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Research paper

# Identification of *mapk* gene family in *Lateolabrax maculatus* and their expression profiles in response to hypoxia and salinity challenges

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ABSTRACT

Mitogen-activated protein kinases (MAPK) superfamily exerts crucial roles in the regulation of intracellular metabolism, gene expression as well as integral activities in diverse cellular processes. In our study, 14 *mapk* genes were identified in spotted sea bass (*Lateolabrax maculatus*) and these genes were divided into three subfamilies according to phylogenetic analysis, including 6 *erk* genes, 3 *jnk* genes and 5 *p38-mapk genes*. Syntenic and tertiary structure analysis confirmed their annotation and orthologies. The expression profiles of several stress-responsive *mapk* genes of spotted sea bass were examined in gill using quantitative real-time PCR after salinity (0‰, 12‰, 30‰, 45‰) and hypoxia challenges (DO = 1.0 ± 0.2 mg/L). After salinity challenge, significant up-regulations were observed in the expressions of *mapk8* (*jnk1*), *mapk9* (*jnk2*), *mapk11* (*p38β*), *mapk14a* (*p38a*) and *mapk14b* (*p38b*). *mapk8* and *mapk9* were more sensitive to hypotonic challenge (0‰) than hyperosmotic (45‰) and isosmotic challenges (12‰), while the highest expression of *mapk11*, *mapk14a and mapk14b* were observed in hyperosmotic challenge (45‰). After hypoxia challenge, the expression levels of *mapk1* (*erk2*), *mapk3* (*erk1*) and *mapk14a* (*p38a*) in treatment group (DO = 1.0 ± 0.2 mg/L) were significantly up-regulated in comparison with control group (DO = 8.0 ± 0.5 mg/L) in time-dependent manner. These results indicated that these *mapk* genes in spotted sea bass may play important roles in response to salinity and hypoxia challenges.

## 1. Introduction

Mitogen-activated protein kinases (MAPK) pathways play important roles in diverse cellular processes including gene transcription, cytoskeletal organization, metabolite homeostasis, cell growth and apoptosis in response to many different extracellular signals (Garrington and Johnson, 1999; Kyriakis and Avruch, 2012; Roux and Blenis, 2004). These multifunction pathways are conserved in evolution from yeast to human, which are ubiquitous in all eukaryotic cells (Waskiewicz and Cooper, 1995; Cowan and Storey, 2003). Based on the consensus of the dual-phosphorylation site, MAPK is divided into three major subfamilies: the extracellular signal regulated kinases (ERK), the c-Jun amino-terminal kinases (JNK), and the p38-MAPKs. The ERKs have a TEY activation domain (Thr-Glu-Tyr) and JNKs contain a TPY activation domain (Thr-Pro-Tyr), whereas p38-mapk genes comprise a TGY activation domain (Thr-Gly-Tyr) (Johnson and Lapadat, 2002). Each MAPK subfamily phosphorylates specific serines and threonines of target protein substrates and mediates biochemically distinct signal cascades.

Because of the importance in controlling cellular responses to the environment and in regulating gene expression, cell growth and apoptosis, *mapk* genes have been studied extensively to define their roles in physiology in mammals (Seger and Krebs, 1995; Waskiewicz and Cooper, 1995; Karin, 1998; Sheikh-Hamad and Gustin, 2004). In general, the ERK subfamily (*erk1*, *erk2*, *erk3*, *erk4*, *erk5*, *erk7* also named as *mapk3*, *mapk1*, *mapk6*, *mapk4*, *mapk7*, *mapk15*) respond to many different stimuli such as growth factors, cytokines, virus and carcinogens, and stimulate transcriptional responses in the nucleus. Activation of ERK pathways lead to the mediation of cell division, development, migration and survival (Cowan and Storey, 2003). Both JNK (*jnk1*, *jnk2*, *jnk3* also named as *mapk8*, *mapk9*, *mapk10*) and p38 (*p38α*, *p38β*, *p38γ*, *p38δ* also named as *mapk14*, *mapk11*, *mapk12*, *mapk13*) are activated by

*Abbreviations:* cpne5, copine-5; dclre1b, 5′ exonuclease appllo; E, Glutmamic acid; ERK, extracellular signal regulated kinases; fance, Fanconin anemia group E protein; G, Glycine; Glu, Glutmamic acid; Gly, Glycine; HIF-1, hypoxia-inducible factor-1; JNK, c-Jun amino-terminal kinases; lhfpl5, tetraspan membrance protein of hall cell stereocilli; MAPK, Mitogen-activated protein kinases; mkrn, E3 unbiquitn protein ligase makorin; P, Proline; Pro, Proline; rpl10a, 60s ribosomal protein L10a; S, Serine; srpk1b, serine/arginine rich-protein specific kinase 1b; T, threonine; Thr, threonine; Tyr, Tyrosine; Y, Tyrosine

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numerous physical and chemical stresses, including hormones, UV irradiation, osmotic shock and heat shock (Minet et al., 2000; Wada and Penninger, 2004; Qi and Elion, 2005; Krens et al., 2006).

Compared with higher vertebrates, although studies about *mapk* genes were barely in teleost, increasing number of publications about roles of *mapk* genes in fishes has been reported over the last 20 years. The three subfamilies have been characterized in several teleost fishes including zebrafish (*Danio rerio*) (Krens et al., 2006; Shi and Zhou, 2010), carp (*Cyprinus carpio*) (Hashimoto et al., 1997; Hashimoto et al., 2000), rainbow trout (*Oncorhynchus mykiss*) (Urushibara et al., 2009), gilthead sea bream (*Sparus aurata*) (Feidantsis et al., 2012), European sea bass (*Dicentrarchus labrax*) (Antonopoulou et al., 2013) and Atlantic salmon (*Salmo salar*) (Holen et al., 2011). Functional studies about *mapk* genes of aquatic animals have been performed in a few of species. For examples, Marques et al. (2008) reported that MAPK pathways in the hearts of zebrafish (*D. rerio*) were involved in the mechanism of increased tolerance of hypoxia. Zhang et al. (2016) reported that ERK and p38 pathway has been implicated in regulating the hypoxia-inducible factor-1 (*HIF-1*) signaling pathway and hypoxia adaptation in dark barbel catfish (*Pelteobagrus vachelli*). For the hypoxia tolerance in channel catfish (*Ictalurus punctatus*), many genes surrounding the identified QTLs are known to be functionally related to cell adaption and response to hypoxic stress, and they are mostly involved in MAPK signaling pathways (Wang et al., 2017). In marine periwinkle (*Littorina littorea* L.), low dissolved oxygen exposure was shown to upregulate the expression of p38 MAPK (Larade and Storey, 2006). Previous study revealed that MAPKs are involved in the induction of expression of *heat shock protein* genes in mantle tissue and posterior adductor muscle of mussels (*Mytilus galloprovincialis*) during hypoxia challenge (Anestis et al., 2010). In addition, differentially expressed genes of oriental river prawn (*Macrobrachium nipponense*) under chronic hypoxia stress were significantly enriched in MAPK signaling (Sun et al., 2015). It was also observed that *jnk* and *p38* genes in killifish (*Fundulus heteroclitus*) were implicated in osmotic regulation (Kultz and Avila, 2001; Marshall et al., 2005). Nevertheless, it is of great significance to further understand the gene structures and functions about teleost *mapk* genes.

