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ORIGINAL ARTICLE

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Identification and expression analysis of two HSP70 isoforms in mandarin fish *Siniperca chuatsi*

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Abstract Heat shock protein 70 (HSP70) plays important roles in multiple cellular stress responses. Two HSP70 isoforms, ScHSP70a and ScHSP70b, were identified from *Siniperca chuatsi* in this study. ScHSP70a and ScHSP70b shared all but one of their 639 amino acids and showed a remarkable homology to HSP70s of other species. Expression profile analysis by qRT-PCR revealed that both isoforms were detected throughout embryonic development, and a striking feature of *ScHSP70a* was its significant up-regulation during the crystal stage. *ScHSP70a* and *ScHSP70b* were expressed ubiquitously and at a low level in tissues under non-stressed conditions. However, they were dramatically induced by heat shock at different levels, and their induction was positively correlated with the increasing rate of temperature. Although insensitive to hypoxia in the heart, both genes were greatly induced by hypoxia in the liver, and the induction was returned to the basal level after re-oxygenation for 24 h. Additionally, *Aeromonas hydrophila* infection also markedly augmented *ScHSP70a* and *ScHSP70b* expression in a time-dependent manner in the head kidney and spleen, and the *ScHSP70a* induction levels were much higher than those of *ScHSP70b*. These results suggest that *ScHSP70a* and *ScHSP70b* contribute differently to embryonic development and protection against damage from high temperature, hypoxia and bacterial infection.

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Introduction

Heat shock proteins (HSPs) are a super family of ubiquitous and evolutionarily highly conserved proteins, which function as chaperone molecules to maintain the cellular protein homeostasis under both normal and physiological stress conditions [1, 2]. HSPs modulate the folding and assembly of nascent proteins, refold or degrade damaged proteins, assist in the membrane translocation of organellar and secretory proteins, as well as regulate signal transduction [3, 4]. According to their sequence homology and approximate molecular weights, HSPs are generally classified into several conserved groups: HSP100, HSP90, HSP70, HSP60 and small HSPs [5]. Of these proteins, HSP70s are thought to be the central components of the cellular network of molecular chaperones and folding catalysts [3]. The HSP70 family is composed mainly of two subgroups, the constitutive heat shock cognate 70 proteins (HSC70s) and the inducible HSP70s. HSC70 is actively expressed in non-stressed cells and remains unchanged or only mildly induced upon stress stimulation [6]. It is involved in many cellular functions, such as protein translocation, cell apoptosis and embryonic development [6]. In contrast, HSP70 can be strongly induced from low basal levels by various environmental stresses, such as heat shock, heavy metals, hypoxia and pathogenic infection. It is required for a variety of cellular processes and for the response to environmental changes [7]

In teleosts, genes or cDNAs of *HSP70* have been identified in a number of species, such as *Oncorhynchus mykiss* [8], *Danio rerio* [9], *Oreochromis mossambicus* [10],

Xiphophorus maculatus [11] and *Ctenopharyngodon idella* [12], and observations of expression patterns in these studies suggested that HSP70 plays a vital role in enhancing the survival and health of cells or whole organisms upon being stressed. It is noted that there are multiple *HSP70* isoforms in mammals [7, 13] and *Drosophila melanogaster* [14], and these isoforms differ with respect to their expression patterns. Human *HSP70* isoforms show different expression profiles in the developmental stages, among different tissues and under stress conditions [13] and *D. melanogaster HSP70* isoforms reveal the characteristics of tissue- and development-specific induction after heat shock and during recovery [14]. In teleosts, however, only a few species such as *Oryzias latipes*, *D. rerio*, *O. mykiss, X. maculatus* and *Fundulus heteroclitus macrolepidotus* have been reported to have two or more *HSP70* isoforms [2, 11, 15, 16], and information about different expression patterns between teleost *HSP70* isoforms during embryonic development, as well as in different tissues upon being stressed, remains rare.

The mandarin fish, *Siniperca chuatsi*, is an economically important fish and has been widely cultured in China [17]. With the development of intensive production in the aquaculture industry, 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 [18, 19]. Understanding the expression characteristics and roles of HSP70 during stresses will contribute to improving the abilities of fish to tolerate stress and resist disease. However, the *HSP70* gene and its response to environmental stresses are basically unknown in *S. chuatsi*. Therefore, in the present study, we identified and characterized two distinct inducible *HSP70* isoforms in *S. chuatsi* and comparatively assessed their expression during embryonic development, on exposure to heat shock, hypoxia, and an *A. hydrophila* challenge, using real-time qRT-PCR. This study provides a better understanding of the correlations between the expression of *HSP70* isoforms in *S. chuatsi* and during its embryonic development, as well as different environmental stresses, and lays the foundation for further study of the mechanism of anti-adverseness in *S. chuatsi*.

Materials and methods

Animals and sampling

Mandarin fish *S. chuatsi* weighing approximately 120–140 g were obtained from BaiRong Aquatic breeding Co., Ltd (Guangdong, China). The fish were maintained in a circulating water system containing a series of 400-l tanks at 25 °C for at least 2 weeks prior to processing. The fish were fed with fresh juvenile *Cirrhinus molitorella* at a ratio of approximately 5 % of the total biomass. For sampling, the fish were anesthetized with tricaine methanesulfonate (MS-222) and tissue samples 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.

Total RNA and genomic DNA isolation

Total RNA was extracted from tissues with EZNA total RNA kit II (Omega Bio-Tek, USA) according to the manufacturer's instructions. Genomic DNA was isolated from the muscle using a TIANamp Genomic DNA Kit (Tiangen Biotec, China). The concentration and quality of RNA/DNA were verified by spectrophotometer and electrophoresis on 1.0 % agarose gel.

Cloning of full-length cDNAs and genomic DNAs of *ScHSP70* isoforms

A schematic diagram of cloning of *HSP70* full-length cDNAs from *S. chuatsi* is shown in Fig. 1. The fish used in cloning was exposed to heat shock at 34 °C for 60 min, and then tissues, including heart, head kidney, liver and muscle were sampled. First-strand cDNA synthesis was performed using 2 μg of DNase I-treated total RNAs with the first-strand cDNA synthesis using M-MLV for RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The cDNAs of the four tissues were mixed and then used as the template for polymerase chain reaction (PCR). Two couples of degenerate primers, HSP-F1/HSP-R1 and HSP-F2/HSP-R2 (Table 1), based on the highly conserved regions of amino acid sequences of HSP70 family members of other fish, were designed to amplify the *HSP70* cDNA fragments from *S. chuatsi* using the nested PCR.

Full-length cDNA sequences of *HSP70s* were obtained by the technique of rapid amplification of cDNA ends (RACE). The 5′-RACE PCR was performed with the purified first-strand cDNA as the template, which had a poly(C) end added to the 5′ terminal, and nested primers HSP70-R1 and HSP70-R2 (Table 1) as the reverse primers and an abridged anchor primer (AAP) or an abridged universal amplification primer (AUAP) as the forward primers. Based on the two distinct 5′-untranslated regions (UTRs) of *HSP70* cDNAs we obtained, forward gene-

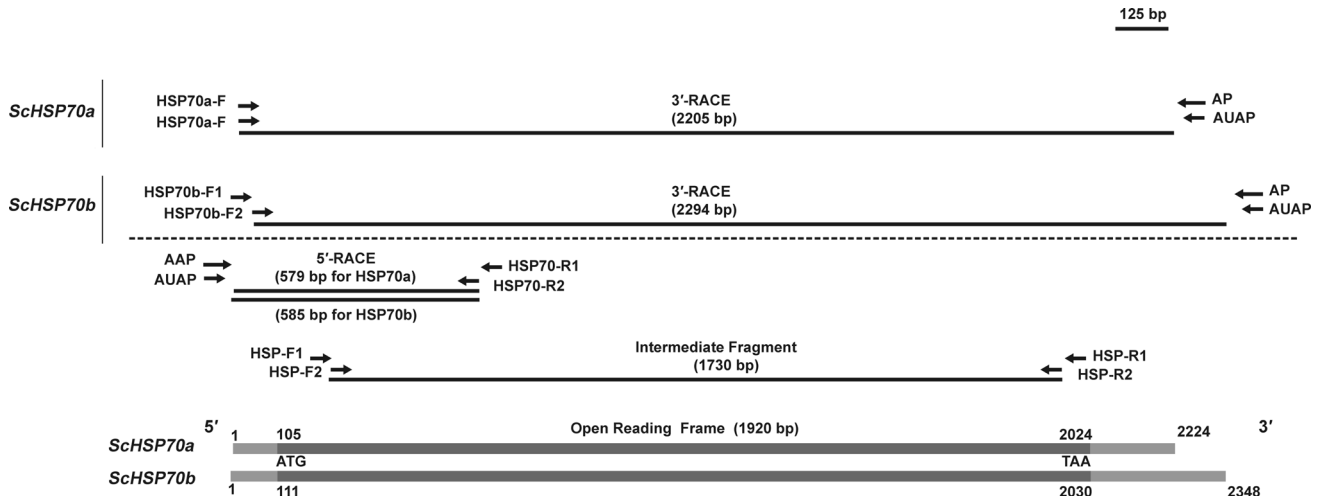


Fig. 1 Cloning strategy of *HSP70a* and *HSP70b* full-length cDNAs from *Siniperca chuatsi*. *Arrows* represent primers for intermediate fragment, 5′- and 3′-end amplification, and *lines* under them indicate their amplification sequences. The open reading frames (ORFs) are indicated by *dark gray boxes*, and the untranslated regions (UTRs) are indicated by *light gray boxes*

specific primers for *ScHSP70a* and *ScHSP70b* (Table 1) were designed for two rounds of 3′-RACE PCR. The anchor primer (AP) and AUAP were used as the reverse primers in the 1st and 2nd rounds, respectively. In addition, in order to verify the correctness of assembly of the intermediate fragment, a 5′-RACE fragment and a 3′-RACE fragment of each of the cDNA gene-specific primers ORF-HSP70a-F/-R for *ScHSP70a* and ORF-HSP70b-F/-R for *ScHSP70b* (Table 1) were designed to amplify their complete ORFs using first-strand cDNA as the template, and the PCR products were sequenced.

