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Identification of differentially expressed genes in the developmental stages from olive flounder *Paralichthys olivaceus* using an annealing control primer system

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We employed a new and improved differential display reverse transcription–polymerase chain reaction (DDRT-PCR) method, which involves annealing control primers (ACPs), to identify the genes that are specifically or prominently expressed in olive flounder (*Paralichthys olivaceus*) juveniles (35 days post-hatch; dph) compared to larval-stage (dph 21) flounder. Using 60 ACPs, we identified eight differentially expressed genes (DEGs) and basic local alignment search tool (BLAST) searches revealed eight known genes. Gene expression levels were confirmed by RT-PCR. Phosphoglucose isomerase (PGI) was highly expressed at 21 dph, while nephrosin, myosin light chain (MLC), myosin heavy chain (MHC), carboxypeptidase A, chymotrypsin B, fish-egg protein, and matrix protein were expressed at 35 dph. PGI, MLC, and MHC expression was further analyzed by RT-PCR. The differentially expressed genes identified in this study may provide insights into the molecular basis of development in olive flounder.

Keywords: annealing control primer; gene expression; *Paralichthys olivaceus*; RT-PCR

Introduction

An important factor governing the successful production of commercially viable numbers of cultured marine fish is early larval survivability. Early developmental stages are a complicated process including cleavage, blastulation, gastrulation, body axis formation, tissue, and organ formation (Drasdo and Forgacs 2000). Olive flounder, *Paralichthys olivaceus*, is one of the most widely cultured fish species and is considered to be an important protein source in Asia. A major bottleneck in flounder farming is the production of juveniles for on-growing. Significant problems include high embryonic larval mortality and the prevalence of body deformities (Kjørsvik et al. 1990; Wieser 1995). While many variables are important for achieving good survival, effective larval feeding and nutrition are most crucial (Shields 2001). Olive flounder are fed enriched rotifers and *Artemia* sp. until 21 days post-hatch (dph) and almost reach metamorphosis at 35 dph once feeding with dry diets begins (Takeuchi 1998). Flounder larvae undergo dramatic changes in body shape, morphology, metabolism, swimming ability, and behavior until they completely metamorphose into juveniles at 35 dph. Although flounder have been studied for many years, little information is available regarding their stage-specific developmental gene expression (Park et al. 2009; Wen et al. 2009; Nam et al. 2010). Therefore, more exhaustive molecular approaches are needed to identify the genes involved in the regulation of biological changes occurring during flounder larval development.

Recently, an improved method to identify differentially expressed genes (DEGs) was developed that uses annealing control primers (ACPs) (Hwang et al. 2003; Kim et al. 2004). This is an easy technique that does not produce false-positives and only allows real products to be amplified. In this study, we used this technique to identify differentially expressed transcripts in larval and early juvenile stages of olive flounder.

Materials and methods

Sample preparation

Larval (21 dph, approximately 8 mm body length) and juvenile (35 dph, approximately 13 mm body length) olive flounder were obtained from Koje Hatchery of the National Fisheries Research and Development Institute. Samples were rapidly frozen in liquid nitrogen, ground with a mortar/pestle, and stored at -80°C.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's

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instructions. First-strand cDNA synthesis was performed with reverse transcriptase. Reverse transcription was performed for 1.5 h at 42°C in a final reaction volume of 20 μl containing 3 μg of purified total RNA, 4 μl of 5 × reaction buffer (Promega, Madison, WI, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM dT-ACP1 (5′-CTGTGAATGCTGCGACTAC-GATIIIII$(T)_{18}$-3′), 0.5 μl of RNasin® RNase Inhibitor (40 U/μl; Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega). First-strand cDNAs were diluted by adding 80 μl of ultra-purified water for the GeneFishing™ PCR and stored at −20°C until use.

