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Four Members of Heat Shock Protein 70 Family in Korean Rose Bitterling (*Rhodeus uyekii*)

Jung Hyun Kim[1], Chun Mae Dong[1], Julan Kim[1], Cheul Min An[1], Hae Ja Baek[2] and †Hee Jeong Kong[1]

[1]Biotechnology Research Division, National Fisheries Research and Development Institute, Busan 46083, Korea
[2]Dept. of Marine Biology, Graduate School, Pukyong National University, Busan 48513, Korea

ABSTRACT : Heat shock protein (HSP) 70, the highly conserved stress protein families, plays important roles in protecting cells against heat and other stresses in most animal species. In the present study, we identified and characterized four Hsp70 (RuHSP4, RuHSC70, RuHSP12A, RuGRP78) family proteins based on the expressed sequence tag (EST) analysis of the Korean rose bitterling *R. uyekii* cDNA library. The deduced RuHSP70 family has high amino acid identities of 72-99% with those of other species. Phylogenetic analysis revealed that RuHsp70 family clustered with fish groups (HSP4, HSC70, HSP12A, GRP78) proteins. Quantitative RT-PCR analysis showed the specific expression patterns of RuHsp70 family members in the early developmental stages and several tissues in Korean rose bitterling. The expression of 4 groups of Hsp70 family was detected in all tested tissue. Particularly, Hsp70 family of Korean rose bitterling is highly expressed in hepato-pancreas and sexual gonad (testis and ovary). The expression of Hsp70 family was differentially regulated in accordance with early development stage of *Rhodeus uyekii.*

Key words : Development, Expression, Korean rose bitterling, HSP 70 family, *Rhodeus uyekii*

INTRODUCTION

The heat shock or stress response refers to the reaction of cellular organisms to adverse environmental stresses such as heat stress and heavy metals, which are highly conserved proteins (Welch et al., 1991). It composed of the rapid and coordinated induction of a group of proteins referred to as the stress proteins and the concomitant reduction of normal cellular proteins. The stress proteins can be divided into two groups of families: the heat shock proteins (HSPs) and the glucose-regulated proteins (GRPs). HSPs are categorized into several families and named according to their function, sequence homology and molecular mass in kilo-Daltons (kDa): HSP100, HSP90, HSP70, HSP60, HSP40 and several smaller HSP groups (Lindquist, 1992).

HSP70 is the largest and most highly conserved of the stress protein families (Sanders, 1993). HSP70 family contains four major members: HSP70, heat shock cognate 70 (Hsc70), HSP75 and GRP78 (Polanowska-Grabowska et al., 1997; Bausero et al., 2005). At least 121 proteins have been isolated in the HSP70 family and cross-hybridization occurs across mammals, fish and molluscs; indeed, humans and molluscs share the same antigenic and ATP binding domains (Margulis et al., 1989; Roberts et al., 2010). Hsp70 family was isolated from a variety of fishes such as rainbow trout (Kothary et al., 1984), *Oryzias latipes* (Arai et al., 1995), Zebrafish

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† Corresponding Author : Hee Jeong Kong, Biotechnology Research Division, National Fisheries Research and Development Institute (NFRDI), 216 Haean-ro, Gijang-up, Gijang-gun, Busan 46083, Republic of Korea. Tel. : +82-51-720-2453, Fax :+82-51-720-2456, E-mail : heejkong@korea.kr

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(Lele et al., 1997), *Oreochromis mossambicus* (Molina et al., 2000) and *Fugu rubripes* (Lim & Brenner, 1999) etc. Hsp70 family also has been reported to be associated with differences in environmental temperatures (Feder & Hofmann, 1999).

Most of the HSPs are also constitutively synthesized in considerable amounts even in the unstressed normal cells (Welch et al., 1991; Roberts et al., 2010; Hunt & Morimoto, 1985), which play a fundamental role in the regulation of normal protein synthesis within the cell. HSP families such as HSP90 and HSP70 are critical to the folding and assembly of other cellular proteins (Gething & Sambrook, 1992). These also have a wider role in relation to the immune, apoptotic and inflammatory processes (Moseley, 2000; Srivastava, 2002; Pockley, 2003). Depletion of either HSP70 or HSP90 in a transgenic zebrafish model caused defects in blood vessel formation through the modulation of VEGF-A-stimulated intracellular signaling, endothelial cell migration, blood vessel development and repair (Bruns et al., 2012).

