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Molecular characterization and expression analyses of two homologues of interferon-stimulated gene ISG15 in *Larimichthys crocea* (Family: Sciaenidae)

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ABSTRACT

In this study, we sequenced and characterized two homologues of interferon-stimulated gene ISG15, termed as LcISG15-1 and LcISG15-2, from the large yellow croaker (*Larimichthys crocea*). The LcISG15-1 encodes 159 amino acids and the LcISG15-2 encodes 155 amino acids, both of which contain two tandem ubiquitin-like domains and the conserved C-terminal LRGG conjugation motif. The sequence analyses showed that both the LcISG15-1 and LcISG15-2 exhibit high similarity with ISG15 from other fishes. A putative IFN-stimulatory response element (ISRE) was detected in promoter regions of both the LcISG15-1 and LcISG15-2. Phylogenetic analyses revealed a close evolutionary relationship of both the LcISG15-1 and LcISG15-2 with other teleostean ISG15. Molecular evolutionary analyses suggested a gene duplication event of ISG15 in the ancestor of the Sciaenidae, with a signature of positive selection was found in the ISG15-2 gene copy of sciaenid fishes. The Real-time PCR analyses showed that the LcISG15-1 and LcISG15-2 were both found to be ubiquitously expressed in ten examined organs in large yellow croaker, with predominant expressions both in peripheral blood. Expression analyses showed that both the LcISG15-1 and LcISG15-2 were rapidly and significantly upregulated *in vivo* after poly (I:C) challenge in liver and spleen organs. However, the LcISG15-1 and LcISG15-2 were both significantly induced after pathogen *Vibrio parahemolyticus* infection only in the liver but not in the spleen. These results indicated that there are two ISG15 homologues in the large yellow croaker, both of which are likely to be involved in host immune defense against viral and bacterial infection.

1. Introduction

The type I interferons (IFNs)-mediated innate immune response acts as the first line of host defense against invading pathogens in fish and other vertebrates [1,2]. Upon infection, viral compounds, known as pathogen-association molecular patterns (PAMPs), are recognized by pattern recognition receptors (PRRs) [3]. The PPRs for sensing PAMPs are also conserved in teleost fishes [4]. Viral nucleic acids (dsRNA, ssRNA and DNA) and/or proteins are mainly recognized by the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and the membrane-anchored Toll-like receptors (TLRs) (such as TLRs 1, 2, 4, 5 and 6 on the cell surface; TLRs 3, 7, 8 and 9 in endosome/endoplasmic reticulum membrane) [5,6]. For example, viral dsRNA and poly (I:C) (a synthetic mimic of virus dsRNA) could be recognized by TLR3 and RLRs [6,7]. The recognition of viral PAMPs by PRRs could trigger the activation of signaling pathways to produce type I IFNs and several other cytokines [8,9]. Secreted type I IFN molecules bind to cell surface IFN receptor (IFNAR) and initiate a signaling cascade through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway [10], leading to the induction of hundreds of interferon-stimulated genes (ISGs) [11]. In addition, type I IFNs could stimulate innate immune cells to enhance antigen presentation and the produce of immune response mediators, such as cytokines and chemokines [12]. Besides, type I IFNs could also affect adaptive immunity, such as inducing antibody production in B cells and amplifying the effector function of T cells [12]. For the major consequence of type I IFNs secretion, the products of ISGs play important roles in the induction of innate and adaptive immune responses [11]. Many of ISGs could establish a cellular antiviral state and restrain pathogens by mechanisms such as inhibition of viral replication, the degradation of viral nucleic acids and the alteration of cellular lipid metabolism [12]. By far, more than 200 ISGs have been described in mammals [13,14]. Thereinto, a protein named ISG15 (IFN-stimulated gene, 15 kDa), which was one of the first ISGs to be identified [15], has drawn much attention after its discovery [16,17].

ISG15 was one of the most upregulated genes upon type I IFNs

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treatment or pathogen infection [18]. ISG15 was first identified as a ~15 kDa protein from human and bovine cell lines treated with IFN [19]. Later, it was realized that the premature form of human ISG15 contained 165 amino acids with a predicted molecular weight of ~17 kDa [20]. The mature form of human ISG15 is generated by removing eight C-terminal amino acids [21,22]. ISG15 contains two tandem ubiquitin-like (UBL) domains joined by a short linker [23] and a highly conserved C-terminal LRGG motif [24,25]. As the first identified ubiquitin-like protein [26], ISG15 could covalently attached to target cellular proteins via LRGG conjugation motif through an E1-E2-E3 enzyme cascade, in a process known as ISGylation [26–28]. Conjugation of ISG15 to targets (ISGylation) are known to be involved in many important functions, such as prevention of infections, DNA repair, autophagy, protein translation and exosome secretion [28]. Besides, ISG15 could also be secreted from cells and act as a cytokine [27]. ISG15 is believed to play an important role in innate immunity, and its expression could be induced by type I IFN [28], viral infections [29,30], poly (I:C) [31], LPS [31] and bacterial [32].

ISG15 has been identified in many teleostean fishes, including *Carassius auratus* [33,34], *Gadus morhua* [35–37], *Salmo salar* [38], *Sebastes schlegeli* [39], *Sciaenops ocellatus* [40], *Oplegnathus fasciatus* [41], *Paralichthys olivaceus* [42], *Cynoglossus semilaevis* [43], *Danio rerio* [44], *Epinephelus coioides* [45], *Scophthalmus maximus* [46], *Dicentrarchus labrax* [47], *Ctenopharyngodon idella* [48], *Solea senegalensis* [49] and *Sparus aurata* [24]. Thereinto, *C. auratus* [33] and *G. morhua* [35] has been reported to possess two and three copies of ISG15 genes, respectively. Similar to mammalian counterparts, teleostean ISG15 homologues also possess two tandem UBL domains and a highly conserved LRGG conjugation motif at the C-terminus [38,40,43,46,47]. Evidence from some studies have shown that teleostean ISG15 could be strongly induced by type I IFNs [33,38], poly (I:C) [36,40,45–47], virus infections [37,42–47], LPS [40] and bacterial [40,43]. Results from several studies have revealed that *in vitro* overexpression of teleostean ISG15 could inhibit viral infections in cultured fish cells [43–45]. In contrast, *in vitro* knockdown of ISG15 by RNAi could promote megalocytivirus infection in primary head kidney lymphocytes of *C. semilaevis* [43]. These results suggested the important role of ISG15 in innate antiviral immunity of teleost fish.

The large yellow croaker, *Larimichthys crocea*, is an economically important marine fish species cultured widely in China [50]. In recent years, the mariculture of the large yellow croaker has been suffered from serious diseases caused by bacterial, parasites and virus [51–54], resulting in huge economic losses. Chen et al. (2003) have reported outbreaks of virus disease in maricultured large yellow croaker caused by iridovirus, with a high mortality rate (75%) [51]. Therefore, to establish effective measures in disease control for maricultured large yellow croaker, it is necessary to understand the molecular processes involved in the antiviral responses in this species. By far, only few studies have been made on ISGs of large yellow croaker. The ISG56 has been characterized in the large yellow croaker [55], and the gene is found to be significantly upregulated in major immune organs of large yellow croaker after poly (I:C) stimulation. Results from a transcriptome study [56] have shown that several genes annotated as ISGs (such as Mx, Viperin and ISG56) are significantly upregulated in spleen of large yellow croaker challenged by poly (I:C). The Viperin of large yellow croaker has been characterized in our recently published study [57], and the gene is found to be strongly induced by poly (I:C), indicating the involvement of the Viperin in antiviral immune responses of large yellow croaker. However, the knowledge for the role of ISG15 in large yellow croaker is still limited. In this study, we characterized two homologues of ISG15 from large yellow croaker and studied molecular evolution and expression profiles of these two genes in this species.

2. Materials and methods

2.1. Ethics statement

Our procedures involving animals were in accordance with the guidelines of Regulations for the Administration of Laboratory Animals (Decree No.2 of the State Science and Technology Commission of the People's Republic of China on November 14, 1988). The fish species used for this study are artificially cultured and no endangered species are involved. Fishes were anaesthetized by immersion in MS222 and were sacrificed humanely.

2.2. Taxonomic coverage

We amplified and sequenced coding regions of two homologues of ISG15 in the large yellow croaker, *Larimichthys crocea*. We also sequenced coding regions of two ISG15 homologues from two close relatives of the large yellow croaker, *L. polyactis* and *Collichthys lucidus*, which both belong to the family Sciaenidae. Besides, we also incorporated available published ISG15 sequences of other species from GenBank database in this study. We obtained sequences from 12 other fish species, including *Sciaenops ocellatus* (GU370888), *Oplegnathus fasciatus* (AB548677), *Oreochromis niloticus* (XM_019365938), *Channa argus* (EF067846), *Paralichthys olivaceus* (AB519717), *Cynoglossus semilaevis* (NM_001301006), *Tetraodon nigroviridis* (CAAE01014999), *Salmo salar* (AY926456), *Oncorhynchus mykiss* (AF483529), *Danio rerio* (NM_001204169), *Carassius auratus* (AY303810 and AY303811) and *Gadus morhua* (DQ398946, FJ883792 and FJ883793). We also collected ISG15 coding sequences from three mammal species, including *Homo sapiens* (NM_005101), *Mus musculus* (NM_015783) and *Rattus norvegicus* (NM_001106700). The detailed information for all species and corresponding accession numbers are listed in Table S1.

