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Molecular cloning and functional analysis of type I *IFNd* of Chinese sea bass (*Lateolabrax maculatus*) under immune stimulation

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Abstract

Chinese sea bass (*Lateolabrax maculatus*) is an economically important marine cultured species in China. Interferons (IFNs) play an essential role in innate antiviral immunity. The study on IFN immune system helps prevent and control viral diseases of *L. maculatus*. We have obtained cloning and characterization of the type I IFNd gene from L. maculatus (*LmIFNd*) in the present study. The full length of cDNA was 1190 bp, including 5'UTR (untranslated region) of 354 bp, 3'UTR of 278 bp, and an open reading frame (ORF) of 558 bp. It encodes 185 amino acids, and the first 20 amino acids are hypothetical signal peptides. The results of amino acid multiple sequence alignment and phylogenetic tree analysis showed that *LmIFNd* and mandarin fish (*Siniperca chuatsi*) *IFNd* were clustered into one branch, and the gene sequence similarity was as high as 88.9%. The expression of *LmIFNd* was tissue-specific and highly expressed in the head kidney, spleen, and gill. After infection with *Rana grylio* virus (RGV), and polyinosinic-polycytidylic acid [Poly(I:C)], the expression of *LmIFNd* in gill, spleen, and head-kidney was up-regulated significantly. Besides, the expression level of *LmIFNd* has increased significantly under the stimulation of *Vibrio harveyi* and *Streptococcus iniae*. The results show that *LmIFNd* may play a protective role in both viral and bacterial infections.

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Introduction

Lateolabrax maculatus is one of the most economically important farmed fish in China. It exhibits a fast growth rate, short culture cycle, delicious meat and high economic benefits. According to the 2019 China Fisheries Statistical Yearbook, the total output of *L. maculatus* culture in China exceeded 160,000 tons in 2018, ranking second among mariculture economic fish, with highest production in Guangdong, Fujian, Zhejiang, Guangxi and Shandong. Most *L. maculatus* culture methods involve high-density intensive culture. However, this scale of aquaculture makes fish especially vulnerable to viral or bacterial infection, resulting in high mortality and huge economic losses to the aquaculture industry. The innate immune system is the first line of defense for resisting invading microorganisms in vertebrates. In addition, IFNs are among the most critical innate immune cytokines. Therefore, studying the IFN immune system of *L. maculatus* helps to prevent and control viral diseases of *L. maculatus*.

Interferons (IFNs) are a type II α-helical cytokine with multiple biological effects. IFNs play a variety of roles in numerous cellular responses by inducing a large number of IFN stimulating factors, including innate immune cell activation and regulation of adaptive immune response, especially antiviral responses (Luo et al., 2018; Teijaro, 2016). When the body is infected by a virus, the cells secrete the bioactive cytokine IFN, to induce innate immunity (Samuel, 2001). According to sequence similarity, genomic organization, receptor usage and biological function, mammalian IFNs can be divided into type I, type II and type III (Pestka S, 2004). Functionally, types I and III IFNs are specialized as innate antiviral factors (Teijaro, 2016), while type II IFNs are considered to be cytokines that regulate innate and adaptive immunity against viral and intracellular bacterial infections (Schoenborn et al., 2007). IFNs have been found in vertebrates ranging from fish to mammals. Type II IFNs are uniquely composed of IFN-γ in mammals, but not in fish. It has been reported that there are two IFN components in fish: IFN-γ and IFN-γ-related genes (Li et al., 2019; Zou et al., 2011). In mammals, IFN-γ acts as a key immunomodulatory factor, participating in the immune response to a variety of pathogens (viruses, bacteria and protozoa), as well as cell growth, cell death and anti-tumor activity (Bae et al., 2016; Li et al., 2019). However, type III IFNs are limited to tissues with a high risk of virus exposure and infection, such as tissues with an epithelial surface, but type III IFN genes have not been found in teleost fish (Secombes et al., 2017). According to the relationship between the amount of cysteine and phylogeny, it is generally believed that type I IFNs are divided into group I and group II. There are two cysteines in group I and four cysteines in group II, and these can be further divided into subgroups a, d, e and h and b, c and f, respectively (Laghari et al., 2018; Milne et al., 2018). The type I IFNs of most fishes consist of three subgroups a, c and d, and the recently discovered *IFNh* is only found in Perciformes (Milne et al., 2018). Type I IFNs are a secreted cytokine that coordinates various immune responses to infection. Although type I IFNs are generally considered to be the most important factor in viral response, most bacterial pathogens can also induce type I IFNs, and some genes induced by type I IFNs also display antibacterial activity, such as p47GTPases (Taylor et al., 2004). The signal pathways of three kinds of IFNs are mediated by three different receptor complexes. Type I IFNs bind to the same protein complex IFN-α receptor (IFNAR) consisting of two subunits of the receptor chains IFNAR1 and IFNAR2. A tetramer consisting of two IFN-γ receptor 2 (IFNGR2) chains and two IFNGR1 chains binds dimers of type II IFN-γ. The interleukin-10 receptor 2 (IL-10R2) associates with IFN-λ receptor 1 (IFNLR1) to bind the type III IFN-λ (Sadler et al., 2008). From an evolutionary point of view, mammalian type I IFNs play a role mainly through the JAK-STAT pathway (Stark GR, 2012). Some studies have shown that tyrosine kinase 2 (TYK2), Janus kinase 1 (JAK1), signal transducers and activators of transcription (STAT1, STAT2) and IFN regulator factor (IRF) 9 which are involved in the type I IFN-mediated signal transduction pathway also exist in fish (Jin et al., 2018), and this mechanism can be preserved in fish (Hou et al., 2017).

