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Research Article

Cloning and Expression Analysis of Skeletal Myosin Heavy Chain (MYHs) Gene from the Most Famous Freshwater Fishes in China-*Culter alburnus*

[1]Kun Wang, [2]Ying Han, [2]XiaoTing Chen, [2]RongWei Zhao and [2]QiRui Hao
[1]Harbin University of Commerce, Haerbin, 150028, China
[2]College of Animal Science and Technology, Northeast Agricultural University, Harbin, China

Abstract: *Culter alburnus*, one of the four most famous freshwater fishes, is a very important freshwater fishing species with high economic value in China. The present study focused on the myosin, major protein in skeletal muscles from *Culter alburnus* in Xingkai Lake. Two types of the gene encoding Myosin Heavy Chain (MYH), a large subunit of the myosin molecule, were cloned from fast skeletal muscle and defined as MYHa (Genbank ID JX272926) and MYHb (Gen bank ID JX402919). The full-length cDNA clones of MYHa and MYHb consisted of 6003 and 5990 bp, which encoded 1933 and 1930 amino acids, respectively. The total levels of the MYHs were significantly higher with the fish age increase. In comparison to the wild and cultured muscles, From 2 to 6 years old, MYHa gene expression of wild population was significantly lower than the cultured population ($p<0.05$), else MYHb gene expression of wild population was higher than cultured population. The two kinds of genotype interaction affect meat quality traits. The present study has therefore, revealed a complex pattern of expression of MYH genes in relation to developmental stage and population. Our work provided a novel myosin heavy chain gene sequence in fish biology and the results indicate that the MYH gene and the protein it encodes are important for the growth and development of fish, as well as its muscle characterization.

Keywords: cDNA cloning, *Culter alburnus* in Xingkai Lake, expression, myosin heavy chain, wild and cultured population

INTRODUCTION

Muscle fiber is a basic unit for constructing muscle and its molecular diversity and composition has direct impacts on muscle growth and meat quality. Myosin amounts to about over 50% of myofibrillar protein in fish skeletal muscles. It is a ubiquitous eukaryotic motor protein that interacts with actin to generate the force for cellular movements ranging from cytokinesis to muscle contraction (Weiss and Leinwand, 1996). Conventional myosin exists as a hexameric protein consisting of two Myosin Heavy chain (MYH) subunits (200 kDa each) and two pairs of non-identical light chain subunits (17-23 kDa). MYH provides both the motor and filament-forming functions and can be divided into two functional domains: a globular, amino-terminal head domain, which contains the motor function and an elongated α-helical coiled-coil carboxyl rod domain, which contains filament-forming properties (Craig and Woodhead, 2006). Therefore, it is reasonably predicted that myosin properties affect the quality of muscle in animals to be consumed as protein resources (Watabe and Ikeda, 2006).

It has been reported that myosin types comprising 24 classes, including a conventional type of class II that contains sarcomeric myosins driving muscle contraction (Warshaw, 2004; Foth *et al*., 2006). It exists as a variety of isoforms adapted to function in individual muscles according to physiological requirements during the development. These isoforms are evolved from primordial Myosin Heavy chain genes (MYHs). Variation in regulation and amino acid sequence among members of the sarcomeric MYH multigene family contributes strongly to the variation in muscle properties, which is important to the performance (Schiaffino and Reggiani, 1996). During muscle fiber development, each MYH shows a complex expression pattern that continues throughout life cycle (Johnston *et al*., 1998; Mascarello *et al*., 1995). More diverse sarcomeric MYHs have been found in fast muscle of fish, such as common carp *Cyprinus carpio* (Kikuchi *et al*., 1999; Muramatsu-Uno *et al*., 2005), medaka *Oryzias latipes* (Liang *et al*., 2007) and torafugu *Takifugu rubripes* (Ikeda *et al*., 2004; Watabe and Ikeda, 2006; Ikeda *et al*., 2007), not only at the genome level, but also at the transcription level. Besides such industrial demands, the expression of skeletal myosin isoforms is closely associated with muscle development and recruitment of muscle fibers and possibly nervous fibers as well. Thus it is important to characterize skeletal myosin isoforms both in the viewpoints of fundamental and practical purposes.