Amplifications for cloning the *ScHSP70* cDNAs were performed as follows: denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55–60 °C for 30 s and 72 °C for 1–2.5 min, and a final extension of 10 min at 72 °C. The PCR amplification products were purified with a Universal DNA Purification Kit (Tiangen Biotec, China) and cloned into pEASY-T1 cloning vector (TransGen Biotec). Recombinants were identified by blue/white screening and confirmed by PCR, and then positive ones were selected and sequenced.

The genomic DNA sequences coding ScHSP70a and ScHSP70b were amplified from total genomic DNA by gene-specific primers ORF-HSP70a-F/-R and ORF-HSP70b-F/-R, respectively, which were located at the terminus of 5′- and 3′-UTRs of *ScHSP70s* cDNA sequences. PCR conditions for the genomic DNA sequence cloning 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 3 min, and a final extension of 10 min at 72 °C. The PCR amplification products were cloned into pEASY-T1 cloning vectors (TransGen Biotec, China) and sequenced.

BLAST searches and sequence alignments

Nucleotide and amino acid sequences that are homologous to ScHSP70 were retrieved by the BLAST programs at the National Center for Biotechnology Information (http://www.blast.ncbi.nlm.nih.gov/). Protein sequence alignments were performed by ClustalX 2.1 (http://www.clustal.org), and then used for constructing the phylogenetic tree with MEGA5.2 software according to the neighbor-joining method [20]. The compute p*I*/MW tool of ExPASy (http://www.expasy.org/proteomics) was used to calculate the molecular weight (MW) and isoelectronic point (p*I*) of deduced protein of *S. chuatsi HSP70s*. The program ScanProsite (http://www.us.expasy.org/tools/scanprosite/) was used to search the protein motifs.

Embryos and tissues

Embryos were obtained by natural crossing. Fertilized eggs were hatched in three vertical fish egg incubators. The clean and oxygenated water with a temperature of 27 °C entered incubators continuously under pressure from below and kept the eggs in a suspended state. Embryos at different embryonic stages including fertilized eggs, 16-cell stage, morula, blastula, gastrula, closure of blastopore, appearance of myomere, tail-bud stage, muscle burl stage, crystal stage, blood circulating stage, pre-hatched larvae and 1-day post-hatched larvae (1 dph) were collected and snap-frozen in liquid nitrogen. Thirty embryos or twelve larvae in every stage from each incubator were collected as one sample.

A range of tissues including brain, heart, gill, head kidney, liver, spleen, muscle, stomach, intestines and

Table 1 Primer sequences used in this study

Primer name	Primer sequence (5′–3′)	Location (bp)	Objective
HSP-F1	GGNACYACCTACTCCTGYGTNGG[a]	144–166[b]	1st round intermediate fragment amplification of *ScHSP70s*
HSP-R1	TTAGTCNAYCTCYTCRATGGTNGG[a]	2,001–2,024[b]	
HSP-F2	ATCATHGCCAAYGACCAGGGNAA	192–214	2nd round intermediate fragment amplification of *ScHSP70s*
HSP-R2	TTRCAYACYTTCTCCARCTCCTT	1,899–1,921	
HSP70-R1	CCGTCGGCTCGTTGATGATCCTCAG	618–623	1st round 5′-RACE for *ScHSP70s*
HSP70-R2	CCTGTCGCTGGGAGTCGTT	561–579	2nd round 5′-RACE for *ScHSP70s*
HSP70a-F	AACTGACGAAAGGAACAACAAGTG	20–44	1st and 2nd round 3′-RACE for *ScHSP70a*
HSP70b-F1	AGATCAGACCGCAGCAGAAGAT	12–33	1st round 3′-RACE for *ScHSP70b*
HSP70b-F2	GATCCTCTTCACAACACAGACCAC	55–78	2nd round 3′-RACE for *ScHSP70b*
ORF-HSP70a-F	AACTGACGAAAGGAACAACAAGTG	20–43	ORF verification and genomic DNA cloning for *ScHSP70a*
ORF-HSP70a-R	CCAGTCGTCTTTATTGCAAGGTC	2,197–2,219	
ORF-HSP70b-F	AGATCAGACCGCAGCAGAAGAT	12–23	ORF verification and genomic DNA cloning for *ScHSP70b*
ORF-HSP70b-R	GACGTTCATCTTTATTGCAAAGTCC	2,318–2,342	
RT-HSP70a-F	CGCTGAGACAAGAAAACTGACG	6–27	Real-time qRT-PCR for *ScHSP70a*
RT-HSP70a-R	CTTGGTTGTTTTGAATTTGTGTCC	81–104	
RT-HSP70b-F	CGCAGCAGAAGATCCAGAGAG	21–41	Real-time qRT-PCR for *ScHSP70a*
RT-HSP70b-R	GGAGACGGTCGTAGTTTAGAGTTGA	79–103	
18S-F	CTGAGAAACGGCTACCACATCC	126–157[c]	Real-time qRT-PCR for *S. chuatsi 18S rRNA*
18S-R	GCACCAGACTTGCCCTCCA	290–308	
AP	GGCCACGCGTCGACTAGTAC$(T)_{18}$		3′-RACE for *ScHSP70s*
AAP	GGCCACGCGTCGACTAGTAC$(G)_{10}$		5′-RACE for *ScHSP70s*
AUAP	GGCCACGCGTCGACTAGTAC		3′- and 5′-RACE for *ScHSP70s*

[a] N, A/C/T/G; Y, C/T; R, A/G, [b] Location in *ScHSP70a* cDNA sequence, [c] GenBank accession number of *S. chuatsi 18S rRNA* gene is AY452490

opisthonephros from the fish (body weight, 150–160 g; body length, 16.0–16.5 cm) reared in non-stressed conditions at 25 °C were sampled and used for analysis the tissue distribution of the two *ScHSP70* isoforms by real-time qRT-PCR.

Heat shock treatment

To examine the expression patterns of two *S. chuatsi HSP70* isoforms in response to thermal stress, the fish were subjected to three regimes of thermal stress: acute, fast and gradual heat shock. For acute heat shock, 20 fish (mean body weight of 140 ± 20 g) maintained in the 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. Six fish were randomly collected after each heat treatment (2, 6 and 12 h), and another six individuals maintained at 25 °C served as the control group.

In the other two regimes of thermal stress, fish were transferred to the tank of 2,000 l in the circulating water system (initial temperature: 25 °C), and then the water temperature was elevated from 25 to 38.8 °C (semilethal temperature acquired from our preliminary experiment) at an average speed of 1.2 °C increase per hour for fast heat shock and 0.2 °C increase per hour for gradual heat shock. Six fish were randomly sampled when the temperature reached 34 °C, six more at 38.8 °C, and another six individuals maintained at 25 °C were taken as the control group.

Hypoxia exposure and re-oxygenation

Twenty-four fish (mean body weight of 130 ± 20 g) acclimated to a normal oxygen environment (dissolved oxygen, DO: ≥5.6 mg/l, temperature: 25 °C) for 1 week were randomly and evenly divided into four equal groups (A–D): A, exposure to a low oxygen level of

0.9 ± 0.1 mg/l of DO for 4 h; B, re-oxygenation under normal oxygen for 24 h after the 4-h hypoxia; C and D, exposure to normal oxygen, served as control groups for A and B, respectively. The dissolved oxygen of 0.9 ± 0.1 mg/l was maintained by controlling the water and nitrogen inflows. Dissolved oxygen was monitored continuously using a YSI Model 550A dissolved oxygen meter (Geo Scientific Ltd, USA).

Bacterial challenge

Sixty healthy *S. chuatsi* (140 ± 15 g, BW) were used for a 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 were used 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 after injection, respectively.

Real-time qRT-PCR

Quantitative real-time PCR (real-time qRT-PCR) was used to quantify the expression of *ScHSP70s* at the transcriptional level according to MIQE validation guidelines [21]. *18S rRNA* was selected as the reference gene, because it was stably expressed among different tissues and upon stress exposure in the present study. Total RNA (0.9 μg) from each sample was reverse-transcribed using a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Specific primers (Table 1) were designed to amplify PCR fragments for *ScHSP70a*, *ScHSP70b* and *S. chuatsi 18S rRNA*. The amplification was performed on the LightCycler® 480 II Real-Time PCR System (Roche) using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Japan) in 384-well plates. The final reaction volume was 10 μl consisting 1.0 μl of cDNA sample (10 ng/μl), 0.4 μl of each gene-specific primer (10 μM), 3.2 μl of nuclease-free H_2O and 5 μl of 2× SYBR Premix Ex Taq II. All reactions were carried out in three technical replicates, from which mean threshold cycle (C_T) values were calculated. Based on Tm value of 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 verify the PCR specificity.