2.3. Isolation, amplification and sequencing

In order to gain information of ISG15 homologue in the large yellow croaker, we conducted genomic blast search analyses of the gene through the draft genome of large yellow croaker which published by our lab [58], using ISG15 sequences from 12 other fish species listed above as query (E-value sets as 1e-10). Ultimately, two potential ISG15 homologues (480 bp and 468 bp, respectively) were detected from the draft genome of large yellow croaker in Scf 1572 (E-value 0.0) (data not shown), indicating the existence of ISG15 gene in this fish species. Thus, a subsequent molecular cloning was conducted to obtain unambiguous sequences of two ISG15 homologues from large yellow croaker. For this purpose, the total RNA was isolated from spleen organ (stored in liquid nitrogen) of the large yellow croaker using Trizol reagent (Invitrogen) following the standard protocol, 5 μg total RNA was reverse-transcribed into cDNA using SuperScript™ III Reverse Transcriptase kit (Invitrogen). Two pair of primers were designed based on sequences obtained from the draft genome to amplify two ISG15 homologues in large yellow croaker (See Table S2 for information on the primers). Polymerase Chain Reaction (PCR) were conducted using Premix Ex Taq™ (TaKaRa) with the following conditions: denaturation at 95 °C for 5 min, 32 amplification cycles (95 °C for 30 s, annealing at 56 °C for 30 s, 72 °C for 40 s), and a final extension at 72 °C for 10 min. All PCR products were isolated using 1% agarose gels and purified with Gel Extraction Kits (Qiagen), ligated into pGEM-T easy vector (Promega), cloned and sequenced using the Terminator kits (Applied Biosystems) on an ABI 3730 DNA sequencer. The sequenced coding sequences of two ISG15 homologues of large yellow croaker were termed as LcISG15-1 (Accession number: MH280016) and LcISG15-2 (Accession number: MH280018) and used for subsequent analyses.

In addition, to examine whether other fish species from the family Sciaenidae also contain two ISG15 homologues, we conducted molecular cloning for *L. polyactis* and *C. lucidus*, two common sciaenid fishes

in the East China Sea. We isolated genomic DNA using DNeasy Blood & Tissue Kit (Qiagen) from the muscle biopsy of *L. polyactis* and *C. lucidus* that were collected and stored in 100% ethanol. Two pairs of primers (Table S2) were designed based on the draft genome sequence of large yellow croaker to amplify coding regions of two ISG15 homologues in these two fish species. PCR conditions were the same as above. The sequenced coding sequences of ISG15 homologues were termed as LpISG15-1 (Accession number: MH280020) and LpISG15-2 (Accession number: MH280021) in *L. polyactis* and ClISG15-1 (Accession number: MH280014) and ClISG15-2 (Accession number: MH280015) in *C. lucidus*, respectively.

Besides, we also isolated genomic DNA from the muscle biopsy of large yellow croaker. Two pairs of primers (Table S2) were designed based on the draft genome sequence of large yellow croaker to amplify 5' flanking regions (both ~800 bp) of the LcISG15-1 and LcISG15-2. The promoter regions of the LcISG15-1 and LcISG15-2 were analyzed by MEME Suit version 4.12.0 (http://meme-suite.org/tools/meme) [59] to predict potential conserved motif. The transcription start site was predicted by NNPP (http://www.fruitfly.org/seq_tools/promoter.html) [60].

2.4. In silico sequence analyses

The nucleotide sequences of 18 species were aligned using MEGA7 [61] and checked for accuracy by eye, and coding sequences were translated to amino acids. The conserved protein domains in ISG15 homologues were predicted using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) [62] and Pfam database search (http://pfam.xfam.org) [63]. The amino acid sequence identity and similarity percentages of ISG15 were calculated by Matrix Global Alignment Tool (MatGAT v2.02) [64]. The genomic sequences of the LcISG15-1 and LcISG15-2 were retrieved from the draft genome database of large yellow croaker, and the corresponding genomic and cDNA sequences were aligned by bl2seq (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to predict exon/intron boundaries. Gene synteny analyses were performed to large yellow croaker, zebrafish, mouse and human, to check whether the genes surrounding ISG15 are evolutionary conserved among vertebrates.

Bayesian phylogenetic tree based on aligned amino acid sequences of ISG15 was reconstructed using MrBayes 3.2.6 [65]. The amino acid substitution model JTT + Γ was selected as the best fit by ProTest3.4 [66]. For Bayesian analysis, 10,000,000 generations of MCMC were performed with sampling frequency as every 100th generation. The first 2,000,000 generations were discarded as burn-in, since the standard deviations of split frequencies were stable below 0.01 after 2,000,000 generations of MCMC performances. All other options and priors were the default settings of MrBayes 3.2.6 software. The Bayesian phylogenetic tree based on aligned nucleotide sequences of ISG15 was also reconstructed based on the nucleotide substitution model of GTR.

In addition, tertiary structures of the LcISG15-1 and LcISG15-2 were predicted using the SWISS-MODEL homology modeling [67] both based on human ISG15 protein model of 3RT3 [68] (31.33% and 31.13% sequence similarity with LcISG15-1 and LcISG15-2, respectively).

2.5. Expression profile of genes in normal and challenged organs

Real-time PCR assays were conducted to examine the expression of LcISG15-1 and LcISG15-2 in major organs of large yellow croaker. Healthy juvenile large yellow croaker individuals (average weight of ~100 g) were obtained from Zhejiang Dahaiyang Technology Co., Ltd (Zhejiang, China). The fishes were acclimated in an aerated seawater (25 °C, ~20‰ salinity) tank with seawater changed once a day and fed twice daily for at least one week before treatment. Individuals of large yellow croaker without any treatment were sacrificed after anaesthetization, and muscle, skin, peripheral blood, spleen, liver, intestine, heart, head kidney, gill and brain organs were collected and stored in liquid nitrogen immediately for RNA preservation. Three untreated individuals were used for replication.

Real-time PCR assays were also performed to examine expression profile changes of the LcISG15-1 and LcISG15-2 in major immune organs challenged by poly (I:C). The liver, an organ contains a large population of innate immune cells, plays an important role in controlling systemic innate immunity [69]. The liver is considered as an important component of immune system in teleost fish [70,71]. The spleen comprises diverse cell populations including macrophage and lymphocytes and is also considered as an important immune organ in fishes [70,71]. The liver and spleen are widely involved in studies of innate immunity of teleost fishes [56,72–74]. For these reasons, in this study, the liver and spleen of large yellow croaker were selected for expression profile analyses. Poly (I:C) is a synthetic double-stranded RNA that is used experimentally to model viral infections [75]. As a viral mimic, poly (I:C) is widely used in mechanism studies of innate antiviral responses in teleost fishes [57,76–79]. Sterile poly (I:C) stock (Sigma-Aldrich) was dissolved in phosphate buffered saline (PBS) (pH 7.4) at the concentration of 1 mg/ml and filtered through a 0.22 μm filter. Each fish was challenged by intraperitoneal injection of poly (I:C) solution at a dose of 0.25 mg per 100 g body weight. The control group was injected an equal volume of sterile PBS solution. Liver and spleen organs were harvested from the immune challenged fishes at time points of 0 (PBS-injected), 3, 6, 12, 24 and 48 h post poly (I:C) injection. For each group, three individuals were collected for replication.

Besides, the expression profile changes of the LcISG15-1 and LcISG15-2 in major immune organs of large yellow croaker challenged by *Vibrio parahemolyticus* were also examined. *V. parahemolyticus*, a Gram-negative bacterium, is a major pathogen causing severe infections in cultured large yellow croaker [80]. *V. parahemolyticus* was cultured in LB medium under 28 °C to mid-logarithmic phase, then centrifuged, washed and resuspended in sterile PBS to 1×10^7 CFU/ml. Each fish of experimental group was challenged by intraperitoneal injection of *V. parahemolyticus* resuspension at a dose of 0.5 ml per 100 g body weight. The control group was injected an equal volume of sterile PBS solution. Liver and spleen organs were collected from fishes at time points of 0 (PBS-injected), 6, 12, 24, 48, 72 and 96 h post *V. parahemolyticus* injection. For each group, three individuals were collected for replication.

Total RNA was isolated from each organ, and cDNA was synthesized from 5 μg total RNA as described above. Gene expression was analyzed using SYBR® Premix Ex Taq™ (TaKaRa) in the ABI 7500 Fast Real-time PCR system (Applied Biosystems) following the protocol (See Table S2 for primer information). For each organ, 45 ng cDNA template was used. The PCR products from Real-time PCR assays were sequenced for confirmation. The β-actin [81,82] was used as an internal control gene. For tissue distribution of the LcISG15-1 and LcISG15-2 in ten organs of healthy large yellow croaker, the amount of each gene was normalized to the β-actin and arbitrarily calibrated to muscle using the $2^{-\Delta\Delta Ct}$ method [83]. For temporal expression of the LcISG15-1 and LcISG15-2 in challenged organs, the expression levels of target genes were normalized to the β-actin and calibrated to the control group. All data were presented as relative mRNA expression (mean ± SD). Statistical analysis for tissue distribution and stimulation was conducted using one-way ANOVA followed by Turkey *post hoc* test and unpaired two-tailed Student's *t*-Test, respectively. A P-value < 0.05 was considered statistically significant.