In this study, we report the identification and molecular characterization of the Korean rose bitterling (*Rhodeus uyekii*) HSP70 family members. We analyzed multiple alignments and phylogenetic tree of the deduced RuHSP70 family sequences and other homologs. We investigated the expression of RuHSP70 transcript during early development and in several tissues of Korean rose bitterling. This is the first report of molecular and functional analyses of the Korean rose bitterling HSP70 gene.

MATERIALS AND METHODS

1. Fish maintenance and sample preparation

Rhodeus uyekii were collected from the Yangchun River, Uiryung-gun, Gyungnam, Republic of Korea. The fish were maintained at the National Fisheries Research and Development Institute (NFRDI) in Busan, Republic of Korea (Kim et al., 2014). The adults were maintained in 40 L glass aquaria at a density of approximately 20 fish per aquarium. The water was renewed weekly and the temperature in the rearing tanks was maintained at 20 ± 1°C. The room was maintained on a 12:12-h light:dark cycle. Adults were fed TetraBits (Tetra) and frozen bloodworms (Advanced Hatchery Technology) twice a day. For RNA extraction, the sample of 10 randomly selected embryo or fish were collected and immediately frozen in liquid nitrogen, and stored at –80°C before use.

2. Identification of Korean rose bittering *R. uyekii* RuHSP70 family

The RuHSP70 family cDNA sequences were isolated from the expressed sequence tag (EST) analysis of the Korean rose bittering *R. uyekii* cDNA library. EST clones were isolated using a Plasmid Miniprep Kit (Qiagen), and sequenced using T3 reverse primers (Promega) and an ABI3730xl automatic sequencer (Applied Biosystems). The nucleotide sequence was analyzed and compared using the BLASTX search program (http://www.ncbilnlm.nih.gov/BLAST/).

3. Multiple sequence alignment and phylogenetic analysis

The relevant sequences were compared using the BLASTX search program (http://www.ncbi.nlm.nih.gov/BLAST/) and retrieved from GenBank for multiple sequence alignments using CLUSTALW (http://www.genome.jp/tools-bin/clustalw). MEGA (ver. 4) was used to assess homologies among the aligned sequences. A phylogenetic tree based on the deduced amino acid sequences was constructed using a neighbor-joining algorithm, and the reliability of the branching was tested using bootstrap resampling with 1,000 pseudo-replicates.

4. Quantitative real-time PCR

Total RNA was prepared from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, treated with DNase I (New England BioLabs, Beverly, MA, USA) and quantitatively

Table 1. Sequences of primers used for the RT-PCR

Gene	Primer	Sequence (5'→3')	Amplicon length(bp)
RuHSP4	Forward	CCT GGC AGG GCG TTC A	74
	Reverse	GGCATCTGAGCA AGATCA AAG A	
RuHSC70	Forward	GGATGACGTCCA AAG AGA AAAGG	82
	Reverse	ATCTTCAACGGTGGACTTCATGT	
RuHSP12A	Forward	AGCGTATAAGGCTGGTCTGGTTT	74
	Reverse	ATGCAGCTTCTGGTTCCAAAG	
RuGRP78	Forward	CCACCAGAGTGAAGGGAAAAAA	74
	Reverse	CGTGAAGCCACGAGGAAAGA	

determined; 500 ng samples were used for reverse transctiption (RT). First-strand cDNA was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Inc.). The PCR primers used for real-time PCR are listed in Table 1. Following an initial 10-min Taq activation step at 95°C, real-time PCR was performed using the following cycling conditions: 40 cycles of 95°C for 10 s, 60°C for 15 s, and fluorescence reading in an SDS 7500 system (Applied Biosystems, Inc.). Transcript levels were quantified as expression relative to the β-actin transcript level.