Corresponding Author: Ying Han, College of Animal Science and Technology, Northeast Agricultural University, 59 Mu-Cai Street, Xiang-Fang Region, Harbin 150030, China, Tel.: 13936621841

The topmouth Culter, *Culter alburnus* (Basilewsky), belongs to Cypriniformes, Cyprinidae, Culter and is widely distributed in big rivers, reservoirs and lakes in China. The culture production has greatly increased over the past decades, accounting for about 1500 tons annually. *Topmouth culter* in Xingkai Lake has already become one of the most important commercial freshwater fishes in Jixi City. Earlier studies were only focused on the biological characteristics and population structure of its closely relevant carp species (Xie *et al*., 2003). In our previous study, the nutrition analysis results showed that wild population had a better nutritional value than cultured population and; the difference increased with the age too (Wang *et al*., 2012). A higher number of MYHs expressed in fish is the result of many environmental factors such as water temperature and physiological requirements at different developmental stages, thus resulting in changes of the composition of muscle-fiber type (Hirayama and Watabe, 1997; Liang *et al*., 2007; Watabe, 2002). There is no molecular biotechnology studies aimed directly to compare wild and cultured population gene expression at the same time. In this study, different types of *Culter alburnus* (MYHs) were cloned from skeletal muscles of adult fish and their abundances were determined in both muscles from the fishes with different ages. The purpose of the present study was to analyze MYHs gene expression and MYHs content on paralleled wild and cultured populations at the same time, in an attempt to detect the meat quality which might be related to the quality decline in the cultured population.

MATERIALS AND RESEARCH METHODS

Fish samples: A total of 58 *Culter alburnus* (age class from 2 to 6 years old) were sampled from 2010-2012 in the Xingkai Lake (between 44°44′-45°24′ N. and 130°59′-132°51′ E., Heilongjiang Province, China). The age was estimated using the FL-age key reported by Santamaria *et al*. (2009). During the same period, the cultured *Culter alburnus* (from 2 to 6 years old) were randomly sampled from Nongken Zhenda Xingkai Lake Large White Fish Research Institute. The farm has the longest history in culturing *Culter alburnus* in the region. During the sampling, muscles were collected in 1.5 mL plastic tubes soon after fish death for molecular biology. Muscles samples destined for molecular biology studies were first stored in the liquid nitrogen until they were transferred to the lab and stored at -80°C.

First strand cDNA: Total RNAs were extracted from the dorsal fast skeletal muscles of *Culter alburnus* in Xingkai Lake. First strand cDNA was synthesized with the first-strand kit PCR for amplification using primer, which was designed with reference to nucleotide sequences of MYHs from *Hypophthalmichthys molitrix* (Fukushima *et al*., 2009). Specific first strand cDNA was synthesized and performed with 3′, 5′-RACE rapid amplification kit (Takara Biotechnology, Dalian, China). The most conserved regions were chosen for primer design. Approximately 5 μg RNA was reversely transcribed by Power Script™ reverse transcriptase with Coding Sequences (CDS). Thirty cycles were performed for PCR using an Ex Taq DNA polymerase (TranGen Biotechnology, Beijing, China). Every cycle consisted of denaturation at 94°C for 30 sec, annealing at 55-58°C for 30 sec and extension at 72°C for 1 min. The reaction mixture for PCR was shown as follows: each of the forward and reverse primers (20 pmol), 1 μg first strand cDNA as a template, 20 nmol dNTP mixture, 10 μL 10×Ex Taq buffer (Takara) and 1 U Ex

Table 1: Nucleotide sequence of primers for determination of 3′end, full-length and 5′end of *Culteralburnus* in Xingkai Lake myosin heavy chain genes