Besides, the expression profile changes of some other immune genes, including interleukin-8 (IL-8), Toll-like receptor 3 (TLR3), STAT1 and STAT2, were also examined in poly (I:C) and bacterial challenged liver and spleen of large yellow croaker (See Table S2 for primer information).

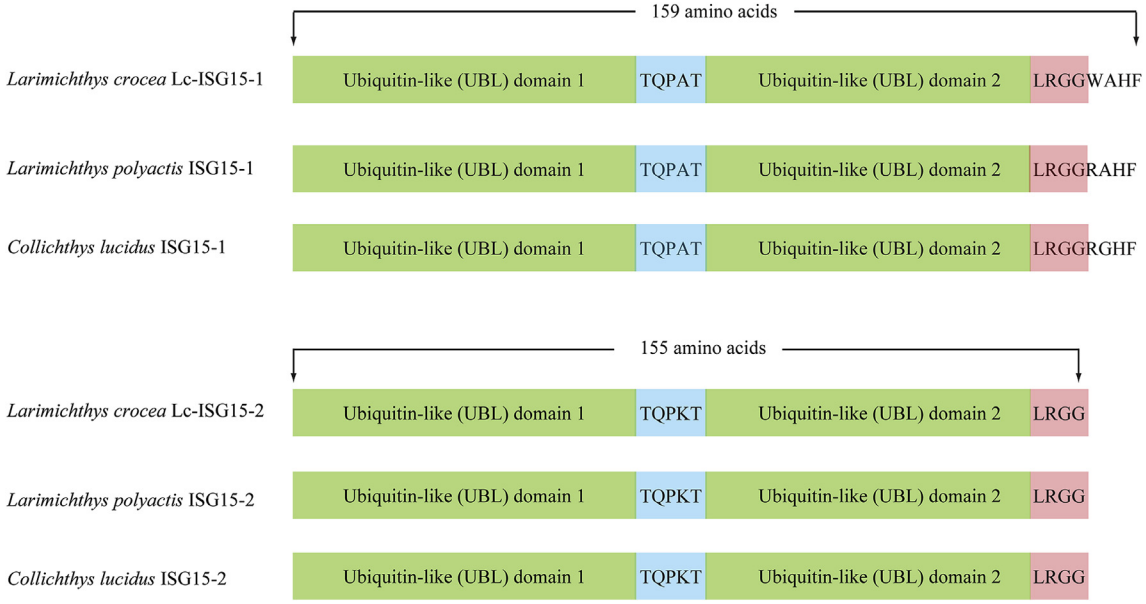

Fig. 1. Schematic diagram of ISG15 protein structure of three sciaenid fishes. The Ubiquitin-like (UBL) domain, short linker and LRGG conjugation motif are highlighted in green, blue and red boxes, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Sequence analyses of the LcISG15-1 and LcISG15-2

The open reading frame of LcISG15-1 comprises 477 nucleotides encoding 159 amino acids (AA) (Fig. 1 and Fig. S1), with a predicted molecular mass of 18.24 kDa and a theoretical isoelectric point (pI) of 9.26. The LcISG15-2 contains 465 nucleotides encoding 155 AA (Fig. 1 and Fig. S1), with a predicted molecular mass of 17.61 kDa and a theoretical pI of 9.36. The sequences of LcISG15-1 and LcISG15-2 were compared with the corresponding gene sequences obtained from draft genome of large yellow croaker. A total of ten bases were found to be different between sequenced LcISG15-1 and the genome sequence, with three base differences causing amino acid changes (Fig. S2A). Nine base differences with five causing amino acid changes were detected between LcISG15-2 and corresponding sequence from genome (Fig. S2B). These base differences between sequenced and predicted sequence are likely to be caused by genome sequencing errors. Besides, these base differences may also partly due to single nucleotide polymorphisms (SNPs). In a recent study conducted by other scholar in our lab, 312G/T in LcISG15-1 and 83C/T and 297G/T in LcISG15-2 were proved to be SNPs (unpublished data). The LcISG15-1 and LcISG15-2 contain two tandem ubiquitin-like domains joined by a five-residue linker and the conserved C-terminal LRGG motif (Fig. 1). In contrast, the LRGG motif of LcISG15-1 is followed by four residues that terminate the protein sequence (Fig. 1). We also successfully amplified and sequenced two homologues of ISG15 from genomic DNA of two sciaenid fishes, *L. polyactis* (termed as LpISG15-1 and LpISG15-2) and *C. lucidus* (termed as ClISG15-1 and ClISG15-2), supporting the existence of two ISG15 homologues not only in the large yellow croaker but also in some (if not all) other fish species from the family Sciaenidae. The LpISG15-1 and ClISG15-1 both comprise 477 nucleotides encoding 159 AA (Fig. 1 and Fig. S1), while the LpISG15-2 and ClISG15-2 both contain 465 nucleotides encoding 155 AA (Fig. 1 and Fig. S2). The ISG15-1 and ISG15-2 homologues of *L. polyactis* and *C. lucidus* all possess two tandem ubiquitin-like domains joined by a five-residue linker and the conserved C-terminal LRGG motif (Fig. 1). The LpISG15-1 and ClISG15-1 both contain four residues after the LRGG motif (Fig. 1).

The sequence lengths of ISG15 homologues in examined fishes are ranged from 152 AA (*G. morhua* ISG15-2) to 167 AA (*G. morhua* ISG15-3) (Table S3). Among 18 examined species, the LcISG15-1 shared maximum identity with the *C. lucidus* ClISG15-1 (96.9%, Table S3). The human and mouse ISG15 were found to be 29.9% and 31.9% identical with the LcISG15-1, respectively (Table S3). The LcISG15-2 shared maximum identity with the *C. lucidus* ClISG15-2 (96.1%, Table S3). The human and mouse ISG15 were found to be 30.5% and 29.7% identical with the LcISG15-2, respectively (Table S3). The LcISG15-1 shared 71.7% sequence identity at amino acid level with the LcISG15-2 (Table S3).

Both the LcISG15-1 and LcISG15-2 are composed of two exons and one intron (Fig. 2A). The exon-intron junctions are both in accordance with the classic GT/AG rule. The first exon of LcISG15-1 and LcISG15-2 were non-coding, as has been observed in ISG15 of zebrafish (Fig. 2A). However, the first exon of human and mouse ISG15 genes contains three nucleotides (ATG) encoding a starting methionine (M) residue (Fig. 2A). Our results of gene synteny analyses showed that the LcISG15-1 and LcISG15-2 are tandemly located in the scaffold 1567 of the large yellow croaker genome with a same transcription orientation (Fig. 2B). The ISG15 gene is immediately downstream of the PXMP2 gene in both large yellow croaker and zebrafish, however, is immediately downstream of the AGRN gene in both human and mouse (Fig. 2B). The gene upstream the ISG15 locus is the GATC gene in large yellow croaker, the SGSM1A gene in zebrafish, the HES4 gene in human and the PERM1 gene in mouse (Fig. 2B), indicating the gene upstream the ISG15 locus is highly variable among teleost fishes and mammals.

In addition, we also amplified and sequenced ~800 bp of 5' flanking regions (~550 bp of promoter regions were included) of both the LcISG15-1 and LcISG15-2 in large yellow croaker. Our results of MEME/MAST motif search revealed the putative presence of IFN-stimulatory response element (ISRE) both in the LcISG15-1 and LcISG15-2 promoter regions (Fig. 3). For the LcISG15-1, the putative ISRE motif (GAAACCGAAACT, *P*-value of MEME/MAST motif search is 9.7e-6) was found to be located at position of −111 to −100 relative to the putative transcription start site of the gene (Fig. 3A). For the LcISG15-2, the putative ISRE motif (GAAACTGAAAGC, *P*-value is 9.7e-6) was detected to be located at position of −91 to −80 relative to the putative transcription start site of the gene (Fig. 3B). The ISRE sites of LcISG15-1 and LcISG15-2 both match exactly the GAAAN$_{1\text{-}2}$GAAA(C/G) consensus

