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Conserved Gene Structure and Function of Interleukin-10 in Teleost Fish

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Abstract: Interleukin-10 (IL-10) is an important immunoregulatory cytokine produced by various types of cells. Researchers describe here the isolation and characterization of olive flounder IL-10 (ofIL-10) cDNA and genomic organization. The *ofIL-10* gene encodes a 187 amino acid protein and is composed of a five exon/four intron structure, similar to other known *IL-10* genes. The ofIL-10 promoter sequence analysis shows a high level of homology in putative binding sites for transcription factors which are sufficient for transcriptional regulation ofIL-10. Important structural residues are maintained in the ofIL-10 protein including the four cysteines responsible for the two intra-chain disulfide bridges reported for human IL-10 and two extra cysteine residues that exist only in fish species. The phylogenetic analysis clustered ofIL-10 with other fish IL-10s and apart from mammalian IL-10 molecules. Quantitative real-time Polymerase Chain Reaction (PCR) analysis demonstrated ubiquitous *ofIL-10* gene expression in the 13 tissues examined. Additionally, the induction of *ofIL-10* gene expression was observed in the kidney tissue from olive flounder infected with bacteria (*Edawardsiella tarda*) or virus (Viral Hemorrhagic Septicemia Virus; VHSV). These data indicate that IL-10 is an important immune regulator that is conserved strictly genomic organization and function during the evolution of vertebrate immunity.

Key words: Interleukin-10, genomic organization, promoter, mRNA expression analysis, *Paralichthys olivaceus*

INTRODUCTION

Interleukins (ILs) are a subgroup of cytokines involved in the intercellular regulation of the immune system. Since the discovery of IL-1 in 1977, >40 cytokines are now designated as ILs (Akdis *et al*., 2011). Numerous interleukin genes including IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-17, IL-18, IL-20-like, IL-21 and IL-22/26 have been identified in various bony fish (reviewed in Secombes *et al*., 2011). Among them, IL-10 is the most important anti-inflammatory cytokine first identified in supernatants of mouse T cells stimulated with ConA and was originally described as a Cytokine Synthesis Inhibitory Factor (CSIF) based on its ability to inhibit the synthesis of IL-2 and IFN-γ (Fiorentino *et al*., 1989). IL-10 is a member of the class II cytokine family which also includes IL-19, IL-20, IL-22, IL-24, IL-26 and interferons based on their structural similarity (Lutfalla *et al*., 2003). However, their sequence relationship is not reflected by a shared biological function (Moore *et al*., 2001; Sabat *et al*., 2007; Wolk and Sabat, 2006).

The IL-10 sequence was identified across mammals and other vertebrates including avian species (Rothwell *et al*., 2004; Yao *et al*., 2012) amphibians and bony fish. IL-10 was first discovered in fish during a fugu genome search (Zou *et al*., 2003). IL-10 has since been cloned in several other fish species including carp (Savan *et al*., 2003), zebrafish (Zhang *et al*., 2005), rainbow trout (Inoue *et al*., 2005), sea bass (Buonocore *et al*., 2007; Pinto *et al*., 2007), cod (Seppola *et al*., 2008), goldfish (Grayfer *et al*., 2011), eel (Van Beurden *et al*., 2011) and grass carp (Wei *et al*., 2013). Fish IL-10 bioactivity was recently reported, recombinant goldfish IL-10 reduced the expression of the pro-inflammatory cytokines IL-1β, TNFα, IL-8 and IL-10 itself but increased Suppressor of Cytokine Signaling 3 (SOCS3) expression (Grayfer *et al*., 2011). Very recently, the bioactivity of recombinant grass carp IL-10 was examined in grass carp Peripheral Blood Lymphocytes (PBLs) and was shown to up-regulate cellular activity (Wei *et al*., 2013). However, knowledge on the functional role of IL-10 in fish immunity is still limited.

Although, pro-inflammatory interleukins such as IL-1β (Emmadi *et al*., 2005), IL-6 (Nam *et al*., 2007) and IL-8 (Lee *et al*., 2001) have been identified and their bioactivity investigated, IL-10 has not been reported in the olive flounder, *Paralichthys olivaceus*. Here, researchers report cDNA and mRNA expression of anti-inflammatory cytokine IL-10 in olive flounder and also gene organization and promoter structures.

