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Molecular Cloning, Characterization and Expression Profile of Myf5 and Myf6 During Growth and Development in the *Seriola lalandi*

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Abstract Myogenic Regulatory Factors (MRFs) is involved in the muscle growth and differentiation. In this study, the cDNA sequence of yellowtail kingfish MRFs genes were cloned by rapid amplification of cDNA ends (RACE) method; then, the characteristics of these genes and the predicted protein sequences were analyzed by bioinformatics methods, the tissue and embryonic stages differential expression pattern were detected by the quantitative real-time PCR. Our results showed that the yellowtail kingfish (YTK) Myf5 cDNA has a full length of 951 bp, encoding 266 amino acids. The yellowtail kingfish Myf6 cDNA has a full length of 1105 bp, encoding 250 amino acids. The proteins contain α-helix, β-strand, and loops. The Neighbour-joining tree revealed that YTK Myf5 and Myf6 are closely related to *Seriola dumerili*. The yellowtail kingfish Myf5 and Myf6 gene expressed significantly higher in muscle than in other tissues ($P<0.05$). In addition, Myf5 and Myf6 in muscle was significantly expressed in 400 g and 500 g fish but not in 50 g, suggesting that myogenic regulatory factors expression had a great relationship with the fish size. Our results also indicated that Myf5 and Myf6 have different functions during embryonic development, because Myf5 showed highest expression level at the neuroembryo period, but Myf6 had the highest expression level at embryo coverage yolk 70% stage. Myf5 gene showed highest expression at 30 d of age, suggesting it played key roles in myogenic period. However, the Myf6 gene was significantly highly expressed at 60 d, revealing this gene functioned in the later muscle formation period.

Key words *Seriola lalandi*; myogenic regulatory factors; gene cloning; expression

1 Introduction

Fish skeletal muscle is predominantly composed of white muscle, which represents up to 60% of total weight in some fish species, and involved in most fish growth, becoming the edible part of the fish (Zhang *et al*., 1996; Almeida *et al*., 2010). Therefore, research on fish skeletal muscle is currently a hot topic in field of aquaculture. Muscle growth and differentiation is regulated by the positive regulators of the Myogenic Regulatory Factors (MRFs) family members including myoblast determination protein (MyoD), myogenin (MyoG), Myogenic factor 5 (Myf5) and Myogenic factor 6 (Myf6; which was also named as MRF4) (Braun and Gautel, 2011). MRFs, the transcription factors share a highly conserved central region termed the basic helix loop helix (bHLH) domain, which could be dimerized with ubiquitous E proteins (Edmondson and Olson, 1993). Many studies have identified that MyoD and Myf5 were required for muscle determination (Rudnicki *et al*., 1993) and involved in myogenic lineage determination (Tajbakhsh *et al*., 1996). MyoG was known as acted as a crucial differentiation factor in the control of myoblast fusion and myofiber maturation (Hasty *et al*., 1993; Nabeshima *et al*., 1993). Meanwhile, Myf6 played a key role initiating and maintaining the myogenic differentiation program, which functioned to be more complicated (Braun and Arnold, 1995; Zhang *et al*., 2015).

MRFs exhibiting functional similarity between higher vertebrates and fish, however, the distinct characteristics of muscle in fish including indeterminate growth, slow and fast muscle type, and contribution of hyperplasia in post-larval growth should be considered (Mommsen, 2001). So far, Myf5 and Myf6 genes had already been identified and characterized in many fish species such as

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zebrafish (*Danio rerio*) (Chen and Tsai, 2002; Hinits *et al*., 2007), striped bass (*Morone saxatilis*) (Tan *et al*., 2002), common carp (*Cyprinus carpio* L.) (Kobiyama *et al*., 1998), rainbow trout (*Oncorhynchus mykiss*) (Johansen and Overturf, 2005) and snow trout (*Schizothorax richardsonii*) (Rajesh *et al*., 2019). The expression for these genes have been all resolved during early embryonic development, such as *D. rerio* (Chen and Tsai, 2002), *C. carpio* L. (Cole *et al*., 2004) and sea perch (*Lateolabrax japonicus*) (Ye *et al*., 2007), and provided a better understanding of Myf5 and Myf6 function during fish development.

Yellowtail kingfish (*Seriola lalandi*, here after referred to as YTK) is distributed in most oceanic areas, including the coasts of Australia, New Zealand, Japan, China, the United States, Chile *etc*. (Ai *et al*., 2020). YTK is well-received by consumers because of its characteristics of balanced nutrition, because of the increased demand for YTK in global, this species has recently been designated as one of the important species for the diversification of aquaculture industry in many countries (Palomino *et al*., 2014; Candebat *et al*., 2020; Dettleff *et al*., 2020).

To gain more insight into the potential function of MRFs, we have cloned Myf5 and Myf6 genes in YTK. Subsequently, we tried to explore tissue distribution of both Myf5 and Myf6 genes, and quantify their transcriptional changes during fish embryonic and juvenile development, as well as in different fish size. Our data will undoubtedly expand our knowledge about fish Myf5 and Myf6 genes, which may provide some novel information for fish muscle growth.

