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Research Article

Expression Pattern of Myogenic Regulatory Transcription Factor mRNAs in the Embryo and Adult *Labeo rohita* (Hamilton, 1822)

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Understanding the regulation of skeletal muscle development is important to meet the increasing demand of Indian major carp *Labeo rohita*. Myogenic regulatory factors (MRFs) along with myocyte specific enhancer factor 2 (MEF2) play the pivotal role in the determination and differentiation of skeletal muscle. The majority of skeletal muscle genes require both MRFs and MEF2 family members to activate their transcription. In this study, the expression pattern of MyoD, myf-5, myogenin, and MEF2A was observed from 6 h after fertilization to 12 months of age using semiquantitative RT-PCR as well as real-time PCR method. MyoD and myf-5 mRNAs were expressed at high level at the early embryonic stages. Myogenin and MEF2A were expressed after MyoD and myf-5 and remained active up to adult stage. Expression of MyoD was lower than that of Myf-5 after the 5th month. Partial sequencing of MyoD, myf-5, and MEF2A was done to draw phylogeny. In phylogenetic study, *Labeo* MyoD, MEF2A and myf-5 were found to be closely related to those of common carp. The present investigation suggests that the four transcription factors play pivotal role in the regulation of muscle growth of *Labeo rohita* in an overlapping and interconnected way.

1. Introduction

Rohu *Labeo rohita* (Hamilton 1822) is one of the most important economic carps in India and other South East Asian countries. With increasing demand, more studies are required on growth and differentiation of skeletal muscle to improve the growth rate of this fish. The understanding of the regulation of embryonic and postnatal skeletal muscle growth and development is extremely important in this regard [1]. During vertebrate embryogenesis, skeletal muscle is derived from somites, which is formed by segmentation of the paraxial mesoderm lateral to neural tube [2]. The trunk musculature of fish is originated from the segmental plate mesoderm flanking the notochord and lying underneath the presumptive nerve cord. Studies in zebrafish (*Brachydanio rerio*) have revealed that the most medial cells in the segmental plate, called adaxial cells, commit to become myoblasts with a slow muscle lineage and the fast muscle fibres are derived from the lateral presomitic mesoderm by fusion of several myoblasts to form multinucleated myotubes [3]. Development and growth of skeletal muscle are complex dynamic processes involving both the recruitment of new muscle fibres (hyperplasia) and growth of existing fibres (hypertrophy) [4]. Fish have an ability to recruit new skeletal muscle fibres throughout the larval life and even during juvenile and adult life [5]. Both hyperplasia and hypertrophy occur during myogenesis in larval and adult muscle growth of fish which reach a large adult size [6].

The expression of genes in skeletal, cardiac, and smooth muscle cells could be controlled by a shared myogenic regulatory programme [7]. Myogenic regulatory factors (MRFs), family of basic helix-loop-helix (bHLH) transcription factors, play the pivotal role in the determination and differentiation of skeletal muscle and they have the property of converting a variety of cells into myoblasts and myotubes [8]. Members of this gene family like MyoD, myf-5, and myogenin have been identified in many fish species and found to be expressed in developing somites and skeletal muscles [9–11] though no report is available in any Indian carp species. MyoD and myf-5 play a common role in establishing myoblast identity, whereas myogenin is involved in terminal differentiation. MRF genes are specifically expressed in myoblast cells and regulate expression of different muscle proteins and enzymes like myosin, troponin, and creatine kinase [12]. MRFs form heterodimers with E-proteins and bind to a consensus DNA sequence known as E box present in the control region of many skeletal muscle genes. Myocyte specific enhancer factor 2 (MEF2), family of transcription factors, is another important regulator of skeletal muscle differentiation. The cloning of genes encoding MEF2 factors revealed that these proteins belong to the MADS box family of transcription factors. Multiple isoforms of MEF2 have been identified in vertebrates, all of which possess a specific DNA binding domain characteristic of the MADS box gene family and a highly conserved MEF2 specific sequence [9]. MyoD and MEF2 family members function in a combined way to activate myogenesis. Consistent with these observations, the majority of skeletal muscle genes require both MyoD and MEF2 family members to activate their transcription [13]. Many studies revealed that dynamics of skeletal muscle growth can be affected by several external factors like photoperiod [14], temperature variation [15], and dietary treatment [4]. But no report is available on age specific expression pattern of these genes during growth of *Labeo rohita* in natural condition.

