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Molecular characterization and expression analysis of four fish-specific CC chemokine receptors CCR4La, CCR4Lc1, CCR4Lc2 and CCR11 in rainbow trout (*Oncorhynchus mykiss*)

Zhitao Qi[a,b], Jason W. Holland[a], Yousheng Jiang[a,c], Christopher J. Secombes[a], Pin Nie[d], Tiehui Wang[a*]

[a]Scottish Fish Immunology Research Centre, School of Biological Sciences, University of Aberdeen, Aberdeen AB24 2TZ, UK

[b] College of Animal Sciences, Yangtze University, Jingzhou, Hubei Province, 434020 China

[c]College of Fishery and Life Science, Shanghai Ocean University, Shanghai, 201306, China

[d]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei province, 430072, China

*Corresponding author.

Tel.: +44 1224 272872;

Fax: +44 1224 272396.

Email: t.h.wang@abdn.ac.uk

Abstract

The chemokine and chemokine receptor networks regulate leukocyte trafficking, inflammation, immune cell differentiation, cancer and other biological processes. Comparative immunological studies have revealed that both chemokines and their receptors have expanded greatly in a species/lineage specific way. Of the 10 human CC chemokine receptors (CCR1-10) that bind CC chemokines, orthologues only to CCR6, 7, 9 and 10 are present in teleost fish. In this study, four fish-specific CCRs, termed as CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, with a close link to human CCR1-5 and 8, in terms of amino acid homology and syntenic conservation, have been identified and characterized in rainbow trout (*Oncorhynchus mykiss*). These CCRs were found to possess the conserved features of the G protein-linked receptor family, including an extracellular N-terminal, seven TM domains, three extracellular loops and three intracellular loops, and a cytoplasmic carboxyl tail with multiple potential serine/threonine phosphorylation sites. Four cysteine residues known to be involved in forming two disulfide bonds are present in the extracellular domains and a DRY motif is present in the second intracellular loop. Signaling mediated by these receptors might be regulated by N-glycosylation, tyrosine sulfation, S-palmitoylation, a PDZ ligand motif and di-leucine motifs. Studies of intron/exon structure revealed distinct fish-specific CCR gene organization in different fish species/lineages that might contribute to the diversification of the chemokine ligand-receptor networks in different fish lineages. Fish-specific trout CCRs are highly expressed in immune tissues/organs, such as thymus, spleen, head kidney and gills. Their expression can be induced by the pro-inflammatory cytokines, IL-1β, IL-6 and IFNγ, by the pathogen associated molecular patterns, PolyIC and peptidoglycan, and by bacterial infection. These data suggest that fish-specific CCRs are likely to have an important role in immune regulation in fish.

Key words: Rainbow trout, CC chemokine receptor, CCR4La, CCR4Lc, CCR11, expression, modulation, pro-inflammatory cytokine, bacterial infection, parasitic infection,

1. Introduction

A hallmark feature of an inflammatory response is the accumulation of leukocytes in injured or infected tissues, where they remove pathogens and necrotic tissue by phagocytosis and proteolytic degradation. This leukocyte trafficking is regulated by the chemokines and chemokine receptors [1]. The mammalian genome encodes approximately 50 different chemokines, which are classified into two major subfamilies (CC and CXC) and two minor subfamilies (CX3C and XC), based on the spacing of the conserved cysteine residues [2, 3]. According to expression patterns and function, they can also be classified as inflammatory, homeostatic or dual-functional chemokines. Inflammatory chemokines are upregulated during inflammation. Homeostatic chemokines are constitutively expressed under normal physiological conditions being involved in homeostatic migration and homing of cells. Some chemokines have both properties, and are thus called dual-function chemokines [4]. The binding of a chemokine with its cognate receptor triggers a cascade of intracellular events that promotes physiological events, from gene transcription to cytoskeleton rearrangement and chemotaxis. In addition to their roles in leukocyte trafficking, chemokine receptor-ligand interactions can give rise to a variety of additional cellular and tissue responses, including cell proliferation, activation and differentiation, extracellular matrix remodeling, angiogenesis, hematopoiesis, embryologic development, lymphocyte development, dendritic cell maturation, inflammation, tumor growth and metastasis [1, 5-10].

Chemokine receptors are seven transmembrane (TM) proteins belonging to the superfamily of G protein-coupled receptors (GPCRs). As the receptors were discovered after the chemokines and most of them are selective for members of one chemokine subfamily, they are classified according to the subfamily of chemokines to which most of their ligands belong. Thus, receptors are named using the prefixes CCR, CXCR, CX3CR, and XCR followed by an identifying number [1, 11]. The extracellular face of the receptor includes an extended, largely unstructured N-terminal region and three connecting loops (extracellular loops, ECL1, 2, and 3), with conserved disulfide bonds connecting the N-terminus to ECL3 and ECL1 to ECL2. The cytoplasmic face of the receptor includes three additional connecting loops (intracellular loops, ICL1, 2, and 3) and the C-terminal region. Upon binding to their cognate chemokine ligands, the receptors undergo conformational changes giving rise to activation of intracellular effectors (G proteins or β-arrestins), initiation of signal transduction pathways and, ultimately, cellular responses.

The N-terminal is a critical determinant of ligand binding and involved in signal transduction. Chemokine receptors, in common with other rhodopsin-like GPCRs, have a conserved DRY

motif after the third TM domain that is critical for signaling. The C-terminal of the receptor, as for many GPCRs, contains key serine and threonine residues which can be phosphorylated by G protein-coupled receptor kinases (GRKs) to induce recruitment of arrestin proteins leading to receptor internalization and signal termination [3]. Chemokine receptors are subject to a variety of post-translational modifications, such as N-glycosylation, tyrosine sulfation, and palmitoylation, that are known to influence chemokine recognition and signaling [1, 12].

The human genome encodes for 18 standard chemokine receptors (CXCR1-6, CCR1-10, XCR1 and CX3CR1), and at least 5 atypical non-signaling chemokine receptors (ACKR1-5) that bind chemokines but do not elicit standard chemotactic responses following ligand binding [4, 10]. Individual chemokine receptors often bind more than one chemokine. Conversely, a single chemokine often binds to more than one receptor [4, 13].

Studies in teleosts have revealed that both chemokines and their receptors have expanded greatly through whole genome duplication (WGD) events and/or species-specific gene duplications. For example, in zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and tetraodon (*Tetraodon nigroviridis*), which have undergone 3R WGD events, express 89, 36, and 20 chemokines [14] and 40, 31, and 24 chemokine receptors, respectively [4, 15]. More genes have been found in 4R WGD salmonid fish, where 48 chemokine receptor loci are present in the Atlantic salmon (*Salmo salar*) genome with 40 supported by transcript expression [16]. Similarly in rainbow trout *Oncorhynchus mykiss*, another economically important salmonid species, a number of chemokines and chemokine receptors have been cloned and functionally characterized [19-28]. Of the CCRs, clear orthologues of mammalian homeostatic CCR7, 9 and 10, and dual functional CCR6 are present in teleosts. However, the inflammatory CCR1, 2, 3 and 5, and dual functional CCR4 and 8, clustered on human chromosome 3, are absent in fish [4]. Conversely, fish-specific CCRs, including CCR4La-c, CCR11 and CCR12 have been identified. Only CCR6, 7 and 9 have been reported in rainbow trout [29-31], although CCR10 and CCR12 sequences are present in GenBank.

In this communication, four fish specific CC chemokine receptors, CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, which share higher identities/similarities to human inflammatory/dual functional CCR1-5 and 8 CCRs, were identified, cloned and sequence characterized in rainbow trout. The expression of these CCRs in healthy and infected (bacterial and parasitic) trout was investigated *in vivo*. The effects of pathogen associated molecular patterns (PAMPs, polyinosinic acid: polycytidylic acid and peptidoglycan), and pro-inflammatory cytokines (IL-1β, IL-6 and IFNγ) on CCR expression was examined, *in vitro*, in head kidney (HK) macrophages.

2. Materials and methods

2.1. Database searching, gene cloning and sequence analysis

Blast (the basic local alignment search tool [32]) search was performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using CCRs from trout and other fish species resulting in the identification of a number of candidate ESTs and genomic loci in rainbow trout. ESTs and genomic loci to known trout CCRs were excluded and four novel candidates were identified. Trout genomic sequences were analyzed by FGENESH software (http://www.softberry.com) to predict the potential 3'-untranslated region (UTR) and 5'-UTR. Primers (Table 1) were designed within the 5'-UTR and 3'-UTR and used for PCR using cDNA samples prepared from HK as template. PCR products were cloned and sequence analyzed as described previously [33, 34].

Open reading frames in sequences were determined using Translate software at the ExPASy server (http://www.expasy.org). Other bioinformatics programs used for sequence analysis were; TMpred program [35] for transmembrane domain prediction, NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) for N-glycosylation prediction, NetPhos 3.1 Server [36] for serine/threonine phosphorylation prediction, SulfoSite [37] for tyrosine sulfation site prediction, PDZPepInt [38] for prediction of potential PDZ domain binding peptides and GPS-Lipid server [39] for palmitoylation site prediction. A multiple amino acid sequence alignment was generated using Clustal Omega [40] and boxshaded at http://www.ch.embnet.org/software/BOX_form.html. A neighbour-joining phylogenetic tree was constructed using MEGA 7.0 [41] with 10,000 bootstrap calculations.

