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Research Article

Characterization of HSP70 and HSP90 genes of tropical abalone (*Haliotis diversicolor squamata*) and their expression under salinity induced stress

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Abstract

Salinity is an environmental factor that affects abalone, *H. diversicolor squamata* stress and survival in the hatchery and grow-out area. To understand the protective mechanism of HSP70 and HSP90 under salinity stress, a completely randomized design, and one way ANOVA test were carried out with 95% confidence interval. To characterize heat shock protein genes, we used target clones and target plus clones to obtain partial length sequences of two heat stress response-related genes: (1) heat shock protein 70 (HSP70) and (2) heat shock protein 90 (HSP90). The HSP70 and HSP90 genes contain 201 bp and 302 bp which encode 38 and 87 amino acids, respectively. The results of multiple sequence alignment showed that HSP70 and HSP90 sequences were highly conserved compared to other species. Real-time polymerase chain reaction (PCR) results showed that HSP70 and HSP90 were salinity dependent and HSP70 and HSP90 gene expression was quantified by Quantitative Real-Time PCR of hemolymph and leg muscles showing 10ppt salinity shock for 12 h showing higher HSP70 and HSP90 mRNA expression levels higher than the control group at 32 ppt and decreased expression thereafter. Experimental results suggest that these two genes may play an important role in responding to environmental stress caused by decreased salinity. Thus, this study established a theoretical foundation for further in-depth study of mechanisms of protection of abalone molecules against salinity stress.

Keywords : *Haliotis diversicolor squamata*, HSP70, HSP90, Salinity stress

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Introduction

There are about 100 species of abalone worldwide, with larger abalones mostly found in temperate zones and smaller specimens usually in tropical and cold areas (NCBI, 2018; Wikipedia, 2021). Indonesia is home to two commercially valuable abalone species, *H. asinina* and *H. diversicolor squamata*. Abalone of Haliotidae family has distinct characteristics, such as a single shell with a nacreous layer on the inside and a coloured and patterned outer layer.

The shell also has a series of 12–23 breathing holes, with the first two often being closed and subsequent ones appearing hollow. The shell of *H. diversicolor squamata* is generally round and reddish with a rough surface, although the colour of the shell can be affected by the species' environment and feeding habits. The tropical abalone species *H. diversicolor squamata* is widespread along the south and east coasts of Bali and south of Java Island. In recent years, abalone has become important because of the increasing trend of shellfish production in Indonesia, including abalone, which has increased by 9.7% per year from 2020, which was 87,000m^3 to 107,000m^3 in 2022 (Directorate General of Aquaculture RI, 2022). Global warming and ocean pollution have become major challenges for aquatic organisms, including abalone in recent years. These environmental stressors can impact marine molluscs, which are often considered ideal indicators of changes in environmental quality because of their presence in coastal and estuarine areas and their ability to filter water (Pascal *et al*., 2004; Jeyachandran *et al*., 2023; Pourmozaffar *et al*., 2023). Various species of bivalves, including abalone, have demonstrated the effects of pollution on immune and stress responses (Galloway and Depledge, 2001; Boutet *et al*., 2004; Lee *et al*., 2023). Fluctuations in physical and chemical quality of water, such as changes in salinity, can significantly stress mollusks and weaken their immune systems (Gajbhiye and Khandeparker, 2017). Low-salinity stress, in particular, can affect the abalone's immune system and make it more susceptible to infection with pathogenic bacteria (Cheng *et al*., 2004; Yasa *et al*., 2020).

Heat shock proteins (HSPs) are a type of stress protein that help reduce biochemical, physiological, and histological changes that cells undergo due to environmental changes (Harsij *et al*., 2021). HSPs in eukaryotic organisms are usually categorized into six main families based on their molecular weight: small HSPs, HSP60, HSP70, HSP90, HSP100, and HSP110 (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). HSPs, such as HSP27, HSP60, HSP70, and HSP90, help cells cope with various stress conditions (Qian *et al*., 2012). They are involved in cell functions, including protein folding, aggregation, stabilization, assembly, and transport (Morimoto, 1993; Sharma *et al*., 2009). HSP also functions as a cellular defence mechanism, preventing protein denaturation and helping to remove

denatured proteins caused by external stress (Feder and Hofmann, 1999; Wang *et al*., 2004). Characterization and gene expression profiling studies of HSP in response to various environmental pressures have been carried out on various aquaculture species such as abalone, *H. diversicolor* (Huang *et al*., 2014), common octopus, *Octopus vulgaris* (Hong *et al*., 2015), *Haliotis discus* (Wang *et al*., 2011), tiger prawns, *Penaeus monodon* (Shi *et al*., 2016) on the other hand, studies on the adaptation of *H. diversicolor squamata* to environmental changes, especially in low-salinity culture systems, are still rarely carried out.

In this study, we sequenced HSP70 and HSP90 DNA from *H. diversicolor squamata* hemocytes, then we used real-time PCR to investigate the relative mRNA expression of HSP70 and HSP90 after different salinity shocks for abalone. In addition, very few studies have been conducted regarding response to salinity shock in tropical abalone and protective activity of HSP70 and HSP90 in *H. diversicolor squamata* during low salinity stress is largely unknown. This study is the first report on genetic characterization of HSP70 and HSP90 in *H. diversicolor squamata* abalone and its expression in response to exposure to low-salinity culture media.

Materials and methods

H. diversicolor squamata seed source

Abalone juveniles which are used in this study were obtained from the abalone hatchery unit at Sukadana Village, Kubu Sub-District, Karangasem Regency in Bali Province, Indonesia in September 2021. It takes 8 months for Abalone seeds production starting from newly hatched larvae. Larval rearing up to juvenile size of 1 cm is carried out on the rearing plate which is hung on the rearing tank with volume of $1m^3$. At this stage abalone were fed with benthic diatoms (*Nitzschia* sp.) attached to the rearing plate. After 1 cm of abalone seed and grading, seeds were transferred into floating baskets and fed with *Ulva* sp. and *Gracilaria* sp. Maintenance with this basket is carried out for 4 months until the abalone seed reaches 3-4cm in size.

