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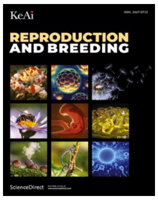

Molecular cloning and expression analysis of the *mstn* and *myod* genes in the hybrids derived from *Megalobrama amblycephala* × *Xenocypris davidi* Bleeker

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ABSTRACT

The hybrids (BY) derived from blunt snout bream (*Megalobrama amblycephala*, BSB, ♀) and Bleeker's yellow tai (*Xenocypris davidi Bleeker*, YT, ♂) have a fast growth rate and body thickness. However, the molecular mechanisms underlying the growth advantage of BY are still unclear. MSTN, a member of the TGF-β superfamily, is a negative regulator of muscle growth and development. MYOD, a member of the myogenic regulatory factor family (MRFs), is a positive regulator of muscle growth and development. MSTN and MYOD interact to co-regulate muscle growth and development. In this study, we cloned the full-length cDNAs of *mstn* and *myod* in BY, analyzed differential expression of the two genes in muscle of BY and its parents, and observed the localization of the two genes during embryonic development of BY. Multiple sequence alignment of amino acids and phylogenetic analysis showed that *mstn* and *myod* were highly conserved in BY, BSB, YT, and other carp family fishes. Quantitative Real-time PCR (qRT-PCR) showed that *mstn* and *myod* were primarily expressed in muscle of BY, and there were significant differences in the expression of the two genes in the red and white muscles of BY, BSB, and YT at different age. The results of in situ hybridization of embryos showed an overlap in the temporal and spatial expression of *mstn* and *myod* in BY embryo development, suggesting that the two genes might interact to co-regulate embryo growth and development. This study provides a theoretical basis for further research on the mechanisms of *mstn* and *myod* in muscle growth of hybrid fishes.

1. Introduction

Distant hybridization can combine two distantly related genomes together, and the genotype and phenotype of the hybrid offspring are different [1]. In previous study, our laboratory successfully produced BY by mating blunt snout bream (*Megalobrama amblycephala*, BSB, ♀) and bleeker's yellow tai (*Xenocypris davidi Bleeker*, YT, ♂), which had advantages of fast growth rate, high back, and thick meat. Hence, BY with many advantages provides good experimental materials for us [2]. Muscle is the most occupied tissue in fish and is the main part being eaten, but there are few studies based on muscle growth and development in hybrid fish. The differentiation and growth of muscle cells are bidirectionally regulated by negative and positive regulators, among which the *mstn* gene of the TGF-β family and *myod* gene of MRFs family play important roles.

Myostatin (*mstn*), also known as transforming growth factor 8 (GDF-8), is one of the members of the TGF-β superfamily. In 1997, McPherron et al. cloned the *mstn* gene for the first time in mice, and the knockout results showed that the weight of muscle tissue increased 2–3 times, so it is speculated that the *mstn* gene can inhibit the proliferation of myoblasts through certain signaling pathways [3]. The *mstn* gene has been cloned in many fish species, such as common carp (*Cyprinus carpio*) [4], rainbow trout (*Oncorhynchus mykiss*) [5], zebrafish (*Danio rerio*) [6], spotted catfish (*Ictalurus punetaus*) [7], cutthroat trout (*Lateolabrax japonicus*) [8], and greater amberjack (*Larimichthys crocea*) [9]. Research has found that in mammals, the expression of *mstn* is tissue specific, with high expression in skeletal muscle and little expression in other tissues [3]. However there are species differences in the spatiotemporal expression characteristics of *mstn* in fish. The *mstn* gene is expressed in various tissues such as liver, heart, muscle, and brain in zebrafish, while

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in carp it is only expressed in muscle and brain. In *Scomberomorus niphonius*, *mstn* is highly expressed in the gills, liver and kidneys, but only slightly expressed in the muscles [10]. Many researchers suggest that myostatin may control muscle mass by inhibiting muscle growth or regeneration [11,12]. The *mstn* gene can also control the proliferation of cardiomyocytes in mice, leading to cardiac hypertrophy and weight loss [13,14], regulate fat metabolism and indirectly control body weight [15,16], and participate in bone formation and regulate bone density [17,18]. In fish research, the physiological activity of the *mstn* gene can also significantly affect the development of skeletal muscle. RNA interference was achieved by microinjecting dsRNA to target the biologically active C-terminal domain of the zebrafish *mstn* gene, and it was found that hyperplasia and hypertrophy occurred in early developmental stages [19]. S Chisada et al. constructed an experimental model of most-deficient medaka using targeted-induced local injury, and found that its body weight increased significantly from late juvenile to adult stage [20]. Transgenic Rainbow trout (*Oncorhynchus mykiss*)overexpressing follistatin has increased upper and lower axis muscles, and it has been reported that the double muscularization of *Oncorhynchus mykiss* is at least partially caused by follistatin binding to MSTN and inactivating MSTN [21].

Myogenic Differentiation Antigen (*myod*) plays a positive role in muscle growth and development and is a member of the Myogenic Regulatory Factors (MRFs) family, which consists of four genes, *myod*, *myf5*, *myog* and *mrf4*, of which *myod* and *myf5* functionally overlap [22,23]. The *myod* was initially isolated from mouse embryonic fibroblasts and was discovered to affect the differentiation fate of myoblasts [24]. Further research has shown that the *myod* have been cloned in a variety of fish species, including common carp (*Cyprinus carpio*) [25], grass carp (*Ctenopharyngodon idellus*) [26], Lanzhou catfish (*Silurus lanzhouensis*) [27], red tilapia (*Oreochromis* sp) [28], and gilthead seabream (*Sparus aurata*) [29]. The proliferation and differentiation of skeletal muscle require a series of transcriptional events to control the spatiotemporal expression of muscle-specific genes. The *myod* plays a crucial role in the development of vertebrate muscles during embryonic and post-embryonic stages. It is considered a key regulator of skeletal muscle formation and differentiation, responding to multiple regulatory programs to activate the transcription of muscle-related genes and drive the differentiation fate of myoblasts. Association of *myod* with core promoter recognition factors or activation of myogenin transcription by TAF3/TRF3 results in up-regulation of *myog* and *mrf4* and a down-regulation of *Myf5*, causing the cells to exit the division phase and differentiate into mononuclear myoblasts, which eventually fuse into myotubes to continue to form relatively stable myofibrils [30–32]. *Myod* is crucial for the repair of skeletal muscle by satellite cells during adulthood following embryonic development. When skeletal muscle is damaged and requires repair, *myod* activates muscle-specific genes in various cell lines, including pigmented, neural, adipose, hepatic, and fibroblast, inducing expression and differentiation to form myoblasts [33,34]. *Myod* is involved in early muscle regeneration in mice, and transcripts were found to be low in adult rats, but preferentially expressed in fast glycolytic muscle. Low levels of *myod* are associated with faster muscle fibre types, and deficiency can alter the muscle fibre type composition towards slower fibre types, although only in certain muscles. Constitutive expression of *myod* increases the amount of fast myosin heavy chain in slow muscle. Compared with controls, the mice that lack *myod* have about three times more satellite cells, which regulate the balance between cell proliferation, self-renewal and differentiation, and determine the size of the satellite cell pool [35].

