenetic relations to other vertebrates

The *S. chuatsi* myogenin gene encodes a protein of 250 amino acids. BLAST analysis of myogenin sequences of 16 species in Gene bank revealed that the bHLH domain in the first exon of these myogenins was highly conserved among the species analyzed (Figure 3). The phylogenetic analysis revealed that among the teleost species, *S.*

*chuatsi* myogenin had the highest sequence identity with myogenin from *Epinephelus coioides*, and followed by *Sparus aurata*, *Takifugu rubripes*, *Salmo salar* and *Danio rerio*. The other vertebrate myogenin sequences were fallen into a group, including mammals and reptile species, while, the amphibians in the middle (Figure 4).

### The temporal and spatial expression of *S. chuatsi* myogenin gene during embryonic development

To examine the temporal and spatial expression of myogenin mRNAs in *S. chuatsi*, whole-mount *in situ* hybridization and RT-qPCR were carried out. Firstly, ten different developmental stages of embryos from fertilized eggs to hatch out fry were assayed with whole-mount *in situ* hybridization. As described in Figure 5 (up panel), no myogenin expression was observed in newly fertilized eggs and balstula stage embryos (Figure 5 up panel 1, 2) and its expression was first detected in the gastrula stage embryos (Figure 5 up panel 3). At the 5 somite stages, myogenin mRNAs were apparently identified in white and red muscles (Figure 5 up panel 4). As the somites were increasing from the anterior to the posterior region until 24 somites, myogenin was correlated expressed with somite formation detected in both medial and lateral regions (Figure 5 up panel 6 and 7). However, after the 24 somite stage, the myogenin expression was decreased in the anterior somites where somatic muscle was differentiated, but the posterior somites still showed a strong expression (Figure 5 up panel 8 and 9).

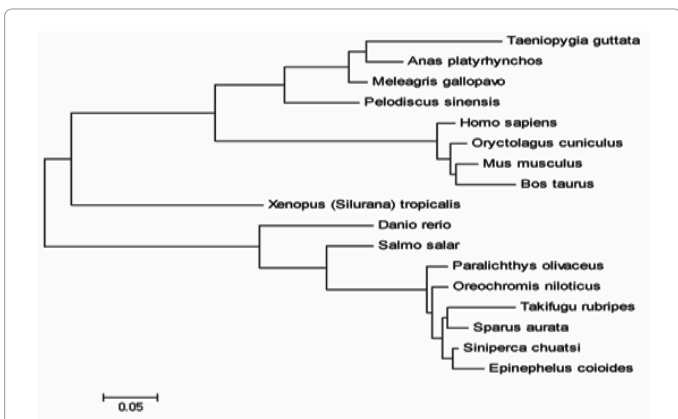


	MEF3	MEF2
<i>Siniperca chuatsi</i>	TGAAGTGTAG ATGTGCAGCA ACAGCTAAAC GTCGTGTCAG GTTCTGCGG GCTCGTGCTA	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	
		E-box2
<i>Siniperca chuatsi</i>	AATTTAACCG TGTGATGCTG CAGCAGGCAG AGGGGTTTAA ATGCCAGCCT ACAGTTGCTC	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	
	E-box1	
<i>Siniperca chuatsi</i>	CACACCAGTT GCTCTCCACA GTCCTTCTAG TCATCACAAA CCGAGGC-----AAGA	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	
		E-box2
<i>Siniperca chuatsi</i>	CAGCCACACA CACTACACT CCGACACACA CCGTCAGGC CAGGACCGA AGA---CCAC	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	
		E-box2
<i>Siniperca chuatsi</i>	ATACAGTAGA GCAG---TTC CAGGGGAAGC AGGAATCTTT GTCCCTT---T-CAGACCA	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	
		E-box2
<i>Siniperca chuatsi</i>	CAGCGTGTCT CAGTATGGAG CTITTCGGAA CCAACCCCTA CTTCCTCCCT GACCAAGCCT	
<i>Epinephelus coioides</i>	.....	
<i>Sparus aurata</i>	.....	

