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Atlantic salmon type I interferon genes revisited

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ABSTRACT

Type I interferons (IFN–I) play a pivotal role in vertebrate innate immunity against viruses. This study is an analysis of IFN-I genes in an updated version of the Atlantic salmon genome published in 2021 (version Ssal_v3.1), revealing 47 IFN-I genes in the Atlantic salmon genome. The GH1 locus of chromosome (Chr) 3 harbors 9 IFNa genes, 5 IFNb genes, 6 IFNc genes, 11 IFNe genes and 1 IFNf gene. The GH2 locus on Chr6 contains 1 IFNa gene, 12 IFNc genes and 1 IFNf gene while Chr19 carries a single IFNd gene. Intraperitoneal injection of Atlantic salmon presmolts with poly I:C, a mimic of virus double-stranded RNA, significantly up-regulated IFNc genes from both Chr3 and Chr6 in heart, with lower expression in head kidney. IFNe expression increased in the heart, but not in the head kidney while IFNf was strongly up-regulated in both tissues. Antiviral activity of selected IFNs was assessed by transfection of salmon cells with IFN-expressing plasmids followed by infectious pancreatic necrosis virus infection, and by injection of fish with IFN-plasmids followed by measuring expression of the antiviral Mx1 gene. The results demonstrated that IFNc from both Chr3 and Chr6 provided full protection of cells against virus infection, whereas IFNe and IFNf showed lesser protection. IFNc from Chr3 and Chr6 along with IFNe and IFNf, up-regulated the Mx1 gene in the muscle, while only the IFNcs caused induction of Mx1 in liver. Overall, this study reveals that Atlantic salmon possesses an even more potent innate immune defense against viruses than previously understood.

1. Introduction

Type I interferons (IFN–I) are cytokines that play crucial roles in innate immunity against viruses in vertebrates. They are induced and secreted when host cells recognize viral RNA through receptors such as RIG-I, MDA5, TLR3, TLR7 and TLR22 [1,2]. In the next step, IFN-I protect cells against virus infection by inducing Mx and other antiviral proteins [3,4]. IFN-I also play a significant role in activating the adaptive immune response, as demonstrated in Atlantic salmon by their ability to enhance the immune response of a DNA vaccine against infectious salmon anemia virus [5,6]. A review of IFN-I functions in Atlantic salmon has been published [7].

While fish and mammalian IFN-I share functional similarities, they have evolved differently [8,9]. Seven IFN-I subtypes have been identified in fish including IFNa, IFNb, IFNc, IFNd, IFNe, IFNf and IFNh [7,9–12]. The first six subtypes are found in salmonids, while IFNh has only been discovered in percomorph species such as large yellow croaker, turbot and Tetraodon, and in Atlantic cod [7,9,11–15]. IFNa, IFNd, IFNe and IFNh possess two conserved cysteines forming one disulfide bridge, while IFNb, IFNc and IFNf possess four conserved cysteines forming two disulfide bridges. The sequencing of fish genomes has facilitated the identification of the repertoire of IFN-I genes in salmonids and other fish species, enhancing our understanding of the evolution of IFN-I in fish [12,15,16].

Atlantic salmon IFNa was one of the first fish IFN-I to be discovered by classical cloning [13]. Subsequent sequencing of genomic BAC clones revealed a large cluster of type I IFN genes in the Atlantic salmon genome, including two IFNa, four IFNb and five IFNc genes [9]. This cluster was found to be linked to the growth hormone 1 (GH1) gene on Chr3, while another IFNc gene was linked to the GH2 gene on Chr6 [7]. A single IFNd gene was discovered in Atlantic salmon, located on Chr19 [10,17]. IFNe and IFNf subtypes were initially discovered in rainbow trout and later in Atlantic salmon [7,14]. The sequencing of the Atlantic salmon genome published in 2015 (ICSASG v2) revealed an even larger repertoire of IFN-I genes on Chr3 and Chr6 [7,18]. However, several IFN-I were placed in unassigned scaffolds, and some IFNs were incorrectly placed on Chr28 instead of Chr3. These and other shortcomings have been rectified in the 2021 version of the Atlantic salmon genome Ssal_v3.1 [19], providing a more complete picture of the repertoire and location of IFN-I genes. In this study, we identified the IFN-I genes at the GH1 and GH2 loci and compared them to previously identified Atlantic

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Abbreviations

Chr	chromosome
IFN-I	type I interferon
IPNV	infectious pancreatic necrosis virus
i.m.	intramuscular
i.p	intraperitoneal
MDA5	melanoma differentiation-associated gene 5
Mx	myxovirus resistance gene
ORF	open reading frame
poly I:C	polyinosinic-polycytidylic acid
RT-qPCR	reverse transcription quantitative PCR
RIG-I	retinoic acid-inducible gene I
TLR	Toll-like receptors

salmon IFN-I, resulting in identification of 47 type I IFN genes in Atlantic salmon, the largest repertoire of IFN-genes reported in any vertebrate.

An important question is whether IFN subtypes serve similar or different functions. Previous studies showed that IFNa, IFNb and IFNc all induced antiviral genes in the muscle of Atlantic salmon and induced antiviral activity in cell lines, while IFNd did not [17]. Furthermore, IFNb and IFNc, but not IFNa, induced antiviral genes systemically in salmon [20]. IFNa, IFNb and IFNc were induced by the double-stranded RNA poly I:C whereas only IFNb and IFNc were induced by the imidazoquinoline R848 [17]. IFNd was neither induced by poly I:C nor R848. Poly I:C mimics viral double-stranded RNA and induces IFN-I through MDA5, TLR3 or TLR22 [2,21–23]. R848 mimics viral single-stranded RNA and induces IFN through the TLR7 pathway [24,25]. Interestingly, IFNa, IFNb and IFNc showed cell specific expression in Atlantic salmon injected with poly I:C and R848 [17]. In this work, we studied expression and antiviral properties of IFNe and IFNf and of IFNc encoded by Chr6, which have not been previously studied functionally in Atlantic salmon.

2. Materials and methods

2.1. Bioinformatics

TBLASTN searches with IFNa1, IFNa3, IFNb1, IFNc1 (Accession no. EU768890), IFNe (XP_014035910) and IFNf (XP_014048243) as queries were performed against the Reference sequence (RefSeq) genome database, Whole-genome shotgun contigs (wgs) database and the nucleotide database in NCBI GenBank with Atlantic salmon as organism. In addition, annotated IFNs displayed in the Atlantic salmon salmon genome database (NCBI RefSeq assembly Ssal_v3.1 (GCF_905237065.1) were identified by using them as queries in BLASTP searches. Multiple alignments of IFNs were performed with the ClustalW method using the MegAlign multiple alignment program (DNASTAR, Inc.). Calculation of percent sequence identity between IFN protein sequences was done based on the multiple alignments using the MegAlign program. Phylogenetic analysis of Atlantic salmon IFN-I proteins was performed by multiple alignment of sequences using the Muscle method in the MEGA 11 program [26,27]. A phylogenetic tree was then constructed from the alignment using the Neighbor-joining method [28]. The optimal tree is shown. The percentage of replicate trees in which the associated IFNs clustered together was revealed by a bootstrap test (1000 replicates) [29]. The evolutionary distances were computed in MEGA11 using the Poisson correction method and are in units of the number of amino acid substitutions per site. The analysis involved 51 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 215 positions in the final dataset.

Table 1
List of primers used qPCR.

