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Molecular characterization and expression of malabar grouper (*Epinephelus malabaricus*) IRF8 gene response to immuno-stimulants and nervous necrosis virus

Thirunavukkarasu Periyasamy and Ming-Wei Lu

Abstract
The interferon regulatory factor (IRF8) is considered to play essential roles in innate and adaptive immune responses. In this present study, the full-length cDNA sequences characterization, tissue distribution and expression in response to immune stimulation and nervous necrosis (NNV) challenges were in Malabar grouper *(Epinephelus malabaricus)*. The full-length cDNA of MgIRF8 was of 2537 bp, including 5'-terminal untranslated region (UTR) of 483 bp and 3'-terminal untranslated region (UTR) 785 bp. It contains an open reading frame (ORF) of 1269 bp including encoding 422 amino acid, molecular weight 47.8 KDa and 5.74 isoelectric points. The putative protein sequence possesses a DNA-binding domain (DBD), IRF-association domain (IAD) and a nuclear localization signal (NLS). Phylogenetic tree analysis classified MgIRF8 into the cluster of fish IRF8 with in vertebrate IRF8 group with IRF4 family. Quantitative real-time PCR analysis revealed a brad expression of IRF8 transcript with the highest expression of IRF8 in developmental stage at 120 h post fertilization and the tissue specific expression resulted showed that spleen, skin, muscle and head kidney were highly expressed. The expression levels of IRF8 after challenged with immuno-stimulation and NNV challenge were examined in brain, spleen and head kidney tissues. These finding contribute to an understanding of host antiviral responses and function of IRF8 in teleost fish.

Keywords: IRF8, innate antiviral response, nervous necrosis virus, *E. malabaricus*

1. Introduction
The transcription factor of Interferon regulatory factors (IRFs) were originally identified as a regulator of the type I IFN [1]. Over the last a few decades, these factors have been the focus of various biological functions in both innate and adaptive immune responses. All IRF family members possess an amino (N) terminal DNA binding domain (DBD) of about 115 amino acids, containing five conserved tryptophan (Trp) residues and forming a helix-loop helix motif [2]. It recognizes the IFN stimulated response element (ISRE) DNA sequence, which has the consensus sequence A/GNGAAANNGAAACT [3]. The carboxyl (C) terminal IAD mediates the interactions of a specific IRF with other family members except IRF1 and IRF2 [4]. The activation of IRFs is triggered by phosphorylation in the C-terminal region which induces conformation changes permitting extensive contacts to a second subunit that is transported into the nucleus and outside the IAD, the C-terminus is not well conserved. According to the characteristic of C-terminal region, IRFs have 11 members and subdivided into four subfamilies, IRF1 (IRF1, IRF2), IRF3 (IRF3 and IRF7), IRF4 (IRF4, IRF8, IRF9 and IRF10) and IRF5 (IRF5, IRF6) [5].
Interferon regulatory factor 8 (IRF8), also known as the interferon consensus sequence-binding protein (ICSBP), was first identified through screening mouse λ expression libraries with interferon consensus sequence as a probe [6]. IR8 is a key element for the differentiation of myeloid progenitor cells toward macrophages and for the development of lymphomyeloid cell lineages, including B and T cells, dendritic cells (Dcs) and macrophages [7, 8]. To perform their transcriptional regulatory functions, IRF8 can heterodimerize with other IRF family members or form homodimers to bind interferon (IFN)-stimulated response elements represented as GAAANNGAAA. Transcriptional regulatory action of IRF8, interacts with PU.1 to bind Ets/IRF composite elements (EICEs) or the IRF/Ets composite sequence (IECS), and associates with AP-1 family members to bind AP-1/IRF composite elements

(AICEs) to exert its transcriptional regulatory action [9, 10]. It has been demonstrated that IRF8 is induced preferentially by IFN-γ rather than by IFN-α/β and the induction by IFN-γ is rapid which doesn't depend on de novo protein synthesis, suggesting a nature of an immediate early gene for IRF8 in host's immune responses [6].

Moreover, the report that ectopic expression of IRF8 enhances IFN-α and IFN-β expression further supports the idea that IRF-8 is capable of stimulating type I IFN transcription [11, 12]. In addition to the establishment of host resistance, type I IFN brings about diverse consequences on innate and adaptive immunity and affects activity and maturation of dendritic cells (Dcs) themselves. Further, IRF8 was reported as an important regulator of cross-talk between the toll-like receptor (TLR) and IFN-γ signaling pathways with respect to LPS-TLR4 and poly (I: C)-TLR3 ligations [13] IFN-γ in concert with LPS, however, can synergistically enhance IRF8 expression indicating cross talk between the STAT1 and NF-kB pathways [14, 15]. Moreover, the crucial role of IRF8 in the development of Dcs subtypes and the amplification of IFN production in plasmacytoid Dcs (pDcs) makes it a likely target for poly (I: C)-activated signaling pathways [8, 16].

Recently, great progress has been made in fish innate immunity, which is similar to mammalian innate immunity. Fish IRF8 is up-regulated in vivo after stimulation with immuno-stimulation and virus, suggesting it participates in cellular antiviral defense. However, precise evidence regarding IRF8 expression and immune response in grouper is also not available. The aim of this study is to investigate the full-length of MgIRF8 characterization, gene expression profiles of embryonic developmental stage and various tissues distributions and then focusing the challenges with immuno-stimulants (poly I: C, and LPS) and nervous necrosis virus infection.

2. Materials and Methods

2.1 Animals

Healthy Malabar grouper *(E. malabaricus)* average sizing from 4 to 5 cm were purchased from commercial form (Kaohsiung, Taiwan) and were kept in laboratory environment (1000-L aquaria) for pathogen free for two weeks before experiment processing. Three to four days of experiments were conducted by following the institutional IACUC guide of the National Taiwan Ocean University, Keelung, Taiwan.

2.2 Cell culture and virus

The grouper kidney cells (GK) [17] and fin (GF-1) [18] cells were established as previously described. The GK and GF-1 cells were grown in L-15 medium (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 ug/mL streptomycin in a humidified incubator under 5% CO_2 at 28 °C. Nervous necrosis virus, which was isolated from M. grouper brain tissue, was propagated in GF-1 cells cultured in 2% FBS-supplemented L-15 medium at 28 °C [18]. The NNV was measure the titer 10^7 $TCID_{50}$ per mL by using the endpoint method described by Reed and Muench [19]. The NNV were incubated at -80 °C until use.