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MATERIALS AND METHODS

Cloning of IL-10 cDNA: Total RNA was extracted from olive flounder PBLs using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Basel, Switzerland). To isolate the *IL-10* precursor gene from olive flounder, researchers designed two degenerate oligonucleotide primers that coded for the conserved amino acid sequence of the *IL-10* precursor gene in five species of fish: common carp (*Cyprinus carpio*, GenBank Accession No. AFV36669), zebrafish (*Danio rerio*, GenBank Accession No. NP_001018621), Atlantic cod (*Gadus morhua*, GenBank Accession No. ABV64720), rainbow trout (*Oncorhyncus mykiss*, GenBank Accession No. NP_001232028) and Fugu (*Takifugu rubripes*, GenBank Accession No. XP_003973743). The sequences were as follows: IL10-deg-F, 5'-TGYTGYWSITTYGTIGA RGGITTYCC-3' and IL10-deg-R, 5'-TCIARYTCICCCAT IGCYTT-3' (where Y is C or T, W is A or T, S is G or C, R is A or G and I is deoxyinosine). The primer set was used for Polymerase Chain Reaction (PCR) with first-strand cDNA from olive flounder PBLs as a template under the following conditions: 30 cycles at 95°C for 30 sec, 45-58°C for 30 sec and 72°C for 1 min. The amplified PCR product was subcloned into a T-easy vector (Promega, Madison, WI) and sequenced using an automatic DNA sequencer (ABI 3130, Applied Biosystems, Foster City, CA). To isolate full-length cDNA from the IL-10 precursor, researchers used a rapid amplification kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. The following primers specific to the 5'- and 3'-ends of the IL-10 precursor were used: IL10-5'RACE, 5'-TTGAAAGACTCCTCCACGCTCTGA; IL10-3'RACE-1, 5'-GCTCAAGGAAGATCTCACCAAAT G-3'; IL10-3'RACE-2, CTTCTTCTCATGCAAGAAACAG; IL10-3'RACE-3, GGAGAATAAAGGTCTATATAA. PCR products were cloned into the T-easy vector (Promega) and each clone was sequenced using the ABI 3130 automatic DNA sequencer. The full-length cDNA sequence of the *IL-10* gene was attained by overlapping all fragments with GENETYX (V. 8.0, Software Development, Tokyo, Japan).

Cloning of the *IL-10* gene: The olive flounder genomic Bacterial Artificial Chromosome (BAC) library (Nam *et al*., 2010) was screened to isolate the *IL-10* gene using the BAC pooling system with PCR primers specific to the IL-10 coding region. PCR-based BAC library screening was carried out as reported previously (Chae *et al*., 2007). The obtained PRP/PACAP genomic BAC clone was purified and used for genomic structure analysis and determination of the nucleotide sequence of the flounder IL-10 5'-flanking region with specific primers for genome walking (IL-10 gw-1, 5'-AAGACAAGATACC TGGGAGG-3').

Quantitative real-time PCR: Total RNA was isolated from several tissues including brain, muscle, liver, intestine, stomach, skin, pyloric coece, kidney, spleen, gill, eye, heart and PBLs of healthy olive flounder. The cDNAs were synthesized by reverse transcription as described above. IL-10 mRNA level was determined by quantitative Real-Time PCR (qRT-PCR) using the LightCycler System (Roche Diagnostics) with FastStart DNA Master SYBR Green I (Roche Diagnostics). The specific primer set used was as follows: flounder IL-10 ORF-F, 5'-ATGACTCTTC GGTCTCTCCT-3' and IL-10 RT-R, 5'-TTGAAAGACTC CTCCACGCTCTGA-3'. Following an initial Taq activation step at 95°C for 10 min, 40 cycles of LightCycler PCR were performed under the following cycling conditions: The 95°C for 10 sec, 55°C for 5 sec and 72°C for 20 sec with fluorescence reading. Immediately following PCR, the machine performed a melting curve analysis by gradually increasing the temperature (0.1°C sec^{-1}) while measuring the intensity of fluorescence emission. IL-10 mRNA expression in tissues was normalized to 18S rRNA expression (18S rRNA-F, 5'-ATGGCCGTTCTTAGTTC CTG-3' and 18S rRNA-R, 5'-CCACGCTGATCCAGTC AGT-3') as a reference gene. The amount of mRNA expression was determined by the $2^{-\Delta\Delta C}{}_{T}$ Method (Livak and Schmittgen, 2001). Each sample was analyzed in triplicate and the data were calculated as the mean±Standard Deviation (SD) of relative mRNA expression. A one-tailed t-test was carried out to determine significant differences ($p<0.05$) between the challenged group at each time after infection and the 0 time group.

