Identification of an IRF10 gene in common carp (Cyprinus carpio L.) and analysis of its function in the antiviral and antibacterial immune response

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Research article

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Abstract

Background: Interferon (IFN) regulatory factors (IRFs), as transcriptional regulatory factors, play important roles in regulating the expression of type I IFN and IFN stimulated genes (ISGs) in innate immune responses. In addition, they participate in cell growth and development and regulate oncogenesis.

Results: In the present study, the cDNA sequence of IRF10 in common carp (Cyprinus carpio L.) was characterized (abbreviation, CcIRF10). The predicted protein sequence of CcIRF10 shared 52.7-89.2% identity with other teleost IRF10s and contained a DNA-binding domain (DBD), a nuclear localization signal (NLS) and an IRF-associated domain (IAD). Phylogenetic analysis showed that CcIRF10 had the closest relationship with IRF10 of Ctenopharyngodon idella. CcIRF10 transcripts were detectable in all examined tissues, with the highest expression in the gonad and the lowest expression in the head kidney. CcIRF10 expression was upregulated in the spleen, head kidney, foregut and hindgut upon polyinosinic:polycytidylic acid (poly I:C) and Aeromonas hydrophila stimulation and induced by poly I:C, lipopolysaccharide (LPS) and peptidoglycan (PGN) in peripheral blood leucocytes (PBLs) and head kidney leukocytes (HKLs) of C. carpio. In addition, overexpression of CcIRF10 was able to decrease the expression of the IFN and IFN stimulated genes PKR and ISG15.

Conclusions: These results indicate that CcIRF10 participates in antiviral and antibacterial immunity and negatively regulates the IFN response, which provides new insights into the IFN system of C. carpio.

Background

Interferon (IFN) regulatory factors (IRFs), as transcriptional regulatory factors, play multiple important roles in host immune responses and other physiological processes; for example, they regulate the expression of type I IFN and IFN-stimulated genes (ISGs) [1], the activation and differentiation of distinct immune cell populations, cell growth, differentiation and apoptosis [2, 3]. The N-terminal regions of IRFs share a highly conserved DNA-binding domain (DBD), which contains five or six tryptophan repeats [4]. The C-terminal regions of IRFs generally possess an IRF-associated domain (IAD), which mediates the interactions between IRFs and other proteins to form transcriptional complexes [5].

To date, eleven kinds of IRFs have been identified in vertebrates [6], which can be divided into four subfamilies, IRF1 (IRF1, 2, and 11), IRF3 (IRF3 and 7), IRF4 (IRF4, 8, 9, and 10) and IRF5 (IRF5 and 6), according to their differences in the C-terminal region [7]. IRF10 is present only in non-mammals and was first identified in the chicken (Gallus gallus). Phylogenetic analysis has shown that GgIRF10 clusters into the IRF4 subfamily and plays a crucial role in the later stages of the immune response to invading pathogens in G. gallus [8]. Functional characterization of fish IRF10 has also begun recently, and this gene has been identified in teleosts including zebrafish (Danio rerio) [9], orange spotted grouper (Epinephelus coioides) [10, 11], Japanese flounder (Paralichthys olivaceus) [12], Atlantic cod (Gadus morhua) [13, 14], grass carp (Ctenopharyngodon idella) [15], rainbow trout (Oncorhynchus mykiss) [15,
16], Asian swamp eel (Monopterus albus) [15, 17], mandarin fish (Siniperca chuatsi) [18], Senegalese sole (Solea senegalensis) [19] and blunt snout bream (Megalobrama amblycephala) [20].

The teleost IRF10 genes are constitutively expressed in a variety of tissues and can be upregulated by the viral mimic polyinosinic:polycytidylic acid (poly I:C) [10-15, 17, 18, 21], lipopolysaccharide (LPS) [12, 17], viruses [12, 18, 19] or bacteria [10, 12, 13, 17, 20], suggesting that IRF10 plays roles in immune responses to both viral and bacterial infections. Furthermore, in zebrafish and orange spotted grouper, IRF10 inhibits IFNφ1 and IFNφ3 promoter activation and negatively regulates fish antiviral gene expression to prevent an excessive immune response, which is a unique regulatory mechanism of IFN responses in teleosts [10, 11].

