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Lineage/species-specific expansion of the Mx gene family in teleosts: Differential expression and modulation of nine Mx genes in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

Myxovirus resistance (Mx) proteins are interferon (IFN)-inducible Dynamin-like GTPases, which play an important role in antiviral immunity. Three Mx genes (Mx1-3) have been cloned previously in rainbow trout. In this study, an additional six Mx genes were cloned that reside in four chromosomal loci. Further bioinformatics analysis suggests the presence of three teleost Mx groups (TMG) each with a characteristic gene organisation. Salmonid Mx belong to TMG1 and TMG2. The increased salmonid Mx gene copies are due mainly to local gene duplications that happened before and after salmonid speciation, in a lineage/species specific manner. Trout Mx molecules have been diversified in the loop 1 and 4 regions, and in the nuclear localisation signal in loop 4. The trout Mx genes were shown to be differentially expressed in tissues, with high levels of expression of TMG1 (Mx1-4) in blood and TMG2 (Mx5-9) in intestine. The expression of the majority of the trout Mx genes was induced by poly IC *in vitro* and *in vivo*, and increased during development. In addition, induction by antiviral (IFN) and proinflammatory cytokines was studied, and showed that type I IFN, IFNγ and IL-1β can induce Mx gene expression in an Mx gene-, cytokine- and cell line-dependent manner. These results show that salmonids possess a large number Mx genes as well as complex regulatory pathways, which may contribute to their success in an anadromous life style.

1. Introduction

Mx (myxovirus resistance) proteins are interferon (IFN)-inducible Dynamin-like GTPases, with an important role in antiviral immunity [1,2]. They are members of a family of large GTPases, and share an N-terminal GTPase domain, a middle domain (MD), and a C-terminal GTPase effector domain (GED). The GTPase domain is the most conserved part that consists of a tripartite GTP-binding motif (GDXXSGKS, DLPG, and TKPD) and a dynamin signature (LPRXXGXXTR). The MD is important for oligomerization and viral target recognition, whilst the GED has a conserved C-terminal leucine zipper that folds back to join the N-terminal GTP-binding domain to establish the enzymatically active centre of Mx proteins [1–3]. Mx proteins form tetramers in solution that oligomerize further into large filaments and rings [3], with both GTPase activity and oligomerization required for antiviral immunity.

A prototype Mx gene has been found in amphioxus, containing the N-terminal GTPase domain [4]. Typical Mx genes are found in all vertebrate groups. The first evidence of Mx genes in fish started with the isolation of an Mx genomic DNA fragment in perch (*Perca fluviatilis*) in 1989 [5]. The first full-length characterisation of Mx genes was reported in rainbow trout *Oncorhynchus mykiss*, that has three Mx genes (Mx1-3) [6,7]. Subsequently Mx genes have been characterised in many fish species, with 1–9 genes present [4,8–21]. However, some fish species such as the Gadiformes have lost their Mx genes [22]. The role of fish Mx proteins in antiviral defence has been established in a few species, such as Japanese flounder *Paralichthys olivaceus*, Atlantic salmon *Salmo salar* and grass carp *Ctenopharyngodon Idella* [23–26].

The multiple copies of mammalian Mx are closely linked and arise from local gene duplications [2]. How multiple fish Mx genes have evolved is currently unclear [4,16]. A recent publication has shown that there are nine Mx genes present on three chromosomes (Ch) in Atlantic salmon with Mx1-3 on Ch12, Mx4-8 on Ch25, and Mx9 on Ch9 [27]. The origin of multiple copies of Mx genes on the same chromosome, that are linked closely and share high sequence identities, is likely to also be via local gene duplications. However, due to the third teleost-wide whole genome duplication (3R WGD) and the salmonid 4R WGD,

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many genes with single copy in mammals are present as four copies on four chromosomes in salmonids [28]. Thus it is possible there could be a fourth chromosome that harbours Mx genes in salmonids, and if discovered this may shed light on how the different Mx-bearing chromosomes evolved in salmonids.

Mammalian Mx gene expression is induced by type I and type III IFNs but not by type II IFNγ or other proinflammatory cytokines [29–31]. Interestingly, the diversified repertoire of Atlantic salmon Mx genes appear to show some differential responsiveness to type I and II IFNs, with those on Ch12 being highly induced by type I IFNs and those on Chr25 being more strongly induced by IFNγ than by type I IFN [27]. This finding is very interesting and raises the question as to whether a diversified Mx repertoire may also be responsive to other cytokines released during innate antiviral defence, and remains to be examined. Hence, in this study we aimed to shed light on Mx gene evolution in actinopterygian fish, in an attempt to establish a better model of their evolution, and to establish whether the increased Mx copy number in salmonids has allowed neo-functionalisation giving a broader responsiveness to a variety of cytokines. We first identified and cloned an additional six Mx genes in rainbow trout, and found that all salmonids with a mapped genome have four chromosomes harbouring Mx genes. We identified three groups of Mx genes present in teleosts in a lineage-specific manner, with some (Ostariophysi) having all three groups, some having two groups (Protacanthopterygii, including salmonids) but the percomorphs possessing only a single group. We next investigated the expression of the nine trout Mx family members individually. We found that the trout Mx genes are differentially expressed constitutively in tissues, that they increase during development, are induced *in vivo* by poly IC, and are modulated *in vitro* by type I and type II IFNs, and by other proinflammatory cytokines in a gene-, cytokine- and cell line-specific manner.

2. Materials and methods

2.1. Rainbow trout

Healthy rainbow trout (~40 g) were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK). The fish were fed twice a day with a commercial diet (EWOS) and maintained in 1-m-diameter fibreglass tanks with recirculating freshwater at 14 °C at the Scottish Fish Immunology Research Centre, University of Aberdeen, UK. Head kidney (HK) swabs were taken routinely and showed no bacterial presence [32]. Fish were given at least two weeks for acclimation prior to use and ranged in size from 100 to 140 g when experiments were performed. All the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals, and were carried out under UK Home Office project licence PPL 60/4013, approved by the ethics committee at the University of Aberdeen.

2.2. Identification, cloning and sequence analysis of Mx cDNA in rainbow trout

Three Mx genes (Mx1-3) are known in rainbow trout [6,7]. To identify additional Mx genes in this species, we searched the recently released rainbow trout reference genome (GCF_002163495.1) using TBLASTN [33] with the known trout Mx genes as query, resulting in the identification of four genomic loci (Chromosomes (Ch)3, 11, 17 and 24) that harbour Mx genes. The Mx genes were then predicted as described previously [34,35]. In addition, potential exons in untranslated regions (UTR) were predicted by using trout RNA-seq datasets (SRP108798) through aligning to the reference genome. Primers (supplementary Table S1) were subsequently designed in the predicted 5′- and 3′-UTR for PCR cloning of the complete coding region of each predicted Mx gene. The general cloning and sequence analysis was as described previously [34,35]. The nucleotide sequences generated were assembled and analysed with the AlignIR programme (LI-COR, Inc.). Homology search was performed using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [33] and the gene organisation was predicted using the Spidey program at NCBI. Protein prediction was undertaken using software at the ExPASy Molecular Biology Server (http://www.expasy.org/tools) [36]. Multiple sequence alignments were generated using CLUSTALW [37]. Amino acid sequence identity/similarity comparison was performed using the scoring matrix BLOSUM62 within the MatGAT program, with a gap open penalty of 10 and gap extension penalty of 1 [38].

2.3. Analysis of Mx genes in other salmonids

The Mx genes in other salmonids were predicted/analysed using recently released genomes of Atlantic salmon (*Salmo salar*, Atlantic, acc. no. GCF_000233375.1), chinook salmon (*Oncorhynchus tshawytscha,* Chinook, acc. no. GCF_002872995.1), coho salmon (*Oncorhynchus kisutch*, Coho, acc. no. GCF_002021735.1), and Arctic charr (*Salvelinus alpinus*, Charr, acc. no. GCF_002910315.2). Each Mx aa and nucleotide sequence was mapped to chromosomes/scaffolds. Similarly, Mx genes were analysed in the pike (*Esox lucius*) reference genome (acc. no. GCF_000721915.3), the closest relative of salmonids that has not undergone the salmonid 4R WGD and that has a sequenced genome. The aa sequences were used for phylogenetic tree analysis using MEGA7.0 software [39] based on aa multiple alignments generated by CLUSTALW. The evolutionary distances were computed using the JTT matrix-based method. A neighbour-joining phylogenetic tree was constructed using pair-wise deletion option.

2.4. Evolutionary analysis of teleost Mx family

Mx genes/proteins were retrieved at NCBI from selected teleost fish, including species known to possess multiple Mx genes. The naming of Mx genes/proteins followed those already published [4,16,40,41] or simple Mx with an acc. no. For phylogenetic tree analysis, Mx protein sequences were extracted from one holostean species, spotted gar (*Lepisosteus oculatus*, Lepisosteiformes) that is an early actinopterygian fish species without the 3R WGD, twenty-one teleosts and three mammals (human *Homo sapiens*, mouse *Mus musculus* and cow *Bos taurus*) as an outgroup. The teleost species included an elopomorph, European eel (*Anguilla anguilla*, Anguilliformes), five Ostariophysi (Otophysi) species including three Cypriniformes (common carp *Cyprinus carpio*, goldfish *Carassius auratus* and zebrafish *Danio rerio*), channel catfish (*Ictalurus punctatus*, Siluriformes), and Mexican tetra or blind cave fish (*Astyanax mexicanus*, Characiformes), five protacanthopterygii (the salmonids and pike described above), and ten percomorphs including two Pleuronectiformes (turbot *Scophthalmus maximus*, and olive flounder *Paralichthys olivaceus*), Atlantic killifish (*Fundulus heteroclitus*, Cyprinodontiformes), stickleback (*Gasterosteus aculeatus*, Gasterosteiformes), Medaka (*Oryzias latipes*, Beloniformes), fugu (*Takifugu rubripes*, Tetraodontiformes), Nile tilapia (*Oreochromis niloticus*, Cichliformes) and three Perciforme fish (gilt-head sea bream *Sparus aurata*, orange-spotted grouper *Epinephelus coioides*, and the Asian sea bass *Lates calcarifer*). A neighbour joining phylogenetic tree was constructed as above. Synteny analysis was performed using the Genomicus program [42] or with information extracted from reference genome sequence at NCBI.

2.5. Real-time PCR analysis of gene expression

Specific primers for each Mx gene were carefully designed based on a multiple cDNA sequence alignment to ensure that at least one primer was isoform specific, and one primer crosses an intron to prevent genomic DNA amplification. The primers for qPCR analysis of Mx genes and other cytokine genes are detailed in Table S1 and S2, respectively. Total RNA preparation, cDNA synthesis and qPCR analysis were as

Table 1
Summary of Mx gene family in salmonids and pike.

Common name	Gene	Chromosome	Location	Genebank accession number (mRNA/protein)
Rainbow trout	Mx1	NC_035093.1 (Ch17)	54,895,743 → 54,907,262	NM_001171901.1
Rainbow trout	Mx2	NC_035093.1 (Ch17)	54,827,385 → 54,839,851	NM_001124751.1
Rainbow trout	Mx3	NC_035093.1 (Ch17)	54,879,332 → 54,883,908	XM_021569609.1
Rainbow trout	Mx4	NC_035093.1 (Ch17)	54,848,974 → 54,863,639	MK301134
Rainbow trout	Mx5	NC_035079.1 (Ch3)	82,015,213 → 81,992,259	MK301135
Rainbow trout	Mx6	NC_035079.1 (Ch3)	82,029,332 → 82,045,373	MK301136
Rainbow trout	Mx7	NC_035087.1 (Ch11)	76,228,384 → 76,215,926	MK301137
Rainbow trout	Mx8	NC_035087.1 (Ch11)	76,240,419 → 76,420,666	MK301138
Rainbow trout	Mx9	NC_035100.1 (Ch24)	21,286,705 → 21,268,999	MK301139
Atlantic salmon	Mx1	NC_027311(Ch12)	66,798,275 → 66,829,177	NM_001123690/NP_001117162
Atlantic salmon	Mx2	NC_027311(Ch12)	66,776,028 → 66,803,979	NM_001139918/NP_001133390
Atlantic salmon	Mx3	NC_027311(Ch12)	66,816,288 → 66,829,177	NM_001123675/NP_001117147
Atlantic salmon	Mx4	NC_027324(Ch25)	47,088,993 → 47,121,652	XM_014174614/XP_014030089
Atlantic salmon	Mx5	NC_027324(Ch25)	47,228,437 → 47,217,827	XM_014174615/XP_014030090
Atlantic salmon	Mx6	NC_027324(Ch25)	47,161,992 → 47,139,132	XM_014174616/XP_014030091
Atlantic salmon	Mx7	NC_027324(Ch25)	47,193,272 → 47,175,785	XM_014174617/XP_014030092
Atlantic salmon	Mx8	NC_027324(Ch25)	47,243,602 → 47,262,616	XM_014174618/XP_014030093
Atlantic salmon	Mx9	NC_027308(Ch9)	117,838,750 → 117,853,816	XM_014214722/XP_014070197
Atlantic salmon	M-x10	NC_027314(Ch15)	5,299,091 → 5,292,439	XM_014143485/XP_013998960
Chinook salmon	Mx1	NW_020142590	72,571 → 83,518	XM_024415949/XP_024271717
Chinook salmon	Mx2	NW_020142590	17,032 → 35,480	XM_024415950/XP_024271718
Chinook salmon	Mx3	NW_020142590	56,817 → 83,518	XM_024415946/XP_024271714
Chinook salmon	Mx4	NW_020133776	172 → 6566	XM_024410424/XP_024266192
Chinook salmon	Mx5	NC_037108(Ch12)	2,001,377 → 2,013,759	XM_024438118/XP_024293886
Chinook salmon	Mx6	NC_037110(Ch14)	41,090,466 → 41,103,747	XM_024445373/XP_024301141
Coho salmon	Mx1	NC_034174(Ch1)	46,664,816 → 46,675,808	LOC109896993
Coho salmon	Mx2	NC_034174(Ch1)	46,587,607 → 46,621,678	XM_020468497/XP_020324086
Coho salmon	Mx3	NC_034174(Ch1)	46,651,400 → 46,656,202	XM_020491468/XP_020347057
Coho salmon	Mx4	NW_018090236	57,121 → 68,549	GDQG01031501//Q6PW23
Coho salmon	Mx5	NC_034181(Ch8)	66,940,031 → 66,941,209	XM_020488627/XP_020344216
Coho salmon	Mx6	NC_034191(Ch18)	53,794,477 → 53,822,277	XM_020508491/XP_020364080
Arctic charr	Mx1	NC_036838(Ch1)	44,797,136 → 44,802,044	XM_023993827/XP_023849595
Arctic charr	Mx2	NC_036838(Ch1)	44,762,649 → 44,772,138	XM_023993825/XP_023849593
Arctic charr	Mx3	NW_019943275	202,142 → 225,309	XM_024139811/XP_023995579
Arctic charr	Mx4	NW_019943275	93,732 → 108,405	XM_024139809/XP_023995577
Arctic charr	Mx5	NW_019943275	87,444 → 170,634	XM_024139810/XP_023995578
Arctic charr	Mx6	NW_019945020	48,231 → 54,383	XM_024143207/XP_023998975
Arctic charr	Mx7	NW_019945020	11,050 → 28,797	XM_024143206/XP_023998974
Arctic charr	Mx8	NW_019946381	2678 → 17,616	XM_024144359/XP_024000127
Arctic charr	Mx9	NW_019942645	359,971 → 369,223	XM_024136430/XP_023992198
Arctic charr	M-x10	NW_019942645	378,229 → 388,058	XM_024136431/XP_023992199
Pike	Mx1	NC_025984(Ch17)	25,113,053 → 25,132,843	XM_013138351/XP_012993805
Pike	Mx2	NC_025980(Ch13)	25,725,708 → 25,740,882	ENSELUG00000023570/ ENSELUT00000043341
Pike	Mx3	NC_025980(Ch13)	25,694,138 → 25,702,004	ENSELUG00000023626/ ENSELUT00000036437

