Molecular Characterization of Rockbream (*Oplegnathus fasciatus*) Cytoskeletal β-actin Gene and Its 5'-Upstream Regulatory Region

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The cytoskeletal β-actin gene and its 5'-upstream region were isolated and characterized in the rockbream (*Oplegnathus fasciatus*). Complementary DNA of the rockbream β-actin represented a 1,125 bp of an open reading frame encoding 375 amino acids, and the rockbream β-actin cDNA and deduced amino acid sequences were highly homologous to those of other vertebrate orthologs. At the genomic level, the β-actin gene also exhibited an organization typical of vertebrate cytoskeletal actin genes (2,159 bp composed of five translated exons interrupted by four introns) with a conserved GT/AG exon-intron splicing rule. The putative non-translated exon predicted in the rockbream β-actin gene was much more homologous with those of teleostean β-actin genes than those of mammals. The 5'-upstream regulatory region isolated by genome walking displayed conserved and essential elements such as TATA, CArG and CAAT boxes in its proximal part, while several other immune- or stress-related motifs such as those for NF-kappa B, USF, HNF, AP-1 and C/EBP were in the distal part. Semi-quantitative RT-PCR assay results demonstrated that the rockbream β-actin transcripts were ubiquitously but differentially expressed across the tissues of juveniles.

Key words: Rockbream (*Oplegnathus fasciatus*), β-actin, Gene structure, mRNA expression, Regulatory region

Introduction

Cytoskeletal β-actin, a highly conserved protein found in a wide spectrum of animal cell types, plays crucial roles in maintaining cell shape and cellular mobility (Reece et al., 1992). Due to its constitutive and housekeeping expression, β-actin mRNA has been used commonly as an invariant standard for various gene expression assays (Pang and Ge, 2002; Andreassen et al., 2005; Cao et al., 2007). More importantly, the functional utility of the β-actin promoter as a strong regulator to drive heterologous expression of its downstream sequence has been reported in various fish species harboring the β-actin regulator-driven gene construct (Liu et al., 1990; Nam et al., 2001, Hwang et al., 2003; Brooks et al., 2007).

Genetic determinants of β-actins have been isolated and characterized from many teleost species. Previous studies have indicated that most fish β-actin genes share high sequence homology and had evolved from a common ancestor (Lee and Gye, 2001; Noh et al., 2003; Kim et al., 2008). However, barring a few studies, postmortem information on the isolation of fish β-actin genes has been limited mainly to the characterization of structural genes or coding regions. The regulatory regions of the fish β-actin genes, however, have been relatively less studied (see Kim et al., 2008). Structural and functional characterization of the 5'-flanking region in the β-actin gene is a prerequisite for employing the β-actin promoter as a regulatory element in germ-line transgenesis and DNA vaccination (Nam et al., 2008; Ruiz et al., 2008).

The objective of this study was to characterize the molecular structure of the cytoplasmic β-actin gene in the rockbream (*Oplegnathus fasciatus*), a highly valued marine food fish in Korea. We thus isolated and characterized rockbream β-actin cDNA and genomic DNA gene; assessed the structure and putative elements of the 5'-flanking regulatory region; and examined the tissue expression of β-actin transcripts

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in a semi-quantitative RT-PCR assay.

Materials and Methods

Isolation of rockbream β-actin cDNA

From our rockbream liver and kidney expressed sequence tag (EST) databases (unpublished data), clones exhibiting significant homology to previously known teleostean β-actin orthologs were surveyed. Based on a contig analysis with 20 selected clones (9 liver and 11 kidney clones) using Sequencher (Gene Codes, USA), the continuous version of full-length rockbream β-actin cDNA was isolated by RT-PCR. DNase-treated liver total RNA (2 µg) was reverse-transcribed using Omni-RTase (Qiagen, Germany) and oligo-d(T)$_{20}$ primer according to the manufacturer's recommendation. The RT-product (2 µl) was subjected to PCR amplification with primers (RB β-ACT 1F: 5'-TTCATTTGCCTGAAACCGGTTC-3' and RB β-ACT 1R: 5'-CATGTCGGAACACATGT GCA-3'). Amplification of the expected 1,856-bp product was carried out using the Expand High Fidelity PCR System (Roche Applied Science, Germany) under the following thermal cycling conditions: 30 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 1 min with an initial denaturation step at 94°C for 3 min. The amplification product was cloned into pGEM T-easy vector (Promega, USA) and sequenced in both directions using the primer walking method.