As a pivotal aquaculture fish species widely cultured in both Asia and Europe, common carp (Cyprinus carpio L.) is also a good model for exploring the immune system of teleosts [22-24]. Several IRFs have been identified in common carp, including IRF1, IRF3, IRF5, IRF7 and IRF9 [25-28]. In this study, we identified the full-length cDNA sequence of IRF10 in C. carpio (named CcIRF10) and investigated its function. We examined the tissue distribution of CcIRF10 in healthy common carp and then evaluated the expression level of CcIRF10 upon viral or bacterial stimulation both in vivo and in vitro to determine its function in the immune response against pathogens. Furthermore, the regulatory role of CcIRF10 in the IFN signalling pathway was also determined in this study. The results will contribute to understanding of the innate immune system of fish.

**Methods**

**Fish feeding and sampling**

*C. carpio* specimens were purchased from a local fish farm (Jinan, Shandong, China), selected for size (approximately 200 g per fish), maintained in recirculating tap water at 20 °C and fed daily for more than one week before challenge and sampling. After treatment, the fish were euthanized by immersion in a solution of tricaine methane sulfonate (MS222, Sigma-Aldrich) at a concentration of 100 mg/l of water.

**Cloning and analysis of CcIRF10 cDNA**

The full-length cDNA sequence of CcIRF10 was obtained using RT-PCR and the rapid amplification of cDNA ends (RACE) method. First, the primers IRF10-F/IRF10-R were used to amplify a partial sequence of CcIRF10. The primers were designed based on the known fish IRF10 cDNA sequences. Then, 3′ and 5′ Full RACE Core Sets (TaKaRa) were used to obtain the full-length cDNA sequence. The PCR products were ligated into the pMD18-T vector (TaKaRa) and transformed into competent *E. coli* DH-5α for sequencing (Invitrogen). The domains of the protein sequence were analysed using the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de). Multiple alignment and phylogenetic analysis were performed using MEGA 5.0. The primers used in this study are listed in Table 1.

**Experimental challenge of C. carpio with poly I:C and Aeromonas hydrophila**
Poly I:C and A. hydrophila challenge experiments were performed in C. carpio as previously described [28-30]. In the poly I:C challenge experiments, 50 fish were intraperitoneally injected with 500 μl of poly I:C solution (2.6 mg/ml in PBS, Sigma). In the A. hydrophila challenge experiment, the bacteria were inactivated in 0.5% formalin at 37 °C for 36 h and then resuspended in PBS. Then, 50 fish were intraperitoneally injected with 500 μl of A. hydrophila (at a dose of 2.0 × 10^8 cells). At 0, 3, 6, 12, 24, 48 and 72 h post injection (hpi), the spleen, head kidney, foregut and hindgut were collected from three fish at every time points (n=3). Total RNA was extracted using TRlzol reagent (TIANGEN), and cDNA was synthesized using a FastQuant RT Kit (TIANGEN).

**In vitro stimulation of PBLs and HKLs**

Peripheral blood leucocytes (PBLs) and head kidney leukocytes (HKLs) were isolated from C. carpio according to a previous report [28, 31]. In brief, C. carpio peripheral blood and head kidney cell suspensions were loaded onto freshly prepared 34%/51% Percoll (Sigma) density gradients and separated via centrifugation at 650 × g for 30 min. The cells were resuspended in cold Leibovitz’s L-15 medium with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. PBLs or HKLs (1 × 10^6) were maintained in a 24-well cell culture plate with 500 μl in each well and treated with 5 μl poly I:C (500 μg/ml), LPS (1 mg/ml) or peptidoglycan (PGN, 1 mg/ml). At 0, 3, 6, 12 and 24 h post stimulation, triplicate cell samples were harvested, and total RNA was extracted (n=3).

**Construction and transfection of CcIRF10 overexpression vectors**

The ORF of CcIRF10 was amplified using Phusion High-Fidelity DNA polymerase (PrimeSTAR) with specific primers. Purified fragments were digested with the SacII and EcoRI restriction enzymes, ligated into the pcDNA3.1-EGFP vector, and transformed into E. coli Top10 cells. The overexpression vector pcDNA3.1-EGFP-CcIRF10 (abbreviation, pclRF10) was verified by sequencing. The plasmids were extracted using an endotoxin-free plasmid isolation kit (TIANGEN) following the manufacturer’s instructions.

Epithelioma papulosum cyprini (EPC) cells were seeded in 24-well plates with 500 μl in each well at a concentration of 4 × 10^5 cells/ml and maintained at 25 °C in M199 medium (HyClone) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) for one day so that they reached approximately 80% confluency. Each well of cells was transfected with 1 μg of plasmids using 2 μl of X-tremeGENE HP DNA Transfection Reagent (Roche). EPC cells transfected with empty plasmids served as controls, and the gene expression of IFN, PKR, ISG15 and TNFα was detected after overexpression of CcIRF10. The primers used in this study are listed in Table 1.

