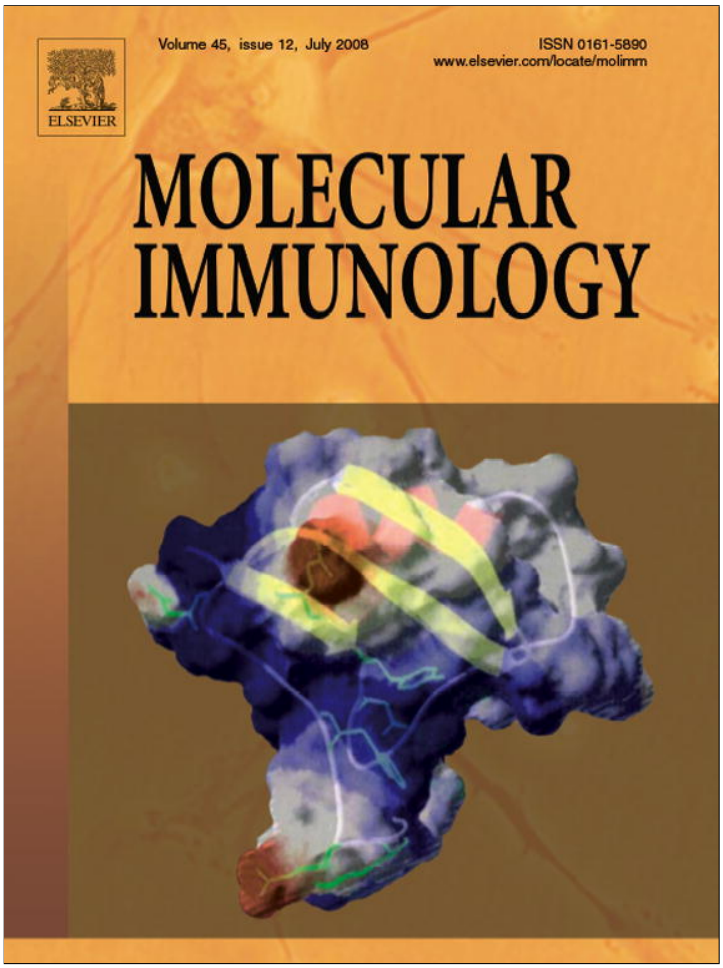

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Short communication

Teleostean IL11b exhibits complementing function to IL11a and expansive involvement in antibacterial and antiviral responses

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Abstract

Interleukin 11 is a class-1 helical cytokine, having the four-helix bundle structure, possessing pleiotropic characteristics involved in physiological processes including blood production, bone formation and placentation. The interleukin 11 paralogues (IL11a and IL11b) have been identified in fish with only IL11a from carp and trout have been characterized and analyzed for its expression thus far. Here, we cloned and studied the structure and expression of IL11b in Japanese flounder (*Paralichthys olivaceus*), and compared this with the existing information on fish IL11 paralogues. Japanese flounder IL11b (*po*IL11b) cDNA is composed of 1536 bp encoding for 201 aa residues with a 23 aa leader peptide, three cysteine residues (C^{12}, C^{183} and C^{198}) and four potential N-linked glycosylation sites. *po*IL11b does not show constitutive expression in tissues of adult fish except for the very slight expression in kidney and spleen, and the very high expression in peripheral blood leukocytes (PBLs). *po*IL11b is transiently up-regulated by bacterial lipopolysaccharide (LPS) and increasingly stimulated by the IFN inducer poly I:C in kidney, spleen and peripheral blood leukocytes of adult fish *in vitro*. It is likewise slightly stimulated by *Edwardsiella tarda* infection but is highly expressed after hirame rhabdovirus (HIRRV) infection in kidney of juvenile fish. The stimulation studies suggest that *po*IL11b, aside from its role in bacterial infection, is well involved in antiviral responses. Moreover, *po*IL11b structure and expression pattern appears to be slightly distinct and opposite to IL11a, respectively, suggesting a complementation of function of the duplicate fish IL11 genes.

Keywords: Interleukin 11b; Gene duplication; Subfunction; Teleost; Immunity

1. Introduction

Interleukin 11 (IL11) is a pleiotropic cytokine that possesses many functions such as production of thrombocytes (thrombocytopoiesis), megakaryocytes (megakaryo-cytopoiesis) and other blood cells, bone formation and osteoblastosis, and in placental development (for reviews see Huising et al., 2006; Du and Williams, 1997). Because of its influence in hematopoiesis, particularly in megakaryocyte and thrombocyte development, it has been used to prevent thrombocyte/platelet loss following cancer therapy (Kurzrock, 2005) and is also being explored as a cure for neonatal thrombocytopenia (Ramasethu, 2004).

Mammalian IL11 is a single copy signal molecule that is expressed by and acts on various types of cells. It is composed of a high number of proline (P), leucine (L) and positively charged amino acids making it a basic molecule. It possesses a 4 α-helix bundle structure even in the absence of disulphide binding cysteine (C) residues, made possible by hydrophobic interactions (Czupryn et al., 1995). Because of the 4 α-helix configuration and the formation of a hexameric complex by IL11, IL11 receptor and gp130 during signal transduction (Heinrich et al., 2003), IL11 has been classified as a member of the class-1 helical cytokines that include interleukin 6 (IL6), ciliary neurotrophic factor (CNTF), leukemia inhibiting factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), cardiotrophin-2 (CT-2) and cardiotrophin-like cytokine (CLC) (Huising et al., 2006).