ACP-based GeneFishing™ PCR

DEGs were screened by the ACP-based PCR method (Kim et al. 2004) using GeneFishing™ DEG kits (SeeGene, Seoul, Korea). Briefly, second-strand cDNA synthesis was conducted at 50°C during one cycle of first-stage PCR in a final reaction volume of 20 μl containing 3–5 μl (about 50 ng) of diluted first-strand cDNA, 1 μl of dT-ACP2 (10 μM), 1 μl of 10 μM arbitrary ACP, and 10 μl of 2 × Master Mix (SeeGene). The PCR protocol for second-strand synthesis was one cycle at 94°C for 1 min, 50°C for 3 min, and 72°C for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles at 94°C for 40 s, 65°C for 40 s, 72°C for 40 s, and a 5-min final extension at 72°C The amplified PCR products were separated on 2% agarose gels stained with ethidium bromide.

Cloning and sequencing

Differentially expressed DNA fragments were separated on agarose gels, extracted from the gel using the GENCLEAN® II Kit (Q-biogene, Carlsbad, CA, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen) according to the manufacturer's instructions. The cloned plasmids were sequenced bidirectionly with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Identified DEGs were confirmed by BLAST searches.

Reverse transcription PCR

Total RNA was used to synthesize the cDNA from larval whole bodies ($n = 30$) at 7, 14, 21, 27, and 35 dph (approximately 4–13 mm body length at 20°C). First-strand cDNA synthesis was conducted using the Advantage RT-for-PCR kit (BD Biosciences, Sparks, MD, USA). DEG expression was confirmed by RT-PCR using each gene-specific primer pair in Table 1. The gene encoding GAPDH DNA was used as an internal control. The cycling conditions were 95°C for 5 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by one cycle at 72°C for 5 min. Amplified PCR products were analyzed on 2.0% agarose gels containing ethidium bromide (100 ng/ml).

Protein alignment and phylogenetic analysis

The nucleotide sequences and deduced amino acid sequences were analyzed using the Genetyx-Win

Table 1. Sequence-specific primers for RT-PCR of differentially expressed genes in olive flounder.

DEG no.	Gene name	GenBank Acc. No.		Primer sequence	Length (bp)
3	Nephrosin	FJ211409	F	GAGCCGACGGGGCTGCAT	321
			R	CTGGCTCATCTGCTTGGC	
8	Myosin light chain	FJ211410	F	GGACGTCATCAAAATCCT	306
			R	CATGATGTGCTTGACGAA	
10	Myosin heavy chain	FJ211411	F	CTGAGCTGTTCAAGATGAAG	318
			R	GATCTGCTCCATCTCCTC	
14	Phosphoglucose isomerase	FJ211412	F	TGGGGAGAGCCAGGAACC	310
			R	TCCAAGTGTGTACGGTGTCA	
15	Carboxypeptidase A	FJ211413	F	TACGGCTACACCAGGACT	305
			R	TATGGGTTCTTGAGGGTGTG	
21	Fish-egg lectin	FJ211414	F	CTACTGCCTGAGAGAGAGT	312
			R	TGCGAGAACTGCTGATGC	
28	Chymotrypsin B	FJ211415	F	GGGGAAGAAGAAGTTGCT	185
			R	ATCCTTCATTAACCAAACTCT	
29	Matrix protein	FJ211416	F	AAGCAAGGGGGATCTCAG	309
			R	GGTTGCATTCCTTCAGCC	
	GAPDH	AB0293370	F	TCCCATGTTCGTCATGGGCGTGA	292
			R	ATTGAGCTCAGGGATGACCTTG	

program ver. 4.0 (Genetyx Co., Tokyo, Japan). Multiple alignments of the proteins were constructed using the CLUSTALW program (Thompson 1994) and visualized with MEGA3.1 (Kumar et al. 2001). A phylogenetic tree based on the deduced amino acid sequences was constructed using the neighbor-joining (NJ) algorithm (Saitou and Nei 1987), and the confidence level of each branching node was tested by bootstrap resampling (1000 pseudo-replicates) using MEGA3.1.