Gene	Sequence	Accession no.
EF1a F	TGCCCCTCCAGGATGTCTAC	BG933853
EF1a R	CACGGCCCACAGGTACTG	
IFNc1.1 F	TACAAGGACAGCTGGTGCGAATAAC	JX524153
IFNc1.1 R	CAAATGCGGTGGCTGGGAATG	
IFNc3.1 F	ATGCCTTGCCAGCTACAACGACATC	XM_014204556
IFNc3.1 R	GGCTGGGAATACCATGAAGACATTA	
IFNc4.10 F	GCTGGTGCGATCAACCCACAACCTA	XM_045721312
IFNc4.10 R	CGTGGCTGGGAATTCCATGAAGACA	
IFNe1.1 F	AACAGATGGCCTTCATTTCGCATAC	XM_014180435
IFNe1.1 R	CCAGGCGGTGGACTCATATTTACCA	
IFNe2.2 F	ATAACGGCCGGATTGTCTTCATTT	XM_014187504
IFNe2.2 R	CAGGCGGTGGAATCATATTTACCA	
IFNf2 F	AACTCGCCAGACCCTCAATCATCT	XM_014204335
IFNf2 R	AAGCGTTGGACGTTGGACCATT	
Mx1 F	TGCAACCACAGAGGCTTTGAA	U66475
Mx1 R	GGCTTGGTCAGGATGCCTAAT	

F = forward, R = reverse.
Primers are specific except as follows: IFNc1.1 primers match IFNc1.2, IFNc1.3. IFNc1.4 and IFNc1.5100 %; IFNc4.10 primers match IFNc4.2 and IFNc4.3100 % and are also likely to amplify IFNc4.5, c4.6, c4.7, c4.8, c4.9 and c4.11, but not IFNc4.1 and IFNc4.4.

2.2. Fish

Atlantic salmon (*Salmo salar* L.) presmolts (30–45 g) were obtained, labelled and kept in 300 l tanks at 10 °C as described [20]. All handling of fish was in accordance with the Norwegian "Regulation on Animal Experimentation" and all fish experiments were submitted to and approved by the Norwegian Animal Research Authority (NARA) before initiation.

2.3. Plasmids expressing IFN

pSF-CMV-TOPO plasmids containing the open reading frames (ORF) of respectively IFNc3.1 (XM_014204556), IFNc4.9 (XM_014204559), IFNe2.2 (XM_014187504) and IFNf2 (XM_014204335) downstream of the CMV promoter were ordered from Oxford Genetics, Oxford UK. pSF-CMV-TOPO plasmid without insert served as control.

2.4. Stimulation of Atlantic salmon with poly I:C and R848 to study IFN gene expression

Groups of presmolts (n = 5) were injected i.p. with 0.2 ml PBS (control), 0.2 ml PBS containing 400 μg poly I:C (GE Healthcare) or 0.2 ml PBS containing 200 μg R848 (InvivoGen). Twenty-four hours post injection, organs (head kidney, spleen, gills, liver, heart, intestine and muscle) were harvested and preserved in RNAlater (Ambion) for RT-qPCR analysis of IFN gene expression.

2.5. Treatment of fish with IFN expression plasmids to study Mx1 gene induction

Groups of Atlantic salmon presmolts (n = 5) were injected i.m. approximately 1 cm below the dorsal fin with 50 μl sterile phosphate-buffered saline (PBS) at pH 7.4 or 50 μl PBS containing 15 μg of the plasmids described above. Seven days later samples from muscle at the injection point and from liver were harvested and stored in RNAlater for RT-qPCR analysis of Mx1 gene expression.

2.6. RT-qPCR measurement of gene expression

Total RNA was extracted from Atlantic salmon tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA yield was determined using Nanodrop ND-1000 Spectrophotometer (Nanodrop Tec., Wilmington, DA, USA). cDNA was synthesized with the QuantiTect

Gene	Location	Or.	Gene Id	Protein acc. no.
GH1	55678562-55682490	+	LOC100136588	
Rdm1	55686098-55695815	+	LOC100196558	
IFNc2.1	55891167-55892144	-	ENSSSAG00000105246	
IFNc1.4	55898884-55899884	-	LOC106600970	XP_014048254
IFNb1.4	55914501-5591656	-	ENSSSAG00000121867	
IFNa2.3	55923899-55926214	-	LOC106600969	XP_014048251
IFNc1.3	55927619-55928609	-	LOC101448043	NP_001266026
IFNb1.2	55939947-55941733	-	LOC101448042	NP_001266024
IFNc1.2	55964936-55966021	+	LOC123723647	XP_045570931
IFNb1.5	55977348-55979195	+	LOC106600866	XP_014048072
IFNc1.1	55990145-55991248	+	LOC123741677	XP_045570937
IFNa2.2	55992543-55994692	+	LOC106600966	XP_045570928
IFNb1.3	56002268-56004143	+	LOC123741673	XP_045570932
IFNc1.5	56026892-56027965	+	LOC106600965	XP_014048249
IFNa2.1	56029920-56031643	+	LOC106600964	XP_014048247
IFNb1.1	56039224-56041047	+	LOC123741674	XP_045570933
IFNe1.1	56075470-56076928	-	LOC106589965	XP_014035910
IFNa1.1	56080395-56084636	+	LOC100137019	NP_001117182
IFNe2.1	56095008-56096192	+	LOC106593342	XP_045570935
IFNe1.2	56103023-56104519	+	LOC123741675	XP_045570934
IFNa1.2	56107758-56111775	+	LOC106600963	XP_014048245
IFNa1.3	56121370-56125158	-	LOC123741678	XP_045570938
IFNe1.3	56128833-56130234	-	LOC123741679	XP_045570939
IFNe2.2	56137434-56138554	-	LOC106596190	XP_014042979
IFNa1.4	56154548-56158768	-	LOC100136436	NP_001117042
IFNe1.4	56161489-56162887	-	LOC106598185	XP_014044720
IFNe2.3	56170913-56172037	-	LOC106593349	XP_014040156
IFNa1.5	56185570-56189780	-	LOC106590949	XP_045570940
IFNe1.5	56192526-56193925	-	LOC106593350	XP_045572134
IFNe2.4	56201947-56203083	-	LOC123741840	XP_045572007
IFNa1.6	56216721-56220929		LOC106600865	XP_045570942
IFNe1.6	56223680-56225082	-	LOC106600962	XP_014048244
IFNe2.5	56233104-56234226	-	LOC123741841	XP_045572008
IFNf1	56257762-56258869	+	LOC106600961	XP_014048243
Gbgt1	56266219-56275391	-	LOC100194757	

Fig. 1. IFN-I genes in Chr3. Gene Id numbers, accession numbers and sequence location numbers were found in the NCBI Genbank except for IFNc2.1 and IFNb1.4 genes, which were found in the Ensembl genome site (Ensembl.org). Or. = gene orientation where + and – indicate forward and reverse direction, respectively. GH1 = Growth hormone 1, Rdm1 = RAD52 motif cont. 1, Gbgt1 = Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1.

Reverse Transcription Kit (Qiagen) starting with 1 μg total RNA following standard protocol. qPCR was performed using 6.1 μl 1:10 dilution of cDNA in a 15 μl reaction mixture containing 7.5 μl 2 × SYBR green PCR Master Mix (Applied Biosystems) and 400 nM forward and reverse primers (Table 1). Each sample was run in duplicate wells on a 7500 Fast Real-Time PCR System (Applied Biosystems). The mixtures were incubated at 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The absence of primer–dimer artifacts was confirmed by melting curve analysis. Relative IFN and Mx1 expression values were normalized against levels of Elongation factor1αB (EF1αB) mRNA. Fold increase of the representative genes were calculated by comparison with gene expression in organs of treated fish versus control fish injected with PBS or pSF-CMV-TOPO plasmid without insert. Relative expression of IFN or Mx genes was calculated by the Pfaffl method using EF1αB as a reference gene [30]. Data were calculated from five samples in each group and expressed as mean ± standard errors. The primers used in RT-qPCR were designed by the Primer Select program (DNASTAR, Inc.) and are listed in Table 1. Unpaired *t*-test with two-tail distribution was used for statistical analysis.