Isolation of rockbream β-actin genomic gene

Based on the full-length cDNA sequence, the continuous fragment from ATG through the stop codon (TAA) was isolated by genomic PCR. Genomic DNA was purified from whole blood using the conventional SDS/proteinase K method and the integrity of the genomic DNA was confirmed by agarose gel electrophoresis. Next, 1 µg of the genomic DNA was subjected to PCR amplification using primers, RB gβ-ACT 1F (5'-TCAGCCATGGAAGA TGAAATCG-3') and RB gβ-ACT 1R (5'-TAAGTA TGCGCCAACCACTG-3'). Thermal cycling conditions for the genomic PCR involved 30 cycles at 94°C for 45 s, 60°C for 30 s and 72°C for 1 min. The PCR product was TA cloned and sequenced (six clones) using several sequencing primers as above.

Isolation of the β-actin 5'-flanking upstream region

To amplify the proximal 5'-flanking region of the rockbream β-actin gene, a forward primer, RB β-ACT 5FW (5'-TGACGCTGGACCAATCAG-3'), was designed based on the conserved sequence of known teleostean β-actin upstream sequences. A reverse primer, RB gβ-ACT 2R (5'-ACCTCATTGG ACTGCATAGC-3'), was designed to be comple-mentary to the intron following the first translated exon of the rockbream β-actin gene. PCR ampli-fication (30 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min with an initial denaturation step at 94°C for 4 min) was carried out using the Expand High Fidelity PCR system. PCR products were cloned into pGEM T-easy vector, and insert sequen-ces were obtained as described above. Based on the sequence information, the distal region of the β-actin upstream sequence was obtained by the genome walking method. Genome walking was performed with the GenomeWalker Universal Kit (Clontech Laboratories, USA) according to the manufacturer's instructions. Briefly, 2.5 µg of genomic DNA was digested by *Eco*RV, *Dra*I, *Pvu*II or *Stu*I restriction endonuclease. After the adaptor ligation step as guided by the instruction manual, two rounds of successive PCR amplifications were carried out using one each of the two primer pairs AP1 (forward primer provided in the kit)/ RB β-actin GW01 (5'-GTGACG GCGCTCAGAGTGAATGTGTAA-3') and AP2/ RB β-actin GW02 (5'-GTGGGCGCTTGTTTTTTATAC TGCGTC-3'). Amplified products were TA cloned and sequenced as above. Based on the contig assem-bly, a continuous version of the 5'-flanking region was isolated again from the genomic DNA and its sequence was confirmed.

Sequence analysis

The amino acid sequence was deduced using the open reading frame (ORF) finder by NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple se-quence alignments of the nucleotide and amino acid sequences were performed using ClustalW (http://align.genome.jp). Transcription factor binding motifs in the 5'-flanking upstream region of rockbream β-actin were predicted using TESS (http://www.cbil. upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and the TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html). The rockbream β-actin cDNA and genomic sequences are available from GenBank under accession numbers FJ975145 and FJ975146, respectively. To perform the molecular phylogenic analysis of the putative non-translated exon (first exon) from the teleostean β-actin genes, publically available β-actin sequences were either downloaded from GenBank or deciphered from the Ensembl genome database (http://www.ensembl.org/index. html). Putative nontranslated exon I sequences were aligned using ClustalW with the default setting for gap penalties in BioEdit 7.0.5 (Hall, 1999). The

aligned data matrix was subjected to neighbor-joining (NJ) analysis and visualized with the Molecular Evolutionary Genetics Analysis (MEGA) program. Two mammalian seque-nces from humans (*Homo sapiens*) and mice (*Mus musculus*) were used as outgroups. The robustness of the tree topologies was evaluated by bootstrap analyses with 1,000 replicates.