The studies of the *mstn* and *myod* have been carried out mainly in mammals, to a lesser extent in scleractinian fishes and to a lesser extent in distant hybrid fish. This study presents the first characterization of the mRNAs of the *mstn* and *myod* in the BY and compares the distribution of *mstn* and *myod* gene expression in different tissues. Additionally, the expression during early embryonic development was explored using in situ hybridization. The results will contribute to a better understanding of the relationship between *mstn* and *myod* genes, enrich the theoretical data on muscle regulators in hybrid fish studies, and lay the foundation for further exploration of the regulatory mechanisms of *mstn* and *myod* genes in BY muscle growth and development.

2. Materials and methods

2.1. Ethics statement

Flowing the guidelines of the Administration of Affairs Concerning Animal Experimentation, approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary if the fish in question are neither rare nor endangered (first- or second-class state protection level). None of the experimental fish involved in this experiment met these criteria. Animals were deeply anesthetized with 50 mg/l MS-222 (Sigma-Aldrich, St. Louis, MO, USA) before necropsy to minimize suffering. All animal care and experimental procedures were approved by the Animal Care Committee of Hunan Normal University.

2.2. Preparation, feeding and collection of experimental fish

The fish used in this study were all from the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University, China. From April to May of 2020, 3 pairs of BSB and 3 pairs of YT were selected as parent injected with oxytocin respectively and then artificially inseminated. The hybrid combinations include female BSB × male BSB, female YT × male YT and female BSB × male YT. The fertilized eggs obtained after hybridization were incubated in a Petri dish, and observed developmental stages, collect and preserve materials. The fertilized eggs hatch into fingerling, then transferred to a special pond and raised for 3 years. During this period, experimental materials are collected and stored at the first, second and third ages.

2.3. RNA extraction, reverse transcription and target fragment amplification

Total RNA from tissues was obtained using Trizol (invitrogen, USA) extraction method according to the manufacturer's protocol, the concentration of total RNA was measured with a UV-Spectrophotometer and the quality of total RNA was detected by agarose electrophoresis. Use Prime Script™ RT reagent Kit with gDNA Eraser (Perfect Real Time, TaKaRa, China) to process total RNA to synthesize cDNA template. Use SMART™ RACE cDNA Amplification Kit (Clonetech, USA) and RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher, USA) to synthesize 5′ and 3′ end cDNA template. These specific steps refer to the manufacturer's protocol, and all cDNA template were stored at −20 °C.

The primers used in the study for cloning the intermediate fragments were designed with reference to the homologous fragments of the CDS region of the following three fish species in NCBI. *mstn* gene: zebrafish (AF019626.1), grass carp (KP719016.1) and bream (KY072939.1); *myod* gene: zebrafish (AF318503.2), grass carp (MG544985.1) and bream (KF601795.1). The primers for cloning the 5′ and 3′ end sequences were designed using primerpremier5.0 software based on the corresponding intermediate fragments obtained.

2.4. Sequence analysis

The ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/) was used for predicting the coding sequences of *mstn* and *myod* of BSB, YT and BY. Similarity searches of their nucleotide and amino acid sequences were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast). The signal peptide was predicted using Signal P 4.1 Server (http://www.cbs.dtu.dk/services/Signal P/). The deduced domains of MSTN and MYOD were predicted through TMHMM Server v.2.0 (https://dtu.biolib.com/DeepTMHMM) and further predict the subcellular localization of MSTN and MYOD through PSORT IIPrediction (http://psort.hgc.jp/form2.html). Three unrooted phylogenetic trees were constructed based on the amino acid sequences of MSTN and MYOD by the neighbor-joining (NJ) algorithm using the MEGA7.0 software (http://www.megasoftware.net). The bootstrap trials were replicated 1000 times to derive the confidence value for the phylogeny analysis.

2.5. qRT-PCR program

16 tissues were selected to explore the expression of *mstn* and *myod* in different tissues in BSB, YT and BY (white muscle, liver, red muscle, spleen, head kidney, mesonephros, skin, gills, cerebellum, cerebrum, midbrain, hypothalamus, pituitary gland, oblongata, Intestine, heart). On the other hand, 3 ages were selected to explore the expression of *mstn* and *myod* in different ages in BSB, YT and BY (1st, 2nd, 3rd age). 3 biological replicates are included for each tissue required for the qRT-PCR experiment, and three repeated experiments were set up to ensure the reliability of the experimental results.

The qRT-PCR primers were designed using the Primer Premier 5 software and were listed in Table 1. Primer specificity was confirmed through qRT-PCR, and NCBI blast was used to ensure sequence similarity above 99 % prior to their application in qRT-PCR. The specific reaction system is as follows: 2 × Power SYBR Green PCR Master Mix (ABI) 5 μl, Dilute cDNA template 10 × 1 μl, Q-x-F 0.4 μl and Q-x-R 0.4 μl (Where x stands for *mstn*, *myod* and *β-actin* genes), ddH2O 3.2 μl, all 10 μl. Used Prism 7500 Sequence Detection System (ABI, Foster City, CA, USA) to detection. The reaction procedure was as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 61 °C for 45 s, 72 °C for 45s, and 72 °C 10 min.

The experimental results were shown as the mean ± standard deviation, then exported in the form of excel table and analyzed using the relative standard curve method ($2^{-\Delta\Delta CT}$ method), graphed using GraphPad Prism 5 software. significance analysis (one-way analysis of variance) using SPSS software. $P < 0.05$ represents a significant difference.

2.6. Embryo in situ hybridization

The embryo material comes from 2.1. After insemination, the embryo material was collected according to the development stage and packed in RNase-free glass tubes. EP tubes can be used in the later stage, and each tube can hold about 20 embryos. Add 4 % PFA, fix at 4 °C overnight, and dehydrate on the next day: dehydrate once successively in 25 % Methanol-75 % PBS, 50 % Methanol-50 % PBS, and 75 % Methanol-25 % PBS, 15 min each time, dehydrate with 100 % Methanol twice, 15 min each time, and finally immerse in 100 % Methanol and store at −20 °C for later use. According to the CDS sequences of BY *mstn* and *myod* gene obtained in the previous 2.2, refer to the previously reported method [36], and use the PCR product as a template to prepare antisense RNA probes (DIG label). Before starting in situ hybridization, the embryos were gradient rehydrated and fixed, and probes were used for hybridization color reaction. Observe the reaction under a microscope and take photos for storage.

Table 1
Primer sequences for qRT-PCR.

Primer name	Primer sequence (5′–3′)
β-*actin*-F	CGGACAATTTCTCTTTCGGCTG
β-*actin*-R	ACCCACACCGTGCCCATCTA
Q-*mstn*-F	CGTTACGGACGGAGGAAGAC
Q-*mstn*-R	TTGACCACACCGAGAGCACC
Q-*myod*-F	TCCACCAACCCGAACCA
Q-*myod*-R	TCAGAGTCTCCGCTGTAATGC

3. Results

3.1. The mstn and myod cDNA cloning

In this study, the *mstn* genes and full-length *myod* cDNA sequences from BY, BSB and YT were successfully obtained. BY *mstn* full-length is 2211bp, open reading frame is 1128bp, encoding a total of 375 amino acids, 5 ′non-coding region 116bp, 3′ non-coding region 967bp (Fig. 1a). The isoelectric point of the protein is 6.22, and the corresponding molecular weight is 42097.11 Da. BY MSTN protein has two typical TGF- β protein domains: the lead peptide (amino acid 1–22) and the active domain of TGF- β family genes (mature MSTN domain). It has a protease hydrolysis site RIRR (position 263–266) and 9 conserved cysteine residues. Among its encoded protein peptide sequences, Leu is the most abundant, followed by Ser and Ile. The protein phosphorylation sites were concentrated in Ser, Thr and Tyr.

