Fish and Shellfish Immunology 84 (2019) 390–403

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Sequence analysis and characterization of type I interferon and type II interferon from the critically endangered sturgeon species, *A. dabryanus* and *A. sinensis*

Qiaoqing Xu[a,b,c,1], Kai Luo[a,e,*,1], Shuhuan Zhang[d], Weihua Gao[a,b,c], Wenbing Zhang[a,e], Qiwei Wei[d,**]

[a] *School of Animal Science, Yangtze University, Jingzhou, 434020, China*
[b] *Guangxi Key Laboratory of Marine Biotechnology, Guangxi Institute of Oceanology, Beihai, 536006, China*
[c] *State Key Laboratory of Developmental Biology of Freshwater Fish, Changsha, 410081, China*
[d] *Key Lab of Freshwater Biodiversity Conservation Ministry of Agriculture, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, 430223, China*
[e] *The Key Laboratory of Mariculture (Education Ministry of China), Ocean University of China, 5 Yushan Road, Qingdao, Shandong, 266003, PR China*

ARTICLE INFO

Keywords:
Interferon
Chinese sturgeon
Dabry's sturgeon
Antiviral and bacterial response

ABSTRACT

In the present study, we identify three type I interferon (IFN) genes (*Ad/As*IFNe1-3) and a type II IFN gene (*Ad/As*IFNγ) from the Dabry's sturgeon (*Acipenser dabryanus*) and the Chinese sturgeon (*Acipenser sinensis*). Sequence analysis revealed that *Ad/As*IFNe1–3 and *Ad/As*IFNγ contain several conserved characteristics, including signal peptides, interferon alpha, beta, and delta (IFabd) domains, and N-glycosylation sites. *Ad/As*IFNe1-3 belongs to the type I IFN group I subgroup, possessing two conserved cysteines residues (C1 and C3), and *Ad/As*IFNγ contained a conserved nuclear localization sequence (NLS) motif. *Ad/As*IFNe1-3 and *Ad/As*IFNγ contain signature motifs indicative of their corresponding IFN group. The *Ad/As*IFNe1-3 and *Ad/As*IFNγ genes were found to consist of 5 exons/4 introns and 4 exons/3 introns, respectively. These IFNs were separated by four phase 0 introns (type I IFN) and three phase 0 introns (type II IFN). The sequences of IFNe1-3 and IFNγ from the Dabry's sturgeon and the Chinese sturgeon were closely aligned, suggested that these two species are closely related. Phylogenetic analysis revealed that *Ad/As*IFNe1-3 and *Ad/As*IFNγ clustered together with the corresponding homologous proteins from other fish species. *Ad*IFNe1-3 were found to be high expressed in early embryonic development, suggesting that *Ad*IFNe1-3 might indicate maternal transmission, while *Ad*IFNγ may not mediate embryonic development. Tissue distribution analysis revealed that *Ad*IFNe1-3 and *Ad*IFNγ carry out biological functions in immune and non-immune tissues compartments. *Ad*IFNe1-3 and *Ad*IFNγ can be stimulated by polyinosinic-polycytidylic acid (poly I:C) and lipopolysaccharides (LPS). *Ad*IFNe1-3 have stronger antiviral activity than *Ad*IFNγ, and *Ad*IFNγ has a stronger antibacterial activity than *Ad*IFNe1-3. The differential responses of these genes to poly I:C and LPS suggest differences in the mechanisms of defense against viruses and bacteria.

1. Introduction

Interferons (IFNs) were first identified in 1957 [1,2]. The IFNs and their receptors are a subset of class 2 alpha-helical cytokines that existed in early chordates as early as 500 million years ago, and represent early elements to emerge in innate and adaptive immunity [3]. In mammals, three distinct IFN families are reported: type I IFN (IFNα, IFNβ, IFNε, IFNτ, IFNκ, IFNω, IFNδ, and IFNζ), type II IFN (IFNγ), and type III IFN (IFNλ1, IFNλ2, IFNλ3, and IFNλ3) [4–9]. On the other hand, only two distinct IFN families have been identified in teleost fish, including type I IFN and type II IFN [10,11]. However, fish IFNs share limited sequence homology with their counterparts in birds and mammals [12]. Mammalian type I and III IFNs (labeled “virus-induced IFNs”) are truly specialized as innate antiviral cytokines; for example,

---

[*] Corresponding author. School of Animal Science, Yangtze University, Jingzhou, 434020, China.
[**] Corresponding author. Key Laboratory of Freshwater Biodiversity Conservation, Ministry of Agriculture of China, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences Wuhan 430223, China.
E-mail addresses: luokai@stu.ouc.edu.cn (K. Luo), weiqw@yfi.ac.cn (Q. Wei).
[1] The two authors contributed equally to this work.

https://doi.org/10.1016/j.fsi.2018.10.038
Received 23 August 2018; Received in revised form 7 October 2018; Accepted 14 October 2018

Table 1
The primers used in this study.

Name	Sequence(5′-3′)	Usage
IFNe1-5R1	AGTCTGTCTGTAGAGGTTGTT	5′-RACE
IFNe1-5R2	ATCCTTCGAGACACAAGAC	5′-RACE
IFNe1-3F1	CTCTGCTCGTGCCTGGGAGGTTAT	3′-RACE
IFNe1-3F2	GGAAAGGATGGATCGGTTCACTGC	3′-RACE
IFNe2-5R1	AGAACCTCCAACTCCT	5′-RACE
IFNe2-5R2	CGGCTCACATCTTTAT	5′-RACE
IFNe2-5R3	GGCATCCCAGAGAAAAGT	5′-RACE
IFNe2-3F1	CCAACCTTCACCGCACGACTTCTG	3′-RACE
IFNe2-3F2	AGATTACCTATTCTGACAGCACGGGG	3′-RACE
IFNe3-5R1	GTGCTGAATCCACTTG	5′-RACE
IFNe3-5R2	CATCCCAGAGAAAAAC	5′-RACE
IFNe3-5R3	GCTCACGGCTACCATCAC	5′-RACE
UPM (Long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE PCR
NUP(Short)	CTAATACGACTCACTATAGGGC	RACE PCR
IFNe1-tF	AACAGCTTACCGCTCACA	ORF cloning
IFNe1-tR	GACTTGATAAGTCTCTCC	ORF cloning
IFNe2-tF	CATTCTCATACTGTAGCTTGTC	ORF cloning
IFNe2-tR	TTCCATACTTCGGTTCTGAC	ORF cloning
IFNe3-tF	AAATGGATCTGAGGAGCTTATGG	ORF cloning
IFNe3-tR	CGTGTGAAGTGTTCCTTGGT	ORF cloning
IFNγ-tF	ATGAATTCACTCCAGAGTTTGT	ORF cloning
IFNγ-tR	TTATGGTGAGTTTACTCGTCT	ORF cloning
IFNγ-1-F	GGATGCGTCTATTCCAGTGAG	cloning the intron 1 of IFNγ
IFNγ-1-R	ACCTCCACCAGCCAAGATG	cloning the intron 1 of IFNγ
IFNγ-2-F	CATCTTGGCTGGTGGAGGT	cloning the intron 2 of IFNγ
IFNγ-2-R	AGACATGCTGGGTGCTGTT	cloning the intron 2 of IFNγ
IFNγ-3-F	AGCACCCAGCATGTCTACAG	cloning the intron 3 of IFNγ
IFNγ-3-R	TTACTCGTCTCCTCCTTCTCTTC	cloning the intron 3 of IFNγ
IFNe1-1-F	GCCGATCTATATTCGCATTCTG	cloning the intron 1 of IFNe1
IFNe1-1-R	GCATGTGTGCTCTTTCTTTAAC	cloning the intron 1 of IFNe1
IFNe1-2-F	GTTAAAGAAAGAGCACACATGC	cloning the intron 2 of IFNe1
IFNe1-2-R	TTAGTCTGTCTGTAGAGGTTGT	cloning the intron 2 of IFNe1
IFNe1-3-F	GTGTTTGAGGTTTATGAAGC	cloning the intron 3 of IFNe1
IFNe1-3-R	GACTCCAATGTCTTGATTTC	cloning the intron 3 of IFNe1
IFNe1-4-F	GAAATCAAGACATTGGAGTC	cloning the intron 4 of IFNe1
IFNe1-4-R	GTGTGTTCTAATATGAGCAG	cloning the intron 4 of IFNe1
IFNe2-1-F	TGAGATGCTGCTTTGTACTGTTC	cloning the intron 1 of IFNe2
IFNe2-1-R	CTGTGAATGTTTGGCGTTTGAG	cloning the intron 1 of IFNe2
IFNe2-2-F	GGTGGAGAGTTTGTGAGAGAG	cloning the intron 2 of IFNe2
IFNe2-2-R	CAGAAGTCGTGCGGTGAAG	cloning the intron 2 of IFNe2
IFNe2-3-F	AACCTTCACCGCACGACTT	cloning the intron 3 of IFNe2
IFNe2-3-R	CGCTTCAGACTCCTGTTCTCT	cloning the intron 3 of IFNe2
IFNe2-4-F	ATGGGAAAGAGAACAGGAGTCT	cloning the intron 4 of IFNe2
IFNe2-4-R	AGGCATTCGCGCTGTAATC	cloning the intron 4 of IFNe2
IFNe3-1-F	GTAGCCGTGAGCCAGAGTT	cloning the intron 1 of IFNe3
IFNe3-1-R	GATGTTCTCCACTTGTTCTTCGT	cloning the intron 1 of IFNe3
IFNe3-2-F	ACGAAGAACAAGTGGAGAACATC	cloning the intron 2 of IFNe3
IFNe3-2-R	GAGCAGTCTGAGTATGGAGGTT	cloning the intron 2 of IFNe3
IFNe3-3-F	TGTGAAGTGGGACCGAGAAA	cloning the intron 3 of IFNe3
IFNe3-3-R	GCTGTGGATCACCTCCTTATC	cloning the intron 3 of IFNe3
IFNe3-4-F	AGGTGATCCACAGCTCAGG	cloning the intron 4 of IFNe3
IFNe3-4-R	TGAAGTGTTCCTTGGTCTCCT	cloning the intron 4 of IFNe3
*Ad*IFNγ-F	GGCTGGTGGAGGTCCATTGTT	Quantitative Real-time PCR
*Ad*IFNγ-R	ACGCTGATCTTCAGTGGTGTCT	Quantitative Real-time PCR
*Ad*IFNe1-F	GCTGCTGCTCATCGCTAGGAT	Quantitative Real-time PCR
*Ad*IFNe1-R	TGTTCCATGCTGTCACCGAGTC	Quantitative Real-time PCR
*Ad*IFNe2-F	AACCTTCACCGCACGACTTCT	Quantitative Real-time PCR
*Ad*IFNe2-R	TGACGCTTCAGACTCCTGTTCT	Quantitative Real-time PCR
*Ad*IFNe3-F	GGTAGCCGTGAGCCAGAGTT	Quantitative Real-time PCR
*Ad*IFNe3-R	GGTAGCAGTTGCAGGACTCAGT	Quantitative Real-time PCR
β-actin-F	CCTTCTTGGGTATGGAATCTTGC	Quantitative Real-time PCR
β-actin-R	CAGAGTATTTACGCTCAGGTGGG	Quantitative Real-time PCR

IFNγ, a regulatory cytokine of innate and adaptive immunity, is mostly active against intracellular bacteria [11,13–19]. Pathogen associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) to induce IFN production; IFN is then recognized by the cognate receptor and associated signaling molecules to trigger antiviral responses and expression of IFN-stimulated genes (ISGs), which can inhibit viral replication in the host [20–25]. The IFN pathway is also involved in many diseases [26–30].

As in mammals, the type I IFN of teleost fish possesses two or four cysteine-containing subgroups, termed group I and group II IFNs, which can be further divided into seven groups based on the conserved cysteines [12]. Group I IFNs include IFNa, IFNd, IFNe, and IFNh which

Table 2
Summary of sequences of IFNe1, IFNe2, IFNe3 and IFNγ in Dabry's sturgeon and Chinese sturgeon.

Sequence features	*Ad*IFNe1	*As*IFNe1	*Ad*IFNe2	*As*IFNe2	*Ad*IFNe3	*As*IFNe3	*Ad*IFNγ	*As*IFNγ
GenBank Accession NO.	MH645438	MH645441	MH645439	MH645442	MH645440	MH645443	MH645436	MH645437
ORF (bp)	579	579	564	564	573	573	528	528
Length of amino acids (aa)	192	192	187	187	190	190	175	175
Molecular weight (kDa)	22.89	22.9	21.95	22.14	22.49	22.56	20.24	20.24
Theoretical pI	9.26	9.34	9.51	9.61	9.54	9.63	9.56	9.56
Signal peptide (aa)	1–21	1–21	1–22	1–22	1–17	1–17	1–23	1–23
IFabd domain (aa)	61–172	61–172	54–171	54–171	48–171	58–171	No	No
N-glycosylation site	85, 101, 155	85, 101, 155, 189	136	136	74, 186	74, 112, 186	99	99
Numbers of α-helix	6	6	6	6	5	5	6	6
Sinature motif (aa)	143–161	143–161	142–160	142–160	142–160	142–160	139–150 158–172	139–150 158–172
NLS	No	No	No	No	No	No		

each contain two conserved cysteines, and group II IFNs consist of IFNb, IFNc, and IFNf which contain four conserved cysteines [31–33]. Salmonids possess six subgroups (IFNa-f), while other teleost species have one or more groups, but no more than six [31]. Cyprinid fish possess IFNa, c, and d subgroups [34]. The numbers of IFN subgroups vary among species, suggesting that teleost IFN systems were more complex than previously thought. The same IFN subgroups have distinct antiviral activities. IFNa1 is effective in enhancing cell protection against Salmonid Alphaviruses (SAV) and Infectious Pancreatic Necrosis Virus (IPNV) but is relatively ineffective against Infectious Salmon Anaemia Virus (ISAV) [35–37]. IFNa2 of goldfish is an intracellular antiviral cytokine against Grass Carp Reovirus (GCRV) and Spring Viremia of Carp Virus (SVCV) [38]. Fish type I IFNs may also play a role in the regulation of inflammation and may have antibacterial effects [39]. Turbot IFN2 was up-regulated during infection with VHSV and with the bacterial pathogen *Aeromonas salmonicida* [40].

