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Abstract

Mx proteins are antiviral GTPases, which are induced by type I IFN and virus infection. Analysis of the Atlantic salmon genome revealed the presence of 9 Mx genes localized to three chromosomes. A cluster of three Mx genes (SsaMx1 – SsaMx3), which includes previously cloned Mx genes, is present on chromosome (Chr) 12. A cluster of five Mx genes (SsaMx4-SsaMx8) is present on Chr25 while one Mx gene (SsaMx9) is present on Chr9. Phylogenetic and gene synteny analyses showed that SsaMx1-SsaMx3 are most closely related to the main group of teleost Mx proteins. In contrast, SsaMx 4-SsaMx9 formed a separate group together with zebrafish MxD and MxG and eel MxB. The Mx cluster in Chr25 showed gene synteny similar to a Mx gene cluster in the gar genome. Expression of Mx genes in cell lines stimulated with recombinant IFNs showed that Mx genes in Chr12 responded more strongly to type I IFN than to type II IFN (IFN gamma) whilst Mx genes in Chr25 responded more strongly to IFN gamma than to type I IFNs. SsaMx9 showed no response to the IFNs.

Keywords	Antiviral; Evolution; Interferon; Innate immunity; Fish; Mx
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Dear Editor

Enclosed is a manuscript by Børre Robertsen, Linn Greiner-Tollersrud and Lars Gaute Jørgensen, entitled "**Analysis of the Atlantic salmon genome reveals a cluster of Mx genes that respond more strongly to IFN gamma than to type I IFN**", which is being submitted for possible publication in Developmental and Comparative Immunology. This work showed that Atlantic salmon possesses three clusters of Mx genes on different chromosomes. A cluster of three Mx genes (SsaMx1 – SsaMx3) is present on chromosome (Chr) 12. Two of these genes have been cloned and studied in a previous work. A cluster of five Mx genes (SsaMx4-SsaMx8) was found on Chr25 while one Mx gene (SsaMx9) was found on Chr9. In this work we have studied the evolution of these Mx genes and studied their expression in response to type I IFN and type II IFN (IFN gamma). As expected for Mx-genes, SsaMx1-SsaMx3 responded more strongly to type I IFN than to IFN gamma. Surprisingly, however, SsaMx4-SsaMx8 responded more strongly to IFN gamma than to type I IFN, which is a novel feature of Mx genes.

Sincerely yours,

Prof. Børre Robertsen

Highlights salmon Mx genes

1. Atlantic salmon was shown to possess 9 Mx genes located on three chromosomes: Chr12 (SsaMx1-SsaMx3), Chr25 (SsaMx4-SsaMx8) and Chr9 (SsaMx9).
2. Phylogenetic and gene synteny analyses showed that SsaMx1-SsaMx3 belong to the main group of teleost Mx genes.
3. SsaMx4-SsaMx9 formed a separate group together with zebrafish MxD and MxG and eel MxB.
4. Expression studies showed that Mx genes in Chr12 responded more strongly to type I IFN than to IFN gamma while Mx genes in Chr25 responded more strongly to IFN gamma than to type I IFNs.
5. SsaMx9 showed no response to IFNs.

Title: Analysis of the Atlantic salmon genome reveals a cluster of Mx genes that respond more strongly to IFN gamma than to type I IFN

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Abstract

Mx proteins are antiviral GTPases, which are induced by type I IFN and virus infection. Analysis of the Atlantic salmon genome revealed the presence of 9 Mx genes localized to three chromosomes. A cluster of three Mx genes (SsaMx1 – SsaMx3), which includes previously cloned Mx genes, is present on chromosome (Chr) 12. A cluster of five Mx genes (SsaMx4-SsaMx8) is present on Chr25 while one Mx gene (SsaMx9) is present on Chr9. Phylogenetic and gene synteny analyses showed that SsaMx1-SsaMx3 are most closely related to the main group of teleost Mx proteins. In contrast, SsaMx 4-SsaMx9 formed a separate group together with zebrafish MxD and MxG and eel MxB. The Mx cluster in Chr25 showed gene synteny similar to a Mx gene cluster in the gar genome. Expression of Mx genes in cell lines stimulated with recombinant IFNs showed that Mx genes in Chr12 responded more strongly to type I IFN than to type II IFN (IFN gamma) whilst Mx genes in Chr25 responded more strongly to IFN gamma than to type I IFNs. SsaMx9 showed no response to the IFNs.

Keywords**:** Antiviral; Evolution; Interferon; Innate immunity; Fish; Mx

Abbreviations

Aa, amino acids; Chr, chromosome; GAS, gamma-activated sequence; IFN, interferon; ISG, interferon stimulated gene; ISRE, interferon-stimulated signalling element; Mx, myxovirus resistance; nt, nucleotides; RT-qPCR, reverse transcription quantitative PCR

1. Introduction

Mx proteins are antiviral GTPases, which play an important role in innate antiviral immunity of vertebrates (Haller et al., 2015; Verhelst et al., 2013). They were first discovered in influenza resistant mice. This resistance was shown to be inherited as a single dominant trait named Mx1+, for myxovirus resistance, and is dependent on a single gene encoding the Mx1 protein (Horisberger et al., 1983). Mx proteins have since been found in most vertebrates and are typically induced by type I IFN, double-stranded RNA (dsRNA) and virus infection (Robertsen, 2018; Verhelst et al., 2013). While Mx proteins are highly conserved, they show antiviral specificity between species and are either localized to the cytoplasma or the nucleus (Haller et al., 2015; Verhelst et al., 2013). Mouse Mx1 protein is localized in the nucleus and mainly inhibits orthomyxoviruses. In contrast, human MxA protein is localized in the cytoplasm and inhibits a variety of RNA viruses. Although the antiviral mechanisms of Mx proteins are yet not fully understood, crystallographic evidence suggests that mammalian Mx proteins form tubular aggregates, which trap virus nucleocapsids resulting in inhibition of transcription of the virus genome (Haller et al., 2010). While mammals possess 1-3 Mx genes, fish possess 0-9 Mx genes dependent on species (Solbakken et al., 2016; Verhelst et al., 2013). Some fish species such as Atlantic cod in fact lack Mx genes (Solbakken et al., 2016). Fish Mx proteins were first characterized in rainbow trout, which was shown to possess three Mx proteins, named RBTMx1, RBTMx2 and RBTMx3 (Trobridge et al., 1997; Trobridge and Leong, 1995). RBTMx1 and RBTMx3 are localized in the cytoplasm while RBTMx2 is localized in the nucleus. Antiviral activity of fish Mx proteins was first established for Atlantic salmon Mx1 protein against IPNV and has since been demonstrated for Mx proteins from several fish species against various virus types (Alvarez-Torres et

