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Molecular cloning and characterization of an inducible heat shock protein 70 gene in Chinese soft-shelled turtle (*Pelodiscus sinensis*)

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ABSTRACT

Heat shock protein 70 (HSP70) is a class of highly conserved proteins which is involved in essential functions as molecular chaperones and in the acquired tolerance processes. The HSP70 family of proteins is encoded by two genes, a heat shock cognate 70 (Hsc70), and an inducible heat shock protein 70 (Hsp70) gene. In this work, the cDNA of Chinese soft-shelled turtle *Pelodiscus sinensis* Hsp70 (designated *PS*-Hsp70) was cloned by the techniques of homological cloning and rapid amplification of cDNA end (RACE). The full length of *PS*-Hsp70 cDNA was 2365 bp, consisting of a 5' -terminal untranslated region (UTR) of 64 bp, a 3' -terminal UTR of 393 bp, and an open reading frame (ORF) of 1908 bp encoding a polypeptide of 635 amino acids with a theoretical molecular weight of 69.81 kDa and an estimated isoelectric point of 5.37. BLAST analysis revealed that the *PS*-Hsp70 gene shared high similarity with other Hsp70 genes. Three classical Hsp70 family signatures sequences were found in the *PS*-Hsp70 deduced amino acid sequence by InterPro analysis. The results indicated that the *PS*-Hsp70 was a member of the heat shock protein 70 family. A semi-quantitive RT-PCR method was used to analyse the tissue specific expression of *PS*-Hsp70. The spatial mRNA expression of *PS*-Hsp70 gene were constitutively in the *Pelodiscus sinensis* tissues including muscle, liver, ovary, testis, kidney, spleen, lung and heart, with the highest level in testis.

KEYWORDS

Inducible heat shock protein 70;
cDNA cloning;
mRNA expression;
Pelodiscus sinensis.

INTRODUCTION

Heat shock proteins (HSPs) are the most abundant and highly conserved multigenic superfamily which are phylogenetically conserved in all living organisms from bacteria to humans[1]. They are a family of proteins that protect organisms from environmentally induced cellular damage with multiple chaperone functions and their expression can be up-regulated by various physiological perturbations or stressors, such as high temperature, oxygen radicals, toxins and other stressors[2]. In addition, HSPs are of great importance in the modulation of the immune system, and have been reported to play important roles in antigen presentation and cross-presentation in recent years[3].

Those HSP proteins have been classified into sev-

eral families according to their apparent molecular mass, such as Hsp90 (85-90 kDa), Hsp70 (68-73 kDa), Hsp60, Hsp47 and low molecular mass Hsps (16-24 kDa)[1,4]. Of these proteins, Hsp70 has been studied extensively because of its essential roles in protein metabolism under normal and stress conditions functioning as a molecular chaperone[2,3]. Hsp70 can be induced by various chemicals and biological stresses such as heat shock, oxidative stress, heavy metals, viral and bacterial infections, crowding and inflammation[1,4-6]. The Hsp70 family of proteins is encoded by two genes, a heat shock cognate 70 (Hsc 70) gene, and an inducible heat shock protein 70 (Hsp70) gene. Geneally, Hsc 70 is constitutely expressed in unstressed cells but slightly induced upon whereas Hsp70 transcript occurs at very low levels in unstressed cells but is greatly induced upon exposure to stress[7,8].

Many cDNAs encoding Hsp70 or Hsc70 have been described in different animals species, including mollusks[9], amphibia[10-12], crustaceans[13] and fishes[7]. However, the studies on Hsp70 mRNA expression level in terrapin animals were scarce. Chang et al.[14] provided the first evidence of increased Hsp70 expression in painted turtle myocardium subjected to a 12 h forced dive, but they did not distinguish the constitutive (Hsp73) and inducible (Hsp72) isoform. Scott et al.[15] reported an increase in the expression Hsp72 but not Hsp73 in brain, heart and skeletal muscle of western painted turtles force-dived for 24 h at 17°C. Similarly, Ramaglia and Buck[16] investigated the expression of the constitutive Hsp73 and inducible Hsp72 in tissues of the anoxia-tolerant western painted turtle *Chrysemys picta bellii* in response to forced dives and following 1h recovery. Li et al.[17] identified an hsc70 gene in Chinese soft-shell turtle *Pelodiscus sinensis* for the first time. To date, in most cases where Hsp70 has been used as a biomarker, protein measurements have been used. However, there is no gene transcription information of *Pelodiscus sinensis* Hsp70 (designated *PS*-Hsp70) available, which could theoretically provide for an earlier and more sensitive method of analysis[7].