The first fish IFNγ gene was identified by gene synteny analysis of the fugu genome [41]. Type II IFN exerts regulatory functions in both innate and adaptive immunity, including activating macrophages, enhancing antigen presentation, and promoting the Th1 T cell response [42–44]. IFNγ also has antiviral and antibacterial effects. In trout RTS-11 cells, IFNγ weakly induces Mx expression [45], and in salmon cells treated with IFNγ, IPNV replication is inhibited [36]. Interestingly, a teleost-specific IFNγ was identified, IFNγ-rel, which does not exist among the various mammalian IFNγ varieties [46]. IFNγ-rel is thought to be involved in immune defense against bacterial infection [47].

The octaploid Dabry's sturgeon (*Acipenser dabryanus*, *Ad*), which is mainly distributed in the mainstream of the upper Yangtze River and its tributaries, is a critically endangered species and was listed as a First Class Protected Animal by the Chinese Government in 1989 [48]. Many viruses that threaten sturgeons have been identified, including white sturgeon (*Acipenser transmontanus*) iridovirus (WSIV), white sturgeon herpesvirus-1,2 (WSHV-1,2), and shovelnose sturgeon (*Scaphirhynchus platorynchus*) iridovirus (SSIV) [49]. However, no systematic studies have detailed IFNs or IFN responses in sturgeons. Better understanding of sturgeon IFNs is urgently required, and will facilitate efforts to protect sturgeon populations from viral and bacterial infection. In the present study, we evaluated the molecular characterization and expression of IFNs from Dabry's sturgeon and Chinese sturgeon (*Acipenser sinensis*, *As*); namely, IFNe1 (*Ad*IFNe1 and *As*IFNe1), IFNe2 (*Ad*IFNe2 and *As*IFNe2), IFNe3 (*Ad*IFNe3 and *As*IFNe3), and IFNγ (*Ad*IFNγ and *As*IFNγ). This study will provide further insights into the nature and expression of sturgeon IFNs.

2. Materials and methods

2.1. Fish

Dabry's sturgeon (250–300 g) and Chinese sturgeon (250–300 g) were supplied by the Yangtze River Fisheries Research Institute (Jingzhou, Tai Lake, China). The fish were the offspring of artificially propagated sturgeons and were maintained in a flow-through water supply kept at 24 ± 1 °C for 7 days. No clinical symptoms of disease were observed during this period. Healthy fish were used for the experiments. The Chinese sturgeon samples were used for cloning the corresponding *As*IFNe1-3 and *As*IFNγ.

2.2. Preparation of embryonic cDNA and tissue cDNA samples

Dabry's sturgeon embryos were collected at Yangtze River Fisheries Research Institute (Jingzhou, Tai Lake, China). Three embryos were collected and pooled at 0, 4, 14, 28, 40, 58, 84, and 107 h post fertilization (hpf) in RNA save reagent (Biological Industries). Four healthy fish were euthanized and eleven tissues (gill, muscle, liver, spleen, head kidney, caudal kidney, intestine, heart, brain, skin, and blood) were collected and homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA).

Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Fermentas). The cDNA was synthesized using Oligo $(dT)_{18}$ primers using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), according to the manufacturer's instruction, as described previously [50,51]. The synthesized cDNA was diluted with TE buffer and kept at −80 °C before quantitative real-time PCR (qPCR).

2.3. Gene sequencing and primer design

The partial cDNA sequences of *Ad/As*IFNe1-3 (*Ad*IFNe1-3 and *As*IFNe1-3) and full-length cDNA of *Ad/As*IFNγ (*Ad*IFNγ and *As*IFNγ) were obtained by RNA-Sequencing analysis (Sangon Biotech Co., Ltd, Shanghai, China) of spleen tissues (SRA accession: SRP152585) [52]. Sequences were searched using a query set of available sequences in the NCBI non-redundant (NR) database (http://ncbi.nlm.nih.gov/). To obtain the full-length cDNA of *Ad*IFNe1-3, 3‘-RACE (rapid-amplification of cDNA ends) and 5‘-RACE were performed using the SMART RACE cDNA Amplification Kit (TaKaRa, Japan) with specific primers (Table 1). All PCR products were ligated into pMD18-T vectors (TaKaRa, Japan) and sequenced. The sequences were further assembled with SeqMan software to obtain the full-length cDNA sequence, and ORF (Open Reading Frame) cloning was performed to verify the full ORF before additional analysis. The ORF cloning primers were also used to clone the full-length cDNA of *As*IFNe1-3 and *As*IFNγ because of the high sequence similarity between these two species (data not published).

The exon and intron structures of IFNe1-3 and IFNγ were amplified by PCR, and amplification products were ligated into pMD18-T vectors (TaKaRa) and sequenced. The sequences were then assembled using SeqMan software.

All primers were designed using Primer Premier 5.0 (Table 1) according to the obtained sequences. The primers used for qPCR were pretested to ensure that each primer pair could amplify the cDNA.

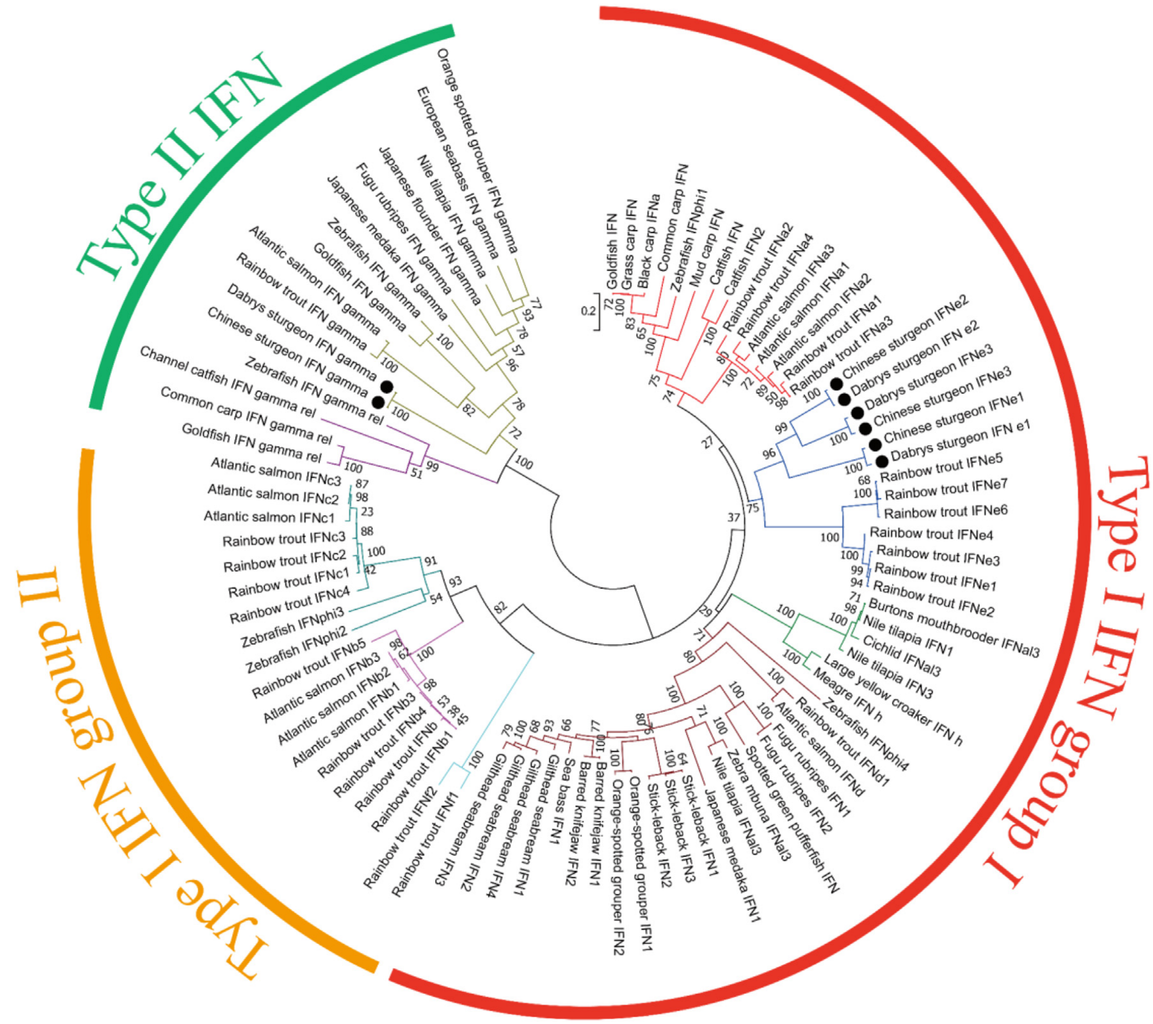

Fig. 1. Neighbor-joining phylogenetic tree of IFNe1, IFNe2, IFNe3 and IFNγ in Dabry's sturgeon and Chinese sturgeon. The tree was constructed using deduced amino acid sequence multiple alignment and the neighbor-joining method within the MEGA 7.0 program The percentage of tree in which the associated taxa clustered together is shown next to the branches based on 1000 bootstrap replications. Accession numbers for sequences used are as follows: Japanese flounder IFNγ, BAG50576.1; European seabass IFNγ, AIK66530.1; Orange spotted grouper IFNγ, AFM31242.1; Nile tilapia IFNγ, NP_001274331.1; Japanese medaka IFNγ, XP_004087027.2; Fugu rubripes IFNγ, CAE82301.2; Rainbow trout IFNγ, NP_001118092.1; Atlantic salmon IFNγ, NP_001165275.1; Goldfish IFNγ, EU909368; Zebrafish IFNγ, NM_212864; Channel catfish IFNγ-rel, NP_001187146.1; Grass carp IFNγ-rel, ACN56578.1; Goldfish IFNγ-rel, ACV41807.1; Zebrafish IFNγ-rel, NP_001018629.1; Common carp IFNγ-rel, CAJ98867.1; Large yellow croaker IFNh, API68650; Meagre IFNh, MG489874; Goldfish IFN, AAR20886; Mud carp IFN, AAY56128; Grass carp IFN, ABC87312; Common carp IFN, ADI81047; Zebrafish IFNphi1, AAM95448; Zebrafish IFNphi2, NP_001104552; Zebrafish IFNphi3, NP_001104553; Sea bass IFN1, CAQ17043; Orange-spotted grouper IFN1, AGL21770; Orange-spotted grouper IFN2, AGJ98284; Stick-leback IFN1, CAM31706; Stick-leback IFN2, CAM31707; Stick-leback IFN3, CAM31708; Burton's mouth brooder IFNal3, XP-005950669; Catfish IFN, AAV97701; Catfish IFN2, AAV97699; Zebra mbuna IFNal3, XP_004556871; Black carp IFNa, AKM15287; Rainbow trout IFNa1, CAM28541; Rainbow trout IFNa2, NP_001153977; Rainbow trout IFNa3, CCV17397; Rainbow trout IFNa4, CCV17398; Rainbow trout IFNb1, NP_001153974; Rainbow trout IFNb2, NP_001158515; Rainbow trout IFNb3, CCV17399; Rainbow trout IFNb4, CCV17400; Rainbow trout IFNb5, CCV17401; Rainbow trout IFNc1, CCV17402; Rainbow trout IFNc2, CCV17403; Rainbow trout IFNc3, CCV17404; Rainbow trout IFNc4, CCV17405; Rainbow trout IFNd1, CAV07949; Rainbow trout IFNe1, CCV17406; Rainbow trout IFNe2, CCV17407; Rainbow trout IFNe3, CCV17408; Rainbow trout IFNe4, CCV17409; Rainbow trout IFNe5, CCV17410; Rainbow trout IFNe6, CCV17411; Rainbow trout IFNe7, CCV17412; Rainbow trout IFNf1, CCV17413; Rainbow trout IFNf2, CCV17414; Barred knifejaw IFN1, AFP94213; Barred knifejaw IFN2, AFP94214; Nile tilapia IFNω1, XP_005950669; Nile tilapia IFNω3, XP_005469255; Nile tilapia IFNal3, XP_003453450; Japanese medaka IFN1, BAU25609; Cichlid IFNal3, XP_013771349; Atlantic salmon IFNa1, ABD39320; Atlantic salmon IFNa2, ABD39321; Atlantic salmon IFNa3, ACE75687; Atlantic salmon IFNb1, ACE75691; Atlantic salmon IFNb2, ACE75693; Atlantic salmon IFNb3, ACE75689; Atlantic salmon IFNc1, ACE75692; Atlantic salmon IFNc2, XP_014048249; Atlantic salmon IFNc3, ACE75688; Atlantic salmon IFNd, DAA64377; Gilthead seabream IFN1, CAT03221; Gilthead seabream IFN2, CAT03222; Gilthead seabream IFN3, CAT03223; Gilthead seabream IFN4, CAT03224; Fugu rubripes IFN1, CAM82750; Fugu rubripes IFN2, CAM82751; Spotted green pufferfish IFN, CAD67779. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.4. Phylogenetic and sequence analysis

The deduced amino acid sequences were predicted using the web-based tool ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). A phylogenetic tree was constructed based on the deduced amino acid multiple alignment with the Neighbor-Joining method using the MEGA software package (version 7.0). A bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed, and the bootstrap values of the major branches are shown as percentages.