2.7. Antiviral activity of novel IFNs in Atlantic salmon kidney cells

Antiviral activity of IFNs was measured by transfection of IFN plasmids or control plasmid into ASK cells (LGC standards ATCC-CRL-2747) followed by infection with infectious pancreatic necrosis virus (IPNV). ASK cells were grown at 20 °C in L-15 medium (Gibco) containing 1x MEM Non-Essential Amino Acid Solution (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin and 8 % FBS Superior.

117 (Biochrom AG) and seeded in 96 well plates (25.000 cells/well). Eight wells were transfected with each plasmid (95 ng/well) using FuGENE® HD Transfection Reagent (Promega). Seventy-two hours post transfection four of the wells were infected with IPNV at a multiplicity of infection (MOI) of 1, and incubated at 19 °C for 8 days with medium containing 2 % FBS, which resulted in near complete lysis of cells transfected with control plasmid. All cells were then washed with PBS and stained by incubation with 1 % (w/v) crystal violet in 20 % ethanol for 10 min. The cells were next washed three times with distilled water and air dried before the stain was dissolved by addition of 100 μl 50 % ethanol containing 0.05 M sodium citrate and 0.05 M citric acid and the absorbance read at 550 nm. Percent cell survival was calculated from absorbance values where 100 % survival represents values of non-

Gene	Location	Or.	Gene Id	Protein acc.no.
IFNf2	45343981-45345088	+	LOC106607408	XP_014059810
Gbgt1	45370902-45394332	+	LOC106607464	
IFNa3.1	45401524-45407691	-	LOC106607463	XP_014059915
IFNc4.11	45453809-45455053	+	LOC123743453	XP_045576589
IFNc4.10	45459992-45461235	+	LOC123743551	XP_045577268
IFNc4.9	45471814-45473005	+	LOC123743454	XP_045576590
IFNc4.8	45484287-45485529	+	LOC106594533	XP_014041375
IFNc4.7	45503055-45504297	+	LOC123743553	XP_045577269
IFNc4.6	45509267-45510509	+	LOC123743554	XP_045577270
IFNc4.5	45521663-45522814	+	LOC106597870	XP_014044467
IFNc4.4	45533225-45534390	+	LOC123743555	XP_045577271
IFNc4.3	45551212-45552377	+	LOC106607529	XP_014060035
IFNc4.2	45557520-45558685	+	LOC106594534	XP_045577272
IFNc4.1	45569003-45570154	+	LOC106607528	XP_014060034
IFNc3.1	45594238-45595213	+	LOC106607525	XP_014060031
GH2	45618189-45621918	-	LOC106607462	XP_014059912

Fig. 2. IFN-I genes in Chr6. Gene Id numbers, accession numbers and sequence location numbers were found in the NCBI Genbank. Or. = gene orientation where + and – indicate forward and reverse direction, respectively. GH2 = Growth hormone 2, Rdm1 = RAD52 motif cont. 1, Gbgt1 = Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1.

Gene	Location	Or.	Gene Id	Protein acc.no.
IFNd	61391384..61414828	+	LOC101448041	NP_001266021.1
cd79b	61414831..61420232	-	LOC106579125	
scn4aa	61442081..61517749	-	LOC106579124	

Fig. 3. IFNd gene in Chr19. Gene Id numbers, accession numbers and sequence location numbers were found in the NCBI Genbank. Or. = gene orientation where + and – indicate forward and reverse direction, respectively. cd79b = CD79b molecule, immunoglobulin-associated beta, scn4aa = sodium channel, voltage-gated, type IV, alpha a.

infected cells transfected with empty vector.

2.8. Statistical analyses

Two-sided unpaired Student *t*-test was used to calculate statistics, where $p \leq 0.05$ was considered to indicate a statistically significant difference.

3. Results and discussion

3.1. Identification of IFN-I genes in Chr3 and Chr6

IFN-I genes identified in Chr3 and Chr6 are depicted in Fig. 1 and Fig. 2, respectively. The GH1 locus of Chr3 harbors 32 complete IFN-I genes: 9 IFNa genes, 5 IFNb genes, 6 IFNc genes, 11 IFNe genes and 1 IFNf gene. Meanwhile, the GH2 locus on Chr6 contains 14 IFN-I genes: 1 IFNa gene, 12 IFNc genes and 1 IFNf gene. IFNd is associated with the CD79b gene on Chr19 (Fig. 3) as previously observed [7,17]. No IFN-I genes were detected on other chromosomes or unplaced scaffolds. The data thus suggest that the Atlantic salmon genome encompasses a total of 47 IFN-genes: 10 IFNa genes, 5 IFNb genes, 18 IFNc genes, 1 IFNd gene, 11 IFNe genes and 2 IFNf genes. A phylogenetic tree illustrating the relationship between previously and newly identified IFN-I proteins is shown in Fig. 4.

The abundance of IFN-I genes in the Atlantic salmon genome poses a challenge regarding nomenclature. Originally, we employed Roman letters to designate IFN-I subtypes, which this still appears practical [9]. However, numbering IFNs within each subtype becomes problematic due to the multitude of genes. Previously, we numbered IFNa, IFNb and IFNc genes based on their positions on BAC clone sequences [9]. Here, we adopt a different numbering system where the first number corresponds to the subcluster to which the IFN-I subtype belongs in the phylogenetic tree (Fig. 4), and the second number distinguishes individual IFNs within each subcluster. To minimize confusion with our previous works, we retain IFNa1 as IFNa1.1, IFNa2 as IFNa2.1, IFNa3 as IFNa3.1, IFNb1 as IFNb1.1 and IFNc1 as IFNc1.1. The numbering system proposed by Liu and colleagues assigns the first number according to the locus (1 or 2) to which the IFN belongs [12]. Except in Fig. 4, we refrain from using the locus or chromosome number to simplify the nomenclature.

The order GH1-IFNc-IFNc-IFNb-IFNa3-IFNc-IFNb-IFNc and the order IFNb-IFNe-IFNa from the BAC clones correspond to the order found in Chr3 (Fig. 1). Notably, the IFNa3 gene in the BAC clone (accession no. EU621898) was interrupted by a GA insert in exon 3 and was therefore excluded from the GH1 gene map displayed previously [9]. Additionally, reexamination revealed the presence of IFNe in the previously studied BAC clone sequence (accession no. EU768890). Discrepancies exist in the individual IFN protein sequences between the BAC clones and their counterparts in Chr3, possibly due to allelic variations or assembly issues with very similar IFN genes.