Results and discussion

Differentially expressed genes in larval and juvenile olive flounder

To explore DEGs at the larval stage and juvenile stages, RNAs extracted from two different stages were subjected to RT-PCR using a combination of 60 arbitrary primers and two anchored oligo(dT) primers (dT-ACP1 and dT-ACP2). This method is depicted schematically in Figure 1. Among the 146 amplicons analyzed, 29 were differentially expressed (data not shown) and eight DNA bands were excised from the gels, sequenced for analysis, and numbered as DEG 3, 8, 10, 14, 15, 21, 28, and 29 (Figure 2A). Almost all DEGs were abundantly expressed in 35 dph larvae, but only DEG 14 was mainly expressed at 21 dph. The identified gene names, GenBank accession numbers, and specific primer sets are summarized in Table 1. A BLAST search for sequence similarity in the NCBI GenBank revealed that eight DEGs, that is, nephrosin (DEG 3), myosin light chain (DEG 8), myosin heavy chain (DEG 10), phosphoglucose isomerase (DEG 14), carboxypeptidase A (DEG 15), fish-egg protein (DEG 21), chymotrypsin B (DEG 28), and matrix protein (DEG 29), showed significant similarities (67–91%) with sequences from other species. These differential

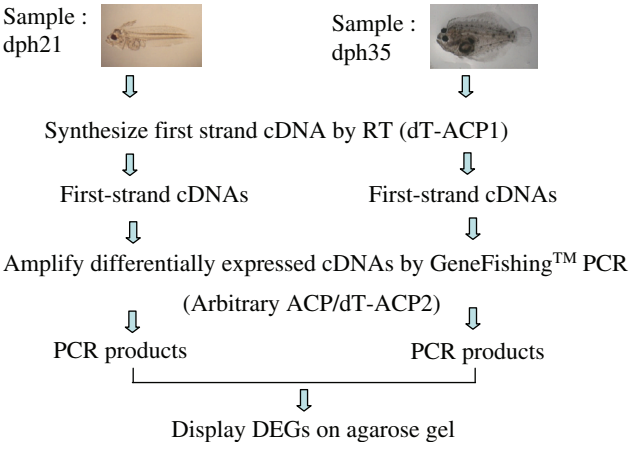

Figure 1. Schematic depiction of the annealing control primer polymerase chain reaction GeneFishing procedure.

display patterns between 21 and 35 dph larvae as assessed three times by ACP RT-PCR were very reproducible.

Confirmation of annealing control primer differential display by RT-PCR

The DEG 3, 8, 10, 14, 15, 21, 28, and 29 expression patterns were confirmed by RT-PCR (Figure 2B). Sequence-specific primers were designed to amplify products with lengths ranging from 150 to 350 bp. The RT-PCR assay revealed that in accordance with the ACP differential display, DEG 14 mRNA levels were significantly higher in 21 dph than 35 dph flounder juveniles and others were expressed relatively less in 21 dph larvae. Among them, PGI and the myosin genes have been previously reported to show developmental stage-specific distribution in other species (Whalen et al. 1981; Xu et al. 1996). However, neither gene has been reported from olive flounder yet, so the differential gene expression involved in flounder development was investigated in the larval developmental stages and various tissues of adults (2 years old), respectively.

Analysis of the differentially expressed DEGS during the developmental stages of olive flounder

Phosphoglucose isomerase

Phosphoglucose isomerase (PGI: EC 5.3.1.9) is a ubiquitous cytosolic enzyme that plays a key role in the glycolysis and gluconeogenesis pathways (Harrison 1974). PGI acts as a potent mitogen/cytokine (i.e., tumor autocrine mobility factor, neuroleukin, and maturation factor; Chaput et al. 1988; Watanabe et al. 1996; Xu et al. 1996) and therefore represents a unique example of a 'moonlighting protein' which exhibits multiple cellular functions (Jeffery 1999). Other previously unknown factors and functions, such as a sperm antigen involved in sperm agglutination from mouse sperm and a novel serine proteinase inhibitor from the skeletal muscle of white croaker, *Argyrosomus argentatus*, were also reported recently (Cao et al. 2000; Yakirevich and Naot 2000). The deduced amino acid sequence of flounder PGI was compared with PGI sequences from other species. The deduced amino acid sequence of the olive flounder PGI had a 74–88% identity with those from others, and the PGI from flathead mullet, *Mugil cephalus*, was more highly related to that of olive flounder than the other species (Figure 3A). To discriminate the specific expression levels, we assessed PGI expression at five different larval stages (7, 14, 21, 28, and 35 dph) (Figure 3B). The PGI transcripts were detected from 14 dph and increased until 21 dph but then declined at 35 dph. At the adult stage, PGI mRNA

