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14-3-3 gene family in spotted sea bass (*Lateolabrax maculatus*): Genome-wide identification, phylogenetic analysis and expression profiles after salinity stress

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ABSTRACT

The tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation (14-3-3) proteins are a group of highly conserved homologous and heterologous proteins involved in a wild range of physiological processes, including the regulation of many molecular phenomena under different environmental salinities. In this study, we identified eleven *14-3-3* genes from the spotted sea bass (*Lateolabrax maculatus*) genome and transcriptomic databases and verified their identities by conducting phylogenetic, syntenic and gene structure analyses. The spotted sea bass *14-3-3* genes are highly conserved based on sequence alignment, conserved domains and motifs, and tertiary structural feature. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *14-3-3* genes in gill of spotted sea bass under normal physiological conditions indicated that the expression level of *14-3-3 zeta* was the highest among tested genes, followed by *14-3-3 theta*. Furthermore, expression profiles of *14-3-3* genes in gill tissue (in vivo and in vitro) indicated that the *14-3-3 zeta* and *14-3-3 theta* genes were significantly induced by different environmental salinities in spotted sea bass, suggesting their potential involvement in response to salinity challenge. Our findings may lay the foundation for future functional studies on the *14-3-3* gene family in euryhaline teleosts.

1. Introduction

Euryhaline teleosts have evolved sophisticated iono−/osmoregulatory mechanisms to maintain their plasma osmotic homeostasis during salinity adaptation. The gill is the most important organ for teleost osmotic regulation, participating in ion and water transport in seawater and freshwater (Lam et al., 2014). Na^+/Cl^- is actively absorbed in plasma-hypoosmotic environments, whereas in hyperosmotic environments, Na^+/Cl^- is secreted by the gill epithelium. To meet the altered requirements of ion transport, the morphology of the gill epithelium of euryhaline fish changes greatly during salinity acclimation (Laurent and Dunel, 1980). The 14-3-3 proteins, also known as tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation proteins, were named according to their special elution and migration characteristics in two-dimensional diethylaminoethyl-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967). At least 50 signaling proteins are ligands for 14-3-3 proteins. Through interactions with effector proteins, the 14-3-3 proteins are phosphoprotein partners and are very potent master regulators that control the activity of many signal transduction pathways in response to a changing environment (Fu et al., 2000). As ubiquitous phosphoprotein partners, 14-3-3 proteins are essential candidates for controlling the reorganization of the gill epithelium and are also involved in regulating many molecular phenomena during acclimation to different environmental salinities (Kültz et al., 2001). For example, 14-3-3 proteins regulate the activity of Na^+/K^+-ATPase and H^+-ATPase, which are essential for the regulation of ion channels and transporters, as well as regulating the cytoskeleton (Kültz et al., 2001). Since the first 14-3-3 protein was discovered by Moore and Perez (Moore and Perez, 1967) in bovine brains in 1967, increasing evidence has suggested that 14-3-3 proteins are important elements for osmoregulation and salinity adaptation (Kültz et al., 2001; Kohn et al., 2003; Scott et al., 2006; Xu and Shi, 2006; Xu and Shi, 2007; Kaeodee et al., 2011). In addition to functions in salinity regulation, 14-3-3 proteins are involved in a wide range of physiological processes, such as stress responses, immune responses, cell signal transduction, cell proliferation, cell differentiation, and

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apoptosis (Koskinen et al., 2004; Angrand et al., 2006; Neal et al., 2009; Matta et al., 2012; Rehman et al., 2014; Gómez-Suárez et al., 2016; Lu et al., 2017; Yang et al., 2017).

As highly conserved homologous and heterologous proteins, members of the 14-3-3 protein family have been found in all eukaryotes, but they have never been found in prokaryotes (Ferl et al., 2002). The 14-3-3 family comprises different members in different species. In plants, there are 5–17 members (Konagaya et al., 2004; Yao et al., 2007; Schoonheim et al., 2009; Zhang et al., 2010). Most mammalian species harbor seven *14-3-3* isoforms (β, θ/τ, ε, ζ, η, γ and σ) (Yang et al., 2017), while only two isoforms are found in insects (Skoulakis and Davis, 1996). In contrast, the number of *14-3-3* genes in teleosts varies significantly by species. In twelve investigated fish species, including *Oncorhynchus mykiss*, *Astyanax mexicanus*, *Danio rerio*, *Gadus morhua*, *Gasterosteus aculeatus*, *Lepisosteus oculatus*, *Oreochromis niloticus*, *Oryzias latipes*, *Poecilia formosa*, *Takifugu rubripes*, *Tetraodon nigroviridis* and *Xiphophorus maculatus*, the numbers of *14-3-3* genes are notably different, ranging from six to eleven (Cao and Tan, 2018).

Previous functional studies on 14-3-3 proteins mainly concentrated on plant and human disease (Layfield et al., 1996; Ostrerova et al., 1999; Wilker and Yaffe, 2004; Yang et al., 2013; Yang et al., 2014). Although 14-3-3 proteins are well known for their functions in the stress response, particularly in salinity regulation, the function of *14-3-3* genes in teleosts has rarely been reported. The spotted sea bass, *Lateolabrax maculatus*, is a euryhaline marine teleost species that can adapt to a wide range of salinities (Du et al., 2013). Thus, the spotted sea bass is an ideal model in which to investigate the mechanisms of acclimation to osmotic stress. To characterize the structures and investigate the functions of *14-3-3* genes in spotted sea bass, in this study, we identified eleven *14-3-3* genes across the spotted sea bass genome by using all available genomic and transcriptomic resources. Comprehensive analysis was performed for these genes in spotted sea bass, including analysis of gene copy numbers, molecular structures and phylogenetic relationships. To investigate the potential involvement of *14-3-3* genes in salinity regulation, we conducted an expression analysis of the spotted sea bass *14-3-3* gene family after salinity treatment. In addition, we performed an in vitro experiment on the short-term effect of salinity stress on the expression levels of the *14-3-3* genes in gill fragments from spotted sea bass and compared the in vitro expression to the expression levels of these genes in gill tissue after salinity stress in vivo. The results of a comparative study with other vertebrates and the expression patterns of each *14-3-3* gene provide insights into the potential functions of *14-3-3* genes in spotted sea bass and provide theoretical guidance for the study of the *14-3-3* gene family in other teleosts.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China (State Science and Technology Commission of the People's Republic of China for No. 2, October 31, 1988. Permit Number: 20141201. http://www.gov.cn/gongbao/content/2011/content_1860757.htm).

2.2. Identification of 14-3-3 gene sequences in spotted sea bass

To identify the *14-3-3* genes, the transcriptome database (NCBI accession numbers: SRR4409341 and SRR4409397) (Zhang et al., 2017) and the whole-genome sequence database (unpublished data) of the spotted sea bass were searched using 14-3-3 protein sequences of teleosts (*Ictalurus punctatus*, *Cyprinus carpio*, *Salmo salar*, *Danio rerio*, *Oncorhynchus mykiss*, *Oreochromis niloticus*, *Oryzias latipes*), a bird (*Gallus gallus*) and mammals (*Homo sapiens, Mus musculus*) as queries at NCBI (http://www.ncbi.nlm.nih.gov). TBLASTN was used to obtain the initial pool of 14-3-3 transcript sequences in the spotted sea bass, and BLASTN was then used to verify the cDNA sequences by comparing the transcriptome sequences with the whole-genome sequences. Open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to predict amino acid sequences, which were verified by Smart-BLAST against the NCBI non-redundant (NR) protein sequence database.