**Real-time PCR analysis**

Real-time PCR was performed as previously described in a Rotor-Gene Q PCR instrument (Qiagen) with TransStart Tip Green qPCR SuperMix (TransGen)[28]. All samples were analysed in triplicate, and the
expression values of all genes were calculated relative to those of the 40S ribosomal protein S11 or the β-actin gene using the $2^{(\Delta \Delta CT)}$ method. The primers used are listed in Table 1.

**Statistical analysis**

Comparisons between the experimental group and the control group were performed using one-way analysis of variance (ANOVA) in GraphPad Prism 5, and a value of $P < 0.05$ was considered to indicate significance.

**Results**

**cDNA cloning and molecular characterization of CcIRF10**

The full-length cDNA of CcIRF10 was found to consist of 1274 bp. The CcIRF10 cDNA (GenBank accession no. MT646905) contains a 78 bp 5'-untranslated region (UTR), a 26 bp 3'-UTR containing mRNA instability motifs ($^{1244}_{1249}$AATAA), and an ORF of 1170 bp that translates into a 390-amino acid putative peptide with a predicted molecular mass of 44.4 kDa. The theoretical isoelectric point is 8.314. The predicted protein sequence contains a DBD (M1-R119) that possesses five tryptophans (Trp13, Trp28, Trp40, Trp60 and Trp79), an IAD (P186-L367) and a nuclear localization signal (NLS) in the DBD (Fig. S1).

The predicted protein of CcIRF10 shares 89.2% identity with that of C. idella IRF10, 52.7-75.6% identity with those of other teleost IRF10s and 50.1% identity with a bird (G. gallus) protein (Table 2). Multiple alignments of CcIRF10 with the amino acid sequences from other vertebrates revealed areas of amino acids conserved in all vertebrates. Significant homology was found in the putative DBD (Fig. 1A). To explore the phylogenetic relationships of IRF10 in vertebrates, a phylogenetic tree including IRF10s from all known species was constructed using the neighbour-joining method. The tree was divided into two branches, teleost and bird. CdRF10 had the closest relationship with C. idella IRF10 (Fig. 1B).

**Tissue distribution of CdRF10**

The expression patterns of CdRF10 in eleven tissues of healthy common carp, including the liver, spleen, head kidney, foregut, hindgut, gills, gonad, skin, muscle, buccal epithelium and brain, were detected by real-time PCR. The results showed that CdRF10 mRNA was detected in all examined tissues, with the highest expression in the gonad, the lowest expression in the head kidney, and moderate expression in the other nine tissues (Fig. 2).

**Gene expression of CdRF10 in response to poly I:C and A. hydrophila stimulation in vivo**

To determine the function of CdRF10 in immune defence in common carp, CdRF10 expression was examined in some immune-related tissues after viral or bacterial challenge. Upon poly I:C stimulation, the peak expression of CdRF10 appeared at 6 hpi in the spleen (4.5-fold, $P < 0.05$), foregut (27.5-fold, $P < 0.05$) and hindgut (7.5-fold, $P < 0.05$). In the head kidney, CdRF10 expression peaked at 3 hpi (7.5-fold, $P < 0.05$) (Fig. 3).
In *A. hydrophila*-infected sh, CcIRF10 was upregulated in the head kidney (6.8-fold, \(P < 0.05\)), foregut (13.7-fold, \(P < 0.05\)) and hindgut (3.0-fold, \(P < 0.05\)) at 6 hpi. In the spleen, CcIRF10 reached its peak at 48 hpi, with a 4.6-fold induction (\(P < 0.05\)) (Fig. 4).

**Expression of CcIRF10 upon poly I:C, LPS and PGN stimulation in vitro**

To examine the CcIRF10 transcription level in response to poly I:C, LPS or PGN challenge *in vitro*, we isolated leukocytes from the peripheral blood and head kidneys of *C. carpio*. As shown in Fig. 5A, real-time PCR data showed that the expression of CcIRF10 in the PBLs was upregulated by poly I:C (2.0-fold, \(P < 0.05\)) and PGN (2.2-fold, \(P < 0.05\)) at 12 h, but not by LPS. In HKLs, CcIRF10 was induced by all PAMPs. Upon PGN stimulation, the expression of CcIRF10 reached its peak at 12 h (3.8-fold, \(P < 0.05\)), and after stimulation by poly I:C (1.7-fold, \(P < 0.05\)) and LPS (4.1-fold, \(P < 0.05\)), the highest expression was found at 24 h (Fig. 5B).