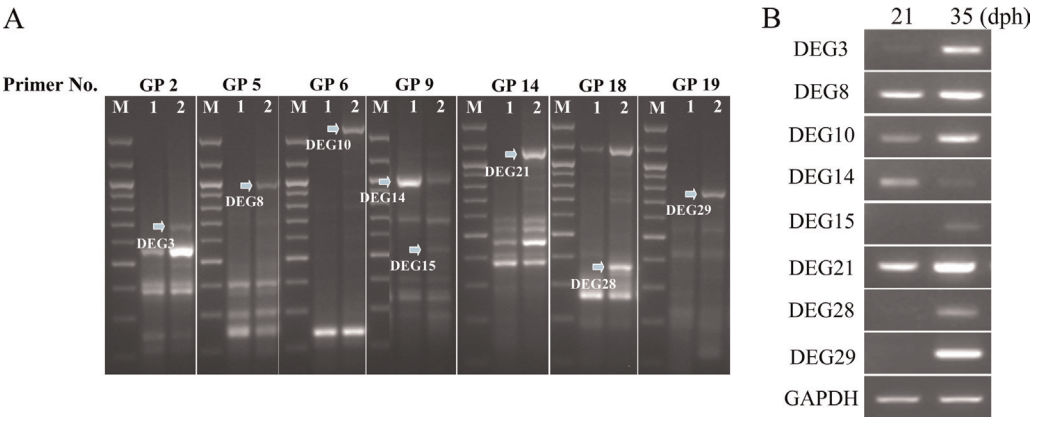

Figure 2. Differential expression of genes during two developmental stages (21 and 35 days post-hatch; dph). (A) PCR products from 21 dph (1) and 35 dph (2) olive flounder were amplified using annealing control primers and separated on 2% agarose gels containing ethidium bromide. Arrows indicate the differentially expressed genes (DEGs) that showed differential expression during the two developmental stages. (B) DEGs expression (DEG 3, 8, 10, 14, 15, 21, 28, and 29) was confirmed by RT-PCR using each gene-specific primer pair.

was predominantly expressed in the muscle (Figure 3C). These results suggest that PGI might play a specific role in the early larval stage-specific distribution and muscle metabolism of olive flounder.

Heavy and light chain skeletal myosin

Myosin, the major striated muscle protein component, consists of two heavy chains (MHCs) and four light chains (MLCs), which combine to form a long coiled