The full length of BY *myod* is 1612bp, the 5 ′non-coding region is 217bp, the 3′ non-coding region is 567bp, and its open reading frame is 828bp, encoding a total of 275amino acids. The isoelectric point of the protein is 5.46, and the relative molecular weight of the protein is 30810.10 Da (Fig. 1b). The protein of BY MYOD has no signal peptide sequence, and in its subcellular localization prediction, it was found to be mainly distributed in the nucleus (95.7 %). Its protein domain has a conserved bHLH structure, in which the basic domain is located at position 1–89, the HLH domain is at position 90–141A, and HelixIII is at position 205-222a. Ser has the highest number of encoded protein peptide sequences, accounting for 34, accounting for 12.4 %, followed by Asp and Pro, with protein phosphorylation sites concentrated in Ser, Thr, and Tyr.

3.2. The mstn and myod cDNA sequence analysis

The amino acid sequences of MSTN from 9 species (Fig. 2a) were compared with the predicted MSTN amino acid sequences of BY, BSB, and YT using the DNAMAN multiple sequence alignment program for amino acid sequence similarity analysis. The results showed that the similarity between BSB and YT's MSTN amino acid sequences was 97.3 %, BY's MSTN amino acid sequence similarity with BSB was 99.7 %, and with YT was 100 %. The MSTN amino acid sequence similarity between BY and its parents and other four fish species was above 96 %. In contrast, the similarity with cattle, goat, Woodchuck, mouse, and human mammalian species was lower, ranging from 65.3 % to 68 %. This indicates that BY and its parents have sequence homology in MSTN. MSTN shows strong conservation in the cyprinidae, suggesting that the function of the *mstn* gene in BY, parents BSB, and YT is similar to that in cyprinid fish. Comparative analysis revealed that the N-terminal of the MSTN polypeptide sequences of these 9 species all possess TGF-β propeptide, the C-terminal region contains a TGF-β or TGF-β-like structure domain encoding mature peptide, and there is also a conserved node composed of 9 cysteine residues and an RXXR proteinase cleavage site (Fig. 2a).

The amino acid sequences encoded by the *myod* genes of BY and its parent were aligned and compared with those of 8 other species (Fig. 2b) through multiple sequence alignment analysis. The results revealed the presence of a Basic domain and a HLH domain in all species. The comparison of amino acid sequences showed high similarity between the MYOD sequences of BY and its parent, with a similarity of 100 % to the BSB and 98.9 % to the YT, while the similarity between BSB and YT was also 98.9 %. Although there were minor nucleotide differences in the CDS sequences of these three fish, the encoded amino acid sequences were nearly identical, with some nucleotide changes resulting in synonymous mutations, suggesting no significant biological differences in the *myod* gene between BY and its parent. The similarity of MYOD amino acid sequences between BY and BSB, zebrafish, koi carp, grass carp, and Topmouth culter were 98.9 %, 94.5 %, 96.4 %, 99.3 %, and 99.3 %, respectively, while the similarity with cattle, Eurasian wild boar, mouse,

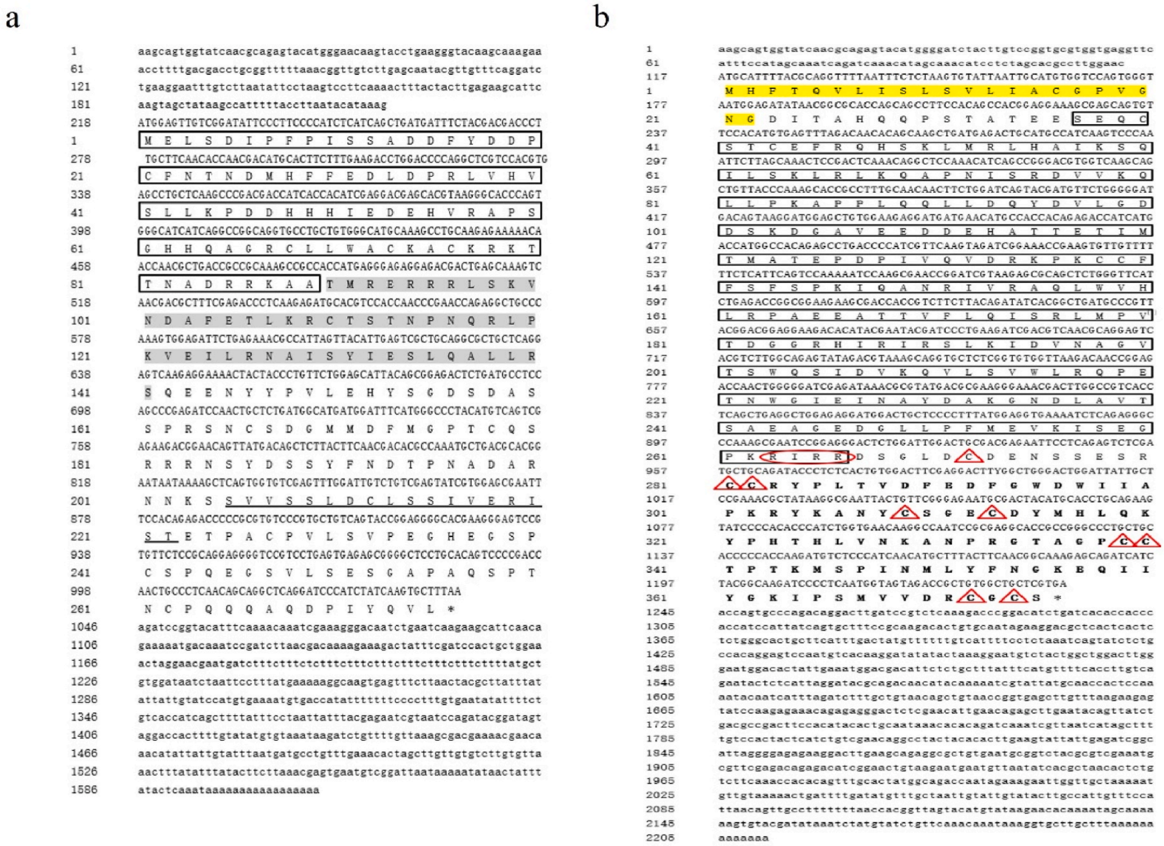

Fig. 1. Nucleotide and deduced amino acid sequences of *mstn* (a) and *myod* (b) cDNA in BY.

and human MYOD sequences was lower at 70.1 %–71.4 %. The YT showed similarities of 94.2 %, 96 %, 98.9 %, and 98.9 % with zebrafish, koi carp, grass carp, and Topmouth culter, while the similarity with the other 4 mammalian MYOD amino acid sequences ranged from 70.4 % to 71.8 % (Fig. 2b).

