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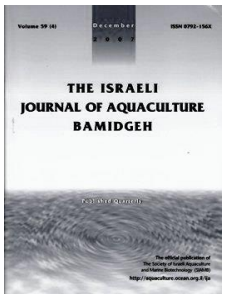

Molecular Cloning, Characterization, and Expression Analysis of HSP60 in Mandarin Fish *Siniperca chuatsi*

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Key words: *Siniperca chuatsi*; HSP60; heat shock; hypoxia; *Aeromonas hydrophila*

Abstract

HSP60 protein plays an important role in stress response, protein folding, and cell signaling. In this study, mitochondrial *HSP60* from *Siniperca chuatsi* was identified, and its cDNA and gDNA structures, amino acid sequence features, and phylogenetic analysis, were described. Expression profiles during embryonic development, in different tissues and under stressful conditions were analyzed using RT-PCR. During embryogenesis, low levels of transcripts of *ScHSP60* were detected during early developmental stages and were upregulated at blastopore closure stages to 1 dpf. *ScHSP60* showed tissue-specific variation, highly expressed in ovaries under non-stressed conditions. Acute heat shock at 34^0C resulted in strong upregulation of *ScHSP60* in heart, liver, and head kidney in a time-dependent manner. However rapid and gradual elevated heat shock did not affect *ScHSP60* expression when temperature reached 34^0C, although it was dramatically induced when temperature reached 38.8^0C. *ScHSP60* was also markedly induced in the liver in a stage-dependent manner under hypoxia. Additionally, *Aeromonas hydrophila* infection augmented *ScHSP60* in head kidney and spleen. Results showed that *ScHSP60* expression is significantly modified under different environmental conditions including high temperatures, hypoxia, and bacterial infection. This study will further clarify the role of fish *HSP60* in embryogenesis and under stressful conditions, and contribute to further investigation to understand stress tolerance and disease resistance of mandarin fish.

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Introduction

Heat shock proteins (HSPs) are highly conserved proteins that function as molecular chaperones to maintain cellular protein homeostasis under both normal physiological, and stress conditions, by facilitating folding of nascent polypeptides and degrading damaged proteins, assisting in intracellular trafficking, and modulating signal transduction as well as regulating immune responses (Roberts et al. 2010; Rupik et al. 2011). The HSP genes are generally divided into several major families: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs based on molecular weight and sequence homology of proteins (Sarkar *et al*. 2011).

The 60 kDa heat shock proteins (HSP60) are conserved in prokaryote and eukaryote cells and are required in the folding, translocation, and assembly of native proteins (Brocchieri and Karlin 2000). HSP60 is encoded by the chromosomal DNA and is typically active in the mitochondria; it has been found in organelles of endosymbiotic origin (Cheng et al., 1990). HSP60 cooperates with other chaperones such as HSP10 and HSP70, facilitates proper folding, assembly and transportation of protein complexes imported into mitochondria (Mayer 2010; Nakamura and Minegishi 2013). Apart from its classical chaperone function, mitochondrial HSP60 is also critically involved in the replication and transmission of mitochondrial DNA (Kaufman *et al*. 2003). HSP60 has been shown to be involved in stress response. The upregulation of HSP60 allows for maintenance of other cellular processes to protect cells from damage during stressful conditions (Vargas-Parada *et al*. 2001). Several studies have shown that HSP60 is necessary for cellular survival under toxic or stressful circumstances (Rossi *et al*. 2002). HSP60 also interacts with both the innate and adaptive immune system in mammals (Quintana and Cohen 2011).

To date, most studies of HSP60 have focused on mammals and typical model organisms, thus reports for teleost HSP60 is limited. HSP60 cDNA sequences from a few of fish species, including *Ctenopharyngodon idellus* (Xu *et al*. 2011), *Tanichthys albonubes* (Liu *et al*. 2011) and *Epinephelus akaara* (Qu *et al*. 2011), were cloned and similarity of amino acid structure to mammals suggested that fish HSP60 may function similarly to that in mammals. Mandarin fish, *Siniperca chuatsi*, an endemic freshwater fish species found in eastern Asian countries has been widely cultured in China. Various environmental stresses, including high temperature, hypoxia, and *Aeromonas hydrophila* infection, the causative agent of outbreaks of bacterial hemorrhagic septicemia disease, have frequently threatened the health of this fish, causing serious economic losses to the aquaculture industry (Lao *et al*. 2008; Chen *et al*. 2012). Understanding the characteristics of HSP60 expression under different conditions of stress will contribute to improving the stress tolerance and disease resistance of *S. chuatsi* as the response of the HSP60 gene to environmental stresses is totally unknown in *S. chuatsi*. In the present study we identified the HSP60 gene in *S. chuatsi*, and investigated its expression patterns during embryogenesis, in different tissues of juvenile fish, both under normal conditions and in response to heat shock, hypoxia, and bacterial infection.

Materials and Methods

Animals. Mandarin fish were obtained from BaiRong Aquatic breeding Co., Ltd (Guangdong, China). The fish (120 ± 20 g, body weight, BW) were reared in a circulating water system containing a series of 2000 L tanks with water temperature of 25°C for more than two weeks in Sun Yat-Sen University, and fed fresh juvenile *Cirrhinus molitorella* at approximately 5% total biomass every day. Before tissue collection, fish were anesthetized with tricaine methanesulfonate (MS-222, XiangBo Biotec, China). Tissues were rapidly removed by team dissection, snap-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. All experiments were approved by the Animal Research and Ethics Committees of Sun Yat-Sen University and performed in accordance with the guidelines of the committee.

Embryo and tissue sampling. Embryos were obtained by artificial fertilization and hatching. Fertilized eggs were hatched in three vertical fish egg incubators (BaiRong Aquatic breeding Co., Ltd). Clean, oxygenated water (temperature 27°C) flowed continuously into incubators. Pressure from below kept the eggs in a suspended state.

Embryos at different stages including unfertilized eggs, fertilized eggs, 16-cell stage, morula, blastula, gastrula, closure of blastopore, appearance of myomere, tail-bud stage, muscle burl stage, blood circulating stage, crystal stage, pre-hatched larvae, 1-day post-hatching (dph) and 7-dph larvae were collected and snap-frozen in liquid nitrogen. Tissues (brain, heart, gill, head kidney, liver, spleen, muscle, stomach, intestines, opisthonephros and ovary) from three female fish (152-160g, BW) reared in non-stressed conditions were sampled and used for RT-PCR analysis of the tissue distribution of the two HSP90 isoforms.