RESULTS AND DISCUSSION

1. Identification of four Hsp70 family cDNA sequences in the Korean rose bittering

The partial sequences of Korean rose bittering Hsp70 family were identified from the expressed sequence tag (EST) analysis of the *R. uyekii* cDNA library. A search using the BLASTX program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and pairwise alignment revealed that the deduced amino acids of EST clones RU-1-2a_I02, RU-1-3a_F01, RU-2-1a_I11 and RU-2-3a_F17 showed the high homology with heat shock protein 70kDa 4-like (HSP4), heat shock cognate 70 (HSC70), heat shock protein 70kDa 12A-like (HSP12A) and glucose regulated protein 78 (GRP78) of other species, respectively. Accession no. XM008284359.1, AY538777.1, NM001045435.1 and N595368.1.

2. Comparison of RuHSP70 family with other homologs

The deduced amino acids of RuHSP70 family were aligned with Hsp70 proteins from other species including Tongue sole, biocolor damselfish, Zebrafish, Amazon molly, Korean rose bittering, Cichlidae, Asiatic ricefish, Prussian carp, Minnows, Grass carp, Channel catfish, Mexican tetra, Zebra mbuna, Cichlid, guppy, Southern platyfish, Alpaca, Mouse, Human, Olive baboon, Cow, Hamster, Sheep, Falcon, Burmese python, Hill pigeon, African elephant (Fig. 1). Pairwise alignment revealed RuHSP70 family showed high amino acid identities of 72-99% with those of other species. In Table 2, RuHSP4 showed high homology with *Stegastes parties* and *Poecilia formosa*. RuHSC70 showed high homology with *Carassius gibelio, Hypophthalmichthys molitrix* and *Pimephales promelas*. RuHSP12A showed high homology with *Danio rerio* and *Astyanax mexicanus*. RuGRP78 showed high homology with *Ctenopharyngodon idell* and *Danio rerio*.

3. Phylogenetic analysis of RuHSP70 family with other homologs

A phylogenetic analysis, based on the deduced amino acid sequence of RuHSP70 family and related sequences,