Semi-quantitative RT-PCR assay

RT-PCR was carried out to determine the relative mRNA expression levels of rockbream β-actin across different tissues. Ten somatic tissues (brain, eye, fin, gill, heart, intestine, kidney, liver, muscle, and spleen) were obtained surgically from juvenile in-dividuals (n=8; mean body weight=159±24 g). Total RNA was extracted from each tissue sample pooled from the eight individuals using the RNeasy Midi Kit (Qiagen, Germany) according to the manufacturer's instructions. Two μg of purified total RNA was reverse transcribed into cDNA using the Omniscript Reverse Transcription kit (Qiagen). The reverse transcriptase (RT) reaction included an oligo-d(T)$_{20}$ primer and a rockbream 18S rRNA primer (Cho et al., 2008) at final concentrations of 1 and 0.05 μM, respectively. All other reaction steps were performed according to the manufacturer's recom-mendations. The cDNA samples from each tissue were diluted twofold (for β-actin) or tenfold (for 18S rRNA) with sterile distilled water, and then 2 μL of diluted cDNA was subjected to PCR thermal cycling. Based on a preliminary assay, PCR in a reaction volume of 50 μL was performed with AccuPower PCR premix (Bio-neer, Korea) using primers qRBβ-ACT 1F (5'-ATG GAAGATGAAATCGCCGC-3') and qRBβ-ACT 1R (5'-AGGGTCAGGATACCTCTCTT-3'). As a nor-malizing standard, rockbream 18S rRNA was also amplified from each cDNA sample using primers qRB18S 1F (5'-TACCACATCCAAGGAAGGCA-3') and qRB18S 1R (5'-TTCCTAGCTGCGGTATTCA G-3'). Amplicon sizes of β-actin and 18S rRNA were 200 and 407 bp, respectively. The reaction was con-ducted for 20 (β-actin) or 18 (18S rRNA) cycles at 94°C for 20 s, 58°C for 20 s, and 72°C for 20 s, with an initial denaturation step at 94°C for 2 min. The level of β-actin transcripts in each sample was normalized against its own 18S rRNA control using the image analysis software Quantity-One imple-mentted in VersaDoc 4000 (Bio-Rad, USA). All assays were performed in triplicate per cDNA sample. Differences in the relative expression levels among samples were assessed by analysis of variance (ANOVA) followed by Duncan's multiple range test at a significance level of P=0.05.

Results and Discussion

Sequence characteristics of cDNA and deduced amino acid

The complementary DNA of rockbream β-actin consisted of a 59 bp of 5'-UTR, a 1,125-bp single ORF encoding 375 amino acids and a 672-bp 3'-UTR excluding a 23-bp poly (A+) tail. A consensus polya-denylation signal (AATAAA) was observed 27 bp before the poly (A+) tail (see GenBank number FJ975145). The theoretical pI and molecular weight values of the rockbream β-actin estimated with its deduced amino acid sequence were 5.30 and 41,767 Da, respectively. A multiple sequence alignment of the rockbream β-actin with other orthologs revealed that it shares high homology with most vertebrate orthologs at both the cDNA and amino acid levels (alignment not shown). Such a high sequence identity indicates that the structure and function of β-actins are highly conserved in the vertebrate lineage and that most nucleotide changes in the coding regions are synonymous (or silent) third-base substitutions as observed in other actin genes (Fang and Brandhorst 1994; Kim et al., 2008).

Gene structure and organization

The rockbream β-actin gene displayed an organi-zation typical of vertebrate cytoplasmic actin genes. The genomic gene sequence of β-actin amplified by PCR contained six exons: one putative non-translated exon (exon I in 91 bp) and five translated exons. The five translated exons (123 bp from the ATG translation start site and 240, 439, 182 and 144 bp for exons II to VI, respectively) were interrupted by four introns (396, 418, 110 and 107 bp), with illustrating the conserved GT/AG junction rule (see GenBank number FJ975146). Translated exon sequences were clearly matched with the cDNA sequence. Moreover, the number of exons in the rockbream β-actin gene is identical to most teleost β-actin genes (Venkatesh et al., 1996; Hwang et al., 2002; Noh et al., 2003; Kim et al., 2008) as well to as warm-blooded vertebrate β- and γ-cytoskeletal actins (Erba et al., 1988). Most β- and γ-cytoskeletal actins of vertebrates are generally agreed to have originated from a common ancestor through divergence based on gene duplication events (see Kim et al., 2008). However, several teleost species are known to exhibit uncommon genomic organization in their β-cytoplasmic actin genes. For example, *Takifugu rubripes* β-cytoplasmic actin 2 and *Pagrus major* β-cytoplasmic actin possess one more intron, and *T. rubripes* β-cytoplasmic (vascular type) actin gene contains eight introns (Venkatesh et al.,

1996). Hence, further mining of different β-actin isoforms from the rockbream genome would be central to gaining deeper insight into the molecular evolution of the actin gene family in this species.