The first attempts at fish type I IFN gene cloning were made in the early 1990s, but were unsuccessful. It was not until 2003 that three different research teams successfully cloned the fish type I IFN gene from Atlantic salmon (*Salmo solar*) (Stein et al., 2007), zebrafish (*Danio rerio*) (Altmann SM, 2003) and spotted green puffer fish (*Tetraodon nigroviridis*) (Lutfalla et al., 2003). Subsequently, the IFN genes of grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), silver crucian carp (*Carassiusaur atus gibelio*), red crucian carp (*Carassius auratus gibelio*) were identified (Casani et al., 2009; Chang et al., 2009; Long et al., 2006; Purcell et al., 2009). In recent years, fish type I IFN has been a focus of research for comparative immunologists, and great progress has been made in the study of type I interferon system. For example, *Ctenopharyngodon idella* recombinant interferon protein (rCiIFN) can induce the expression of some well-known ISGs and IFNs in vivo and in vitro. In addition, both intraperitoneal injection and oral administration of rCiIFN can effectively improve the survival rate of fish infected with GCHV (Dongming Li et al., 2013). In mandarin fish (*Siniperca chuatsi*), the expression of three IFNs (IFNc, IFNd and IFNh) induced by intraperitoneal injection of Poly(I:C), which mimics dsRNA virus infection, was significantly induced in the head kidney (Laghari et al., 2018).

The present study provides the first report of the cDNA sequence of *LmIFNd* and the characteristics of *LmIFNd* expression in different tissues and pathogeny microbiology, which provides a molecular theoretical basis for research on the immunity of *L. maculatus*.

Materials and Methods

Ethics statement. *L. maculatus* is not an endangered or protected species, and there is no requirement for permission to undertake experiments involving this species in China.

Experimental animal. The *L. maculatus* for the experiment were provided by the Zhuhai Experimental Base of the South China Sea Fisheries Research Institute, China Academy of Fisheries Sciences (Guangdong, China). The fish were 35 ± 5 cm long and weighed 365 ± 10 g. Groups of 15 fish were reared in yellow round vats with a water temperature of 24 ± 1 °C and a salinity of 2.88‰. The seawater was continuously aerated and was changed every day. *L. maculatus* were acclimatized for one week prior to the experiment.

Virus preparation. Diseased tissue, 0.4 g *Rana grylio* virus (RGV), and 4 ml aseptic PBS were placed in a sterile and enzyme-free glass grinding tube, ground slowly on ice, frozen and thawed twice at -20 °C. The homogenate was then transferred into a centrifuge tube at 4 °C, and centrifuged at 3000 r/min for 20 min. The supernatant was removed and centrifuged at 8000 r/min for 25 min. The supernatant was filtered with 0.45 μm and 0.22 μm filters, respectively, diluted 10 times with aseptic PBS and prepared at -20 °C.

Bacteria preparation. *Vibrio harveyi* and *Streptococcus iniae* were provided by the Department of fishery biological disease prevention and control, South China Sea Fisheries Research Institute, China Academy of Fisheries Sciences. During the experiment, the two strains were activated by marking on the plate, and single colonies of *V. harvei* and *S. iniae* were inoculated into liquid LB and BHI mediums, respectively, at 28 °C, 180 rpm, and 0.6 OD600. The bacteria were collected and washed twice with aseptic PBS. Then the bacterial body weight was prepared into a bacterial suspension and placed at 20 °C until required.

Design of injection experiment. To detect the mRNA expression pattern of *LmIFNd* after viral and bacterial infection, the fish were divided into five groups. Group 1 (PBS group) comprised 30 fish that received an intraperitoneal injection with 800 μL sterile PBS (control). Group 2 (RGV group) contained 30 fish that received an intraperitoneal injection with 800 μL RGV virus (3.0×10^8 copies/μL). Group 3 (Poly(I:C) group) comprised 30 fish that received an intraperitoneal injection with 800 μL Poly(I:C) (1 mg/mL). Group 4 (*V. harvei* group) comprised 30 fish that received an intraperitoneal injection with 800 μL *V. harvei* (5×10^9 cfu/mL). Group 5 (*S. iniae* group) contained 30 fish that received an intraperitoneal injection with 800 μL *S. iniae* (5×10^9 cfu/mL).

sample collection. To collect tissue samples from healthy *L. maculatus*, three healthy *L. maculatus* were randomly selected and anesthetized with 50 mg/ml MS222 for 2 min. Blood, spleen, head-kidney, liver, gill, heart, muscle, small intestine, skin, stomach and

other tissues were removed and immediately put into liquid nitrogen for temporary storage, and then transferred to a -80°C refrigerator until further analysis.