Teleost fish IL11 orthologue was first reported in trout (Wang et al., 2005). Subsequent cloning studies on carp coupled with *in silico* analysis of zebrafish (*Danio rerio*), tiger pufferfish (*Takifugu rubripes*) and green spotted pufferfish (*Tetraodon nigroviridis*) genomes allowed for the identification

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of a duplicated fish IL11 gene (IL11a and IL11b) (Huising et al., 2005). In both these reports, a partial expressed sequence tag (EST) fragment (AU090873) from Japanese flounder has been included in the phylogenetic analysis and was shown to cluster with fish IL11b. The IL11a and IL11b genes from carp, trout, zebrafish, tiger pufferfish and green-spotted pufferfish have been thoroughly characterized for its genomic and secondary protein structures. However, only IL11a from carp and trout has been so far investigated for its expression where it was found to be ubiquitously expressed in all tissues including brain, intestine, skin, muscle, liver, spleen, head kidney, kidney, thymus and gills except peripheral blood leukocytes (PBLs). IL11a expression has likewise been observed to be enhanced by lipopolysaccharide (LPS), bacteria (*Aeromonas salmonicida* MT423), concanavalin A, poly I:C and recombinant IL-1β, albeit at different levels, but significantly inhibited by cortisol (Wang et al., 2005; Huising et al., 2005). It is not known whether IL11b is functional and to what extent is its role in fish immunity in relation to a functional duplicate IL11a given that there is only a single IL11 protein in higher vertebrates.

Japanese flounder is one of the economically important culture species that is being impacted by diseases such as *Edwardsiella tarda* and hirame rhabdovirus (HIRRV) (Oh and Choi, 1998; Pluma, 1999). As such, it has been the subject of extensive studies on host–pathogen interactions and immunology at the molecular level.

Here, we report the cloning of the full Japanese flounder IL11b cDNA (*po*IL11b) and the analysis of its expression constitutively and in response to LPS and poly I:C stimulation *in vitro*, and to *E. tarda* and HIRRV infection *in vivo*. *po*IL11b amino acid structure is generally conserved compared to other vertebrate IL11 orthologues. However, IL11bs has two extra C residues compared with fish IL11as. Further, comparative analysis between the IL11 duplicates showed different and opposite expression patterns, constitutively and in response to immunostimulation. These suggest that there is complementation of function of the duplicate genes. Moreover, *po*IL11b is significantly up-regulated by both bacterial and viral agents *in vitro* and *in vivo* exhibiting its involvement in a wide range of immunological responses.

2. Materials and methods

2.1. Molecular cloning

The full-length Japanese flounder IL11 type b cDNA was cloned by first selecting an EST clone (Accession no: AU090873) showing putative homology to published fish IL11. An anti-sense primer (5′-GTCCACCTGATGGATCATCG-3′) was then designed from the upstream portion of this clone and, in partner with M13 reverse primer (5′-AGCGGATAACAATTTCACACAGG-3′) amplified a resulting ∼200 bp fragment using a previously constructed λZipLox vector-based Japanese flounder cDNA library (GIBCO BRL/Life technologies) as template.

2.2. In silico analysis

The nucleotide sequence, translated amino acids, isoelectric points (p*I*) and average molecular weight were analyzed and determined using GENETYX 7.0.3 (GENETYX Corporation). SignalP (http://www.cbs.dtu.dk/services/SignalP/) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) servers were used to predict signal peptide cleavage and *N*-glycosylation sites, respectively. Identities were calculated using BLASTp (BLOSUM 62) implemented in BLAST 2 SEQUENCES (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) and the complete multiple amino acid alignments were carried out in CLUSTAL X 1.81 using default parameters. Protein domain prediction was carried out using ProDom (http://prodom.prabi.fr/prodom/current/html/home.php) and 123D+ (http://123d.ncifcrf.gov/123D+.html) servers. For phylogenetic analysis, we used the neighbor joining (NJ) algorithm implemented in the MEGA3 (http://www.megasoftware.net/index.html/) employing the Poisson correction method with 1000 bootstrap re-sampling and with complete deletion of gap sites. The bootstrap consensus tree was shown.

2.3. Constitutive expression in tissues

For RT-PCR analysis of constitutive expression, total RNA was extracted from brain, eyes, gills, kidney, heart, intestine, peripheral blood leukocytes, liver, muscle, skin, spleen, stomach from three apparently healthy Japanese flounder. cDNA synthesis was done in each of the tissue samples. Resulting cDNA were amplified using the following *po*IL11b primers (IL11F, 5′-CACTGGAGTCAGAGGAGGTC-3′ and IL11R, 5′-TGACTCTCCTGCCTCCAGAG-3′). PCR conditions were: initial denaturation at 95 °C for 5 min, 30 cycles (95 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min), and final elongation at 72 °C for 5 min. The PCR products were electrophoresed on a 1.0% agarose gel containing ethidium bromide. Resulting bands were photographed with a densitometer and were semi-quantitatively assessed for their relative expression following Santos et al. (2006) using the ImageJ software (Abramoff et al., 2004).