Molecular features of the 5'-upstream region

From both the PCR isolation and genome walking method, a total of 3.95 kb of the upstream sequence from the ATG translation start codon was obtained (Fig. 1). A conserved TATA box (TATAAA) was identified 1,600 bp upstream from the ATG, and two CAAT boxes (CCAAT) were predicted at positions, -1,781 and -1,663. A consensus CC(A/T)$_6$GG motif (CArG box; CCTTTTATGG) was identified at two locations: 33 bp upstream from the TATA box and in the intron between non-translated exon and the first translated exon (221 bp prior to the ATG start codon). The CArG box is known to be a *trans*-acting factor-binding site (Reece et al., 1992) and its presence in the proximal promoter as well as in the intron following a non-translated exon is a common feature of many teleost β-actin genes (Takagi et al., 1994; Noh et al., 2003; Kim et al., 2008). Overall, the proximal region of rockbream β-actin promoter was highly homologous to the equivalent regions of the major teleost β-actin genes (Takagi et al., 1994; Venkatesh et al., 1996).

Besides the essential motifs in the proximal regions, several other transcription factor binding sites were predicted (see Fig. 1), including motifs/elements for nuclear factor kappa B (NF-κB), upstream stimulatory factor (USF), hepatocyte nuclear factor (HNF), CCAAT/enhancer binding protein (C/EBP), activeting protein-1 (AP-1) and metal responsive transcripttion factor-1 (MTF-1). Although the specific roles of these transcription factors in the regulation of the rockbream β-actin gene are not yet clear, many of them have been reported to be involved in stress-and/or inflammation-mediated pathways (Anderson, 2000; Bayele et al., 2006; Cho et al., 2009). The presence of these motifs is generally in agreement with recent studies reporting that β-actin gene expression is unstable and/or responsive to various physiological states, suggesting that caution is needed to employ this gene as an internal control (Arukwe, 2006; Filby and Tyler, 2007; Small et al., 2008). Hence, a heterologous expression study should follow to elucidate the functional role of the transcription factor binding motifs in the modulation of rockbream β-actin gene. In addition, the examination of β-actin transcripts in response to different stimulatory treatments, including bacterial or viral challenges,

would be valuable to hypothesize the possible orchestration of these transcription factors in β-actin gene transcription.

Typing of non-translated exon

An *in silico* analysis based on the alignment of the rockbream β-actin 5'-upstream sequence with those from other teleost and representative mammalian species successfully allowed us to successfully identify a putative non-translated exon although the transcription initiation site of the rockbream β-actin gene was not empirically determined (Fig. 2). The presumed rockbream β-actin non-translated exon was located at positions -1,569 to -1,481 bp from the ATG codon. As shown in Fig. 2, the non-translated exon of the rockbream β-actin shares high sequence identity with those of teleost species. Furthermore, the overall sequence homology among species was generally in accord with their known taxonomic positions based on our phylogenetic analysis.

Tissue distribution and basal expression in juveniles

Rockbream β-actin transcripts were detected ubiquitously in all tissues tested, but the basal expression levels varied among tissues based on the semi-quantitative RT-PCR analysis (Fig. 3). Our external control, the 18S rRNA, was quite stable without any notable fluctuation among samples as evidenced by the narrow range in deviations from the mean of the ten tissue types examined (<6%) in the scanning densitometry assay. When normalized to the 18S rRNA control, the β-actin transcripts were expressed most abundantly in the kidney and spleen, followed by the brain. The eye, fin, gill, heart, and intestine displayed moderate levels expression, while the lowest expression was detected in the liver and muscle. Unlike muscular alpha actins, β-actin, a cytoskeletal actin, is known to be expressed in almost all animal tissue types, and thereby the ubiquitous distribution of β-actin transcripts in this study was as expected (see also Reece et al., 1992; Hennessey et al., 1993). However, a quantitative comparison of β-actin transcripts across tissue types has not been performed extensively in fish (Venkatesh et al., 1996; Kim et al., 2008). Although, we did not estimate the absolute copy numbers by real-time RT-PCR, the difference in the basal expression level of β-actin between the kidney (highest expression) and muscle (lowest) was more than tenfold under our semi-quantitative RT-PCR conditions. Differential expression of β-actin mRNAs across tissue types, particul-

