Fish and Shellfish Immunology 84 (2019) 848–856

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Molecular characterization of p38 MAPK from blunt snout bream (*Megalobrama amblycephala*) and its expression after ammonia stress, and lipopolysaccharide and bacterial challenge

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ARTICLE INFO

Keywords:
Blunt snout bream
p38 MAPK
Ammonia stress
Aeromonas hydrophila challenge
Lipopolysaccharide

ABSTRACT

p38 mitogen-activated protein kinase (MAPK) is an important protein which plays a key role in regulating the innate immunity, so exploring its molecular characterization is helpful in understanding the resistance against microbial infections in cultured fish. Here, a full-length cDNA of p38 MAPK was cloned from liver of blunt snout bream (*Megalobrama amblycephala*) which covered 2419 bp with an open reading frame of 1086 bp encoding 361 amino acids. p38 MAPK contained the characteristic structures of Thr-Gly-Tyr (TGY) motif and substrate binding site Ala-Thr-Arg-Trp (ATRW), which are conserved in MAPK family. To investigate p38 MAPK functions, two *in vivo* experiments were carried out to examine its expression following ammonia exposure and bacterial challenge. Also, an *in vitro* experiment was conducted to assess the role of p38 MAPK in inflammation of primary hepatocytes induced by lipopolysaccharide (LPS). The results showed the ubiquitous expression of p38 MAPK in all the tested tissues with varying levels. p38 MAPK mRNA expression was significantly up-regulated by ammonia stress and *Aeromonas hydrophila* challenge, and altered in a time-dependent manner. Moreover, the results indicated that the inflammatory response induced by LPS in hepatocytes is p38 MAPK dependent as knockdown of p38 MAPK using siRNA technology depressed the expression of IL-1β and IL-6. The findings in this study showed that p38 MAPK has anti-stress property, and plays key role in protection against bacterial infection and inflammation in blunt snout bream.

1. Introduction

Mitogen-activated protein kinase (MAPK) superfamily are members of serine/threonine protein kinase that play important roles in cellular response to extracellular stimuli [1]. This superfamily has four main subgroups including extracellular signal-regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK/big MAP kinase 1 (BMK1), and p38 MAPK [2,3]. p38 MAPK is involved in inflammation, environmental stresses and microbial infections in various organisms [4–6]. It has been demonstrated that p38 MAPK is activated during viral infection and replication in mammals [7–9]. Overexpression of p38 MAPK inhibits viral gene transcription and protein synthesis as well as apoptosis in fish cells [10]. So, p38 MAPK is probably involved in cellular immune responses to microbial infection in fish. On the other hand, fish is subjected to various environmental stresses such as ammonia stress which adversely impact fish immune function [11]. To date, there is little available information about the role of p38 MAPK in ammonia stress in fish.

Blunt snout bream (*Megalobrama amblycephala*) is an herbivorous freshwater fish native to China. Due to its fast growth, tender flesh, and high disease resistance, it has been a favorable candidate for aquaculture in China. However, it is prone to bacterial and viral diseases and is relatively sensitive to ammonia exposure [12]. Also, induction of oxidative stress and inflammation by dietary oxidized lipids has been a common issue in its culture [13]. Therefore, to overcome such issues in *M. amblycephala* culture research on immune-related and anti-stress genes is imperative. The goals of the present study were to: (1) achieve the molecular characterization of p38 MAPK; (2) examine p38 MAPK expression following ammonia exposure and *Aeromonas hydrophila* challenge; and (3) explore the role of p38 MAPK in inflammation induced by lipopolysaccharide (LPS). The outcome of this research could contribute to our understanding on fish resistance against environmental stresses and bacterial infection.

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https://doi.org/10.1016/j.fsi.2018.10.074
Received 25 August 2018; Received in revised form 22 October 2018; Accepted 26 October 2018
Available online 28 October 2018
1050-4648/

2. Materials and methods

2.1. Experimental fish and sample collection

Juvenile blunt snout bream (mean body weight, 50 g) were used to clone p38 MAPK sequence and study its expression after ammonia stress and bacterial challenge. Fish were obtained from a commercial farm in Wuhan (Hubei province, China) and reared in a recirculating aquaculture system in laboratory under the following conditions: water temperature, 25–27 °C; DO, 5.0–6.0 mg l^{-1}; pH, 7.2–7.6; photoperiod, 12: 12 h (dark: light). During the adaptation period fish were fed a commercial diet twice daily (09:00 and 17:00). For sampling, six fish were captured and quickly anesthetized with tricaine methane sulfonate (MS-222, 200 mg l^{-1}), and then gill, liver, brain, kidney, head kidney, spleen, intestine, muscle and heart were sampled, frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Molecular cloning of p38 MAPK

Total RNA was extracted using Trizol Reagent (Invitrogen, USA) following the manufacturer's instructions. RNA purity was verified by measuring the absorbance at 260 and 280 nm by a ND1000 spectrophotometer (NanoDrop Technologies, USA) and the first strand cDNA was synthesized by First cDNA Synthesis Kit (Fermentas) following the manufacturer's protocol. Partial fragment of p38 MAPK was acquired from our previous results by high-throughput sequencing. A pair of designed primers was used to verify it. PCR was carried out at the following program: 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min, followed by a 10 min extension at 72 °C. The products were extracted and inserted into pMD18-T prior to sequencing (Invitrogen Biotechnology Co., Ltd, Shanghai, China). The full-length cDNA of p38 MAPK was obtained by 5′-rapid amplification of cDNA ends (RACE) and 3′RACE according to our previous method [14]. The p38 MAPK primers for the RACE are listed in Table 1. The full-length cDNA sequence of p38 MAPK was assembled by identifying the overlapping region of each fragment.