The putative amino acid similarity and identity were calculated using MatGAT 2.01 software [53]. The domains were predicted with SMART (http://smart.embl.de/). The signal peptides were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/

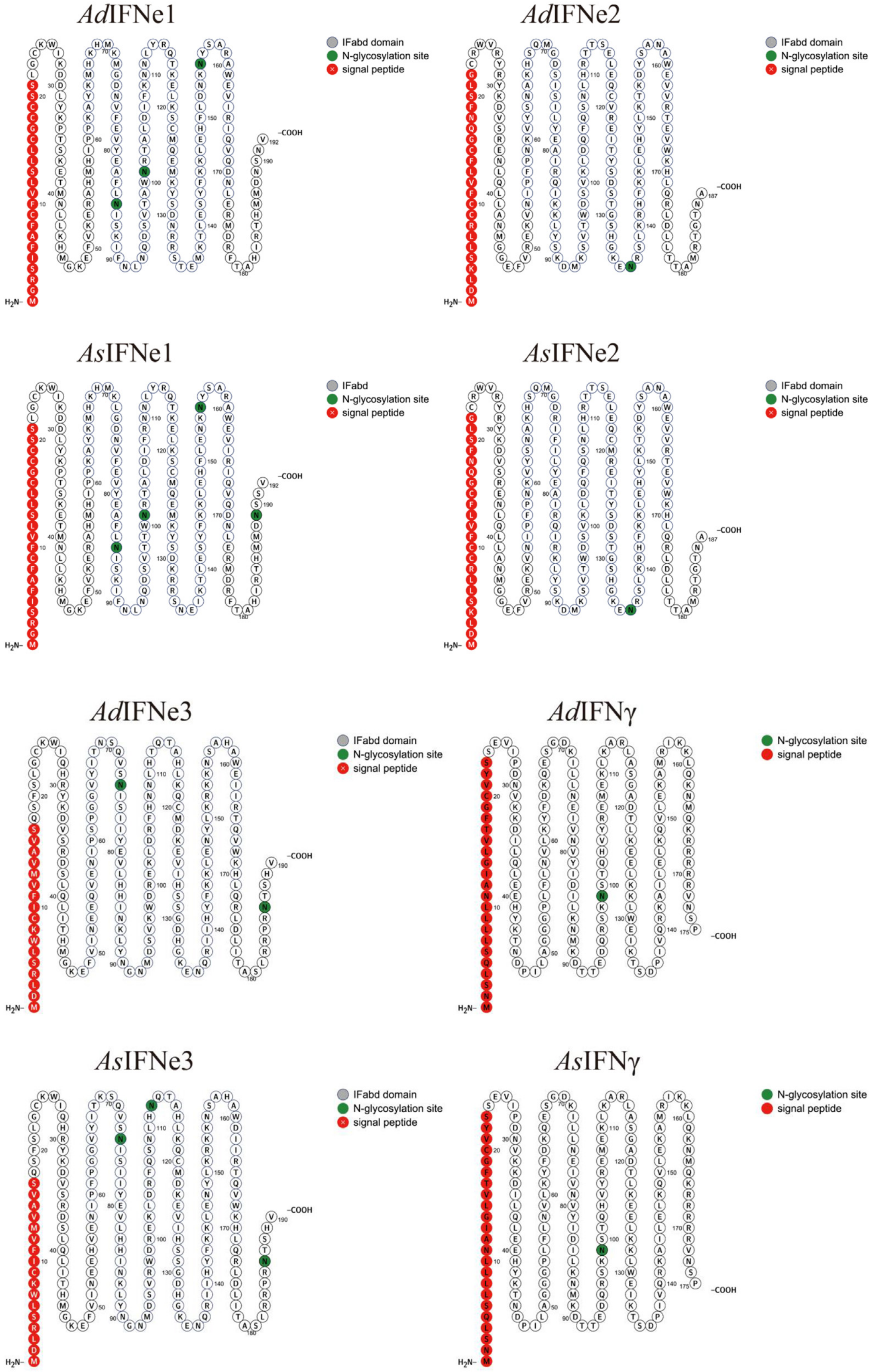

Fig. 2. Deduced amino acid (aa) sequences of IFNe1, IFNe2, IFNe3 and IFNγ in Dabry's sturgeon and Chinese sturgeon. The putative amino acid sequences were shown in the cycle. The signal peptide, N-glycosylation site and IFabd domain were marked with white character and red back, bottle green back, and bottle green cycle, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

). The properties of the proteins were determined using various software programs: computed theoretical isoelectric point (pI) and molecular weight (Mw) tool (https://web.expasy.org/compute_pi/); ExPASy Prosite (https://prosite.expasy.org/) for identifying N-glycosylation sites, and nuclear localization sequence (NLS). The amino acid secondary structures were predicted using PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/). Multiple alignments of amino acids were carried out using Clustal Omega

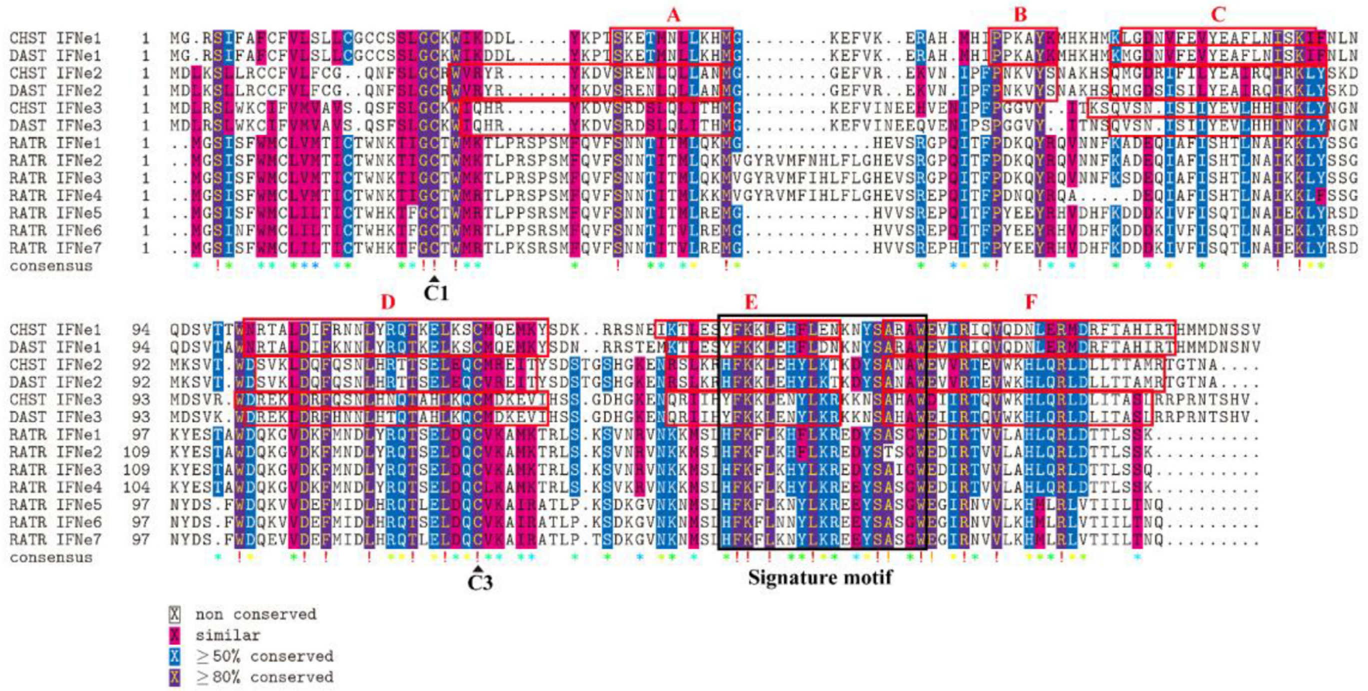

Fig. 3. Multiple alignment of IFNe1, IFNe2 and IFNe3 from Dabry's sturgeon and Chinese sturgeon with that of other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter and that is a sequences positions in which all sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The A-F alpha-helix were marked with red frame. Signature motif and conserved cysteine residues were marked with black frame and upward triangle, respectively. The abbreviations were Chinese sturgeon (CHST), Dabry's sturgeon (DAST), Rainbow trout (RATR), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(https://www.ebi.ac.uk/Tools/msa/clustalo/) and visualized using R software (version 3.5, https://www.r-project.org/) and Bioconductor (version 3.7, msa package: https://bioconductor.org/packages/release/bioc/html/msa.html) [54]. The shading mode was set to be similar for all amino acid sequences.

2.5. Isolation of primary cells and injection

Primary spleen leukocytes were isolated from four Dabry's sturgeons (250–300 g). Spleens were harvested from the freshly euthanized fish and were gently dissociated on sterile conditions in a 100 μm cell strainer. Isolated cells were washed three times with M199 media (Life Technologies) supplemented with 100 U/mL penicillin and streptomycin (HyClone, USA). The cells were seeded into a fresh 6-well plate at a density of 1.5×10^6 cells per well and were maintained at 24 °C in M199 media supplemented with 10% Fetal Bovine Serum(FBS, Life Technologies, USA).

Primary leukocytes were then stimulated with polyinosinic-polycytidylic acid (poly I:C; Sigma, USA) and lipopolysaccharides (LPS; from *Escherichia coli* 055:B5, Sigma, USA) suspended in phosphate-buffered saline (PBS) for 3, 6, 12, 24, 48, and 72 h at a dose of 100 μg/mL and 50 μg/mL, respectively. Control groups were treated with an equal volume of PBS. After stimulation, media was removed from the 6-well plates and cells were harvested and lysed with 1 mL of Trizol reagent (Invitrogen) to extract total RNA. RNA extraction and cDNA synthesis were performed as previously described [48].

2.6. Quantitative real-time PCR (qPCR)

The expression of *As*IFNe1-3 and *As*IFNγ was quantified by qPCR using KAPA SYBR® FAST qPCR Master Mix (KAPA BIOSYSTEMS) and a Step-one Plus real-time PCR system (ABI). β-actin was included as a common reference gene. For comparison of the relative expression levels of different genes, a standard curve was established using a series of 10-fold dilutions of purified PCR products of each gene amplified from cDNA. A serial dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as reference for quantification.

The primers used for qPCR analysis and the accession numbers are listed in Tables 1 and 2. The amplification reaction was performed in a reaction volume of 20 μL containing 4 μL of the corresponding cDNA, 10 μL of KAPA SYBR® FAST qPCR Master Mix, 0.5 μL of each target gene primer, and 5 μL of sterile water. The following thermocycling conditions were used to determine the expression profiles for each gene: 95 °C for 3 min; followed by 45 cycles of 95 °C for 15 s, 62 °C for 30 s; with subsequent incubations at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s to detect fluorescence.

2.7. Statistical analysis

The expression data of each gene was normalized to that of the reference gene, β-actin. Fold change in gene expression was obtained by comparing the normalized gene expression level of the treated groups with the corresponding untreated groups (defined as 1). SPSS 19.0 was used for statistical analysis. One way-analysis of variance (ANOVA) followed by a least significant difference (LSD) post hoc test (when appropriate) was used to analyze the expression data, with $P < 0.05$, $P < 0.01$, or $P < 0.001$ between control and treatment groups being considered significant, as described previously [50].