Tissue samples were collected from three *L. maculatus* from the PBS group, RGV group, Poly(I:C) group, *V. harvei* group and *S. iniae* group. Gill, head kidney and spleen tissues were removed and immediately put into liquid nitrogen for temporary storage at 0 h (the immediately after infection), 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h.

Total RNA extraction and cDNA Synthesis.

Approximately 50 mg of total RNA was extracted from each tissue sample using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The integrity of RNA was detected by 1.2% agarose gel electrophoresis, and total RNA concentration was estimated at 260 nm using a NanoDrop-2000 (Thermo Fisher, USA). Reverse transcription with 1 μg of total RNA was performed using TaKaRa kit (Primer Script RT Reagent kit with gDNA Eraser, TaKaRa, Japan) following the manufacturer's instructions. The synthesized cDNA was stored at -20 °C.

The full-length cDNA cloning of LmIFNd. The partial cDNA sequence of type I IFNd was obtained from the transcriptome database. The open reading frame (ORF) sequence was amplified using IFN-OF/OR primers (**Table 1**), which was synthesized by Sangon Biotech. The cDNA template for rapid amplification of the cDNA ends (RACE) was synthesized according to the instructions of the SMARTTM RACE 5'/3' kit (Clontech, Japan). This step reaction was performed by touchdown PCR and nested PCR using the 5'IFNR1/ 5'IFNR2 /3'IFNF1 primers and UPM/NUP (**Table 1**). The RACE - PCR products were purified using an agarose gel DNA purification kit (Sangon Biotech, China). The product was ligated to PMD18-T vector and transformed into *Escherichia coli* DH5 α competent cells. The positive colonies were selected and sent to the Guangzhou Ruibo Biological Company for sequencing.

The 5 'and 3' end sequences and the ORF of *LmIFNd* were spliced to get the full length of *LmIFNd*. Using the specific primers CF1 and CR1, the 5'RACE SMARTTM cDNA (diluted 10 times), was used as the template to verify the correctness of the spliced IFN full-length cDNA sequence.

Bioinformatics analysis of LmIFNd. The *LmIFNd* cDNA sequences were compared and analyzed using an online BLAST analysis tool https:/blast.ncbi.nlm.nih.gov/ Blast.cgi. The ORF and the coding amino acid sequence was predicted by NCBI online ORF Finder https://www.ncbi.nlm.nih.gov/orffinder/. The ExPaSy calculation tool was used to calculate the theoretical isoelectric point and molecular weight http://web.expasy.org/compute_pi/. The SignalIP4.1 tool was used to predict the signal peptide http://www.cbs.dtu.dk/services/SignalP/. And SMART http://smart.embl-heidelberg.de/ was used to predict the *LmIFNd* domain. Multiple sequence alignment of *LmIFNd* was performed using Clustal Omega and Jalview software, and the phylogenetic tree was constructed using neighbor joining by Mega7.0. All of the IFN alignment sequence information was downloaded from the GenBank database. The GenBank accession numbers of the IFNs from different species are as follows: *O. mykiss* IFNa3 (ACJ03567), *S. salar* IFNa1 (NP_001117182), *S. salar* IFNa2 (NP_001117042), *S. salar* IFNa3 (ACE75687), *O. mykiss* IFNb3 (NP_001153974.1), *O. mykiss* IFNb2 (NP_001158515), *O. mykiss* IFNb3 (CCV17399), *S. salar* IFNb1 (ACE75691.1), *S. salar* IFNb2 (ACE75693), *S. salar* IFNb3 (ACE75689), *O. mykiss* IFN f1 (CCV17413), *O. mykiss* IFN f2 (CCV17414), *Paralichthys olivaceus* IFN3 (BBA46271), *Siniperca chuatsi* IFNc (AVJ47959), *O. mykiss* IFNc1 (CCV17402), *O. mykiss* IFNc2 (CCV17403.1), *S. salar* IFNc1 (ACE75692.1), *S. salar* IFNc2 (ACE75694.1), *S. salar* IFNc3 (ACE75688), *O. mykiss* IFNd (NP_001152811.1), *O. mykiss* IFNe1 (CCV17406), *O. mykiss* IFNe2 (CCV17407), *O. mykiss* IFNe3 (CCV17408), *O. mykiss* IFNe4 (CCV17409.1), *O. mykiss* IFNe5 (CCV17410), *Larimichthys crocea* IFNh (API68650), *S. chuatsi* type IFNh (AVJ47961), *S. chuatsi* IFNd (KY768915), *C. carpio* (BAG68522), *Hyporthodus septemfasciatus* (BAJ79339), *T. nigroviridis* IFN (CAD67779), *Ictalurus punctatus* IFN (AAP92146), *C. idella* IFN (ABC87312), *Carassius auratus* IFN

(AAR20886.1), *Epinephelus coioides* IFN2 (KC495073), *E. coioides* IFN1 (KC495072), *L. crocea* IFNd (API68651), *Lateolabrax maculatus* IFNd (MT462156), *Argyrosomus regius* IFNd (MG489873), *P. olivaceus* IFN1 (BAH84776.1), *P. olivaceus* IFN2 (AHB59752.1), *P. olivaceus* IFN4 (BBA46272.1), *Nothobranchius guentheri* IFN1 (KP324755), *Mus musculus* IFN-beta (AAA37891.1), *Homo sapiens* interferon beta (NP_002167.1).