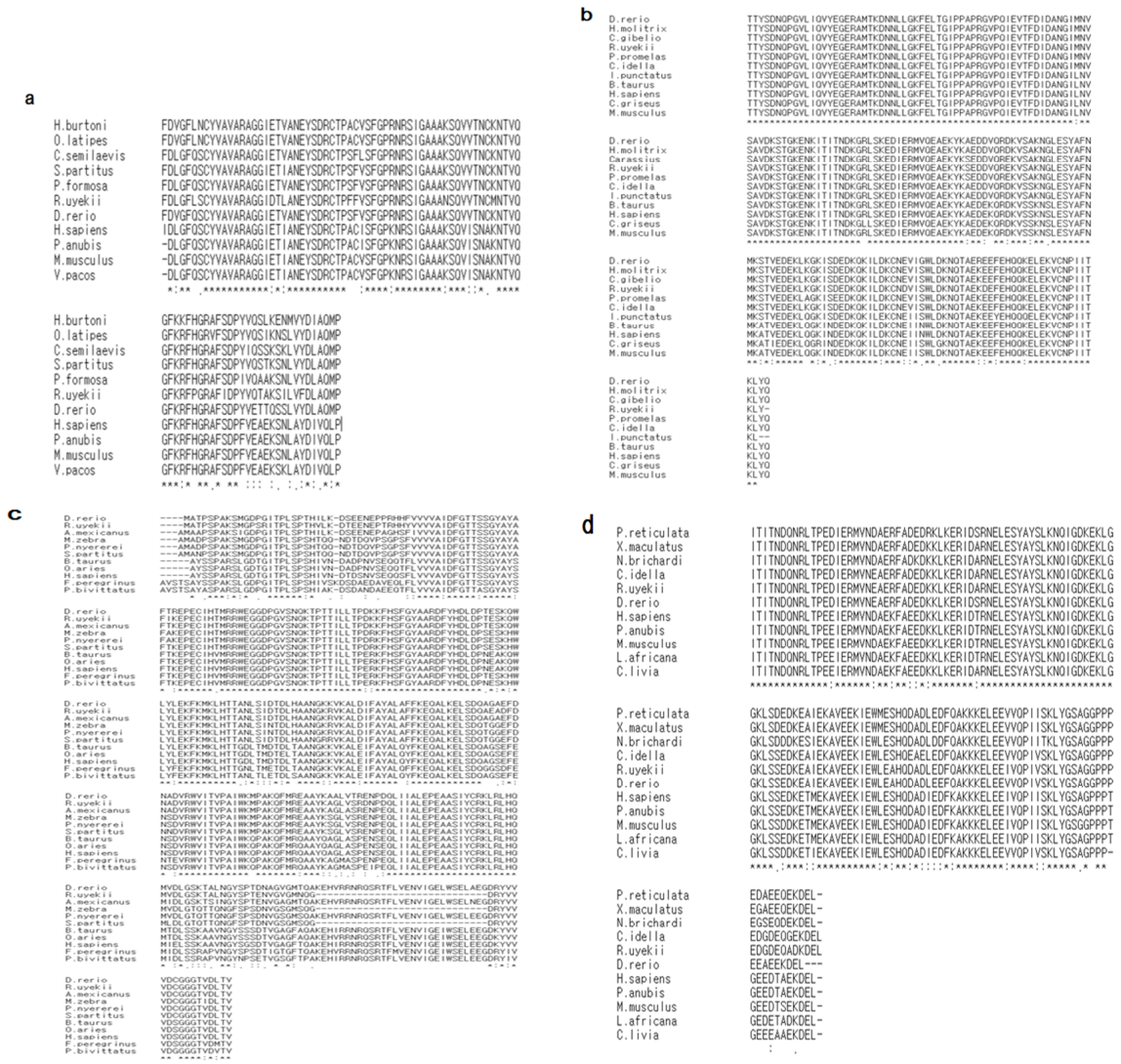

Fig. 1. Multiple alignment of the amino acid sequences of the Korean rose bitterling heat shock protein 70 family (RuHSP70 family) and related sequences. A multiple alignment of amino acid sequences of Hsp70 family was produced using ClustalW 1.81. GenBank accession numbers for the analyzed sequences are the following: (a) *Haplochromis burtoni* (XP-005921284), *Oryzias latipes* (XP-004073389), *Cynoglossus semilaevis* (XP-008331072), *Stegastes partitus* (XP-008296412), *Poecilia formosa* (XP-007562090), *Danio rerio* (NP_999881), *Homo sapiens* (AAH02526), *Papio anubis* (XP-003900145), *Mus musculus* (EDL33602), *Vicugna pacos* (XP-006212876). (b) *Anio rerio* (NP-001103873), *Hypophthalmichthys moliteix* (ACJ03595), *Carassius gibelio* (AAO43731), *Pimephales promelas* (AAS46619), *Ctenopharyngodon idella* (ACJ03596), *Ictalurus punctatus* (ABD77547), *Bos taurus* (AAI54390), *Homo sapiens* (AAH08907), *Cricetulus griseus* (EGW02963), *Mus musculus* (BAE29904). (c) *Danio rerio* (NP_001038900), *Astyanax mexicanus* (XP-007244701), *Maylandia zebra* (XP-004572562), *Pundamilia nyererei* (XP-005747498), *Stegastes partitus* (XP-008305029), *Bos taurus* (AAI54390), *Ovis aries* (XP-004020391), *Homo sapiens* (XP-005269729), *Falco peregrinus* (XP-005239629), *Python bivittatus* (XP-007433493). (d) *Poecilia retculata* (XP-008422585), *Xiphophorus maculatus* (XP-005803813), *Neolamprologus brichardi* (XP-006789208), *Ctenopharyngodon idella* (ACJ65009), *Danio rerio* (AAH63946), *Homo sapiens* (EAW87621), *Papio anubis* (XP-003911999), *Mus musculus* (AAA37315), *Loxodonta africana* (XP-003407784), *Columba livia* (XP-005513063). Identical residues are indicated by asterisks (*); conservative substitutions are indicated by dots (.:).