Comparison of Figs. 1 and 2 reveals the same order of the following genes in Chr3 and Chr6: Growth hormone (GH), IFNc, IFNa, IFNf and globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 (Gbgt1). Furthermore, incomplete IFNb and IFNe genes were identified at the GH2 locus (see Sections 3.3 and 3.5). This supports the notion that an ancestral chromosome containing GH, IFNa, IFNb, IFNc, IFNe and IFNf underwent duplication during the salmonid specific fourth round vertebrate whole-genome duplication (Ss4R-WGD), which occurred

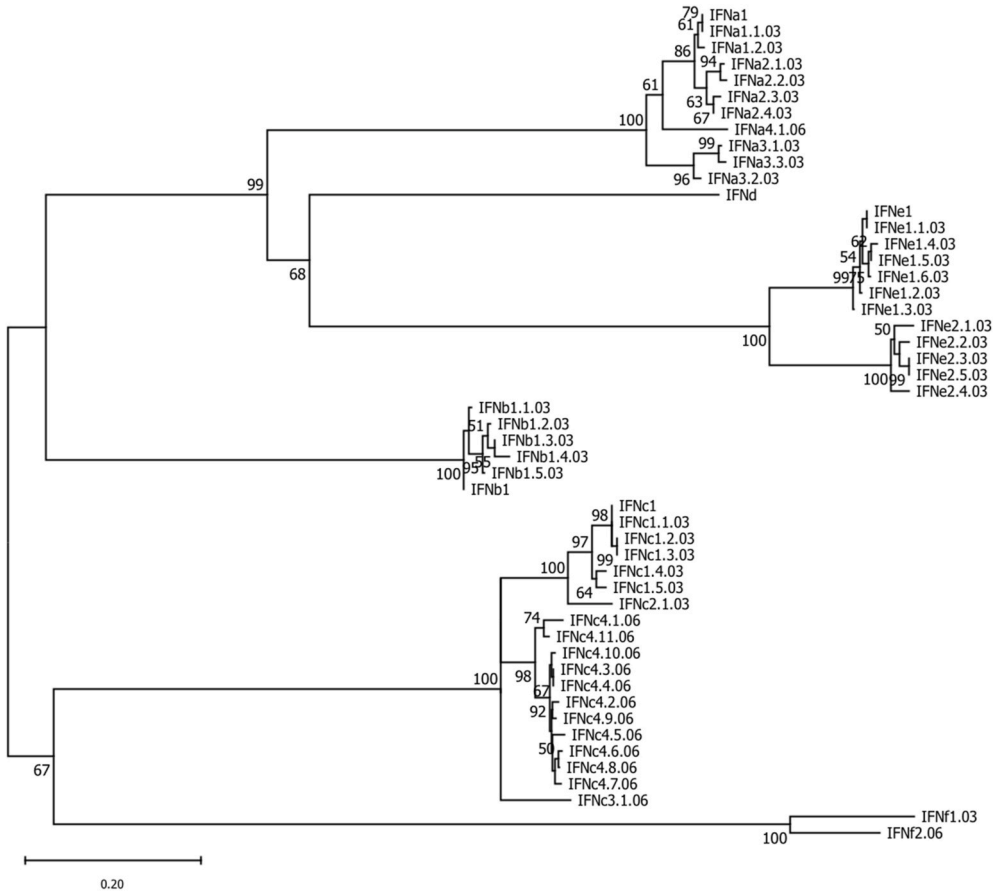

Fig. 4. Unrooted phylogenetic tree of Atlantic salmon type I IFNs. IFNs identified in Chr3 (Fig.1) are added 03 at the end of the name, while IFNs identified in Chr6 (Fig.2) are added 06 at the end of the name. IFNa1, IFNb1, IFNc1 and IFNe1 are IFN-I proteins previously identified in a BAC clone [9]. The tree was constructed from a multiple alignment of protein sequences using the Neighbor-joining method in the MEGA11 program. The optimal tree is shown. The percentage of replicate trees in which the associated IFNs clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Values less than 50 % are not shown. The evolutionary distances are in units of the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 215 positions in the final dataset.

about 80 million years ago [31]. Subsequently, intact IFNb and IFNe genes were lost at the GH2 locus as suggested previously [12]. Several duplications of IFN genes have since occurred in both Chr3 and Chr6. Blocks of IFNa-IFNe-IFNe and IFNb-IFNc appear to have undergone several duplications at the GH1 locus. In contrast, a single IFNc gene appears to have undergone multiple duplications at the GH2 locus.

3.2. IFNa genes

Previous studies have indicated that Atlantic salmon harbors at least three distinct IFNa genes named IFNa1, IFNa2 and IFNa3 linked to GH1 [9,13,32]. In the current investigation, we identified nine IFNa genes in the GH1 locus of Chr3 and one IFNa gene in the GH2 locus of Chr6. A comparison of IFNa proteins in Chr3 and Chr6 with previously identified IFNa1 is illustrated in the phylogenetic tree (Fig. 4). Gene classification into IFNa1, IFNa2 and IFNa3 was conducted based on a Clustal W alignment (Fig. 5). The percentage sequence identity between the IFNa proteins is presented in Suppl. Table 1. In the phylogenetic tree (Fig. 4), the IFNa proteins at the GH1 locus of Chr3 form two main clusters. The larger cluster comprises two IFNa1 proteins (a1.1, a1.2) and 4 IFNa2 proteins (a2.1, a2.2, a2.3 and a2.4), while the other cluster comprises three IFNa3 proteins. IFNa4.1 from Chr6 forms a single branch and has 82–89 % sequence identity with IFNas in Chr3. The previously identified IFNa1 exhibits 100 % sequence identity with IFNa1.1 and the previous IFNa2 shares 100 % identity with IFNa2.1. IFNa1 and IFNa2 proteins exhibit 95–97 % sequence identity. The differentiation between IFNa1 and IFNa2 can be questioned, but we retained this nomenclature to maintain consistency with previous research. The previous IFNa3 displays 99 % sequence identity with IFNa3.1 indicating it is likely an allelic variant. The IFNa in Chr6 is designated IFNa4.1, showing greater similarity to IFNa1 and IFNa2 than to IFNa3 proteins. The primary distinction between IFNa3 and IFNa1/IFNa2 lies in the C-terminal region with IFNa3 featuring FKAKIH or FKAN, while IFNa1/IFNa2 exhibit LAAQMY. IFNa4.1 possesses the C-terminal LAGQMY. Incomplete IFNa genes were also identified in Chr3 and Chr6 but were not further investigated.

3.3. IFNb genes

The current study reveals the presence of 5 complete IFNb genes (IFNb1.1-IFNb1.5) at the GH1 locus of Chr3 (Fig. 1). A comparison of IFNb proteins in Chr3 and the previously identified IFNb1 is shown in the phylogenetic tree (Fig. 4), Clustal W alignment (Fig. 6) and as percent sequence identity between the IFNb proteins (Suppl Table S2).

The IFNb proteins exhibit a high degree of sequence similarity, with identities ranging from 96 to 99 %. The previous IFNb1 shares 99 % identity with IFNb1.1, suggesting it is likely an allelic variant of b1.1. Although no complete IFNb genes were detected in Chr6, a TBLASTN search with IFNb revealed several IFNb exon 5 sequences in Chr6 (not shown), indicating the presence of IFNb before the Ss4R-WGD.