The lengths of mRNAs, 5′-untranslated regions (5'-UTRs), 3′-untranslated regions (3'-UTRs), and the number of amino acids encoded by *14-3-3* genes were obtained from the transcriptome database and genome database of spotted sea bass. The molecular weight (MW, kDa) and isoelectric point (pI) of each putative 14-3-3 proteins were calculated using the ExPASy Prot-Param tool (https://web.expasy.org/protparam/). The subcellular localization of all putative 14-3-3 proteins in spotted sea bass was predicted by two tools: subcellular localization predictor (http://cello.life.nctu.edu.tw/) (Yu et al., 2006) and the pSORT prediction software (https://www.genscript.com/wolf-psort.html) (Horton et al., 2007).

2.3. Phylogenetic and syntenic analysis of 14-3-3 genes in spotted sea bass

To investigate the phylogenetic relationship and classification of *14-3-3* genes in spotted sea bass, 41 amino acid sequences of *14-3-3* genes from selected vertebrates retrieved from NCBI, including mammals (*H. sapiens, M. musculus*, *Bos taurus*, *Papio anubis*), one bird (*G. gallus*), teleost fishes (*C. carpio, S. salar, Callorhinchus milii, D. rerio*, *Sinocyclocheilus grahami*), and one insect (*Bombyx mori*) were used for phylogenetic analysis. Multiple-sequence alignments of protein sequences were created using the ClustalX1.83 program. The phylogenetic tree was constructed using MEGA 6 by employing the neighbor-joining (NJ) method with the following parameters: Poisson correction, pairwise deletion, and bootstrapping (1000 replicates). The tree was displayed with Interactive Tree Of Life (iTOL, http://itol.embl.de/). To provide additional evidence for orthologs and identification, syntenic analysis was conducted for some *14-3-3* genes that were not well annotated by phylogenetic analysis. Syntenic analysis was conducted by comparing the genomic regions that harbored *14-3-3* genes in spotted sea bass with those in other vertebrates. The neighboring genes of spotted sea bass *14-3-3* genes were identified from the spotted sea bass genome assembly with the FGENESH program and were verified by BLAST analysis against the NCBI nonredundant database. Genomicus (Louis et al., 2015), the NCBI database (http://www.ncbi.nlm.nih.gov) and Ensembl genome browser (http://www.ensembl.org/) were used to determine the conserved syntenic pattern of *14-3-3* genes among various vertebrates.

2.4. Multiple alignment and gene structure analysis of 14-3-3 genes

Multiple-protein sequence alignments of all predicted spotted sea bass 14-3-3 proteins were performed using DNAMAN 6.0 (Lynnon Biosoft, USA) with the default parameters. To confirm the presence of the conserved domains, all spotted sea bass 14-3-3 proteins were analyzed with the NCBI conserved domain database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The DNA and cDNA sequences corresponding to each predicted *14-3-3* gene from the spotted sea bass genome and transcriptome databases were used to determine the sizes of the exons and the positions of exon-intron boundaries. Exon-intron structure schematic diagrams of the *14-3-3* genes were generated using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). Conserved motifs among the 14-3-3 proteins in spotted sea bass were predicted with Multiple Expectation Maximization for Motif Elicitation (MEME) software (http://meme-suite.org/tools/meme) (Bailey et al., 2009). The parameters were set as follows: distribution of motif occurrences, zero or one per sequence; width of motifs, 6 to 50 residues; other parameters,

default.

The model of the three-dimensional structure of spotted sea bass 14-3-3 proteins was built with the program Swiss-Model (https://swissmodel.expasy.org/) using the crystal structure of human protein as template, and all structure figures were prepared with Swiss PDB viewer 4.1.0 software.

2.5. Salinity stress challenge and sample collection

Fish for the salinity stress test (80.25 ± 10.50 g) were acquired from Shuangying aquaculture farms (Dongying, Shandong Province, China) and were temporarily housed in a 5 × 5 × 1 m cement pond for one week. Fish were fed twice a day during temporary housing. The salinity was 30.0 ± 0.5‰, and the other abiotic factors (temperature 18.5 ± 0.5 °C, dissolved oxygen ≥5.0 mg/L and pH 8.0 ± 0.3) were within the appropriate ranges for fish.

After one week of acclimation, juveniles were randomly assigned to four groups, namely, a 0 ppt group (low-salinity group), a 12 ppt group (isosmotic group), a 30 ppt group (control group) and a 45 ppt group (high-salinity group) in twelve 120 L tanks. All treatment experiments were conducted in triplicate. The sampling times were 0, 1, 3, 6, 12, 24, and 72 h, and 6 fish per salinity group were sampled at each time point. All fish sampled were quickly anesthetized with MS-222 (200 mg/L) and sampled immediately. Gill tissues were collected, temporarily placed in liquid nitrogen and then stored at −80 °C for subsequent RNA extraction.

2.6. In vitro salinity stress test of gill fragments

A gill fragment in vitro culturing system was used as described previously with slight modification (Mazon et al., 2004). Briefly, the gill arches were excised and washed in phosphate-buffered saline (PBS): 2.6 mM KCl, 1.5 mM KH_2PO4, 137 mM NaCl, 6.4 μM Na_2HPO_4, pH 7.3. The gill filaments were separated from the gill arch just above the septum. The gill filaments were washed 3 times in PBS containing penicillin (200 IU/mL) and streptomycin (200 μg/mL). Two or three gill filaments from each fish were placed in 2 mL of medium 199 (M199) in sterile 24-well culture dishes at 28 °C for 2 h before the different salinity treatments.

Triplicates were set as the biological replication. Four culturing system salinity treatments (0 ppt (low-salinity group), 12 ppt (isosmotic group), 30 ppt (control group), and 45 ppt (high-salinity group)) were performed. Before processing, sterile 10% sodium chloride solution with different concentrations was added to the culture medium to achieve the final salinity (0 ppt, 12 ppt, 30 ppt, and 45 ppt). The gill fragments were harvested after incubation for 1 h in the four salinity treatments and were stored at −80 °C for subsequent RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2.7. Total RNA extraction and qRT-PCR

The tissue samples were ground into powder with a mortar and pestle in liquid nitrogen. Following the manufacturer's protocol, total RNA was isolated from the gill for salinity treatment using TRIzol reagent (Invitrogen, USA). The concentration and integrity of tissue total RNA were measured using the Agilent 2100 Bioanalyzer System (Agilent Technologies, USA). Before first-strand cDNA was synthesized, gDNA was removed by using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara). Then, first-strand cDNA was synthesized using random primers and M-MLV reverse transcriptase (TaKaRa) according to the manufacturer's instructions. The cDNA products were stored at −20 °C for subsequent qRT-PCR.

qRT-PCR was used to determine the expression of the spotted sea bass *14-3-3* genes after salinity treatment in the gill. Primers (Table S1) were designed based on the spotted sea bass *14-3-3* gene sequences using Primer5 software (PREMIER Biosoft International). All cDNA samples were diluted to 50 ng/μL. 18S ribosomal RNA was used as an internal reference gene for gene expression normalization as described in previous studies (Aitken, 2006). Each reaction for qRT-PCR consisted of a total volume of 20 μL containing 10 μL of SYBR® FAST qPCR Master Mix (2×), 0.4 μL of ROX, 2 μL of template cDNA, 0.4 μL of each primer and 6.8 μL of nuclease-free water. PCR amplification was carried out in a 96-well optical plate with denaturation at 95 °C for 5 s followed by 40 cycles of 95 °C for 5 s and 59 °C for 30 s with a final dissociation curve to verify the specificity of the amplified products. qRT-PCR was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems), and the $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of the genes.