2.3. Sequence analysis of p38 MAPK

The open reading frame (ORF) of p38 MAPK was predicted using the program ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf. html). The nucleotide sequence of the ORF was translated into the amino acid sequence using ExPASY translation tool of EMBL (http://web.expasy.org/translate/). The similarity of p38 MAPK with other species was analyzed using the BLASTP search program at the NCBI (http://www.ncbi.nlm.nih.gov/blast). The bioinformatics analysis of p38 MAPK was performed with the ExPASy Proteomics Sever (http://tw.expasy.org/tools/pi_tool.html), SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP) and SMART program (http://www.smart.embl-heidelberg.de). The pair-wise and multiple sequence alignment were analyzed using the ClustalW version 4.0 [15]. The phylogenetic relationship of p38 MAPK was determined by MEGA software version 4.0 with the Neighbor-Joining Method [16]. Bootstrap values were determined based on 1000 replications.

Table 1
The primers used for cloning and expression analysis.

Primer	Sequence (5′-3′)	Application
Primers used for RACE		
F1	TGCAGGCATGTCGCAGAA	Verification of the known sequence
R1	AAGGTCCACAGCTAGTGG	
5′-RACE		
GSP1	AGTTCTGATATCGCAC	5′-RACE (1st round PCR)
GSP2	CCTCCCATATCGTCTTGTT	Used with AAP for first PCR of 5′RACE
GSP3	CCTGTCGATAGAAAGTGGG	Used with AUAP for nested PCR of 5′ RACE
3′-RACE		
GSP1	CTCCAGGACCTGAGCTGTTAATGA	3′-RACE (1st round PCR)
GSP2	TCATCGGAGTCTGCTCGTACTTAC	3′-RACE (2nd round PCR)
Real time PCR		
p38MAPK-F	ACACAGTGGGACGGTTCG	Real-time PCR
p38MAPK-R	CCTGTGATTCAAGGCGGT	
IL1β-F	TTCTTCCCCTCACCTGGTCT	Real-time PCR
IL1β-R	CCAGCGCGAAGTTTGTCAAT	
IL6-F	AAGACAACCGCACACTCGAT	Real-time PCR
IL6-R	CTGGGTCTCTTCACGCCTTT	
Rpl13a-F	TCTGGAGGACTGTAAGAGGTATGC	Real-time PCR
Rpl13a-R	AGACGCACAATCTTGAGAGCAG	

Rpl13a: reference gene.

2.4. Ammonia exposure

In this experiment, two different ammonia concentrations treatment of 0 (normal freshwater as control) and 25 mg l^{-1} were used according to a previous study [17]. During the exposure time, pH of water in each aquarium was recorded at every 6 h using a pH meter. The total ammonia nitrogen levels were measured by nesslerization and adjusted by adding NH_4Cl solution [18]. For each treatment, liver, gill and kidney were sampled at 0, 3, 6, 12, 24 and 48 h after ammonia exposure, frozen in liquid nitrogen and stored at −80 °C until used. Expression of p38 MAPK was analyzed as described earlier.

2.5. Bacterial challenge

After 2 weeks of acclimation period, 100 fish were captured randomly, stocked into two tanks (50 fish in each tank) and challenged with *Aeromonas hydrophila* as described in our previous research [19]. The fishes in one of the tanks were intraperitoneally injected with the bacterium suspension, and the fishes in the other tank received the same amount of phosphate buffered saline (PBS) as control group. After injection, all fish were transferred into two fiberglass-reinforced plastic tanks containing 200 L of aerated freshwater, and water temperature was maintained at 26 ± 1 °C. Head kidney, spleen, gill, and intestine were collected at 0, 6, 12, 24, 48 and 72 h post injection and stored at 80 °C until used.

2.6. LPS challenge

This *in vitro* study was carried out using primary hepatocytes of blunt snout bream as described in the following sections.

2.6.1. Isolation of hepatocytes

Prior to isolation of hepatocytes, fish were anesthetized with MS-222 (tricaine methanesulfonate; Sigma, USA) (100 mg l^{-1}) and bled by cutting the gill arches. Then, liver was rapidly isolated and washed several times in ice-cold PBS containing antibiotic (100 IU ml^{-1} penicillin G sodium and 100 IU ml^{-1} streptomycin). After removal of PBS by sterile pipette, the samples were cut into small pieces (about 1 mm^3) and digested with pancreatin at 28 °C for 30 min. Thereafter cell suspension was centrifuged at 500 × *g* for 10 min and washed twice. The harvested cell pellets were re-suspended in Leibovitz's L-15 medium (L15 medium) (HyClone™, USA) with 15% fetal bovine serum (Biological Industries, USA) at a density of 1×10^6 ml^{-1}. For each test three different fish were used and each time the livers were pooled to make a single sample.

2.6.2. Cell treatment

Two milliliter of isolated hepatocytes was seeded in each well of 6-well culture plates. After 24 h, all cells attached and cultured in 2 ml of the following media: control group (L15 medium), LPS group (L15 + 1 μg ml^{-1} LPS), and si-RNA group (L15 + 1 μg ml^{-1} LPS + siRNA-p38MAPK). Hepatocytes of si-RNA group were transfected with small interfering RNA (siRNA) duplexes (5′-Chol, 2′-Ome)

for p38 MAPK (siRNA-p38MAPK), which is designed to knockdown p38 MAPK expression. The sequences of siRNA-p38MAPK duplexes were as follows: sense sequence, 5′-GGAACUGUUUGCUCUGCAUTT-3'; anti-sense sequence, 5′-AUGCAGAGCAAACAGUUCCTT-3'. The sequences of negative control siRNA duplexes were as follows: sense sequence, 5′-UUCUCCGAACGUGUCACGUTT-3'; anti-sense sequence, 5′-ACGUGA CACGUUCGGAGAATT-3'. The delivery of siRNA duplexes was carried out using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were collected for analysis. The supernatant was removed by sterile pipette, and then cells were harvested by trypsinization (0.25% trypsin–EDTA) at 25 °C in 5 min. All the tests were performed in three replicates.