The standard curve for *ScHSP70a*, *ScHSP70b* and *S. chuatsi 18S rRNA* was generated through a 10-fold dilution series of plasmids pEASY-T1 (TransGen Biotec, China) containing the target or reference genes. Amplification efficiencies (E) of real-time qPCR were determined by employing the equation $E = 10^{(-1/\text{slope})} - 1$. Here, amplification efficiencies of *ScHSP70a*, *ScHSP70b* and *S. chuatsi 18S rRNA* were 100, 99.4 and 104.6 %, respectively.

The relative transcript amounts of *HSP70s* of the experimental fish were normalized to the level of *18S rRNA* in the same sample by the $2^{-\Delta Ct}$ method and the relative expression levels of HSP70s after exposure to heat shock, hypoxia and bacterial challenge were determined by the $2^{-\Delta\Delta Ct}$ method and indicated as n-fold differences relative to the control group.

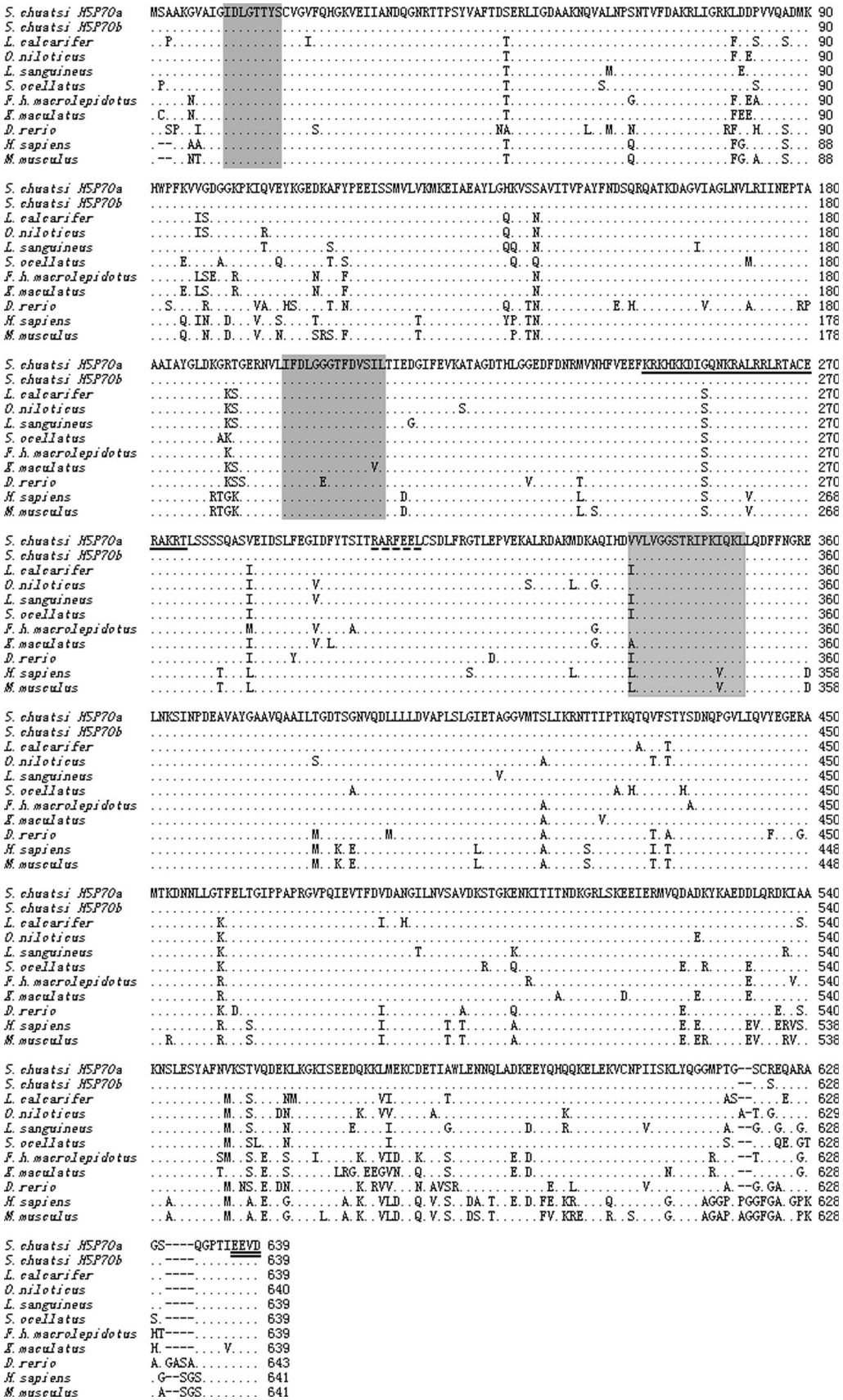
Statistical analysis

Statistical analyses were performed using SPSS software version 18.0 (SPSS Inc., Chicago, IL, USA). Data presented in this study were analyzed using one-way ANOVA followed by Tukey's test and expressed as mean ± SD.

Results

Cloning and characterization of two distinct *ScHSP70* isoforms

Considering the possibility that more than one isoform of *HSP70* exists in *S. chuatsi*, we carefully designed a RACE strategy to clone their cDNA sequences (Fig. 1). Firstly, a mixture of total cDNAs from multiple tissues of the heat-treated fish was used as the PCR amplification template to avoid certain isoforms being expressed in a tissue-specific manner. Secondly, the primers for intermediate fragment cloning were designed at conserved regions of the HSP70 but with high degeneracy. As a result, two intermediate cDNA fragments with extremely high nucleotide sequence identity (>99 %) were isolated, and their 5′-UTRs were amplified by the same 5′-RACE primers. The sequence differences between their 5′-UTRs allowed us to further design their specific 3′-RACE primers. Finally, two full-length cDNAs were successfully obtained. BLAST analysis of nucleotide and deduced amino acid sequences of the two cDNAs revealed that they belong to the HSP70 family. Sequence alignment showed that they share 99.8 % identity with a single substitution of Arg-Ser at position 623, indicating that these two transcripts represent distinct isoforms of HSP70. Thus, in the present study, they are designated as *ScHSP70a* and *ScHSP70b* and deposited in GenBank under the accession numbers JX500752 and KF150224, respectively.


S. chuatsi HSP70a MSAAKGVAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDSERLIGDAAKNQVALNPSNTVFDAKRLIGRKLDDPVVQADMK 90
S. chuatsi HSP70b 90
L. calcarifer 90
O. niloticus 90
L. sanguineus 90
S. ocellatus 90
F. h. macrolepidotus 90
M. maculatus 90
D. rerio 90
H. sapiens 88
M. musculus 88
S. chuatsi HSP70a HWPFKVVGDGGKPKIQVEYKGEDKAFYPEEISSMVLVKMKEIAEAYLGHKVSSAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
S. chuatsi HSP70b 180
L. calcarifer 180
O. niloticus 180
L. sanguineus 180
S. ocellatus 180
F. h. macrolepidotus 180
M. maculatus 180
D. rerio 180
H. sapiens 178
M. musculus 178
S. chuatsi HSP70a AAIAYGLDKGRTGERNVLIFDLGGGTFDVSILTIEDGIFEVKATAGDTHLGGEDFDNRMVNHFVEEFKRKHKKDIGQNKRALRRLRTACE 270
S. chuatsi HSP70b 270
L. calcarifer 270
O. niloticus 270
L. sanguineus 270
S. ocellatus 270
F. h. macrolepidotus 270
M. maculatus 270
D. rerio 270
H. sapiens 268
M. musculus 268
S. chuatsi HSP70a RAKRTLSSSSQASVEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKMDKAQIHDVVLVGGSTRIPKIQKLLQDFFNGRE 360
S. chuatsi HSP70b 360
L. calcarifer 360
O. niloticus 360
L. sanguineus 360
S. ocellatus 360
F. h. macrolepidotus 360
M. maculatus 360
D. rerio 360
H. sapiens 358
M. musculus 358
S. chuatsi HSP70a LNKSINPDEAVAYGAAVQAAILTGDTSGNVQDLLLLDVAPLSLGIETAGGVMTSLIKRNTTIPTKQTQVFSTYSDNQPGVLIQVYEGERA 450
S. chuatsi HSP70b 450
L. calcarifer 450
O. niloticus 450
L. sanguineus 450
S. ocellatus 450
F. h. macrolepidotus 450
M. maculatus 450
D. rerio 450
H. sapiens 448
M. musculus 448
S. chuatsi HSP70a MTKDNNLLGTFELTGIPPAPRGVPQIEVTFDVDANGILNVSAVDKSTGKENKITITNDKGRLSKEEIERMVQDADKYKAEDDLQRDKIAA 540
S. chuatsi HSP70b 540
L. calcarifer 540
O. niloticus 540
L. sanguineus 540
S. ocellatus 540
F. h. macrolepidotus 540
M. maculatus 540
D. rerio 540
H. sapiens 538
M. musculus 538
S. chuatsi HSP70a KNSLESYAFNVKSTVQDEKLKGKISEEDQKKLMEKCDETIAWLENNQLADKEEYQHQQKELEKVCNPIISKLYQGGMPTG--SCREQARA 628
S. chuatsi HSP70b 628
L. calcarifer 628
O. niloticus 629
L. sanguineus 628
S. ocellatus 628
F. h. macrolepidotus 628
M. maculatus 628
D. rerio 628
H. sapiens 628
M. musculus 628
S. chuatsi HSP70a GS----QGPTIEEVD 639
S. chuatsi HSP70b 639
L. calcarifer 639
O. niloticus 640
L. sanguineus 639
S. ocellatus 639
F. h. macrolepidotus 639
M. maculatus 639
D. rerio 643
H. sapiens 641
M. musculus 641

