Fish & Shellfish Immunology 58 (2016) 650–662

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular and functional characterization of caspase-8 from the big-belly seahorse (*Hippocampus abdominalis*)

Minyoung Oh [a, b], Don Anushka Sandaruwan Elvitigala [a, b, c], S.D.N.K. Bathige [a, b], Seongdo Lee [a, b], Myoung-Jin Kim [a, b, *], Jehee Lee [a, b, **]

[a] Department of Marine Life Sciences, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[b] Fish Vaccine Development Center, Jeju National University, Jeju Self-Governing Province, 63243, Republic of Korea
[c] Department of Zoology, University of Sri Jayewardenepura, Gangodawila, Nugegoda, 10250, Sri Lanka

ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form
6 October 2016
Accepted 7 October 2016
Available online 11 October 2016

Keywords:
Caspase-8
Big-belly seahorse
Immune challenge
mRNA expression

ABSTRACT

Apoptosis is a physiological process that can also participate in host immune defense mechanisms, including tumor growth suppression along with homeostasis and maturation of immune cells. Caspases are known to be involved in cellular apoptotic signaling; among them, caspase-8 plays an important role in the initiation phase of the apoptotic death cascade. In the current study, we molecularly characterized a caspase-8 homolog (designated as HaCasp-8) from *Hippocampus abdominalis*. The *HaCasp-8* gene harbors a 1476 bp open reading frame (ORF) that codes for a protein of 492 amino acids (aa) with a predicted molecular mass of 55 kDa. HaCasp-8 houses the typical domain architecture of known initiator caspases, including the death effector domain and the carboxyl-terminal catalytic domain. As expected, phylogenetic analysis reflected a closer evolutionary relationship of HaCasp-8 with its teleostean similitudes. The results of our qPCR assays confirmed the ubiquitous expression of *HaCasp-8* in physiologically important tissues examined, with pronounced expression levels in ovary tissues, followed by blood cells. *HaCasp-8* expression at the mRNA level was found to be significantly modulated by lipopolysaccharide, polyinosinic:polycytidylic acid, *Streptococcus iniae,* and *Edwardsiella tarda* injection. Overexpression of HaCasp-8 could trigger a significant level of cell death in HEK293T cells, suggesting its putative role in cell death. Taken together, our findings suggest that HaCasp-8 is an important component in the caspase cascade, and its expression can be significantly modulated under pathogen stress conditions in the big-belly seahorse.

1. Introduction

Apoptosis is a physiologically important process that can also participate in the regulation of host immune defense mechanisms, including the suppression of tumor growth along with immune cell homeostasis and maturation [1,2]. Cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases (caspases) are a family of cysteine proteases that take part in vital roles in apoptotic cell death, necrosis, and inflammation [3,4]. Caspases can be divided into two functional groups: pro-inflammatory caspases (caspases 1, 4, 5, 11, and 12) and apoptotic caspases (caspases 2, 3, 6, 7, 8, 9, and 10) [5,6]. Apoptotic pathway caspases can be further divided into initiator (caspases 2, 8, 9, and 10) and effector (caspases 3, 6, and 7) types, which are involved in upstream and downstream of the death cascade, respectively [7].

Caspase-dependent apoptosis consists of two major pathways: an intrinsic pathway and an extrinsic pathway [6,8]. The intrinsic pathway is basically triggered in mitochondria and is characterized by the liberation of different proteins, such as cytochrome C due to the permeability of mitochondrial membrane. Subsequently, cytochrome C binds with the C-terminal of Apaf1 [9] and, then tends to undergo oligmerization, accompanied by synchronized recruitment of procaspase-9 to the CARD motif at the Apaf-1 N-terminus, forming the apotosome [10]. Within the apoptosome, procaspase-9 is proteolytically activated and converted into mature caspase-9

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju, 63243, Republic of Korea.
** Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju, 63243, Republic of Korea.
E-mail addresses: mj.kim.lucky@gmail.com (M.-J. Kim), jehee@jejunu.ac.kr (J. Lee).

http://dx.doi.org/10.1016/j.fsi.2016.10.015
1050-4648/

[11]. Mature caspase-9 can activate procaspase-3 to execute apoptosis [12]. The extrinsic pathway is initiated when Fas ligand binds with CD95 (a Fas receptor) on the cell surface [13–15]. The Fas-associated protein with death domain (FADD) of the Fas receptor then binds with the procaspase-8 death domain [13,14] to form the death-inducing signaling complex (DISC), in which activated caspase-8 is formed to activate procaspase-3 [16,17]. Once capsase-3 is activated, it can induce apoptosis [18].

Currently, 15 caspases have been identified from mammals, of which 11 are from humans. Intriguingly, 14 caspases were reported from Atlantic salmon [19,20]. Caspases are generally synthesized as zymogenic forms that harbor a pro-domain region [7]. The pro-domain at the N-terminus of procaspase is cleaved at a specific aspartic acid residue, resulting in a mature caspase consisting of p20 (large subunit) and p10 (small subunit) subunits that can be further processed by proteolysis to form a biologically active heterodimer [7,21].

Caspase-8, which is classified as initiator caspase, comprises two death effector domains (DEDs) in its amino terminus and one carboxyl-terminal catalytic domain (CASc) at the carboxyl terminus, contained p20 (large subunit) and p10 (small subunit) subunits [22]. Typically, caspase-8 is involved in extrinsic apoptotic signaling pathway of mammals and stimulates various cell receptors called death receptors, one of which is Fas, to initiate the apoptosis [23].

Upon host cell invasion by pathogens, Toll-like receptor (TLR) 3 or TLR4 can recognize their molecular motifs and mediate the activation of caspase-8, which triggers apoptotic cell death [24]. Caspase-8 is also known to be essential for lymphocyte activation and inflammatory gene expression [25,26]. Moreover, as reported previously, caspase-8 was found to be crucial for the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via antigen receptors, Fc receptor, or TLR4 in T cells, B cells, and natural killer cells [26]. Human caspase-8 is believed to act as a tumor suppressor, although this has not been clearly elucidated to date [27].

Although much information has been reported for the mammalian caspase-8 counterparts, knowledge on those of teleostean origin remains incomplete. However, there are several reports on caspase-8 from the fish species *Cyprinus carpio* [28], *Dicentrarchus labrax* [29], and *Danio rerio* [30]. Therein, transcriptional modulation of fish caspase-8 against pathogen and chemical stress was noted, further deciphering the apoptogenic property of some caspase-8 counterparts.

Big-belly seahorse (*Hippocampus abdominalis*) is an economically important teleost that is used for traditional oriental medicine in China, Japan, and Korea [31–33]. The increasing demand for the species has influenced its mariculture industry. However, regular pathogen infections leading to significant mortality of this seahorse have adversely affected its commercial farming [34,35]. Thus, investigation of plausible ways to develop disease resistance strategies in this fish species is necessary to maintain a disease-controlled seahorse aquaculture industry. Molecular-level investigation of the seahorse immune system is one of the initial steps of this process that in turn can lay the foundation for designing therapeutic methodologies to target the different immune components of this aquacrop.

In this study, we have molecularly characterized caspase-8 from *Hippocampus abdominalis* and deciphered its mRNA expression dynamics in response to the stress mounted by lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (poly I:C), *Edwardsiella tarda* (*E. tarda*), and *Streptococcus iniae* (*S. iniae*). Moreover, we analyzed the cell viability with the overexpression of caspase-8 *in vitro* using a cellular approach in order to determine its contribution to the cell death.

2. Materials and methods

2.1. cDNA database

A cDNA database of the big-belly seahorse was established using the 454 GS FLX™ sequencing platform as described previously [36].

2.2. Sequence characterization and in silico analysis of HaCasp-8

Analysis of our previously constructed seahorse cDNA database using the NCBI-BLASTX program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) led to the identification of a caspase-8 homolog, which was designated as HaCasp-8 (GenBank ID: KU363232). The complete open reading frame (ORF) of HaCasp-8 was determined and its corresponding amino acid sequence was derived, with further prediction of some of its physicochemical properties using the DNAssist 2.2 software [37]. The derived amino acid sequence was analyzed using the SMART online server (http://smart.embl-heidelberg.de/). Multiple sequence alignment and pairwise sequence alignment were carried out using the Clustal W2 and EMBOSS needle programs, respectively (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic reconstruction was generated using the MEGA 5.2 software, applying the neighbor-joining platform with the support of 5000 bootstrap replicates.