2.3 Bioinformatics analysis of MgIRF8

The cDNA sequence containing the complete open reading farm (ORF) of MgIRF8 was obtained from transcriptome annotation established in our previous study [20] and feeding other vertebrae orthologs as keys. According to the IRF8 sequence were retrieved and sequence-specific primers were designed according to our database. The detected amino acid sequences of M. grouper IRF8 similarity and identity were analyzed using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments M. grouper IRF8 gene sequences from different species were performed with the Clustal W program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The protein domain topology was predicted by the SMART program (http://smart.embl-heidelberg.de/). The molecular weight and isoelectric point of the protein were predicted by the Compute pI/Mw tool (http://www.expasy.ch/). Phylogenetic trees were constructed according to the amino acid sequences of grouper and other vertebrae orthologs by the neighbor-joining method using MEGA7 software with 1000 bootstraps.

2.4 Developmental Stage and Tissue distribution of MgIRF8

Fertilized eggs obtained by artificial fertilization were incubated at 17 ± 1 °C in clean sea water with continuous aeration. Three pools of samples at embryonic and post embryonic stage samples were collected at 0, 6, 12, 24, 48, 72 and 120 h post fertilization [21]. The healthy M. grouper eye, brain, heart, head kidney, trunk kidney, liver, spleen, gas bladder, gill, muscle and skin samples were collected from each of three individuals. The collected tissue samples were frozen rapidly in liquid nitrogen and stored at -80 °C until use.

2.5 Expression of IRF8 by Immuno-stimulation in vivo

In order to determine the IRF8 upon immuno-stimulation experiments, 100 uL of lipopolysaccharide (LPS) suspended in phosphate buffered saline (PBS) (10 ug/mL; *Escherichia coli* 0127:B8, Sigma Aldrich, USA) and 100 uL of polyinosinic:polycytidylic acid (poly I: C) in PBS (5 ug/uL, Sigma Aldrich, USA) were intraperitoneally injected to each fish from three separate fish groups, respectively. Brain, spleen and head kidney tissue samples were collected at 0, 6, 12, 24, 48 and 72 h post stimulation were collected and immediately frozen rapidly in liquid nitrogen and stored at -80 °C until RNA extraction.

2.6 Expression of IRF8 by Nervous necrosis virus in vivo

NNV challenge experiment was performed according to the two experimental groups. Healthy M. grouper were intraperitoneally injected with 0.1 mL of NNV containing 10^5 $TCID_{50}$ per mL, and 0.1 mL of PBS as a control. Brain, spleen and head kidney tissue samples were collected from the fish of the infection group and the control group (three fish each) at 0, 6, 12, 24, 48 and 72 h post-infection. The tissue samples were stored at -80 °C until use.

2.7 RNA isolation and cDNA synthesis

The total RNA was extracted from selected tissues using TRIzol reagent, according to the manufacturer's instruction. A Nanodrop spectrophotometer method by analysis the RNA sample quality, (OD260/280 nm). The total RNA (1 ug) was used to synthesize the first-stand cDNA with Hi-Script Reverse Transcriptase (BIONOVAS, CA), which was used as a template to amplify the IRF8 and β-actin genes.

2.8 Real-time PCR analysis

Gene expression profiles were analyzed by quantitative real-time PCR performed using an ABI Applied Biosystems 7500 real time PCR system V2.0.6 (Applied Biosystems, USA) equipped with analytical software version 7500HD. The qPCR reaction was performed in a final volume of 20 ul containing 1 ul of first-strand cDNA, 10 ul of 2X SYBR Green qPCR Master Mix, 8 ul of distilled water and 1 ml of each primer (10 pmol/ul) (IRF8-GGCTGTGTTTAAAGGCA AGTT/ CTCCTCCTCAGGCACAAT and b-actin- CACAG TGCCCATCTACGAG/ CCATCTCCTGCTCGAAGTC). The thermal cycler conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10s, 56 °C for 15s, and 72 °C for 15s. The DNA melting curve analysis was used to verify the specificity of the primers. The house keeling gene b-actin was used as an internal control for cDNA normalization.

2.9 Statistical analysis

The means of triplicates were representative of more than three independent and were subjected to Student's t-test. The data were represented as mean ± SD, and significant differences were achieved $p<0.05$*. High significance was achieved $p<0.01$** and $p<0.001$***.

3. Results

3.1 Sequence characterization MgIRF8

The full-length cDNA sequence of IRF8 (Genbank ID: MF490283) was identified by homology screening of the M. grouper cDNA transcriptome database. Full-length IRF8 sequence is 2537 bp including a 5' untranslated region (UTR) of 483 bp, a ORF of 1269 bp that encodes 422 amino acid (AA) and a 3' UTR of 785 bp (Fig. 1). The amino acids representing a protein with a 47.8 KDa molecular weight, and its putative isoelectric point (pI) of 5.74. In the 3' UTR, one poly-(A)-tail is located end of these sequences, respectively. Sequencing and Blastx analysis indicated that MgIRF8 shared high similarity to other reported IRF8 genes.

Table 1: The amino acid identities (%) and similarities (%) and the gap of Malabar grouper IRF8 to reprehension in other species.

Common name	Species	Accession no.	AA	Identity (%)	Similarity (%)	Gap
Malabar grouper	*Epinephelus malabaricus*	MF490283	422	100	100	0
Sea bream	*Oplegnathus fasciatus*	AFU81290.1	422	92	96	0
Large yellow croaker	*Larimichthys crocea*	XP_010739904.2	422	91	95	0
Turbot fish	*Scophthalmus maximus*	AFE88896.1	420	90	94	2
Japanese puffer	*Takifugu rubripes*	XP_003970043.1	421	83	91	1
Southern platyfish	*Xiphophorus maculatus*	XP_005805008.1	422	83	91	0
The rainbow trout	*Oncorhynchus mykiss*	ALS92677.1	444	72	79	31
The grass carp	*Ctenopharyngodon idella*	AMT92197.1	428	67	78	29
Zebrafish	*Danio rerio*	NP_001002622.1	423	65	76	34