In our study, we have assessed the expression of four important regulatory transcription factors in *Labeo rohita*. mRNA expression pattern of MyoD, myf-5, myogenin, and MEF2A genes from 6 h postfertilization to 12 months of age was monitored at 16 time points by semiquantitative RT-PCR followed by qRT-PCR at 5 time points. To the best of our knowledge, this is the first study on the expression pattern of myogenic regulatory factors both in embryo and adult *Labeo rohita*. This study is important for a better understanding of the molecular mechanisms of regulation of skeletal muscle growth of this important Indian major carp.

2. Materials and Methods

2.1. Chemicals and Reagents. TRI reagent for RNA isolation was procured from Sigma-Aldrich Corp (St. Louis, MO, USA). Reverse transcriptase and all chemicals of PCR mix were purchased from Fermentas (USA). All other chemicals used were of analytical grade and purchased from Sisco Research Laboratories (Mumbai, India) and Merck (Darmstadt, Germany). Custom designed primers were synthesized from Sigma-Aldrich Corp (St. Louis, MO, USA).

2.2. Maintenance of Fish and Sample Collection. Fish were maintained and cultured in a pond at a local fish farm, in Birbhum, India. Embryos were collected and pooled for RNA isolation. Fingerlings of same batch were maintained in a stocking pond provided with standard diet. Fish from 1 month of age to 12 months age were collected in the first week of every month from the stocking pond. Dorsal skeletal muscle was dissected from each fish and processed for RNA isolation. Length and weight of each fish were recorded monthly for 12 months. From each age group 6 individuals were taken randomly for further experiments.

2.3. RNA Extraction. Approximately 100 mg of pooled whole embryo or dorsal white muscle fragments (1-month–12-month sample) was mechanically homogenized with 1 mL of the TRI reagent (Sigma, St. Louis, MO, USA) and the total RNA was extracted according to the manufacturer's instructions and stored in DEPC treated nuclease-free water at −20°C. RNA was pooled for each age group. Samples were subjected to electrophoresis on 1% agarose gels to confirm the integrity of the 28S and 18S rRNA bands. RNA quality was assessed as the 260/280 nm absorbance ratio and RNA was quantified from absorbance at 260 nm.

2.4. cDNA Synthesis through RT-PCR. Single-strand cDNA was reverse-transcribed from equal amounts of total RNA (5 μg) using an oligo-dT_{18} primer and reverse transcriptase (Fermentas) through RT-PCR. The reaction mixture contained 4 μL of reverse transcriptase buffer (5 × RT Buffer), 0.5 μL (20 U) of ribonuclease inhibitor (RiboLock, Fermentas), 2 μL of dNTP Mix (10 mM each), 0.5 μg of oligo-dT_{18} primer, and 1 μL (200 U) of RevertAid H minus reverse transcriptase (Fermentas). Reaction was carried out following manufacturer's protocol.

2.5. PCR Amplification of Myogenic Regulatory Genes. For partial amplification of MyoD, myf-5, myogenin, and MEF2A gene specific primer pairs were designed using Primer 3 software (version 0.4.0) based on sequences of carp available in GenBank (http://www.ncbi.nlm.nih.gov). β-Actin was amplified simultaneously as an internal control and the primers for β-actin were adopted from Li et al. 2004 [16]. The primer sequences are given in Table 1.

The PCR was performed following the procedure as per the manufacturer's instruction for 35 cycles. All test samples were amplified simultaneously from equal volume of first strand cDNA with the particular primer pair using a master PCR mix. For each reaction, master PCR mix contained PCR buffer, 0.2 mM of dNTPs, 25 mM $MgCl_2$, 0.2 mM of each primer, template cDNA, and 1.0 unit of *Taq* DNA polymerase (Fermentas). PCR reactions were run in a programmable thermal cycler (GeneAmp 9700, ABI) with simultaneous NTC (no template control). The PCR products were run in 1.5% agarose gel and visualized in a gel documentation system (Gel Doc EZ Imager, Bio-Rad) after staining with ethidium bromide. The densitometric quantification was done using ImageJ (NIH) software.

Table 1: Primers used for polymerase chain reaction amplification of *Labeo* MyoD, myf-5, myogenin, MEF2A, and β-actin.