Soft-shelled turtle (*Pelodiscus sinensis*) is a commercially important aquatic reptile species because of its high nutritional and medicinal value in Asian countries including China, Japan and Korea. In recent years, the farming of this species has developed rapidly in China with a yield of more than 265,721 tons in 2010[18]. However, adverse environment conditions such as water pollution and temperature change may cause immune suppression in soft-shelled turtles, thus increasing susceptibility to disease and in turn resulting in high mortality[19]. Since the Hsps have the above multiple functions, it is believed that understanding the response of *Pelodiscus sinensis* to environmental challenge can provide us further information of anti-stress mechanism in turtles' culturing industry and help us select appropriate biomarkers to evaluate the environmental impact, and develop strategies to control diseases. The main objectives of the present study were to clone the inducible Hsp70 gene from the liver of *Pelodiscus sinensis* and to investigate the expression pattern of Hsp70 gene in the tissues.

MATERIALS AND METHODS

Turtles and samples collection

About 10 healthy adult Chinese soft-shell turtles, *Pelodiscus sinensis* (1 year old, body weight of 500 ± 20g) were obtained from a commercial turtle farm in Zhejiang, China. Turtles were raised in cylindrical aquaria (90cm Length, 70cm diameter), for 2 weeks prior to processing. There were 5 individuals in each aquarium. Water temperature was maintained at 28±0.5°C by a thermo-controlled heater. For gene cloning and mRNA expression analysis, the turtles were sacrificed and different tissues were quickly dissected, frozen in liquid nitrogen, and stored at -80°C until further analysis.

Oligonucleotide design

Oligonucleotide primers for the amplification of the *PS*-Hsp70 cDNA were designed based on the conserved sequence of the known Hsp70s such as American alligator and lizard. The primers are given in TABLE 1. Those primers used for amplification of the 5' ends of the *PS*-Hsp70 cDNA were designed from the Hsp70 subclone sequence (TABLE 1). The β-actin primers, which served as a reference for the loading amount of total RNA of the tissues, were designed based on the *Pelodiscus sinensis* β-actin sequence (GenBank accession no. AY998617.1).

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TABLE 1 : The primers used for HSP70 gene cloning and expression in *Pelodiscus sinensis*

Primer	Sequence (5'—3')	PCR objective
Hsp70 F1	CCCAAGTAATGCCCTTTTCG	cDNA homolog cloning
Hsp70 R1	CCCAAGTAATGCCCTTTTCG	
3'RACE1	GAGACTTACAAGGCGGAGGATGA	3'RACE
3'RACE2	GCAGACCGTAGAGGACGAGAAG	
5'RACE1	TCGATACCCAAGGACAGAGGAGT	5'RACE
5'RACE2	TGCACGTTCTCAGACTTGTCCC	
Hsp70F2	GACAAGAGCACGGGTAAAGA	RT-PCR
Hsp70R2	CTGACCACCTCCTGGCATTTG	
β-actin F	AACTGGGATGACATGGAGAAGA	
β-actin R	AACATGATCTGGGTCATCTT	

Total RNA isolation and synthesis of the cDNA first strand

Total RNA was isolated from the examined tissues (weight 50 mg) of the turtles using TRIzol reagent (Invitrogen, CA, USA) following the protocol of the manufacturer, and resuspended in DEPC-treated water and stored at –80°C. RNA concentration and quality were assessed by spectrophotometry and 1% agarose gel electrophoresis. The cDNA was synthesized from 2 μg of total RNA by M-MLV reverse transcriptase (TaKaRa Biotechnology, Dalian) at 42°C for 1 h with Oligo dT primer following the protocol of the manufacturer. The cDNA was used as the template for PCR reactions in gene cloning and expression analysis.