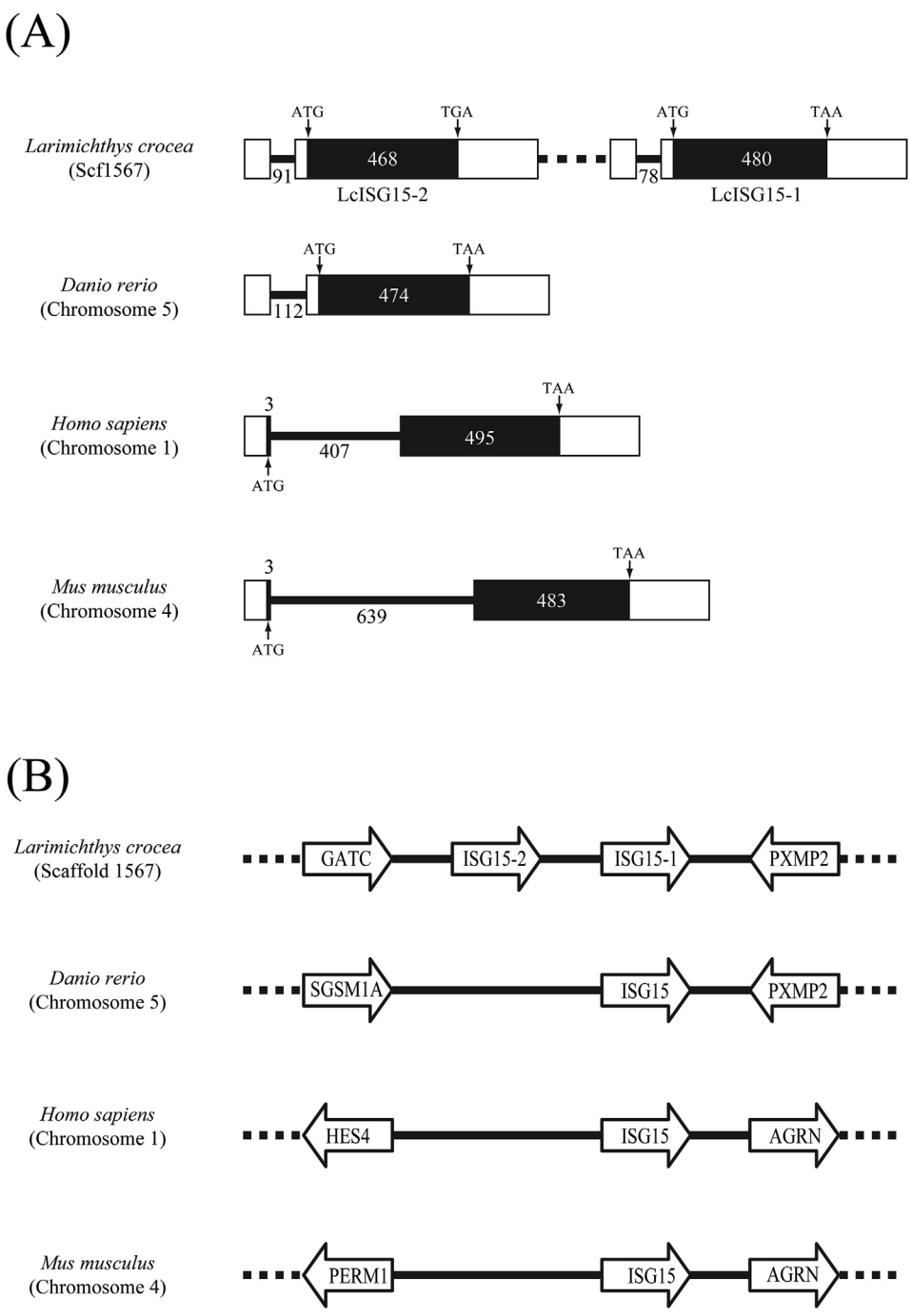

Fig. 2. Genomic structure comparison of ISG15 homologues among selected vertebrates. (A) Genomic structure comparison of LcISG15-1 and LcISG15-2 with other ISG15 homologues. The accession numbers of the genomic sequences used are: *Larimichthys crocea* (NW_017608003), zebrafish (NC_007116), mouse (NC_000070) and human (NG_033033). Exons and introns are denoted by boxes and lines, respectively. Coding regions in exons are filled with black. Start codon sequences (ATG) and stop codons (TAA) are indicated. (B) Gene synteny analyses of ISG15 among vertebrate genomes. Genes are transcribed in the direction of the arrows.

sequence which is present in all promoters known to be induced by type I IFN [84]. Besides, a putative TATA box was found to be located at position −18 to −23 and −19 to −24 in the promoter region of the LcISG15-1 and LcISG15-2 (Fig. 3), respectively.

3.2. Phylogenetic reconstruction and molecular evolutionary analyses

The Bayesian phylogenetic tree based on amino acid sequences of ISG15 homologues revealed a tree topology that all the sequences were found to be divided into two groups, consisting of teleostean and mammalian branches (Fig. 4). In the ISG15 amino acid tree, all fish species grouped together to form the teleostean clade with a high nodal support [Bayesian posterior probability (BPP) of 100%] (Fig. 4). All ISG15 homologues from four sciaenid fishes (*L. crocea*, *L. polyactis*, *C. lucidus* and *S. ocellatus*) grouped together to form a monophyletic group of the family Sciaenidae with a 100% BPP nodal support (Fig. 4). Thereinto, all ISG15-1 (including the *S. ocellatus* ISG15) and all ISG15-2 sequences of examined sciaenid fishes grouped separately with their own homologues to form two distinct clades, the ISG15-1 clade (100% BPP) and the ISG15-2 clade (100% BPP), *prior to* the forming of the Sciaenidae clade (Fig. 4). However, in accordance with the results from previous studies [33,35], the reported three and two ISG15 homologues in *G. morhua* (AcISG15-1, AcISG15-2 and AcISG15-3) and *C. auratus* (CaISG15-1 and CaISG15-2) respectively, were both grouped together with their own homologues to form a single clade (Fig. 4). The repeated Bayesian phylogenetic analyses based on nucleotide sequences of ISG15 homologues revealed a quite similar tree topology (Fig. S3). The reported *S. ocellatus* ISG15 [40] was found to be clustered with ISG15-1 sequences of sciaenid fishes (100% BPP) (Fig. 4), indicating that the *S. ocellatus* ISG15 is homologous to our newly sequenced LcISG15-1,

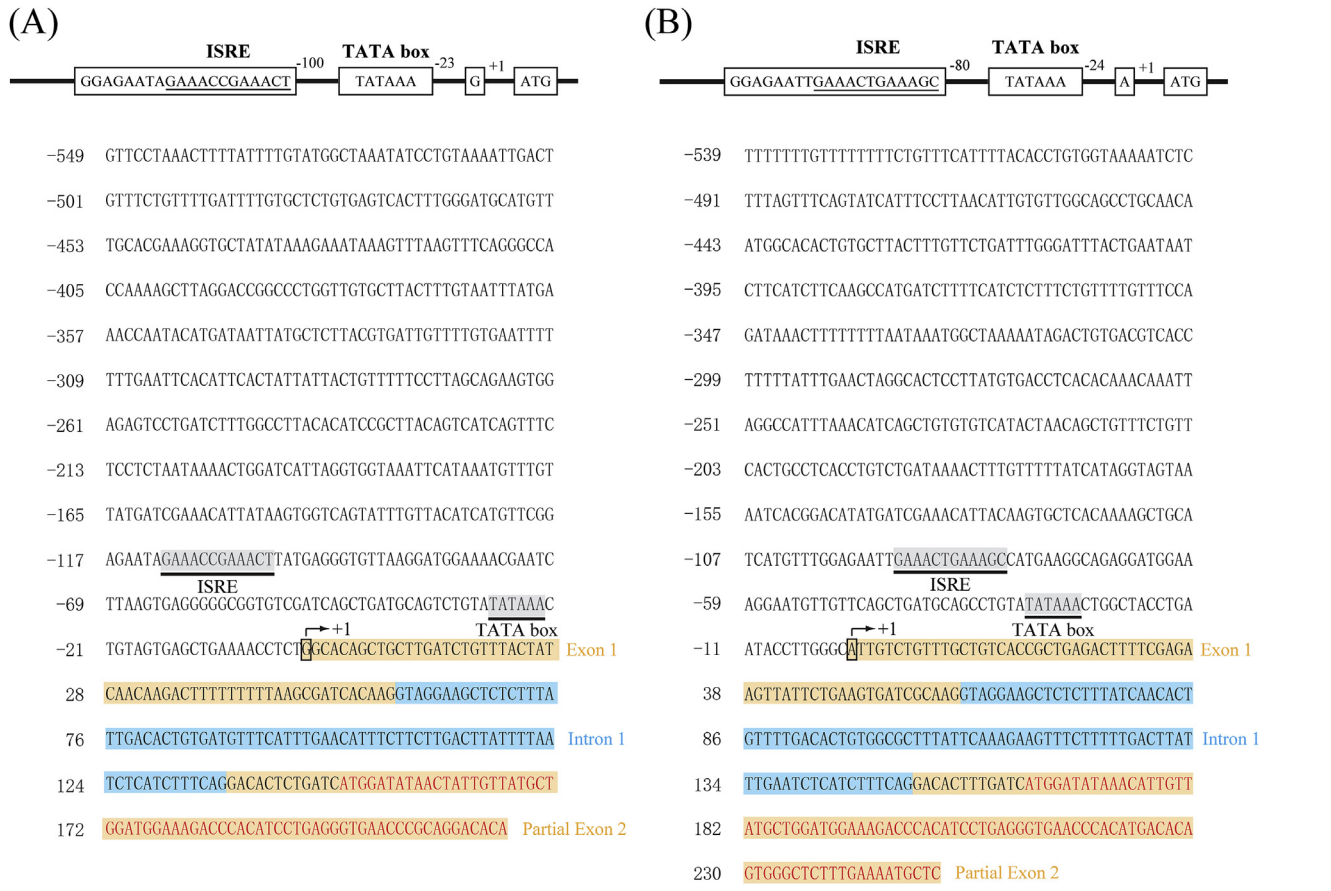

Fig. 3. Sequence analyses of LcISG15 promoter regions. (A) Sequence analysis of the LcISG15-1 promoter region (GenBank accession number: MH280017). (B) Sequence analysis of the LcISG15-2 promoter region (GenBank accession number: MH280019). The putative transcription start site are indicated with boxes. ISRE motif and TATA box in promoter regions of LcISG15-1 and LcISG15-2 are marked, underlined and shaded. The sequences of exons and introns are highlighted with yellow and blue boxes, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

LpISG15-1 and ClISG15-1, thus could be referred to as SoISG15-1. Moreover, the separate grouping of ISG15-1 and ISG15-2 homologues of sciaenid fishes, indicating that a gene duplication event of ISG15 may have occurred in the ancestor of the family Sciaenidae before the diversification of sciaenid fishes. However, further studies are needed to confirm this, because the Sciaenidae is a large family includes roughly 70 genera and 270 species worldwide [85].