H. diversicolor squamata with total length and weight of (32.97±1.83 mm and 5.13±0.83 g) respectively were collected from floating baskets and distributed in 20cm PVC pipe and lied in $1m^3$ fiberglass tank with flow through system for 1 week (temperature, 29–30°C; salinity, 32–33ppt) in laboratory. The abalone were fed every day with fresh *Gracilaria* sp. before doing the research.

Characterization of Hsp70 and Hsp90 genes

Haemolymph collection and sample preparation

The abalone haemolymph was withdrawn from the cephalic arterial sinus, accessed from the anterior at the angle between foot and head using a

microsyringe fitted with a 25-gauge needle. the hemolymph from normal and healthy abalone were sampled for extracting RNA, and stored in -80°C freezer before using.

HSPs gene expression analysis under salinity induced stress

Low salinity shock experiment

For low salinity challenge experiment, 20 cm long 3" PVC pipe which contained 30 abalone per pipe was used as experimental unit. Salinity values of 10 ppt (low salinity) and 32 ppt (control) were conducted on 4 rectangular glass aquaria (100L). In each aquarium three PVC pipes were put in as replicates. During the salinity treatment, the abalone was observed for stress response, survival, and hemolymph was taken at 0, 2, 4, 6, 12, 24 and 48h for gene expression analysis.

RNA extraction and HSP70 and HSP90 genome amplification

Total RNA was extracted from hemolymph abalone using spin column method with Quick-RNA™ MiniPrepPlus Kit (R1058) (Zymo Research). For sample preparation DNA/RNA shield™ (1X) was added to a hemolymph sample, and resuspended in a 1500 µl microtube. For every 300 µl of sample, 30 µl PK digestion buffer and 15 µl Proteinase K were added, mixed and then incubated at 55ºC until dissolved. 30 minutes, after incubation, the sample was vortexed and then centrifuged at 16.000xg for 2 minutes and transferred the aqueous supernatant into an RNase-free tube. An equal volume of RNA lysis buffer was added and well mixed.

For RNA purification, samples were lysed in RNA lysis buffer onto a Spin-Away™ filter in a yellow tube and centrifuged to remove most of the gDNA. Then it was transferred again to the green Zymo-Spin™ III CG column in a collection tube, centrifuged, and discarded. 50 µl of DNase/RNase-free water was directly added to the column matrix and centrifuge at 16,000xg. The eluted RNA was stored at -20°C. RNA integrity was assessed by electrophoresis on 1% TBE agarose gel. RNA purity was verified measuring the absorbance at 260 and 280 nm with NDD 2000 (Nano Drop Technologies, USA). The cDNA was obtained by ReverTra Ace® qPCR RT master mix with gDNA Remover (Toyobo, Japan). Firstly, RNA templates were incubated at 65°C for 5 minutes, then RNA templates were mixed with master mix I and incubated again at 35°C for 5 minutes. The mixture was then mixed again with master mix II and incubated at 37°C for 5 minutes. The mixture was incubated at 50°C for 5 minutes. Then it was heated at 98°C for 5 minutes. Finally, the mixture was left at room temperature before use.

Real-time PCR reaction was performed in a 20 µL reaction system with a mixture of 2 µL Thunderbird SYBR® qPCR Mix (Toyobo, Japan), 2 µL forward primary (10 µM), 2 µL reverse primer (10 µM), 2 µL cDNA template equivalent to total RNA total

50 ng, and 4 µL free water nuclease. Gene-specific primers were listed in Table 1. The thermal cycling condition was 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Melt curve analysis was added (65°C to 95°C, with 0.5°C / s addition). The average cycle threshold (Ct) value of each triplicate reaction was calculated using Applied Biosystem system software with β-actin gene as reference gene. The expression level of HSP70 and HSP90 mRNA were determined using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The housekeeping gene β-actin (GenBank: AM236595) was selected as internal control.

Table 1: Primer used for Real Time PCR.

Primer	Sequence (5'-3')	Gene bank Accession number	Reference
HSP90 F	CCAGGAAGAATATGCCGAGT	AM283515	Farcy *et al*., 2007
HSP90 R	CACGGAACTCCAACTGACC		
HSP70 F	CCGCTCTAGAACTAGTGGAT	AM283516	Farcy *et al*., 2007
HSP70 R	CCGCCAAGTGGGTGTCT		
β-actin F	GGGTGTGATGGTCGGTAT	AM236595	Farcy *et al*., 2007
β-actin R	AGCGAGGGCAGTGATTTC		

Results

Agarose gel electrophoresis

Genomic DNA for polymerase chain reaction (PCR) analysis was obtained using nucleic acid extraction kit II according to the manufacturer's instruction. Amplicon of HSP70 and HSP90 genes were electrophored and compared with Marker at 1% TBE agarose gel with the size of 874bp and 1813bp, respectively, as shown at Figure 1.

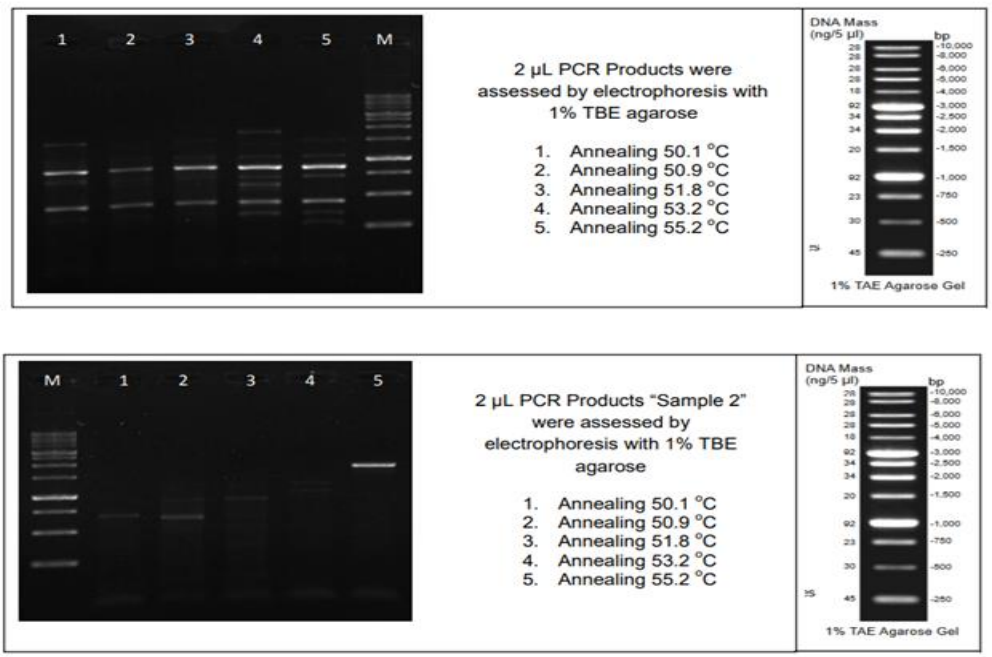


Figure 1: Amplicon of abalone hemolymph after PCR using HSP70 and HSP90 primer on 1% TBE agarose, M: marker; 1: HSP70; 2: HSP90.