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-3952  ctgatttaacacaaagacacagaaacaaactctggtttcactctttaatcaggagcacatgaagaagaacacgccattaaacgtttttaca
                                                              USF
-3862  cctgaatcaaaggaggctgggccggaacaatgactcacatgaatgaatgtgcaaacgtacagactgacatacacactgtgaacttgagag
       USF                            AP-1 & USF
-3772  gcactccctctaggccggattaaatattttccagccaatccctctttacgccatcacagcgtcaacatggagaaacaatcctctcagctt
-3682  gtttcccttcaaatctctgttaacagcatcacctggacctgcacctttttttattattacactccctcatcacagaaatgcacctttatc
                                      USF
-3592  gccactgaaaccattatataaatgagtagacgagtttcaggctgacacaggtgagacagggataaaccaggtttgagatttgacctttagcg
-3502  atgagatttaaggcctgtaaatcaataagtgggcccagcagacctttgatctccttaaacactcttccttccatccgtaaaccctctaaa
-3412  cagcgtctgcatgagactgcacagacagtctttcaccgagtcaaaactctgactcaacatggacaaattgacaggtctagctctgattgt
                                                      AP-1
-3322  tcccttttttcttttttttaaatgtttatcaccatcttataattatctaagtctggaagcaggactggacttaaactgtgtttgtaaaaga
                                                                               HNF
-3232  actaaaatgtgagcctgcagggtgagagggaacatacacgagccatcgtcatctttctgcatattaaaagaccgctgtttcacactgcat
-3142  ggacactgaggtgtagctcattcaaagggtcatatgaaccatgtgaggacgacagtaatgtccttttaaagccaacttgacagaatgaa
-3052  tgaagacaaggaagagaagacaagaacgaaaaaaaaacagatacacaggagttgatgcaactgagagaggaagggaagtggtaaaggag
-2962  gaagagaaggagttgctgcatgtctcagtttaaatggcaccgaggacgtccagccgctctgctaacacatgcactcatggtgtaggagc
                                                                    USF & MRE
-2872  acaaagtgaataaaagtgtgcaccagatgttacatattagggagaagatggatgatagaaggatggagggcaaatgagagaaaaagaaat
                         MRE
-2782  tcacaagtgaaaaaaggaaaatgcttaagagtggggcggtatcacttaagataagactttggtctgatggtgtatggatccttaatatga
-2692  cagtgaatggaagttttactatagcctgcaggcccatggccattcaagagagtgtcacatgggcctcatagagggtcaaaatgacatata
                                                            USF
-2602  ataatgacataataggtgtttctacatgattgaaactgaaaacctctatagtactttcactaagtgtgtatttaggtgattttttttggg
                                                                                           C/EBP &
-2512  aattttcacaaagtatattgcctgaggggtattatttaactctcttttttaacacctcatctcagtatactgcaagcactaaaacctgaga
       NF-kB
-2422  gtgctgacaaatcccatcctctcctgagacgacacacctttgtcaaactattgtatctcaggcagaaagagagaggcagcaagcagccag
-2332  tggactggatatttaggaggagcccaagaccatgtagtcacagagcagcacaaatactcataataatcaataaataaatgaacattatca
                                                            HNF
-2242  gcgagcgttggcagtatacggagaaggggtggcctacataagtggtcattccccggcctgcatgtggcagggcagctccaccaccctgtg
-2152  tgtgttatgtatgtcatgtgtttgacctggtgatgcagtggctgtgagcctacagcaacaccagcatatcgcctcctcaccctctgaaa
-2062  aatatgaaatagtgtcaggttaatgtaaagagaagagccgggagcgagggcagcctttggaggaaagtctccggcctttgcaggcttcac
