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Functional analysis of two MyoDs revealed their role in the activation of myomixer expression in yellowfin seabream (*Acanthopagrus latus*) (Hottuyn, 1782)

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ABSTRACT

Myoblast determination protein (MyoD), a muscle-specific basic helix-loop-helix (bHLH) transcription factor, plays a pivotal role in regulating skeletal muscle growth and development. However, the regulation mechanism of MyoD has not been determined in marine fishes. In the present study, we isolated the *MyoD1* (*AlMyoD1*) and *MyoD2* (*AlMyoD2*) genomic sequences and analyzed the expression patterns in different tissues of yellowfin seabream (*Acanthopagrus latus*). The open reading frame (ORF) sequences of *AlMyoD1* and *AlMyoD2* encoded 297 and 271 amino acids possessing three common characteristic domains, respectively, containing a myogenic basic domain, a bHLH domain, and a ser-rich region (helix III). Phylogenetic and genome structure analyses exhibited classic phylogeny and highly conserved exon/intron architecture. Furthermore, the *AlMyoD1* and *AlMyoD2* transcription levels were higher in white muscle than in the other tissues. In order to further study *AlMyoD* function in muscle, promoter sequence analysis found that several E-box binding sites were present. Additionally, binding sites of *Almyomixer* involved in mammal myoblast fusion, which expression was also the highest in white muscle, were found in the promoter of *AlMyoD*. Pomoter activity assays further confirmed that both *AlMyoD1* and *AlMyoD2* can dramatically activate *Almyomixer* expression, and the AlMyoD1 M2 and AlMyoD2 M5 E-box binding sites were functionally important for *Almyomixer* transcription based on mutation analysis and electrophoretic mobile shift assays (EMSA). In summary, two MyoDs play a core role in *Almyomixer* regulation and may promote myofibre formation during muscle development and growth by regulating *Almyomixer* expression.

1. Introduction

The growth of skeletal muscles is controlled by myogenic regulatory factors (MRF) that contain myoblast determination protein (MyoD), myogenic factor 5 (Myf5), myogenin and muscle-specific regulatory factor 4 (MRF4) in vertebrates. The MRF proteins share similar amino acid structural domains, including a highly conserved basic helix-loop-helix domain (bHLH) of approximately sixty amino acids involved in protein dimerization and DNA binding [1,2]. Gene knockout studies have indicated that those MRFs possess hierarchical relationships and different functions in mice (*Mus musculus*) [3–5]. MyoD and Myf5 are myogenic determining genes that are involved in early myoblast differentiation and determination. *M. musculus* lacking a functional MyoD or Myf5 gene shows no observably abnormal condition in skeletal muscles [6,7].

MRFs can discriminate a particular consensus motif identified as the E-box (CAnnTG) present in the promoters of numerous muscle-specific related genes [2,8,9]. Furthermore, MRFs bind to the E-box, and their co-factors, known as E-proteins, regulate the transcription of muscle-specific related genes and myogenic differentiation. MyoD also has the ability to convert non-muscle cells into skeletal muscle cells [10]. Moreover, myoblast-myoblast fusion is essential for muscle development [11–13]. Myomixer, a muscle-specific membrane micropeptide, is initially regarded as a transcriptional target of MyoD in *M. musculus* [14]. Myomixer, Minion, Myomerger and Myomaker, which can mediate myoblast fusion in vertebrates is reported [15–19]. Myomixer cooperates with the transmembrane protein myomaker to activate embryonic myoblast fusion and skeletal muscle formation in mammals [19,20]. In freshwater fishes, it is found that a downstream gene myomixer is triggered by MyoD activation in MyoD-dependent signalling pathway [21].

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Interestingly, both myomakers and myomixers are over-expressed in non-myoblasts, and they can fuse into multicellular cells [16,18,19]. Consequently, three conserved E-boxes in the promoters of the zebrafish (*Danio rerio*) and fugu (*Takifugu rubripes*) myomixer genes are identified, suggesting that myomixer may be transcriptionally regulated by MyoD during myogenesis [21]. Nevertheless, no information about myomixer is dominated by MyoD in marine fish.

Although four members of the MRF gene family have been identified in fish, amphibians, birds, and mammals [22], only one MRF gene has been detected in invertebrates containing nematodes (*Caenorhabditis elegans*) [23], Drosophila (*Drosophila melanogaster*) [24], sea urchin (*Lytechinus variegatus*) [25,26], jellyfish (*Podocoryne carnea*) [27], and ascidians (*Halocynthia roretzi*) [28]. Moreover, the yellowfin seabream (*Acanthopagrus latus*) (Hottuyn, 1782), sparidae, and Perciformes are found in the Indo-western Pacific region and considered to be important aquaculture fish in southern China due to their economic value. However, the muscle growth rate is overly slow in *A. latus*. Consequently, this species has provided a specific model for investigating regulatory mechanisms in muscle development in marine fish. Bioinformatic analysis found several E-box sites in the promoter of myomixer in *A. latus*, but, it is unclear whether *A. latus* myomixer is involved in myoblast fusion and how the gene plays its regulatory role. Therefore, to explore the potential function of *Almyomixer* and transcriptional regulation of two AlMyoDs, the present study focused on illuminating the importance of MyoD in the activation of myomixer expression. First, we identified three genes from *A. latus*, *MyoD1* (*AlMyoD1*), *MyoD2* (*AlMyoD2*), and *myomixer* (*Almyomixer*). Second, to authenticate whether MyoDs were the key elements in the *Almyomixer* promoter, promoter activity assays employing mutations to potential MyoD binding sites were performed. Finally, the role of the MyoD1 M2 and MyoD2 M5 binding site in the *Almyomixer* promoter was investigated using an electrophoretic mobility shift assay (EMSA). These approaches contributed to the identification of myomixer function in marine fish and indicated that two MyoDs played a core role in the regulation of myomixer expression.

2. Materials and methods

2.1. Ethics statement

All trials in the present study were allowed by the Animal Care and Use Committee of South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (No. SCSFRI96-253), and the experiments were implemented based on the regulations and guidelines established by this committee.

2.2. Animals and tissue collection

A. latus adult fish (body weight: 289 ± 18.5 g) were collected from Yangjiang Marine Fish Farm in Guangdong Province, China. For the study, fish tissues (n = 3) containing heart, eye, skin, brain, fin, spleen, small intestine, gill, white muscle, kidney, liver, gonad, and stomach were sampled, flash-frozen in liquid nitrogen, and stored at −80 °C until use.

