Newly identified gene *muscle segment homeobox C* may play a role in intermuscular bone development of *Hemibarbus labeo*

Y.P. Lv[1]*, W.J. Yao[2]*, J. Chen[2] and B.L. Bao[2]

[1]College of Ecology, Lishui University, Lishui, China
[2]Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources,
Shanghai Ocean University, Ministry of Education, Shanghai, China

*These authors contributed equally to this study.
Corresponding author: B.L. Bao
E-mail: blbao@shou.edu.cn

ABSTRACT. Intermuscular bones, ossified from tendons within the myosepta, occur only in teleost fish. Current understanding of the homology and origins of intermuscular bones in fishes is based mainly on morphological data. To date, there is no published data regarding molecular mechanisms of intermuscular bone formation. In this study, we cloned the gene muscle segment homeobox C (*MsxC*). *MsxC* potentially plays a role in intermuscular bone development of *Hemibarbus labeo*, an important species of cyprinid fish in the Chinese aquaculture industry. Sequence analysis of *MsxC* revealed motifs characteristic of the homeobox domain family. Whole-mount *in situ* hybridization showed that *MsxC* was primarily expressed in the myosepta and brain. *MsxC* was expressed in the myosepta from 26 to 41 days after hatching (DAH); this coincided with the onset of intermuscular bone ossification, which occurred between 35 and 62 DAH. Evidence for localization of *MsxC* expression by *in situ* hybridization correlated

with its detection by quantitative real-time PCR. In vertebrates, *MsxC* plays a role in the regulation of mesenchymal cell differentiation during bone formation. We therefore conclude that *MsxC* may have a role in epithelium-mesenchyme interactions during intermuscular bone formation in *H. labeo*.

Key words: Intermuscular bone; *MsxC*; Mesenchyme; Ossification; *Hemibarbus labeo*

INTRODUCTION

Three sets of intermuscular bones may be present in teleosts, and these are classified in reference to their site of attachment (Johnson and Patterson, 2001) as follows: the epineural bones, attached to the neural arches; epicentral bones, attached to the centra; and epipleural bones, attached to the hemal arches or ribs. Intermuscular bones are present only in teleosts, and it is thought they are ossified from tendons in myosepta (Patterson and Johnson, 1995; Danos and Ward, 2012). The development of intermuscular bones has been described as undergoing intramembranous ossification (Bird and Mabee, 2003). This process is initiated by mesenchymal stem cells, an unspecialized cell population that can develop into osteoblasts (Brighton and Hunt, 1991).

Cyprinid fish species are extensively cultivated throughout the world. *Hemibarbus labeo* is one such species. The natural distribution of *H. labeo* is primarily in Asia, and the species has an important role in the freshwater fish farming industry of China (Feng et al., 2006). As for other cultivated cyprinid fishes, *H. labeo* has many intermuscular bones. This quality makes the flesh of the fish difficult to consume for children and the elderly, and unsuitable for processing into fillets. Breeding cyprinid fish with less or no intermuscular bones is becoming an exciting goal of the aquaculture industry in China (Li et al., 2013).

In vertebrates, the muscle segment homeobox (*Msx*) gene family is related to the *Drosophila* muscle segment homeobox *(Msh)*-like gene family (Lord et al., 1995). *Msh* and *Msx* are known to display distinct patterns of expression in the neural crest (Ramos and Robert, 2005); the facial mesenchyme of neural crest origin (Francis-West et al., 1998); and the distal mesoderm of developing limbs, where they play an important role in the induction of epithelial-mesenchymal interactions contributing to organogenesis (Thesleff et al., 1995; Bendall and Abate-Shen, 2000). Phylogenetic analysis indicates that MsxC is not orthologous with Msxl or Msx2, but more closely related to mouse Msx3 (Ekker et al., 1997). *MsxC* is expressed in discrete stripes within the cranial mesoderm (Phillips et al., 2006), as well as in the pharyngeal arches and fins. During fin regeneration in zebrafish, *MsxC* expression is restricted to the distal tip of the blastemal mesenchyme, suggesting a role for this gene in epithelial-mesenchyme interactions (Akimenko et al., 1995). Up-regulation of *MsxC* is observed in developing and regenerating sword rays in two species of swordtails (*Xiphophorus* spp), which suggests that it may constitute a general feature of swordtail ray development (Zauner et al., 2003; Offen et al., 2008). Although intermuscular bones and fins have different patterning, morphogenesis, and histogenesis, it is important to note that both contain osteoblasts, which are differentiated from mesenchymal cells.