2.4. Expression in tissues after in vitro immunostimulation

Primary cultures of kidney and spleen were prepared by first dissecting out the tissues from about 3 kg fish and placed in a solution containing heparin, FBS, RPMI and penicillin/streptomycin. These were then mash-filtered using a sterile mesh net, suspended in 1× phosphate buffer saline (PBS), centrifuged then re-suspended in a medium containing FBS, RPMI and penicillin/streptomycin. On the other hand, PBLs were isolated using Percoll solution, centrifuged then re-suspended in a medium similar to the kidney and primary cultures. 1 ml of each primary culture suspensions was equally placed in cell culture plates and then treated with immunostimulants accordingly. The following final concentration of 0.5 mg/ml LPS and poly I:C and with PBS were used and sampling was done at 1, 3 and 6 h post-induction. Total RNA was extracted from the treated and control cells for cDNA synthesis and subsequent RT-PCR anal-

ysis *po*IL11b primers (same as above). Primers for Japanese flounder granulocyte colony stimulating factor, CSF3 (Santos et al., 2006), Mx (Caipang et al., 2003) and β-actin (Katagiri et al., 1997) genes were used for comparative analysis and as controls. PCR conditions were: initial denaturation at 95 °C for 5 min, 26 cycles (95 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min), and final elongation at 72 °C for 5 min.

2.5. Expression in tissues after infection in vivo

Apparently healthy juvenile fish samples weighing about 2 g each were used in the *in vivo* studies. Five fish individuals were placed in re-circulating aquarium tanks. For the *E. tarda* experiment, one tank containing 15 fish samples were infected with the bacteria (2.5×10^7 CFU/ml) by immersion while one untreated tank was used as a control. Kidney from five fish samples was sampled at 1 day, 3 days and 7 days post-infection and also from the control. For the HIRRV, 15 fish samples were intramuscularly injected with the virus (3.2×10^3 $TCID_{50}$) and another 15 with PBS for the control. Kidney from five samples was taken at 1 day, 3 days and 7 days post-injection. For each of the sampling time points, kidney was pooled and then total RNA was extracted for cDNA synthesis and subsequent RT-PCR analysis. *po*IL11b, CSF3, Mx and β-actin gene primers (same as above) were used for the analysis. PCR conditions were: initial denaturation at 95 °C for 5 min, 26 cycles (95 °C: 30 s; 55 °C: 30 s; 72 °C: 1 min), and final elongation at 72 °C for 5 min.

3. Results

3.1. Japanese flounder IL11b

The complete Japanese flounder interleukin 11 type b (*po*IL11b) cDNA was composed of 1536 bp encoding for a putative protein of 201 aa residues (Fig. 1). The nucleotide sequence showed four mRNA destabilizing AUUUA motifs and a typical poly-adenylation signal "AATAAA". The polypeptide on the other hand had a predicted signal sequence of 23 aa cleavage of this peptide results to a mature *po*IL11b protein containing 178 aa with a predicted molecular mass of about 20 kDa and predicted isoelectric point (p*I*) of 6.94. It was leucine (L) and serine (S) rich at 16% and 15%, respectively, had four C residues and four predicted N-linked glycosylation sites. Using the ProDom and 123D+ servers, *po*IL11b protein has been identified as homologous to the mammalian IL11 motif and belongs to the structural classification of proteins family of long-chain cytokines (A.26.1.1), respectively.