Primer name	Sequence (5’-3’)	Primer name	Sequence (5’-3’)
M1-1F	CTACGGTGACTTCAAGCAGAGAT	M3-1F	GGAGCTTAAGAGGCAGATTGAA
M1-1R	CCAGGTCATCAATGTCTTTCTTC	M3-1R	CTCTCTGGCTGTTCCTCTTGAT
M1-2F	TCGAGATGAATAAGAAGCGTGA	M3-2F	ACGTCAAGGGAACCATCAAG
M1-2R	TCTCCACATCAATCATGAGGTC	M3-2R	TCAAGAGAGCCCTGCATTTT
M1-3F	AGAGCAATCATGGGAGATGG	M3-3F	CCAGAGGCAAACAAGCTTTC
M1-3R	GACAGGTTCTGCCTTCTTCG	M3-3R	CGGTCTGAATCTCTGCCTTC
M1-4F	CGAAGAAGGCAGAACCTGTC	M3-4F	CTGAGCTGGGAGAGCAAATC
M1-4R	CACCATCTCATTTCCGACCT	M3-4R	GCCTCAACTTGTTCCTCTGC
M1-5F	GGCCAGACAGTACCACAGGT	M3-5F	CAAGAATCCGTCATGGACCT
M1-5R	GGAAGGAACCACCCTTCTTC	M3-5R	GAAAGCTTGTTTGCCTCTGG
M1-6F	CGAGAGACTGGAGGATGAGG	M3-6F	ATCGCCTACCTTCTGGGTTT
M1-6R	GGAATTCAGCTTCACGCTTC	M3-6R	TCTGGAAAGCATTGCACTTG
M1-7F	CCCGTCATGACTGTGATTTG	M3-7F	TCACTACGCTGGAACTGTGG
M1-7R	CCTCATCCTTCTCAGCAAGC	M3-7R	ACTCCCTCCTCTCCGTCATT
M1-8F	TGCACCTTGATGATGCTCTC	M3-8F	TCTTGAGGAGATGCGTGATG
M1-8R	CAGCTTGTTGACCTGGGATT	M3-8R	CCTGGAGGGCTTTCTTCTCT
M1-9F	TGAGACCCTGAAGAGGGAGA	M3-9F	GGCACATCAGCAGACACT
M1-9R	TGTTTGCTCCAGTGCAGTTC	M3-9R	GCTTCACGCTTCTTATTC
M1-10F	TGATGCCATCCAACG	M3-10F	GCAGGACACCAGTTC
M1-10R	CTGCTCCGCCACTTT	M3-10R	GCATCACGGCTCTTA
M1-5’out R	AAGCCCAGAATGTCAATGGCAGTAT	M3-5’outR	ACTCCCAAACAATGCCCTCCT
M1-5’in R	CTGCCCTTCTTCGGTCCAGACATTG	M3-5’in R	CTGGTTCTCACGCTGCTTCTGTTTA
M3-3’out F	GAACAGATCAAGAGGAACAGCC	M3-3’outF	CGTTCAGGGACAACTCAAGGA
M3-3’in F	TCTCAGAGGACAGGAGGACAT	M3-3’in F	AGCAGGACACCAGTTCTCACC

Taq DNA polymerase. The final volume was adjusted to 100 µL with sterilized water.

The products were isolated using the Gel Extraction Kit (Takara Biotechnology, Dalian, China), cloned into the pMD18-T vector (Takara Biotechnology, Dalian, China) and transformed into Escherichia coli strain DH5α-competent cells according to the manufacturer's instructions. Putative clones were screened by PCR using the above primers under the same cycle conditions and the selected clones were sequenced using the dideoxy chain-termination method on an automatic 3730xl DNA Analyzers (ABI, Foster City, CA, USA). To obtain the full-length cDNA sequence, 5′ RACE and 3′ RACE were performed using the gene specific primers and adaptor Primers (UPM) listed in Table 1. The PCR cycling conditions were 1 cycle at 94°C for 3 min; 20 cycles at 94°C for 30 sec, 55°C for 40 sec and 72°C for 60 sec for outer PCR; 25 cycles at 94°C for 30 sec, 56°C for 40 sec and 72°C for 60 sec for inner PCR; followed by 1 cycle at 72°C for 10 min. The purified fragments were then cloned and sequenced.