2.2. Fish

Rainbow trout (~300g) were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, UK). Fish were maintained in 1-m-diameter aerated fiberglass tanks with a re-circulating water system at 14 ± 1 °C and fed twice daily with standard commercial pellets (EWOS). Prior to any experiments, fish were acclimated for at least 2 weeks and screened for potential bacterial infection by taking head kidney swabs. For the challenge experiment the fish were kept in the same facility for three months before use.

2.3. Tissue distribution of expression of the novel CCRs

To detect the transcript level of CCRs in healthy fish, six individuals (mean ± SEM = 142 ± 9 g) were anaesthetized, killed and seventeen tissues (tail fins, adipose fin, thymus, gills, brain, scales, skin, muscle, liver, gonad, spleen, HK, caudal kidney, intestine, heart, blood and adipose tissue) sampled. RNA and cDNA preparation and real-time PCR analysis was

performed as described previously [24, 42]. Expression levels of each gene were normalized to the expression level of the house-keeping gene, EF-1α.

2.4. Expression of novel CCRs after bacterial infection

For the infection group, trout were injected intraperitoneally (ip) with *Yersinia ruckeri* (strain MT3072), a Gram-negative salmonid pathogen, at a dose of 0.5×10^6 cfu in 0.5 mL PBS. The dose and volume (0.5 ml) were as used in our previous studies [43], and induce mortalities from day 3. Control fish were injected with PBS alone (0.5 mL per fish). HK tissue from six fish in each group was sampled at 6 h, 24 h, 48 h and 72 h post-injection. Real-time PCR quantification of expression was as described previously [43] and expressed as fold change relative to time-matched controls.

2.5. Expression of novel CCRs after parasitic infection

Caudal kidney tissues were collected from fish infected with the myxozoan parasite *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease (PKD), as described previously [44]. The severity of clinical pathology was analyzed and a kidney swelling index assigned to each fish according to the kidney swelling index system devised by Clifton-Hadley and colleagues [45]. In brief, fish kidneys were graded using the following criteria: Grade 0 fish exhibited normal / healthy kidneys that appeared slightly concave. The kidney tissue of grade 1 fish no longer appeared concave, although there was no indication of kidney swelling. Grade 1-2 fish exhibited moderately low level swelling of the caudal kidney tissue with the remaining kidney resembling the kidneys of grade 1 fish. Grade 2 fish exhibited markedly swollen kidneys particularly of the caudal kidney tissue, whilst grade 3 fish exhibited gross swelling throughout the kidney with clear signs of tissue discolouration and appearance of ascitic fluid in the peritoneal cavity. Collected caudal kidney samples were analyzed for CCR expression by real time-PCR as described above. Gene expression, at each swelling grade, was expressed as average expression level relative to levels in un-infected controls.

2.6. Expression of novel CCRs in primary HK macrophage

Primary HK macrophages were isolated, cultured as described previously [46], and stimulated with PAMPs and recombinant cytokines, including polyinosinic acid: polycytidylic acid (PolyIC, 50 μg/ml, Sigma), peptidoglycan (PGN, 5μg/ml, Invivogen), rIL-1β (20 ng/ml) [47], rIL-6 (100 ng/ml) [46], rIFN-γ (20 ng/ml) [34], for 4 h, 8 h, and 24 h. Incubation with the stimulants was terminated by dissolving the cells in TRI reagent (Sigma). RNA preparation and real-time PCR analysis were performed as described above. The expression level of each treatment group was expressed as a fold change relative to time-matched controls.

206

207 **2.7. Statistical analysis**

208 All data were expressed as mean + SEM. SPSS statistics package 24 (SPSS Inc., Chicago,
209 Illiois) was used for statistical analysis. The data from the infection studies was analyzed
210 using one way-analysis of variance (ANOVA) and the LSD post hoc test. Data from *in vitro*
211 studies was analyzed by paired-sample T-test, as described previously [33]. Statistical
212 significance was set with a p value $\leqslant 0.05$.

3. Results

3.1. Sequence analysis of novel CCRs in rainbow trout

Four genomic loci have been identified in the rainbow trout genome that could encode for four novel CCRs. Primers were designed at the predicted 5'- and 3'-UTR to enable the cloning of full length cDNA sequences (supplementary Figs. S1-4). Each sequence translated into a complete ORF. Three sequences have at least one in-frame stop codon upstream of the ORF, as summarized in Table 2. The proteins encoded were termed as CCR4La, CCR4Lc1, CCR4Lc2 and CCR11 according to the unified nomenclature [4] and our analysis of chemokine receptors. The sequences were found to be orthologous to the recently reported Atlantic salmon CCR4, CCR2a, CCR2b and CCR5, respectively [16]. Importantly, the salmon genes were not found to be orthologous to the well-studied mammalian genes encoding CCR4, CCR2 and CCR5, thus introducing ambiguity into CCR nomenclature. For clarification, the current state of CCR nomenclatures in humans, rainbow trout, Atlantic salmon and zebrafish are presented in Table 3. It is noteworthy that trout CCR4La, CCR4Lc1 and CCR11 are located at the same genomic scaffold 1743.

The cDNA sequences of CCR4La, CCR4Lc1, CCR4Lc2 and CCR11 exhibit: An ORF of 1227 bp, 1038 bp, 1038 bp and 1062 bp encoding for 408 aa, 345 aa, 345 aa and 353 aa; 6, 1, 2 and 2 potential N-glycosylation sites in the predicted extracellular regions; 3, 2, 1 and 4 potential tyrosine sulfation sites; 6, 3, 3, and 4 predicted palmitoylation sites, respectively (Table 2, Figs. S1-4). Each translation contained a predicted extracellular amino-terminal domain (N-terminus), three ECLs, three ICLs and a cytoplasmic carboxyl domain (C-terminus), separated by seven transmembrane regions (Figs. 1, S1-4). Multiple serine/threonine phosphorylation sites and a PDZ binding motif were predicted in the cytoplasmic tail of each receptor (Table 2, Figs. 1, S1-4).

Trout CCR4La shares highest amino acid identities to salmon CCR4a (88.7%) and salmon CCR4b (77.9%). It also shares higher identities/similarities (48.0-55.1%/65.9-70.8%) to CCR4La or CCR4Lb molecules found in other teleost fish than to any other CCR protein (Table 4). Similarly, trout CCR11 shares highest identities to salmon molecules and higher identities to fish CCR11 (Table 4). The trout CCR4Lc1 and CCR4Lc2 share 92.2% identity with similar identities to salmon CCR2a and 2b (92.5-94.5%). As with CCR4La, they share higher identities to fish CCR4Lc molecules than to other CCRs (Table 4). All of the trout CCRs exhibit higher identities to human CCR1-5 and 8 than to human CCR6, 7, 9 and 10. For example, trout CCR4Lc1 and 2 exhibit 38.0-41.5% identity to human CCR1-5 and 8 relative to 31.2-33.7% to CCR6,7,9 and 10 (Table 4). Trout CCR4La, CCR4Lc and CCR11 were

found to have low sequence identity when compared to each other.

Multiple alignments of the fish CCR4La/b, CCR4Lc and CCR11 molecules from selected fish species (salmonids, zebrafish, medaka, tetraodon and platyfish *Xiphophorus maculatus*) revealed general conservation of chemokine receptors, including the seven transmembrane domains that separate the N-terminal, the three ECLs, three ICLs and the C-terminal tail with a well conserved DRY motif in ICL2 (Figs. 2-4). Each extracellular region (the N-terminal, and three ECLs) had a conserved cysteine residue that is known to form two disulfide bonds between the N-terminal and ECL3, and ECL1 and ECL2 (Fig. 1) to stabilize the receptor conformation [24]. The exceptions are zebrafish CCR4Lb, medaka CCR4La and medaka CCR4Lb, in which one of the conserved cysteines is missing. The predicted tyrosine sulfation and cysteine palmitoylation sites in the trout sequences were conserved in most fish species, although their actual positioning was not conserved. N-glycosylation sites were predicted in the N-terminals, and in some ECL2s in most fish molecules. Multiple serine/threonine residues, which could be phosphorylated after receptor activation, and a PDZ binding peptide motif were found in the cytoplasmic tail in most fish species (Figs. 2-4). Furthermore, di-leucine motifs (L/I)(L/I) that are well conserved in salmonid CCR4La/b and CCR11, were also found in CCR4La/b and some CCR11 molecules from other fish species (Figs. 2 and 4).