Nucleotide and homology sequence of H. diversicolor squamata HSP70 and HSP90 compared to other sequences in gene bank

Nucleotide sequences and deduced amino acid sequences of HSP70 and HSP90 are shown with single letter representation below with the respective codons. BLAST results analysis showed the HSP70 sequence homolog with 24 sequences with an identity value of 87-95%. The highest similarity with *H. diversicolor*, *H. tuberculata*, *H. rufescens*, *H. discus hannai* and *H. fulgens* hsp70 was 95% and the lowest homology with *Providencia rettgeri* strain 151 was 30% similarity. The nucleotide sequence of *H. diversicolor squamata* HSP70 after BLAST analysis shared high sequence similarity with other known HSP70 (over 90%) with *H. diversicolor* (FJ812176.1), *H. tuberculata* (AM283516.1), *H. rufescens* (JN129486.1), *H. discus hannai* (DQ329856.1), and *H. fulgens* (MH221528.1).

Family signature of HSP70 and HSP90 sequences and phylogenetic analysis

Homology was done through multiple sequences alignment by CLUSTAL O (1.2.4), and the result of homologous analysis showed that three conserved amino acid motifs of HSP70 protein family had highly conserved sequences during species evolution (Fig. 2). HSP70 amino acid sequences among the species we chosen were highly homologous (higher than 80%). The amino acid sequence of HSP70 shared high similarity with other HSP70s from *H. diversicolor* (95%) and *Galeopterus variegatus* (78%).

Otherwise the HSP90 sequence was homolog with 11 sequences with a similarity value of around 11-35%. HSP90 only shared sequence similarity (over 20%) with other abalone species like *H. diversicolor* (KC161208.1) 22%, *H. midae* (JN793423.1) 22% and *H. tuberculata* (AM283515.1) 35% (Fig. 3).

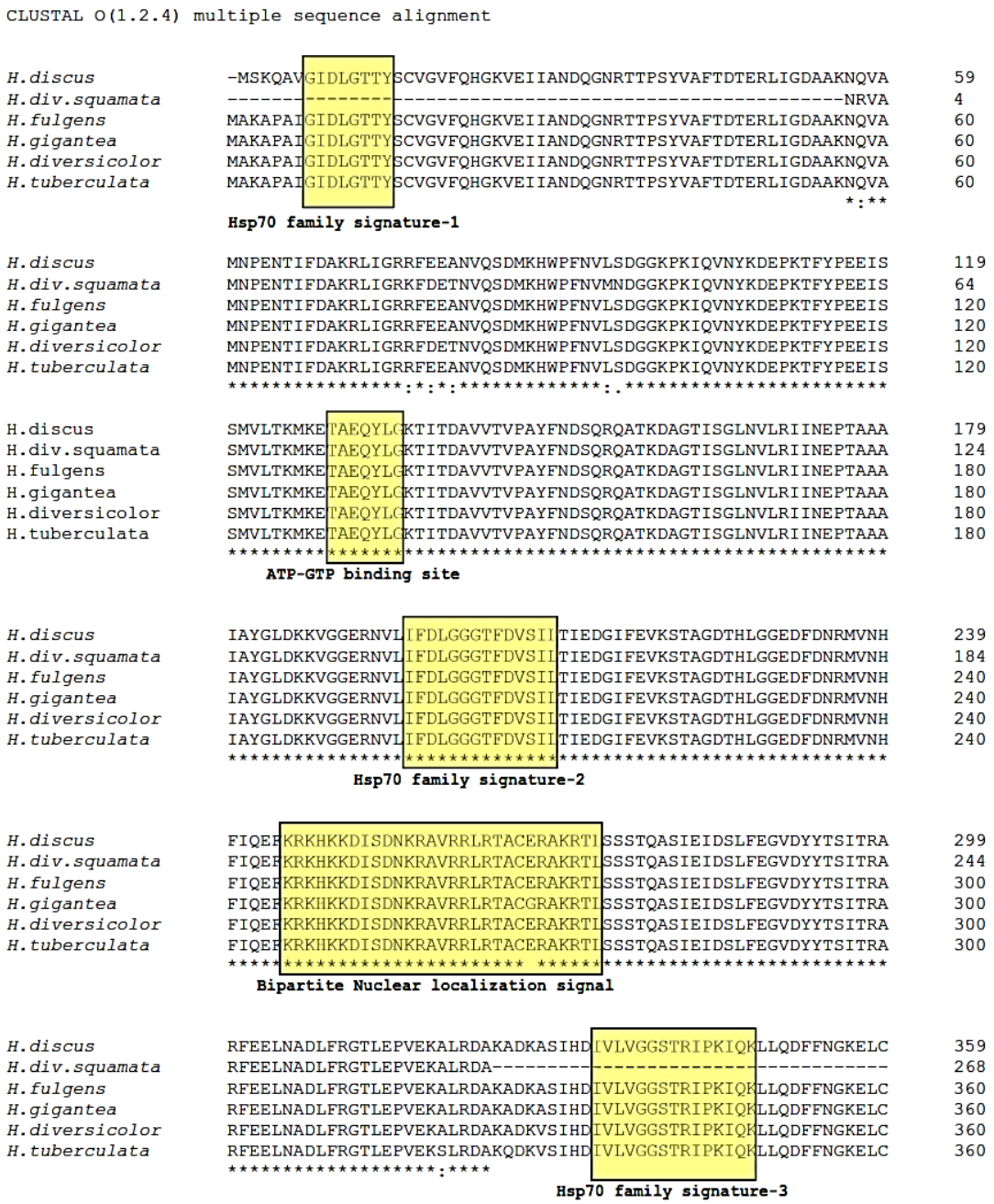
Characterization of partial-length H. diversicolor squamata HSP70

The partial length HSP70 cDNA from *H. diversicolor squamata* was obtained by 5’ and 3’ RACE-PCR. Sequence analysis of HSP70 cDNA revealed that the cDNA was 874 bp long encoding 268 amino acids, with a calculated molecular mass of 30170.04 kDa and an isoelectric point of 6.19. Deduced amino acid sequence of HSP70, includes ATP-GTP binding site, HSP70 family signature 2, and bipartite nuclear localization signal. This sequence is missing HSP70 family signature 1, HSP70 family signature 3, Glycosylation motifs 1 and 2, and EEVD consensus sequence.