2.3. Immune challenge and tissue collection

Big-belly seahorses were purchased from Korea Marine Ornamental Fish Breeding Center (Jeju Island, Republic of Korea) and acclimated to the laboratory conditions in aquarium tanks filled with aerated seawater (salinity, 34‰), at 20 °C for 1 week prior to the experiment. During the acclimatization period, the animals were fed with a commercial fish feed. To determine the tissue-specific expression patterns of HaCasp-8 in healthy seahorses, gill, liver, testis, ovary spleen, intestine, stomach, kidney, skin, muscle, heart, and pouch brain tissues and blood cells were collected from six seahorses (3 males and 3 females) having an average total body weight of 8 g. Blood was harvested by cutting the edge of the tails of the animals, and peripheral blood cells were collected by centrifugation at 3000*g* for 10 min at 4 °C. The collected blood cells and tissues were snap-frozen in liquid nitrogen and stored at −80 °C.

Seahorses with an average body weight of 3 g were used for the immune challenge experiments. The seahorses in the control group were intraperitoneally injected with 100 μL of PBS. The immune challenge groups were injected intraperitoneally with LPS (1.25 μg/μL, from *Escherichia coli* 055:B5; Sigma, USA), poly I:C (1.5 μg/μL), *Edwardsiella tarda* (5×10^3 CFU/μL), or *Streptococcus iniae* (10^5 CFU/μL), all prepared in PBS in a total volume of 100 μL. Blood and kidney tissues were sampled from five individuals in a time-course manner (0, 3, 6, 12, 24, and 72 h post injection).

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissues of six healthy seahorses and from blood cells and kidney tissues of five immune-challenged seahorses corresponding to each time point, using the RNAiso Plus Kit (TaKaRa, Japan) followed by clean-up with an RNeasy spin column (Qiagen, USA). The RNA concentration was determined at 260 nm using a multiplate reader (Thermo Scientific, USA). RNA samples were diluted to 2.5 μg/μL, and 2.5 μg was used to synthesize cDNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara, Japan) following the manufacturer's protocol. The synthesized cDNA was diluted 40-fold and stored at −20 °C.

2.5. Quantitative real-time PCR

Evaluation of the mRNA expression was carried out via quantitative real-time polymerase chain reaction (qPCR) using a Thermal Cycler Dice™ TP800 system (TaKaRa). The qPCR reaction volume was 10 μL in total, containing 3 μL of cDNA template, 5 μL of 2× TaKaRa Ex Taq™ SYBR premix, 0.5 μL of forward primer (10 pmol/μL), 0.5 μL of reverse primer (10 pmol/μL), (See Table 1) and 1 μL of nuclease-free water. The thermal cycling profile was as follows: one cycle of 95 °C for 10 s, followed by 35 amplification cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [38]. Seahorse 40S ribosomal protein S7 (GenBank ID: KP780177) was used as the internal control for relative quantification. The relative expression level corresponding to each immune challenge experiment was normalized to the PBS-injected control, further comparing with the un-injected control as a basal level. All data were presented as the mean ± standard deviation (SD). In the case of the challenge experiment, $P < 0.05$ was considered as statistically significant compared with the expression level of un-injected control, based on a one-way analysis of variance test and followed by Duncan's multiple range test conducted by the SPSS 16 program.

2.6. Construction of the recombinant vector

The coding region from the predicted cleavage site of the mature protein encoded by *HaCasp-8* (831 bp) was PCR amplified from cDNA synthesized from seahorse blood cells and then cloned into the pcDNA 3.1 His C vector (Life Technologies, USA) between the *Eco*RI and *Xho*I sites using standard restriction digestion reactions. The recombinant vector (HaCasp8-pcDNA) was then transformed into *Escherichia coli* DH5α cells, and the DNA sequence of the putative transformed colonies was confirmed by sequencing (Macrogen, Seoul, Republic of Korea).

2.7. Cell culture and transfection

Human embryonic kidney 293T (HEK293T) cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Seoul, Republic of Korea) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, at 37 °C in the presence of 5% CO_2. The HEK293T cells were seeded in 24-well pates and grown to 80% confluency at 37 °C in 5% CO_2. Thereafter, the HaCasp8-pcDNA recombinant vector construct or an empty vector (Mock control) was transfected into the HEK293T cells using the X-tremeGENE9 (Sigma, USA) reagent according to the vendor's protocol. An untransfected control was also used in the experiment, following the aforementioned assay procedure without using any vector.

2.8. Cell viability assay

Transfected HEK293T cells along with untransfected HEK293T cells as a control were subjected to a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 24 h post transfection. Briefly, HEK293T cells were treated with 100 μL of MTT reagent (2 mg/mL) for 3 h. After removing the media, the cells were incubated in 300 μL dimethyl sulfoxide overnight. The optical density (OD) of the resultant solutions were measured at 540 nm to measure the formazan quantity formed, which can be directly correlated with the cell viability by comparing the OD values of the samples with that for the untransfected control. This assay was carried out in triplicates to ensure the reliability of outcomes.

2.9. Cell counting

Trypan Blue exclusion method was performed to determine the viable cell count in the cell suspension. Briefly, transfected HEK293T cells along with untransfected HEK293T cells as a control, were collected after 30 h post transfection by centrifugation at 1000 rpm for 1 min. Cells which are attached to the bottom of the flask were collected after incubating them with trypsin. Collected cells were resuspended in 200 μL of sterile PBS. Aliquot of cell suspension (10 μL) was mixed with same amount of 0.4% of Trypan Blue (Sigma, USA) and, live and dead cells were counted using hemocytometer.

3. Results and discussion

3.1. Sequence characterization and phylogenetic analysis of HaCasp-8

The identified cDNA contig sequence of *HaCasp-8* contained a 5′ untranslated region (UTR) of 97 bp and a 3′ UTR of 146 bp. Its ORF was 1476 bp long, coding for a 492-amino-acid (aa) sequence with a predicted molecular mass of 55 kDa. Our *in silico* analysis confirmed that HaCasp-8 harbors the functionally important domain architecture that can be commonly identified in initiator caspases, including two DEDs (residues 3–76 and 93–172 aa) and a single CASc domain (residues 232–483 aa) contained p20 large subunits (residues 239–363) and p10 small subunits (residues 400–483) (Fig. 1). Moreover, a pentapeptide active-site motif (^{357}QACQG^{361}) was also identified in *HaCasp-8*, which was highly conserved among other teleostean counterparts (Fig. 2). A cleavage site at the N-terminal aspartic acid residue (D-216) was identified, being a typical well-conserved feature of procaspases (Fig. 1). Multiple sequence alignment confirmed that the caspase family active-site residues were thoroughly conserved in *HaCasp-8* (Fig. 2). Based on the EMBOSS pairwise alignment algorithm, *HaCasp-8* showed a sequence identity of 56.4–35.9% and a similarity range of 71.2–51.7% with its vertebrate homologs (Table 2). The highest sequence identity (56.4%) was reported with caspase-8 from *Oplegnathus fasciatus* and the highest similarity (71.2%) was shown with caspase-8 from *Dicentrarchus labrax*. Moreover, human caspase-8 shared 36.1% identity and 51.7% similarity with *HaCasp-8*. Altogether, these *in silico* data suggest the homology of HaCasp-8

Table 1
Oligonucleotide primers used in this study.

Purpose	Orientation	Primer sequences (5′-3′)
40S ribosomal protein S7 q-PCR	Forward	GCGGGAAGCATGTGGTCTTCATT
40S ribosomal protein S7 q-PCR	Reverse	ACTCCTGGGTCGCTTCTGCTTATT
Caspase8 q-PCR	Forward	TCGGCATGGCCACTGTGT
Caspase8 q-PCR	Reverse	AGATCGTCCGAGTTGCTTGCAC
Caspase8 cloning (sequence conformation)	Forward	GCCACTTTACCGATAAAGACG
Caspase8 cloning (sequence conformation)	Reverse	TCACAAGAGTCGTTTTCAGATTTT
Caspase 8 cloning (transfection)	Forward	GAGAGAgaattcGCCGAAACGAACCCCGAG
Caspase 8 cloning (transfection)	Reverse	GAGAGActcgagTTAGGAGAGTTTCACTTGGTCCTCATCAG