Spatial and temporal expression analysis of LmIFNd. The distribution and expression of *LmIFNd* in healthy *L. maculatus* and the *LmIFNd* mRNA expression patterns in different tissues at different time points after viral and bacterial infection were analyzed by real-time PCR (qRT-PCR) using qF1/qR1 primers (**Table 1**). 18sRNA was the internal reference gene, qRT-PCR amplifications were carried out on a 384-well rotor with a total volume of 12.5 μL, containing 6.25 μL of TB Green Premix Ex Taq II (TaKaRa) (2X), 0.5 μL of each specific primer, 2 μL cDNA of template, and 3.25 μL of DEPC water. The PCR reaction conditions were 95°C for 30 s, followed by 40 cycles of 94°C for 5 s, 60 °C for 30 s. At the end of the reaction, melting-curve analysis for each amplification was performed from 75–95 °C to ensure specificity. Each experiment was repeated three times and three parallel reactions were carried out each time.

Statistical analyses. The relative mRNA expression data were analyzed by the $2^{-\Delta\Delta Ct}$ method after normalization of 18S RNA. One-way analysis of variance (ANOVA) was performed to calculate *P* values using SPSS v22.0 software. The results of the analyses were plotted with GraphPad prism8 software.

Results

Molecular characteristics of the full-length LmIFNd gene. The full-length cDNA of the *LmIFNd* (GenBank No. MT462156) is 1190 bp, which contains 5'UTR (untranslated region) of 354 bp, 3'UTR of 278 bp and an ORF of 558 bp. It encodes 185 amino acids. The predicted molecular weight of the *LmIFNd* protein is 20.977 kDa and its theoretical isoelectric point is 6.35. There are nine unstable ATTTA mRNA sequences and one tailed signal AATAAA in the 3'UTR. The first 20 amino acids (MLNRIFFVCLFLGLYSAASS) of the N-terminal of the *LmIFNd* protein was predicted as a signal peptide by SignalIP. The functional domain predicts that the protein has an extracellular IFabd domain, which is a cytokine receptor binding site. Online prediction of glycosylation sites showed that there were two potential N-glycosylation sites (Asn47 and Asn50). In addition, *LmIFNd* has two conserved cysteine residues (**Figure 1A**).

Multiple sequence alignment and evolutionary tree analysis. The alignment of the deduced amino acid sequences of *LmIFNd* with known IFNs from other species is shown in **Figure 1B**. *LmIFNd* has the highest genetic similarity with *A. regius* IFNd (89.9%). The similarity with *S. chuatsi*, *D. labrax*, *L. calcarifer* and *L. crocea* was 88.9%, 88.8%, 88.3% and 88.2%, respectively. It was more than 80% consistent with *S. aurata*, *P. olivaceus* and *E. septemfasciatus*, indicating that the IFN gene is conservative in fish. Amino acid alignment analysis revealed that the amino acid sequence of *LmIFNd* is similar to that of type I IFN of other known species, with two cysteine residues.

The results of the *LmIFNd* phylogenetic tree showed (**Figure 1C**) that fish and mammals are divided into two branches. The members of the first group of fish type I IFN a, d, e and h were clustered into a small branch, and *LmIFNd* was closest to *S. chautsi* IFNd, followed by *L. crocea* and *A. regius* IFNd, and distant from *O. mykiss* IFNd. The IFN genes of fish and mammals come from the same group but have evolved differently.

Tissue distribution of LmIFNd. The spatial expression pattern of *LmIFNd* mRNA was determined by qRT-PCR using 18s RNA as an internal control. The brain was set to the calibrator and the variation in expression level of *LmIFNd* from the brain was defined as 1.00. The highest relative expression was in the head-kidney, followed by spleen and gill,

a relatively low expression pattern was detected the brain, skin, intestine, and heart. (**Figure 2**).

Induced expression of LmIFNd by virus challenge. To investigate the correlation with virus infection, the expression of *LmIFNd* was investigated in vivo during RGV infection and poly(I:C) stimulation. Under the stimulation of RGV and Poly(I:C), the relative expression of *LmIFNd* in the gill, head-kidney and spleen was significantly upregulated compared with the control group, but the expression pattern was different. In the gill, the relative expression of *LmIFNd* increased significantly at 6 h, peaked at 12 h after RGV stimulation, then decreased significantly after 24 h. However, in the Poly(I:C) group, the relative expression level of *LmIFNd* began to increase significantly at 6 h, and reached its peak at 48 h (**Figure 3A**). In the head-kidney, the relative expression of *LmIFNd* peaked at 6 h and remained significantly higher than the PBS group from 12–24 h (**Figure 3B**). In the spleen, the relative expression levels of *LmIFNd* began to increase significantly at 6 h. However, levels began to fluctuate after 6 h in the RGV group, and were significantly higher than those in the control group at 96 h. In the Poly (I:C) group, the expression of *LmIFNd* decreased to the level of the PBS group after 6 h (**Figure 3C**).