2 Material and Methods

2.1 Experimental Fish and Sample Processing

YTK were taken from Dalian Fugu Aquatic Products Co., Ltd., and reared under standard conditions (18–25℃; salinity at 28–30 ppt; pH 8.0–8.3), feeding with 2%–5% of the body weight of the fish. One year old YTK (body weight 0.5–0.55 kg, body length 38–40 cm), 2 year old YTK (body weight 2–2.5 kg, body length 50–52 cm), 3 year old YTK (7–7.5 kg, body length 93–95 cm) were chosen as samples. After MS-222 (260 mg L^{-1}) anesthesia, rapid dissection, 11 tissues including brain, pituitary, liver, muscles, spleen, kidney, gills, head, stomach, intestinal and head kidney were collected. Tissues were put into liquid nitrogen for quick freezing, and then transferred to −80℃ for storage.

According to the division of embryonic stages of YTK (Xu *et al*., 2019), microscope (NIKON MSZ800, Japan) was used to observe the embryonic development, record the time sequence of development, and collect embryo samples at 18 developmental stages, from fertilized eggs to newly hatched larvae. At the same time, samples of 14 growth and development stages including hatchling juveniles (1 d to 60 d old) were collected. All of the samples were quickly frozen in liquid nitrogen, used for gene expression analysis during embryonic and juvenile juvenile development. All experimental methods were performed following the guidelines for the care and use of experimental animals of China (China's national standard: GB/T358922018).

2.2 Total RNA Extraction and First Strand cDNA Synthesis

RNAiso Plus (TaKara, Japan) was used to extract total RNA from muscle tissue, and the integrity of RNA was detected by 1.2% agarose gel electrophoresis. The RNA concentration was measured using Nanodrop2000 (Thermo, USA). The PrimeScript RT reagent Kit reverse transcription kit (TaKara, Japan) was used to synthesize the first strand of cDNA for amplification of conserved regions. The first strands of 5' RACE and 3' RACE cDNA were synthesized by SMARTerTM RACE cDNA Amplification Kit (Clontech, USA) for subsequent full-length RACE experiments.

Table 1 Nucleotide sequences of primers used for PCR amplification of *S. lalandi*

Primer name	Sequence (5'–3')	Purpose
Myf5-F1	ACCATCCCAGGTCTACT	Fragment amplification
Myf5-R1	CGCCCAAACTCTCATTC	Fragment amplification
Myf5-5'-F1	TGACCTTCTTCAGCCGCCG	5'RACE, 1st PCR
Myf5-5'-F2	ACGTGCTCGTCCTCCTCTG	5'RACE, 2nd PCR
Myf5-3'-F1	CCAACCCGAGCCAACGCCTG	3'RACE, 1st PCR
Myf5-3'-F2	AAAGAATGAGAGTTTGGGCG	3'RACE, 2nd PCR
Myf6-F1	CGCTACTTGGAGGAAGG	Fragment amplification
Myf6-R1	GTCGCTGGTGATGCTGT	Fragment amplification
Myf6-5'-R1	AGGGCTCAGACGACTTCTTCCA	5'RACE, 1st PCR
Myf6-5'-R2	GGCTGTCGTTGCCGTTGT	5'RACE, 2nd PCR
Myf6-3'-F1	CCAACCCAAACCAGAGGC	3'RACE, 1st PCR
Myf6-3'-F2	CCGACCTCTGCTGACCAT	3'RACE, 2nd PCR
18S-F	TACCACATCCAAAGAAGGCA	qRT-PCR
18S-R	TCGATCCCGAGATCCAACTA	qRT-PCR
Myf5 -F1	CCTGCCCAAGGTGGAGAT	qRT-PCR
Myf5-R1	GCCGCCCAAACTCTCATT	qRT-PCR
Myf6-F1	CCAACCCAAACCAGAGGCTAC	qRT-PCR
Myf6-R1	AGGGCTCAGACGACTTCTTCCAG	qRT-PCR

The primers used for gene cloning are shown in Table 1. Amplification of intermediate fragments using muscle cDNA as template, PCR reaction system (25 μL): 2 μL 10 × PCR buffer, 2 μL MyoD1-F, 2 μL MyoD1-R, 0.5 μL Taq enzyme, 2.5 μL dNTP mixture, 2 μL template, and 14 μL ddH_2O. The PCR product was detected by 1.2% agarose gel electrophoresis. The target band was recovered by EZNA™ Gel Extraction Kit Manual (Omega, USA), and quickly connected to the pEASY-T1 vector, and then transferred to Transl T1 competent cells (whole-type gold, China) were cultured in a 37℃ incubator overnight, and positive clones were picked and sent to Biotech (Shanghai) Co., Ltd. for sequencing. The full length gene sequences were obtained using nested PCR reaction (primers are shown in Table 1).

2.3 Protein Sequence Alignment and Phylogenetic Analysis

Expasy online database prediction (www.expasy.org/tools/protparam.html) was used to predict the structure, molecular weight and isoelectric point of related genes. The software DNAMAN6.0, SignalP4.1 (http://www.cbs.dtu.dk/services/SignalIP) was used for amino acid sequence derivation, splicing and homology analysis to predict the signal peptide position. PSORT II software (https://psort.hgc.jp/form2.html) was used for subcellular localization analysis. NCBI database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used for domain prediction, and ClustalW online software (http://www.genome.jp/tools-bin/clustalw) was chosen to analyze amino acid sequence alignment, MEGA 6 software was used for system evolution analysis (the self-expansion value was 1000). A neighbour-joining tree was constructed based on the deduced amino acid sequences of other reported species using MEGA 6.0 software.