Characterization of partial-length H. diversicolor squamata HSP90

The partial length HSP90 cDNA from *H. diversicolor squamata* was obtained by 5’ and 3’ RACE-PCR. HSP70 cDNA sequence analysis revealed that the cDNA was 874 bp long, and had encoded 268 amino acids, an isoelectric point of 6.19 with a predicted molecular

mass of 30170.04 kDa. Deduced amino acid sequence of HSP70, includes ATP-GTP binding site, HSP70 family signature 2, and bipartite nuclear localization signal. This sequence consisted of HSP90 family signature 1,2,3,4,5 and Lysine-rich nuclear localization signal. The sequence is only missing MEEVD consensus sequence.



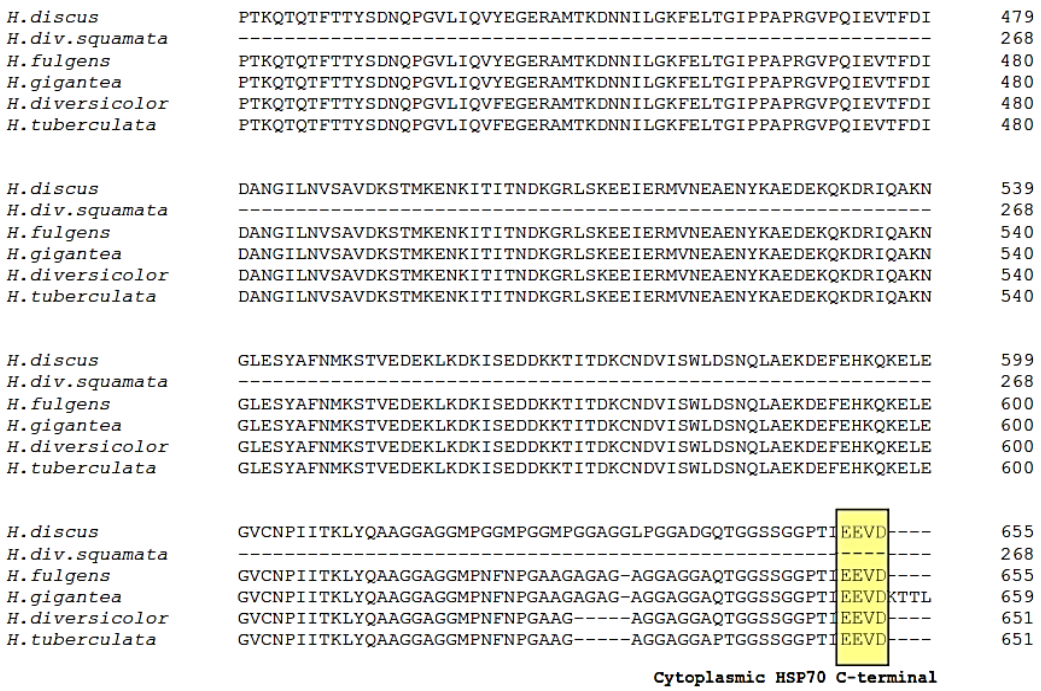


Figure 2: Multiple sequence alignment of HSP70 *H. diversicolor squamata* by CLUSTAL O (1.2.4) with different species. The characteristic motifs of the Hsp70 family are underlined as follows: three signature at positions 55-65 (IDLGTTYSCV), 123-136 (IFDLGGGTFDVSIL), and 325-340 (IVLVGGSTRIPKIQK); a putative ATP-GTP binding site at 131-137(TAEQYLG); a putative bipartite nuclear localization signal at 247-275 (KRKHKKDISDNKRAVRR); and cytoplasmic HSP70 carboxyl terminal region at 651-654(EEVD). Moreover, two glycosylation domains, (KSI) and (NVSA) were also found at residues 362-364 and 488-491.

To examine the relationships among various HSP70 and HSP90, phylogenetic trees were generated by ETE3 3.1.2 (Huerta-Cepas *et al*., 2016) as implemented on the GenomeNet (https://www.genome.jp/tools/ete/) method using different HSP70 and HSP90 family members selected from vertebrate and invertebrate species. The phylogenetic tree of HSP70 revealed that these proteins were divided into two clusters, one comprising vertebrate and mollusk proteins, and the other one containing Reptilia and bird proteins such as *Anolis carolinensis* and *Meleagris gallopavo*. In the vertebrate cluster, there were Mammalia, Reptilia, birds, amphibians, bony fishes, and insects. As expected, HSP70 was divided into the mollusk cluster and closely positioned to *Haliotis diversicolor* (Fig. 4A).

Interestingly, the phylogenic tree of HSP90 was very similar to that of HSP70 proteins were also divided into two main clusters. Mollusks, including *H. tuberculata*, *H. diversicolor squamata*, *Crassostrea virginica*, and *Chlamys* formed a sub-cluster, vertebrates including mammals, Amphibia formed the second sub-cluster, and bony fishes formed the other sub-cluster. These three sub-clusters grouped together to form a big cluster. Reptilia including *Varanus komodoensis*, and *Chelonia mydas* formed the other cluster (Fig. 4B).