2.8. Statistical analysis

The data were statistically analyzed using one-way ANOVA and Duncan's multiple range tests in SPSS 19.0 software (SPSS, Chicago, IL, USA). The values are presented as the mean ± SEM (standard error of mean). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Identification of 14-3-3 genes in spotted sea bass

A total of eleven *14-3-3* genes were identified in the spotted sea bass transcriptome and whole-genome sequencing databases, including *14-3-3 beta-b*, *14-3-3 beta-a*, *14-3-3 beta/alpha*, *14-3-3 epsilon-1*, *14-3-3*

Table 1
Summary of spotted sea bass *14-3-3* genes.

Gene name	mRNA (bp)	5'-UTR (bp)	3'-UTR (bp)	ORF (bp)	MW (kDa)	pI	Localization	Accession
14-3-3 beta-a	735	–	–	735	27.81	4.65	Cy[a], Ec[a], Pp[a]	MK075249
14-3-3 beta-b	1287	136	416	735	27.69	4.71	Cy[a,b]	MF977443
14-3-3 beta/alpha	2391	221	1993	801	30.43	4.72	Cy[a]	MF977444
14-3-3 epsilon-1	2102	199	1135	768	29.08	4.71	Cy[a]	MF977446
14-3-3 epsilon-like	1129	202	159	768	29.18	4.59	Cy[a]	MF977447
14-3-3 epsilon-2	876	62	46	768	29.05	4.83	Cy[a]	MF977448
14-3-3 gamma	1098	180	174	744	28.26	4.86	Cy[a,b]	MF977452
14-3-3 theta	1419	270	396	753	28.38	4.71	Cy[a,b]	MF977445
14-3-3 zeta	1732	101	893	738	27.83	4.64	Cy[a,b]	MF977449
14-3-3 zeta-like (partial)	658	26	–	630	23.89	5.22	Cy[a]	MF977450
14-3-3 zeta/delta	1072	116	218	738	27.77	4.73	Cy[a,b]	MF977451

Abbreviations: mRNA: messenger RNA; ORF, open reading frame; MW, molecular weight; pI, isoelectric point; bp, base pairs; UTR, untranslated region; Ec, extracellular space; Pp, periplasm; Cy, cytoplasm.

[a] Localization prediction by CELLO v.2.5 (http://cello.life.nctu.edu.tw/).

[b] Localization prediction by pSORT (http://www.genscript.com/wolf-psort.html).

epsilon-2, *14-3-3 epsilon-like*, *14-3-3 zeta*, *14-3-3 zeta-like*, *14-3-3 gamma*, *14-3-3 theta* and *14-3-3 zeta/delta*. Their sequence information has been submitted to GenBank, and their accession numbers are provided in Table 1. All 14-3-3 proteins were highly conserved and had one conserved 14-3-3 domain.

The characteristics of all eleven *14-3-3* genes are summarized in Table 1, including the gene name; mRNA, 5'-UTR, and 3'-UTR lengths; MW; pI; and subcellular localization (Table 1). The complete encoding sequence was obtained for most of the genes, except for the *14-3-3 zeta-like* gene (Table 1, Fig. 3). The transcript lengths of the *14-3-3* genes ranged from 658 bp (*14-3-3 zeta-like*) to 2,391 bp (*14-3-3 beta/alpha*), the number of amino acids in the predicted 14-3-3 proteins ranged from 209 (*14-3-3 zeta-like*) to 266 (*14-3-3 beta/alpha*), the putative MWs ranged from 23.89 (*14-3-3 zeta-like*) to 30.43 kDa (*14-3-3 beta/alpha*), and the theoretical pIs ranged from 4.59 (*14-3-3 epsilon-like*) to 5.22 (*14-3-3 zeta-like*) (Table 1). Subcellular location prediction for the putative 14-3-3 proteins showed that most of them were mainly localized in the cytoplasm (Cy), except for 14-3-3 beta-a, which was distributed in the cytoplasm, extracellular space (Ec) and periplasm (Pp) (Table 1).

3.2. Gene copy number of 14-3-3 genes in spotted sea bass

The copy number of the *14-3-3* genes was investigated in spotted sea bass, and other selected vertebrates (Table 2). Among these genes, three *14-3-3* genes had two or more copies in the spotted sea bass genome, including *14-3-3 beta* (2 copies), *14-3-3 epsilon* (3 copies), and *14-3-3 zeta* (2 copies). The remaining *14-3-3* genes of spotted sea bass were present in a single copy, including *14-3-3 beta/alpha*, *14-3-3 beta*, *14-3-3 epsilon*, *14-3-3 gamma*, *14-3-3 theta*, *14-3-3 zeta* and *14-3-3 zeta/delta*.

The identities and copy numbers of the *14-3-3* genes varied significantly between species. In general, 11 *14-3-3* genes were detected in spotted sea bass (Lmu) and zebrafish (Dre); 10 in tetra (Ame) and catfish (Ipu); 8 in tilapia (Oni); 7 in fugu (Tru), human (Hsa) and mouse (Mmu); and 6 in chicken (Gga), frog (Xtr) and medaka (Ola) (Table 2). For mammals, birds and amphibians, each 14-3-3 gene existed as only one copy. However, duplicates of *14-3-3* genes existed for several genes in the teleost species analyzed. For instance, *14-3-3 beta* had two copies in spotted sea bass and three copies in zebrafish, *14-3-3 epsilon* had three copies in spotted sea bass and two copies in zebrafish, and *14-3-3 gamma* had one copy in spotted sea bass and two copies in zebrafish. Notably, the *14-3-3 sigma* gene was found only in mammals, and *14-3-3 beta/alpha* was a teleost-specific gene with different copy numbers in the tested teleosts, such as a single copy in spotted sea bass and fugu, 2 copies in medaka, 4 copies in tilapia and catfish, and 5 copies in tetra. In addition, *14-3-3 zeta/delta* was detected only in several teleosts, including spotted sea bass, catfish and tilapia, and all these species had a single copy.

Table 2
Copy numbers of *14-3-3* genes in spotted sea bass and several other vertebrates. Spotted sea bass (Lmu), zebrafish (Dre), tetra (Ame), catfish (Ipu), tilapia (Oni), fugu (Tru), medaka (Ola), human (Hsa), mouse (Mmu), chicken (Gga) and frog (Xtr).

Name	Hsa	Mmu	Gga	Xtr	Lmu	Dre	Ame	Ipu	Oni	Tru	Ola
14-3-3 beta/alpha	0	0	0	0	1	0	5	4	4	1	2
14-3-3 beta	1	1	1	1	2	3	1	0	0	1	0
14-3-3 epsilon	1	1	1	1	3	2	1	1	2	2	1
14-3-3 gamma	1	1	1	1	1	2	2	2	1	1	1
14-3-3 theta	1	1	1	1	1	2	1	1	0	1	1
14-3-3 zeta	1	1	1	1	2	1	0	0	0	1	1
14-3-3 eta	1	1	1	1	0	1	0	1	0	0	0
14-3-3 zeta/delta	0	0	0	0	1	0	0	1	1	0	0
14-3-3 sigma	1	1	0	0	0	0	0	0	0	0	0
Total	7	7	6	6	11	11	10	10	8	7	6

3.3. Phylogenetic and syntenic analysis of 14-3-3 genes in spotted sea bass

A phylogenetic tree was constructed to analyze the evolutionary relationships among the *14-3-3* genes of spotted sea bass and other vertebrates (Fig. 1). The results indicated that the *14-3-3 beta, 14-3-3 beta/alpha, 14-3-3 epsilon, 14-3-3 zeta, 14-3-3 gamma*, *14-3-3 theta*, and *14-3-3 zeta/delta* genes of spotted sea bass were categorized into the same clades as those of the other tested species and had the closest relationships with teleost sequences. However, some of the genes had multiple copies that were not sufficiently classified into the same groups and needed further analysis.