**Figure 2:** Comparison of the *S. chuatsi* myogenin promoter sequences with other fish species, *Sparus aurata* (EF462192) and *Epinephelus coioides* (HM190251). The two E-boxes, E-box 1 (CAGTTG) and E-box 2 (CAGTTG), MEF2 (CTAAATTTAA) and MEF3 (CAGGGTTT) binding sites are underlined. The nucleotide sequence and location of the regulatory elements are highly conserved among the three fish species.

<i>Siniperca chuatsi</i>	CLPWAKLQKPKTVYNDKRRRAATMREKRRKKNVAFDALRSTLMPNGRLPKVEILRSALQTEGLQYSSLNQDTEFGQGLHYR
<i>Xenopus tropicalis</i>	.....V.....S.....L.....E.....L.....R.....T.L.A.....-..RD..RFI
<i>Takifugu rubripes</i>	.....L.....K.....T.L.A.....F.
<i>Taeniopygia guttata</i>	.....V.....S.S.....L.....E.....L.....R.....S.L.T.....-..RE..RE..L.
<i>Sparus aurata</i>	.....R.....N.
<i>Salmo salar</i>	.....K.....E.....R.....E.....DQ.T.....
<i>Pelodiscus sinensis</i>	.....I.....S.L.....L.....E.....L.....R.....S.L.T.....-..RD..RMR.
<i>Paralichthys olivaceus</i>	.....R.....
<i>Oryctolagus cuniculus</i>	.....V.....S.S.V.....L.....E.....L.....R.....L.....E--R--D..R.
<i>Oreochromis niloticus</i>	.....L.....R.....L.....
<i>Mus musculus</i>	.....V.....S.S.V.....L.....E.....L.....R.....L.....E--R--D..R.
<i>Meleagris gallopavo</i>	.....I.....S.L.....L.....E.....L.....R.....S.L.T.....-..RE..RE..L.
<i>Homo sapiens</i>	.....V.....S.S.V.....L.....E.....L.....R.....L.....E--R--D..R.
<i>Epinephelus coioides</i>	.....R.....A.....
<i>Danio rerio</i>	.....V.....S.....K.....L.....E.....R.....E.H.Q.N.....
<i>Anas platyrhynchos</i>	.....I.....S.L.....L.....E.....L.....R.....S.L.T.....-..RE..RD..R.
<i>Bos taurus</i>	.....V.....S.S.V.....L.....E.....L.....R.....L.....E--R--D..R.

**Figure 3:** Protein sequence comparison of myogenin from 16 vertebrates. The highly conserved basic helix-loop-helix domains are underlined, and the basic regions are marked in square box. The conserved residues are indicated by dots. The Gene bank accession number for the selected vertebrate myogenin proteins are: *Epinephelus coioides* (HM190251), *Sparus aurata* (EF462192), *Oreochromis niloticus* (GU246725), *Paralichthys olivaceus* (EF144128), *Takifugu rubripes* (AY566282), *Danio rerio* (CAQ14920), *Xenopus (Silurana) tropicalis* (CAJ82458), *Taeniopygia guttata* (XP\_002195870), *Salmo salar* (NP\_001117072), *Pelodiscus sinensis* (BAJ53267), *Oryctolagus cuniculus* (ACN53836), *Mus musculus* (NP\_112466), *Meleagris gallopavo* (AAT39143), *Anas platyrhynchos* (ADG85647), *Bos Taurus* (BAE93440), and *Homo sapiens* (NP\_002470).