There is little data published on molecular mechanisms of intermuscular bone formation, and understanding of the homology and origins of intermuscular bones in fishes is

therefore based largely on morphological data (Patterson and Johnson, 1995; Gemballa and Britz, 1998; Johnson and Patterson, 2001). In consideration of the possible role of MsxC in epithelial-mesenchyme interactions, in this study we investigated the expression pattern of *MsxC* during segmental ossification of intermuscular bones in *H. labeo*. Our findings suggest that *MsxC* may have a role in intermuscular bone development.

MATERIAL AND METHODS

Fish maintenance

Larval *H. labeo* were reared in our laboratory at Lishui University (Lishui, Zhejiang, China) according to previously described methods (Feng et al., 2006; Xu et al., 2009). Larvae were reared at 22°C and fed commercial feed (Kesheng Feed Stock Co., Zhejiang, China) for the duration of the experiment. Fish were anesthetized by immersion in 0.1 μg/μL tricaine methane-sulfonate (MS 222), and fish from each group were preserved as follows for further experimentation: for bone staining, 40 fish were fixed in 4% paraformaldehyde (PFA) at room temperature; for RNA whole-mount *in situ* hybridization, 40 fish were fixed overnight in 4% PFA, then stored in methanol at 4°C; and for total RNA extraction, 100 fish were frozen in liquid nitrogen.

Total RNA extraction and reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from fish at different developmental stages using TRIzol Reagent, following the manufacturer protocol (Invitrogen, Carlsbad, CA, USA). Assessment of RNA quality was performed by electrophoresis on a 1.0% agarose (w/v) gel, and total RNA concentrations were determined using a NanoDrop ND-2000 spectrophotometer (Implen, Munich, Germany). Synthesis of cDNA was carried out from 1 μg total RNA, using the TaKaRa PrimerScript™ First Strand cDNA Synthesis Kit, following the manufacturer protocol (Ta-KaRa, Kyoto, Japan).

Cloning and sequencing of full-length *MsxC* cDNA

A PCR fragment was generated for the *MsxC* gene (326 bp; 608-943 referring to *H. labeo MsxC;* GenBank accession No. KC693187). Primers are listed in Table 1. A full-length cDNA *MsxC* was also generated, using the SMARTer™ RACE cDNA Amplification Kit, following the manufacturer protocol (Clontech, San Francisco, CA, USA). Two *MsxC*-specific primers were designed based on the PCR fragment sequence (GSP1 and GSP2; Table 1). For 5'/3'-RACE, part of the *MsxC* gene was amplified with 5'/3'-RACE-Ready cDNA, under the following conditions: 5 cycles of 30 s at 94°C and 3 min at 72°C; 30 s at 94°C; 3 min at 70°C; then 25 cycles of 30 s at 94°C and 3 min at 68°C. Amplified products were cloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

Sequence analysis

A search for published sequences that were similar to cloned *MsxC* was conducted with the BLAST algorithm at the National Center for Biotechnology Information (http://

www.ncbi.nlm.nih.gov/BLAST/). The predicted amino acid (aa) sequence of MsxC was obtained using the Simple Modular Architecture Research Tool (http://smart.embl-hei-delberg.de/). Deduced aa sequence was analyzed using ExPASy tools (http://us.expasy.org/). Multiple sequence alignment of Msx was carried out using ClustalW (http://www.ebi.ac.uk/clustalw/), and a cladogram was constructed, based on aa sequence alignment, using the neighbor-joining algorithm embedded in the MEGA 4 program (http://www.megasoftware.net/). Branching reliability was tested by bootstrap resampling with 1000 pseudoreplicates.

Table 1. Nucleotide sequences and annealing temperatures of oligonucleotide primers.