Alignment of fish IL11 orthologues and paralogues revealed some interesting results (Fig. 2). The *po*IL11b leader peptide of 23 aa was 3-less than the other fish IL11a and 1-less than mam-

```
CCACGCGTCCGTGTACACACACTGGAGTCAGAGGAGGTCGGTTCACTGCAGCAGGAGAAG    60
AAGAAAGATGAAATTGCTTCATGACTCCATCCCATGTCTTTTCCACCTGCTGCTATTGGC   120
       M  K  L  L  H  D  S  I  P  C  L  F  H  L  L  L  L  A
TGAGCTGTTTGTCCCGTCATCGTCTCGTCCCGTCCACACCTCCTCCCTCTGTAGGATGTT   180
 E  L  F  V  P  S  S  S  R  P  V  H  T  S  S  L  C  R  M  F
TGGATCGATGATCCATCAGGTGGACAAGCTGACGGACATCTCCAAAAACCTCCATGAGCT   240
 G  S  M  I  H  Q  V  D  K  L  T  D  I  S  K  N  L  H  E  L
GTCGGACAACAACGAGCTCCTGAACTCTGCGGATAACAAACTTCCTGATCTTCCTCACAT   300
 S  D  N  N  E  L  L  N  S  A  D  N  K  L  P  D  L  P  H  M
GCAACACTCTGCGGCACATTTTTTTAATTCACTGAAGATGAACGAGTCCCTCTCTGAGCT   360
 Q  H  S  A  A  H  F  F  N  S  L  K  M  N  E  S  L  S  E  L
CTACCTGCTCGCTCAGGCCTTCAGGCTGCACGTCGACTGGCTGAAGACGGAAAAAGACAA   420
 Y  L  L  A  Q  A  F  R  L  H  V  D  W  L  K  T  E  K  D  N
CTTCAGTTTACCCAGTCAGTCAGCAGAGGACGCCAGCACTCATCTGCTGCAGCTGTCCAA   480
 F  S  L  P  S  Q  S  A  E  D  A  S  T  H  L  L  Q  L  S  N
CCTGCTCAACATGTCACTGCACCAGATGAGTGCAGAGACGCCTCAGCCGCCGGCTCCCTC   540
 L  L  N  M  S  L  H  Q  M  S  A  E  T  P  Q  P  P  A  P  S
CCTCCCTGTCGTCTCCTCGGCCTTCGACCTTCTCCAGTTCTCCATCGAGATCTCTGAACG   600
 L  P  V  V  S  S  A  F  D  L  L  Q  F  S  I  E  I  S  E  R
GCTAAAAGTCTTCTGTAATTGGTCAAAAAGAGTTCTACGATCTCTAAAACTCCCGCGCTG   660
 L  K  V  F  C  N  W  S  K  R  V  L  R  S  L  K  L  P  R  C
CCGCAGACAGTGAGAAGCAGCGGCTCATGTCTTCCTAACCACTGTTAACGTAAAAGATTT   720
 R  R  Q  *
TCATGCAGCAGCGAATCAGCAGCAAAACTCTGGAGGCAGGAGAGTCACTTTGGTTCAACA   780
ACAATAGATATATACTTTGCACTGTGCTCAGAGGATGTGAACCCTCGACCACACACACGT   840
TCACTTATCGGTTTTTATTTCAACGCCTTGTTGATGAATTGGTACAAAATCTGGACGTTGA   900
CAGTGAAGAATTTTCATGACTTCGGTGATTTGCTTCCTCAGACGGCATCAGGTTTACATG   960
TGGTTTTTTGAAATAAGTCTGAGTTTGGTTCGGATGTGTTTTGTTTGGACAAGTTAGGAC  1020
AAAATGGTGAATGTTGAAGTAACTATTGGTGGTGGGAACATTATGGGAAGTTTACCCCCA  1080
GATGATCAACTTTTAAAGGTCGACAAAAATATGGTCTCAAAAAACAGTCGCAAATAATAG  1140
AGACTAAAGTTCGTGTTGATCAGGAAATGATTGTGTGAGGTGCAGCGCTCAGTGGCACGG  1200
TTACGTTTACGCCCTTTAAGACTGTTTACATGATTCAAAGTACGAGAAAAAATATGAATG  1260
AATGCTGTTATTAATATTTTTCTACTGACTGTTTTATATTTAAGAGGCTATTTATGATAT  1320
TTGTTACATAAAGAACTTGACAGTGTTTAGTGTGAGATGCAGGCATCTCATTGTTTTCTA  1380
TGAAATATTTAATATTAATATTAATAATAATAATAACGTATTGTCATGATGTAGACGGTT  1440
ACACTTCTATTTATACTTTTTTATACCTTTTTGTTTTTCTACCATGTTTGAAGTCATTAAT  1500
AAAAATTTACATTAAAACATAAAAAAAAAAAAAAAA                          1536
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Fig. 1. Complete cDNA sequence of Japanese flounder IL11 type b (*po*IL11b). Start and stop codons are in bold, italized; predicted signal peptide is underlined, potential N-linked glycosylation sites are boxed; the mRNA destabilizing motif ATTTA are in bold, underlined; and the poly(A) signal AATAAA is in bold. Cysteine residues are shaded black.

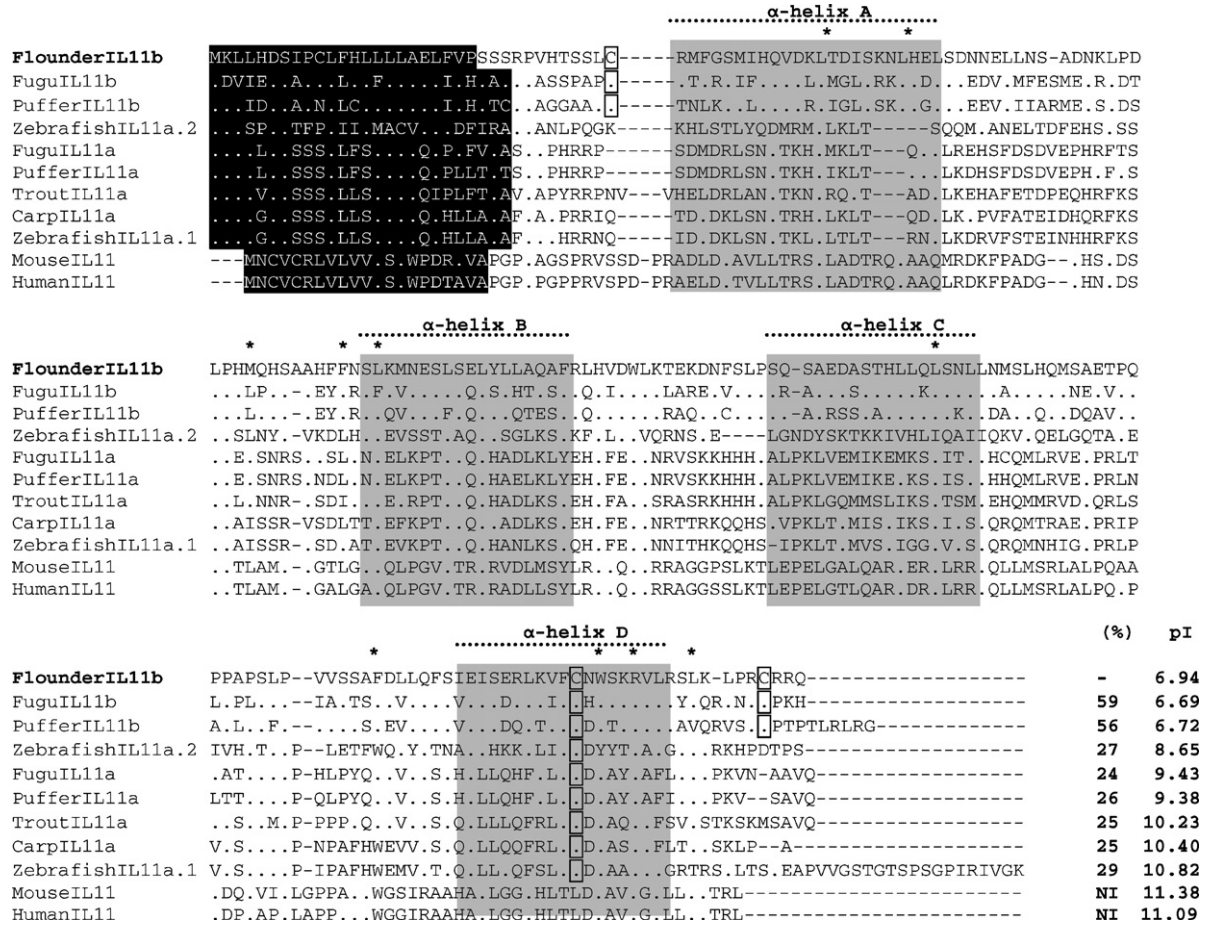

Fig. 2. Alignment of interleukin 11 orthologues and paralogues. Signal peptides and charged amino acids (aspartic acid, D; glutamic acid, E; lysine, K; arginine, R) and the polar histidine (H) are shaded in black while important functional residues are denoted by asterisks (*). α-Helices A–D are shaded gray. Conserved cysteines are boxed while other conserved residues are represented by dots (. . .). Gaps (−) were introduced to maximize alignment. NI means no significant identity.