3.2 Homology sequence and Multiple sequence alignment

A pairwise alignment of the MgIRF8 protein sequence with other IRF8 members revealed that MgIRF8 shared the highest identity (92%) and similarity (96%) with the *O. fasciatus* IRF8 homolog. The next highest identities were with *L. crocea* (91%) and *S. maximus* (90%). The teleost IRF8 homologs showed 65-92% amino acid sequence identity range whereas, zebrafish to M. grouper and zebrafish had less than 65% amino acid identity with IRF8 counterpart (Table 1). The multiple alignment revealed that three conserved domains: an N-terminal DNA-binding domain (DBD) characterized by a five tryptophan pentad-repeat, a C-terminal IRF association domain (IAD) and a bipartite nuclear localization signal (NLS) in the DBD (Fig. 2). Alignment protein sequences of IRF8 revealed that N-terminal region showed highly conserved from fish then human, and even the sequences of teleost IRF8 were shorter, respectively (Fig. 2). The MgIRF8 and IRF8 motifs are highly conserved among invertebrates, mammals and fish.

A.

```
3                                                                    tag
36    aaccagcgcttgtgtcaggccatttcttgtaaatgcccgcagttaggagaaaaatcactt
92    ccggaatgggccggccttcagaagcttgagcgcgctgattggtcagagcgcggctgcgtt
148   gtcaaatcatctcccagctatttcattcataacacggagcacaataaactcgataggcat
204   gtgtcggggagtgctcatttgattacatctgtcatccgtcaacatcttaggatatcacac
260   acagggtcaagagctcaaccgcgtatcaaagtgtaagtgcttttacttttgatttctaat
316   ctaaaggtgaaggctggagaattaaaggtgtaagtgttgccttaattaaagatacattag
372   agcataataatatctcagacaaaactaaatagtagcctaacatttatatgctatcatga
428   cttttatcctgctcatttaggttcaaggcagcaatccaaaagcaacttttctccatcaag
484   ATGTCAAACACGGGAGGTCGCAGACTGAAGCAGTGGCTGATGGAGCAGATCCAGAGCGGC
        M  S  N  T  G  G  R  R  L  K  Q  W  L  M  E  Q  I  Q  S  G
554   CAATACTCCGGACTGCAGTGGGAGGATGAAAGTCGCACTATGTTCCGAATTCCATGGAAA
        Q  Y  S  G  L  Q  W  E  D  E  S  R  T  M  F  R  I  P  W  K
604   CACGCAGGGAAACAGGATTACAACCAAGAAATAGACGCATCCATTTTTAAGGCCTGGGCT
        H  A  G  K  Q  D  Y  N  Q  E  I  D  A  S  I  F  K  A  W  A
664   GTGTTTAAAGGCAAGTTTAAGGAGGGGGACAAGGCTGAGCCTGCAACATGGAAGACCAGA
        V  F  K  G  K  F  K  E  G  D  K  A  E  P  A  T  W  K  T  R
724   CTCCGCTGTGCCCTGAATAAAAGCCCTGACTTTGAGGAGGTGACGGAAAGGTCGCAGCTG
        L  R  C  A  L  N  K  S  P  D  F  E  E  V  T  E  R  S  Q  L
784   GACATCTCTGAGCCCTATAAAGTCTACCGCATTGTGCCTGAGGAGGAGCAGAAGCATGGC
        D  I  S  E  P  Y  K  V  Y  R  I  V  P  E  E  E  Q  K  H  G
844   AAAAGCTCAGTGATGGCCATGGCGGCTACCACCAGCACTGGTGATATCACTGACATGGAC
        K  S  S  V  M  A  M  A  A  T  T  S  T  G  D  I  T  D  M  D
904   TGCAGCCCTGCAGAGATAGAGGAGTTTATAAAAGAGGAGGAAGGCCGTAGTATCCAGGCC
        C  S  P  A  E  I  E  E  F  I  K  E  E  E  G  R  S  I  Q  A
964   AGTCCAGAGTATTGGTCCCAGGGCAGCATCAATGCTTTCCCTCTGCATCAGGACCCTTTG
        S  P  E  Y  W  S  Q  G  S  I  N  A  F  P  L  H  Q  D  P  L
1024  CCATCAGGCGGTCTCAGCTCAGCTTTCTCCCAAATGCTGATCAGTTTCTACTACGGAGGA
        P  S  G  G  L  S  S  A  F  S  Q  M  L  I  S  F  Y  Y  G  G
1084  AAGCTGATGCACACCACAGCGATTACTCATCCTGAAGGCTGCCGGATCTCTCCACAGCAG
        K  L  M  H  T  T  A  I  T  H  P  E  G  C  R  I  S  P  Q  Q
1144  CACCTGGGTCGTGGCACCATGTACAGTTCAGACAGCATGCAGAGTGTTCATTTCCCTCCC
        H  L  G  R  G  T  M  Y  S  S  D  S  M  Q  S  V  H  F  P  P
1204  CCCGAGCTCATCGAGTACGACCGCCAGCGTCACGTCACACGCAAGCTCCTGGGCCACCTG
        P  E  L  I  E  Y  D  R  Q  R  H  V  T  R  K  L  L  G  H  L
1264  GAGAGAGGCGTGCTGGTCCGTGCCAACCAAGAGGGCATCTTCATCAAGAGGCTGTGCCAG
        E  R  G  V  L  V  R  A  N  Q  E  G  I  F  I  K  R  L  C  Q
1324  AGCCGTGTCTTCTGGAGCGGGCTGGGAGAAGTGGGCTCACCATTCAGCCCCATGCCTTGT
        S  R  V  F  W  S  G  L  G  E  V  G  S  P  F  S  P  M  P  C
1384  AAACTTGAAAGGGATGCTGTTGTCAAGATTTTGACACAGGGAAGTTTCTTCAAGCTCTA
        K  L  E  R  D  A  V  V  K  I  F  D  T  G  K  F  L  Q  A  L
1444  CAGATGTACCAGGAGGGTCAGTTTCCTGCTCCTGATCCGACAGTGACTCTGTGTTTCGGA
        Q  M  Y  Q  E  G  Q  F  P  A  P  D  P  T  V  T  L  C  F  G
1504  GAGGAGCTCAGTGATCTCAGCAATGCCAAAAGCAAACTGATCATTGTGCAGATCACTGTG
        E  E  L  S  D  L  S  N  A  K  S  K  L  I  I  V  Q  I  T  V
1564  GTGAACTGCCAGCACCTGCTGGAGGCAGTGAACATGCGCCGCTCCCAGCCCTACTGCAAC
        V  N  C  Q  H  L  L  E  A  V  N  M  R  R  S  Q  P  Y  C  N
1624  AACCCAAACCTGGAGATGTCTGATAATGTGGCCAGTGACCAGATGGCCCGTATCTACCAG
        N  P  N  L  E  M  S  D  N  V  A  S  D  Q  M  A  R  I  Y  Q
1684  GACTTGTGCAGCTACAGCGGCCCCCAGAGGCCAGCCTGCTACAGGGACAACATGCCCATC
        D  L  C  S  Y  S  G  P  Q  R  P  A  C  Y  R  D  N  M  P  I
1744  ACTGCCTGATCTGTGAGCAGCAACACACCTGCCAGGAGCTCTCCAGCGTGTATCAGACTG
        T  A  *
1804  agtgtacaacatattagggacattccttatttttactgacatacactgacaagatgaattc
1864  agacaaagcacagtattgtaatcagttttaacttcatacttttacgcttataccaatca
1924  cagtaaggacaagaaaatctcatacaatagcatattaaagctgcagctccaacccgttct
1984  caattttaggatgtccctggtcccttgtatgtcagtaatgtttgtatttttttttacaagt
2044  gcagtgctaacaaaaactctgtgtttaacttctgcttttttattgcatgggagtatcatc
2104  atccagctcaagagacaaaagtagcgtgttataatatatgttacacacaacttgtgtgtt
2164  gtaaagccttaagaacagattcactgtggtgacttccatcacagaacttttctcatatata
2224  tctgtaaagactagactcaaagcctcggagcagtgtgacagcagttggatatatgcaatg
2284  tagttctcaaagctgaatttatgtgtttgttttgaatttgagttgctttctcatagat
2344  aactgtatggataaacagacaaaattaatttttatctatttttacagtaaatgaaaaa
2404  tcttttcatgagttgataaaaaaagtgtggaattgtaggtgcaatgtggcattatattac
2464  agatagggttttactatcagactgatttttttataaactgcatttctagtaaactagtttgt
2524  actgaaaaaaaaaa
```