Using MEGA7, an NJ amino acid system evolution tree was constructed for MSTN of 18 species including BY, BSB, YT, rainbow trout, human, mouse and others. The results revealed that BY and its parents species clustered closely together, showing a closer evolutionary relationship with bony fish such as *Megalobrama Pellegrini*, grass carp and common carp, and a more distant relationship with mammals like human, cattle, and others (Fig. 3-a). Another NJ amino acid system evolution tree was built for MYOD of BY and its parent species, along with 17 species including Eurasian wild boar, grass carp, zebrafish and others (Fig. 3-b). The analysis indicated that BY clustered closely with BSB in the MYOD tree, forming a branch with Topmouth culter and grass carp. The results revealed that BY and its parents showed the closest evolutionary relationship with *Schizothorax prenanti* and common carp, and a more distant relationship with mammals like human, wild boar, and amphibian species *Xenopus laevis*.

3.3. Expression levels of mstn and myod in different tissues

The qRT PCR method was used to analyze the expression of BY *mstn* in various tissues. The results showed that the expression of *mstn* in white muscle was the highest, with significant difference in expression levels compared to other tissues. In addition, *mstn* was also highly expressed in intestinal tract, cerebellum and red muscle (Fig. 4a). There is muscle-specific expression of *myod* in BY. The expression of *myod* is the highest in red muscle, the second in white muscle, and low in other tissues (Fig. 4b).

3.4. Expression levels of mstn and myog in different ages

The qRT-PCR results of *mstn* expression in white muscle (Fig. 5a) at different ages (1 year old, 2 years old, 3 years old) show that there are significant differences among BSB, YT, and BY. The expression level of BSB was the highest in 1 year old fish, the expression level of YT was the highest in 2 year old fish, and the expression level of BY was the highest in 3 year old fish. In red muscle (Fig. 5b), YT shows significantly higher levels of *mstn* expression than BY and BSB at 1 and 2 years of age, but BY exhibits significantly higher expression levels than its parents at 3 years of age. During the growth and development of BY, the expression levels of the *mstn* gene in both white and red muscles gradually increase over the years. In BSB, the expression of the *mstn* gene in white muscle decreases gradually over the years, while its expression in red muscle peaks at 2 years old. In YT, the expression levels of the *mstn* gene in both white and red muscles reach their highest levels at 2 years old.

The qRT-PCR results of *myod* expression in white muscle (Fig. 5c) showed that in YT, the expression levels of *myod* in white muscle at ages 1, 2, and 3 were significantly higher compared to the same age groups of BY and BSB. Additionally, at 3 years old, the expression of *myod* in white muscle of BY was higher than that in BSB. The qRT-PCR results of *myod* expression in red muscle (Fig. 5d) showed that in 1 year old and 2 year old, the expression level of *myod* in YT was significantly higher than in the same age groups of BY and BSB, but in 3 year old, the expression level of *myod* in BY was significantly higher than in BSB and YT. During the growth and development of BY, the expression of *myod* in white muscle at ages 1, 2, and 3 remained relatively stable, while the expression in red muscle increased yearly. In the growth and development process of BSB the expression level of *myod* in white muscle was highest at age 1, and decreased in ages 2 and 3, while the expression level in red muscle remained relatively low during ages 1, 2, and 3. In the growth and development process of YT, the expression level of *myod*