2.7. Quantitative real-time PCR (qPCR)

The RNA and first strand cDNA preparation were performed as described in Section 2.2. Expression of genes was determined by qPCR in CFX96 system (Bio-Rad, USA). The primers were designed based on the nucleotide sequence (Table 1). Amplification reactions were carried out in triplicate wells, each well with a final volume of 20 μl, containing 2.0 μl cDNA sample, 0.4 μl of each primer, 10 μl SYBR premix Ex Taq (TaKaRa) and 7.2 μl ddH_2O. The PCR conditions were applied as follow: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. The result was analyzed by the comparative CT method ($2^{-\triangle\triangle CT}$ method).

2.8. Statistical analysis

All the data were analyzed using SPSS version 16.0 for Windows software (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) or student's t-test were used to analyze differences among treatments. The level of significance was set at $P < 0.05$. All data were presented as means ± S.E. (standard error of the mean).

3. Results

3.1. Cloning and characterization of p38 MAPK

The full length cDNA of p38 MAPK is 2419 bp containing 5′ UTR of 307 bp, open reading frame (ORF) of 1086 bp and 3′UTR of 1026 bp. This cDNA was submitted to GenBank with accession number of MH791036. The initiation codon (ATG) and stop codon (TAA) present at positions 308 bp and 1394 bp. The ORF encodes a predicted protein of 361 amino acids with no signal peptide. The calculated molecular mass is 41.48 kDa and the estimated pI is 5.10. There are three catalytic sites at residues 72, 151, 153. The p38 MAPK protein contains a functional domain (STKc) at sites 25–309 (Fig. 1). It contains glycine-rich (GxGxxG) ATP binding loop, ED site (Glu and Asp), the putative dual phosphorylation motif Thr-Gly-Tyr (TGY) and the substrate binding site Ala-Thr-Arg-Trp (ATRW).

3.2. Homology and phylogenetic analysis

Multiple sequence alignment of *M. amblycephala* p38 MAPK amino acid sequence with other species showed well conserved regions across the sequence (Fig. 2). Functional sites of p38 MAPK, including the TGY motif, ATRW site and ED motif, also display well conservation (Fig. 2). The homology levels of *M. amblycephala* p38MAPK with other species are shown in Fig. 3 p38 MAPK of *M. amblycephala* shared the highest identity to *Sinocyclocheilus grahami* (99%), following *Cyprinus carpio* and *Danio rerio* (Fig. 3). Phylogenetic analysis indicates p38 MAPK of *M. amblycephala* has the closest relationship to *Sinocyclocheilus grahami*, and then with *Cyprinus carpio* (Fig. 3).

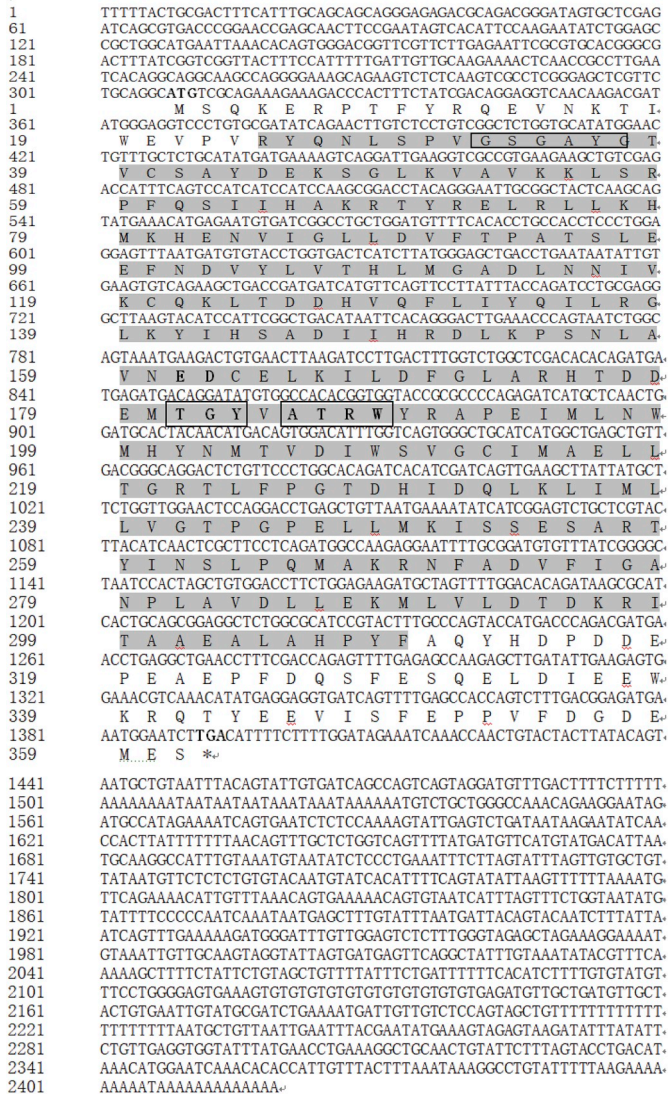

Fig. 1. Nucleotide and deduced amino acid sequences of p38 MAPK. The start codon (ATG) and stop codon (TGA) are indicated in bold. Nucleotides and amino acids are numbered on the left of the sequences. The MAPK superfamily homology domain of p38 MAPK is shaded. The conserved TGY motif and ATRW site appear as underlined and boxed.

