

## Research Article



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

Yasa N.S.<sup>1\*</sup>; Anshory L.<sup>2</sup>; Murwantoko M.<sup>3</sup>

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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

1-Research Center for Fishery, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 47 Cibinong, Bogor Jawa Barat 16912, Indonesia.

2-Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Jl. Raya Bogor Km. 46 Cibinong, Bogor Jawa Barat 16911, Indonesia.

3-Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Jl. Flora, Bulaksumur, Yogyakarta, 55281, Indonesia.

\*Corresponding author's Email: ngur002@brin.go.id

## 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<sup>3</sup> to 107,000m<sup>3</sup> 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<sup>3</sup>. 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<sup>3</sup> 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 55°C 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  $\mu$ L 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  $\beta$ -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  $\beta$ -actin (GenBank: AM236595) was selected as internal control.

**Table 1: Primer used for Real Time PCR.**

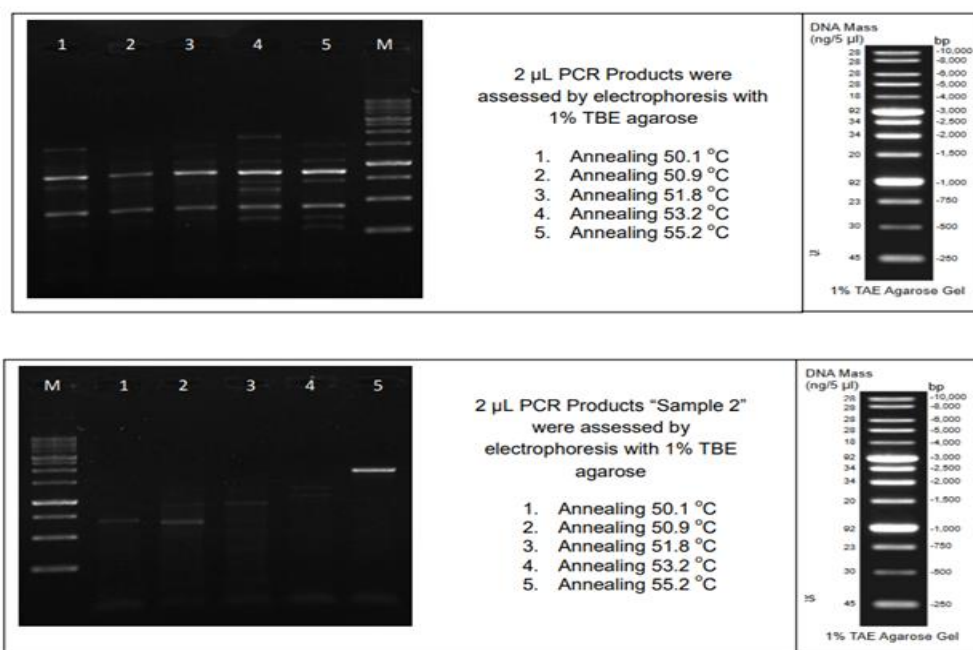
Primer	Sequence (5'-3')	Gene bank Accession number	Reference
HSP90 F	CCAGGAAGAATATGCCGAGT	AM283515	Farcy <i>et al.</i> , 2007
HSP90 R	CACGGAACCTCCAACCTGACC		
HSP70 F	CCGCTCTAGAACTAGTGGAT	AM283516	Farcy <i>et al.</i> , 2007
HSP70 R	CCGCCAAGTGGGTGTCT		
$\beta$ -actin F	GGGTGTGATGGTCGGTAT	AM236595	Farcy <i>et al.</i> , 2007
$\beta$ -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.