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CLUSTAL O(1.2.4) multiple sequence alignment

H.midae          KKKKKKKKKKKKKKKKKKXLSIPTRKFS-EN-FPTPLMLWTRFVTNLLQ-THPNWMPTK  111
H.discus         ------------------ALTL-QPTGEEHSGERLRN-L-FFSIVPLN-FKMPEPQEAQME   39
H.tuberculata    ---------------------HEATGEQQVEERLRT-L-FFSIIPLNLVKMPEPQEAQMD   37
H.asinina        ------------------LTL-QPTGEQQVAERLRT-S-FFSITLPN-VKMPEPQEAQMD   38
H.div.squamata   ------------------------------------------------------------    0
H.diversicolor   ------------------LTL-QPTGEQLVEERLRT-L-FFSITPLN-VKMPEPQEAQMD   38

H.midae          TCRSELFLIRR--PRPLSSRIAVLV-PRLILSTTLV----------LLPSLEQKLSWRPC  158
H.discus         EAEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLDAC   99
H.tuberculata    EGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLDGN   97
H.asinina        EGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLDAS   98
H.div.squamata   ------------------LIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLDAQ   42
H.diversicolor   EGEVETFAFQAEIAQLMSLIINTFYSNKEIFLRELISNSSDALDKIRYESLTDPSKLDAN   98
                                     * .:  : *:   *:            ** :  .

                                                Hsp90 family signature-1

H.midae          RQVLTSL-LD----SLVWVSTPPTLLQRGWLSSRNTVMMNSTSGSHQ-LEGLS-PSKVQM  211
H.discus         KDLQIRIVPDKENKTLVIQDSGIGMTKADLVNNLGTI---AKSGTKAFMEALQAGADISM  156
H.tuberculata    KDMQIRIVPDKENKTLVIEDSGIGMTKADLVNNLGTI---AKSGTKAFMEALQAGADISM  154
H.asinina        KDLQIRIVPDKESKTLIIEDSGIGMTKADLVNNLGTI---AKSGTKAFMEALQAGADISM  155
H.div.squamata   KDLQIRIVPDKENKTLVIEDSGIGMTKADLVNNLGTI---AKSGTKAFMEALQAGADISM   99
H.diversicolor   KDLQIRIVPDKENKTLVIEDSGIGMTKADLVNNLGTI---AKSGTKAFMEALQAGADISM  155
                 :::   :  *    :*:  .:   : : . :.. .*:   :.**::  :*.*.  :.:.*

                                                Hsp90 family signature-2

H.midae          IPASHVELESPF----------T-RKTRPNIWRNVASRRS-RSTPSSLATPSS-WLRRRG  258
H.discus         IGQFGVGFYSAYLVAERAVVESKHNDDEQYIWESSAGGSFTIKN-------SNDPTLPRG  209
H.tuberculata    IGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIKT-------SNDPTLGRG  207
H.asinina        IGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIRS-------SNDPTLPRG  208
H.div.squamata   IGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIKS-------SNDPSLPRG  152
H.diversicolor   IGQFGVGFYSAYLVAERVVVESKHNDDEQYIWESSAGGSFTIKS-------SNDPSLPRG  208
                 *    * : * :          . .. .  **.. *.      .       *.     **

                Hsp90 family signature-3

H.midae          TRRCLMMRKRRRKKMRRRRKMR--KR----------------RRKKMRR-----------  289
H.discus         TRITLYMKEDQVEYLEERRVKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEEEEKKEDE  269
H.tuberculata    TRITLYMKEDQVEYLEERRVKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEEEKKEDEE  267
H.asinina        TRITLYMKEDQAEYLEERRIKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEDEKKEDEE  268
H.div.squamata   TRITLYMKEDQAEYLEERRIKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEDEKKEDEE  212
H.diversicolor   TRITLYMKEDQAEYLEERRVKEIVKKHSQFIGYPIKLMVEKERDKEVSDDEEDEKKEDEE  268
                 **  * *:: : : :..**  .  *:                 * *::

H.midae          RRKMRKRMRINPR-RILMRMRMKIKAKTRRRRRSRKNTQKMKS-TRPNHCGPEMQMISPR  347
H.discus         EKKEDEENEEKPKVEDLDEDEDEDKNKD--KKKKKKIKEKYTEDEELNKTKPLW---TRN  324
H.tuberculata    KKDDEEESEDKPKVEDLDEDEDEDKSKD--KKKKKKIKEKYTEDEELNKTKPLW---TRN  322
H.asinina        KKEDEEENEDKPKVEDLDEDEDEDKSKD--KKKKKKIKEKYTEDEELNKTKPLW---TRN  323
H.div.squamata   KKEDEEENEDKPKVEDLDEDEDEDKSKD--KKKKKKIKEKYTEDEELNKTKPLW---TRN  267
H.diversicolor   KKEDEEENEDKPKVEDLDEDEDEDKSKD--KKKKKKIKEKYTEDEELNKTKPLW---TRN  323
                 .:.  :. . :*: . * . . : * *   :::.:* .:* ..  . *:  *     : .