Experimental challenge: For the bacterial experimental challenge, healthy juvenile olive flounder fish (mean weight 100 g) obtained from the Genetic and Breeding Research Center were used. The fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester; Sigma, St. Louis, MO) and infected with *Edwardsiella tarda* by intraperitoneal injection of a sublethal dose (1.2×10^6 cfu/0.1 mL/fish) suspended in Phosphate-Buffered Saline (PBS). Tissues were collected from three fish at 0, 1, 6, 12, 24, 48 and 72 h postinjection, frozen in liquid nitrogen and kept at -80°C until use.

Olive flounder were infected with a dose of 1×10^6 $TCID_{50}$ of VHSV, administered by immersion at 16°C (Kong *et al*., 2011). After infection for 1.5 h, the virus was removed by replacing the water in the tank. Kidney was removed from three fish each at 1, 3, 6, 9, 12, 24 and 48 h post-infection, frozen in liquid nitrogen and kept at -80°C until use.

Computational analysis: Sequence analysis was performed with GENETYX. The putative signal peptide was predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The 5'-flanking region of the *IL-10* precursor gene was analyzed using the Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/cgi-bin/tess/tess) to identify transcription factor binding elements. An unrooted phylogenetic tree was reconstructed using the maximum-likelihood method in MEGA5 with 500 bootstrap replicates.

RESULTS AND DISCUSSION

Characterization of ofIL-10 cDNA: Olive flounder IL-10 full-length cDNA (GenBank Accession No. KF025662) was composed of a 150-bp 5'-Untranslated Region (UTR) a 564 bp Open Reading Frame (ORF) encoding a predicted protein of 187 amino acids and a 3'UTR of 334 bp. Analysis of the ofIL-10 predicted polypeptide revealed a 22-amino acid (aa) signal peptide sequence which would generate a mature protein with a predicted molecular mass of 19.3 kDa and a pI value of 5.71. The amino acid sequence of ofIL-10 shared 28-78% identity with those of other known vertebrate IL-10s (Table 1) and contained four conserved cysteine residues and one potential N-glycosylation site. Similar to all other IL-10s described to date, ofIL-10 contained the conserved IL-10 family signature motif, G-X-X-K-A-X-X-(D/E)-X-D-(I/L/V)-(F/L/Y)-(F/I/L/M/V)-X-X-(I/L/M/V)-(E/K/Q/R)inthiscase, G-L-Y-K-A-M-G-E-L-E-L-L-F-N-Y-I-E. The 3'UTR contains four mRNA instability motifs (AUUUA) and doublet polyadenylation signals (AAUAAAUAAA) 9 bp upstream of the poly(A)-tail (Fig. 1). Multiple copies of AUUUA motifs are found in the 3'UTR of IL-10s in most fish species including goldfish (Grayfer *et al*., 2011), trout (Harun *et al*., 2011), common carp (Sabat *et al*., 2007), zebrafish (Zhang *et al*., 2005) and fugu (Zou *et al*., 2003). However, the 3'UTR of grass carp IL-10 mRNA did not contain the AUUUA motif thus, the expression of mRNA lacking an AUUUA motif may be subject to a different regulatory mechanism (Wei *et al*., 2013). Two IL-10 paralogs (IL-10a and IL10b) exist in trout and seven AUUUA motifs are found in IL-10b but not in IL-10a (Inoue *et al*., 2005). The expression of trout IL-10a and IL-10b mRNA is differentially modulated by cytokine or bacterial infection (Harun *et al*., 2011) suggesting that fish IL-10 including olive flounder IL-10 may also be regulated at the transcriptional level.

Table 1: Amino acid homology of olive flounder IL-10 with known IL-10 sequences

Species	Amino acid identity (%)	Accession No.
Homo sapiens	31.1	CAG46825
Canis lupus familiaris	32.2	ABY86619
Equus caballus	30.5	NP_001075959
Macaca mulatta	31.6	ABI63893
Bos taurus	28.3	P43480
Sus scrofa	30.1	NP_999206
Ovis aries	28.9	CAG38358
Gallus gallus	35.8	CAF21727
Oreochromis niloticus	47.0	XP_003441414
Dicentrarchus labrax	72.6	ABH09454
Takifugu rubripes	65.9	XP_003973743
Tetraodon nigroviridis	60.3	CAD67773
Gadus morhua	63.5	ABV64720
Oncorhynchus mykiss	57.8	NP_001232028
Anguilla anguilla	48.0	AEL9992
Cyprinus carpio	47.8	AFV36669
Ctenopharyngodon idella	47.5	AEA50953
Hypophthalmichthys molitrix	47.5	AAY99196
Carassius auratus	47.0	ADU34193
Danio rerio	46.5	NP_001018621