3. Results

3.1. Sequences features of IFNe1-3 and IFNγ

To obtain the full Open Reading Frame (ORF) length of the *Ad*IFNe1, *Ad*IFNe2, and *Ad*IFNe3, 5'-RACE and 3'-RACE was carried out

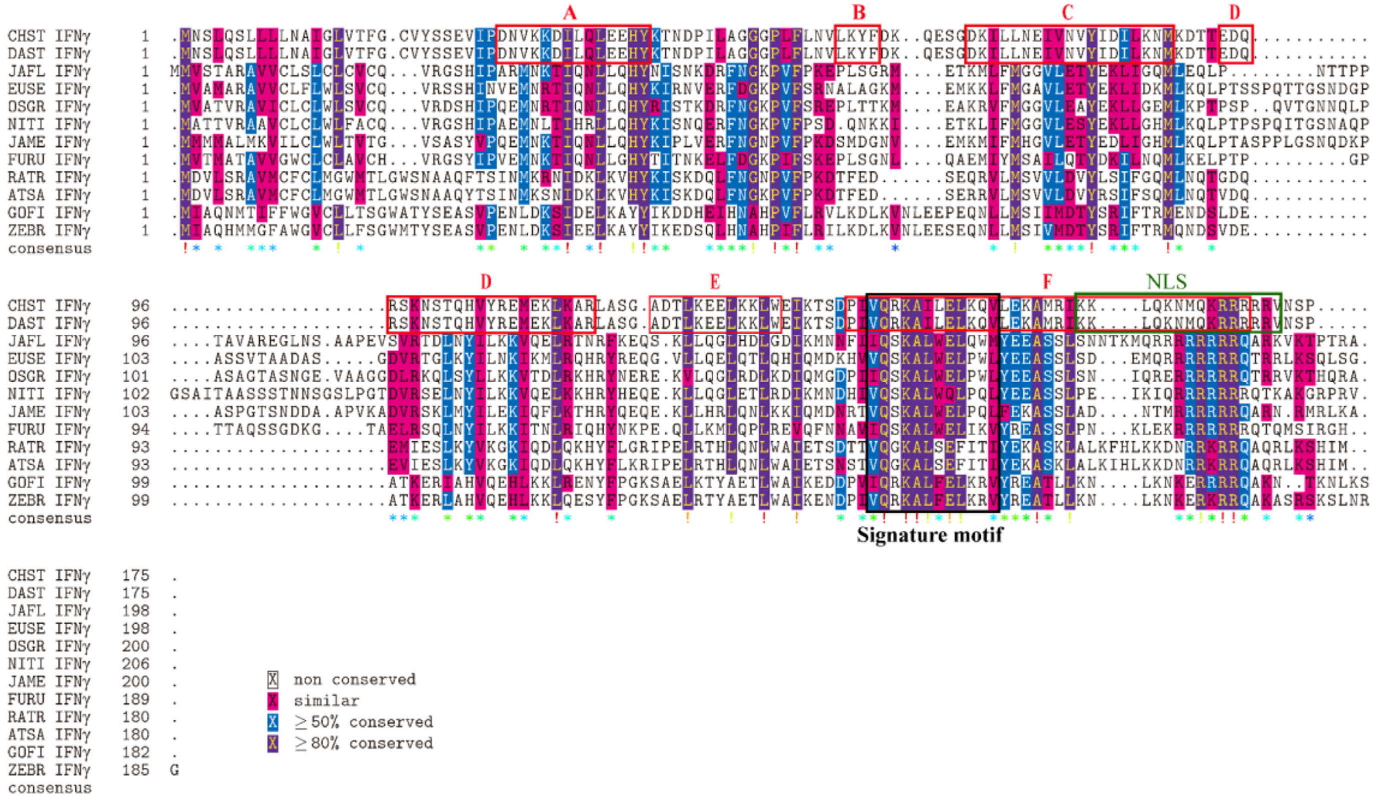

Fig. 4. Multiple alignment of IFNγ from Dabry's sturgeon and Chinese sturgeon with that of other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter and that is a sequences positions in which all sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The A-F alpha-helix were marked with red frame. Signature motif and nuclear localization sequence (NLS) were marked with black and green frame, respectively. The abbreviations were Chinese sturgeon (CHST), Dabry's sturgeon (DAST), Japanese flounder (JAFL), European seabass (EUSE), Orange spotted grouper (OSGR), Nile tilapia (NITI), Japanese medaka (JAME), Fugu rubripes (FURU), Rainbow trout (RATR), Atlantic salmon (ATSA), Goldfish (GOFI), and Zebrafish (ZEBR), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Amino acid identity and similarity of *Ad*IFNe1-3 and *Ad*IFNγ putative peptides compared with that of other teleost fish.

Species	*Ad*IFNe1		*Ad*IFNe2		*Ad*IFNe3		Species	*Ad*IFNγ	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)		Identity (%)	Similarity (%)
*Ad*IFNe1	–	–	42.6	66.7	39.2	58.9	*Ad*IFNγ	–	–
*Ad*IFNe2	42.6	66.7	–	–	52.3	75.8	*As*IFNγ	100	100
*Ad*IFNe3	39.2	58.9	52.3	75.8	–	–	Japanese flounder IFNγ	20.6	43.9
*As*IFNe1	95.8	98.4	41.5	67.2	39.2	58.9	European seabass IFNγ	22.8	40.9
*As*IFNe2	43.6	66.7	97.9	98.9	52.3	75.3	Orange spotted grouper IFNγ	21.6	41
*As*IFNe3	38.7	59.4	53.3	75.3	95.8	98.4	Nile tilapia IFNγ	22.6	43.7
Rainbow trout IFNe1	29.3	52.6	33.5	55.1	30.3	52.6	Japanese medaka IFNγ	22	42.5
Rainbow trout IFNe2	27.1	50.5	31.2	53	28.8	51.0	Fugu rubripes IFNγ	21.8	45.5
Rainbow trout IFNe3	27.1	51.0	32.2	53	29.3	51.0	Rainbow trout IFNγ	28.3	57.2
Rainbow trout IFNe4	26.5	51.3	30.3	53.9	29.8	52.8	Atlantic salmon IFNγ	27.7	56.1
Rainbow trout IFNe5	27.1	51.6	32.6	52.4	29.1	51.1	Goldfish IFNγ	31.1	55.5
Rainbow trout IFNe6	27.1	51.0	32.6	52.4	28.6	51.1	Zebrafish IFNγ	29.7	54.1
Rainbow trout IFNe7	27.6	51.0	33.5	54.5	30.2	52.1	–	–	–

using spleen cDNA as template. Due to the high similarity of many genes among these two species, specific primers were used to amplify the corresponding *As*IFNe1-3 and *As*IFNγ sequences (Supplementary Fig.1-4). The full ORFs were verified by PCR amplification. The sequence features of *Ad*IFNe1-3, *As*IFNe1-3, *Ad*IFNγ, and *As*IFNγ are summarized in Table 2.

The ORF of *Ad*/*As*IFNe1, *Ad*/*As*IFNe2, *Ad*/*As*IFNe3, and *Ad*/*As*IFNγ were 579 bp, 564 bp, 573 bp, and 528 bp, and encoded 192 amino acids (aa), 187 aa, 190 aa, and 175 aa, respectively. The molecular weight (kDa)/theoretical pI of *Ad*IFNe1, *As*IFNe1, *Ad*IFNe2, *As*IFNe2, *Ad*IFNe3, *As*IFNe3, *Ad*IFNγ and *As*IFNγ were 22.89/9.26, 22.9/9.34, 21.95/9.51, 22.14/9.61, 22.49/9.54, 22.56/9.63, 20.24/9.56 and 20.24/9.56, respectively.

3.2. Phylogenetic analysis, amino acid characterization, and molecular cloning

To analyze the evolutionary relationship of the newly identified *Ad*/*As*IFNe1-3 and *Ad*/*As*IFNγ, a phylogenetic tree was established using the putative amino acid sequences (Fig. 1). The tree revealed that *Ad*/*As*IFNe1-3 and *Ad*/*As*IFNγ cluster together with the corresponding homologous proteins from other fish species.

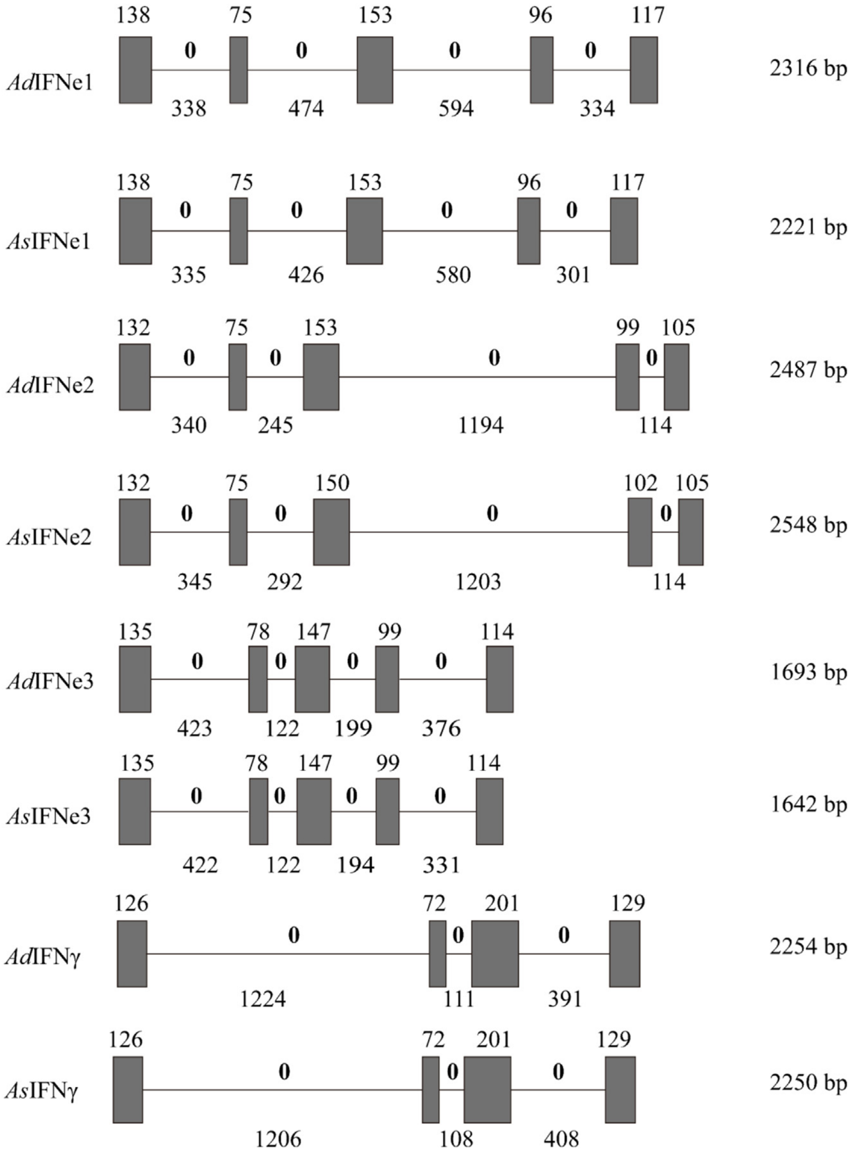

Fig. 5. Schematic diagrams of exon-intron structure of IFNe1, IFNe2, IFNe3, and IFNγ from Dabry's sturgeon and Chinese sturgeon. Boxes represent exons and horizontal lines connecting exons represent introns. The number in the box and under the lines represented the nucleotide length (base pairs). The intron phase is indicated above the lines with boldface.

*Ad/As*IFNe1-3 and *Ad/As*IFNγ all possess a signal peptide (Fig. 2), and the signal peptide of *Ad/As*IFNe1, *Ad/As*IFNe2, *Ad/As*IFNe3, and *Ad/As*IFNγ were found to be 1–21 aa, 1–22 aa, 1–17 aa, and 1–23 aa, respectively. *Ad/As*IFNe1-3, but not *Ad/As*IFNγ, contain a conserved interferon alpha, beta, and delta (IFabd) domain, approximately 120 aa in length (Table 2, Fig. 2). The IFabd domain of *Ad/As*IFNe1, *Ad/As*IFNe2, *Ad*IFNe3, and *As*IFNe3 were 61–172 aa, 54–171 aa, 48–171 aa and 58–171 aa, respectively. *Ad/As*IFNe1-3 and *Ad/As*IFNγ also possess one to four N-glycosylation sites (Fig. 2).

The putative amino acid sequences of *Ad/As*IFNe1-3 were aligned with that of rainbow trout IFNe proteins; *Ad/As*IFNe1-3 retain two conserved cysteines (C1 and C3) (Fig. 3). Six alpha-helices (A-F) were observed in *Ad/As*IFNe1-2, and *Ad/As*IFNe3 only possessed five alpha-helices. *Ad/As*IFNe1-3 also contain a conserved type I IFN signature motif. The alignment of *Ad/As*IFNγ was carried compared to homologous proteins in other fish (Fig. 4). Six α-helices were identified in *Ad/As*IFNγ, and *Ad/As*IFNγ also possessed a type II IFN signature motif and a conserved NLS (Fig. 4).

The amino acid homology of full-length *Ad/As*IFNe1-3 and *Ad/As*IFNγ proteins was analyzed using the MatGAT 2.01 software (Table 3). Within each IFNe subgroup, the sequence identity and similarity between Dabry's sturgeon and Chinese sturgeon was high, ranging from 95.8% to 97.9% aa identity and 98.4%–98.9% aa similarity, respectively. In contrast, the IFNe intergroup sequence aa identity and aa similarity is relatively low in these two sturgeons, ranging from 38.7% to 53.3% aa identity and 58.9%–75.8% aa similarity. *Ad/As*IFNe1-3 have low identity and similarity with IFNe proteins of the rainbow trout, ranging from 26.5% to 33.5% aa identity and 50.5%–55.1% aa similarity. The amino acid sequence of *Ad/As*IFNγ was identical, and exhibited 100% aa identity and similarity between Dabry's sturgeon and Chinese sturgeon. *Ad/As*IFNγ have low identity and similarity with homologous IFNγ proteins from other fish (Table 3), ranging from 20.6% to 31.1% aa identity and 40.9%–57.2% aa similarity, respectively.