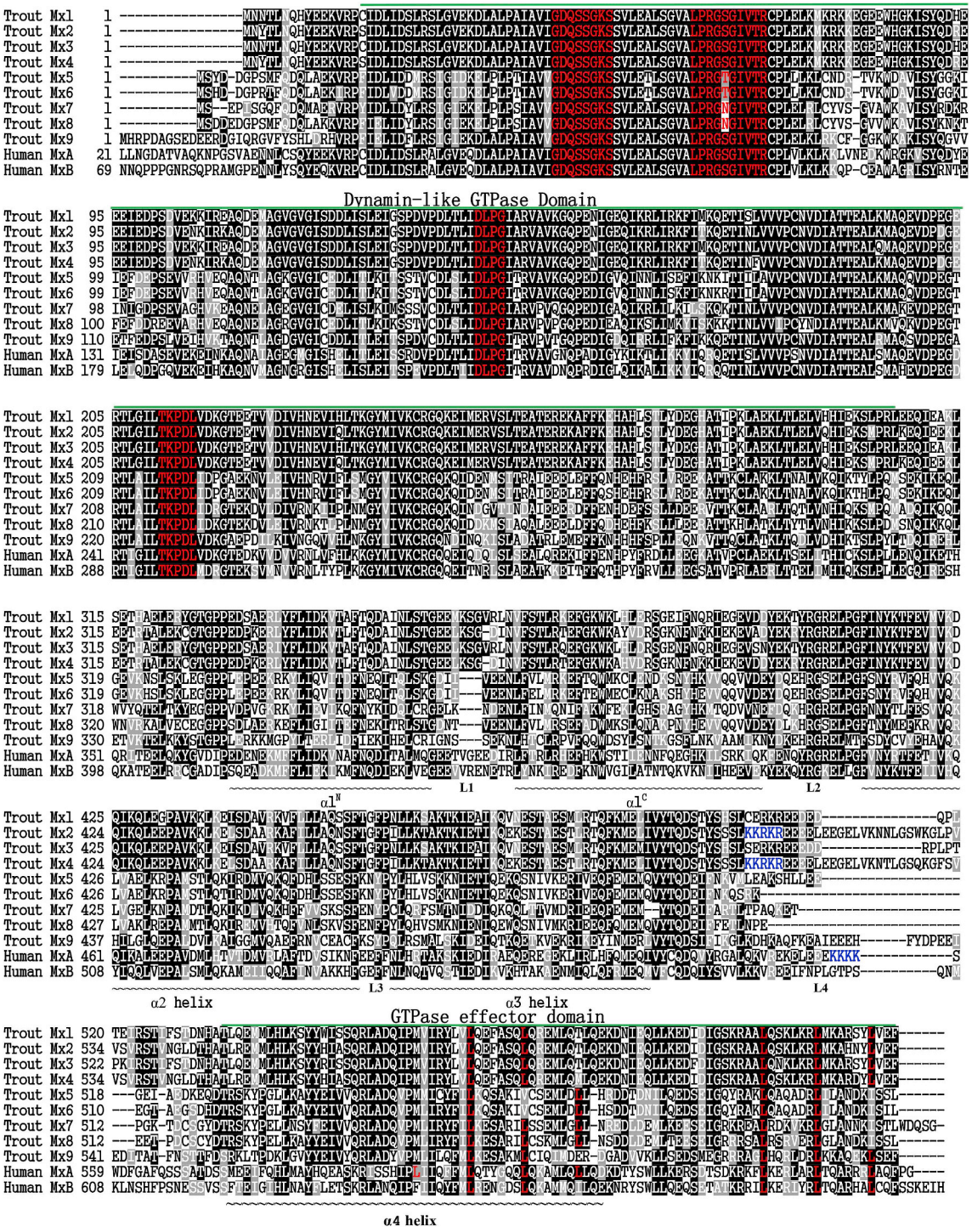

Fig. 1. Amino acid multiple alignment of rainbow trout Mx family. The multiple alignment was produced using ClustalW, and conserved amino acids shaded using BOXSHADE (version 3.21). Human MxA and MxB were included in the alignment for comparison. The N-terminal GTPase domain and C-terminal GTPase effector domain are indicated above the alignment. The conserved tripartite GTP-binding motif (GDXXSGKS, DLPG, and TKPD) and a dynamin signature (LPRXX-GXXTR) in the GTPase domain, and leucine residues that form leucine zipper folds in the GTPase effector domain are in red. The four α-helices and the four loops connecting them are shown under the alignment as defined by Gao et al. [55]. The potential nuclear localisation signal (KKRKR) in trout Mx2 and 4, the four lysine residues of human MxA in L4 are in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

described previously [43]. The expression of each gene was first normalized to that of the house keeping gene elongation factor-1α (EF-1α). To directly compare the expression level of the different Mx paralogues, a reference was constructed using equal molar amounts of PCR product from each gene, including EF-1α.

2.6. Tissue distribution of rainbow trout Mx gene family

Six healthy rainbow trout (~140 g) were killed and seventeen tissues (blood, thymus, gills, scales, skin, muscle, tail fins, adipose fin, brain, adipose tissue, spleen, liver, heart, intestine, gonad, head kidney (HK) and caudal kidney) were collected and processed as described

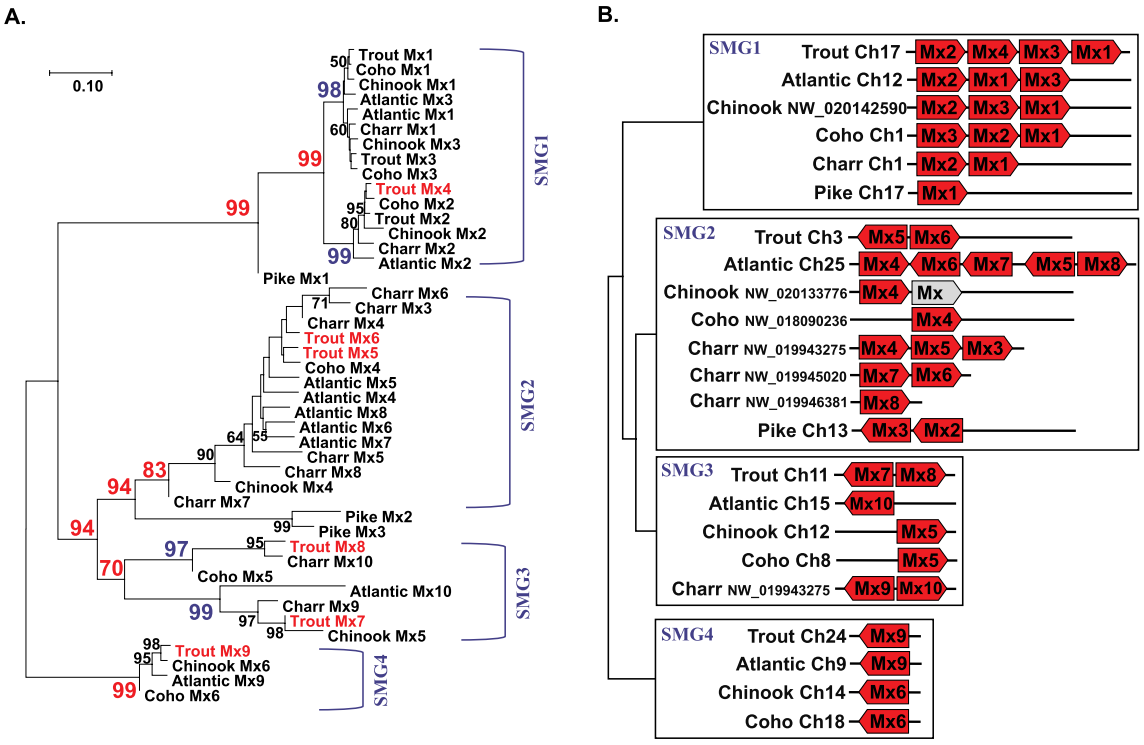

Fig. 2. Phylogenetic tree (A) and chromosome localisation (B) of salmonid Mx genes. A. The phylogenetic tree was constructed using a multiple alignment of salmonid and pike Mx aa sequences and the neighbour-joining method within the MEGA7.0 program. The evolutionary distances were computed using the JTT matrix-based method with all ambiguous positions removed for each sequence pair. The percentage (> 50%) of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The accession number for each sequence is given in Table 2. Four salmonid Mx groups (SMG)1–4 are indicated on the right. **B.** the chromosome localisation of Mx genes in salmonids and pike.

previously [34,35]. The relative expression level of Mx genes in each sample was normalized against the expression level of EF-1α and expressed as arbitrary units (AU) where 1 AU = the expression level of EF-1α/1,000,000.

2.7. Ontogeny of the expression of the Mx gene family

To investigate if the expression of Mx is correlated to immune capacity in early life, the ontogeny of the expression of Mx genes was examined. Archived samples from a previous experiment were used in this study as detailed in Wang et al. [44]. Briefly, eyed eggs, immediate post-hatch fry, pre-first feeding (Pre-feeding) fry at the stage of full disappearance of the yolk sac, and fry 3 weeks following first feeding were sampled and cDNA prepared. Six samples for each developmental stage were prepared. The qPCR quantification of gene expression was as described above.

2.8. Production of recombinant trout type I IFNa

The cDNA sequence encoding the mature peptide of trout IFNa was amplified from a poly IC stimulated cDNA sample using the primers IFNaF (TGTGACTGGATCCGACACCAT) and IFNaR (GTACATCTGTGCT GCAAGGATATCC). The amplified product was cloned to a pTriEx vector (Novagen) as described previously [45]. Sequence analysis of the construct used for recombinant protein production revealed that it encodes a His-tag (MAHHHHHHHHG) at the N-terminus followed by the 152 aa mature peptide identical to XP_021480273. Thus, the recombinant trout IFNa was 163 aa with a calculated molecular weight of 19.5 kDa and a theoretical pI of 9.17. A sequence confirmed plasmid was transformed into BL21 Star (DE3) competent cells (Invitrogen). The protein was produced, purified under denaturing conditions, refolded, and quantified as described previously [34,43,45]. The refolding buffer was phosphate buffered saline (PBS, pH7.4, Sigma, UK) containing 10% glycerol, 0.5 M arginine monohydrochloride, and 5 mM 2-mercaptoethanol (2-ME). The purified protein was buffer changed using a centrifugal concentrator (10 kDa cutoff, Thermo Scientific). The storage buffer was PBS (pH7.4) containing 10% glycerol, 2 mM EDTA, 10 mM arginine monohydrochloride, 10 mM glutamine, and 5 mM 2-ME. After sterilization with a 0.2 μm filter, the recombinant protein was aliquoted and stored at −80 °C ready for bioactivity analysis.