2.4 Expression Analysis of Myf5 and Myf6 by Real-Time Quantitative PCR

According to the Myf5 and Myf6 sequences and 18S rRNA (EU047719.1), the real-time quantitative PCR primers of YTK Myf5 and Myf6 genes were designed by the Premier 5.0 software (shown in Table 1). Total RNA was extracted from different tissues, embryos and juveniles of different developmental stages with RNAiso Plus (TaKara, Japan) according to the manufacturer instructions. Total RNA was first treated with DNase I (Invitrogen, CA, USA) to remove any genomic DNA contamination. The cDNA was synthesized using the Takara PrimerScript™ First Strand cDNA Synthesis kit (TaKaRa, China) according to the manufacturer's instructions. Then, the mRNA expression levels of Myf5 and Myf6 genes were evaluated using a relative quantitative real-time RT-PCR assay with SYBR Premix Ex Taq™ II (TaKaRa, China). The real-time quantitative PCR reaction condi-

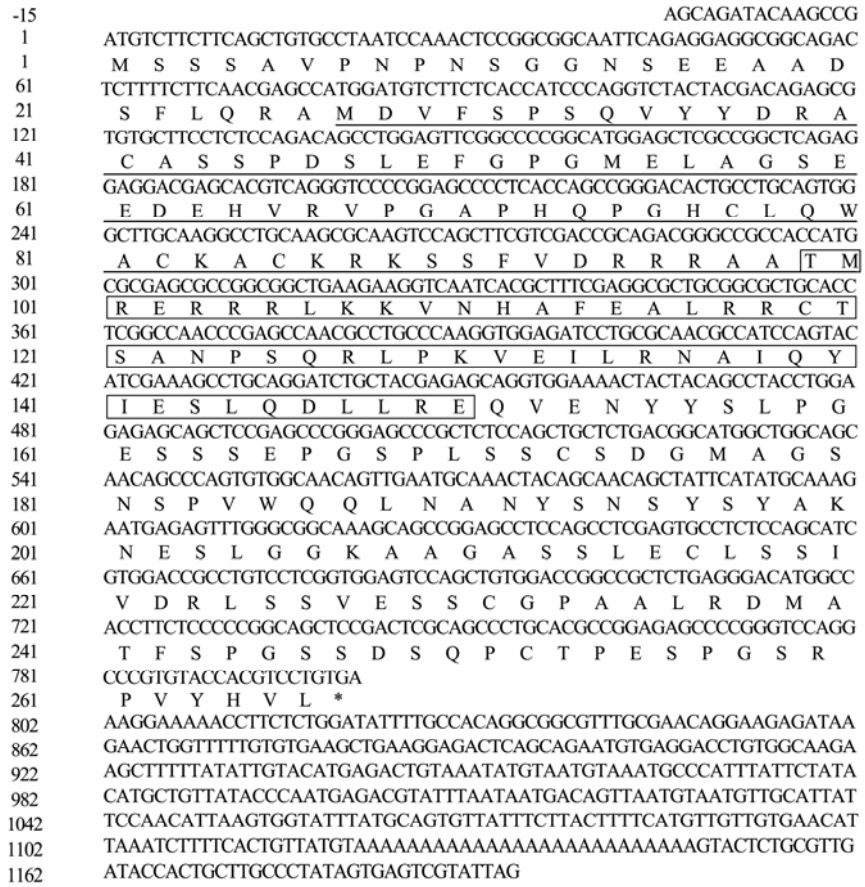

Fig.1 The full-length cDNA sequence of *S. lalandi* Myf5 gene and deduced amino acid sequence. The underline represents Basic region, and the box represents HLH region.

tions were shown as follows: pre-denatured at 95℃ for 30 s, denatured at 95℃ for 5 s, and annealed at 60℃ for 20 s, 72℃ for 30 s, 40 cycles. The relative expression of Myf5 and Myf6 mRNA of different tissues was calculated by $2^{-\triangle\triangle Ct}$ method.

2.5 Statistical Analysis

The mean ± standard deviation (Mean ± SD) is used to represent the obtained experimental data. For comparison between multiple sets of data, SPSS 19.0 statistical software is used for data analysis (one-way ANOVA, Duncan comparative analysis, $P<0.05$).

3 Result

3.1 Gene Identification and Sequences Analysis

The full length of Myf5 cDNA obtained 951bp (GenBank accession number: MK101226), including 15 bp5' UTR, 801bp ORF and 330 bp 3'UTR, encoding 266 amino acids, the protein predicted molecular weight 28749.81 and isoelectric point 6.0. There are two distinct domains in Myf5, one is the Basic region (27–98), and the other is its HLH domain (99–150) (Fig.1). The full length of Myf6 cDNA obtained 1105 bp (GenBank accession number: MK036016), including 166 bp 5'UTR, 753 bp ORF and 186 bp 3'UTR, encoding 250 amino acids, the protein predicted molecular weight 27323.58, and isoelectric point 6.82, Myf6 also contained two distinct domains, the HLH domain (102–153) and its extended structure basic region (2–101, Fig.2), the protein predicted molecular weight 26586.50, and isoelectric point 4.91.