Spotted sea bass, *Lateolabrax maculatus*, is a euryhaline marine teleost naturally distributing along China's coastline and the borders of Vietnam and Korea (Shao et al., 2009; Zhang, 2001). It's considered as one of the leading aquaculture marine fish in China because of its high yield, high nutritive value and pleasant taste. In this study, a complete set of *mapk* genes were identified and annotated from spotted sea bass. To study orthologies and paralogies of these genes and confirmed gene annotations, phylogenetic and syntenic analysis were conducted. With the interest of understanding the involvement of *mapk* genes of spotted sea bass in response to hypoxia and salinity stress, the mRNA expression patterns of *mapk* genes in gill tissues were determined after salinity and hypoxia challenges.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments involving animals were conducted according to the guidelines and approved by the respective Animal Research and Ethics Committees of Ocean University of China. The field studies did not involve endangered or protected species and experiments were performed in accordance with relevant guidelines.

### 2.2. Gene identification and sequence analysis

To identify *mapk* genes in *L. maculatus*, reference genome (unpublished) and transcriptomic database (SRR4409341, SRR4409397) were searched by TBLASTN using sequences of *mapk* genes from human (*Homo sapiens*) and zebrafish (*D. rerio*) retrieved from the GenBank (NCBI) as queries, with a cutoff E-value of $1e^{-5}$. To remove duplicates and obtain a unique set of sequences, initial sequence pool was aligned by ClustalW2 program (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Open reading frame (ORF) were predicted and the retrieved sequences were translated by ORF Finder (https://www.ncbi.nlm.nih.gov/gorf/gorf.html). Predicted ORFs were then validated by BLASTP against NCBI non-redundant protein database (nr). The conserved domains were identified and predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/).

### 2.3. Phylogenetic analysis

Phylogenetic analysis was conducted using the amino acid sequences of *mapk* genes from *L. maculates* and several representative vertebrates retrieved from NCBI, including human (*H. sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish (*D. rerio*), tilapia (*Oreochromis niloticus*), Atlantic salmon (*S. salar*) and killifish (*F. heteroclitus*). Multiple amino acid sequences were aligned by ClustalW2 program (https://www.ebi.ac.uk/Tools/msa/clustalw2/) with default parameters (Edgar, 2004). The phylogenetic tree was constructed using MEGA 7 with neighbor-joining method. JTT (Jones-Taylor-Thornton) + I (invariant sites) + G (gamma distribution for modeling rate heterogeneity) model was selected and bootstrapping with 1000 replications was conducted to evaluate the phylogenetic tree (Tamura et al., 2011).

### 2.4. Syntenic analysis

To provide additional evidence for the annotation, syntenic analysis was conducted for the duplicated copies, *mapk14a* and *mapk14b* respectively. The neighboring genes of *mapk14* were identified from *L. maculatus* reference genome and further confirmed by BLAST against non-redundant (nr) database. The conserved syntenic regions of *mapk14* in other species were determined by Genomicus (Louis et al., 2015) and Ensembl genome databases (http://www.ensembl.org/).

### 2.5. Tertiary structure analysis of spotted sea bass mapk genes

The amino acid sequences of human, zebrafish and spotted sea bass *mapk* genes were submitted to Swiss-Model (http://swissmodel.expasy.org/) (Biasini et al., 2014) to construct the three-dimensional (3D) protein structure models and images were created by Swiss-Pdb Viewer 4.10 software.

### 2.6. Salinity challenge

To investigate the expression patterns of *L. maculatus mapk* genes under salinity challenge, acute salinity stress experiment was performed in Dongying Shuangying Aquaculture Company, Shandong Province, China. 300 fish individuals (body length: 21.27 ± 0.54 cm, body weight: 142.76 ± 17.44 g) were randomly collected and acclimated for 7 days in a square tank [5 × 5 × 1 m (L × W × H)]. Water temperature (13.5–14.5 °C), pH (7.8–8.15), salinity (30–33 ppm) and DO (6.7–7.5 mg/L) were stabilized during the acclimation.

After acclimation, these 300 fish were randomly transferred to tanks with 0‰, 12‰ (isotonic point), 30‰ and 45‰ salinities, respectively. 12 rectangular tanks (100 × 65 × 60 cm, water volume 300 L) were used for salinity challenges experiment at a density of 25 fish per tank (3 replicated tanks per salinity treatment). The desired salinity was adjusted by adding NaCl to seawater (30‰) or mixing seawater (30‰) with fresh water (0‰). 3 individuals per tank were sampled at each time points including 0 h, 1 h, 3 h, 6 h, 12 h, 24 h and 72 h following exposure to different salinities. Sampled fish were anesthetized with tricaine methanesulfonate (MS-222), and gill tissues were quickly dissected and flash frozen in liquid nitrogen for RNA extraction. No fish died during the experimental processes.

### 2.7. Hypoxia challenge

For hypoxia challenge experiment, 150 fish individuals (body length: 21.75 ± 0.77 cm, body weight: 162.08 ± 22.81 g) were randomly assigned to control and hypoxia groups at a density of 25 fishes per tank (3 replicated tank per treatment). Oxygen level of control group was kept at 8.0 ± 0.5 mg/L under normoxic conditions. The hypoxia dissolved oxygen value (1.0 mg/L) was chosen on the basis of the previous studies about the hypoxia stress of spotted sea bass (Chang et al., 2018). Before the experiment, oxygen level of hypoxia group was reduced to 1.0 ± 0.2 mg/L by bubbling nitrogen gas. Hypoxia was maintained by continuous bubbling of nitrogen gas for 24 h. Oxygen concentration was measured with YSI DO200 oxygen meter (YSI EcoSense, OH, USA). 3 individuals per tank were sampled at each time points including 0 h, 1 h, 3 h, 6 h, 12 h, and 24 h after hypoxia challenge. Individuals were anesthetized with MS-222, gill tissues were quickly dissected and flash frozen in liquid nitrogen for RNA extraction. No fish died during the experimental processes.