Table 2. Pairwise ClustalW analysis of the deduced amino acid sequences of RuHSP70 family with those of other species

Species	GenBank no.	Identity(%)
(A) RuHSP4		
Haplochromis burtoni Heat shock 70 kDa protein 4-like	XP_005921284	80
Oryzias latipes Heat shock 70 kDa protein 4-like	XP_004073389	81
Cynoglossus semilaevis Heat shock protein 105 kDa isoform X1	XP_008331072	84
Stegastes partitus Heat shock 70 kDa protein 4-like	XP_008296412	87
Poecilia formosa Heat shock 70 kDa protein 4-like	XP_007562090	87
Danio rerio Heat shock protein 4a	NP_999881	84
Homo sapiens Heat shock 70kDa protein 4	AAH02526	72
Papio anubis Heat shock 70 kDa protein 4	XP_003900145	73
Mus musculus Heat shock protein 4, isoform CRA_b	EDL33602	74
Vicugna pacos Heat shock 70 kDa protein 4 isoform X2	XP_006212876	74
(B) RuHSC70		
Danio rerio Heat shock cognate 71 kDa protein	NP_001103873	97
Hypophthalmichthys molitrix Heat shock protein 70	ACJ03595	98
Carassius gibelio Heat shock cognate 70 kDa protein	AAO43731	99
Pimephales promelas Heat shock cognate 70 kDa protein	AAS46619	98
Ctenopharyngodon idella Heat shock protein 70	ACJ03596	97
Ictalurus punctatus Heat shock cognate 70 kDa protein	ABD77547	95
Bos taurus HSPA8 protein	AAI54390	92
Homo sapiens HSPA8 protein	AAH08907	92
Cricetulus griseus Heat shock cognate 71 kDa protein	EGW02963	91
Mus musculus Unnamed protein product	BAE29904	95
(C) RuHSP12A		
Danio rerio Heat shock protein 12A	NP_001038900	92
Astyanax mexicanus Heat shock 70 kDa protein 12A isoform X1	XP_007244701	91
Maylandia zebra Heat shock 70 kDa protein 12A-like isoform X4	XP_004572562	85
Pundamilia nyererei Heat shock 70 kDa protein 12A-like isoform X5	XP_005747498	84
Stegastes partitus Heat shock 70 kDa protein 12A isoform X3	XP_008305029	85
Bos taurus Heat shock 70 kDa protein 12A isoform X1	XP_002698580	76
Ovis aries Heat shock 70 kDa protein 12A	XP_004020391	75
Homo sapiens Heat shock 70 kDa protein 12A isoform X1	XP_005269729	75
Falco peregrinus Heat shock 70 kDa protein 12A	XP_005237627	74
Python bivittatus Heat shock 70 kDa protein 12A-like isoform X1	XP_007433493	74

Table 2. Continued

Species	GenBank no.	Identity(%)
(D) RuGRP78		
Poecilia reticulata 78 kDa glucose-regulated protein	XP_008422585	89
Xiphophorus maculatus 78 kDa glucose-regulated protein-like	XP_005803813	90
Neolamprologus brichardi 78 kDa glucose-regulated protein-like	XP_006789208	88
Ctenopharyngodon idella GRP78	ACJ65009	95
Danio rerio Heat shock protein 5	AAH63946	93
Homo sapiens Heat shock 70kDa protein 5	EAW87621	84
Papio anubis 78 kDa glucose-regulated protein-like	XP_003911999	84
Mus musculus Immunoglobulin heavy chain binding protein	AAA37315	82
Loxodonta africana 78 kDa glucose-regulated protein	XP_003407784	86
Columba livia 78 kDa glucose-regulated protein	XP_005513063	86