Structure analysis of *ofIL-10* gene promoter region: Sequence analysis of the 5'-flanking 926 bp upstream from the transcription start site of the *IL-10* gene reveals multiple potential cis-acting regulatory elements by computer-assisted analysis (Fig. 2). A TATAA box was located from -31 to -26 and a Sp1 binding site was found between -119 and -107. An NF-κB binding site was identified at position -552/-540. Three C/EBP binding sites were found at positions -575/-568, -605/-597 and -898/-888. Three STAT (putative signal transducer and activator of transcription) binding sites were located at -48/-43, -715/-709 and -779/-774 regions. One IRF motif was located at position -78/-68. Three putative C/EBP (CCAAT enhancer binding protein) sites were located in the regions of -575/-567, -715/-709 and -897/-888. All these elements were also found in vertebrate IL-10 promoters including humans (Eskdale *et al*., 1997; Brightbill *et al*., 2000; Brenner *et al*., 2003; Cuesta *et al*., 2003), mice (Liu *et al*., 2006), pigs (Quan *et al*., 2012), chickens (Rothwell *et al*., 2004) and fish (Zou *et al*., 2003; Seppola *et al*., 2008) suggesting that IL-10 promoters have a high level of homology, especially around certain putative binding sites for transcription factors.

Multiple alignments and phylogenetic analysis of ofIL-10: Olive flounder IL-10 was compared to other IL-10 molecules by pairwise alignments and their phylogenetic

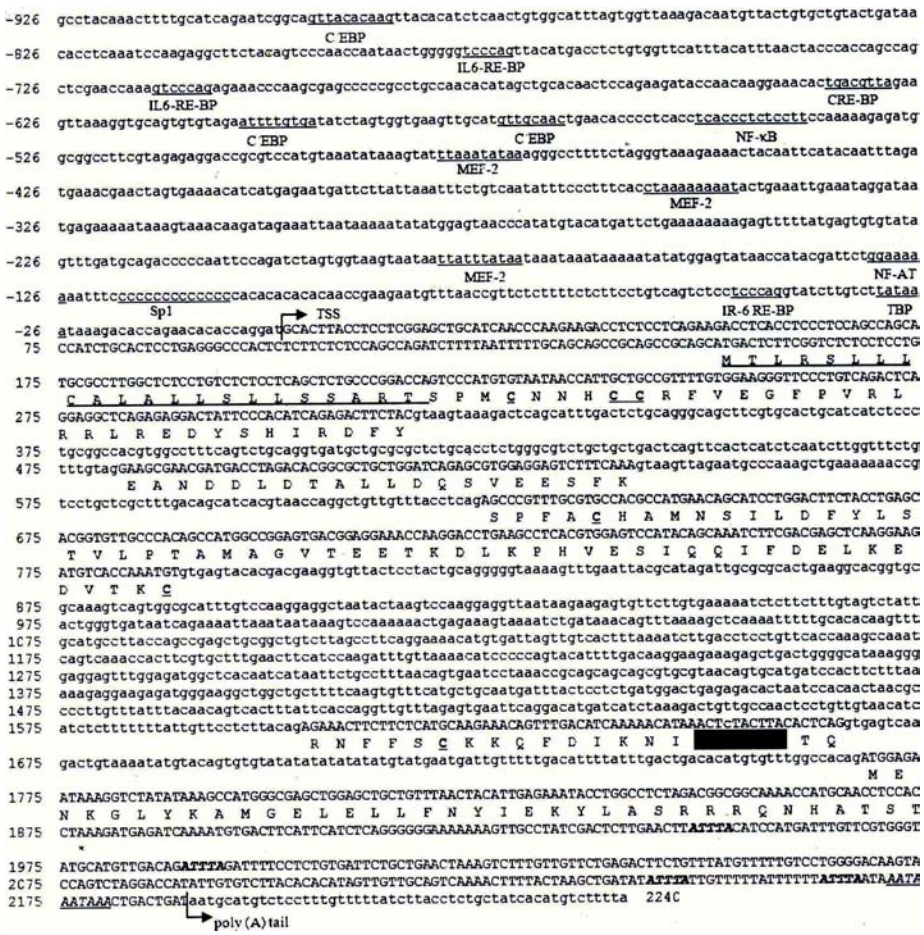

Fig. 1: DNA sequence of the flounder *IL-10* gene. Nucleotide numbering is from the proposed transcriptional start site. Underlined regions indicate consensus sequences as described in the text. Cysteine residues are underlined and bold; a potential N-glycosylation site is shaded in gray. The poly (A) signal sequence is in italics and underlined