### 2.8. RNA extraction and quantitative real-time PCR analysis (qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions and digested with RNase-free DNase I (Takara, Otsu, Japan) to remove genomic DNA contamination. RNA concentration and integrity were measured using Biodropsis BD-1000 spectrophotometric absorbance (Beijing Oriental Science & Technology Development Ltd., Beijing, China) and 1.5% agarose gel electrophoresis (AGE). Equal amounts of RNA from the gill tissues of 9 fish individuals from 3 replicated tanks under the same conditions and time points were pooled as one sample to minimize the variation among individuals, and such pools were obtained for each salinity treatment and hypoxia treatment group. cDNA synthesis was then performed using PrimeScript™ RT reagent Kit (Takara, Otsu, Japan) following the manufacturer's instructions. Primers for tested *mapk* genes were designed by Primer 6 software on the basis of the least conserved regions of these genes. The 18 s rRNA was set as an internal reference gene. Prior to qPCR, the specificity of these primers was verified by dissociation curve analysis. qPCR was performed on the Applied Biosystems 7300 machines (Applied Biosystems, CA, USA) under following conditions: 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 60 °C for 30 s, followed by 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Each PCR reaction consisted of 2 μL cDNA, 10 μL SYBR premix Ex Taq, 0.4 μL of each forward and reverse primer, 0.4 μL ROX Reference Dye, 6.8 μL water to a final volume of 20 μL. Gene expression level was determined by the cycle threshold ($C_t$) values of each sample and 18S generated by qPCR. $2^{-\Delta\Delta CT}$ method was used for subsequent analysis and One-Way ANOVA were conducted followed by Duncan's multiple tests to identify significance differences when $P$-value $< 0.05$. To confirm expression patterns, each sample was repeated in triplicate analysis (technical replicates). The primers were listed in Table 1.

**Table 1**
Primers used for qPCR.

| Gene name | Primer (5′-3′) |
|---|---|
| *mapk1* (*erk2*) | F: TTCACACCAGCCGTGTGCTA |
| | R: AGCATGGAGGTTGTGTGGCT |
| *mapk3* (*erk1*) | F: AGCCAGCGCGTAGCTATCAA |
| | R: ATGTTGTCGATGTGCCGTGC |
| *mapk8* (*jnk1*) | F: CCAACAGACCAGACATCAA |
| | R: GTATTCACACCACAGAAGAC |
| *mapk9* (*jnk2*) | F: ATGTCCTATCTGCTCTACCA |
| | R: GTGTGCCAAGAACCTCAA |
| *mapk11* (*p38β*) | F: CGCAGAAGTACATCCAGTC |
| | R: CAGTCCAGAACCAGCATAC |
| *mapk14a* (*p38a*) | F: GTGTCCGTCCTGTAAGTAG |
| | R: AGTAATCTGGCTGTGAATGA |
| *mapk14b* (*p38b*) | F: CCAAGAGGAACTTCGCAGAC |
| | R: GATCCAGCAGCTTTCAGGAC |
| | CCAAGAGGAACTTCGCAGAC |
| | CCAAGAGGAACTTCGCAGAC |
| | CCAAGAGGAACTTCGCAGAC |
| 18 s | F: GGGTCCGAAGCGTTTACT |
| | R: ACCTCTAGCGGCACAA |

## 3. Results

### 3.1. Identification and annotation of mapk genes in L. maculatus

A total of 14 *mapk* genes were identified and further divided into three subfamilies based on dual-phosphorylation site, including six extracellular signal regulated kinases (*mapk1* (*erk2*), *mapk3* (*erk1*), *mapk4* (*erk4*), *mapk6* (*erk3*), *mapk7* (*erk5*), *mapk15* (*erk7*)), three c-jun amino-terminal kinases (*mapk8* (*jnk1*), *mapk9* (*jnk2*), *mapk10* (*jnk3*)), and five *p38-mapk* genes (*mapk11* (*p38β*), *mapk12* (*p38γ*), *mapk13* (*p38δ*), *mapk14a* (*p38a*), *mapk14b* (*p38b*)). The cDNA sequences of these *mapk* genes had been submitted to GenBank database. The characteristics of spotted sea bass *mapks* transcripts were summarized in Table 2. In details, the transcript lengths of *L. maculatus mapk* genes ranged from 951 bp to 6724 bp and the deduced sizes of amino acid varied from 316aa to 1,131aa (Table 2). The middle amino-acid residues of dual-phosphorylation activation sites were different among the three subfamilies, as Thr-Glu-Tyr for ERK, Thr-Pro-Tyr for JNK and Thr-Gly-Tyr for p38. However, *mapk4* (*erk4*) and *mapk6* (*erk3*), lacking TEY activation motif, display SEG as activation site. The activation sites of spotted sea bass *mapk* genes were relatively conserved with previous research in zebrafish (Krens et al., 2006).

### 3.2. Gene copy numbers of mapk genes

The copy numbers of *mapk* genes in spotted sea bass and several representative vertebrates were summarized in Table 3. In general, the number of *mapk* genes were conserved across a broad spectrum of species from mammals, to birds, and to fishes, where only one copy of each gene was present except for *mapk14*. The total gene numbers of *mapk* were slightly varied from 12 to 14 among different species except blackstripe livebearer (*Poeciliopsis prolifica*), of which only 4 *mapk* genes were reported probably due to the incompleteness of its genomic information. However, *mapk2* and *mapk5* were absent in most animals expect a few teleosts such as blackstripe livebearer (*P. prolifica*). Similar with zebrafish (*D. rerio*), large yellow croaker (*Larimichthys crocea*) and Atlantic salmon (*S. salar*), spotted sea bass retained duplicated copies of *mapk14* (*mapk14a* and *mapk14b*), which were teleost-specific.

### 3.3. Phylogenetic analysis

The identification and annotation of *mapk* genes in spotted sea bass were further confirmed by phylogenetic analysis depending on the inclusion of *mapk* genes from human, mouse, chicken, zebrafish and several other teleost species. For delineating the evolution history, the phylogenetic tree was constructed using the amino acid sequences of *L. maculatus* and selected species. As shown in Fig. 1, *mapk* genes of spotted sea bass were clustered with respective counterparts as expected and 13 clades were generated. The 13 clades were divided into three subfamilies, ERK, JNK and p38, which were consistent with their annotation. The simple bars outside the tree, standing for the size of deduced amino acid of these genes, were similar among the same clade. As a result, the phylogenetic analysis further confirmed the annotation of spotted sea bass *mapk* genes. In general, *mapk* genes were relatively conserved during evolution history.