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-73     GGCGGTATTGAA
-60     GCTGCCCTTAAGCAGTGGTAACAACGCAGAGTCCATGGGGAGAGAGAGGGGCGCAACAAT
1       ATGATGGACCTTTTTGAGACCAACACTTATCTTTTCAGTGATTTACGCTACTTGGAGGAA
1       M  M  D  L  F  E  T  N  T  Y  L  F  S  D  L  R  Y  L  E  E
61      GGGGATCATGGACCACTACAACACTTGGACATGGCGGGGGTGTCCCCTCTGTACAACGGC
21      G  D  H  G  P  L  Q  H  L  D  M  A  G  V  S  P  L  Y  N  G
121     AACGACAGCCCGCTGTCTCCGGGCCAGGATAACGTTCCGTCCGAGACCGGCGGGGAGAGC
41      N  D  S  P  L  S  P  G  Q  D  N  V  P  S  E  T  G  G  E  S
181     AGCGGGGAGGAGCACGTCCTCGCGCCGCCAGGACTCCGCGCGCACTGCGAGGGCCAGTGC
61      S  G  E  E  H  V  L  A  P  P  G  L  R  A  H  C  E  G  Q  C
241     CTCATGTGGGCCTGCAAGATCTGCAAGAGAAAGTCGGCGCCCACCGACAGACGCAAGGCC
81      L  M  W  A  C  K  I  C  K  R  K  S  A  P  T  D  R  R  K  A
301     GCCACGCTCAGGGAGAGGAGGAGGCTCAAGAAGATCAACGAGGCCTTCGACGCGCTGAAG
101     A  T  L  R  E  R  R  R  L  K  K  I  N  E  A  F  D  A  L  K
361     AGGAAGACCGTGGCCAACCCAAACCAGAGGCTACCCAAGGTGGAGATTTTACGCAGCGCC
121     R  K  T  V  A  N  P  N  Q  R  L  P  K  V  E  I  L  R  S  A
421     ATCAGCTACATTGAGAGATTACAGGACCTGCTGCAAACGCTGGACGAGCAGGAGAAAAGC
141     I  S  Y  I  E  R  L  Q  D  L  L  Q  T  L  D  E  Q  E  K  S
481     CAAAACGGATCATCCCATAACTCTAAAGAACTCAGTGTGGCCAGTCATGAGTACCACTGG
161     Q  N  G  S  S  H  N  S  K  E  L  S  V  A  S  H  E  Y  H  W
541     AAGAAGTCGTCTGAGCCCTGGCCGACCTCTGCTGACCATTCCAATGCAGCAATGATAGAC
181     K  K  S  S  E  P  W  P  T  S  A  D  H  S  N  A  A  M  I  D
601     CAGAGAGAAGGAACCAGTGAGTCCTCCGCATCCTCCAGCCTCCTGCGTCTGTCCTCCATC
201     Q  R  E  G  T  S  E  S  S  A  S  S  S  L  L  R  L  S  S  I
661     GTGGACAGCATCACCAGCGACGAGAAGATCAGCTTCAGCGGGGACGTGTCAGAGAACTGA
221     V  D  S  I  T  S  D  E  K  I  S  F  S  G  D  V  S  E  N  *
721     GACGTCGGCGAAGGCCTTTAAGCCGACAGCGTATTTAGGATTTTCAAAATTTCCATTGTT
781     TATCTCTAAATTATTTCCACGATAGTCAGCCGTCCTGGCTTCTGTTTTGTCGTGTGGCCT
841     TCATTTGTGCAGCAGGAGCAGCAAATATGTACATATTTTATAATAATGTCAAGTTTAGAT
901     TTATTTATTTTGTATTCAAAGGCAGATGAGCTCTCTAAAAAAAACAAAAAAACCTGAGAT
961     TGTATTTAATTATTTGTACATAACTGGCCGTGAAAATAAAATGCTACTTTTGCAATATAA
1021    AAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCCGGGTTGGTACCCCTGGTTAAGGGCAA
1081    CTTGGCGTAATCCTGGGTATAAGTGGTTCCTGGGGGGAAATTGGTATCCGCTCCACATTCC
1141    CACCCACATACGGAGCGGAAGCATAAAAGGGTAAGCCCGGGGTGGCTAATGGATGAACAC
1201    TTAATTGGCGTGGCCTTACTGGCCGCTTTCCCAGCGGGAAAACTGGCGTGGCCAGTGGCT
1261    TAATGGATCCGCCCACCGGGGGGGGAAAAGCCGGTTGGCTAATGGGGCCTCCTCCGCTTCC
1321    CCCCCCACTGAATCCGTGCGCTCCGTCCGTTCGCTGGCGGCCGACGGTATCCACTTCCTT
1381    CAAGGGGGGTATACCGGTATCCCCCAGAATCCAGGGGATAACCGCAGGAAAGAAACCATT
1441    GTGAAGCAAAGGCCAGCAAAACGGCCAGGAACCCGTAAAAAGGCGCGTTTGCCTGGCCGT
1501    TTTTCCAATAGGCCTCCGCCCCCCCTTGACGAGCATCCCAAAAATCGACGCTCAAGGTCA
1561    GAAGGTGGGCGAAACCCGAAAGGACTATTAAGAAACAGGCGTTTCCCCTGAGCTCTCGTG
1621    CGCTCTCTCTGTTCGACTGCGCTTACGAAACTGTCGCATCTCCTCGAGCTGCCCTTTCTC
1781    TAGCTCAGCGTAGGATTCTCAGTTCGGGTAGTCGTACGCTCAAGCTGGCTGGGTGACGAC
1841    TTGGGATCG
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Fig.2 The full-length cDNA sequence of *S. lalandi* Myf6 gene and deduced amino acid sequence. The underline represents Basic region, and the box represents HLH region.