2.9. Stimulation of cell lines with PAMPs and recombinant cytokines

Three trout cell lines, a macrophage-like cell line RTS-11 from spleen [46], a fibroblast-like cell line RTG-2 from gonad [47], and an epithelial-like cell line RTGill from gills [48] were used for *in vitro* stimulation. All the cells were maintained at 20 °C in Leibovitz medium (L-15) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (P/S), and 10% (for RTG-2 and RTGill cell lines) or 30% (for RTS-11 cells) foetal bovine serum (FBS). The cells were seeded at 1×10^6 cells/ml (RTS-11) or 0.5×10^6 cells/ml (RTG-2 and RTGill) in L-15 containing 10% FCS at 2 ml/well in 12-well cell culture plates overnight before stimulation.

RTS-11 cells were first stimulated with pathogen-associated molecular patterns (PAMPs), the bacterial cell wall component lipopolysaccharide (LPS, from *E. coli* strain 055:B5, Sigma) and the viral dsRNA mimic polyinosinic: polycytidylic acid (poly IC, Sigma). The stimulants were added to the cells at 25 μg/ml for LPS and 50 μg/ml for poly IC, or medium alone as control. The treatments were terminated by dissolving the cells in TRI reagent (Sigma, UK) 4 h, 8 h and 24 h post-stimulation. Total RNA isolation and gene expression analysis was as described above.

The RTS-11 cells were then stimulated with five trout recombinant cytokines, IFNγ (20 ng/ml) [49], IFNα (25 ng/ml) prepared above, IL-1β (25 ng/ml) [50], IL-6 (100 ng/ml) [51] and TNFα (50 ng/ml) [52], or medium alone as control. The treatments were terminated at 4 h, 8 h

Table 2
Comparison of trout Mx aa sequence identities to Mx from other salmonids, spotted gar and mammals. The amino acid number for each sequence is also shown. Only full-length aa sequences were included in the analysis.

		No. of aa	SMG1				SMG2		SMG3		SMG4
			Trout Mx1	Trout Mx2	Trout Mx3	Trout Mx4	Trout Mx5	Trout Mx6	Trout Mx7	Trout Mx8	Trout Mx9
SMG1	Trout Mx1	**621**	**100.0**	**86.5**	**96.3**	**86.5**	46.2	46.3	47.9	46.4	47.7
	Trout-Mx2	**635**	**86.5**	**10 0.0**	**86.3**	**98.4**	45.6	45.0	47.0	45.1	47.3
	Trout Mx3	**623**	**96.3**	**86.3**	**100.0**	**86.0**	45.9	46.0	48.1	46.6	47.9
	Trout Mx4	**635**	**86.5**	**98.4**	**86.0**	**100.0**	45.8	45.5	47.0	45.2	47.0
	Atlantic Mx1	**623**	**95.3**	**85.8**	**97.3**	**85.4**	45.7	45.9	47.9	46.4	47.4
	Atlantic Mx2	**638**	**85.4**	**94.0**	**85.4**	**93.9**	45.4	44.8	46.4	44.7	46.7
	Atlantic Mx3	**623**	**96.3**	**86.5**	**95.8**	**86.6**	46.5	46.0	48.1	46.7	48.3
	Chinook Mx1	**621**	**98.2**	**86.8**	**96.0**	**86.5**	46.2	46.2	47.9	46.7	47.9
	Chinook Mx2	**633**	**83.7**	**95.0**	**83.4**	**95.1**	43.6	43.5	45.4	43.5	45.6
	Chinook Mx3	**623**	**95.2**	**86.0**	**97.8**	**86.2**	46.2	46.2	48.1	46.3	47.8
	Coho-Mx2	**648**	**84.3**	**95.2**	**84.1**	**95.7**	44.7	44.3	45.8	44.2	45.9
	Coho Mx3	**623**	**96.8**	**85.5**	**97.8**	**85.4**	46.0	46.2	47.9	46.3	47.8
	Charr Mx1	**623**	**96.0**	**86.6**	**97.9**	**86.2**	45.7	46.0	48.1	46.1	47.6
	Charr Mx2	**638**	**85.6**	**94.8**	**85.6**	**95.1**	45.4	44.9	47.0	45.3	47.3
SMG2	Trout Mx5	**614**	46.2	45.6	45.9	45.8	**100.0**	**93.6**	**61.2**	**69.4**	51.4
	Trout Mx6	**606**	46.3	45.0	46.0	45.5	**93.6**	**100.0**	**61.4**	**69.7**	50.8
	Atlantic Mx4	**606**	45.6	45.4	45.2	45.6	**86.0**	**88.3**	**62.2**	**70.1**	49.7
	Atlantic Mx5	**608**	45.4	44.7	45.5	44.8	**90.1**	**88.8**	**62.7**	**70.8**	50.2
	Atlantic Mx6	**627**	45.3	44.4	45.0	44.7	**83.9**	**84.4**	**61.3**	**67.9**	49.5
	Atlantic Mx7	**603**	46.4	45.0	46.1	45.5	**86.6**	**87.1**	**61.8**	**69.9**	49.8
	Atlantic Mx8	**607**	47.0	45.5	46.7	45.6	**87.0**	**90.0**	**62.5**	**71.2**	50.6
	Coho Mx4	**614**	46.0	45.2	45.7	45.3	**95.1**	**91.7**	**61.1**	**69.9**	51.6
	Charr Mx4	**607**	46.5	45.9	46.2	46.1	**90.9**	**89.8**	**61.2**	**69.3**	50.2
	Charr Mx5	**612**	45.1	43.8	45.2	44.2	**82.7**	**85.0**	**60.6**	**68.2**	49.7
SMG3	Trout Mx7	**613**	47.9	47.0	48.1	47.0	**61.2**	**61.4**	**100.0**	**68.6**	49.1
	Trout Mx8	**608**	46.4	45.1	46.6	45.2	**69.4**	**69.7**	**68.6**	**100.0**	49.8
	Charr Mx9	**549**	42.3	41.7	42.4	41.5	**53.6**	**53.4**	**80.7**	**57.8**	44.5
	Charr Mx10	**608**	46.1	45.2	46.4	45.4	**69.3**	**69.6**	**68.7**	**93.8**	49.8
SMG4	Trout Mx9	**640**	47.7	47.3	47.9	47.0	51.4	50.8	49.1	49.8	**100.0**
	Atlantic Mx9	**642**	48.1	47.6	48.2	47.1	51.6	50.8	49.5	49.5	**94.9**
	Chinook Mx6	**638**	47.4	47.2	47.8	46.9	51.1	50.5	49.3	49.4	**97.0**
	Coho Mx6	**646**	43.2	42.3	43.2	42.1	45.2	44.8	45.4	43.6	**80.2**
Gar	Gar Mx1	**619**	**73.9**	**71.3**	**74.8**	**71.3**	46.6	47.1	50.8	47.0	48.1
	Gar Mx2	**684**	**48.5**	**47.8**	**48.5**	**47.8**	38.7	38.6	41.2	39.2	39.4
	Gar Mx3	**616**	**47.6**	**44.3**	**47.0**	**44.4**	41.3	40.8	41.1	40.8	37.3
Mammals	Human MxA	**670**	**52.6**	**52.1**	**52.4**	**52.0**	40.5	40.4	42.1	41.2	43.1
	Mouse-Mx1	**631**	**51.8**	**51.2**	**52.0**	**51.6**	40.4	40.4	42.1	40.9	43.3
	Cow Mx1	**648**	**51.8**	**52.0**	**52.1**	**51.7**	39.5	40.7	42.7	41.5	42.8

High aa identities within each SMG, and between SMG1 and gar and mammalian Mx are in bold.

and 24 h and gene expression analysed as above.

Finally, RTG-2 and RTGill were stimulated with IFNγ (20 ng/ml) and IFNα (25 ng/ml) for 4 h and gene expression analysed as above.

2.10. Modulation of Mx gene expression in vivo by poly IC

Poly I:C (Sigma, UK) was dissolved at 10 mg/ml in sterile cell culture-grade water, stored at −80 °C and diluted to 5 mg/ml in PBS before use. Trout (~100 g, N = 24) were injected intraperitoneally (ip) with 1 mg poly IC in 0.2 ml of PBS, or the same amount of PBS as control. Six fish from each group were killed at 6 h and 24 h post injection, and spleen, HK, gills and intestine were collected for gene expression analysis as described previously [53]. The time points chosen were based on past studies of the rapid PAMP response *in vivo* in rainbow trout [54]. The expression was expressed as AU after normalisation with EF-1α, where 1 AU = the average expression level in control fish at 6 h in each tissue.

2.11. Statistical analysis

The data were analysed statistically using the SPSS Statistics package 24 (SPSS Inc., Chicago, Illinois). The analysis of real-time PCR data was as described previously (43). To improve the normality of data, real-time quantitative PCR measurements were scaled, with the lowest expression level in a data set defined as 1, and log2 transformed. One way-analysis of variance (ANOVA) and the LSD post hoc test were used to analyse the gene expression data, with $P \leq 0.05$ between treatment and control groups considered significant.

3. Results

3.1. Identification, cloning and sequence analysis of Mx gene family in rainbow trout

In addition to the known Mx1-3 in rainbow trout, six additional Mx genes (Mx4-9) have been identified and cloned in this study (Supplementary Figs. S1-S6, acc. nos. MK301134-MK301139). Mx4, as with Mx1-3, was located on Ch17 and was located between Mx2 and Mx3. Mx5-6, Mx7-8 and Mx9 were located on Ch3, Ch11 and Ch24, respectively (Table 1).

Each trout Mx cDNA sequence had a complete open reading frame that encoded for 635, 614, 606, 613, 608, and 640 aa for Mx4-9, respectively. Each trout Mx had a N-terminal dynamin GTPase domain, and a C-terminal GTPase effector domain, that were well conserved as shown in a multiple alignment of the 9 trout and two human Mx proteins (Fig. 1). The tripartite GTP-binding motif (GDXXSGKS, DLPG, and TKPD) in all trout Mx were identical to human MxA and MxB. The dynamin signature (LPRXXGXXTR), and the leucine residues that form leucine zipper folds in the GTPase effector domain, were also conserved (Fig. 1). The middle domain and the GTPase effector domain of Mx fold

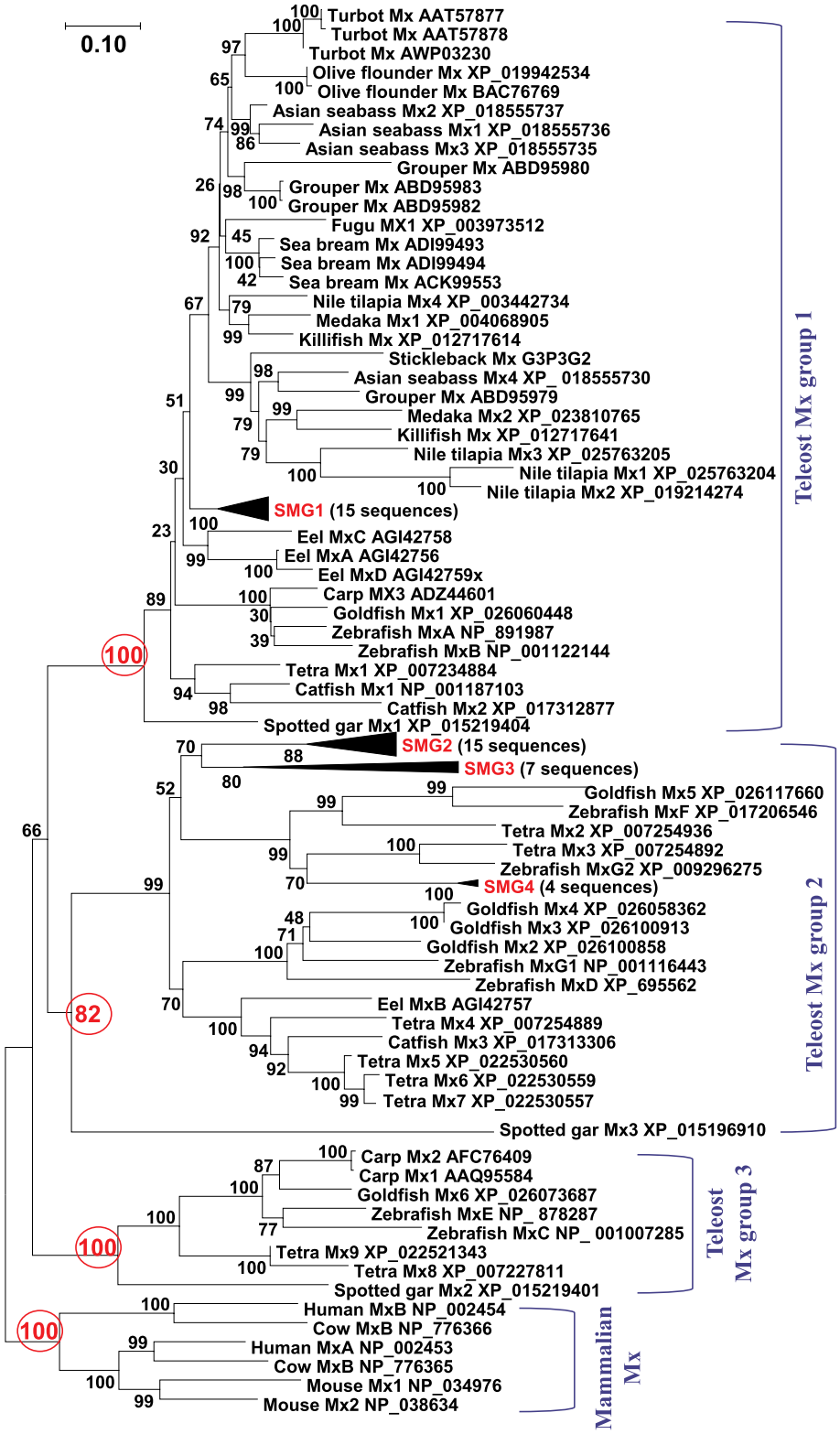

Fig. 3. Phylogenetic tree analysis of bony fish Mx. The phylogenetic tree was constructed using amino acid multiple alignments of Mx from selected teleosts and mammals, and the neighbour-joining method within the MEGA7.0 program. The evolutionary distances were computed using the JTT matrix-based method with all ambiguous positions removed for each sequence pair. Node values represent percent bootstrap confidence derived from 10,000 replications. The accession number for each sequence is given after the species and molecule names. The salmonid clades are highlighted and condensed under the name of SMG (salmonid Mx group)1–4, which share the same topologies as in Fig. 2. The root bootstrap values of mammalian Mx and teleost Mx group 1–3 are highlighted in red with the tentative groupings indicated on the right of the tree. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

into a four-helical bundle that constitutes a stalk that mediates oligomerization and transmits conformational changes from the G domain to the target structure [55]. The regions forming the helix, and loops L2 and L3 were all conserved. However, relatively large differences were present in loops L1 that connects the N and C-terminal of the helix α1 and introduces a kink, and L4 that connects the helix α3 and α4 (Fig. 1). Potential nuclear localisation signals (KKRKR) are present in trout Mx2 and Mx4 in L4, where a lysine motif (KKKK) is also present in human MxA that contribute to membrane association of MxA [2].