Gene		Primer sequence	Annealing temperature	Source sequence for primer designing (accession numbers are given)	Position of primers in source sequence
MyoD	Forward	5′-CGACTGAGCAAAGTCAACGA-3′	59.0°C	*Ctenopharyngodon idella* mRNA for MyoD	296–315
	Reverse	5′-TTCCGTCTTCTCGACTGACA-3′		(GenBank accession: JQ793893.1)	561–542
myf-5	Forward	5′-GCCAGGTCACTGTCTGCAAT-3′	58.5°C	*Cyprinus carpio* mRNA for myf-5	202–221
	Reverse	5′-GCTCAGAGCTGCTTTCCATT-3′		(GenBank accession: AB012883.1)	479–460
Myogenin	Forward	5′-GGCTTCGACCAAACAGGATA-3′	59.0°C	*Cyprinus carpio* mRNA for myogenin	232–251
	Reverse	5′-GCTCCTGGTGAGGAGACAAG-3′		(GenBank accession: AB012881.1)	376–357
MEF2A	Forward	5′-ACGGATCATGGATGAGAGGA-3′	58.8°C	*Cyprinus carpio* mRNA for MEF2A	199–218
	Reverse	5′-TGACCGAAACAGTCATCTGG-3′		(GenBank accession: AB012884.1)	492–473
β-Actin	Forward	5′-TGGAATCCTGTGGCATCCATGAAAC-3′	66.0°C	Li et al. 2004 [16]	
	Reverse	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′			

Table 2: Primers used for qRT-PCR of *Labeo* MyoD, myf-5, myogenin, MEF2A, and GAPDH.

Gene		Primer sequence	Annealing temperature	Source sequence for primer designing (accession numbers are given)	Position of primers in source sequence
MyoD	Forward	5′-TCCAAGCGCTGCTAAGAAGT-3′	59.0°C	*Labeo rohita* partial mRNA for MyoD	132–151
	Reverse	5′-CATCATGCCATCAGAGCAGT-3′		(GenBank accession: KC344537.1)	241–222
myf-5	Forward	5′-GCAGGCTGAAGAAGGTGAAC-3′	59.0°C	*Labeo rohita* partial mRNA for myf-5	96–115
	Reverse	5′-GGCTTCCTCAGGATCTCAAC-3′		(GenBank accession: KC344536.1)	195–176
Myogenin	Forward	5′-GGCTTCGACCAAACAGGATA-3′	59.0°C	*Cyprinus carpio* mRNA for myogenin	232–251
	Reverse	5′-GCTCCTGGTGAGGAGACAAG-3′		(GenBank accession: AB012881.1)	376–357
MEF2A	Forward	5′-TGACTGTGAGATTGCCCTGA-3′	59.0°C	*Labeo rohita* partial mRNA for MEF2A	94–113
	Reverse	5′-CGTGGGGTTCGTTGTATTCT-3′		(GenBank accession: KC344535.1)	206–187
GAPDH	Forward	5′-AGGGGCTCAGTATGTTGTGG-3′	59.0°C	*Cyprinus carpio* partial mRNA for GAPDH	257–276
	Reverse	5′-CTCTCTTGGCACCACCCTTA-3′		(GenBank accession: AJ870982.1)	342–323

The PCR products of MyoD, myf-5, and MEF2A were subjected to partial sequence analysis to confirm the genes. Sequencing was done by Xcelris Genomics Pvt Ltd. Nucleotide sequences were translated into amino acid sequences for further analysis. Sequence alignments were obtained using Clustal Omega software (EMBL-EBI; http://www.ebi.ac.uk). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [17]. Phylogenetic tree was reconstructed using neighbour-joining distance algorithms from translated amino acid sequences in PAM matrix. Statistical consistency was evaluated by 1000 bootstrap resamplings of the data.

2.6. Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed using a Bio Rad CFX96 Real-Time PCR System (Bio Rad Laboratories Inc., Hercules, CA, USA) and an iQ SYBER Green super Mix Kit (Bio Rad Laboratories Inc., Hercules, CA, USA), which were used in accordance with the manufacturers' instructions. Standard reaction mixtures (15 μL) were assembled using 7.5 μL of iQ SYBER Green super Mix 2x, 300 nM of each primer, and 100 ng of template cDNA. For amplification of MyoD, myf-5, myogenin, MEF2A, and GAPDH gene specific primer pairs were designed (Table 2) using Primer 3 software (version 0.4.0) based on sequences of carp available in GenBank (http://www.ncbi.nlm.nih.gov).