3.2 Phylogenetic Analysis of Myf5 and Myf6

The multiple alignment results of amino acid sequences

of YTK Myf5 and Myf6 of different species are shown in the figure below (Fig.3). The deduced amino acid sequence with other fish species showed well conserved regions across the sequence (Fig.3). The Neighbour-joining tree constructed based on multiple sequence alignment revealed that YTK is closely related to *S. dumerili* (Fig.4). Phylogenetic analysis showed that YTK Myf5 and Myf6 shared high amino acid identity (>90%) with *Seriola dumerili*, while only 52.24%–62.10% identity with mammals.

Fig.3 Comparison of deduced amino acids equences of Myf5 and Myf6 of *S. lalandi* with other published Myf5 and Myf6. The Basic region is indicated by single line. The predicted bHLH domain is indicated by double line. Identical amino acid residues are represented by stars.

3.3 Tissue Distribution Using Real-Time PCR (qRT-PCR)

The qRT-PCR showed that Myf5 and Myf6 were expressed highest in muscle tissues, indicating that Myf5 and Myf6 mainly played roles in muscles (Fig.5A and 5B). In addition, Myf5 is highly expressed in gill and stomach, but Myf6 is highly expressed in head and stomach (Figs.5A and 5B). Both of the Myf5 and Myf6 genes expressed in kidney are low (Fig.5A and 5B).

3.4 Expression Levels of Myf5 and Myf6 in YTK in Different Tissues in Various Sizes

Our results showed that Myf5 mRNA had no significant difference in the 50 g weight fish in brain, pituitary, liver and muscle. The highest expression level of Myf5 was shown in the 400 g weight fish ($P<0.05$; Fig.6A) in both of pituitary and muscle. In addition, the expression of Myf5 gene in the pituitary is significantly higher than that in brain, liver and muscle in 500 g fish samples ($P<0.05$; Fig.6A). Meanwhile, for Myf6 gene, there was no significant change in the expression level of YTK Myf6 mRNA in the brain ($P<0.05$; Fig.6B). The expression level of Myf6 gene in 400 g fish was also significantly higher in the pituitary and muscle than that in the brain and liver, which consistent with the Myf5 gene results. The highest expression level of Myf6 gene in the 500 g stage of the body weight was seen in muscle ($P<0.05$; Fig.6B).

3.5 Expression Levels of Myf5 and Myf6 in YTK During Embryogenesis

The gene expression level of Myf5 began to increase from middle gastrula embryo stage, reached its highest level at neural embryo stage, and then began to decrease ($P<0.05$; Fig.7A). However, the expression level of Myf6 was higher at fertilized egg stage, and sharply decreased at embryo encompassing yolk 50% stage, then reached