3.2. Sequence analysis of Mx family in salmonids

Nine Mx genes (Mx1-9) have been described recently in Atlantic salmon [27] that map to three chromosomes (Ch9, 12 and 25, Table 1). In addition, a partial sequence for Atlantic Mx10 (XP_013998960) has been mapped to Ch15 (Table 1). At least 6 Mx genes each in chinook

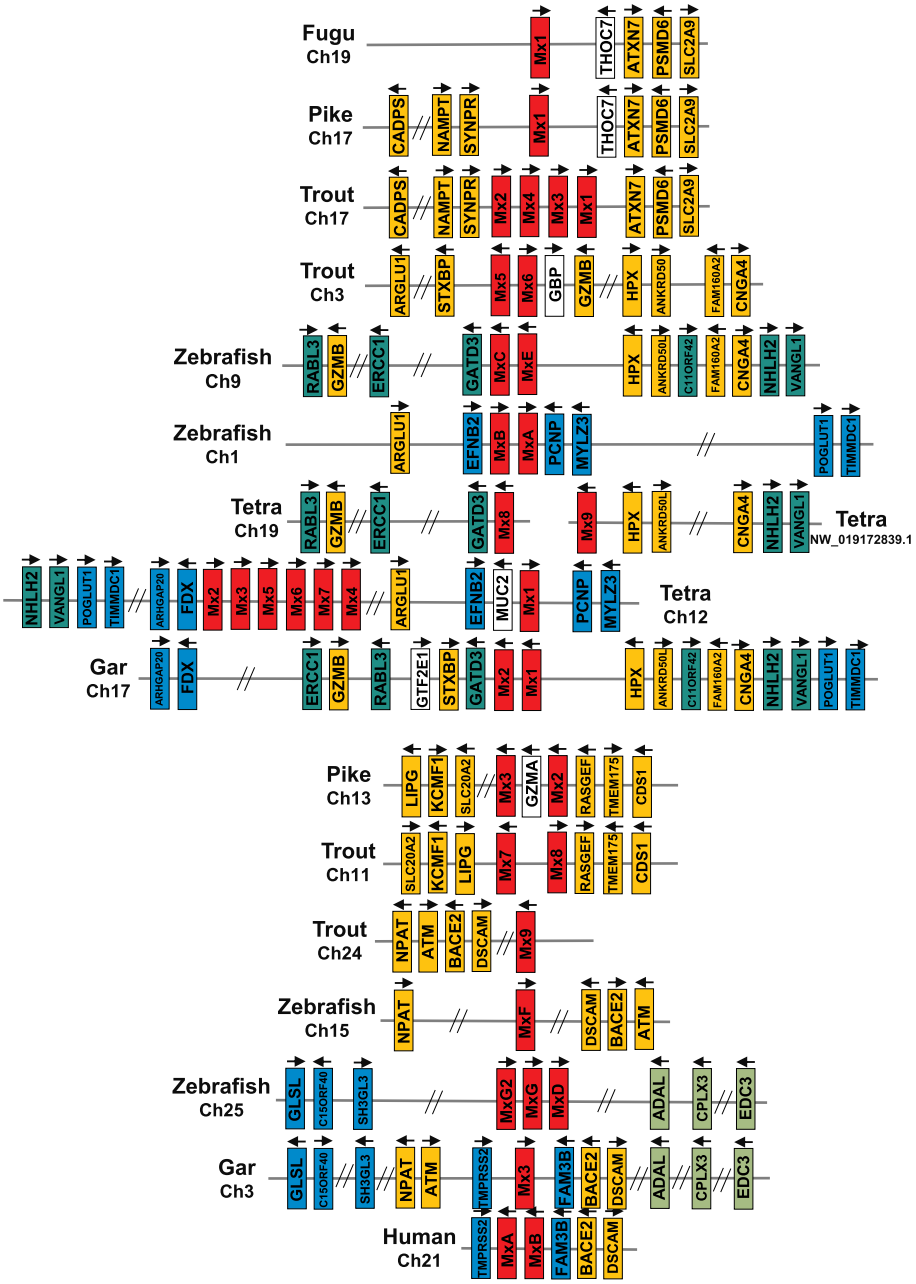

Fig. 4. Synteny analysis of Mx loci in bony fish and human. The synteny was predicted using the Genomicus program [42] or information extracted from recently released reference genomes at NCBI.

salmon and coho salmon, and 10 Mx genes in Arctic charr could be identified and mapped to chromosomes or scaffolds (Table 1). Partial sequences for three pike Mx genes were also found, with Mx1 on Ch17 and Mx2-3 on Ch13 (Table 1).

A phylogenetic tree constructed using all the known salmonid Mx and the three pike Mx protein sequences showed that the salmonid Mx family clustered into four separate clades (Fig. 2A). Moreover, salmonid Mx genes are located on four cognate chromosomes, at least in rainbow trout, Atlantic, chinook and coho salmon, in which their genome sequences have been mapped to chromosomes. The Mx genes on the same chromosome are grouped together (Fig. 2B), as seen also in Atlantic salmon [27], suggesting that multiple genes on the same chromosome originate from local gene duplication events in each species. Thus there are four salmonid Mx groups (SMG, Fig. 2). Pike Mx1 was grouped with SMG1, whilst pike Mx2 and Mx3, which are linked on Ch13, were grouped with SMG2. SMG1 consisted of Mx1-4 of trout, Mx1-3 of Atlantic, chinook and coho salmon, and Mx1-2 of charr. SMG2 consisted of trout Mx5-6, Atlantic salmon Mx4-8, chinook and coho salmon Mx4, and charr Mx3-8. SMG3 contained trout Mx7-8, Atlantic salmon Mx10, chinook and coho salmon Mx5 and char Mx9-10. SMG4 had trout and Atlantic salmon Mx9, and chinook and coho salmon Mx6 (Fig. 2).

It is notable that trout Mx1/3 and Mx2/4, along with their cognate salmonid Mx molecules formed two separate branches with high bootstrap support in SMG1 (Fig. 2A), suggesting that the existence of these genes, or their ancestral gene predates salmonid speciation. A similar situation was also observed with trout Mx7 and Mx8 in SMG3 (Fig. 2A). Although more Mx genes might still be found, the data for salmonids with an advanced (sequenced) genome suggests that the distinct numbers of Mx genes in SMG1-3 are due to species-specific independent local gene duplication or deletion events after salmonid speciation.

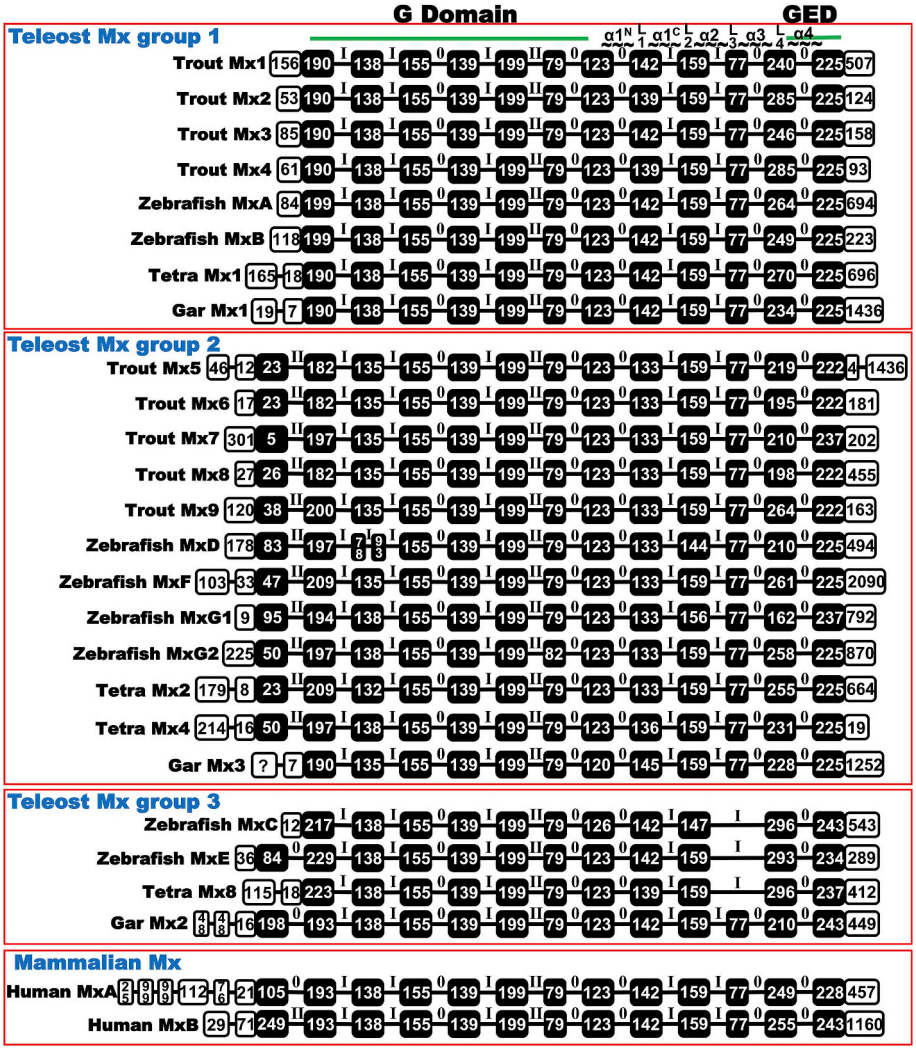

Fig. 5. Comparison of gene organisation of the Mx gene family in rainbow trout, other bony fish and humans. Boxes represent exons, and lines between exons represent introns. The black and white boxes represent non-coding and amino acid (aa) coding regions, respectively. The sizes (bp) of each exon are numbered in the boxes. The gene organisation of rainbow trout Mx genes was predicted using the Splign program based on the sequence information from Table 1 and Figures S1–S6 in Supplementary Material. The information of other species was extracted from recently released reference genomes at NCBI.

In agreement with four SMGs in the phylogenetic tree, the Mx aa sequences within each SMG share high aa identities (Table 2). In SMG1, trout Mx1-4 share high aa sequence identities between each other (86.3–98.4%) in a similar range to SMG1 Mx from different salmonids (83.4–98.2%), but have relatively low identities to Mx from SMG2 (43.8–47.0%), SMG3 (41.5–48.1%) and SMG4 (42.1–48.1) (Table 2). Similarly, Mx sequences share high identities within SMG2 (82.7–93.6), SMG3 (57.8–93.8%) and SMG4 (80.2–97.0%). However, the identities of Mx between SMGs are similarly low (41.5–51.6%) with the exception of Mx molecules in SMG2 and SMG3 that share moderate 53.4–71.2% aa identities (Table 2). Furthermore, the Mx bearing chromosomes in rainbow trout (Ch3, 11, 17 and 24) and Atlantic salmon (Ch9, 12, 15 and 25) do not share syntenic origins [56,57]. These data suggest that the four Mx-bearing chromosomes do not appear to originate from the salmonid 4R WGD.

3.3. Phylogenetic tree analysis of Mx in vertebrates

To understand how the four SMGs evolved, we analysed the Mx gene family in other vertebrates with a focus on teleost Mx genes. Three Mx genes, Mx1-2 on Ch17 and Mx3 on Ch3, are present in spotted gar, an early Actinopterygian (Holostei) that has not undergone the 3R WGD that may represent an ancestral state [4,27]. A neighbour-joining phylogenetic tree was constructed based on a multiple alignment of Mx proteins from selected mammalian and teleost fish species. In agreement with previous studies, mammalian Mx form an independent group separate from all teleost Mx molecules (Fig. 3). Three teleost Mx groups (TMG) can be identified, with a gar Mx at the root of each clade. TMG1 contained gar Mx1, salmonid SMG1 and Mx from all the major teleost groups (Fig. 3). TMG2 contained gar Mx3, salmonid SMG2, SMG3 and SMG4, and Mx molecules from European eel, zebrafish, goldfish, Mexican tetra and catfish. TMG3 contained gar Mx2, and Mx from Cypriniformes (zebrafish, common carp and goldfish) and Characiformes (tetra) (Fig. 3). This phylogenetic tree may suggest that the 3R WGD duplicated 6 Mx genes (from the 3 ancestral Mx genes present in gar) that have subsequently undergone lineage specific deletion, with Cypriniformes and Characiformes species retaining a copy of each of the duplicates, protacanthopteryggii such as the salmonids and pike retained two whilst in the majority of teleosts, the percomorphs only one is present. Within a species the numbers of Mx genes might be increased again by local gene duplication.