Induced expression of LmIFNd by bacteria challenge. To explore whether *LmIFNd* is involved in antibacterial activity, the expression of *LmIFNd* in vivo after *V. harvei* and *S. iniae* stimulation was explored. Under the stimulation of *V. harvei* and *S. iniae*, the temporal and spatial expression patterns of *LmIFNd* were significantly different in the gill, head-kidney and spleen. In the gill, the relative expression of *LmIFNd* in the *S. iniae* group began to increase, and peaked at 6 h before gradually decreasing. The relative expression of *LmIFNd* began to increase at 6 h and changed significantly at 12 h in the *V. harvei* group (**Figure. 4A**). In the head-kidney, the relative expression of *LmIFNd* in the *S. iniae* group increased significantly after 6 h, decreased at 12 h and 24 h, and then increased significantly at 48 h. Similarly, the expression of *LmIFNd* in the *V. harvei* group also increased significantly at 6 h and peaked at 48 h (**Figure 4B**). In the spleen, the expression levels of the *V. harvei* group and the *S. iniae* group increased significantly at 6 h. After that, the relative expression of the *V. harvei* group decreased to initial levels, while levels in the *S. iniae* group remained significant at 12 h, 24 h and 48 h (**Figure 4C**).

Table 1 Primer sequences.

Primer name	*Primer sequence 5′-3′*	*Primer use*
OF	ATGCTCAACAGGATCTTCTTTGTCT	ORF Verification
OR	TGACAGATTTTAGTTGGTGGTGAGTA	ORF Verification
3′F1	TGCCTGTTTCTCGGGCTGTA	3′RACE
5′R1	GACGTGGCGCGACAGTCTCTTGA	5′RACE
5′R2	GCAGCCACCTCCTCCAGAACCTGA	5′RACE
UPM-long	CTAATACGACTCACTATAGGGC-AAGCAGTGGTATCAACGCAGAGT	3′ &5′ RACE
UPM-short	CTAATACGACTCACTATAGGGC	3′ &5′ RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	3′ &5′ RACE
CF1	CGCTGCTACAGTATAAATGAGCG	IFN Full-length verification
CR1	GACTGCATGTAAGCAAATAAATGAACACA	IFN Full-length verification
qF1	CTTCGCCCCTGTATTGGGAG	Real-time PCR
qR1	CCATCTGGTCTGCTCTCATCA	Real-time PCR