### 3.4. Syntenic analysis

In general, only one copy of each gene was present for all *mapk*

**Table 2**
Characteristics of *mapk* genes in spotted sea bass.

| Gene name | Synonyms | Orthologs | Subfamily classification | Activation sites[a] | mRNAsize | Size of amino acid | Accession number |
|---|---|---|---|---|---|---|---|
| *mapk1* | *erk2*<br>*p42-mapk* | *h_erk1*<br>*m_erk2*<br>*z_erk2* | ERK | TEY | 1947 | 369 | MF802841 |
| *mapk3* | *erk1*<br>*p44-mapk* | *h_erk1*<br>*m_erk1*<br>*z_erk1* | ERK | TEY | 951 | 316 | MG876756 |
| *mapk4* | *erk4*<br>*p63-mapk*<br>*erk3-related* | *h_erk4*<br>*m_erk4*<br>*z_erk4* | ERK | SEG | 1290 | 414 | MF802843 |
| *mapk6* | *erk3*<br>*p97-mapk* | *h_erk3*<br>*m_erk3*<br>*z_erk3* | ERK | SEG | 5587 | 761 | MF802844 |
| *mapk7* | *erk5*<br>*bmk1* | *h_erk5*<br>*m_erk5*<br>*z_erk5* | ERK | TEY | 6724 | 1131 | MF802845 |
| *mapk8* | *jnk1* | *h_jnk1*<br>*m_jnk1*<br>*z_jnk1* | JNK | TPY | 4864 | 384 | MF802846 |
| *mapk9* | *jnk2* | *h_jnk2*<br>*m_jnk2*<br>*z_jnk2* | JNK | TPY | 3494 | 420 | MF802847 |
| *mapk10* | *jnk3* | *h_jnk3*<br>*m_jnk3*<br>*z_jnk3* | JNK | TPY | 1380 | 459 | MG876757 |
| *mapk11* | *p38β* | *h_p38β*<br>*m_p38β*<br>*z_p38β* | p38 | TGY | 3876 | 361 | MF802849 |
| *mapk12* | *p38γ*<br>*erk6* | *h_p38γ*<br>*m_p38γ*<br>*z_p38γ* | p38 | TGY | 2316 | 361 | MF802850 |
| *mapk13* | *p38δ* | *h_p38δ*<br>*m_p38δ*<br>*z_p38δ* | p38 | TGY | 1508 | 363 | MF802851 |
| *mapk14a* | *p38a* | *h_p38α*<br>*m_p38α*<br>*z_p38a* | p38 | TGY | 3424 | 361 | MF802852 |
| *mapk14b* | *p38b* | *h_p38α*<br>*m_p38α*<br>*z_p38b* | p38 | TGY | 1086 | 361 | MG876758 |
| *mapk15* | *erk7* | *h_erk7*<br>*h_erk8*<br>*m_erk7*<br>*z_erk7* | ERK | TEY | 3138 | 616 | MF802853 |

[a] Activation site is the dual-phosphorylation site of amino acids (T-Thr, E-Glu, Y-Tyr, P-Pro, G-Gly, S-Ser). Orthologs abbreviations: h: human; m: mouse; z: zebrafish.

genes in spotted sea bass except for *mapk14* (Table 3). The phylogenetic relationships of these *mapk* genes were conserved between spotted sea bass with other tested vertebrates, which well supported their annotations (Fig. 1). In that case, syntenic analysis was only conducted for the *mapk14* to provide additional evidence for the annotation of duplicated copies. As shown in Fig. 2, the conserved syntenic blocks for

**Table 3**
Copy number of *mapk* genes among a variety of vertebrate species.

| Name | Human | Mouse | Chicken | Zebrafish | Tilapia | Atlantic salmon | Killifish | Large yellow croaker | Japanese pufferfish | Blackstripe livebearer | Swamp eel | Silver Perch | Spotted sea bass |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mapk1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| mapk3 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 1 |
| mapk4 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| mapk6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk7 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk8 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| mapk9 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk10 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk11 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk12 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk13 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| mapk14 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 2 | 0 | 1 | 1 | 2 |
| mapk15 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| Total | 13 | 13 | 10 | 14 | 12 | 13 | 13 | 14 | 13 | 4 | 12 | 12 | 14 |

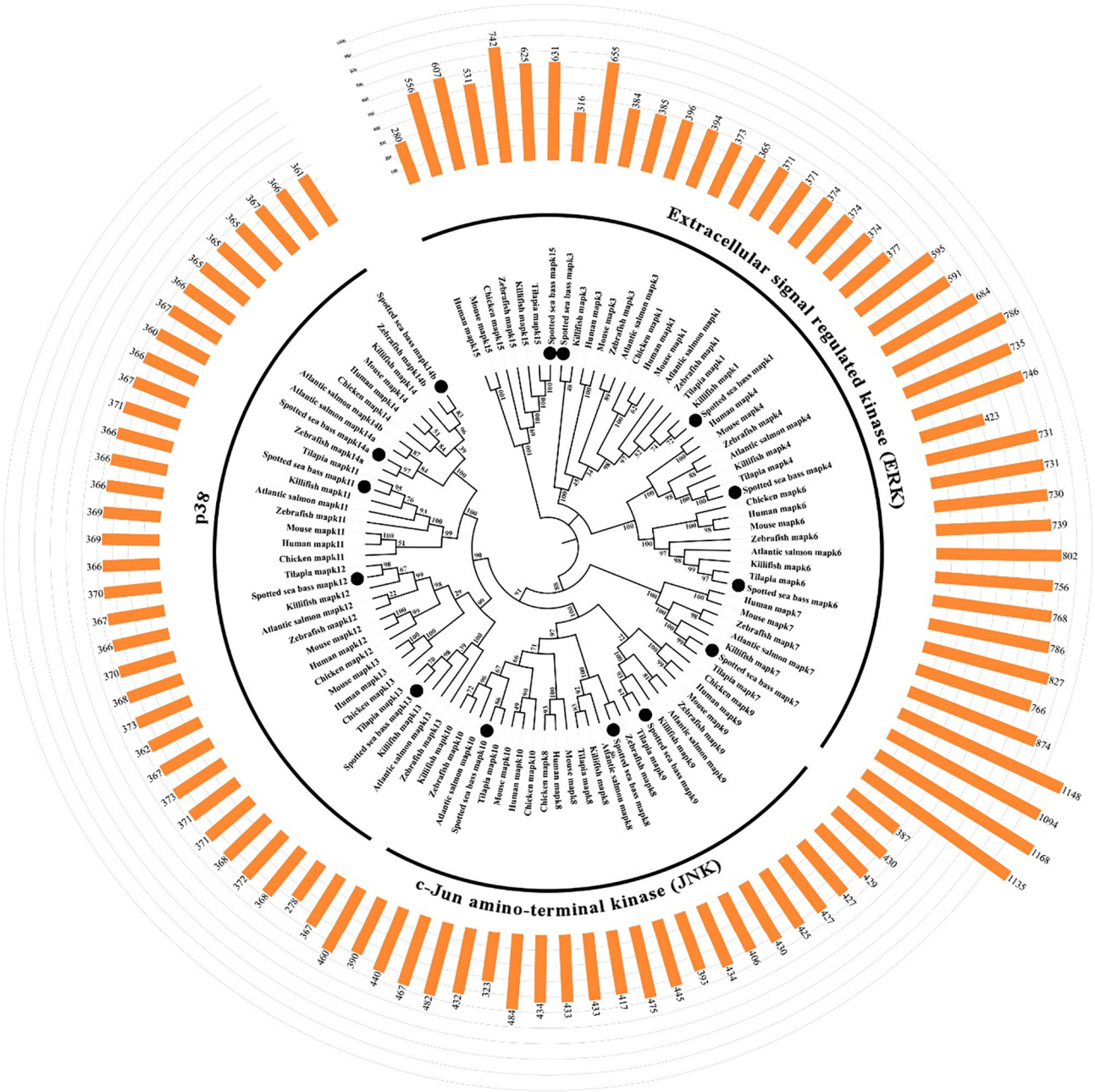


**Fig. 1.** Phylogenetic analysis of spotted sea bass *mapk* genes. The phylogenetic tree was constructed by the amino acid sequences from several representative mammals and teleosts with 1000 bootstrap replications in MEGA 7. *mapk* genes of spotted sea bass were labeled with black dot. The phylogenetic tree was divided into three subfamilies (ERK, JNK and p38) with covered lines. The simple bars outside phylogenetic tree stood for the size of amino acid of these genes.