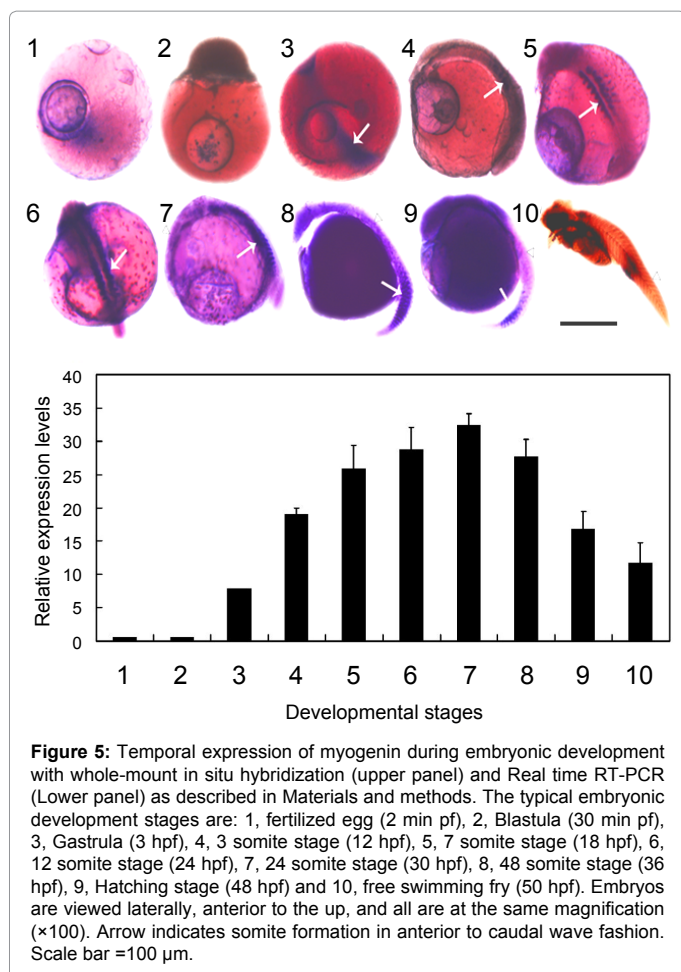


**Figure 4:** Phylogenetic analysis of the *S. chuatsi* myogenin gene sequence relative to myogenin genes from other vertebrates. The deduced protein sequences were searched from Gene bank same as in Figure 3 and the phylogenetic tree was constructed with the neighbor-joining method in MEGA version 3.0 based on Poisson-corrected pairwise distances between protein sequences. Note *S. chuatsi*, *Epinephelus coioides*, *Sparus aurata* and *Takifugu rubripes* are in the same branch.

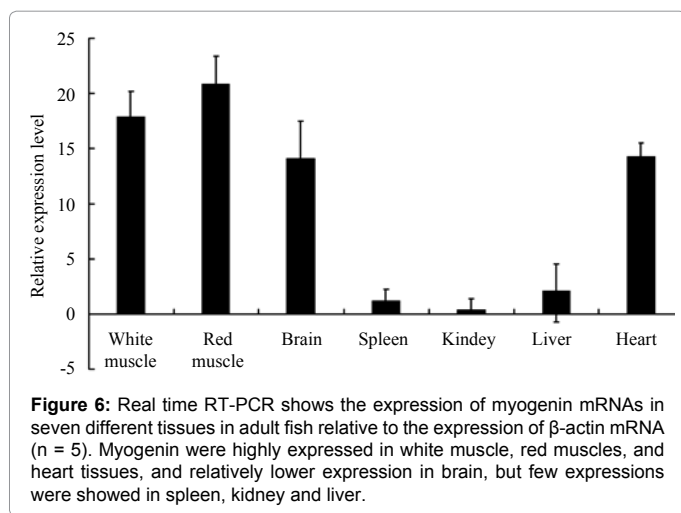
At the free-swimming fry, (after hatching about 12 hours), few of myogenin expression signal was detected (Figure 5 up panel 10). The developmental profile of myogenin in *S. chuatsi* was then confirmed by RT-qPCR (Figure 5, lower panel). The quantitative results of *S. chuatsi* myogenin expression during developmental stages were basically consistent with those observed in whole-mount *in situ* staining (Figure 5).