3.2. Phylogenetic tree analysis

To further understand the relationship of CCRs from teleosts and other vertebrates, phylogenetic tree analysis was performed using an amino acid multiple alignment of CCR molecules from selected fish species and mammals. As shown in an unrooted phylogenetic tree (Fig. 5), 14 CCR orthologous groups namely, CCR1-12, CCR4La/b and CCR4Lc were present in fish and mammals with high bootstrap support (98-100%), the only exception being mammalian CCR2/5 owing to mammalian-specific genetic conversion [48]. The homeostatic molecules, CCR7, 9 and 10, and the dual functional CCR6 were conserved in fish and mammals and form a distinct sub-family with high bootstrap support (99%) and separated from the rest of CCRs. The inflammatory CCR1, 2, 3 and 5 molecules, and dual-functional CCR4 and 8 were found to be mammalian specific, whilst CCR11-12, CCR4La/b and CCR4Lc were found to be fish-specific. Furthermore, trout CCR4La, CCR4Lc1 and 2, and CCR11, cloned in this report, clustered with orthologues from other fish species, thus supporting our proposed nomenclature. This tree also supports the notion that the fish specific CCRs are phylogenetically closer to mammalian CCR1-5 and 8, than they are to the CCR6, 7, 9-10 subfamily, as suggested by homology analysis (Table 4).

3.3. Gene organization analysis

A detailed analysis of gene organisation of CCRs and their conservation in different animal lineages is lacking due to the need for mRNA/cDNA sequence information [49]. The cloning of novel trout CCR cDNAs has enabled the determination of the gene organisation. Trout CCR4La has a four exon/three intron gene organisation with the coding region spanning the last three exons separated by two phase II introns (Fig. 6A). The three coding exon structure was also observed in salmon CCR4a and zebrafish CCR4La/b, although the second coding exon was found to be missing in salmon CCR4b and CCR4La genes from fugu and platyfish (Fig. 6A). Both trout CCR4Lc1 and c2 have a two exon structure with the first exon being noncoding and the last exon encoding the complete ORF. This gene organisation might be preserved in salmon CCR2a-b and zebrafish CCR4Lc, but it is different in CCR4Lc from fugu, platyfish and medaka, which have a three coding exon structure (Fig. 6B). Similarly, trout CCR11 has a two exon structure with the protein encoded by the last exon, a structure that may also be preserved in salmon CCR5a-b. However, zebrafish CCR11a-d has two coding exons separated by a phase I intron, whilst fugu and platyfish CCR11 orthologues have a three coding exon structure with both introns in phase I. (Fig. 6C).

3.4. Tissue distribution of transcript expression of the four trout CCRs

The transcriptional levels of the four novel trout CCRs were examined in seventeen tissues from six healthy fish by real-time PCR (Fig. 7). Expression of all four CCRs was detectable in all tissues examined albeit at different levels. The highest expression levels were detected in spleen and thymus, and lowest in liver for all four receptors (Fig. 7). High expression levels were also detected in other immune organs, such as HK and gills, and non-immune tissue, eg. gonad. Expression patterns and levels of trout CCR4La, 4Lc1 and 4Lc2 were similar. It is noteworthy that CCR11 expression in intestine, skin and scales was relative low (Fig. 7).

3.5. Modulation of the expression of trout CCRs by bacterial and parasitic infection

CCR transcriptional levels were also investigated *in vivo* following bacterial and parasitic infection. The bacterium *Yersinia ruckeri* is the causative agent of ERM or yersiniosis, and is responsible for significant economic losses in salmonid aquaculture worldwide [43]. Disease symptoms were observed from day 3 in naïve fish after ip injection and modulation of immune gene expression has been observed previously from 6 h to 72 h [43]. Thus the expression of CCRs in the current study was examined from 6 h to 72 h post ip challenge in HK, a major immune tissue in fish. CCR4La expression remained unchanged at 6 h, but increased significantly by 24 h (5-fold) and 48 h (3-fold) and returned to control levels by 72 h post challenge (Fig. 8A). An increased expression of CCR11 (7-fold), CCR4Lc1 (10-fold) and CCR4Lc2 (15-fold) was only observed at 24 h post challenge (Fig. 8B-D).

Proliferative Kidney Disease of salmonid fish is a slow progressive disease of major economic importance to aquaculture. The causative agent, a myxozoan parasite *Tetracapsuloides bryosalmonae*, primarily targets the kidney of infected fish where it causes a chronic lymphoid hyperplasia with an anti-inflammatory phenotype [44]. Expression of trout CCRs was examined in caudal kidney tissue in fish exhibiting a range of clinical disease (kidney swelling grade) collected during a natural exposure to the parasite, as described previously [44]. The expression of trout CCR4La remained unchanged, whilst a small but significant increase (2-fold) of CCR11 was observed in infected fish with swelling grade of 2 (Fig. 9A-B). In contrast, expression of CCR4Lc1 and CCR4Lc2 decreased significantly in infected fish from grade 1 to grade 3 (Fig. 9C-D).

3.6. Modulation of the expression of trout CCRs in primary HK macrophages

Modulated CCR expression by bacterial and parasitic infection prompted further investigation regarding CCR expression in primary HK macrophages post stimulation with PAMPs (PolyIC, and PGN) and recombinant proinflammatory cytokines (rIL-1β, rIL-6 and rIFNγ). In general, CCR expression exhibited a U-shaped response profile after stimulation, the dynamics of which was CCR-specific (Fig. 10). PolyIC down-regulated CCR4La expression at 8 h, but up-regulated expression at 24 h. It also upregulated CCR4Lc1 expression at 4 h and 24 h but had no significant effects on CCR11 and CCR4Lc2 expression at all three time points (Fig. 10). PGN up-regulated CCR4La, CCR4Lc2 and CCR11 expression at 4 h, an effect that was lost by 8 h post stimulation with the expression returning to control levels or even increasing in the case of CCR4Lc1 at 24 h. PGN had no significant effects on CCR4Lc2 expression. rIL-1β, rIL-6 and rIFNγ, had similar effects on CCR expression with all three cytokines upregulating CCRL4a, CCR4Lc1 and CCR11 at 4 h, whilst suppressing expression levels by 8 h and with no significant effects observed at 24 h post stimulation (Fig. 10A-C). CCR4Lc2 was less responsive, with only decreased expression observed at 8 h following rIL-1β and rIFNγ stimulation (Fig. 10D).

4. Discussion

4.1. Nomenclature of Chemokine receptors with particular reference to the CCRs

Four novel fish-specific CCRs have been characterized in rainbow trout and named as trout CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, a nomenclature that fits with the outcome of the phylogenetic analysis conducted in this study and the naming system proposed by Nomiyama et al. [4]. It is now clear that there are well-conserved CCRs (CCR6-7 and CCR9-10) from fish to mammals with others being either mammalian-specific (CCR1-5 and CCR8) or fish-specific (CCR4La/b, CCR4Lc and CCR11-12). Fish-specific CCRs in different fish species have been assigned a variety of names causing a degree of ambiguity in the comparative study of chemokine biology [2, 16, 49, 50].

From our phylogenetic analysis, fish-specific CCRs are apparently more closely related to the mammalian CCR1-5 and 8 subfamily than the CCR6-7 and 9-10 subfamily. Consistent with this concept, trout CCRs cloned in this study exhibited similarly high amino acid identity/similarity to human CCR1-5 and 8 compared to CCR6-7 and 9-10. The mammalian specific CCR1-5 and 8 subfamily are located at the same genomic locus (eg. Human chromosome 3). Fish specific CCRs were also found to be located in the same locus (eg in zebrafish chromosome 16) [49]. Trout CCR4La, CCR4Lc1 and CCR11 were located at the same genomic scaffold. Similarly, their salmon counterparts (CCR4, 2 and 5) were located at the same loci in two separate contigs (acc. nos. AGKD03026506 and AGKD03006887) that have arisen from the 4R salmonid WGD [16]. These data suggest that mammalian and fish-specific CCRs arose from a common ancestral gene that expanded by local lineage-specific gene duplications with further expansion in salmonids facilitated by the 4R WGD event. Overall, consistent with our analysis, we adopted the nomenclature proposed by Nomiyama et al. [4] in naming fish specific CCR4La, CCR4Lc, CCR11 and CCR12.

4.2. The molecular features of functional importance

All newly identified trout CCRs were found to possess the conserved G protein-linked receptors (GPLR) family features. Firstly, all trout CCRs possess; an extracellular N-terminal, seven TM domains, three ECLs and three ICLs, and a cytoplasmic carboxyl tail. Secondly, four cysteine residues involved in forming two disulfide bonds were present in the extracellular domains of the novel CCRs [51]. Thirdly, a DRY motif was present in the second ICL [52] with the extended DRYLAIV motif present in salmonid CCR4La, CCR4Lc1 and CCR4Lc2 and in most other fish species. However, the DRYLAIV motif differed in salmon CCR11 (DRYVVIV) and orthologues in other fish species. The triggering of classical downstream signaling, such as calcium mobilization and chemotaxis, requires the coupling of

chemokine receptors to Gαi proteins. The DRYLAIV motif is essential for G protein coupling, and is highly conserved in classical chemokine receptors and less so in atypical chemokine receptors [53]. The implication on down-stream signaling of fish CCR11 remains to be determined.

