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Characterization of *Caspase* Gene Family Members in Spotted Sea Bass (*Lateolabrax maculatus*) and Their Expression Profiles in Response to *Vibrio harveyi* Infection

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Abstract The *caspase* gene family is a crucial gene cluster that regulates apoptosis which contribute to programmed cell death, cell proliferation and differentiation, and several immune responses. In our study, a complete set of 12 caspase genes were identified in spotted sea bass *Lateolabrax maculatus*. These genes were divided into three subfamilies: 2 inflammatory caspases (*casp-1* and *casp-14-like*), 5 apoptosis initiators (*casp-2*, *casp-8a*, *casp-8b*, *casp-9*, and *casp-10*), and 5 apoptosis executioners (*casp-3a*, *casp-3b*, *casp-3-like*, *casp-6*, and *casp-7*). Their phylogenetic relationships, synteny and gene structures were systematically analyzed. Furthermore, the relative expression profiles of the caspase family members in the liver, intestine, head kidney, and spleen were measured by qPCR after infection with *Vibrio harveyi*. The results showed that the overall mRNA levels of the caspase genes were dramatically increased after *V. harveyi* infection, and the expression patterns varied among genes and tissues. More caspase genes underwent pronounced expression changes in the head kidney and spleen than in the liver or intestine, mainly after 48 h of the challenge. Specifically, *casp-3a*, *casp-3b*, *casp-3-like*, *casp-6*, *casp-7*, *casp-8a*, *casp-8b*, *casp-10*, and *casp-14-like* in the head kidney, and *casp-3-like*, *casp-6*, *casp-7*, and *casp-14-like* in the spleen, were the most responsive caspase genes which may contribute significantly to immune regulation in spotted sea bass. Additionally, the apoptosis level in head kidney and spleen after infection were examined using the Caspase assay. Our study provides a systemic overview of the caspase gene family in spotted sea bass after *V. harveyi* infection and lays a foundation for further deciphering the biological roles of these caspase genes.

Key words *caspase* gene family; spotted sea bass; *Vibrio harveyi*; gene expression; apoptosis

1 Introduction

The caspase (*casp*) family is a group of conserved cysteine-dependent aspartate-specific proteases that have essential functions in mediating apoptosis, pyroptosis, necroptosis, and inflammatory responses (Man *et al*., 2017). The principal morphology of caspases consists of two domains: the prodomain and the interleukin-1 beta converting enzyme (ICE) homologues (CASc) domain. The prodomain is composed of various death domain superfamily members, such as the death domain (DD), death effector domain (DED), caspase recruitment domain (CARD), and pyrin domain (PYD). The CASc domain consists of the large subunit (p20), small subunit (p10) and proteolytic cleavage site (Takle and Andersen, 2007). Based on their functional and structural similarities, mammalian caspase members can be classified into 3 subfamilies: apoptosis initiators (*casp-2*, *casp-8*, *casp-9*, and *casp-10*), apoptosis executioners (*casp-3*, *casp-6*, and *casp-7*) and inflammatory mediators (*casp-1*, *casp-4*, *casp5*, *casp-11*, and *casp-12*) (Eckhart *et al.*, 2008; Spead *et al*., 2018; Zeng *et al*., 2021). Additional orthologs of caspases, including *casp-15* to *-23*, have been reported in specific mammalian or teleost species (Eckhart *et al*., 2008).

Generally, the initiator caspases induce apoptosis through the mitochondrial or Bcl-2-regulated intrinsic pathway, or the death receptor-induced extrinsic pathway (Fan *et al*., 2005). During the intrinsic apoptosis pathway, varieties of stimuli such as cellular stresses induce the assembly of apoptosome complex and activate apoptosis initiators like *casp-9*. Alternatively, apoptosis can be triggered via the extrinsic pathway, which is initiated by ligand binding to death receptors, forming the death-inducing signaling complex (DISC) and then apoptosis initiators such as *casp-8* and *casp-10* are activated (Sakamaki and Satou, 2009; Rami-

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rez and Salvesen, 2018; Van Opdenbosch and Lamkanfi, 2019). Subsequently, the executioners including *casp-3*, *casp-6*, and *casp-7* are stimulated by the initiator caspases, promoting the process of cell death by driving the execution phase of apoptosis (Slee *et al*., 2001). Caspase genes are also involved in pyroptosis cascade, which is a form of the inflammatory programmed cell death pathway. It has been demonstrated that inflammatory caspases regulate inflammation by mediating the cleavage of gasdermin D directly (*casp-4*, *casp-5*, and *casp-11*), or through the formation of casp-1-containing inflammasome complex, resulting in pyroptosis cascade (Man *et al*., 2017).

In recent decades, although not as well characterized as the mammalian model, caspase genes have been identified in several teleost species, such as *casp-1*, *-2*, *-3*, *-8*, and *-9* in striped snakehead (*Channna striata*) (Kumaresan *et al*., 2016), *casp-1*, *-2*, *-3*, and *-9* in tongue sole (*Cynoglossus semilaevis*) (Long and Sun, 2016), *casp-1*, *-2*, *-3*, *-6*, *-8*, and *-10* in Japanese flounder (*Paralichthys olivaceus*) (Kurobe *et al*., 2007; Li *et al*., 2017, 2019), *casp-1*, *-2*, *-3*, *-6*, *-7*, and *-8* in puffer fish (*Takifugu obscurus*) (Fu *et al*., 2018, 2019, 2020), *casp-3*, *-8*, and *-9* in large yellow croaker (*Pseudosciaena crocea*) (Mu *et al*., 2010; Li *et al*., 2011; Yang *et al*., 2021) and *casp-3* and *casp-8* in European sea bass (*Dicentrarchus labrax*) (Reis *et al*., 2007, 2010). Novel caspase members have been found in zebrafish, including *casp-17*, *-19a*, *-19b*, *-20*, *-21*, *-22*, and *-23* (Spead *et al*., 2018). Recently, a complete set of 18 casp genes in rainbow trout were published (Zeng *et al*., 2021).

Spotted sea bass, *Lateolabrax maculatus*, is an economical marine species that has been found in various Asian countries. In China, the annual production of spotted sea bass exceeds 150000 tons, and spotted sea bass has become one of the most popular marine commercial fishes (Fan *et al*., 2019; Tian *et al*., 2019). However, *Vibrio harveyi*, which is a gram-negative bacterium that infects various marine species, causes high mortality rates and results in serious economic loss to the spotted sea bass industry (Austin and Zhang, 2006; Tian *et al*., 2019; Mao *et al*., 2020). In this study, the complete *casp* family gene set was identified in spotted sea bass, the gene structures and phylogenetic relationships were systematically characterized. The participation of the genes in the immune response was evaluated by detecting the variations of gene expression after *V. harveyi* infection.

2 Materials and Methods

2.1 Ethics Statement

All fish experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China (permit number: 20141201). The study did not involve endangered or protected species.