To quantify the spatial expression pattern in *S. chuatsi* adult fish, the myogenin mRNA levels were assayed by real-time PCR in seven tissues





**Figure 5:** Temporal expression of myogenin during embryonic development with whole-mount in situ hybridization (upper panel) and Real time RT-PCR (Lower panel) as described in Materials and methods. The typical embryonic development stages are: 1, fertilized egg (2 min pf), 2, Blastula (30 min pf), 3, Gastrula (3 hpf), 4, 3 somite stage (12 hpf), 5, 7 somite stage (18 hpf), 6, 12 somite stage (24 hpf), 7, 24 somite stage (30 hpf), 8, 48 somite stage (36 hpf), 9, Hatching stage (48 hpf) and 10, free swimming fry (50 hpf). Embryos are viewed laterally, anterior to the up, and all are at the same magnification ( $\times 100$ ). Arrow indicates somite formation in anterior to caudal wave fashion. Scale bar = 100  $\mu$ m.



**Figure 6:** Real time RT-PCR shows the expression of myogenin mRNAs in seven different tissues in adult fish relative to the expression of  $\beta$ -actin mRNA ( $n = 5$ ). Myogenin were highly expressed in white muscle, red muscles, and heart tissues, and relatively lower expression in brain, but few expressions were showed in spleen, kidney and liver.

including the white muscle, red muscle, kidney, spleen, liver, brain and heart. As showing in Figure 6, myogenin was strongly expressed in the four tissues, red muscle, white muscle, heart and brain, but almost no expression was detected in spleen, kidney and liver tissues.

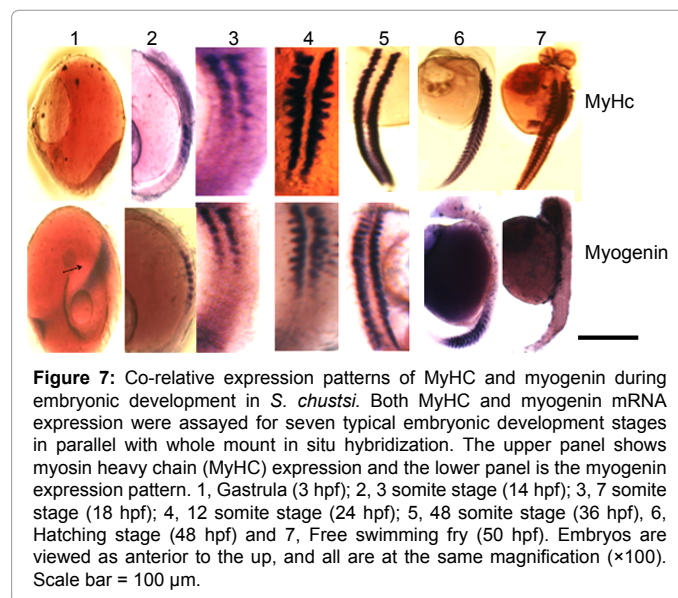
### Correlative expression between myogenin with myosin heavy chain gene

Earlier reports demonstrated that myosin structures genes,

myosin heavy chain (MyHC) and myosin light chain (My LC) genes, are definitive molecular markers of slow and fast twitch fates during fish myotome development [27,28]. To investigate how the muscle structural gene, MyHC, and myogenin, are concomitantly expressed during *S. chuatsi* embryonic development, both MyHC and myogenin mRNA expression were assayed with whole-mount in situ hybridization. As shown in Figure 7, myogenin mRNA expression signals were firstly detected at gastrula stages, but no signal was identify for MyHC gene in this stage (Figure 7, 1 up panel). Both myogenin and MyHC expression were correlatively and strongly expressed until 24 somatic stages (Figure 7, 3-5). After the 24 somatic stages, the MyHC expression was persistent in all late stages as well as in hatched free-swimming fries (Figure 7, 6-7). However, myogenin expression was only at the caudal region, and almost very weak mRNA signal was observed at the free-swimming fry (Figure 7, 6-7).