The N-terminal region of chemokine receptors is important for ligand binding [1]. Thus, any post-translational modification of the N-terminal region of CCRs is likely to influence ligand binding and downstream signaling. Putative N-glycosylation sites and tyrosine sulfation sites are predicted in the N-terminal region of trout CCRs and in other fish CCRs. N-glycosylation is a post-translational modification, which has distinct functional consequences, including the determination of protein conformation, stability, trafficking, ligand-receptor binding affinity and intracellular signaling. N-glycosylation of CXCR3 is known to influence its binding to CXCL10 [54]. Tyrosine sulfation is a post-translational modification of secreted and transmembrane proteins, including chemokine receptors, by the addition of a negatively charged sulfate to their hydroxyl groups. Sulfation of these receptors has been shown to increase chemokine binding affinity and potency [55]. Thus, N-glycosylation and tyrosine sulfation of fish-specific CCRs may have a role in the regulation of ligand binding.

S-palmitoylation, a process by which palmitate is reversibly attached to proteins via a thioester linkage, effectively increases the hydrophobicity of its modified substrate. Thus, S-palmitoylation can regulate membrane association of various cellular proteins. Palmitoylation of human CCR5 is involved in ligand induced receptor phosphorylation, desensitization and internalization [12]. Multiple cysteine palmitoylation sites were predicted in the trout CCRs and that were conserved in CCR orthologues in other fish species. This suggests palmitoylation of fish-specific CCRs is involved in ligand induced receptor phosphorylation, desensitization and internalization.

After ligand binding and activation, chemokine receptors typically undergo internalization, followed by either degradation or recycling to the plasma membrane. The process starts with receptor activation by the ligand and phosphorylation of serine or threonine residues near the C-terminus of the receptor, leading to receptor desensitization. Phosphorylated receptors, containing the “di-leucine” motif, facilitate the recruitment of endocytosis-related molecules adaptin 2 (AP2) and β-arrestin, leading to internalization of the receptor to form clathrin-coated vesicles. Studies of receptors CCR5 and CXCR2 have suggested that a PDZ ligand domain at the C-terminus can direct receptor sorting between recycling or degradation pathways [1]. Multiple serine/threonine residues and a PDZ binding motif are present in the cytoplasmic tail of fish-specific CCRs. In addition, di-leucine motifs (L/I)(L/I) are well

conserved in salmonid CCR4La/b and CCR11 and are also present in CCR4La/b, and some CCR11 molecules in other fish species. This suggests that multiple regulatory mechanisms likely take place during fish specific CCR / ligand interactions.

4.3. Implications of diversified CCR gene organization

Whilst all mammalian-specific CCRs in humans, with the exception of CCR3, being encoded by a single exon, each fish-specific CCR4L and CCR11 is encoded by 1 to 3 exons in a CCR-specific and species-specific manner. This suggests that intron insertions occurred independently in different CCRs and in different species/lineages during teleost evolution. Intron insertion (retention) is energetically costly to cells, although the selective advantages of carrying additional introns has been proposed to be in the regulation of alternative splicing, positive regulation of gene expression, and regulation of nonsense-mediated decay [56]. Alternative splicing is a controlled molecular mechanism producing multiple variant proteins from a single gene in a eukaryotic cell. For example, CXCR3, exists in three differentially spliced forms—CXCR3A, CXCR3B, and CXCR3Alt. CXCR3A and CXCR3B differ only in the lengths of their N-terminal regions, with CXCR3Alt being a truncated protein. These splice variants have been reported to show specific expression profiles in particular cell types and activate different signaling pathways [57]. It has been estimated that 95% of multi-exon genes in the human genome may undergo alternative splicing [56]. Interestingly, the intron insertion in fish specific CCRs has occurred at the 5'-end encoding mainly the N-terminal. Alternative splicing of these exons may produce CCRs with different N-termini that could affect ligand binding specificity/affinity.

4.4. The expression of the trout CCRs

CCR expression has been studied in several fish species but in only a limited number of tissues. Liu and colleagues [49] examined zebrafish CCR expression in 6 tissues by RT-PCR. Grimholt and colleagues [16] investigated salmon chemokine receptor expression in 11 tissues by RNAseq using a single fish and by RT-qPCR using a single pooled sample of three fish. In this study, we have examined fish-specific CCR expression in 17 tissues including thymus, a tissue not examined in previous studies. The highest CCR expression levels in the present study were detected in thymus and spleen, with high expression levels also seen in other immune organs, such as HK and gills. These observations suggest that fish-specific CCRs may have important roles in immune regulation. High levels of CCR expression were also seen in other tissues, such as gonad, suggesting the presence of migrating CCR expressing leukocytes in non-immune tissues of the organism.

Due to the additional WGD, salmonids often possess two paralogues of a gene relative to 3R

teleost fish [42, 58-62]. Thus, two loci for CCR4La, CCR4Lc and CCR11 have been identified in the salmon genome [16]. In this study we were unable to identify additional CCR4La and CCR11 loci in the current version of the trout genome of 1.9 Gb [63], and in the NCBI EST database. These paralogues may have been lost in trout after the 4R WGD event, or are present in the genome but are expressed at low levels, as suggested by their expression levels in salmon where CCR4b and CCR5b transcripts were absent from RNAseq transcriptomes [16].

4.5. The inflammatory characteristics of the trout CCR4La, CCR4Lc1, CCR4Lc2 and CCR11

Some chemokines and receptors are constitutively expressed in specific tissues and cell types, where they contribute to homeostatic functions such as T cell development, stem cell migration, and lymphoid organogenesis. Others are induced at sites of injury or infection as part of the inflammatory response. Moreover, a few chemokines and their receptors appear to have both homeostatic and pro-inflammatory functions. Of the 10 human CCRs, CCR7, 9 and 10 are homeostatic, CCR1, 2, 3 and 5 are inflammatory, and CCR4, 6 and 8 have dual functionality [1, 4]. The close link of trout CCR4La, CCR4Lc and CCR11 to human CCR1-5 and 8, as revealed by phylogenetic tree and homology analysis, may suggest that they are of an inflammatory nature. This notion is supported by their induction in HK macrophages stimulated with the inflammatory cytokines IL-1β, IL-6 and IFNγ, and by PAMPs. In all cases, a U-shaped time course was observed, suggesting that the transcription of these receptors is tightly regulated to allow proper control over the inflammatory response. Differences between the 4R WGD paralogues CCR4Lc1 and CCR4Lc2, in responses to PAMPs and proinflammatory cytokines, is noteworthy and perhaps indicates functional diversification.

The notion that trout CCR4La, 4Lc and 11 are inflammatory in nature was also supported by their induction during bacterial infection and modulation during PKD. *Y. ruckeri* infection elicits an acute inflammatory response whereby proinflammatory cytokines and chemokines, such as IL-1β, IL-6, IL-8, TNFα and IFNγ, are highly induced [43]. Cell differentiation and movement of lymphoid and monocytic cells have also been observed in immune organs after *Y. ruckeri* infection [64]. The increased expression of the trout CCRs after *Y. ruckeri* infection may indeed be upregulated directly by bacterial infection, or indirectly by the upregulated proinflammatory cytokines. However, the contribution of cell trafficking events to changes in CCR gene expression after infection cannot be excluded.

The characteristic kidney swelling associated with PKD is due to the predominant increase of proliferating lymphocytes accompanied with the over-expression of immunoglobulin isotypes

and dysregulated TH-like responses [44]. The expression of anti-inflammatory cytokines, including IL-10, TGF-β1 and nIL-1Fm are upregulated, whilst lacking the classical signs of a pro-inflammatory response characterised by upregulated IL-1β and TNFα transcription. Thus, PKD appears to be associated with a prevailing anti-inflammatory phenotype [44]. The expression of both CCR4Lc1 and CCR4Lc2 decreased in infected fish, with CCR11 exhibiting only a minor transcriptional increase and CCR4La remaining refractory to infection. These expression patterns may reflect the lack of pro-inflammatory signals, or be partly due to the decreased ratio of receptor-expressing inflammatory cells owing to the *in situ* proliferation of lymphoid cells during PKD.

4.6. Conclusion

In conclusion, four fish specific CCRs (CCR4La, CCR4Lc1, CCR4Lc2 and CCR11), that are closely linked to mammalian CCR1-5 and 8, have been characterized in rainbow trout. These novel CCRs possess the conserved G protein-linked receptor (GPLR) family features, including an extracellular N-terminal, seven TM domains, three ECLs and three ICLs, and a cytoplasmic carboxyl tail with multiple serine/threonine phosphorylation sites. Four cysteine residues that are known to be involved in the formation of two disulfide bonds are present in the extracellular domains with a DRY motif present in the second ICL. The signaling mediated by these receptors may be regulated by N-glycosylation, tyrosine sulfation, S-palmitoylation, a PDZ ligand motif and di-leucine motifs. Studies of intron/exon structure revealed a diversified gene organization with intron insertion being receptor and species-specific. The fish-specific trout CCRs are highly expressed in immune tissues/organs, such as spleen, thymus, HK and gills with expression being inducible in the presence of proinflammatory cytokines, PAMPs and bacterial infection. Overall, this study suggests that fish-specific CCRs are involved in inflammation with potentially important roles in fish immune regulation.

