www.trjfas.org
ISSN 1303-2712
*Turkish Journal of Fisheries and Aquatic Sciences 18:1421-1430(2018)* DOI: 10.4194/1303-2712-v18_12_10

RESEARCH PAPER

# Molecular Cloning and Functional Analysis of IRF-1 in the Asian Swamp Eel (*Monopterus albus*)

**Lihai Xia[1], Kai Luo[1], Qiaoqing Xu[1], Hanwen Yuan[1,2], Weihua Gao[1,*],Wenbing Zhang[3,*]**

[1] Engineering Research Center of Ecology and Agricultural Use of Wetland, Ministry of Education, Yangtze University, Jingzhou 434024, China.
[2] College of Marine and Biotechnology, Guangxi University for Nationalities, Guangxi 530006, China.
[3] The key laboratory of Mariculture(Education Ministry of China), Ocean University of China, 5 Yushan Road, Qingdao, Shandong 266003, PR China.

* Corresponding Author: Tel.: +86.716 8062788; Fax: +86.716 8066301;
E-mail: xivisfnf@qq.com

Received 21 November 2017
Accepted 08 February 2018

**Abstract**

Interferon regulatory factors (IRFs) have been studied in mammals and various fish species, but not in the Asian swamp eel (*Monopterus albus*). Complete IRF-1 cDNA sequence and genomic DNA sequence of *M. albus* were cloned. The IRF-1 gene, named *maIRF1*, spanned 3.5 kb from the transcription start site to the polyadenylation signal. The *maIRF* mRNA contained an open reading frame of 885 nucleotides, which translated into 294 amino acids. The overall amino acid cDNA sequences shared 42.5-81.0% identity with IRF-1 in Mandarin (*Siniperca chuatsi*), Crucian carp (*Carassius auratus*), Atlantic salmon (*Salmo salar*), Grass carp (*Ctenopharyngodon idella*), Zebrafish (*Danio rerio*) and Fugu (*Takifugu rubripes*), and 36.6-38.0% identity with IRF-1 in Chicken (*Gallus gallus*), Frog (*Xenopus tropicalis*), Human (*Homo sapiens*) and Mouse (*Mus musculus*). Quantitative real-time PCR revealed that *maIRF1* expression changed little during the three different growth stages (female, intersex, male). Moreover, *maIRF* was constitutively expressed in all analyzed organs, being most predominantly expressed in the trunk kidneys and blood and the least in muscle tissue. The expression levels of *maIRF1* were tested in trunk kidney and spleen after challenge with synthetic double-stranded RNA poly I:C and lipopolysaccharide (LPS). It was found that *maIRF1* expression changed significantly 24-72 h after injection ($P<0.05$), which demonstrated that *maIRF1* plays an important role in congenital immunity and adaptive immunity of *M. albus*.

*Keywords*: IRF-1; *Monopterus albus*; expression; infection; poly I:C.

## Introduction

In recent years, the Asian swamp eel (*Monopterus albus*) has become one of the most economically important freshwater fishes in East Asia. *M. albus* is a good vertebrate model to study comparative genomics, evolution and developmental biology due to its unique characteristics, such as a relatively small genome size and natural sex reversal from female via intersex into male during its life span (Zhou, Cheng, & Tiersch, 2002). The IRF family of transcription factors consists of nine members (IRF-1 to IRF-9) in mammals and an additional one (IRF-10) in chicken and fish (Tamura, Yanai, Savitsky, & Taniguchi, 2008; Huang, Qi, Xu, & Nie, 2010), of which IRF-1 was first discovered in 1998 and has been defined as a transcription regulator of IFN-β (Fujita *et al*. 1998; Fujiki, Shin, Nakao, & Yano, 1999). IRF-1 is constitutively expressed in most cell types and plays a key role in the inducible expression of major histocompatibility complex (MHC) class I, interferons (IFNs), nitric oxide synthase (iNOS), the development of T cells, IL-12, and T helper differentiation (Taniguchi, Ogasawara, Takaoka, & Tanaka, 2001; Takaokass *et al*. 2008). IRF-1 can control the transcription of genes involved in mediating antiviral, immunomodulatory, and antiproliferative effects (Tamura *et al*. 2008). For example, IRF-1 mediates cell fate by facilitating apoptosis with or without functional p53 in breast cancer (Schwartz, Shajahan, & Clarke, 2011). More recently, it was found that overexpression of IRF-1 induces autophagy in human hepatocellular carcinoma cells together with IFN-γ (Li *et al*. 2012). In addition, IRF-1 is a key transcription factor for the induction of RIG-I by 25-hydroxycholesterol, and the IRF-1/RIG-I axis mediates 25-hydroxycholesterol-induced IL-8 production in atherosclerosis (Wang, Xia, Liu, & Gu, 2012).

As IRF-1 plays an critical role in immune pathways, it has been cloned successfully in Japanese flounder (*Paralichthys olivaceus*)(Yabu *et al*. 1998), fugu (*Takifugu rubripes*)(Richardson, Tay, Goh, Venkatesh, & Brenner, 2001), rainbow trout (*Oncorhynchus mykiss*)(Collet *et al*. 2003), zebrafish (*Danio rerio*)(Song *et al*. 2004), turbot (*Scophthalmus*

*maximus*)(Ordás, Abollo, Costa, Figueras, & Novoa, 2006), sea bream (*Sparus aurata*)(Ordás *et al*. 2006), mandarin fish (*Siniperca chuatsi*)(Sun, Chang, Song, Yao, & Nie, 2007), crucian carp (*Carassius carassius*)(Shi *et al*. 2008), orange-spotted grouper (*Epinephelus coioides*)(Shi, Zhu, Yin, Zhang, & Gui, 2010), Atlantic cod (*Gadus morhua*)(Feng *et al*. 2009), large yellow croaker (*Pseudosciaena crocea*)(Yao, Kong, Huang, & Wang, 2010), and Atlantic salmon (*Salmo salar*) (Bergan, Kileng, Sun, & Robertsen, 2010). Many studies have shown that IRF-1 plays an important role in fish defense against viral infections (Caipang, Hirono, & Aoki, 2005; Collet *et al*. 2007; Shi *et al*. 2008) and that it is up-regulated by salmon IFNα1 and by salmon anemia virus compared with IRF-3 and IRF-7 (Bergan *et al*. 2010). Therefore, IRF-1 might be useful as an adjuvant in the development of DNA vaccines against commercially important viral pathogens (Caipang *et al*. 2005). In order to further study its anti-viral role, Shi *et al*. (2008) found two nuclear localization signals (NLS) in carp IRF-1, any one of which is sufficient for nuclear translocation. It is worth noting that IRF-1 has a potential role in blood-borne pathogen clearance, such as bacterial antigens (Feng *et al*. 2009). However, the constitutive expression profiles and immune responses of IRF-1 shows species-specific variations in fishes (Collet *et al*. 2007; Bergan *et al*. 2010; Yao *et al*. 2010).