Syntenic analysis was performed to provide additional evidence for the annotation of ambiguous *14-3-3* genes, including *14-3-3 epsilon* (3 copies), *14-3-3 beta* (2 copies), *14-3-3 zeta* (2 copies) and *14-3-3 beta/alpha*. The positions of these *14-3-3* genes and their neighboring genes were identified from spotted sea bass genome scaffolds. As shown in Fig. 2, for the *14-3-3 epsilon-1* gene, a conserved synteny was identified among spotted sea bass, zebrafish, and humans that included *vps53* upstream and *crk* and *gemin4* downstream (Fig. 2A). Another annotated spotted sea bass paralog, the *14-3-3 epsilon-2* gene, shared similar neighbor genes with that of barramundi perch, including *tecta*, *sc5d* and *usp28* (Fig. 2A).

As shown in Fig. 2B, the *14-3-3 beta-a* gene was located between *rims4* and *tomm34* in the genome, an arrangement highly conserved with that of zebrafish. In addition, the spotted sea bass *14-3-3 beta-b* gene had similar neighboring genes to those in humans, including *spoll, cdh4, mtg2, lsm14b, taf4* and *col9a3*. Furthermore, the *14-3-3 beta/alpha* gene in spotted sea bass was located between *psmg2*, *lixll*, *sf3b4* and *vps72* in the genome, which was in accordance with the location of medaka *14-3-3 beta/alpha-B*. The spotted sea bass *14-3-3 zeta* gene had similar neighboring genes to those of the human and medaka *14-3-3 zeta* genes, including *taf2*, *enpp2*, *ndufaf6*, *tp53inp1* and *ccne2* (Fig. 2C). We did not find the genomic position of another copy of the *14-3-3 zeta* gene in spotted sea bass. As this copy grouped with the zeta of other species in an evolutionary tree, we named it *14-3-3 zeta-like*.

3.4. Multiple alignment and gene structure analysis of 14-3-3 genes in spotted sea bass

The predicted 14-3-3 amino acids of spotted sea bass contained nine α-helices, five conserved blocks, several conserved residues and a special sequence of a nuclear export signal (NES) involved in DNA damage (Fig. 3). Except in the N- and C-terminal regions, the amino acid sequences of the eleven proteins are highly conserved (Fig. 3). The *14-3-3 epsilon-1* and *14-3-3 beta-a* genes had six exons and six introns; the *14-3-3 epsilon-2* gene had six exons and five introns; the *14-3-3 beta-b*, *14-3-3 zeta* and *14-3-3 theta* genes had five exons and five introns; the *14-3-3 beta/alpha* gene had three exons and two introns, and the *14-3-3 gamma* gene had two exons and two introns (Fig. 4A).

To explore the structural diversity and predict the functions of the 14-3-3 proteins, a total of eleven conserved motifs in the spotted sea bass 14-3-3 proteins were identified by MEME software and annotated with the InterPro database (Fig. 4B, Table S2). The results indicated that all 14-3-3 proteins are highly conserved, with five (1-5) out of eight motifs annotated as 14-3-3 proteins (IPR000308), as they are basic characteristics of the 14-3-3 family. Except for 14-3-3 zeta-like, which is a fragment, all the 14-3-3 proteins identified contained these conserved domains. Moreover, all the 14-3-3 proteins also had motifs 6 and 7. However, 14-3-3 beta-b, 14-3-3 gamma and 14-3-3 zeta had no motif 8. The three remaining motifs, including motif 9, motif 10 and motif 11, were relatively nonconserved. This result is consistent with the results of the amino acid sequence alignment.

Prediction of the tertiary structures of the spotted sea bass 14-3-3 proteins showed a group of highly conserved proteins (Fig. 5). The proteins were highly helical and dimeric, and each monomer contained nine unique α-helices. The overall three-dimensional structure of the

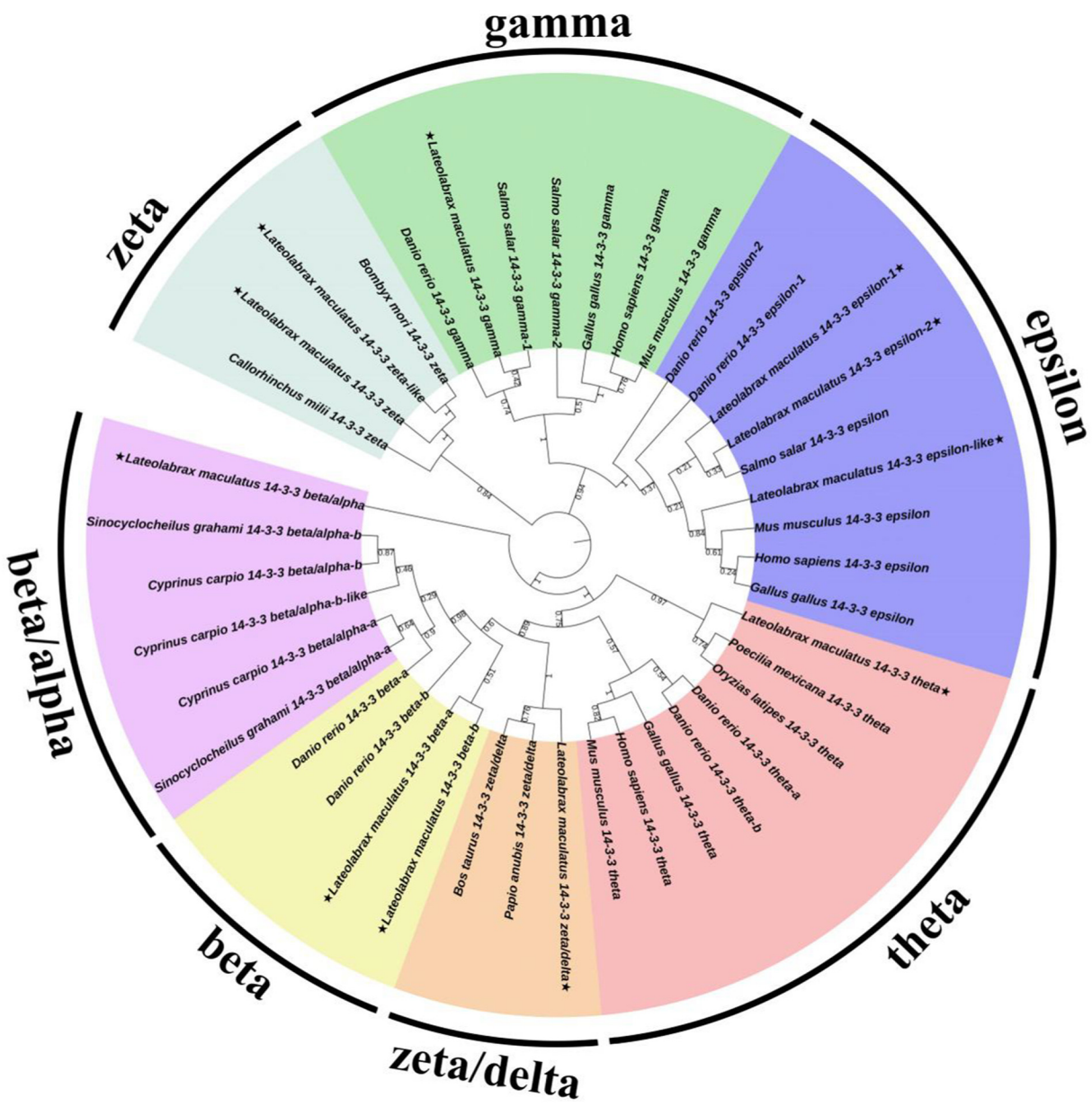

Fig. 1. Phylogenetic analysis of spotted sea bass *14-3-3* genes. The phylogenetic tree was constructed based on the *14-3-3* gene-encoded amino acids via the neighbor-joining method with 1000 bootstrap replicates in MEGA6, ClustalX1.83 and iTOL online software. The percentages of the bootstrap values are given next to the branches. The 14-3-3 proteins of spotted sea bass are indicated by stars (★).