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728

729 **Figure legend**

730

731 **Fig. 1. Schematic representation of key features of the trout CCRs located across the cell**
732 **membrane.** The N-terminus and three extracellular loops (ECL1-3) are located outside the
733 cell, whereas the C-terminus and three intracellular loops (ICL1-3) are within the cell. The
734 CCRs have seven transmembrane helices (TM1-7). Each receptor has multiple potential
735 N-glycosylation sites and tyrosine sulfation sites in the N-terminus, a DRY motif in ICL2, and
736 multiple serine/threonine phosphorylation sites, di-leucine motifs and a PDZ binding motif
737 predicted in the C-terminus. The conserved cysteine residues located in each of the
738 extracellular regions, that potentially form two disulfide bonds, are also indicated.

739

740 **Fig. 2. Multiple alignment of trout CCR4La with CCR4La and CCR4Lb from selected**
741 **teleosts**. The multiple alignment was produced using Clustal Omega and conserved amino
742 acids shaded using BOXSHADE (version 3.21). The shading at the N-terminus and
743 C-terminus has been removed to illustrate other features. The N-terminal, seven
744 transmembrane domains (TM1-7), three extracellular loops (ECL1-3), three intracellular
745 loops (ICL1-3) and the C-terminal are marked above the alignment. The conserved cysteine
746 residues in the extracellular regions that form disulfide bonds are indicated by black arrow
747 heads, and predicted palmitoylation sites indicated by red arrows below the alignment. The
748 DRY motifs in ICL2 region are within the red box. Putative sulfated tyrosine residues in the
749 N-terminus are in red and underlined, and potential N-glycosylation sites are in purple.
750 Serine/threonine residues in the cytoplasmic tail that may be phosphorylated and bind
751 β-arrestin are highlighted in yellow, and an amino acid motif predicted to bind PDZ
752 domain-containing proteins is in red and underlined. The di-leucine motif is in bold and
753 underlined. Note that the N-terminals of medaka CCR4La and b are not complete.
754 Salmon-a=Salmon CCR4a and salmon-b=salmon CCR4b. The accession numbers for
755 sequences used in this alignment are given in Fig. 5.

756

757 **Fig. 3. Multiple alignment of trout CCR4Lc1 and CCR4Lc2 with CCR4Lc from selected**
758 **teleosts.** The multiple alignment was produced using Clustal Omega and the conserved amino
759 acids shaded using BOXSHADE (version 3.21). The shading at the N-terminus and
760 C-terminus has been removed to illustrate other features. The N-terminal, seven
761 transmembrane domains (TM1-7), three extracellular loops (ECL1-3), three intracellular
762 loops (ICL1-3) and the C-terminal are marked above the alignment. The conserved cysteine
763 residues in the extracellular regions that form disulfide bonds are indicated by black arrow
764 heads, and predicted palmitoylation sites indicated by red arrows below the alignment. The
765 DRY motifs in ICL2 region are in red box. The putative sulfated tyrosine residues in the

766 N-terminus that potentially is sulphated are in red, and underlined and potential
767 N-glycosylation sites are in purple. The serine/threonine residues in the cytoplasmic tail that
768 may be phosphorylated and bind β-arrestin are highlighted in yellow, and an amino acid motif
769 predicted to bind PDZ domain-containing proteins is in red and underlined. The C-terminal
770 amino acids (326-384) of zebrafish CCR4Lc were removed from the alignment.
771 Salmon-c1=Salmon CCR2a and salmon-c2=salmon CCR2b. The accession numbers for
772 sequences used in this alignment are given in Fig. 5.
773
774 **Fig. 4. Multiple alignment of trout CCR11 with CCR11 from selected teleosts**. The
775 multiple alignment was produced using Clustal Omega and the conserved amino acids shaded
776 using BOXSHADE (version 3.21). The shading at the N-terminus and C-terminus has been
777 removed to illustrate other features other characteristics. The N-terminal, seven
778 transmembrane domains (TM1-7), three extracellular loops (ECL1-3), three intracellular
779 loops (ICL1-3) and the C-terminal are marked above the alignment. The conserved cysteine
780 residues in the extracellular regions that form disulfide bonds were indicated by black arrow
781 heads, and predicted palmitoylation sites indicated by red arrows below the alignment. The
782 DRY motifs in ICL2 region are within the red box. The Putative sulfated tyrosine residues in
783 the N-terminus that potentially is sulphated are in red and underlined and potential
784 N-glycosylation sites are in purple. The serine/threonine residues in the cytoplasmic tail that
785 may be phosphorylated and bind β-arrestin are highlighted in yellow, and an amino acid motif
786 predicted to bind PDZ domain-containing proteins is in red and underlined. The di-leucine
787 motif is in bold and underlined. The C-terminal amino acids (358-492) of zebrafish CCR11c
788 were removed from the alignment. Note that the N-terminals of medaka CCR11a and b are
789 not complete. Salmon-a=Salmon CCR5a and salmon-b=salmon CCR5b. The accession
790 numbers for sequences used in this alignment are given in Fig. 5.
791
792 **Fig. 5. An unrooted phylogenetic tree of vertebrates CCRs.** The tree was constructed using
793 amino acid multiple alignments and the neighbour-joining method within the MEGA7
794 program. Node values represent percent bootstrap confidence derived from 10,000 replicates.
795 Evolutionary distances were computed using the JTT matrix-based method and pairwise
796 deletion option. The accession number for each sequence is given after the species name and
797 molecular type. Trout CCR4La, CCR4Lc1, CCR4Lc2 and CCR11 are marked in red.
798 Bootstrap values at the roots of the clades from different lineages are highlighted with a circle.
799 Molecular groups are indicated on the right.
800
801 **Fig. 6. Comparison of gene organizations of teleost CCR4La/b (A), CCR4Lc (B) and**
802 **CCR11 (C).** Gene organization was predicted using the Splign program based on sequences

from the Ensembl database. Black and white boxes represent amino acid coding regions and untranslated regions within exons, respectively, and black bars represent introns. Exon size (bp) is numbered in the boxes. Intron phase (0, I or II) is also denoted. Dotted boxes denote uncertainty of exon presence or size. Detailed genomic sequences used for this analysis are given in Fig. S5.

Fig. 7. Constitutive expression of trout CCR4La (A), CCR11 (B), CCR4Lc1 and CCR4Lc2 (C) *in vivo*. Transcript levels of trout CCRs were determined by real time RT-PCR in 17 tissues from six fish. Transcript levels were first calculated using a serial dilution of references and normalized against the expression level of EF-1α. Results represent the means + SEM of six fish.

Fig. 8. Modulation of trout CCR4La (A), CCR11 (B), CCR4Lc1 (C) and CCR4Lc2 (D) expression by *Y. ruckeri* infection. Rainbow trout were injected ip with *Y. ruckeri* or PBS as vehicle control. HK tissue was collected at 6 h, 24 h, 48 h and 72 h post-challenge and gene expression expressed as fold change, calculated as the average expression level of infected fish normalized to expression levels in time-matched controls. Results are presented as means + SEM of five fish. Significance of LSD post hoc tests after one way-analysis of variance between infected and time-matched control fish is shown above the bars.***** $p \leq 0.05$,** $p \leq 0.01$, *** $p \leq 0.001$.

Fig. 9. Modulation of trout CCR4La (A), CCR11 (B), CCR4Lc1 (C) and CCR4Lc2 (D) expression by parasite infection. Kidneys from rainbow trout infected with *Tetracapsuloides bryosalmonae* or from unexposed (control) fish were collected during a natural infection. Gene expression was expressed as fold change, calculated as the average expression level of infected fish normalized to expression levels in controls. Results are presented as means + SEM. Numbers of fish analyzed were 11, 5, 9, 10 and 9 representing control, grade 1, 1-2, 2 and 3, respectively. Significance of LSD post hoc tests after one way-analysis of variance between infected and control fish is shown above the bars as ***** $p \leq 0.05$, ** $p \leq 0.01$.

Fig. 10. Modulation of trout CCR4La (A), CCR11 (B), CCR4Lc1 (C) and CCR4Lc2 (D) expression in primary HK macrophages. Four day old primary HK macrophages were stimulated with PolyIC (50 μg/ml), peptidoglycan (PGN, 5μg/ml), rIL-1β (20 ng/ml), rIL-6 (100 ng/ml) and rIFNγ (20 ng/ml) for 4 h, 8 h and 24 h. Gene expression was expressed as fold change, calculated from the average expression of each treatment group normalized to expression levels in time matched controls. Results are presented as means + SEM of cells

839 from four fish. Significant results of a paired sample t-test between stimulated samples and
840 controls at the same time point is shown above the bars as: *$p<0.05$.

Table 1

Primers used for cloning and expression analysis.