2.2 Identification of *Caspase* Sequences from Spotted Sea Bass

The candidate caspase sequences from spotted sea bass were identified by using the amino acid sequences from human, cattle (*Bos taurus*), house mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), black rock fish (*Sebastes schlegelii*) and giant grouper (*Epinephelus lanceolatus*) retrieved from the NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/) and UniProt database (https://www.uniprot.org/). TBLASTN with a cutoff e-value of 1e-5 was used to search the reference genome (PRJNA408177) and RNA-Seq databases (PRJNA515783, PRJNA515986). The open reading frame was determined through the ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/) and verified by Smart BLAST of the NCBI nonredundant database.

2.3 Phylogenetic and Synteny Analysis

The amino acid sequences of caspase genes from spotted sea bass and various representative vertebrates were used to construct a phylogenetic tree. Multiple sequence alignment was performed using ClustalW with default parameters. The phylogenetic analysis was conducted through the MEGA7 program (Kumar *et al*., 2016) with the neighbor-joining method, as well as through Jones-Taylor-Thornton (JTT) model with 1000 bootstrapped replications. The tree was illustrated with Tree of Life (iTOL, https://itol.embl.de/) version 5.6.3 (Letunic and Bork, 2007).

Synteny analysis was conducted to provide further evidence for the gene annotations of caspases from spotted sea bass. Information on neighboring genes of representative teleosts including zebrafish, Nile tilapia and European sea bass was determined from the Genomicus database (https://www.genomicus.bio.ens.psl.eu/genomicus-104.02/cgi-bin/search.pl) (Louis *et al*., 2015). The information of giant grouper was retrieved from NCBI annotation data base (www.ncbi.nlm.nih.gov/genome/browse/#!/proteins/8795/519138%7CEpinephelus%20lanceolatus/). The neighbor genes of caspases in spotted sea bass were identified from the reference genome assembly.

2.4 Gene Structure Analyses

The theoretical isoelectric point (pI) and molecular weight (Mw) were predicted with the pI/Mw tool of the online service Expasy (https://web.expasy.org/compute_pi/). The three-dimensional (3D) protein structure of casp genes was predicted by Swiss-Model (https://swissmodel.|expasy.org/) (Waterhouse *et al*., 2018) and visualized with the PyMOL Molecular Graphics System software, Version 2.0 (Schrödinger, LLC).

2.5 Experimental Fish

Spotted sea bass were obtained from Shuangying Aquaculture Company in Dongying City, Shandong Province, China. Seventy-five fish (body weight: 60.28 g ± 19.78 g; body length: 13.21 cm ± 1.75 cm) were acclimated in a total of 15 glass tanks (46-L in volume) at a density of 5 fish per tank for 1 week before the formal experiment. The water salinity, dissolved oxygen, pH and temperature were maintained at 30, 6−7 mg L^{-1}, 7.8 and 14−15℃, respectively. Feed was withheld 72 h before bacterial injection.

2.6 Bacterial Challenge and Experimental Design

V. harveyi was kindly received from the Fish Immunology Laboratory (School of Marine Science and Engineering, Qingdao Agricultural University, Shandong, China) and confirmed by amplification of the *toxR* gene, which is considered an effective taxonomic marker to identify *Vibrio* spp., following the procedure from Pang *et al*. (2006). Single colony of *V. harveyi* was selected and grown in Luria-Bertani (LB) broth supplemented with 1.5% NaCl and incubated overnight at 32℃. Bacterial cells were harvested by centrifugation at 5000 r min^{-1}, at 25℃ for 10 min. The bacterial pellet was washed two times and diluted with 1.5% NaCl and used as an original stock. The *V. harveyi* concentration was determined by measuring the standard curve generated by the Vibrio TCBS plate count protocol.

At the beginning (0 h), every fish in the tanks 1−3 (control group) were intraperitoneally injected with 0.1 mL normal saline solution (0.85%). At the same time, every fish in tanks 4−15 (treatment groups) were injected with 0.1 mL *V. harveyi* solution at a concentration of 2×10^9 CFU mL^{-1} dissolved in 1.5% saline solution. Shortly after injection, three fish in each tank of control group were randomly collected and euthanized with MS-222 in a dose of 210 mg L^{-1} (Leary *et al*., 2020). At 12 h, 24 h, 48 h, and 72 h after injection, three fish in tanks 4−6, 7−9, 10−12, and 13−15 were respectively collected with the same method as described above. Tissue samples, including the head kidney, spleen, liver, and intestine were quickly frozen in liquid nitrogen and stored at −80℃ before the RNA extraction.

2.7 RNA Extraction and Quantitative Real Time PCR (qPCR) Analysis

Total RNA from each tissue sample was extracted by TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol and subsequently reverse transcribed into complementary DNA (cDNA) using a Prime Script RT reagent kit (Takara, Otsu, Japan). The concentration of RNA and cDNA were validated by using a Biodropsis BD-1000 spectrophotometric absorbance machine (Beijing Oriental Science & Technology Development Ltd., Beijing, China).

Primers were designed using the Primer5 software (Palo Alto, CA) according to MIQE guidance (Bustin *et al*., 2009), and they are listed in Table 1.

Table1 Primer list for qPCR

Name	Primer sequence (5'→3')	Amplicon size (bp)	PCR efficiency (%)
Casp-1 F	CTTGCCAGGGTGAGGACTAA	341	100
Casp-1 R	CAGAATGCGTTGGTGGTAGA		
Casp-2 F	CGTGGTTTCGCTTTGGTGAT	247	107.8
Casp-2 R	CTCCGTGGGATAGCAGACAC		
Casp-3a F	ACAGATGTAGATGCTGCCAACG	266	115.2
Casp-3a R	GGCTTTCCCACCATTGATTT		
Casp-3b F	ACAAGAACTTCCACCGTAGCA	216	103.4
Casp-3b R	CATCTCCGTGACTTAGCAACA		
Casp-3-like F	GCAGCGTGTCACAGCATC	245	115
Casp-3-like R	GCACGGCATCTCCTTCTT		
Casp-6 F	AGTGACTGCCTGTGATGC	155	115.2
Casp-6 R	AGCCGTTTATGGTCTCCC		
Casp-7 F	GCCCGCTGAACTTGGAGGT	429	97
Casp-7 R	CTTAGCAGGATACAGGCGAAACA		
Casp-8a F	CCTTGGTGGTATGCGTGC	293	101.2
Casp-8a R	GCAAACGAAGGCATCACCAT		
Casp-8b F	CAAGATAAGAAGTGGAACCGACAA	238	106.1
Casp-8b R	GCAAACGAAGGCATCACCAT		
Casp-9 F	GGACCCATCAAACCTCTACG	339	97.3
Casp-9 R	TCGGGATGGACAGGGAC		
Casp-10 F	TGCTATTAGAAGTAGGGAAGGC	250	92.84
Casp-10 R	GGAGAGATGGTTGTAGACGC		
Casp-14-like F	TGTATGCAGTGTGTCCAGGG	194	90
Casp-14-like R	CTGAGAACGTGATGCTCGGT		
18s rRNA F	GGGTCCGAAGCGTTTACT	179	94
18s rRNA R	ACCTCTAGCGGCACAA		

The efficiency of each pair of primers was examined with the standard curve using the following formula (Rasmussen, 2001):

$$\text{Efficiency}\ (\%) = 10^{(-1/\text{slope})}\times 100\ .$$

The melting temperature (Tm) value for each pair of primers was precisely verified by gradient PCR and interpreted with agarose gel electrophoresis. qPCR analysis was performed using Applied Biosystems 7300 machines (Applied Biosystems, CA, USA), and 18S rRNA was set as the internal reference gene (Wang *et al*., 2018). All samples, including 3 biological replications, were repeated in triplicate. The thermocycle program was performed using the following conditions: 95℃ for 30 s, followed by 40 cycles of 95℃ for 10 s, 60℃ for 30 and 72℃ for 30 s for *casp-3-like* and *casp-14-like*. An annealing temperature of 55℃ was used

for *casp-1*, *-2*, *-9*, *-10*, *-8a*, *-8b*, *-3a*, *-3b*, *-6*, and *-7*. The relative expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ formula (Schmittgen and Livak, 2008) and the relative expression values of control fish at 0 h was used as the calibrator for each time point. The results are graphically illustrated as the mean ± standard error mean (SEM) using GraphPad Prism version 8.0.2 for Windows (La Jolla, CA, USA).