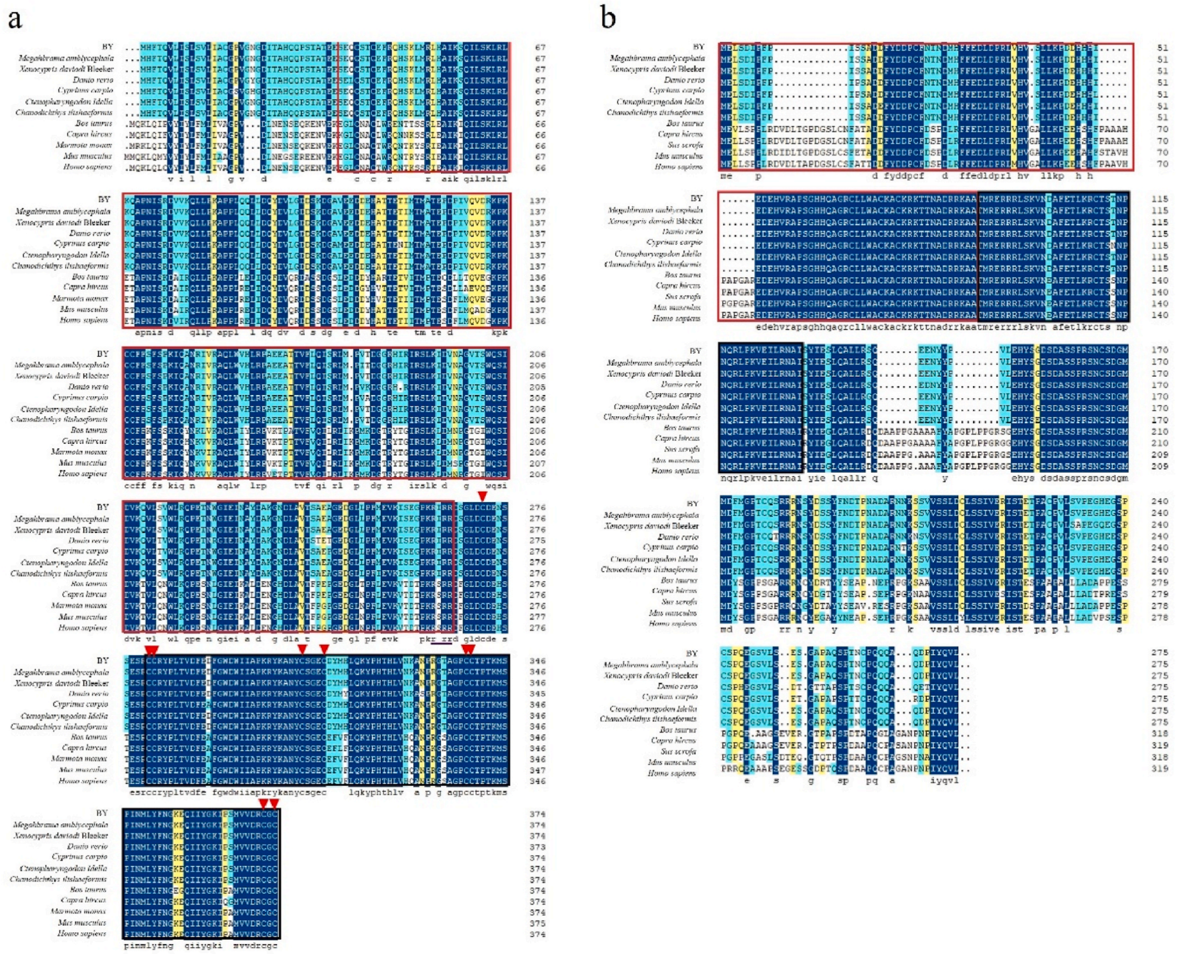

Fig. 2. Comparison of MSTN (a) and MYOD (b) amino acid sequences among BY and other species. a. The red box indicates the leader peptide sequence, the black box indicates the mature peptide sequence, the purple underlines indicate the conserved protease hydrolysis sites, the red triangular box represents the conserved cysteine residues, and the lowercase letters indicate the same amino acids. *Homo sapiens*: ABI48494.1, *Danio rerio*: AAB86693.1, *Cyprinus carpio*: XP_018960963.2, *Ctenopharyngodon idella*: XP_051761551.1, *Chanodichthys ilishaeformis*: AGK84721.1, *Mus musculus*: NP_034964.1, *Marmota monax*: KAI6070397.1, *Bos taurus*: ACN65829.1, *Capra hircus*: AFX59924.1, *Erythroculter ilishaeformis*: AHI13616.1, *Sus scrofa*: NP_001002824.1. b. The red box indicates the basic domain, the black box indicates the HLH domain, and the lowercase letters indicate the same amino acids. *Homo sapiens*: CAA40000.1, *Danio rerio*: NP_571337.2, *Cyprinus carpio*: XP_018923874.2, *Ctenopharyngodon idella*: XP_051740096.1, *Erythroculter ilishaeformis*: AHI13616.1, *Mus musculus*: AAA39798.1, *Sus scrofa*: NP_001002824.1, *Bos taurus*: BAC76802.1.

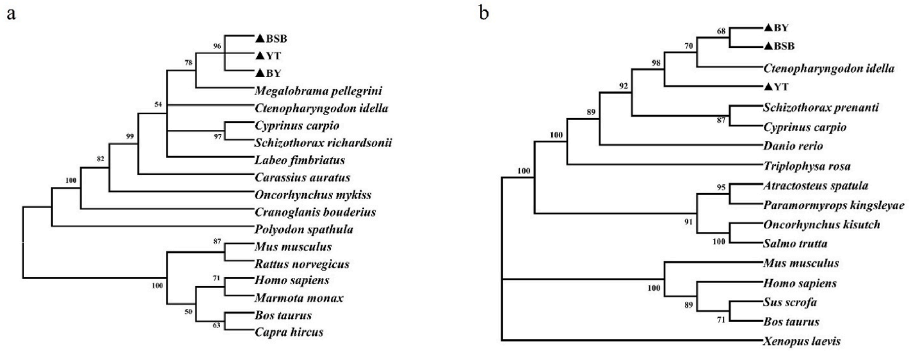

Fig. 3. Phylogenetic tree of MSTN (a) and MYOD (b) protein sequences. a. *Megalobrama Pellegrini*: ADD71156.1, *Ctenopharyngodon idella*: XP_051761551.1, *Labeo fimbriatus*: AEN75197.1, *Cyprinus carpio*: ACY01745.1, *Schizothorax richardsonii*: QCC30165.1, *Homo sapiens*: ABI48494.1, *Mus musculus*: NP_034964.1, *Oncorhynchus mykiss*: NP_001117755.1, *Cranoglanis bouderius*: AYF53235.1, *Carassius auratus*: XP_026127226.1, *Rattus norvegicus*: AAB86691.1, *Marmota monax*: KAI6070397.1, *Bos taurus*: ACN65829.1, *Capra hircus*: AFX59924.1. b. *Ctenopharyngodon idella*: AWG41987.1, *Cyprinus carpio*: XP_042609345.1, *Schizothorax prenanti*: AFL56775.1, *Danio rerio*: CAA85407.1, *Triplophysa rosa*: KAI7789209.1, *Atractosteus spatula*: MBN3324017.1, *Paramormyrops kingsleyae*: XP_023697965.1, *Oncorhynchus kisutch*: XP_020311898.1, *Salmo trutta*: ABD24216.1, *Mus musculus*: AAA39798.1, *Homo sapiens*: CAA40000.1, *Sus scrofa*: NP_001002824.1, *Bos taurus*: BAC76802.1, *Xenopus laevis*: XP_023697965.1.

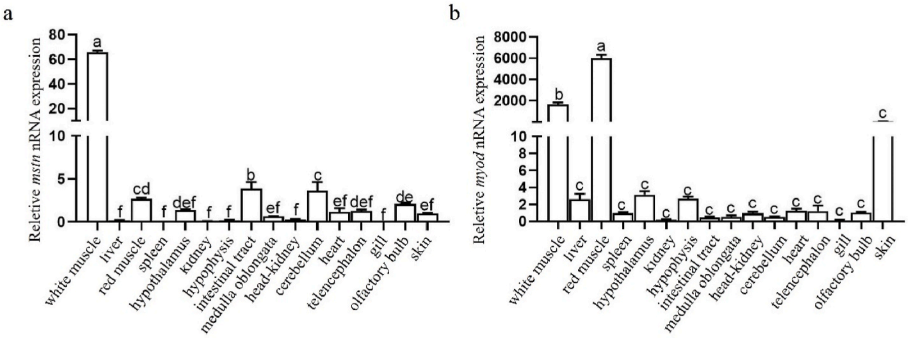

Fig. 4. Results of qRT-PCR of *mstn* (a) gene and *myod* (b) gene in different tissues of BY. Different letters indicate significant differences ($P < 0.05$).

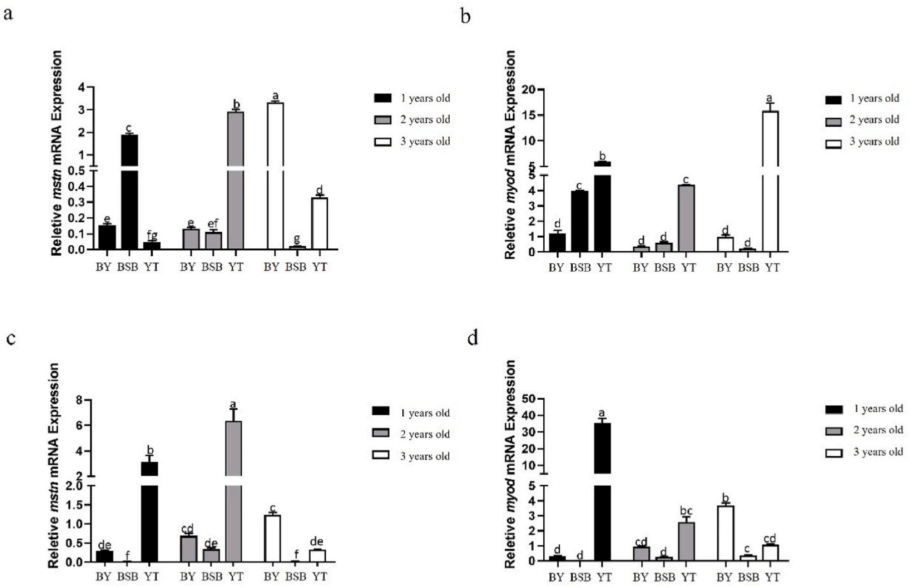

Fig. 5. Results of qRT-PCR of *mstn* and *myod* in the white muscle and red muscle of BY, BSB and YT at different ages. a. Expression of *mstn* gene in white muscle. b. Expression of the *mstn* gene in red muscle. c. Expression of *myod* gene in white muscle. d. Expression of the *myod* gene in red muscle. Different letters indicate significant differences ($P < 0.05$).

in white muscle was highest at age 3, while the expression level in red muscle was lowest at age 3.