Heat shock treatment. To investigate the expression patterns of *ScHSP60* in response to thermal stress, the fish were subjected to three regimes of thermal stress: acute, rapidly elevated, and gradually elevated. For acute thermal stress, 20 fish (140 ± 20 g, BW) maintained in water at 25°C were carefully and directly shifted to a tank of 2,000 L in the circulating water system at a constant temperature of 34°C. Another 20 individuals were shifted to a tank of the same volume in the other circulating water system at 25°C and served as the control group. Six individuals from the treatment and control groups were randomly collected and sampled at 2, 6, and 12 h, respectively. In the other two regimes of thermal stress, fish were transferred to the 2,000 L tank in the circulating water system (initial temperature: 25°C); then the water temperature was elevated from 25 to 38.8°C at an average speed of 1.2°C increase per hour for rapid elevated thermal stress treatment, and 0.2°C increase per hour for gradual elevated thermal stress. Six fish were randomly sampled when the temperature reached 34°C, six more at 38.8 °C, and another six individuals were maintained at 25°C (control group).

Hypoxia exposure. Forty-eight fish (132 ± 17 g, BW) acclimated to the normal oxygen environment (dissolved oxygen, DO: >5.6 mg/L, temperature: 25°C) were randomly distributed into eight tanks of 400 L each (A-D, and A′-D′): A-C, exposed to a low oxygen level of 0.9 ± 0.1 mg/l of DO for 2, 6 and 12 h, respectively; D, re-oxygenation under normal oxygen for 24 h after 6-h hypoxia; A′-D′, exposed to normal oxygen, were referred to as control groups for A-D, respectively. Dissolved oxygen of 0.9 ± 0.1 mg/l was maintained by controlling water and nitrogen inflows. Dissolved oxygen was monitored in real-time using a YSI Model 550A dissolved oxygen meter (Geo Scientific Ltd, USA).

Bacterial infection. Bacterial infection was conducted in a series of 400 L tanks with six individuals per tank. Sixty healthy fish (140 ± 15 g, BW) were used for the challenge of virulent bacteria *A. hydrophila*, provided by MOE Key Laboratory of Aquatic Product Safety, Sun Yat-Sen University. Each fish was intraperitoneally injected with a total of 0.5 mL of *A. hydrophila* (5×10^8 CFU/mL), which was diluted with sterile phosphate buffer solution (PBS, pH 7.4). Another 30 fish injected with the same volume of aseptic PBS served as the control group. Six individuals from the treatment and control groups were randomly collected and tissues were sampled at 6, 12, 24, 48 and 72 h post injection, respectively.

Total RNA and genomic DNA isolation. Total RNA was extracted from tissues ground with liquid nitrogen using E.Z.N.A. total RNA kit II (Omega Bio-technology, USA) according to the manufacturer's instructions. Genomic DNA was isolated from the muscle using a TIANamp Genomic DNA Kit (Tiangen Biotec, China). Quality and quantity of RNA and DNA were assessed by the OD 260/OD280 measurement method and electrophoresis in 1% agarose gel.

Cloning of cDNAs and genomic DNAs. First-strand cDNA was synthesized from a pool of total RNA of liver and head kidney with the RT-PCR kit of first-strand cDNA synthesis (Invitrogen, Carlsbad, USA). Two pairs of degenerate primers, HSP60-F1/-R1 and HSP60-F2/-R2 (Table 1), designed according to the highly conserved regions of HSP60 amino acid sequences from other fish species, were used to obtain the mandarin fish HSP60 cDNA fragments using nested PCR.

The technique of rapid amplification of cDNA ends (RACE) was used to obtain full-length cDNA sequences. 3′-RACE PCR was performed with forward gene-specific primers for *ScHSP60* (Table 1) and reverse primers anchor primer (AP) and abridged universal amplification primer (AUAP) in the 1st and 2nd round, respectively. The 5′-RACE PCR was

performed with the template of purified first-strand cDNA which had a poly (C) end added to the 5′-terminal with a terminal deoxynucleotidyl transferase (TaKaRa, Japan), and the reverse gene-specific nested primers for *HSP60* (Table 1) as well as the forward primers abridged anchor primer (AAP) and AUAP in the 1st and 2nd round, respectively.

Table 1. Primers and their applications in this study.

Primer name	*Primer sequences (5′ – 3′)*	*Objective*
HSP60-F1	TGGCNGTCACNATGGGNCCRAA *	1st round intermediate fragment amplification
HSP60-R1	CTGGCATNTCCTTCTCCTCCTTNG *	
HSP60-F2	GTNCAGGAYGTNGCCAACAACAC*	2nd round intermediate fragment amplification
HSP60-R2	GTCCTCACNACCTTNGTGGGRT*	
HSP60-3′F1	CGGTTACATCTCCCCTTACTTCATC	1st round 3′-RACE
HSP60-3′F2	AGAAGGACCGTGTGACAGATGCT	2nd round 3′-RACE
HSP60-5′R1	GAGCATCTGTCACACGGTCCTT	1st round 5′-RACE
HSP60-5′R2	GGTAGGCATCCTGGAACTCACACT	2nd round 5′-RACE
g-HSP60-F	GTCGTTAGATCATTCCGTCTTGC	Genomic DNA cloning for *HSP60*
g-HSP60-R	ACAGTGACACAATCCTGGCTAGG	
RT- HSP60-F	TGTCCCGTTCACCACGCTAT	Real-time quantitative
RT- HSP60-R	TTGATGGTTGATTGAACAGAGCC	PCR
18S-F	CTGAGAAACGGCTACCACATCC	Real-time quantitative
18S-R	GCACCAGACTTGCCCTCCA	PCR

* N = G+A+C+T; R = G+A; Y = C+T.

Amplifications for cloning the cDNAs were performed as follows: denaturation at 94°C for 3 min, followed by 35-38 cycles at 94°C for 30 s, 54-60°C for 30 s and 72°C for 1-2 min, and a final extension of 8 min at 72°C. The amplified PCR products were purified with the TIANgel Midi Purification Kit (Tiangen Biotec, China) and cloned into a pEASY-T1 cloning vector using the TA cloning kit (TransGen Biotec). The positive recombinants were identified and sequenced.

Genomic DNA (gDNA) sequences coding *ScHSP60* was amplified from total DNA by gene-specific primers gHSP60-F/-R, which were located at the terminus of 5′- and 3′-terminal untranslated regions (UTRs) of the cDNA sequences. PCR conditions for cloning of the genomic DNA were as follows: initial denaturation at 94°C for 3 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 8 min, and a final extension of 10 min at 72°C.