2.8 Detection of Caspase 3/7 Activity

To analyze the apoptosis level of the infected tissue samples, the Caspase assay was performed using the Caspase 3/7 Activity Apoptosis Assay Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. Briefly, 0.2 g tissue sample (head kidney and spleen) was ground into powder with liquid nitrogen, and lysed in the 150 μL lysis buffer at room temperature for 15 min. Then the mixture was centrifuged at 800 × *g* for 10 min, and the supernatant was transferred into a 96-well plate and incubated at 25℃ for 1 h. Meantime, 3 blank controls were set which contained only Caspase3/7 detection buffer. The fluorescence value of Caspase3/7 was measured at Ex/Em=490/525 nm by the microplate reader (MD Spectra Max Plus 384).

2.9 Statistical Analyses

The raw data of fold change values at different time courses in each tissue were validated for parametrical statistics. Shapiro-Wilk's test ($P>0.05$) (Shapiro and Wilk, 1965; Mohd Razali and Yap, 2011) and Levene's test were used to verify the normality of the distribution and equality of variances in the samples (homogeneity of variance) ($P>0.05$) (Nordstokke and Zumbo, 2010; Nordstokke *et al*., 2011). All normalized data were conducted using analysis of variance (ANOVA). The significant differences between obtained data at each time course were interpreted with post hoc multiple comparisons by Duncan's multiple range test using SPSS software version 23.0 (SPSS, Chicago, IL) (Walter and Duncan, 1955). Difference was considered Significant when $P<0.05$.

3 Results

3.1 Characterization of Caspase Genes in Spotted Sea Bass

In total, 12 caspase genes were identified in spotted sea bass. These genes were further divided into three subfamilies depending on their deduced functions: inflammatory caspases (*casp-1* and *casp-14-like*), initiator caspases (*casp-2*, *casp-8a*, *casp-8b*, *casp-9*, and *casp-10*) and executioner caspases (*casp-3a*, *casp-3b*, *casp-3-like*, *casp-6*, and *casp-7*). The number of amino acid residues encoded by spotted sea bass caspase genes ranged from 254 to 539, with the relative molecular weights (Mw) ranging from 29.11 kDa to 60.47 kDa. Detailed information on the pI (isoelectric points), Mw, predicted protein size, chromosome location and GenBank accession number of the caspase genes in spotted sea bass is provided in Table 2.

Table 2 Summary of the characteristics of the caspase gene family identified in spotted sea bass

Name	Chromosome location	Protein length (aa)	Mw (kDa)	pI	Subfamily classification	Accession number
Casp-1	Chr5: 16706493-16709872	428	48.43	5.94	Inflammatory mediator	MZ318677
Casp-14-like	Chr13: 8545457-8547210	254	29.11	9.26	Inflammatory mediator	MZ318688
Casp-2	Chr7: 21422753-21431634	468	53.16	6.14	Initiator	MZ318678
Casp-8a	Chr10: 14398202-14403514	497	56.03	5.55	Initiator	MZ318684
Casp-8b	Chr10: 14391290-14394315	388	43.35	6.34	Initiator	MZ318685
Casp-9	Chr9: 7171262-7173484	435	48.89	5.99	Initiator	MZ318686
Casp-10	Chr11: 24163787-24171392	539	60.47	5.50	Initiator	MZ318687
Casp-3a	Chr3: 4989214-4994726	285	31.70	6.32	Executioner	MZ318679
Casp-3b	Chr14: 22659311-22664379	298	32.80	5.06	Executioner	MZ318680
Casp-3-like	Chr17: 21609996-21612468	273	29.86	8.41	Executioner	MZ318681
Casp-6	Chr3: 7581322-7586021	355	40.77	6.23	Executioner	MZ318682
Casp-7	Chr21: 11416541-11427059	312	35.22	5.24	Executioner	MZ318683

3.2 Phylogenetic and Syntenic Analyses

Phylogenetic analysis was performed by using the predicted caspase amino acid sequences from spotted sea bass and selected vertebrates. As shown in Fig.1, the caspase genes in spotted sea bass were clustered with corresponding teleost counterparts, which are consistent with their annotations, and categorized into three subfamilies as expected (Fig.1). The results demonstrate that the caspase family was conserved during evolution.

Syntenic analysis was conducted for *casp-3a*, *casp-3b*, *casp-3-like*, *casp-8a*, *casp-8b*, and *casp-14-like*, which had multiple gene copies or could not be accurately annotated based on the phylogenetic analysis. To further confirm their annotations, the caspase genes and their neighboring genes in spotted sea bass are shown in Fig.2. The results showed that for the three *casp-3* genes (*casp-3a*, *casp-3b*, and *casp-3-like*), conserved genomic neighborhoods were found between spotted sea bass, zebrafish, Nile tilapia, and European sea bass (Fig.2A). The synteny results of *casp-8a* and *casp-8b* were elucidated in zebrafish and spotted sea bass, which had notably conserved patterns (Fig.2B). Similarly, a highly conserved syntenic block between spotted sea bass and giant grouper was identified in the genomic region sur-

rounding *casp-14-like* (Fig.2C). Additionally, the predicted three-dimensional (3D) structures of *casp-3a*, *casp-3b*, *casp-3-like* and *casp-14-like* were constructed in spotted sea bass, and the results demonstrate that the tertiary structure was highly conserved as illustrated in Fig.3. In this case, all phylogenetic, syntenic and tertiary structure analyses support the accuracy of our annotation and nomenclature of *casp* genes from spotted sea bass.

3.3 Domain Structure Analysis and Multiple Sequence Alignment of the CASc Catalytic Domain

The *casp* genes from spotted sea bass were found to contain an N-terminal prodomain of varying size and a catalytic CASc domain, which was consistent with caspase orthologs of mammalian and other vertebrate species (Fig.4). As results showed, *casp-1*, *casp-2*, *casp-8b* and *casp-9* of spotted sea bass had a caspase-recruitment domain (CARD) in their N-terminal region, while *casp-8a* and *casp-10* had two death-effector domains (DEDs) instead. The structural features may facilitate these proteins to combine with different adapter proteins and trigger distinct downstream signaling pathways. All members in the executioner subgroup, such as *casp-3a*, *casp-3b*, *casp-3-like*, *casp-6* and *casp-7*, were characterized by a short prodomain (Fig.4).