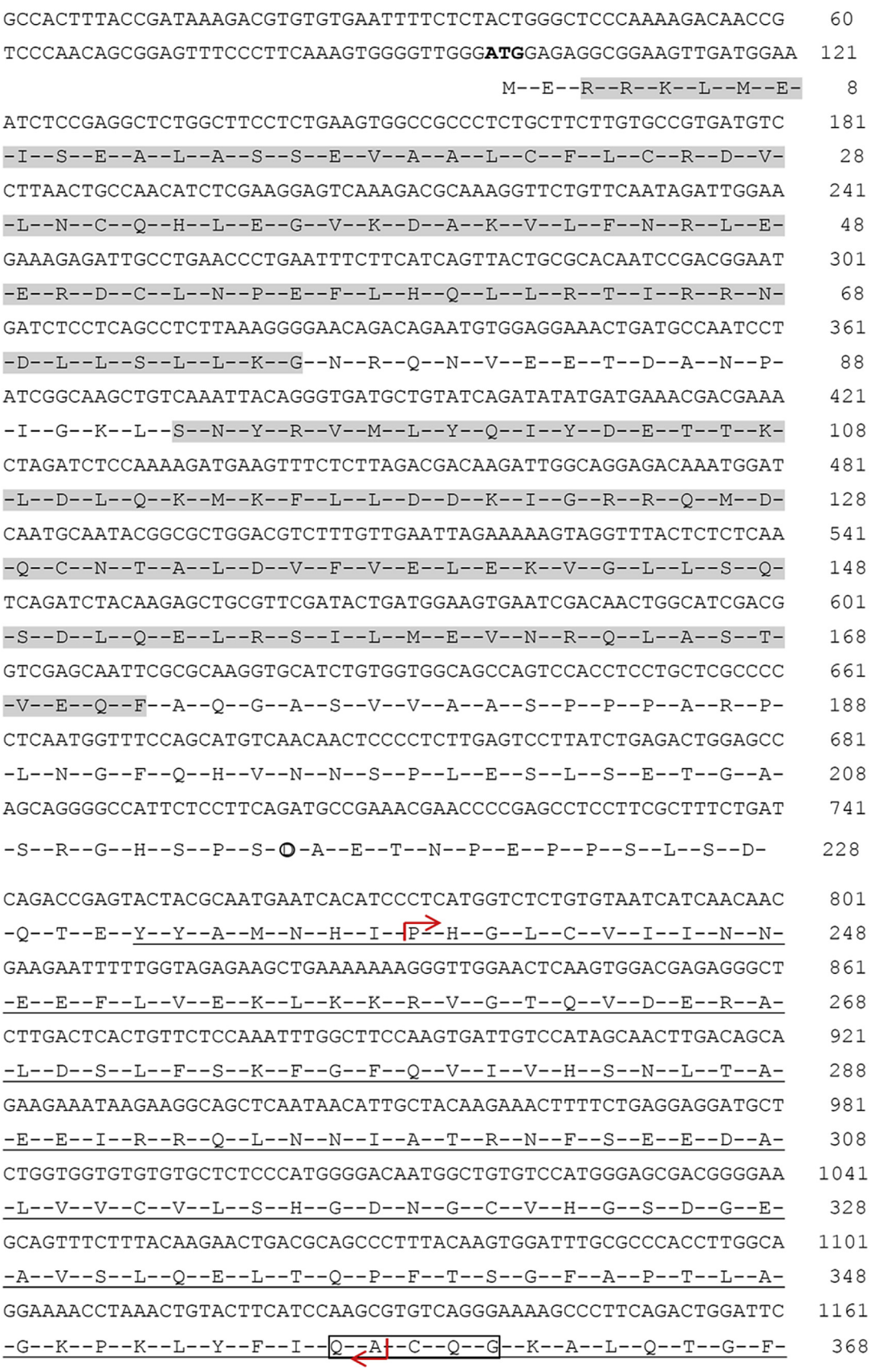

Fig. 1. The complete cDNA and amino acid sequences of HaCasp-8 from *Hippocampus abdominalis*. The start codon (ATG) and stop codon (TAA) are in bold fonts. The death effector domains are marked in gray. The carboxyl-terminal catalytic domain is underlined. The cleavage site at the aspartic acid reside is encircled and the pentapeptide active-site motif (QACQG) is boxed. The red arrow and blue arrow indicated p20 large subunits and p10 small subunits, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

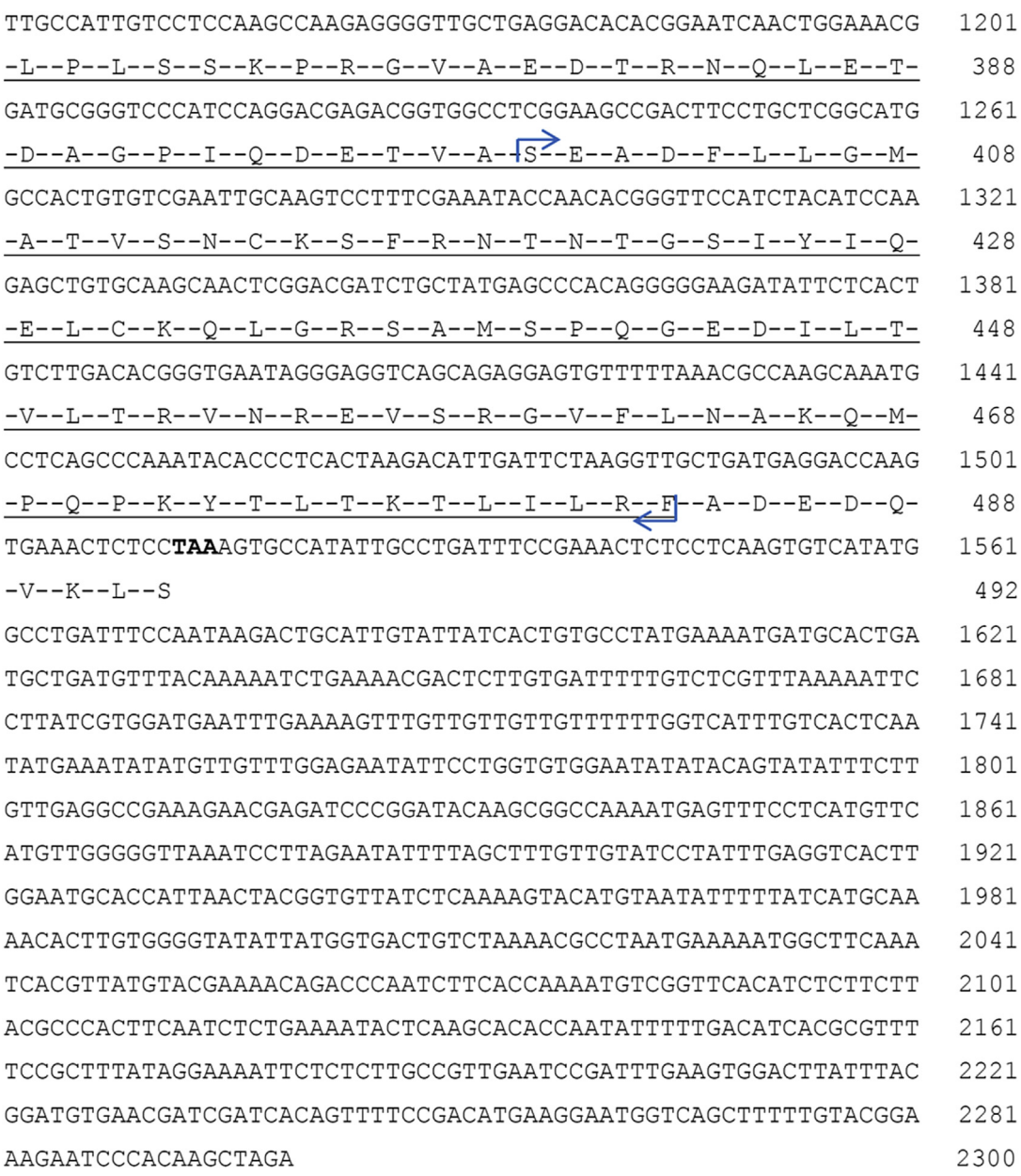

Fig. 1. (*continued*).

with the known initiator caspases, especially with teleostean caspase-8 counterparts.

According to the phylogenetic reconstruction, two basic clusters were clearly identified, one of which was formed by teleostean caspase-8 homologs (fish clade), and the other by mammalian caspase-8 homologs (Fig. 3). Within the fish clade, freshwater fish counterparts were clustered closely and independently. However, the clustering pattern of the marine fish counterparts deviated from the expected topology. *HaCasp-8* was positioned within the fish clade but with relatively distance evolutionary relationship with the other teleostean counterparts. Nevertheless, the clustering pattern hints the homology and fairly closer evolutionary relationship of *HaCasp-8* with its known teleostean homologs.