Gene cloning and sequencing

Initially, PCR was performed using the cDNA prepared above as template, with the degenerated primers of Hsp70 F1 and Hsp70 R1 (TABLE 1) designed according to the conserved regions of other known Hsp70 gene sequences in order to obtain the partial fragment of Hsp70 gene from the turtle. The obtained PCR products were separated by 1.2% agarose gel electrophoresis, and then purified by PCR purification kit. The purified PCR product was ligated with the pMD18-T vector (TaKaRa Biotechnology, Dalian), and transformed into the competent *Escherichia coli* cells. The positive clones were sent for sequenceing. Sequences generated were analyzed for similarity with other known sequences using the BLAST programs (http://www.ncbi.nim.nih.gov/).

3' and 5' rapid amplification of cDNA end

Rapid amplification of cDNA end (RACE) technique was used to extend the cDNA end of Hsp70 sequence including the 3'- and 5'-UTR in accordance to the procedures of 3'-Full RACE Core Set and 5'-Full RACE Kit provided by the manufacturer (TaKaRa Biotechnology, Dalian), using gene-specific primers shown in TABLE 1.

For 3' RACE, 2 μL of RT product was then amplified by PCR with nest forward primer (3' RACE1 and 3' RACE2) of the obtained partial sequence of the Chinese soft-shell turtle and adaptor primer (TABLE 1). After an initial 3 min denaturation at 94 °C, 30 cycles were performed as follows: 30 s denaturation at 94 °C, 30 s annealing at 55°C and 2 min elongation at 72 °C followed by a final 10 min at 72 °C. The obtained PCR products were separated on 1% agarose gel, and then purified. The purified PCR product was ligated using TaKaRa DNA Ligation Kit (TaKaRa Biotechnology, Dalian), and transformed into E. coli competent cells JM109. The recombinants were identified through blue-white colour selection and screened with M13 primers. The positive clones were then sequenced.

For 5' RACE, the first strand cDNA obtained was tailed with poly (C) at the 50 ends using terminal deoxynucleotidyl transferase. Outer PCR was performed with primer 5' RACE1 and Outer Primer. The

PCR temperature profile was initial 3 min denaturation at 94 °C, 30 amplification cycles were performed as follows: 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 2 min elongation at 72°Cÿthen a final elongation at 72 ÚC for 10 min. Inner PCR was performed with primer 5' RACE2 and Inner Primer. The PCR were performed under hot-start condition (94 °C, 3min) with *TaKaRa LA Taq* (TaKaRa) for 30 cycles of 94°C (30 s), 60°C (30 s), and 72°C (2 min), and then 10min at 72°C. Finally, the products were gel-purified, cloned and sequenced as for 3' RACE.

Sequence and phylogenetic analysis

The similarity analysis of nucleotide and protein sequence was carried out by using BLAST program at NCBI (http://ncbi.nlm.nih.gov/BLAST). Multiple sequences alignments were performed using the CLUSTAL W program at the European Bioinformatics Institute (http://www.ebi.ac.uk). The motif sequences were searched using the InterPro software. A phylogenetic tree was computed using MEGA4 software according to the neighbourjoining (NJ) method based on the sequences of *PS*-Hsp70 and other known Hsp70 sequences.