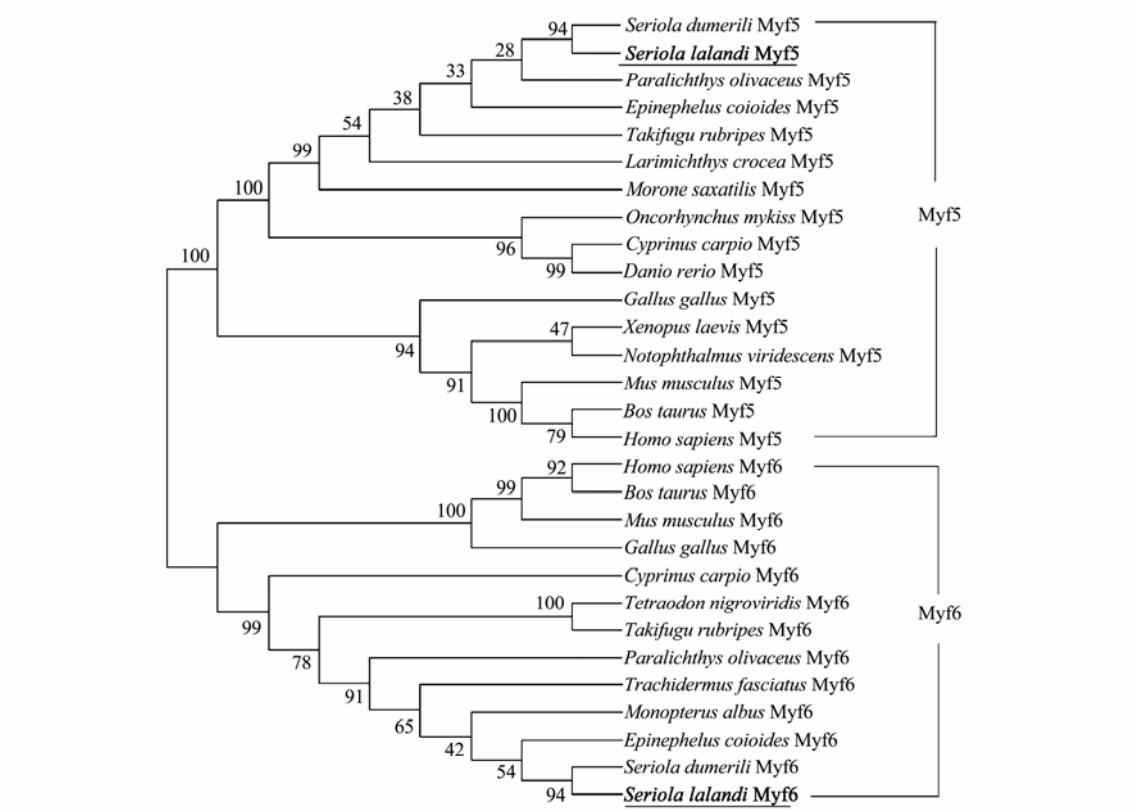

Fig.4 Phylogenetic tree for Myf5 and Myf6 amino acid sequences of mammals and teleosts. The tree was generated by MEGA 6.0 software using the neighbor-joining method, following Clustal X. Scale bar indicates an evolutionary distance of 0.01 amino acid substitution per position in the sequence. Bootstrap values are indicated (1000 replicates). GenBank ID for Myf5 mammalians: *Homo sapiens* NP_005584.2, *Bos taurus* AAA51415.1, *Mus musculus* NP_032682.1; for birds: *Gallus gallus* CAA51712.1; for amphibian: *Xenopus laevis* CAA40062.1, *Notophthalmus viridescens* CAA58045.1; for fish: *Takifugu rubripes* AAR20811.1, *Oncorhynchus mykiss* AAV30214.1, *Paralichthys olivaceus* ABI96686.1, *Morone saxatilis* AAL66387.1, *Epinephelus coioides* AMR58947.1, *Cyprinus carpio* BAA33566.1, *Seriola lalandi* XP_0232688 83.1, *Danio rerio* AAI65074.1, *Seriola dumerili* XP_022605279.1, *Larimichthys crocea* XP_019132416.2. GenBank ID for Myf6 mammalians: *Homo sapiens* CAG46563.1, *Bos taurus* NP_861527.1, *Mus musculus* NP_032683.1; for birds: *Gallus gallus* NP_001025917.1; for fish: *Seriola lalandi* XP_023268934.1, *Seriola dumerili* XP_022605203.1, *Paralichthys olivaceus* 961011.1, *Takifugu rubripes* NM_001032771.1, *Epinephelus coioides* HM190248.1, *Cyprinus carpio* GU 339054.1, *Tetraodon nigroviridis* AY576806.1, *Monopterus albus* AIS22055.1, *Trachidermus fasciatus* AFP28938.1.

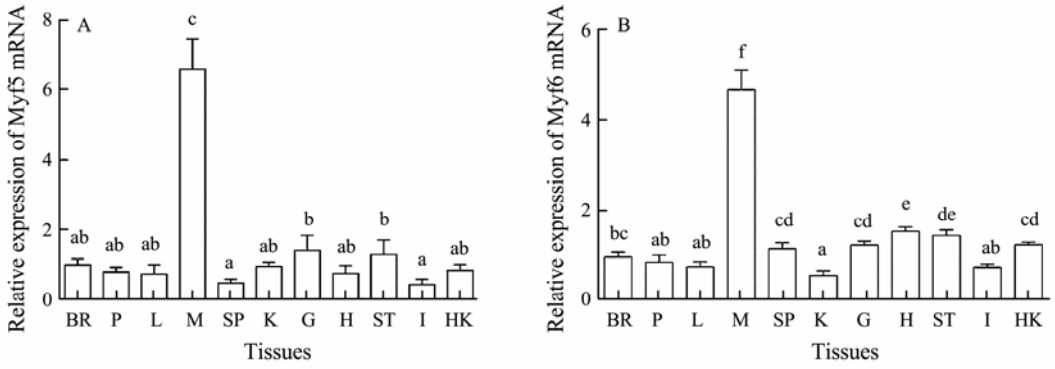

Fig.5 The expression levels of *S. lalandi* Myf5 (A) and Myf6 (B) mRNA in different tissues. BR, brain; P, pituitary; L, liver; M, muscle; SP, spleen; K, kidney; G, gill; H, head; ST, stomach; I, intestinal; HK, head kidney.

the highest value at yolk 70% stage, and then decreased from yolk 100% stage ($P<0.05$; Fig.7B).