However, data on immune genes of *M. albus* are scarce compared to other commercially important fishes. Therefore, a better understanding of the immune response of *M. albus* may contribute to develop strategies for disease management and improved culture efficiency. In the present study, the cDNA sequence and genomic structure of *IRF-1* from *M. albus* were cloned. Furthermore, *IRF-1* expression levels were examined at different developmental stages and in different organs. Its expression was also analyzed in trunk kidney and spleen tissue following challenges with synthetic double-stranded RNA poly I:C and lipopolysaccharide (LPS).

## Material and Methods

### Ethical Clearance

All the experiment was conducted by the correspondence author with the help of other authors, and all the operation complied with the Ethical Clearance in accordance with the National Regulations of the research laboratory.

### Cloning cDNA Sequence and Genomic Sequence

Based on the sequences from Atlantic salmon, mandarin fish and orange-spotted grouper of IRF-1 homologues from the NCBI website, a pair of degenerate primers (F1 and R1) was designed to obtain the internal region of *maIRF1*. An Asian swamp eel, weighing about 100 g was injected with 400 mg poly I: C (Sigma, USA) and 24 h later, trunk kidney (80-90mg) was sampled from which the RNA was isolated using Trizol (Invitrogen, USA) and reverse transcribed into cDNA by Powerscript II reverse transcriptase with Random primer and CDS primer which used to amplified the internal region and 3′-RACE respectively.

For 3′-RACE, the PCR was initially performed with primers UPM/3-F1 followed by a nested PCR with primers UPM/3-F2. For 5′-RACE, the adaptor primers AAP and AUAP were used. The RNA from *M. albus* trunk kidney tissue was reverse-transcribed at 42°C using the gene-specific primer 5-R1. After synthesis of the first strand cDNA, the purified cDNA was used in the TdT-tailing reaction. Tailed cDNA was then amplified by primers 5-R2 and AAP. A dilution of the original PCR (0.1%) was re-amplified using AUAP and a nested 5-R3 primer. All primers are shown in Table 1.

The genomic DNA was purified from muscle tissue of *M. albus* using a Wizard Genomic DNA Purification Kit (Promega). Based on the cDNA full-length sequence, primers were designed to obtain the full-length genomic sequence of *maIRF1*. All the primer sequences have been stated in Table. 1.

### Sequence Analysis

Protein prediction was performed using software at the ExPASy Molecular Biology Server. The intron/exon structure of the identified genomic sequences was determined by aligning the full-length cDNA to the genomic sequence using ClustalW2. A multiple alignment was generated using the ClustalW program. Sequence identities were calculated using the Megalign program within DNAStar. Phylogenetic analysis was performed using the neighbor-joining method within the MEGA molecular evolutionary genetic analysis software package. Data were analyzed using Poisson correction, and gaps were removed by pairwise deletion. The degree of confidence for each branch point was determined by bootstrap analysis (1,000 times).

### RNA Extraction and cDNA Synthesis for Expression Analysis

*M. albus* (3 individuals) of 150-200 g body weight were obtained from the aquaculture base of Yangtze University, China. After acclimatization, gonad(G), trunk kidney(TK), brain(BR), liver(L), intestine(I), spleen(SP), blood(BL), skin(SK), and muscle(M) from three fish were used for RNA isolation using Trizol reagent (Invitrogen, USA) in order to analyze the expression of *maIRF1* in healthy *M. albus.*

Three growth stages (female, intersex and male)

**Table 1**. Oligonucleotide primers used to amplify *maIRF1* gene

| Name | Sequence (5′–3′) | Usage |
|---|---|---|
| F1 | TCCGC(T)TGTGCAATGAACTC | Cloning for the internal fragment |
| R1 | CTTCATTGCTCAGG(T)AACC | |
| UPM Long | CTAATACGACTCACTATAGGGCAAGCAGTG GTATCAACGCAGAGT | 3′-Race PCR Universal primers |
| UPM Short | CTAATACGACTCACTATAGGGC | |
| AAP | GGCCACGCGTCGACTAGTACGGGIIGGGIIGG GIIG | 5′-Race PCR Universal primers |
| AUAP | GGCCACGCGTCGACTAGTAC | |
| 5-R1 | GCTGCTGGCAAATC | 5′ RACE 1st round PCR |
| 5-R2 | ATGGTTTCTTCTGGCACTGAT | 5′ RACE 2nd round PCR |
| 5-R3 | TCCTCCAGCTTGACCTTCTTCC | 5′ RACE 3rd round PCR |
| 3-F1 | ATGAACTCACTGCCTGACATCG | 3′ RACE 1st round PCR |
| 3-F2 | CCCGATTACGACTATACTGACG | 3′ RACE 2nd round PCR |
| D-F1 | ACTTCACTCAAGGAGGGTTC | For the 1st to 3rd intron |
| D-R1 | ATGCTCTTGTCTTTTACCTCC | |
| D-F2 | TCCGTTGTGCAATGAACTC | For the 4th to 5th intron |
| D-R2 | GGTTTCTTCTGGCACTGAT | |
| D-F3 | CATCAGTGCCAGAAGAAACCAT | For the 6th to 9th intron |
| D-R3 | AGTAGGCTATGATGATGAC | |
| β-actin F | CAGTCCTCCTAAGGCGATAA | Real-time quantitative PCR control |
| β-actin R | GCATCATCTCCAGCAAAGC | |
| RT-F1 | GGGAGCCAGTGGAGTGAAT | Real-time quantitative PCR |
| RT-R1 | AAGGATGGAAGGCAGGAAT | |

were tested. After seven days of acclimatization in a quarantine tank, trunk kidney and spleen tissues were sampled for RNA isolation using Trizol, in order to study the *maIRF1* expression model at different developmental stages.

To study the effect of immunostimulants on the expression of *maIRF1*, three groups of fish (three fish each, about 45 cm) were injected intraperitoneally with either 500 µl poly I:C (2 mg/ml), 500 µl LPS (2 mg/ml), or 500 µl PBS. At 6 h, 12 h, 24 h, 48 h, and 72 h after injection, total RNA was extracted from trunk kidney and spleen using Trizol reagent (Invitrogen, USA) as described by the manufacturer.

After treatment with RNase-free DNase I, 2 µg of total RNA was reverse-transcribed with Revert AidTM First Strand cDNA Synthesis Kit (Fermentas). All cDNA samples were stored at -20°C until used in real-time PCR assays.