CLUSTAL O(1.2.4) multiple sequence alignment

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H.discus      -MSKQAVGIDLGTYYSCVGVFQHGKVEI IANDQGNRTTPSYVAFDTERLIGDAAKNQVA 59
H.div.squamata -----NRVA 4
H.fulgens     MAKAPAI GIDLGTYYSCVGVFQHGKVEI IANDQGNRTTPSYVAFDTERLIGDAAKNQVA 60
H.gigantea    MAKAPAI GIDLGTYYSCVGVFQHGKVEI IANDQGNRTTPSYVAFDTERLIGDAAKNQVA 60
H.diversicolor MAKAPAI GIDLGTYYSCVGVFQHGKVEI IANDQGNRTTPSYVAFDTERLIGDAAKNQVA 60
H.tuberculata MAKAPAI GIDLGTYYSCVGVFQHGKVEI IANDQGNRTTPSYVAFDTERLIGDAAKNQVA 60

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**Hsp70 family signature-1**

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H.discus      MNPENTIFDAKRLIGRRFEEANVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPPEEIS 119
H.div.squamata MNPENTIFDAKRLIGRRFDETNVQSDMKHWPFNVMNDGGKPKIQVNYKDEPKTFYPPEEIS 64
H.fulgens     MNPENTIFDAKRLIGRRFEEANVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPPEEIS 120
H.gigantea    MNPENTIFDAKRLIGRRFEEANVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPPEEIS 120
H.diversicolor MNPENTIFDAKRLIGRRFDETNVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPPEEIS 120
H.tuberculata MNPENTIFDAKRLIGRRFEEANVQSDMKHWPFNVLSDGGKPKIQVNYKDEPKTFYPPEEIS 120

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H.discus      SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 179
H.div.squamata SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 124
H.fulgens     SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 180
H.gigantea    SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 180
H.diversicolor SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 180
H.tuberculata SMVLTMKMETAEQYLCKTTIDAVVTVPAYFNDSQRQATKDAGTISGLNVLRI INEPTAAA 180

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**ATP-GTP binding site**

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H.discus      IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 239
H.div.squamata IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 184
H.fulgens     IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 240
H.gigantea    IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 240
H.diversicolor IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 240
H.tuberculata IAYGLDKKVGGERNVLI FDLGGGTFDVSII TIEDGIFEVKSTAGDTHLGGEDFDNRMVNH 240

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**Hsp70 family signature-2**

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H.discus      FIQEEKRKHKKDISDNKRAVRRRLTACERAKRTISSSTQASIEIDSLFEGVDYYTSITRA 299
H.div.squamata FIQEEKRKHKKDISDNKRAVRRRLTACERAKRTISSSTQASIEIDSLFEGVDYYTSITRA 244
H.fulgens     FIQEEKRKHKKDISDNKRAVRRRLTACERAKRTISSSTQASIEIDSLFEGVDYYTSITRA 300
H.gigantea    FIQEEKRKHKKDISDNKRAVRRRLTACGRAKRTISSSTQASIEIDSLFEGVDYYTSITRA 300
H.diversicolor FIQEEKRKHKKDISDNKRAVRRRLTACERAKRTISSSTQASIEIDSLFEGVDYYTSITRA 300
H.tuberculata FIQEEKRKHKKDISDNKRAVRRRLTACERAKRTISSSTQASIEIDSLFEGVDYYTSITRA 300

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**Bipartite Nuclear localization signal**

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H.discus      RFEELNADLFRGTLEPVEKALRDAKADKASIHDI VLVGGSTRIPKIQKLLQDFNKGKELC 359
H.div.squamata RFEELNADLFRGTLEPVEKALRDA----- 268
H.fulgens     RFEELNADLFRGTLEPVEKALRDAKADKASIHDI VLVGGSTRIPKIQKLLQDFNKGKELC 360
H.gigantea    RFEELNADLFRGTLEPVEKALRDAKADKASIHDI VLVGGSTRIPKIQKLLQDFNKGKELC 360
H.diversicolor RFEELNADLFRGTLEPVEKALRDAKADKASIHDI VLVGGSTRIPKIQKLLQDFNKGKELC 360
H.tuberculata RFEELNADLFRGTLEPVEKSLRDAKQDKVSIHDI VLVGGSTRIPKIQKLLQDFNKGKELC 360

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**Hsp70 family signature-3**