B.

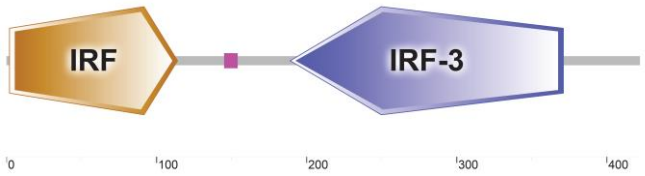

Fig 1: (A) Nucleotide sequence of MgIRF8 cDNA and the deducted amino acid sequence. Nucleotides are numbered in the 5'-3'-direction. The open reading frame sequences are shown in capital letters. The start codons (ATG) and stop codons (TGA) are boxed. The stop codon is indicated by an asterisk. The poly-(A) tail is underlined at the end of the nucleotide sequence. (B) Illustration of the IRF8 protein structure of M. grouper by SMART model online software

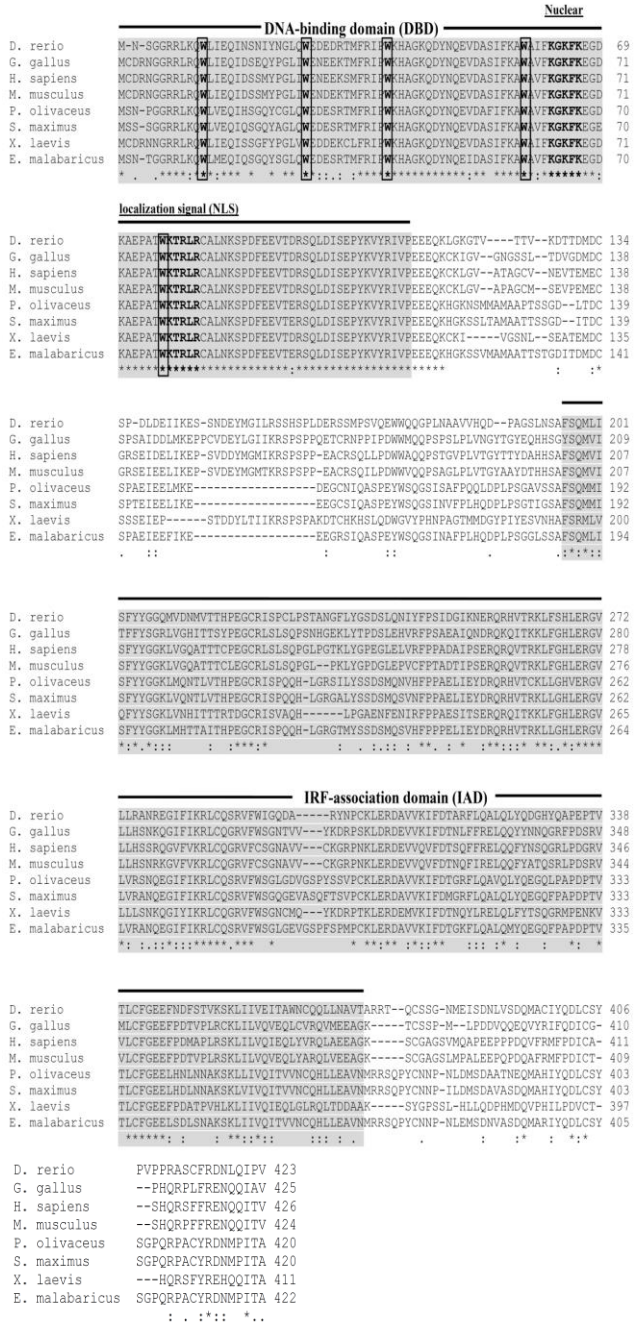

Fig 2: Multiple alignment of MgIRF8 amino acid with other IRF8 proteins. The putative DA-binding domain (DBD) and IRF-association domain (IAD) are shaded in gray. The bipartite nuclear localization signal (NLS, KGGFGn^{10}KTRLR) is bold and indicated underlined. The served tryptophan (W) residues composing a "tryptophan cluster' are boxed in the DBD. The residues identical in all sequences are shown with asterisks (*), whereas those with strong homologies and weak similarities are marked by colons (:) and dots (.), respectively. The accession numbers of the sequences are listed in Table 1

3.3 Phylogenetic Analysis

A phylogenetic tree was constructed by the neighbor-joining (NJ) method based on the multiple alignments of the predicted amino acid sequences of MgIRF8 with other counterparts (Fig. 3). The full amino acid sequence showed that MgIRF8 was clustered with *M. miiuy* and *O. fasciatus* IRF8. The cluster degree closely related to the *M. miiuy* IRF8 (Fig. 3). IRF8 protein was firstly clustered with other vertebrate proteins and significantly diverged from vertebrate IRF8s. The results indicated that the evolutionary relationship revealed in the Phylogenetic tree of MgIRF8 was in agreement with the member of fish IRF8 family.