◀**Fig. 2** Alignment of amino acid sequences of ScHSP70s and other vertebrate HSP70s. Identical amino acid residues are indicated by *dots*. Missed amino acids are marked by *dashes*. Three HSP70 proteins family signature sequences are indicated by *shaded* regions. The putative bipartite nuclear localization signal is *underlined* and the non-organellar motif RARFEEL is marked by a *dashed underline*. The C-terminal EEVD motif is marked by *double underlines*. The GenBank accession numbers of the HSP70s are as follows: *Siniperca chuatsi* HSP70a (AFU54391), *Siniperca chuatsi* HSP70b (AGS83422), *Lates calcarifer* (AEH27544), *Oreochromis niloticus* (ACI42865), *Lutjanus sanguineus* (ADO32584) *Sciaenops ocellatus* (ADL18372), *Fundulus heteroclitus macrolepidotus* (ABB17042), *Xiphophorus maculatus* (BAB72168), *Danio rerio* (BAB72170), *Homo sapiens* (NP_005336), *Mus musculus* (NP_034609)

The full length of *ScHSP70a* cDNA sequence was 2,224 bp [excluding the poly(A) tail], containing an open reading frame (ORF) of 1,920 bp, a 5′-UTR of 104 bp and a 3′-UTR of 200 bp with a polyadenylation signal (AATAAA) located 14 nucleotides upstream from the poly(A) tail. The deduced amino acid sequence encoded a protein of 639 amino acids with a calculated molecular mass of 70.20 kDa and a p*I* of 5.41. The complete *ScHSP70b* cDNA was 2,348 bp in length [excluding the poly(A) tail], consisting of a 5′-UTR of 110 bp, and 3′-UTR of 318 bp with a polyadenylation signal (AATAAA) located 16 bases before the poly(A) tail and an ORF of 1,920 bp encoding a protein of 639 amino acids with a calculated MW of 70.13 kDa and a p*I* of 5.35.

ScHSP70a and ScHSP70b were composed of an N-terminal ATPase domain (amino acids, aa 1–383), a peptide (substrate)-binding domain (aa 387–545) and a C-terminal domain (aa 539–622) [6]. Three signature motifs of the HSP70 family were identified in both ScHSP70a and ScHSP70b sequences (signature 1, IDLGTTYS; signature 2, IFDLGGGTFDVSIL; and signature 3, VVLVGGSTRIPKIQK) (Fig. 2). The other conserved motifs, such as the cytoplasmic characteristic EEVD motif known to be the site of interaction with some co-chaperones, the bipartite nuclear localization signal (KRKHKKDIGQNKRALRRRTACERAKRT), that is involved in the selective translocation of HSP70 into the nucleus, and the non-organellar stress protein motif RARFEEL were also identified in both ScHSP70 isoforms (Fig. 2).

Using the ORF primers (Table 1) close to the 5′- and 3′-terminals, genomic DNA fragments of *ScHSP70a* (GenBank accession no. KF500540) and *ScHSP70b* (GenBank accession no. KF500541) were isolated, with lengths of 2,980 and 2,661 bp, respectively. Genomic DNA structure analysis revealed that both genes contained no introns in their coding regions, which meant the uninterrupted ORF of 1,920 bp encoded the entire protein. However, there were two introns with lengths of 780 and 331 bp within the two 5′-UTRs, respectively (Fig. 3).

Sequence comparison and phylogenetic analysis

The homology analysis based on the amino acid sequences revealed that the predicted HSP70a and HSP70b protein of *S. chuatsi* displayed similar amino acid residues to those of other vertebrates (Fig. 2). The highest identity (95 %) was with *Lates calcarifer*, *Oreochromis niloticus* and *Lutjanus sanguineus*. Identities with the other species were 94 % (*Sciaenops ocellatus)*, 93 % (*F. h. macrolepidotus*), 92 % (*X. maculatus*) and 87 % (*D. rerio*). They also shared 87 and 85 % identities with human and mouse HSP70s, respectively.

In the phylogenetic tree constructed with HSP70s and HSC70s in vertebrates (Fig. 4), the fish HSP70 family members were clearly clustered into two distinct groups, fish HSC70 group and fish HSP70 group, respectively. And ScHSP70a and ScHSP70b identified in the present study were clustered into the fish HSP70 group, revealing that the ScHSP70a and ScHSP70b were HSP70s rather than HSC70s. In addition, ScHSP70a and ScHSP70b showed distant relationship to the mammalian MHC-linked HSP70 group and mammalian HSP70B′ group.

Expression of *ScHSP70a* and *ScHSP70b* during embryonic developmental stages

The mRNA expression levels of *ScHSP70a* and *ScHSP70b* were detected throughout the embryonic developmental stages from fertilized eggs to 1-day post-hatched larvae

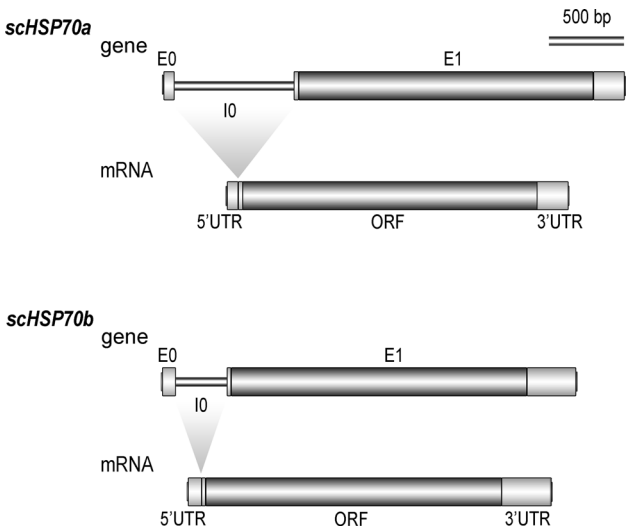
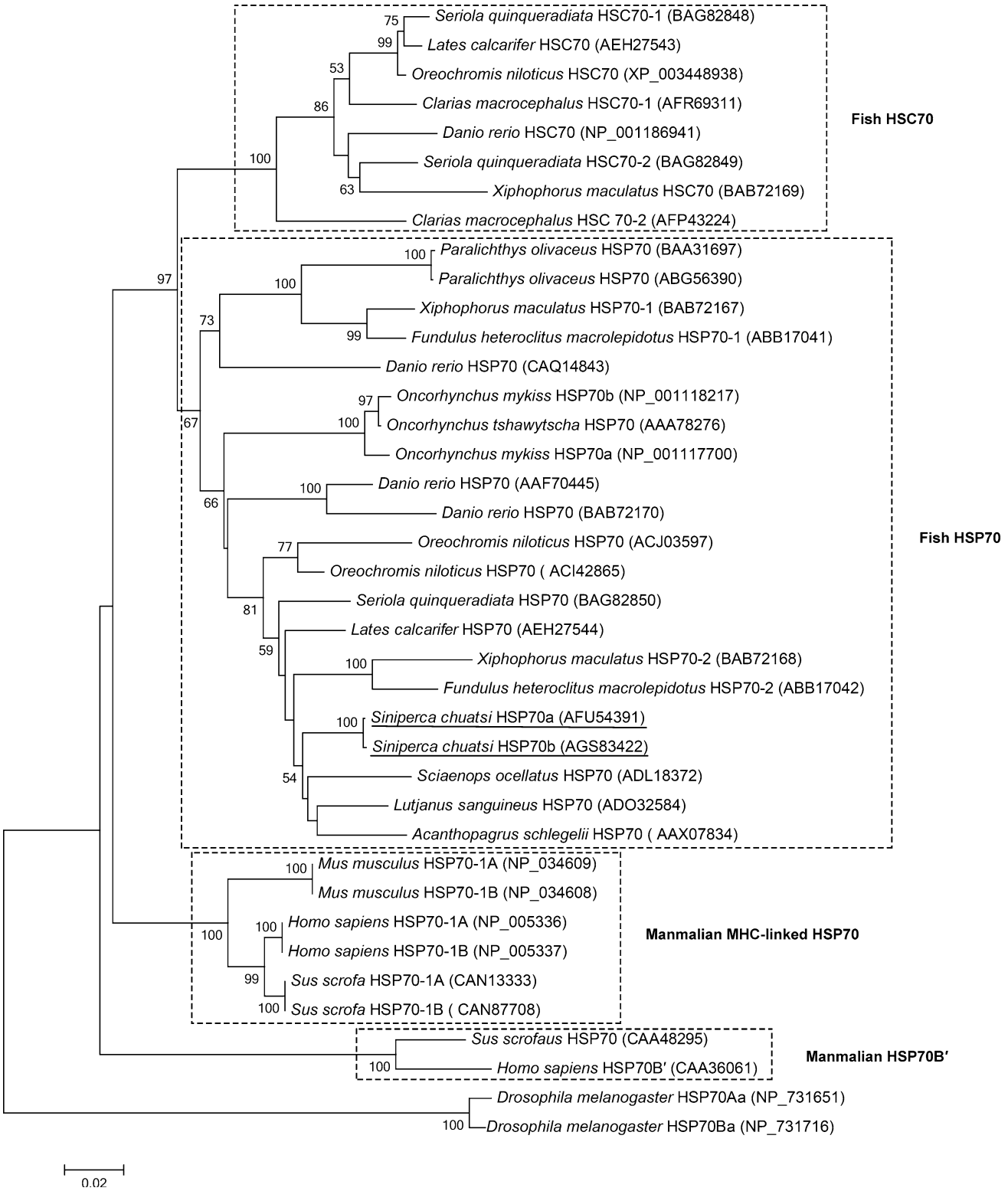