                                        Lysine-rich nuclear localization signal

H.midae          KNMLSSTSP-QMIGRTILLSSTSP-----------WKASLEFRALLFLPKRAPFDMFENK  395
H.discus         ADDI-----TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENK  379
H.tuberculata    ADDI-----TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENK  377
H.asinina        ADDI-----TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENK  378
H.div.squamata   ADDI-----TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENK  322
H.diversicolor   ADDI-----TQEEYAEFYKSLTNDWEDHLAVKHFSVEGQLEFRALLFLPKRAPFDMFENK  378
                  : :      *     :  * *.             :..********************
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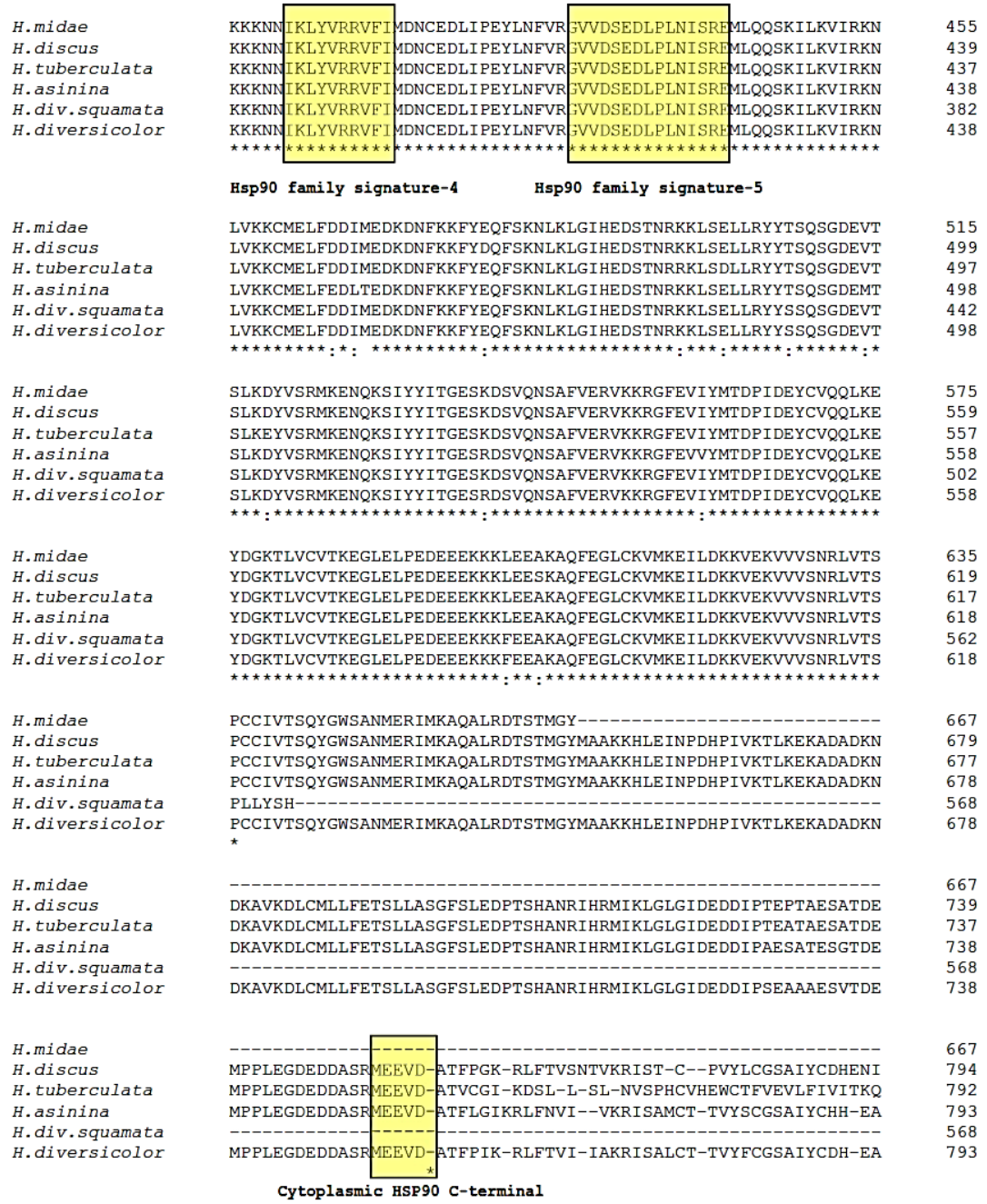


Figure 3: Multiple sequence alignment of HSP90 *H. diversicolor squamata* by CLUSTAL O (1.2.4) with different species. The characteristic motifs of the HSP90 family are underlined: five signatures at positions 55-65 (SNKEIFLRELISNSSDALKIR), 123-136(LGTIAKSGT), 325-340 (IGQFGVGFYSAYLVAR), 356-364 (IKLVYRRVF), and 382-395 (GVVDSEDLPLNISR) a putative Lycine-rich nuclear localization signal at 131-137(KDKKKKKKIKEK), and cytoplasmic HSP90 C-terminal region at 651-654 (MEEVD).

Protein folding, transport, and remodeling processes of macromolecular complexes are mediated by HSP70 and HSP90. Proteins that use the nucleotide-binding domain (NBD) of HSP70 to exchange

ADP for ATP, control the activity of these molecules. A nucleotide-binding domain (NBD), a protein substrate-binding domain (SBD), and the C-terminal domain, which is referred to as the lid for the substrate binding domain, make up the three primary functional domains of HSP70 and HSP90 proteins. The interaction of ATP and ADP at the nucleotide-binding domains causes the lid to transition from an open to a closed conformation, acting as a lid on the SBD (Fig. 5).