As predicted, the CASc domain of spotted sea bass caspase genes was composed of two subunits: the large subunit (p20), which contained the caspase active site (QACNG, N represents G for *casp-9*, Q for *casp-8a*, *-8b*, and *-10*, and R for the other casp genes), and the small subunit (p10) (Fig.5). The sequence alignments of the predicted CASc catalytic domains and the active site (QACNG) are illustrated in Fig.5.

3.4 Relative Expression Changes of Caspase Genes After the *V. harveyi* Infection

To investigate the potential involvement of caspase genes from spotted sea bass in innate immunity, the mRNA expression level of *casp* genes was measured in four classical immune organs, including liver, intestine, head kidney and spleen at 5 time points (0, 12, 24, 48 and 72 h) after the *V. harveyi* infection. Our qPCR results show that caspase genes exhibited different expression profiles in a time-dependent manner that varied among genes and tissues (Fig.6). Notably, 12 h after injected with *V. harveyi*, fish

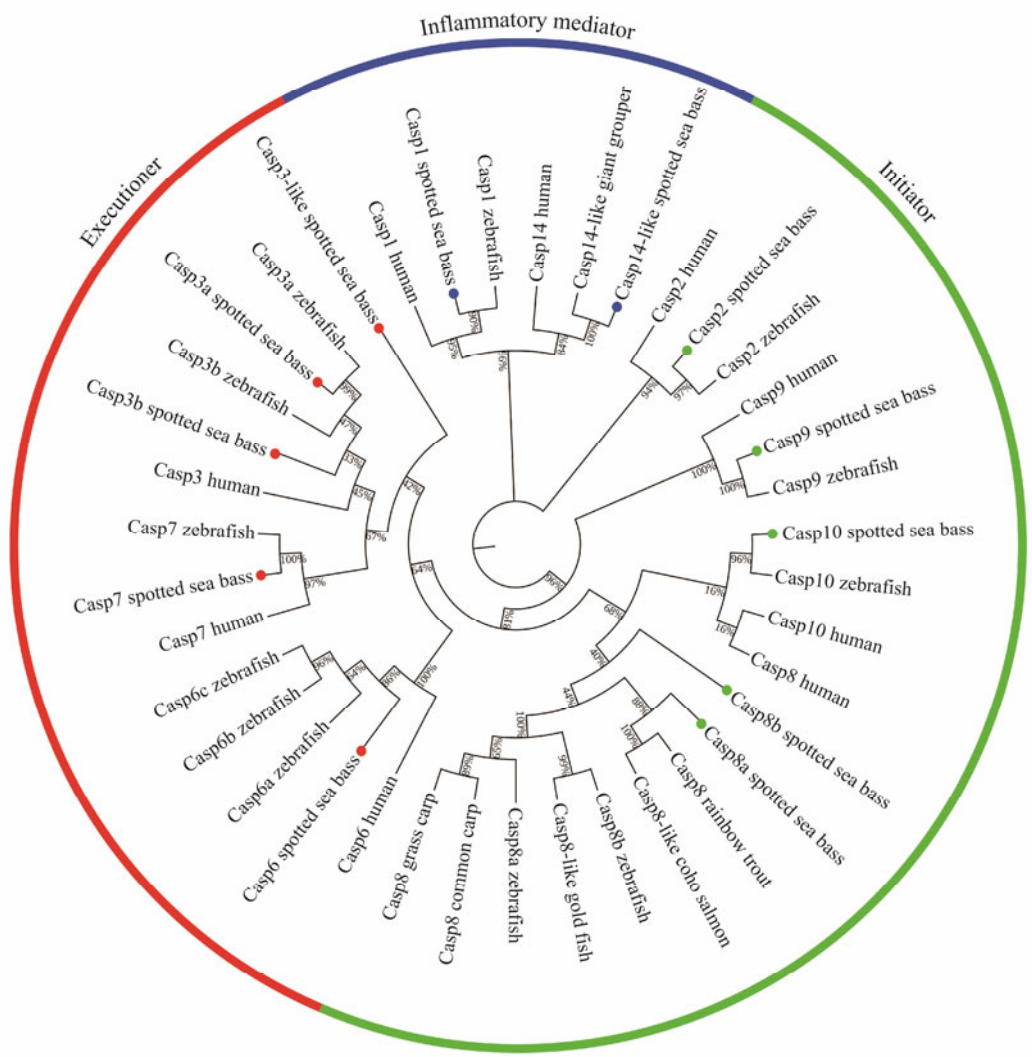

Fig.1 Phylogenetic analysis of the caspase gene family in spotted sea bass and selected vertebrate species. The phylogenetic tree was constructed by using amino acid sequences from spotted sea bass and other selected vertebrates. Those values on each node represented bootstrapping values, and the colors are referred as subfamily classification.