was performed. RuHSP70 family was divided into two distinct groups, one as fisheries and the other one as mammals. The tree indicated clear clustering of RuHSP4 sequences into two groups: Amazon molly; Bicolor damselfish; Tongue sole; Zebrafish; Cichlidae; Asiatic ricefish VS alpaca; mouse; olive baboon and human. The tree indicated clear clustering of RuHSC70 sequences into two groups: Prussian carp; Minnows; Zebrafish; Cuvier et valenciennes; Grass carp; Channel catfish VS Cow; Human; Hamster and Mouse. The tree indicated clear clustering of RuHSP12A family sequences into two groups: Zebrafish; Mexican tetra; Bicolor damselfish; Zebra mbuna; Cichlid VS Falcon; Burmese python; Human; Cow and Sheep. The tree indicated clear clustering of RuGRP78 sequences into two groups: Grass carp; Zebrafish; Guppy; Southern platyfish VS Hill pigeon; African elephant; mouse; olive baboon and human (Fig. 2).

4. Tissue distribution of Hsp70 family mRNA in Korean rose bitterling

Tissue distribution of Hsp70 family mRNA in Korean rose bitterling were investigated by quantitative real-time PCR. The expression levels of Hsp70 family mRNA were quantified after normalization to β-actin as an internal reference gene. The expression of RuHSP4 mRNA was detected in all tissue examined; highly in the ovary, testis and hepatopancreas. Levels of the RuHSP4 mRNA in the ovary, testis and hepatopancreas were 14.6, 11.3 and 9.8 folds that in gill where expression was the lowest, respectively. The expression of RuHSC70 mRNA was detected in all tissue examined; highly in the hepatopancreas, testis and spleen. Levels of the RuHSC70 mRNA in the hepatopancreas, testis and spleen were 2.7, 1.8 and 1.1 folds that in muscle where expression was the lowest, respectively. The expression of RuHSP12A mRNA was detected in all tissue examined; highly in the ovary, testis and brain. Levels of the RuHSP12A mRNA in the ovary, testis and brain were 2.8, 1.1 and 1 folds that in gill where expression was the lowest, respectively. The expression of RuGRP78 mRNA was detected in all tissue examined; highly in the hepatopancreas, spleen and ovary. Levels of the RuGRP78 mRNA in the hepatopancreas, spleen and ovary were 42.1, 28.2 and 12.7 folds that in fin where expression was the lowest, respectively (Fig. 3). Overall,