Sequence analysis: The Molecular weight (Mw) and the Isoelectric point (pI) were predicted using the Compute pI/Mw tool (Gasteiger *et al*., 2005). Using the PBILLYON-GERLAND information database for protein Secondary structure prediction sequences, mainly Hopfield Neural Network (HNN) (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). The amino acid sequences were aligned using the Clustal W program (Thompson *et al*., 1994). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei, 1987). One hundred bootstrap analyses were performed for the phylogenetic analysis.

Real-time PCR: To examine the MYH expression pattern in muscles at different ages, cDNA of pooled samples from five wild and cultured fishes were used as a template for RT-PCR analysis, using 96 micro well plates and an ABI Prism 7500 Sequence Detector System (Applied Bio systems) according to the manufacturer's instructions. Total RNA from muscle (2.5 g) was reverse-transcribed as described previously. Therefore, the RNA was amplified by the regular PCR with β-actin primers in order to confirm that the isolated RNA was free of DNA. The cDNA sequences obtained in the present study were used as a template for MYH primers (Table 2). Several primers were then designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3) for the target genes. To evaluate PCR efficiency, uniformity and linear dynamic range of each real-time PCR assay, a standard curve for each gene of interest was constructed using serial dilutions of pooled cDNAs (1:1; 1:10; 1:100; 1:1000; 1:10,000) with nuclease-free water. The amplification Efficiency (E) was calculated using the following equation: $E = (10^{-1/slope})-1$. Reaction efficiencies for all genes were greater than 90%. Transcription was performed in a 20 µL volume reaction, contained SYBR Premix Ex TaqII 10 uL, ROX Reference Dye or Dye II 0.4 uL, 0.8 uL (final concentration 10 M) of each forward/reverse primer set and 8 uL of diluted (1:3) cDNA template. Samples were heated for 10 min at 95°C and then underwent 40 cycles, each one consisting of 15 sec at 95°C and 1 min at 60°C. The quantification of the β-actin gene was used as endogenous control for the normalization of initial amounts of RNA. PCR reactions were performed in triplicate. Blank reagent (negative control) was prepared with RNase-free water instead of the template (cDNA). The method, a procedure allowing a direct comparison of relative gene expression values between samples in different conditions, was used to define relative gene expression profiles in relation to a fixed internal reference or housekeeping gene.

Table 2: Primers for relative muscles MYH levels analysis by real-time PCR

Primer name	Sequence (5'-3')	Amplicon length (bp)
β-actin	F: ACTTCGAGCAGGAGAT	150
	R: ACAGTGTTGGCATACAG	150
MYHa	F: TGGAGGCTTATGGATTCACTT	116
	R: GGTAGCTCCTCTCAGCAGAC	116
MYHb	F: CTTGAGGAGATGCGTGATGAG	147
	R: GAATGAGCGGATGTTGTATTGG	147

Statistical analysis: Different wild versus cultured at same time examined using a t-test with a significance level of pb 0.05. Differences among MYHs levels were examined using a one-way ANOVA, followed by Student-Newman-Keuls (SNK) multiple comparison procedure, with a significance level of pb 0.05.

RESULTS

cDNA clones of MYHs: First strand cDNA was prepared from dorsal fast skeletal muscles of *Culter alburnus* from age 2 to 6. As a result, two DNA fragments of 453 and 516 bp were amplified by 3'RACE PCR with primers, 532 and 588 bp were amplified by 5'RACE PCR with primers (Table 1). The nucleotide sequences of the 3' regions of MYH clones indicated that there were two types of MYHs, subsequently designated MyHC fast type 1 (MYHa) and MyHC fast type b (MYHb). PCR performed with primer sets of primers yielded the amplified products of MYHa and MYHb clones of 5018 and 4886 bp long, respectively. So the full-length cDNAs encoding MYHa (Genbank ID JX272926) and MYHb (Genbank ID JX402919) were 6003 and 5990 bp long, respectively. The open reading frame of MYHa and MYHb were 5802 and 5893 bp long and encodes a peptide of 1933 and 1930 amino acids, respectively (Fig. 1). The molecular weight of the deduced protein is about 221.2 and 221.6 k, pI was 5.61 and 5.58,