Semi-quantitative RT-PCR analysis

The normal mRNA expression levels of *PS*-Hsp70 gene in different tissues were measured by semi-quantitative RT-PCR. Three turtles were sacrificed and total RNA was isolated as described above, from various tissues including muscle, liver, ovary, testis, kidney, spleen, lung and heart. The same amount of total RNAs (100 ng) from each tissue was reverse transcribed to first-strand cDNA. Two *PS*-Hsp70 gene-specific primers Hsp70F2 and Hsp70R2 were used. Two turtle β-actin primers F and R were used to amplify a fragment as an internal reference to calibrate the cDNA template for corresponding samples. PCR was conducted in a Mastercycler pro S (Eppendorf, Germany) in a 20 µl reaction volume containing 2 µl of 10×PCR buffer (plus $MgCl_2$), 1.6 µl of dNTP (2.5 mmol l^{-1}), 1µl of each primer (10 pmol μl^{-1}), 0.2µl of *Taq* polymerase (TaKaRa, Japan), 1µl of cDNA product, and 13.2 µl of ddH_2O. The PCR conditions for *PS*-Hsp70 gene were 94°C for 5min followed by 33 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. After the final cycle, samples were incubated for a further 10min at 72 °C then held at 4°C prior to analysis. The PCR conditions for β-actin gene were 94°C for 3min followed by 30 cycles of 94°C for 30, 55°C for 30 s, 72°C for 1 min, and then an additional extension at 72 °C for 5 min. The PCR products were revealed by 2.5% agarose gel electrophoresis. The gels were developed by ethidium bromide (EtBr) staining and scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories Inc., USA). The data obtained from RT-PCR analysis for the expression of *PS*-Hsp70 were subjected to one-way analysis of variance using the General Linear Model (GLM) Procedure of SAS. Significant difference among groups was shown as $P<0.05$.

RESULTS

cDNA cloning and sequencing of *PS*-Hsp70

The PCR product amplified by the degenerated primers was about 684bp, and its nucleotide sequence was significantly similar to other known Hsp70s. *PS*-Hsp70 gene-specific primers 3'RACE1 and 3'RACE2 were designed to clone the 3end of *PS*-Hsp70 cDNA, and a fragment of 519 bp was amplified using 3' RACE technique. By 5'RACE-PCR with specific primers of 5'RACE1 and 5'RACE2, an 1150 bp fragment was amplified. A 2365 bp nucleotide sequence representing the complete cDNA sequence of *PS*-Hsp70 gene was obtained by cluter analysis of the above sequences. The complete cDNA of the *PS*-Hsp70 gene was deposited in GenBank under accession No. JN582024.

Characterization of *PS*-Hsp70

The sequence of *PS*-Hsp70 consisted of 2365 bp including a 5'-terminal untranslated region (UTR) of 64 bp, a 3'-terminal UTR of 393 bp with a canonical polyadenylation signal sequence AATAAA and a poly (A) tail, and an open reading frame (ORF) of 1908 bp encoding a polypeptide of 635 amino acids with a theoretical molecular weight of 69.81 kDa and an estimated isoelectric point of 5.37. Three Hsp70 family signatures, signature 1 (IDLGTTYSCV), signature 2 (IFDLGGXTFDVSIL) and signature 3 (IVLVGGSTRIPKIQK) were found in the deduced *PS*-Hsp70 amino acid sequences. The putative ATP-GTP binding site motif (AEAYLG) and the putative bipartite

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nuclear localization signal (KK and RRLRT) were also observed. In addition, the cytoplasmic Hsp70 carboxyl terminal region of EEVD was also highly conserved and was included at residues 632-635 (Figure 1).

Homology analysis of *PS*-Hsp70

The BLAST program analysis showed that the nucleotide sequence of the *PS*-Hsp70 gene shared homology with other known Hsp70 genes, indicating that the cloned gene encodes Hsp70 protein. Compared to other Hsp70 genes, the nucleotide sequence had 84-88% identity. The deduced amino acid sequence of the Chinese soft-shell turtle *PH*-Hsp70 protein shares identities of 94% with birds, 88-92% with mammals, 88%