Relative gene expression values were obtained using Bio Rad CFX manager software (Version 2.1).

3. Results

3.1. Fish Growth. Mean weight of fish increased at a steady rate up to 6 months of age. During winter season (6 months to 8 months of age), the rate of monthly weight gain was reduced marginally which again recovered during 9th- to 12th-month period. There was a net increase in mean length in all the time points. After 12 months the average weight of fish was around 700 g and length was approximately 40 cm (Figure 1).

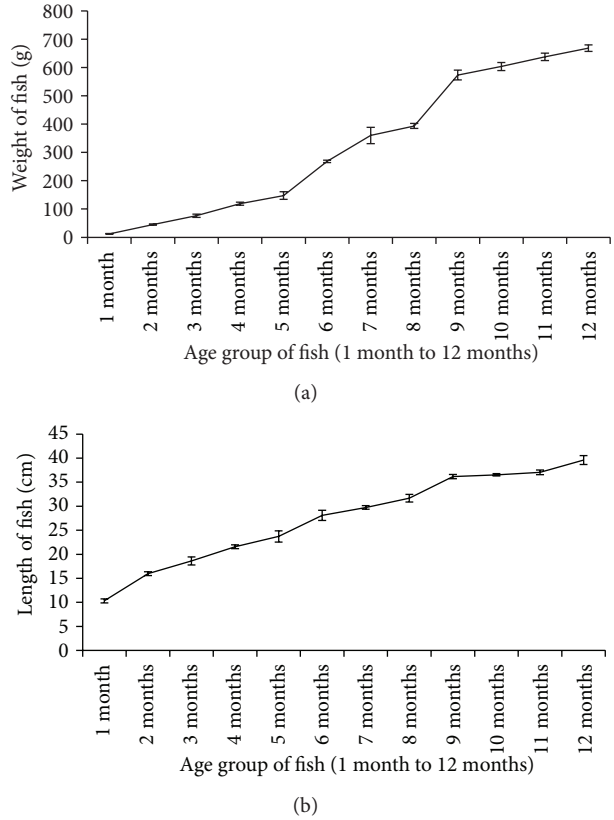

Figure 1: Monthly growth of *Labeo rohita* from 1 month to 12 months of age. (a) Mean weight of fish. (b) Mean length of fish. Values are expressed as mean ± SEM ($N = 6$).

3.2. mRNA Transcription Pattern of Myogenic Regulatory Factors

3.2.1. Reverse Transcriptase PCR (RT-PCR). MyoD mRNA expression was found to be high in the embryo stages and decreased gradually after 5 months of age reaching the minimum at 12 months. The highest expression was obtained at 24 h after fertilization (Figure 2). Expression of myf-5 also reached its maximum at 24 h after fertilization and decreased gradually to reach the basal level (Figure 2). Myogenin expression did not show any marked alteration and maintained a steady expression pattern up to 12 months with slight modulation (Figure 2). MEF2A had a steady expression pattern with slight elevation during 6 h after fertilization to 1 month of age. After 1 month, it decreased slightly and then became almost stable throughout the rest part of the experimental period (Figure 2).

3.2.2. Quantitative Real-Time PCR (qRT-PCR). Quantitative PCR was done at 5 time points (6 h, 24 h, 1 month, 6 months, and 12 months). All the four transcription factors showed the expression pattern similar to that of RT-PCR (Figure 3) and corroborated with RT-PCR results.

3.3. Construction of Phylogenetic Tree. Partial nucleotide coding sequences of three genes (MyoD, myf-5, and MEF2A) were submitted to GenBank (http://www.ncbi.nlm.nih.gov). The GenBank accession numbers are KC344537.1 for MyoD, KC344536.1 for myf-5, and KC344535.1 for MEF2A. Molecular phylogenetic relationship (based on translated amino acid sequences) of *Labeo* MyoD, myf-5, and MEF2A with that of other closely related species was depicted by reconstructing phylogenetic tree using neighbor-joining method (Figure 4).

4. Discussion

The growth pattern of the experimental fishes followed similar annual pattern of growth of *Labeo* species *in situ* as reported by Jhingran [18] (Figure 1). After fertilization, differentiation of skeletal muscle is initiated by MyoD, which binds directly to the regulatory regions of a wide number of genes and regulates their expression during differentiation [19, 20]. Both MyoD and myf-5 are necessary for the initiation of myogenesis in vertebrates. Disruption of both genes in mice results in the absence of skeletal muscle cells [21].