spotted sea bass *14-3-3* genes was highly conserved compared to that of mammalian *14-3-3* genes. The nine α-helices were represented by H1-H9, which corresponded to those of mammalian *14-3-3* genes (Rosenquist et al., 2000; Kültz et al., 2001; Ferl et al., 2002; Yaffe, 2002). Spatial arrangement analysis of the nine α-helices showed that H1, H2, H4, H6 and H8 are located on the outside of the 14-3-3 proteins and that the remaining four helices (H3, H5, H7, H9) are seated in the inner face of the 14-3-3 proteins, forming an amphipathic substrate-binding groove (Kültz et al., 2001). The positions of the nine α-helices differed slightly among the different 14-3-3 protein members in spotted sea bass, which might be related to the distinct roles of the different genes in this family. In addition, the conserved sequence called the C-terminal NES, which was involved in DNA damage, was found near the C-terminus in all 14-3-3 proteins, which was consistent with the results reported by Lopez-Girona et al. (Lopez-Girona et al., 1999) and Rittinger et al. (Rittinger et al., 1999).

3.5. Expression patterns of 14-3-3 genes in the gill under normal and salinity-challenge conditions

To further test the *14-3-3* gene response to salinity challenge, we first analyzed the expression profile of these genes in the gill under normal conditions. As shown in Fig. S1, *14-3-3 theta* and *14-3-3 zeta* were significantly highly expressed in the gill, whereas no *14-3-3 epsilon-2* or *14-3-3 gamma* mRNA expression was detected in the gill. Thus, for the subsequent analyses of gene expression variation after salinity challenge, we selected only the *14-3-3* genes with detectable expression.

Based on the results of the *14-3-3* gene expression profile in the gill, we tested the variations in *14-3-3 epsilon-1*, *14-3-3 epsilon-2*, *14-3-3 epsilon-like*, *14-3-3 beta-a*, *14-3-3 beta-b*, *14-3-3 beta/alpha*, *14-3-3 theta* and *14-3-3 zeta* after salinity challenge. As shown in Fig. 6, the mRNA expression of *14-3-3 theta* and *14-3-3 zeta* in the gill of fish transferred from 30 ppt to 0, 12, and 45 ppt showed that these genes were

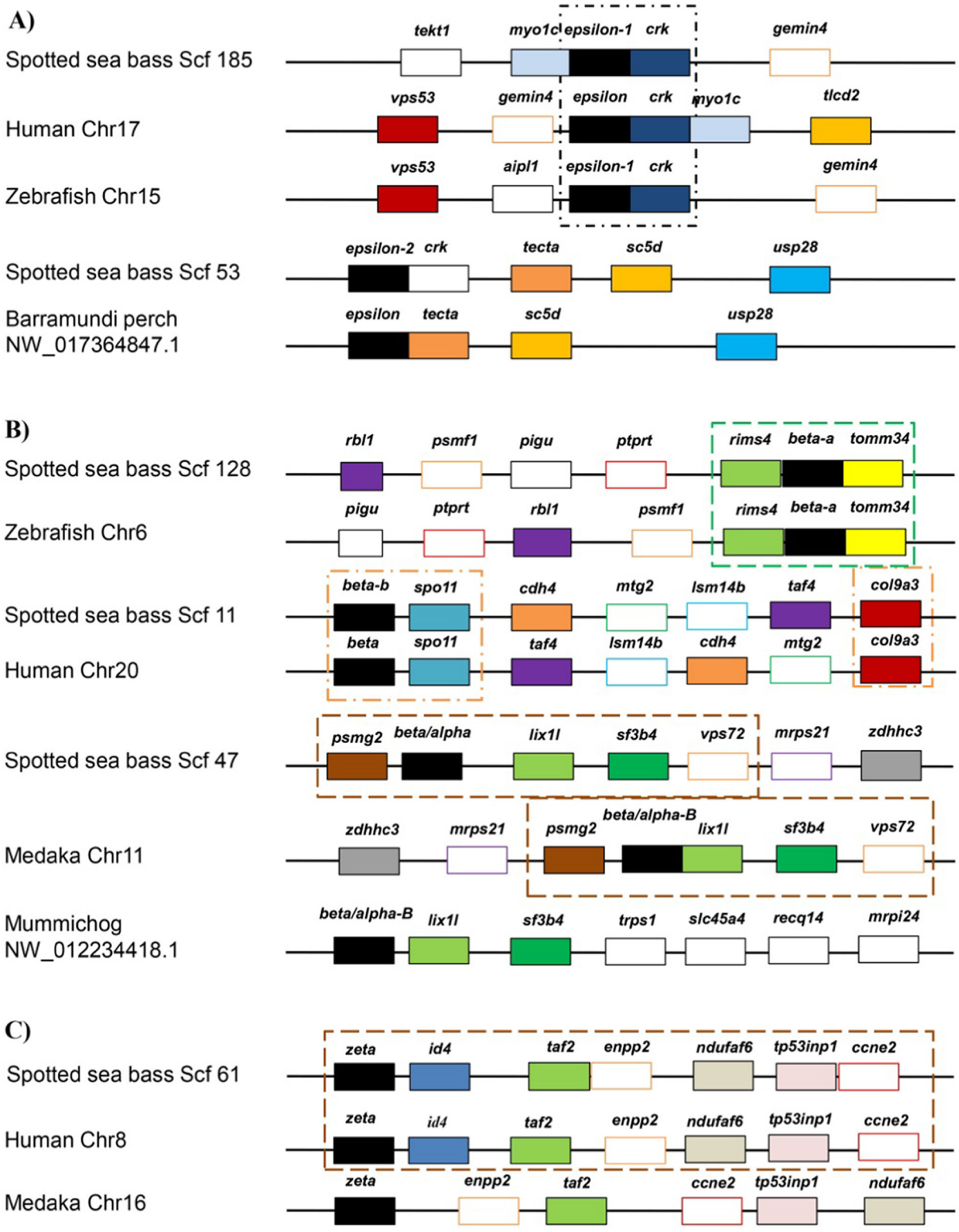

Fig. 2. Syntenic analysis of spotted sea bass *14-3-3* genes. (A) *14-3-3 epsilon-1* and *14-3-3 epsilon-2*; (B) *14-3-3 beta-a*, *14-3-3 beta-b* and *14-3-3 beta/alpha*; (C) *14-3-3 zeta*. These syntenies were generated with the information obtained from NCBI and Ensembl. Squares of the same color represent the same genes between different species, and the gene in question is highlighted in black.

osmoregulated. In all tested salinity challenges, the expression of both *14-3-3 theta* and *14-3-3 zeta* decreased significantly at all time points, and the lowest expression levels appeared at 12 ppt, which is the isotonic point for the spotted sea bass. The *14-3-3 epsilon-1*, *14-3-3 epsilon-2*, *14-3-3 beta/alpha*, *14-3-3 beta-a* and *14-3-3 beta-b* genes were all expressed in a similar pattern, but because of the lower basal expression of these genes in the gill, the variations were less important for the response to the salinity challenge. The high abundance and preferential osmotic regulation of *14-3-3 theta* and *14-3-3 zeta* mRNA in the gill clearly indicates that these *14-3-3* genes play an important