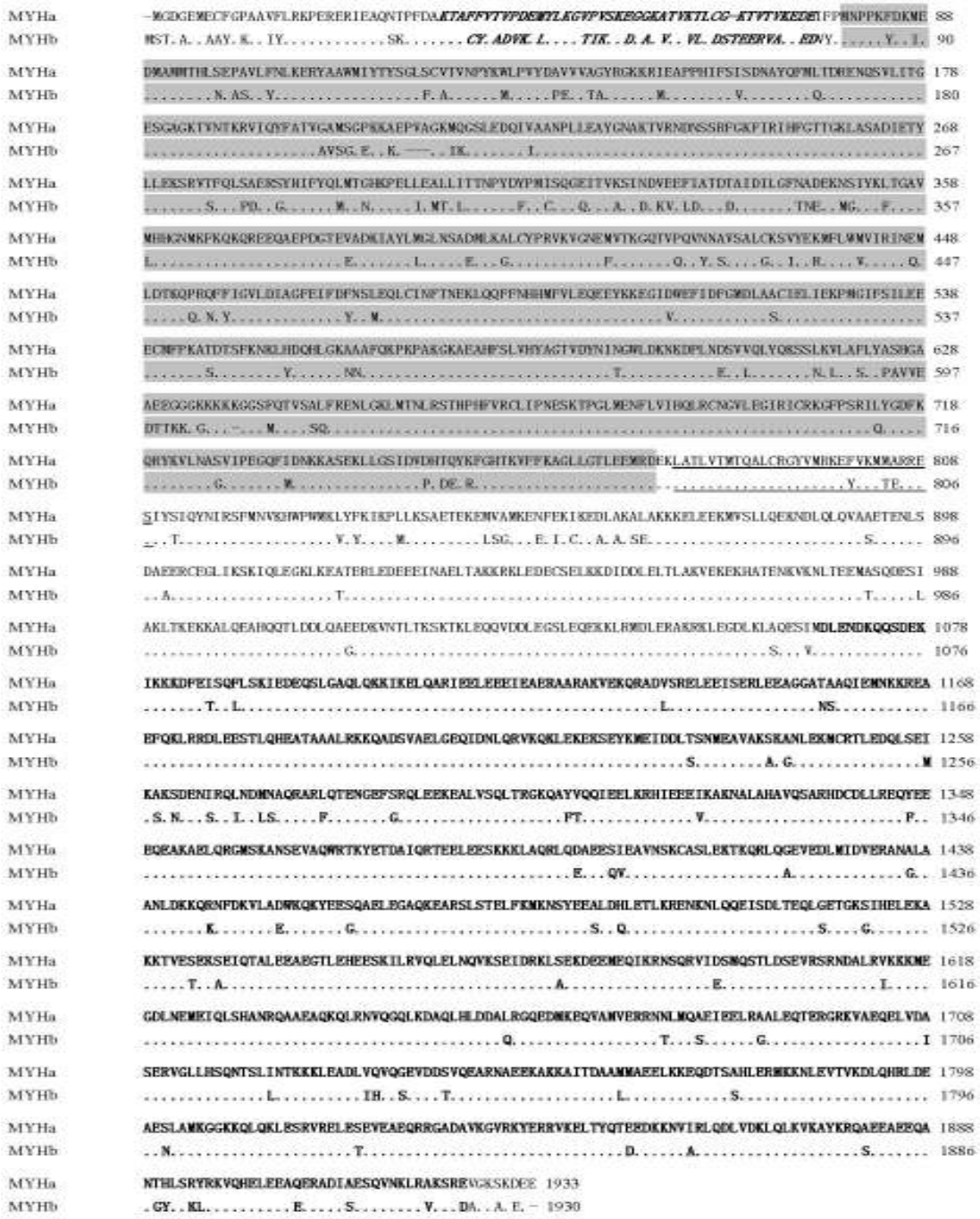

Fig. 1: The full-length deduced amino acid sequences of myosin heavy chains of *Culter alburnus* in Xingkai Lake

respectively by ExPASY software on line. A search of the encoded protein sequence in the NCBI conserved domain database indicated the presence of all known protein domains for MYH protein, i.e., the myosin motor domain in N-terminal region, the DIL domain in the C-terminal region and the chromosome segregation domain, an actin-binding site between the two domains (Fig. 1).