al., 2013; Caipang et al., 2003; Larsen et al., 2004; Lester et al., 2012; Lin et al., 2006; Wu et al., 2010). Atlantic salmon was originally found to possess three Mx proteins named ASMx1, ASMx2 and ASMx3 where ASMx1 and ASMx2 have 96 % sequence identity and show strong homology to RBTMx1 while ASMx3 is homologous to RBTMx3 (Robertsen et al., 1997; Trobridge and Leong, 1995). In this work we screened the Atlantic salmon genome for Mx genes and found that salmon possesses 9 Mx genes localized to three chromosomes. A cluster of three Mx genes is present on chromosome (Chr) 12 and includes the previously cloned salmon Mx genes. A cluster of five Mx genes is present on Chr25 while one Mx gene is present on Chr9. We have compared the salmon Mx genes and the proteins sequences and analysed their evolution. Moreover, we have demonstrated that the three groups of salmon Mx proteins have different expression properties in response to type I IFN and type II IFN. Mammalian type I IFNs signal through a heterodimeric receptor composed of the IFNAR1 and IFNAR2 chains (Stark et al., 1998). This results in phosphorylation and dimerization of signal transducer and activator of transcription (STAT) 1 and STAT2 proteins, which interacts with IRF9 to form transcription factor ISGF3. Subsequently, ISGF3 translocates into the nucleus and activates transcription of hundreds of IFN-stimulated genes (ISGs) by binding to the interferon-stimulated signalling element (ISRE). Type II IFN is identical to IFN gamma (IFNg), which signals through another heterodimeric receptor resulting in phosphorylation and dimerization of STAT1 only (Stark et al., 1998). The STAT1 homodimer typically activates gene transcription by binding to gamma-activated sequences (GAS). Accordingly, type I IFNs and IFNg show different patterns of gene activation. The ISRE consensus sequence is present in the promoter of both mouse Mx1 and rainbow trout Mx1 (Collet and Secombes, 2001; Hug et al., 1988). The predominant Atlantic

88 salmon type I IFNs are IFNa, IFNb and IFNc, which all induce the salmon Mx1 gene
89 (Svingerud et al., 2012). Atlantic salmon Mx1 is induced much more strongly by
90 IFNa than IFNg (Sun et al., 2011). Surprisingly, the present work shows that Mx
91 genes encoded by Chr25 are more strongly induced by IFNg than by IFNa.
92
93 **2. Materials and methods**
94 *2.1. Bioinformatics*
95 All sequences annotated as Mx were extracted from the Atlantic salmon genome
96 (NCBI Reference Sequence Database (RefSeq) assembly accession:
97 GCF_000233375.4). To identify Mx genes in the Atlantic salmon genome,
98 TBLASTN using the salmon ASMx1 sequence (GenBank accession U66475) as
99 query was performed against Atlantic salmon chromosomes in the NCBI database.
100 Nuclear localization signal (NLS) in salmon Mx proteins was predicted using the
101 NucPred program at http://www.sbc.su.se/~maccallr/nucpred/. Multiple alignment of
102 the salmon Mx genes was performed with the ClustalW method in the MegAlign
103 program (DNASTAR, Inc.). The alignment was used to obtain sequence distances (%
104 identity). Phylogenetic analysis of vertebrate Mx genes was performed by multiple
105 alignment of sequences using Clustal W in the MEGA7 program (Kumar et al., 2016).
106 A phylogenetic tree was the constructed from the alignment using the Neighbor-
107 joining method (Kumar et al., 2016; Saitou and Nei, 1987).
108
109 *2.2. Cells*
110 ASK cells derived from Atlantic salmon (*Salmo salar* L.) head kidney (Devold et al.,
111 2000) were purchased from American Type Culture Collection. SSP-9 cells derived
112 from head kidney of Atlantic salmon were obtained from Dr. Perez-Prieto (Centro de

Investigaciones Biológicas, CSIC, C/Ramiro de Maeztu 9, 28040, Madrid, Spain) (Rodriguez Saint-Jean et al., 2014). Both cell lines 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 (Biochrom AG).

2.3. Stimulants

Poly I:C (polyinosinic polycytidylic acid) was obtained from GE Healthcare Life Sciences. Recombinant IFNa and IFNc were produced in HEK293 cells as described (Svingerud et al., 2012). IFNg was produced in *E. coli* (Sun et al., 2011).

2.4. Stimulation of cells

SSP-9 cells ($1.2x10^5$ cells/well) and ASK cells (10^5 cells/well) were seeded in 1 ml medium in 24 well culture plates. Cells in triplicate wells were stimulated extracellularly with 10 µg/ml poly I:C, 1000 U/ml IFNa, 1000 U/ml IFNc or 1 ng/ml IFNg for 24 and 48 h. Cells were stimulated intracellularly with 1 µg/ml poly I:C for 24 and 48 h using FuGene HD transfection Reagent according to the the manufacturers (Promega).

2.5. Gene expression analysis by reverse transcription quantitative PCR (RT-qPCR)

RNA was isolated from cells using RNeasy Mini Kit (Qiagen) by lysing cells in each well with 350 µl RLT buffer and extracting RNA as described by the manufacturer. cDNA was synthesized with the QuantiTect Reverse Transcription Kit (Qiagen) starting with 100 ng total RNA following standard protocol. qPCR was performed using 6.0 µl 1:5 dilution of cDNA in a 15-µl reaction mixture containing 7.5 µl Fast

SYBR® Green Master Mix (Thermo Fisher Scientific) 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 running melting curve step. Relative expression values were normalized against the levels of Elongation Factor 1αB (EF1αB) mRNA. Fold increase of the representative genes was calculated by comparison of gene expression in treated versus untreated cells. Relative expression of Mx genes was calculated by the Pfaffl method using EF1αB as a reference gene (Pfaffl, 2001). Data were calculated from triplicates of three samples in each group, and expressed as mean ± standard errors. The primers used in RT-qPCR are listed in Table 1. Unpaired t-test with two-tail distribution was used for statistical analysis, $p \leq 0.05$.

3. Results

3.1. Identification of Mx genes in the Atlantic salmon genome

To identify Mx genes in the Atlantic salmon genome, TBLASTN using the salmon ASMx1 gene as query, was performed against Atlantic salmon chromosomes (Chr) in the NCBI GenBank database. The search resulted in identification of three Mx genes in Chr12, five Mx genes in Chr25 and one Mx gene in Chr9. All genes and the deduced proteins are listed in Table 2. Chr12 contains one Mx gene, which encodes a protein corresponding to the previously cloned ASMx1 and ASMx2 proteins (Robertsen et al., 1997). This confirms that they are encoded by alleles of the same gene rather than separate genes. We propose to name the encoded protein SsaMx1,

which is homologous to rainbow trout (RBT) Mx1 (Accession no. AAA87839). Another Mx gene in Chr 12 encodes a protein corresponding to ASMx3, which is homologous to RBTMx3 (Accession no. AAC60215). We propose to name this protein SsaMx3. The third Mx gene in Chr 12 encodes a protein homologous to the RBTMx2 protein (Accession no. AAC60214), and has not been identified before. We propose to name this gene SsaMx2. While SsaMx1 and SsaMx3 both contain 623 amino acids (aa), SsaMx2 contains 638 aa due to an insert as described for RBTMx2. A nuclear localization signal (NLS) RKRKR was predicted for SsaMx2 at amino acid number 506-510, similar to the NLS of RBTMx2 (Trobridge et al., 1997). None of the other salmon Mx proteins appeared to contain NLS.