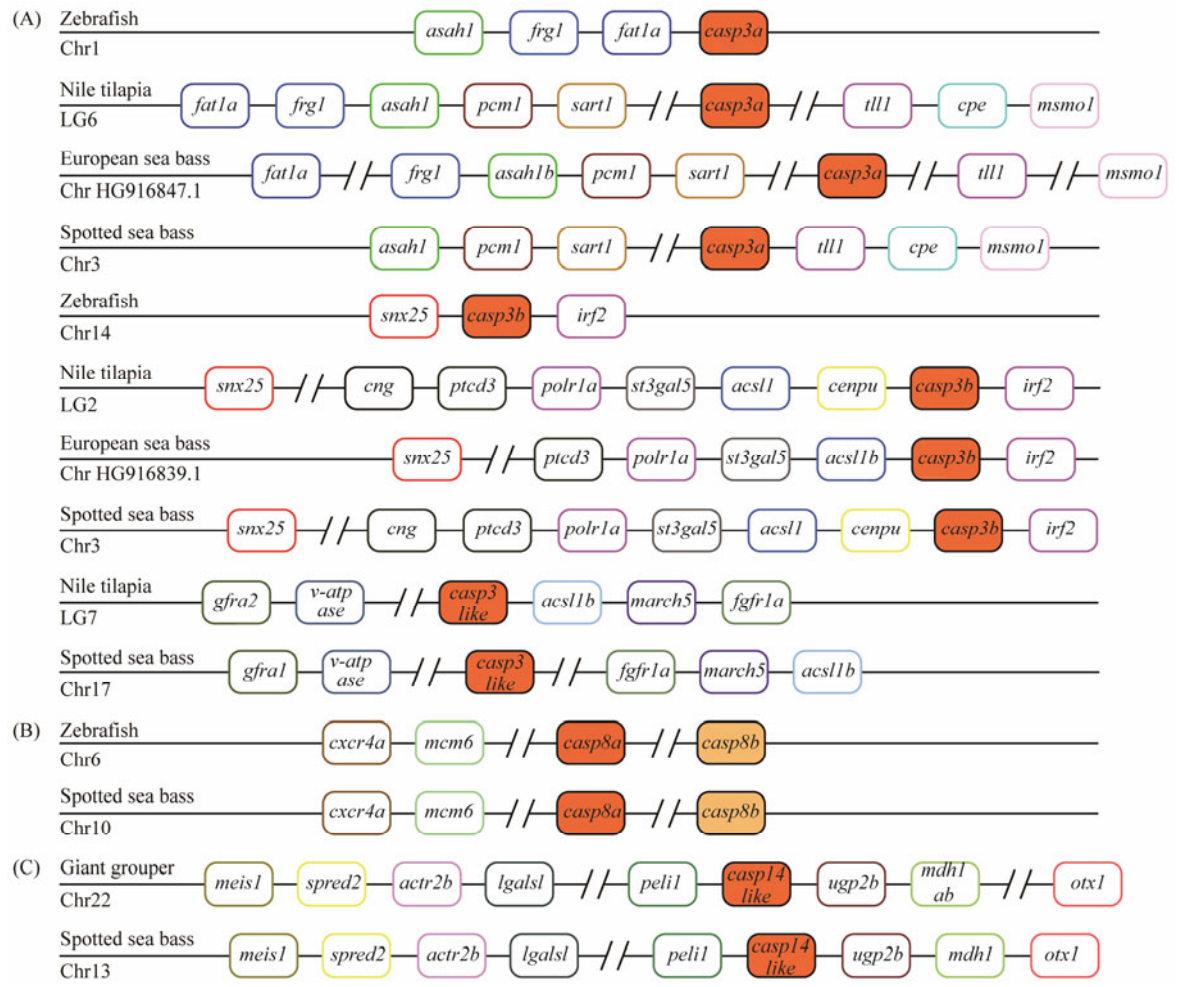

Fig.2 Syntenic analysis of caspase genes in selected vertebrates. (A) *casp-3*; (B) *casp-8*; (C) *casp-14*.

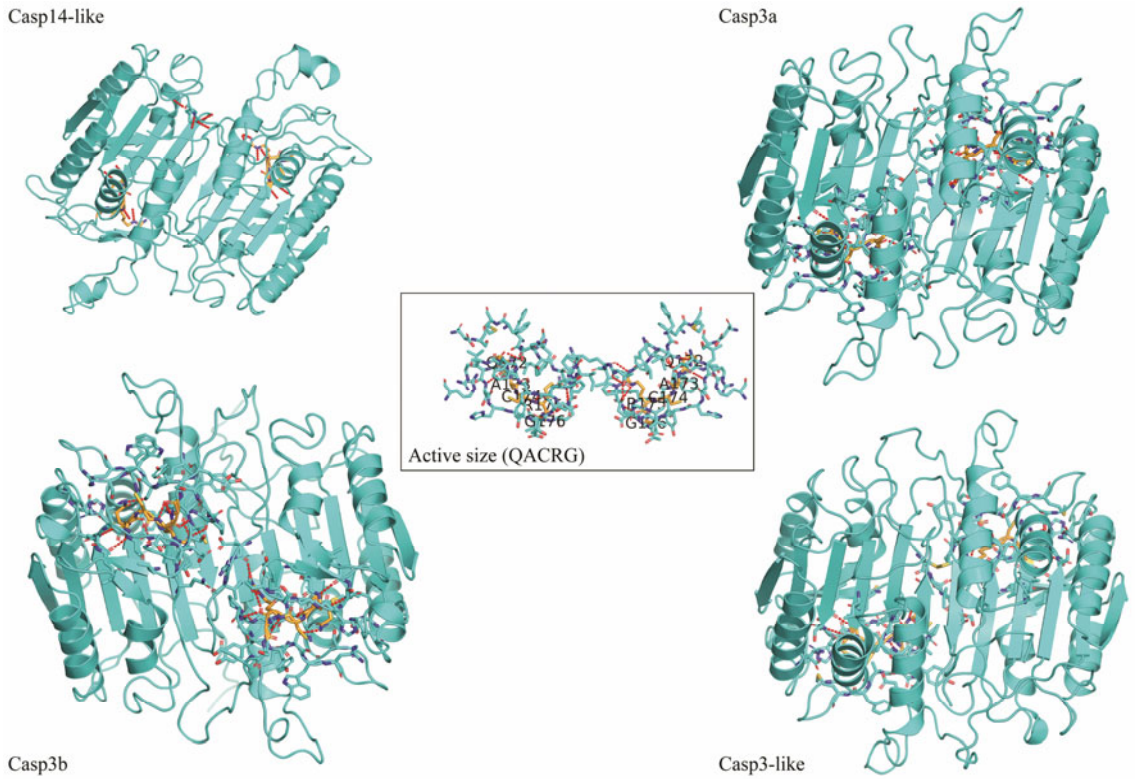

Fig.3 Comparison of three-dimensional structures (3D) of Casp-3 members and Casp-14-like. The caspase active site is enlarged in the middle.