Gene	Primer	Sequence (5' to 3')	Usage
CCR4La	F1	CACACCAGAGTGTCACACCCAG	PCR cloning
	R1	GCAGTTAAGTTGCTGTTCACACGGC	PCR cloning
	QF	CCAGTTATGCATATGGCACACATTTTG	Real-time PCR*
	QR	AGGATGACCCACAGGACCAGAAC	Real-time PCR
CCR4Lc1	F1	ATAGTTATCTAGAGCACACCTTAC	PCR cloning
	R1	GTCTTCTGCTCTACTTGCTGCTTTC	PCR cloning
	QF	TGTACATCAGAAAAGAAGGTATTGGGTAAG	Real-time PCR
	QR	TGCCAGTGCTACAAGGGCTTT	Real-time PCR
CCR4Lc2	F1	ATAGAGTAGACAAAACCTAAGAG	PCR cloning
	R1	GAACAGAAATTGGTCTTCTGCTCAATG	PCR cloning
	QF	TGTACATCAGAAAAGAAGAGATTGGGTAAG	Real-time PCR
	QR	CCAGTGCTACAGGGGCTGC	Real-time PCR
CCR11	F1	AGACTCAGAGAAGAAACACCAAAGAGC	PCR cloning
	R1	GAAATCCTACTTACATTTGTTTGTAGT	PCR cloning
	QF	GCTAATTGATCATTAATTATACCTGACAAGGA	Real-time PCR
	QR	ATGACGCCCACGATGAAGAC	Real-time PCR
EF-1α	F	CAAGGATATCCGTCGTGGCA	Real-time PCR
	R	ACAGCGAAACGACCAAGAGG	Real-time PCR

Note

* The primer amplification efficiencies of real-time PCR were 1.98, 2.01, 1.95, 1.98 and 1.90 for EF-1α, CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, respectively.

Table 2

Summary of sequence analysis of four novel CCRs in rainbow trout.

Features	**CCR4La**	**CCR4Lc1**	**CCR4Lc2**	**CCR11**
GenBank Acc. No.	KM516348	KM516343	KM516344	KM516345
cDNA length	1,292	1,219	1,244	1,222
ORF (bp)	1227	1038	1038	1062
In frame stop codon[1]	0	2	1	1
ORF (aa)	408	345	345	353
N-glycosylation sites[2]	6	1	2	2
Sulfation sites[3]	3	2	1	4
Phosphorylation sites[4]	6	4	5	8
Palmitoylation sites[5]	6	3	3	4
PDZ binding motif[6]	1	1	1	1
Genome location	Scaffold 1743	Scaffold 1743	Scaffold 1620	Scaffold 1743

Notes

[1] In frame stop codons before the main ORF.

[2] Potential N-glycosylation sites in extracellular regions.

[3] Potential tyrosine sulfation sites predicted at the N-terminal.

[4] Potential serine/threonine phosphorylation sites at the C-terminal tail.

[5] Predicted palmitoylation sites.

[6]Predicted PDZ binding peptide at the C-terminal tail.

Table 3

The CC ckemokine receptors known in humans, rainbow trout, Atlantic salmon and zebrafish in relation to the unified nomenclature of chemokine receptors (Nomiyama et al., 2011). '-' denotes absence. The trout molecules shaded were cloned in this report.

CCR	Humans	Rainbow trout	Atlantic salmon	Zebrafish
CCR1	CCR1	-	-	-
CCR2	CCR2	-	-	-
CCR3	CCR3	-	-	-
CCR4	CCR4	-	-	-
CCR4La/b	-	CCR4La	CCR4a CCR4b	CCR4La CCR4Lb
CCR4Lc	-	CCR4Lc1 CCR4Lc2	CCR2a, CCR2b	CCR4Lc
CCR5	CCR5	-	-	-
CCR6	CCR6	CCR6a1, CCR6a2	CCR6.1a, CCR6.1b CCR6.2	CCR6a CCR6b
CCR7	CCR7	CCR7	CCR7a CCR7b	CCR7
CCR8	CCR8	-	-	-
CCR9	CCR9	CCR9a CCR9b	CCC9.1a CCR9.1b CCR9.2a CCR9.2b	CCR9a CCR9b CCR9c
CCR10	CCR10	CCR10	CCR10	CCR10
CCR11	-	CCR11	CCR5a CCR5b	CCR11a CCR11b CCR11c CCR11d
CCR12	-	CCR12	CCR3a CCR3b	CCR12

Table 4

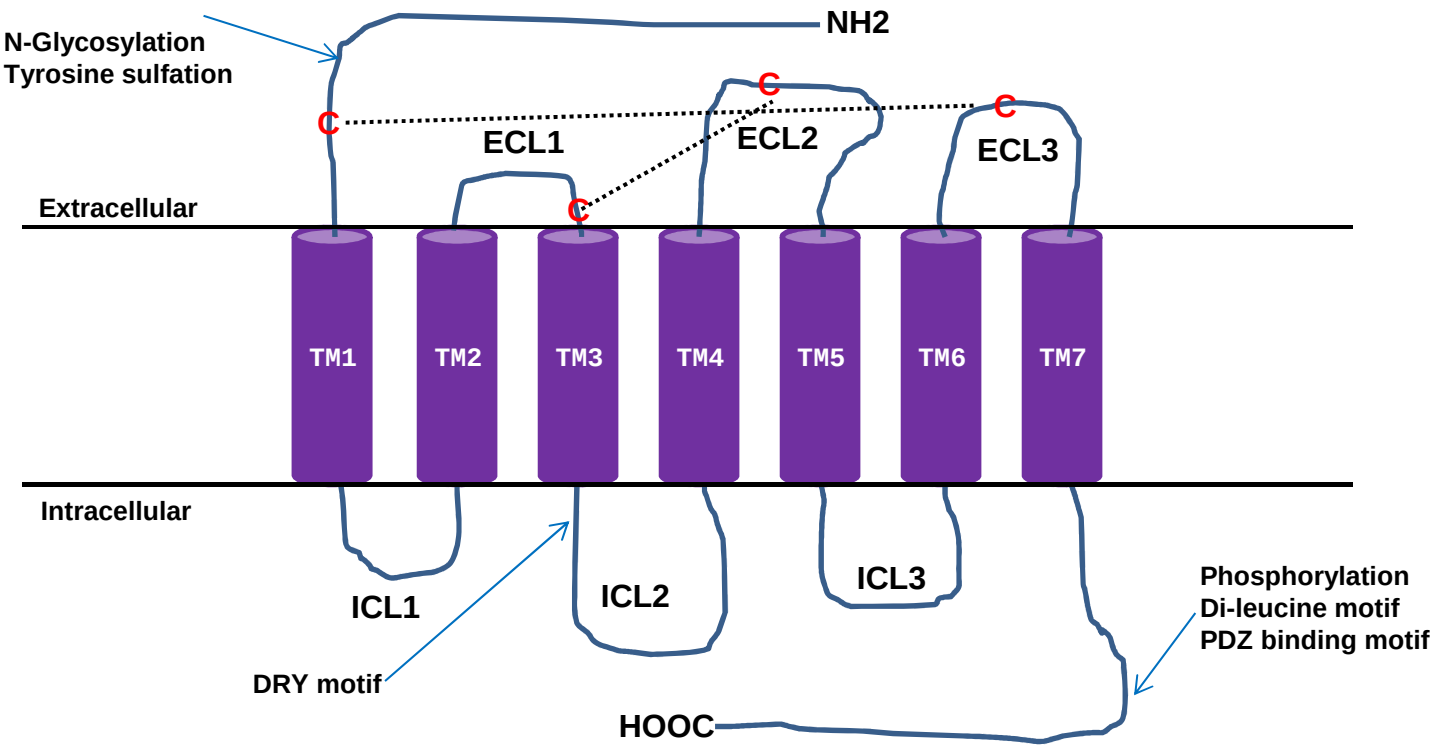
Comparison of the amino acid identity/similarity of rainbow trout CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, with relevant molecules from selected fish species and human CCRs. The homologies of the same molecules between trout and other fish species are in bold and underlined, and between trout and human CCR1, 2, 3, 4, 5, and 8 are in bold and italics.