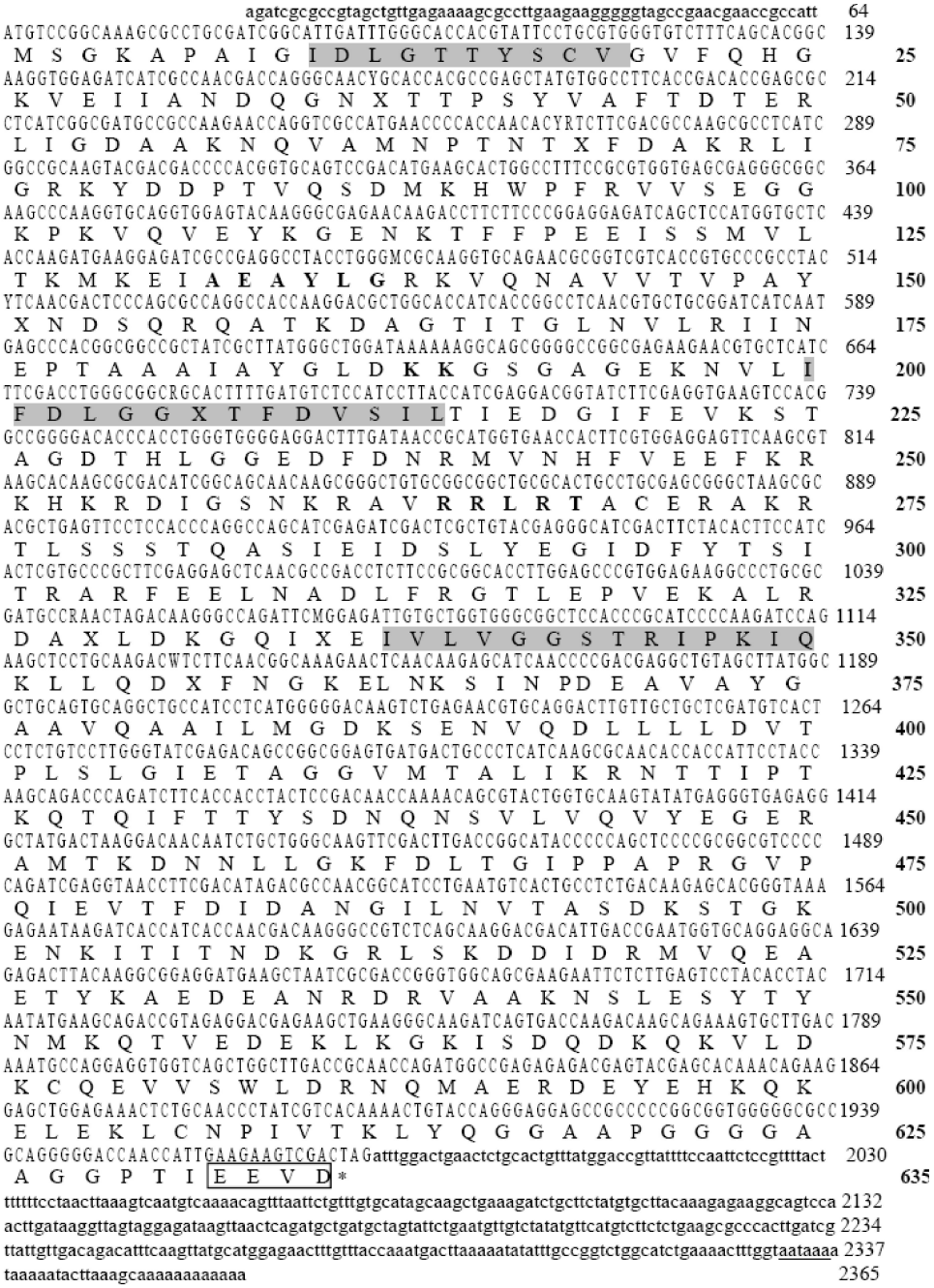

Figure 1 : Nucleotide and deduced amino acid sequences of *PS*-Hsp70 from *Pelodiscus sinensis*. In the nucleotide sequence the lower case indicates 5' and 3'UTRs and the upper case indicates the coding region. The stop codon is indicated with an asterisk and in the 3'UTR the consensus polyA signal is underlined. The characteristic Hsp70 family signatures are shown in grey; the putative ATP-GTP binding site motif and the putative bipartite nuclear localization signalare in bold; the cytoplasmic C- terminal region of EEVD is framed

with amphibians, 87% with fish and 92-94% with reptiles.

Based on the amino acid sequences of Hsp70, a phylogenic tree was constructed using the program MEGA 4.0 by the neighbor-joining method (Figure 2). In general, species from the same animal classes are clustered into groups, except the amphibians and reptiles. Reptile (Chinese soft-shell turtle *Pelodiscus sinensis* and snake *Oxyuranus scutellatus scutellatus*), which are closer to birds and mammals, divert from amphibians and reptiles in the phylogenetic tree. In amphibians, *Rana lessonae*.