2.3. Gene cloning and bioinformatics of A. latus MyoDs and myomixer

Total RNA (1 μg) was isolated from *A. latus* white muscle by TRIzol Reagent (Takara, Japan). The Prime ScriptTM RT reagent Kit (Takara) was used to synthesize cDNA according to the manufacturer's instructions. Two putative *MyoDs* and myomixer sequences were acquired according to genomic data of *A. latus* (Sequence Read Archive under BioProject PRJNA566024). To confirm the veracity of the supposed sequence, gene-specific primers were designed (Table 1). The PCR protocol used was described previously [29]. The amplified products were purified using a DNA Purification Kit (Tiangen, China), inserted into the pEASY-T1 vector (TransGen Biotech, China), and sequenced (Invitrogen, China). Confirmed plasmids were transformed into competent Trans1-T1 cells (TransGen Biotech, China). A Blast search on the presumptive two *MyoDs* and *myomixer* ORF sequences further pinpointed accuracy and validity.

Table 1
Primers used for sequence cloning, deletion mutant construction, and qRT-PCR.

Subject and primers	Nucleotide sequence
Primers for sequence cloning	
MyoD1-ORF-F	CGCGGATCCATGGAGCTGTCGGATATCT
MyoD1-ORF-R	CCGCTCGAGCTATAGGACTTGATAGATCA
MyoD2-ORF-F	CGCGGATCCATGGATCTGTCCGACCTTCC
MyoD2-ORF-R	CCGCTCGAGTCAGAGCGGCTCCTGGATGCT
Myomixer-ORF-F	ATGCCAGCAGTTTTCATCTTG
Myomixer-ORF-R	TCAGTTCTCCACCTTCTTGTG
Deletion mutant construction	
Myomixer-pF1	CGGGGTACCTTATGTTCTTACCAAACAAG
Myomixer-pF2	CGGGGTACCAAGCGCCATTAACAATGTAG
Myomixer-pF3	CGGGGTACCTTTAAGGAGCATAGCCAC
Myomixer-pF4	CGGGGTACCCCCTGGATTTTGTTTTTACA
Myomixer-pF5	CGGGGTACCGGAAGTTTTGACAGCCAGGC
Myomixer-pR	CCGCTCGAGCATGCTGCCTGACTGTGCAG
Primers for qRT-PCR	
Myomixer-F	CCAGCAGTTTTCATCTTG
Myomixer-R	GTGCCACTGAGCCTGC
MyoD1-R	ATCTCTTTCCCCATCTCTGC
MyoD1-F	TTCGTGGTCTTCCGCTTG
MyoD2-R	AGACCACCTTCATCACCATC
MyoD2-F	GGCGTCGTTTACTTTACTCA
EF1α-F	AAGCCAGGTATGGTTGTCAACTTT
EF1α-R	CGTGGTGCATCTCCACAGACT
EMSA assays	
Myomixer-P1-MS2-WT	GGGATGTCTCAACTGCTTCCTCAA
Myomixer-P1-MS2-MUT	AAAGCACTCTGGTCATCCTTCTGG
Myomixer-P1-MS5-WT	CTTGCAAAAACATGTGTGTTTATA
Myomixer-P1-MS5-MUT	TCCATGGGGGTGCACACACCCGCG

2.4. Real-time quantitative PCR (qRT-PCR) analysis

Specific primers for qRT-PCR were designed using Primer Premier 5.0 (Premier Biosoft, USA) based on cloned nucleotide sequences (Table 1). The MyoDs and elongation factor 1-alpha (*EF-1α*) were tested and used as target and reference genes, respectively. The programme parameters were 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 20 s. Amplification efficiencies of the reference and target genes were examined by the slope of the log-linear portion of the calibration curve, with PCR efficiency = $10^{(-1/\text{Slope})}$-1. The mRNA levels of target genes were counted using the $2^{-\Delta\Delta Ct}$ method [32].

Table 2
Sequences of putative binding sites on *AlMyomixer-P1* promoter.

Putative binding sites	Nucleotide sequence	Mutated pattern
M1	TCAACACAACAAGTGTAAAGAGTC	Deletion
M2	GGGATGTCTCAACTGCTTCCTCAA	Deletion
M3	TGTCTCTGACAAATGTACCCTTTA	Deletion
M4	GGTAATGAGCATGTTGTATTCTAC	Deletion
M5	CTTGCAAAAACATGTGTGTTTATA	Deletion
M6	CCATATTTCCAAATGTCTCAGTTG	Deletion

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2.5. Cloning of the myomixer promoter and construction of deletion mutants

To clone the candidate promoter of *Almyomixer*, genomic DNA was extracted from the white muscle tissue of *A. latus*, as described in other marine fish previously [33]. The coded sequence upstream of the myomixer gene was acquired from genomic data of *A. latus*. To identify the core promoter region of *Almyomixer*, the forward primers (Myomixer-pF1, Myomixer-pF2, Myomixer-pF3, Myomixer-pF4, and Myomixer-pF5) were designed with a 5′ *Kpn* I site, and the common reverse primer (Myomixer-pR) was designed with a 3′ *Xho* I site (Table 1). These primers were used to gain a full-length promoter fragment (Myomixer-pF1, 2035 bp and four truncated fragments (i) Myomixer-pF2, 1205 bp; (ii) Myomixer-pF3, 653 bp; (iii) Myomixer-pF4, 488 bp and (iv) Myomixer-pF5, 211 bp) (Fig. 6). The truncated mutants were augmented using PrimeSTAR Master Mix (Takara, Japan). The programme parameters were 95 °C for 4 min followed by 30 cycles of 95 °C for 40 s, 56 °C for 40 s, and 72 °C for 1 min. Then, the PCR products were purified by the general DNA Purification Kit (Tiangen, China). All purified PCR products and a pGL3-basic (Promega, USA) vector were digested with *Kpn* I and *Xho* I and connected by T4 DNA ligase (Takara, Japan) overnight at 16 °C. Recombinant plasmids were collected using the EndoFree Plasmid Giga Kit (Tiangen, China), and recombinants were validated by sequencing, as described above.

2.6. Plasmid construction, cell culturing, transfection and luciferase assay

To acquire the recombinant plasmids containing MyoD1 and MyoD2, the coding sequences for *MyoD1* and *MyoD2* were inserted into the pCDNA3.1-Flag vector (Invitrogen, USA) using gene-specific primers (Table 1). Moreover, to determine the underlying function of MyoD1 and MyoD2 binding sites on the core myomixer promoter, six truncated mutations of recombinant plasmids were structured. The transcription factor binding site prediction (TFBS)-JASPAR database (http://jaspar.genereg.net/), TRANSFAC®, and MatInspector® were used to search for possible binding sites in the myomixer promoter sequence with MyoD1 and MyoD2, respectively. According to the manufacturer's protocol, truncated mutants were produced with a Mutadirect™ site-directed mutagenesis kit (SBS Genetech, Shanghai, China) from the deletion mutant pGL3-basic-myomixer-p1, which was defined as wild-type plasmid. The predictions of six binding sites (M1, M2, M3, M4, M5, and M6) were directly deleted, and the homologous TF binding site schematic diagram and sequences are displayed in Fig. 7A and Table 2, respectively. Furthermore, to obtain the TF binding site mutations, the method of PCR augmentation referred to a previous study [34]. The effect of TF binding site mutations on promoter activity of *Almyomixer* was clarified by dual luciferase assay as below.