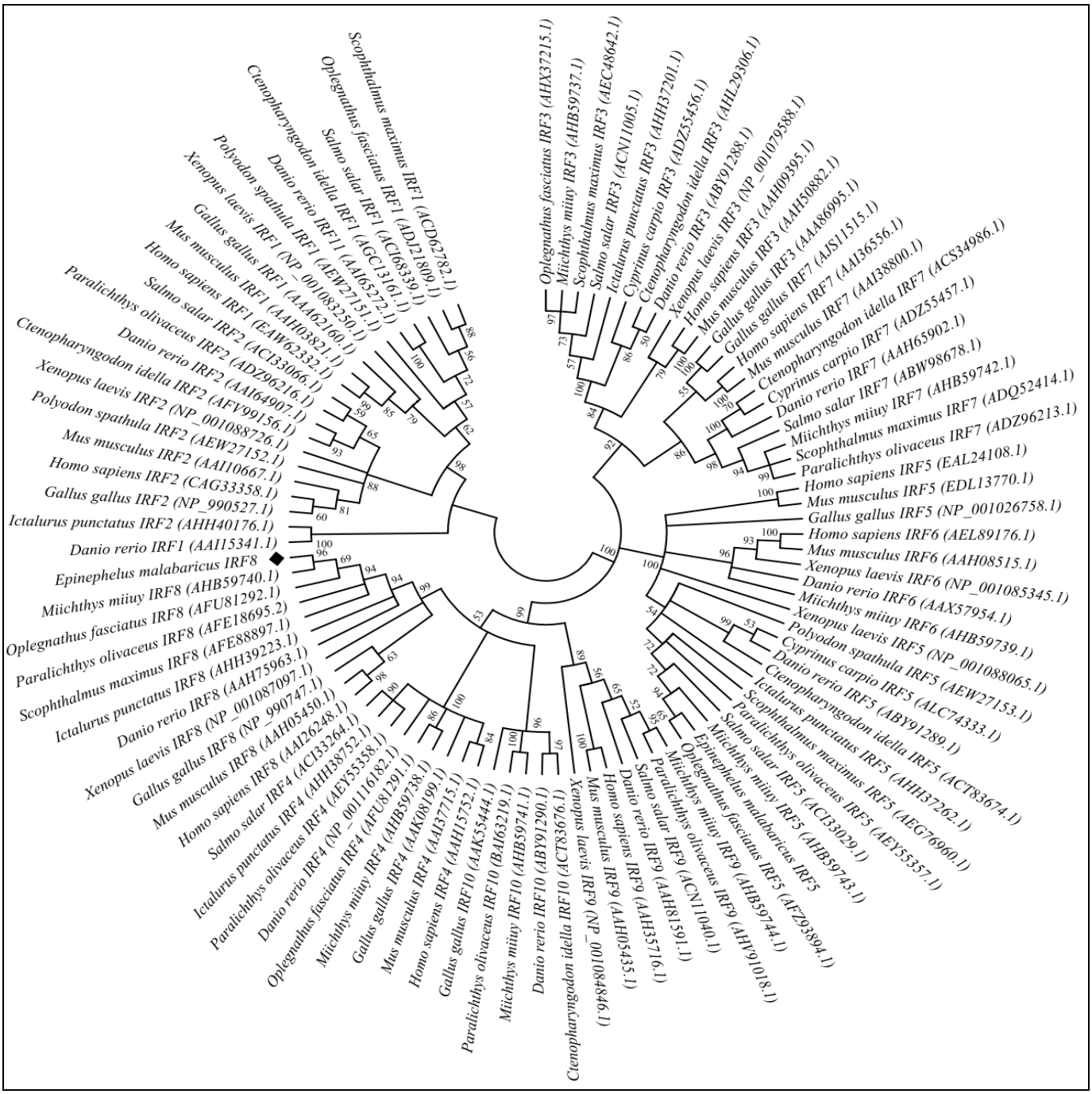

Fig 3: Phylogenetic analysis of MgIRF8 with other members of the IRF8. The phylogenetic tree was constructed using MEGA software 7.0 by Neighbor-joining method. Bootstrap values are shown next to the branches based on 1000 replications.

3.4 Tissues distribution of MgIRF8

To further understand the potential function of IRF8 mRNA transcript in developmental stage and different tissues from healthy M. grouper was examined by quantitative real-time PCR analysis (Fig. 4). The transcripts of MgIRF8 gene level were significantly expressed at 72 h (0.018-fold) up to 120 h (0.04-fold) post fertilization (Fig. 4A). As shown in Fig. 4B, MgIRF8 transcripts could be found tissue specific and the spleen (0.075-fold) was the highest expression and gradually decreased in eye, gill (0.048-, 0.041-fold) and intestine is lowest expression (0.021-fold). However, the overall expression of MgIRF8 mRNA was higher in spleen in detected differential tissues.