3.4. Synteny analysis of Mx locus in vertebrates

Despite much analysis, the evolutionary relationship of Mx genes in different vertebrates is still unclear [4,16]. In the present study we

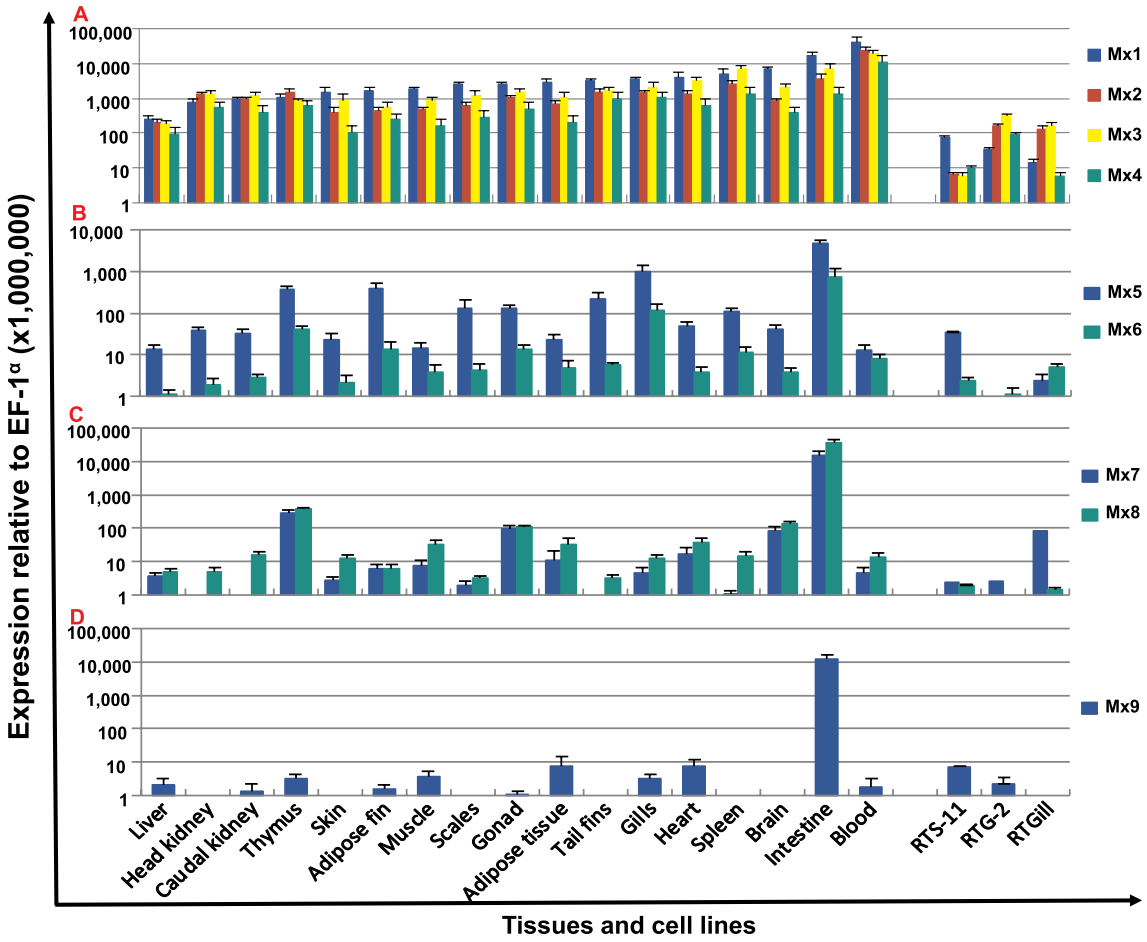

Fig. 6. Transcript expression of rainbow trout Mx gene family in tissues and cell lines. The expression level of Mx1-4 (A), Mx5-6 (B), Mx7-8 (C) and Mx9 (D) was determined by RT-qPCR in 17 tissues from six fish and four replicates of each cell line. The transcript level was calculated using a serial dilution of references that contained equal molar amounts of the probes for each gene and was normalized against the expression level of EF-1α. The results are presented as the average + SEM.

performed a synteny analysis using the most advanced genomes available. Pike Ch17 (Mx1) and trout Ch17 (Mx1-4), and pike Ch13 (Mx2-3) and trout Ch11 (Mx7-8) share a considerable syntenic relationship (Fig. 4). However, trout Ch3 (Mx5-6) and Ch24 (Mx9) share no clear syntenic relationships to pike Mx loci, but have a good relationship instead with gar Ch17 (Mx1-2) and Ch3 (Mx3), respectively (Fig. 4).

Interestingly, the Gar Mx3 (Ch3) locus also has considerable synteny with zebrafish Ch15 (MxF) and Ch25 (MxD, G1 and G2), in addition to the trout Mx9 locus, and all the Mx residing in these loci belong to TMG2. Furthermore, zebrafish Ch15 and 25 combined share a perfect match syntenically to gar Ch3, suggesting a break of the ancestral gar-like derived chromosome in zebrafish. The gar Mx3 locus also shares synteny with the tetrapod Mx locus, eg. human Ch21 (MxA and MxB) (Fig. 4), as also reported by Robertsen et al. [27]. This suggests that a gar Mx3-like ancestral locus gave rise to the teleost Mx group loci in zebrafish and salmonids, and led to the tetrapod Mx locus.

The gar Mx1-2 locus shares synteny to tetra Ch12 (Mx1-7) and the combined tetra Ch19 (Mx8) and scaffold NW_019172839 (Mx9) of tetra, indicating the retention of two 3R derived Mx loci in this species. Similarly, the gar Mx1-2 locus shares synteny to both zebrafish Ch1 (MxA/B) and Ch9 (MxC/E) (Fig. 4). It is notable that the tetra Ch12 Mx locus has Mx genes that belong to TMG1 (Mx1) as well as TMG2 (Mx2-7), whilst the zebrafish TMG2 resides in Ch15 and 25 derived from gar Ch3. Taken as a whole, two ancestral gar-like Mx loci gave rise to the current teleost Mx loci in a lineage-specific manner.

3.5. Gene organisation analysis of Mx genes in vertebrates

To shed more light on the evolution of the three teleost Mx groups, we analysed the gene organisation of all trout Mx genes in comparison with Mx genes from other teleosts and humans. All exon-intron boundaries of trout Mx genes conformed to the consensus sequences (GT/AG). In TMG1, trout Mx1-4 genes all had a 12 coding exon/11 intron structure with identical intron phase. The coding region of exons were identical with the exception of exon 8 and 11 (Fig. 5). A similar gene organisation of the coding region was observed with other TMG1 genes, eg. zebrafish MxA and MxB that are on the same chromosome, tetra Mx1 and gar Mx1, with the exception of a non-coding exon in the 5′UTR of the gar and tetra Mx gene in this group (Fig. 5).

Trout Mx5-9 belong to TMG2. They all had 13 coding exons with the coding regions of exons well conserved, with the exception of the first and the last two exons (Fig. 5). Compared to TMG1 genes, the last eleven intron phases were identical to that of TMG1 genes. The main difference in gene organisation was an extra N-terminal coding exon that brought a phase II intron in Mx5-9 that was missing in Mx1-4. This gene organisation was conserved in other TMG2 Mx genes except gar Mx3, that shared the same gene organisation with TMG1 Mx genes (Fig. 5).

Human MxA and MxB also had the same 13 coding exon structure as TMG2 but with the first intron in phase 0 (Fig. 5). Interestingly, some TMG3 Mx genes had the same gene organisation as in humans (zebrafish MxE and gar Mx2), and others (eg. zebrafish MxC and tetra Mx8) had the same as in TMG1 (Fig. 5). Some of TMG3 Mx genes have lost

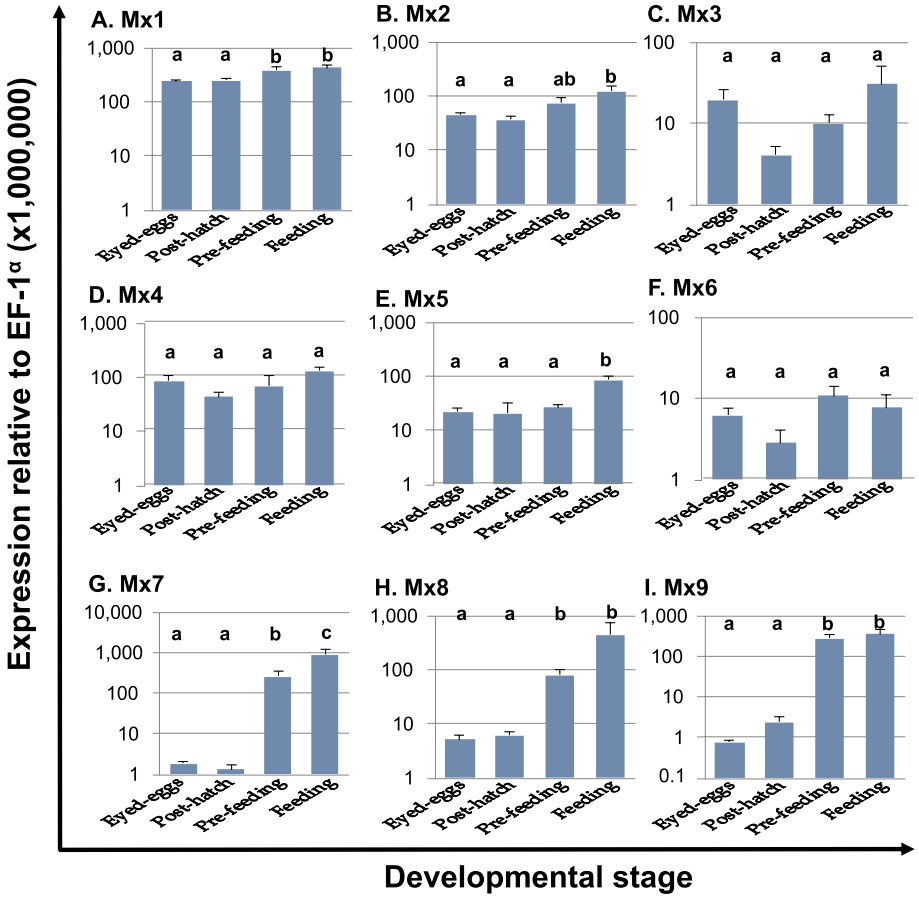

Fig. 7. The expression ontogeny of rainbow trout Mx gene family. cDNA samples were prepared from eyed-eggs, immediately post-hatch, pre-first feeding fry or fry 3 weeks after first feeding. Six independent samples for each developmental stage were prepared for real-time quantification of gene expression as described in Fig. 6. The results are presented as the average + SEM. Different letters over bars indicate significant differences ($p \leq 0.05$, one way-ANOVA).

the third last exon.

In general, the exon size and intron phase in the regions encoding for the N-terminal GTPase domain, the middle domain and the C-terminal GTPase effector domain are well conserved. The noticeable variations in size were the fifth last exon that encodes the L1 loop, and the second last exon that encodes for the L4 loop (Fig. 5).

3.6. The expression of rainbow trout Mx family in tissues and cell lines

The expression of each trout Mx gene was comparatively studied using gene specific primers and serial dilutions of references, and expressed as arbitrary units (AU) relative to EF-1α expression. Thus the AU of the relative expression is on an equal molar basis. The expression of paralogues on the same chromosome was grouped together, and the tissues were ordered according to the average expression level of Mx1 (Fig. 6). The expression level of Mx1-4 on Ch17 was medium (AU = 100 to 1000) to high (AU > 1000) across tissues (Fig. 6A). Mx5 and Mx6 expression was detectable in all the seventeen tissues but at low levels (AU < 100). The exceptions were Mx5 in intestine that was at a high level, and Mx5 in thymus, gills, adipose fin, tail fins, spleen, scales and gonad, and Mx6 in intestine and gills that was at medium expression levels (Fig. 6B). Mx8 expression was also detectable in all tissues examined but at low levels except for high level expression in intestine and medium level expression in thymus, brain and gonad. Mx7 expression was undetectable in head/caudal kidney and tail fins, but had high level expression in intestine, medium level in thymus, with low levels in other tissues (Fig. 6C). Mx9 expression was also high in intestine but low or undetectable in other tissues (Fig. 6D).

Each Mx gene was differentially expressed across different tissues. In the same tissue, the majority of Mx genes had varying expression levels, as shown by the ratio of different genes and paired-samples T tests (Table S3). In general, the expression of the Mx1-4 and Mx5-9 was more similar within each group than between groups. It is noteworthy that blood expressed highest levels of Mx1-4 but low levels of Mx5-9. In contrast, intestine expressed highest levels of Mx5-9 genes amongst the tissues examined (Fig. 6).