Amino acid residues in MYHa identical to those in MYHb are indicated by dots and hyphens denote deletions. Regions highlighted in gray, bold, underlined and in bold italics indicate the location of MYSc, myosin tail 1, IQ and myosin-N domain, respectively.

Homology and phylogenetic analysis of MYHs: The degree of conservation for the *Culter alburnus* MYHs with those from other fish species and animals was tested by DANMAN software analysis. The amino acid composition of the *Culter alburnus* MYHs showed a high conservation with *Hypophthalmichthys molitrix*.

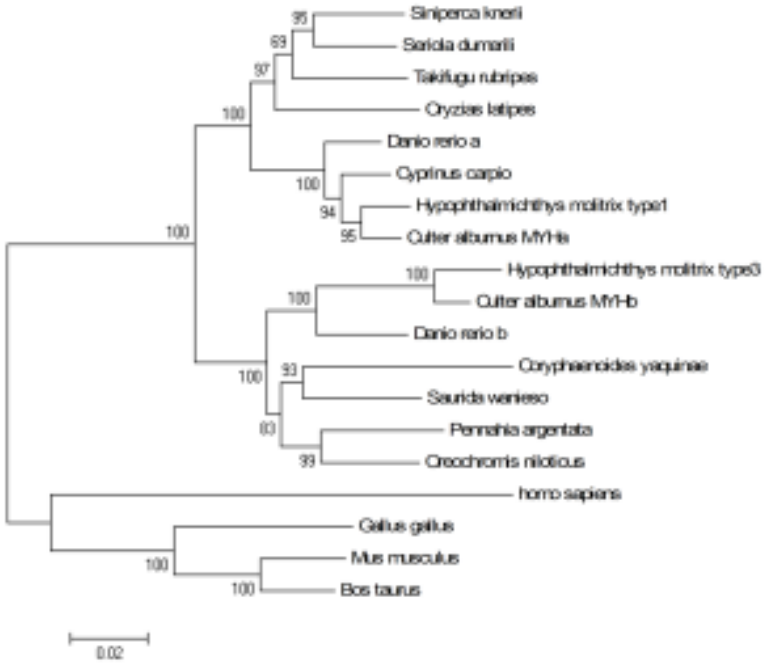

Fig. 2: Phylogenetic tree constructed by neighbour-joining method using the complete amino acid sequences for fast myosin heavy chain from various teleosts and higher animal. Complete amino acid sequences of MYHs were deduced from cDNA nucleotide sequences with accession numbers in the NCBI GenBank databases for *Homo sapiens* (AAD 29948.1); *Musmusculus* (NP 109604.1); *Bostaurus* (NP 77654.1); *Gallus gallus* (AAB 47555.1); *Daniorerio* (XP 001924009.1); *Pennahia argentata* (BAB12571.1); *Siniperca knerii* (ABO31103.1); *Oryziaslatipes* mMYH1 (BAF34701.1); *Serioladumerili* (BAA92289.1); *Coryphaenoidesyaquina* (BAG16351.1); *Hypophthalmichthys molitrix* type1 (BAF93222.1); *Hypophthalmi chthysmolitrix* type3 (BAF93224.1); *Takifugurubripes* (BAH56385.1); *Saurida wanieso* (BAF49658.1); *Oreochromis niloticus* (XP003439494.1); *Cyprinuscarpio* (Q90339.2); *Daniorerio* (XP_002667379.1)