To reveal the intron-exon structure of *Ad/As*IFNe1-3 and *Ad/As*IFNγ, the introns were cloned by PCR (Fig. 5, Supplementary Fig.1-4). Like other teleost fish type I IFN, *Ad/As*IFNe1-3 possess five exons and four introns, and *Ad/As*IFNγ has four exons and three introns. Exon length in *Ad/As*IFNe1-3 and *Ad/As*IFNγ was conserved, and the third and fourth exons were shifted only three amino acids between *Ad* and

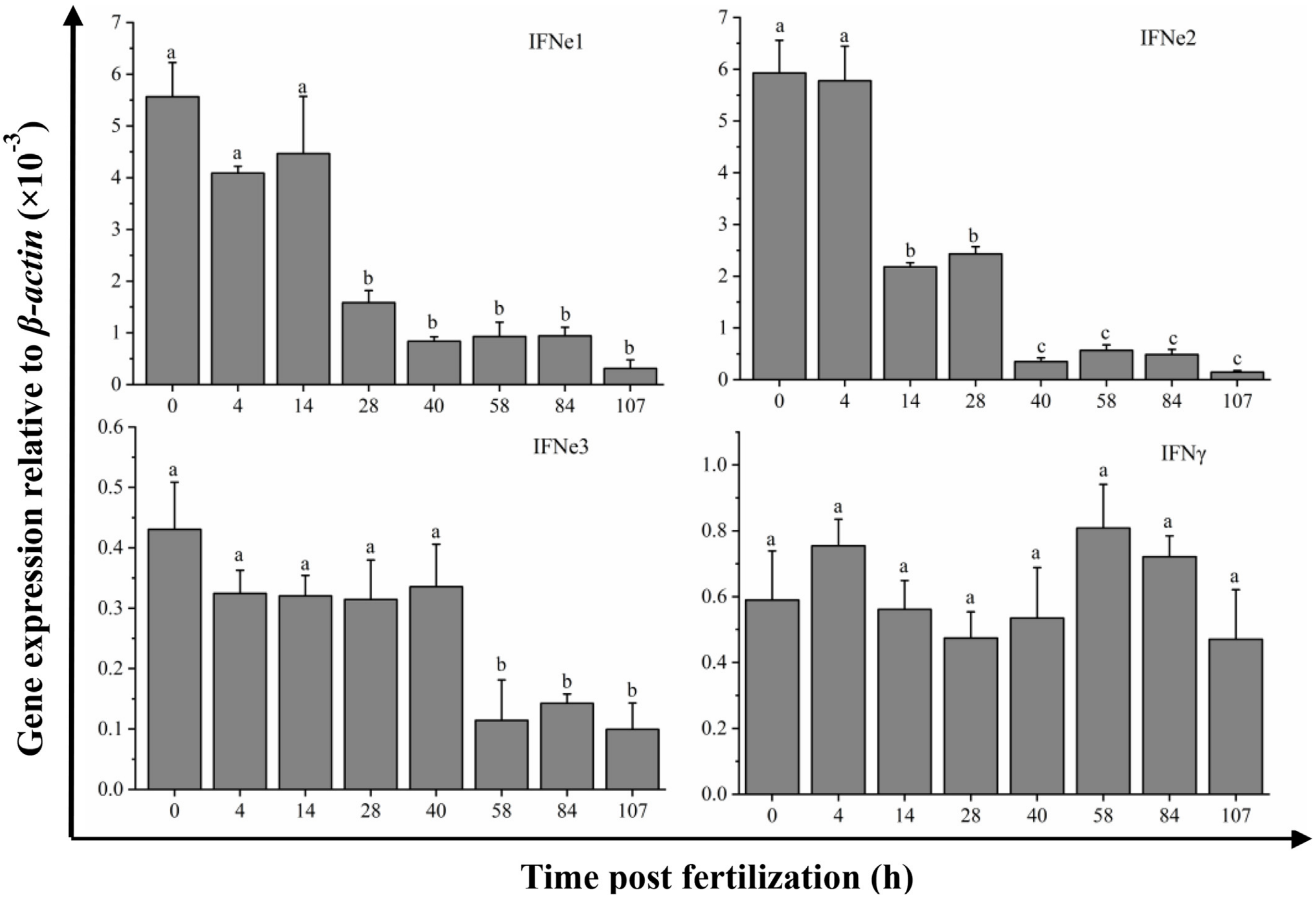

Fig. 6. Expression profiles of IFNe1, IFNe2, IFNe3, and IFNγ in Dabry's sturgeon during ontogeny. Dabry's sturgeon eggs were fertilized by artificial insemination, and then 3 embryos were collected and pooled at 0, 4, 14, 28, 40, 58, 84, and 107 h post fertilization. The expressions of the IFNe1, IFNe2, IFNe3, and IFNγ were normalized to that of β-actin. Data are presented as the mean ± SE (n = 4), and a, b, c indicate significant differences.

As IFNe2. The ORFs of *Ad/As*IFNe1-3 and *Ad/As*IFNγ genes are each separated by four phase 0 introns (Fig. 5).

3.3. Expression profiles of IFN genes during early ontogeny

To determine the importance of *Ad*IFNe1-3 and *Ad*IFNγ in embryonic defenses against pathogens, Dabry's sturgeon embryos in various embryonic stages (0, 4, 14, 28, 40, 58, 84, and 107 hpf) were harvested and RNA was extracted to analyze IFN gene expression by qPCR (Fig. 6). The *Ad*IFNe1-3 genes generally followed the same expression profile. *Ad*IFNe1, *Ad*IFNe2, and *Ad*IFNe3 were highly expressed at 0–14 hpf, 0–4 hpf, and 0–40 hpf, respectively, illustrating that *Ad*IFNe1-3 may be maternally transmitted transcripts ($P < 0.05$). During embryonic development, the expression levels of *Ad*IFNe1 and *Ad*IFNe2 were higher than *Ad*IFNe3, while the expression levels of *Ad*IFNγ were fairly low and showed no significant changes ($P > 0.05$).

3.4. Baseline expression of AdIFNe1-3 and AdIFNγ

To further explore the baseline expression patterns of *Ad*IFNe1-3 and *Ad*IFNγ, tissues samples were taken from the gill, muscle, liver, spleen, head kidney, caudal kidney, intestine, heart, brain, skin, and blood of healthy Dabyr's sturgeon (Fig. 7). These genes were basally expressed in a wide range of tissues at different expression levels. *Ad*IFNe1-3 were highly expressed in the blood, and *Ad*IFNe2 also exhibited high expression in muscle tissue. The baseline expression of *Ad*IFNγ was similar in the 11 tested tissues, and was highest in the muscle, followed by liver, blood, and heart.

3.5. Expression of AdIFNe1-3 and AdIFNγ in response to poly I:C and LPS in vitro

To understand how *Ad*IFNe1-3 and *Ad*IFNγ are regulated in response to poly I:C and LPS, fish were challenged with poly I:C and LPS for 3, 6, 12, 24, 48, and 72 h at doses of 100 μg/mL and 50 μg/mL, respectively. *Ad*IFNe1 and *Ad*IFNe2 were significantly upregulated from 6 h to 48 h after poly I:C administration (Fig. 8), and both reached their highest expression levels at 12 h with 18.82-fold change and 28.48-fold change, respectively. *Ad*IFNe3 levels were strikingly upregulated from 6 h to 24 h, and had highest fold-change at 12 h (21.77-fold change, $P < 0.05$). At 72 h, expression levels of *Ad*IFNe1-3 had returned to the level of the untreated groups. Meanwhile, *Ad*IFNγ expression was prominently elevated from 12 h to 72 h, and peaked at 48 h with a 5.03-fold change. After LPS treatment, mRNA levels of *Ad*IFNe1-3 and *Ad*IFNγ were upregulated. *Ad*IFNe1, *Ad*IFNe2, and *Ad*IFNe3 transcripts were dramatically upregulated at 24–48 h, 12–48 h, and 12–24 h, and peaked at 48 h (6.23-fold), 48 h (4.55-fold), and 12 h (6.21-fold), respectively. Simultaneously, the mRNA levels of *Ad*IFNγ was higher than *Ad*IFNe1-3, and was upregulated from 6 h to 48 h, peaking at 12 h (12.21-fold) after LPS challenge.

4. Discussion

Teleost fish have type I and type II IFNs [11]. Type I IFN family is classified into two groups based on cysteine patterns in the mature peptide, with group I and group II containing either two or four cysteines, respectively [12]. Recent studies have shown that group I IFNs

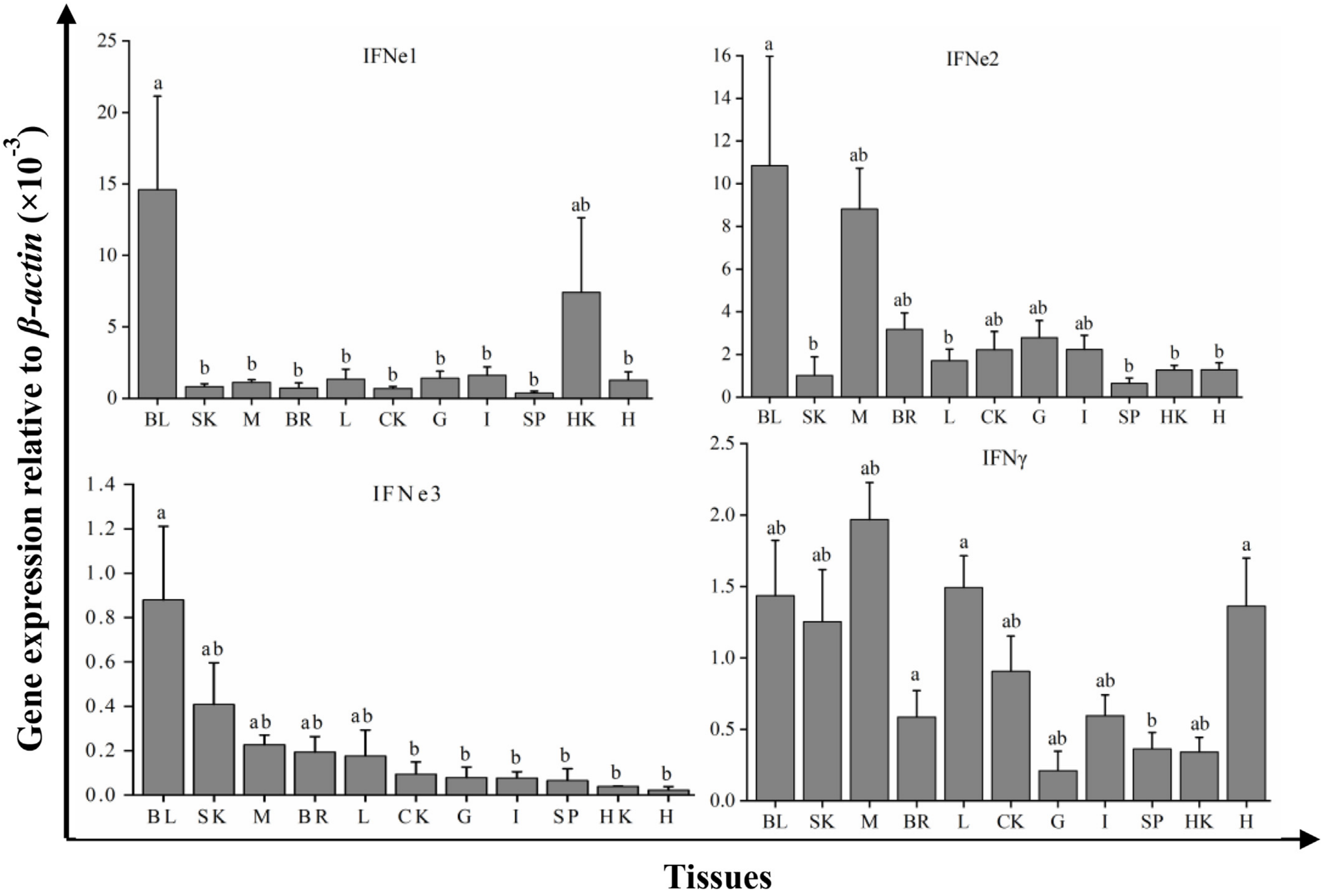

Fig. 7. Tissue distribution of IFNe1, IFNe2, IFNe3, and IFNγ mRNA in Dabry's sturgeon. Gill, G. Muscle, M. Liver, L. Spleen, SP. Head kidney, HK. Caudal kidney, CK. Intestine, I. Heart, H. Brain, BR. Skin, SK. Blood, BL. The data analysis was performed using quantitative real-time PCR, where gene expression was normalized to that of β-actin. Data are presented as the mean ± SE (n = 4).

consist of three subgroups (IFN-a, IFN-d, IFN-e) and group II also has three subgroups (IFN-b, IFN-c, IFN-f) [31]. In addition, IFN h was described recently, and was assigned to a novel subgroup of group I IFN in fish [32,55]. IFNγ and a fish-specific IFNγ-rel are type II IFNs [34,56,57]. Multiple IFN genes are commonly present, usually as a result of tandem gene duplication within the IFN locus/loci. This results in the potential capacity of subfunctionalization and neofunctionalization [58], whereby each IFNs genes may acquire distinctive roles in the immune response. Hence in the current study, three IFNes and one IFNγ genes were identified and their expression patterns were analyzed to provide further insights into the functions of IFNs in Dabry's sturgeon.

4.1. Sequences features

The ORF of *Ad*IFNe1-3 and *Ad*IFNγ display high identity/similarity to *As*IFNe1-3 and *As*IFNγ with 97.6%, 99.3%, and 97.9%, respectively (Supplementary Fig.1-4). This demonstrated that the Dabry's sturgeon is closely related to Chinese sturgeon. Each cDNA sequence contained an in-frame stop codon upstream of the ORF, and a polyadenylation signal (AATAA motif) before the poly A tail, demonstrating that the complete ORFs of IFNe1-3 and IFNγ were obtained. The amino acid homology was analyzed using the MatGAT 2.01 software (Table 3). Within each IFNe subgroup, the sequence identity and similarity between Dabry's sturgeon and Chinese sturgeon was high. In contrast, the IFNe intergroup sequence aa identity and similarity is relatively low in these two sturgeons. *Ad/As*IFNe1-3 have low aa identity and similarity with IFN protein in rainbow trout, and the amino acid sequence of *Ad* and *As* IFNγ was identical among these two sturgeons. The *Ad/As*IFNγ sequences have rather lower aa identity and similarity with that of homologous IFNγ proteins from other fish (Table 3).