3.3. Tissue distribution of p38 MAPK

To detect tissue distribution of p38 MAPK, expression of p38 MAPK was analyzed in gill, liver, brain, kidney, head kidney, spleen, intestine, muscle and heart and blood. As shown in Fig. 4, p38 MAPK mRNA ubiquitously expressed in all the tested tissues. The highest transcription was observed in spleen followed by gill and head kidney, respectively.

3.4. Expression of p38 MAPK following ammonia stress

As shown in Fig. 5, remarkable increases in the expression of p38 MAPK in gill, liver and kidney were found after ammonia stress. A significant ($P < 0.05$) increase in expression of p38 MAPK in gill was found after 6 h of ammonia exposure which maintained till 24 h. Also, its expression in liver enhanced after 12 h of exposure and a further increase was detected after 24 h. While, expression of p38 MAPK in kidney only increased after 48 h of ammonia stress. The enhancement in expression of p38 MAPK in gill was remarkably faster (5.32 folds at 6 h) than liver (5.64 folds at 12 h) and kidney (7.52 fold at 24 h).

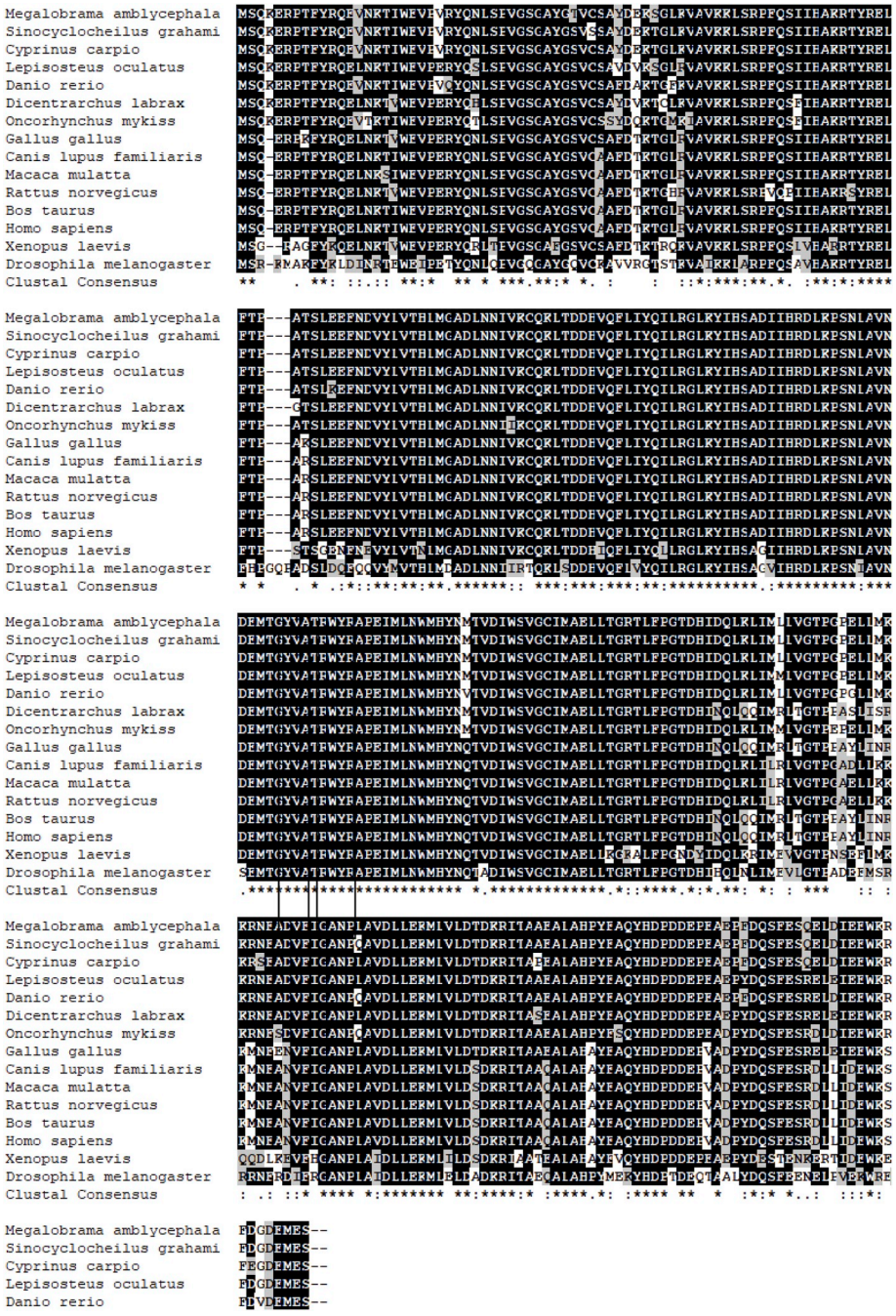

Fig. 2. Multiple-sequence alignment of the deduced amino acid sequence of p38 MAPK with other anmials. Completely conserved residues across all species aligned are shaded in black. The predicted phosphorylation motif TGY and substrate-binding site ATRW are indicated by boxes. The arrows indicate the conserved residues, which are important for docking.

3.5. Expression of p38 MAPK following bacterial challenge

Expression of p38 MAPK in head kidney, spleen, intestine and gill after *A. hydrophila* challenge is shown in Fig. 6 p38 MAPK expression in head kidney, intestine and gill significantly increased after 12 h and maintained until 24 h in head kidney and intestine, and until 48 h in gill. Whereas expression of p38 MAPK in spleen enhanced after 48 h of bacterial challenge (7.12 folds).