3.2. Tissue-specific expression of HaCasp-8

In order to investigate the basal transcription of *HaCasp-8* in different tissues under the physiological conditions of healthy big-belly seahorses, the tissue-specific relative mRNA expression was assayed by qPCR (Fig. 4), using 40S ribosomal protein S7 from *Hippocampus abdominalis* as the internal reference gene. The lowest expression level in the liver was calculated as the normalizing standard. According to the findings of the assay, the highest expression level of *HaCasp-8* was in ovary tissue, followed by blood cells. In fish, the basal mRNA expression level of *Megalobrama amblycephala* caspase-8 was abundant in the liver [38]. As reported previously, caspase-8 tissue expression was found to be diverse in mollusks. Caspase-8 from *Haliotis discus discus* was reported to be pronouncedly expressed in the gills [39]. On the other hand, the caspase-8 from *Crassostrea gigas* was noted to be highly expressed in the hemolymph [40], whereas counterparts from *Mytilus galloprovincialis* and *Mytilus coruscus* were more prominently expressed in the digestive gland and mantle, respectively [41].

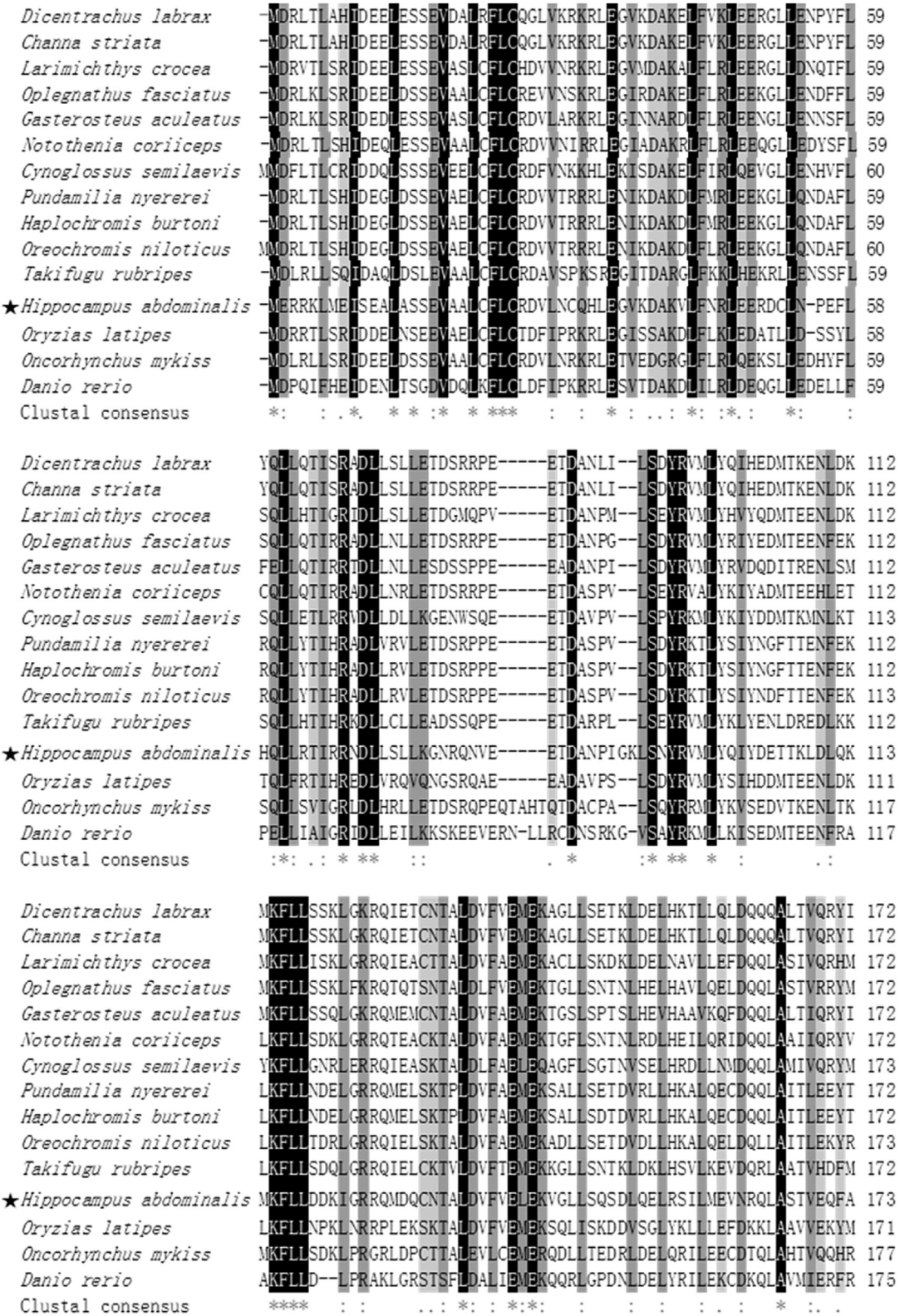

Fig. 2. Multiple amino acid sequence alignment of HaCasp-8 with other caspase sequences. The caspase family active site is boxed with dotted lines and the pentapeptide active-site motif is boxed.

Since apoptosis is known to play a key role in development, gonadal cells, including ovarian cells (especially ovarian follicles) can characteristically undergo marked apoptosis under the control of sex hormones [42]. Moreover, in fish, severe apoptosis has been observed in diplotene oocytes at the transition from ovary-like gonadal tissues to testes during sexual differentiation in pre-sumptive male fish [43]. On the other hand, blood cells can frequently undergo apoptosis due to the effect of many factors,

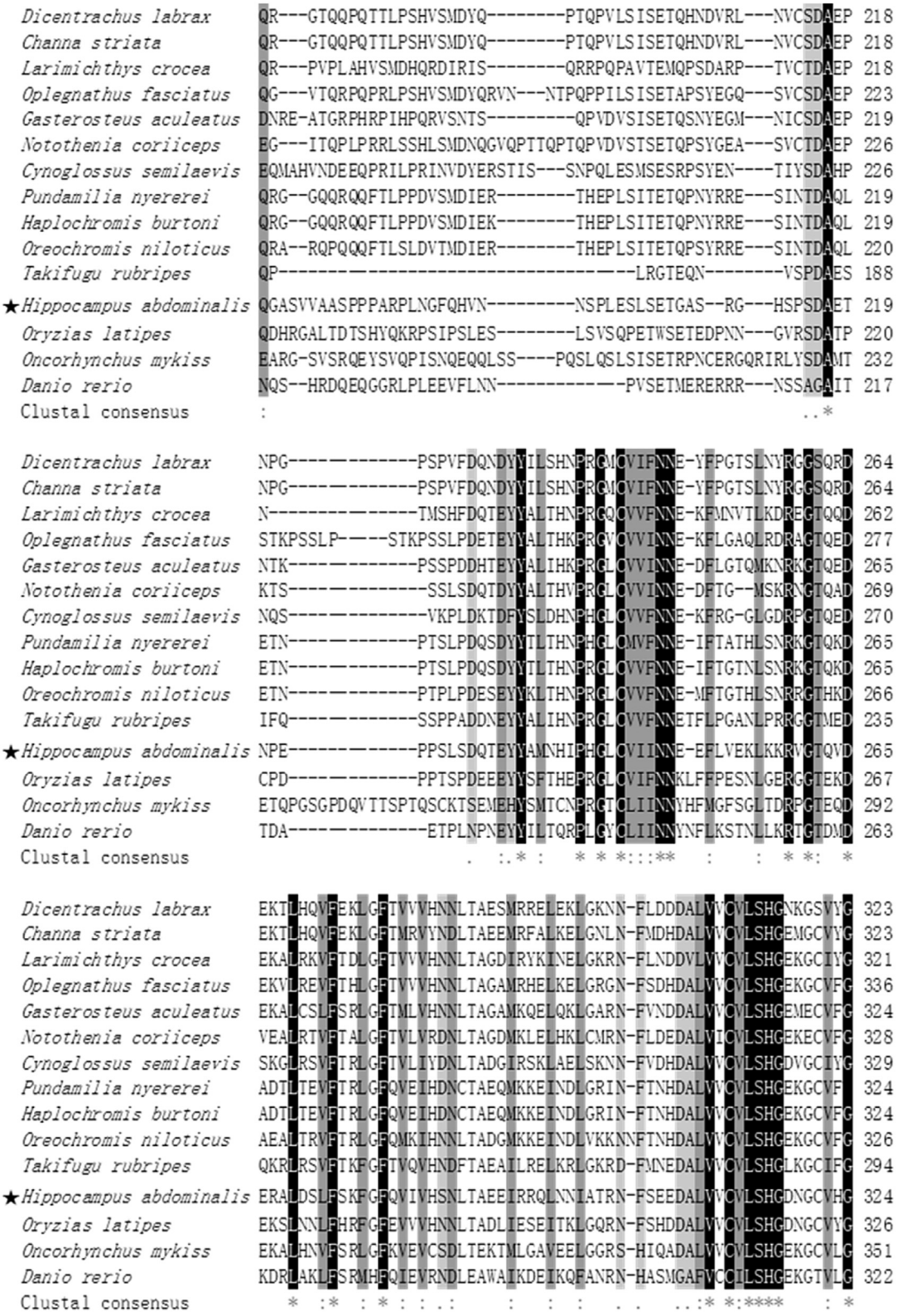

Fig. 2. (*continued*).

including reactive oxygen intermediates, aging conditions, and cytosolic calcium increase [44]. Thus, it is not unusual to observe a pronounced level of caspase-8-like molecules in fish ovary and blood cells, complying with our observations.