Primer	Nucleotide sequence (5'-3')	Tm °C
GSP1	GACACCCTCGTGCACTTCTCCGCCAAGG	70
GSP2	TGGACGGTCCGTAGAGGGGCGGAGAACC	70
MsxC-F	CCACGAACACCGTTTACAACCTCACAG	62
MsxC-R	ACCGAATAGTCCCGGGACGGGTAG	63
qβactin-F	AGGATGCGGAAACTGGCAAAGG	56
qβactin-R	GAGGAGGGCAAAGTGGTAAACG	56
qMsxC-F	AGGAGGCTCAACCCAACGATAA	56
qMsxC-R	GTCGGGTAGGTTGGAGACACTC	56

Alizarin red staining

To visualize intermuscular bone development, Alizarin red staining was performed on PFA-fixed fish as previously described (Walker and Kimmel, 2007). Briefly, specimens were washed twice with PBS to remove PFA, then once for 30 min in TBST [50 mM Tris; pH 7.4; 150 mM NaCl; 0.1% Triton X-100 (v/v)]. Specimens were digested in 1% trypsin, transferred to 2 mg/mL Alizarin red in 1% KOH for 1 h, then cleared with 20% glycerol in 1% KOH for 40 min. Alizarin red-stained *H. labeo* were photographed and then preserved in solution [4:1 of 100% glycerol:95% ethanol (v/v)]. Specimens were examined using a conventional dissecting microscope SMZ1500 (Nikon, Tokyo, Japan) and photographs were obtained using the NIS-Element D software (Nikon).

Quantitative real-time PCR

Expression levels of *MsxC* mRNA in fish at 2, 7, 10, 26, 32, 41, 44, and 47 days after hatching (DAH) were assessed by quantitative real-time PCR. Following extraction, 1 µg total RNA was used to synthesize first strand cDNA, as described above. For quantification of *MsxC* expression, gene-specific primers qMsxC-F and qMsxC-R were used (Table 1). A second pair of primers, qβactin-F and qβactin-F, was used to amplify reference *β-actin* (Table 1). Amplification and relative quantification of cDNA was carried out using SYBR green master mix on a CFX96™ Real-time PCR System (both Bio-Rad, Hercules, CA, USA). Transcript levels of *MsxC* were determined relative to 100 copies of β-actin mRNA. Relative gene expression levels versus β-actin were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Data were analyzed by a Least Significant Difference *t*-test or one-way ANOVA, using SPSS 13.0 (IBM, New York, NY, USA). Results were considered statistically significant if $P < 0.01$. All data are reported as means ± SE for the PCR in triplicates.

Probe synthesis and whole-mount *in situ* RNA hybridization

A 326-bp PCR fragment was generated for *MsxC* as described for cloning and sequencing above and cloned into the pGEM-T vector (Promega). The cloned product was used as template to create DIG-labeled antisense and sense riboprobes [using, respectively, RNA polymerases and labeling mix (Roche, San Francisco, CA, USA)], to be used as controls. *In situ* hybridization was performed at 53°C using a modified protocol (Thisse and Thisse, 2008). Briefly, specimens were bleached in 3% H_2O_2 in PBS prior to digestion in 100-150 μg/mL proteinase K. Specimens were incubated with anti-DIG antibody (1:2000), stained with Fast Red (Roche), and examined and photographed using a conventional dissecting microscope as described above. In order to precisely localize signal sites of *MsxC,* selected specimens were embedded in medium (Leica Instruments, Germany); cut transversely into 10 μm frozen sections; and photographed by microscopy (ECLIPSE 80i, Nikon).

RESULTS

Cloning and phylogenetic analysis of *MsxC* from *H. labeo*

Full-length *MsxC* cDNA was 2180 nucleotides in length (GenBank accession No. KC693187), comprising a 145-bp 5' untranslated region (UTR); a 759-bp open reading frame encoding 252 aa; and a 1276-bp 3'-UTR, which included the consensus polyadenylation signal.

Multiple sequence alignment showed that the deduced aa sequence of MsxC had significant identity with that of other known Msx proteins. The highest sequence identity was to MshC in *Danio rerio* (93%), and the lowest sequence identities were to Xhox-7 in *Xenopus laevis* and MsxA in *Petromyzon marinus* (both 55%). In general, homology between aa sequences was higher at N-termini than C-termini. Further analysis of MsxC aa sequence revealed structural features that included a homeobox domain present in other Msxs (Figure 1).

A cladogram was created that divided the Msx family into two groups (Figure 2). The first group contained MsxA, Msx1, Msx2, Hox7, and Hox8; the second group contained MsxB, MsxC and Msx3. There was a strong evolutionary relationship between MsxC and Msx3, and *H. labeo* MsxC was most closely related to *D. rerio* MsxC. Furthermore, *H. labeo* MsxC clustered with other MsxC sequences, providing supporting evidence for its classification as an MsxC.