Clusters together with reptiles, while Xenopus laevis diverts from reptile, forming a separate branch.

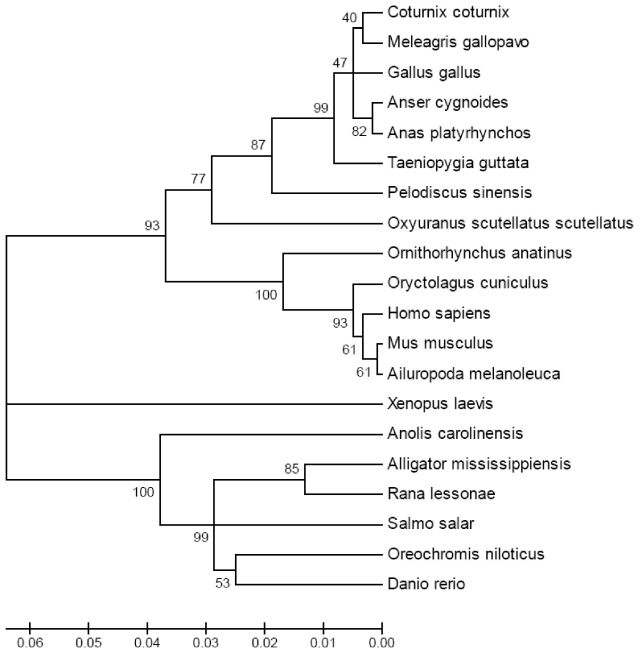

Figure 2 : A phylogenetic tree of Hsp70 family members constructed with the neighbor-joining method

Tissue specificity of *PS*-Hsp70 gene expression

A product of 262 bp of expected size was amplified from most of the examined tissues including liver, heart, kidney, muscle, ovary, testis, lung and spleen. The mRNA expression levels varied significantly among the tissues. There was a high level in liver and testis, lower in ovary, muscle, spleen and lung, while lowest in heart (Figure 3).

DISSCUSSION

In this study, we determined the complete cDNA

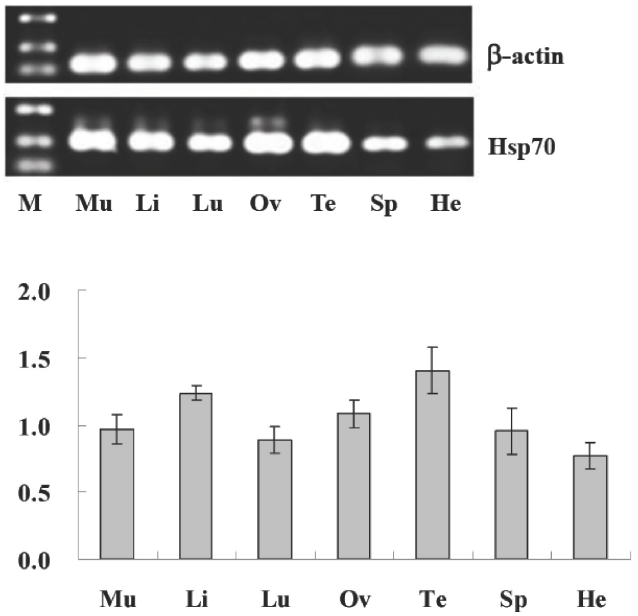

Figure 3 : Semi-quantitative RT-PCR analysis of Hsp70 expression in various tissues of *Pelodiscus sinensis*. M, DL 2000 marker; Mu, muscle; Li, liver; Lu, lung; Ov, ovary; Te, testis; Sp, spleen; He, heart. -actin, which served as a reference of the loading amount of total RNA for each tissue was also included. PCR products of cDNA were revealed by 1.5% agarose gel electrophoresis

sequence of inducible Hsp70 gene from the Chinese soft-shelled turtle (*Pelodiscus sinensis*), which is commercially cultured as a health-promoting aquatic food in Asian countries. Meanwhile, the Hsp70 was cloned for investigating the diversity of its cDNA nucleotide and deduced protein sequence in an ectothermic reptilian species for comparison with those of other vertebrate classes, because reptiles occupy a key position in evolutionary history of the vertebrates[20]. Such information is important for understanding the phylogenetic diversity and evolution of Hsp70 molecules in vertebrates.