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H.discus      KSINPDEAVAYGAAVQAAI LHGDKSEEVQDLLLLLDVTPLSLGIETAGGVMTVLIKRNNTTI 419
H.div.squamata ----- 268
H.fulgens     KSINPDEAVAYGAAVQAAI LHGDKSEEVQDLLLLLDVTPLSLGIETAGGVMTVLIKRNNTTI 420
H.gigantea    KSINPDEAVAYGAAVQAAI LHGDKSEEVQDLLLLLDVTPLSLGIETAGGVMTVLIKRNNTTI 420
H.diversicolor KSINPDEAVAYGAAVQAAI LHGDKSEEVQDLLLLLDVTPLSLGIETAGGVMTVLIKRNNTTI 420
H.tuberculata KSINPDEAVAYGAAVQAAI LHGDKSEEVQDLLLLLDVTPLSLGIETAGGVMTVLIKRNNTTI 420

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<i>H. discus</i>	PTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNILGKFELTGIPAPRGVFPQIEVTFDI	479
<i>H. div. squamata</i>	-----	268
<i>H. fulgens</i>	PTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNILGKFELTGIPAPRGVFPQIEVTFDI	480
<i>H. gigantea</i>	PTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNILGKFELTGIPAPRGVFPQIEVTFDI	480
<i>H. diversicolor</i>	PTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNILGKFELTGIPAPRGVFPQIEVTFDI	480
<i>H. tuberculata</i>	PTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNILGKFELTGIPAPRGVFPQIEVTFDI	480
<i>H. discus</i>	DANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAENYKAEDEKQKDRIQAKN	539
<i>H. div. squamata</i>	-----	268
<i>H. fulgens</i>	DANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAENYKAEDEKQKDRIQAKN	540
<i>H. gigantea</i>	DANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAENYKAEDEKQKDRIQAKN	540
<i>H. diversicolor</i>	DANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAENYKAEDEKQKDRIQAKN	540
<i>H. tuberculata</i>	DANGILNVSAVDKSTMKENKITITNDKGRLSKEEIERMVNEAENYKAEDEKQKDRIQAKN	540
<i>H. discus</i>	GLSYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEHKQKELE	599
<i>H. div. squamata</i>	-----	268
<i>H. fulgens</i>	GLSYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEHKQKELE	600
<i>H. gigantea</i>	GLSYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEHKQKELE	600
<i>H. diversicolor</i>	GLSYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEHKQKELE	600
<i>H. tuberculata</i>	GLSYAFNMKSTVEDEKLKDKISEDDKKTITDKCNDVISWLDSNQLAEKDEFEHKQKELE	600
<i>H. discus</i>	GVCNPIITKLYQAAGGAGGMPGMPGMPGGAGGLPGGADGQTGGSSGGPTI <u>EEVD</u> ---	655
<i>H. div. squamata</i>	-----	268
<i>H. fulgens</i>	GVCNPIITKLYQAAGGAGGMPNFNPGAAGAGAG-AGGAGGAQTGGSSGGPTI <u>EEVD</u> ---	655
<i>H. gigantea</i>	GVCNPIITKLYQAAGGAGGMPNFNPGAAGAGAG-AGGAGGAQTGGSSGGPTI <u>EEVD</u> KTTL	659
<i>H. diversicolor</i>	GVCNPIITKLYQAAGGAGGMPNFNPGAAG----AGGAGGAQTGGSSGGPTI <u>EEVD</u> ---	651
<i>H. tuberculata</i>	GVCNPIITKLYQAAGGAGGMPNFNPGAAG----AGGAGGAPTGGSSGGPTI <u>EEVD</u> ---	651

## Cytoplasmic HSP70 C-terminal

**Figure 2:** Multiple sequence alignment of HSP70 *H. diversicolor* 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 (K RKHKKDISDNKRAVRR); 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 phylogenetic 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).