Sequences and phylogenic analysis. The search for nucleotide and amino acid sequence similarities was conducted with the BLAST programs at the National Center for Biotechnology Information. Multiple sequence alignments of amino acids were performed by ClustalX 2.1. Protein phylogenetic analysis was conducted with MEGA 6.0 using the neighbor-joining method with 1000 iterations. The molecular weight (Mw) and isoelectronic point (pI) of deduced amino acids were computed by pI/Mw tool of ExPASy. The motifs of ScHSP60 were searched using the ScanProsite program, and signal sequences were searched using SignalP software.

Real-time quantitative PCR. Real-time quantitative PCR was used to quantify mRNA expression with a LightCycler® 480 II Real-Time PCR system (Roche) according to MIQE validation guidelines (Bustin *et al.*, 2009). In the present study, *18S rRNA* was selected as the reference gene for its stable expression in all situations. Specific primers (Table 1) were designed to amplify *ScHSP60* and *18S rRNA* fragments. Total RNA was reverse-transcribed using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan), and cDNA equivalent to 10 ng of total RNA was used for each reaction. Amplification was performed using SYBR®Premix Ex Taq™II (Tli RNaseHPlus) (TaKaRa, Japan) in 384-well plates. All reactions were carried out in three replicates, from which the mean threshold cycle (C_T) values were calculated. Based on Tm values of the primer pairs, cycling conditions were designed as: initial denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 20 s and extension at 72°C for 30 s, then a dissociation curve step (95°C for 1 s, 60°C for 20 s, and 95°C for 1 s) was conducted to evaluate the specificity.

Ten fold serial dilutions of recombinant plasmid pEASY-T1 containing *ScHSP60* and *18S rRNA* genes were used to construct standard curves. Amplification efficiencies (E) were determined by the equation $E=10(^{-1/Slope})-1$. Here, amplification efficiencies of *ScHSP60* and *18S rRNA* were 98.8 and 104.6%, respectively.

Statistical analysis. The relative transcript levels of *ScHSP60* were normalized to the level of *18S rRNA* in the same sample by the $2^{-\Delta Ct}$ method and the relative expression levels to the control were determined by the $2^{-\Delta\Delta Ct}$ method. Statistical analyses were performed using IBM SPSS software version 22. Data presented in this study were analyzed using one-way ANOVA followed by Tukey's test and expressed as mean ± SD.

Results

Cloning and sequence analysis of ScHSP60 cDNA. The full length of *ScHSP60* cDNA sequence was 2505 bp, containing an open reading frame (ORF) of 1731 bp, a 5′-UTR of 85 bp and a 3′-UTR of 689 bp with a polyadenylation signal (AATTAAA) located 13 nucleotides upstream from the poly (A) tail. The deduced amino acid sequence encoded a protein of 576 amino acids with calculated molecular weight of 61.11 kDa and theoretical isoelectric point of 5.56. The cDNA sequence of *ScHSP60* was deposited in the GenBank database with accession number KU997668.

As shown in Fig. 1, ScHSP60 sequence contains the classical mitochondrial HSP60 signature motif AAVEEGIVPGGG (amino acid, aa, 430-441) and a typical GGM repeat site at the C terminus. Using SignalP 4.1 Server, a presequence of 28 amino acids at the N terminus was predicted that is required for import into the mitochondria. A conserved domain search on the NCBI database identified a conserved ATP-binding/$Mg2^{+}$-binding site (aa, 195-213).