3.3. Transcriptional modulation of HaCasp-8 upon immune challenge

We analyzed the transcriptional modulation of *HaCasp-8* in

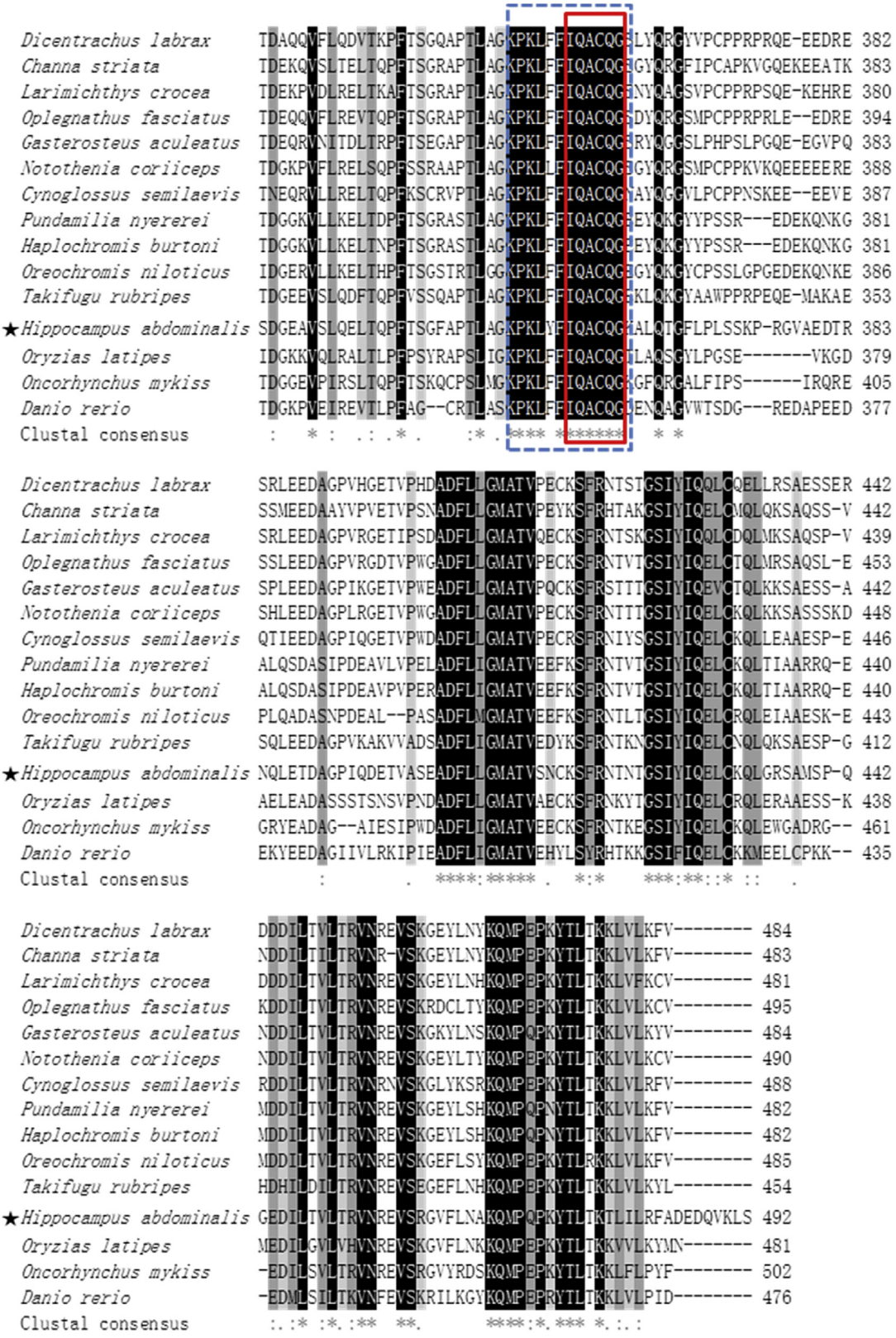

Fig. 2. (*continued*).

healthy seahorses after stimulating their immune systems with live pathogens or associated molecular stimulants, to elucidate its potential significance in host immune responses. In blood cells, all four treatments (LPS, poly I:C, *S. iniea*, and *E. tarda*) could mount inductive transcriptional responses (Fig. 5A). Upon LPS treatment, the basal transcript level of *HaCasp-8* was elevated at the early phase [3 h post treatment (p.t.)], and late phase (48 h p.t), albeit with significant down-regulation at the middle (12 h p.t.) phase. On

Table 2
Pairwise sequence alignment of *HaCasp-8* with known caspase-8 homolog sequences taken from the NCBI GenBank database.

Common name	Species	GenBank accession number	Identity (%)	Similarity (%)	Amino acids
Rock bream	*Oplegnathus fasciatus*	AHH30803	56.4	70.7	495
Sea bass	*Dicentrachus labrax*	ACO53629	55	71.2	484
Stickleback	*Gasterosteus aculeatus*	NP_001254591	54.7	71.1	484
Common snakehead	*Channa striata*	CCV01626	54	69.2	483
Yellow croaker	*Larimichthys crocea*	XP_010752886	53.9	67.7	481
Black cod	*Notothenia coriiceps*	XP_010773156	52.5	68.2	490
Tiger puffer	*Takifugu rubripes*	XP_003975926	51.8	64.9	454
Tongue sole	*Cynoglossus semilaevis*	XP_008332470	51.1	67.6	488
Burton's mouthbrooder	*Haplochromis burtoni*	XP_005951292	50.9	65.7	482
Python Island	*Pundamilia nyererei*	XP_005740222	50.7	66.1	482
Asiatic ricefish	*Oryzias latipes*	NP_001098258	50	67.3	481
Nile tilapia	*Oreochromis niloticus*	XP_003457507	49.8	64.8	485
Rainbow trout	*Oncorhynchus mykiss*	NP_001268251	47.6	61.4	502
Zebrafish	*Danio rerio*	AAS91705	38.9	54.8	476
Human	*Homo sapiens*	AAD24962	36.1	51.7	479
Cattle	*Bos taurus*	ABQ12952	36.2	52.3	485
Rat	*Mus musculus*	AAH49955	35.9	54.8	480