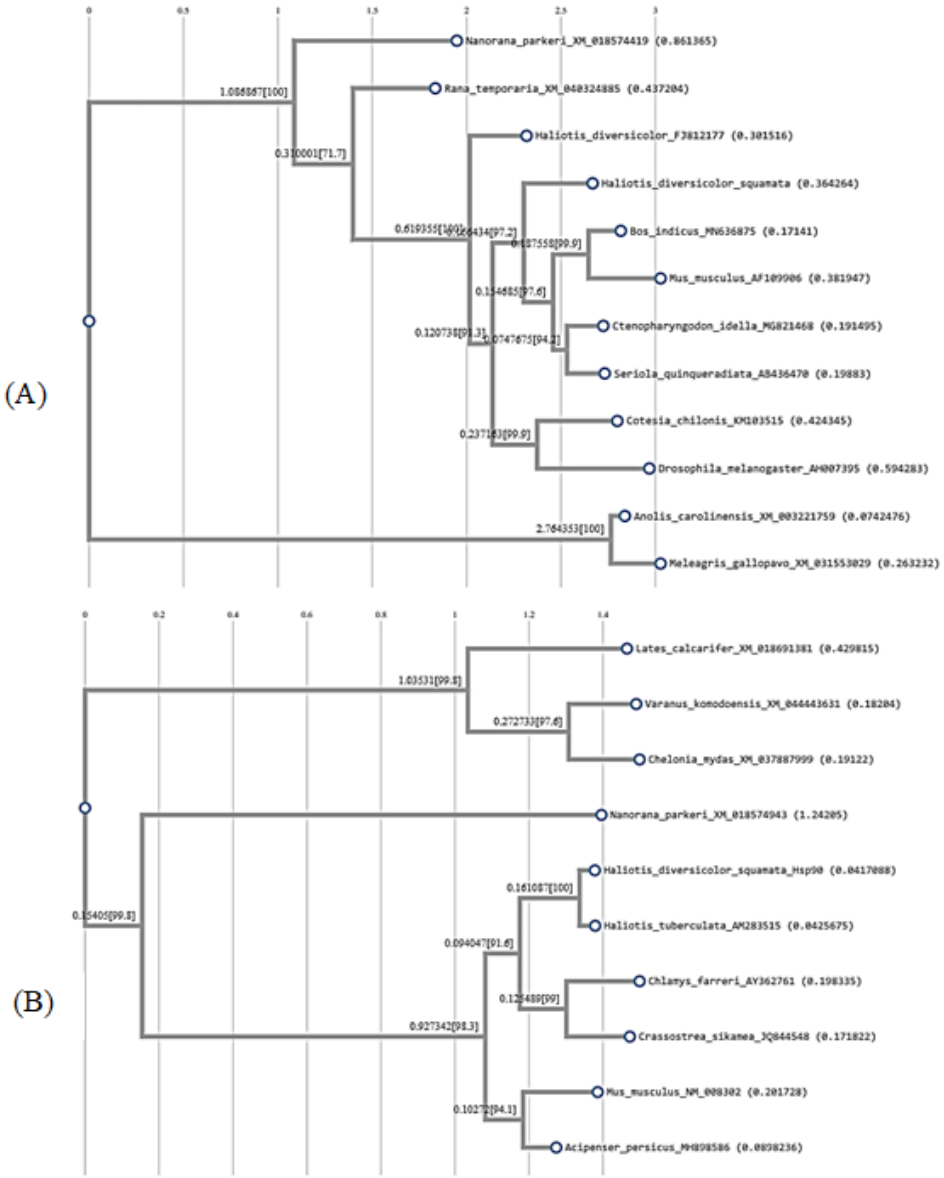


Figure 4: Phylogenetic tree of *H. diversicolor squamata* HSP70 (A) and HSP90 (B) constructed with neighbor-joining distance method.

HSP70 and HSP90 mRNA expression after low salinity challenge

The HSP70 and HSP90 expression pattern of *H. diversicolor squamata* in

hemocytes and gill from unchallenged abalone were determined by qRT-PCR. β–actin and was used as a reference gene; all primers used for real-time PCR are listed in Table 1. The temporal expression of HSP70 and HSP90 in hemocytes and gill after low salinity treatment was investigated for a better understanding after salinity treatment, the expression levels of HSP70 continued to increase and reached a peak at 12 h after 10 ppt salinity exposure.

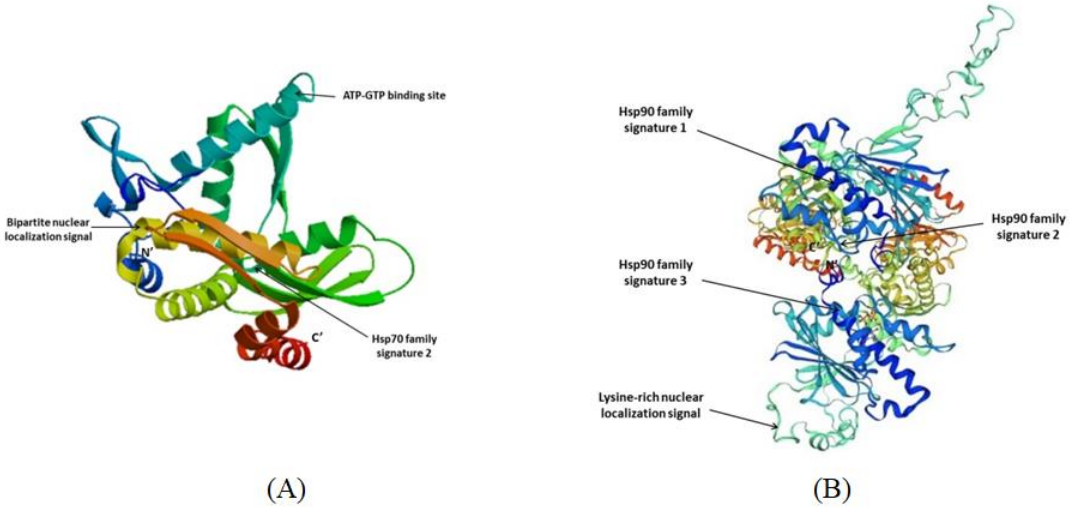
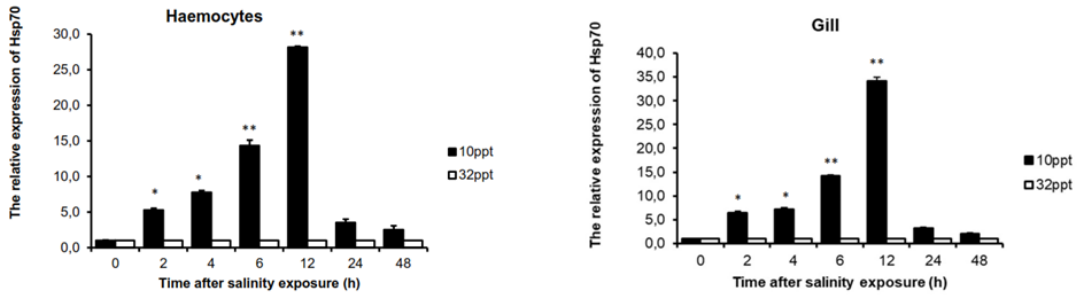
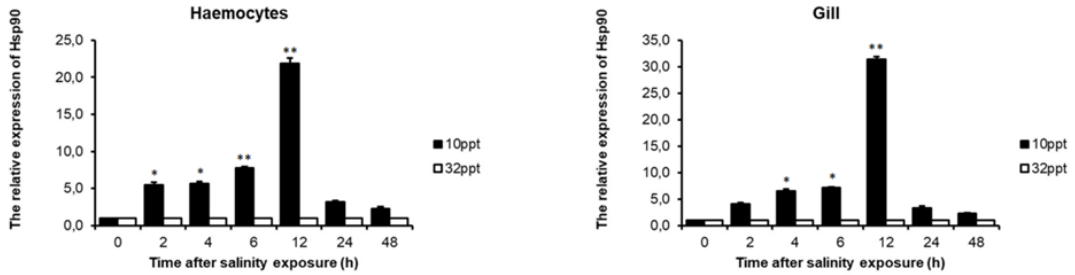


Figure 5: Three-dimensional structure of *H. diversicolor squamata* HSP70(A) and HSP90(B) from N-terminal (N') to C-terminal (C') was predicted using SWISS-Modell prediction algorithm program (https://swissmodel.expasy.org) based on similarities with other homologous sequences.