### Real-Time Quantitative PCR

Primer premier 5.0 was used to design forward and reverse primers. The primers that performed best in real-time PCR were: forward (RT-F1), reverse (RT-R1); *β-actin* forward (β-actin F) and reverse (β-actin R). The annealing temperature for both *maIRF1* and *β-actin* was 58°C, and resulting amplicons were 187 and 114 bp long, respectively. The *maIRF1* and *β-actin* cDNA fragments were generated by PCR. Amplicons were gel-purified, cloned into a pMD18-T vector (Takara, Japan) and transformed into *E. coli* strain DH5α competent cells. Cloned amplicon sequences were confirmed by sequencing. Plasmid DNA was obtained using a plasmid mini kit I (Omega) according to the manufacturer's instructions. The concentrations of plasmid DNA was measured by spectrophotometry. Serial tenfold dilution was used in PCR to establish a standard curve. PCR reactions were performed using Chromo 4™ Continuous Fluorescence Detector (MJ Research). Amplifications were carried out at a final volume of 20 µl containing 1 µl DNA sample, 10 µl 2 × SYBR green Real time PCR Master Mix (Toyobo, Japan), 2 µl of each primer and 5 µl $H_2O$. PCR amplification consisted of 5 min at 95°C, followed by 45 cycles consisting of 10 s at 94°C, 15 s at 58°C, 20 s at 72°C and plate reading at 80°C. A reaction carried out without template DNA was used as negative control. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in triplicates. Standard curves were run on the same plate.

Statistical analysis was performed using a one-way analysis of variance (ANOVA). A probability level of $P<0.05$ was considered significant. The expression level of *maIRF-1* gene was calculated with reference to the expression of *β-actin*, as described by Xu, Chang, Xiao, Huang, & Nie, (2010). All statistical analyses were based on comparisons between the control and injection groups.

## Results

### Cloning and Characterization of maIRF1

The *maIRF1* cDNA contained 1674 bp with a 241 bp 5′-UTR and an 885 bp open reading frame

encoding a 294 aa peptide. The encoded polypeptide had a predicted molecular weight 33.67 kDa and theoretical pI of 4.76. The 3′-UTR consisted of 648 nucleotides with a typical polyadenylation signal (AATAAA). It also contained an mRNA instability motif (ATTTA) (Figure 1). The nucleotide sequence of *maIRF1* has been submitted to the GenBank nucleotide databases under accession no. JQ861979.

The predicted amino acid sequence for the protein showed significant similarity with fish and mammalian IRF-1 (Figure 2). The N-terminal region of this amino acid sequence (aa 1-115) matched with IRF-1 of mammals, birds, amphibians and fishes. However, the C-terminal region (aa 116-332) was more similar to fish IRF-1. The phylogenetic tree analysis supported the contention that the *maIRF-1* have closet relationship with mandarin, and subjected to the same brands with other teleost (Figure 3).

In the first 115 residues, which compose the DNA binding domain (DBD), the sequence of the predicted maIRF1 proteins showed the highest identity with mandarin fish IRF-1 (95.5%), 79.5-81.3% to other fish IRF-1, 75.9% to chicken IRF-1, 68.8% to frog IRF-1, and 70.5% to mammalian IRF-1. In the second conserved IRF-associated domain (IAD), the *maIRF1* sequence had 84.0% similarity to mandarin fish IRF-1, but the identity between *maIRF1* and other vertebrate IRF-1 was very low (Table 2). Besides, *maIRF1* contains three basic amino acid sequences ($^{74}$KTWKANFR$^{81}$, $^{94}$KDKSINK100$^{116}$ and KARDKRSKTKETKQRKKVK$^{135}$).

**Characterization of maIRF1 Genomic DNA**

The *maIRF1* gene (GenBank accession no. JQ861980) extended over approximately 4.3 kb of genomic sequence. The exon-intron arrangement of the *maIRF1* genomic locus was similar to those of other vertebrate IRF-1 loci, with 10 exons and nine introns, which was similar to fugu IRF-1 with a relatively compact genomic size (Figure 4). The first exon was 133 bp in length. Exon 2 was 93 bp long and contained 5′ UTR and 29 aa of ORF. Exons 3 and 4 encoded 33 aa and 56 aa, respectively. Exons 2-4 encoded N-terminal conserved DBD domain. Exons 5-10 were 41, 124, 120, 29, 118, and 746 bp long, respectively. Exon 8 encodes the IAD domain. The interleaved nine introns were 639, 400, 97, 175, 130, 209, 141, 185, and 617 bp long, respectively (Figure 4). Although IRF-1 genomic DNA of zebrafish and rat had only nine exons, that in the others species contained ten exons (Figure 4). All exon-intron junctions followed the consensus rule of the splice acceptor-AG/GT-splice donor for splicing (Table 3).

**Expression of maIRF1 at Different Growth Stages**

Natural sex reversal occurs in *M. albus* from functional females via an intersex stage into males during development. Thus, *maIRF1* expression in the three developmental stages was studied in order to explain whether *maIRF1* transcription levels had changed during the sex reversal. Quantitative RT-PCR of trunk kidney and spleen tissue revealed that *maIRF1* expression had remained broadly consistent during the sex reversal (Figure 5).

**maIRF1 Transcription in Different Organs and Inductive Expression**

The *maIRF1* gene was constitutively expressed in all tested tissues/organs, with the highest expression levels in trunk kidney, blood, skin, and intestines (Figure 6). The lowest *maIRF1* expression levels were found in brain and muscle tissue. After poly I:C stimulation, the transcription levels of *maIRF1* increased dramatically at 12 h post-stimulation (hps) and remained high at 48 hps in trunk kidney ($P<0.05$). In spleen, a significantly increased expression of *maIRF1* was detected at 12 hps and 24 hps ($P<0.05$), while it decreased to control level at 48 hps ($P>0.05$) (Figure 7). LPS also induced *maIRF1* expression. Transcription of *maIRF1* was increased 2.34-fold in trunk kidney and 1.89-fold in spleen at 48 hps compared with the control ($P<0.05$).

## Discussion

In this study, we have cloned and sequenced the entire cDNA of the interferon regulatory factor 1 (IRF-1) of *M. albus*. The amino-terminal end of the translated proteins, in which the DNA binding domain (DBD) is located, was very similar to IRF-1 of other fishes, mammals, amphibians, and birds. In its DBD, *maIRF1* showed six invariant tryptophan repeats, which constitute the signature for the IRF family and is conserved in all IRF-1(Collet *et al*. 2003; Richardson *et al*. 2001; Yabu *et al*. 1998). However, the carboxy-terminal end was less conserved and was more similar to fish IRF-1 than to IRF-1 in chicken, frog, human and mouse. In addition, these IRF-1 possess a low amount of basic amino acid residues in their carboxy-terminal ends, while IRF-2 is rich in basic amino acid residues, which constitutes a transcriptional repression domain (Childs, & Goodbourn, 2003). A phylogenetic tree constructed with IRF-1 sequence data from fishes, frogs, birds, and mammals demonstrated that all fish IRF-1 were clustered together. Therefore, we identified the IRF from *M. albus* as IRF-1. As other fish IRF-1, *maIRF1* possessed a characteristic polyadenylation signal and an mRNA instability motif (ATTTA), although these were located in different positions (Yabu *et al*., 1998). The predicted length of the maIRF1 polypeptide (294 aa) was very similar to that of fugu (296 aa). In contrast, fish IRF-1 is shorter than those from birds and mammals. Further analysis showed that maIRF1 contains a basic K- and R-rich sequence ($^{116}$KARDKRSKTKETKQRKKVK$^{135}$), which