malian IL11s. Three C residues of *po*IL11b were shown to be part of the mature peptide. One C residue (C^{183}) was well conserved in fish IL11s but the other two C residues (C^{12} and C^{198}) were only conserved among IL11bs [*po*IL11b, tiger pufferfish (*tr*IL11b) and green-spotted pufferfish (*tn*IL11b)] and not with IL11as (*tr*IL11a, *tn*IL11a, *cc*IL11a, *om*IL11a, *dr*IL11a.1 and *dr*IL11a.2). It is important to note that there are two zebrafish *dr*IL11s identified so far that are homologous to type a. The putative *po*IL11b helices (A–D) were mainly composed of charged residues aspartic acid (D), glutamic acid (E), lysine (L) and the polar histidine (H). Some residues that have been reported in mammals to be important for receptor binding were conserved in *po*IL11b including L^{28} and L^{34} in Helix A, L^{173} at the start of Helix B, L^{115} in Helix C, and tryptophan (W^{165}), arginine (R^{168}), L^{171} and L^{173} in Helix D. Another important residue which was conservatively replaced to phenylalanine (F) is the hydrophobic L^{170}. The comparative identities among fish and mammalian IL11 orthologues as well as isoelectric points were also interesting (Fig. 2). Between *po*IL11b and the other fish IL11b, the identities were significantly conserved at more than 50%. *po*IL11b has limited identity compared to IL11a orthologues (24–29%), and no significant identity with mammalian IL11s. Teleost fish IL11bs were also neutral compared to the IL11as, which are basic including the mammalian IL11s.

With the complete *po*IL11b amino acid sequence and the limited identities especially between Japanese flounder *po*IL11b and two zebrafish IL11as, we ran a phylogenetic analysis with fugu IL6 as an outgroup. Our results confirmed the clustering between IL11as and IL11bs and hence the orthology of *po*IL11b. Moreover, we also show a, lineage-specific, secondary duplication for zebrafish IL11a (Fig. 3).

3.2. *poIL11b tissue distribution*

At 30 PCR cycles, Japanese flounder *po*IL11b was remarkably expressed in PBLs and slightly in kidney and spleen but not in brain, eyes, gills, heart, intestine, liver, muscle, skin and stomach (Fig. 4).

3.3. *poIL11b expression is regulated by LPS and poly I:C treatment*

We checked the expression of *po*IL11b vis-a-vis with the Japanese flounder granulocyte colony stimulating factor (*po*CSF3), Mx (*po*Mx) and β-actin genes in primary cultures of kidney, PBLs and spleen of adult fish following LPS and poly I:C treatment (Fig. 5). Expectedly *po*IL11b, identified as a pro-inflammatory cytokine, was induced by LPS although not

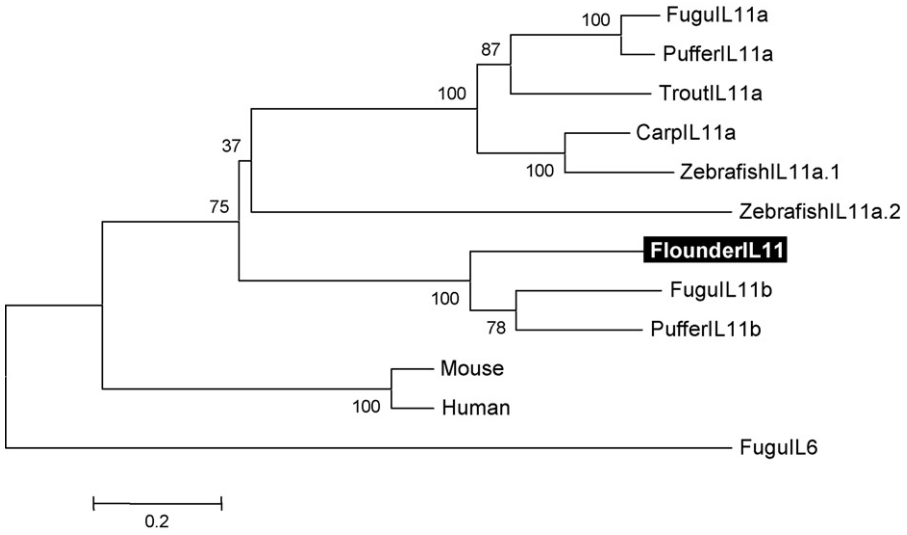

Fig. 3. Neighbor joining tree of the Japanese flounder IL11 type b and other IL11 orthologues and paralogues. The Poisson correction method was utilized. Accession numbers include: Japanese flounder IL11b-AB299205; Fugu IL11a-BN000713; Fugu IL11b-BN000714; Green-spotted pufferfish IL11a-BN000715; Green spotted pufferfish-AY374548; Zebrafish IL11a-BN000717; Zebrafish IL11b-BN000718; Trout IL11a-AJ535687; Carp IL11a-AJ632159; Mouse IL11-P47873; Human IL11-P20809.

comparable to *po*CSF3 in the three tissues studied, where the latter was highly induced. Surprisingly, however, it was highly stimulated by poly I:C in a time-series manner similar to *po*Mx expression pattern, a gene that is well known to be highly induced by poly I:C and virus. Interestingly, *po*CSF3, was highly induced at 6 h post-stimulation by poly I:C in PBLs but not in kidney or spleen.

3.4. *poIL11b involvement in E. tarda and HIRRV infection*