The nomenclature of the Mx proteins encoded by Chr25 is suggested to be SsaMx4 to SsaMx8, and SsaMx9 for the Mx protein encoded by Chr9 (Table 2). The Mx proteins encoded by Chr25 vary in size from 603 to 627 aa while SsaMx9 in Chr9 contains 642 aa and is the largest of the salmon Mx proteins. A multiple alignment of the salmon Mx proteins (Fig. 1) showed that they all contain conserved regions which are typical for vertebrate Mx proteins (Verhelst et al., 2013). These include a GTPase region in the N-terminal half with the highly conserved tripartite GTP-binding sequence element consisting of GDQSSGKS, DLPG and TKPD; the dynamin signature LPRGS/TGIVTR; and a leucine zipper motif in the C-terminal (Verhelst et al., 2013). Besides the leucine zipper motif, the C-terminal half of Mx proteins from Chr12 is very different from the Mx proteins of Chr25 and Chr9. This may be of importance for their antimicrobial activities since it has been shown that the C termini of mammalian Mx proteins are responsible for recognition of viral targets and for their differential antiviral activities (Verhelst et al., 2013). Mx proteins encoded by Chr12 showed 86-97 % identity among themselves. Overall, Mx proteins encoded by

188 Chr12 showed only 45 - 48 % amino acid (aa) sequence identity with SsaMx9
189 encoded by Chr9, and 44 - 50% identity with the Mx protein encoded by Chr25
190 (Table 3). SsaMx9 showed 51 - 52 % sequence identity with Mx proteins in Chr25.
191 Mx proteins encoded by Chr25 have 87 to 91 % aa sequence identity among
192 themselves.

193 A phylogenetic analysis was conducted to study the relationship between the
194 three groups of salmon Mx genes and other vertebrate Mx genes. This was done by
195 first creating a multiple alignment using the Clustal W program. A phylogenetic tree
196 was then constructed from the alignment with the Neighbor-joining method using
197 lamprey (*Petromyzon marinus*) Mx as an outgroup (Fig.2). The tree shows that
198 vertebrate Mx genes form three major groups. The first group includes most teleost
199 Mx genes including SsaMx1, SsaMx2 and SsaMx3. The second group contains Mx
200 genes of tetrapods (amphibians, reptiles, birds and mammals). The third group
201 includes SsaMx4 to SsaMx8 of Chr25 and SsaMx9 in Chr9 plus zebrafish MxD and
202 MxG and eel MxB. Zebrafish MxC and MxE formed a minor group together with one
203 of the gar (*Lepisosteus oculatus*) Mx proteins in linkage group (LG) 17. The other gar
204 Mx protein in LG17 did not group with any of the three main groups while the gar Mx
205 in LG3 grouped with the lamprey Mx.

206 Gene synteny studies supported that salmon Mx genes encoded by Chr12 are
207 related to the main group of teleost Mx genes except zebrafish, being linked to the
208 SYNPR, THOC7 and ATXN7 genes (Fig.3). In contrast, salmon Mx genes in Chr25
209 are flanked by the STXBP5L and HPX genes similar to the two Mx genes in LG17 in
210 the gar genome. Salmonids thus seem to have kept one of the ancestral Mx clusters.
211 The seven Mx genes found in zebrafish are organised among four clusters where Mxc
212 and Mxe are linked to HPX whilst the other zebrafish Mx clusters show no likeness in

213 gene synteny with other Mx genes (Solbakken et al., 2016). SsaMx9 in Chr9 neither
214 showed obvious likeness in gene synteny with other Mx genes. Interestingly, the gar
215 Mx gene in LG3 is flanked by the FAM3B and TMPRSS2 genes similar to the Mx
216 genes of tetrapods.

217 The presence of salmon Mx genes on three chromosomes may be the result of
218 the teleost and salmonid specific whole genome duplications (WGD). However, it is
219 interesting to note that even the spotted gar, which is a non-teleost bony fish that
220 originates from fish before the teleost specific WGD, possesses Mx gene clusters in
221 two linkage groups. FAM3B and TMPRSS2 as flanking genes to Mx have been
222 maintained in gar LG3 and tetrapods, but not in the major teleost groups (Solbakken
223 et al., 2016). The possibility exists, however, that rearrangements in an ancestral
224 teleost have replaced FAM3B and TMPRSS2 with SYNPR, THOC7 and ATXN7 as
225 flanking genes since as described below, SsaMx1, SsaMx2 and SsaMx3 respond
226 similarly to type I IFN as their mammalian homologs. In teleosts, Mx flanked by
227 STXBP5L and HPX has apparently only been observed in Atlantic salmon, but
228 zebrafish also possesses two Mx genes linked to HPX (Solbakken et al., 2016).
229

230 *3.2. Expression of Mx genes in response to stimulation with poly I:C, IFNa and IFNc*
231 TBLASTN with each salmon Mx protein as query against the Atlantic salmon EST
232 database showed that SsaMx1, SsaMx2 and SsaMx3 gave at least 36 positive hits
233 with ≥ 64 % sequence identity and an E-value ≤ 2e-36. In contrast, SsaMx4, SsaMx5,
234 SsaMx6, SsaMx7 and SsaMx9 gave no positive hits and SsaMx8 gave one positive
235 hit. This suggests that SsaMx4-SsaMx9 are even more strictly regulated than
236 SsaMx1-SsaMx3. To compare expression properties of the different Mx genes,
237 Atlantic salmon cell lines were stimulated with IFNa, IFNc, IFNg and poly I:C, which

238 mimics viral dsRNA. In the first experiment SSP-9 cells were stimulated
239 extracellularly with 10 µg/ml poly I:C, 1000 U/ml IFNa or 1000 U/ml IFNc, or
240 stimulated intracellularly by transfection with 1 µg/ml poly I:C. Mx expression was
241 measured by RT-qPCR after 24 and 48 hours (Fig. 4). The results showed that Mx
242 genes in Chr12 were increased by all treatment using the primer pair SsaMx123,
243 which amplifies all three Mx genes, SsaMx1, SsaMx2 and SsaMx3. SsaMx4 and
244 SsaMx5 in Chr25 were also increased by these treatments, but to a lesser extent than
245 Mx genes in Chr12. In general, the responses were higher at 48 h than at 24 h for all
246 Mx genes in Chr12 and Chr25. The difference in response at 24 vs 48 h were largest
247 for poly I:C, possibly due to induction of IFNa. In contrast, SsaMx9 in Chr9 showed
248 no significant response to any of the treatments ($p \leq 0.05$).
249 In the next experiment we wanted to compare the response of the different Mx
250 genes to IFNa and IFNg. For this purpose, we also designed specific primers for
251 SsaMx2 and SsaMx8 and used both SSP-9 and ASK cells. As expected, SsaMx123
252 and SsaMx2 primers showed stronger up-regulation of Mx in response to IFNa than to
253 IFNg in both cell types. Surprisingly, however, SsaMx4, SsaMx5 and SsaMx8
254 showed a much stronger response to IFNg than to IFNa in both cell types. SsaMx8
255 showed by far the strongest response to both IFNg and IFNa, followed by SsaMx5
256 and SsaMx4. The fold up-regulation of SsaMx8 with IFNg and IFNa was 3867 vs 162
257 in ASK cells and 1621 vs 54 in SSP-9 cells. In contrast, the fold up-regulation of
258 SsaMx2 with IFNg and IFNa was 27 vs 1153 in ASK cells and 5 vs 49 in SSP-9 cells.
259 Even if the concentrations of IFNa and IFNg are not comparable, the ratios of the
260 responses to these IFNs are clearly different for Mx genes in Chr12 and Chr25.
261 SsaMx9 responded significantly neither to IFNa nor to IFNg ($p \leq 0.05$). Taken
262 together, SsaMx1, SsaMx2 and SsaMx3 are typical type I IFN responsive genes,