*mapk14a* and *mapk14b* were identified between spotted sea bass and zebrafish. Syntenies were clearly conserved for *mapk14a*, which was closely linked to *srpk1b*, *lhfpl5a* and *copine-5* in zebrafish and spotted sea bass. Similarity, *rpl10a*, *fance* and *mkrn* were found on the upstream of *mapk14b* and *lhfpl5b*, *cpne5a* were located on the downstream of *mapk14b* both in zebrafish and spotted sea bass. Therefore, the syntenic analysis showed *mapk14a* and *mapk14b* were relatively conserved in evolution and supported the annotation of *mapk14* of spotted sea bass.

### 3.5. Tertiary structure of spotted sea bass mapk genes

Predicted 3D protein structures of *mapk* genes of human, zebrafish and spotted sea bass were shown in Fig. 3 and Supplemental Figs. 1–13. Analysis of spotted sea bass *mapk* genes revealed conserved protein tertiary structure in comparison of the corresponding genes in human and zebrafish. Tertiary structures of *mapk* genes were mainly composed of three structures: α helices, β strands and phosphorylation lips. The C-terminal domain was full of α helices, and the β strands lay predominantly in the N-terminal domain with the phosphorylation lip locating in the middle of α helices and β strands. The phosphorylation lip, a regulatory loop, started from Leu to Val and contained active phosphorylation site (Thr-X-Tyr). The lips in JNK subfamilies were longer than p38, but shorted than ERK.

### 3.6. qPCR analysis of selected mapk genes after salinity challenge

Previous studies reported that osmotic stress could induce the activation of JNK and p38 signaling pathway (Cowan and Storey, 2003;

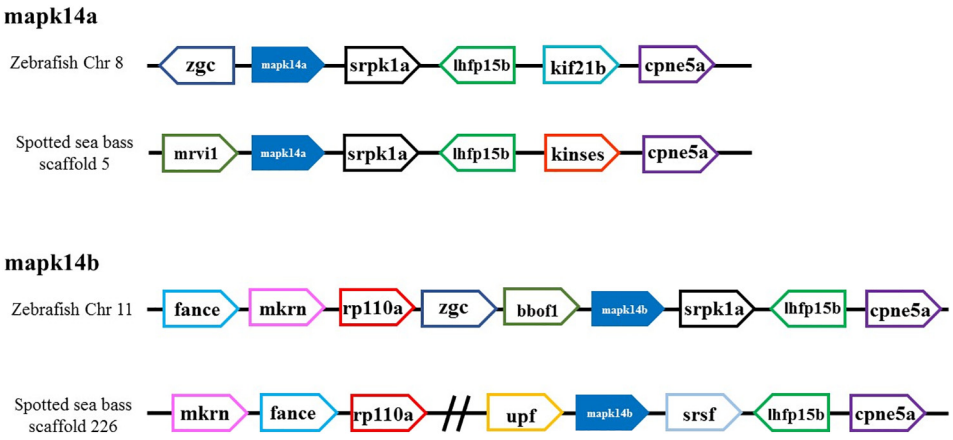


**Fig. 2.** Syntenic analysis of *mapk14* (*mapk14a* and *mapk14b*) in zebrafish and spotted sea bass. Abbreviations: *rpl10a*: *60 s ribosomal protein L10a*; *fance*: *Fanconin anemia group E protein*; *mkrn*: *E3 unbiquitn protein ligase makorin*; *lhfpl5*: *tetraspan membrance protein of hall cell stereocilli*; *cpne5*: *copine-5*; *dclre1b*: *5′ exonuclease appllo*; *srpk1b*: *serine/arginine rich-protein specific kinase 1b*.

Krens et al., 2006). Among which, *mapk8* (*jnk1*) and *mapk9* (*jnk2*) are widely expressed in many tissues while *mapk10* (*jnk3*) is brain-specific (Davis, 2000), while *mapk14* (*p38α*) and *mapk11* (*p38β*) are widely expressed isoforms involved in the regulation of response to stress. In the present study, qPCR analysis was employed to detect the expression pattern of the five *mapk* genes (*mapk8*, *mapk9*, *mapk11*, *mapk14a*, *mapk14b*) after salinity challenge in gill tissue at seven time-points, including 0 h, 1 h, 3 h, 6 h, 12 h, 24 h and 72 h. Overall, all the five *mapk* genes were significantly up-regulated at 3 h post challenge in comparison with control group (30‰) ($P < 0.05$). As shown in Fig. 4AB, similar expression patterns were observed in *mapk8*, *mapk9*, in which, the hypotonic treatment (0‰) showed stronger effect on *mapk8*, *mapk9* mRNA expression when compared with the hypertonic (45‰) and isotonic treatment (12‰) groups. The highest expression levels of *mapk8* (4.79-fold at 0‰, 2.27-fold at 12‰ and 3.20-fold at 45‰, respectively) and *mapk9* (6.16-fold at 0‰, 2.43-fold at 12‰ and 4.82-fold at 45‰, respectively) were found at 6 h after salinity challenge. However, the expressions of *mapk11*, *mapk14a* and *mapk14b* were more sensitive to hypertonic challenge (45‰) than hypotonic (0‰) and isotonic (12‰) challenges (Fig. 4CDE). The expression peak of *mapk11* (2.30-fold at 0‰, 1.77-fold at 12‰ and 3.28-fold at 45‰, respectively), *mapk14a* (3.50-fold at 0‰, 2.81-fold at 12‰ and 4.45-fold at 45‰, respectively) and *mapk14b* (3.34-fold at 0‰, 3.22-fold at 12‰ and 4.42-fold at 45‰, respectively) and are all appeared at 12 h after salinity challenge. Compared to the other two salinity challenge group, the isotonic conditions (12‰) showed the lowest gene expression level at the same time point.