4.2. Phylogenetic analysis, amino acid characterization

A phylogenetic tree was constructed, and showed that *Ad/As*IFNe1-3 and *Ad/As*IFNγ clustered together with homologous IFN proteins from other fish (Fig. 1). *Ad/As*IFNe1-3 exhibit a close relationship with IFNe proteins from rainbow trout. This phenomenon might be attributed to the fact that IFNe variants had primarily been reported in salmonids, and limited information regarding teleost IFNe proteins was available. *Ad/As*IFNe1-3 belong to the group I subgroup of type I IFN, which is the same as rainbow trout IFNe1-7 [31]. *Ad/As*IFNe1-3 and *Ad/As*IFNγ all possess a signal peptide (Fig. 2), suggesting that these IFN proteins could be secreted out of cells to act on adjacent cells. Intracellular IFNs are able to elicit cellular responses through intracellular IFN receptors [59], and IFNs can exert their functions in intracellular and extracellular signaling pathways to combat various infections. *Ad/As*IFNe1-3, but not *Ad/As*IFNγ, contain a conserved IFabd domain, made up of approximately 120 aa (Table 2, Fig. 2). The IFabd domain is a typical type I IFN characteristic domain, which is found in many IFNs [55,60]; this domain covers a similar region in the IFN subgroups within teleost and human IFNs. *Ad/As*IFNe1-3 and *Ad/As*IFNγ possess one to four N-glycosylation sites; although the sequences of the *Ad* and *As* IFNs were very similarly, the number of N-glycosylation sites differed between the IFNs from Dabry's and Chinese sturgeon (Fig. 2). *Ad*IFNe1 possesses three glycosylation sites, while *As*IFNe1 has four; *Ad*IFNe3 possesses two glycosylation sites while *As*IFNe3 has three.

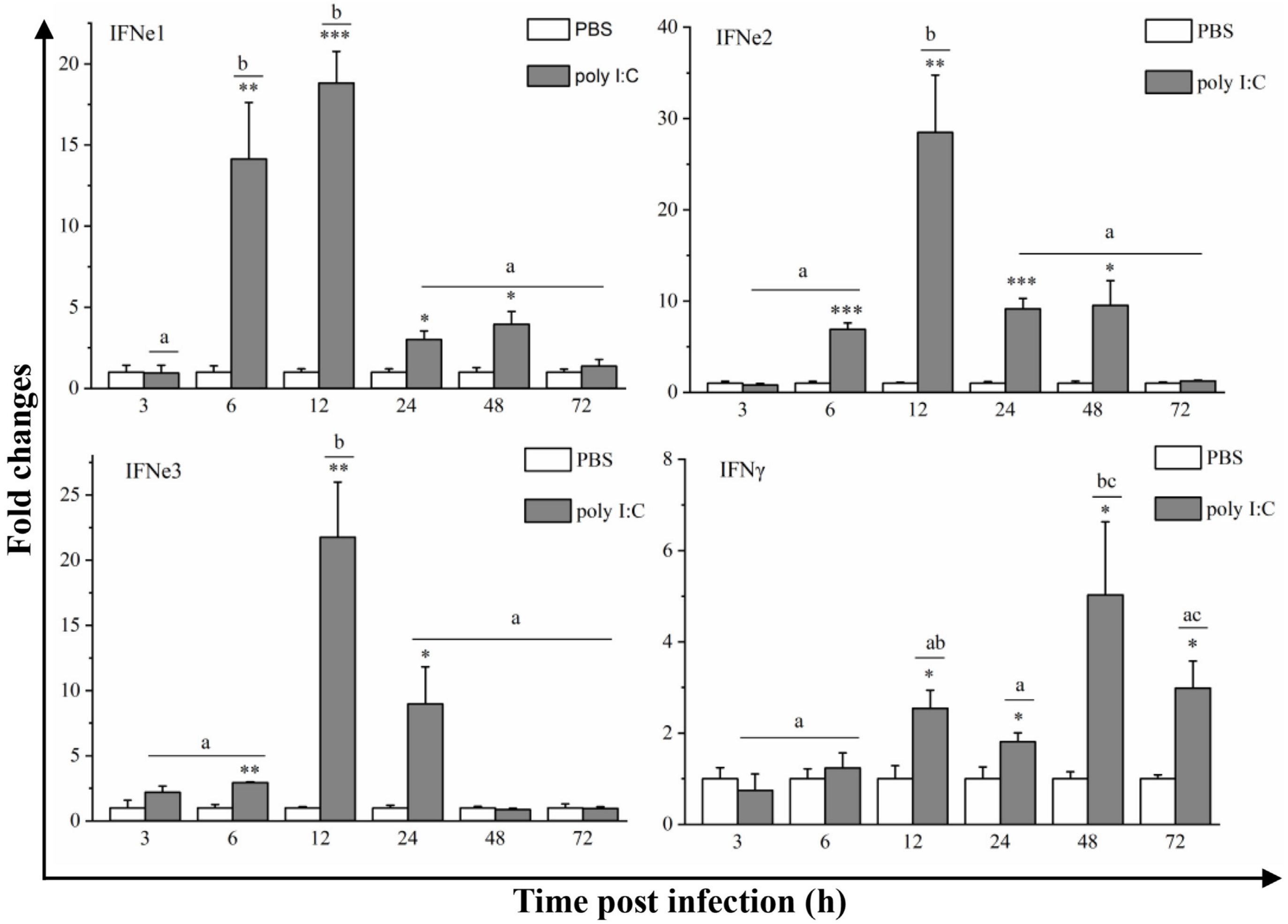

Fig. 8. Poly I:C-induced expression patterns of *Ad*IFNe1, *Ad*IFNe2, *Ad*IFNe3, and *Ad*IFNγ in primary spleen leukocytes. The cells were stimulated with 100 μg/mL poly I:C and used for qPCR analysis. Gene expression levels were normalized to that of β-actin, and are presented as the fold-change compared with the respective control group (which was set to 1). Data are presented as the group means ± SE of four independent wells of cells from four fish. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The letters on the line represented differences among different time points.

Glycosylation sites influence protein conformation by preventing formation of disulphide bonds [61]. Although the Dabry's and Chinese sturgeon demonstrate a close phylogenetic relationship, the Chinese sturgeon is more susceptible to infection than the Dabry's sturgeon. The differences in glycosylation sites in IFNe1 and IFNe3, and the IFabd domain in IFNe3 may influence the functional differences in immunity between these two species, which merits further investigation.

Six alpha-helices (A-F) were observed in *Ad/As*IFNe1-2 and *Ad/As*IFNγ, while *Ad/As*IFNe3 only possessed five alpha-helices (Figs. 3–4). The α-helices are arranged in an antiparallel manner to form the core structure; these regions are well conserved among fish IFNs [62–64]. Among these α-helices, helix F is the most conserved in *Ad/As*IFNe1-3 and *Ad/As*IFNγ. The putative amino acid sequences of *Ad/As*IFNe1-3 were aligned with the sequences of rainbow trout IFNe variants, showing that *Ad/As*IFNe1-3 retain two conserved cysteines (C1 and C3). The first C1 appeared almost immediately following the signal peptide and the C3 was found approximately 80% of the way down the IFNe1-3 sequences (Fig. 3). C1 and C3 reside at the N-terminal region preceding helix A and the C-terminal helix D, respectively, and can form a putative intramolecular disulphide bond in both group I and group II IFNs in fish [31]. Clearly, *Ad/As*IFNe1-3 have a variable number of amino acid residues upstream of C1. The type I IFN signature motif, which is also observed in other teleost type I IFNs [33], was found in *Ad/As*IFNe1-3. *Ad/As*IFNγ also possesses a type II IFN signature motif and a conserved NLS (Fig. 4). In mammals, NLS motif is important for establishing an IFNγ dependent antiviral cellular state [65]. Similarly, removal of the NLS motif of IFNγ abolished its biological activity in teleost fish [45]. Therefore, the NLS is one of the most important components in *Ad/As*IFNγ.

4.3. Intron-exon structure of IFN

Like other teleost fish type I IFN proteins [10], *Ad/As*IFNe1-3 possess five exons and four introns(Fig. 5). The ancestral gene organization of type I IFN (5 exons/4 introns) is seen in type I IFNs in bony fish and in some amphibian IFNs [66–68]. All IFN introns possess the classic 5′ GT/AG 3′ intron splice motifs (Supplementary Fig.1-4). In amphibians, both intron-containing and intronless genes are present [66,67]. However, in amniotes (mammals, birds and reptiles), only the intronless type I IFNs were identified, with apparent loss of the intron-containing IFN [69–71]. This indicated that a retroposition event might have occurred in amphibians, resulting in the generation of intronless type I IFN genes [66]. By means of a retroposition event, 5 exons/4 introns of teleost type I IFN may have partially lost their introns to form the intronless IFN in amphibians, and then introns became completely lost in amniotes. *Ad/As*IFNe1-3 and *Ad/As*IFNγ demonstrated generally conserved exon length, while the third and fourth exon was shifted just three amino acids between *Ad*IFNe2 and *As*IFNe2. Whether this shift

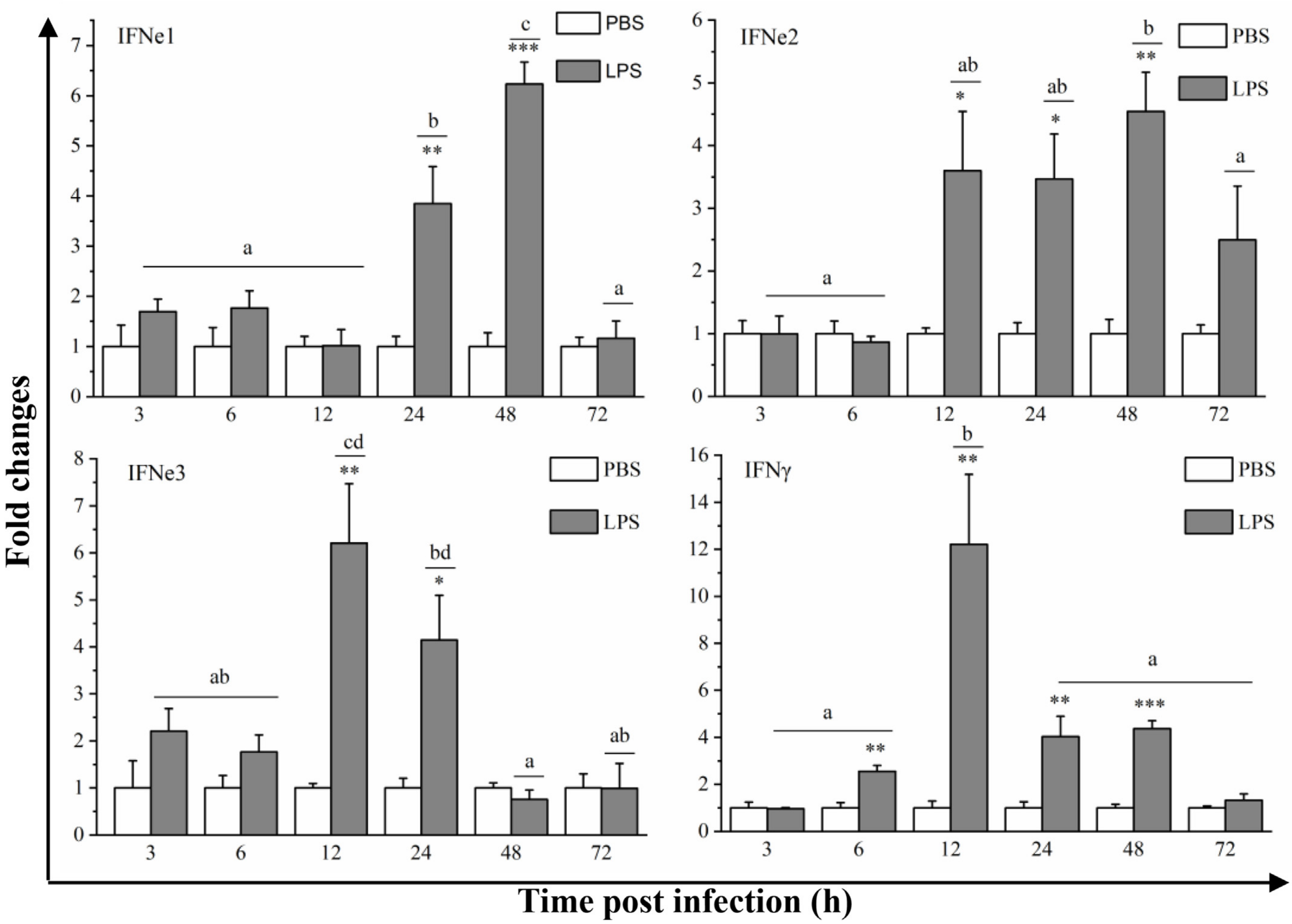

Fig. 9. LPS-induced expression patterns of *Ad*IFNe1, *Ad*IFNe2, *Ad*IFNe3, and *Ad*IFNγ in primary spleen leukocytes. The cells were stimulated with 50 μg/mL LPS and used for qPCR analysis. Gene expression levels were normalized to that of β-actin, and are presented as the fold-change compared with the respective control group (which was set to 1). Data are presented as the group means ± SE of four independent wells of cells from four fish. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). The letters on the line represented differences among different time points.

generated the distinctive functions between *Ad*IFNe2 and *As*IFNe2 should be further analyzed. *Ad/As*IFNγ have four exons and three introns. Type II IFN genes are universally organized into 4 exons/3 introns spanning from cartilaginous fish to mammals, with loss of the canonical third intron [72]. The introns phase was also calculated, and the ORF of all *Ad/As*IFNe1-3 and *Ad/As*IFNγ genes is separated by four and three phase 0 introns, respectively (Fig. 5). This characterization was presented in all teleost IFN genes [73–78]. The genes of *Ad*IFNe1-3 and *Ad*IFNγ shared high identity/similarity with the corresponding genes in Chinese sturgeon, respectively (Supplementary Fig.1-4). This further revealed that Dabry's sturgeon is closely related to the Chinese sturgeon.