HEK293T cells were cultured in DMEM (Gibco, USA) supplemented with 10% foetal bovine serum (FBS) (Invitrogen, USA), 100 $\mu g \cdot mL^{-1}$ streptomycin, and 100 $U \cdot mL^{-1}$ penicillin (Thermo Fisher Scientific, USA) at 37 °C in a humidified incubator under 5% CO_2. Transfection and dual luciferase reporter assays were described by Li et al. (Genecreate, China) [35]. Relative luciferase activities (firefly and renilla luciferase activities) were measured and calculated by the VICTOR™ X2 Multi-label Plate Reader (PerkinElmer, Inc., Waltham, MA, USA).

2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [36]. Briefly, the lysates of HEK293T cells transfected with pcDNA3.1-Flag-MyoD1 or

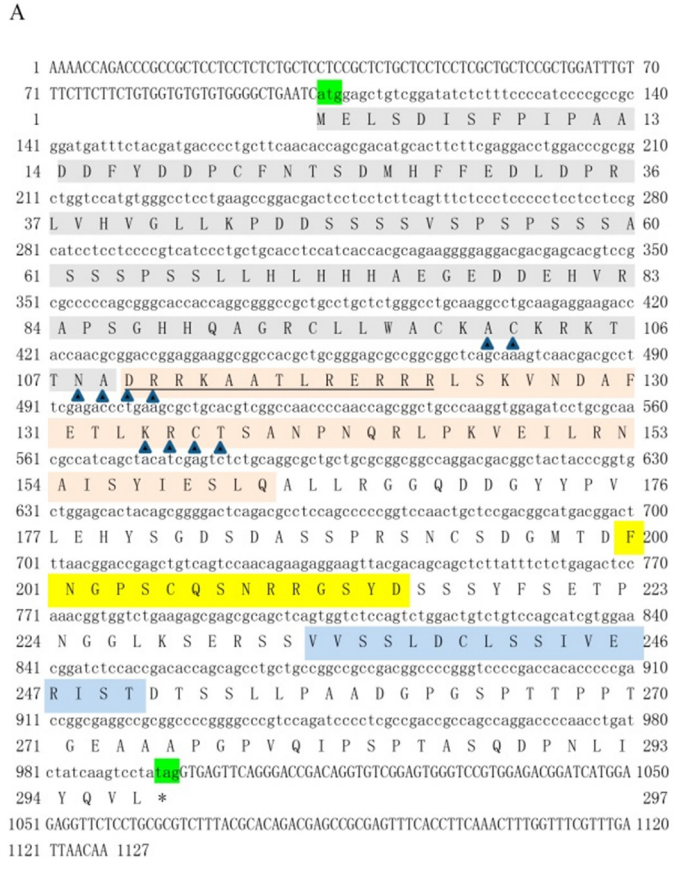

Fig. 1. Sequences characterization of *MyoD1* (A) *and MyoD2* (B) gene in *Acanthopagrus latus*. A. The potential binding domain of cyclin-dependent kinase (cdk) 4 is indicated by a yellow box. The DNA binding region was A^{101}-C^{102}, N^{108}-D^{110}, R^{112}, and K^{134}-T^{137}. B. The DNA binding region was A^{101}-C^{102}, N^{108}-D^{110}, R^{112}, K^{134}-T^{137}. Myogenic basic domain is shaded. The highly conserved basic helix-loop-helix (bHLH) domain, which contains a basic region (underline), is shown with a pink box. Helix III is shown with yellow and blue boxes.

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pcDNA3.1-Flag-MyoD2 were prepared for DNA-protein conjugation reactions. To mark wild-type and mutated oligonucleotides (Table 1) for EMSA biotin-labelled probes, the EMSA Probe Biotin Labelling Kit (Beyotime, China) was used according to the manufacturer's instructions. DNA-protein binding reactions were carried out using an EMSA/Gel-Shift Kit (Beyotime, China) at 25 °C. To observe the specific DNA/protein binding reactions, competition assays were implemented with 100× excessive unmarked wild-type or mutant probes. Then, completed reactions were distributed through non-denaturing 4% PAGE gels after 20 min incubation. The proteins were developed by autoradiography using a LightShift® Chemiluminescent EMSA Kit (Pierce, USA).

2.8. Statistical analysis

All trials were performed in triplicate. All values are displayed as the mean ± SD. Significant differences were calculated by one-way ANOVA tests. $P < 0.05$ was considered to be statistically significant.