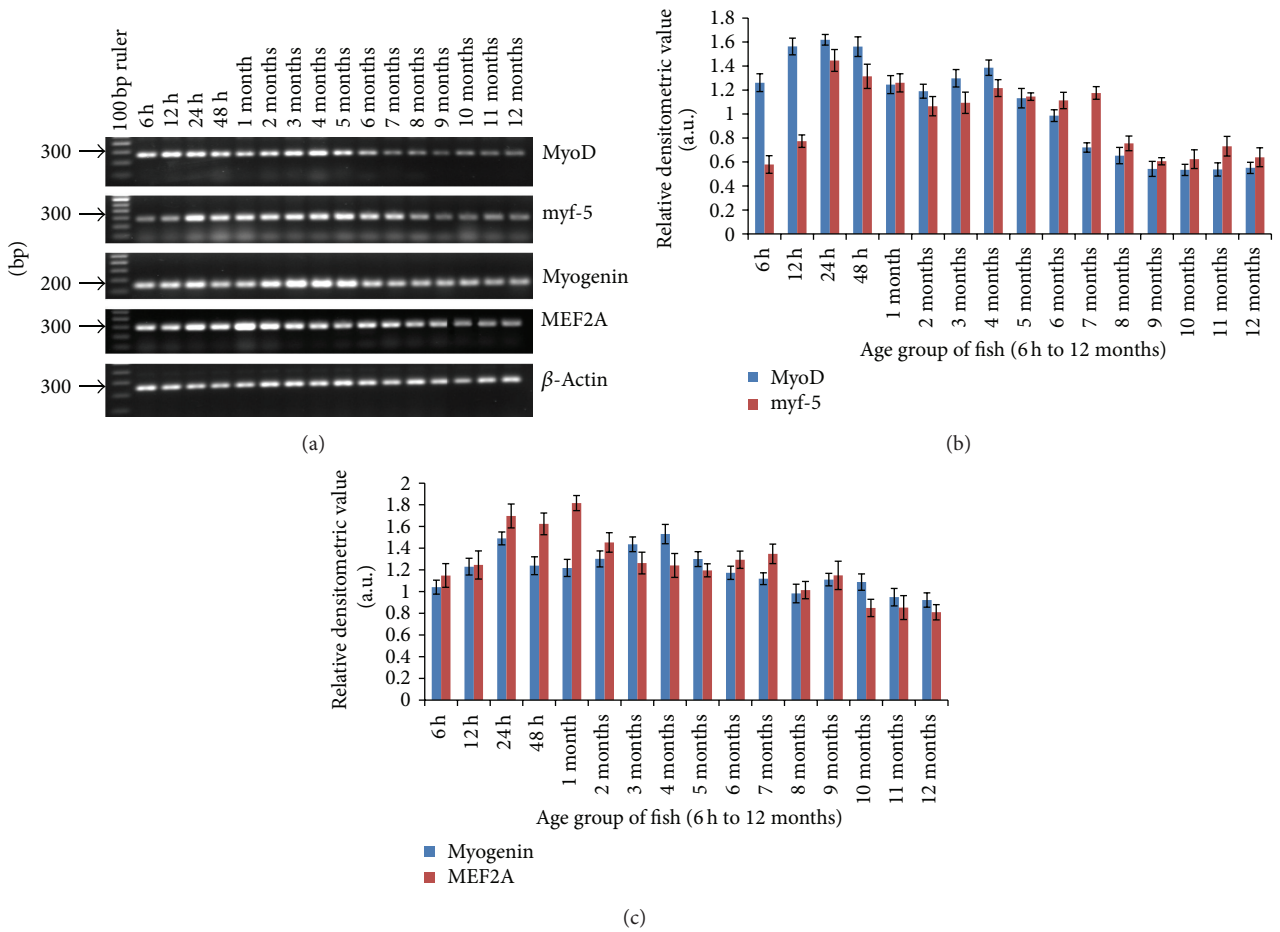

Figure 2: mRNA transcription pattern of myogenic regulatory factors by RT-PCR (a) expression of MyoD, myf-5, myogenin,and MEF2A mRNA at different time points of growth (6 hours to 12 months), (b) relative densitometric analysis of MyoD and myf-5 expression, and (c) relative densitometric analysis of myogenin and MEF2A expression. Values are expressed as mean ± SEM ($N = 6$).