homology between fish species is higher compared to the homology between fish and tetrapods. All the IL-10 molecules have a signal peptide, six α-helices and four well-conserved cysteine residues known to form two disulfide bonds in mammals. Figure 3a illustrates the olive founder IL-10 primary sequence as compared with other known IL-10 sequences. The mature ofIL-10 encodes six cysteines of which C^8, C^{58}, C^{108} and C^{114} are conserved across all vertebrates; C^4 and C^9 are conserved within the fish IL-10 sequences. Rainbow trout IL-10 disulfide bonds were predicted by the DISULFIND program which showed that two additional cysteine residues at the N-terminus result in an alternative disulfide bridge scheme (Harun *et al*., 2011). The phylogenetic tree constructed using the maximum-likelihood method in the program MEGA5 (Tamura *et al*., 2011) divides vertebrate IL-10 into two distinct clades: fish and other vertebrates including mammals and birds. Moreover, the fish IL-10 clade was subdivided into seawater fish and freshwater fish. Tree nodes were critically evaluated by performing 1,000 bootstrap replicates (Fig. 3b).

Gene organization of IL-10: All vertebrate *IL-10* genes reported to date have a very similar genomic organization consisting of five exons and four introns. To determine the genomic sequence and organization of the *IL-10* gene, a BAC clone was isolated containing the *IL-10* gene using a PCR-based BAC Library Screening Method (Chae *et al*., 2007). The genomic DNAs of the coding region and 5'-flanking region were amplified from IL-10 BAC DNA by PCR. Sequence analysis showed that the genomic DNA sequence of the ofIL-10 was 2,189 bp in length and composed of five exons and four introns (GenBank accession no. KF025663) which is similar to other species' *IL-10* gene organization (Fig. 4). The ofIL-10 introns were conserved phase 0 which means that