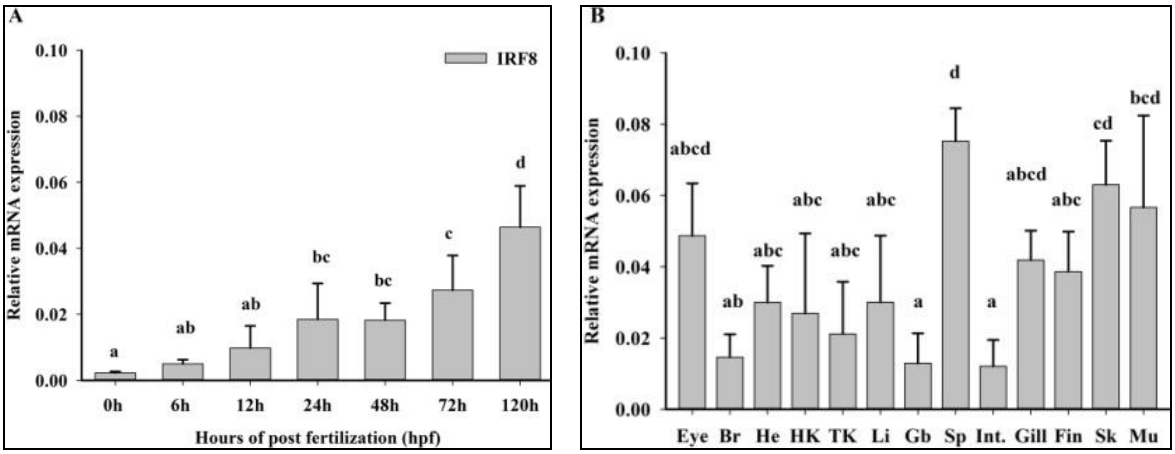

Fig 4: Embryonic developmental stage and tissue distributions of MgIRF8 in healthy M. grouper. (A) Relative expression levels of MgIRF8 in embryonic developmental stage at 0, 3, 6, 12, 24, 48, 72 and 120 h post fertilization. (B) Relative expression levels of the MgIRF8 in eyes, brain, heart, head kidney, trunk kidney, liver, gas bladder, intestine, spleen, gills, muscle and skin were analyzed by quantitative real-time PCR. M. Grouper β-actin expression was used as an internal control and the data were shown as mean ± SD (N=3). Values marked by different letters are significantly different from each other

3.5 Expression pattern of IRF8 upon Immuno-stimulation *in vivo*

In order to determine the innate immune responses of MgIRF8 in brain, spleen and head kidney of immuno-stimulation were shown in Fig. 5 & 6. In brain and spleen, the expression of IRF8 was significantly up-regulated to 8.78-fold and 2.02-fold by poly (I: C) stimulation at 12 hps (Fig. 5A). However, in brain a significant increase of MgIRF8 expression was observed at 6 hps after poly (I: C) stimulation. As sown in Fig. 5C, the expression level were significantly increased 6.26- to 12.36 fold at 12-24 h after poly (I: C) stimulated spleen tissues. Considering that MgIRF8 is an important antiviral function, we therefore tested the expression profiles of MgIRF8 following the immune and non-immune tissues expression profiles under LPS stimulations at indicated time points. The experiment showed that LPS challenge could induce IRF8 expression in brain and spleen tissues only (Fig. 6). In spleen, 3.71-, 3.40- and 1.90-fold were significantly increased at 6-24 h post stimulation by LPS treatment (Fig. 6B). However, a high expression of MgIRF8 was detected at 72 hps LPS stimulation. As it shown in Fig. 5 & 6, the transcription of MgIRF8 significantly higher level in immuno-stimulated samples compared with that of the control groups.

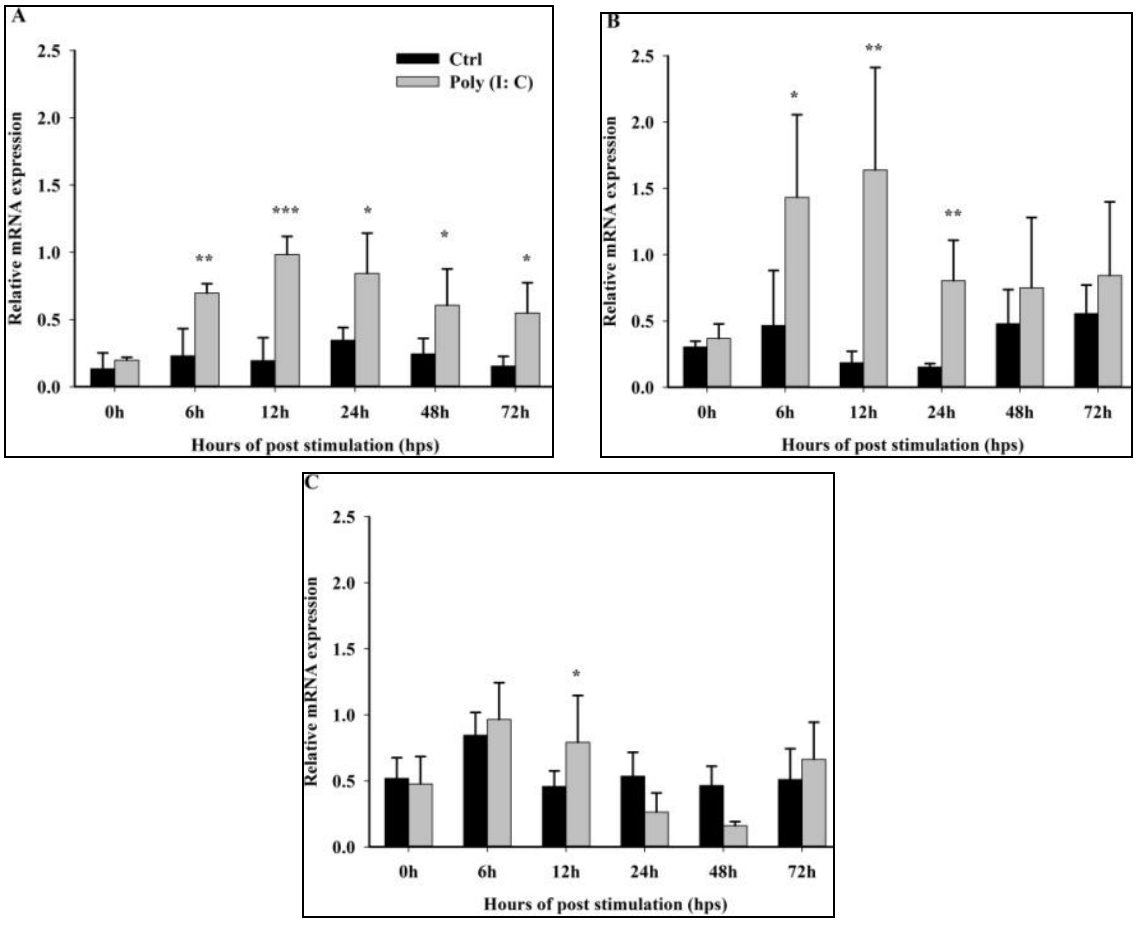

Fig 5: Relative mRNA expression of MgIRF8 in the brain (A) spleen (B) and head kidney (C) after poly (I: C) stimulations with M. grouper was

detected using quantitative real-time PCR at 0, 6, 12, 24, 48, and 72 hps. The mRNA levels of MgIRF8 were normalized by β-actin mRNA levels in the same sample and expressed as the fold changes of the control group. Data presented are expressed as mean ± SD (N = 3). The statistical analysis was conducted with student t-test. Significant differences at P< 0.05 and P< 0.001 are labeled with * and **, respectively