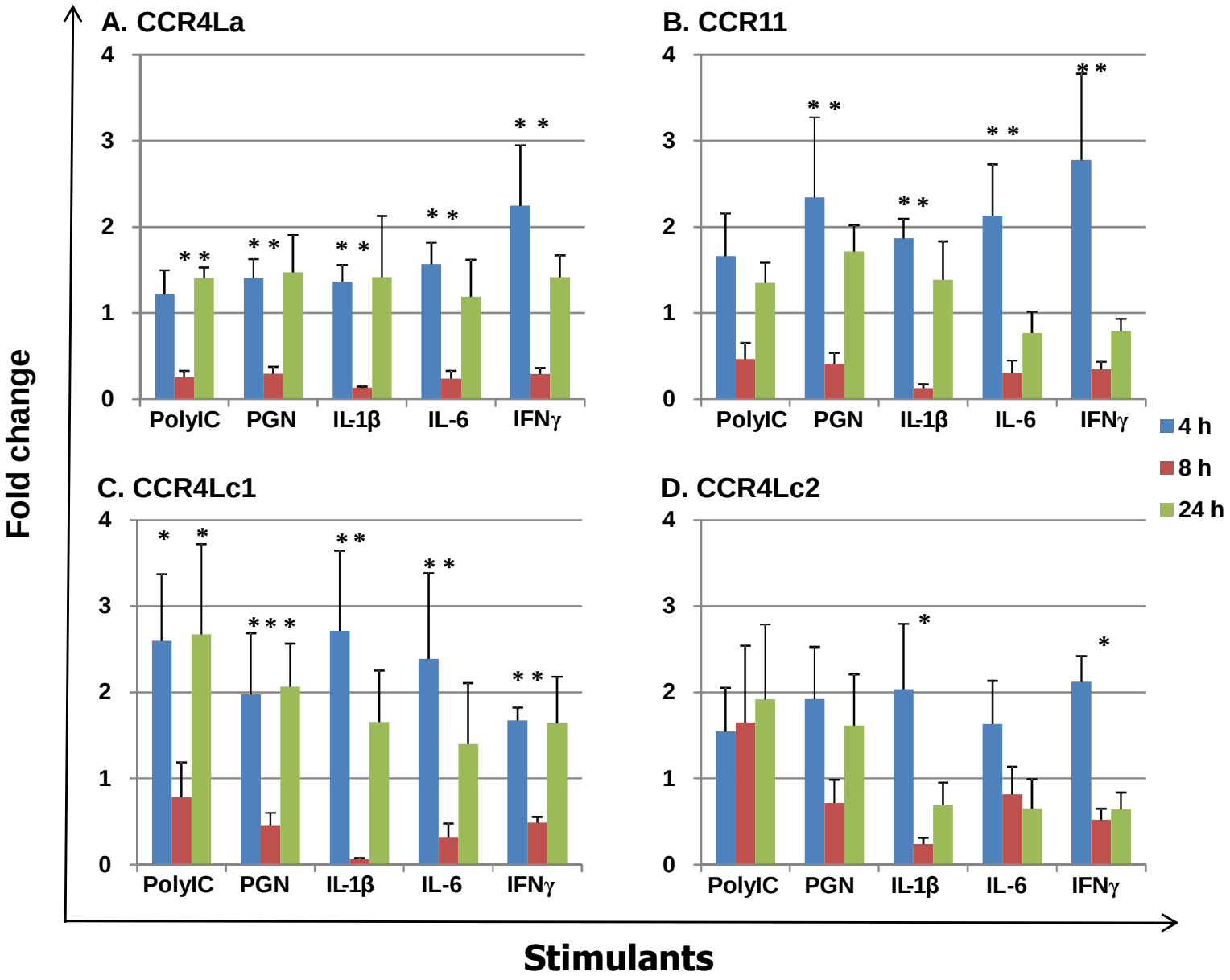
		Trout CCR4La	Trout CCR4Lc1	Trout CCR4Lc2	Trout CCR11
Trout CCRs	Trout CCR4La		36.5/56.9	25.2/55.6	34.2/54.2
	Trout CCR4Lc1	36.5/56.9		**92.2/95.1**	38.2/59.2
	Trout CCR4Lc2	25.2/55.6	**92.2/95.1**		37.0/60.1
	Trout CCR11	34.2/54.2	38.2/59.2	37.0/60.1	
CCR4La/b	Salmon CCR4a	**88.7/91.2**	36.3/57.1	35.6/56.4	35.6/53.8
	Salmon CCR4b	**77.9/82.1**	39.8/61.8	39.0/61.3	38.9/59.3
	Zebrafish CCR4La	**50.6/68.4**	35.1/55.4	36.3/56.2	32.7/56.5
	Zebrafish CCR4Lb	**48.4/67.1**	33.0/50.1	33.0/50.3	30.9/50.6
	Medaka CCR4La*	**52.8/67.4**	36.5/57.7	37.8/57.7	34.4/57.7
	Medaka CCR4Lb*	**48.0/65.9**	36.2/57.4	36.1/57.7	34.9/56.3
	Platyfish CCR4La	**55.1/70.8**	36.6/62.1	36.2/62.6	36.4/61.6
	Tetraodon CCR4La	**49.4/62.3**	34.6/59.7	34.3/58.8	35.3/60.1
CCR4Lc	Salmon CCR2a	35.4/55.4	**92.5/95.4**	**92.5/96.3**	39.3/60.3
	Salmon CCR2b	35.9/55.6	**94.5/97.4**	**93.3/95.9**	39.0/59.5
	Zebrafish CCR4Lc	28.5/51.0	**47.1/62.8**	**47.1/63.0**	33.3/55.5
	Medaka CCR4Lc	33.4/48.8	**46.2/64.9**	**47.8/65.8**	34.3/57.2
	Platyfish CCR4Lc	35.0/53.9	**54.1/73.0**	**53.0/71.9**	36.7/61.8
	Tetraodon CCR4Lc	31.7/49.0	**47.8/64.3**	**48.0/64.9**	34.1/55.5
CCR11	Salmon CCR5a	35.7/55.6	39.0/58.7	37.6/59.3	**87.7/91.9**
	Salmon CCR5b	35.0/53.7	39.6/58.6	37.9/58.9	**93.8/96.0**
	Zebrafish CCR11a	33.4/49.8	37.8/58.3	37.1/58.8	**45.8/65.4**
	Zebrafish CCR11b	35.3/52.5	38.3/59.3	37.9/59.3	**51.3/72.6**
	Zebrafish CCR11c**	27.8/43.7	29.0/42.9	26.9/41.9	**36.3/51.0**
	Zebrafish CCR11d	33.7/52.9	38.6/59.0	37.5/58.5	**49.4/72.0**
	Medaka CCR11a*	31.6/48.8	35.5/54.5	35.9/54.2	**46.5/63.2**
	Medaka CCR11b*	29.6/47.1	36.6/55.4	35.7/54.5	**43.9/59.8**
	Platyfish CCR11	33.6/54.4	34.8/57.2	35.2/57.2	**46.8/67.7**
Human CCRs	Human CCR1	***36.3/55.6***	***40.8/58.9***	***40.4/57.7***	***41.0/63.7***
	Human CCR2	***35.1/53.7***	***37.5/55.9***	***38.0/57.0***	***40.2/60.4***
	Human CCR3	***35.7/54.9***	***41.3/58.6***	***40.2/59.2***	***42.3/61.1***
	Human CCR4	***37.0/53.4***	***41.5/59.4***	***41.3/60.3***	***42.5/62.5***
	Human CCR5	***32.6/52.2***	***39.2/58.8***	***38.9/58.8***	***43.3/66.3***
	Human CCR6	32.2/51.7	31.2/51.3	31.7/51.9	34.7/56.1
	Human CCR7	29.7/50.2	33.4/54.0	33.2/54.0	30.1/52.9
	Human CCR8	***34.6/51.5***	***38.9/57.2***	***39.8/56.1***	***35.9/60.8***
	Human CCR9	30.3/50.5	32.2/52.6	32.3/52.3	33.5/53.4
	Human CCR10	26.9/41.9	32.7/49.7	33.7/51.7	30.5/49.4

Note

*N-terminal amino acid sequence is not complete.