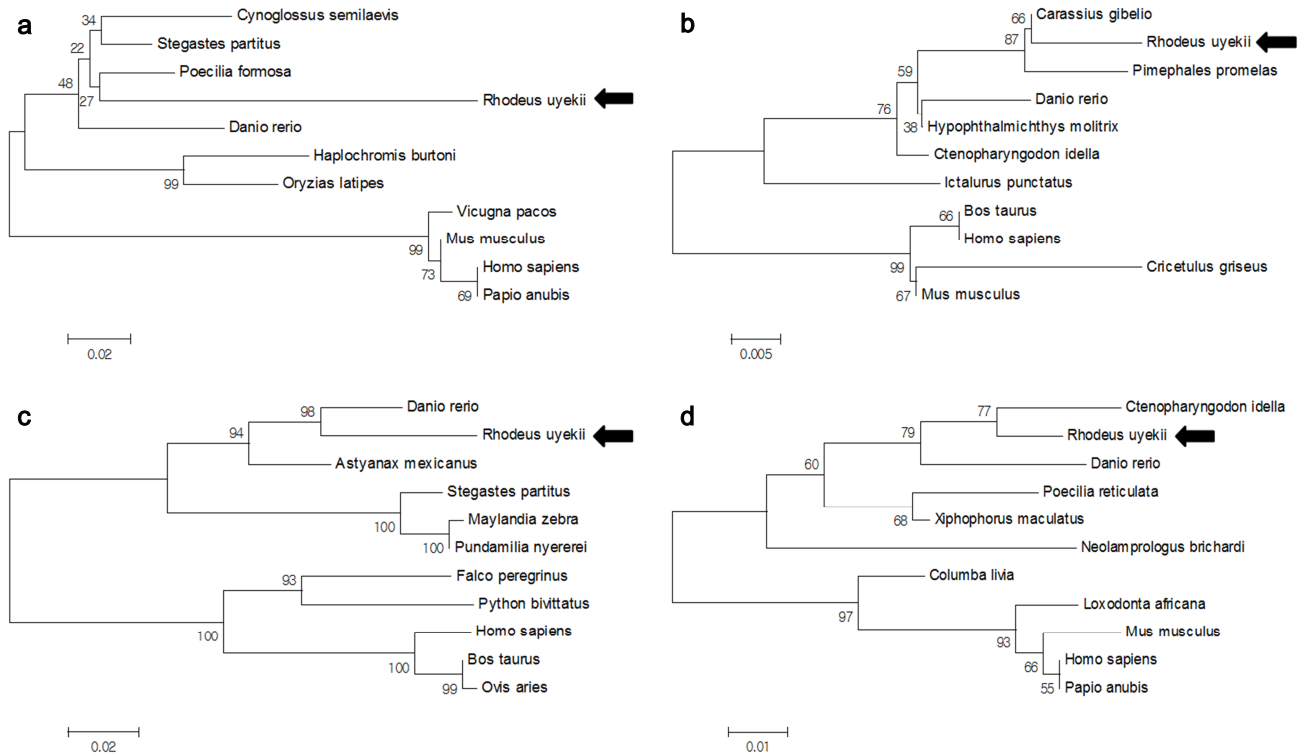

Fig. 2. Phylogenetic analysis of the Korean rose bitterling Hsp70 family and related sequences. GenBank accession numbers for the analyzed sequences are the following: (a) *Haplochromis burtoni* (XP-005921284), *Oryzias latipes* (XP-004073389), *Cynoglossus semilaevis* (XP-008331072), *Stegastes partitus* (XP-008296412), *Poecilia formosa* (XP-007562090), *Danio rerio* (NP-999881), *Homo sapiens* (AAH02526), *Papio anubis* (XP-003900145), *Mus musculus* (EDL33602), *Vicugna pacos* (XP-006212876). (b) *Danio rerio* (NP-001103873), *Hypophthalmichthys molitrix* (ACJ03595), *Carassius gibelio* (AAO43731), *Pimephales promelas* (AAS46619), *Ctenopharyngodon idella* (ACJ03596), *Ictalurus punctatus* (ABD77247), *Bos taurus* (AAI54390), *Homo sapiens* (AAH08907), *Cricetulus griseus* (EGW02963), *Mus musculus* (BAE29904). (c) *Danio rerio* (NP-00038900), *Astyanax mexicanus* (XP-007244701), *Maylandia zebra* (XP-004572562), *Pundamilia nyererei* (XP-005747498), *Stegastes partitus* (XP-008305029), *Bos taurus* (XP-002698580), *Ovis aries* (XP-004020391), *Homo sapiens* (XP-005269729), *Falco peregrinus* (XP-005237627), *Python bivittatus* (XP-007433493). (d) *Poecilia reticulate* (XP-008422585), *Xiphophorus maculatus* (XP-005803812), *Neolamprologus brichardi* (XP-006789208), *Ctenopharyngodon idella* (ACJ65009), *Danio rerio* (AAH63946), *Homo sapiens* (EAW87621), *Papio anubis* (XP-003911999), *Mus musculus* (AAA37315), *Loxodonta africana* (XP-003407784), *Columba livia* (XP-005513063).

we found that Hsp70 family of Korean rose bitterling is highly expressed in hepatopancreas and sexual gonad (testis, ovary). This expression pattern is consistent with high previous results. The results showed that Hap 70 family was highly expressed in hepatopancreas and sexual gonad (testis, ovary) in *Rhynchocypris kumgangensis* (Im et al., 2013) and *Paphia undiata* (Wu et al., 2014).