Ossification patterns of intermuscular bone during postembyro development

Results of Alizarin red staining are shown in Figure 3. Ossification of intermuscular bones in *H. labeo* was observed in fish aged 35 to 62 DAH. At 35 DAH, all bones appeared ossified, with the exception of the intermuscular bones (Figure 3a). From this stage onwards, intermuscular bones began to ossify in the tail region; specifically, ossified epineural bone was observed in each myosepta between the 37th and 41st myotomes, and ossified epipleurals were found between the 39th and 40th myotomes (Figure 3b). In 47-DAH fish, epipleural bones had a simple linear morphology (type I; Figure 3c). However, Y-shaped, ossified intermuscular bones were observed at 62 DAH (Figure 3d and e). As *H. labeo* development continued, ossified intermuscular bones were observed in the myosepta closer to the head, and by 62 DAH, all intermuscular bones were ossified (Figure 3f). No epicentrals were observed in any fish examined.

Figure 1. Alignment of amino acid sequence of MsxC of *Hemibarbus labeo* and other known sequences. The following species are shown: Xl-Xhox7 = *Xenopus laevis* Xhox-7.1, AAB19630.1; Dl-Msx1 = *Dicentrarchus labrax* Msx1 protein, CBN81149.1; Am-Msx1 = *Ambystoma mexicanum* Msx1 protein, AAS17879.1; Hs-Msx-2 = *Homo sapiens* Msx-2 homolog, AAD14169.1; Hg-MSX-2 = *Heterocephalus glaber* homeobox protein MSX-2, EHB18669.1; Bt-MSX-2 = *Bos taurus* homeobox protein MSX-2, NP_001073082.1; Cl-MSX-2 = *Canis lupus* familiaris homeobox protein MSX-2, NP_001003098.1; Mm-Hox8 = *Mus musculus* Hox8, CAA41945.1; Ms-MSX-2 = *M. musculus* homeobox protein MSX-2, NP_038629.2; Rn-MSX-2 = *Rattus norvegicus* homeobox protein MSX-2, NP_037114.2; Gg-homeodomain = *Gallus gallus* homeodomain protein, CAA44007.1; Pm-MsxA = *Petromyzon marinus* MsxA, ABW76121.1; Mum-MSX3 = *M. musculus* homeobox protein MSX-3, NP_034966.1; Hl-MsxC = *H. labeo* MsxC, KC693187; Dar-C = *Danio rerio* Muscle segment homeobox C, AAH71302.1; Xh-MsxC = *Xiphophorus helleri* homeobox protein MsxC, AAO06122.1; and Dr-MSH-B, *D. rerio* homeobox protein MSH-B, NP_571335.1. Identical amino acids are highlighted in black and similar amino acids are highlighted in gray.
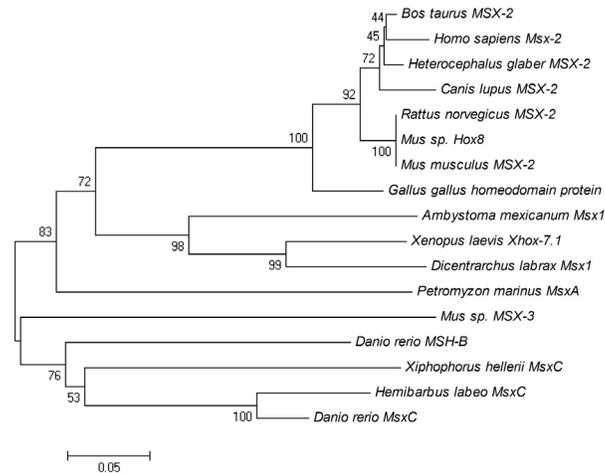
Figure 2. Phylogenetic tree showing the relationship between MsxC and Msxs from other species. Numbers on the branches represent the bootstrap value. The origins and accession No. of the TRAF6 protein sequences are: *Xenopus laevis* Xhox-7.1 = AAB19630.1; *Dicentrarchus labrax* Msx1 = CBN81149.1; *Ambystoma mexicanum* Msx1 = AAS17879.1; *Homo sapiens* Msx-2 = AAD14169.1; *Heterocephalus glaber* H MSX-2 = EHB18669.1; *Bos taurus* MSX-2 = NP_001073082.1; *Canis lupus* MSX-2 = NP_001003098.1; *Mus* sp Hox8 = CAA41945.1; *Mus musculus* MSX2 = NP_038629.2; *Rattus norvegicus* MSX-2 = NP_037114.2; *Gallus gallus* homeodomain protein = CAA44007.1; *Petromyzon marinus* MsxA = ABW76121.1; *Mus* sp MSX-3 = NP_034966.1; *H. labeo* MsxC = KC693187; *Danio rerio* MsxC = AAH71302.1; *Xiphophorus helleri* MsxC = AAO06122.1; and *D. rerio* MSH-B = NP_571335.1.
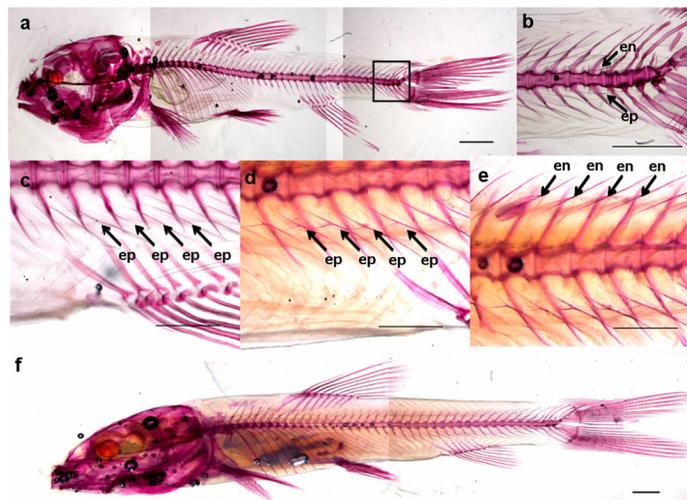
Figure 3. Distribution and morphological specification of intermuscular bones during early development of *Hemibarbus labeo*. Representative images of *H. labeo,* stained with Alizarin red to visualize developing intermuscular bones (arrows), are presented. The entire skeleton of *H. labeo* at 35 DAH is shown (**a**), with the rectangular area containing the intermuscular bones enlarged (**b**). Type I epipleurals are shown at 47 DAH (**c**), as well as type Y epipleurals (**d**) and type Y epineurals at 62 DAH (**e**). The entire skeleton of *H. labeo* at 62 DAH is also shown (**f**). Scale bar = 500 μm; ep = epipleurals; en = epineurals.