3.6. Expressions of p38 MAPK in LPS treated hepatocytes

Expression of p38 MAPK and pro-inflammatory cytokines including IL-1β and IL-6 in LPS treated hepatocytes is shown in Fig. 7. LPS stimulation enhanced the expression levels of p38 MAPK, IL-1β and IL-6 genes. Furthermore, knockdown of p38 MAPK with siRNA reduced the expression levels of IL-1β and IL-6.

4. Discussion

In the present study, p38 MAPK was cloned from liver of *M. amblycephala*. The ORF of p38 MAPK encodes a predicted protein of 361 amino acids with no signal peptide. As reported for all the p38 subfamily members, the dual phosphorylation site of TGY motif and substrate binding site of ATRW are highly conserved [20,21]. The dual phosphorylation of both Thr and Tyr in TGY motif is required for all p38 MAPK activation. The ATRW domain is the kinase interaction motif

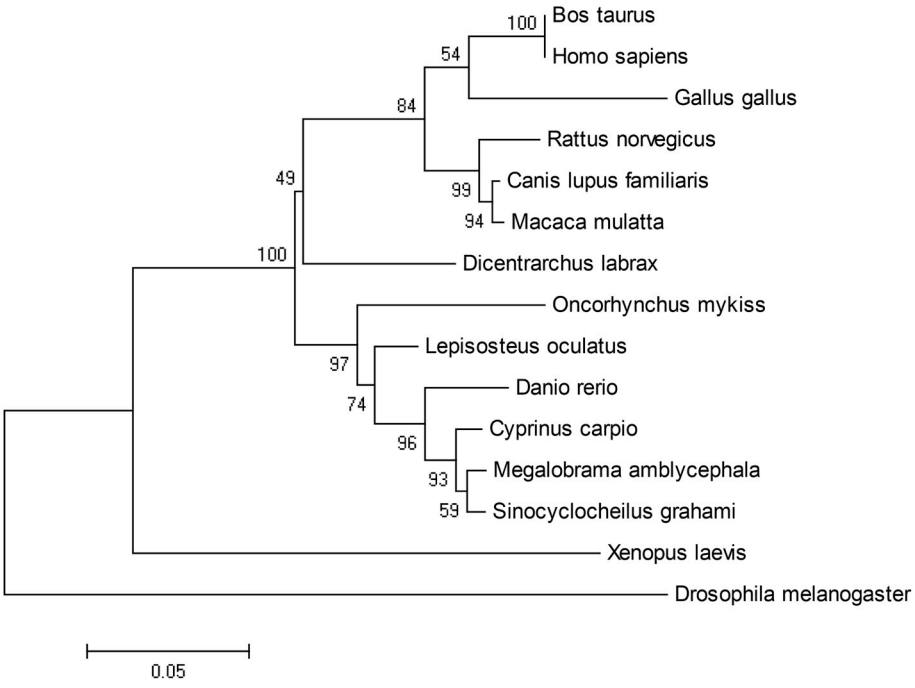

Fig. 3. Phylogenetic tree of p38 MAPK with other reported p38 MAPK. The phylogenetic tree of the alignment amino acid sequences was constructed by the neighbor-joining method using the MEGA 4.0 program. The bar shows the genetic distance (0.05). The GenBank accession numbers are as followed:*Sinocyclocheilus graham* (XP_016136592.1); *Cyprinus carpio* (XP_018961799.1); *Danio rerio* (XP_005167118.1); *Dicentrarchus labrax* (CBN80892.1); *Oncorhynchus mykiss* (XP_021472540.1); *Gallus gallus* (XP_004934853.1); *Canis lupus familiaris* (AAC36131.1); *Macaca mulatta* (AFI33384.1); *Rattus norvegicus* (AAB51285.1); *Bos Taurus* (NP_001095644.1); *Homo sapiens* (NP_001306.1); *Xenopus laevis* (NP_001165343.1); *Drosophila melanogaster* (NP_477361.1).

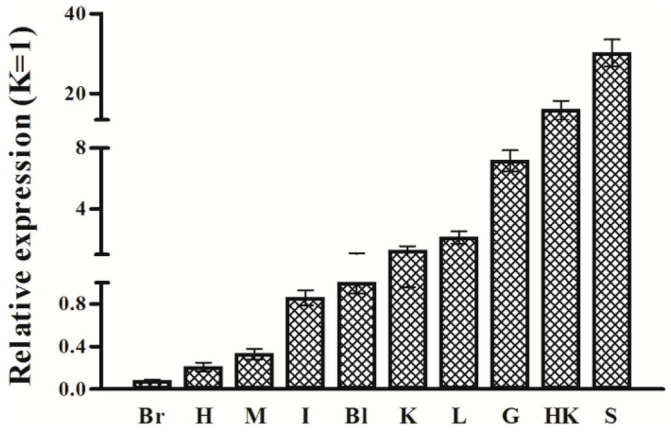

Fig. 4. Tissue distribution of p38 MAPK in blunt snout bream. Expression values were normalized by β-actin transcript and data were presented as the means ± SE (n = 6). Expression levels in kidney was used as control and set to 1. *Br = brain, H = heart, M = muscle, I = intestine, Bl = blood, K = kidney, L = liver, G = gill, Hk = head kidney, S = spleen.*

(KIM) docking site binding to the linear KIM sequences and MAPK phosphatases [22]. Moreover, CD motif and ED site are important for interaction of p38 MAPK with substrate, activators and regulators [22–24]. In this study, p38 MAPK contained all these conversed structures as other fish species [10,25]. Phylogenetic analysis also revealed that blunt snout bream p38 MAPK is close to *Sinocyclocheilus graham* and *Cyprinus carpio* with a similarity coefficient of 99% and 98%, respectively. In mammals, p38 MAPK family includes four highly homologous isoforms: p38α, p38β, p38γ and p38; whereas to date only p38 has been identified in blunt snout bream.