263 which respond less to IFNg as observed before (Sun et al., 2011). In contrast,
264 SsaMx4, SsaMx5 and SsaMx8 are more typical IFNg responsive genes than type I
265 IFN responsive genes. This is apparently the first identification of vertebrate Mx
266 genes that are more responsive to IFNg than to IFNa. In mammals, Mx genes are
267 strictly induced by type I and type III IFN and are not induced by IFNg or other
268 cytokines (Haller et al., 2015; Verhelst et al., 2013). Type III IFN has not yet been
269 identified in fish. In Atlantic salmon, Mx genes of Chr12 are up-regulated by IFNg,
270 but this in part due to up-regulation of IFNa (Sun et al., 2011). Some type I IFN
271 induced genes such as viperin, may be up-regulated by IFNg through induction of
272 IRF-1 (Stirnweiss et al., 2010). Whether this is the case for the salmon Mx genes in
273 Chr25 is not known.
274
275 *3.3. ISRE and GAS motifs in Mx promoter regions*
276 In mammals, ISRE and GAS sequences are the main promoter elements, which
277 control transcription of genes induced by type I IFNs and IFNg, respectively.
278 Henceforth, it was of interest to search for such motifs in promoter regions of the
279 salmon Mx genes. The ISRE consensus sequence of mammalian ISGs is $GAAAN_{1-}$
280 $_{2}GAAA$ or its inverse complement (Hug et al., 1988). The GAS consensus sequence
281 is $TTCN_{2-4}GAA$ (Decker et al., 1997). The promoter of rainbow trout Mx1 gene
282 contains the element GAAAGTGAAAC, which matches the ISRE consensus (Collet
283 and Secombes, 2001). To identify ISRE elements in salmon Mx genes, 500
284 nucleotides (nt) upstream of the ATG translation start site were screened manually for
285 the ISRE and GAS consensus sequences (Fig. 6). For SsaMx1 an ISRE motif was
286 found 304-312 nt upstream of ATG while a putative GAS motif was found 254-263 nt
287 upstream of ATG. For SsaMx2, an ISRE motif was found 365-374 nt upstream of

ATG and a putative GAS motif was found 414-423 nt upstream of ATG. For SsaMx3, an ISRE motif was found 245-254 nt upstream of ATG and a putative GAS motif was identified 274-283 nt upstream of ATG. Compared to the ISRE element in the rainbow trout Mx1 promoter, SsaMx2 and SsaMx3 possess identical ISRE sequences while the ISRE element of SsaMx1 lacks one G.

NCBI gene bank predicts a 10083 nt intron interruption of the SsaMx8 mRNA 5´-UTR region. A similar prediction is made for the mRNA of SsaMx4, but not for SsaMx5. Accordingly, the transcription start sites of these Mx genes are uncertain and need to be confirmed by experimental determination in order to confirm the respective promoter regions. In the present work the sequence 500 nt upstream of the transcription start sites of SsaMx4 - SsaMx8 predicted by NCBI GenBank, were analysed for ISRE and GAS motifs. The SsaMx8 sequence contained two putative GAS motifs and two ISRE motifs while the SsaMx4 sequence contained one GAS and one ISRE motif (Fig.6). No ISRE or GAS motifs were detected in the 500 nt sequences upstream of the predicted mRNAs for SsaMx5, SsaMx6, SsaMx7 or SsaMx9 (not shown).

At present the true role of ISRE-elements and GAS elements for the type I IFN response and IFNg response in salmonids is not known. The reporter studies of rainbow trout Mx1 has apparently only been performed with a sequence 584 nt upstream of translation start site (Collet and Secombes, 2001). The importance of GAS-elements in the promoters of IFNg responsive genes in rainbow trout could not be established (Castro et al., 2008). In fact, promoter regions containing ISRE motifs responded stronger to IFNg than promoter regions containing GAS motifs. Thus, while STAT1 phosphorylation and dimerization in response to IFN gamma has been demonstrated in salmonids (Skjesol et al., 2010), the IFNg responsive elements have

yet to be defined. Whether the strong response of SsaMx8 to IFNg is due to possession of two ISRE and/or two GAS motifs has to be studied in reporter assays. In the case of the IFNg responsive genes, it must be taken into account that IFNg may also up-regulate genes through induction of transcription factors, such as known for IRF-1 (Stirnweiss et al., 2010; Storm van's Gravesande et al., 2002).

4. Concluding remarks

The prominent antiviral properties of Mx proteins opens the possibility for an antiviral role also for the Mx proteins of Chr25, which might be examined by establishment of cell lines constitutively expressing these proteins. On the other hand, Mx genes induced by IFNg might have different functions compared to type I IFN induced Mx genes. In mammals, the GTPases guanylate binding proteins (GBPs) and p47 immunity regulated GTPases (IRGs) are among the most abundant IFNg-induced proteins. Both provide cell-autonomous resistance against a variety of intracellular bacterial and eukaryotic pathogens (Kim et al., 2011; Pilla-Moffett et al., 2016). GBPs also possess antiviral activity although the antiviral properties are weak compared to those of the Mx proteins (Haller et al., 2015). It would thus be interesting to examine if the Mx proteins encoded by Chr25 possess similar antimicrobial activities as GBPs and IRGs. The antimicrobial activity of GBPs and IRGs are linked with their ability to associate with pathogen-containing vacuoles, followed by recruitment of certain binding partners (Pilla-Moffett et al., 2016). Recently, it was found that GBPs protect against bacterial infections by interacting with phagocyte oxidase, antimicrobial peptides and autophagy effectors (Kim et al., 2011). Targeting of GBPs to membrane-bound compartments is due to isoprenylation, but is dispensable for targeting of GBPs to pathogen-containing vacuoles is dispensable (Pilla-Moffett et al., 2016). Rainbow

338 trout GBP also possesses an isoprenylation motif CaaX at the C-terminus (Robertsen
339 et al., 2006). None of the salmon Mx proteins contain CaaX motifs (Fig. 1). It would
340 still be important to examine if SsaMx4-SsaMx8 reside in the cytoplasma or if they
341 are associated with pathogen-associated vacuoles.
342
343

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439

440 **Figure legends**

441 Figure 1. Multiple alignment of Atlantic salmon Mx proteins. Amino acids that are
442 identical with those of SsaMx1 are shaded with black.