3.5. In situ hybridization of mstn and myod in embryos

Using whole mount in situ hybridization technology, a spatial and temporal expression localization study was conducted on the *mstn* and *myod* genes during BY embryo development. As shown in (Fig. 6), the results indicate that signals were observed in the blastodisc due to the presence of *mstn* and *myod* transcripts. The signals persisted during the cleavage stage and were prominently detectable in the blastoderm cells. In general, expression of *mstn* and *myod* genes was widespread. At the 64 cell stage Fig. 6A and the 1 K cell stage (Fig. 6B), signals for both genes appeared in dividing cell clusters, with *myod* gene expression levels higher than those of *mstn* gene. Subsequently, as the embryo developed to the early gastrula stage (Fig. 6C), the expression of *mstn* and *myod* genes was observed in the blastoderm cells.

During the neuro embryonic stage (Fig. 6 continued D), the blastopore begins to close, and the *myod* and *mstn* genes are expressed in cells oriented towards segmental body development, with a more concentrated expression of the *myod* gene. As these cells expand and develop, the embryo enters the 16 segment stage (Fig. 6 continued E), at which point both *mstn* and *myod* gene expression is significantly upregulated, consistent with the quantitative results mentioned earlier, and the expression sites become more specific. Both genes are expressed in muscle cells of the segmental plate on either side of the notochord, known as paraxial cells [29]. As shown in the picture the expression of *mstn* is more pronounced in cells near the central part of the body, while *myod* is more prominently expressed in muscle cells near the tail. Both genes are expressed in the paraxial cells and lateral cells that will develop into red muscle (slow muscle) and white muscle (fast muscle) in the future [37].

During the embryonic development up to the organ formation period (Fig. 6 continued F), a decrease in the expression levels of both *mstn* and *myod* mRNA was observed in mature muscle cells in the anterior part of the body, but there was a very noticeable strong signal of *mstn* and *myod* mRNA at the tail bud. In the incubation period (Fig. 6 continued G), differences in the expression patterns of *mstn* and *myod* genes started to emerge. The former was expressed almost throughout the trunk, including the brain (telencephalon, midbrain, diencephalon, midbrain-hindbrain boundary, hindbrain), eyes, spinal cord, and tail, with the strongest signal in the tail. On the other hand, the *myod* gene was mainly expressed in the most posterior body segments and showed a significant decrease in signal intensity in the spinal cord region.

When the embryo reached the membrane emergence stage (Fig. 6 continued H), *mstn* expression in brain tissue was mainly concentrated at the diencephalon and midbrain-hindbrain boundary, with continued expression in the eyes. The signal in the spinal cord and tail tended to

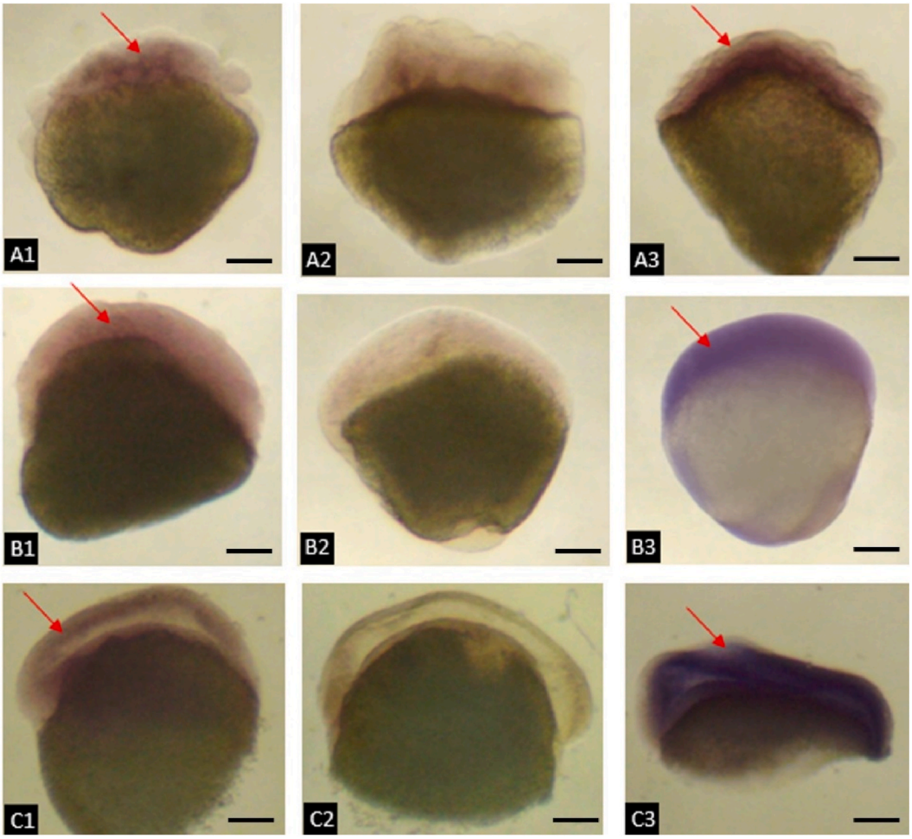

Fig. 6. aSpatial and temporal expression patterns of *mstn* and *myod* of BY during early embryogenesis. A-C represents all stages of the embryonic development. Group 1 represents the whole-mount in situ hybridization experimental group of *mstn* mRNA, group 2 represents the negative control group, group 3 represents the whole-mount in situ hybridization experimental group of *myod* mRNA, and purple is the hybridization signal area (red arrow). A. 64 cell stage. B. 1 K cell phase. C. early gastral stage. Scale bar = 200 μm.

shift posteriorly, although the tail remained a region of concentrated expression. The *myod* expression pattern did not change significantly compared to the organogenesis stage. Moving on to the appearance stage of eyeball pigment (Fig. 6 continued I), both *mstn* and *myod* were expressed in the eyes, with *mstn* also showing expression in the diencephalon, hindbrain, and other muscle cells. However, the signal at the tail end segments became weaker. The *myod* gene exhibited strong expression in the notochord and tail regions.

4. Discussion

In this study, the full-length cDNA sequences of the *mstn* and *myod* genes of BY were obtained Using RACE technology. The amino acid sequence similarity and phylogenetic relationship were analyzed, and it was found that there is sequence homology between the MSTN and MYOD of BY and its parents. The NJ phylogenetic tree also shows that they are closely clustered together. Those results indicate that the mstn and myod genes are relatively stable in the genetic process of distant hybrids and that no significant variation has occurred during their evolution. In addition, the *mstn* and *myod* genes have evolved conservatively in the Cyprinidae family, which is distantly related to mammals. This is generally consistent with the relationship between system development and traditional species evolution, suggesting that the *mstn* and *myod* genes are helpful for studying systematic evolutionary relationships between species.