**mRNA expression of cytokines in EPC cells overexpressing CcIRF10**

To investigate the regulatory role of CcIRF10 in the IFN signalling pathway, the gene expression of IFN, two IFN stimulated genes (PKR and ISG15) and TNFα was detected after overexpression of CcIRF10 in EPC cells (Fig. S2 and S3). The results showed that the gene expression of IFN, PKR and ISG15 was reduced in the cells transfected with pcDNA3.1-EGFP-CcIRF10, which were 83%, 0.87% and 0.69% of the expression in control cells, respectively (\(P < 0.05\), Fig. 6 A-C). However, the expression of TNFα, a non-ISG, was not changed in the cells (Fig. 6 D).

**Discussion**

IRF10, which has been reported to play important roles in immune responses to both viral and bacterial infections in teleosts, can inhibit the activation of IFN promoters and negatively regulate fish antiviral gene expression to prevent an excessive immune response [10, 11, 32]. In the present study, a non-mammalian IRF10 gene was cloned from common carp, and the antiviral and antibacterial immune functions of CcIRF10 were investigated. The predicted CcIRF10 protein contains two conserved functional domains, the N-terminal DBD and the C-terminal IAD, suggesting that the functions of IRF10 may be conserved throughout vertebrates. The DBD contains a highly conserved five-tryptophan repeat that can bind to IFN-stimulating response elements (ISREs) and IRF-regulatory elements (IRF-Es) in target promoters [4, 33]. Notably, the five well-conserved tryptophan residues in *D. rerio*, *C. idella*, *G. gallus*, and *C. carpio* are all at positions 10, 25, 37, 57 and 76 in the N-terminus. The IAD, which is responsible for homo-/heterodimer interactions of the IRFs and association with other transcription factors, is less conserved than the DBD [5]. Phylogenetic analysis of predicted IRF10 protein sequences of *C. carpio* and other vertebrate species supported the division of IRF10 into two branches: teleost and bird. These results match the established evolutionary relationships among teleost and other vertebrate species and support the authenticity of the nomenclature for IRF10.
IRF10 was first identified in *G. gallus* and is highly expressed in white blood cells and splenic lymphocytes, whereas low expression levels are found in other tissues [8]. In the present study, constitutive expression of the *CcIRF10* gene was detectable in all eleven tissues of *C. carpio* when analysed by real-time PCR, although there were differences in the levels of expression. This ubiquitous tissue expression pattern supports the findings of previous studies on IRFs in teleosts, including *M. albus* [17], *G. morhua* [13], *D. rerio* [34], *O. mykiss* [35], turbot (*Scophthalmus maximus*) [36], *P. olivaceus* [12, 37], rock bream (*Oplegnathus fasciatus*) [38], blunt snout bream (*Megalobrama amblycephala*) [39] and half-smooth tongue sole (*Cynoglossus semilaevis*) [40]. *CcIRF10* was found to be most highly expressed in gonads (Fig. 2), which is different from the expression patterns in *P. olivaceus*, *C. idella* and *M. albus*. In *P. olivaceus*, the expression of IRF10 has been found to be very high in the gills, intestine, trunk kidney, heart, stomach, head kidney and PBLs; in *C. idella*, IRF10 expression has been found to be high in all tested tissues, with the highest expression in the thymus and gills; and in *M. albus*, the highest expression level has been observed in intestine, whereas the lowest level has been found in the liver [12, 15, 17]. However, *D. rerio* IRF10 is highly expressed in the testis, which is also a reproductive organ [9]. These results suggest that IRF10 may not only play an important role in the immune system but also likely participate in the regulation of the reproductive system in teleosts.

Previous studies on *C. carpio* have shown that the expression of IRF1, IRF3, IRF5 and IRF7 is upregulated upon stimulation with poly I:C or viruses [25-27]. In the present study, following poly I:C injection, the induction of *CcIRF10* in the foregut (27.5-fold) was much stronger than that in the other tissues (4.5- to 7.5-fold), revealing the important role of *CcIRF10* in the mucosal immune system response to poly I:C (Fig. 3). Moreover, similar results were observed in PBLs and HKLs with poly I:C stimulation (Fig. 5). The expression of IRF10 in *P. olivaceus* is also upregulated by poly I:C stimulation in PBLs [12]. In *G. morhua*, two isoforms of IRF10 have been identified, and the expression of the long isoform reaches its peak at 6 hpi at 16 °C and 24 hpi at 10 °C, whereas the highest expression of the short isoform is observed at 6 hpi at both 16 °C and 10 °C after poly I:C stimulation [13, 41]. Moreover, IRF10 in *P. olivaceus*, *C. idella*, *M. albus* and zebrafish embryo fibroblast-like ZF4 cells can be upregulated by viruses (viral haemorrhagic septicaemia virus [VHSV] or grass carp haemorrhagic virus [GCHV]) or poly I:C [9, 12]. The observed induction of IRF10 expression by various viruses and poly I:C suggests that the fish IRF10 may play a crucial role in protecting the host from viral infection.