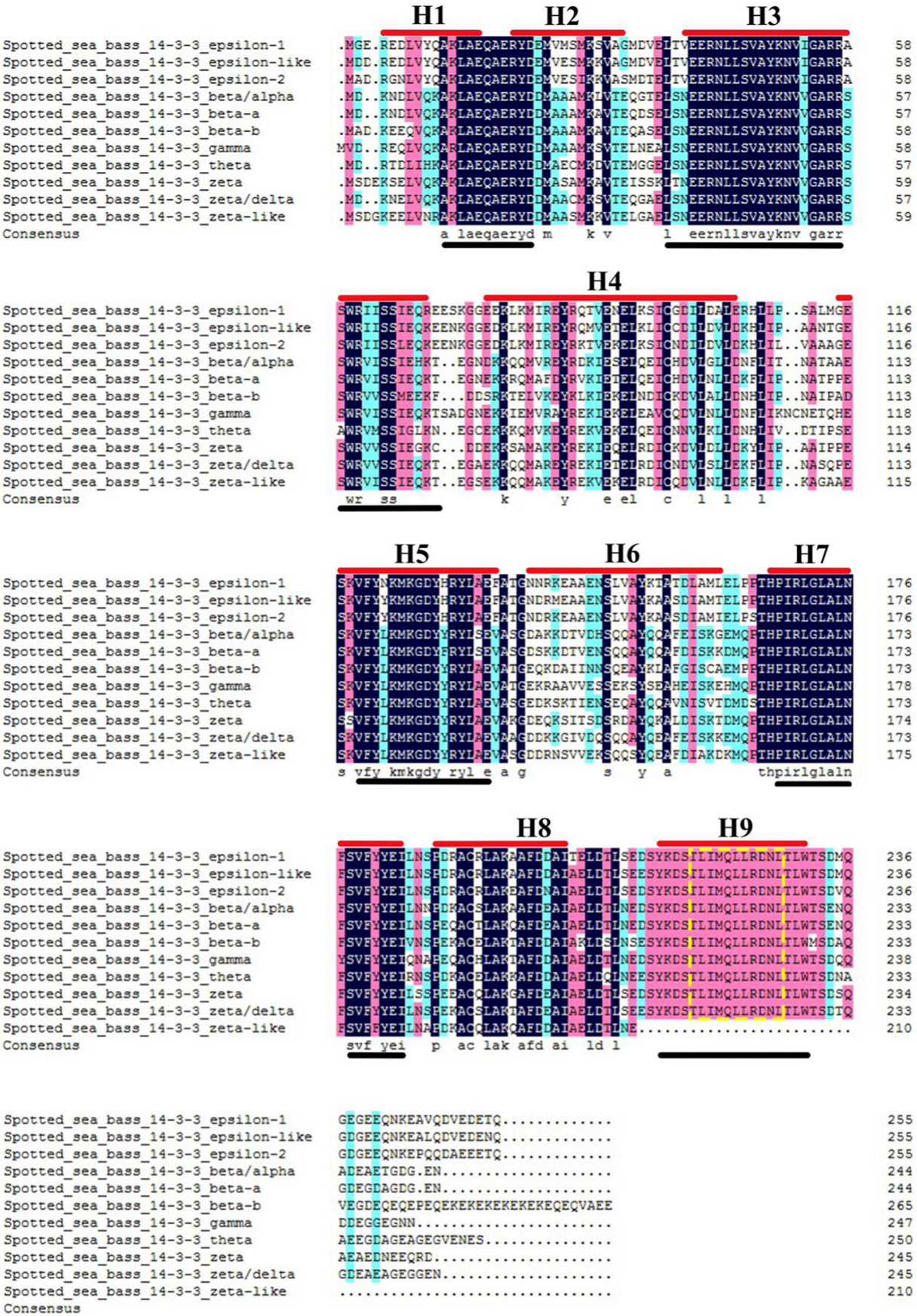

Fig. 3. Sequence alignment of spotted sea bass 14-3-3 proteins. Identical amino acid residues are shaded in black, while relatively conserved residues are shaded in pink and blue. The α-helices (H1-9) of the 14-3-3 proteins are marked with red lines. The black lines mark the five conserved blocks identified by Wang and Shakes in 1996. The yellow box indicates the nuclear export signal (NES) (sequence, LIMQLLRDMLTL) involved in DNA damage.

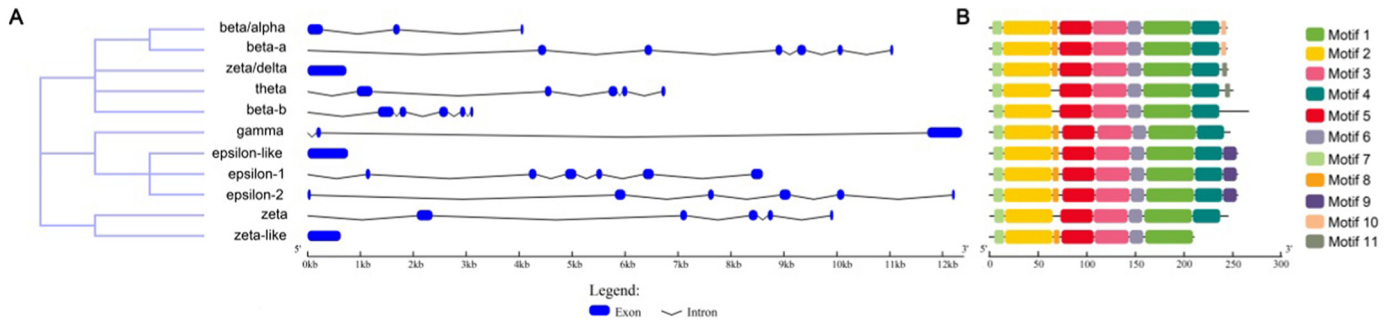

Fig. 4. Gene structure and motifs of spotted sea bass 14-3-3 genes and proteins. (A) Exon-intron structure analyses were performed using the Gene Structure Display Server database. The blue boxes indicate exons; the black lines indicate introns. (B) Eleven motifs were identified by the MEME software in the amino acid sequences of the 14-3-3 proteins. The width of each motif ranged from 6 to 50 amino acids. The differently colored blocks represent the different motifs.