The amino acid sequence analysis results indicate that the MSTN of BY lacks transmembrane domains, the first 22 amino acids serve as signal peptide sites, suggesting that MSTN is a typical secretory protein, and its signal peptide assists in transporting the MSTN precursor protein between ribosomes and the endoplasmic reticulum [10]. Differences in the amino acid sequences of the signal peptide domains between fish and humans may affect the structure of the mature peptides, resulting in functional differences in the actions of MSTN between fish and mammals. The highly conserved features of the C-terminal domain may contribute to the slow evolution of *mstn*, thereby maintaining its ability to inhibit muscle growth. The amino acid sequence results showed that MYOD lacks a signal peptide sequence. Additionally, its subcellular localization prediction predominantly indicated nuclear distribution (95.7 %). This suggests that MYOD encodes a non-secretory protein, consistent with the structural characteristic of the bHLH domain beginning at the first amino acid. Structural domain analysis of MYOD protein in BY revealed the presence of conserved bHLH domain and domain HelixIII. Among them, the Basic domain represents an extension of the HLH helical structure, while the domain HelixIII may be associated with chromatin remodeling [38]. Comparison of the HLH domain revealed high similarity between species, with the Basic domain being relatively conserved in the common carp, consistent with the observation by Guo Y et al. [39,40] that the amino acid peptide chain encoded by the *myod* gene tends to elongate as animals evolve from lower to higher orders. Furthermore, the bHLH domain is a conserved structural domain of the MRFs family, and structural differences outside this domain may lead to functional differences between the *myod* gene and its family genes, thereby better regulating the growth and development of skeletal muscles [41].

The qRT-PCR results showed that *mstn* and *myod* genes were differently expressed in different tissues, and the expression site of *mstn* was wider than that of *myod*. The expressions of *mstn* and *myod* in different tissues may have some correlation with their roles in each tissue. In adult zebrafish, *mstn* transcripts are present in various tissues, with the highest expression in the brain, muscles, heart, and testis [42]. In roughskin

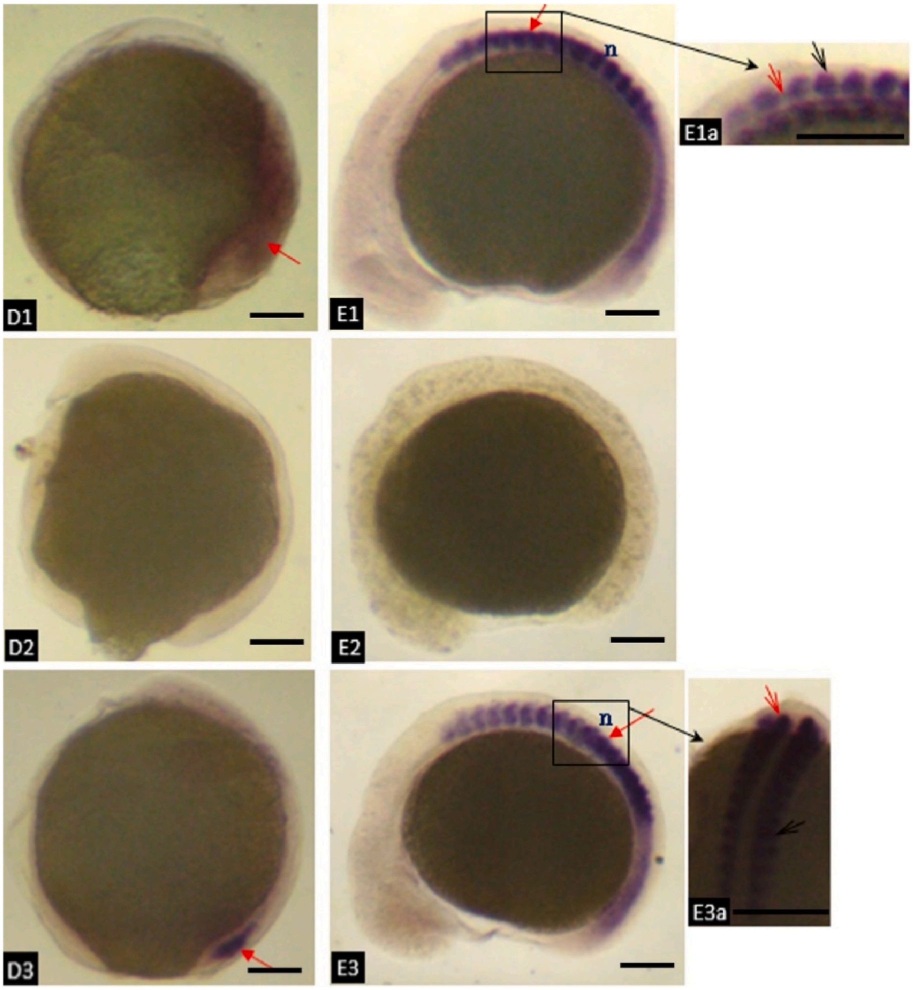

Fig. 6. bD. neuro embryonic stage. E. 16 segment stage. Group 1 represents the whole mount in situ hybridization experimental group of *mstn* mRNA, group 2 represents the negative control group, group 3 represents the wholemount in situ hybridization experimental group of *myod* mRNA and purple is the hybridization signal area (red arrow), n represents notochords. E1a and E3a are enlarged diagrams of the segment signal region of group E1 (*mstn* whole-mount in situ hybridization experimental group) and group E3 (*myod* whole-mount in situ hybridization experimental group), respectively. The signal sites of the paraxial cells in the red arrow example and the signal sites of the lateral cells in the black arrow example. Scale bar = 200 μm.

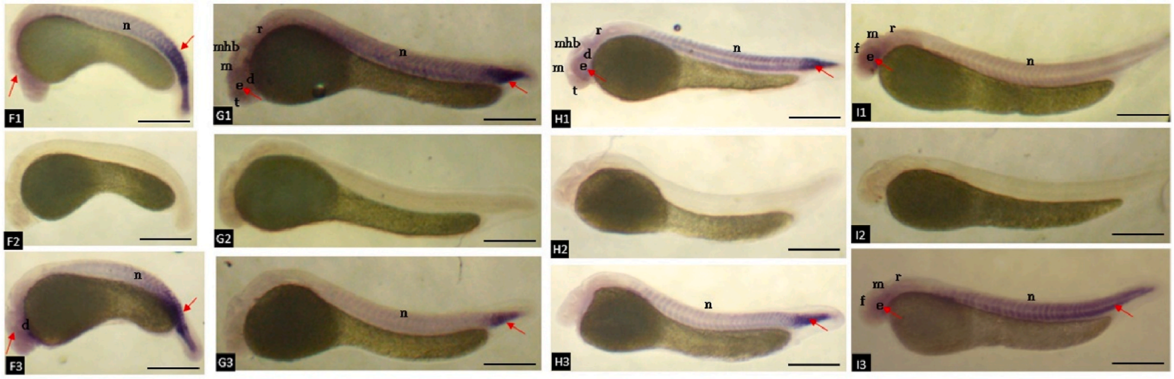

Fig. 6. cF. organ formation period, d represents diencephalon, n represents as above. G. incubation period, e represents the eye, t represents the telencephalon, m represents the midbrain, mhb represents the junction of midbrain and hindbrain, r represents the hindbrain, d and n represent the same anterior. H. Membrane emergence stage, e, t, m, d, mhb, r, n, as above. I. the appearance stage of eyeball pigment, f represents the forebrain e, m, r, n as above. Scale bar = 600 μm.