The constitutive expression of the trout Mx gene family was also examined in three trout cell lines. The macrophage-like cell line RTS-11 expressed all the Mx genes at low level (Fig. 6). The fibroblast-like cell line RTG-2 and epithelial-like cell line RTGill expressed medium levels of Mx2 and Mx3, and low levels of other Mx genes but Mx5 and Mx8 in RTG-2 and Mx9 in RTGill were not detectable (AU < 1).

3.7. Transcript expression of Mx gene family during developmental stages

The high levels of expression of Mx gene family members in blood and intestine suggest an important role in immune defence. We next examined the expression of these genes in eyed-eggs, immediately post-hatch fry, pre-first feeding fry or fry 3 weeks after first feeding, which represent a critical period when the fish encounter potential pathogens from the environment and food [44]. The expression levels of all Mx genes were maintained from eyed-eggs till post-hatch. The expression of Mx1, Mx8 and Mx9 was increased in pre-feeding fry and maintained at the same levels in post-feeding fry (Fig. 7). Mx5 expression was low in eyed-eggs and post-hatch fry but increased significantly in pre-feeding fry and increased further in post-feeding fry. The expression of Mx2 and Mx5 was only increased in post-feeding fry whilst that of Mx3, Mx4 and Mx6 was unchanged across the different developmental stages (Fig. 7).

3.8. Modulation of the expression of trout Mx and proinflammatory cytokine genes in vivo by poly IC

Poly IC, a known strong inducer of Mx expression, was used to investigate its ability to modulate Mx expression *in vivo*. The expression of

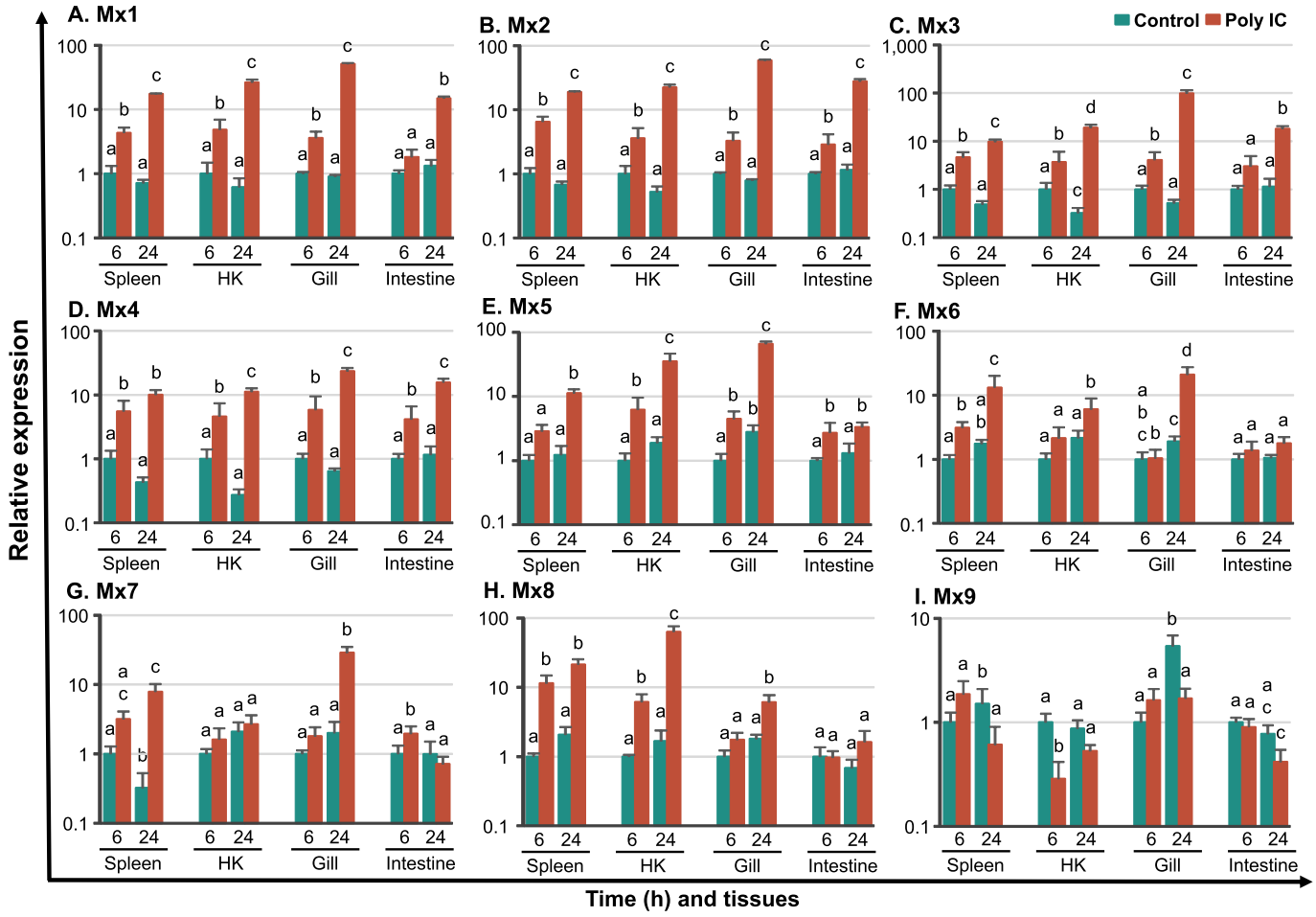

Fig. 8. Modulation of Mx gene expression *in vivo* by poly IC. Rainbow trout were injected ip with 1 mg poly IC in 0.2 ml PBS or 0.2 ml PBS as control. The spleen, head kidney (HK), gills and intestine were taken at 6 h and 24 h post injection. The quantification of Mx gene expression was as described in Fig. 6. The relative expression is shown, where the average expression level in the control fish at 6 h in each tissue was defined as 1. The results are presented as the mean + SEM of six fish. Different letters over bars in the same tissue indicate significant differences ($p \leq 0.05$, one way-ANOVA).

Mx genes was examined in two major systemic lymphoid tissues, the spleen and HK, and two mucosa-associated lymphoid tissues, the gills and intestine. The expression of Mx1-4 was induced in all four tissues at both 6 h and 24 h post poly IC injection, with the exception of Mx1 and Mx3 in intestine at 6 h (Fig. 8A–D). As seen *in vitro*, poly IC did not increase Mx9 expression *in vivo* (Fig. 8I). The induction of other Mx (5–8) genes was time- and tissue-dependent (Fig. 8E–H). In the spleen, poly IC increased Mx5 and Mx7 expression at 24 h and Mx6 and Mx8 expression at both time points. In the HK, poly IC increased Mx5 and Mx8 expression at both time points, and Mx6 expression at 24 h, but had no effect on Mx7 expression. In the gills, poly IC increased the expression of Mx6-8 at 24 h, and Mx5 at both time points. In the intestine that has high constitutive expression of Mx5-8, poly IC increased Mx5 expression at both time points and Mx7 expression at 6 h, but had no effects on Mx6 and Mx8 (Fig. 8E–H). In summary, poly IC was a strong inducer of Mx gene expression *in vivo* with highest induction seen at 24 h post-injection (Fig. 8).

In addition to inducing Mx gene expression, poly IC also induced the expression of many pro-inflammatory cytokines, including IL-1β1-2, IL-6, IL-8, TNFα1-2, IFNa1, IFNγ and CXCL11, at least at one time point, in all the four tissues examined (Fig. 9). In contrast to the later (at 24 h) peak induction of Mx gene expression, poly IC induced an early (6 h) induction of the majority of the proinflammatory cytokines studied (eg IL-1β1-2, IL-6, IL-8, TNFα2 and IFNa1) (Fig. 9).

3.9. Modulation of trout Mx expression in RTS-11 cells by PAMPs

The expression of all trout Mx genes was detectable in the macrophage cell line RTS-11 (Fig. 6). Thus we examined the modulation of trout Mx gene family members first in this cell line using poly IC and LPS, classical viral and bacterial PAMPs. Poly IC was a strong inducer of Mx gene expression. It significantly induced the expression of Mx2, Mx3 and Mx4 from 4 h to 24 h, that of Mx1, Mx5 and Mx6 from 8 h to 24 h, and that of Mx7 and Mx8 at 8 h, but had little effect on Mx9 expression (Fig. 10). As expected, LPS had only minor effects on Mx gene expression; it induced a small upregulation of Mx4 at 8 h and Mx5 at 24 h, and a small downregulation of Mx1 at 24 h and Mx2 at 4 h (Fig. 10).

3.10. Modulation of Mx expression by proinflammatory cytokines in RTS-11 cells

The early peak induction of proinflammatory cytokine expression and late peak induction of Mx genes may suggest that poly IC can induce Mx indirectly via proinflammatory cytokines as well as by virus sensing pathways. Indeed, IFNγ has been shown recently to modulate some of the Mx isoforms in Atlantic salmon [27]. Hence, the possibility of modulation of Mx gene expression by IFNa1, IFNγ, IL-1β, IL-6 and TNFα was studied using RTS-11 cells. Mx9 expression was refractory to all the cytokines (Fig. 11I). However, the expression modulation of the other Mx genes was cytokine-specific. IFNa induced the expression of Mx1-4 and Mx6 from 4 h to 24 h, Mx5 at 4 h and 24 h, and Mx7 at 8 h,

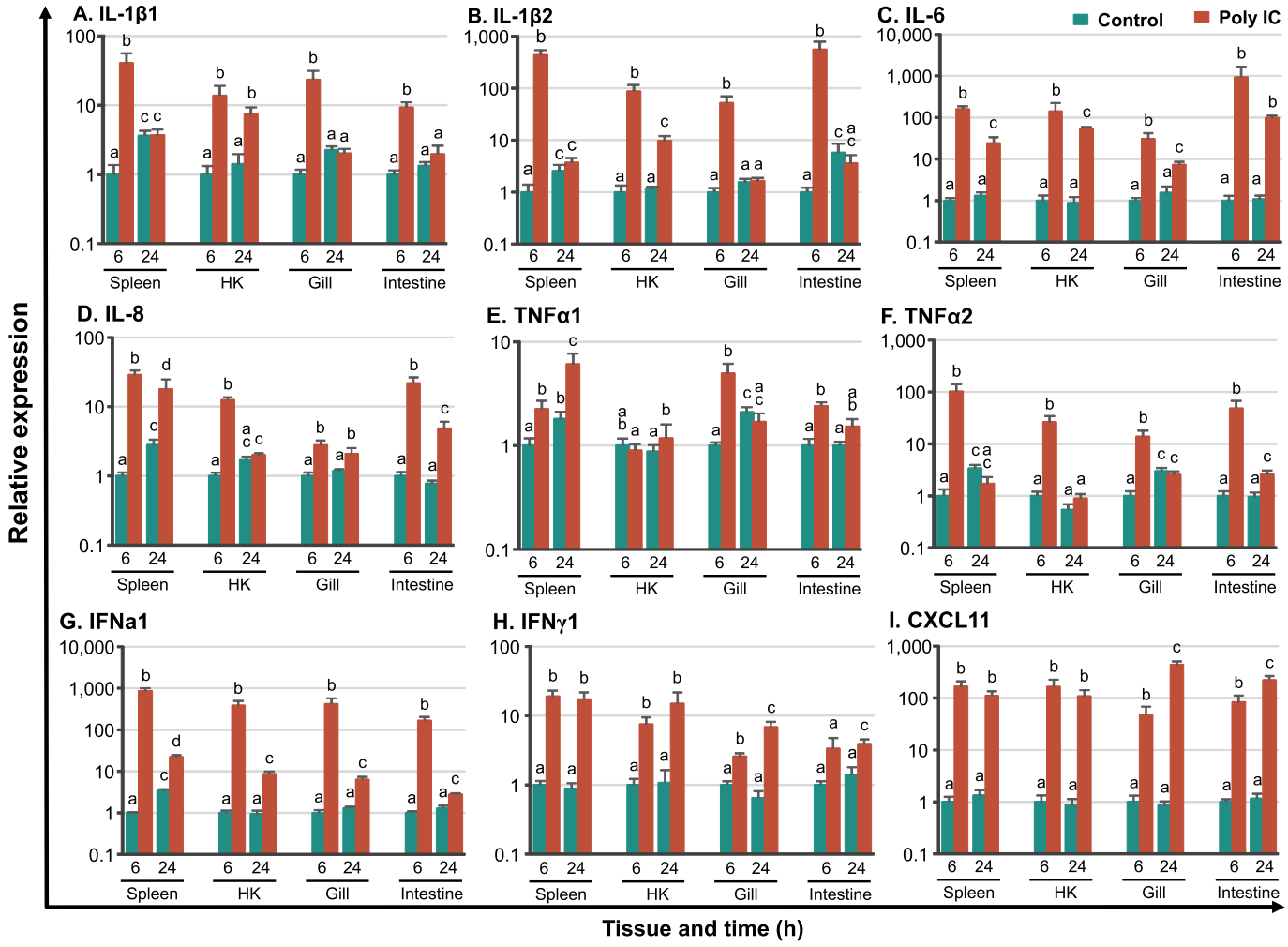

Fig. 9. Modulation of proinflammatory cytokine gene expression *in vivo* by poly IC. Rainbow trout were injected ip with 1 mg poly IC in 0.2 ml PBS or 0.2 ml PBS as control. The spleen, head kidney (HK), gills and intestine were taken at 6 h and 24 h post injection. The quantification of gene expression was as described in Fig. 6. The relative expression is shown, where the average expression level in the control fish at 6 h in each tissue was defined as 1. The results are presented as the mean + SEM of six fish. Different letters over bars in the same tissue indicate significant differences ($p \leq 0.05$, one way-ANOVA).

but had no effects on Mx8 (Fig. 11). IFNγ induced the expression of Mx2-6 from 4 h to 24 h, Mx7-8 at 24 h, but decreased Mx1 expression at 24 h (Fig. 11A–H). IL-1β induced the expression of Mx3-4 and Mx8 from 4 h to 24 h, Mx2 and Mx6 at 4 h and 8 h, Mx5 at 24 h, but had no effects on the expression of Mx1 and Mx7 (Fig. 11A–H). IL-6 increased the expression of Mx3 at 8 h and 24 h, Mx5 at 24 h, and Mx6 at 4 h, but decreased the expression of Mx 1 and Mx4 at 24 h, and Mx8 at 8 h and 24 h. It had no effects on Mx2 and Mx7 (Fig. 11A–H). TNFα induced the expression of Mx2 and Mx4 at 8 h, Mx3 from 4 h to 24 h, Mx5 at 24 h, but decreased Mx1 expression at 24 h and Mx5 expression at 8 h. It had no effects on Mx6-8 (Fig. 11A–H). It is noteworthy that IFNa is a strong inducer of the expression of Mx1-4 and Mx7, IFNγ is a strong inducer of Mx5-6 expression and IL-1β a strong inducer of Mx8 (Fig. 11A–H). In conclusion, all the Mx genes except Mx9 can be modulated by multiple antiviral and proinflammatory cytokines in an Mx- and cytokine-dependent manner.