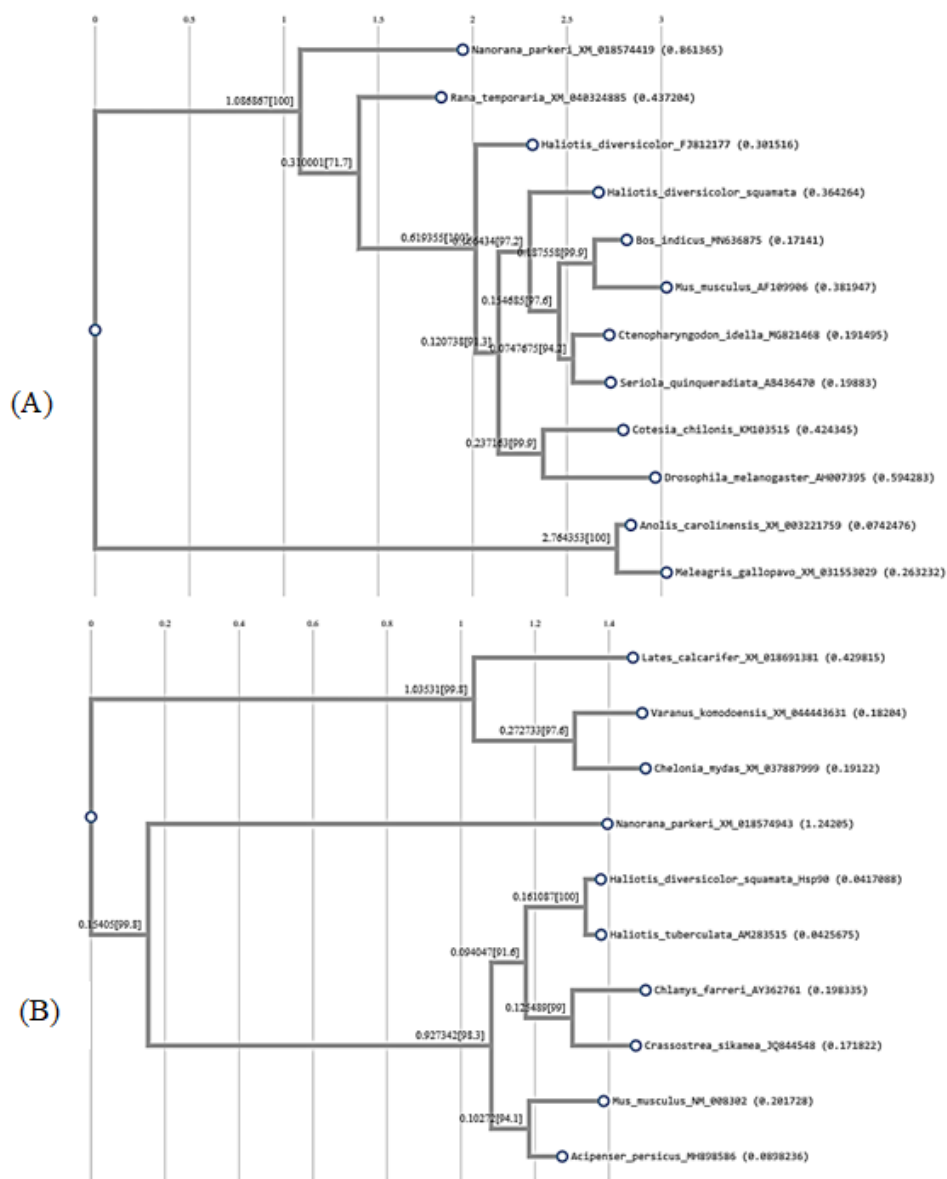






ADP for ATP, control the activity of these molecules. A nucleotide-binding domain (NBD), a protein substrate-binding domain (SBD), and the C-terminal domain, which is referred to as the lid for the substrate binding domain, make up the three primary functional domains of HSP70 and HSP90

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).