Fig. 3 Genomic structures of *ScHSP70a* and *ScHSP70b*. *Wide boxes* represent exons, and *narrow boxes* indicate introns. The ORFs are indicated by *dark gray boxes*, and the UTRs are indicated by *light gray boxes*. *Boxes* between *wide* and *narrow* represent the terminus region in the UTRs of the cDNAs which were not amplified in the genomic DNA. *E* Exon, *I* intron, *UTR* untranslated regions


Seriola quinqueradiata HSC70-1 (BAG82848)
Lates calcarifer HSC70 (AEH27543)
Oreochromis niloticus HSC70 (XP_003448938)
Clarias macrocephalus HSC70-1 (AFR69311)
Danio rerio HSC70 (NP_001186941)
Seriola quinqueradiata HSC70-2 (BAG82849)
Xiphophorus maculatus HSC70 (BAB72169)
Clarias macrocephalus HSC 70-2 (AFP43224)
Fish HSC70
Paralichthys olivaceus HSP70 (BAA31697)
Paralichthys olivaceus HSP70 (ABG56390)
Xiphophorus maculatus HSP70-1 (BAB72167)
Fundulus heteroclitus macrolepidotus HSP70-1 (ABB17041)
Danio rerio HSP70 (CAQ14843)
Oncorhynchus mykiss HSP70b (NP_001118217)
Oncorhynchus tshawytscha HSP70 (AAA78276)
Oncorhynchus mykiss HSP70a (NP_001117700)
Danio rerio HSP70 (AAF70445)
Danio rerio HSP70 (BAB72170)
Oreochromis niloticus HSP70 (ACJ03597)
Oreochromis niloticus HSP70 (ACI42865)
Seriola quinqueradiata HSP70 (BAG82850)
Lates calcarifer HSP70 (AEH27544)
Xiphophorus maculatus HSP70-2 (BAB72168)
Fundulus heteroclitus macrolepidotus HSP70-2 (ABB17042)
Siniperca chuatsi HSP70a (AFU54391)
Siniperca chuatsi HSP70b (AGS83422)
Sciaenops ocellatus HSP70 (ADL18372)
Lutjanus sanguineus HSP70 (ADO32584)
Acanthopagrus schlegelii HSP70 (AAX07834)
Fish HSP70
Mus musculus HSP70-1A (NP_034609)
Mus musculus HSP70-1B (NP_034608)
Homo sapiens HSP70-1A (NP_005336)
Homo sapiens HSP70-1B (NP_005337)
Sus scrofa HSP70-1A (CAN13333)
Sus scrofa HSP70-1B (CAN87708)
Manmalian MHC-linked HSP70
Sus scrofaus HSP70 (CAA48295)
Homo sapiens HSP70B′ (CAA36061)
Manmalian HSP70B′
Drosophila melanogaster HSP70Aa (NP_731651)
Drosophila melanogaster HSP70Ba (NP_731716)
0.02

◀**Fig. 4** Phylogenic tree for the HSP70 and HSC70 amino acid sequences of vertebrates. The tree was generated by MEGA 5.2 software using the neighbor-joining method, following ClustalX 2.1. *Scale bar* indicates an evolutionary distance of 0.02 amino acid substitutions per position in the sequence. Bootstrap values of greater than 50 % are indicated (1,000 replicates). The four clusters of fish HSC70, fish HSP70, mammalian MHC-linked HSP70 and mammalian HSP70B′ are *boxed*. ScHSP70a and ScHSP70b identified from *Siniperca chuatsi* in this study are *underlined*. Sequence database accession numbers in GenBank are indicated in *parentheses*. *HSC70* Heat shock cognate 70 protein, *MHC* major histocompatibility complex

(Fig. 5). During embryonic developmental stages, the expression of both genes showed high values and no significant changes from fertilized eggs to blastula. The expression level of *ScHSP70a* mRNA decreased from the stage of gastrula to tail-bud stage, significantly increased during the muscle burl stage and crystal stage, and then decreased again and was maintained at low levels until 1 dph. The mRNA expression level of *ScHSP70b* also decreased from the stage of gastrula to a low level, at which it was maintained until 1 dph except for a moderate increase at pre-hatched larvae stage.

Tissue expression of *ScHSP70a* and *ScHSP70b*

Under normal physiological conditions, transcripts of *ScHSP70a* and *ScHSP70b* were universally detected in brain, heart, gill, head kidney, liver, spleen, muscle, stomach, intestines and opisthonephros (Fig. 6) at substantially different levels. *ScHSP70a* was expressed at the highest level in the intestines, with lower levels in the head kidney and opisthonephros, and at the lowest level in muscle. *ScHSP70b* had the highest level in the brain, followed by the intestines, and was almost undetectable in the liver. In general, mRNA expression of *ScHSP70a* was higher than that of *ScHSP70b* in all tissues except for the brain in which the expression of the two *HSP70* isoforms was nearly equal.

Expression patterns of *ScHSP70a* and *ScHSP70b* after heat shock exposure

To investigate the expression patterns of *ScHSP70a* and *ScHSP70b* after heat shock exposure, the fish were exposed to three regimes of heat shock: acute, fast and gradual 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 °C [22] with an upper lethal limit of 34 °C under acute heat stress, and approximately 39 °C under fast and gradual heat shocks measured in the present study. The expression levels of *ScHSP70a* and *ScHSP70b* in heart, liver and head kidney in the time-course of acute heat shock exposure are shown in Fig. 7. The results showed that acute heat shock for 2 h at 34 °C resulted in a strong up-regulation of *ScHSP70a* by averages of 663.7-, 184.0- and 89.1-fold of that in controls in heart, liver and head kidney, respectively. Meanwhile, *ScHSP70b* expression was also dramatically increased by averages of 4,782.3-, 2,063.3- and 877.4-fold in heart, liver and head kidney, respectively. Although *ScHSP70b* showed lower basal expression levels under non-stressed conditions, its increased level was more remarkable than that of *ScHSP70a* in heart and liver following 2-h heat shock treatment. The time-course

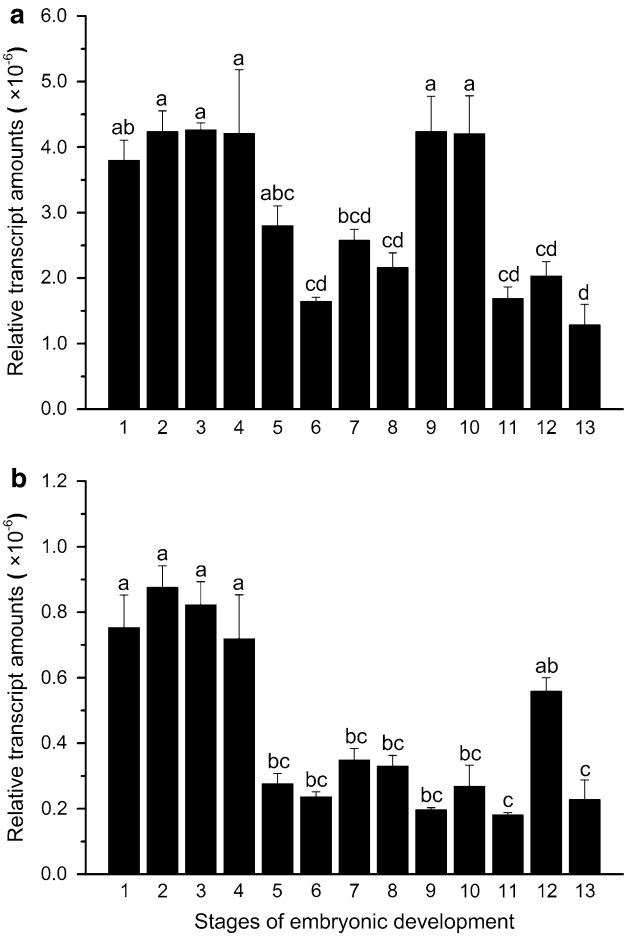


Fig. 5 Expression of *ScHSP70a* **a** and *ScHSP70b* **b** during embryonic developmental stages of *Siniperca chuatsi*. The mRNA levels were quantified by real-time qRT-PCR and normalized by *18S rRNA*. Thirty embryos or 12 larvae in each stage were collected as one sample. *Each bar* represents the mean ± SD of three samples. *Different letters* above the *bars* indicate significant difference ($P < 0.05$). *1* Fertilized eggs, *2* 16-cell stage, *3* morula, *4* blastula, *5* gastrula, *6* closure of blastopore, *7* embryo body stage, *8* tail-bud stage (optic vesicle), *9* muscle burl stage, *10* crystal stage, *11* blood circulating stage, *12* pre-hatched larvae, *13* 1 day post-hatched larvae (dph)