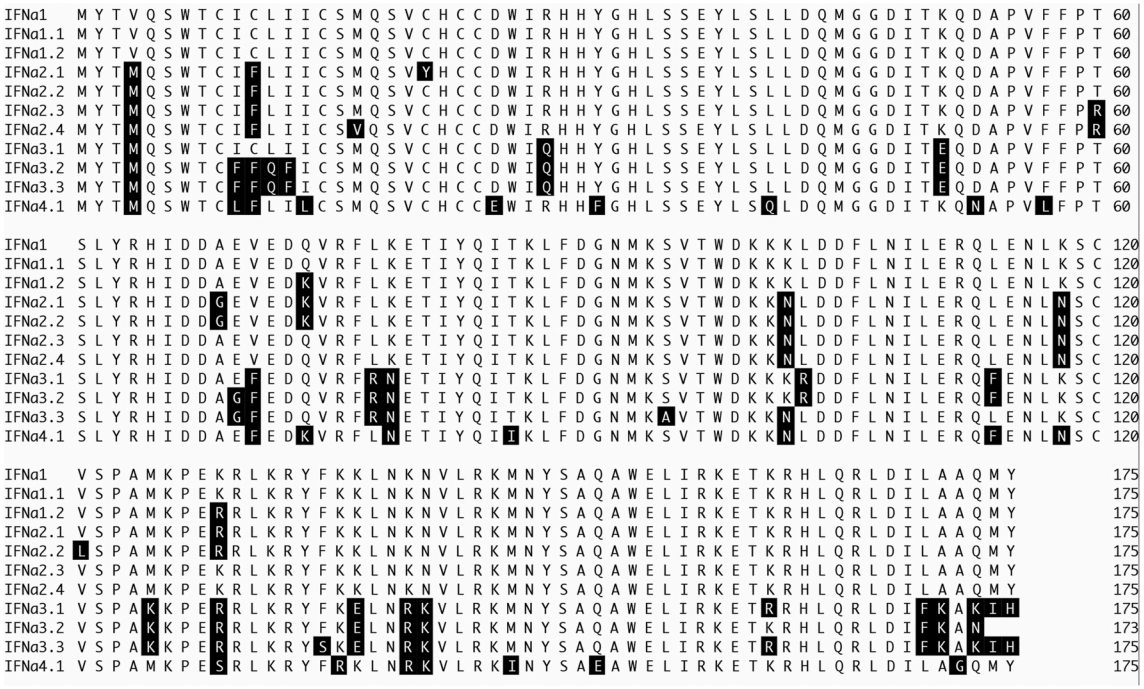

Fig. 5. Multiple alignment of IFNa proteins encoded by Chr3 (Fig.1) and Chr6 (Fig.2) compared with the previously identified IFNa1 protein. Amino acids that are identical with those of IFNa1 are shaded with black.

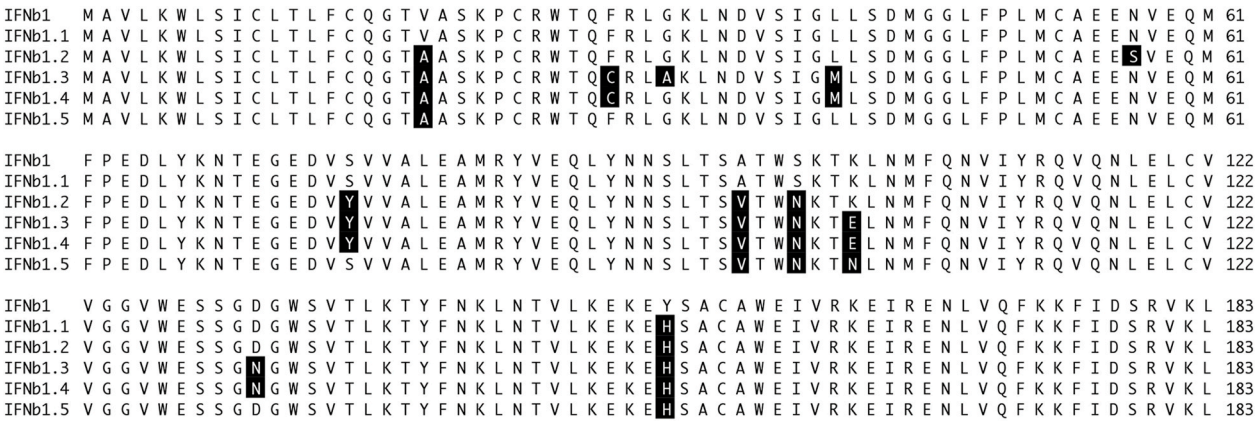

Fig. 6. Multiple alignment of IFNb proteins encoded by Chr3 (Fig.1) compared with the previously identified IFNb1 protein. Amino acids that are identical with those of IFNb1 are shaded with black.

3.4. IFNc genes

Six IFNc genes were identified in the GH1 locus of Chr3, while 12 IFNc genes were found in the GH2 locus of Chr6 (Figs. 1 and 2). The encoded IFNcs alongside the previously known IFNc1, are compared in the phylogenetic tree (Fig. 4). The tree delineates two significant clusters of IFNc genes: one in Chr3 (IFNc1.1-IFNc1.5) and another in Chr6 (IFNc4.1-IFNc4-11). Furthermore, IFNc2.1 in Chr3 and IFNc3.1 in in Chr6 form distinct branches. The IFNc proteins are compared in the Clustal W alignment (Fig. 7) and as percent sequence identity (Suppl Table S3). All IFNc consists of 187 amino acids, except for IFNc2.1, which comprises 186 amino acids and IFNc4.2, which contains 195 amino acids. The previously identified IFNc1 shares 100 % sequence identity with IFNc1.1 in Chr3. IFNc proteins from Chr3 exhibit a high degree of sequence similarity ranging from 96 to 99 %, except for IFNc2.1, which shows 90–92 % sequence identity with the other IFNc proteins encoded by Chr3. Similarly, IFNc4.1 to IFNc4.11 proteins encoded by Chr6 share considerable similarity, with sequence identities ranging from 93 to 97 %, while IFNc3.1 shows 86–87 % sequence identity with the other IFNc proteins encoded by Chr6. Moreover, IFNc proteins from Chr3 show 82–84 sequence identity with those of Chr6. The main difference between IFNc proteins encoded by Chr3 and Chr6 lies in their leader sequences.

3.5. IFNe genes

Examination of the GH1 locus of Chr3, revealed the presence of 10 complete IFNe genes, expressing IFNe belonging to two distinct clusters in the phylogenetic tree (Fig. 4). An alignment of IFNe members is depicted in Fig. 8. Members of the first cluster (IFNe1.1-IFNe1.5) share 95–99 % sequence identity (Suppl Table S4), while the members of the second cluster (IFNe2.1-IFNe2.5), show 95–100 % sequence identity. Notably, IFNe1 members exhibit 75–77 % sequence identity with IFNe2 members. Characteristic differences are observed in the C-terminal: IFNe1 members possess the sequence DTTLSSQ, whereas IFNe2 members have the sequence VTIPID. Reexamination of the previously studied