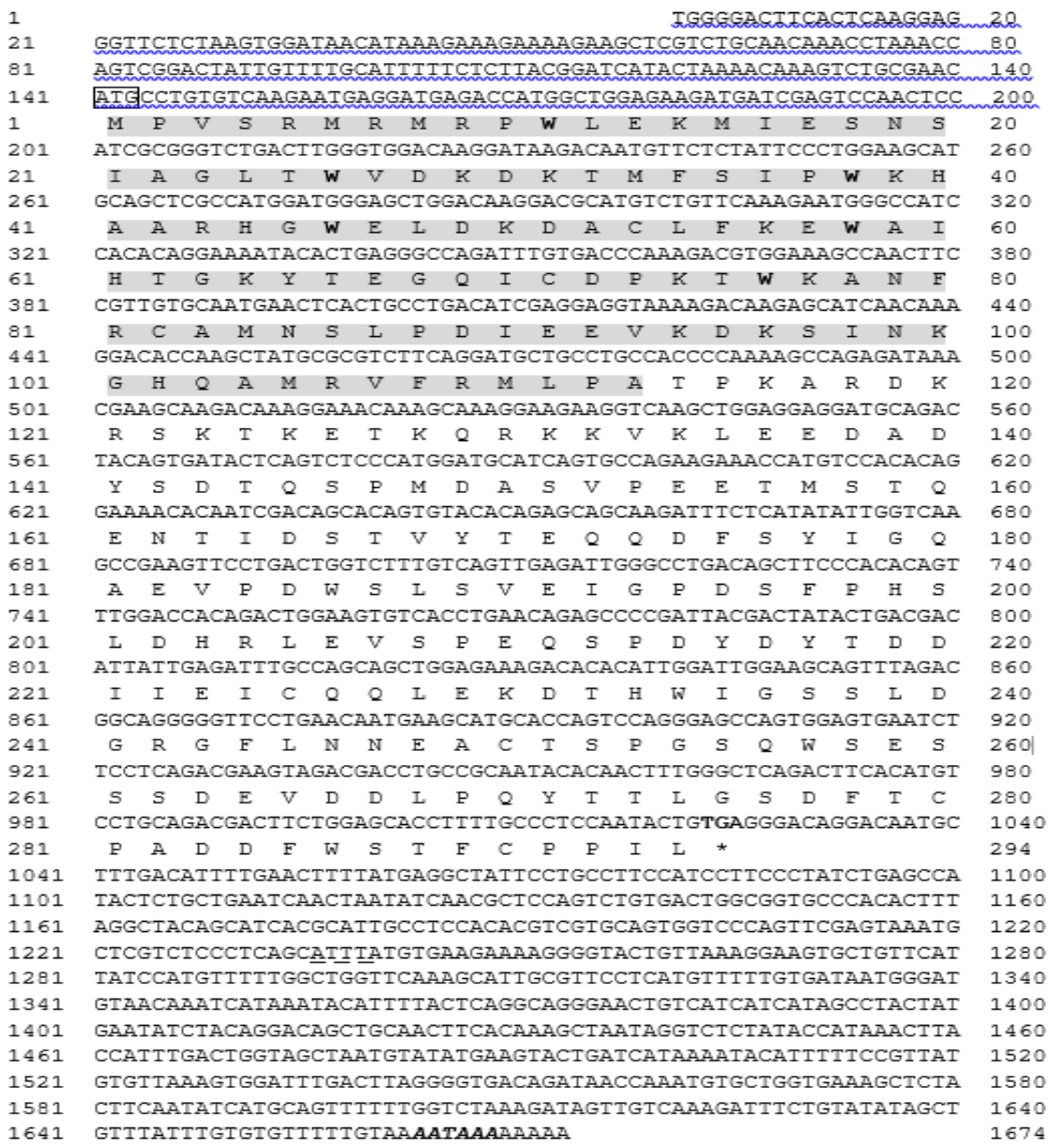


**Figure 1.** Nucleotide sequence (GenBank accession no. being) and deduced amino acid sequence. The nucleotides (upper row) and deduced amino acids (lower row) are numbered at the both sides of sequences.
The start codon (ATG) is boxed and the stop codon (TAG) indicated by an asterisk. The bold and italic indicates the position of polyadenylation signal. The mRNA instability motif is underlined.

appears to be the structural and functional equivalent of the nuclear localization signals (NLS) in mammalian IRF-1 (Schaper *et al*. 1998). In addition, the region rich in basic amino acids is also found within the DBD of maIRF1 ($^{74}$KTWKANFR$^{81}$ and $^{94}$KDKSINK$^{100}$), which is similar to the second NLS in crucian carp IRF-1(Shi *et al*. 2008). Collective data suggest that each fish IRF family member is a true ortholog of its mammalian counterpart, although alternatively spliced variants found in some IRF family members can complicate the assignment of orthologous pairs. It is worth noting that the existence of two turbot IRF-1 genes might represent two different loci (Ordás *et al*. 2006). In our study, a comparison of the genomic organization showed that all IRF-1 genes have an identical disposition of exons and introns (Figure 3). Although fish IRF-1 genes have a very compact size as a result of short introns, exon sizes are similar among all IRF-1 genes studied. These results further indicate that vertebrate IRF-1 genes are evolutionarily conserved.

The expression of IRF-1 has been investigated in many species and IRF-1 is constitutively expressed at low levels in a variety of tissues, such as large yellow croaker (*Pseudosciaena crocea*) IRF-1 expression level was low in blood, skin, muscle, intestine, liver,