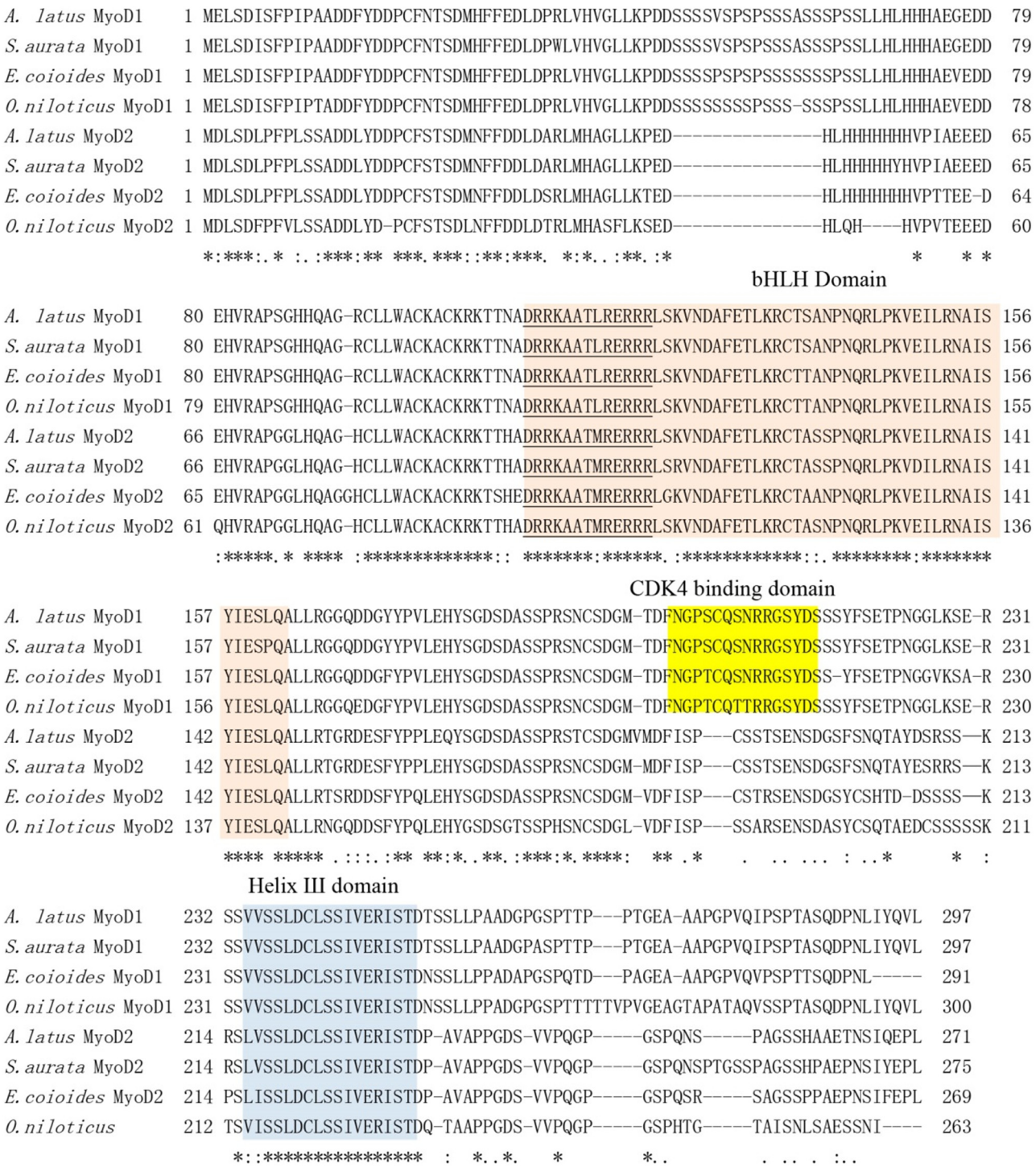

Fig. 2. Amino acid alignment of MyoD1 and MyoD2 proteins from *A. latus*, *Oreochromis niloticus*, *Epinephelus_coioides* and *Sparus aurata*. Conserved sequences are marked with asterisks. Dashes represent gaps created to maximize the degree of identity among all compared sequences. The accession numbers of the MyoD sequences used are listed in Tables S1 and S2.

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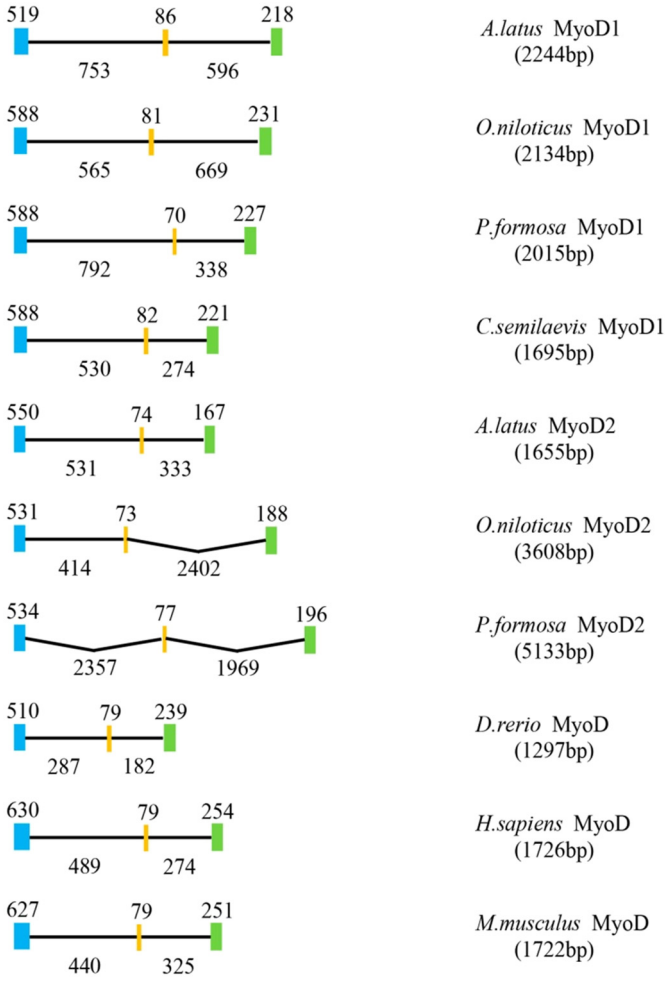

Fig. 3. Genomic structure of MyoD1 and MyoD2 in vertebrates. The lengths of exons and introns of each MyoD gene are displayed proportionally. Different colour boxes and lines represent exons and introns, respectively. The identical colour boxes represent homologous sequences.

3. Results

3.1. Sequence characterization of AlMyoDs and Almyomixer

The genomic sequences of *AlMyoD1* and *AlMyoD2* were obtained. The predicted open reading frames (ORFs) of *AlMyoD1* (Fig. 1A) and *AlMyoD2* (Fig. 1B) are 894 bp and 816 bp long and encode 297 and 271 amino acids, respectively (accession nos. MN266856 and MN266857). The assumed two MyoD proteins possess a conserved myogenic basic domain, basic helix-loop-helix (bHLH) domain, and a Ser-rich region (helix III) (Fig. 1). Moreover, a potential binding domain of cyclin-dependent kinase 4 (cdk4) is also located in MyoD1. The DNA binding regions of AlMyoD1 and AlMyoD2 are A^{101}-C^{102}, N^{108}-D^{110}, R^{112}, K^{134}-T^{137} and A^{101}-C^{102}, N^{108}-D^{110}, R^{112}, K^{134}-T^{137}, respectively. The predicted molecular weights of AlMyoD1 and AlMyoD2 are 32.07 kDa and 29.74 kDa, respectively, and a theoretical isoelectric point of 5.33 and 5.55, respectively. Additionally, the *A. latus* putative genomic sequence of myomixer is 228 bp and includes only one exon with 228 bp. This nucleotide sequence translated to a peptide sequence of 75 amino acids with a predicted molecular weight of 8.34 kDa and a theoretical isoelectric point of 11.24 (accession no. MN266855, Fig. S1).