Myogenic cells undergo active proliferation before cell cycle arrest and fusion into myotubes. MyoD and Myf-5 play the central role in specifying muscle lineage and MyoD is the key regulator of maintaining balance between the differentiation and proliferation [22]. In the present study, the expression of MyoD and myf-5 was higher in the embryonic stages of *Labeo rohita*. Both the genes followed nearly similar pattern of expression as depicted by semiquantitative and quantitative PCR (Figure 3). In mouse, myf-5 was reported as the first expressed MRF in the myotomal muscle [23] and in common carp a high level of mRNA transcripts of myf-5 was detected at 30 h after fertilization [9]. In our study, considerable amount of MyoD mRNA transcript was detected at 12 h after fertilization stage (Figure 2(b)) and myf-5 had the highest level of expression at 24 h after fertilization (Figure 2(b)). So in this particular fish, the MyoD and myf-5 expression pattern is not similar with the same in other fish species reported earlier [9, 24].

Postnatal muscle growth involves hypertrophy of muscle fibres which require additional nuclei to maintain a relatively constant nuclear to cytoplasmic ratio. These nuclei are provided by activated myogenic stem cells which also express myogenic bHLH proteins. myf-5 and MyoD expressions in skeletal muscles are followed by upregulation of myogenin and of MEF2 family factors, which enhance expression of muscle differentiation genes [25]. We observed that myogenin mRNA transcript was present in a considerable amount at all stages with highest value at 24 h (Figure 3). MEF2 family of transcription factors specifically bind to an A/T rich sequence present in many muscle specific promoters and enhancers [26]. In zebrafish, knockdown of MEF2A has been shown to downregulate a large set of genes encoding contractile proteins such as troponins, myosin heavy and light chains, and α-tropomyosin [27]. In *Labeo rohita*, MEF2A expressed at all the stages showing the high level of expression at 1 month. This expression pattern of myogenin and MEF2A is similar to the pattern in common carp described by Kobiyama et al. 1998 [9]. Muscle growth in fish involves the production of new muscle fibres in addition to muscle fibre hypertrophy [6]. The continued expression of myogenin and MEF2A in *Labeo rohita* reflects activated myogenic cells which help to maintain continuous hypertrophy as well as hyperplasia of skeletal muscle. In embryonic stages, most of the muscle cells remain in the early differentiation stage and