5. Expression analysis of the HSP70 family mRNA during early development

The expression of Hsp70 family mRNA during early development of Korean rose bitterling was determined by quantitative real-time PCR at 1, 3, 6, 15 and 21 days post-fertilization (dpf). After the first dpf, the relative expression of RuHSP4 sharply decreased and was maintained at similar

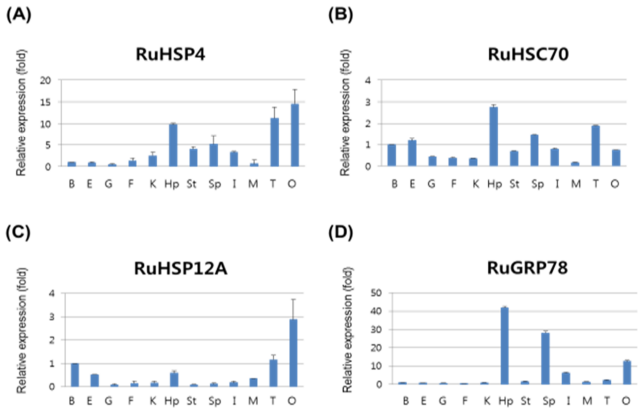

Fig. 3. Tissue distribution of The Korean rose bitterling RuHSP70 family. Quantitative real-time RT-PCR was performed on equal amounts of total RNA isolated from tissues of normal conditioned fish. Korean rose bitterling β-actin was used as an internal control. Expression levels of (A) RuHSP4, (B) RuHSC70, (C) RuHSP12A, (D) RuGRP78 transcript were quantified by expression relative to the β-actin transcript level. B, brain; E, eye; G, gill; F, fin; K, kidney; Hp, hepatopancreas; St, stomach; Sp, spleen; I, intestine; M, muscle; T, testis; O, ovary. The values represent the mean±SD (n=3).

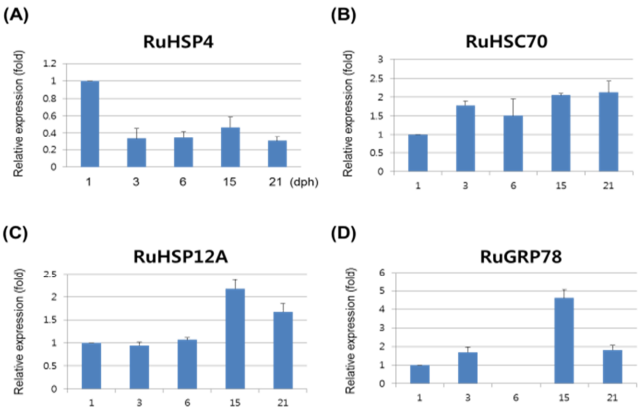

Fig. 4. Development stage of the Korean rose bitterling RuHSP70 family. Quantitative real-time RT-PCR was performed on equal amounts of total RNA isolated from during development stage of whole body in fish. Korean rose bitterling β-actin was used as an internal control. Expression levels of (A) RuHSP4, (B) RuHSC70, (C) RuHSP12A, (D) RuGRP78 transcript were quantified by expression relative to the β-actin transcript lever. The values represent the mean±SD (n=3).

level, until the last dpf. The expression of RuHSC70 mRNA was detected from 1 dpf and moderately increased until 21 dpf during the early development. The relative expression of RuHSP12A and RuGRP78 increased significantly at the 15 dpf and decreased slightly at 21dpf (Fig. 4).

The results of previous research are similar to our study. It is consistent that the expression of Hsp70 family gene was increased after fertilization in *Oncorhynchus tshawytscha* (Kong et al., 1996), *Oncorhynchus mykiss* (Currie & Tufts., 1997; Currie et al., 2000; Ojima et al., 2005a, b) and *Salmo salar* (Lund et al., 2002). We expected that differences in the expression are due to differences on the role of Hsp70 family mRNA at various stage of development. Further investigations are required to determine the function of Hsp70 family in development stage of Korean rose bitterling under *in vivo* and *vitro* conditions.

In this study, the partial sequences of Korean rose bittering Hsp70 family were identified from the expressed sequence tag (EST) analysis of the *R. uyekii* cDNA library. We found 4 members (RuHSP4, RuHSC70, RuHSP12A, RuGRP78) of Hsp70 family in *R. uyekii*. Pairwise alignment and phylogenetic analysis revealed RuHSP70 family showed RuHsp70 family has been conserved during evolution. The expression patterns of RuHsp70 family suggest that they play a unique or specific role during early development. Further investigations are required to elucidate the functional role of HSP70 families in *R. uyekii*.

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