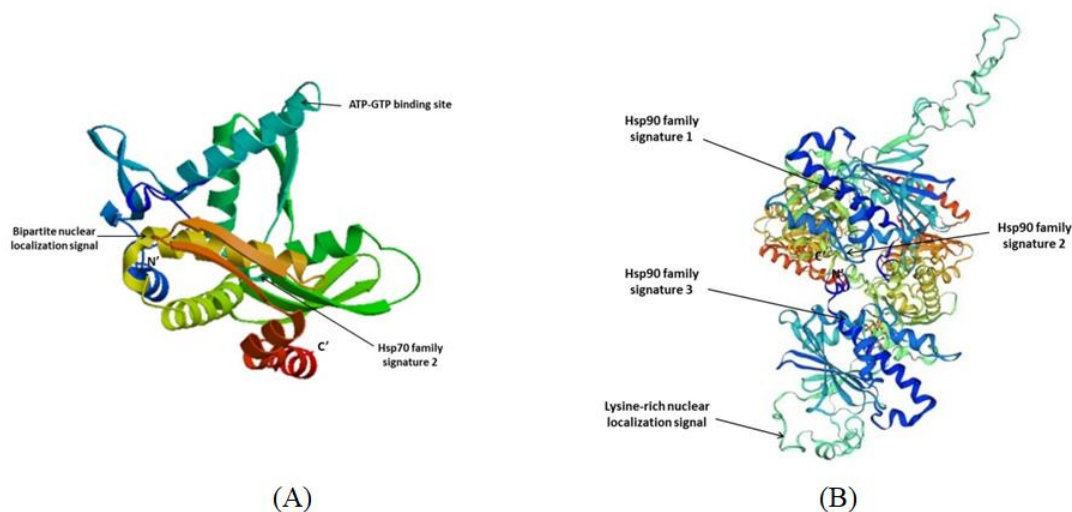
**Figure 4: Phylogenetic tree of *H. diversicolor squamata* HSP70 (A) and HSP90 (B) constructed with neighbor-joining distance method.**

*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.  $\beta$ -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

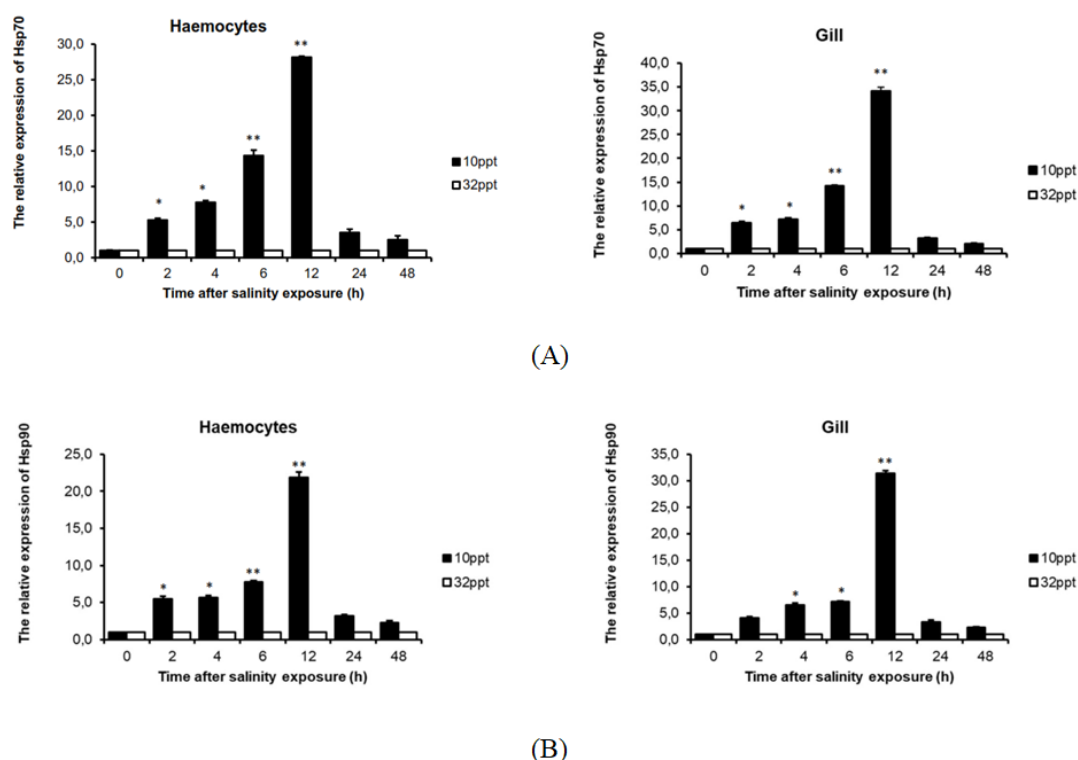
treatment was investigated for a better understanding after salinity treatment, the expression levels of HSP70 continued to increase and reached a peak at 12 h after 10 ppt salinity exposure.