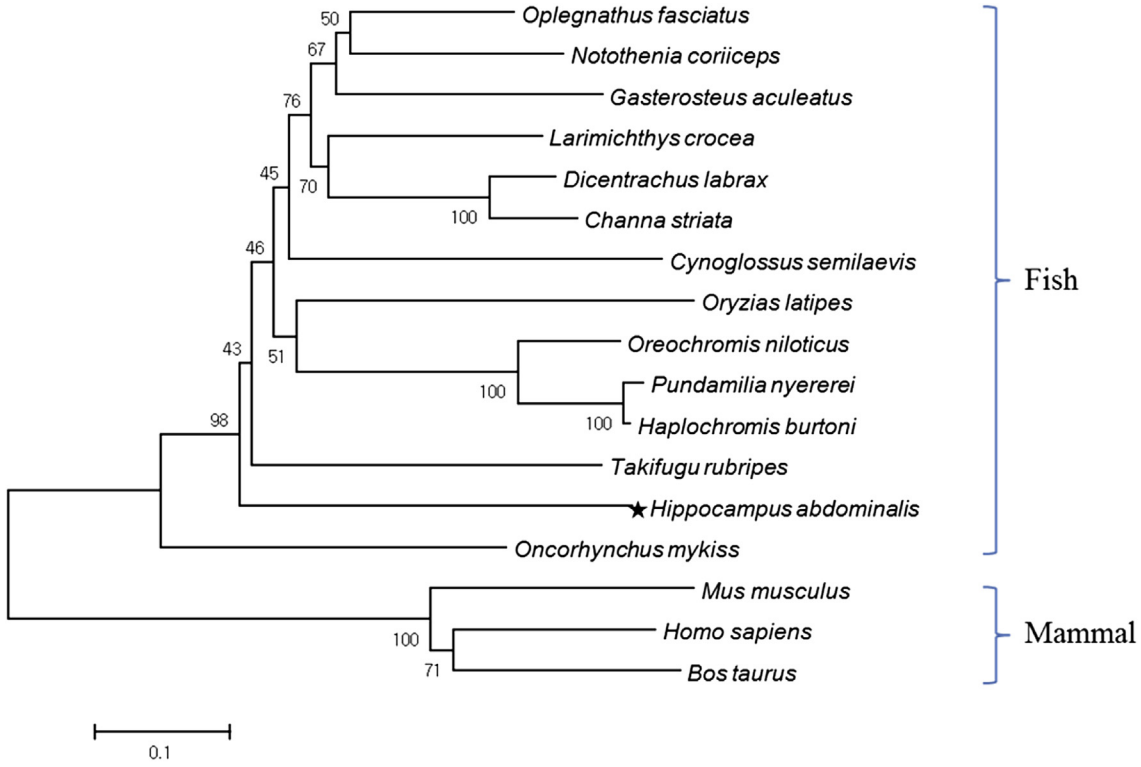

Fig. 3. Phylogenetic analysis of caspase-8 homologs. The phylogenetic tree was constructed by the MEGA 5 software, with the support of 5000 bootstrap replicates. The NCBI GenBank accession numbers of the homologs used are given in Table 2.

the other hand, with *E. tarda* treatment, the transcription was up-regulated at early (3 h p.t.) and late phase (48 h p.t.) as well as middle phase (12 h p.t.). Intriguingly, the basal transcript level of *HaCasp-8* was elevated by *S. iniae* at all the time points considered. Moreover, poly I:C treatment also induced increases from basal transcription at all the time points considered, except at 6 h p.t., reflecting that the virus genome itself may alter the expression pattern of *HaCasp-8*.

Similarly, in kidney tissues, LPS and *E. tarda* treatments could mount significant transcriptional up-regulation of *HaCasp-8* at the early and late phases after treatment; however, at the much later phase (72 h p.t.), significant down-regulation occurred, plausibly to propagate infection by suppressing the level of apoptosis (Fig. 5B). *S. iniae* treatment could also elicit a significant inductive transcriptional response at 6, 12, and 24 h p.t. in kidney tissues, whereas poly I:C treatment significantly elevated the transcription at 12, 24, and 48 h p.t. In agreement with our data, sea bass (*Dicentrarchus labrax* L.) capsase-8 mRNA expression was shown to be up-regulated under *Photobacterium damselae* subsp. *piscicida* infection [29]. Meanwhile, in invertebrates such as disk abalone (*Haliotis discus discus*) and oyster (*Crassostrea gigas*), the caspase-8 counterparts were transcriptionally up-regulated upon bacterial and viral invasion, respectively [39,40].

Much evidence for the involvement of caspase-8 in the host immune defense against bacterial or viral infections exists to date [26,45,46]. For example, caspase-8 is known to temporarily associate with the IkappaB kinase (IKK) complex through interactions with TLR3 and TLR4 in response to poly I:C or LPS stimulation,

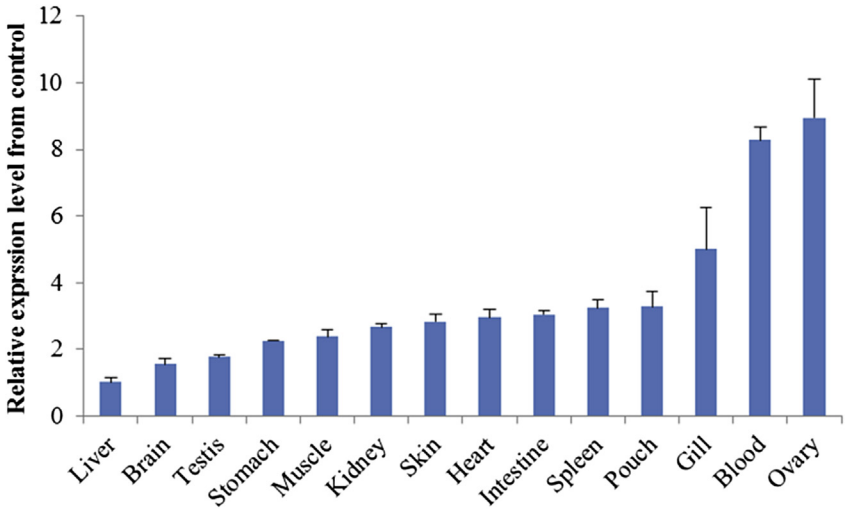

Fig. 4. Transcriptional distribution of *HaCasp-8*, as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method, using seahorse 40S ribosomal protein S7 as the internal reference gene. Fold changes in expression are shown relative to the mRNA expression level in the liver. Error bars represent the standard deviation (n = 3).

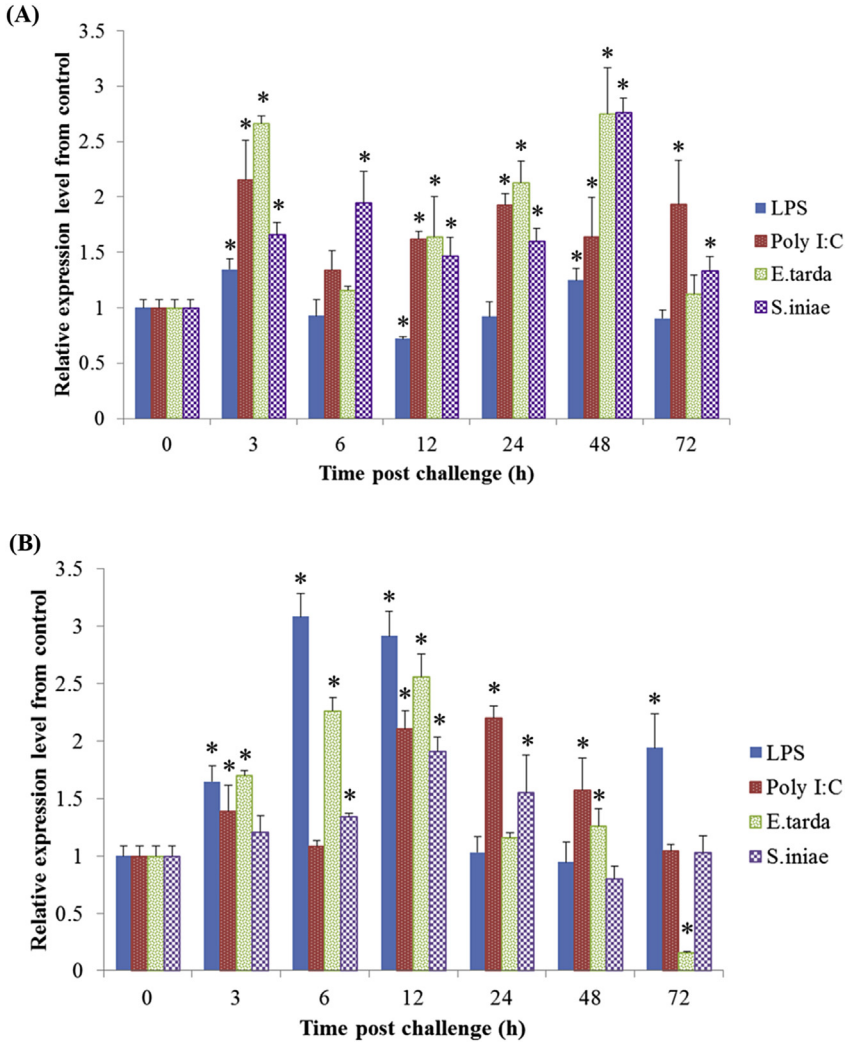

Fig. 5. Transcriptional modulation of *HaCasp-8* in (A) blood and (B) kidney tissue upon different immune challenges, as determined by qPCR. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method, using seahorse 40S ribosomal protein S7 as the reference gene, with normalization to the expression levels of corresponding PBS-injected controls at each time point. The relative fold change in expression at 0 h post-injection was used as the basal line. Error bars represent the standard deviation (n = 3). Significant differences between the challenged group and the control group are indicated with an asterisk (*, $P < 0.05$).