-1972  agggaggagtccaggatgttaagctccttcagttatcatataaagtatgtccagactgctcagagcaggtgcagggcggacattttaact
-1882  cttttaaaagtttatacatcatttaacccgcatataaaacagaatatcaagagattaggcttactaggcctcacaacatcaatcccttccc
-1792  ttttattttctccaatcctttgctattatcgcctccctctctctccctctctctctctctccctcaaatcgcgagcgcccagtgcacc
                  CAAT box
-1702  acagtgtgcacgcgacgtgccccagtgcatgacgctggaccaatcagagggcgctattccgaaagtttacctttttatggctcgagccggg
          MRE                                 CAAT box                         CArG box
-1612  cagctgacgcagtataaaaacaagcgcccacagcttacacATTCACTCTGAGCGCCGTCACACGCAGCTTGTGCGGGATATCATTTGCC
                         TATA box                        Non-translated exon I
-1522  TGAAACCGGTTCCCTTAAAGCGAAAAGCCCCCCCACCCAAAGgtaaggagacagaaacggttatttaaatatttctaattttagaatgat
-1432  atttatcacgtaaacaaagtctgtatttgtttttttaaatgcaatttgtgtaaaaatgtgtcatttttgaacgcaaaagacggcagggacca
-1342  cgaggttctttgtcctgtcatacaccaactgtactgctaattaaacttaatggttttaaaaaggtaattatgatagaaacaatgtattt
-1252  gtgcttgtttttaaagtatgtagtggcgttggttagagaccgccaaacttgcggctcacaggtgcttttggctgtagccggcttatggt
-1162  taatggtactttgttaagtcaacggtcctactttaaactggtcatcgtgtaagttagtggtgcaacatttgctgcatcccagcgttggc
-1072  ttgtctctcttccccagcagggtagtttgaaagaggcttttgagttgaggtcgtctggatcccggctgctccctttgtgcgcccgatg
- 982  cggcggggtgtgacctactttagcatattagcctagccacatcatgctaacaccgcccttcagacttgctgcagcagcaatttgaatcaa
- 892  gatacatttttaccacagacacttgtgcacacttcttgggcgtgttaaatttcactttatcctctgaataatttacatttatataaggc
- 802  taatcctctaaataagaaagtgccaagaataggaggcctgcaaaaagcataaattgattagtggattaattaaataaacctgtcttgctg
- 712  tgaattctcgccagttggcacatgaggggaaaagaggaaactgcgaatgacaaatcatcctcttagaaaaggcgtggctttcaggaaatg
- 622  gagcatcagtcaaggacttgtgaccattcatgagtcaacatataagttcattatgcaacttcatttattttaatccagtgtgtgaaggtt
- 532  ggtgccagtatttagaaaatgcagaatagctgttaattatatctgtagtcgtgtgtcagagaggcagtatgaatgaatgaggttgaatat
- 442  gacgcagtgatattgaagggaggctctcaggaggggtattaaaaccagtatgattgggcaacaatgggaggggggcagcggaggaagtta
- 352  accgtagtgaaccggagggagtggtcgcgcggcgcccggtctgatagtctcgaatgttccttttttttttctgccgctctggccgcgcgcca
- 262  tggcgccggtagccgcaaagctgctcaaaaccgaaccatgtccttatatggtaataacagaatgcagcgccacttccttttgtctggctt
                                               CArG box
- 172  ggccggaatgcggggcacgagctccacccagcggacagtctcgtgaactgccgctagttgcgttaacaggaagtgagagctcaaagtgtg
                                                                                           +1
- 82   cagcggacttttgttgggaggcggatgtgagactttgagtgtttttaacggtgtttttgtttttctcctctttaacagTTCAGCCATGGAAGA
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Fig. 1. The 5'-flanking upstream sequence of the rockbream *Oplegnathus fasciatus* β-actin gene. Potential transcription factor binding motifs or elements are indicated by bold italics, and a putative non-translated exon is noted by uppercase letters and underlined. Figures on the left are distance from the translation start site (ATG; +1).
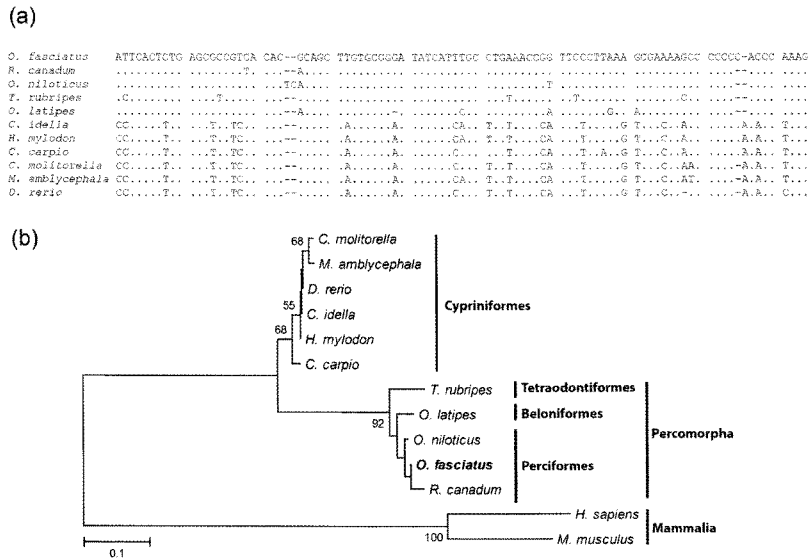
(a)