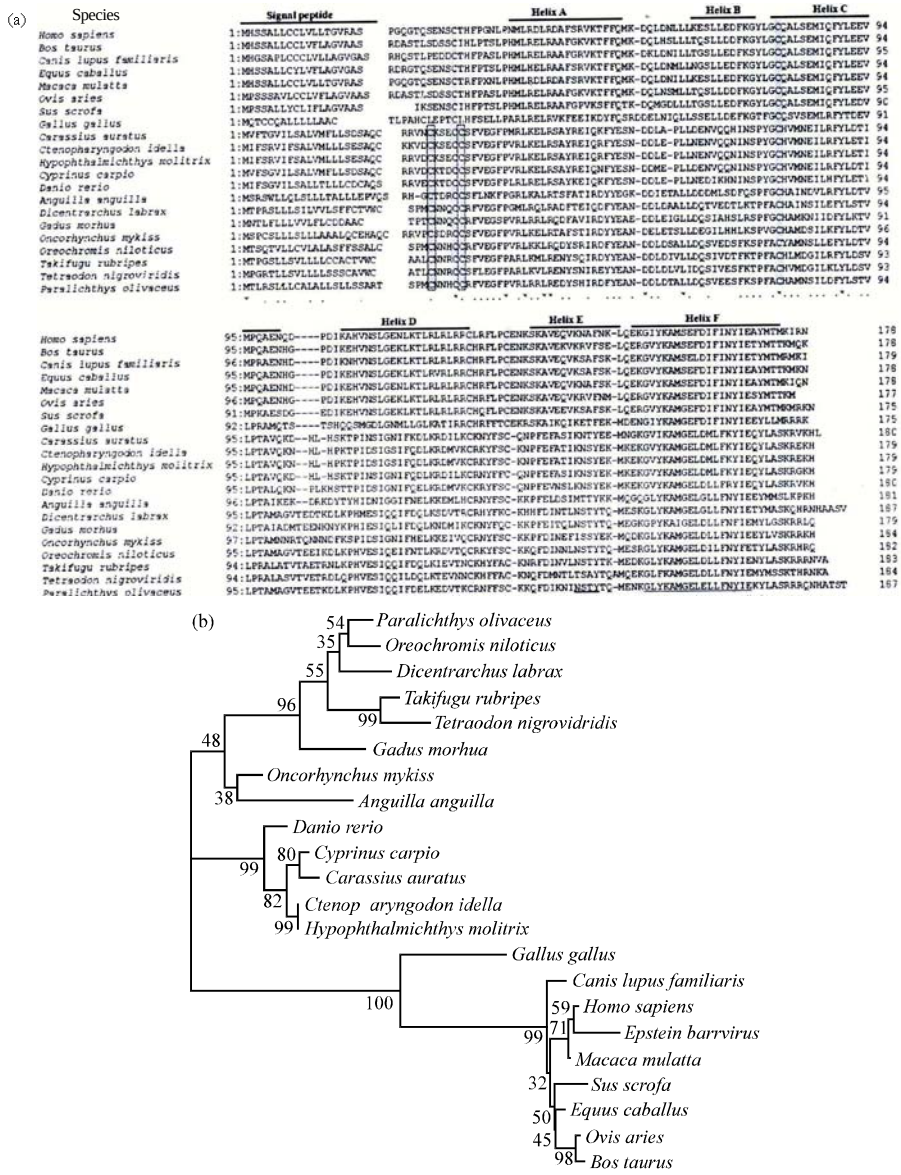

Fig. 2: Amino acid sequence comparison between ofIL-10 and known IL-10 (A) and phylogenetic analysis of IL-10 in vertebrates (B); a) Dashes denote gaps introduced to maximize alignments. Identical and similar amino acids conserved among species are indicated by asterisks (*) and dots (•), respectively. The four conserved cysteine residues that pair to make up the two disulfide bridges are shaded in gray, the two cysteine residues conserved in fish IL-10s are shaded in gray and dashed. The six α-helical domains predicted in human IL-10 are marked by the bar above; b) An unrooted phylogenetic tree constructed using the Maximum-Likelihood Method in MEGA5. The numbers indicate the frequencies with which the phylogram topology represented here was replicated for 500 bootstrap interactions. Amino acid sequences were extracted from GenBank and the alignment was produced with CLUSTAL W and further edited in Genetyx, V. 8.0. The accession numbers for the sequences are *Homo sapiens*, CAG46825; *Bos taurus*, P43480; *Canis lupus* familiaris, ABY86619; *Equus caballus*, NP_001075959; *Macaca mulatta*, ABI63893; *Ovis aries*, CAG38358; *Sus scrofa*, NP_999206; *Gallus gallus*, CAF21727; *Carassius auratus*, ADU34193; *Ctenopharyngodon idella*, AEA50953; *Hypophthalmichthys molitrix*, AAY99196; *Cyprinus carpio*, AFV36669; *Danio rerio*, NP_001018621; *Anguilla anguilla*, AEL99923; *Dicentrarchus labrax*, ABH09454; *Gadus morhua*, ABV64720; *Oncorhynchus mykiss*, NP_001232028; *Oreochromis niloticus*, XP_003441414; *Takifugu rubripes*, XP_003973743; *Tetradon nigroviridis*, CAD67773; *Paralichthys olivaceus*, KF025662

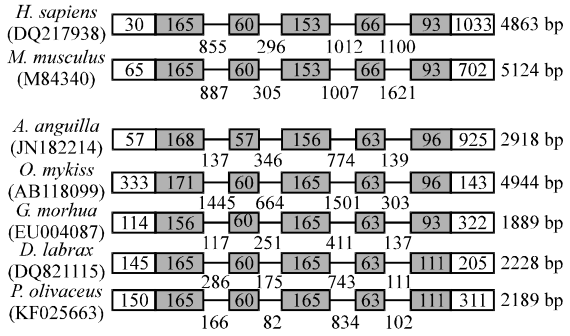

Fig. 3: Schematic representation of the genomic intron-exon organization of IL-10s. The lengths of the exons and introns are not proportional. Exons are shown as boxes with sizes (in base pairs) given inside, open and gray boxes represent the coding region and untranslated region, respectively. Horizontal lines represent introns with their nucleotide lengths provided below. Numbers show the base pairs of introns and exons. The accession numbers of the IL-10 genomic sequences used in the GenBank database are as follows: *Homo sapiens*, DQ217938; *Mus musculus*, M84340; *Anguilla anguilla*, JN182214; *Oncorhynchus mykiss*, AB118099; *Gadus morhua*, EU004087; *Dicentrarchus labrax*, DQ821115; *Paralichthys olivaceus*, KF025663