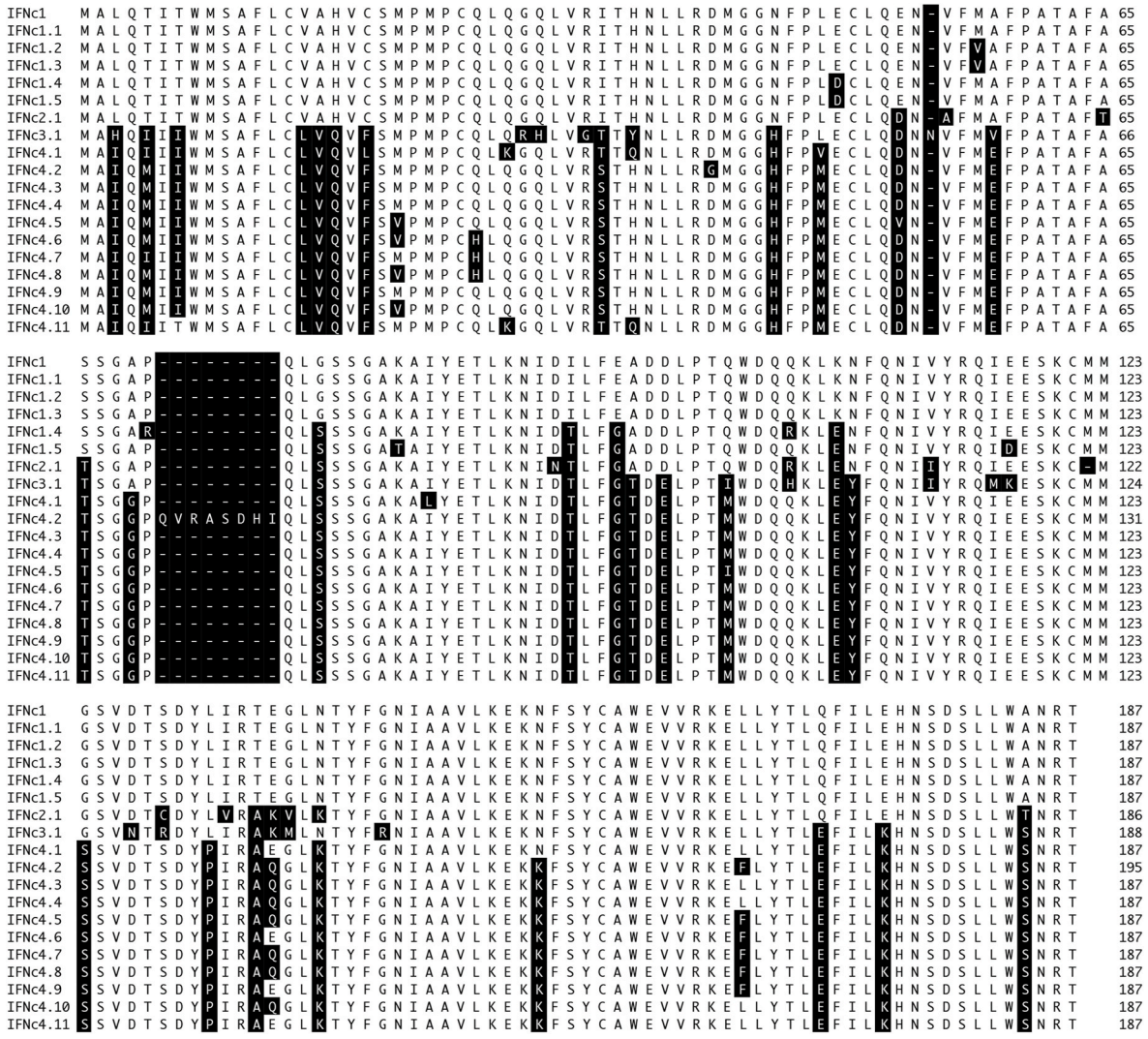

Fig. 7. Multiple alignment of IFNc proteins encoded by Chr3 (Fig.1) and Chr6 (Fig.2) compared with the previously identified IFNc1 protein. Amino acids that are identical with those of IFNc1 are shaded with black.

BAC clone sequence (EU768890) revealed the presence of one IFNe gene, named IFNe1, situated between the IFNa1 and IFNb1 gene and which encodes a protein sequence identical to IFNe1.1. The pattern of IFNe genes in Chr3 suggests that an ancestral IFNe gene has duplicated followed by duplication of blocks of IFNa1-IFNe1-IFNe2 genes.

In contrast to the GH1 locus, the GH2 locus only exhibit an IFNe pseudogene (Ensembl accession no. ENSSSAG00000100953), supporting the presence of IFNe before the Ss4R-WGD event.

3.6. *IFNf genes*

Two IFNf genes were found in the Atlantic salmon genome, IFNf1 at the GH1 locus of Chr3 and IFNf2 at the GH2 locus of Chr3 (Fig. 2). An alignment of IFNf1 and IFNf2 proteins is shown in Fig. 9, which demonstrates 79 % sequence identity between the two IFNs.

3.7. *Expression of IFNc, IFNe and IFNf genes*

To investigate the expression of IFNc, IFNe and IFNf genes, groups of Atlantic salmon presmolts were injected i.p. with PBS, poly I:C or R848. After 24 h, organs were harvested and expression of IFNs was assessed. In the initial experiment, we evaluated the expression of IFNc1.1 (representative of IFNc1.1-IFNc1.5); IFNc3.1; IFNc4.10 (representative of IFNc4.1 – IFNc4.11); IFNe1.1 (representative of IFNe1.1-IFNe1.6) and IFNe2.2 (representative of IFNe2.1-IFNe2.5) in response to poly I:C (Fig. 10A). As anticipated from previous research, poly I:C elicited strong expression of IFNc1.1 in heart and comparatively lower expression in head kidney [17]. IFNc4.10 exhibited similar expression to IFNc1.1, while IFNc3.1 displayed a much weaker increase in both organs. Both IFNe1.1 and IFNe2.2 demonstrated increased expression in the heart, but not in the head kidney. In the subsequent experiment, we examined the expression of IFNe2.2 and IFNf2 in various organs following injection of poly I:C and R848. As depicted in Fig. 10B, poly I:C induced a significant up-regulation of IFNf in all organs except intestine, with the highest increase observed in heart and head kidney. The primers designed for IFNf2 each exhibit only one mismatch for IFNf1 and are thus likely to amplify both IFNf cDNAs. Poly I:C did not induce significant up-regulation of IFNe in any organs except in heart. R848 had no significant effect on either IFNf or IFNe.

Taken together, the IFNc genes in the large cluster of Chr6 (IFNc4.1 – IFNc4.11) exhibited similar expression as IFNc genes in the main cluster of in Chr3 (IFNc1.1-IFNc1.5) in response to poly I:C, while IFNc3.1 in Chr6 showed a much weaker response. The pronounced increase in IFNc transcripts in response to poly I:C may partially result from the fact that the primers amplify cDNA from multiple genes in each cluster. Both IFNe1 and IFNe2 genes responded to poly I:C in heart, while no

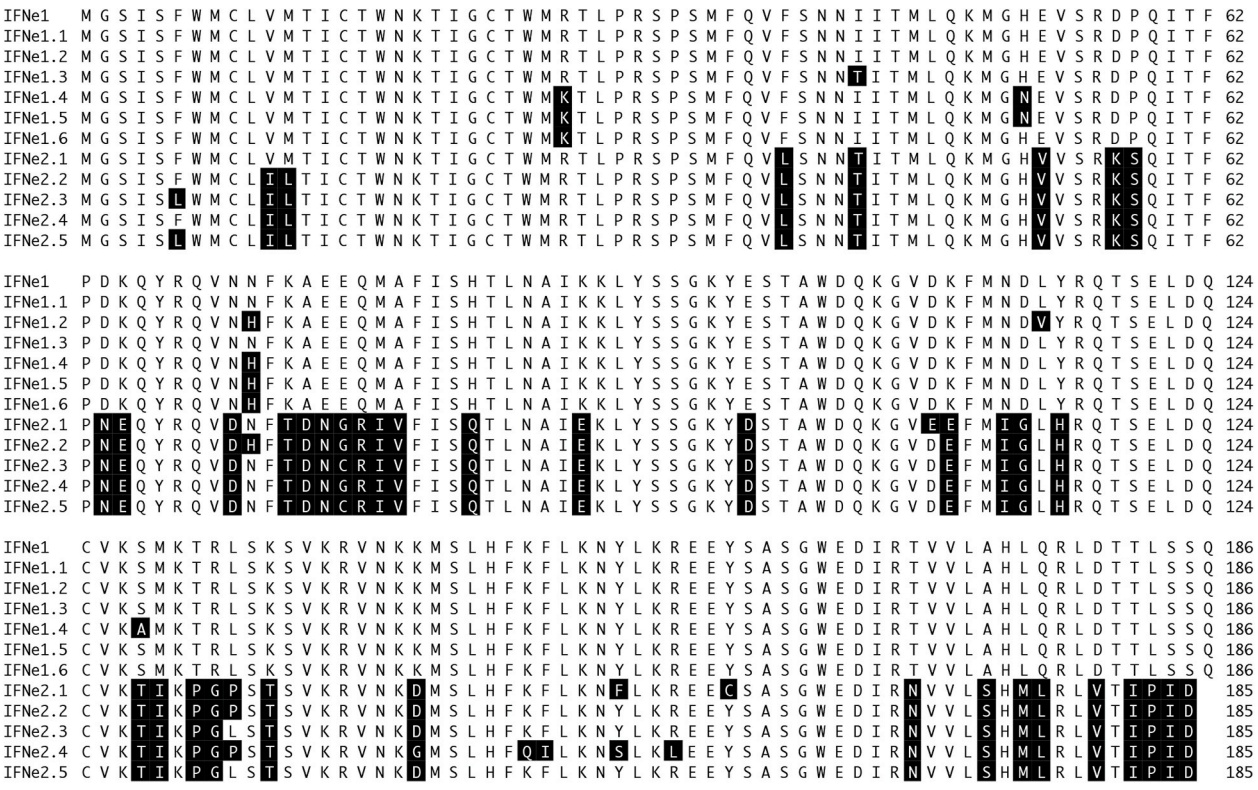

Fig. 8. Multiple alignment of IFNe proteins encoded by Chr3 (Fig.1) compared with the IFNe1 protein identified in a BAC clone [9]. Amino acids that are identical with those of IFNe1 are shaded with black.