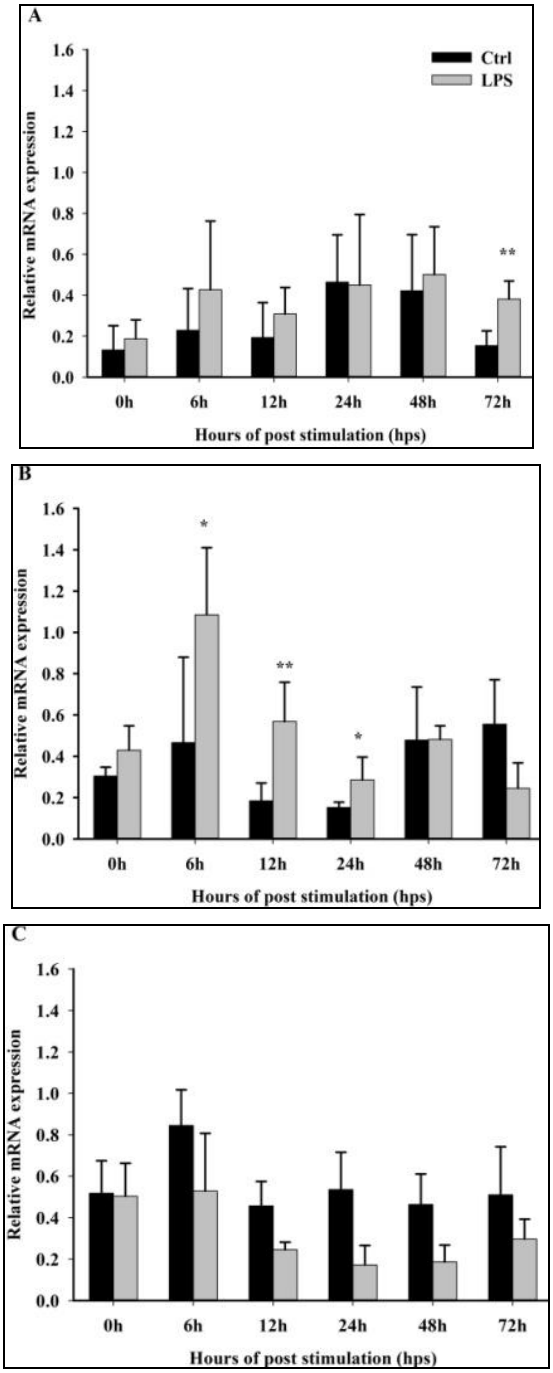

Fig 6: Quantitative real-time PCR analysis of MgIRF8 in the brain (A) spleen (B) and head kidney (C) after LPS stimulations with Malabar grouper at 0, 6, 12, 24, 48, and 72 hps. The mRNA levels of MgIRF8 were normalized by β-actin mRNA levels in the same sample and expressed as the fold changes of the control group at the same time point. Data presented are expressed as mean ± SD (N = 3). The statistical analysis was conducted with student t-test. Significant differences at P< 0.05 and P< 0.001 are labeled with * and **, respectively

3.6 Expression pattern of IRF upon NNV challenge *in vivo*

Based on the nervous necrosis virus infection, the immune response gene expression of IRF8 was quantified by real-time PCR analysis (Fig. 7). The expression level of IRF8 increased significantly from 24 to 72 h after NNV challenge. In brain, the result showed that the highest expression level of 2.67-fold at 48 h post injection (Fig. 7A). In head kidney, the expression level of IRF8 was up-regulated by NNV at 12-24h (1.33, 1.47-fold) and subsequently decreased after 24 h post infection (Fig. 7C). At 12 phi, the infected spleen tissues were showed that the highest expression levels (2.17-fold). The expression levels of MgIRF8 had significant in immune and non-immune tissues post infection of NNV comparison with the control group.