It was 28–fold in hemocytes and 35-fold in gill as much as the level observed in the control group (p<0.05) (Fig. 6A). Then the mRNA expression levels of HSP70 were dropped as time progressed at 24h until 48h with a similar value to the control. HSP90 mRNA was up-regulated in salinity challenge experiments and the expression level reached peak values (20–fold higher compared with that of the control) in hemocytes and 30-fold in 12h after 10 ppt salinity exposure (p<0.05). The expression level declined at 24h close to normal condition (Fig. 6B). Compare with the results of HSP70 and HSP90 gene expression, HSP70 was more sensitive to salinity exposure than HSP90 gene in 12h treatment at 10 ppt salinity exposure both in hemocytes and gill.



(A)



(B)

Figure 6: HSP70 of hemocytes and gill (A) and HSP90 of hemocytes and gill (B) relative expression levels during salinity challenge of the abalone. Bars with different asterisk indicate statistically significant differences (p<0.05) in the relative expression.

Discussion

In this study, the partial cDNA sequences of HSP70 and Hsp90 genes from *H. Diversicolor squamata* were cloned and showed high similarity to those from other species. A homology study revealed 24 sequences with an identity value of 87-95% as the HSP70 sequence homolog. The highest similarity was with *H. diversicolor*, *H. tuberculata*, *H. rufescens*, *H. discus hannai*, and *H. fulgens* HSP70 with a value of 95% and the lowest homology was with *Providencia rettgery* strain 151 with 30% similarity. Otherwise HSP90 sequence was homolog with 11 sequences with similarity value of around 11-35%. HSP90 only shared sequence similarity (over 20%) with other abalone species, like *H. diversicolor* (KC161208.1) 22%, *H. midae* (JN793423.1) 22%, and *H. tuberculata* (AM283515.1) 35%.

The evolutionary relationship between, HSP70 and HSP90 was established by constructing a phylogenetic tree using 12 homologous sequences. The tree formed three distinct clades of HSP70 and HSP90 family; further, each HSP family was formed in two branches, which include invertebrates (Insecta and mollusks) and vertebrates (reptilian, amphibians, fishes, and mammals) (Fig. 4). In the tree of HSP70 and HSP90 *H. diversicolor squamata* was most closely related to *H. diversicolor* into a clade. Otherwise, both of the trees showed that reptilia were always farthest from branches and formed a separate group.

In addition to having a family signature, HSP70 also had an ATP-GTP binding site that functioned for ATP binding and had a different role to play, whether it was directly involved with ATP binding or aided development of an ATP-binding cassette transporter, as can be seen from the three-dimensional structures of HSP70 and HSP90. Each dimer subunit had a connection site that the ATP molecule attached to, demonstrating that ATP was nearby both subunits during catalysis. Walker A motif residues are the two binding motifs that directly interact with ATP (Walker *et al*., 1982). Additionally, the carboxyl-terminal domain controls the bipartite nuclear localization signal necessary for p53 nuclear import (Liang and Clarke, 1999). Unlike HSP90, which has 4 family signatures and also a Lysine-rich nuclear localization signal which functions to mediate the interaction between STAT Dimeric and Importin α5 (Fagerlund *et al*., 2002).

HSP70 and HSP90 are ubiquitously expressed with different expression levels under normal conditions. In this study, the mRNA expression levels of HSP70/90 were detected in hemocytes of abalone *H. diversicolor squamata*. The expression pattern of both genes exposed to low salinity challenge were almost similar. The maximum expression of HSP70 and HSP90 was observed at 12 hours after exposure to salinity stress, and decreased very rapidly and reached similar level as the control after 24 hours post exposure.

It is known that decrease in salinity affects metabolic functions and physiological parameters in aquatic animals (Bussell *et al*., 2008; Roberts *et al*., 2010; Pourmozaffar *et al*., 2019). When abalones are exposed to salinity stress, ROS are generated, which are highly impairing normal cell function and indirectly act as DNA damage signaling molecules (Zhou *et al*., 2009). HSP70 and HSP90 are common molecular chaperones involved in the folding and processing of various cellular regulators (Frydman, 2001; Sharma, *et al*., 2009). The induced increase in HSP expression levels was found to be one of the approaches protecting the organism from further damage (Li and Xiang, 2013). In our study, under low salinity stress, we found that HSP70 and HSP90 mRNA levels increased in both tested tissues (hemocytes and gill) at 12h after challenge and decreased thereafter (Fig. 6). The above results showed that the transcription rates of HSP70 and HSP90 had the same pattern in both genes. Elevated salinity stress dramatically increased the expression of HSP70 and HSP90 after 6h. Based on the current data, we suspect that, among the two known HSP genes in *H. diversicolor squamata*, HSP70 plays a more important role in protecting cells from damage due to acute salinity stress than HSP90, because its expression level was higher than HSP90 at the same salinity concentration and this may be the best candidate gene for use as a biomarker to assess salinity stress in *H. diversicolor squamata* abalone cultures.

In another mollusk, *H. discus hannai*, HSP70 expression analysis

showed that HSP70 was expressed in several organs or tissues, indicating that HSP70 was synthesized under unstressed conditions, but its levels were relatively higher in the mantle (Cheng *et al*., 2007). Similar results were found in other mollusks, such as *Ruditapes philippinarum* where they were ubiquitously expressed in four collected tissues, and the highest level of the two genes were observed in digestive gland (Liu *et al*., 2004). PuHSC70 mRNA form *Paratapes undulatus* was expressed in all tested tissues, and the highest expression level was detected in digestive gland (Wu *et al*., 2014). However, a different result was observed in *Magallana hongkongensis*, the highest HSP70 expression level was detected in muscle (Zhang *et al*., 2012).

In conclusion, the study provides insights into the expression profiles and potential roles of HSP70 and HSP90 in *H. diversicolor squamata* under salinity stress. HSP70 is suggested to play a more significant role in protecting cells from damage, and it may serve as a potential biomarker to assess salinity stress in *H. diversicolor squamata* abalone cultures.

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