3.6 Expression Levels of Myf5 and Myf6 During Development

Our results showed that the expression level of Myf5 mRNA increased after hatching out of the membrane. Then increased significantly after 20 d, peaked at 30 d of age, then significantly decreased and maintained a lower expression level ($P<0.05$; Fig.8A). The expression level of Myf6 mRNA was low at 0–40 d, and began to increase significantly at 45 d, and maintained a high expression

until it reached a peak at 60 d (P <0.05; Fig.8B).

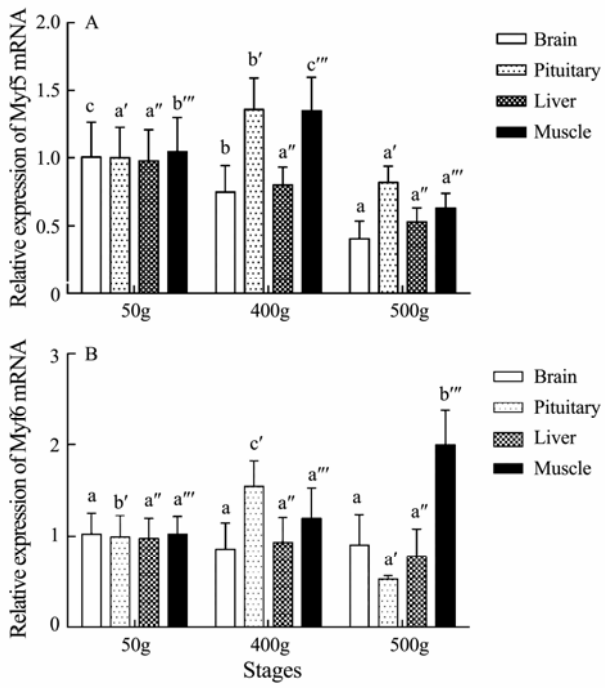

Fig.6 The expression levels of *S. lalandi* Myf5 (A) and Myf6 (B) mRNA in brain, pituitary, liver and muscle in different size.

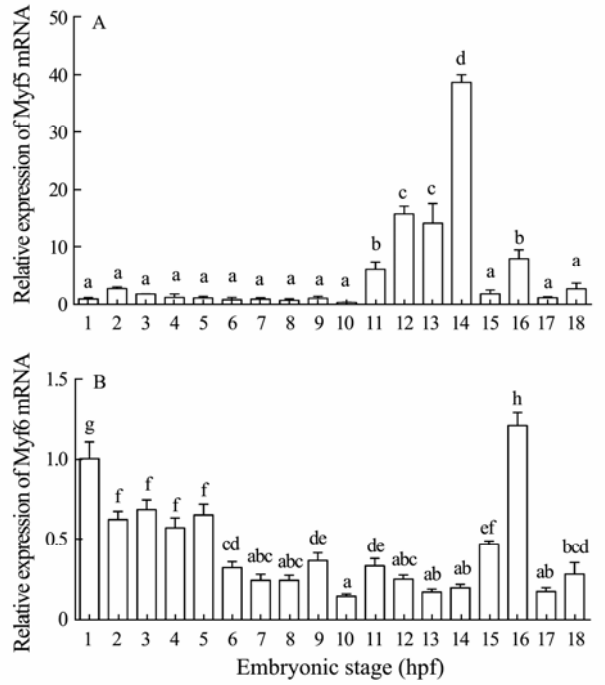

Fig.7 The expression levels of *S. lalandi* Myf5 (A) and Myf6 (B) mRNA in different embryonic stages. (1), Fertilized egg; (2), 2 cells egg; (3), 4 cell egg; (4), 8 cell egg; (5), 16 cell egg; (6), 32 cell egg; (7), multi-cell stage; (8), mulberry embryo; (9), high blastocyst; (10), low blastocyst; (11), early gastrula embryo; (12), middle gastrula embryo; (13), late gastrula embryo; (14), neural embryo stage; (15), embryo encompassing yolk 50%; (16), embryo encompassing yolk 70%; (17), embryo encompassing yolk 100%; (18), newly hatched larva.

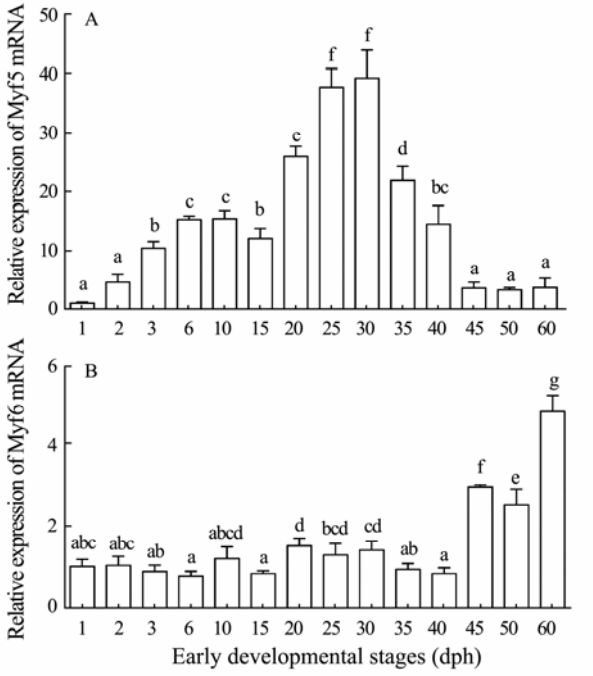

Fig.8 The expression levels of *S. lalandi* Myf5 (A) and Myf6 (B) mRNA in larval and juvenile development stages.