The aligned teleost MyoDs show the highest sequence homology in the bHLH and adjacent Cys-rich region, in addition to the conserved Ser-rich region (helix III) in the C-terminal end (Fig. 2). A BLAST analysis indicates that the AlMyoD1 protein sequence shares high sequence identity with MyoD1 sequences from other teleosts, including the gilthead seabream (*Sparus aurata*, 99%), grouper (*Epinephelus coioides*, 94%), large yellow croaker (*Larimichthys crocea*, 93%), tilapia (*Oreochromis niloticus*, 91%), and tongue sole (*Cynoglossus semilaevis*, 91%), and low sequence identity with humans (*Homo sapiens*, 58%) and mice (*M. musculus*, 58%) (Table S1). Furthermore, the AlMyoD2 protein sequence shares high sequence identity with MyoD2 sequences from other teleosts, containing *S. aurata* (94%), mandarin fish (*Siniperca chuatsi*, 89%), *E. coioides* (87%), and yellowtail amberjack (*Seriola lalandi dorsalis*, 84%), and low sequence identity with *M. musculus* (64%) and *H. sapiens* (62%) (Table S2).

3.2. Two AlMyoD structural and phylogenetics analyses

Similar to other species *MyoD1* and *MyoD2* genes, three exons and two introns were identified in *AlMyoD* genomic DNA (Fig. 3). Exons are apparently conserved, while the sequence differences of introns are considerable in *A. latus*, *O. niloticus*, *P. formosa*, *C. semilaevis*, *D. rerio*, *H. sapiens* and *M. musculus MyoDs*. Moreover, phylogenetic tree analysis shows that *AlMyoDs* clusters with several MyoD sequences from other osteichthyes and, more distantly, with amphibian, avian, and mammalian MyoD (Fig. 4). *AlMyoDs* are closely grouped together with Sparidae, such as *S. aurata*.

3.3. Tissue expression of AlMyoDs and Almyomixer

The mRNA levels of *AlMyoDs and Almyomixer* are determined by qRT-PCR in various kinds of tissues. There are similar expression patterns between *AlMyoD1* and *AlMyoD2*. The highest *AlMyoD* transcriptions were detected in the white muscle and heart followed by the brain and eye, whereas relatively low *AlMyoD1* and *AlMyoD2* expression levels were detected in the gill and stomach, respectively (Fig. 5A). Nevertheless, the expression levels of *Almyomixer* in the white muscle and eye are considerably higher than in other tissues, and the lowest mRNA levels are in the kidney and spleen ($P < 0.05$) (Fig. 5B).

3.4. Two AlMyoDs activate Almyomixer expression

The amplified candidate *Almyomixer* promoter (2035 bp) is an upstream non-transcribed sequence. To investigate the binding region of MyoD1 and MyoD2 in the *Almyomixer* promoter, a full-length candidate promoter and four truncated mutants were inserted with a promoterless luciferase reporter vector, pGL3-basic (Promega, USA). The promoter activity of construct myomixer-p2 (−1205 bp to +1 bp) memorably reduces compared to the activity of myomixer-p1 (−2035 bp to +1 bp) with co-transfection of MyoD1 or MyoD2. The results indicate that the region between −2035 to −1205 bp may contain some important cis-regulated element sites (Fig. 6). Therefore, the region between −2035 to −1205 bp is considered the core regulatory region of the *Almyomixer* promoter with two MyoDs, and the sequence of construct myomixer-p1 (−2035 bp to +1 bp) was used for further functional analysis.

To explore the MyoD1 and MyoD2 binding sites in the *Almyomixer* promoter, the predicted binding sites that contain the E-box (CAnnTG) are mutated (Fig. 7A, Table 2). The effects on promoter activity were investigated in 293 T cells transfected with each mutant and MyoD1 or MyoD2. The results reveal that mutation of the M2 binding site (GGGA TGTCTCAACTGCTTCCTCAA, −1945 bp to −1922 bp) causes a prominent reduction in promoter activity (Fig. 7B), showing that M2 is the MyoD1 binding site in the *Almyomixer* promoter. Moreover, the M4 binding site (GGTAATGAGCATGTTGTATTCTAC, −1639 bp to −1616 bp), M5 binding site (CTTGCAAAAACATGTGTGTTTATA, −1548 bp to −1525 bp), and M6 binding site (CCATATTTCCAAATGTCTCAGTTG, −1488 bp to −1465 bp) caused a significant reduction in promoter activity (Fig. 7B), suggesting that M4, M5, and M6 are the potentially MyoD2 binding site on the *Almyomixer* promoter, and the degree of decrease of M5 is notably greater

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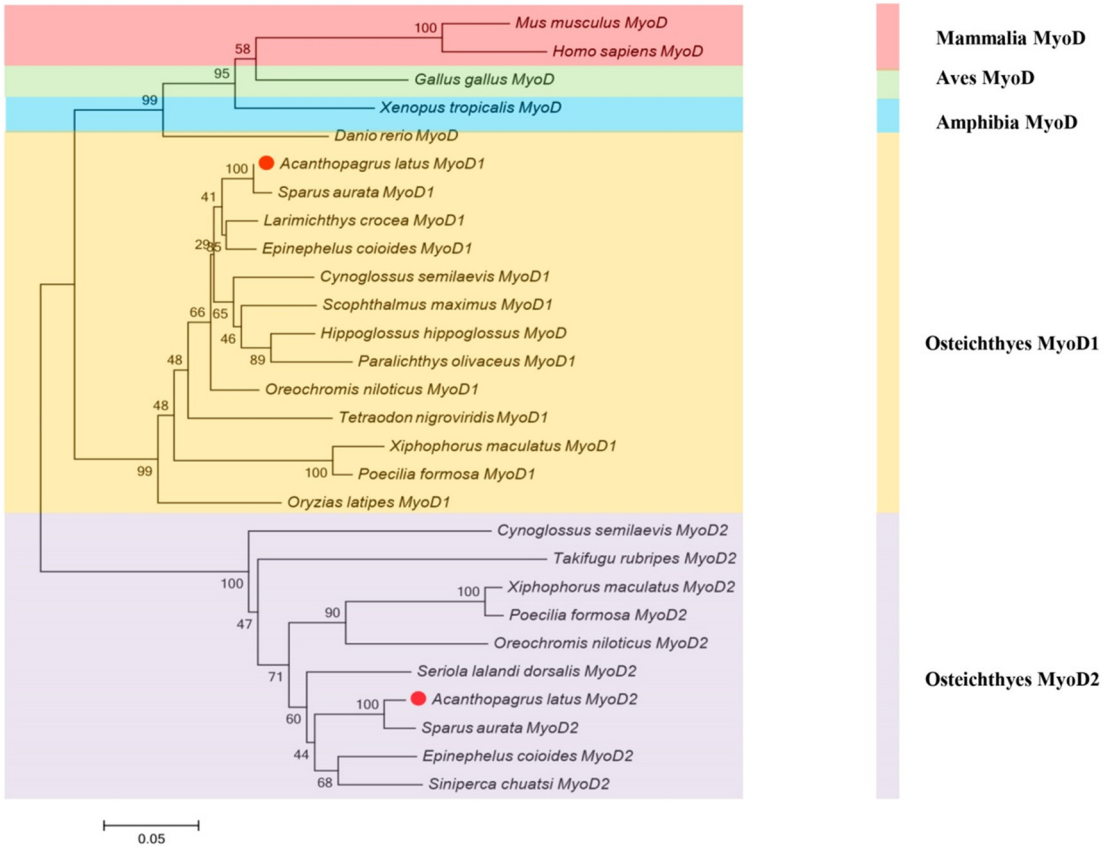

Fig. 4. Phylogenetic relationship of *A. latus* MyoDs amino acid sequences with their counterparts from other species. The main topology was produced by MEGA 6 software with the maximum likelihood (ML) method with 1000 bootstrap replicates. The accession numbers of the sequences used are from Tables S1 and S2.

than that of M4 and M6. Notably, two other predicted binding sites do not induce luciferase activity with MyoD1, suggesting that these two sites are not required for triggering *Almyomixer* expression with MyoD1.