4.4. Expression pattern of AdIFN

In mammals, IFNε is constitutively expressed in the female reproductive tract (FRT), and plays critical role in protecting the FRT mucosa from viral and bacterial infection [79]. Fish IFN also function similarly in ovarian tissue [80]. To determine the importance of *Ad*IFNe1-3 and *Ad*IFNγ in embryonic defense against pathogens, Dabry's sturgeon embryos of different embryonic stages were collected and pooled, and their mRNA was extracted and gene expression was evaluated using qPCR (Fig. 6). *Ad*IFNe1-3 generally followed the same expression profile. *Ad*IFNe1, *Ad*IFNe2, and *Ad*IFNe3 were highly expressed in early embryonic development, illustrating that *Ad*IFNe1-3 may be maternally transmitted transcripts. The expression level of *Ad*IFNe1 and *Ad*IFNe2 were higher than *Ad*IFNe3, suggesting that the *Ad*IFNe1 and *Ad*IFNe2 may play more important roles in embryonic development. Surprisingly, the expression levels of *Ad*IFNγ were low, and showed no significant changes over embryonic development, suggesting that *Ad*IFNγ may not mediate embryonic development. *Ad*IFNe1-3 and *Ad*IFNγ were constitutively expressed in a wide range of tissues at different expression levels (Fig. 7). *Ad*IFNe1-3 were both highly expressed in blood, and *Ad*IFNe2 also had high mRNA expression in muscle. The high expression of these genes in blood suggested their possible function in blood immunity [81–83]. The baseline expression of *Ad*IFNγ was similar in the 11 tested tissuest. These results suggest that *Ad*IFNe1-3 and *Ad*IFNγ have potentially different roles in un-stimulated tissues, and may play vital roles in normal conditions.

IFN can be stimulated by poly I:C and LPS [55,64,84]. Poly I:C, a extracellular dsRNA, can activate IFNα/β through the TLR3 (Toll-like receptor 3) pathway, and can stimulated host antiviral defense through the JAK/STAT pathway [85]. In teleost fish, TLR22 can also recognize dsRNA (including poly I:C) on the cell surface, and the trigger IFN signaling [86]. LPS in one of the main PAMPs produced by bacteria [87]. Treatment with poly I:C and LPS can imitate the immune responses seen in viral and bacterial infection, respectively [48]. Therefore, we examined the mRNA levels of *Ad*IFNe1-3 and *Ad*IFNγ *in vitro* using primary spleen leukocytes. After poly I:C administration (Fig. 8), *Ad*IFNe1-3 were significantly upregulated, and then returned to the levels of the untreated groups. *Ad*IFNe1-3 fold changes were lower than IFNd of black carp (bcIFNd) when stimulated by poly I:C, and the bcIFNd showed faster response than *Ad*IFNe1-3 [84]. In rainbow trout, IFNe variants were also highly expressed at 2 h after poly I:C treatment [31]. These differences may due to the specific cells used for each experiment. Similar to *Ad*IFNe1-3, IFNd of meagre fish peaked at 12 h after poly I:C administration [55]. The fold-change of *Ad*IFNe2 was higher than *Ad*IFNe1 and *Ad*IFNe3 at 12 h, suggesting that *Ad*IFNe2 has a stronger antiviral function than the other two IFNe members. The expression level of *Ad*IFNe1 was higher than *Ad*IFNe2-3 at 6 h, illustrating that *Ad*IFNe1 plays a prominent antiviral function in the early stages of infection. Meanwhile, *Ad*IFNγ was highly expressed, suggesting that *Ad*IFNγ is also involved in the antiviral response, although its effect may be subtler than *Ad*IFNe1-3 and the time to implement the effects of *Ad*IFNγ was longer than that of *Ad*IFNe1-3. In trout RTS-11 cells, IFNγ weakly induces Mx expression [45]. After LPS challenge, the mRNA levels of *Ad*IFNe1-3 and *Ad*IFNγ were upregulated (Fig. 9). Simultaneously, the mRNA level of *Ad*IFNγ was higher than that of *Ad*IFNe1-3 and was upregulated from 6 h to 48 h. These results reveal that Dabry's sturgeon type I IFNs possess antibacterial functions. In contrast, meagre IFNc, IFNd, and IFNh were not stimulated by LPS [55]. Japanese eel type I IFN can be induced by LPS treatment of spleen cells for 24 h, and can also be stimulated by *Aeromonas hydrophila* infection [60]. It is speculated that different IFN types have distinctive functions among species. According to expression levels of *Ad*IFNs response to LPS, we found that *Ad*IFNγ has stronger and longer effects than *Ad*IFNe1-3, and that *Ad*IFNe2 has longer but subtler effects than *Ad*IFN1/3.

5. Conclusion

In summary, our findings indicate that *Ad*IFNe1-3 and *Ad*IFNγ possess prototypical type I and II IFN sequence characteristics. Based on mRNA levels of *Ad*IFNe1-3 and *Ad*IFNγ response to poly I:C and LPS, we speculated that *Ad*IFNe1-3 have stronger antiviral capability than *Ad*IFNγ, and *Ad*IFNγ has stronger antibacterial activity than *Ad*IFNe1-3. The differential responses of these genes to poly I:C and LPS indicated differences in the mechanisms of defense of the various IFN group members against viral and bacterial infection.

Acknowledgments

This work research was financially supported by grants from the Key Lab of Freshwater Biodiversity Conservation, Ministry of Agriculture, China (LFBC0904), open research fund program of Guangxi Key Laboratory of Marine Biotechnology (GLMBT-201802), State Key Laboratory of Developmental Biology of Freshwater Fish (2018KF011) and the National Basic Research Program of China (973 Program) (2015CB150702).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.038.

References

[1] A. Isaacs, J. Lindenmann, R.C. Valentine, Virus interference. II. Some properties of interferon, Proc. Biol. Sci. 147 (927) (1957) 268–273.
[2] A. Isaacs, J. Lindenmann, Virus interference. I. The interferon, Proc. Biol. Sci. 147 (927) (1957) 258–267.
[3] S. Pestka, The interferons: 50 years after their discovery, there is much more to learn, J. Biol. Chem. 282 (28) (2007) 20047–20051.
[4] C. Odendall, E. Dixit, F. Stavru, H. Bierne, K.M. Franz, A.F. Durbin, S. Boulant, L. Gehrke, P. Cossart, J.C. Kagan, Diverse intracellular pathogens activate type III interferon expression from peroxisomes, Nat. Immunol. 15 (8) (2014) 717–726.
[5] L. Xu, L. Yang, W. Liu, Distinct evolution process among type I interferon in mammals, Protein Cell 4 (5) (2013) 383–392.
[6] J.R. Schoenborn, C.B. Wilson, Regulation of Interferon-γ during Innate and Adaptive Immune Responses vol. 96, (2007), pp. 41–101.
[7] L.B. Ivashkiv, L.T. Donlin, Regulation of type I interferon responses, Nat. Rev. Immunol. 14 (1) (2014) 36–49.
[8] F. McNab, K. Mayer-Barber, A. Sher, A. Wack, A. O'Garra, Type I interferons in infectious disease, Nat. Rev. Immunol. 15 (2) (2015) 87–103.
[9] T.R. O'Brien, L. Prokunina-Olsson, R.P. Donnelly, IFN-lambda4: the paradoxical new member of the interferon lambda family, J. Interferon Cytokine Res. 34 (11) (2014) 829–838.
[10] C.J. Secombes, J. Zou, Evolution of interferons and interferon receptors, Front. Immunol. 8 (2017) 209.
[11] C. Langevin, E. Aleksejeva, G. Passoni, N. Palha, J.P. Levraud, P. Boudinot, The antiviral innate immune response in fish: evolution and conservation of the IFN system, J. Mol. Biol. 425 (24) (2013) 4904–4920.
[12] J. Zou, C. Tafalla, J. Truckle, C.J. Secombes, Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates, J. Immunol. 179 (6) (2007) 3859–3871.
[13] Y.B. Zhang, J.F. Gui, Molecular regulation of interferon antiviral response in fish, Dev. Comp. Immunol. 38 (2) (2012) 193–202.
[14] J.M. Gonzalez-Navajas, J. Lee, M. David, E. Raz, Immunomodulatory functions of type I interferons, Nat. Rev. Immunol. 12 (2) (2012) 125–135.
[15] G. Trinchieri, Type I interferon: friend or foe? J. Exp. Med. 207 (10) (2010) 2053–2063.
[16] A.J. Sadler, B.R. Williams, Interferon-inducible antiviral effectors, Nat. Rev. Immunol. 8 (7) (2008) 559–568.
[17] C.E. Samuel, Interferons, interferon receptors, signal transducer and transcriptional activators, and interferon regulatory factors, J. Biol. Chem. 282 (28) (2007) 20045–20046.
[18] L.C. Platanias, Mechanisms of type-I- and type-II-interferon-mediated signalling, Nat. Rev. Immunol. 5 (5) (2005) 375–386.
[19] K. Schroder, P.J. Hertzog, T. Ravasi, D.A. Hume, Interferon-gamma: an overview of signals, mechanisms and functions, J. Leukoc. Biol. 75 (2) (2004) 163–189.
[20] W.M. Schneider, M.D. Chevillotte, C.M. Rice, Interferon-stimulated genes: a complex web of host defenses, Annu. Rev. Immunol. 32 (2014) 513–545.
[21] K. Honda, A. Takaoka, T. Taniguchi, Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors, Immunity 25 (3) (2006) 349–360.
[22] M.R. Capobianchi, E. Uleri, C. Caglioti, A. Dolei, Type I IFN family members: similarity, differences and interaction, Cytokine Growth Factor Rev. 26 (2) (2015) 103–111.
[23] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (4) (2001) 778–809.
[24] S. Pestka, C.D. Krause, M.R. Walter, Interferons, interferon-like cytokines, and their receptors, Immunol. Rev. 202 (2004) 8–32.
[25] D. Santhakumar, D. Rubbenstroth, L. Martinez-Sobrido, M. Munir, Avian interferons and their antiviral effectors, Front. Immunol. 8 (2017) 49.
[26] K.M. Kanninen, A.R. White, Type-I interferons in Parkinson's disease: innate inflammatory response drives fate of neurons in model of degenerative brain disorder: an editorial comment on 'Type-I interferons mediate the neuroinflammatory response and neurotoxicity induced by rotenone', J. Neurochem. 141 (1) (2017) 9–11.
[27] V. Galani, S.S. Papadatos, G. Alexiou, A. Galani, A.P. Kyritsis, In vitro and in vivo preclinical effects of type I IFNs on gliomas, J. Interferon Cytokine Res. 37 (4) (2017) 139–146.
[28] T. Blank, M. Prinz, Type I interferon pathway in CNS homeostasis and neurological disorders, Glia 65 (9) (2017) 1397–1406.
[29] D. Califano, Y. Furuya, S. Roberts, D. Avram, A.N.J. McKenzie, D.W. Metzger, IFN-γ increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells, Mucosal Immunol. 11 (1) (2017) 209–219.
[30] P. Niedzwiedzka-Rystwej, W. Ratajczak, B. Tokarz-Deptula, W. Deptula, Mechanisms of type I interferon action and its role in infections and diseases transmission in mammals, Acta Biochim. Pol. 64 (2017) 1–7.
[31] J. Zou, B. Gorgoglione, N.G. Taylor, T. Summathed, P.T. Lee, A. Panigrahi, C. Genet, Y.M. Chen, T.Y. Chen, M. Ul Hassan, S.M. Mughal, P. Boudinot, C.J. Secombes, Salmonids have an extraordinary complex type I IFN system: characterization of the IFN locus in rainbow trout *oncorhynchus mykiss* reveals two novel IFN subgroups, J. Immunol. 193 (5) (2014) 2273–2286.
[32] Y. Ding, J. Ao, X. Huang, X. Chen, Identification of two subgroups of type I IFNs in perciforme fish large yellow croaker *Larimichthys crocea* provides novel insights into function and regulation of fish type I IFNs, Front. Immunol. 7 (2016) 343.
[33] Y. Hu, T. Yoshikawa, S. Chung, I. Hirono, H. Kondo, Identification of 2 novel type I IFN genes in Japanese flounder, *Paralichthys olivaceus*, Fish Shellfish Immunol. 67 (2017) 7–10.
[34] J. Zou, C.J. Secombes, Teleost fish interferons and their role in immunity, Dev. Comp. Immunol. 35 (12) (2011) 1376–1387.
[35] O. Kileng, M.I. Brundtland, B. Robertsen, Infectious salmon anemia virus is a powerful inducer of key genes of the type I interferon system of Atlantic salmon, but is not inhibited by interferon, Fish Shellfish Immunol. 23 (2) (2007) 378–389.
[36] B. Sun, I. Skjaeveland, T. Svingerud, J. Zou, J. Jorgensen, B. Robertsen, Antiviral activity of salmonid gamma interferon against infectious pancreatic necrosis virus and salmonid alphavirus and its dependency on type I interferon, J. Virol. 85 (17) (2011) 9188–9198.
[37] T. Svingerud, J.K. Holand, B. Robertsen, Infectious salmon anemia virus (ISAV) replication is transiently inhibited by Atlantic salmon type I interferon in cell

culture, Virus Res. 177 (2) (2013) 163–170.