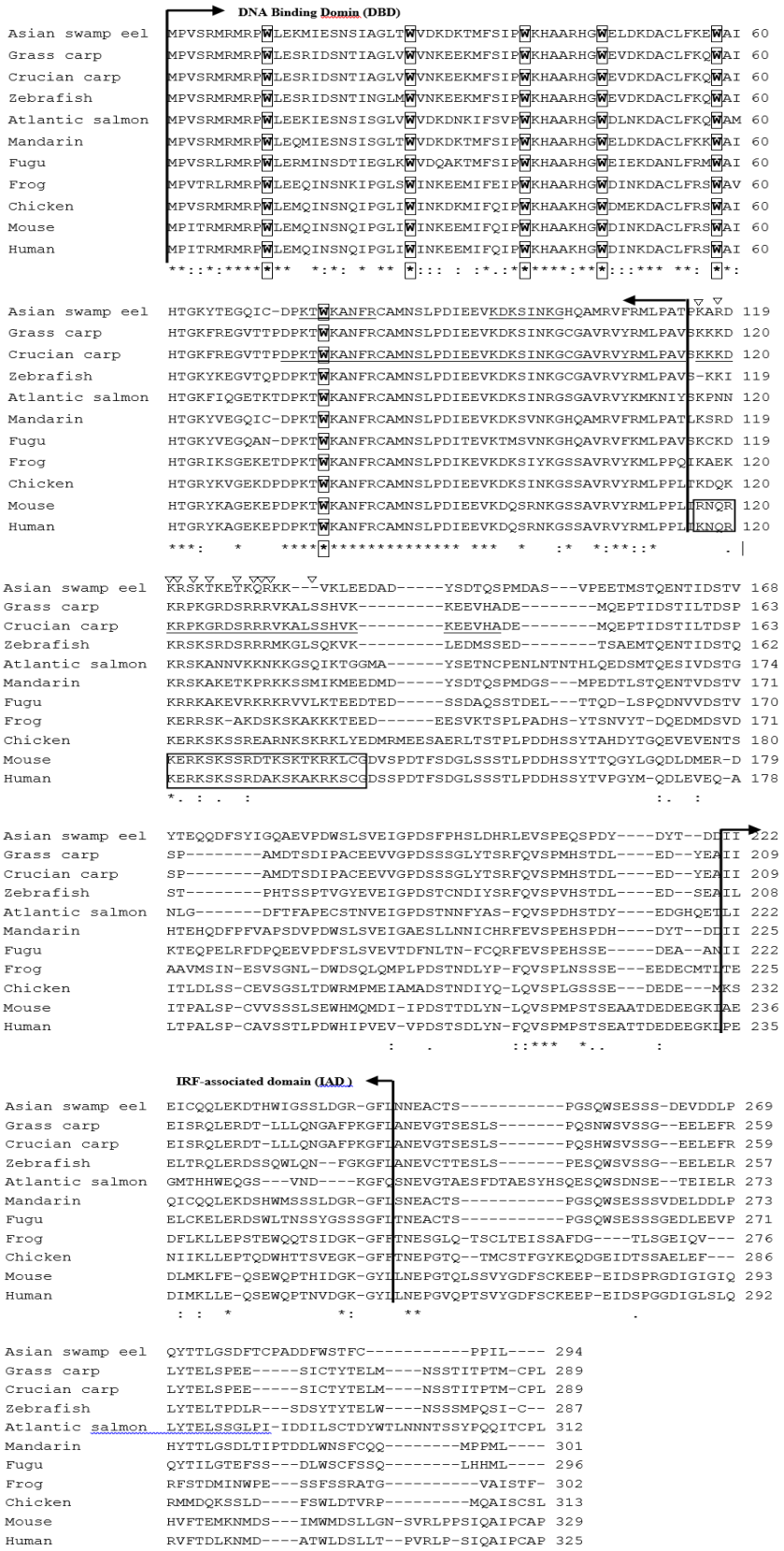


**Figure 2.** Multiple alignment of maIRF1 amino acid sequence with other IRF-1 proteins. Symbol (*) represents identical residues, (:) conservative substitution and (.) similar residues. Missing amino acids are denoted by hyphens. The DNA-binding domain (DBD) and IRF association domain (IAD) are indicated by lines above the aligned sequences. The conserved tryptophan (W) residues that comprise a ‘‘tryptophan cluster’’ are highlighted in boxed. The NLS of mammalian IRF-1 proteins is boxed. Two NLS of crucian carp IRF-1 is underlined. The basic amino acid K and R are indicated by open triangles where maIRF1 contains a basic K- and R-rich sequence.

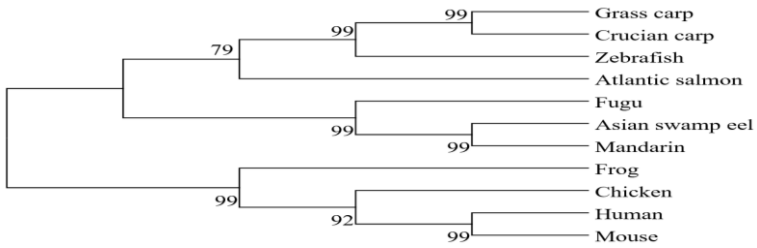


**Figure 3.** Phylogenetic tree of IRF-1 in vertebrates. A neighbor-joining phylogenetic tree of IRF proteins was constructed based on the analysis of protein sequences by the computer program CLUSTERW and MEGA 3.1. The sequence of the IRFs used for the analysis are derived from the GenBank.
Asian swamp eel, *Monopterus albus* IRF-1 maIRF1 (GenBank No: JQ861979); Mandarin, *Siniperca chuatsi* IRF1 (GenBank No: AY647431.1); Crucian carp, *Carassius auratus* IRF1 (GenBank No: EF174419); Fugu, *Takifugu rubripes* IRF1 (GenBank No: AF242474_1); Atlantic salmon, *Salmo salar* IRF1 (GenBank No: NP_001239290); Zebrafish, *Danio rerio* IRF1 (GenBank No: NP_001035442); Grass carp, *Ctenopharyngodon idella* IRF1 (GenBank No: ADF57571.1); Frog, *Xenopus tropicalis* IRF1 (GenBank No: AAH75398); Chicken, *Gallus gallus* IRF1 (GenBank No: NP_990746); Mouse, *Mus musculus* IRF1 (GenBank No: CAB9163); Human, *Homo sapiens* IRF1 (GenBank No: NP_002189).

**Table 2.** Pairwise identity of *maIRF1* with other IRF-1 proteins

| Gene | Species | Identity percentage | | |
|---|---|---|---|---|
| | | Whole | DNA-binding domain (DBD) | IRF association domain (IAD) |
| Teleost | | | | |
| IRF-1 | Mandarin (*Siniperca chuatsi*) | 81.0 | 95.5 | 84.0 |
| IRF-1 | Crucian carp (*Carassius auratus*) | 48.8 | 81.3 | 48.0 |
| IRF-1 | Atlantic salmon (*Salmo salar*) | 42.5 | 76.8 | 31.6 |
| IRF-1 | Grass carp (*Ctenopharyngodon idella*) | 48.8 | 81.3 | 45.8 |
| IRF-1 | Zebrafish (*Danio rerio*) | 48.4 | 81.3 | 41.7 |
| IRF-1 | Fugu (*Takifugu rubripes*) | 57.5 | 79.5 | 36.0 |
| Avian | | | | |
| IRF-1 | Chicken (*Gallus gallus*) | 38.8 | 75.9 | 32.0 |
| Amphibian | | | | |
| IRF-1 | Frog (*Xenopus tropicalis*) | 37.4 | 68.8 | 36.0 |
| Mammal | | | | |
| IRF-1 | Human (*Homo sapiens*) | 38.1 | 70.5 | 25.0 |
| IRF-1 | Mouse (*Mus musculus*) | 36.0 | 70.5 | 25.0 |