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1       ACCATACCGCTGTCCTTTAGTCCAGTCGTTAGATCATTCCGTCTTGCAAACGTGCACGTTGTAGCAGCTACTTCACCTCACAAAGATGTT
1                                                                                       M  F
91      TCGTCTACCAACAGTCATGAGGCAGGTGAGGCCTGTGTGCAGGGCGCTGGCTCCTCACCTCACACGAGCCTACGCTAAGGATGTAAAGTT
3         R  L  P  T  V  M  R  Q  V  R  P  V  C  R  A  L  A  P  H  L  T  R  A  Y  A  K  D  V  K  F
181     TGGCGCTGATGCTCGTGCCCTCATGCTGCAGGGAGTGGACCTCCTGGCTGACACTGTGGCTGTCACCATGGGCCCAAAGGGTCGCACTGT
33        G  A  D  A  R  A  L  M  L  Q  G  V  D  L  L  A  D  T  V  A  V  T  M  G  P  K  G  R  T  V
271     CATCATTGAGCAGAGCTGGGGCAGCCCTAAGGTCACCAAGGACGGTGTGACGGTGGCCAAGAGCATCGAGCTGAAGGACAAGTACAAGAA
63        I  I  E  Q  S  W  G  S  P  K  V  T  K  D  G  V  T  V  A  K  S  I  E  L  K  D  K  Y  K  N
361     CATCGGCGCCAGGTTGGTGCAGGATGTGGCCAACAACACCAACGAGGAGGCCGGTGACGGCACTACCACCGCCACAGTGCTGGCTCGTGC
93        I  G  A  R  L  V  Q  D  V  A  N  N  T  N  E  E  A  G  D  G  T  T  T  A  T  V  L  A  R  A
451     CATTGCCAAGGAGGGCTTCGACACCATCAGCAAAGGCGCCAACCCTGTCGAGATCCGCCGTGGAGTCATGGCAGCCGTAGACACCATCAT
123       I  A  K  E  G  F  D  T  I  S  K  G  A  N  P  V  E  I  R  R  G  V  M  A  A  V  D  T  I  I
541     CAGCGAGCTGAAAAAGCTCTCCAAACCAGTCACAACACCTGAGGAGATCGCGCAGGTTGCTACAATCTCAGCCAATGGAGACGTGGAGAT
153       S  E  L  K  K  L  S  K  P  V  T  T  P  E  E  I  A  Q  V  A  T  I  S  A  N  G  D  V  E  I
631     TGGTAACATCATATCCAATGCCATGAAGAGAGTTGGCCGCAAGGGAGTGATCACTGTCAAGGATGGGAAGACTCTGCACGATGAGCTGGA
183       G  N  I  I  S  N  A  M  K  R  V  G  R  K  G  V  I  T  V  K  D  G  K  T  L  H  D  E  L  E
721     GATCATTGAGGGTATGAAGTTCGACCGCGGTTACATCTCCCCTTACTTCATCAACACTGCCAAAGGCCAGAAGTGTGAGTTCCAGGATGC
213       I  I  E  G  M  K  F  D  R  G  Y  I  S  P  Y  F  I  N  T  A  K  G  Q  K  C  E  F  Q  D  A
811     CTACCTGCTGCTGAGTGAGAAGAAGATCTCCAGTGTCCAGAGCATCGTGCCAGCCCTGGAGATTGCCAATCAGCACCGCAAACCACTGGT
243       Y  L  L  L  S  E  K  K  I  S  S  V  Q  S  I  V  P  A  L  E  I  A  N  Q  H  R  K  P  L  V
901     TATTGTGGCTGAGGATGTGGACGGAGAGGCCCTCAGCACCTTGGTTCTCAACAGGCTGAAGGTTGGGCTTCAGGTGGTAGCTGTTAAAGC
273       I  V  A  E  D  V  D  G  E  A  L  S  T  L  V  L  N  R  L  K  V  G  L  Q  V  V  A  V  K  A
991     CCCCGGCTTCGGAGACAACAGGAAGAATCAGCTAAGGGACATGGCCATCGCCACCGGAGGCACTGTGTTTGGAGATGACGCCGTGGGCCT
303       P  G  F  G  D  N  R  K  N  Q  L  R  D  M  A  I  A  T  G  G  T  V  F  G  D  D  A  V  G  L
1081    GGCGCTCGAGGACATCCAGGCTCATGACTTTGGAAAGGTTGGTGAAGTGCAGATTACCAAAGATGACACCTTGCTGCTGAAGGGAGGCGG
333       A  L  E  D  I  Q  A  H  D  F  G  K  V  G  E  V  Q  I  T  K  D  D  T  L  L  L  K  G  G  G
1171    CAGCCCAGCTGAAGTTGAGAAACGGGCGGCAGAGATCATGGAGCAGCTGGAGAGCACCACTAGTGACTATGAGAAAGAGAAACTCAACGA
363       S  P  A  E  V  E  K  R  A  A  E  I  M  E  Q  L  E  S  T  T  S  D  Y  E  K  E  K  L  N  E
1261    GAGGCTGGCCAAGCTGTCTGACGGAGTGGCTGTACTCAAGATCGGAGGAACAAGTGATGTGGAGGTGAATGAGAAGAAGGACCGTGTGAC
393       R  L  A  K  L  S  D  G  V  A  V  L  K  I  G  G  T  S  D  V  E  V  N  E  K  K  D  R  V  T
1351    AGATGCTCTGAATGCCACACGGGCAGCTGTGGAGGAGGGTATCGTACCAGGTGGAGGCTGCGCCCTGCTGCGCTGCATCCCAGCACTCGA
423       D  A  L  N  A  T  R  A  A  V  E  E  G  I  V  P  G  G  G  C  A  L  L  R  C  I  P  A  L  D
1441    TACCATCAAAACTGTCAACGCTGACCAGACGATCGGTGTGGACATCATCAGACGGGCGCTCCGTATCCCTGCCATGACCATCGCTAAGAA
453       T  I  K  T  V  N  A  D  Q  T  I  G  V  D  I  I  R  R  A  L  R  I  P  A  M  T  I  A  K  N
1531    TGCAGGTGTAGAGGGTTCGCTAGTAGTGGAGAAGATCCTGCAGGGACCACCTGAGATCGGCTACGATGCCATGCAGGGAGAGTACGTCAA
483       A  G  V  E  G  S  L  V  V  E  K  I  L  Q  G  P  P  E  I  G  Y  D  A  M  Q  G  E  Y  V  N
1621    CATGGTGGAGAAGGGCATCATCGACCCCACCAAGGTGGTGAGGACGGCCCTGTTGGACGCTGCAGGAGTAGCGTCCCTGCTGTCCACTGC
513       M  V  E  K  G  I  I  D  P  T  K  V  V  R  T  A  L  L  D  A  A  G  V  A  S  L  L  S  T  A
1711    TGAGGCCGTAGTCACAGAGATACCCAAGGAGGAGAAGGAGATGCCAGGAGGCGGCATGGGAGGCATGGGCGGCATGGGAGGCATGGGAGG
543       E  A  V  V  T  E  I  P  K  E  E  K  E  M  P  G  G  G  M  G  G  M  G  G  M  G  G  M  G  G
1801    CGGCATGGGTTTCTAAGCCAATCCTCCTGACTCGCTGTACAGACTAATAGGCAGAGTGGTGCTGAGGAGCACAACGAGAAACCCTCCCAC
573       G  M  G  F  *
1891    CTTCACCAAACTCTGCCCACACCGACGGACTGACTGGCCATGAGTATGTTGGAGCTGCAAATGGTTGATGACCCCCTCCCCTCCCCACTA
1981    ACTTCACACGATTCTGCCTCCTTCCTCTTTGTCTGGGTTTCTGGAGGAGCCTGACTCCGTGCCCCCTCTGTCCCGTTCACCACGCTATGGG
2071    AGCCCATCAAGCCCAATTACACCACAGTCCCCTTCACACCTCTAATGGCTCTGTTCAATCAACCATCAACATCTGCACAAAACTCATGTG
2161    AAACACTCAACAGAAAGAAGAATGCTTATTCAGATTTACGTACAGATTTGATCTCTGTAAGAAGTAGTAAAGTGGTCTAAGAAGAAACTA
2251    ACATAGTACACATAATGATGACGCTCTATTACTGCAAAGAGAGCAGGGCTTGAACGAGAAAAGATGCCTAGCCAGGATTGTGTCACTGTC
2341    CTTTATTTTTGTCATAGTATTTTGTTAATTGTTTTGTTTTTTTATATATATATACATCTGTCATGTTTTGTTTCCCCCAGCTAATATA
2431    TTATGTGGGTTAAAATCTGTCATTTGATTTAATTAAACCGATTTTTTCTTAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig.1 Nucleotide sequence and deduced amino acid sequences of *S.chuatsi* HSP60. The polyadenilation signal at N terminus is *shaded* and the GGM repeat site at C terminus is *boxed*. The typical mitochondrial HSP60 signature motif is *boxed* and *shaded*. The ATP-binding/$Mg2^{+}$-binding site is *underlined*, and the polyadenylation signal is *double lined*.

Sequence identities and phylogenetic analysis of ScHSP60. The homology analysis based on the amino acid sequences revealed that ScHSP60 displayed highly identity (> 90%) with its counterparts in other fish species, especially *Epinephelus coioides*, up to 95.5%. The ScHSP60 also shared more than 86% identities with its orthologs in birds and mammals, and 74.9% identities with *Bactrocera dorsalis* (Table 2).

Table 2. Identity analysis of HSP60 amino acid sequences from vertebrates. Data are expressed as percentage of amino acid sequence identity.