Fig. 9. Alignment of IFNf1 and IFNf2 proteins encoded by Chr3 (Fig.1) and Chr6 (Fig.2), respectively. Amino acids in IFNf2 that are different from those of IFNf1 are shaded with black.

significant increase was observed for IFNe2 in other organs. The strong expression of IFNf and the weak expression of IFNe in response to poly I:C align with the observations in rainbow trout cell lines [14], indicating an essential role for IFNf in the first line of defense against RNA viruses in salmonids.

Despite the weak expression of IFNe in response to poly I:C and R848, the importance of IFNe in antiviral defense is supported by the presence of multiple copies of IFNe genes in salmonids. IFNe may play an important role in specialized cells or be induced through pathways other than the MDA5, TLR3, TLR7 or TLR22 pathways. This study underscores that IFNc genes in Chr6, IFNe genes and IFNf genes are all upregulated by poly I:C, suggesting induction via the viral RNA receptors MDA5, TLR3 or TLR22. The absence of up-regulation of IFNe and IFNf genes by R848, suggests that they are not induced via TLR7 or TLR8. So far, IFNb1 and IFNc1 in Chr3 are the only IFN genes demonstrated to be up-regulated by R848 in Atlantic salmon [17]. Unfortunately, the effect of R848 on IFNc genes in Chr6 was not studied in the present work.

3.8. Antiviral activity of novel IFNs

Plasmids expressing IFNc3.1, IFNc4.9, IFNe2.2 and IFNf2 under the control of the CMV promoter were made to investigate the antiviral activity of these IFNs. Transfection of HEK293 cells with the plasmids, following the methodology employed in previous studies of IFNa, IFNb and IFNc [17], failed to yield antiviral activity in medium supernatants (data not shown). This could possibly be attributed to HEK293 cells' inability to recognize the leader sequences or to improper folding and degradation of the IFNs. Consequently, Atlantic salmon ASK cells were transfected with the plasmids, and antiviral activity was assessed by measuring cell survival after infection with IPNV. A plasmid lacking IFN gene insert served as the control. The results indicated that IFNc3.1 and IFNc4.9 provided complete protection of the cells, whereas IFNe2.2 and IFNf2 exhibited weaker protective effect (Fig. 11). This confirms that IFNcs encoded by Chr6, IFNe and IFNf all possess antiviral activity. The difference in antiviral activity may be due to that ASK cells possess IFN receptors better suitable for IFNc than for IFNe and IFNf, or could be attributed IFNc being more efficiently produced following plasmid transfection of ASK cells.

3.9. In vivo induction of the Mx1 gene by IFNc3.1, IFNc4.9, IFNe2.2 and IFNf2

To further explore the antiviral potential of these IFNs, Atlantic salmon presmolts were injected i.m. with plasmids expressing IFNc3.1, IFNc4.9, IFNe2.2, or IFNf2. A plasmid expressing IFNc1.1 (previously named IFNc1) served as a positive control, as it was previously demonstrated to induce antiviral genes both at the muscle injection site

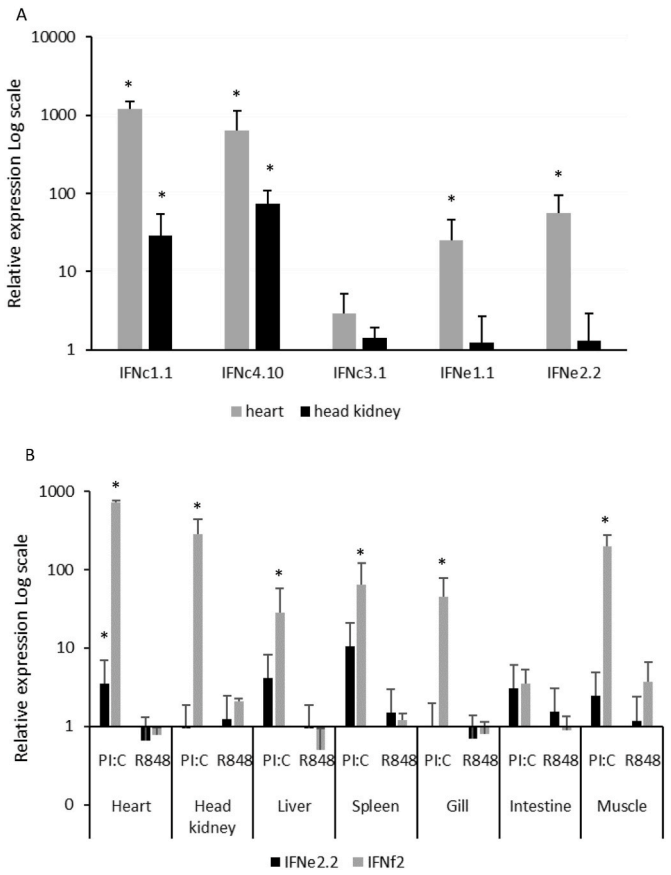

Fig. 10. Expression of IFNc, IFNe and IFNf in organs of Atlantic salmon injected with poly I:C or R848. Groups of Atlantic salmon presmolts (n = 5) were injected with 10 mg/kg poly I:C, 5 mg/kg R848, or PBS. Expression of IFNs were measured in organs by RT-qPCR 24 h after injection. Bars show fold increase in transcripts (mean ± SD) compared to PBS injected fish. A. Fold up-regulation of IFNc1.1, IFNc3.1, IFNc4.10, IFNe1.1 and IFNe2.2 in heart and head kidney after injection of poly I:C compared to PBS. B. Fold up-regulation of IFNf2 and IFNe2.2 in various organs after injection of poly I:C or R848. Significant up-regulation ($p < 0.05$) compared to PBS-injected fish is indicated with a star (*).

and in internal organs [20]. Seven days post-injection, samples were collected from the muscle injection site and internal organs to measure expression of the antiviral Mx1 gene by RT-qPCR. Mx1 was selected because it is a classical IFN-I induced gene [33]. As shown in Fig. 12, IFNc1.1, IFNc3.1, IFNc4.9, IFNe2.2 and IFNf2 all elicited up-regulation of the Mx1 gene in the muscle, indicating their antiviral activity. Notably, only IFNc1.1 and IFNc4.9 induced Mx1 expression in the liver. This demonstrates functional similarity between IFNc1s of Chr3 and IFNc4s of Chr6 in eliciting systemic Mx1 induction in salmon. Conversely, IFNc3.1 from Chr6, IFNe2.2 and IFNf2 did not trigger systemic induction of Mx1.

3.10. Concluding remarks

The abundance of IFN-I genes in the Atlantic salmon genome stems from the salmonid specific whole-genome duplication and the substantial expansion of IFNc genes in Chr3 and Chr6 as well as IFNe genes in Chr3. Conversely, IFNe and IFNb genes have been lost from Chr6, a phenomenon expected in the process of rediploidization following whole genome duplication.