4 Discussion

In the present study, we identified and characterized two important myogenic regulators of muscle growth, Myf5 and Myf6 in yellowtail kingfish (*S. lalandi*). In addition, we have elucidated the transcriptional regulation of the two genes in different stages and tissues. It has been found that the sequence of Myf5 and Myf6 were similar to most cyprinids fish species (Fig.3), suggesting a good conservation in the size and constitution of the protein (Rajesh *et al*., 2019). In this study, the cDNA coding regions of the Myf5 and Myf6 have been determined in the YTK. YTK Myf5 and Myf6 shared high amino acid identity (>90%) with greater amberjack (*Seriola dumerili*), while only 52.24–62.10% identity with mammal such as mouse and human Myf5 and Myf6. The polyadenylation signal (AATAAA) is located in the region 18 bp upstream of the poly (A) tail of the Myf6 sequence, but lost in Myf5 sequence (Figs.1 and 2). The deduced Myf5 and Myf6 protein in YTK had very similar functional domain (helix-loop-helix domain (bHLH)) with other species Myf5 and Myf6 (Fig.3). In addition, both of the two proteins contained a myogenic basic domain and a specific myogenic determination factor 5 domain in C terminal structure. Phylogenetic analysis of Myf5 and Myf6 with other teleosts indicated that both of Myf5 and Myf6 cluster with *Seriola* (Fig.3), especially *S. dumerili*.

In addition, the tissue-specific expression pattern revealed that YTK Myf5 and Myf6 mRNAs were highest in muscle. This was also observed in *Sparus aurata* (Codina *et al*., 2008), *Megalobrama amblycephala* (Zhu *et al*., 2014), *Siniperca chuatsi* (Chu *et al*., 2014) and *Schizothorax prenanti* (Lin *et al*., 2016). Meanwhile, the results of real-

time fluorescence quantification showed that these two genes are also expressed in other tissues, including brain, muscle, spleen, kidney, heart and kidney, as reported by other studies (Zhang *et al*., 2006; Zhu *et al*., 2016) demonstrated that the expression in the eyes could be attributed to the presence of a small group of extraocular muscles. However, comparing younger fish and elder fish, it has been found that only 400 g YTK and 500 g YTK showed higher expression of Myf5 and Myf6 in muscle, respectively (Fig.6). Generally, YTK is considered as fast growers (Bowyer *et al*., 2012), fish apparently indicated size dependent differential growth in terms of absolute weight gain (Rajesh *et al*., 2019). Our results showed that expression of myogenic regulatory factors had a great relationship with the size of fish, so it is particularly important to analyze the expression level in growth and development stages.

Studies of *Lateolabrax japonicus* gene expression during development showed that Myf5 increased at the early gastrointestinal embryo stage, which increased gene expression began after the multi-cell stage (Ye *et al*., 2007). Our results indicated that *S. lalandi* had the highest Myf5 expression during the neuroembryo period, which was similar to the results of Chinese perch (*Siniperca chuatsi*) (Zhu *et al*., 2016). In our study, Myf6 had the highest expression level at embryo coverage yolk 70% stage, it was different from the *S. chuatsi* result which Myf6 was reached higher level at muscular effect stage, but sustained lower level at other stages (Zhu *et al*., 2016). These results indicated that Myf5 and Myf6 have different functions during the embryonic development. It has been well known that Myf5 is participated in establishment and maintenance of muscle progenitor lineage, therefore, higher level of Myf5 expression after neurula stage confirmed this (Emerson, 1990). In this study, the Myf6 expressed at embryo coverage yolk 70% stage suggested Myf6 was mainly involved in terminal differentiation myotubes (Hinits *et al*., 2007). During juvenile stage, it has been found that the expression level of Myf5 gene was highest at 30 d of age (metamorphosis period of YTK). It has been suggested that Myf5 gene played key roles in myogenic period, when requiring a large amount of skeletal muscle formation (Rudnicki *et al*., 1993; Sabourin and Rudnicki, 2000). However, the expression level of Myf6 gene was the highest at 60 d, indicating that the gene functions in the late stage of muscle formation.

5 Conclusions

We have cloned and characterized Myf5 and Myf6 of the yellowtail kingfish (*Seriola lalandi*) and confirmed that it is played key roles in myogenesis. Our alinment suggested that Myf5 and Myf6 shared an analogous structure in highly conserved bHLH domains with other vertebrates. Tissue expression patterns revealed a peak level of these two genes mRNA in muscle. For different size, 400 g YTK and 500 g YTK showed higher expression of Myf5 and Myf6 in muscle, respectively. Spatiotemporal expression patterns indicated the different levels of two genes mRNA during embryo and juvenile development. *S. lalandi* Myf5 had the highest expression during the neuroembryo period and 30 d of age, however, Myf6 had the highest expression level at embryo coverage yolk 70% and 60 d stage. Our data presents a first step in elucidating the potential biological role of yellowtail kingfish Myf5 and Myf6. It furthermore provides information that Myf5 and Myf6 played different roles during embryo and juvenile development.

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