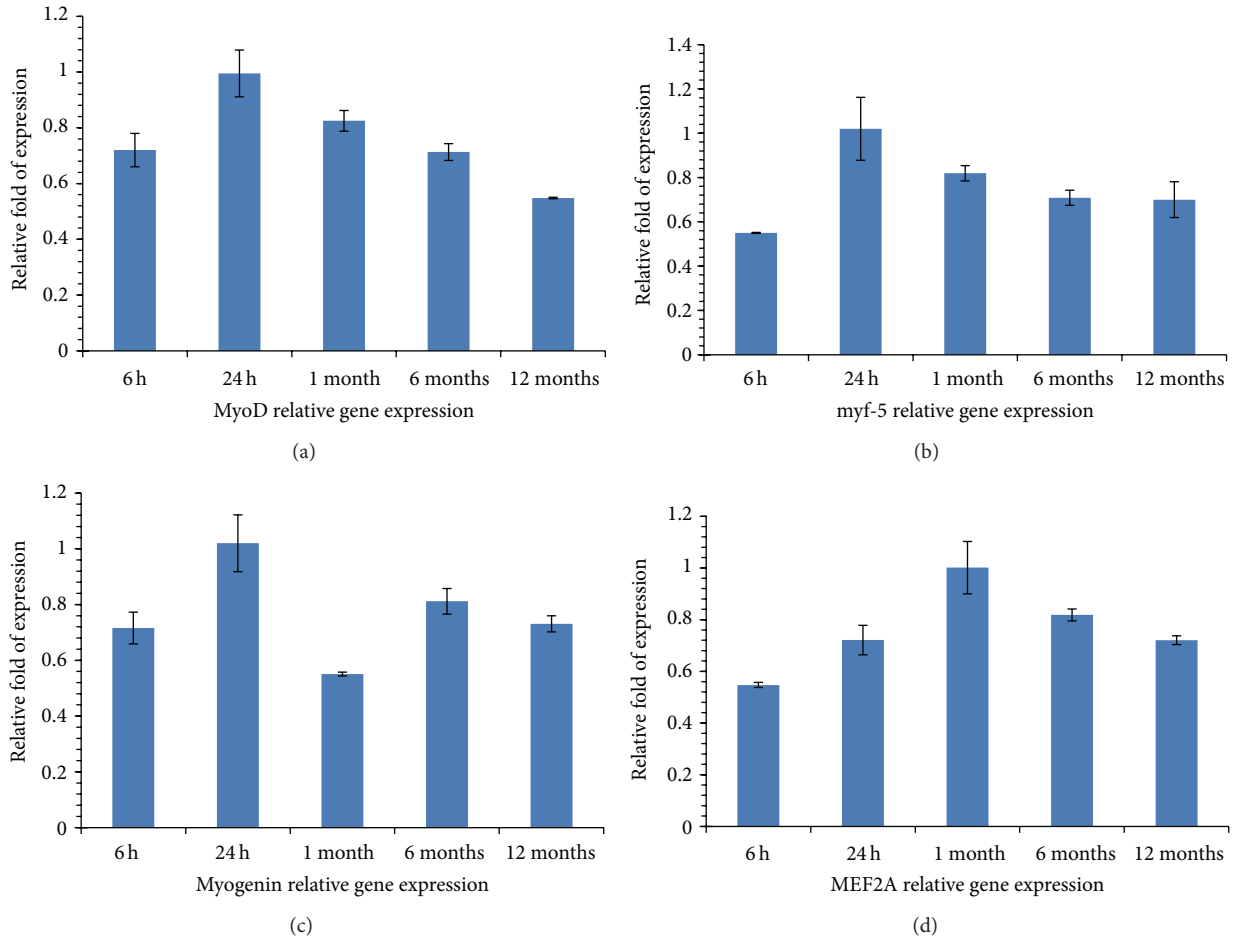

Figure 3: mRNA expression of four myogenic regulatory factors in *Labeo rohita* at 6 h, 24 h, 1 month, 6 months, and 12 months of age (a) MyoD, (b) myf-5, (c) myogenin, and (d) MEF2A.

proliferation stage, while most muscle cells of adult are in the terminal differentiation stage [24]. Our results clearly showed that MyoD and myf-5 mRNAs are expressed at high level in the early embryonic stages, whereas myogenin and MEF2A are expressed after MyoD and myf-5 expression and remained active in adult stage to maintain differentiation and growth of skeletal muscle fibres. MyoD is reported to play an important role in the arrest of cellular growth. Its highest level was detected at early G1 phase and the lowest level was at G1 to S phase transition. MyoD and its cofactors play a critical role in myoblast cell cycle withdrawal. When MyoD is maximal and myf-5 is down, cells exit their cycle into differentiation. The opposite pattern is observed in quiescent nondifferentiating myoblasts, a high myf-5 and no MyoD [22]. In *Labeo rohita*, both the weight rate and length rate between the 5th and 6th month were significantly greater than those of before 5th month. The expression of MyoD was lower than that of myf-5 after 5th month. Considering two relationships (expression of myf-5/MyoD with muscle cells proliferation/differentiation and muscle cells proliferation/differentiation with muscle hypertrophy/hyperplasia), it may be supposed that in this fish species the muscle development after 5th month was properly balanced by both hypertrophy and hyperplasia. The four transcription factors therefore play pivotal role in the regulation of muscle growth in an overlapping and interconnected way.

Molecular phylogenetic tree showed that *Labeo* MyoD, MEF2A, and myf-5 are more closely related to that of common carp (*Cyprinus carpio*) than any other species. Previous report showed high similarities of MyoD, myf-5, and MEF2A sequences between carp and zebrafish but the similarities were more prominent in the bHLH region (MyoD and myf-5) and MADS box region (MEF2A) than total coding sequence [28].