Generally, p38 MAPK is ubiquitously expressed in various organs including brain, muscle, kidney, gill and heart [10,25,26]. The findings in this study revealed that p38 MAPK mRNA could be found in all the tested tissues. The expression level of p38 MAPK was the highest in spleen followed by gill, head kidney, liver and intestine, respectively. This expression pattern is due to the fact that spleen and head kidney are major lymphoid organs in fish [27,28]. Spleen is the major immune organ with abundant IgM^+ mature B cells [29], and head kidney has the highest concentration of developing B lymphoid cells [30] and assumes immune function [31]. Moreover, there are several reports indicating that salmon p38 MAPK is mainly expressed in the immune organs such as head kidney and spleen, and its expression could be up-regulated by immune stimuli [25]. Likewise, the expression pattern observed for p38 MAPK in the current study may indicate its role in immune function of *M. amblycephala*. The higher expression of p38 MAPK in gills compared to head kidney could be due to the fact that p38 MAPK is not only involved in immune function but also its distribution in ionocytes and accessory cells of teleost fish implies its ionoregulatory function [32]. Presence of p38 MAPK has been reported throughout the ionocytes specifically in areas in which sodium potassium 2 chloride cotransporter (NKCC) are absent [32]. It has been suggested that osmotic stress may activate stress-activated protein kinase (SAPK) pathway for signal transmission and in this regard p38-related MAPK is one the responsible molecules which is activated by phosphorelation through sequential actins of kinases [33,34].

Ammonia occurs in the aquatic environment resulting from agricultural run-off and decomposition of biological waste. It is toxic to all vertebrates as it may cause convulsions, coma and death [35]. Moreover, slight ammonia stress often reduces growth and induces oxidative stress in cultured fish [11,36]. There are several reports indicating that expression of p38 MAPK significantly increases under various stresses [37]. So, in the current study we examined the effects of ammonia exposure on the p38 MAPK expression, and the results showed that its expression in gill was up-regulated after 6–24 h of ammonia exposure. Gill has been recognized as the major site that is influenced by various toxicants, and ammonia exposure induces histopathologic damages to gill [38]. The up-regulation of p38 MAPK implies its role in gill damage; however, the detailed mechanism is still unknown. Expression of p38 MAPK in liver was also up-regulated between 12 and 24 h after ammonia exposure. Some studies showed that ammonia exposure induces oxidative stress and apoptosis in fish liver [39,40]. Thus, we assume that the up-regulation of p38 MAPK is associated with the oxidative stress and apoptotic events. It has been reported that thermal stress induces anti-apoptotic events via the p38 MAPK pathway [41]. Based on these results, p38 MAPK is capable of modulating stress responses, and plays key roles in protection of organisms against environmental hazards.

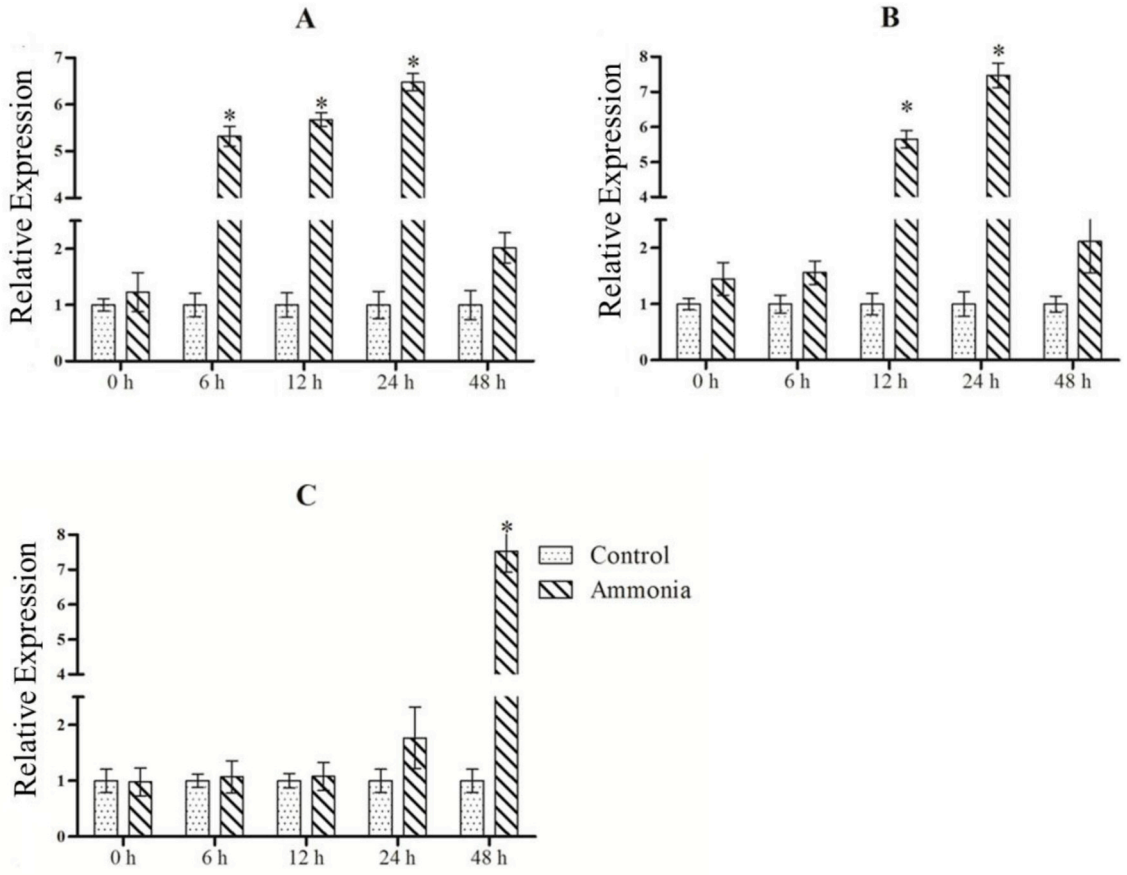

Fig. 5. Relative expression of p38MAPK in response to ammonia stress in gill (A), liver (B) and kidney (C). All values represent the mean ± SE (n = 6). *: the difference between treated samples and control sample was statistically significant ($P < 0.05$).