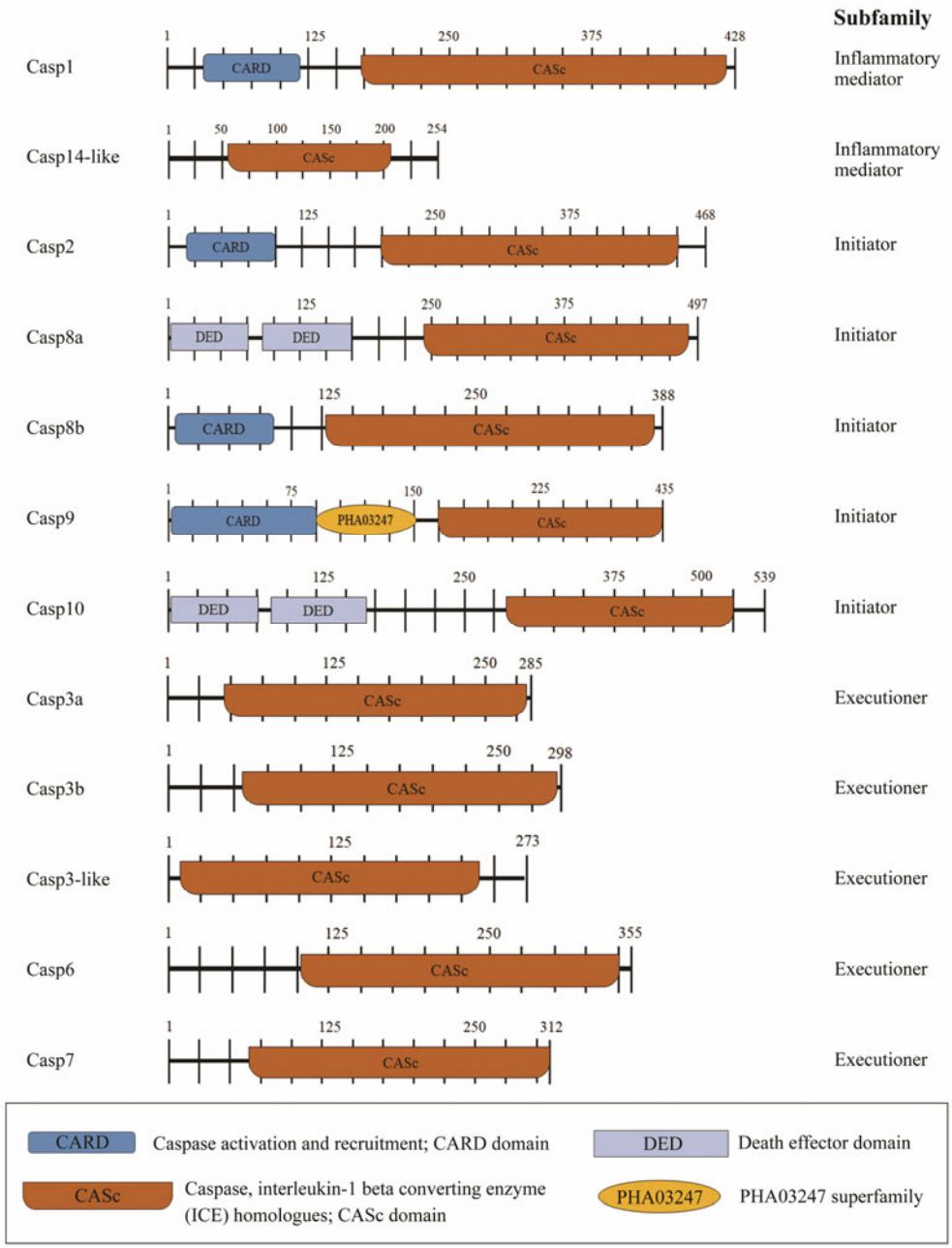

Fig.4 Schematic representation of domain structures of *casp* genes in spotted sea bass. The functional domains are marked with different color blocks.

clearly exhibited skin ulcer, hemorrhage and caudal fin erosion or fin necrosis, which are identified as the important clinical signs caused by the target bacterium (Austin and Zhang, 2006; Tian *et al*., 2019; Mao *et al*., 2020). However, no mortality was observed in these bacteria injected fish.

In detail, among the inflammatory caspase members, *casp-1* was dramatically upregulated in the head kidney at 48 h (7.07-fold), while *casp-14-like* was highly induced in the head kidney and spleen after 12 h (7.98-fold and 16.11-fold, respectively), and the highest expression values occurred at 48 h (16.51-fold and 19.97-fold, respectively) after infection. Then the expressions of *casp-1* and *casp-14-like* genes in these two tissues returned to normal levels at 72 h (Fig.6A). Consistent with our findings, various studies have demonstrated potential functions of teleost *casp-1* in immune responsive organs by testing its expression variation after bacterial challenge trials. For example, after challenged with *Aeromonas hydrophila*, the *casp-1* mRNA level of the striped snakehead (*Channa striata*) increased significantly in the head kidney and trunk kidney but showed a moderate change in the liver and spleen (Kumaresan *et al*., 2016). The expression patterns of *casp-1* after infection by *Edwardsiella tarda* showed high upregulation in the head kidney and spleen in tongue sole (Long and Sun, 2016). In addition, the *casp-1* mRNA expression in Japanese flounder exhibited significant increases in the head kidney after a challenge of bacterium *E. tarda* and in head kidney macrophages (HKMs) after challenges with LPS and poly (I:C). Further study demonstrated that inflammatory *casp-1* was involved in extracellular ATP-mediated immune signaling by interacting with apoptosis-associated speck-like (ASC) protein (Li *et al*., 2017). *Casp-1* also sig-

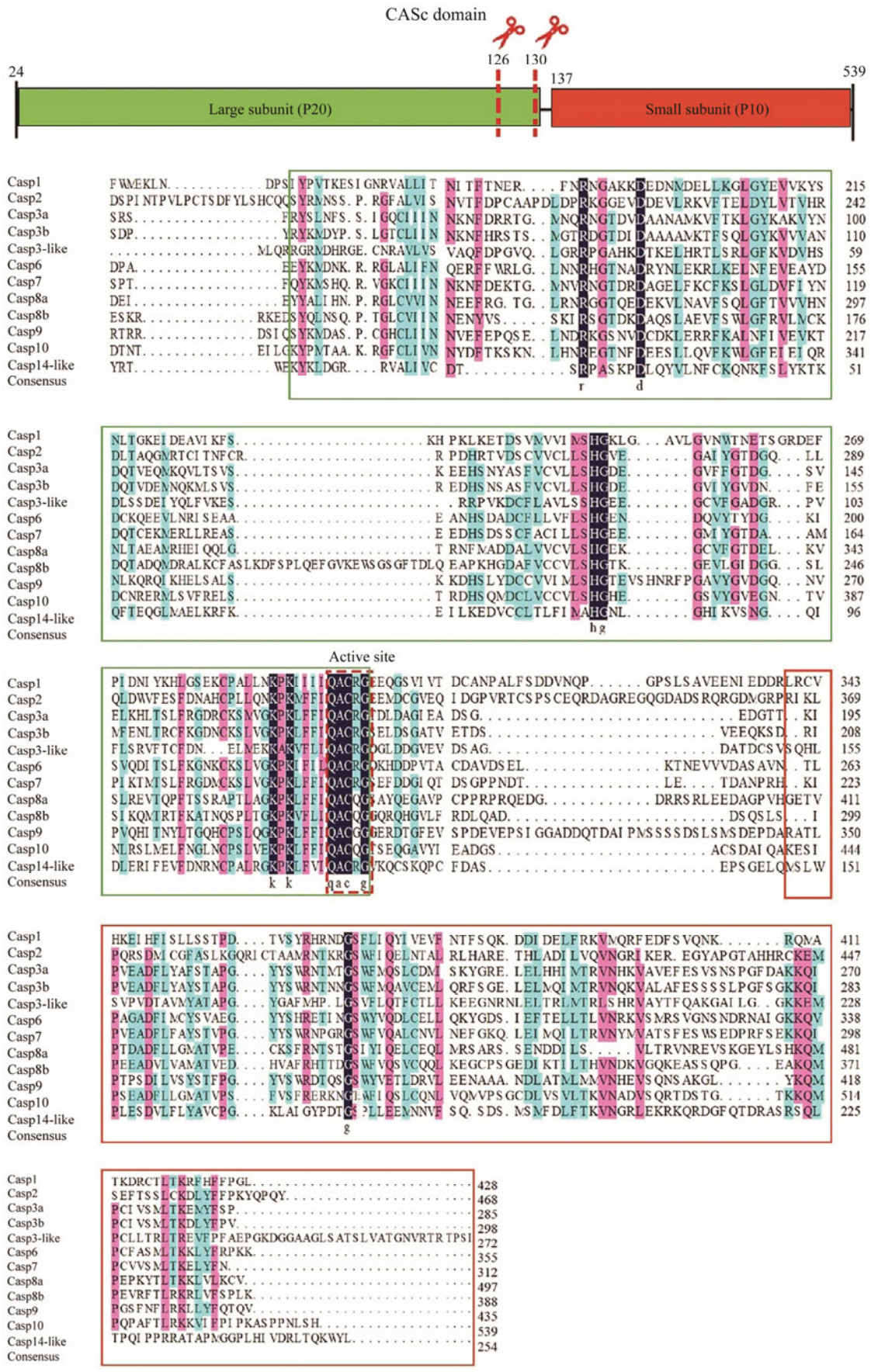

Fig.5 Schematic of the CASc catalytic domains in conserved regions in the multiple sequence alignment of spotted sea bass caspase gene families. The result was visualized using the DNAMAN software. The active site is labeled with a red box (QACNG). The corresponding sequences of large subunits (p20) and small subunits (p10) of catalytic CASc domain were marked with green and red boxes, respectively. The homology level was highlighted by different colors of shading: black for 100%, 100%＜pink＜75% and 75%＜blue＜50%.