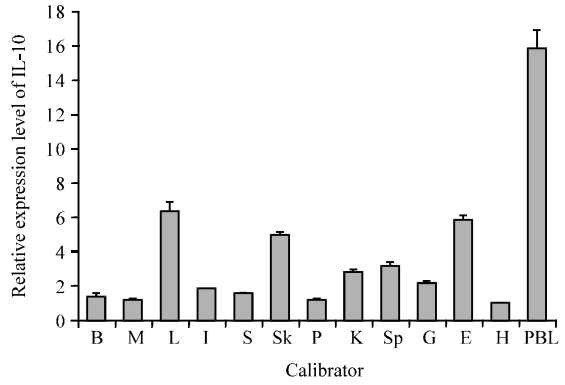

Fig. 4: Tissue distribution of ofIL-10 transcript in unchallenged olive flounder by real-time PCR. The relative IL-10 mRNA expression in various tissues of unchallenged fish was calculated by the $2^{-\Delta\Delta C}$T Method using the olive flounder 18s rRNA as an internal control and the heart as a calibrator. B: Brain; M: Muscle; L: Liver; I: Intestine; S: Stomach; Sk:, Skin; P: Pyloric coece; K: Kidney; Sp: Spleen; G: Gill; E: Eye; H: Heart; PBL: Peripheral Blood Leukocyte

they do not disrupt amino acid codons as shown in several other cytokine genes (Lutfalla *et al*., 2003). *ofIL-10* gene organization was compared to other species *IL-10* gene (Fig. 4). Although, fish IL-10 introns were smaller than those of mammals, exon size was relatively larger. The length of exons 2, 3 and 4 were conserved among vertebrates, however, exon 1 and exon 5 varied in length because of the 5'UTR and 3'UTR, respectively. Overall, the fish *IL-10* gene, except in rainbow trout is approximately half the length of mammalian genes.

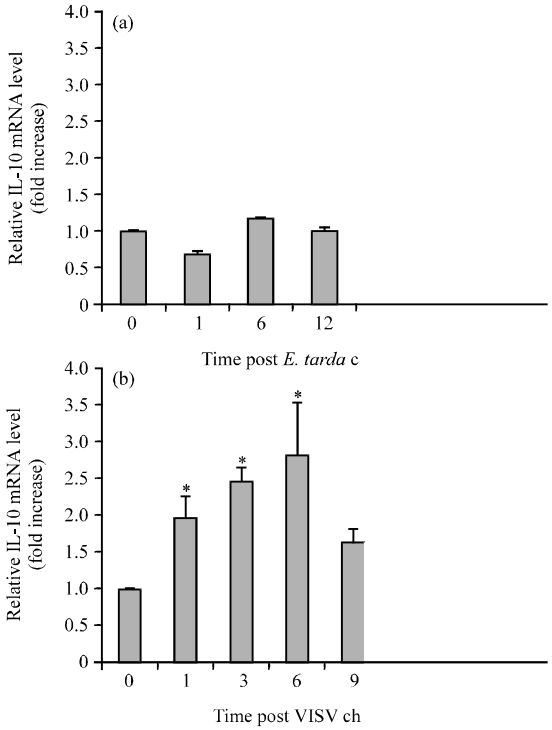

Fig. 5: ofIL-10 transcript expression in the kidney after pathogen challenge. a) Modulation of IL-10 expression was investigated after *E. tarda* challenge in the kidney; b) Modulation of IL-10 was investigated after VHSV challenge in the kidney. Fold changes in mRNA expression in fish challenged with pathogen were calculated by the $2^{-\Delta\Delta C}$T Method by using olive flounder 18s rRNA as an internal control. The relative fold change in expression was compared to that at 0 h. The error bars represent the mean±SD (n = 3). Significant differences compared to 0 h are indicated with an asterisk for $p<0.05$

Tissue distribution of ofIL-10 in normal fish: Olive flounder mRNA expression was assessed by real-time quantitative RT-PCR in 13 tissues (brain, muscle, liver, intestine, stomach, skin, pyloric coece, kidney, spleen, gill, eye, heart and peripheral blood leukocytes) of normal fish. The olive flounder IL-10 mRNA was constitutively