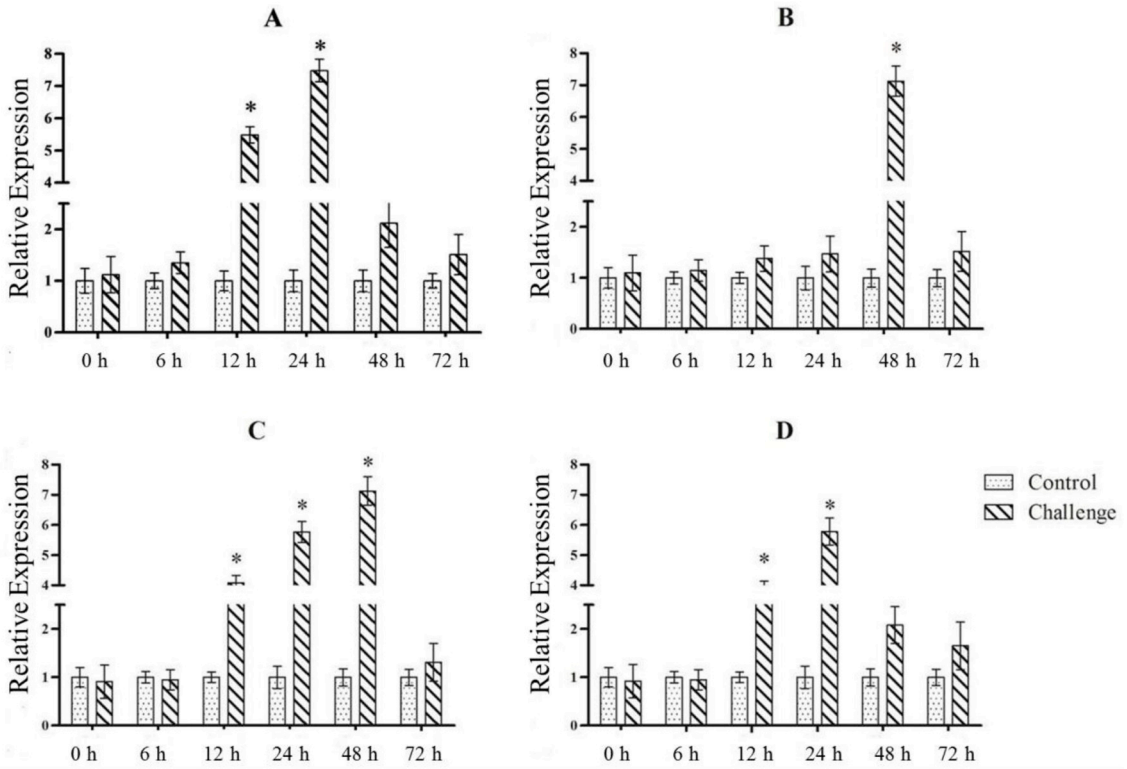

Fig. 6. Relative expression of p38 MAPK in head kidney (A), spleen (B), gill (C) and intestine (D) after *A. hydrophila* challenge. All values represent the mean ± SE (n = 6). *: the difference between treated samples and control sample was statistically significant ($P < 0.05$).