(b)

Fig. 2. Sequence comparison and phylogeny of non-translated exons from representative teleostean β-actin genes. (a) Multiple sequence alignment of teleost β-actin non-translated exons. GenBank accession number or Ensembl ID for each sequence is FJ975146 (*Oplegnathus fasciatus*), AY971579 (*Rachycentron canadum*), AY116536 (*Oreochromis niloticus*), U37499.1 (*Takifugu rubripes*), S74868 (*Oryzias latipes*), M25013 (*Ctenopharyngodon idella*), EF554926 (*Hemibarbus mylodon*), M24113 (*Cyprinus carpio*), DQ241809 (*Cirrhinus molitorella*) and AY170122 (*Megalobrama amblycephala*), and ENSDARG00000037870 (*Danio rerio*). Dots indicate the identical nucleotides to the *O. fasciatus* sequence at the respective positions. Dashes are gaps introduced for an optimal alignment. (b) Phylogenetic tree drawn with the Molecular Evolutionary Genetics Analysis (MEGA) tool using a neighbor-joining algorism. Two mammalian non-translated exon sequences (M10277 for *Homo sapiens* and X03672 for *Mus musculus*) are used as outgroups. Bootstrap values are shown at each node when the confidence level is above 50%.
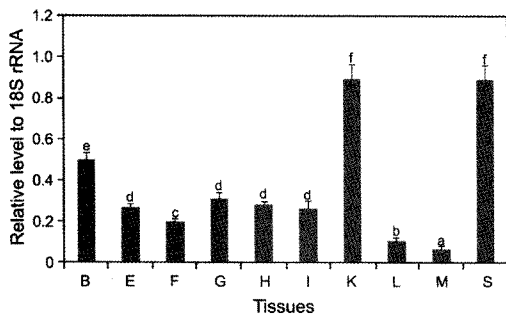
Fig. 3. Tissue distribution and basal expression levels of β-actin transcripts in rockbream *Oplegnathus fasciatus* juveniles based on the semi-quantitative RT-PCR assay. Tissues are brain (B), eye (E), fin (F), gill (G), heart (H), intestine (I), kidney (K), liver (L), muscle (M) and spleen (S). The level of β-actin transcripts in each tissue was normalized against 18S rRNA control based on triplicate amplifications. Mean ± SDs with same letters are not significantly different based on ANOVA followed by Duncan's multiple range test at $P = 0.05$.

arly with regard to the low expression in the liver and muscle, has been reported previously in *Hemibarbus mylodon* (Kim et al., 2008). Data from the previous and present studies strongly suggest that β-actin control may be inappropriate for normalizing the relative levels of a target gene transcript among different tissue types.

Further studies are needed to evaluate the functional utility of the 5'-flanking region for driving heterologous expression in its own and/or related fish species. In addition, careful preexamination of the β-actin gene expression under different biotic and abiotic conditions may be needed before employing β-actin as an invariant control for certain gene expression assays in this species.

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in the phylogenetic analysis.

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