3.11. Modulation of Mx expression by type I and type II IFNs in RTG-2 and RTGill cell lines

The cytokine-dependent Mx modulation may also be cell-type dependent. Thus, the IFN modulated Mx gene expression was further studied in the fibroblast-like cell line RTG-2 and the epithelial like cell line RTGill. In RTG-2 cells, both IFNγ and IFNa induced the expression of Mx1-6, with similar potency for Mx5 and Mx6. However, IFNa was more potent for Mx1-4 (Fig. 12). In RTGill, both IFNγ and IFNa induced the expression of Mx2 and Mx3, with IFNγ more potent for Mx2, but had no effects on Mx1 and Mx4 (Fig. 12A–D). Only IFNγ but not IFNa induced the expression of Mx5 and Mx6 in RTGill cells (Fig. 12E–F). The expression of Mx7-9 was low and refractory (data not shown).

4. Discussion

This study reveals that at least 9 active Mx genes are present in the rainbow trout genome, the same number as reported recently in Atlantic salmon [40]. However, in this study we show that there are in fact 4 Mx loci present in salmonids and that the number of Mx genes at each locus differs between these two species at 3 of these loci. Multiple Mx genes are also present in other salmonids at four chromosomal loci. The salmonid Mx genes at the same genomic locus share high sequence identities within and between species, suggesting they arose from local gene duplication events. It seems that local Mx gene duplication/gene loss is common with some duplication events likely to have happened before salmonid speciation, eg. duplication of Mx1/3 and Mx2/4 in SMG1, and Mx7 and Mx8 in SMG3, but others after salmonid speciation, eg. Atlantic salmon Mx4-8 in SMG2. The four Mx bearing chromosomal loci could have arisen from the 3R and 4R WGDs as seen with other genes when mammals have one and salmonids have 4 [28].

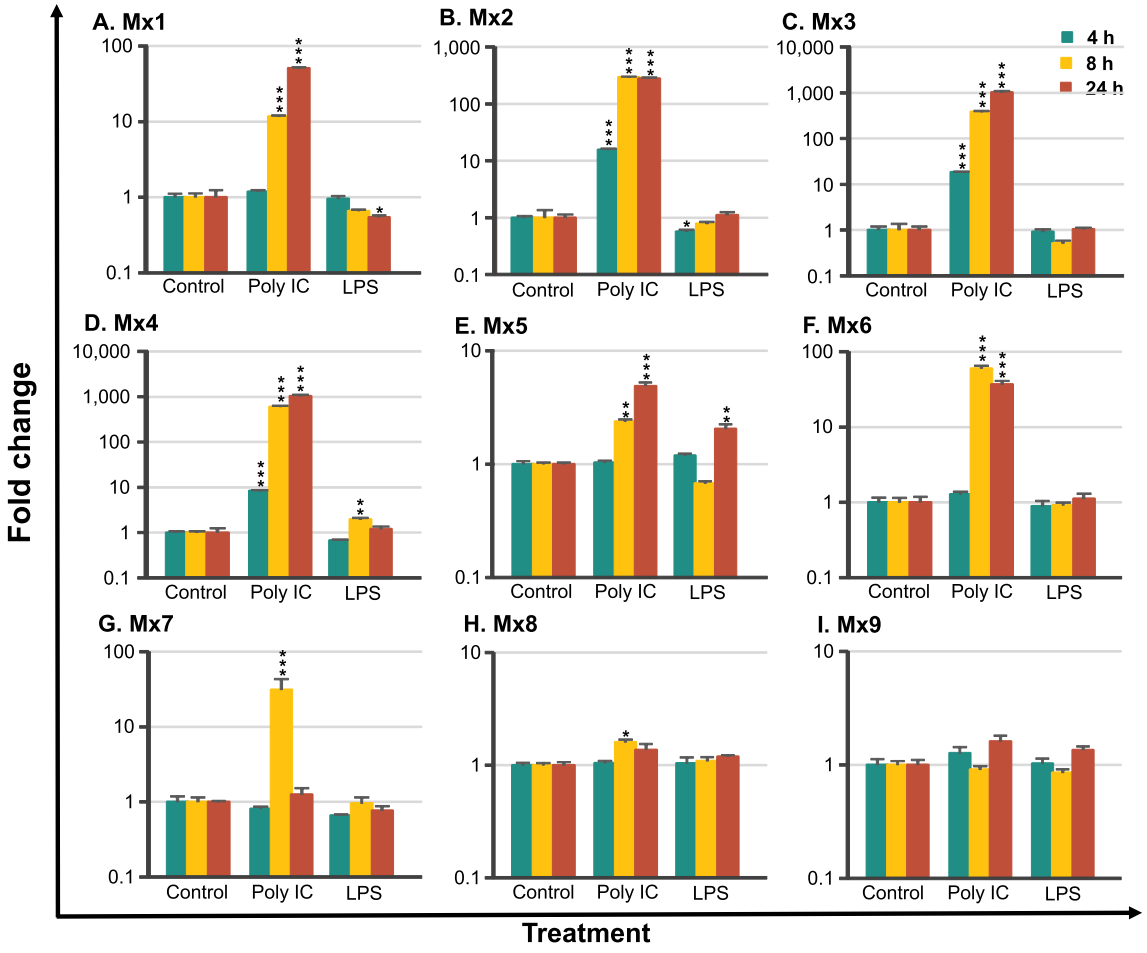

Fig. 10. Modulation of Mx gene expression in RTS-11 cells by poly IC and LPS. Overnight culture of RTS-11 cells were stimulated with poly IC (50 μg/ml), LPS (25 μg/ml), or medium as control for 4 h, 8 h and 24 h, and the expression of trout Mx genes was quantified by RT-qPCR as described in Fig. 6. The data are presented as the mean (+SEM, N = 4) fold change calculated as the average expression level of stimulated samples divided by that of time-matched controls. The relative significance of a LSD post hoc test after a significant one-way ANOVA between the stimulated and time-matched controls is shown above the bars as * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

However, sequence homology, synteny and phylogenetic tree analysis do not clearly support this, and past models [4] do not adequately explain their evolutionary path in bony fish.

Multiple Mx genes (up to 10) can be found in many teleost species. Our phylogenetic tree analysis indicates that three TMG exist. TMG1 are present in different teleost lineages, but TMG2 and TMG3 are found in only more basal teleosts [57]. Each TMG has a unique gene organisation in terms of coding exon number and the first intron phase. For example, whilst TMG1 has a 12 coding exon structure with the first intron in phase I, TMG2 has 13 coding exons with the first intron in phase II, and TMG3 has either 13 coding exons with the first intron in phase 0 (as seen with mammalian Mx genes) or the same organisation as in TMG1. Interestingly, the spotted gar possesses three Mx genes, with one present in each TMG.

Although the four cognate Mx chromosomal loci between salmonids are well conserved, no clear syntenic conservation have been observed in trout and other salmonid species between the four Mx loci. However, a syntenic relationship between the two gar Mx loci and those in zebrafish/tetra is apparent. For example, the Gar Mx1-2 locus and tetra Mx loci on Ch12 and Ch19, that harbour Mx genes in all the three teleost Mx groups. However, the zebrafish cognate Mx loci of gar Mx1-2 only have Mx genes that belong to TMG1 and TMG3, and the zebrafish TMG2 locus shares synteny to the gar Mx3 locus. The two gar Mx loci also share apparent synteny with two of the trout Mx loci whilst the other two show a syntenic relationship with the two pike Mx loci. This complex syntenic relationship may suggest that the current Mx genes in 3R or 4R teleosts may have arisen from the three Mx genes present at two chromosomal loci as seen in spotted gar, with the 3R duplicated Mx loci retained/lost in a lineage specific manner (Fig. 13). This model differs from that in Qi et al. [4] in taking into account the number of loci present in actinopterygians as well as Mx copy number.

The increased copy number of Mx genes seen in many teleosts may confer increased expression level and hence heightened antiviral defence. The duplicated copies may also acquire novel sequence properties that confer anti-viral specificity and efficiency. The nine trout Mx genes have considerable variation in the nucleotide sequence coding for the L1 and L4 loops in the stalk, as seen in the multiple aa alignments and their gene organisation. Both L1 and L4 are at the surface of the stalk [3] that can interact with surrounding proteins and may be involved in interaction with viral components. L4 of mammalian Mx is a critical determinant of viral substrate specificity [58,59]. The diversification of these regions might have been driven by past virus exposure and life history traits of different species. For example, zebrafish and tetra have short life cycles but live in diverse changing water environments. Their survival depends heavily on innate immunity against viral pathogens. Salmonids survive successfully in both fresh and marine waters, and may encounter a larger virus repertoire compared to species living in only fresh water or marine water. Hence, the increase of copy number and types of Mx genes in these species may confer a fitness advantage.

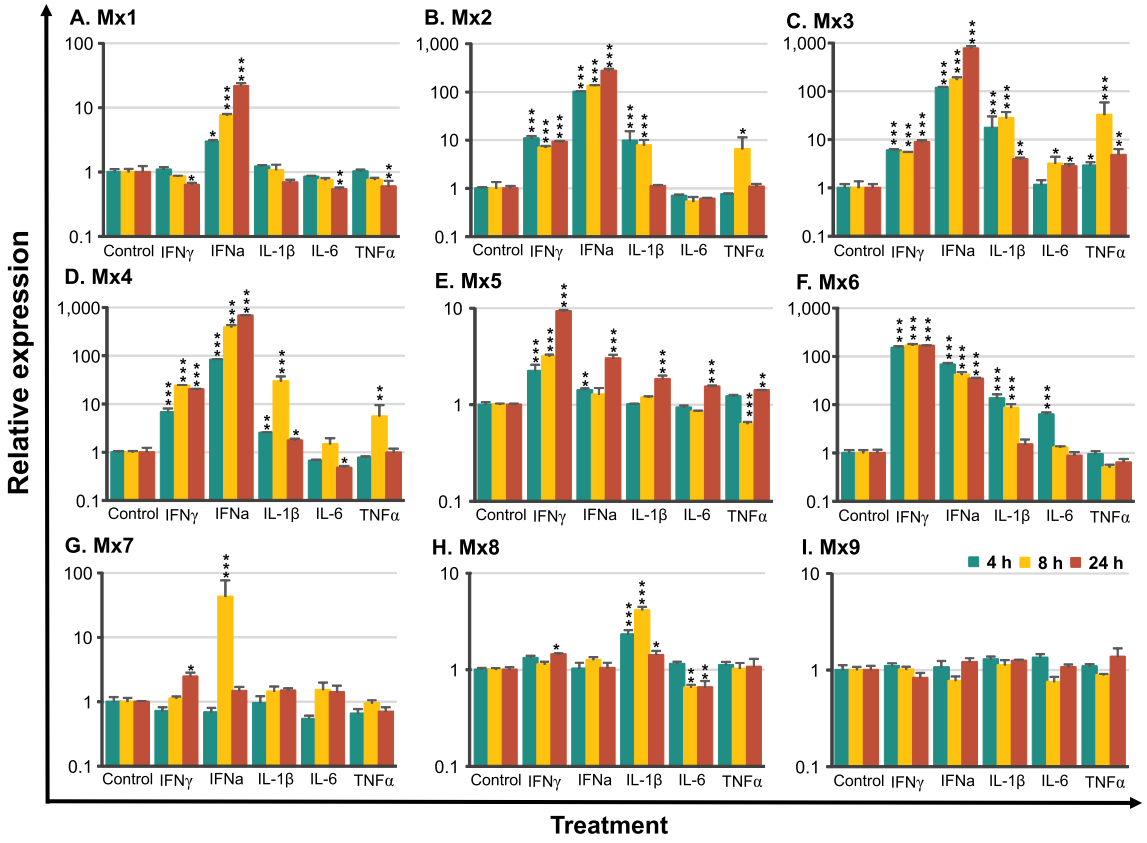

Fig. 11. Modulation of trout Mx expression in RTS-11 cells by pro-inflammatory cytokines. Overnight culture of RTS-11 cells were stimulated with IFNγ (20 ng/ml), IFN-a (25 ng/ml), IL-1β (25 ng/ml), IL-6 (100 ng/ml), TNFα (50 ng/ml), or medium as control for 4 h, 8 h and 24 h, and the expression of trout Mx genes was quantified by RT-qPCR as described in Fig. 6. The data are presented as the mean (+SEM, N = 4) fold change, calculated as the average expression level of stimulated samples divided by that of time-matched controls. The relative significance of a LSD post hoc test after a significant one-way ANOVA between the stimulated and time-matched controls is shown above the bars as * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$.