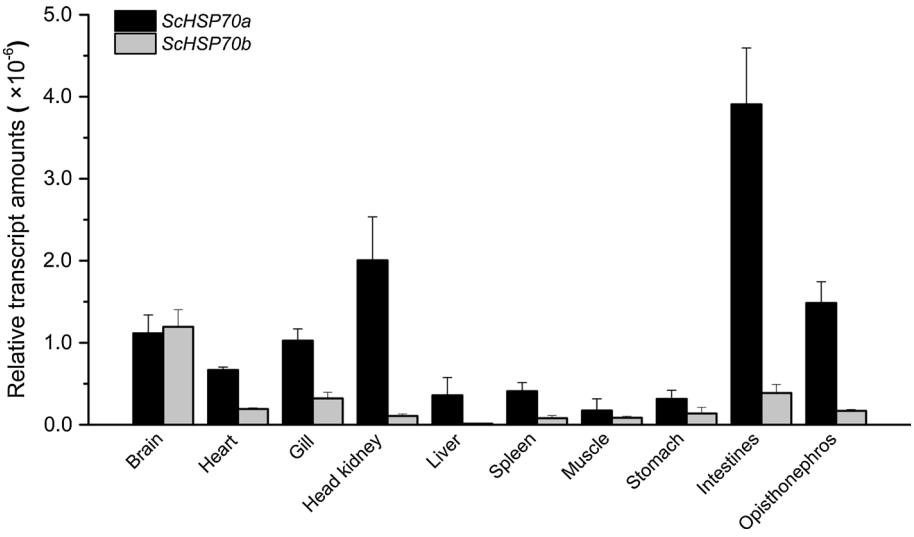


Fig. 6 Tissue distribution of relative expression levels of *ScHSP70a* and *ScHSP70b*. The relative mRNA expression was quantified by real-time qRT-PCR and normalized with *18S rRNA*. Data presented are expressed as mean ± SD, $n = 3$ fish

results revealed that the expression levels of *ScHSP70a* and *ScHSP70b* peaked after 2 h of heat shock exposure, but began to decrease towards control levels thereafter. Compared to *ScHSP70b*, it took more time for *ScHSP70a* to return its expression to the control levels.

The expression levels of *ScHSP70a* and *ScHSP70b* in heart under fast heat shock (elevating temperature rate: ~1.2 °C/h) and gradual heat shock (elevating temperature rate: ~0.2 °C/h) were also investigated. Results showed that the expression of both genes was significantly up-regulated by the two regimes of heat shock when the temperature elevated from 25 °C to 34 and 38.8 °C (Fig. 8). Under fast heat shock from 25 to 34 °C, the mRNA levels of *ScHSP70a* and *ScHSP70b* were increased by averages of 906.3- and 3,240.6-fold, respectively, which continued to be increased with the temperature elevating to 38.8 °C, by averages of 3,708.5- and 18,450.2-fold, respectively. Likewise, gradual heat shock also resulted in up-regulation of *ScHSP70a* and *ScHSP70b*, but was much lower than those under fast heat shock, by averages of 208.2- and 108.5-fold, respectively, at 34 °C and by 2,017.1- and 8,031.7-fold, respectively, at 38.8 °C.

Expression of *ScHSP70a* and *ScHSP70b* after hypoxia exposure and re-oxygenation

In order to know whether hypoxia influences the expression of *ScHSP70a* and *ScHSP70b*, their mRNA levels in heart and liver in response to hypoxia and re-oxygenation were detected and the results are shown in Fig. 9. In heart, *ScHSP70a* and *ScHSP70b* mRNA levels tended to increase after hypoxia treatment but this increase did not reach significance. Although *ScHSP70a* was not significantly induced in the heart by hypoxia, its mRNA level was increased to 2.1-fold during re-oxygenation compared with the control. Meanwhile, the re-oxygenation treatment had no effects on *ScHSP70b* mRNA expression in the heart. However, the expression of both *ScHSP70a* and *ScHSP70b* was greatly increased in liver upon 4-h acute hypoxia exposure, by 286.2- and 1,303.0-fold, respectively, and declined back to their baselines during re-oxygenation over 24 h.

Expression of *ScHSP70a* and *ScHSP70b* after *A. hydrophila* infection

The expression patterns of *ScHSP70a* and *ScHSP70b* in head kidney and spleen from 6- to 72-h post-infection with *A. hydrophila* were examined by real-time qRT-PCR, and the results are shown in Fig. 10. Since there were no significant changes in the mRNA levels of both *ScHSP70* isoforms during the 72 h after injection with PBS ($P > 0.05$), their respective average expression level was used as a control. Analysis showed that treatment with *A. hydrophila* induced *ScHSP70a* and *ScHSP70b* mRNA expression from 6- to 24-h post-infection in head kidney and spleen. During the time-course experiment, the *ScHSP70a* mRNA level was initially markedly increased and reached the highest level of 25.1-fold in the head kidney and 5.4-fold in the spleen at 6 h in comparison to control groups, and gradually decreased to the baselines thereafter; the expression of *ScHSP70b* was also significantly up-regulated and reached the highest level of 15.4- and 10.7-fold in head kidney and spleen, respectively, at 6 h post-infection compared with control groups, and then fluctuated from 6- to 48-h post-induction until returned to the baselines. In addition, the induction of

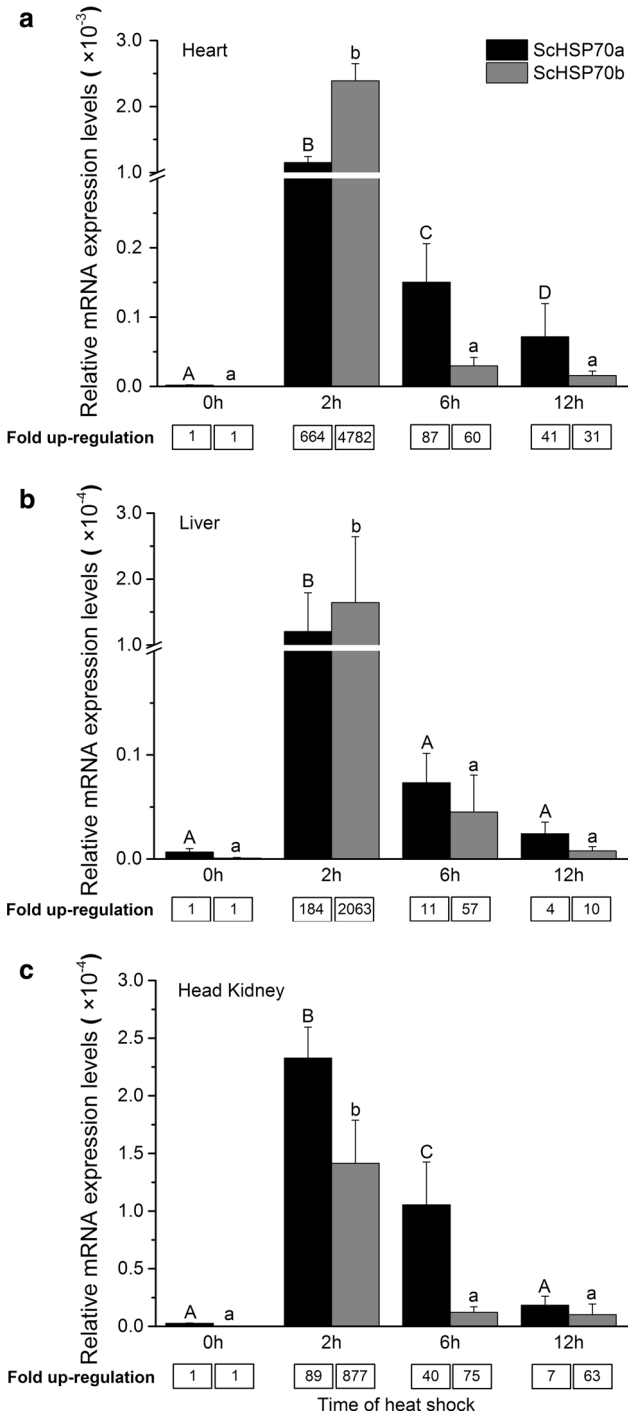


Fig. 7 Expression of *ScHSP70a* and *ScHSP70b* during acute heat shock exposure. The mRNA levels in heart (**a**), liver (**b**) and head kidney (**c**) in the time-course were measured by real-time qRT-PCR and normalized by *18S rRNA*. Fish that were maintained at 25 °C over the entire experimental period were taken as the control group. Data presented are expressed as mean ± SD ($n = 6$). *Different letters* above the *bars* represent significant difference ($P < 0.01$)

ScHSP70a mRNA was much higher than that of *ScHSP70b* at any time point post-infection in both immune tissues.