3.5. Binding of AlMyoDs to Almyomixer promoters

To further confirm the MyoD1 and MyoD2 binding motif in the *Almyomixer* promoter, an EMSA assay was performed. Four oligonucleotide probes (Myomixer-P1-MS2-WT, Myomixer-P1-MS2-MUT, Myomixer-P1-MS5-WT, and Myomixer-P1-MS5-MUT) were synthesized and incubated with HEK293T cell lysates containing recombinant MyoD1 and MyoD2 *in vitro* according to the predicted MyoD1 and MyoD2 binding sites (Table 2). Recombinant MyoD1 and MyoD2 bind to the oligonucleotide probes of Myomixer-P1-MS2-WT/MUT and Myomixer-P1-MS5-WT/MUT, respectively. Mutations in nucleotides in the MyoD1 and MyoD2 binding sites (Table 1) result in the separation of the DNA-rMyoD compound (Fig. 8), showing that MyoD1 and MyoD2 are particularly interacting with the M2 and M5 sites in the *Almyomixer* promoter, respectively. The formation of DNA-rMyoD1 and DNA-rMyoD2 compounds is specific, since it can only be blocked by excessive amounts of unlabelled control probes (100×).

4. Discussion

The present study provides insight into the mechanisms underlying the transcriptional regulation of myomixer by two MyoDs in *A. latus*. To this end, the sequence and functional characterization, tissue expression patterns and regulation relationship between two AlMyoDs and *Almyomixer* were determined. The *AlMyoD1* and *AlMyoD2* ORFs encoded a protein that was 69–99% and 70–94% identical to MyoD1 and MyoD2 proteins from other teleosts, respectively. The conserved myogenic basic and bHLH domains were in the N-terminal region, and the helix III domain was in the C-terminal region, which was structural analogous to that of *S. aurata* MyoD1 and MyoD2 [37], Atlantic halibut (*Hippoglossus hippoglossus*) MyoD1 and MyoD2 [38,39], amphioxus (*Branchiostoma belcheri*) MyoD [22], flounder (*Paralichthys olivaceus*) MyoD [40], and Atlantic salmon (*Salmo salar*) MyoD [41]. To inhibit phosphorylation of the retinoblastoma protein in *M. musculus*, MyoD accelerated the vital periods of myoblast cell cycle withdrawal and terminal differentiation by directly reciprocity with cdk4 [42]. A conserved binding domain in the C-terminus (YSGPPC/SS/G-RRR/QN-YD/E) was shown to inhibit cell growth and promote terminal differentiation and was involved in the MyoD-cdk4 interaction but not for the bHLH domain [43,44]. As shown in Fig. 1, this underlying binding domain was well-conserved in teleost MyoD1, while the MyoD2 paralogs apparently lacked this motif [39]. It is tempting to conjecture that the AlMyoD1 comprising this putative binding site might be involved in secluding cdk4-related kinases to generate terminal differentiation. Moreover, several conserved DNA binding sites were found in both MyoDs (Fig. 1 and Fig. 2). Phylogenetic analysis indicated a typical phylogeny, revealing that the amino acid sequences of two AlMyoDs were closely matched to MyoDs of *S. aurata* but then appeared to separate from other fish, amphibian, avian, and mammalian species. A genome structure analysis revealed that all *MyoDs* included 3 exons and 2 introns in metazoans, suggesting that the function of *MyoDs* was conservative.

In post-hatching *H. hippoglossus*, MyoD was expressed solely in the muscle, suggesting that MyoD played an important role in muscle growth in growing fish [40]. However, in adult *S. aurata*, MyoD1 was expressed in both slow and fast muscles, whereas MyoD2 was exclusively expressed in fast muscles [37]. In the present study, a tissue-specific expression pattern revealed that the highest *AlMyoD* mRNA expression was detected in the white muscle and heart, which was in accordance with the result of *M. amblycephala*, *C. idellus* MRFs [29,45]. Nevertheless, the transcription levels of *Almyomixer* in the white muscle

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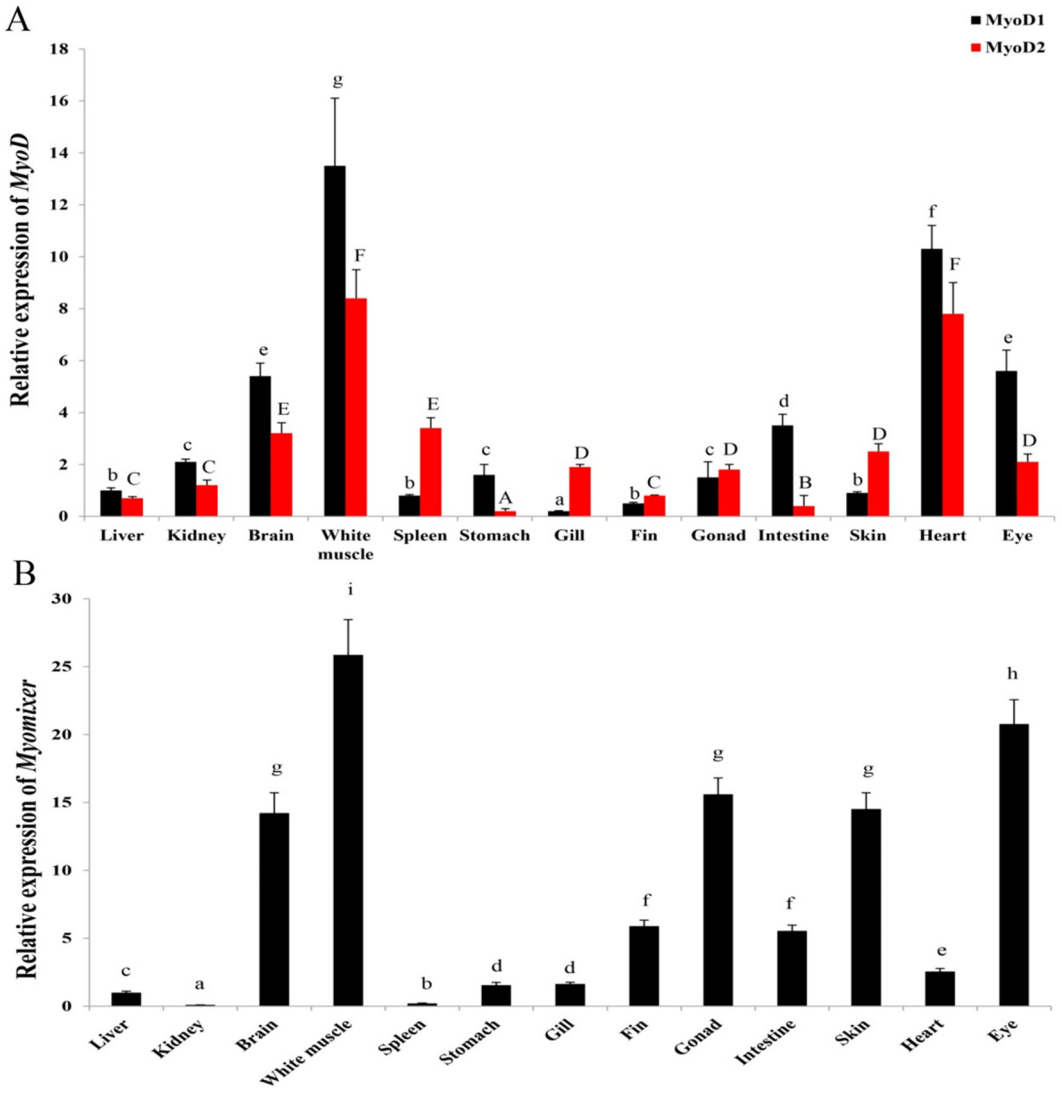