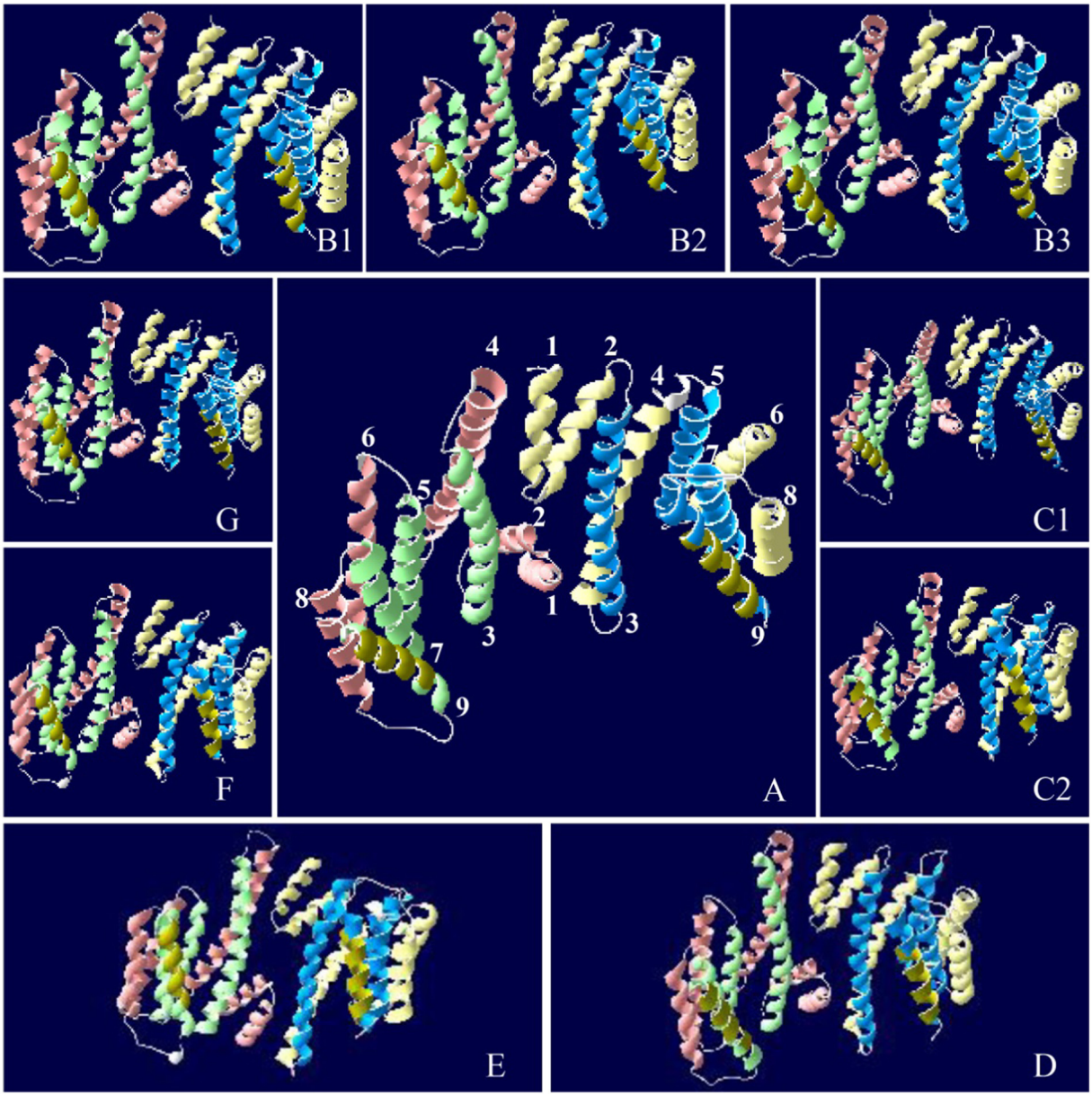

Fig. 5. Three-dimensional structural model of L. *maculatus* 14-3-3 proteins with white loops and colored helices. The helices were named H1-H9 ((A) 14-3-3 beta/alpha). The spotted sea bass 14-3-3 proteins are homologous. One monomer is in red and blue, and the other is in yellow and green. H1, H2, H4, H6, and H8 are located on the outside of the 14-3-3 proteins, and the remaining four helices (H3, H5, H7, and H9) form an amphipathic substrate-binding groove. The NES is marked with gray-green. (B1) Spotted sea bass 14-3-3 epsilon-1. (B2) Spotted sea bass 14-3-3 epsilon-like. (B3) Spotted sea bass 14-3-3 epsilon-2. (C1) Spotted sea bass 14-3-3 beta-a. (C2) Spotted sea bass 14-3-3 beta-b. (D) Spotted sea bass 14-3-3 gamma. (E) Spotted sea bass 14-3-3 theta. (F) Spotted sea bass 14-3-3 zeta/delta. (G) Spotted sea bass 14-3-3 zeta.

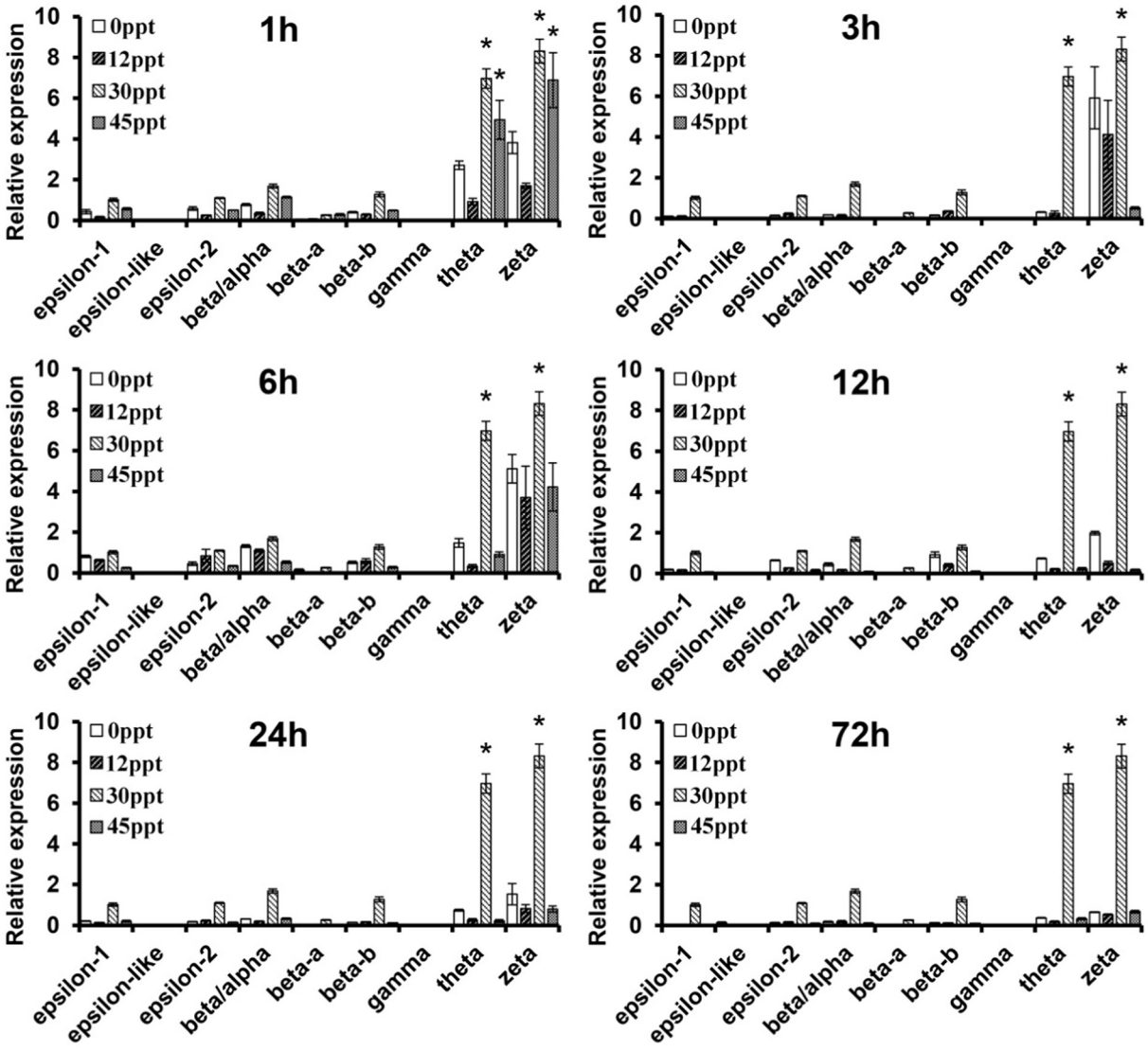

Fig. 6. Relative expression of the selected spotted sea bass *14-3-3* genes after salinity stress. qRT-PCR was used for the expression analysis in gill tissue. After standardization to the 18 s gene expression, the expression of the *14-3-3* genes was normalized to that of the *epsilon-1* gene in the 30 ppt salinity groups. The results are shown as the means ± standard error of mean (SEM). Asterisks in the same gene indicate significant differences ($P < 0.05$).

osmoregulatory role in the euryhaline spotted sea bass.

In vitro culture of gill fragments showed that the expression levels of *14-3-3 beta/alpha*, *14-3-3 theta* and *14-3-3 zeta* decreased significantly when the gill filaments were cultured in low-salinity medium (Fig. 7), indicating their potential involvement in response to salinity challenge.