To check whether the ISG15 gene duplication in sciaenid fishes is associated with selection pressure change (especially positive selection), we applied the test 2 of branch-site model A [86] using PAML CODEML [87] to detect positively selected sites (thus positive selection) along the ancestral branches leading to Sciaenidae, sciaenid ISG15-1 clade and ISG15-2 clade (branches marked with A, B and C in Fig. S4, respectively). Results of all tests 2 of the branch-site model A are shown in Table S4. No evidence of positive selection was detected on ancestral branches leading to Sciaenidae and sciaenid ISG15-1 clade (branch A and B in Fig. S4). However, statistically supported evidence of positive selection ($2\Delta\ell = 4.04$, df = 1, P-value < 0.05) was detected on the ancestral branch leading to sciaenid ISG15-2 clade (Table S4). Five positively selected sites were detected on the ancestral branch of sciaenid ISG15-2 clade, of which two had Bayes Empirical Bayes (BEB) values > 0.95 (131L and 150G) (Table S4). These results showed that the ISG15-2 gene copy has undergone positive selection in sciaenid fishes after ISG15 gene duplication in the family of Sciaenidae.

3.3. Tertiary protein structure prediction

Based on the template 3RT3 (human ISG15, Fig. S5A), the potential tertiary structures of the LcISG15-1 (2–153 AA) (Fig. S5C) and LcISG15-2 (1–153 AA) (Fig. S5D) were predicted using the SWISS-MODEL Automatic Modeling Model. Besides, the potential 3D structure of zebrafish ISG15 (Fig. S5B) was also predicted and compared with those of LcISG15-1 and LcISG15-2. The human ISG15 3D structure contains two UBL domains (N-terminal UBL and C-terminal UBL), each of which assumes a β-grasp fold that is similar to that found in ubiquitin [88] (Fig. S5A). Both UBL domains of human ISG15 contain four β-sheets (β1-β4 and β5-β8 for each), one α-helix (α1 and α2 for each) and two 3_{10} helices (3_{10} helix 1–2 and 3_{10} helix 3–4 for each) (Fig. S5A). The two β-grasp domains are connected by a six-residue extended linker peptide (colored in blue) (Fig. S5A). The spatial structures of the LcISG15-1, LcISG15-2 and zebrafish ISG15 were highly related to that of human, except for the lacking of β3, 3_{10} helix 1 and 4 in zebrafish ISG15 (Fig. S5B) and 3_{10} helix 4 in both LcISG15-1 and LcISG15-2 (Figs. S5C and D).

3.4. Spatial expression of LcISG15-1 and LcISG15-2 in normal organs

Our results showed that the LcISG15-1 and LcISG15-2 were both ubiquitously expressed in all ten examined organs (muscle, skin, brain, intestine, head kidney, spleen, gill, liver, heart and peripheral blood) (Fig. 5). The expression of the LcISG15-1 was found to be predominantly in the peripheral blood (36-fold), followed by heart (8-fold) and liver (4-fold) (Fig. 5). The highest level of the LcISG15-2 was also expressed in peripheral blood (18-fold) (Fig. 5). The expression levels of the LcISG15-1 and LcISG15-2 in major immune organs, such as spleen and head kidney, were all less than 2-fold change (Fig. 5).

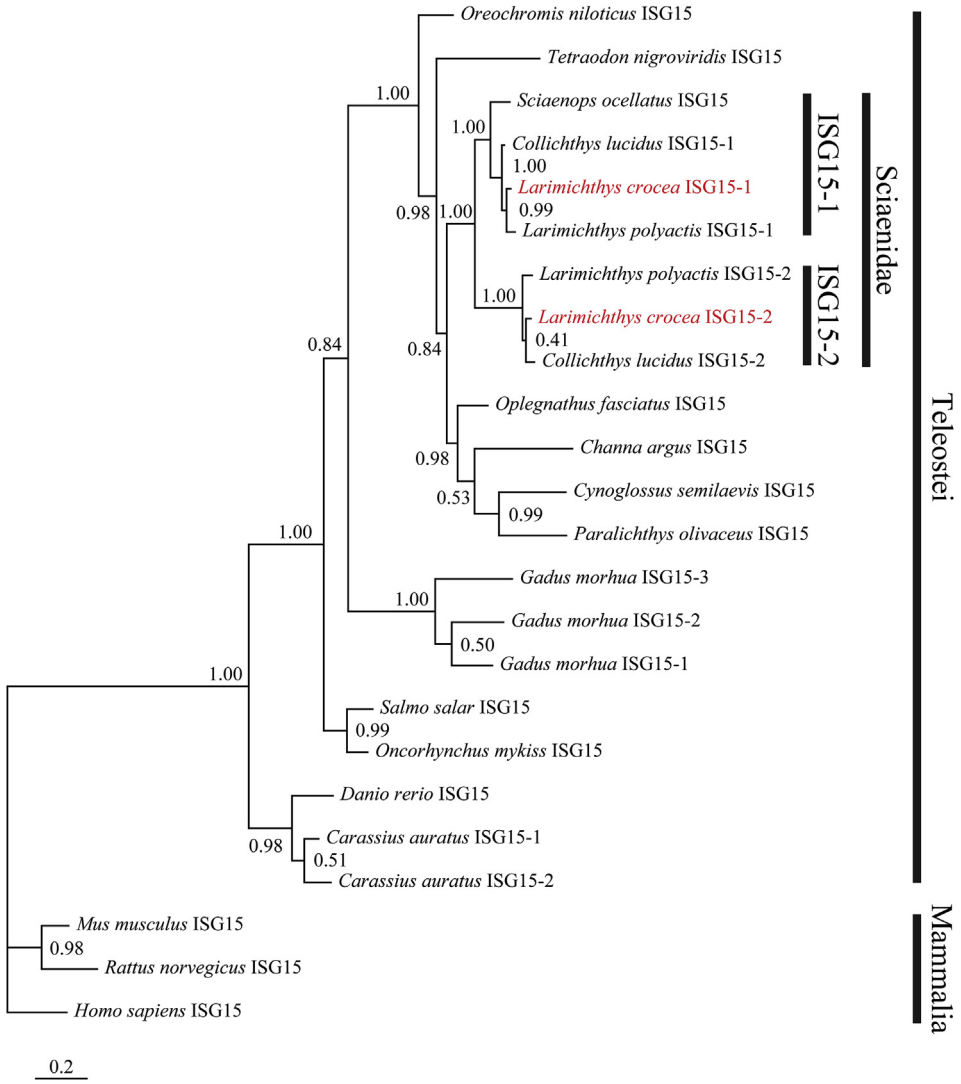

Fig. 4. Bayesian phylogenetic tree based on full ISG15 amino acid sequences of 18 species, under the model of JTT + Γ. Values on the nodes are posterior probabilities. The species of *Larimichthys crocea* is highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Temporal expression of LcISG15-1 and LcISG15-2 after immune stimulation

Our results showed that expression levels of two ISG15 homologues were both significantly changed after stimulation by poly (I:C) (Fig. 6). The expressions of the LcISG15-1 were quickly upregulated after the challenge with poly (I:C) in the two immune organs, and peaks appeared at 12 h in liver (Fig. 6A) and at 6 h in spleen (Fig. 6B). The least levels of the LcISG15-1 were noticed both at 0 h (and 48 h) in liver and spleen (Fig. 6). The peak levels of the LcISG15-1 reached 280-fold in liver and 104-fold in spleen of the control one (Fig. 6). Similarly, the expression levels of the LcISG15-2 were also quickly upregulated after poly (I:C) stimulation, with the peaks appeared also at 12 h in liver and at 6 h in spleen (Fig. 6). The least levels of the LcISG15-2 were found both at 0 h (and 48 h) in liver and spleen (Fig. 6). The peak levels of the LcISG15-2 reached 644-fold in liver and 132-fold in spleen of the control one (Fig. 6).