Fig. 5. Relative expression levels of *AlMyoD* and *Almyomixer* in different tissues. The twelve tissues are heart, gonad, eye, skin, brain, fin, spleen, small intestine, gill, white muscle, kidney, liver, and stomach. Different letters indicate significant differences ($P < 0.05$).

were considerably higher than in other tissues, showing that those three genes played important roles in muscle development.

Generally, the expression of several genes in eukaryotic cells relies on RNA polymerases and transcription factors binding to special sequences in the promoters of target genes [46–48]. Furthermore, the integrity and activity of a promoter could activate gene transcription. The transcription factors MyoDs regulate skeletal muscle growth in mammals and alter the expression of the muscle-related genes in teleosts and avians [49,50]. Several studies have demonstrated that MyoD increases the downstream gene *myomaker* expression by binding its CANNTG motifs (E-boxes) in the promoter region [16,18,19,49,50]. Moreover, in *A. latus*, numerous E-box motifs were also observed in the promoter of myomixer. MyoDs contain a highly conserved bHLH domain, which is required for heterodimerization with specific DNA binding to E-boxes found in regulatory regions of their target genes [1,51–53]. Consequently, to confirm whether *Almyomixer* could be regulated by two AlMyoDs, dual-luciferase reporter assays were conducted. The results of truncated mutants showed that *Almyomixer* reporter activity was induced by the overexpression of two AlMyoDs. The core binding region in the *Almyomixer* promoter was −2035 to −1205 bp (Fig. 6). This result was the first evidence showing that the transcription of *Almyomixer* could be upregulated by two AlMyoDs.

Furthermore, to further determine the active MyoD1 and MyoD2 binding sites on the *Almyomixer* promoter, six vectors with point mutations were constructed. The deletion of the MyoD1 M2 binding site (GGGATGTCTCAACTGCTTCCTCAA), the MyoD2 M5 binding site (CTTG CAAAAACATGTGTGTTTATA), and the MyoD2 M6 binding site (CCATAT TTCCAAATGTCTCAGTTG) resulted in significantly reduced promoter activity (Fig. 7), suggesting that the MyoD1 binding M2 site and MyoD2 binding M5/6 site were essential for *Almyomixer* promoter activity. Furthermore, the sequences of those three binding sites were representative E-box motifs. Consequently, the M2 and M5 binding sites of MyoDs were chosen for the compound probe sequences. The EMSA assay showed that MyoD1 and MyoD2 specifically bound to the *Almyomixer* promoter at the binding M2 and M5 sites, respectively (Fig. 8). Briefly, MyoD1 and MyoD2 could control myomixer expression by binding the M2 and M5 binding sites in fish, respectively.

5. Conclusions

The sequence and expression characteristics and regulatory function of AlMyoD1 and AlMyoD2 were described. Both AlMyoD1 and AlMyoD2 possessed representative features of the MyoD family. Moreover, the expression of *AlMyoD1*, *AlMyoD2* and *Almyomixer* was the highest in white muscle. Furthermore, two AlMyoDs activated *Almyomixer*

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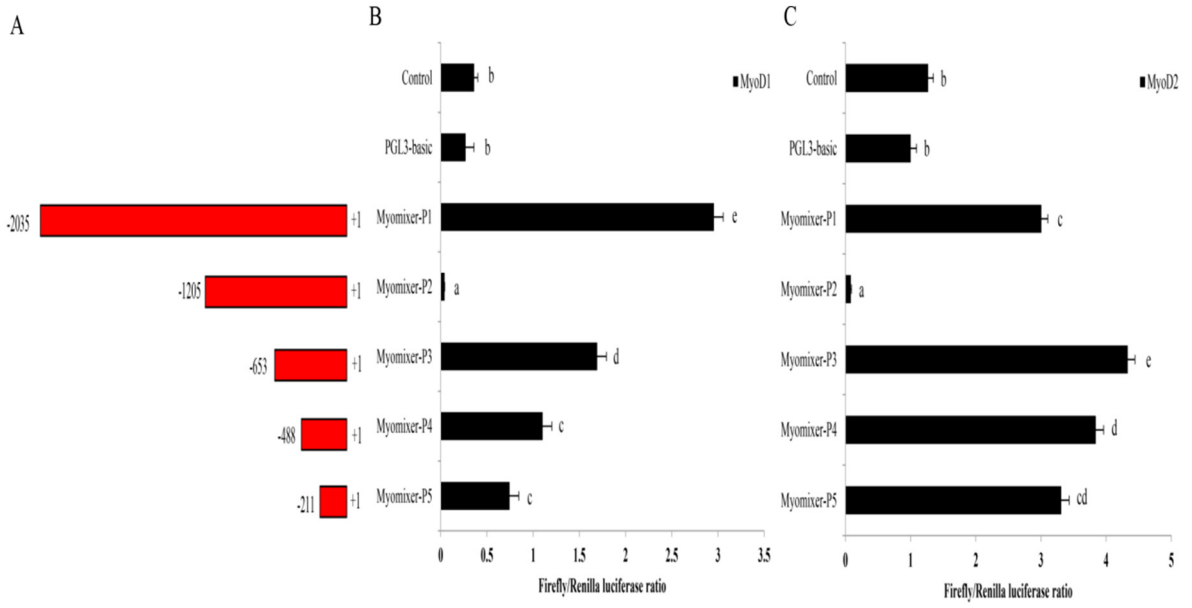