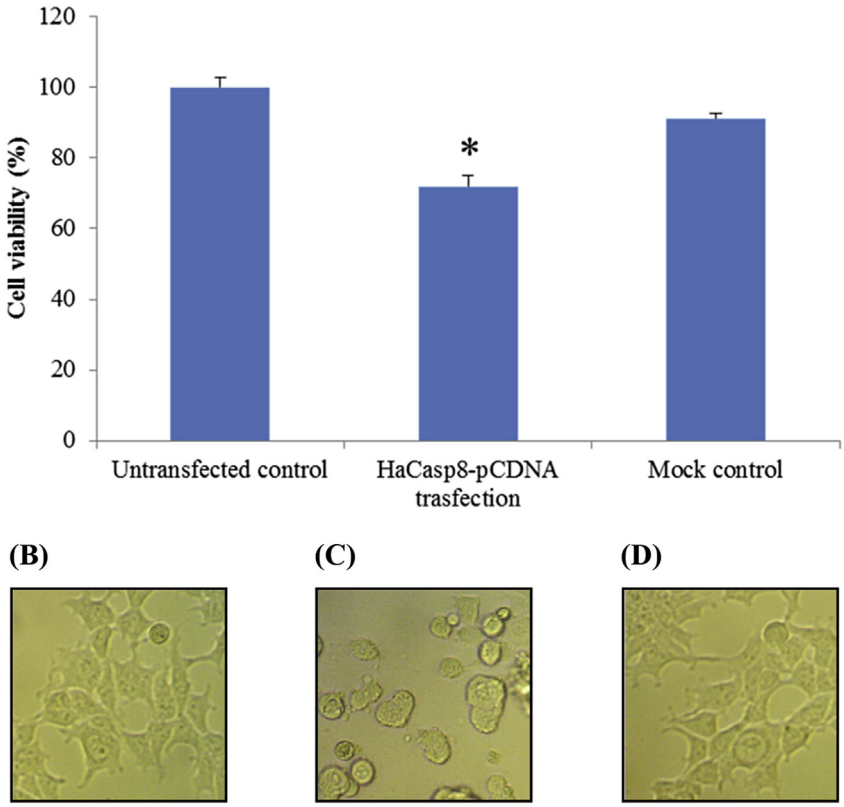

Fig. 6. Cell death inductive activity of HaCasp-8. (A) Viability of HaCasp8-pcDNA-transfected HEK293T cells relative to mock and untransfected controls, as determined by MTT assay. Error bars represent the standard deviation (n = 3). Cell viability (%) significantly different ($P < 0.01$) from the negative control is denoted by an asterisk (*). Microscopic observation of the morphology of (B) untransfected control cells, (C) HaCasp8-pcDNA-transfected cells, and (D) mock control cells.

respectively [46]. The IKK complex can activate NF-κB-mediated pro-inflammatory cytokine expression [47]. The outcomes of our transcriptional analysis in the big-belly seahorse together with the known knowledge on caspase-8 to date suggest that upon bacterial or viral pathogen invasion, caspase-8-mediated apoptosis may be induced as an immune response by the host defense system. However, further studies are merited to validate this suggestion, especially the research centering on HaCasp-8 activity *in vivo*.

3.4. Cell death induced by HaCasp-8

In order to determine the potential apoptogenic property of HaCasp-8 in viable cells, the protein was overexpressed in HEK293T cells and the cell viability was measured using the standard MTT assay. Results showed only a 75% viability of HaCasp8-pcDNA-transfected cells compared with the untransfected control (Fig. 6A). As expected, the cell viability for the mock control and

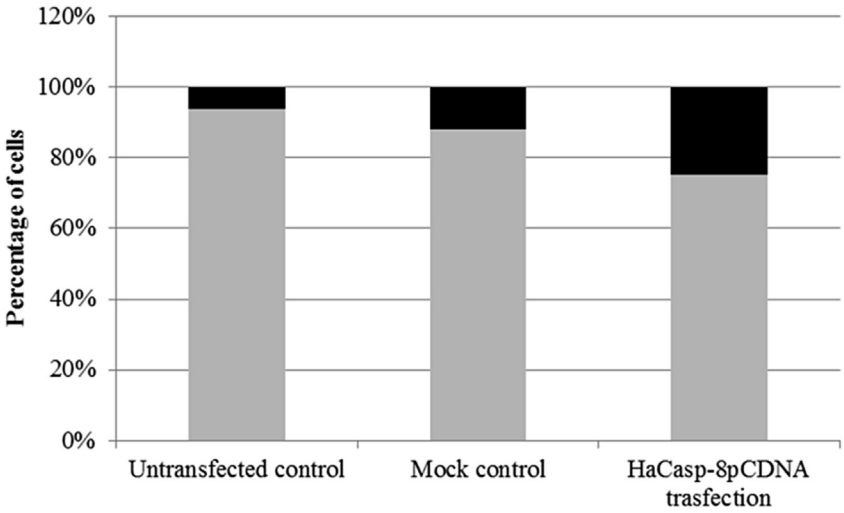

Fig. 7. Counting of live and dead cells. Viable cells were determined in HaCasp8-pcDNA-transfected HEK293T cells relative to mock and untransfected controls by Trypan Blue exclusion method. Gray and black colors marked in the each bar indicated the percentage of live and dead cells, respectively.

untransfected experiments was almost similar. Our results were further confirmed by light microscopic observation, where the morphology of vector-transfected cells was found to be altered compared to the control cells (Fig. 6B, C, and D). In addition, viable cells were determined using Trypan Blue exclusion method. According to the results, 6% and 12% of dead cells were observed for untransfected control and mock control, respectively. In the case of HaCasp8-pcDNA-transfected cells, remarkably higher percentage (25%) of dead cells were noted (Fig. 7). Even though numerical value is not dramatic; seahorse caspase-8 may have slightly low, but possible effect in mammalian cells. However, the result of the DNA laddering assay conducted with mammalian cells [48], revealed that the apoptosis may not be the real mechanism for cell death that have been occurred in this study. Thus, the outcomes of this assay together with our *in silico* data infer that HaCasp-8 is involved in the induction of cell death in seahorses.

4. Conclusion

In conclusion, we have identified a caspase-8 homolog from a seahorse (*Hippocampus abdominalis*). Our *in silico* data reflected the typical caspase family characteristics of *HaCasp-8*. Moreover, *HaCasp-8* was found to be ubiquitously expressed in tissues of healthy animals, with pronounced expression in the ovary, followed by blood. Transcription of *HaCasp-8* was found to be significantly modulated by pathogen stress conditions. Our cell transfection assay along with the cell viability assay demonstrated the putative cell death inductive property of HaCasp-8. Collectively, our results suggest that HaCasp-8 may be an important molecule in the seahorse cell signaling pathway, which in turn plausibly plays an indispensable role in the host immune response against pathogen infection.

Acknowledgment

This research was a part of the project titled 'Fish Vaccine Research Center', funded by the Ministry of Oceans and Fisheries, Korea.