However, the expression profile changes of the LcISG15-1 and LcISG15-2 in spleen and liver from large yellow croaker infected with fish pathogen *V. parahemolyticus* were quite different (Fig. 7). Our results showed that expression levels of two ISG15 homologues were both significantly changed in liver after *V. parahemolyticus* infection, but both in a relatively mild degree (Fig. 7A). The expression of the LcISG15-1 in liver was significantly upregulated after bacterial infection, with the peak appeared at 48 h (and 96 h) (Fig. 7A). The least level of the LcISG15-1 was noticed at 6 h (and 12 h) (Fig. 7A). The peak level the LcISG15-1 reached only 3-fold in liver of the control one (Fig. 7A). Similarly, the expression level of the LcISG15-2 in liver was also significantly upregulated after infection, with the peak appeared at 24 h (and 96 h) (Fig. 7A). The least level of the LcISG15-2 was found at 0 h (and 12 h) (Fig. 7A). The peak level the LcISG15-2 reached 7.3-fold in liver of the control one (Fig. 7A). However, in spleen, our results showed that the expression levels of two ISG15 homologues were both not significantly changed after *V. parahemolyticus* infection (Fig. 7B).

Our results showed that the IL-8, a proinflammatory cytokine, is significantly upregulated in spleen but not in liver both after poly (I:C) and *V. parahemolyticus* challenge (Fig. 8). These results are in accordance with those from a previous study [89]. The TLR3 is significantly upregulated both in liver and spleen after stimulation by poly (I:C) (Fig. 8A and B). However, the TLR3 is slightly upregulated in liver or not significantly changed in spleen after *V. parahemolyticus* infection (Fig. 8C and D). These results strengthened the fundamental role of TLR3 as a pattern recognition receptor in viral dsRNA sensing [90]. The STAT1 and STAT2, two genes involved in type I interferon-mediated

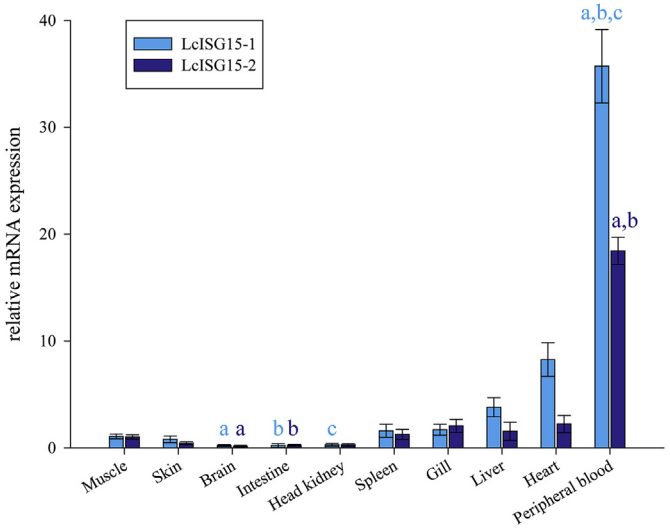

Fig. 5. Tissue distribution analyses of LcISG15-1 and LcISG15-2 in ten organs of healthy *Larimichthys crocea*. Three individuals were used for replication. Error bars show the standard deviation (SD). Data shown with different letters indicate significant expression levels at $P < 0.05$.

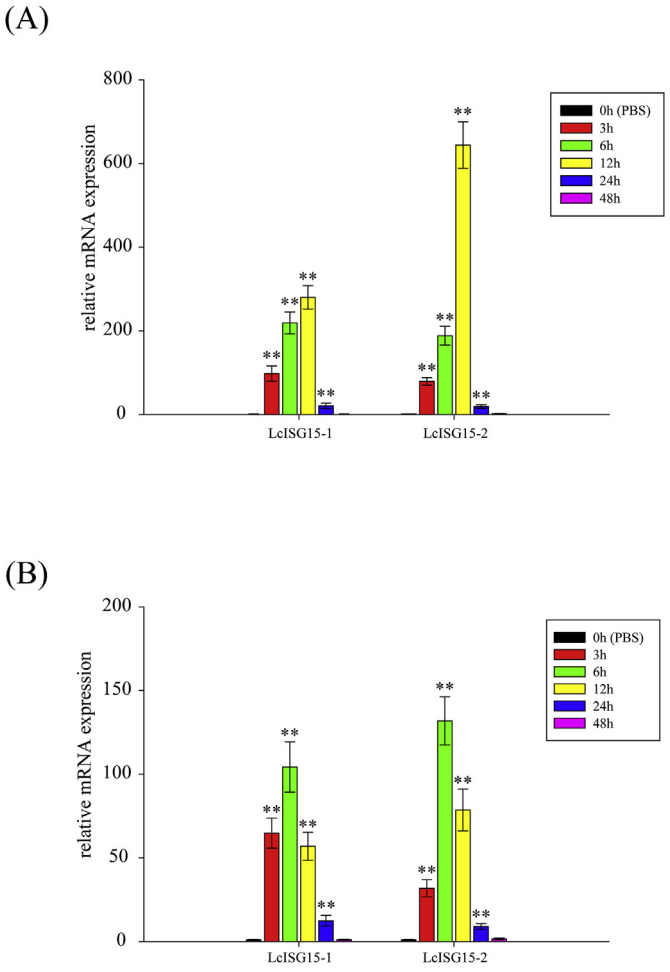

Fig. 6. Expression analyses of the LcISG15-1 and LcISG15-2 in organs post poly (I:C) challenge. (A) Expression analysis of the LcISG15-1 and LcISG15-2 in liver of *Larimichthys crocea* post poly (I:C) challenge. (B) Expression analysis of the LcISG15-1 and LcISG15-2 in spleen of *Larimichthys crocea* post poly (I:C) challenge. Three individuals were used for replication. Error bars show the standard deviation (SD). '**' and '*' indicate statistical significance of $P < 0.01$ and $P < 0.05$, respectively.

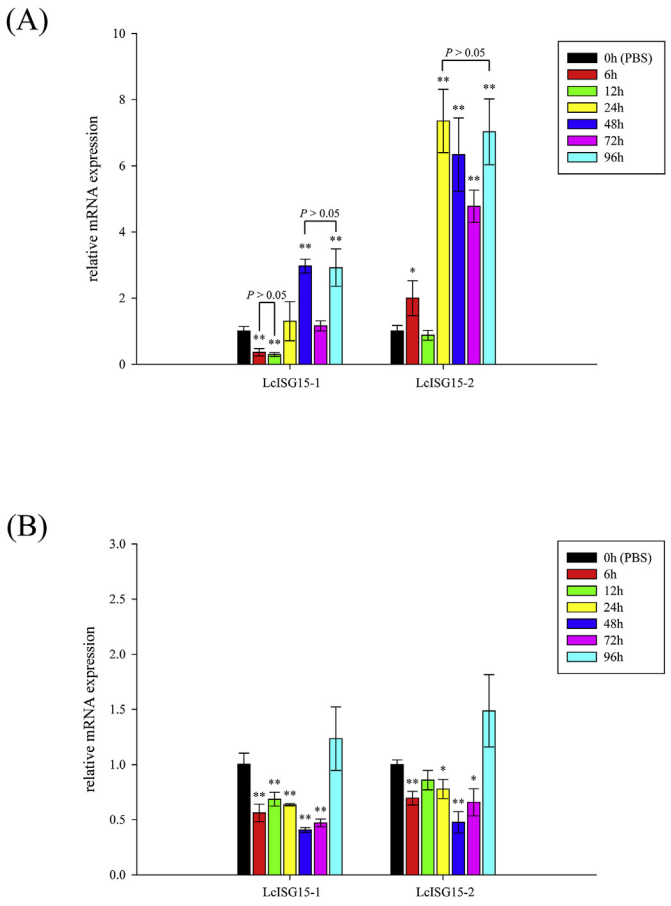

Fig. 7. Expression analyses of the LcISG15-1 and LcISG15-2 in organs post *Vibrio parahemolyticus* infection. (A) Expression analysis of the LcISG15-1 and LcISG15-2 in liver of *Larimichthys crocea* post *Vibrio parahemolyticus* infection. (B) Expression analysis of the LcISG15-1 and LcISG15-2 in spleen of *Larimichthys crocea* post *Vibrio parahemolyticus* infection. Three individuals were used for replication. Error bars show the standard deviation (SD). '**' and '*' indicate statistical significance of $P < 0.01$ and $P < 0.05$, respectively.

pathway [1], were both significantly upregulated in liver and spleen after stimulation by poly (I:C) and *V. parahemolyticus* (Fig. 8). These results indicated that the poly (I:C) and *V. parahemolyticus* stimulation both induced the production of type I IFNs and activated the type I interferon-mediated signaling.