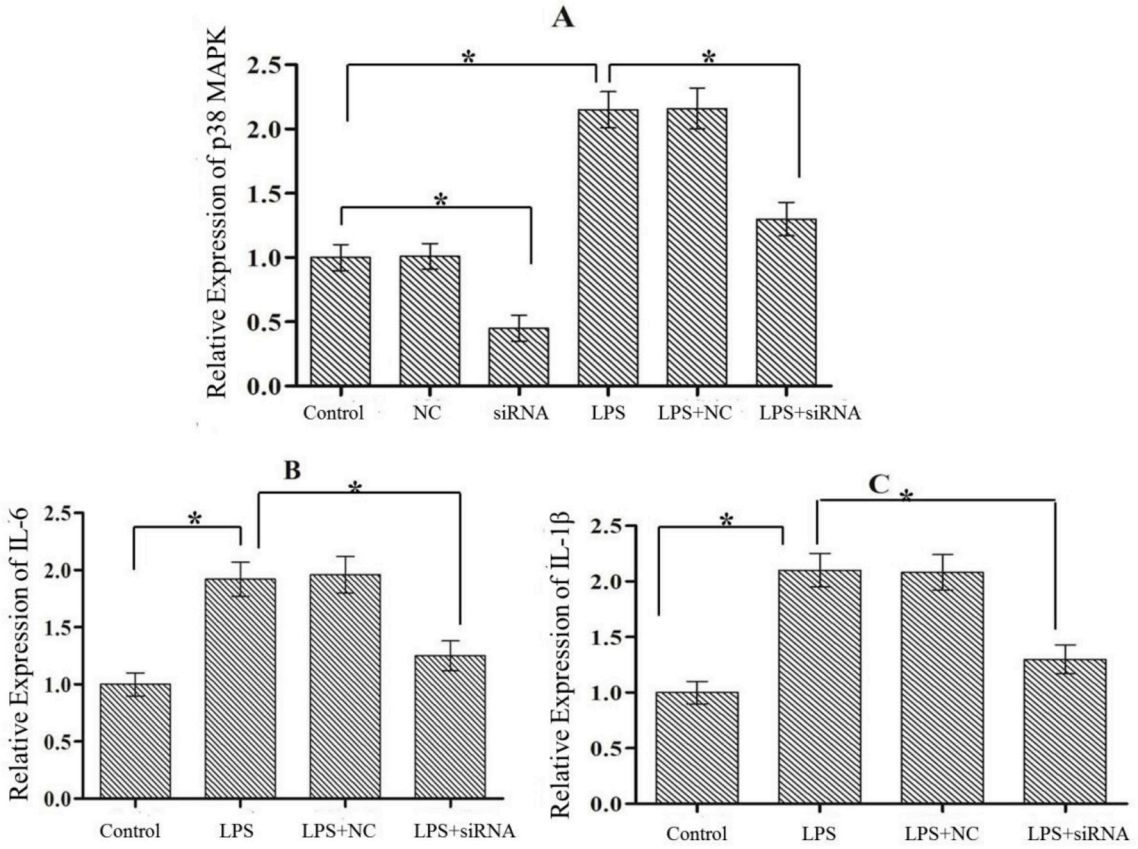

Fig. 7. Relative expression of p38MAPK and pro-inflammatory cytokines (IL-1β and IL-6) in LPS treated hepatocytes. Bars with asterisk are significantly different ($P < 0.05$).

Control: hepatocytes were only cultured with L15 media.
NC: hepatocytes were cultured with L15 media + negative control siRNA duplexes.
si-RNA: hepatocytes were cultured with L15 media + siRNA-p38MAPK duplexes.
LPS: hepatocytes were cultured with L15 media + LPS.
LPS + NC: hepatocytes were cultured with L15 media + LPS + negative control siRNA duplexes.
LPS + siRNA: hepatocytes were cultured with L15 media + LPS + siRNA-p38MAPK duplexes.

Considerable attention has been paid to the role of p38 MAPK against bacterial and viral infections [42,43]. Cai et al. (2011) showed that p38 MAPK is activated by bacterial and viral infections in grouper [44]. Also, enhanced expression of p38 MAPK in immune tissues after infection with gram-negative (*Edwardsiella tarda*) and gram-positive (*Streptococcus iniae*) bacteria has been reported in *Oplegnathus fasciatus* [45]. There is accumulating evidence that viral infection activates p38 MAPK signaling pathway [10,26]. Overexpression of p38 MAPK delayed the occurrence of cytopathic effect in fathead minnow epithelial cells after Singapore grouper iridovirus infection [10]. Furthermore, the viral gene transcription and protein synthesis were reduced by overexpression of p38 MAPK in cells [10]. In the present study, p38 MAPK expression in head kidney, gill and intestine was up-regulated and reached its peak after 24 h of *A. hydrophila* challenge, while expression of p38 MAPK in spleen was up-regulated almost at 48 h. A clear time-dependent expression pattern was observed as p38 MAPK expression significantly increased and then returned to normal levels. The expression profiles suggested that p38 MAPK contributes to immunity against pathogens. This may give us a clue to understand the relationship between p38 MAPK and pathogen infection. However, the mechanism of action of p38 MAPK during infection remains unknown and needs further research.

In other animals, the importance of the p38 MAPK pathway in pro-inflammatory genes expression has been demonstrated [46,47]. There has been accumulating evidence that p38 MAPK plays a crucial role in macrophage-mediated inflammation [48]. p38α participates in the expression of pro-inflammatory mediators in macrophages such as IL-1β, TNF-α, PGE2, and IL-12 [49–51] as well as COX-2, IL-8, IL-6, IL-3, IL-2, and IL-1, all of which contain AU-rich elements in their 3′ untranslated domain to which p38 attaches [52]. Accordingly, in this study we investigated the role of the p38 MAPK pathway in pro-inflammatory cytokines (IL-1β and IL-6) expression in hepatocytes following LPS stimulation. The findings showed that the pro-inflammatory genes expression was enhanced after LPS stimulation and this was along with up-regulated expression of p38 MAPK. To confirm whether p38 MAPK is essential for induction of inflammation by LPS-stimulation, we used RNA interference (RNAi) technology to knockdown p38 MAPK expression. The results indicated that pro-inflammatory responses were mediated through the p38 MAPK pathway as knockdown of p38 MAPK decreased the expressions of both IL-1β and IL-6. It has been reported that the p38 MAPK pathway can regulate gene expression by one of two mechanisms, activation of transcription and stabilization of mRNA transcripts [53,54]. This study provides evidence for the use of p38 MAPK inhibitors as potential therapeutics for the treatment of inflammatory responses in fish.

In conclusion, a p38 MAPK was identified and characterized in *M. amblycephala*. It is ubiquitously expressed in various tissues and its expression can be up-regulated in immune organs after ammonia stress and *A. hydrophila* challenge. Moreover, our results indicated that the inflammatory response induced by LPS in hepatocytes is p38 MAPK dependent.

Acknowledgments

This work was funded by Nature Science Foundation of Fujian Province (2017J05056), Central Public-interest Scientific Institution Basal Research & Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, P. R. China, CAFS (NO. 2018HY-XKQ01) and PHD Support Program from Henan University of Science and Technology (China, 13480074). Also, the financial support of the Ministry of Education, Youth and Sports of the Czech Republic – projects "CENAKVA" (No. CZ.1.05/2.1.00/01.0024), "CENAKVA Center Development" (No. CZ.1.05/2.1.00/19.0380), and "CENAKVA II" (No. LO1205 under the NPU I program) and by NAZV project QJ1510119 is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.074.

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