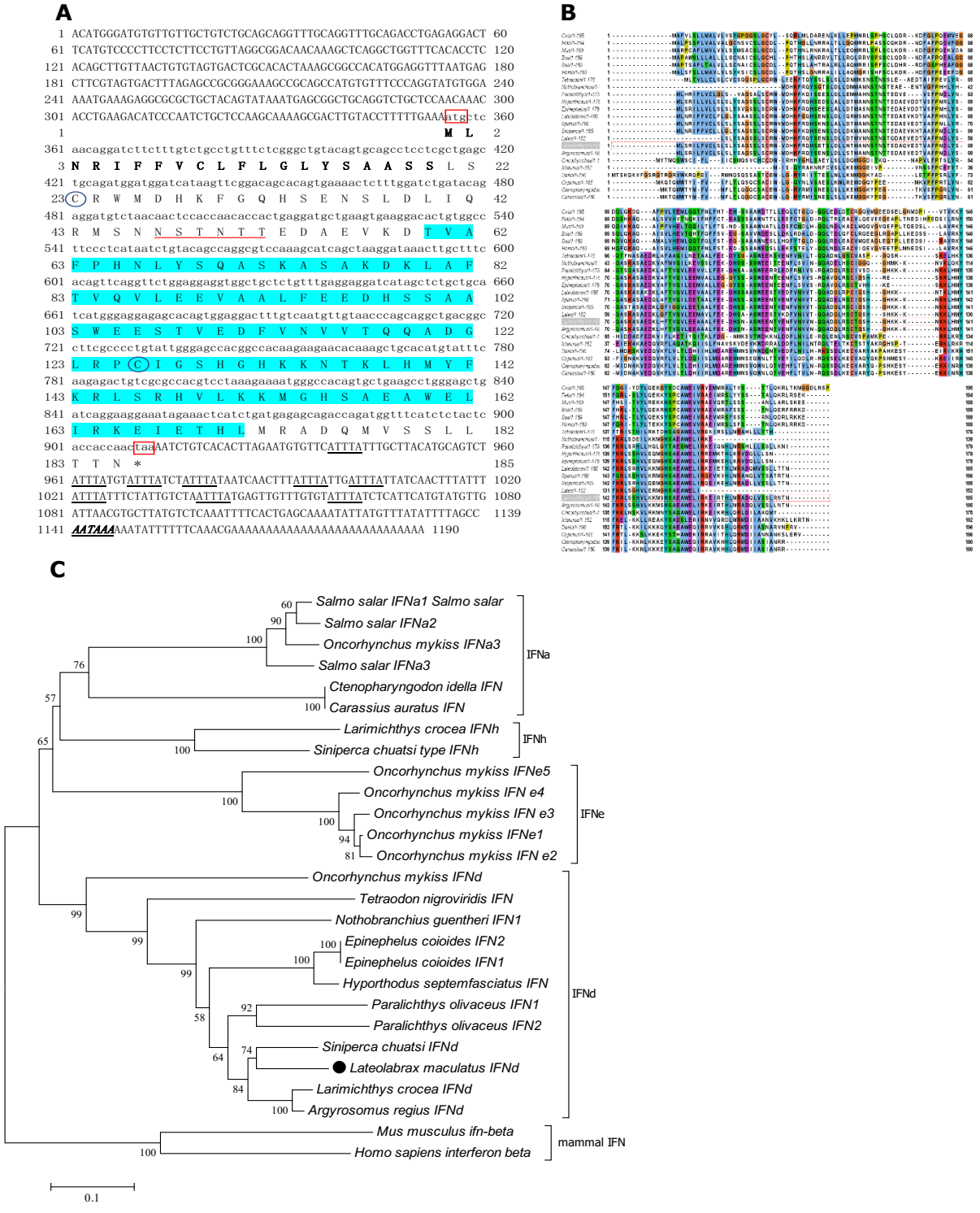

Figure 1 Molecular characterization of *LmIFNd* cDNA. (A)The start codon(ATG) and the stop codon(TAA) are circled in a red box, the bold font indicates the predicted signal peptide, the amino acid drawing the wavy line is the predicted N glycosylation site, the circle is cysteine, the blue background is the IFabd domain, the single underline is the unstable sequence, and the double underline indicates the trailing signal.(B) Multiple alignment of the amino acid sequence of *LmIFNd* and IFNs in the other species.(C) Phylogenetic tree of IFN in Fish and mammals.

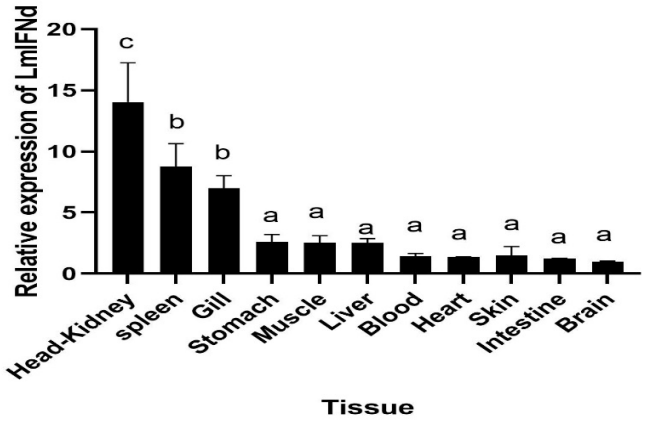

Figure 2 Relative expression of levels of *LmIFNd* mRNA in different tissues
The relative expression levels in the different tissues were calculated by the $2^{-\Delta\Delta Ct}$ method and expressed as the mean ± standard deviation (n=3). Significant different letters above the vertical bars indicate difference (*P < 0.05*)