*A. hydrophila*, a well-known fish-pathogenic bacterium, is primarily found in temperate and freshwater environments and causes infections in various organisms. Fish are becoming increasingly susceptible to *A. hydrophila* because of the increasingly intensive rearing methods used in aquaculture [42]. Moreover, *A. hydrophila* breakouts have caused great economic losses around the world [43]. To gain insights into the immune mechanism of *CcIRF10* in the antibacterial response, its expression pattern in response to *A. hydrophila* was investigated using real-time PCR. When fish were challenged with *A. hydrophila*, the levels of *CcIRF10* were upregulated in all four tissues, with the highest induction in the foregut (Fig. 4). IRF10 of *P. olivaceus* can also be induced by *Edwardsiella tarda* and *Streptococcus iniae* [37]. Upon *Aeromonas salmonicida* infection, the greatest expression of the long isoform of *G. morhua* IRF10 occurs at 24 hpi, whereas the highest expression of the short isoform is observed at 6 hpi at 10 °C and 16 °C,
suggesting the distinct roles of the two isoforms in the immune system of _G. morhua_ [13, 41]. It should be noted that _E. coioides_ IRF10 is responsive to both poly I:C stimulation and _Vibrio parahaemolyticus_ infection but increases to a greater extent after poly I:C stimulation [10]. In accordance with these results, our study showed that the fold change in CclRF10 induced by poly I:C stimulation (4.5- to 27.5-fold) was greater than that induced by _A. hydrophila_ infection (3.0- to 13.7-fold). LPS is the major component of the outer membranes of gram-negative bacteria, but it has been reported that lower vertebrates (such as fish) may be resistant to the toxic effects of LPS [44]. Therefore, it was unsurprising that CclRF10 was not upregulated by LPS in the PBLs (Fig. 5A). PGN, a major component of the bacterial cell wall, consists of sugars and amino acids. Similar to the findings of a previous study regarding head kidney macrophages of _O. mykiss_, CclRF10 was upregulated by PGN stimulation _in vitro_ [15]. Hence, our _in vivo_ and _in vitro_ findings, together with the previous analogous results, suggest that CclRF10 is more susceptible to poly I:C than to _A. hydrophila_ infection and _plays a substantial role in the foregut, which is a mucosal immune organ_. Moreover, fish IRF10 may play essential roles in both antiviral and antibacterial defence, as reported for _G. gallus_ IRF10.

In mammals, IFNs are natural glycoproteins produced by cells of the innate and adaptive immune systems in most vertebrates in response to challenge by viruses, bacteria, fungi, parasites, and tumour cells [45]. In addition, IFNs can also be produced by non-immune cells such as fibroblasts and epithelial cells [45]. Similarly, in fish, IFNs play a crucial role in innate immunity [46, 47]. To investigate the regulatory role of CclRF10 in the IFN response, we detected the mRNA expression of type I IFN, ISGs (PKR and ISG15) and TNFα in CclRF10-transfected EPC cells. The transcription of PKR, ISG15 and TNFα was downregulated in EPC cells transfected with the pclRF10 plasmid (Fig. 6). This result is in line with the findings of a previous study that overexpression of _D. rerio_ IRF10 downregulated the expression of IFN stimulated genes induced by poly I:C and promoted the replication of spring viremia of carp virus (SVCV) in EPC cells [9]. Moreover, this study found that the ISRE site in the promoter was responsible for DrIRF10-mediated inhibition of gene expression of IFNs and ISGs [9, 48]. The mechanism involved in the inhibition of IFN signalling pathway in common carp may be similar, which needs our further study. However, _G. gallus_ IRF10 can upregulate the expression of MHC class I and GBP [8], suggesting that the function of IRF10 might be different between fish and birds.