### Discussion

In the present study, we isolated the *S. chuatsi* myogenin gene and determined its nucleotide and inferred amino acid sequences, as well as its temporal and spatial expression profile during embryonic development and in different tissues. To our knowledge, this is the first



**Figure 7:** Co-relative expression patterns of MyHC and myogenin during embryonic development in *S. chuatsi*. Both MyHC and myogenin mRNA expression were assayed for seven typical embryonic development stages in parallel with whole mount in situ hybridization. The upper panel shows myosin heavy chain (MyHC) expression and the lower panel is the myogenin expression pattern. 1, Gastrula (3 hpf); 2, 3 somite stage (14 hpf); 3, 7 somite stage (18 hpf); 4, 12 somite stage (24 hpf); 5, 48 somite stage (36 hpf), 6, Hatching stage (48 hpf) and 7, Free swimming fry (50 hpf). Embryos are viewed as anterior to the up, and all are at the same magnification ( $\times 100$ ). Scale bar = 100  $\mu$ m.

Primer name	Sequence(5'-3')	Information
MG1	ATGGAGCTTTTCGAGACC	The exon1 and intron1 amplification
MG2	TATTTGGGAATGTCCACAGG	The exon1 and intron1 amplification
MG3	GGCCTTAAAGCCCCTAGAG	The 5'-flanking region amplification
MG4	GGAAGAAGTAAGGGTTGGT	The 5'-flanking region amplification
MG5	CTGTGGACATTCCCAAGTAG	The 3'-flanking region amplification
MG6	AGCATTAGAAACACGCACAAA	The 3'-flanking region amplification
MG7	CTATTTGGGAATGTCCACAGG	The myogenin cDNA amplification
MG8	ACCAACCCTTACTTCTTCCCT	Whole-mount <i>in situ</i> hybridization
MG9	TCCCTGCTGTCCCGTCTC	Whole-mount <i>in situ</i> hybridization
MG10	GCACCCTGATGAACCCAA	Quantitative real-time PCR assay
MG11	CTCGCTTGATGACGACACTC	Quantitative real-time PCR assay
Actin1	CCTTCTGGGTATGGAGTC	Quantitative real-time PCR assay
Actin2	GTCAGCAATGCCAGGGTA	Quantitative real-time PCR assay

**Table 1:** Primers used for myogenin gene isolation, whole-mount in situ hybridization and quantitative real-time PCRs in *Siniperca chuatsi*.

myogenin gene identified and characterized in the Chinese mandarin fish. Sequence BLAST analysis indicated that the obtained *S. chuatsi* myogenin had a high similarity with that of other vertebrates; especially it had more than 90% sequence identity with myogenin from *Epinephelus coioides*, *Sparus aurata* and *Takifugu rubripes*. The *S. chuatsi* myogenin gene contains 3 exons and 2 introns, which has identical gene structure compared to the myogenin gene from the channel catfish, zebra fish and humans [13,16,29]. However, the myogenin gene in *S. chuatsi* encoded a protein with 250 AA, compared to 253 AA in channel catfish (Gene bank accession number: AY534327), 256 AA in zebra fish (Gene bank accession number: AF202639) and 225 AA in humans (Gene bank accession number: AF050501).

The myogenin promoter sequence was highly conserved in vertebrates and it contained two putative E-box sites, one MEF2 and one MEF3 binding sites [13,14,17]. Du et al. [13] reported that in zebra fish, myogenin expression is controlled by the cooperation of E-box, MEF2 and MEF3 elements. Deletion analysis revealed that the muscle specific activity depends on the presence of both MEF2 and MEF3 binding sites within the 550 bp myogenin promoter sequence. Durr et al. [30] reported that the two promoter-proximal E-box elements could enhance promoter activity in muscle and mediate the trans-activation by myogenic factors, but no enhancer activity could be achieved by a single E-Box. Mutations in the myogenin promoter that abolish binding sites for myogenic HLH proteins or myocyte enhancer factor-2 (MEF-2) suppressed transcription of a linked lac Z transgene in subsets of myogenic precursors in mouse embryos [31]. In the present study, we reported here the *S. chuatsi* myogenin contained similar upstream cis-acting elements. It remains to be determined whether all of the elements are required for the muscle specific expression and whether they function in co-operation or independently in controlling myogenin expression.