### 3.7. qPCR analysis of selected mapk genes after hypoxia challenge

It has been reported that hypoxia induced the activation of ERK1/ERK2, p38 pathway and increases their mRNA abundance (Qiu et al., 2016). In order to examine their potential evolvement in response to hypoxia stress in spotted sea bass, the expression profiles of *mapk1* (*erk2*), *mapk3* (*erk1*), *mapk11* (*p38β*), *mapk14a* (*p38a*) and *mapk14b* (*p38b*) were examined in gill at 0 h, 1 h, 3 h, 6 h, 12 h and 24 h after hypoxia challenge. As shown in Fig. 5AB, the expression levels of *mapk1*, *mapk3* were significantly up-regulated (1.82-fold and 1.54-fold

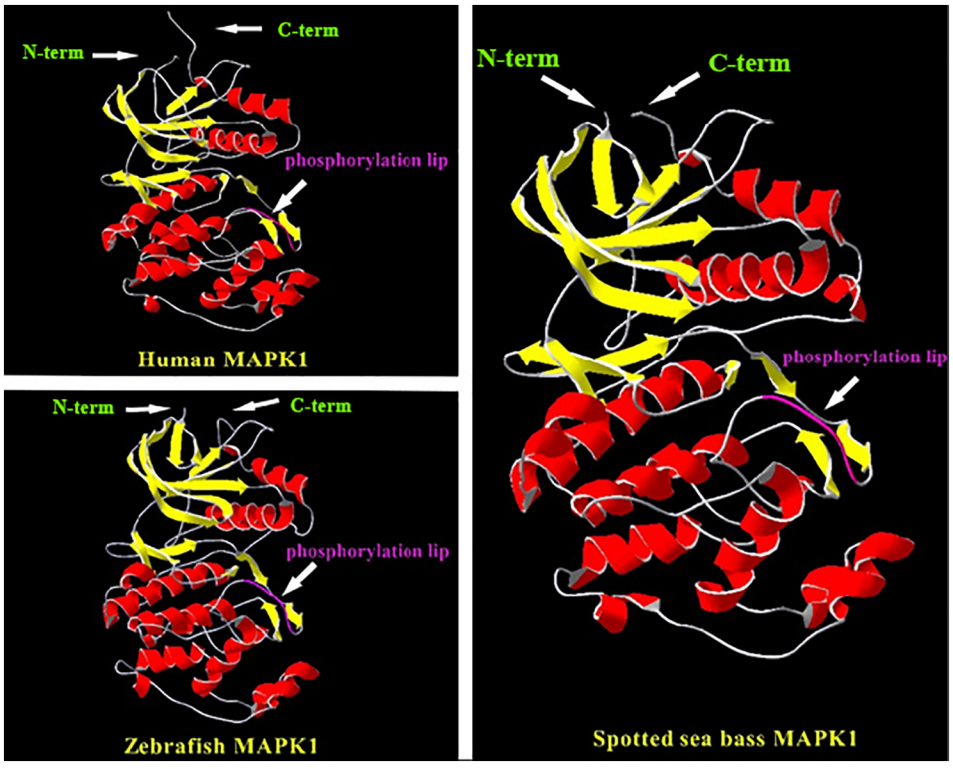


**Fig. 3.** Comparison of the tertiary structures of *mapk1* from human, zebrafish and spotted sea bass. α helices were colored in red, β strand were in yellow and phosphorylation lip was in magenta. The N-term and C-term were labeled with white arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

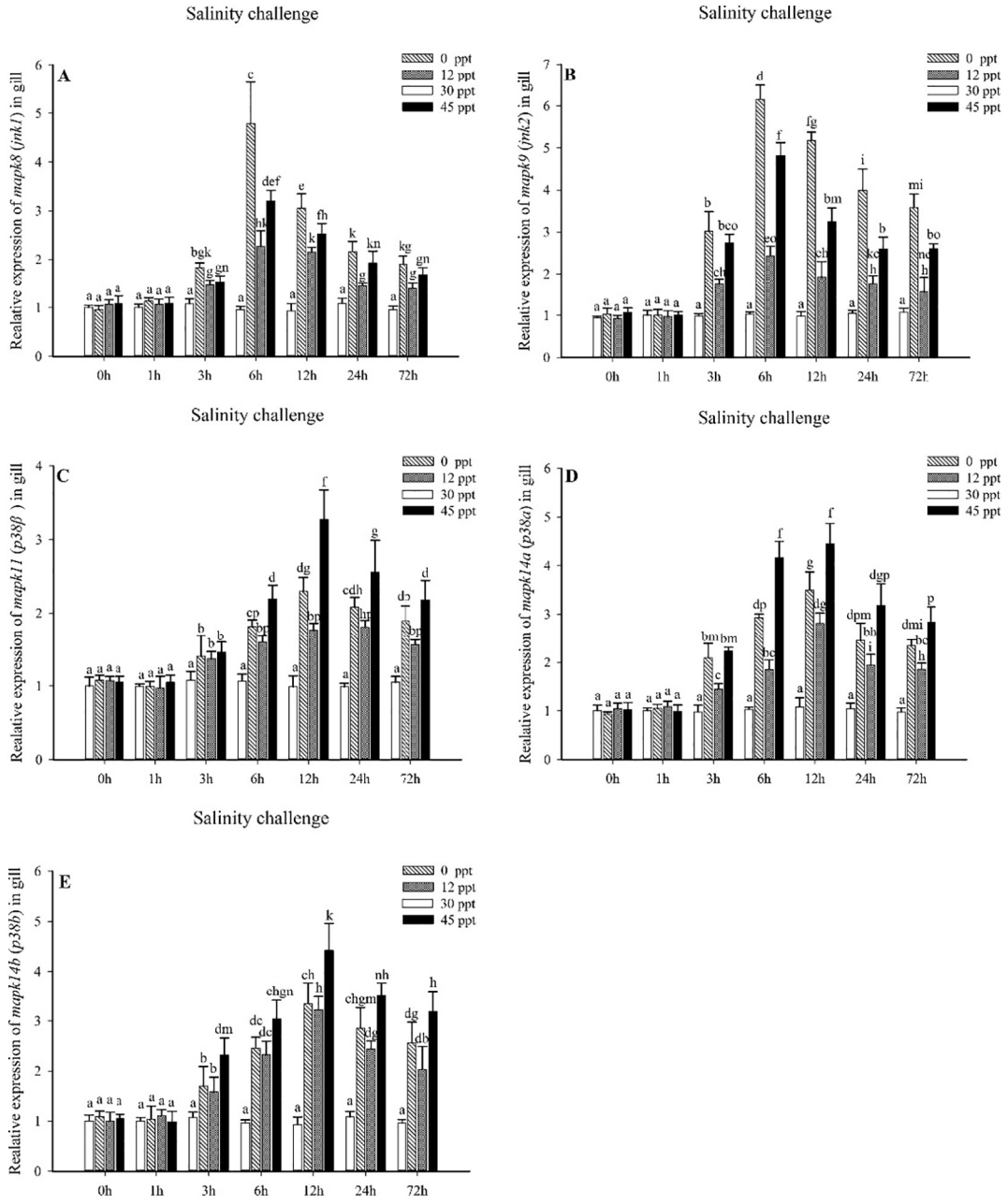


**Fig. 4.** Expression patterns of *mapk8* (*jnk1*), *mapk9* (*jnk2*), *mapk11* (*p38β*), *mapk14a* (*p38*a) and *mapk14b* (*p38b*) in the gill tissue of spotted sea bass following salinity challenge. qPCR analysis was used for determining the expression patterns of the five *mapk* genes at the time points of 0 h,1 h, 3 h, 6 h, 12 h, 24 h and 72 h after salinity challenge. The relative expression values were calculated by the expression of control group (30‰) as reference and normalized by 18S rRNA. Various letters indicated significant difference ($P < 0.05$).

increment, respectively) since 6 h ($P < 0.05$) and lasted till 24 h after hypoxia challenge. The *mapk14a* expression was significantly enhanced since 3 h (1.73-fold increment) after hypoxia challenge and reached the peak level at 12 h (3.74-fold increment, Fig. 5D), No significant differences were observed in the expression of *mapk11* and *mapk14b* ($P > 0.05$). This result suggested that *mapk1*, *mapk3* in ERK pathway and *mapk14a* in p38 pathway may implicated in tolerance of hypoxia in spotted sea bass.