443

444 Figure 2. Phylogenetic tree of vertebrate Mx proteins. Included in the analysis were
445 Mx proteins from representative species of teleost fish, mammals (human and mouse),
446 birds (chicken *Gallus gallus*), reptiles (green anole lizard *Anolis carolinensis*) and
447 amphibians (frog *Xenopus laevis*). Mx proteins from the non-teleost bony fish spotted
448 gar (*Lepisosteus oculatus*) and of lamprey (*Petromyzon marinus*) were also included
449 and the latter was used to root the tree. The evolutionary history of the Mx genes was
450 inferred using the Neighbor-Joining method within the MEGA7 program and shows
451 the bootstrap consensus tree. The percentage of replicate trees in which the associated
452 taxa clustered together in the bootstrap test (1000 replicates) are shown next to the
453 branches. The evolutionary distances were computed using the Poisson correction
454 method and are in the units of the number of amino acid substitutions per site. All
455 positions containing gaps and missing data were eliminated. NCBI accession numbers
456 are shown for all species except lamprey and stickleback Mx, which are from the
457 Ensemble database. Accession numbers for the salmon Mx proteins are shown in
458 Table 2. RBT = rainbow trout.

459

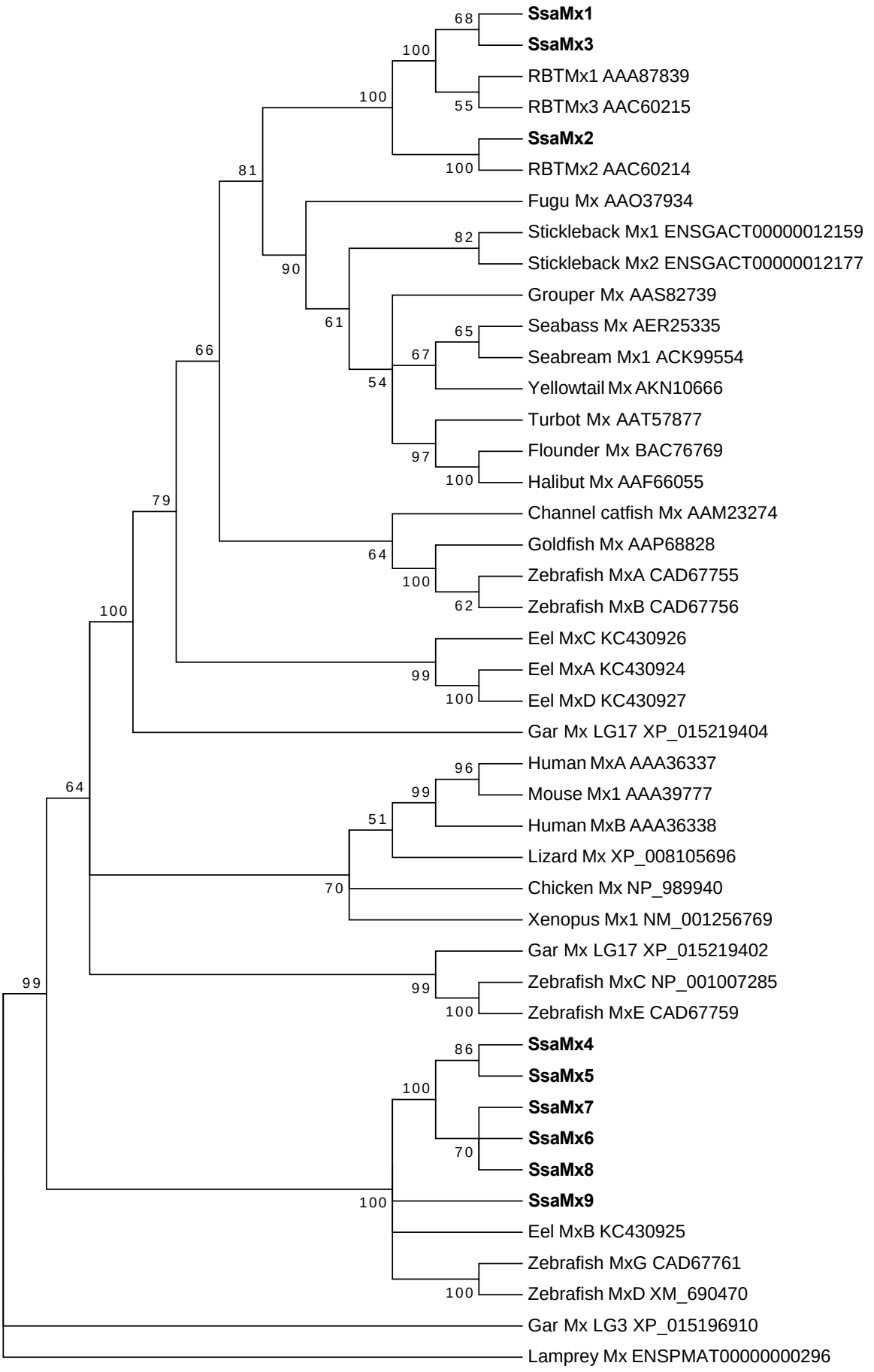
460 **Figure 3.** Local gene synteny analysis of Atlantic salmon Mx regions compared to
461 Mx regions in selected teleost species, the non-teleost bony fish spotted gar
462 (*Lepisosteus oculatus*), frog (*Xenopus tropicalis*) and human. Gene synteny in the
463 Atlantic salmon and gar linkage groups were obtained from NCBI GenBank using the
464 Mx accession numbers depicted in Fig.2. Gene synteny for stickleback Mx1 and Mx2,
465 zebrafish Mxc and Mxd, frog and human were obtained from Solbakken et al
466 (Solbakken et al., 2016).

467

Figure 4. Expression of Mx genes in SSP-9 cells in response to IFNa, IFNc and poly I:C. Cells in triplicate wells were stimulated extracellularly with 10 µg/ml poly I:C (PICs), 1000 U/ml IFNa or 1000 U/ml IFNc for 24 and 48 h. Cells were stimulated intracellularly by transfection with 1 µg/ml poly I:C (PICt) for 24 and 48 h. Expression of genes were measured by RT-qPCR. Data are presented as mean fold increase in transcripts +/- SD relative to non-treated cells. SsaMx1,2,3 means increase in transcripts using the primer set that up-regulates all three genes SsaMx1, SsaMx2 and SsaMx3.

Figure 5. Expression of Mx genes in ASK and SSP-9 cells in response to IFNa (1000 U/ml) or IFNg (1ng/ml). Cells in triplicate wells were stimulated for 24 h. Expression of genes were measured by RT-qPCR. Data are presented as mean fold increase in transcripts +/- SD relative to non-treated cells.