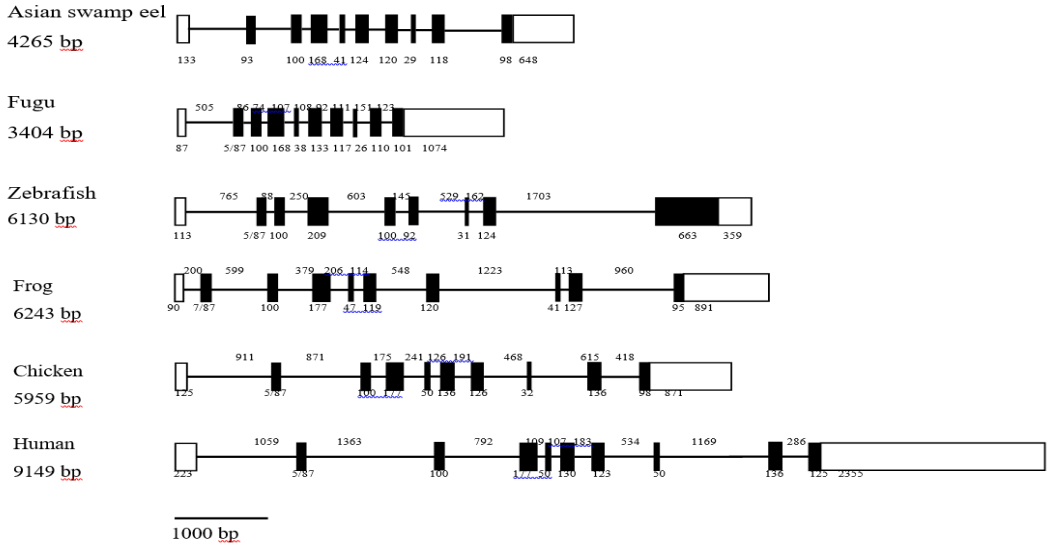


**Figure 4.** Schematic diagram of exon-intron arrangement of IRF-1 genes from Fugu (*Takifugu rubripes*, Gene ID 6116), Zebrafish (*Danio rerio*, Gene ID 792160), Frog (*Xenopus tropicalis*, Gene ID 448320), Chicken (*Gallus gallus*, Gene ID 396384), Rat (*Rattus norvegicus*, Gene ID 24508), Mouse (*Mus musculus*, Gene ID 16362), Pig (*Sus scrofa*, Gene ID 396611) and Human (*Homo sapiens*, Gene ID 3659). Boxes represent exons and lines adjacent to exons represent introns. Open reading frames and untranslated regions are shown as black boxes and white boxes, respectively. The number of nucleotides in each intron and exon is, respectively, shown above and below the corresponding element.

**Table 3.** Intron-exon junctions and flanking sequences of *maIRF1* gene

| Exon no. | Exon size (bp) | Position in DNA | Splice acceptor | Splice donor | Intron no. | Intron size (bp) |
|---|---|---|---|---|---|---|
| 1 | 133 | 1-133 | | AGTCTgtaag | 1 | 639 |
| 2 | 93 | 773-865 | tgcagGCGAA | ACAAGgtgag | 2 | 400 |
| 3 | 100 | 1266-1365 | tccagGATAA | CACAGgtgag | 3 | 97 |
| 4 | 168 | 1463-1630 | tgcagGGAAA | CAGAG**gt**gag | 4 | 175 |
| 5 | 41 | 1806-1846 | cac**ag**ATAAA | AGAAG**gt**aaa | 5 | 130 |
| 6 | 124 | 1977-2100 | tgc**ag**GTCAA | GCAAG**gt**gag | 6 | 209 |
| 7 | 120 | 2310-2429 | tac**ag**ATTTC | CCCCG**gt**aag | 7 | 141 |
| 8 | 29 | 2571-2599 | tcc**ag**ATTAC | TTGAG**gt**aag | 8 | 185 |
| 9 | 118 | 2785-2902 | ttc**ag**ATTTG | CTCAG**gt**aaa | 9 | 617 |
| 10 | 746 | 3520-4265 | tac**ag**ACGAA | | | |

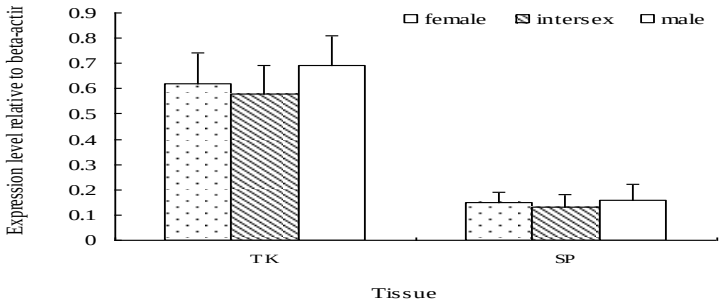


**Figure 5.** Expression of *maIRF1* in Trunk kidney (TK) and Spleen (SP) Asian swamp eel during three development stages (female, intersex and male).

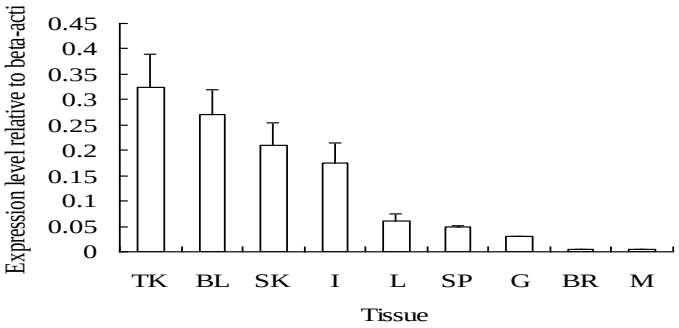


**Figure 6.** Expression of *maIRF1* in different tissues. Real-time quantitative PCR was performed with total RNA isolated from Trunk kidney (TK), Blood (BL), Skin (SK), Intestines (I), Liver (L), Spleen (SP), Gonad (G), Brain (BR) and Muscle (M).

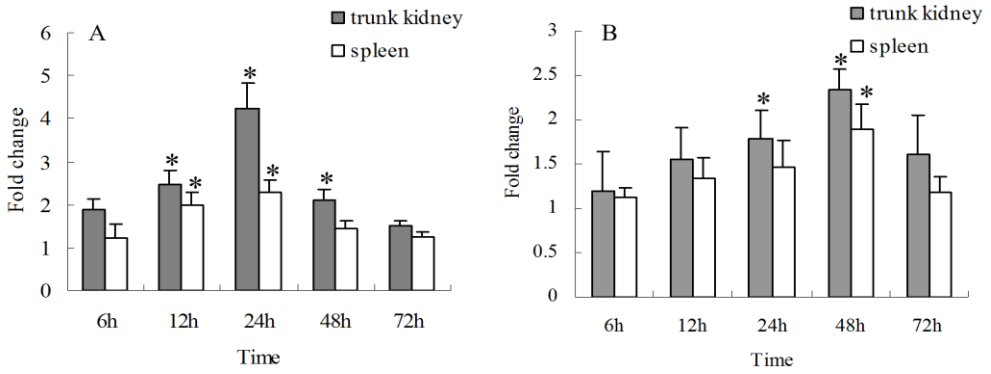


**Figure 7.** Expression pattern of *maIRF1* gene induced by poly I:C (A) and LPS (B) in spleen and trunk kidney. The fish were injected intraperitoneally with either 50 µl Poly I:C, 50 µl LPS or 50 µl PBS. Three individuals were sampled at 6h, 12h, 24h, 48h and 72h post- injection (hpi) on each occasion, with trunk kidney and spleen dissected out. Bars represent standard deviations from three induced fish. The asterisk (*) indicates the significant difference between induced and corresponding control group ($P<0.05$).