sculpin (*trachidermus fasciatus*), *mstn* is mainly distributed in muscle, brain, intestine, and liver [43]. In grouper (*Epinephelus coioides*), *mstn* was detected in 10 tissues, including eye, brain, gill, heart, and muscle [44]. In common carp (*Cyprinus carpio*), *mstn* was only expressed in the brain and muscle [4]. In this study, we found a wider range of tissue expression of BY *mstn* compared to mammals, consistent with the results of a previous study on tissue expression of the fish *mstn* gene [10]. *mstn* is highly expressed in muscle of BY, especially in white muscle, which is compatible with its primary biological function of negatively regulating muscle growth and development. In addition, *mstn* was also expressed in the cerebellum, which is consistent with the results in crabs of Fukuan Du et al. [45], and the brain tissues of different fishes have been proven to be an essential site of *mstn* expression [9]. So we speculate that *mstn* gene may have a certain biological function in the developmental regulation of the nervous system and the immune system in the BY adult fish.

Compared with the expression of the *mstn* gene, *myod* of BY has muscle-specific expression. The muscles of fish can be divided into red muscle (slow muscle) and white muscle (fast muscle) [46]. The expression of *myod* in red muscles of BY was higher than that in white muscles, indicating that *myod* plays a specific biological role in determining muscle differentiation and the growth and development of fast and slow muscles. Previous research has shown that the MYOD protein is present in different amounts in fast and slow muscles , and is required for the balance of normal fiber type in rodents [47]. Xungang Tan et al. suggested that each species may have evolved a unique muscle structure to best adapt to its ecological environment [29]. According to previous reports, *myod* was specifically expressed in the muscles of turbot, rainbow trout and red sea bream. There were two subtypes in red sea bream and rainbow trout, among which *myod*1 was expressed in both slow and fast muscles of adult fish but mainly expressed in slow muscles, which was consistent with the results of our study, and the expression of *myod* genes in the different muscles of different adult fishes may reflect the differences in the species of the two types of muscle fibers in different fishes [29,48,49].

Previous studies have found that the SNP locus of *mstn* of *Ictalurus punctatus* and *Oreochromis niloticus* var is related to individual size, weight, fat deposition and other factors [50–52], and there is a regulatory relationship between *mstn* and *myod* [53], indicating that the growth and development of fish is related to these two genes. In this study, the muscles of BY, BSB and YT at different ages, the expression characteristics of *mstn* and *myod* are different, and the expression trends are also different, which may affects their growth and development. For example, the expression of *mstn* was lowest in BY at 2 years of age. Shi Junxin et al. found that the expression of *mstn* was lower in *Scomberomorus niphonius* at 2 years of age [54]. Sun Ranran et al. found that the expression of *mstn* in *Seriola aureoensis* was also lowest at 2 years of age, which is the rapid growth period, which is consistent with our study [52]. This shows that the expression of *mstn* in fish will changes with the growth process. Previous studies have shown that fish muscle growth is a multifactorial process regulated by GH, IGF-I and other secretory metabolites [52]. Furthermore, MYOD is not the only muscle growth factor; other members of the MRF family such as MYOD and MYF5 have overlapping functions [23]. The interaction between myogenic regulatory factors such as MYOG and MRF4 and different growth environments are all crucial factors that influence the growth and development of fish skeletal muscle. Comprehensive considerations are needed to draw more accurate conclusions. Therefore, the effects of *mstn* and *myod* on the growth and development of BY, BSB, and YT in this study need further study.

Through whole embryo in situ hybridization experiments, the spatiotemporal expression results of BY embryo *mstn* showed significant expression signals in the early stages of development, which may indicate that the maternally derived transcripts of that period were still preserved. This similar phenomenon was observed in zebrafish [55] and tilapia [56], where *mstn* is expressed in their ovaries, unfertilized eggs, and even in newly fertilized eggs at the 2-cell stage. Grass carp and yellowtail kingfish also have similar expressions [26,51]. In our study, *mstn* was strongly expressed in the myotomes that were about to differentiate into muscles, further indicating that it plays an essential role in the developmental differentiation of muscles. This is consistent with the results of in situ hybridization between blunt snout bream and grass carp [26,57], indicating that *mstn* affects the growth of various muscles and regulates the development of many parts during the embryonic period. At the later stage of embryonic development, *mstn* is also significantly expressed in the eyes, suggesting that it may be involved in the growth of the fish eyes' longitudinal and annular muscles [58]. In general, the expression site of BY *mstn* has some similarities with Cyprinid fish, but there are some differences in the expression timing, which may be related to the species differences of fish. In this study, it was found that the *myod* in situ hybridization signal could be clearly seen on the side of the embryo shield when BY embryos developed to the neurula stage, and its expression level in the subsequent newborn somites was higher than that in the anterior mature somites. This is consistent with the developmental process of the somites, which is similar to its expression in flounder embryos [49]. It has been reported that *myod* transcription in *Gadus morhua* [59], *Clupea pallasi* [60] and *Danio rerio* [61] first occurs in the adaxial myoblasts of the unsegmented precursor mesoderm. In contrast, *myod* in *Salmo salar* [62] is first expressed in the myogenic precursors of the somite plate. Those indicating that the expression of *myod* during this period may be involved in the decision to initiate early muscle differentiation of BY. In summary, both are widely expressed at various stages of BY embryonic development, suggesting that they regulate skeletal muscle growth during embryonic development and contribute to the dynamic balance of other tissues to control growth [58].

In order to explore the biological characteristics of the *mstn* and *myod* genes in BY and the parental species, this study utilized PCR and RACE techniques to obtain the full-length cDNA sequences of *mstn* and *myod* in these three fish species. Subsequently, qRT-PCR analysis was conducted to examine the expression of *mstn* and *myod* in the muscle tissues of adult BY, BY at different ages, and the parental species. Finally, through qRT-PCR and whole-mount in situ hybridization, the spatiotemporal expression of these two genes during early embryonic development in BY was compared and studied. In summary, this study lays the foundation for further investigation into the developmental regulation of skeletal muscle in BY and the growth differences between BY and its parent species.

CRediT authorship contribution statement

Siyu Fan: Writing – original draft, Visualization, Validation, Data curation, Conceptualization. **Ting Li:** Writing – original draft, Visualization, Validation, Data curation, Conceptualization. **Zhong Tang:** Visualization, Validation, Methodology. **Zhifeng Zhou:** Visualization, Validation, Methodology. **Xin Deng:** Investigation, Formal analysis. **Lu Huang:** Investigation, Formal analysis. **Xinge Ouyang:** Methodology, Investigation. **Faxian Yu:** Methodology, Investigation. **Xiangqiong Yang:** Visualization, Software. **Liran Zhang:** Software, Investigation. **Min Tao:** Writing – review & editing, Supervision, Resources, Project administration, Formal analysis.

Declaration of competing interest

Min Tao is an editorial board member for Reproduction and Breeding and was not involved in the editorial review or the decision to publish this article. All authors declare that they have no conflict of interest.

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