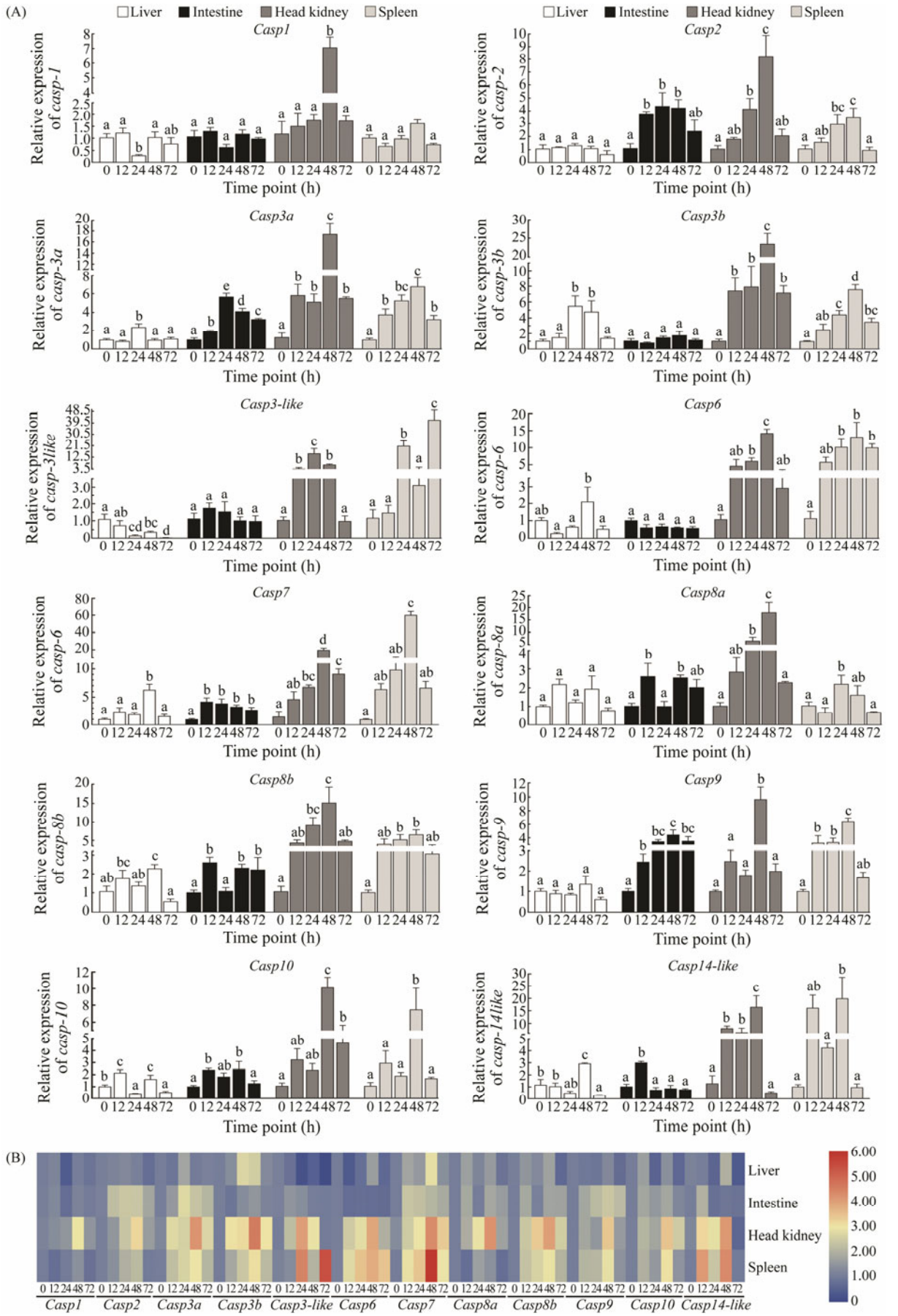

Fig.6 (A) Expression patterns of caspase genes in the liver, intestine, head kidney, and spleen of spotted sea bass at 0, 12, 24, 48 and 72 h after the *V. harveyi* infection, and different small letters represent significant differences in different periods within the same tissue ($P<0.05$); (B) The relative gene expression changes within each tissue are illustrated in heat map schematic, and the warmer colors indicate the higher expression levels.

nificantly contributes to the innate immune system and mediates the pyroptosis cascade (Winkler and Rösen-Wolff, 2015), which indicates that *casp-1* may play a crucial role in the proinflammation of the head kidney after 48 h and may contribute to pyroptosis in spotted sea bass. This hypothesis need to be further investigated in the future. However, for *casp-14*, functional studies remain scarce in teleosts, and our findings indicate that this gene may be essential to defend against bacterial infection in spotted sea bass.

It has been demonstrated that the initiator caspases regulate apoptosis through both intrinsic pathways (*casp-2* and *casp-9*) and extrinsic pathways (*casp-8* and *casp-10*) in higher vertebrates (Paroni *et al*., 2002; McIlwain *et al*., 2013). In our results, all caspase genes of apoptosis initiators (*casp-2*, *casp-8a*, *casp-8b*, *casp-9*, and *casp-10*) were significantly differentially expressed ($P<0.05$) in the intestine, head kidney, and spleen after infection to different extents (Fig. 6A). The mRNA expression levels of the 5 initiator caspase genes significantly increased at 12 h in the intestine (2.40- to 3.73-fold). Moreover, the mRNA expression levels in the head kidney were remarkably induced and achieved the highest values by 48 h (8.24- to 18.02-fold upregulation compared to the control group). Significant variations in the expression levels of the initiator caspases in the spleen (1.59- to 7.47-fold changes) were found at different time points after infection, and the highest expression changes (7.47-fold) was detected for *casp-10* at 48 h (Fig.6A). The induction of initiator caspase gene expression in immune organs or cells by bacterial infection has been illustrated in several teleosts. For example, the upregulated expression of *casp-2* and *casp-9* in the kidney and spleen after an *E. tarda* infection was reported for tongue sole (Long and Sun, 2016). In rainbow trout, *casp-8* and *casp-10a* exhibited different expression profiles between the groups challenged with *V. anguillarum* and the control group without infection (Zeng *et al*., 2021). Likewise, *casp-8* mRNA levels were stimulated with LPS and poly (I:C) treatment in HKMs of Japanese flounder (Li *et al*., 2019). For European sea bass, which is closely related to spotted sea bass, increased expression of *casp-8* in the spleen has been observed after a *Photobacterium damselae* ssp. *piscicida* (Phdp) infection and is known to trigger the selective apoptosis of macrophages and neutrophils (Reis *et al*., 2010). Furthermore, *casp-8* is a crucial initiator caspase and a molecular switch for apoptosis, pyroptosis and necroptosis. For example, *casp-8* can initiate extrinsic apoptosis and inhibit necroptosis mediated by *Ripk-3* and *Mlkl* (Fritsch *et al*., 2019). Moreover, the enzymatic function of *casp-8* is necessary to stimulate *NF-κB*, induces several key immune responses such as cytokine secretion and trigger the pattern-recognition receptors (PRRs) such as antigen receptors, Fc receptors or Toll-like receptors (Su *et al*., 2005; Philip *et al*., 2016; Fritsch *et al*., 2019). Overall, the above experimental evidence supports the involvement of teleost *casp* genes in the apoptotic pathway caused by bacterial infection.