4. Discussion

The interferon-stimulated gene ISG15, which is the first identified ubiquitin-like protein [26], plays an important role in the early innate immune response against viral [33,37,38,42–47] and bacterial [34,36,40,43] infections in teleosts. ISG15 homologues have been identified in a large number of fish species [33–47], however, most of the fishes are reported to contain only one copy of ISG15 gene [38–47]. By far, to our knowledge, two and three copies of ISG15 genes have been found in crucian carp [33] and Atlantic cod [35], respectively. In this study, based on a genomic blast search analyses of the ISG15 gene through the published draft genome sequence [58], we described molecular characterization and expression analyses of two homologues of ISG15, termed as LcISG15-1 and LcISG15-2, in large yellow croaker. Our results of sequence analyses showed that the LcISG15-1 and LcISG15-2 were both highly similar to teleost ISG15 homologues. The LcISG15-1 and LcISG15-2 shared 86.2% and 72.8% sequence identity with ISG15 of *S. ocellatus* (Table S3), respectively. The genomic structure analyses showed that, identical to ISG15 of zebrafish (Fig. 2A) and

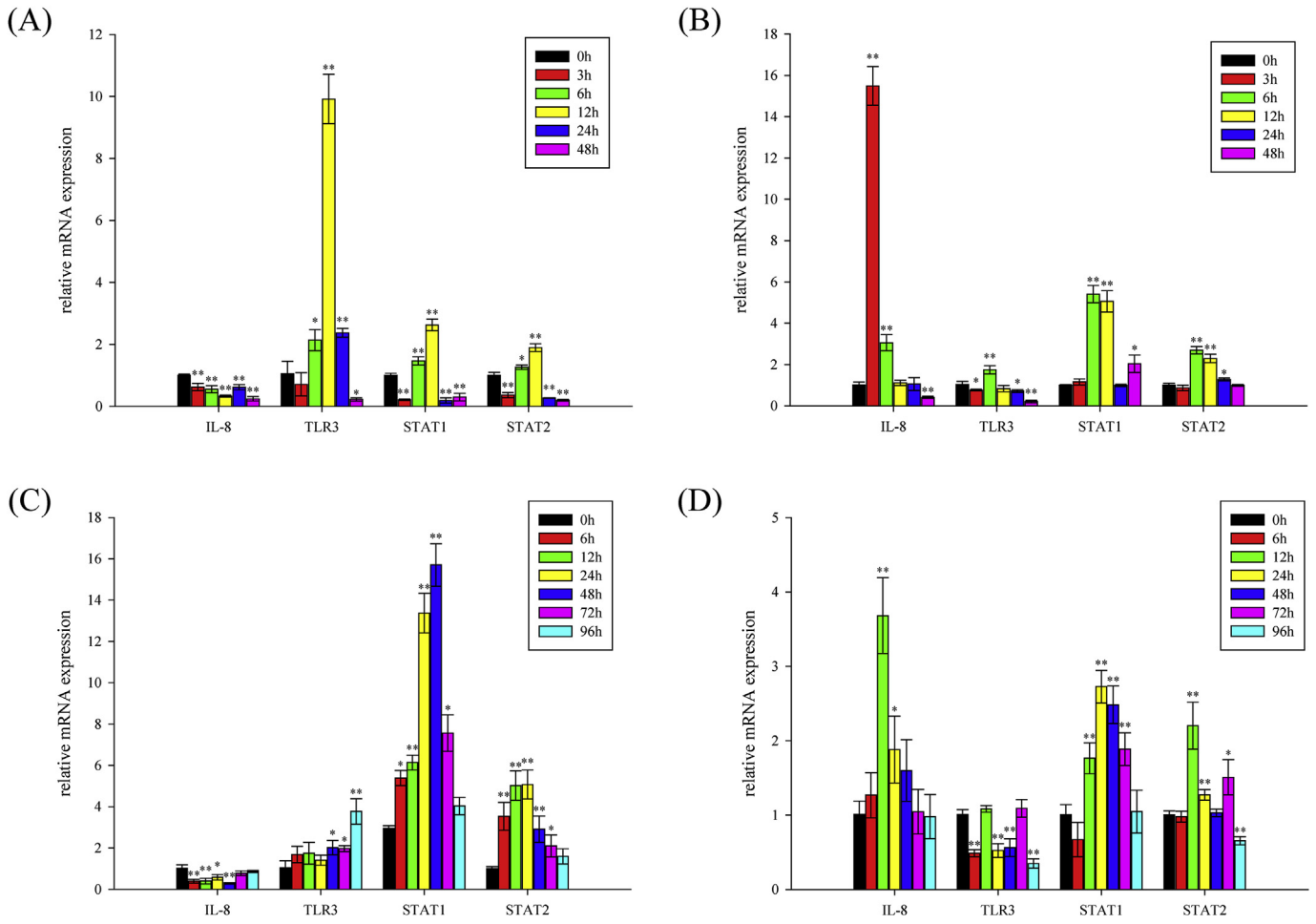

Fig. 8. Expression analyses of other immune-relevant genes in organs post stimulation. Expression analysis of other immune-relevant genes in (A) spleen and (B) liver of *Larimichthys crocea* post poly (I:C) stimulation. Expression analysis of other immune-relevant genes in (C) spleen and (D) liver of *Larimichthys crocea* post *Vibrio parahemolyticus* infection. Three individuals were used for replication. Error bars show the standard deviation (SD). '**' and '*' indicate statistical significance of $P < 0.01$ and $P < 0.05$, respectively.

other teleost fishes [42], the LcISG15-1 and LcISG15-2 both contain two exons, with the coding sequences located in exon 2 (Fig. 2A). Besides, our results of sequence analyses also showed that conserved domains reported in teleost fishes [38,42,43,47] and mammals [18,68,91] were all presented in the LcISG15-1 and LcISG15-2: two tandem ubiquitin-like domains, a short linker and a highly conserved C-terminal LRGG motif (Fig. 1 and Fig. S1). Our phylogenetic reconstruction analyses revealed a tree topology in which the LcISG15-1 and LcISG15-2 were grouped with ISG15 genes of other fishes from the clade Teleostei (Fig. 4 and Fig. S3), indicating the LcISG15-1 and LcISG15-2 were homology to fish ISG15. In addition, our results showed that an IFN-stimulatory response element (ISRE), which were well conserved among ISG15 of teleost fishes and mammals [42], was also found in the promoter regions of both LcISG15-1 and LcISG15-2 (Fig. 3). Taken together, our results showed that the LcISG15-1 and LcISG15-2 were actually homology to teleostean and mammalian ISG15 genes.

Our results of phylogenetic reconstruction analyses showed that all ISG15-1 (including the *S. ocellatus* ISG15) and all ISG15-2 sequences of sciaenid fishes grouped separately to form the ISG15-1 clade and the ISG15-2 clade, before clustered together to form the Sciaenidae clade (Fig. 4 and Fig. S3). These results indicated that a gene duplication event of ISG15 may have occurred in the ancestor of the Sciaenidae before the diversification of sciaenid fishes. Our results of molecular evolutionary analyses showed that the ISG15-2, but not the ISG15-1 gene copy, has undergone positive selection in sciaenid fishes after ISG15 gene duplication in the family of Sciaenidae. A total of five positively selected sites (81F, 86N, 88L, 131L and 150G, amino acid positions referred to the LcISG15-2) were detected on the ancestral branch of sciaenid ISG15-2 clade (Table S4). Thereinto, the Asparagine (N) at position 86 (referred to Arg87 of human ISG15) and Leucine (L) at position 131 (referred to Glu132 of human ISG15) were both predicted to be involved in interactions between ISG15 and the E1-activating enzyme UbE1L [91]. Thus, these two positively selected amino acid sites might assign some significance to the process of ISGylation in sciaenid ISG15-2 proteins. Further studies are needed to determine how these amino acid residues affect the protein function of ISG15-2 in sciaenid fishes.

Our results of Real-time PCR showed that the LcISG15-1 and LcISG15-2 were both ubiquitously expressed in all ten examined organs, with predominant expressions both in the peripheral blood, followed by heart and liver (Fig. 5). Similarly, the ISG15 gene in other teleost fishes, including *S. schlegeli* [39], *S. ocellatus* [40], *O. fasciatus* [41], *P. olivaceus* [42], *C. semilaevis* [43], *E. coioides* [45], *S. maximus* [46] and *S. senegalensis* [49], were all found to be constitutively expressed in all tested organs, with predominant expressions detected in peripheral blood [40], peripheral blood leucocytes (PBLs) [39,41,42], heart [43,49], gills [41,42], spleen [39,41,42,45], head kidney [45,46] and liver [40,49]. The high expression levels of the LcISG15-1 and LcISG15-2 in peripheral blood (a leucocytes-rich tissue) (Fig. 5) indicated that both the LcISG15-1 and LcISG15-2 might be released from leucocytes as cytokines and may both play roles in leucocyte proliferation. Because it has been reported that the human ISG15 is

observed to be secreted from human PBLs and is involved in the stimulation of lymphocyte proliferation [92]. Besides, our results showed that the expressions of the LcISG15-1 in peripheral blood, heart and liver were all stronger than those of LcISG15-2 (Fig. 5), suggesting a relatively more substantial role of the LcISG15-1 in leucocytes-rich organs without immune stimulation.