**Conclusions**

In the present study, the full-length cDNA sequence of IRF10 from common carp was identified and characterized. _In vivo_ and _in vitro_ studies indicated that CclRF10 participates in both antiviral and antibacterial immune responses. Furthermore, overexpression of CclRF10 was able to decrease the expression of the IFN and IFN stimulated genes PKR and ISG5, indicating that CclRF10 might negatively regulate the IFN response of _C. carpio_. The study will provide a valuable experimental foundation for future studies on the immune system of common carp and a theoretical basis for the prevention of fish disease.
Abbreviations

IFN: interferon; IRF: interferon regulatory factor; ISG: IFN-stimulated gene; DBD: DNA-binding domain; NLS: nuclear localization signal; IAD: IRF associated domain; poly I:C: polyinosinic:polycytidylic acid; LPS: lipopolysaccharide; EPC: epithelioma papulosum cyprinid; RACE: rapid amplification of the cDNA ends; SMART: simple modular architecture research tool; PBL: peripheral blood leucocyte; HKL: head kidney leukocyte; PGN: peptidoglycan; ORF: open reading frame; ANOVA: one-way analysis of variance; UTR: untranslated region; hpi: hour post injection; ISRE: IFN stimulating response element; IRF-E: IRF regulatory element; VHSV: viral hemorrhagic septicemia virus; GCHV: grass carp haemorrhagic virus; SVCV: spring viremia of carp virus; RLR: retinoic acid-inducible gene I (RIG-I)-like receptor.

Declarations

Acknowledgments

Not applicable.

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Availability of data and materials

The dataset supporting the conclusions of this article is available in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore/MT646905) and the accession number is MT646905.

Authors’ contributions

HL and GWY conceived and designed the experiments. YYZ, SJS, HPZ and HW performed the experiments. RRL and XPC analyzed the data. YYZ and HL wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol was approved by the Animal Experimental Ethics Committee of Shandong Normal University (Permit Number: AEECSDNJ2017007).

Competing interests

The authors declare that they have no competing interests.
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**Tables**

**Table 1 Primers used in the present study.**

| Name of primer | Sequence[5’-3’] | GenBank accession No. |
|----------------|----------------|-----------------------|
| **CcIRF10-F**  | TAGCGCAGATAGACAGCG | MT646905             |
| **CcIRF10-R**  | CACACCTTTCCTCAGGTG |                      |
| **CcIRF10 ORF-F** | CCGGAATTCTAGGAACAGGTCGAGGCA |             |
| **CcIRF10 ORF-R** | TCCCCGCGGTTCACTGTTTCTGTGTTG |         |
| **CcIRF10 RT-F** | GCTGTGGATGGAAGTGTAATGG |             |
| **CcIRF10 RT-R** | CCAGGTTCCTCAGGAGAAAC |             |
| **CcS11-F**    | CCGTGCGTTAGACATCGTTACA |             |
| **CcS11-R**    | TCAGAGACTTACCTGCTGTCT |             |
| **EPC-IFN-F**  | TCAATCTCATGGATGACTCAGGAC | FN178457 |
| **EPC-IFN-R**  | TGTTATTGCCACACGCATTCTT |             |
| **EPC-TNFα-F** | ACAGGTTGTGATGTCGAGGAGGAGA | JN412133 |
| **EPC-TNFα-R** | TCTGAGACTTCTGGAAGCTGTAAG |             |
| **EPC-ISG15-F** | GTGAGCGGTGACACAGGTCGTTT | KM099174 |
| **EPC-ISG15-R** | GCGAACCCGTGTTACCGACGACAG |             |
| **EPC-PKR-F**  | AGGCTTGTGACACACAGACAGCTGAA | KM099176 |
| **EPC-PKR-R**  | CGTCCACAGGTGGTCGAAGTGATTG |             |
| **EPC-EF1a-F** | AAGACCGTTGAGAAGAAAG | AY643400 |
| **EPC-EF1a-R** | GAGTGCCCAAGTGTAGA |             |
| Species                      | Protein length | GenBank accession numbers |
|------------------------------|---------------|--------------------------|
| *Cyprinus carpio*            | 389           | MT646905                 |
| *Ctenopharyngodon idella*    | 397           | ACT83676                 |
| *Danio rerio*                | 392           | ABY91290                 |
| *Epinephelus coioides*       | 398           | AKC01040                 |
| *Monopterus albus*           | 410           | AKB09095                 |
| *Miichthys miuy*             | 402           | AHB59741                 |
| *Paralichthys olivaceus*     | 404           | BAI63219                 |
| *Gallus gallus*              | 416           | AAK55444                 |