4. Discussion

We carried out a comprehensive analysis of the spotted sea bass *14-3-3* gene family. Phylogenetic and syntenic analyses identified the spotted sea bass *14-3-3* genes, especially the duplicated genes, with the correct annotations. The copy numbers of the *14-3-3* genes in spotted sea bass and selected vertebrates indicated that nearly all of the genes are conserved in mammals (humans and mice), birds (chickens), and amphibians (frogs). However, there was a great difference in the copy numbers of the *14-3-3* genes in teleost fishes, which had more copies of some *14-3-3* genes than did mammals, birds and amphibians. 14-3-3 protein conserved domain and motif analyses showed that each 14-3-3 protein in spotted sea bass shared the same conserved domains and motifs (Fig. 3B). Therefore, the conserved domains and motifs further support the results of the phylogenetic and syntenic analyses.

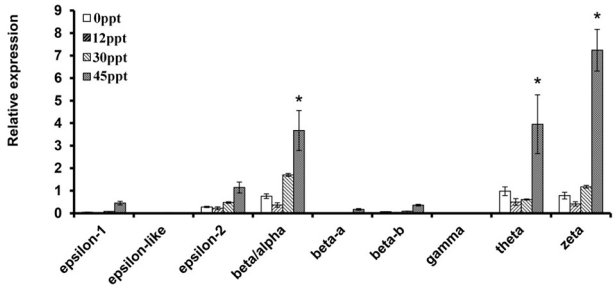

Fig. 7. Relative expression of the spotted sea bass *14-3-3* genes in in vitro salinity stress tests of gill fragments as assessed by qRT-PCR. After standardization to *18 s* gene expression, the expression of the *14-3-3* genes was normalized to that of the *14-3-3 epsilon-1* gene at 30 ppt. The results are shown as the means ± standard error of mean (SEM). An asterisk on a column indicates a significant difference ($P < 0.05$).

The first crystal structures of mammalian 14-3-3 proteins suggested that each monomer in 14-3-3 dimers consists of nine antiparallel helices constituting a protective fence around an amphipathic groove (Liu et al., 1995). Thereafter, in vertebrates, yeast and plants, the residues

located on the dimer surface were found to be invariable, indicating the conservation of 14-3-3 proteins in structure and function. Additionally, many studies had shown that 14-3-3 proteins include nine α-helices (Wang and Shakes, 1996; Rosenquist et al., 2000; Kültz et al., 2001; Ferl et al., 2002; Yaffe, 2002). Similarly, in the spotted sea bass, the 14-3-3 protein family was highly conserved, with nine α-helices. On the other hand, in the structure of 14-3-3 proteins, five conserved blocks have been found in an analysis of 46 different 14-3-3 sequences (Wang and Shakes, 1996). In this study, we also found five blocks in all the 14-3-3 s, and their amino acid sequences were highly conserved. Moreover, we found a number of conserved residues located both inside and outside of the five blocks. These residues may be related to some of the functions of these proteins, including stabilization of the monomer structure, target protein binding, and dimer formation (Rosenquist et al., 2000). The interaction between 14-3-3 proteins and other proteins may be the result of the phosphorylation of one of the above residues. It has been reported that dimer formation involves an interaction between N-terminal parts, including the conserved block 1 and some conserved residues in H3 and H4 (Jones et al., 1995; Liu et al., 1995; Luo et al., 1995; Wu et al., 1997; Abarca et al., 1999; Rosenquist et al., 2000). As in the above studies, these domains were also found to be highly conserved in this study.

The physiological systems are challenged or stressed when the teleosts experience environmental disturbances. Salinity adaptation by euryhaline teleosts is a complex process involving a set of physiological responses by osmoregulatory organs to milieus with differing osmoregulatory requirements (Hwang and Lee, 2007; Evans, 2008). The 14-3-3 proteins are involved in a wide range of physiological processes as regulatory proteins, such as stress responses, cell signal transduction, cell proliferation, cell differentiation and apoptosis (Dougherty, 2004; Mackintosh, 2004; Van-Hemert et al., 2004; Aitken, 2006). Thus far, different *14-3-3* genes have been found in virtually all tissues of different species, and their functions differ. Many studies and specific reviews have shown the functional specificity of different *14-3-3* isoforms by calculating their affinities (Rosenquist et al., 2000; Mhawech, 2005; Xu and Shi, 2006; Wanna et al., 2010). In this study, we found eleven *14-3-3* genes in spotted sea bass, and the expression of nine genes was analyzed in the gill (Table 2; Fig. 6-7, Fig. S1-S2). The *14-3-3* genes had different expression levels in the gill tissue and in response to salinity stress. The expression level of *14-3-3 zeta* in the gill was the highest, followed by *14-3-3 theta*. Only trace amounts of the other *14-3-3* genes were observed.

During the 72h of salinity stress, the *14-3-3 zeta* and *14-3-3 theta* genes had high initial expression but significantly downregulated 72 h after the salinity changed from 30 ppt to 0 ppt, 12 ppt or 45 ppt. These results suggest that fish may respond to salinity stress by downregulating the *14-3-3 theta* and *14-3-3 zeta* genes, enabling fish to adapt to extreme changes in salinity. Similarly, when *Penaeus monodon* were transferred from 3 ppt to 25 ppt or 40 ppt, a strong decrease in *14-3-3 zeta* and *epsilon* mRNA expression was observed in the gills (Kaeodee et al., 2011). In contrast, *14-3-3 epsilon* mRNA expression in shrimp transferred from 40 ppt to 3 ppt increased significantly, and *14-3-3 epsilon* played a major role in the low-salinity stress response of *P. monodon* (Kaeodee et al., 2011). In *Fundulus heteroclitus* gills, 14-3-3a transcript levels significantly increased after transfer from seawater to freshwater (Kültz et al., 2001). It is worth noting that expression of the *epsilon-like* gene in the gill was lowest in the control group after salinity stress, while it was significantly upregulated upon high- or low-salinity stress (Fig. S2). After 72 h of salinity stress, the expression of the *epsilon-like* gene increased 68- to 126-fold and 38-fold in the low- and high-salinity groups, respectively. In our experiments, the induction of *epsilon-like* in the gills may have been related to increases in the activity of H + -ATPase on the cell membrane. However, the expression of the *14-3-3 epsilon-like* gene in gill tissue was extremely low, indicating that this gene may not play a regulatory role in salinity stress. The above evidence suggests that different *14-3-3* genes have different functions and that some genes may also be functionally replaced by other genes in response to environmental stress.

Short-term in vitro tissue culture provides an effective way to study isolated tissue function. In vitro tissue culture has some advantages, including eliminating the variable internal factors that may disturb experiments in vivo, promoting research on the effects of environmental factors on specific tissues under stringently controlled conditions and reducing the number of experimental animals required for a given series of experiments (Mazon et al., 2004). The gill is the most critical tissue in fish responses to salinity stress. In the in vitro culture system, because of the lack of a blood circulation system, the cultured gill filaments were not able to be cultured for a long time in the high-salinity challenge. Additionally, according to our results, the responses of the *14-3-3* genes to salinity were less significant than those in the in vivo salinity challenge. However, the various trends of the *beta/alpha*, *theta* and *zeta* expression levels were consistent with those in vivo.

In conclusion, the present study provides initial insight into the *14-3-3* family members in the spotted sea bass, including their identification, expression profiles in the gill and direct responses to salinity changes. Our findings suggest that the *14-3-3 theta* and *14-3-3 zeta* genes of the *14-3-3* gene family are able to mediate the physiological response to salinity challenge in euryhaline teleosts.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.05.005.

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