heart, kidney, and brain (Feng *et al*. 2009; Yao *et al*. 2010). In most fishes, IRF-1 expression was predominant in gill, spleen, trunk kidney, and pronephros tissues (Sun *et al*. 2007; Collet *et al*. 2003; Richardson *et al*. 2001; Yao *et al*. 2010). Taxonomically, the teleost *M. albus* belongs to the family Synbranchidae of the order Synbranchiformes (Neoteleostei, Teleostei, Vertebrata), and its gills are difficult to sample. In the present study, *maIRF1* transcripts were abundant in trunk kidney and blood, and the lowest expression was found in brain and muscle, which is similar to previous studies (Caipang, Hirono, & Aoki, 2009; Sun *et al*. 2007).

IRF-1-induced expression has been studied in various fishes (Sun *et al*. 2007; Bergan *et al*. 2010). In most experiments, the Mx protein was used as positive control to evaluate the effects of poly I:C. The Mx protein has been regarded as an indicator of type I interferon response in vertebrates, and its antiviral function also been confirmed in fishes (Larsen, Røkenes, & Robertsen, 2004). In mandarin fish, the transcription of IRF-1 could be detected within 12 h after stimulation, which is earlier than a significant increase of Mx protein. Although the transcription of Mx protein could increase by a maximal 10-fold after stimulation, the elevated transcription levels of IRF-1 were less than fourfold (Sun *et al*. 2007). Similarly, IRF-1 was earliest up-regulated by recombinant salmon IFNα1 and infection with infectious salmon anemia virus (ISAV), while IRF-3 and IRF-7 had a similar activation profile induced at a slightly later time point (Bergan *et al*. 2010). In our study, the *β-actin* gene was used as control to estimate *maIRF1*-induced change due to the unknown Mx gene sequence in *M. albus*. Transcription of *maIRF1* was elevated in trunk kidney and spleen tissue 24-72 h after poly I:C injection, which has also been found in turbot and sea bream (Ordás *et al*. 2006). These findings suggested that IRF-1 played a vital role in the early stages of an antiviral defense, which is the main difference between IRF-1 and other IRFs.

Apart from its antiviral role, IRF-1 expression also changes following stimulation with bacteria or bacterial antigens. Yabu *et al* (1998) demonstrated the inducible expression of an IRF1 in the liver of Japanese flounders following intramuscular injection with *Edwardsiella tarda*. *Atlantic cod* IRF-1 expression was significantly elevated in spleen and head kidney at 24 h following *A. salmonicida* stimulation (Feng *et al*. 2009). However, Collet and Secombes (2001) reported that IRF-1 expression was induced only by poly I:C and not by LPS in raibow trout gonad cells. Similarly, IRF-1 expression in head kidney of turbot following *Vibrio pelagius* challenge was not significantly changed at the single time point examined (8 h post challenge) (Ordás *et al*. 2006). In our study, LPS could induce *maIRF1* expression in trunk kidney and spleen after 24 hours, which suggests that the *IRF1* gene may play an important role in fish resistance against bacterial infection. In summary, our study demonstrated that *maIRF1* may play diverse roles in resistance against the viral and bacterial infection.

## Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31101928, 31460689).

## References

Zhou, R.J., Cheng, H.H., & Tiersch, T.R. (2002). Differential Genome Duplication and Fish Diversity. Reviews in Fish Biology & Fisheries, 11(4) 331-337. http://dx.doi.org/10.1023/A:1021395506705.

Tamura, T., Yanai, H., Savitsky, D., & Taniguchi, T. (2008). The IRF Family Transcription Factors in Immunity and Oncogenesis. Annual Review of Immunology, 26(1), 535-584. http://dx.doi.org/10.1046/annurev.immunol.26.021607.090400.

Huang, B., Qi, Z.T., Xu, Z., & Nie, P. (2010). Global Characterization of Interferon Regulatory Factor (IRF) Genes in Vertebrates: Glimpse of the Diversification in Evolution. BMC Immunology, 11(1) 22. http://dx.doi.org/10.1186/1471-2172-11-22.

Fujita, T., Sakakibara, J., Sudo, Y., Miyamoto, M., Kimura, Y., & Taniguchi, T. (1998). Evidence for a Nuclear Factor(s), IRF-1, Mediating Induction and Silencing Properties to Human IFN-beta Gene Regulatory Elements. Embo Journal, **7**(11), 3397-3405. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC454838/

Fujiki, K., Shin, D.H., Nakao, M., & Yano, T. (1999). Molecular Cloning of Carp (*Cyprinus carpio*) CC Chemokine, CXC Chemokine Receptors, Allograft Inflammatory Factor-1, and Natural Killer Cell Enhancing Factor by Use of Suppression Subtractive Hybridization. Immunogenetics, 49(10), 909-914. http://dx.doi.org/10.1007/s002510050573.

Taniguchi, T., Ogasawara, K., Takaoka, A., & Tanaka, N. (2001). IRF Family of Transcription Factors as Regulators of Host Defense. Annual Review of Immunology, 19(1), 23-55. http://dx.doi.org/10.1146/annurev.immunol.19.1.623.

Takaokass, A., Tamura, T., & Taniguchi, T. (2008). Interferon Regulatory Factor Family of Transcription Factors and Regulation of Oncogenesis. Cancer Science, 99(3), 467–478. http://dx.doi.org/10.1111/j.1349-7006.2007.00720x

Schwartz, J.L., Shajahan, A.N., & Clarke, R. (2011). The Role of Interferon Regulatory Factor-1 (IRF1) in Overcoming Antiestrogen Resistance in the Treatment of Breast Cancer. International Journal Breast Cancer, 38, 91-102. http://dx.doi.org/10.4061/2011/912102.

Li, P., Du, Q., Cao, Z., Guo, Z., Evankovich, J., Yan, W., & Geller, D.A. (2012). Overexpression of IRF-1 Induced Autophagy Induces Autophagy in Human Hepatocellular Carcinoma Cells Togther with Interferon-Gamma. Cancer Letters, 314(2) 213-222. http://dx.doi.org/10.1016/j.canlet.2011.09.031.