For the executioner caspase groups, spotted sea bass contained three *casp-3* genes (*casp-3a*, *casp-3b*, and *casp-3-like*), as well as *casp-6* and *casp-7*. As shown in Fig.6A, the *casp-3* genes in spotted sea bass displayed diverse expression profiles after infection. In the liver, significant mRNA upregulation was detected for *casp-3a* and *casp-3b*, while downregulation was observed for *casp-3-like*. The opposite expression patterns suggest a compensatory mechanism for *casp-3* genes in the liver. Among the *casp-3* genes, a significant expression change in the intestine was found for only *casp-3a*, and the intestinal mRNA values for *casp-3b* and *casp-3-like* remained constant. All three *casp-3* genes in the head kidney and spleen showed highly induced expression after the stimulation with bacteria. They tended to increase firstly and then decrease subsequently, except that *casp-3-like* showed increased expression in the spleen until 72 h post-challenge. The highest expression values of *casp-3a* and *casp-3b* occurred in the head kidney at 48 h post-challenge (17.43- and 23.44-fold of those of the control, respectively) (Fig.6A). Several teleost species such as the European sea bass, tongue sole, large yellow croaker, and walking catfish (*Clarias batrachus*) have one unique copy of the *casp-3 gene*, the expression of which was also significantly increased in different immunity-related tissues after bacterial challenge (Reis *et al*., 2007; Li *et al*., 2011; Banerjee *et al*., 2012; Kumaresan *et al*., 2016; Long and Sun, 2016). These expression changes were reasonable because *casp-3* is known as the most important effector caspase activated by *casp-8* and *casp-9* in the apoptotic pathway (Kuribayashi *et al*., 2006; McComb *et al*., 2019; Ponder and Boise, 2019). Similarly, the expression levels of *casp-6* and *casp-7* greatly increased in the head kidney and spleen in the *V. harveyi*-infected fish; notably, the expression values of *casp-6 and casp-7* at 48 h reached 19.75-fold and 60.09-fold, respectively (Fig.6A). The important roles of *casp-6* in the apoptotic signaling pathway were reported in several teleost species such as rainbow trout, walking catfish and Japanese flounder (Laing *et al*., 2001; Banerjee *et al*., 2012; Li *et al*., 2019). However, the specific function of *casp-7* in the regulation of fish immunity must be further investigated.

In a summary, the overall relative expression levels of caspases were dramatically increased after *V. harveyi* challenge, which suggests the essential contribution of the caspase family to immune functions against *V. harveyi* infection in spotted sea bass. As illustrated in Fig.6B, more caspase genes in the head kidney and spleen were affected by bacterial infection than those in the liver and intestine, and the highest expression variation was observed for two executioners (*casp-7* and *casp-3-like*) in the spleen at 48 h (60.09-fold) and 72 h (41.87-fold), respectively. Thus, head kidney and spleen might be the most responsive immune organs and *casp-7* and *casp-3-like* might be the most important caspase genes related to *V. harveyi* infection in spotted sea bass.

3.5 Changes of Caspase 3/7 Activity After the *V. harveyi* Infection

Since *casp-3* and *casp-7* play important roles in the initiation process of apoptosis, they have been widely accepted as the reliable indicator of apoptosis. To investigate the degree of apoptosis of spotted sea bass after *V. harveyi* in-

fection, the caspase activity was measured in head kidney and spleen at 3 time points (0, 48 and 72 h). The results show that the caspase activity from spotted sea bass exhibited different expression profiles between two tissues (Fig.7). In detail, the fluorescence value of caspase-3/-7 at 72 h was significantly higher than those at 0 h (2.00-fold) and 48 h (1.87-fold), while there was no significant change between the 48 h and 0 h time points, indicating that the high apoptosis level appeared in the head kidney at 72 h after the infection, as shown in Fig.7A. In spleen, the fluorescence value increased firstly and then decreased, exhibiting a higher apoptosis level at 48 h after the infection, as shown in Fig.7B. In general, our results proved that the caspase family was involved in the process of apoptosis, with a crucial contribution against *V. harveyi* infection in spotted sea bass.

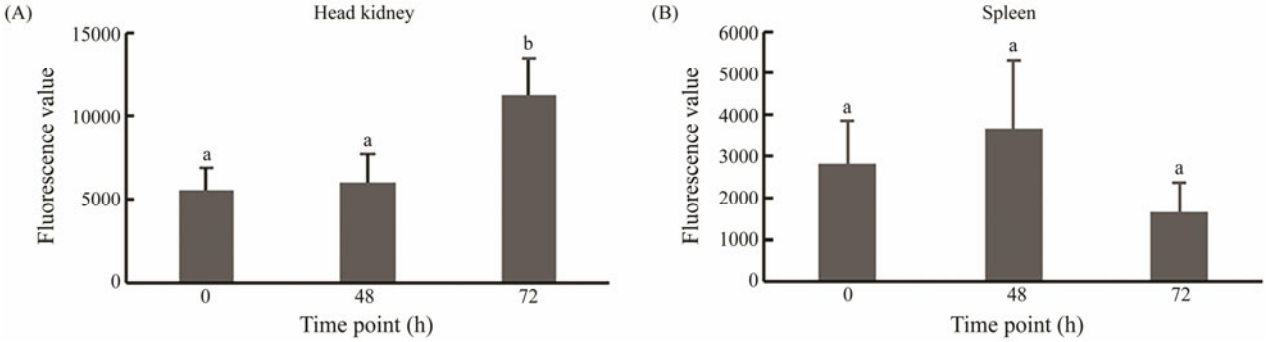

Fig.7 Fluorescence value of Caspase-3/-7 in the head kidney (A) and spleen (B) of spotted sea bass at 0, 48, 72 h after the *V. harveyi* infection.

4 Conclusions

In conclusion, we systematically identified a 12-member caspase gene family in spotted sea bass and characterized the expression patterns of these genes under *V. harveyi* induction. The confirmed genes in spotted sea bass were significantly homologous with those in other vertebrate species. The relative expression profiles after bacterial challenge show that these genes were ubiquitously expressed in crucial immune responsive organs (liver, intestine, head kidney, and spleen) in a tissue- and time-dependent manner. The tissue-based analysis shows a large responsive signal for *casp* gene family members in the head kidney and spleen. The results also indicate that spotted sea bass may present apoptosis signals after 48 h of challenge, and these signals are applicable for maintaining culture systems for disease management and control. Nonetheless, the caspase protein function requires further examination in the future.

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