## 4. Discussion

MAPKs have major roles in regulation of intracellular metabolism, gene expression and integral actions in many areas including growth and development, disease, apoptosis and cellular responses to external stresses (Cowan and Storey, 2003). Despite their importance, *mapk* gene family have not been systematically studied in spotted sea bass, an important type of commercial fishes in Asia. In our study, 14 *mapk* genes in spotted sea bass (*mapk1*, *mapk3*, *mapk4*, *mapk6*, *mapk7*, *mapk8*, *mapk9*, *mapk10*, *mapk11*, *mapk12*, *mapk13*, *mapk14a*, *mapk14b* and *mapk15*) were identified from genomic and transcriptomic databases. Phylogenetic analysis was conducted to determine the annotations of these genes and tertiary structures of spotted sea bass *mapks* were constructed in comparison to these of human (*H. sapiens*) and zebrafish (*D. rerio*). Finally, in order to provide insight into the function of several stress-related *mapk* genes under acute stresses, the expression profiles of these genes were determined in gill tissues following salinity and hypoxia challenges.

*mapk* gene family of spotted sea bass were classified into three

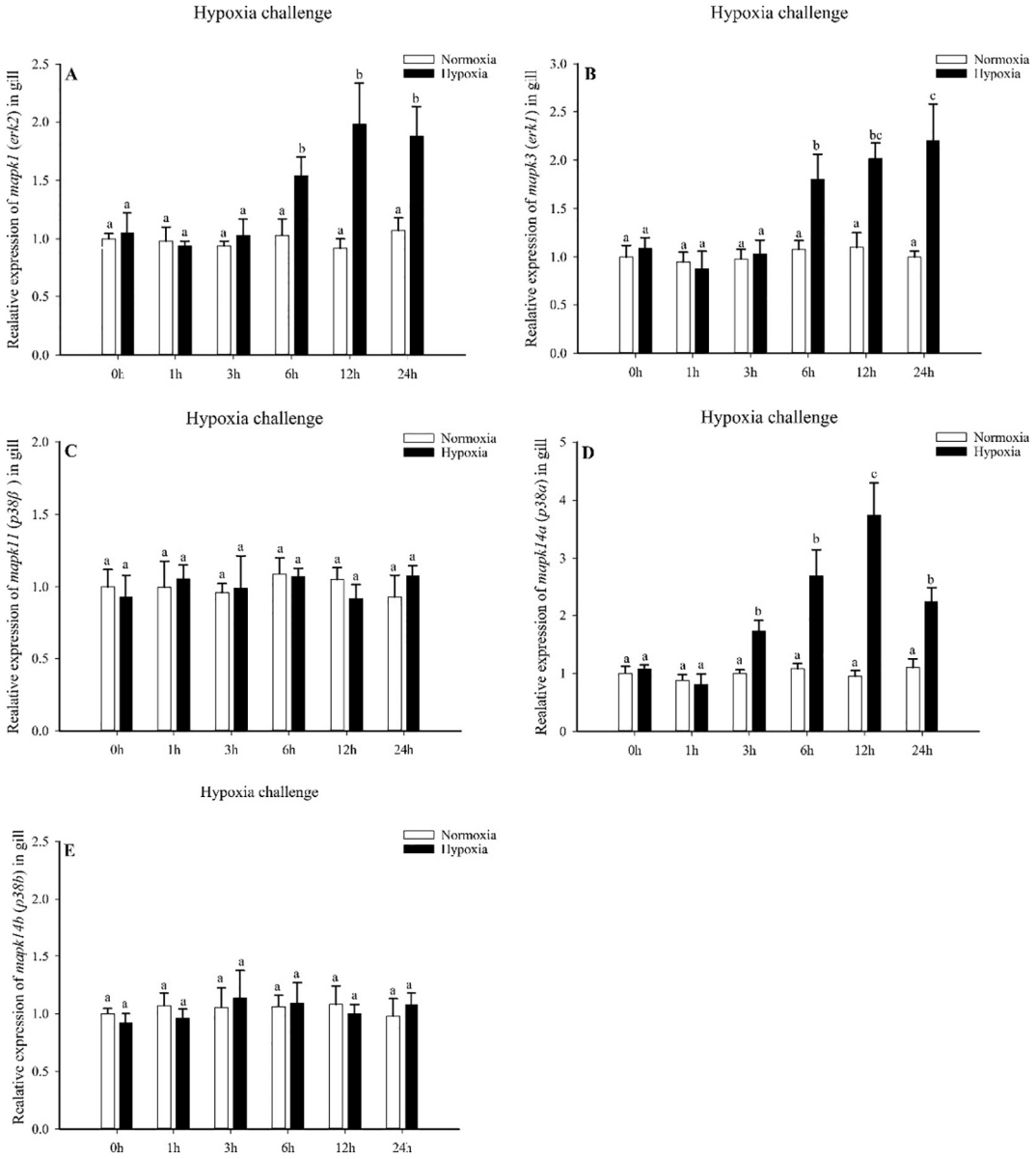


**Fig. 5.** Expression patterns of *mapk1* (*erk2*), *mapk3* (*erk1*), *mapk11 (p38β)*, *mapk14a* (*p38a*) and *mapk14b* (*p38b*) in the gill tissue of spotted sea bass following hypoxia challenge. qPCR analysis was used for determining the expression patterns of the five *mapk* genes at the time points of 1 h, 3 h, 6 h, 12 h and 24 h after hypoxia challenge. The relative expression values were calculated by the expression of control group (Normoxia) as reference and normalized by 18S rRNA. Various letters indicated significant difference ($P < 0.05$).

subfamilies depending on phylogenetic relationships, which consistent with studies in mammalians and other teleost species (Cowan and Storey, 2003; Niswander and Dokas, 2007). Additional evidences based on syntenic and CDS structure analysis further supported their annotations. These results revealed that *mapk* gene family appears to be a conserved gene family in evolution.

Analysis of the copy numbers in representative vertebrates provided insights into the evolution of *mapk* genes. The number of *mapk* genes varies slightly among different species. For example, 13 *mapk* genes were identified in mammalian species such as human (*H. sapiens*) and mouse (*M. musculus*), while only 10 *mapk* genes were found in chicken (*G. gallus*) (Widmann et al., 1999; Saelzler et al., 2006). 12–14 *mapk* genes were identified in teleost species including zebrafish (*D. rerio*), tilapia (*O. niloticus*), Atlantic salmon (*S. salar*) and killifish (*F. heteroclitus*). *mapk2* and *mapk5* were absent in most vertebrates expect a few teleosts such as blackstripe livebearer (*P. prolifica*). The only duplicated copy was *mapk14* (*mapk14a* and *mapk14b*), which was teleost-specific presenting in zebrafish (*D. rerio*), Atlantic salmon (*S. salar*), large yellow croaker (*L. crocea*), Japanese pufferfish (*Fugu rubripes*) and spotted sea bass. Syntenic analysis provided additional orthology evidence and supports the phylogenetic analysis, which verified the identification and annotation of *mapk14* in spotted sea bass.