Wang, F., Xia, W., Liu, F.J., & Gu, J. (2012). Interferon Regulator Factor 1/Retinoic Inducible Gene I

(IRF1/RIG-I) Axis Mediates 25-Hydroxycholesterol-Induced Interleukin-8 Production in Atherosclerosis. Cardiovascular Research, 93(1), 190-199. http://dx.doi.org/10.1093/cvr/cvr260.

Yabu, T., Hirose, H., Hirono, I., Katagiri, T., Aoki, T., & Yamamoto, E. (1998). Molecular Cloning of a Novel Interferon Regulatory Factor in Japanese Flounder, *Paralichthys olivaceus*. Mol Mar Biol Biotechnol, 7(2), 138-144.

Richardson, M., Tay, B.H., Goh, B.Y., Venkatesh, B., & Brenner S. (2001). Molecular Cloning and Genomic Structure of a Gene Encoding Interferon Regulatory Factor in the Pufferfish (*Fugu rubripes*). Marine. Biotechnology, 3(2) 145-151. http://dx.doi.org/10.1007/s101260000066.

Collet, B., Hovens, G.C.J., Mazzoni D., Hirono I., Aoki, T., & Secombes, C.J. (2003). Cloning and Expression Analysis of Rainbow Trout *Oncorhynchus mykiss* Interferon Regulatory Factor 1 and 2 (IRF-1 and IRF-2). Developmental & Comparative Immunology, 27(2), 111-126. http://dx.doi.org/10.1016/S0145-305X(02)00072-1

Song, H.D., Sun, X.J., Deng, M., Zhang, G.W., Zhou, Y., Wu, X.Y., & Chen, Z. (2004). Hematopoietic Gene Expression Profile in Zebrafish Kidney Marrow. Proceedings of the National Academy of Sciscience USA, 101(46) 16240-16245. http://dx.doi.org/10.1073/pnas.0407241101.

Ordás, M.C., Abollo, E., Costa, M. M., Figueras, A., & Novoa, B. (2006). Molecular Cloning and Expression Analysis of Interferon Regulatory Factor-1 (IRF-1) of Turbot and Sea Bream. Molecular Immunology, 43(7), 882-890. http://dx.doi.org/10.1116/j.molimm.2005.06.034.

Sun, B.J., Chang, M.X., Song, Y., Yao, W.J., & Nie, P. (2007). Gene Structure and Transcription of IRF-1 and IRF-7 in the Mandarin Fish *Siniperca chuatsi*. Veterinary Immunology & Immunopathology, 116(2), 26-36. http://dx.doi.org/10.1016/j.vetimm.2007.01.001.

Shi, Y., Zhang, Y.B., Zhao, Z., Jiang, J., Zhang, Q.Y., & Gui, J.F. (2008). Molecular Characterization and Subcellular Localization of *Carassius auratus* Interferon Regulatory Factor-1. Developmental & Comparative Immunology, 32(2), 134-146. http://dx.doi.org/10.1016/j.dci.2007.05.004.

Shi, Y., Zhu, X.P., Yin, J.K., Zhang, Q.Y., & Gui, J.F. (2010). Identification and Characterization of Interferon Regulatory Factor-1 from Orange-Spotted Grouper (*Epinephelus coioides*). Molecular Biology Reports, 37(3) 1483-1493. http://dx.doi.org/10.1007/s11033-009-9544-0.

Feng, C.Y., Johnson, S.C., Hori, T.S., Rise, M., Hall, J.R., Gamperl, K., & Rise, M.L. (2009). Identification and Analysis of Differentially Expressed Genes in Immune Tissues of Atlantic Cod Stimulated with Formalin-killed, Atypical *Aeromonas salmonicida*. Physiological Genomics, 37(3), 149-163. http://dx.doi.org/10.1152/physiolgenomics.90373.2008.

Yao, C.L., Kong, P., Huang, X.N., & Wang, Z.Y. (2010). Molecular Cloning and Expression of IRF1 in Large Yellow Croaker, *Pseudosciaena crocea*. Fish & Shellfish Immunology, 28(4), 654-660. http://dx.doi.org/10.1016/j.fsi.2009.12.026.

Bergan, V., Kileng, O., Sun, B.J., & Robertsen, B. (2010). Regulation and Function of Interferon Regulatory Factors of *Atlantic salmon*. Molecular Immunology, 47(11), 2005-2014. http://dx.doi.org/10.1016/j.molimm.2010.04.015

Caipang, C.M., Hirono, I., & Aoki, T. (2005). Induction of Antiviral State in Fish Cells by Japanese flounder, *Paralichthys olivaceus*, Interferon Regulatory Factor-1. Fish & Shellfish Immunology, 19(1), 79-91. http://dx.doi.org/10.1016/j.fsi.2004.12.008.

Collet, B., Munro, E.S., Gahlawat, S., Acosta, F., Garcia, J., Roemelt, C., & Ellis, A.E. (2007). Infectious Pancreatic Necrosis Virus Suppresses Type I Interferon Signalling in Rainbow Trout Gonad Cell Line but not in *Atlantic salmon* Macrophages. Fish & Shellfish Immunology, 22(1), 44-56. http://dx.doi.org/10.1016/j.fsi.2006.03.011.

Xu, Q.Q., Chang, M.X., Xiao, F.S., Huang, B., & Nie, P. (2010). The gene and virus-induced expression of IRF-5 in grass carp *Ctenopharyngodon idella*. Veterinary Immunology & immunopathology, 134(3-4), 269-278. http://dx.doi.org/10.1016/j.vetimm.2009.10.020.

Childs, K.S., & Goodbourn, S. (2003). Identification of Novel Co-repressor Molecules for Interferon Regulatory Factor-2. Nucleic Acids Research, 31(12), 3016-3026. http://dx.doi.org/10.1093/nar/gkg431.

Schaper, F., Kirchhoff, S., Posern, G., Koster, M., Oumard, A., Sharf, R., & Hauser H. (1998). Functional Domains of Interferon Regulatory Factor I (IRF-1). Biochemical Journal, 335(Pt 1), 147-157. http://dx.doi.org/10.1042/bj3350147.

Caipang, C. M. A., Hirono, I., & Aoki, T. (2009). Modulation of the Early Immune Response Against Viruses by a Teleostean Interferon Regulatory Factor-1 (IRF-1). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 152(3), 440-446. http://dx.doi.org/10.1016/j.cbpa.2008.11.022

Larsen, R., Røkenes, T.P., & Robertsen, B. (2004). Inhibition of Infectious Pancreatic Necrosis Virus Replication by *Atlantic salmon* Mx1 Protein. Journal Virology, 78(15), 7938–7944. http://dx.doi.org/10.1128/JVI.78.15.7938-7944.2004.

Collet, B., & Secombes, C.J. (2001). The Rainbow Trout (*Oncorhynchus mykiss*) Mx1 Promoter. Structural and Functional Characterization. European Journal Biochemistry, 268(6), 1577-1584. http://dx.doi.org/10.1046/j.1432-1033.2001.02021.x.