**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.



**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 rettgeri* 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  $\alpha 5$  (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 from *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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#### References

- Boutet, I., Tanguy, A. and Moraga, D., 2004.** Response of the Pacific oyster *Crassostrea gigas* to hydrocarbon contamination under experimental conditions. *Gene*, 329, 147–157.  
<https://doi.org/10.1016/j.gene.2003.12.027>.
- Bussell, J.A., Gidman, E.A., Causton, D.R., Gwynn-Jones, D., Malham, S.K., Jones, M.L.M., Reynolds, B. and Seed, R., 2008.** Change in the immune response and metabolic fingerprint of the mussel, *Mytilus edulis* (Linnaeus) in response to lowered salinity and physical stress. *Journal of Experimental Marine Biology and Ecology*, 358(1), 78–85.  
<https://doi.org/10.1016/j.jembe.2008.01.018>.
- Cheng, W., Hsiao, I. S., Hsu, C.H. and Chen, J.C., 2004.** Change in water temperature on the immune response of Taiwan abalone *Haliotis diversicolor supertexta* and its susceptibility to *Vibrio parahaemolyticus*. *Fish and Shellfish Immunology*, 17(3), 235–243.  
<https://doi.org/10.1016/j.fsi.2004.03.007>.
- Cheng, P., Liu, X., Zhang, G. and He, J., 2007.** Cloning and expression analysis of a HSP70 gene from Pacific abalone (*Haliotis discus hannai*). *Fish and Shellfish Immunology*, 22(1–2), 77–87.  
<https://doi.org/10.1016/j.fsi.2006.03.014>.
- Directorate General of Aquaculture RI, 2021.** One Data. Aquaculture

- Production of Indonesian Ministry of Marine Affairs and Fisheries.
- Fagerlund, R., Melén, K., Kinnunen, L. and Julkunen, I., 2002.** Arginine/Lysine-rich nuclear localization signals mediate interactions between dimeric STATs and Importin  $\alpha 5$ . *Journal of Biological Chemistry*, 277(33), 30072–30078. <https://doi.org/10.1074/jbc.M202943200>.
- Farcy, E., Serpentine, A., Fiévet, B. and Lebel, J.M., 2007.** Identification of cDNAs encoding HSP70 and HSP90 in the abalone *Haliotis tuberculata*: Transcriptional induction in response to thermal stress in hemocyte primary culture. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 146(4), 540–550. <https://doi.org/10.1016/j.cbpb.2006.12.006>.
- Feder M.E. and Hofmann G.E., 1999.** Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61, 243–282. <https://doi.org/10.1146/annurev.physiol.61.1.243>.
- Frydman, J., 2001.** Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annual Review of Biochemistry*, 70, 603–647. <https://doi.org/10.1146/annurev.biochem.70.1.603>.
- Gajbhiye, D.S. and Khandeparker, L., 2017.** Immune response of the short neck clam *Paphia malabarica* to salinity stress using flow cytometry. *Marine Environmental Research*, 129, 14–23. <https://doi.org/10.1016/j.marenvres.2017.04.009>.
- Galloway, T.S. and Depledge, M.H., 2001.** Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology*, 10(1), 5–23. <https://doi.org/10.1023/a:1008939520263>.
- Harsij, M., Paknejad, H., Khalili, M., Jafarian, H. and Nazari, S., 2021.** Histological study and evaluation of Hsp70 gene expression in gill and liver tissues of goldfish (*Carassius auratus*) exposed to Zinc oxide nanoparticles. *Iranian Journal of Fisheries Sciences*, 20 (3), 741-760. <https://doi.org/10.22092/ijfs.2021.124052>
- Hong, J., Mao, Y., Niu, S., Sun, T. and Su, Y., 2015.** Molecular characterization and expression of HSP70, HSF and HSBP genes in *Octopus vulgaris* during thermal stress. *Acta Oceanologica Sinica*, 34(8), 62–72. <https://doi.org/10.1007/s13131-015-0708-6>.
- Huang, Y., Cai, X., Zou, Z., Wang, S., Wang, G., Wang, Y. and Zhang, Z., 2014.** Molecular cloning, characterization and expression analysis of three heat shock responsive genes from *Haliotis diversicolor*. *Fish and Shellfish Immunology*, 36(2), 590–599.