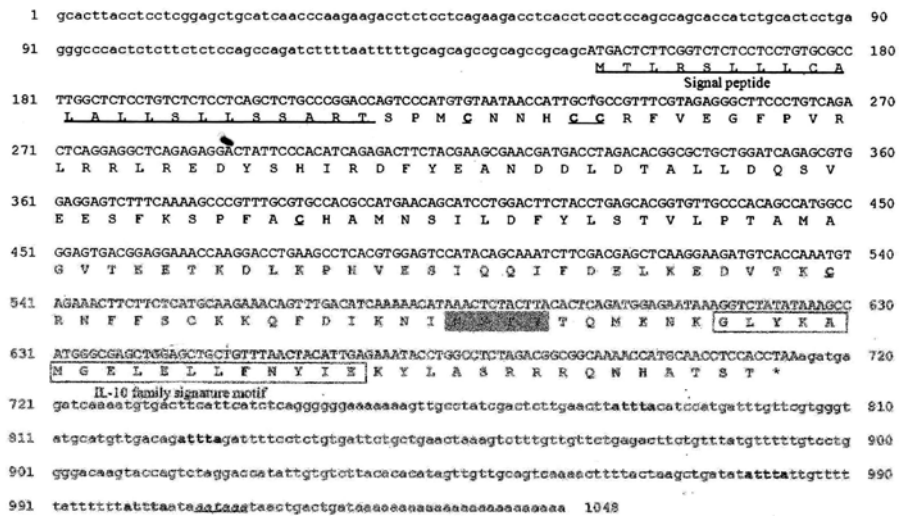

Fig. 6: cDNA and deduced amino acid sequences of ofIL-10. The signal peptide is underlined and cysteines are bold and underlined. Potential N-glycosylation site is grey shaded and IL-10 family signature motif is indicated in boxed. Within the 3'UTR the putative ATTTA instability motifs are bolded. The poly (A) signal sequence is in italic and underlined

expressed in all tested tissues in a tissue-specific fashion (Fig. 5). The greatest expression was observed in PBLs (up to 16 fold higher than levels observed in the heart) followed by liver, eye and skin whereas the lowest expression was observed in the heart. This finding is not consistent with the results for other fish species IL-10 mRNA expression which have a quite different expression pattern. Goldfish IL-10 mRNA expression is reportedly highest in spleen with only a modest level of expression in kidney, brain and gills (Grayfer *et al*., 2011). In fugu, IL-10 expression is reportedly highest in liver and kidney and not detectable in spleen, gill or brain (Zou *et al*., 2003). In carp, IL-10 expression is highest in head kidney and is also detectable in intestine, gills and spleen but not detectable in liver, brain or muscle (Savan *et al*., 2003). In zebrafish, IL-10 expression is detectable in head kidney, intestine and gills but not in spleen (Zhang *et al*., 2005). The reason for these discrepancies is not known although the tissue expression pattern may be fish-specific.

ofIL-10 mRNA expression profiles after pathogen infection: To investigate the immune responses of olive flounder through the transcriptional regulation of ofIL-10, the expression of ofIL-10 mRNA was analyzed using real-time PCR of kidney tissue following infection with either bacterial (*E. tarda*) or viral (Viral Hemorrhagic Septicemia Virus; VHSV) pathogens. The relative expression levels of ofIL-10 at each time point were calculated by normalizing the results with the 0 time point. As shown in Fig. 6, ofIL-10 mRNA expression was highly up-regulated (2.7 fold) at 24 h post-infection with *E. tarda*. In contrast, significant expression of ofIL-10 was initially observed at 1 h post-infection and the highest level of up-regulation was noted at 6 h (3 fold) post-infection following VHSV challenge (Fig. 6). Overall, these results show that IL-10 is up-regulated in fish in response to bacteria or virus but its role during the immune response should be further investigated.

CONCLUSION

This report described the sequences of the full-length cDNA and a BAC clone positive for the of *IL-10* gene. The ofIL-10 conserves well gene organization and shows similarity in the predicted amino acid sequence with other vertebrate *IL-10* gene. Along with the identification of important regulatory motives in the promoter region of ofIL-10, ofIL-10 is expected to be involved in the inflammatory response in olive flounder. Quantitative real-time PCR analysis demonstrated the presence of ubiquitous *ofIL-10* gene expression in the 13 tissues examined whereas challenge experiments with *E. tarda* or VHSV induced ofIL-10 expression. These results suggest that IL-10 is expected to be involved in the inflammatory response in olive flounder. The mechanism of expression regulation and functional analysis is under further investigation.

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