[38] J. Yan, L. Peng, M. Chi, J. Xiao, J. Li, S. Liu, H. Feng, IFNa2 of triploid hybrid of gold fish and allotetraploid is an intracellular antiviral cytokine against SVCV and GCRV, Fish Shellfish Immunol. 62 (2017) 238–246.

[39] T. Decker, M. Muller, S. Stockinger, The yin and yang of type I interferon activity in bacterial infection, Nat. Rev. Immunol. 5 (9) (2005) 675–687.

[40] P. Pereiro, M.M. Costa, P. Diaz-Rosales, S. Dios, A. Figueras, B. Novoa, The first characterization of two type I interferons in turbot (*Scophthalmus maximus*) reveals their differential role, expression pattern and gene induction, Dev. Comp. Immunol. 45 (2) (2014) 233–444.

[41] J. Zou, Y. Yoshiura, J.M. Dijkstra, M. Sakai, M. Ototake, C. Secombes, Identification of an interferon gamma homologue in Fugu, *Takifugu rubripes*, Fish Shellfish Immunol. 17 (4) (2004) 403–409.

[42] J. Zou, C.J. Secombes, The function of fish cytokines, Biology 5 (2) (2016) 23.

[43] S. Yoon, A. Alnabulsi, T.Y. Wang, P.T. Lee, T.Y. Chen, S. Bird, J. Zou, C.J. Secombes, Analysis of interferon gamma protein expression in zebrafish (*Danio rerio*), Fish Shellfish Immunol. 57 (2016) 79–86.

[44] Y. Shibasaki, C. Hatanaka, Y. Matsuura, R. Miyazawa, T. Yabu, T. Moritomo, T. Nakanishi, Effects of IFNgamma administration on allograft rejection in ginbuna crucian carp, Dev. Comp. Immunol. 62 (2016) 108–115.

[45] J. Zou, A. Carrington, B. Collet, J.M. Dijkstra, Y. Yoshiura, N. Bols, C. Secombes, Identification and bioactivities of IFN-gamma in rainbow trout *Oncorhynchus mykiss*: the first Th1-type cytokine characterized functionally in fish, J. Immunol. 175 (4) (2005) 2484–2494.

[46] Y. Shibasaki, T. Yabu, K. Araki, N. Mano, H. Shiba, T. Moritomo, T. Nakanishi, Peculiar monomeric interferon gammas, IFNγrel 1 and IFNγrel 2, in ginbuna crucian carp, FEBS J. 281 (4) (2014) 1046–1056.

[47] B. Swain, M. Basu, S.S. Lenka, S. Das, P. Jayasankar, M. Samanta, Characterization and inductive expression analysis of interferon gamma-related gene in the indian major carp, rohu (*Labeo rohita*), DNA Cell Biol. 34 (5) (2015) 367.

[48] K. Luo, S. Zhang, D. Tang, L. Xia, W. Gao, G. Tian, Z. Qi, Q. Xu, W. Zhang, Analysis of the expression patterns of the cytokine receptor family B (CRFB) and interferon gamma receptor (IFNGR) in Dabry's sturgeon (*Acipenser dabryanus*), Dev. Comp. Immunol. 84 (2018) 420–426.

[49] H. Yu-ping, W. Di, A review of sturgeon virosis, J. For. Res. 16 (1) (2005) 79–82.

[50] K. Luo, Y. Li, L. Xia, W. Hu, W. Gao, L. Guo, G. Tian, Z. Qi, H. Yuan, Q. Xu, Analysis of the expression patterns of the novel large multigene TRIM gene family (finTRIM) in zebrafish, Fish Shellfish Immunol. 66 (2017) 224–230.

[51] K. Luo, Y. Li, K. Ai, L. Xia, J. Zhang, W. Hu, W. Gao, L. Guo, Z. Qi, H. Yuan, Q. Xu, Bioinformatics and expression analysis of finTRIM genes in grass carp, *Ctenopharyngodon idella*, Fish Shellfish Immunol. 66 (2017) 217–223.

[52] K. Luo, J. Di, P. Han, S. Zhang, L. Xia, G. Tian, W. Zhang, D. Dun, Q. Xu, Q. Wei, Transcriptome analysis of the critically endangered Dabry's sturgeon (*Acipenser dabryanus*) head kidney response to Aeromonas hydrophila, Fish Shellfish Immunol. 83 (2018) 249–261.

[53] J.J. Campanella, L. Bitincka, J. Smalley, MatGAT, An application that generates similarity/identity matrices using protein or DNA sequences, BMC Bioinf. 4 (2003) 29.

[54] U. Bodenhofer, E. Bonatesta, C. Horejs-Kainrath, S. Hochreiter, msa: an R package for multiple sequence alignment, Bioinformatics 31 (24) (2015) 3997–3999.

[55] D.J. Milne, C. Campoverde, K.B. Andree, X. Chen, J. Zou, C.J. Secombes, The discovery and comparative expression analysis of three distinct type I interferons in the perciform fish, meagre (*Argyrosomus regius*), Dev. Comp. Immunol. 84 (2018) 123–132.

[56] D. Igawa, M. Sakai, R. Savan, An unexpected discovery of two interferon gamma-like genes along with interleukin (IL)-22 and -26 from teleost: IL-22 and -26 genes have been described for the first time outside mammals, Mol. Immunol. 43 (7) (2006) 999–1009.

[57] R. Savan, S. Ravichandran, J.R. Collins, M. Sakai, H.A. Young, Structural conservation of interferon gamma among vertebrates, Cytokine Growth Factor Rev. 20 (2) (2009) 115–124.

[58] X. He, J. Zhang, Rapid subfunctionalization accompanied by prolonged and substantial neofunctionalization in duplicate gene evolution, Genetics 169 (2) (2005) 1157–1164.

[59] M.X. Chang, J. Zou, P. Nie, B. Huang, Z. Yu, B. Collet, C.J. Secombes, Intracellular interferons in fish: a unique means to combat viral infection, PLoS Pathog. 9 (11) (2013) e1003736.

[60] J. Feng, P. Lin, Y. Wang, S. Guo, Z. Zhang, L. Yu, Identification of a type I interferon (IFN) gene from Japanese eel and its expression analysis in vivo and in vitro, Agri Gene 5 (2017) 19–26.

[61] J. Meng, P. Parroche, D.T. Golenbock, C.J. Mcknight, The differential impact of disulfide bonds and N-linked glycosylation on the stability and function of CD14, J. Biol. Chem. 283 (6) (2008) 3376–3384.

[62] O.J. Hamming, G. Lutfalla, J.P. Levraud, R. Hartmann, Crystal structure of Zebrafish interferons I and II reveals conservation of type I interferon structure in vertebrates, J. Virol. 85 (16) (2011) 8181–8187.

[63] R. Radhakrishnan, L.J. Walter, A. Hruza, P. Reichert, P.P. Trotta, T.L. Nagabhushan, M.R. Walter, Zinc mediated dimer of human interferon-α2b revealed by X-ray crystallography, Structure 4 (12) (1996) 1453–1463.

[64] Y. Xiang, W. Liu, P. Jia, Y. Li, Y. Jin, L. Chen, J. Zhang, K. Jia, M. Yi, Molecular characterization and expression analysis of interferon-gamma in black seabream *Acanthopagrus schlegelii*, Fish Shellfish Immunol. (2017) 140–148.

[65] P.S. Subramaniam, M.G. Mujtaba, M.R. Paddy, H.M. Johnson, The carboxyl terminus of interferon-γ contains a functional polybasic nuclear localization sequence, J. Biol. Chem. 274 (1) (1999) 403–407.

[66] Z. Gan, S.N. Chen, B. Huang, J. Hou, P. Nie, Intronless and intron-containing type I IFN genes coexist in amphibian *Xenopus tropicalis*: insights into the origin and evolution of type I IFNs in vertebrates, Dev. Comp. Immunol. 67 (2017) 166–176.

[67] Y. Sang, Q. Liu, J. Lee, W. Ma, D.S. McVey, F. Blecha, Expansion of amphibian intronless interferons revises the paradigm for interferon evolution and functional diversity, Sci. Rep. 6 (2016) 29072.

[68] Q. Chen, J. Ma, Y. Fan, Y. Meng, J. Xu, Y. Zhou, W. Liu, X. Zeng, L. Zeng, Identification of type I IFN in Chinese giant salamander (*Andrias davidianus*) and the response to an iridovirus infection, Mol. Immunol. 65 (2) (2015) 350–359.

[69] K.E. Goossens, A.C. Ward, J.W. Lowenthal, A.G.D. Bean, Chicken interferons, their receptors and interferon-stimulated genes, Dev. Comp. Immunol. 41 (3) (2013) 370–376.

[70] Y. Sang, J. Bergkamp, F. Blecha, Molecular evolution of the porcine type I interferon family: subtype-specific expression and antiviral activity, PloS One 9 (11) (2014) e112378.

[71] L. Xu, L. Yang, W. Liu, Distinct evolution process among type I interferon in mammals, Protein & Cell 4 (5) (2013) 383–392.

[72] C.D. Krause, Intron loss in interferon genes follows a distinct set of stages, and may confer an evolutionary advantage, Cytokine 83 (2016) 193–205.

[73] P. Boudinot, J. Zou, T. Ota, F. Buonocore, G. Scapigliati, A. Canapa, J. Cannon, G. Litman, J.D. Hansen, A tetrapod-like repertoire of innate immune receptors and effectors for coelacanths, J. Exp. Zool. B Mol. Dev. Evol. 322 (6) (2014) 415–437.

[74] D. Aggad, M. Mazel, P. Boudinot, K.E. Mogensen, O.J. Hamming, R. Hartmann, S. Kotenko, P. Herbomel, G. Lutfalla, J.P. Levraud, The two groups of zebrafish virus-induced interferons signal via distinct receptors with specific and shared chains, J. Immunol. 183 (6) (2009) 3924–3931.

[75] B. Sun, B. Robertsen, Z. Wang, B. Liu, Identification of an Atlantic salmon IFN multigene cluster encoding three IFN subtypes with very different expression properties, Dev. Comp. Immunol. 33 (4) (2009) 547–558.

[76] D. Casani, E. Randelli, S. Costantini, A.M. Facchiano, J. Zou, S. Martin, C.J. Secombes, G. Scapigliati, F. Buonocore, Molecular characterisation and structural analysis of an interferon homologue in sea bass (*Dicentrarchus labrax L.*), Mol. Immunol. 46 (5) (2009) 943–952.

[77] Y.M. Chen, C.E. Kuo, G.R. Chen, Y.T. Kao, J. Zou, C.J. Secombes, T.Y. Chen, Functional analysis of an orange-spotted grouper (*Epinephelus coioides*) interferon gene and characterisation of its expression in response to nodavirus infection, Dev. Comp. Immunol. 46 (2) (2014) 117–128.

[78] E.H. Stolte, H.F. Savelkoul, G. Wiegertjes, G. Flik, B.M. Lidy Verburg-van Kemenade, Differential expression of two interferon-gamma genes in common carp (*Cyprinus carpio L.*), Dev. Comp. Immunol. 32 (12) (2008) 1467–1481.

[79] K.Y. Fung, N.E. Mangan, H. Cumming, J.C. Horvat, J.R. Mayall, S.A. Stifter, N.A. De Weerd, L.C. Roisman, J. Rossjohn, S.A. Robertson, Interferon-ε protects the female reproductive tract from viral and bacterial infection, Science 339 (6123) (2013) 1088–1092.

[80] Y. Valero, P. Morcillo, J. Meseguer, F. Buonocore, M.A. Esteban, E. Chaves-Pozo, A. Cuesta, Characterization of the IFN pathway in the teleost fish gonad against vertically transmitted viral nervous necrosis virus, J. Gen. Virol. 96 (8) (2015) 2176–2187.

[81] M.E. Reid, C. Lomas-Francis, M.L. Olsson, The Blood Group Antigen Factsbook, Academic Press, 2012.

[82] M. Balázs, F. Martin, T. Zhou, J.F. Kearney, Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses, Immunity 17 (3) (2002) 341–352.

[83] J.A. Green, S.R. Cooperband, S. Kibrick, Immune specific induction of interferon production in cultures of human blood lymphocytes, Science 164 (3886) (1969) 1415–1417.

[84] H. Wu, L. Liu, S. Wu, C. Wang, C. Feng, J. Xiao, H. Feng, IFNb of black carp functions importantly in host innate immune response as an antiviral cytokine, Fish Shellfish Immunol. 74 (2018) 1–9.

[85] Q. Hou, R. Gong, X. Liu, H. Mao, X. Xu, D. Liu, Z. Dai, H. Wang, B. Wang, C. Hu, Poly I:C facilitates the phosphorylation of *Ctenopharyngodon idellus* type I IFN receptor subunits and JAK kinase, Fish Shellfish Immunol. 60 (2017) 13–20.

[86] A. Matsuo, H. Oshiumi, T. Tsujita, H. Mitani, H. Kasai, M. Yoshimizu, M. Matsumoto, T.J.T.J.o.I. Seya, Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from, birnaviruses 181 (5) (2008) 3474–3485.

[87] S. Boltana, N. Roher, F.W. Goetz, S. Mackenzie, PAMPs, PRRs and the genomics of gram negative bacterial recognition in fish, Dev. Comp. Immunol. 35 (12) (2011) 1195–1203.