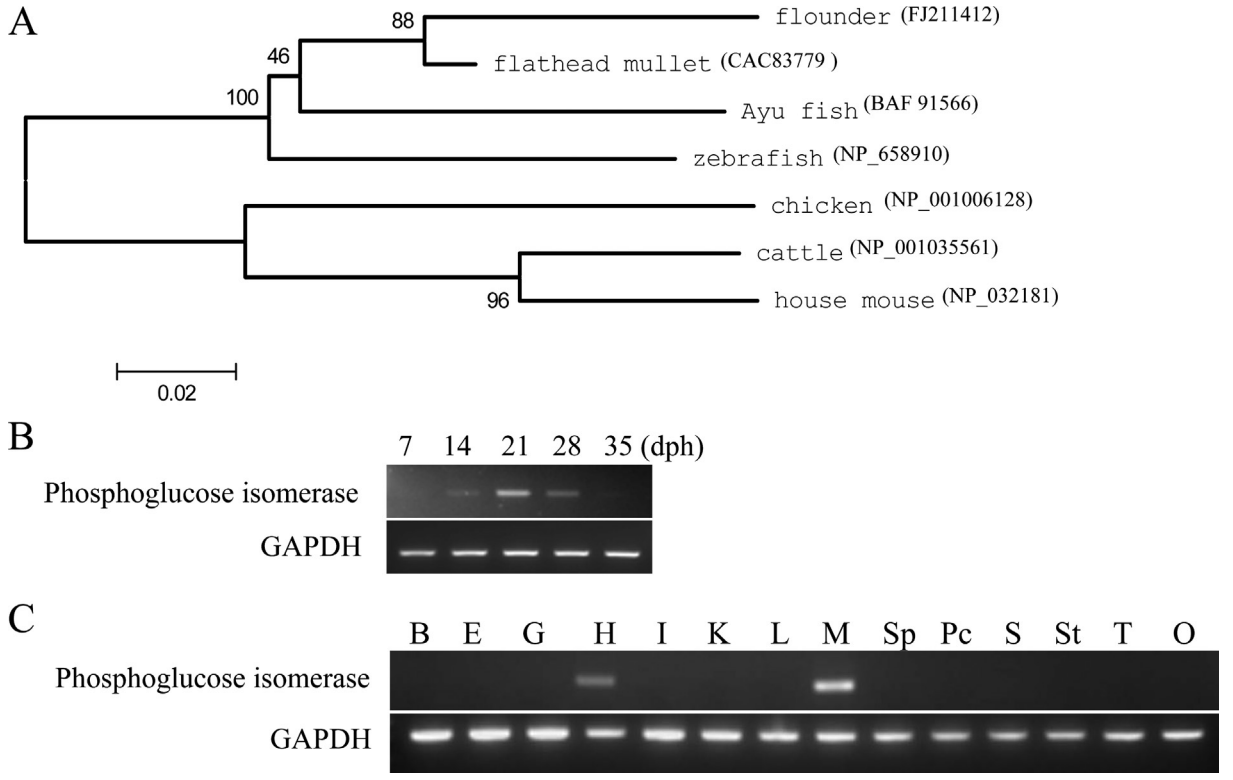

Figure 3. Phylogenetic analysis of the deduced amino acid sequences and RT-PCR analysis of phosphoglucose isomerase (PGI) gene expression. (A) Phylogenetic analysis based on the PGI amino acid sequences from flounder and other species using the neighbor-joining method in MEGA 3.1. GenBank accession numbers are shown in parentheses. (B) RT-PCR assay of PGI gene expression at five different developmental stages (7, 14, 21, 28, and 35 days post-hatch). (C) Expression of PGI mRNA in various tissues of olive flounder. B, brain; E, eye; G, gill; H, heart; I, intestine; K, kidney; L, liver; M, muscle; Sp, spleen; Pc, pyloric ceca; S, skin; St, stomach; T, testis; O, ovary.