MsxC expression during postembryonic development

Data from quantitative real-time PCR are presented in Figure 4. *MsxC* was expressed at all developmental stages examined, but expression levels varied in a time-dependent manner. Specifically, *MsxC* transcripts were weakly expressed at 2 DAH, and significantly increased in expression at 7 DAH (P < 0.01). Following this, there was significantly decreased expression at 10 DAH (P < 0.01). *MsxC* expression levels were increased at 32 DAH (P < 0.01), and dropped again in the following days.
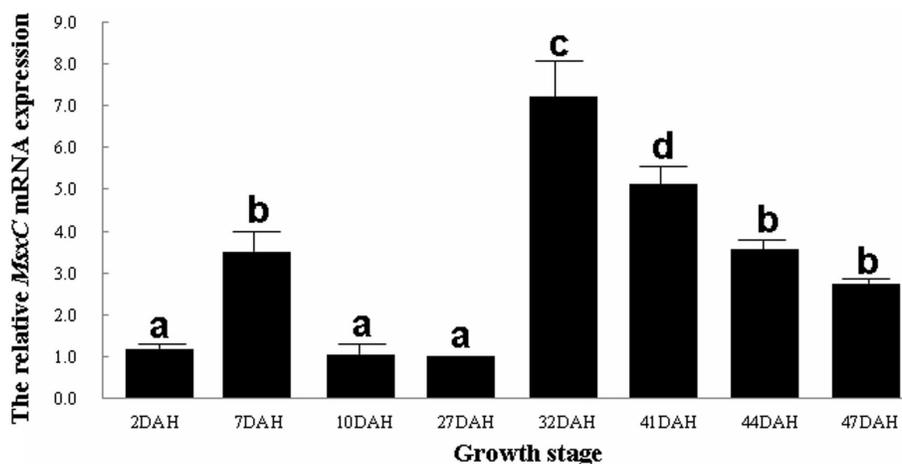
Figure 4. Quantitative real-time PCR analysis of *MsxC* expression during *Hemibarbus labeo* development. Each bar represents the means ± SE of *MsxC* expression (N = 9), relative to 100 copies of *β-actin*. Letters above the bars represent significant differences (P < 0.01).