- Huerta-Cepas, J., Serra, F. and Bork, P., 2016.** ETE 3: Reconstruction, analysis, and visualization of phylogenomic data. *Molecular Biology and Evolution*, 33(6), 1635–1638.  
<https://doi.org/10.1093/molbev/msw046>.
- Jeyachandran, S., Chellapandian, H., Park, K., Kwak, I.S., 2023.** A Review on the Involvement of Heat Shock Proteins (Extrinsic Chaperones) in Response to Stress Conditions in Aquatic Organisms. *Antioxidants*, 12, 1444.  
<https://doi.org/10.3390/antiox12071444>
- Lee, H.B., H. S. Jang, Y. Dae Oh, Y. Ho Lee, H. Kyu Lim., 2023.** Analysis of the physiological responses of Pacific abalone (*Haliotis discus hannai*) to various stressors to identify a suitable stress indicator. *Journal of the World Aquaculture Society*, 1–20.  
<https://doi.org/10.1111/jwas.12969>.
- Li, F. and Xiang, J., 2013.** Recent advances in researches on the innate immunity of shrimp in China. *Developmental and Comparative Immunology*, 39(1–2), 11–26.  
<https://doi.org/10.1016/j.dci.2012.03.016>.
- Liang, S.H. and Clarke, M.F., 1999.** A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. *Journal of Biological Chemistry*, 274(46), 32699–32703.  
<https://doi.org/10.1074/jbc.274.46.32699>.
- Liu, J., Yang, W.J., Zhu, X.J., Karouna-Renier, N.K. and Rao, R.K., 2004.** Molecular cloning and expression of two HSP70 genes in the prawn, *Macrobrachium rosenbergii*. *Cell Stress Chaperones*, 9(3), 313–323.  
<https://doi.org/10.1379/CSC-40R.1>
- Livak, K.J. and Schmittgen, T.D., 2001.** Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta C_T}$  method. *Methods*, 25(4), 402–408.  
<https://doi.org/10.1016/meth.2001.1262>.
- Morimoto, R.I., 1993.** Cell in stress: transcriptional activation of heat shock genes. *Science*, 259(5100), 1409–1410.  
<https://doi.org/10.1126/science.8451637>.
- NCBI, 2018.** Collaborative Collection Management Solution. [https://www.ncbi.nlm.nih.gov/arcotools/database/museum/name/Haliotis\\_squamata](https://www.ncbi.nlm.nih.gov/arcotools/database/museum/name/Haliotis_squamata).
- Parsell, D.A. and Lindquist, S., 1993.** The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annual Review of Genetics*, 27, 437–496.  
<https://doi.org/10.1146/annurev.ge.27.1.437>.
- Pascal, H., Garello, G., Gnassia-Barelli, M., Roméo, M. and Girard, J.P., 2004.** Effect of three xenobiotic compounds on Glutathione S-Transferase in the clam *Ruditapes decussatus*. *Aquatic*