Compared with higher vertebrates, the function studies about *mapk* genes in fish species lag far behind. In this study, in order to investigate the potential roles of *mapk* gene family of spotted sea bass during salinity and hypoxia challenges, we performed qPCR to examine mRNA expression profiles of stress responsive *mapk* genes. *mapk* genes have been reported played central roles in responses to salinity challenge in fishes, including the regulation of intracellular levels of inorganic ions and organic osmolytes, integrating and amplifying signals from osmosensors to activate appropriate downstream targets mediating physiological acclimation (Kultz and Avila, 2001; Fiol and Kültz, 2007; Zhou et al., 2016). We selected *mapk8* (*jnk1*), *mapk9* (*jnk2*), *mapk11* (*p38β*), *mapk14a* (*p38a*) and *mapk14b* (*p38b*) for investigation because it was

demonstrated that *jnk* and *p38* genes were involved in hypotonic and hypertonic regulation in mussels (*M. galloprovincialis*), killifish (*F. heteroclitus*), medaka (*Oryzias latipes*) (Gaitanaki et al., 2004; Kultz and Avila, 2001; William et al., 2011; Marshall et al., 2005; Krens et al., 2006). In our study, *mapk8*, *mapk9*, *mapk11*, *mapk14a* and *mapk14b* were differentially expressed among hypotonic, isotonic and hypertonic stresses, indicating their potential functions in osmotic responses in spotted sea bass.

As shown in Fig. 4, freshwater (0‰) and high salinity seawater (45‰) represented typical hypotonic and hypertonic environments, and salinity seawater (12‰) was the isotonic environment for spotted sea bass (Zhang et al., 2018). Compared with isotonic environments, hypotonic and hypertonic environments are more stressed for spotted sea bass. Isotonic salinities could minimize osmoregulatory stress and cost (Sampaio and Bianchini, 2002; Urbina and Glover, 2015). Hence, expression levels of salinity-stress responded genes in hypotonic and hypertonic environments were higher than isotonic environment. Similar result was also reported in Brazilian flounder (*Paralichthys orbignyanus*), which the mRNA expression levels of osmoregulation-related genes were higher in hyperosmotic environment than isotonic environment (Meier et al., 2009). The expression levels of *mapk11*, *mapk14a* and *mapk14b* were more drastically induced by hypertonic stress (45‰). This observation suggested that these *p38-mapk* genes (*mapk11*, *mapk14a*, *mapk14b*) were more sensitive to hypertonic environment compared with hypotonic (0‰) and isotonic (12‰) environments. *p38-mapk* genes are critically involved in the activation of nonselective cation (NSC) channels on osmotic shrinkage, which play an important role in the volume regulation (Shen et al., 2002). It is generally thought that *p38-mapk* genes could be implicated in the regulatory volume increase and osmolyte transport response to restore cell volume (Han et al., 1998; Nielsen et al., 2008; Hdud et al., 2014), which was paralleled by these findings in rat (*Rattus norvegicus*) kidney cells (Roger et al., 1999), tumor cells (Pederson et al., 2002) and turbot (*Scophthalmus maximus*) (Ollivier et al., 2006). *mapk8* (*jnk1*), *mapk9* (*jnk2*) shared the similar expression patterns after salinity challenge (Fig. 4AB), which they were more sensitive to hypotonic challenge (0‰). Similar result was also found in gill epithelium of killifish (*F. heteroclitus*) (Kultz and Avila, 2001; Marshall et al., 2005). *jnk* genes contribute to the protein synthesis-independent early phase in hypotonic stress-induced $Na^+$ transport (Taruno et al., 2007).

Blaschke et al. (2002), Ossum et al. (2006) and Lan et al. (2011) reported that *p42-mapk* (*erk2*), *p44-mapk* (*erk1*), *p38-mapk* genes play a critical role in hypoxia adaptation. Hence, the expression patterns of *mapk1* (*erk2*), *mapk3* (*erk1*), *mapk11* (*p38β*), *mapk14a* (*p38a*) and *mapk14b* (*p38b*) were determined after hypoxia challenge in spotted sea bass. In present study, *mapk14a* (*p38a*) and *mapk14b* (*p38b*) are both orthologs of *mapk14* (*p38a*) showed completely different expression pattern. The expression of *mapk14a* (*p38a*) was significantly increased, while *mapk14b* (*p38b*) were not induced after hypoxia challenge, and the expression level of *p38β* were not affected by hypoxia. This finding was consistent with the previous report that *mapk14* (*p38α*) was implicated in hypoxia response (Conrad et al., 1999). *mapk14* (*p38α*) strongly inhibits *cyclin D1* gene expression, which plays a role in regulating progression through the $G_1$ phase of the cell cycle (Baldin et al., 1993; Conrad et al., 1999). In addition, Emerling et al. (2005) demonstrate that p38 mitogen-activated protein kinase is essential for *HIF-1* activation in mouse embryonic fibroblasts. *Mapk1* (*erk2*) and *mapk3* (*erk1*) are found to be significantly up-regulated indicating these *mapk* genes of spotted sea bass might be implicated in hypoxia response. Similar as study in rat (*R. norvegicus*) pulmonary arterial smooth muscle cells, hypoxia increased the relative mRNA expression levels of *erk1*, *erk2*, *p38-mapk* genes (Qiu et al., 2016). *erk1* and *erk2* could result in the transactivation of *HIF-1*, which is able to directly phosphorylate the carboxy-terminal domain of *HIF-1* and regulate its activity (Minet et al., 2000; Minet et al., 2001). The transcription factor *HIF-1* is a key regulator responsible for the induction of genes that facilitate adaptation and survival of cells and the whole organism from normoxia to hypoxia (Wang et al., 1995; Semenza, 1998).

## 5. Conclusion

In conclusion, a complete set of 14 *mapk* genes were identified and annotated in spotted sea bass. Phylogenetic and syntenic analyses were conducted to provide sufficient evidences for the annotation and orthologies of these genes. Together with the results of the tertiary structure analysis, *mapk* genes of spotted sea bass were conserved in evolution. In addition, *mapk8* (*jnk1*), *mapk9* (*jnk2*), *mapk11* (*p38β*), *mapk14a* (*p38*a) and *mapk14b* (*p38b*) were significantly up-regulated after salinity challenge, indicating their potential roles in osmotic responses. Three *mapk* genes, including *mapk1* (*erk2*), *mapk3* (*erk1*) and *mapk14a* (*p38a*), were differentially expressed after hypoxia challenge, suggesting their involvement in hypoxia tolerance.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.10.033.

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