The full-length cDNA of the *PS*-Hsp70 gene is 2365 bp, which encodes a polypeptide of 635 amino acids with several highly conserved motifs, such as three conserved Hsp70 family signatures, an ATP-GTP binding site motif and a bipartite nuclear localization signal sequence (Figure 1). The two specific motifs indicative of the *PS*-Hsp70 cytosolic localization: a non-organellar stress protein motif RARFEEL and a cytosolic Hsp70-specific motif of EEVD which is highly conserved throughout all plant and animal Hsp70 family members[9,21]. Wang et al.[9] were also suggested that the *PS*-Hsp70 was a member of the Hsp70 family and also

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was a cytosolic Hsp70 homolog. The EEVD C-terminal domain was found in nearly all the species that have been reported and is thought to mediate interactions with chaperones co-factors and is involved in binding with other co-chaperones[21]. In *PS*-Hsp70ÿno tetrapeptide GGXP repeats at the C-terminus was revealed. In contrast, the previously identified *Pelodiscus sinensis* heat shock cognate 70 (*PS*-Hsc70) gene contained two repeats of GGMP[17]. The absence of GGXP repeats in *PS*-Hsp70 in the present study was in agreement with previous studies in mollusks[9,22] and crustaceans[13]. At present, there are no studies available on the molecular characterization of both the turtle Hsp70 and turtle Hsc70 genes. According to the reports of Simoncelli et al.[23] in *Rana lessona*e, most of the differences between the Hsp70 and Hsc70 sequences occurred within the C-terminal domain. And it has also been suggested that in mammal' changes in the sequence of the carboxy terminus, including the α–helix subdomain and the tetrapeptide GGXP repeats, could be partially responsible for the functional differences observed between inducible and constitutive HSP70 members[24]. Such structural variations of Hsp70 and Hsc70 in *Pelodiscus sinensis* may therefore affect the expression profiles as hypothesized in other vertebrates[24] and also explain the specific functions between them.

The Hsp70 family is a group of abundantly expressed and highly conserved proteins. Multiple sequence alignment of *PS*-Hsp70 with other known Hsp70 amino acid sequences revealed that they were highly conserved. To our surprise, in the phylogenetic tree of vertebrate Hsp70 proteins, the putative amino acid sequence of *PH*-Hsp70 was closer to that of the birds than those of mammals, amphibian and reptiles. It happens that for turtle Hsc70 phylogenetic analysis, turtle is also phylogenetically close to birds[17], which indicates the close evolutionary relationship between reptiles and birds. Similar results were found with TSHβ, IgJ, and MyD88 genes[25-27] in exactly the same animal and with Hsp70 and Hsp90 genes in other reptiles[12]. The karyotypes of birds and turtle may explain this phenomenon. In the work of Matsuda et al.[28], it found that homology between the turtle and chicken chromosomes were highly conserved, with the six largest chromosomes being almost equivalent to each other.

In the present study, tissue-specific differences in levels of inducible Hsp70 were also observed. The highest expression levels were in testis not liver, the lowest in kidney. Intermediate levels were detected in liver, heart, lung, ovary, and spleen (Figure 3). Our findings are almost in agreement with those found in red claw crayfish[29] which the highest level is in testis. Interestingly, similar results were also observed in mammalian in which the highest expression level of another heat shock protein family Hsp90 is also in testis, such as rabbits[30] and pigs[31]. Besides, Jiang et al.[32] also reported that the highest expesssion level of Hsp90 in black tiger shrimp was in ovary. Therefore, the consistent high expression levels of HSPs in gonads may indicate that HSPs play a role in the sex determination in organisms. Further research is needed to investigate the expression response of *PS*-HSP70 gene to various environmental stresses and to confirm the function of HSP70 in *Pelodiscus sinensis*.

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