Our results showed that expression levels of the LcISG15-1 and LcISG15-2 were both significantly changed after stimulation by poly (I:C) (Fig. 6). The expression levels of the LcISG15-1 and ISG15-2 were both quickly upregulated after poly (I:C) stimulation, with the peaks both appeared at 12 h in liver and at 6 h in spleen (Fig. 6). These results indicated that the spleen of large yellow croaker seems to respond to poly (I:C) stimulation much quicker than the liver in the genes of LcISG15-1 and LcISG15-2. The results from our recently published work revealed a similar expression pattern for another interferon-stimulated gene LcViperin, that the inductions of LcViperin were reached the maximum both at 6 h in spleen and at 12 h in liver [57]. Our previous study focused on genes involved in RIG-I-like receptor signaling pathway of large yellow croaker showed that expression levels of the LcMDA5 and LcMAVS were also reached the maximum both at 6 h in spleen but at 12 h in liver [79]. These results might reflect the fact that the regulation of ISG15 expression is mediated by the RIG-I-like receptor signaling pathway and the TLR3-mediated signaling pathway. The overexpression of Japanese flounder MDA5 in HINAE cells could significantly induce the expression of ISG15 [93]. The overexpression of MAVS in rock bream heart cells could promoter the expression of ISG15 [77]. Besides, the overexpression of Japanese flounder TLR3 in YO-K cells also significantly induced ISG15 expression [94]. The expression levels of the LcISG15-1 and ISG15-2 were also both significantly upregulated in liver after *V. parahemolyticus* challenge, though in a relatively mild degree, with the peaks appeared at 48 h (and 96 h) and at 24 h (and 96 h) in liver, respectively (Fig. 7A). However, the expression levels of the LcISG15-1 and ISG15-2 were both not significantly changed in spleen after pathogen *V. parahemolyticus* infection (Fig. 7B). Different patterns of ISG15 homologues expression induced by poly (I:C) and *V. parahemolyticus* in large yellow croaker might be due to different activation mechanisms effected by these two stimuli. It is suggested that the infection of bacterial (especially gram-negative bacteria) could activate the ISGylation of ISG15 via the TLR4 signaling pathway which recognizes the LPS on the bacterial cell surface [32]. Unlike the fast response of fish immune system to poly (I:C), the *in vivo* bacterial infection via intraperitoneal injection may undergo a complex process such as adherence, invading and recognition by PPRs [95–97], before activating innate antibacterial responses and inducing immune-relevant genes. For example, the *S. ocellatus* ISG15 was significantly induced in kidney after bacterial (*Listonella anguillarum* and *Streptococcus iniae*) and poly (I:C) challenge, with the highest expression levels of ISG15 appeared in kidney at 48, 72 and 4 h post *L. anguillarum*, *S. iniae* and poly (I:C) treatment, respectively [40].

Expression of teleostean ISG15 is known to be induced by pathogen infection. The *D. labrax* ISG15 was found to be significantly induced in kidney and brain after challenges with poly (I:C) and red-spotted grouper nervous necrosis virus (RGNNV) [47]. The *S. maximus* ISG15 could be significantly upregulated by poly (I:C) and virus infection, with the peaks appeared at 2 d in spleen and all at 1 d in head kidney, gills and muscle after poly (I:C) stimulation, but at 4 d and 7 d respectively in spleen and head kidney after turbot reddish body iridovirus (TRBIV) infection [46]. The study focused on *G. morhua*, a fish species which has been reported to possess three ISG15 homologues, showed that all three ISG15 gene copies (AcISG15-1, AcISG15-2 and AcISG15-3) were significantly induced in head kidney post poly (I:C) injection [35]. The expression levels of *P. olivaceus* ISG15 appeared to reach peaks at 6 h in head kidney and at 3 h in both PBLs and spleen after poly (I:C) stimulation [42]. The study focused on *C semilaevis* revealed that the ISG15 could be significantly upregulated in both kidney and spleen infected by either megalocytivirus or *V. anguillarum* [43]. These results suggested that the temporal expression pattern of teleostean ISG15 varied from organ to organ, differed among fish species and was affected by stimulus type.

In contrast to the induction of LcISG15-1 and LcISG15-2 by poly (I:C) in both liver and spleen, our results showed that the expression levels of the two ISG15 homologues in large yellow croaker were significantly upregulated only in liver but not in spleen after *V. parahemolyticus* infection (Fig. 7B). These results indicated that the LcISG15-1 and LcISG15-2 are involved in host immune defense against *V. parahemolyticus* infection mainly in liver rather than spleen. However, the study focused on *C semilaevis* [43] showed that the ISG15 could be significantly upregulated in spleen after challenge with *V. anguillarum*, a pathogen species also belongs to the genus *Vibrio* [98]. The *S. ocellatus* ISG15, which is homology to LcISG15-1 (Fig. 4 and Fig. S3), could not be significantly induced in liver after challenges with *L. anguillarum*, *S. iniae* and LPS [40]. These results indicated that the pattern of teleostean ISG15 expression induced by bacterial infection also varied among fish species and was affected by pathogen type. Besides, our results showed that the LcISG15-1 and LcISG15-2 exhibited a relatively much stronger induction upon poly (I:C) stimulation in liver than that in spleen, with the peak levels reached 280-fold and 644-fold respectively for LcISG15-1 and LcISG15-2 in liver and 103-fold and 132-fold respectively for LcISG15-1 and LcISG15-2 in spleen of the control one (Fig. 6). The highest expression levels of LcISG15-1 and LcISG15-2 in liver post stimulation indicated that the liver plays a pivotal role in innate immunity of large yellow croaker. The liver is considered as an important component of immune system in teleost fish [70,71]. It is well known that the liver is an organ with predominant innate immunity and contains a large population of innate immune cells, including macrophages, natural killer and natural killer T [69]. The ISG15 is observed to be released by human lymphocytes and monocytes and epithelial carcinoma cells [92,99], and could perform as a cytokine to induce natural killer proliferation and IFN-γ production by T lymphocytes [92,100,101]. The human ISG15 is synthesized as a 17-kDa precursor protein (pre-ISG15), that is rapidly converted to a 15-kDa mature protein by the removal of eight amino acids from the carboxy terminus of the precursor [21]. Like those of human, rodents and some fish species (such as *C. semilaevis* [43], *P. olivaceus* [42] and *S. senegalensis* [49]), the LcISG15-1 contains a C-terminal extension (^{156}WAHF^{159}) after the LRGG motif (Fig. 1). It has been thought that the cleavage of C-terminal extension is required for the formation of biologically active human ISG15, because only the mature ISG15 but not the pre-ISG15 has the capability of inducing lymphocyte proliferation [92]. However, results from the study focused on *C. semilaevis* showed that the recombinant *C. semilaevis* ISG15 (CsISG15) containing seven-residue sequence of C-terminal extension (Fig. S1) still could activate head kidney (HK) macrophages and enhance the expression of immune genes in HK lymphocytes [43]. Moreover, evidence from the study of *S. ocellatus* ISG15 showed that the recombinant *S. ocellatus* ISG15 (SoISG15) containing three-residue sequence of C-terminal extension (Fig. 1) could also activate HK macrophages and enhance its bactericidal activity [40]. Thus, it is highly likely that both the LcISG15-1 and LcISG15-2 could act as cytokine to activate and enhance the activity of innate immune cells (such as macrophages) in immune organs, especially in liver of large yellow croaker.

Our results showed that the LcISG15-2 exhibited a much stronger induction than LcISG15-1 upon stimulation by poly (I:C) in liver and spleen (Fig. 6) and by *V. parahemolyticus* in liver (Fig. 7A), suggesting an important role of LcISG15-2 in host innate immune response in large yellow croaker. Although the cleavage of C-terminal extension is not necessary for the extracellular cytokine activity of ISG15 in some fish species [40,43], the exposure of LRGG motif after processing of C-terminal extension is required for the intracellular conjugation of ISG15 to its target proteins (ISGylation) [21,102], a process which is induced in various viral/bacterial infections and in response to IFN treatment [103]. In contrast to the LcISG15-1, the LcISG15-2 does not contain C-

terminal extension after LRGG motif (Fig. 1). Without additional processing of C-terminal extension, the synthesized intracellular LcISG15-2 could be directly and rapidly involved in the ISGylation pathway to conjugate to target proteins, a process which has been reported to contribute largely to antiviral activity of ISG15 [30,104]. This possibility may explain why the ISG15-2, but not the ISG15-1 gene copy, has been targeted by positive selection in sciaenid fishes after ISG15 gene duplication in the family of Sciaenidae. And the positively selected amino acid sites, especially 86N and 131L which were predicted to be involved in ISGylation process, may further enhance the ISGylation and innate immune activity of ISG15-2 in sciaenid fishes.

5. Conclusion

In this study, we sequenced and characterized two homologues of ISG15 gene, termed as LcISG15-1 and LcISG15-2, in the large yellow croaker. Our results of sequence analyses showed that both the LcISG15-1 and LcISG15-2 were evolutionary conserved in protein sequences and functionally significant domains. Transcriptional analyses revealed ubiquitous expressions of both the LcISG15-1 and LcISG15-2 in ten examined organs, with predominant expressions both in peripheral blood. Expression analyses showed that both the LcISG15-1 and LcISG15-2 were rapidly and significantly upregulated *in vivo* after poly (I:C) challenge in liver and spleen. However, the LcISG15-1 and LcISG15-2 were both significantly induced after pathogen *Vibrio parahemolyticus* infection only in the liver but not in the spleen. Combined with the existence of a putative IFN-stimulatory response element (ISRE) in the promoter regions of both ISG15 homologues, our results indicated that the LcISG15-1 and LcISG15-2 are both likely to be involved in host immune defense against viral and bacterial infection.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.018.

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