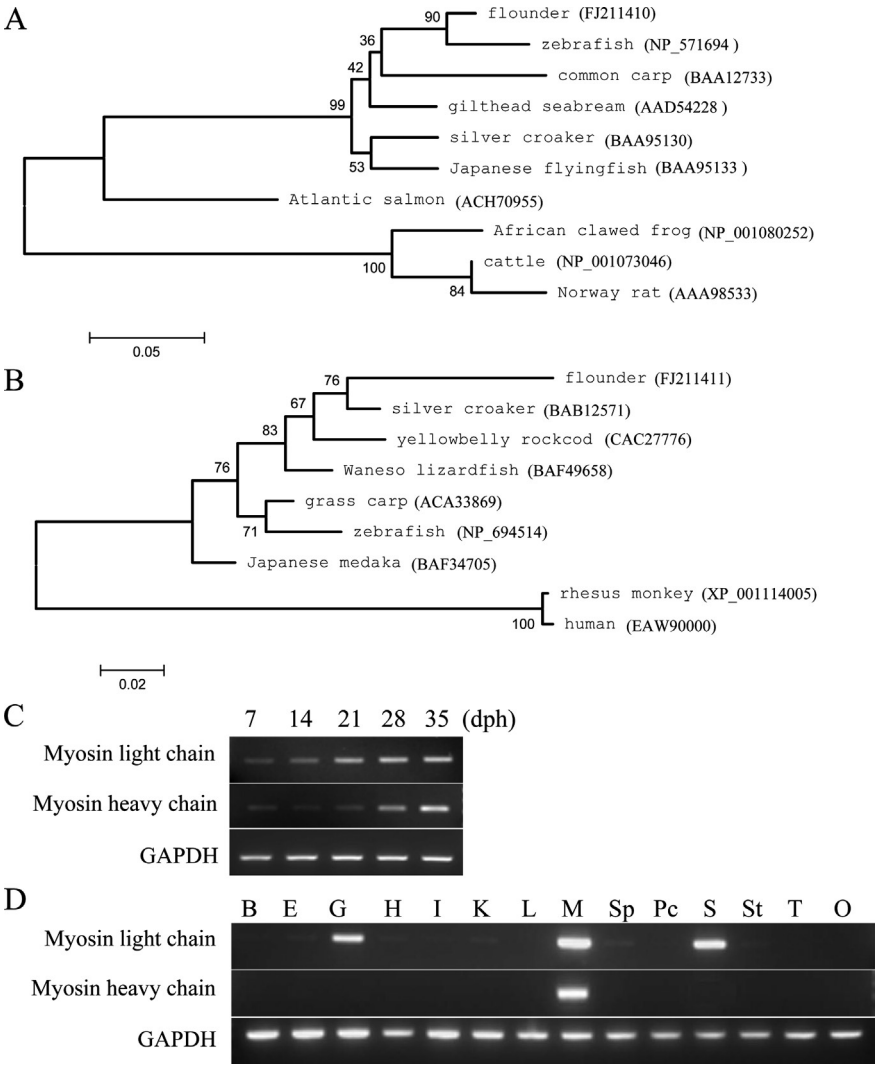

Figure 4. Phylogenetic analysis of the deduced amino acid sequences and RT-PCR analysis of the myosin light chain (MLC) and myosin heavy chain (MHC) (A and B). Phylogenetic analysis based on the deduced amino acid sequences of MLC and MHC from olive flounder and other organisms using the neighbor-joining method in MEGA 3.1. GenBank accession numbers are shown in parentheses. (C) RT-PCR assay for MLC and MHC gene expression at the five different developmental stages (7, 14, 21, 28, and 35 days post-hatch). (D) MLC and MHC mRNA expression in various tissues of the olive flounder. B, brain; E, eye; G, gill; H, heart; I, intestine; K, kidney; L, liver; M, muscle; Sp, spleen; Pc, pyloric ceca; S, skin; St, stomach; T, testis; O, ovary.

α-helical tail with two heads. Each head contains an actin-binding site and ATPase activity. The formation of the head structure involves the N-terminal half of two MHCs and one pair of light chains (Lowey 1994). Both MHCs and MLCs exist in multiple isoforms, which show tissue-specific and/or developmental stage-specific distribution, and their expression is known to be environmentally and hormonally controlled (Gauthier et al. 1982; Izumo et al. 1986; Yamano et al. 1994; Hill et al. 2000). The phylogenetic analysis of the MLC and MHC amino acid sequences from olive flounder and other animals is shown in Figure 4A,B. The deduced amino acid sequences of olive flounder MLC and MHC showed 84–91% identities with other MLCs and MHCs. The phylogenetic analysis indicated that the MLC and MHC of olive flounder were closer to that of zebrafish, *Danio rerio*, and silver croaker, *Pennahia argentata*, respectively, than other species. Analysis of gene expression using RT-PCR indicated that the MLC and MHC genes were detected from the early stage of development and gradually increased by 35 dph (Figure 4C). In adult fish, MHC mRNA was only expressed in muscle, whereas the MLC transcript was detected in muscle, gill, and skin (Figure 4D).

The distinct differences in PGI, MLC, and MHC gene expression during development suggest that they play important roles during development and growth of olive flounder, and also may be used as molecular markers of

development. Although the detailed functions of these genes and their products remain to be determined, their identification provides insights into the molecular mechanisms involved in developmental processes.

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