Myogenin is a member of the MyoD family of transcription factors that plays a central role in muscle cell differentiation and activation of muscle specific gene expression [5,29]. Comparison of the *S. chuatsi* myogenin protein sequence with 16 other vertebrate myogenin proteins revealed that the myogenin protein sequences are highly conserved from lower to higher vertebrates. At the conserved bHLH domain, the *S. chuatsi* shares 100% homology with that of *Paralichthys olivaceus*, *Epinephelus coioides* and *Sparus aurata*, and has only one amino acid difference to those of *Takifugu rubripes*, *Salmo salar* and *Oreochromis niloticus*. The bHLH domain provides a basic cascade for ubiquitous bHLH protein binding to form complexes that bind to DNA sequence E-box, and to activate muscle-specific transcription [32]. The myogenin protein sequence alignment and phylogenetic analysis indicated that the *S. chuatsi* myogenin shared the highest identity with *Paralichthys olivaceus*, *Epinephelus coioides* and *Sparus aurata*, but it had a relatively lower identity to *Meleagris gallopavo*, *Pelodiscus sinensis*, *Xenopus (Silurana) tropicalis*, *Mus musculus* and *Homo sapiens*, respectively. The data suggested that vertebrate myogenin genes are highly conserved during evolution, and the high level of sequence conservation suggests a high evolutionary constraint and the importance of its function in vertebrates [7,33].

The four member of the MyoD family of transcription factors are concomitantly expressed and control muscle cell determination and differentiation. Generally the MyoD and Myf5 are expressed first in pre-somitic cells and developing somites and play redundant roles in myoblast determination, while myogenin and MRF4 are expressed later and involved in myoblast differentiation [9]. In the present study, we applied the whole-mount *in situ* hybridization and RT-qPCR

techniques to assay the expression profile of the *S. chuatsi* myogenin during embryonic development. Our results demonstrated that the myogenin mRNA expression was first identified in early gastrula stage embryos, and increased significantly during somitogenesis. At about 24 somite stage, its expression was decreased in the anterior somites, but the posterior somites still showed a strong expression (Figure 5). At the free-swimming stages, few of myogenin expression signals could be detected. Our observation is consistent to those from the flounder (*Paralichthys olivaceus*) and the sea bream (*Sparus aurata*) [17] but appeared to be different with the rainbow trout (*Oncorhynchus mykiss*), in which myogenin expression remained relatively high at later spawning stage [34]. The difference may be explained by species specificity. Another question raised from our observation is why myogenin expression first decreases in anterior somites but not in somites at the caudal region after 24 somite formation. We suggest that myogenin is an activator for muscle cell differentiation but not a structural component for somite formation. It is known that myogenesis in developing somites occurs in an anterior to posterior wave fashion. Myogenin expression is expected to decrease after completing its function in myogenic differentiation in the anterior somites.

To determine the expression pattern of myogenin in adult fish, we analyzed myogenin expression in seven tissues by Real-time PCR. Xu et al. [17] reported that the myogenin was detected only in skeletal muscle in the adult flounder by RT-PCR. However, in our study the *S. chuatsi* myogenin was not only detected in white and red muscle, but also in heart and brain, but almost no expression was detected in spleen, kidney and liver. Furthermore, the myogenin gene showed the highest levels of expression in the brain compared with other tissues. This pattern of diverse expression in different tissues suggests that the biological actions of myogenin in *S. chuatsi* might not just be restricted to skeletal muscles, it may play a role in other tissues as well. We suggest that myogenin is an activator for muscle cell differentiation but not a structural component for somite formation. Thus, its expression is only accompanying with newly developing muscle somites. To confirm this hypothesis, we conducted a parallel whole-mount *in situ* hybridization with both myosin heavy chain (MyHC) and myogenin and the results clearly supported the hypothesis. During the *S. chuatsi* somatic development, the MyHC mRNA showed a strong expression in all developmental stages, but the myogenin is accompanying with newly developing somites with an anterior to caudal wave pattern (Figure 7).

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