At 26 cycles, Japanese flounder *po*IL11b was expressed, albeit faintly, in the kidney of juvenile Japanese flounder infected with *E. tarda* starting at day 3, in contrast to *po*CSF3 where there was visible expression starting at day 1 and moderate expression at day 3 and day 7. On the other hand, kidney of juvenile fish infected with HIRRV showed increasingly high amounts of *po*IL11b transcripts as compared to PBS-injected controls starting from day 1 until day 7 (Fig. 6). This result was confirmed by controls through *po*Mx expression, where poly I:C is known to stimulate it, and by the constant expression of β-actin. Consequently, from this *in vivo* experiment, we noticed that *po*IL11b is not constitutively expressed in the kidney of juvenile Japanese flounder.

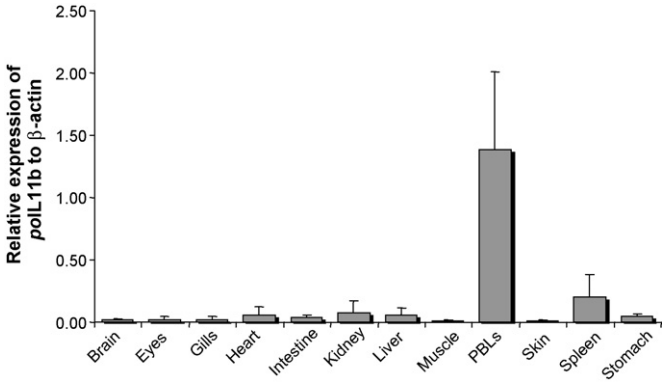

Fig. 4. Constitutive expression of *po*IL11b gene in various tissues in Japanese flounder relative to β-actin expression as determined by semi-quantitative RT-PCR.

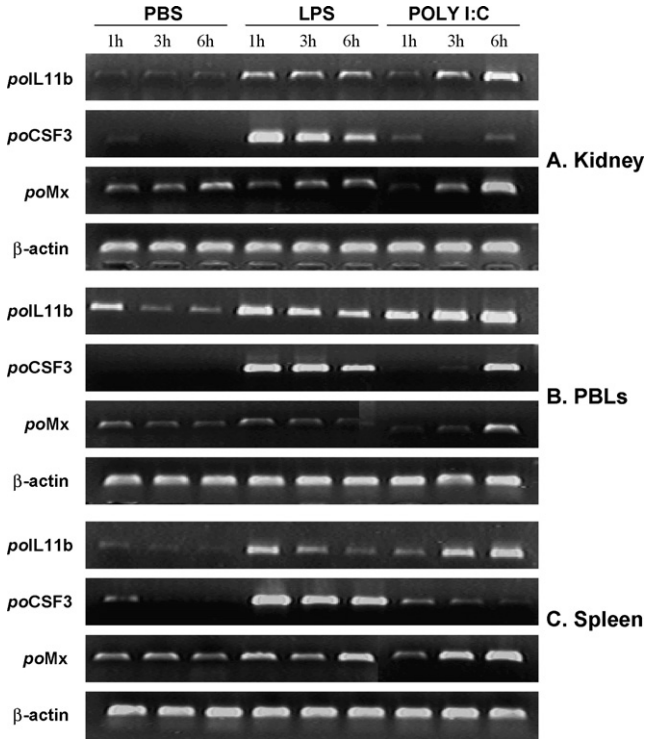

Fig. 5. Expression of *po*IL11b, poCSF3, poMx and β-actin genes *in vitro* in primary cultures of (A) kidney, (B) peripheral blood leukocytes (PBLs) and (C) spleen following PBS, LPS and poly I:C treatment *in vitro* at 1 h (1 h), 3 h (3 h) and 6 h (6 h) sampling.

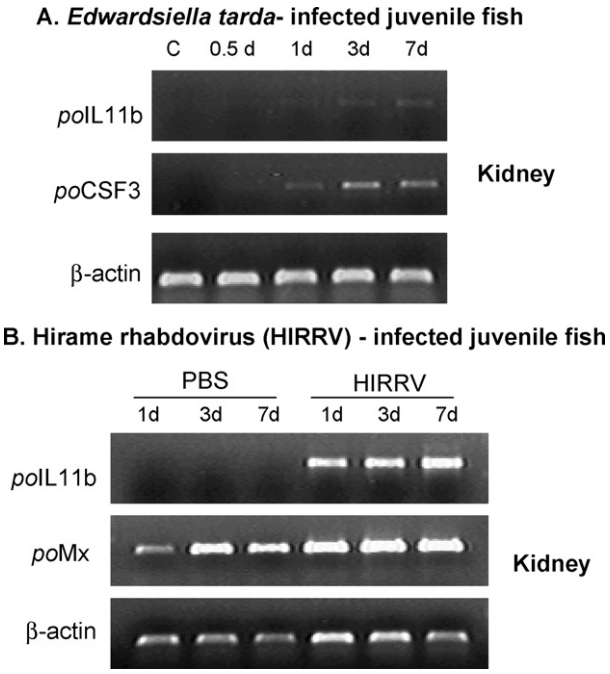

Fig. 6. Expression of *po*IL11b gene in kidney in response to *Edwardsiella tarda* and hirame rhabdovirus (HIRRV) infection *in vivo*. Sampling was conducted at 0 day (control, C), half day (0.5d), 1 day (1d), 3 days (3d) and 7 days (7d) post-infection for *E. tarda*. For HIRRV experiment, sampling was done at 1 day (1d), 3 days (3d) and 7 days (7d) post-HIRRV infection and for PBS.

4. Discussion

Based on our analysis, the full Japanese flounder IL11 cDNA we report here is of type b (*po*IL11b) confirming previous partial third party annotation of this gene (TPA:CAJ57445) and results of phylogenetic analyses where the *po*IL11 EST fragment was included (Huising et al., 2005, 2006). This suggests that there is also an existing IL11a orthologue in Japanese flounder that is yet to be identified as dictated by the Whole Genome Duplication (WGD) theory in teleosts (Christofells et al., 2004; Jaillon et al., 2004). Interestingly, the zebrafish possesses two *dr*IL11as as confirmed by identity, multiple alignment, isoelectric point and phylogenetic analysis. *dr*IL11a.1 is shown to be the paralogue of teleost IL11b and that *dr*IL11a.2 is the a paralogue of *dr*IL11a.1 at 31% identity. These results indicate that *dr*IL11 gene has undergone an additional, secondary tandem duplication that is species-specific.

The general structure of *po*IL11b is conserved based on consensus domain predictions. It is similar with the other fish and mammalian IL11s because it is L residue rich and is made-up of 4 α-helices composed of charged amino acid residues that appear to be found at the hydrophilic surface of the protein molecule (Czupryn et al., 1995). Important receptor-binding residues in *po*IL11b are also conserved suggesting that IL11 duplicates follow the same general receptor-binding mechanism with that of mammalian IL11, which utilizes an IL11 receptor and gp130, and further shows that both paralogues could indeed be utilizing similar receptors that were recently predicted in pufferfish (Jaillon et al., 2004). A new type-1 cytokine receptor (Japanese flounder gp130 homologue, JfGPH) discovered recently could also act as a receptor for IL11s (Santos et al., 2007). Earlier studies reported that the helix A of mammalian IL11 particularly the P^{13}, E^{16}, L^{17}, R^{25}, L^{28}, T^{31}, R^{32}, L^{34} and R^{39} residues has been implicated in gp130 binding site (Site II) critical for bioactivity while helix D particularly its R^{150}, H^{153}, D^{164}, W^{165} and R^{168} residues has been reported as a primary receptor-binding site (Site I) (Du and Williams, 1997). In a more recent study, the R^{168}, L^{171} and L^{173} residues in the helix D at the COOH-terminal have been found to be very important for binding of mouse IL11 to IL11 receptor, while the R^{111} and L^{115} residues were extremely crucial for binding to gp130. Furthermore, the W^{146} within the CD loop was determined to be critical for biological activity but not necessary for binding using A residue replacement experiment (Barton et al., 1999).