1	2	3	4	5	6	7	8	9	10	11	12		
	95.5	94.8	94.4	94.4	93.6	90.9	91.5	84.5	87.9	86.0	74.9	**1**	*S. chuatsi*
		96.5	95.5	96.5	95.3	91.0	92.3	84.9	88.4	86.8	74.4	**2**	*E. coioides*
			96.5	95.8	96.0	90.6	91.8	85.2	88.8	86.7	74.6	**3**	*C. variegatus*
				96.0	94.6	90.8	91.6	85.6	90.0	87.4	74.6	**4**	*O. niloticus*
					95.1	90.4	91.6	85.7	88.8	87.0	74.7	**5**	*C. striata*
						90.8	91.8	85.7	88.2	87.4	74.4	**6**	*K. marmoratus*
							96.2	84.3	87.7	85.4	73.4	**7**	*T. albonubes*
								86.1	88.9	87.7	74.1	**8**	*D. rerio*
									90.4	90.2	75.7	**9**	*X. laevis*
										93.9	75.7	**10**	*G. gallus*
											75.3	**11**	*H.sapiens*
												12	*B. dorsalis*

To evaluate the evolutionary relationship of HSP60s of mandarin fish and other species, we constructed a phylogenetic tree using software MEGA 6.0 by the neighbor-joining method (Fig. 2). According to the phylogenetic tree, vertebrates were divided into two groups based on HSP60s. The mammals, birds and amphibians were clustered into one group, the teleosts were separated and clustered into another group. In addition, the teleosts were further divided into two subgroups supported statistically (Bootstrap value=100%). Species from Perciformes and Cyprinodontiformes were clustered with a subgroup containing the ScHSP60 identified in this study, and species from Cypriniformes were clustered with the other subgroup.

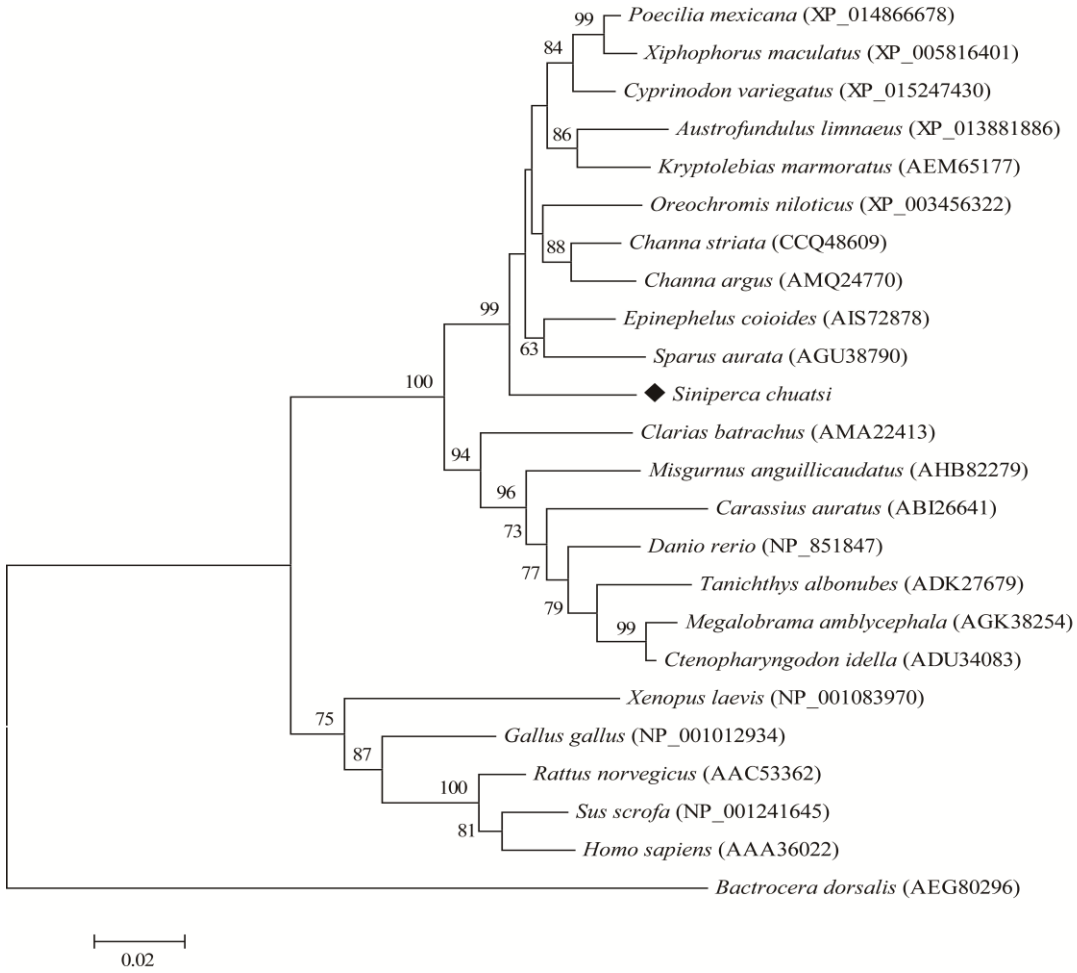

Fig. 2 Phylogenetic tree of the vertebrate HSP60 proteins constructed with MEGA 6.06 software using neighbor-joining method. Bootstrap support values (1,000 replicates) of greater than 50% are indicated at the nodes. Scale bar indicates an evolutionary distance of 0.02 amino acid substitution per position in the sequence. Sequence database accession numbers in GenBank are indicated in parentheses.

Cloning and sequence analysis of ScHSP60 gDNA. Using specific primers close to the terminus of full-length cDNA, *ScHSP60* genomic sequence was obtained and deposited in GenBank under the accession number KU997669. The coding region of *ScHSP60* contains 10 exons (174，336，96，94，169，100，246，175，179 and 162 bp) interrupted with 9 introns (155，103，108，152，159，130，95，1271 and 1462 bp). *ScHSP60* gene contains an intron (length 1538 bp) within its 5′-UTR. All the introns obey the GT-AG rule.

Alignment of genomic sequences of HSP60s in *S. chuatsi* and other vertebrates reported in Ensemble database showed that the genomic structure of HSP60 is highly conservative (Fig. 3). The genomic sequences of coding regions of fish HSP60s all consist of 10 exons interrupted with 9 introns. In comparison with fish species, an extra intron was insert in the second exon in Amphilia (*Xenopus laevis*), birds (*Gallus gallus*) and mammals (*Homo sapiens*).