- Toxicology*, 68(1), 87–94.  
<https://doi.org/10.1016/j.aquatox.2004.03.001>.
- Pourmozaffar, S., T. Jahromi, S., Rameshi, H., Sadeghi, A., Bagheri, T., Behzadi, S., Gozari, M., Zahedi M.R. and Abrari Lazarjani, S., 2019.** The role of salinity in physiological responses of bivalves. *Reviews in Aquaculture*, 12(3), 1548-1566.  
<https://doi.org/10.1111/raq.12397>.
- Pourmozaffar, S. S. T. Jahromi, M. Gozari, H. Rameshi, M. Gozari, M. K. Pazir, B. Sarvi, M. Abolfathi, R. Nahavandi. 2023.** The first reporting of prevalence Vibrio species and expression of HSP genes in rayed pearl oyster (*Pinctada radiata*) under thermal conditions. *Fish and Shellfish Immunology* 139. 108907.  
<https://doi.org/10.1016/j.fsi.2023.108907>.
- Qian, Z., Liu, X., Wang, L., Wang, X., Li, Y., Xiang J. and Wang, P., 2012.** Gene expression profiles of four heat shock proteins in response to different acute stresses in shrimp, *Litopenaeus vannamei*. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 156(3–4), 211–220.  
<https://doi.org/10.1016/j.cbpc.2012.06.001>.
- Roberts, R.J., Agius, C., Saliba, C., Bossier, P. and Sung, Y.Y., 2010.** Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. *Journal of Fish Diseases*, 33(10), 789–801.  
<https://doi.org/10.1111/j.1365-2761.2010.01183.x>.
- Sharma, S.K., Christen, P. and Goloubinoff, P., 2009.** Disaggregating chaperones: an unfolding story. *Current Protein and Peptide Science*, (10)5, 432–446.  
<https://doi.org/10.2174/138920309789351930>.
- Shi, J., Fu, M., Zhao, C., Zhou, F., Yang, Q. and Qiu, L., 2016.** Characterization and function analysis of Hsp60 and Hsp10 under different acute stresses in black tiger shrimp, *Penaeus monodon*. *Cell Stress and Chaperones*, 21(2), 295–312. <https://doi.org/10.1007/s12192-015-0660-6>.
- Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J., 1982.** The P Loop A Common Motif in ATP and GTP Binding Protein. *EMBO J.* 1, 945-951.
- Wang, W., Vinocur, B., Shoseyov, O. and Altman, A., 2004.** Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science*, 9(5), 244–252.  
<https://doi.org/10.1016/j.tplants.2004.03.006>.
- Wang, N., Whang, I., Lee, J.S. and Lee, J., 2011.** Molecular characterization and expression analysis of a heat shock protein 90 gene from disk abalone (*Haliotis discus*). *Molecular Biology Reports*, 38(5), 3055–3060.  
<https://doi.org/10.1007/s11033-010-9972-x>.

- Wikipedia,** 2021.  
<https://en.wikipedia.org/wiki/Abalone>, 2021.
- Wu, C., Wang, J., Xu, W., Zhang, W. and Mai, K., 2014.** Dietary ascorbic acid modulates the expression profile of stress protein genes in hepatopancreas of adult Pacific abalone *Haliotis discus hannai* Ino. *Fish and Shellfish Immunology*, 41(2), 120–125. <https://doi.org/10.1016/j.fsi.2014.08.026>.
- Yasa, N.S., Murwantoko, Isnansetyo A, Handayani N.S.N., Triastutik G, Anshory L., 2020.** Physiological, biochemical and HSP70 and HSP90 gene expression profiles of tropical Abalone *Haliotis squamata* in response to *Vibrio alginolyticus* infection. *Indonesian Journal of Biotechnology*, 25(1), 12-20. <https://doi.org/10.22146/ijbiotech.51322>.
- Zhang, X.Y., Zhang, M.Z., Zheng, C.J., Liu, J. and Hu, H.J., 2012.** Identification of two hsp90 genes from the marine crab, *Portunus trituberculatus* and their specific expression profiles under different environmental conditions. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 150(4), 465–473. <https://doi.org/10.1016/j.cbpc.2009.07.002>.
- Zhou, J., Wang, W.N., Wang, A.L., He, W.Y., Zhou, Q.T., Liu, Y. and Xu, J., 2009.** Glutathione S-transferase in the white shrimp *Litopenaeus vannamei*: characterization and regulation under pH stress. *Comparative Biochemistry and Physiology, Part C: Toxicology and Pharmacology*, 150(2), 224–230. <https://doi.org/10.1016/j.cbpc.2009.04.012>.