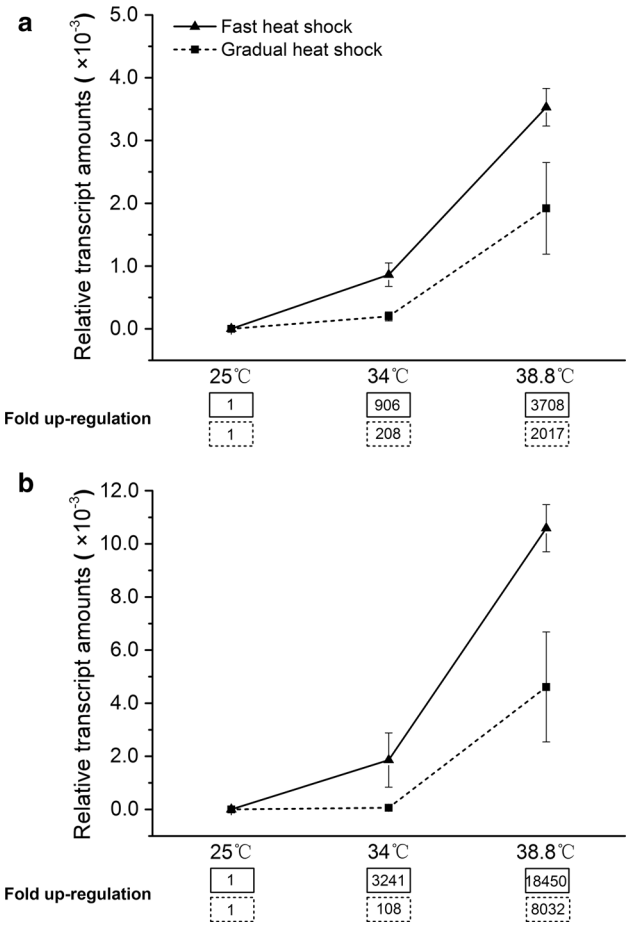


Fig. 8 Expression of *ScHSP70a* **a** and *ScHSP70b* **b** under fast and gradual heat shock regimes. The elevating temperature rate in the fast and gradual heat shock regimes were 1.2 and 0.2 °C/h, respectively. The mRNA levels were quantified by real-time qRT-PCR and normalized by *18S rRNA*. Data presented are expressed as mean ± SD ($n = 6$)

Discussion

The present study is the first report of cloning, characterization and expression analysis of two distinct *S. chuatsi* HSP70 isoforms, ScHSP70a and ScHSP70b. According to the deduced amino acid sequences, ScHSP70a and ScHSP70b share all but one (Arg-Ser at position 623) of their 639 amino acids, being more than 99 % identical. Their deduced amino acid sequences show a remarkable homology (84–95 %) to HSP70s of other species, as much as 95 % with that of *L. calcarifer*, *O. niloticus* and *L. sanguineus*. They have perfectly conserved N-terminal ATPase and peptide-binding domains, and a less conserved C-terminal domain compared with HSP70s from other species. Their C-terminal EEVD motif, non-organellar RARFEEL motif and bipartite nuclear localization signal indicate they are nuclear-cytosolic HSP70s [23]. Another striking feature of *ScHSP70a* and *ScHSP70b* genes is the absence of introns in their coding regions. Such a gene

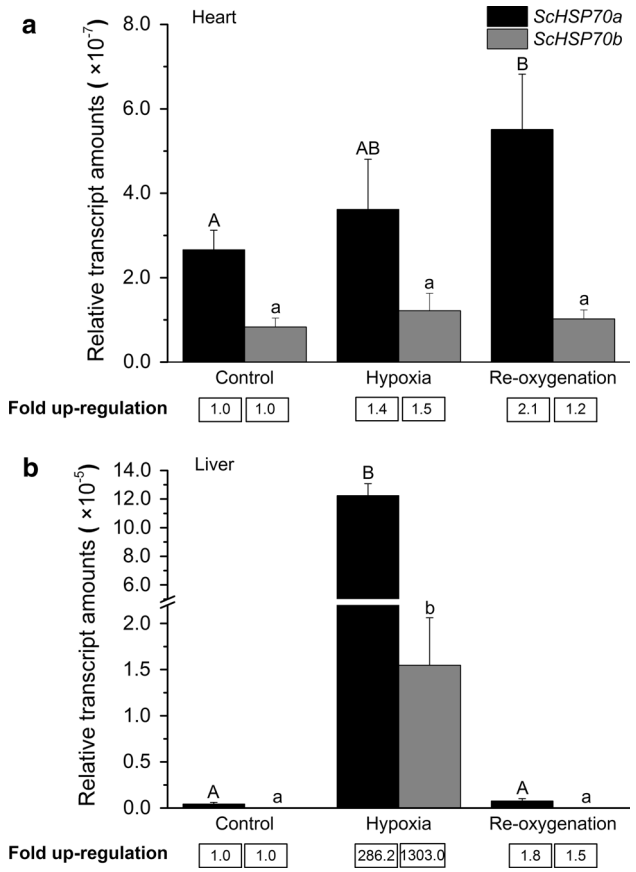


Fig. 9 Expression of *ScHSP70a* and *ScHSP70b* in response to hypoxia. The mRNA levels in heart (**a**) and liver (**b**) were measured by real-time qRT-PCR and normalized by *18S rRNA*. Fish were sampled after continuous exposure to severe hypoxia conditions (DO 0.9 ± 0.1 mg/l) for 4 h. After 4 h of hypoxia the DO levels were adjusted back to normoxic levels within 20 min and fish were sampled after 24 h of re-oxygenation. Data presented are expressed as mean ± SD ($n = 6$). *Different letters* above the *bars* represent significant difference ($P < 0.05$)

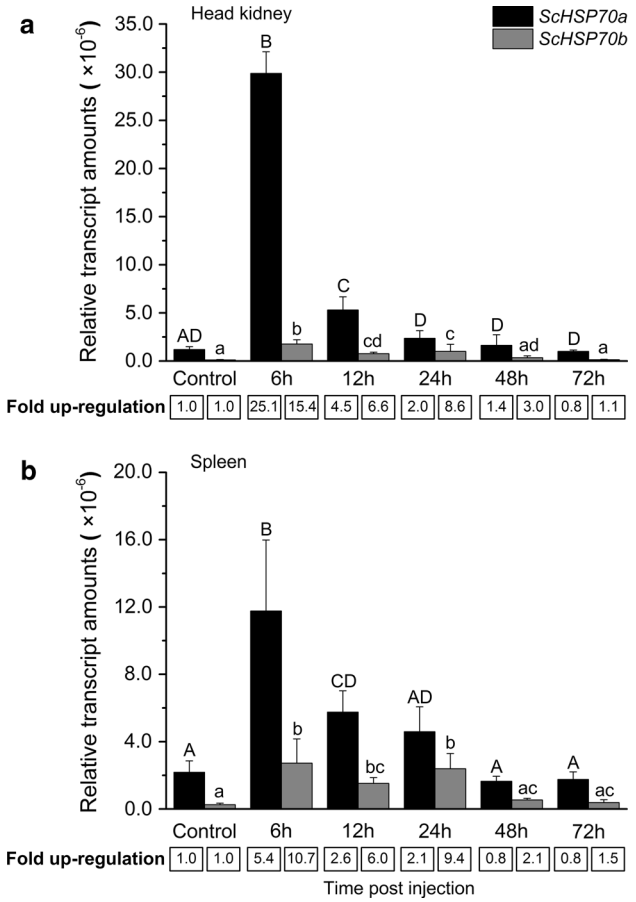


Fig. 10 Expression of *ScHSP70a* and *ScHSP70b* after *A. hydrophila* infection. The mRNA levels in head kidney (**a**) and spleen (**b**) in the time-course were quantified by real-time qRT-PCR and normalized by *18S rRNA*. Data presented are expressed as mean ± SD ($n = 6$). *Different letters* above the bars indicate significant difference among different time points ($P < 0.05$)

structure is characteristic of inducible *HSP70* genes, rather than the constitutively expressed *HSC70* genes [24]. It has been suggested that lack of introns in the *HSP70* gene allows the mRNA to be rapidly translated into a nascent protein by circumventing the block in RNA splicing upon cellular stress [25, 26]. Real-time qRT-PCR experiments revealed that *ScHSP70a* and *ScHSP70b* mRNAs are expressed at low levels in various tissues under non-stressed conditions, but are dramatically induced after heat shock. Taken together, these observations strongly suggest that ScHSP70a and ScHSP70b sequences identified in the present study correspond to the inducible nuclear-cytosolic HSP70.