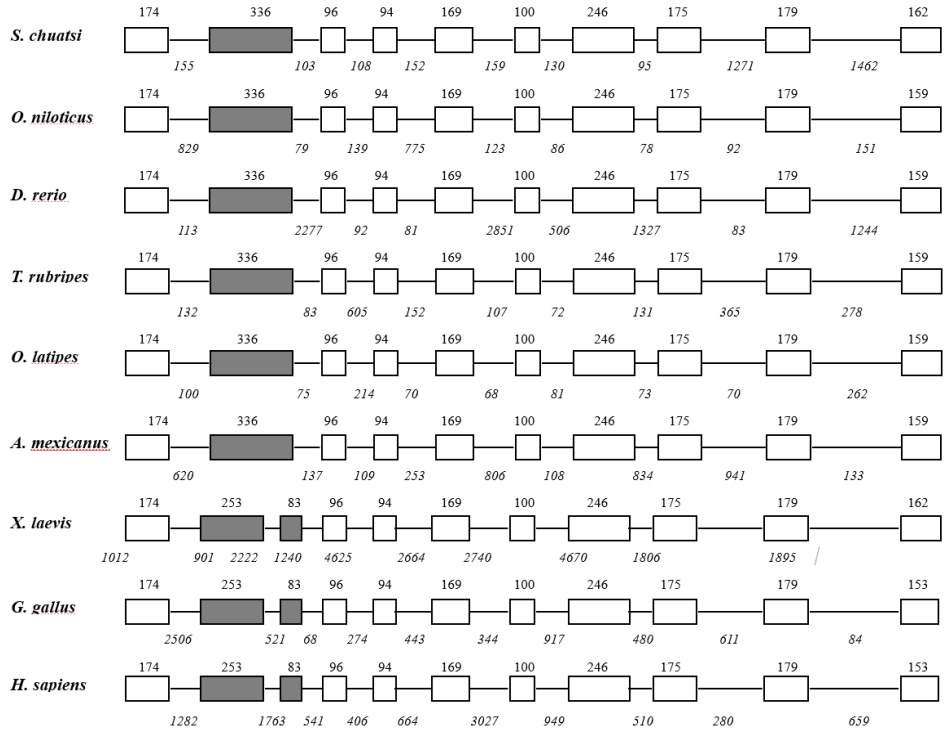

Fig. 3 Comparison of genomic structures of *HSP60* in *S. chuatsi* and other vertebrates, including *Oreochromis niloticus*, *Danio rerio*, *Takifugu rubripes*, *Xiphophorus maculatus*, *Oryzias latipes*, *Astyanax mexicanus*, *Xenopus laevis*, *Gallus gallus* and *Homo sapiens*. The *rectangles* represent exons, and the *lines* indicate introns. *Figures* above the *rectangles* and below the *lines* represent length (bp) of exons and introns, respectively.

Expression patterns of ScHSP60 during embryonic development

ScHSP60 transcripts could be detected throughout the embryonic developmental stages (Fig. 4). The transcript levels of *ScHSP60* had no significant changes from fertilized eggs to gastrula, but were significantly increased from the stage of closure of blastopore to 1 dph. The highest level appeared at metameres appearance stage and muscle burl stage, approximately 4 times ($P < 0.05$) of that in the fertilized eggs.

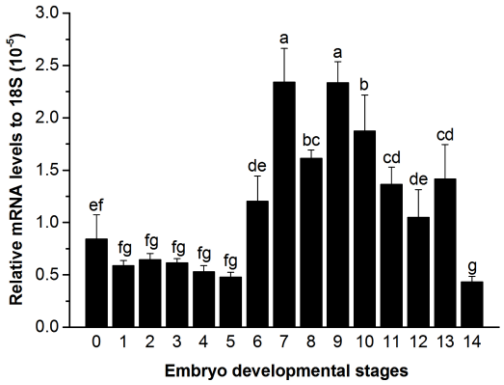

Fig. 4 Expression patterns of HSP60 during embryonic developmental stages of *S.chuatsi*. Each bar represents the mean ± SD of three samples. Different letters above the bars indicate significant difference ($P < 0.05$). 0: Unfertilized eggs; 1, Fertilized eggs; 2, 16-cells stage; 3, Morula; 4, Blastula; 5, Gastrula; 6, Closure of blastopore; 7, Metameres appearance; 8, Tail-bud stage; 9, Muscle burl stage; 10, Crystal stage; 11, Blood circulating stage; 12, pre-hatched larvae; 13, 1 dph larvae; 14, 7 dph larvae.

Tissue expression of ScHSP60. Under normal physiological conditions, the highest level of *ScHSP60* was found in the tissue-specific expression profile of the ovaries. In non-gonadal tissues, *ScHSP60* was highest in opisthonephros, followed by brain, head kidney, and spleen; lowest level was found in muscle, only 1.7% of that in opisthonephros (Fig.5).

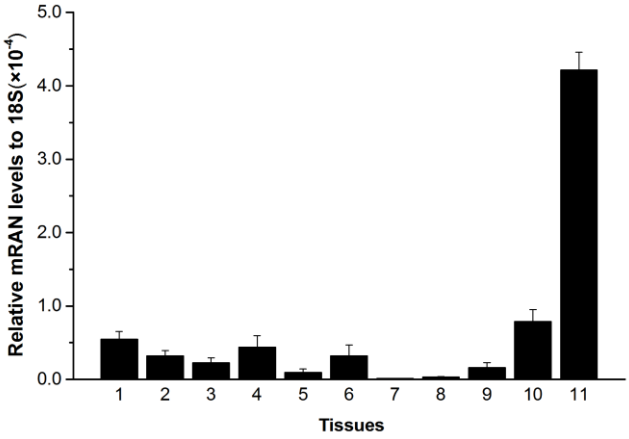

Fig.5 The mRNA expression of HSP70 family members in various tissues of *Siniperca chuatsi*. The mRNA expression was quantified by real-time RT-PCR and normalized with *18S rRNA*. Data presented are expressed as mean ± SD, n = 3 fish. 1: Brain；2: Heart；3: Gill；4: Head kidney; 5: Liver; 6: Spleen; 7: Muscle; 8: Stomach; 9: Intestines; 10: Opisthonephros; 11: Ovary.

Expression patterns of ScHSP60 after thermal stress exposure. To investigate the expression patterns of ScHSP60 after heat shock exposure, the fish were exposed to three regimes of heat shock: acute, rapid elevated and gradual elevated heat shock. The experimental temperatures in the three regimes were based on the fact that *S. chuatsi* has a natural temperature range of between 15 and 32 ℃ with an upper lethal limit of 34℃ under acute heat stress, and approximately 39℃ under rapid and gradual heat shocks measured in the present study. The expression levels of *ScHSP60* in heart, liver and head kidney under acute heat shock exposure are shown in Fig. 6a. The results showed that acute heat shock at 34℃ significantly induced the expression of *ScHSP60* in these tissues. In heart, heat shock for 6 h resulted in the highest up-regulation of *ScHSP60,* 11.7-times that of the control. In liver and head kidney, heat shock for 2 h resulted in the highest up-regulation by averages of 2.8- and 2.6-times that of in controls. And when heat shock for 12 h, the *ScHSP60* levels began to recover towards its baseline in heart and head kidney, and recovered and even below the baseline in liver.