To determine the spatial and temporal expression pattern of *MsxC* in larval and juvenile fish, and whether *MsxC* expression was localized to the myosepta, we performed experiments on whole-mount fish. The expression pattern of *MsxC* dynamically changed at different developmental stages. *MsxC* expression was observed mainly in the brain at 3, 7, and 10 DAH (Figure 5a-f). Alongside continual expression in the brain, expression signal was detected in the epaxial and hypaxial myoseptal regions from 26 DAH (Figure 5g); this expression was more obvious at 32 DAH (Figure 5h and i). At 41 DAH, *MsxC* expression in myosepta had visibly decreased (Figure 5l) and was undetectable at 47 DAH in all tissues except the brain (Figure 5j and k). Whole-mount *in situ* hybridization results correlated well with data from quantitative real-time PCR. No signal with sense probe was detected in larvae, including 10 and 41 DAH larvae (Figure 5m and n), demonstrating that there was no nonspecific staining. Examination of cross sections revealed *MsxC* was expressed in skin and myosepta (Figure 5o). These *MsxC* expressing cells were also symmetrically located in myosepta (Figure 5p and q).

DISCUSSION

During development of *H. labeo*, epineurals and epipleurals developed and ossified predominantly in a posterior to anterior order over time. The primary morphology of inter-

muscular bones was type I; ossified intermuscular bones with further complicated morphology formed gradually from type I. The ossification pattern of intermuscular bones in *H. labeo* described here is similar to that observed in other cyprinid fish species (Bird and Mabee, 2003; Ke et al., 2008).
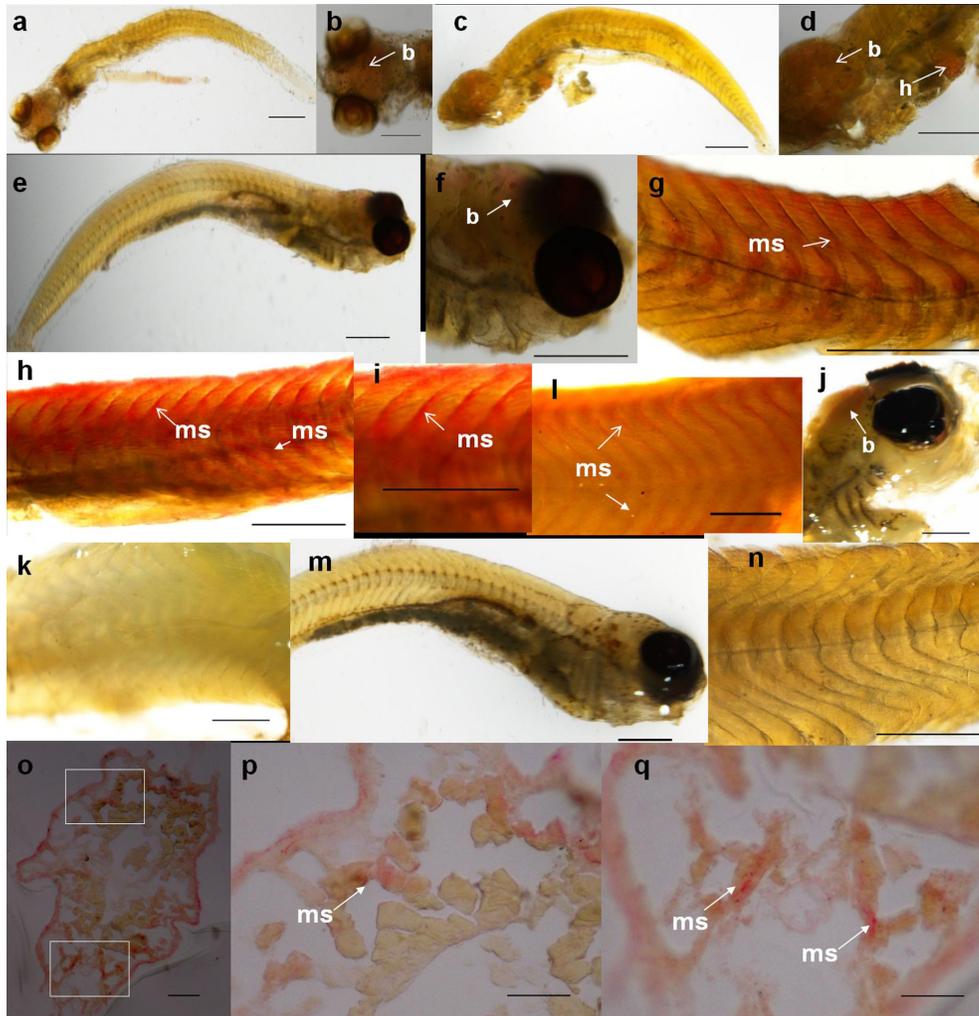
Figure 5. *In situ* hybridization analysis of *MsxC* expression during *Hemibarbus labeo* development. The signal of *MsxC* expression probed with antisense strand is shown in white (arrows). Larvae at various days after hatching (DAH) are shown, with brain enlarged in the adjacent panels, as follow: 3 DAH (**a**), brain (**b**); 7 DAH (**c**), brain (**d**); and 10 DAH (**e**), brain (**f**). Larvae with a view of the trunk are shown for 26 DAH (**g**), 32 DAH (**h**), and 41 DAH (**l**); **i**. enlarged view of the trunk from a 32 DAH larva. A 47-DAH larva with a view of brain (**j**) and trunk (**k**) is shown. The negative controls with sense probe for 10 (**m**) and 41 (**n**) DAH larvae are shown. Transverse sections view through the body of 32 DAH *H. labeo* (**o-q**). All panels show tissue sections, with dorsal up for all cross sections. **p.** and **q.** Enlarged views of the rectangle area in **o. a.-n.** Scale bar = 500 μm; **o.-q.** scale bar = 100 μm; b = brain; h = heart; mt = myotome; ms = myosepta.