The evolution of multiple IFN-I genes in Atlantic salmon may be driven by its anadromous lifestyle, involving encounters with various pathogenic viruses. Atlantic salmon undergoes a complex lifecycle, hatching in rivers, transitioning between freshwater habitats, subsequently adapting to sea water and migrating through estuaries to the sea feeding in distant ocean habitats before it returns to the rivers to spawn [34]. Salmon farming history has revealed susceptibility to numerous pathogenic RNA viruses [7,35]. Smoltification and spawning, which reduce fish immunity, may also have influenced the evolution of the complex IFN-I system in Atlantic salmon and other anadromous salmonids [36,37]. Further genomic sequencing of other salmonids would clarify whether Atlantic salmon's IFN-I gene count is unique and whether anadromous salmonids harbor more IFN-I genes than non-anadromous counterparts.

The functional significance of numerous IFN-I subtypes and the high gene count within each subtype in Atlantic salmon and other salmonids is beginning to be understood. Multiple genes within each IFN subtype may confer a stronger antiviral response due to increased IFN protein production. Evidence suggests that the different IFN-I subtypes possess both shared and distinct properties regarding induction, antiviral activity and cell-specific expression [17]. Different viruses may induce different IFN-I subtypes, as suggested by their varied induction by poly I:C and R848, mimicking double-stranded and single-stranded virus RNA,

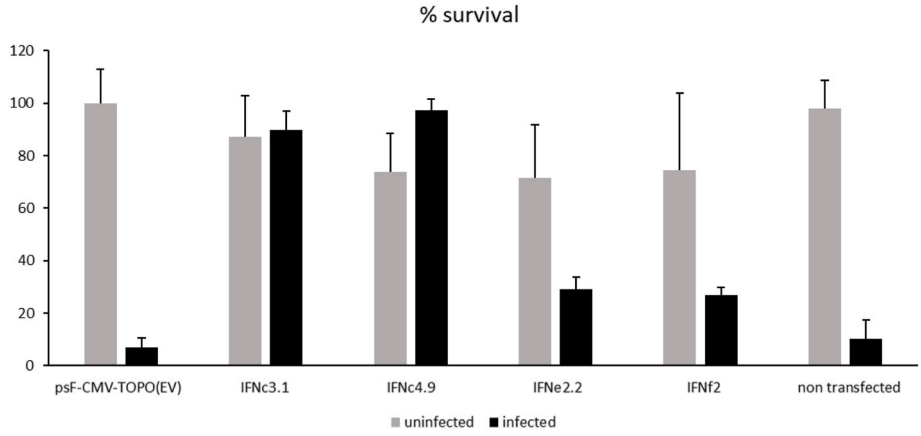

Fig. 11. Antiviral activity of IFNc3.1, IFNc4.9, IFNe2.2 and IFNf2 in ASK cells.
Cells were seeded in 96-well plates and transfected with expression plasmids encoding IFNc3.1, IFNc4.9, IFNe2.2 or IFNf2, or plasmid without insert (pSF-CMV-TOPO). Eight wells were transfected with each plasmid (95 ng/well). Seventy-two hours post transfection, four of the wells were infected with IPNV (MOI 1) and incubated for 8 days resulting in near complete lysis of cells transfected with control plasmid. Cell survival was measured by crystal violet staining (OD at 550 nm) where 100 % represents non-infected cells transfected with empty vector. Values are mean±SD (n = 4). Differences between survival of infected cells transfected with plasmid without insert and infected cells transfected with IFN-plasmids were significant ($p < 0.05$).

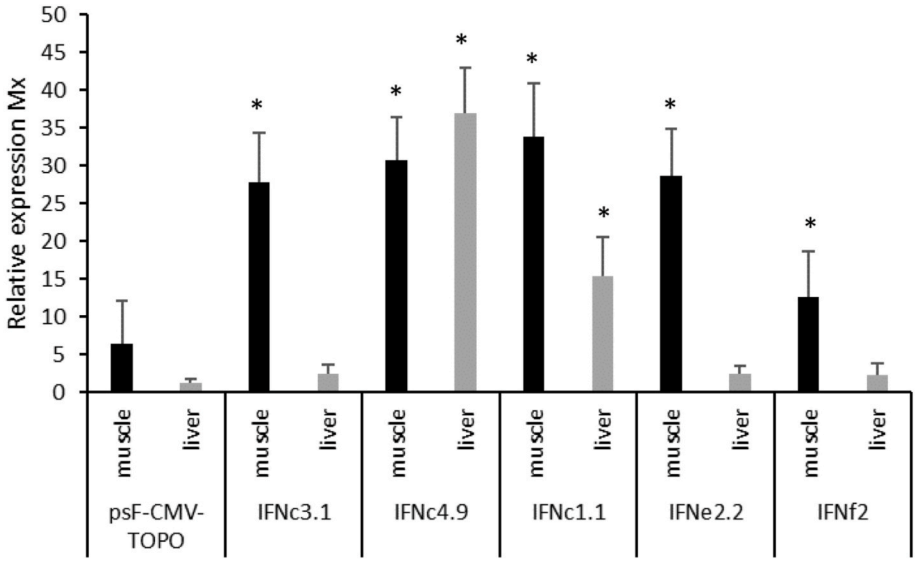

Fig. 12. Expression of the Mx1 gene in response to IFNc1.1, IFNc3.1, IFNc4.9, IFNe1.1, IFNe2.2 and IFNf2 in live Atlantic salmon. Groups of salmon presmolts (n = 5) were injected i.m. with PBS, control plasmid without insert (psF-CMV-TOPO) or psF-CMV-TOPO plasmids expressing IFNc1.1, IFNc3.1, IFNc4.9, IFNe2.2 and IFNf2. Seven days later muscle at the injection site and liver were sampled for measurement of Mx1 expression by RT-qPCR. Fold increase in Mx1 transcription (mean ± SD) is presented relative to PBS injected fish. Statistically significant differences ($p < 0.05$) between IFN plasmid groups and control plasmid group are indicated with a star (*).

respectively, as shown in current and previous studies [7,17]. Present research confirms the antiviral activity of IFNc encoded by Chr6, IFNe and IFNf as previously demonstrated for IFNa, IFNb and IFNc encoded by Chr3 [7,17]. However, antiviral properties of Atlantic salmon IFNd have not been identified [17]. Similar to IFNa [17], IFNe and IFNf appear to induce antiviral genes locally but not systemically. In contrast, the major IFNcs from both Chr3 and Chr6 induce antiviral genes systemically. Previous research even indicates that injection of Atlantic salmon with an IFNc1-expressing plasmid protects against infectious salmon anemia virus infection for at least 8 weeks [20]. Coupled with the abundance of IFNc genes, this suggests a prominent role for IFNcs in salmon innate immunity against viruses.

The presence of six IFN-I subtypes raises questions about whether they signal through the same or different IFN-I receptors as discussed previously [7,38]. Atlantic salmon appears to possess more IFN-I receptors than zebrafish, which have two receptors [39]. Different cell types may express distinct IFN-I receptors, implying some level of cell specificity in induction of antiviral genes by different IFN-I subtypes. Such diversity could be advantageous, as widespread induction of antiviral genes in all cell types might be detrimental to fish health.

Lastly, it is plausible that some IFN-I subtypes have functions beyond antiviral activity. For instance, IFNa, IFNb and IFNc, have been shown to enhance the adaptive immune response against infectious salmon anemia virus in Atlantic salmon when co-injected as expression plasmids with a DNA vaccine against the virus [6]. Whether IFNd, IFNe or IFNf possesses similar adjuvant properties remains to be explored.

CRediT authorship contribution statement

Børre Robertsen: Conceptualization, Data curation, Investigation, Project administration, Writing – original draft, Writing – review & editing. **Linn Greiner-Tollersrud:** Data curation, Investigation, Writing – review & editing.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109694.

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