References

[1] I. Sokolova, Apoptosis in molluscan immune defense, Invertebr. Surviv. J. 6 (2009) 49–58.
[2] J.F. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br. J. cancer 26 (4) (1972) 239.
[3] M.G. Grütter, Caspases: key players in programmed cell death, Curr. Opin. Struct. Biol. 10 (6) (2000) 649–655.
[4] K.M. Boatright, G.S. Salvesen, Mechanisms of caspase activation, Curr. Opin. cell Biol. 15 (6) (2003) 725–731.
[5] A. Nadiri, M.K. Wolinski, M. Saleh, The inflammatory caspases: key players in the host response to pathogenic invasion and sepsis, J. Immunol. 177 (7) (2006) 4239–4245.
[6] M.R. Wilson, Apoptotic signal transduction: emerging pathways, Biochem. cell Biol. 76 (4) (1998) 573–582.
[7] K. Nakajima, A. Takahashi, Y. Yaoita, Structure, expression, and function of the *Xenopus laevis* caspase family, J. Biol. Chem. 275 (14) (2000) 10484–10491.
[8] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science 281 (5381) (1998) 1305–1308.
[9] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (1) (2007) 99–163.
[10] P. Li, D. Nijhawan, I. Budihardjo, S.M. Srinivasula, M. Ahmad, E.S. Alnemri, X. Wang, Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, Cell 91 (4) (1997) 479–489.
[11] L. Dorstyn, S. Kumar, A cytochrome c-free fly apoptosome, Cell death Differ. 13 (7) (2006) 1049–1051.
[12] R. Hakem, A. Hakem, G.S. Duncan, J.T. Henderson, M. Woo, M.S. Soengas, A. Elia, J.L. de la Pompa, D. Kagi, W. Khoo, Differential requirement for caspase 9 in apoptotic pathways in vivo, Cell 94 (3) (1998) 339–352.
[13] M.P. Boldin, T.M. Goncharov, Y.V. Goltseve, D. Wallach, Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1-and TNF receptor–induced cell death, Cell 85 (6) (1996) 803–815.
[14] M. Muzio, A.M. Chinnaiyan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, FLICE, a novel FADD-homologous ICE/CED-3–like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex, Cell 85 (6) (1996) 817–827.
[15] M.E. Peter, P.H. Krammer, Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis, Curr. Opin. Immunol. 10 (5) (1998) 545–551.
[16] I. Lavrik, A. Krueger, I. Schmitz, S. Baumann, H. Weyd, P. Krammer, S. Kirchhoff, The active caspase-8 heterotetramer is formed at the CD95 DISC, Cell Death Differ. 10 (1) (2003) 144–145.
[17] M. Muzio, B.R. Stockwell, H.R. Stennicke, G.S. Salvesen, V.M. Dixit, An induced proximity model for caspase-8 activation, J. Biol. Chem. 273 (5) (1998) 2926–2930.
[18] I.N. Lavrik, A. Golks, P.H. Krammer, Caspases: pharmacological manipulation of cell death, J. Clin. Investig. 115 (10) (2005) 2665.
[19] H. Takle, Ø. Andersen, Caspases and apoptosis in fish, J. Fish Biol. 71 (sc) (2007) 326–349.
[20] M. Lamkanfi, W. Declercq, M. Kalai, X. Saelens, P. Vandenabeele, Alice in caspase land. A phylogenetic analysis of caspases from worm to man, Cell death Differ. 9 (4) (2002) 358–361.
[21] P. Fuentes-Prior, G. Salvesen, The protein structures that shape caspase activity, specificity, activation and inhibition, Biochem. J. 384 (2004) 201–232.
[22] K. Sakamaki, S.I. Tsukumo, S. Yonehara, Molecular cloning and characterization of mouse caspase-8, Eur. J. Biochem. 253 (2) (1998) 399–405.
[23] A. Ashkenazi, Targeting the extrinsic apoptosis pathway in cancer, Cytokine Growth Factor Rev. 19 (3–4) (2008) 325–331.
[24] S. He, Y. Liang, F. Shao, X. Wang, Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3–mediated pathway, Proc. Natl. Acad. Sci. 108 (50) (2011) 20054–20059.
[25] H.J. Chun, L. Zheng, M. Ahmad, J. Wang, C.K. Speirs, R.M. Siegel, J.K. Dale, J. Puck, J. Davis, C.G. Hall, Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency, Nature 419 (6905) (2002) 395–399.
[26] L. Salmena, B. Lemmers, A. Hakem, E. Matysiak-Zablocki, K. Murakami, P.B. Au, D.M. Berry, L. Tamblyn, A. Shehabeldin, E. Migon, Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity, Genes & Dev. 17 (7) (2003) 883–895.
[27] T. Teitz, T. Wei, M.B. Valentine, E.F. Vanin, J. Grenet, V.A. Valentine, F.G. Behm, A.T. Look, J.M. Lahti, V.J. Kidd, Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN, Nat. Med. 6 (5) (2000) 529–535.
[28] D. Gao, X. Zhang, C. Zhu, Y. Wang, W. Min, Cadmium triggers kidney cell apoptosis of purse red common carp (*Cyprinus carpio*) without caspase-8 activation, Dev. Comp. Immunol. 41 (4) (2013) 728–737.
[29] M.I. Reis, C. Costa-Ramos, A. do Vale, N.M. dos Santos, Molecular cloning of sea bass (*Dicentrarchus labrax* L.) caspase-8 gene and its involvement in Photobacterium damselae ssp. piscicida triggered apoptosis, Fish shellfish Immunol. 29 (1) (2010) 58–65.
[30] S.-i. Sakata, Y. Yan, Y. Satou, A. Momoi, P. Ngo-Hazelett, M. Nozaki, M. Furutani-Seiki, J.H. Postlethwait, S. Yonehara, K. Sakamaki, Conserved function of caspase-8 in apoptosis during bony fish evolution, Gene 396 (1) (2007) 134–148.
[31] J.H. Wallace, H. Kok, L. Beckley, B. Bennett, S. Blaber, A. Whitfield, South African estuaries and their importance to fishes, South Afr. J. Sci. 80 (5) (1984) 203–207.
[32] C.M. Woods, Natural diet of the seahorse *Hippocampus abdominalis*, N. Z. J. Mar. Freshw. Res. 36 (3) (2002) 655–660.
[33] A.C. Vincent, The International Trade in Seahorses, Traffic International Cambridge, UK, 1996.
[34] J.L. Balcázar, A. Gallo-Bueno, M. Planas, J. Pintado, Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from captive-bred seahorses with disease symptoms, Antonie Leeuwenhoek 97 (2) (2010) 207–210.
[35] A.C. Vincent, R.S. Clifton-Hadley, Parasitic infection of the seahorse (*Hippocampus erectus*)-a case report, J. Wildl. Dis. 25 (3) (1989) 404–406.
[36] M. Oh, N. Umasuthan, D.A.S. Elvitigala, Q. Wan, E. Jo, J. Ko, G.E. Noh, S. Shin, S. Rho, J. Lee, First comparative characterization of three distinct ferritin subunits from a teleost: evidence for immune-responsive mRNA expression and iron depriving activity of seahorse (*Hippocampus abdominalis*) ferritins, Fish Shellfish Immunol. 49 (2015) 450–460.
[37] H.-G. Patterton, S. Graves, DNAssist: the integrated editing and analysis of molecular biology sequences in windows, Bioinformatics 16 (7) (2000) 652–653.
[38] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, methods 25 (4) (2001) 402–408.
[39] Y. Lee, M. De Zoysa, I. Whang, S. Lee, Y. Kim, C. Oh, C.Y. Choi, S.-Y. Yeo, J. Lee, Molluscan death effector domain (DED)-containing caspase-8 gene from disk abalone (*Haliotis discus discus*): molecular characterization and expression analysis, Fish shellfish Immunol. 30 (2) (2011) 480–487.
[40] C. Li, T. Qu, B. Huang, P. Ji, W. Huang, H. Que, L. Li, G. Zhang, Cloning and characterization of a novel caspase-8-like gene in Crassostrea gigas, Fish shellfish Immunol. 46 (2) (2015) 486–492.
[41] D. Zhang, H.-W. Wang, C.-L. Yao, Molecular and acute temperature stress response characterizations of caspase-8 gene in two mussels, *Mytilus coruscus* and *Mytilus galloprovincialis*, Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 177 (2014) 10–20.

[42] A. Hsueh, K. Eisenhauer, S.-Y. Chun, S.-Y. Hsu, H. Billig, Gonadal cell apoptosis, Recent Prog. Hormone Res. 51 (1995) 433–455.
[43] D. Uchida, M. Yamashita, T. Kitano, T. Iguchi, Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish, J. Exp. Biol. 205 (6) (2002) 711–718.
[44] A. Chukhlovin, Apoptosis and red blood cell echinocytosis: common features, Scanning Microsc. 10 (3) (1995) 795–803.
[45] H. Su, N. Bidère, L. Zheng, A. Cubre, K. Sakai, J. Dale, L. Salmena, R. Hakem, S. Straus, M. Lenardo, Requirement for caspase-8 in NF-κB activation by antigen receptor, Science 307 (5714) (2005) 1465–1468.
[46] B. Lemmers, L. Salmena, N. Bidère, H. Su, E. Matysiak-Zablocki, K. Murakami, P.S. Ohashi, A. Jurisicova, M. Lenardo, R. Hakem, Essential role for caspase-8 in Toll-like receptors and NFκB signaling, J. Biol. Chem. 282 (10) (2007) 7416–7423.
[47] S.L. Doyle, C.A. Jefferies, L.A. O'Neill, Bruton's tyrosine kinase is involved in p65-mediated transactivation and phosphorylation of p65 on serine 536 during NFκB activation by lipopolysaccharide, J. Biol. Chem. 280 (25) (2005) 23496–23501.
[48] Y. Rahbar Saadat, N. Saeidi, S. Zununi Vahed, A. Barzegari, J. Barar, An update to DNA ladder assay for apoptosis detection, Bioimpacts 5 (1) (2015) 25–28.