Further extensive studies are warranted to evaluate the regulation of MRF gene expression pattern in fish which may provide insight into the signaling pathway controlling muscle cell differentiation through regulatory transcription factors. Information is available on tissue specific embryonic expression pattern of these transcription factors. Contrastingly, studies on long term age specific pattern of expression are lacking. To the best of our knowledge this is the first report on myogenic regulatory transcription factors in any

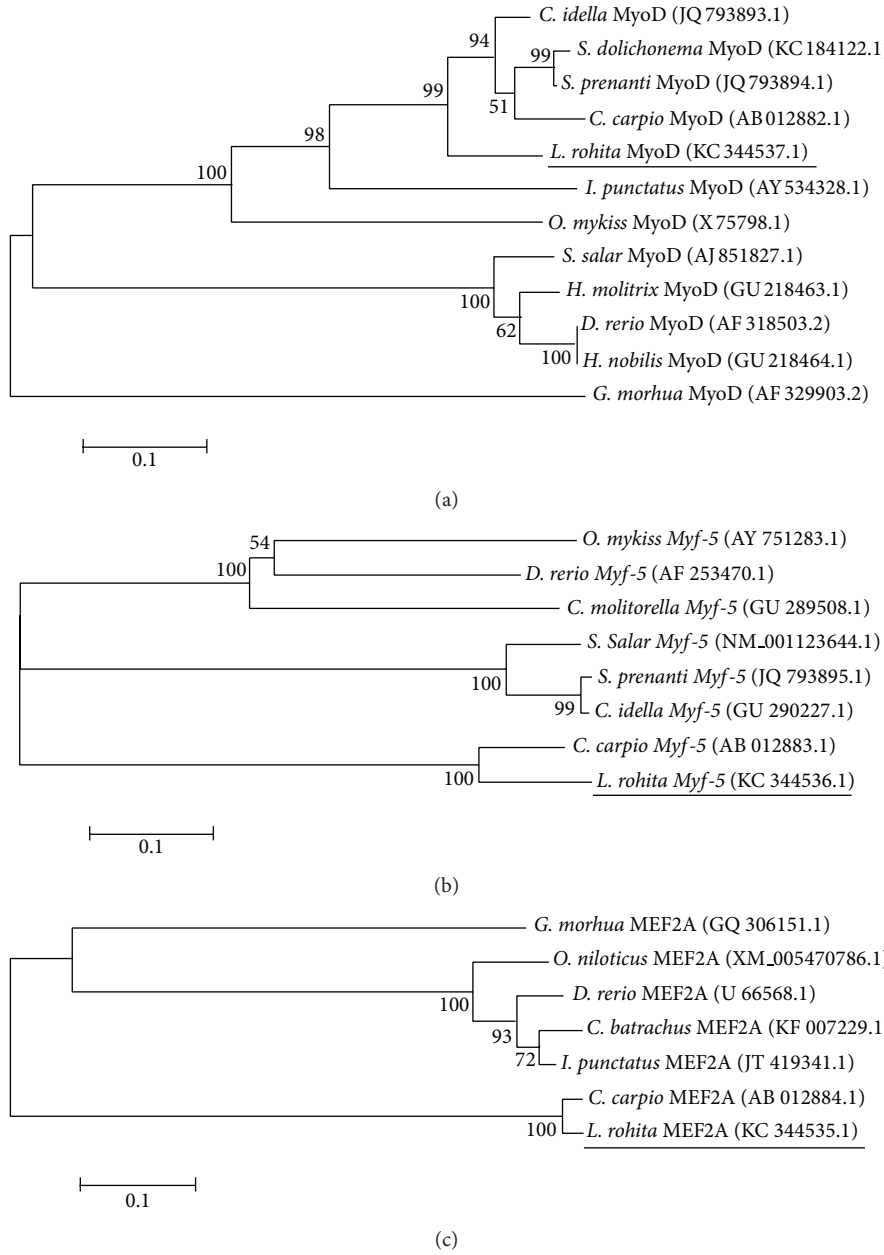

Figure 4: Molecular phylogenetic tree of (a) MyoD, (b) myf-5, and (c) MEF2A. This dendrogram is based on the translated amino acid sequences. GenBank accession numbers are given in the brackets and sequences acquired from this study are underlined accordingly.

Indian carp describing monthly expression pattern of these transcription factors in juvenile and adult *Labeo rohita*.

5. Conclusion

This study demonstrated the mRNA transcription pattern of MyoD, myf-5, myogenin, and MEF2A in embryo and adult *Labeo rohita* and depicted probable phylogenetic relationship of this fish with other related species with respect to MyoD, myf-5, and MEF2A gene. MyoD was expressed first in the embryo along with myf-5 to initiate myogenesis. MEF2A and myogenin were expressed throughout the developmental period to help in differentiation of muscle cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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