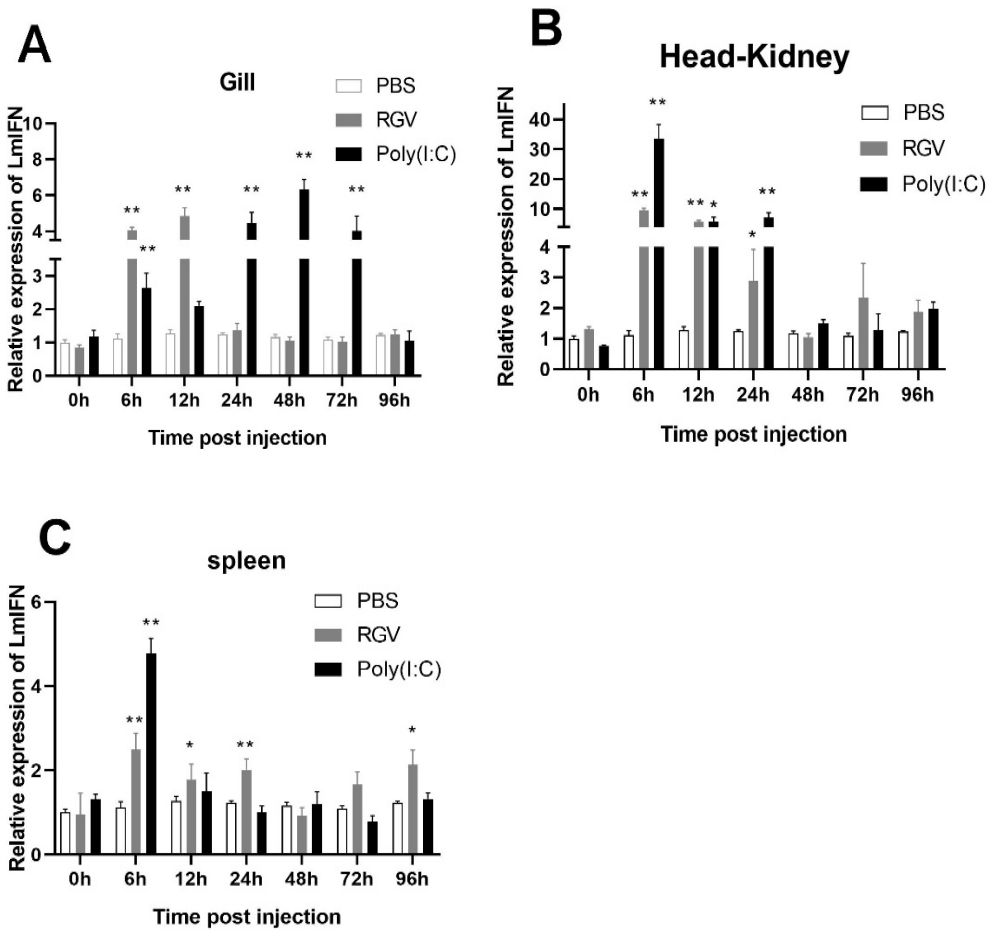

Figure 3 Spatiotemporal expression of *LmIFNd* mRNA in gill(A), head kidney(B) and spleen(C) of *L. maculatus* stimulated by RGV and Poly(I:C). The bars represent the mean ± SD (n = 3). * represents significant difference (*P < 0.05*), and ** represents highly significant difference (*P < 0.01*).

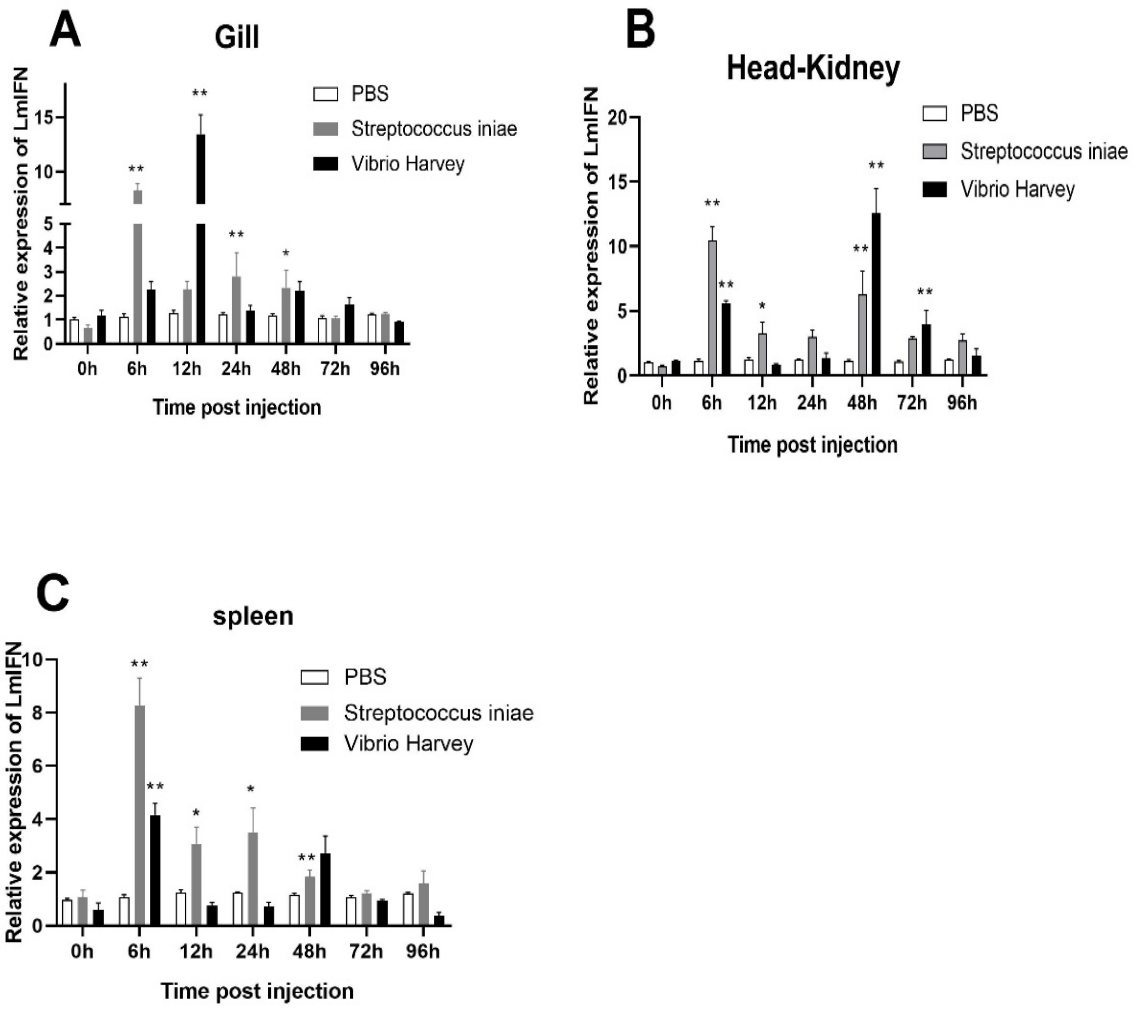

Figure 4 Spatiotemporal expression of *LmIFNd* mRNA in gill (A), head kidney (B) and spleen (C) of *L. maculatus* stimulated by *Streptococcus iniae* and *Vibrio Harvey*. The bars represent the mean ± SD (n = 3). * represents significant difference ($P < 0.05$), and ** represents highly significant difference ($P < 0.01$).