Despite the conserved IL11 structures, we were, however, able to spot some structural differences between fish IL11b and IL11a, which we think would have significant implication in their individual function. Firstly, there are two extra C residues conserved only among fish IL11b. These residues, particularly C^{183} and C^{198}, could potentially form a di-sulphide bond in this area and influence the stability of the peptide particularly the COOH-terminal of the protein. This could consequently affect the bioactivity of the molecule as this region is the primary receptor-binding site (Site I). Secondly, IL11b orthologues are neutral compared to the basic nature of IL11a and even mammalian IL11s, which is a result of the presence of lesser-charged amino acid residues in the helices and thus more neutral p*I*. IL11bs are therefore more cytokine-like in terms of pH and the fish IL11as including mammalian IL11s are unusually basic. Thirdly, the *po*IL11b mRNA is composed of four destabilizing AUUUA motifs (Akashi et al., 1994) in its 3′ UTR region and based on the presence of one poly (A) signal could only produce a single transcript. The DNA sequence of the trout IL11a on the other hand, has several AUUUA motifs and four poly (A) signals that potentially could produce different sized transcripts with varying stability depending on which transcript is produced (Wang et al., 2005). Assuming that the largest of the potential trout IL11a transcripts is transcribed (since this is the one cloned in the study), such IL11a is far more unstable than the *po*IL11b, which has only four ATTTA motifs. Taken together, these structural data suggests that fish IL11a and IL11b may very well exhibit different functional attributes.

The difference between IL11a and IL11b becomes clearer when expression data from both published reports and this study is compared, and that the difference appears to be complementary. First, studies show that carp *cc*IL11a and trout *om*IL11a is expressed significantly high in tissues except in PBLs (Wang et al., 2005; Huising et al., 2005). In contrast, we found that IL11b is highly expressed only in PBLs and very slightly in spleen and kidney. Second, *in vitro* studies showed that for LPS treatment, there is significant immediate expression of IL11b and is then dissipated afterwards while trout IL11a is persistently expressed at the duration of sampling. For poly I:C, a viral mimic (Fortier et al., 2004), the effect is more dramatic and increasing from 0 h to 6 h post-treatment, and approximates that of *po*Mx expression. This is in contrast to the expression of rainbow trout IL11a where it was observed to be slightly up-regulated by poly I:C

at 3 h and 7 h sampling but then disappears at 24 h. Third, the results showed that *E. tarda* infection up-regulates *po*IL11b but not significantly, at least in the kidney of juvenile fish, unlike trout IL11a, which is up-regulated in the liver, head, kidney and spleen of trout, 1 day after challenged with bacterial *Aeromonas salmonicida* MT423. HIRRV infection, on the other hand, stimulated high expression of *po*IL11b at 1–7 days sampling, again mirroring the expression of the antiviral Mx.