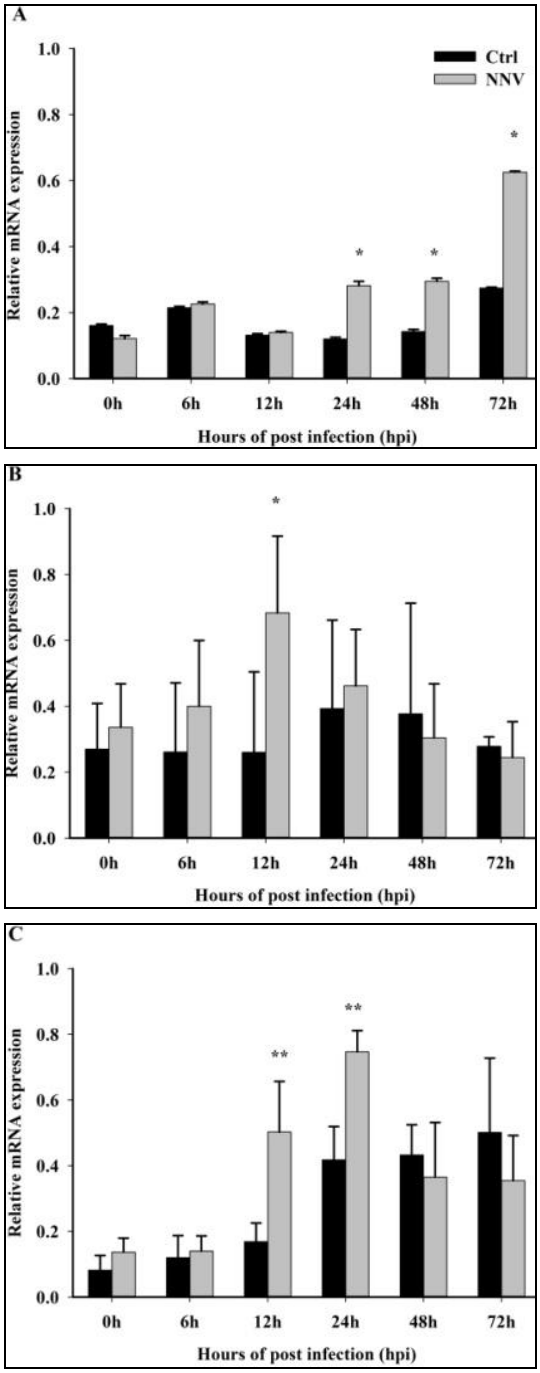

Fig. 7: Real-time quantitative PCR was performed using primer specific MgIRF8 on cDNA from brain (A) spleen (B) and head kidney (C) after NNV infections with M. grouper was detected at 0, 6, 12, 24, 48, and 72 hpi. The mRNA levels of MgIRF8 were normalized by β-actin mRNA levels in the same sample and expressed as the fold changes of the control group. Data presented are expressed as mean ± SD (N = 3). The statistical analysis was conducted with student t-test. Asterisks denote significances in expression between MgIRF8 with in a sample samples, P< 0.05* and P< 0.001** are labeled

4. Discussion

In the present study, the full-length cDNA of IRF8 sequences was isolated and identified from the spleen of the M. grouper. The full-length IRF 2537 bp, a 5'UTR of 483 bp and a 3'-UTR of 785 bp with a poly (A) tail, including an ORF of 1269 pb encoding a poly peptide of 422 amino acid (Fig. 1). The IRF8 showed high identity with *O.s mykiss* [22], *O. fasciatus* [23], *S. maximus* [24], *P. olivaceus* [25] and *G. morhua* [26]. Moreover, the sequence homologs comparison from fish to mammalian IRF8 based on the conserved regains, and it may have a similar function to that of other vertebrate IRF8.

The deducted IRF8 amino acid sequence verified that the conserved a DNA-binding domain (DBD) and interferon-activated domain (IAD). IRF family members shows that they share a DBD for about 120 amino acids, characterized by a cluster of five conserve tryptophan residues, which forms a 'winged' helix-turn-helix motif that binds to the IRSE and IRF regulatory element (IRF-E) sites in target promoter [27, 28]. A C-terminal IRF association domain (IAD), which was originally identified in IRF family members except IRF1 and IRF2 and plays an important role in the formation of IRF homo/hetero-dimmers show distinct DNA-binding specificities and affinities for stimulate or suppress gene transcription [29]. The third conserved domain NLS was found to locate in the DBD, and its related to nuclear translocation and reservation of IRFs. Is has been highly conserved in IRF1, IRF3, IRF4, IRF5, IRF8, and IRF9 [30] NLSs are essential for nuclear translocation and retention of IRFs in virus infected cells [1], but its function has not yet been clarified for IRF8 so far.

In vertebrates, IRF8 transcripts are predominantly expressed in lymphomyeloid-rich tissues such as monocyte/macrophage lineage, B lymphocytes, activated T lymphocytes, DCs and microglia, the tissue resident myeloid cells of the brain [6, 31]. Indeed, in our experiments, the MgIRF8 transcript was detected in differential tissues from healthy M. grouper, higher level of IRF8 were quantified in the spleen and head kidney, and lower level in the gill, heart and eye, respectively (Fig. 4B). However, the gene expression profile in various tissue distributions similarly displayed in grouper fish then known fish species [22-26], which may due to the physiological status similar functions. To our knowledge, we are the first group have been studied the transcript level in developmental stage, and the IRF8 transcripts were significantly observed at 24-72 hpf (Fig. 4A). These results suggest that the IRF8 of M. grouper may play an important role in fish development.

Previous studies have demonstrated that antiviral activity of IRF8 can be induced by poly (I: C) and various viruses [22-26]. In the present study, the transcription profile of IRF8 was analyzed by real-time PCR method in immune challenges such as poly (I: C) and LPS (Fig. 5 & 6). Up on poly (I: C) stimulation, the enhance MgIRF8 transcription was significantly higher in spleen and head kidney at 24h (Fig. 5A-C). These results are similarly observed in the other fish species such as *O. mykiss* [22], *O. fasciatus* [23], *S. maximus* [24], *P. olivaceus* [25] and *G. morhua* [26]. Moreover, IRF8 gene was significantly increased in gill and muscles after immune stimulation of poly (I: C), suggesting this immune response pathways for the activation of IRF8 fish species [24, 25]. In addition, MgIRF8 transcription after LPS challenge in lymphoid and non-lymphoid tissues were significantly up-regulated at 24, 48 hps (Fig. 6A-C). In spleen, MgIRF8 was significantly up-regulated up to 24 hps, and thereafter no significantly observed until 72 hps. Similar transcription profiles were studied in LPS challenge in *O. mykiss* [22] and *O. fasciatus* [23], which indicates that they may have similar roles in M. grouper.

Previous studies have shown that the transcription of IRF8 was analyzed in lymphatic and non-lymphatic tissues such as spleen, head kidney, gill and muscle in experimental infected fish species [23-26]. In grouper, IRF3, IRF7 and IRF5 (Unpublished) have been experimentally demonstrated that the immune response against NNV infection [32, 33], but not IRF8 gene. In our present study, a potential induction of IRF8 was analyzed in the spleen, head kidney and brain at 24h post infection after NNV infection, which is similar to the observation for the IRF8 expression from infected fish species [23-26]. Thus, we suggested that IRF8 functionally in diverse lineages in vertebrates to against virus infection.

5. Conclusion

In conclusion, IRF8 has been identified from M. grouper transcriptome databank and characterization and functional domain organization by bioinformatics tool. The tissues expression profiles revealed that IRF8 expressed in various healthy tissues and involved in the immune response to poly (I: C), LPS stimulation and NNV infection. These finding could provide the important of innate immune response in vertebrates IRF members.

6. Acknowledgement

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