Mx antiviral effects depend on where the Mx protein is present. Thus, the mouse Mx1 protein which is localized in the nucleus mainly inhibits orthomyxoviruses that replicate in the nucleus, whereas mouse Mx2 is confined to the cytoplasm and inhibits viruses with an exclusively cytoplasmic replication phase [60]. There is a potential NLS in the L4 of some salmonid Mx proteins eg. trout Mx2, and Mx4, but not Mx1 and Mx3. This NLS may indeed be functional as trout Mx2 is found in the nucleus, and Mx1/Mx3 in the cytoplasm [7]. This suggests the nuclear presence of trout Mx4. Taken as a whole, salmonids, such as rainbow trout are equipped with a battery of diversified Mx genes with their protein products present in the cytoplasm and nucleus to protect themselves from viral attack during their life cycle.

Investigation of Mx isoform expression will help understand their functional roles. Although multiple Mx genes have been identified in several teleost species, a comparative expression study in healthy fish at the individual gene level is lacking [17,27]. Our results show that the nine trout Mx genes were differentially expressed across different tissues and cell lines, as outlined below, suggesting a level of neofunctionalisation of trout Mx paralogues through variation in expression patterns. The high levels of Mx1-4 transcript in blood and Mx5-9 in intestine is of particular interest. Many different viruses can infect hosts via the intestine to cause acute infectious gastroenteritis, or get access to the blood by physical breaches (wounds) or during viremia [61]. So preventing their spread at these sites is a good antiviral strategy. The differential expression of Mx genes in the cell lines may suggest that specific cell types preferentially express a particular Mx gene or a set of Mx genes to defend against potential cell type-tropic viruses. However, the three cell lines examined expressed relatively low levels of Mx genes compared to the tissues analysed, perhaps due to the need for humoral factors present *in vivo* to maintain high level Mx gene expression.

The expression of Mx genes was also studied during development, and several were increased in pre-feeding and post-feeding fry (eg Mx1, Mx2, Mx5, Mx7-9). First feeding is a critical stage in the life of a fish, when potential food borne viruses are met directly for the first time and when the adaptive immune system has not fully developed. Indeed, it was the genes preferentially expressed in the intestine in adults that were increased in the post-hatch fry.

Next we studied whether the Mx genes could be modulated by PAMPS or cytokines. In agreement with Mx induction in other species, poly IC was a strong inducer of trout Mx1-8 gene expression *in vitro* and *in vivo*, with Mx9 more refractory. Although the induction patterns *in vivo* were gene- and tissue-dependent, highest expression was seen at 24 h with most of the Mx genes. Injection of poly IC also induced the expression of proinflammatory cytokines, such as IL-1β, IL-6 and TNFα as well as type I and type II IFNs. In contrast to the late peak of induction of Mx gene expression, poly IC caused an early peak of expression in the cytokines studied. Therefore, the later peak in Mx expression could be influenced by such molecules. To test this hypothesis, we stimulated RTS-11 cells with these cytokines to see if they could modulate Mx expression. Seven of the nine Mx genes were induced by type I IFNa and type II IFNγ, and six were induced by IL-1β. In contrast, IL-6 and TNFα had only minor effects on Mx expression. This cytokine mediated induction was gene-dependent. IFNa was a strong inducer of Mx1-4 and Mx6-7. Past studies have shown Mx1-3 to be modulated by type I IFNs, and so it was no surprise that Mx4 as an additional SMG1/TMG1 member was also induced. Mx6 and Mx7 on the other hand are TMG2 genes. Studies with two other cell lines confirmed the induction of Mx6 by type I IFN as well as a small induction of Mx5 (as seen in

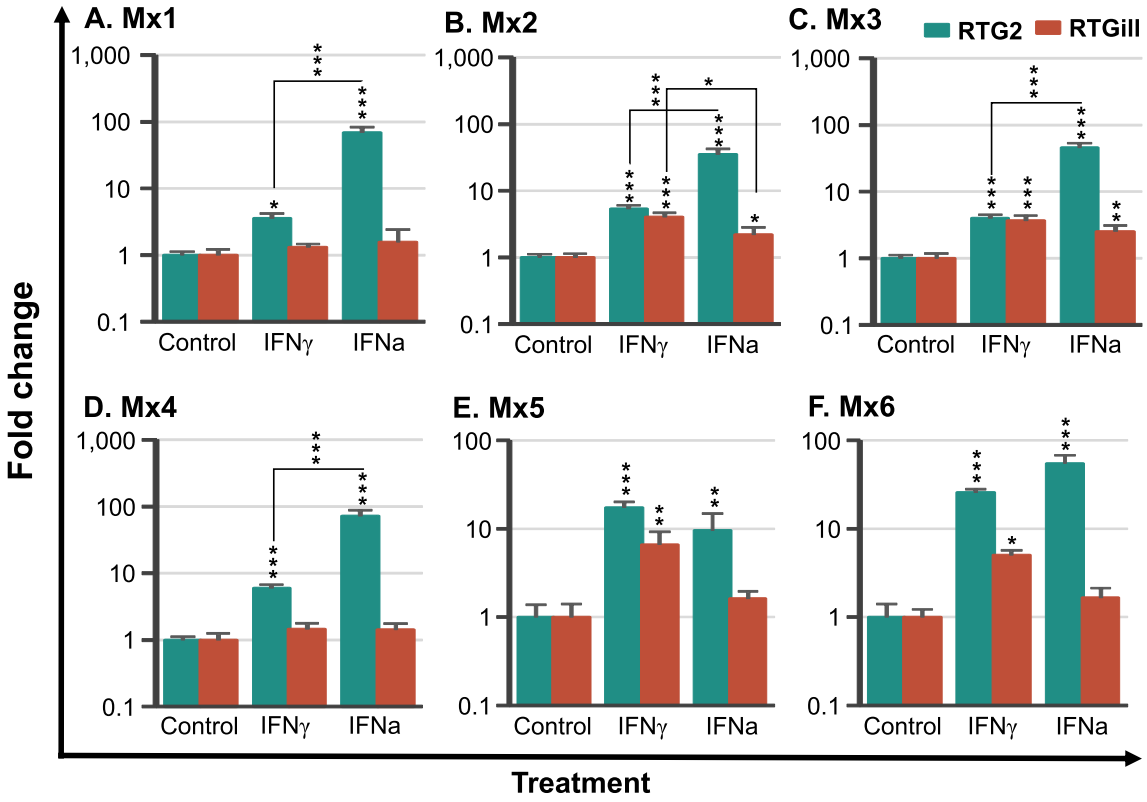

Fig. 12. Modulation of trout Mx expression in RTG-2 and RTGill cell lines by IFNs. Overnight cultures of cells were stimulated with IFNγ (20 ng/ml), IFN-a (25 ng/ml), or medium as control for 4 h, and the expression of trout Mx genes was quantified by RT-qPCR as described in Fig. 6. The data are presented as the mean (+SEM, N = 4) fold change, calculated as the average expression level of stimulated samples divided by that of controls. The relative significance of a LSD post hoc test after a significant one-way ANOVA between the stimulated and controls is shown above the bars as * $p \leq 0.05$; ** $p \leq 0.01$ and *** $p \leq 0.001$. The line-connected groups are significantly different.

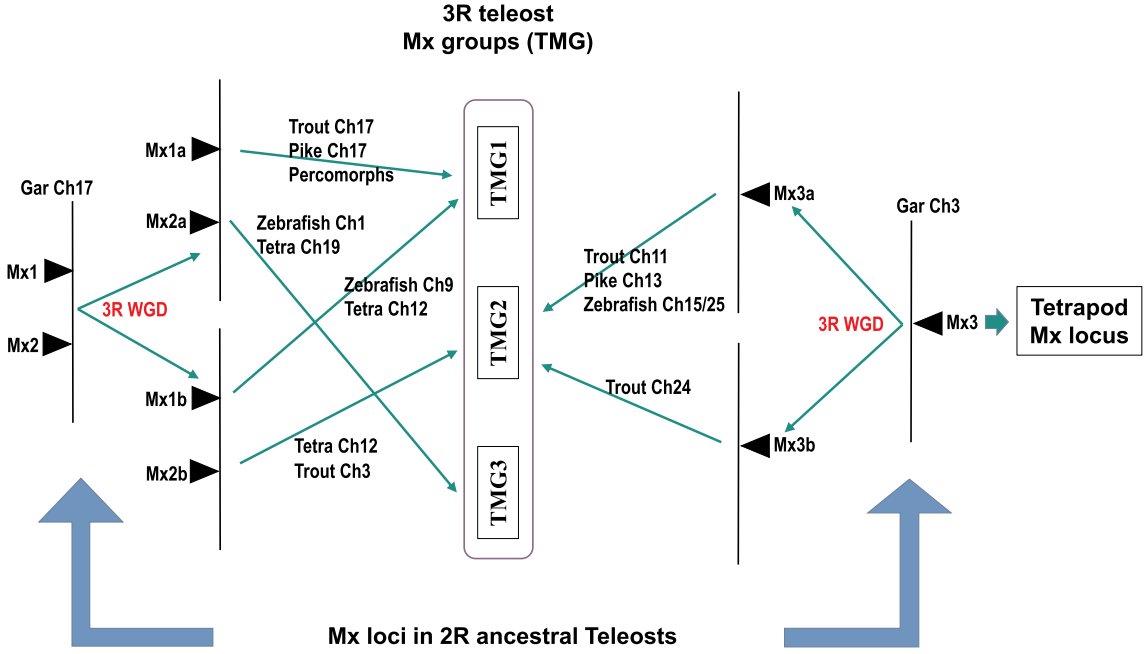

Fig. 13. Hypothetical evolutionary pathways of teleost Mx gene family. Three Mx loci (Mx1-3) were present on two chromosomes in ancestral 2R actinopterygians. 3R WGD is expected to have produced 6 Mx loci on four chromosomes that were retained in a lineage specific manner to give rise to the three extant teleost Mx groups. The ancestral vertebrates that evolved into the tetrapod lineage appear to have possessed a cognate Mx locus of gar Ch3. Arrow heads indicate ancestral Mx genes. Representative chromosomal loci are shown.

RTS11 cells) but Mx7 was not expressed in these cells. IFNγ was a strong inducer of Mx5-6, although some induction of Mx1-4 was also seen in the different cell lines. Trout Mx5 and Mx6 are on the same locus as Mx4-8 in Atlantic salmon, that were also responsive to IFNγ [27], and are SMG2/TMG2 genes. The SMG3 (Mx7-8) and SMG4 (Mx9) genes did not show this responsiveness. IL-1β was able to induce Mx8 (SMG3) in RTS-11 cells although some induction of Mx2-4 and Mx5-6 was also seen, suggesting a broader responsiveness across SMGs. In common with salmon, no induction of Mx9 was found with these PAMPS/cytokines and its role, if any, in antiviral defence remains to be elucidated.

Such findings contrast with mammalian Mx genes that are strictly induced by type I and type III IFNs but are not induced by IFNγ or other proinflammatory cytokines [29–31]. Salmonids possess multiple type I (IFNa-f) and type II (IFNγ1-2 and IFNγrels) IFNs, but type III IFN has not been identified in any fish species [62]. In addition to the induction of Mx genes by type I and type II IFNs, this study confirms that some proinflammatory cytokines also influence Mx expression in fish. IL-1β in particular has a clear impact on Mx gene expression in trout and was the only cytokine that induced Mx8 expression. Thus, it is apparent that cytokines other than IFNs can have a role in antiviral defence.

5. Conclusions

Up to 10 Mx genes are present in salmonids that reside in four chromosomal loci. Three teleost Mx groups (TMG) can be identified with characteristic gene organisations, each with a spotted gar Mx gene at the root in the phylogenetic tree. Synteny analysis suggests that the current Mx genes in 3R or 4R teleosts may be evolved from the three Mx genes present at two chromosomal loci in spotted gar, with the 3R duplicated Mx loci retained/lost in a lineage specific manner. Salmonid Mx belong to TMG1 and TMG2. The increased salmonid Mx gene copies are due to local gene duplications that have happened before and after salmonid speciation in a lineage/species specific manner. Salmonids are equipped with a diversified battery of Mx genes, with their protein products present in both cytoplasmic and nuclear locations to protect against viral attack during their life in freshwater and seawater.

Trout Mx genes are differentially expressed in tissues with high levels of expression of TMG1 (Mx1-4) in blood and TMG2 (Mx5-9) in the intestine. The expression of most of the trout Mx genes was induced by poly IC (*in vitro* and *in vivo*), and increased during early developmental stages. In addition to induction by type I IFN, IFNγ and IL-1β also induced Mx expression in rainbow trout and are cytokines that are highly modulated by viral infection. These results show that salmonids possess a large number Mx genes as well as complex regulatory pathways to induce Mx gene expression for antiviral defence, which may contribute to their success in an anadromous life style.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.303.

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