The expression levels of *ScHSP60* in heart under fast elevated heat shock (elevating temperature rate: ~1.2℃/h) and gradual elevated heat shock (elevating temperature rate: ~0.2 ℃/h) were also investigated (Fig. 6b). Under fast elevated heat shock from 25 to 34℃, the mRNA level of ScHSP60 was increased by averages of 3.1 times, which continued to be increased with the temperature elevating to 38.8℃, by averages of 18.6 ℃。Likewise, gradual elevated heat shock also resulted in elevation of *ScHSP60*, but was lower than those under rapid elevated heat shock, by averages of 2.4 times at 34 ℃ and 16.7- times at 38.8 ℃.

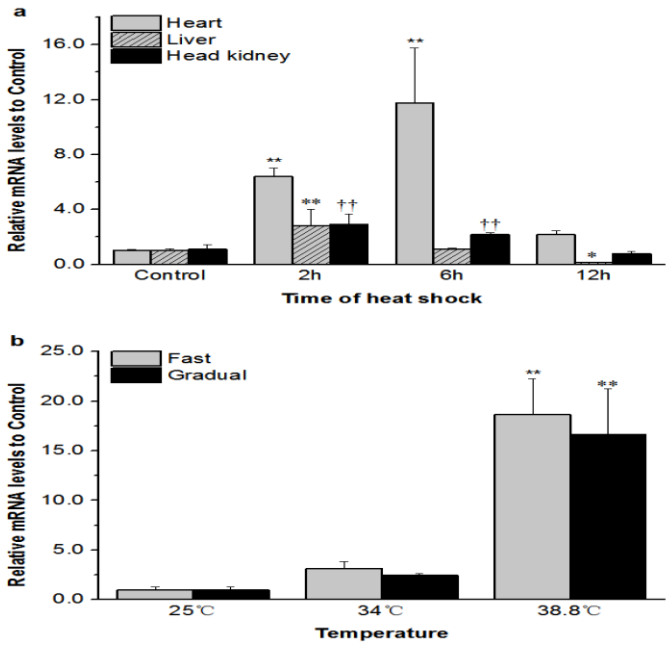

Fig. 6 Expression of *S. chuatsi HSP60* during three regimes of heat shock exposure. **a**, acute heat shock: fish maintained at 25°C were carefully and directly shifted to 34°C. **b,** rapid elevated and gradual elevated heat shock: The elevating temperature rate in the rapid elevated and gradual elevated heat shock regimes were 1.2°C/h and 0.2°C/h from 25°C, respectively. The mRNA levels were measured by real-time RT-PCR and normalized by *18S rRNA*. The fish that maintained at 25 ℃ over the entire experimental period were taken as the control group. Data presented are expressed as mean ± SD (n = 6). Symbols of "*"/"*" / "†" ($P < 0.05$) and "**" / "**"/"††" ($P < 0.01$) above the bars represent significant difference.

Expression of ScHSP60 after hypoxia exposure. In order to determine whether hypoxia influences the expression of *ScHSP60*, its mRNA levels in response to hypoxia in heart (data not shown) and liver (Fig. 7) were detected. Since there were no significant changes in the mRNA levels of *ScHSP60* throughout the experiment, the average expression level was used as a control. Although *ScHSP60* was not significantly changed in the heart, the mRNA level was markedly increased in the liver by hypoxia in a time-dependent manner, increasing 1.9 and 4.6 times compared with the control group at 6 and 12 h, respectively. In addition, the induced *ScHSP60* mRNA level after 6h-hypoxia exposure returned to its baseline during re-oxygenation over 24 h.

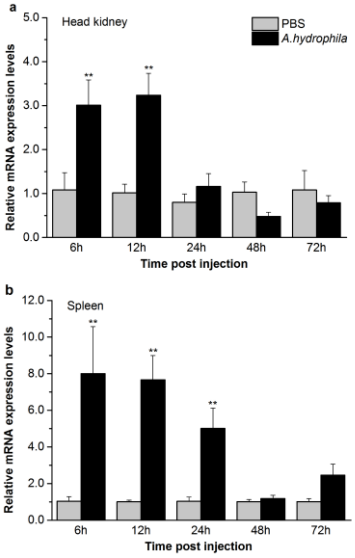

Fig.7 Expression of HSP60 in liver of *S. chuatsi* in response to hypoxia and re-oxygenation. The mRNA levels in the time-course were qualified by RT-PCR and normalized by *18S RNA*. After 6 h of hypoxia the DO levels were adjusted back to normoxic levels within 20 min and fish were sampled after 24 h of re-oxygenation (Re). Data presented are expressed as mean ± SD (n = 6). *Asterisk* (P < 0.05) and *double asterisks* (*P* < 0.01) above the bars indicate significant difference compared with the control group.

Expression of ScHSP60 after A. hydrophila infection. The expression patterns of *ScHSP60* in head kidney and spleen from 6 to 72 h post-infection with *A. hydrophila* were examined by real-time quantitative PCR (Fig. 8). Results showed that infection with *A. hydrophila* significantly (*P* < 0.01) increased the *ScHSP60* expression in the head kidney and spleen. During the time-course experiment, *ScHSP60* mRNA level initially increased significantly and reached the highest level of 3.2 times in the head kidney at 12 h and gradually decreased to baseline thereafter. Similarly, its level in the spleen was markedly increased at 6 h, up to 7.7 times of that in the control group, and then decreased gradually, returning to the baseline at 48 h.

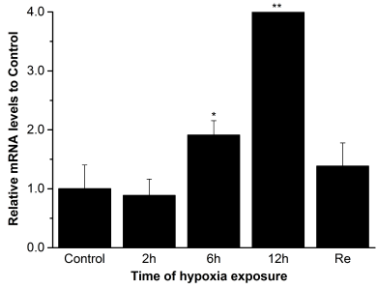

Fig. 8 Expression of *ScHSP60* in the head kidney and spleen after *A.hydrophila* infection. The mRNA levels in the time-course study were qualified by RT-PCR and normalized by *18S rRNA*. Data presented are expressed as mean ± SD (n = 6). Single *asterisk* (*P* < 0.05) and *double asterisks* (*P* < 0.01) above the bars indicate significant difference compared with the control group.