Figure 6. ISRE and GAS motifs in promoter regions of Mx genes.
ISRE motifs are shown in bold upper case letters while GAS motifs are shown in bold lower case letters. For SsaMx8, an additional ISRE is underlined. For SsaMx1, SsaMx2 and SsaMx3, the 500 nt sequence upstream of the translation start site is shown where normal upper case letters indicate mRNA. For SsaMx4 and SsaMx8 the 500 nt sequences upstream of the putative intron in the 5´-untranslated region of mRNA are shown.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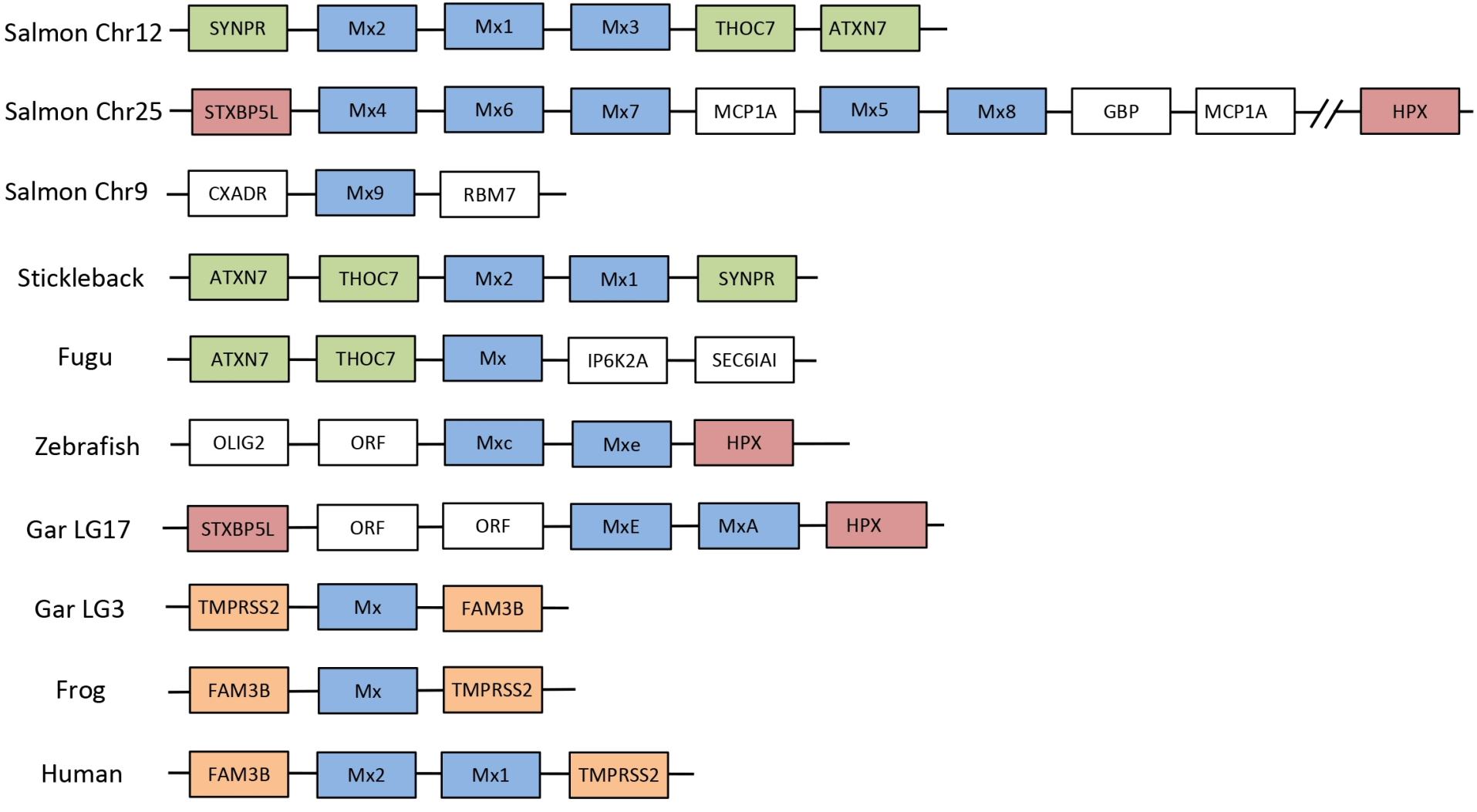
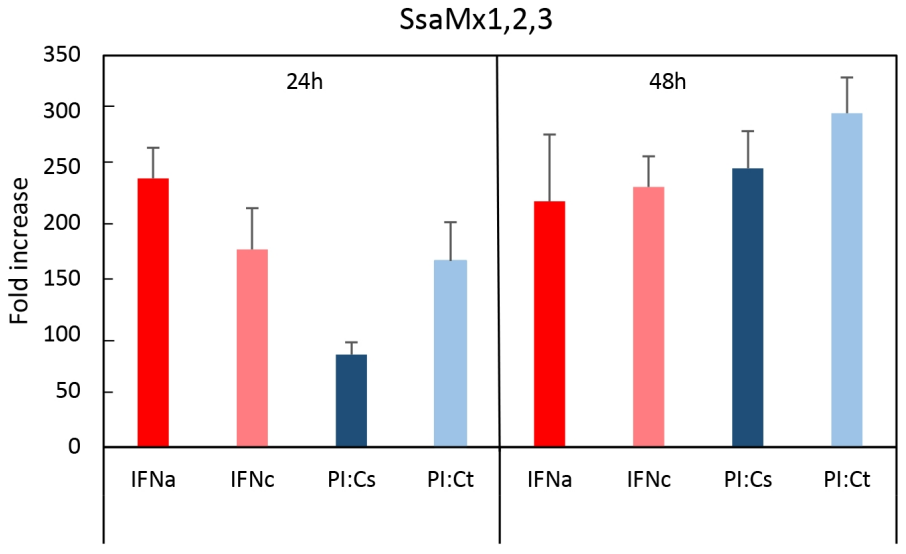
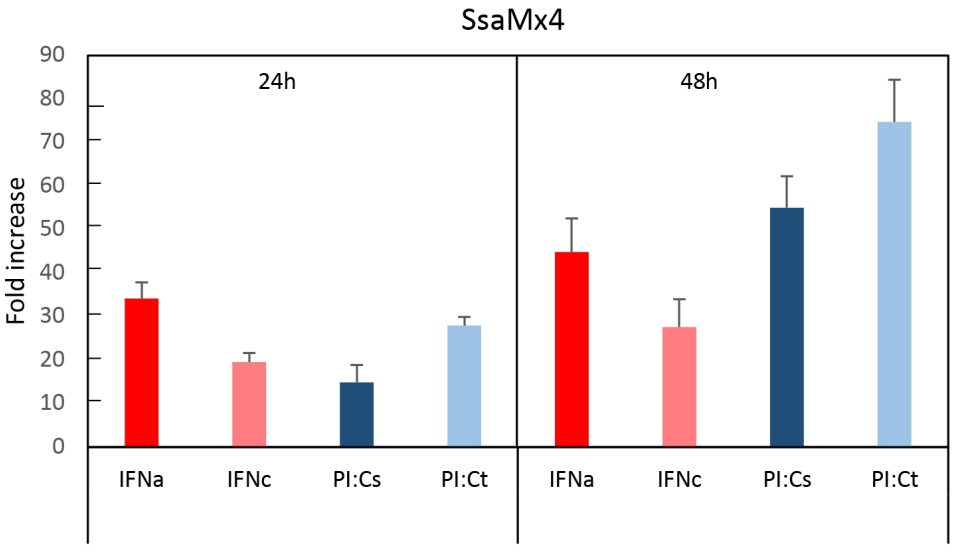
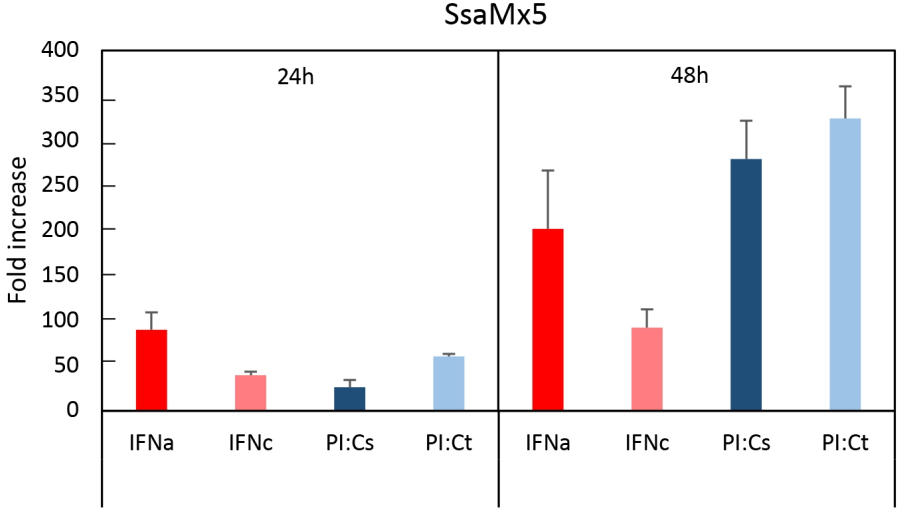
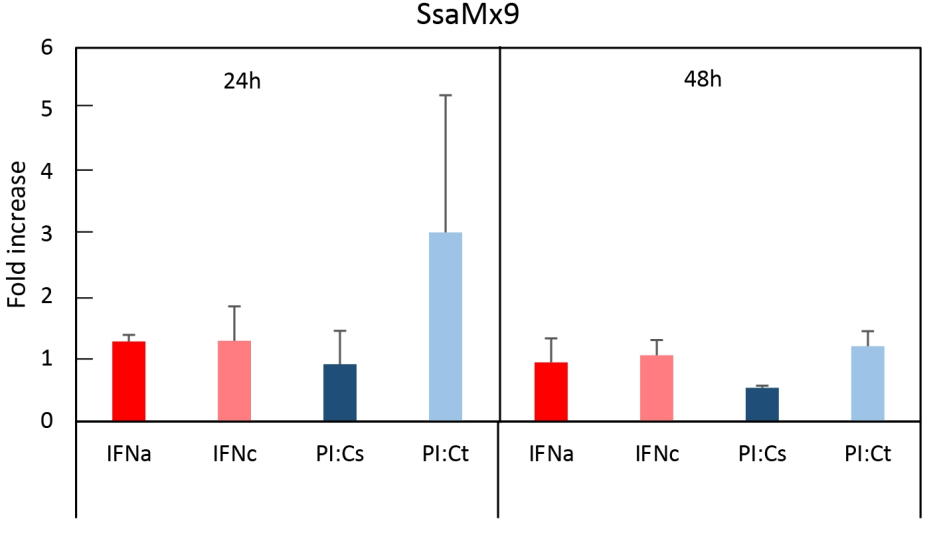
SsaMx3
RBTMx1 AAA87839
RBTMx3 AAC60215
SsaMx2