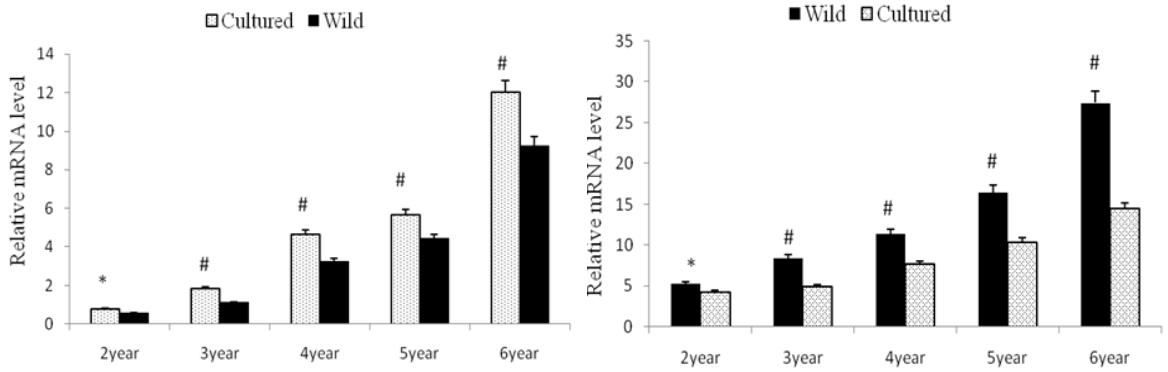

Fig. 3: Comparison of MYHa (a) and MYHb (b) mRNA level in wild and cultured population of *Culter alburnus* in Xingkai Lake
*: Significantly difference ($p<0.05$); #: Significantly difference extremely ($p<0.01$); The following figures are the same

fast skeletal MyHC, as well as *Danio rerio* and *Cyprinus carpio* MYHs from the higher vertebrates such as the MYHs isoform from the chicken fast MYHs family and mammalian fast skeleton MYHs isoforms The Neighbor-Joining (NJ) tree based on deduced amino acid sequences in domains of MYHs (Fig. 2). This tree demonstrated that the two *Culter alburnus* MYHs were divided into two clades. The two MYHs formed one clade with the other fishes, clearly different from the higher vertebrates.

Expression of MYHs: To validate the assembled cDNA sequence, RT-PCR was conducted using primers flanking the ORF. When the extracted intestinal RNA sample was examined on a UV spectrometer, the readings was ranged between 1.8-2.0 for $OD_{260/280}$,

which indicated that the RNA had a high purity and could be used for RT-PCR. The most interesting result to be found was the significant expression of this fast skeletal MYHs transcript during early myogenesis and all stages of muscle growth studied. Furthermore, this MYHs cDNA was present in both the two cDNA libraries. The level trend of MYHa and MYHb gene from *Culter alburnus* in Xingkai Lake were consistent (Fig. 3). Total levels of the MYHs were significantly higher with the age increase. From 2 to 6 years old, MYHa gene expression of wild population was significantly lower than the cultured population ($p<0.05$), else MYHb gene expression of wild population was higher than cultured population. The two kinds of genotype interaction affect meat quality traits.

DISCUSSION

In fish different MyHCs are expressed in skeletal muscle which is composed of different fibre types (Gauvry and Fauconneau, 1996). In the present study, we first applied cDNA cloning and 3', 5' RACE techniques to clone the MYHs gene from *Culter alburnus* in Xingkai Lake. Two types of myosin heavy chain isoforms from skeletal muscles had been isolated from cDNA clones, which encoded 1933 and 1930 amino acids, respectively. Furthermore, there were only two divergent nucleotides in the 3' un-translated region and the end of the coding region which was known to be different in genes expressed sequentially during development. Therefore, the two MYHs could be isomorphous isomer of the same gene rather than a duplicated gene expressed in the same muscle. A comparison of the deduced amino acid sequences indicated that the two MYHs were the fast type. They showed 98 and 97% similarity in sequence to a fast skeletal muscle MYH type1 and type3 mRNA from *Hypophthalmichthys molitrix*. The high degree of identity between the two cDNA sequences can be expected, as the two fish species belong to the same genes.

For conventional myosin (Class II), the N-terminal portion of MYH forms a globular head including actin and ATP-binding sites, where as the C-termimal portion of the two MYH associate to form a coil-coil rod that is involve in filament formation (Zhang *et al*., 2009). In this study, the myosin motor domain in N-terminal region, the DIL domain in the C-terminal region and the chromosome segregation domain, an actin-binding site between the two domains. Further studies are needed on the subfragment-1 loop region of the myosin molecule, because this region is hyper variable and largely associated with functional and structural relation-ships of myosin heavy chain isoforms (Hirayama *et al*., 2000).