Discussion

HSP60 is a molecular chaperone located mainly in the mitochondria. In the present study, the HSP60 cDNA and gDNA from mandarin fish was identified and characterized. The full length of *ScHSP60* cDNA is 2505 bp with an ORF of 1731 bp encoding 576 amino acids. ScHSP60 sequences have three major functional domains: the apical domain, the equatorial domain, and the intermediate domain (Brocchieri and Karlin 2000). ScHSP60 has the classical mitochondrial HSP60 signature motif and a mildly hydrophobic conserved GGM repeat at C-terminal that are required for the rapid folding of some proteins (Tang *et al*. 2006). Sequence alignment shows ScHSP60 is highly homologous

with HSP60 genes from other vertebrates. The phylogenetic analysis revealed that ScHSP60 was clustered with its counterparts in other fish species. Alignment of gDNA sequences of HSP60s from *S. chuatsi* and other vertebrates reported in Ensemble database revealed that numbers of nucleotide bases of all corresponding exons of these fish species are identical except for one; *S. chuatsi* has 3 additional nucleotide bases, more than other fish, encoding one more Gly in the Gly-Gly-Met belonging to the GGM repeat, probably derived from slipped-strand mispairing during gene duplication. On the basis of cDNA and gDNA characterization, multiple alignment, and phylogenetic analysis, we were able to confirm that the newly identified ScHSP60 is a member of the mitochondrial HSP60 family.

To date there has been limited information regarding HSP60 in embryonic development of teleosts. In the present study, we had *ScHSP60* transcripts in unfertilized eggs and embryos before the midblastula transition (MBT) period, until activation of zygotic transcription (Kane and Kimmel 1993), suggesting maternal heredity. *ScHSP60* expression significantly increased from the stage of blastopore closure to 1 dph, at high levels at metameres appearance stage, and muscle burl stage, indicating that mitochondrial HSP60 plays an important part in embryonic development of mandarin fish. This was consistent with the brief summary of HSP60 at significantly higher levels at early stages of oocyte differentiation in teleosts (Lubzens *et al*. 2010). However, in a study of HSP60 of *C. idella*, maternal original *CiHSP60* transcripts were reduced markedly at the gastrula stage and kept at low levels of less than 1/10 of the fertilized eggs (Xu *et al*. 2011). Almost the opposite expression patterns of *HSP60* between *C. idella* and *S. chuatsi* may be due to species variance, and more studies on *HSP60* expression during embryonic development are needed on other fish species.

ScHSP60 showed a tissue-specific variation pattern in different tissues under normal conditions. The much higher expression level in the ovaries (phase III during ovarian development) indicated that *ScHSP60* may play a crucial physiological role in ovary development. In addition, relatively high levels of *ScHSP60* were found in head kidney and spleen, two important immune organs for mandarin fish. Similar results were also found in *C. idella HSP60* (Xu *et al*. 2011), suggesting that *HSP60* may be related to immune function.

Temperature is a major factor for survival of animals. One major type of cellular damage resulting from heat stress is denaturation of proteins, and adaptation to this stress leads to upregulation of heat shock proteins, which interact with stress-denatured proteins to maintain or restore their native structure and prevent aggregation and degradation (Purohit *et al*. 2014). In our previous study, we reported elevated mandarin fish *HSP70* when exposed to heat shock (Wang *et al*. 2014). *ScHSP60* was also upregulated during three regimes of thermal stress: acute, fast, and gradual heat shock. It has been demonstrated that heat stress induces apoptosis through activation of reactive oxygen free radicals (ROS) (Pallepati and Averill-Bates 2011), and HSP60 can inhibit the generation of ROS (Magnoni *et al*. 2014). HSP60 may inhibit cell apoptosis by suppressing ROS generation caused by heat stress. In addition, compared with elevation under acute shock at 34°C, *ScHSP60* expression strongly upregulated at 38.8°C and did not significantly change at 34°C when exposed to rapid or gradual heat shock, suggesting that induction of *HSP60* by heat shock requires drastic changes of temperature or large temperature differences.

There is sparse information of HSP60 expression under hypoxia in fish. In mammals, mice *HSP60* was up-regulated in hypoxic preconditioning and ischemia, and involved in the development of hypoxic preconditioning (Jiang *et al.* 2011). In this study, the expression of *ScHSP60* in liver significantly increased with 6-12 h of hypoxia, suggesting it is also involved in the cellular process of hypoxic response.

HSP60 is a node in intracellular molecular networks, and is also a linking molecule in intercellular immune networks (Quintana and Cohen 2011). Inside both prokaryotic and eukaryotic cells, HSP60 functions as a molecular chaperone coming into contact with most intracellular proteins (Borges and Ramos 2005). In the extracellular environment, HSP60 alone or combined with microbial proteins acts as a connecting link between

immune cells to coordinate activity of the immune system (Quintana and Cohen 2011). Most studies on immune functions of HSP60 have focused on mammals. There are few reports regarding HSP60 in fish immune response. The expression of HSP60 in the liver, gills and spleen of *E. akaara*, was upregulated when infected with *Vibrio* (Qu *et al*. 2011). HSP60 expression in multiple tissues including liver, heart, intestine, head kidney, spleen, and gills of grass carp was significantly increased after 3 days of infection by *A. hydrophila* (Xu *et al*. 2011). Our study showed that the different stages of development affected upregulation of *ScHSP60* in the head kidney and spleen. In the head kidney, *HSP60* reaction manifested primarily in the early stage of infection, while in the spleen, HSP60 mRNA was higher during long-term infection. These results suggest that ScHSP60 stimulated immune response to protect fish from damage caused by *A. hydrophila*.

In summary, HSP60 cDNA and gDNA were identified and characterized in mandarin fish *S. chuatsi*. Characteristic amino acid sequence features, DNA structure, and phylogenetic analysis showed that *ScHSP60* belongs to the mitochondrial HSP60 family. Furthermore, mRNA expression profiles of *ScHSP60* during embryonic development, in different tissues and after exposure to different environmental stresses were analyzed using real-time RT-PCR. Expression profiles found in this study suggest that ScHSP60 is involved in embryogenesis and the stress responses of high temperature, hypoxia, and bacterial infection. The identification and expression analysis of ScHSP60 will benefit to further clarify the important roles of HSP60 in embryogenesis, complex environmental, physiological, and stressful conditions in aquatic vertebrates, and contribute to further studies to enhance stress tolerance and disease resistance of mandarin fish.

Acknowledgements

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