It has been suggested that *HSP70* isoforms differ with respect to their expression patterns during developmental stages, in different tissues and upon stresses. For instance, human *HSP70-1a* and *HSP70-1b* genes are expressed at their highest levels in juvenile tissues while *HSP70-6* is most expressed in the neonate [7]. *HSP70-1a* and *HSP70-1b* are heat-inducible while *HSP70-1t* is not induced by heat stress and *HSP70-6* is only induced after severe stress insults [7]. In RTG-2 cells of *O. mykiss*, *HSP70a* and *HSP70b* displayed similar profiles in both the temperature-shift and time-course heat shock experiments, but at different expression levels [15]. Cadmium slightly induced the expression of *HSP70-1* in platyfish cultured tailfin cells, but it did not alter the *HSP70-2* expression [11]. To investigate the expression patterns of the *ScHSP70a* and *ScHSP70b* identified in the present study, their mRNA levels during embryonic development, in different tissues and under different stress conditions, including heat shock, hypoxia and *A. hydrophila* infection were determined by real-time qRT-PCR.

During embryonic development, *ScHSP70a* and *ScHSP70b* showed similar expression patterns in the early

embryonic developmental stages from fertilized eggs to the tail-bud stage. Their mRNAs, maintained at relatively high levels from fertilized eggs until the blastula stage, are considered as maternal origin according to the demonstration that zygotic transcription is activated until the midblastula transition (MBT) period [27]. Thereafter, their mRNA levels decreased, and were maintained at low levels until the tail-bud stage. At the later embryonic developmental stages, however, the expression patterns of *ScHSP70a* and *ScHSP70b* differed obviously, from the muscle burl stage to crystal stage in particular. The low mRNA expression level of *ScHSP70b* was maintained until 1 dph except for a moderate increase at pre-hatched larvae stage, while the expression of *ScHSP70a* was significantly increased from the muscle burl stage to crystal stage, suggesting that ScHSP70a plays a particular role during these stages. This speculation can be supported by the observation that zebrafish HSP70 is required for embryonic lens formation [28, 29].

ScHSP70a and *ScHSP70b* showed different expression patterns in tissue distribution and after exposure of heat shock, hypoxia and *A. hydrophila* infection. Both genes were expressed at low levels in all tissues tested, while in most cases *ScHSP70a* levels were much higher than *ScHSP70b* levels. Although expressed at low levels under non-stressed conditions, both *ScHSP70a* and *ScHSP70b* were strongly heat-inducible. Their mRNA levels were dramatically increased in heart, liver and head kidney after exposure to 34 °C for 2 h. The quick and high expression of *ScHSP70a* and *ScHSP70b* suggests that they are directly involved in the resistance of organisms to heat stress. In the time-course experiments, *ScHSP70a* and *ScHSP70b* revealed very similar expression patterns. Their mRNA induction levels were initially markedly increased and then decreased gradually towards their control levels following the non-lethal acute heat shock. This tendency is generally consistent with the induction pattern of *HSP70a* and *HSP70b* in RTG-2 cells of rainbow trout upon heat stress [15]. Another finding was that ScHSP70b had much higher increasing rates than ScHSP70a in all the three tissues, indicating the induction of ScHSP70b is more sensitive than that of ScHSP70a on exposure to heat shock. It is clear that transcriptional induction of *HSP70* requires the binding of activated heat shock transcription factors (HSFs) to heat shock elements (HSEs) located within the *HSP70* gene promoter regions [30, 31]. Therefore, it can be speculated that the different induction levels between *ScHSP70a* and *ScHSP70b* may be related to the characteristics of their HSEs. Meanwhile, their induction varies among tissues. Both *ScHSP70a* and *ScHSP70b* are more stongly expressed in heart than in liver and head kidney after heat shock, which may be associated with the different HSF activities in these tissues.

The expression patterns of *ScHSP70a* and *ScHSP70b* in heart under fast and gradual heat shock regimes were further investigated. Their expression levels up-regulated gradually with the increasing temperature under both temperature regimes; however, their induction rate during gradual heat shock was slower than during fast heat shock. These data indicate that the induction of *ScHSP70s* is positively correlated with the elevated temperature as well as the increasing rate of temperature.

It is well established that HSP70 protects mammalian cells against ischemic and hypoxic injury [32, 33]. However, in fish, less is known about the relationship between *HSP70* gene expression and hypoxic stress [34]. Previous reports showed that hypoxia significantly induced the expression of *HSP70* mRNA in embryos of *D. rerio* [35], as well as in gill, spleen and liver of *Sebastes schlegeli* [36]. The combined expression of HSP70/HSC70 protein in blood, brain and muscle of juvenile *O. niloticu*s was also highly induced during hypoxia [37]. These findings suggest that a particular level of HSP70 expression is required during hypoxia [34]. Similarly, mRNA expression of both *ScHSP70a* and *ScHSP70b* was also elevated in liver of *S. chuatsi* after hypoxia treatment in this study, which indicates that ScHSP70a and ScHSP70b may contribute to the resistance of *S. chuatsi* for hypoxic injury. However, no significant changes of *ScHSP70a* and *ScHSP70b* expression were observed in heart after hypoxia exposure compared to those under normoxic conditions, revealing their expression upon hypoxia is in a tissue-specific manner. Stenslokken et al. [34] demonstrated that distinct *HSP70* isoforms in *Carassius carassius* showed different expression profiles under hypoxia, which was also found in our study. The mRNA accumulation of *ScHSP70a* in liver was far greater than that of *ScHSP70b* following hypoxia exposure. In heart, the *ScHSP70a* level was significantly enhanced after re-oxygenation for 24 h, while *ScHSP70b* expression was still maintained at the control level. These differences between *ScHSP70a* and *ScHSP70b* expression under hypoxia/re-oxygenation suggest that ScHSP70a may play more roles in cytoprotection against hypoxia injury. It is documented that *HSP70-2* in human hepatocellular carcinoma (HCC) and hepatoma (HepG2) cells was strongly increased under hypoxic conditions, and *HSP70-2* up-regulation is due to the direct binding of hypoxia-inducible factor to hypoxia-responsive elements (HREs) in *HSP70-2* promoter [38]. However, whether this mechanism is true for teleost fish still needs further elucidation.

In mammals, HSP70 is demonstrated to protect the organism against pathogens not only by functioning as a molecular chaperone, but by mediating the innate immune responses [39]. Enhanced HSP70 can protect cells by inhibiting the production of pro-inflammatory cytokines and promoting the expression of anti-inflammatory

cytokines [40, 41]. HSP70 is released into extracellular compartments and functions as an endogenous "danger signal" to promote immune responses that are involved in protection of the cytoplasmic components against bacterial infection [42, 43]. Moreover, HSP70 serves as a ligand for Toll-like receptors to modulate pathogen-associated molecular pattern (PAMP)-induced Toll-like receptor (TLR) signaling, which is beneficial to stimulation of innate immunity and elimination of pathogens [39]. In fish, organisms responding to pathogens by altering *HSP70* mRNA synthesis has also been reported. Infection with *Vibrio harveyi* increased *L. sanguineus HSP70* mRNA level in the head kidney [44]. *HSP70* mRNA expression was augmented in *Sparus sarba* after a *Vibrio alginolyticus* challenge [45], as was the case for *Epinephelus coioides*, where *HSP70* mRNA expression peaked at 24 h post-infection with live *V. alginolyticus* [46]. After being challenged with *A. hydrophila*, hepatic *HSP70* mRNA expression of *Megalobrama amblycephala* Yih was significantly increased at first, reaching a peak at 6 h post-infection and then decreasing [47]. Likewise, a clear time-dependent mRNA expression pattern of *ScHSP70a* in both head kidney and spleen of *S. chuatsi* was observed in this study when infected with *A. hydrophila*. On the other hand, *ScHSP70b* showed a different expression pattern after *A. hydrophila* infection. Although *ScHSP70b* was also up-regulated at 6 h post-infection in both immune tissues, its induction fluctuated from 6- to 48-h post-induction until it returned to the control levels. The difference in the expression profiles of *ScHSP70a* and *ScHSP70b* post-infection may be affected by complicated factors that are not clear. Still, their up-regulation after *A. hydrophila* infection indeed indicates that the two ScHSP70 isoforms participate in the fish immune response during bacterial infection. Further investigation might help the development of better disease management strategies for *S. chuatsi*.

In summary, two distinct *HSP70* isoforms, *ScHSP70a* and *ScHSP70b*, were identified and characterized from the mandarin fish *S. chuatsi* for the first time. Their amino acid sequence characteristic features, DNA structures and mRNA expression patterns strongly suggest that the two *ScHSP70* isoforms correspond to the inducible nuclear-cytosolic HSP70. Furthermore, mRNA expression patterns of *ScHSP70a* and *ScHSP70b* during embryonic development and after exposure to different environmental stresses were analyzed by real-time qRT-PCR. The results showed that both genes were expressed at low levels in various tissues under normal physiological conditions, but were strongly induced by stress stimulation in a time-dependent or tissue-specific manner. These observations indicate that they participate in different ways in embryonic development and in protection against damage from high temperature, hypoxia and bacterial infection. The identification and expression analysis of the two *ScHSP70* genes have laid the foundation for study of the anti-adverse mechanism as well as possible improvement of stress tolerance and disease resistance of *S. chuatsi*.

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