In this study, an *Msx* homolog was identified and characterized from *H. labeo*. Alignment with established isoforms of Msxs from multiple vertebrate revealed that the approximate length of *H. labeo* MsxC sequence was conserved, with several blocks of conserved sequence (described by Holland, 1991) distributed throughout. The high level (55-93%) of aa sequence identity between MsxC and other Msxs indicated that *H. labeo MsxC* is a new member of the *Msx* family. Results also showed that MsxC identified in this study was more closely related to MsxB and MsxC and to mouse Msx3 than to other Msx genes. *MsxC* contains a homeobox domain, which binds DNA through a helix-turn-helix structure (Figure 1). This suggests that *MsxC* may perform similar functions to other *Msx*s, such as induction of epithelial-mesenchymal interactions that contribute to organogenesis in other vertebrates (Thesleff et al., 1995; Bendall and Abate-Shen, 2000).

Previous studies have demonstrated that *Msx*s function during multiple phases of vertebrate development; this is exemplified by their expression patterns and actions during early, middle, and late stages of craniofacial, limb, and nervous system development (Wang et al., 1996; Francis-West et al., 1998; Cheng et al., 2004) and fin regeneration (Akimenko et al., 1995; Bendall and Abate-Shen, 2000). In our study, the highest expression level of *MsxC* was observed just before ossification of intermuscular bones, indicating that *MsxC* may be involved in intermuscular bone development. Alizarin red staining results indicated that ossification of intermuscular bone began in the posterior part of the body at 35 DAH, and each of the intermuscular bones followed anteriorly. *MsxC* mRNA was detected in fish sampled at 2, 7, 10, 27, 32, 41, and 47 DAH, with the highest level detected in 32 DAH fish. Moreover, whole-mount *in situ* hybridization results correlated well with data from qRT-PCR. *MsxC* was expressed in myosepta from 26 to 41 DAH (Figure 5); this timing overlapped with intermuscular bone formation, providing further support for a role for *MsxC* in intermuscular bone development. The findings presented in this study provide a basis for further investigation of *MsxC* up- and down-signal pathways, and the role of *MsxC* in the regulation of intermuscular bone development in major teleost fish groups.

ACKNOWLEDGMENTS

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