RBTMx2 AAC60214
Fugu Mx AAO37934
Stickleback Mx1 ENSGACT00000012159
Stickleback Mx2 ENSGACT00000012177
Grouper Mx AAS82739
Seabass Mx AER25335
Seabream Mx1 ACK99554
Yellowtail Mx AKN10666
Turbot Mx AAT57877
Flounder Mx BAC76769
Halibut Mx AAF66055
Channel catfish Mx AAM23274
Goldfish Mx AAP68828
Zebrafish MxA CAD67755
Zebrafish MxB CAD67756
Eel MxC KC430926
Eel MxA KC430924
Eel MxD KC430927
Gar Mx LG17 XP_015219404
Human MxA AAA36337
Mouse Mx1 AAA39777
Human MxB AAA36338
Lizard Mx XP_008105696
Chicken Mx NP_989940
Xenopus Mx1 NM_001256769
Gar Mx LG17 XP_015219402
Zebrafish MxC NP_001007285
Zebrafish MxE CAD67759
SsaMx4
SsaMx5
SsaMx7
SsaMx6
SsaMx8
SsaMx9
Eel MxB KC430925
Zebrafish MxG CAD67761
Zebrafish MxD XM_690470
Gar Mx LG3 XP_015196910
Lamprey Mx ENSPMAT00000000296