MYH gene of mammals and fish are clustered in corresponding groups, MYHa and MYHb are clustered in respective groups, MYHa of *Culter alburnus* is clustered with MYHa type1 of *Hypophthalmichthys molitrix*, MYH of carp and MYHa of *Danio rerio*, MYHb of *Culter alburnus* is clustered with MYHa type 3 of *Hypophthalmichthys molitrix* and *Danio rerio*, then are clustered as MYH group together with other fish. The research result about MYH gene showed that in different taxonomic status of fish, the amino acid homology of MYH is very high, which suggest that the MYH is conservative during evolutionary process, the study on MYH gene of fish is helpful to describe the molecular mechanism of fish muscle fiber and meat quality genes.

Identifying the expression law is an important way to study gene function. Regulation of myosin isoforms expression at the level of gene expression presumably employs the same mechanisms in fish as in higher vertebrates (Nihei *et al*., 2006). However, differences in other aspects of myogenesis such as myosin isoforms expression in the different stages and the population variations are of interest because they reflect variations in biological mechanisms and may be of practical importance in aquaculture. Finally, the implication of the continuous expression of this MYHs gene during development could be analyzed taking into account the hyperplasic growth of fish muscle. The persistence of this early MYHs isoform in growing fish muscle could be related to this process. From 2 to 6 years old, MYHa gene expression of wild population was significantly lower than the cultured population ($p<0.05$), else MYHb gene expression of wild population was higher than cultured population. The two kinds of genotype interaction affect meat quality traits.

Hu *et al*. (2008) detected the MyHC mRNA expression of Laiwu and Duroc pigs. The results showed that MyHC II a, II x mRNA expression level of Laiwu pigs were higher than Duroc pigs, negatively correlated with muscle fiber diameter. MyH II b mRNA expression levels were significantly lower, positively correlated with muscle fiber diameter. Therefore, different types of expression were not consistent, two or more interacting genotypes affect muscle fiber diameter.

In fish, the expression of MYH genes is modulated by a variety of factors, especially environmental factor (Ennion *et al*., 1999; Wilkes *et al*., 2001) and nutrition level (Overturf and Hardy, 2001). In addition, as observed here for sunfish, myotomal muscle of grass carp shows a scaling response of increased expression of type MyHC with growth (Fukushima *et al*., 2009). These examples demonstrate that MyHC expression in fish muscle is plastic (Coughlin *et al*., 2012). It showed that high nutritional level and proper environment are helpful to the growth and development of muscle. In the studies cited above, during aquaculture production, especially in the intensive production condition, the nutrition and environmental conditions need should be

ensured, in this way, their genetic potential can be realized sufficiently. The present results show that MyHC expression patterns in fish also depend heavily on the functional roles of the muscle. Our analysis did not reveal sufficient differences in MYH to account for longitudinal variations inactivation and relaxation kinetics within a developmental stage. The lack of variation in MYH expression between anterior and posterior red muscle regions of the animal does correlate with the uniform V max of this muscle (Coughlin *et al*., 2001). It is likely that other sarcomeric proteins have a greater effect on shifts in activation and relaxation along the length of fish. An alternative to the 'functional' hypothesis is that isoforms polymorphism confers regulatory advantages at the level of gene expression rather than controlling changes in protein function. That is to say, although the MYHs isoforms themselves may be very similar in their functional properties, they differ significantly in the regulatory regions of their genes. The cellular environment is probably sufficiently different in the newly developing or growing muscle fibres to necessitate the existence of isogenes that differ in their regulatory regions. Thus, it may be necessary to build the initial myofibrillar infrastructure using a MYHs isoform that is transiently but strongly expressed (Steven *et al*., 1995). Thereafter, this MYHs isoform can be readily exchanged for the molecules of the adult type genes. A study of the promoter region of the different MYHs isogenes should shed light on the developmental expression of such isoforms of the MYHs gene family.

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