Discussion

IFN is a kind of innate immune cytokine that is induced and plays an important role in various protective responses of the body to viral and microbial infection (Pereiro et al., 2014). Type I IFN is the main coordinating factor in the antiviral response of mammals and fish (Pereiro et al., 2014). In this study, the full-length *LmIFNd* cDNA sequence of *L. maculatus* was cloned using the RACE technique, which predicted to encode 185 amino acids with a molecular weight of 20.977 kDa and a theoretical isoelectric point of 6.35. The *LmIFNd* protein consists of a predictive signal peptide and a mature region, which is secreted into extracellular space through a classical protein secretion pathway, which is consistent with the IFN gene of most fish. However, there are type I IFN variants lacking a signal peptide in some fish (Mossman et al., 2013). *LmIFNd* has two cysteine residues and two glycosylation sites (Pereiro et al., 2014). Two Cys residues of *LmIFNd* may form disulfide bonds to stabilize the protein structure. Studies have shown that the function of ZfIFN lacking glycosylation sites in *D. rerio* still have antiviral activity, indicating that

glycosylation sites in *LmIFNd* may not be related to antiviral function. But the specific functions of glycosylation sites in IFNs require further study (Altmann SM, 2003).

The results of tissue distribution showed that the highest relative expression was in the head-kidney, followed by the spleen and gill. A relatively low expression pattern was detected in the brain, skin, intestine, and heart. The results showed that *LmIFNd* was ubiquitously expressed in all tissues, although the expression levels varied. This means that *LmIFNd* may play a multifunctional role in different tissues of *L. maculatus*. In *S. chuatsi*, the expression of *IFNc*, *IFNd* and *IFNh* was highest in the head-kidney and lowest in the skin and muscle (Laghari et al., 2018), which was similar to the results of the current study. In turbot (*Scophthalmus maximus*), the expression of *IFN1* was highest in the gill and lowest in the liver. The expression of *IFN2* in was highest in the muscle and skin, but lowest in the head-kidney and liver (Pereiro et al., 2014). These results for *S. maximus* were significantly different from the results of the current study, possibly due to differences in immune function between species.

Type I IFN is a key molecule in the body's protective immune response against viruses, which controls the specific response of downstream genes and induces high levels of ISG expression, thus reducing virus proliferation (Platanias, 2005). Many studies have shown that IFN expression could be induced by viruses and Poly (I:C). For example, expression levels of Turbot IFN1 increased after viral infection, revealing a fold-change of about 150 at 72 h (Pereiro et al., 2014). It has been proven that the relative expression level of *IFNa* in the head kidney cells of sevenband grouper (*Epinephelus septemfasciatus*) stimulated by Poly(I:C) was significantly upregulated compared with a control group. Poly(I:C) could upregulate the expression of CiIFN mRNA in all detected tissues and peaked in the head kidney at 24 h after treatment, with expression levels 23.90 times higher than the control group (Li et al., 2012). In the current study, it was found that the relative expression levels of *LmIFNd* were upregulated in the gill, head-kidney and spleen after injection with RGV and Poly (I:C). The fold-change was highest in the head kidney, similar to findings for *E. septemfasciatus* and *C. idella*, indicating that the head kidney plays an important role in antiviral immunity. This indicated that *LmIFNd* may be involved in defending RGV infection and plays an important role against virus infection in *L. maculatus*.

At present, the defense mechanism of type I IFNs against bacterial pathogens is still poorly understood. It has been shown that many bacteria can induce the upregulation of IFN expression, but there are species differences in their stimulating effects on the body. It has been reported that the expression level of type I IFN in fish increased significantly after lipopolysaccharide (LPS) or bacterial stimulation (Monroe et al., 2010). In *D. rerio*, injection of IFN recombinant protein can protect *D. rerio* from death caused by *S. iniae* stress (Lopez-Munoz et al., 2009). However, in *S. maximus*, when the bacterial suspension was administrated, a significant upregulation of IFN expression was recorded at 72 h, but the administration of the plasmids encoding the *S. maximus* IFNs did not significantly affect the survival of individuals after a highly lethal dose of *Aeromonas salmonicida*. This result showed that there was no protective effect on the invasion of *A. salmonicida* (Pereiro et al., 2014). In this study, the gram-positive bacteria *V. harvei* and the gram-negative bacteria *S. iniae* were injected into *L. maculatus*. The expression of *LmIFNd* was upregulated in the immune tissues (gill, head-kidney and spleen) after stimulation of *V. harveyi*, indicating that *LmIFNd* may have a protective against bacterial infection.

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