Fig. 6. Promoter activity analysis of the *Almyomixer* gene. (A) The structure of the *Almyomixer* promoter. Five recombinant plasmids were denoted myomixer-p1 (−2035 to +1), myomixer-p2 (−1205 to +1), myomixer-p3 (−653 to +1), myomixer-p4 (−488 to +1) and myomixer-p5 (−211 to +1). (B & C) The transcriptional activity of the *Almyomixer* promoter. These plasmids were transfected along with the transcription factor MyoD1 (B) and MyoD2 (C) into HEK 293 T cells. Dual-luciferase activity was driven by the *Almyomixer* promoter upon the transfection of pcDNA3.1-MyoD1 or pcDNA3.1-MyoD2 or pcDNA3.1 into HEK 293 T cells. Data are presented as the means of three replicates ± SD. Different letters indicate significant differences ($P < 0.05$).

expression by binding with the E-box on its promoter. The EMSA assays further showed that *AlMyoD1* and *AlMyoD2* bound effectively to the M2 and M5 binding sites in the *Almyomixer* promoter, respectively. Thus, a positive feedback mechanism mediated by myomixer-induced MyoD activation was proposed in *A. latus*. Our findings might help to elucidate the molecular basis of myoblast fusion involving myomixer function and help to characterize the regulatory functions of MyoDs in marine fishes.

Abbreviations

MyoD	myoblast determination protein
EMSA	electrophoretic mobile shift assays
bHLH	basic helix-loop-helix
Myf5	myogenic factor 5
MRF4	muscle-specific regulatory factor 4
ORF	open reading frame
MRFs	myogenic regulatory factors
qRT-PCR	quantitative real-time polymerase chain reaction
FBS	fetal bovine serum

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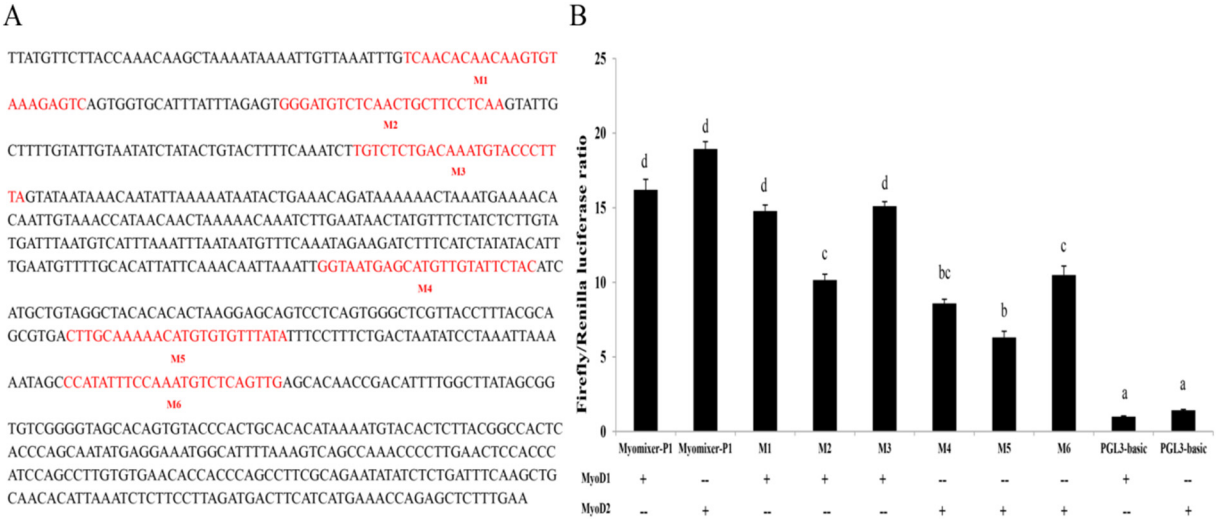

Fig. 7. Construction of truncated mutants for the identification of predicted transcription factor (TF) binding sites in the *Almyomixer* promoter. (A) The nucleotide sequence and predicted binding sites in the core region of the *Almyomixer-p1* promoter. Effects of six mutants on *Almyomixer-p1* promoter activity transfected with pcDNA3.1-MyoD1 (B) or pcDNA3.1-MyoD2 (C) or pcDNA3.1. Binding sites are shown with boxes. Data are presented as the means of three replicates ± SD. Different letters indicate significant differences ($P < 0.05$).

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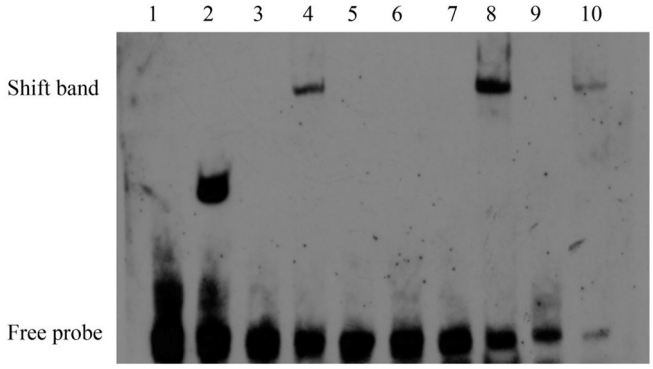

Fig. 8. Binding reactions of AlMyoDs and *Almyomixer* promoters. Biotin-labelled EMSA probes were incubated with lysates of HEK293T cells containing MyoD1 and MyoD2 protein, respectively. WT, wild-type probe; MT: mutated probe. 1, negative control; 2, positive control; 3, plus Myomixer-P1-MS2-WT; 4, Myomixer-P1-MS2-WT plus MyoD1-flag; 5, plus Myomixer-P1-MS2-MUT; 6, Myomixer-P1-MS2-MUT plus MyoD1-flag; 7, plus Myomixer-P1-MS5-WT; 8, Myomixer-P1-MS5-WT plus MyoD2-flag; 9, plus Myomixer-P1-MS5-WT; 10, Myomixer-P1-MS5-WT plus MyoD2-flag.

Author contributions

K.C.Z., S.G.J., and D.C.Z. designed the research and wrote the paper. K.C.Z. performed the research. H.Y.G. and N.Z. analyzed the data. B.S.L. and L.G. contributed reagents/materials/analysis tools.

Declaration of competing interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.11.139.

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