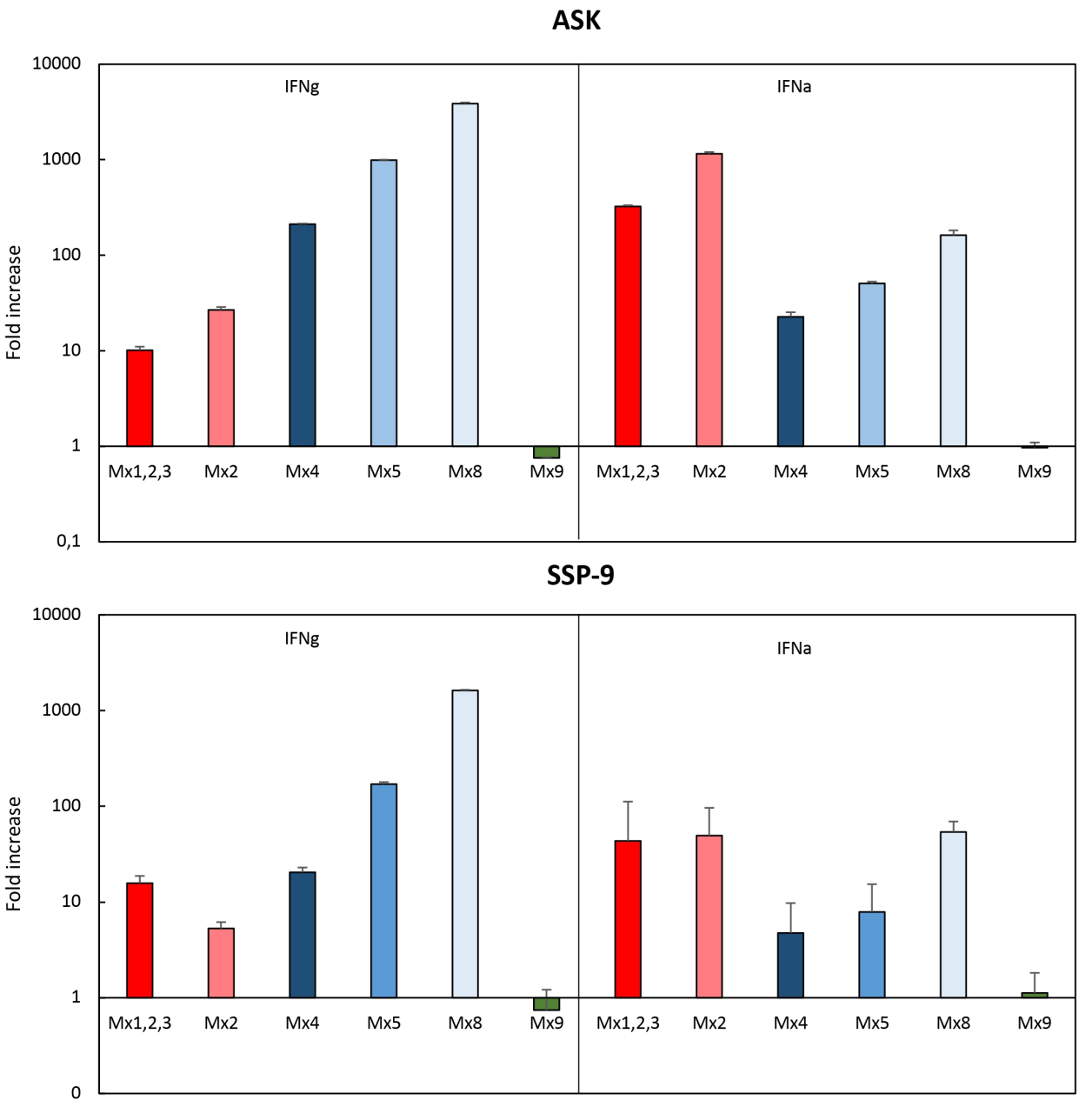

Salmon Chr12
SYNPR
Mx2
Mx1
Mx3
THOC7
ATXN7
Salmon Chr25
STXBP5L
Mx4
Mx6
Mx7
MCP1A
Mx5
Mx8
GBP
MCP1A
HPX
Salmon Chr9
CXADR
Mx9
RBM7
Stickleback
ATXN7
THOC7
Mx2
Mx1
SYNPR
Fugu
ATXN7
THOC7
Mx
IP6K2A
SEC6IAI
Zebrafish
OLIG2
ORF
Mxc
Mxe
HPX
Gar LG17
STXBP5L
ORF
ORF
MxE
MxA
HPX
Gar LG3
TMPRSS2
Mx
FAM3B
Frog
FAM3B
Mx
TMPRSS2
Human
FAM3B
Mx2
Mx1
TMPRSS2


SsaMx1,2,3
Fold increase
350
300
250
200
150
100
50
0
24h
48h
IFNa
IFNc
PI:Cs
PI:Ct
IFNa
IFNc
PI:Cs
PI:Ct


SsaMx4
Fold increase
90
80
70
60
50
40
30
20
10
0
24h
48h
IFNa
IFNc
PI:Cs
PI:Ct
IFNa
IFNc
PI:Cs
PI:Ct


SsaMx5
Fold increase
400
350
300
250
200
150
100
50
0
24h
48h
IFNa
IFNc
PI:Cs
PI:Ct
IFNa
IFNc
PI:Cs
PI:Ct


SsaMx9
Fold increase
6
5
4
3
2
1
0
24h
48h
IFNa
IFNc
PI:Cs
PI:Ct
IFNa
IFNc
PI:Cs
PI:Ct


ASK
IFNg
IFNa
Fold increase
10000
1000
100
10
1
0,1
Mx1,2,3
Mx2
Mx4
Mx5
Mx8
Mx9
SSP-9
IFNg
IFNa
Fold increase
10000
1000
100
10
1
0
Mx1,2,3
Mx2
Mx4
Mx5
Mx8
Mx9

SsaMx1

acctcacagtttaggttgacctatttggattttgttggagtgttaaaccaagtggttttt
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gaa**GAAATGAAAgtggaaa**aaccgcttaagtttcgatttccagggatgacactgaacaca
cgaaaccggttgtttcacccattataaaacgtgtcgagacgtaaaatctcctgtatcgga
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TCACTAAATAATAATTCACA

SsaMx2

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acgtacaggctgcaagtcaattaattactttctgtcctcaggatggttgagctattgata
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GAACCTGTACAGCCAGTAGTCAGTTGCAGTGTAGTCAGTTGCAGTTGCAGTGTATTAACC
ATTTTTTTTTATCGCAAATC

SsaMx3

cagtgatatattgggggggacaaatcatatttttcccaggatgggggggtcgtgtccgcc
tctgcatcacccctggtgagacaaaaccgcgactcgtcagtgaagagcacttttgccag
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acat**GAAAGTGAAA**cacaccgtgtaagattcga**ttcccaggaa**tgatacgccctctgttt
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SsaMx4

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ttcttggaaatcatgtgacccttaagaaccaatgaggttgtatctgagatgtgattaggc
actttgtaacagatggagtataatggtatgctttccggtaatggaccaccg**TTTCACTTT**
Cattttaccttctataaaag

SsaMx8

gtcactgagtaggctgatacccatttcattggtgcacaatctgtagtttagcctgtatag
Tcccaatctgtatgttcaatatgcacaataggctgtgtagtgagctgatttctcacagtc
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gcagtataattattcccccattgtaaagtcaattacatccacagtgggatttcatcagtt
ttttttgtatctatctgtagtagaaacaaccttga**TTTCA<u>TTTCACTttc</u>tgggaa**atca
tgtgacccttaagaaccaatgaggttatatctgaactttaattatgcactgtgtaacaga
tgcagaataatggtatgat**ttcccagaa**tggacaatcaaaacgtttcactttcattttgt
ttccatgaaaggtgatagac

Fig.6.

Table 1. Primers used in qPCR

Primer	Sequence
SsaMx123F	TGCAACCACAGAGGCTTTGAA
SsaMx123R	GGCTTGGTCAGGATGCCTAAT
SsaMx2F	CTGAGGAAGAGGAAGAGGGA
SsaMx2R	CAGATAACACTTTCTGACTCCCA
SsaMx4F	CAGTGAAATGCTGGATCTGCTACACAG
SsaMx4R	GATCCAATTGGGCCTGCAACTTTG
SsaMx5F	ATTCTGGAGGAGGGGGAAATAGCAG
SsaMx5R	CAGCCAGTCTTTGCACCACAATCTCATA
SsaMx8F	GGAGCCCAAATCAATCATCTCATTAGG
SsaMx8R	ATTTTCAGGGCCTCTGTTGTTGCTATG
SsaMx9F	ATGTTGTCAAACTGCTGAGCGAAGACTCTA
SsaMx9R	CACTAAGTTTTTCCTGGGCCTTTTTCAGA
EF1αBF	TGCCCCTCCAGGATGTCTAC
EF1αBR	CACGGCCCACAGGTACTG

F = forward, R = reverse

Table 2. Mx genes in the Atlantic salmon genome

	Gene ID	Protein[a]	Length[b]
Chr.12			
SsaMx1	LOC100136920	NP_001117162	623
SsaMx2	LOC100136920	NP_001133390	638
SsaMx3	LOC100136587	NP_001117147	623
Chr.25			
SsaMx4	LOC106586887	XP_014030089	606
SsaMx5	LOC106586888	XP_014030090	608
SsaMx6	LOC106586889	XP_014030091	627
SsaMx7	LOC106586890	XP_014030092	603
SsaMx8	LOC106586891	XP_014030093	607
Chr.09			
SsaMx9	LOC106612969	XP_014070197	642

[a] GenBank accession numbers

[b] Number of amino acids

Table 3. Sequence pair distances of Atlantic salmon Mx proteins (% identity)

	SsaMx number								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
SsaMx1	100								
SsaMx2	86	100							
SsaMx3	97	87	100						
SsaMx4	49	47	49	100					
SsaMx5	49	47	49	91	100				
SsaMx6	47	44	47	87	88	100			
SsaMx7	50	47	50	88	89	88	100		
SsaMx8	49	47	50	90	90	89	90	100	
SsaMx9	48	45	48	52	52	51	52	52	100

Calculated from the alignment in Fig. 1 using the MegAlign program.