The fish IL11 paralogues appears to have complementing function, a characteristic that is very similar to fish M17s and MSHs, also members of the IL6-cytokine subfamily (Hwang et al., 2007), that follows the Duplication–Degeneration–Complementation (DDC) model (Force et al., 1999). There is evidence to support, at least at the transcriptional level, of the partitioning of ancestral functions of these molecules. This model was initially supported by the analysis of the *engrailed* gene in zebrafish. Since then, a number of duplicate genes have been reported to follow such model including among others the *protocadherin 15* and αB-crystallin genes in zebrafish (Seiler et al., 2004; Smith et al., 2006) and the pro-opiomelanocortin genes in green-spotted pufferfish (Souza et al., 2005).

Focusing on its immune function, *po*IL11b was indeed shown to be involved in both bacterial and antiviral responses based on its *in vitro* and *in vivo* expression in immune-related tissues that include the kidney, spleen and PBLs. Bacterial (LPS and *E. tarda*) and viral (poly I:C and HIRRV) mimics/agents have been shown to regulate numerous immune-related genes, including cytokines, during infection (Matsuyama et al., 2007; Yasuike et al., 2007). *po*IL11b is yet another cytokine molecule that responds to these agents and as such underscores its important role in host–pathogen interactions. This result adds to the growing information about fish IL11s. For example, trout *om*IL11a has been reported to be induced by a plasmid construct containing the glycoprotein gene of VHSV and a CMV promoter although the induction is significant only at day 3 in spleen and curiously only at day 10 in kidney (Jiménez et al., 2006). The non-expression of *po*IL11b, in juvenile Japanese flounder indicates that during development, it is functional only at the adult stage. A similar pattern has been observed with other genes related to hematopoiesis where they are expressed at specific stages in zebrafish development (Davison and Zon, 2004). Furthermore, the viral challenge experiment and poly I:C treatment in trout, suggests that IL11a is not very much involved in antiviral responses unlike the *po*IL11b. It is therefore likely that IL11b has more antiviral function as compared to antibacterial responses and such broad involvement in pathogen responses makes it a good vaccine adjuvant candidate as compared to its counterpart IL11as in fish.

It is interesting to note that the Japanese flounder IL6-cytokine *po*CSF3, which was immediately up-regulated by LPS in kidney, PBLs and spleen similar to what was reported previously (Santos et al., 2006), was strongly expressed only in PBLs and only at 6 h following stimulation by poly I:C. This suggests that *po*CSF3 is differentially regulated by bacterial and viral infection, similar to *po*IL11b, and that its function is tissue-specific. The reason for such kind of expression pattern is not yet clear and would have to be studied further.

In conclusion, whether the observed difference in structure and expression between IL11a and IL11b are linked remains to be confirmed. Also, complementation of function of both duplicates at the protein level within a species should be investigated further. Nevertheless, we can conclude that both IL11 paralogues in fish are 'functional' at the expression level and in a manner that is complementary to each other. This has implication to for example strategies on the development of vaccines against fish pathogens. Combinatorial use of these genes for therapy may prove to be more effective than used separately.

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