**Zebrafish CCR11c has an unusually large C-terminal tail.




A. CCR4La
B. CCR11
C. CCR4Lc1
D. CCR4Lc2
Fold change
Stimulants
PolyIC
PGN
IL-1β
IL-6
IFNγ
4 h
8 h
24 h
0
1
2
3
4

```
                                              N-terminus
Trout-a        1 -------------------------MNITGYPVHTTAGGNTTTIPFSSVSVENGNSSSYSYENSSSYAY-----------
Salmon-a       1 -------------------------MNTTGYPVHTTEGGNTTTIPFSSVSVENGNSSSYAYENSYS--------------
Salmon-b       1 -------------------------MNITGYPVHTTA-------------------------------------------
Zebrafish-a    1 ---------------------------------------MSSTIALLSIAAQLLLTD---MEDSSIPDLNDHTLYI----
Zebrafish-b    1 MTTTTGFVRGRAVSEISNKTAEHLSSTLARQTKTHKRAKLSFSFPFLLLSNIRSTDS---MADGLLQPLSDTIEELNGWT
Medaka-a       1 ------------------------------------------------------------------------------NN
Medaka-b       1 --------------------------------------------------------------------------------
Platyfish-a    1 --------------------------------------------------------------------------------

                                                                           TM1            ICL1
Trout-a       45 ENSSSYAYENSSSYAYGTHFADT-FEVTTYDYGDYDDGVCKYKPYGANFLPVLYSLFFILGFLGNVLVLWVILQGVKLRN
Salmon-a      42 -------------YAYGTHFADA-FEVTTYDYSDYDDGICEYKPHGASFLPVLYSLFFILGFLGNVLVLWVILLGVKLCS
Salmon-b      13 ---------------STHFADA-FEVTTYDYNNYDDGVCKYNAHGASFLPVLYSLFFILGFLGNVLVLWVILLRVRLRS
Zebrafish-a   35 --SNVNGPVTDQPTTPVLMITDYSYDDYYNSV-DPDSLPCVYPAHGASILPVLYSLFFVLGFLGNTLVIRLVLKS--LRS
Zebrafish-b   78 KEASPGHPDRNQPLHHLLNLSQQFG--TNSSSSLQNYINYNYS-HGASILPVLYSLFFVVGFLGNALVIWVVLMGVKLRS
Medaka-a       3 NFPSFVFLFKSSSGHPTTTFSDDATTEYDYDYF-LQFETCYYEKLGARFIPAMYSMFFLLGLLGNSLVIWVVVCGARLRS
Medaka-b       1 --ISFTVDDNVLFYLPKL------------PFS-ANSQIAFKLNLSVMVDPGLYIMFFLLGLLGNSLVIWVVVCGARLRS
Platyfish-a    1 MNSTESDLFTSDGYNSSMPFTDGTTIEYFYPGDDEDYQTCYYVRHGAYFLPPLYAIFFLLGLLGNSLVIWIACGVRLRS

                           TM2                ECL1             TM3                   ICL2
Trout-a      124 MTDVCLLNLALADLLLVCTLPFLAHHATDQWVFGDVMCKVVLGAYHIGFYSGIFFITLMSVDRYLAIVHAVYAMRARTRK
Salmon-a     108 MTDVCLLNLALADLLLVCTLPFLAHHATDQWVFGDIMCKVVLSAYHIGFYSGIFFITLMSVDRYLAIVHAVYAMRARTRK
Salmon-b      76 MTDVCLLNLALADLLLVCSLPFLAHHARHQWVFGDVMCKVVLSAYHVGFYSGIFFITLMSVDRYLAIVHAVYAMRARTRK
Zebrafish-a  110 MTDICLLNLAIADLLLVSSLPFLAHYARDQWIFGGPMCTIVLSVYHIGFYSGIFFIVLMSVDQYLAVVHAVFALKVRTRT
Zebrafish-b  155 MTDICLLNLAIADLLLVSSLPFLAHYARDQWIFGDHMCTMVLSVYHIGFYSGIFFIVMMSVDRYLAVVHAVFALKVRTKT
Medaka-a      82 MTDMCLLNLAIADLLLVCSLPFLAYQARDQWLFGDAMCKIVLGVYHVVFYSGIFFICLMSIDRYLAIVHAVYAMKARTLF
Medaka-b      66 MTDMCLLNLAIADLLLVCSLPFLAYQARDQWLFGDAMCKIVLGVYNVVFYSGIFFICLMSIDRYLAIVHAVYAMKARTLF
Platyfish-a   81 MTDVCLVNLAIADLLMVCSLPFLAHQARDQWLFGDAMCKMVLGIYHIAFYCGIFFICLMSIDRYLAVVHAVYALKARTRT

                          TM4                  ECL2                         TM5
Trout-a      204 YGAIAAVVTWLAGFLASFPEALFLKVEKH-NEKENCRPVYDD----------RALVIFGLFKMNTLGLLIPLVIMGFCYT
Salmon-a     188 YGAIAAVVTWLAGFLASFPEALFLKVEKH-NEKENCRPVYDG----------HAWGIFGLFKMNTLGLLIPLVIMGFCYT
Salmon-b     156 YGAIAAVVTWLAGFLASFPEALFLKVEKN-NEKENCRPVYDG----------HSWGIFALFKRIIFGLLIPLIIMGFCYT
Zebrafish-a  190 YGFLASLVIWVAAVAASFPELIYIDTTDI-NNQTLCTSYPTTD-----QSSYHDSKTNGIFKMNIIGLIIPLSVIGFCYS
Zebrafish-b  235 YGILASLVIWVAAVTASFPELIHLKTTVT-NNQTLCASYPTTD-----QWSYHDSKTAGIFKMNVIGLILPLSVIGFCYS
Medaka-a     162 FGRIAAAVTWTAGFLASFPELIFIKQQTT-TNKTEKT-----------DSSSHFWTIFSIFKMNIMGLFIPLCIMTFCYS
Medaka-b     146 FGRIAAAVTWTAGFLASFPELIFIKQQEE-GDRHHCLSVYPDSGAGE-DDSSHFWRIFGIFKMNIMGLFVPLCIMVYCYS
Platyfish-a  161 CGIAAAAVTWLAGFLASFPDLIFLKTQTSVNGSQYCYPEYPQKSPNDVSGNLHFWSVFSLLKMNILGLFIPIFILGFCYS

                       ICL3               TM6                  ECL3                     TM7
Trout-a      273 QIVRRLLSRPSSKKQAIRLILIVVVVFFCCWTPYNMTSFFKALELSEVY-SSCESSKAIRLTLQITEAMAYSHSCLNPIL
Salmon-a     257 QIVKRLLSCPSSKKQTIRLILIVVVVFFCCWTPYNMTAFFKALELSEVY-SSCESSKAIRLTLQITEAMAYSHSWLNPIL
Salmon-b     225 QIVRRLLSAPSSKKQAIRLILIVVVVFFCCWTPYNMTAFFKALELSEVY-SSCESSKAIRLTLQITEAMAYSHSCLNPIL
Zebrafish-a  264 MILIKLLNVRSSRKQAIRLVVVVMVVFFCCWVPYNIAAFFKALELKRVIPHSCESSKAITLSLQITEAVAYSHSCINPFL
Zebrafish-b  309 MILIKLLTVRSSRRQAMRLVVVVMVVFFCCWAPYNIAAFFKALELKKVLTHSCESSKAITLSLQITEAVAYSHSCVNPFL
Medaka-a     230 RIIWKLLDSHSSRKQPIRLVLLVIAVFFCCWVPYNISSLFKGLELLQIY-MGCESSNSIRLALQVTEVIAYSHSCLNPIL
Medaka-b     224 QIIWKLLDSHSSRKQTIRVVILVIVVFFCCWVPYNITSMVKGLELLQIY-TGCESSKAITLALQVTEVIAYSHSCLNPIL
Platyfish-a  241 QIICRLLSTQSSKKQAIRLVVVVVAVFFCCWVPYNVVAFFKTLELLQVY-ATCESSKAVRLALQITEVIAYSHSCLNPIL

                                          C-terminus
Trout-a      352 YVFLGQKFRRHLIRLINKVPCRMCQFMKNYLPLDFRASRTGPVYSQTTSVDERSTAV----------
Salmon-a     336 YVFVGQKFRRPLIRLINKAPRRMCQFMKNYLPWDFRASRTGSVYSQTTSMDERSTAV----------
Salmon-b     304 YVFVGQKFRRPLIRLINKAPCRMCQFMKNYLPRDFRVSRTGSIYSQTTSMDERSTAVGTAT------
Zebrafish-a  344 YVFVGEKFRKHLFRLLNRTPFSRLQFMKSYVQT-T-----ASVYSQTTSLDARSSASV---------
Zebrafish-b  389 YVFVGEKFRKHLFRLLNRTPFSRLQFMKSYIIQAT-----GSVYSQTTSMDERSSVAV---------
Medaka-a     309 YVFVGEKFRRQLLRLISKTPCLLCQMIKVYIPQNR---IFGSTYSQNTSMEERSTAV----------
Medaka-b     303 YVFVGEKFRRQLLRLISKTPCLICQMIKVYIPQER---IFGSTYSQSTRMNERSTAKNISETLDQIT
Platyfish-a  320 YVFVGEKFRRHLVRLINRAPCSLCQVIKMYIPQDR---GT-SVYSQTTSVDERNTVV----------
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                  N-terminus                          TM1              ICL1        TM2
Trout-c1      1 MNTTQATSTDNYYGH---GNYESPCSTGT-SLTQGSNYQPILFYLVFTLGLTGNSLVLWVLLKYMKLKTMTDICLLNLAL
Trout-c2      1 MNTTEATSTDDYSGDDYNG---SPCSTGT-SLTQGSNYQPILFYLVFTLGLTGNSLVLWVLLKYMKLKTMTDICLLNLAL
Salmon-c1     1 MNTTEATSTDDYSGDNYYGNMISPCSTGT-SLTQGSNYQPILFYLVFTLGLTGNSLVLWVLLKYMKLKTMTDICLLNLAL
Salmon-c2     1 MNTTEATSTDDYYG------YDSPCSTGT-SLTQGSNYQPILFYLVFTLGMTGNSLVLWVLLKYMKLKTMTDICLLNLAL
Zebrafish     1 -----------------------MCNTE--EGSLNPHIKAAIFYIVFVLGLVGNIIVLWVLLKSMHVKNMTNLCLLNLAM
Medaka        1 -----------------MYDYTNNCDNSSADLQDGSKFFLVLYCIMFGFGLIANCTVLWVLIKHIKLRMMTDVLLLNLVL
Platyfish     1 MGNTMNVTENLT----EYYDYDSDCNETSWVFTSGSVLIPVLYYMLFCVGLIGNAIVLWVLLRYTKIRTMTDVFLLNLVL
Tetraodon     1 –MNTSGVNFSLY-PDIYDYDYNSTCDQDPNPVLSDTVL-RLFYCVVFGFGLIGNSTVIWVLLQFIKLKTMADVCLLNLAL

                        ECL1             TM3                     ICL2             TM4
Trout-c1     77 SDLLLALSLPLWAHHAQGHEFKGDSPCKIMAGAYQVGFYSSILFVTLMSVDRYLAIVHAVAAMRARTLRYGALASIIVWV
Trout-c2     77 SDLLLALSLPLWAYHAQGHEFEGNSPCKIMAGVYQVGFYSSILFVTLMSVDRYLAIVHAVAAMRARTLRYGALASIIVWV
Salmon-c1    80 SDLLLALSLPLWAYHAQGHEFEGDSPCKIMAGVYQVGFYSSILFVTLMSVDRYLAIVHAVTAMRARTLRYGTLASIIVWV
Salmon-c2    74 SDLLLALSLPLWAYHAQGHEFEGDSPCKIMAGVYQVGFYSSILFVTLMSVDRYLAIVHAVAAMRARTLRYGTLASIIVWV
Zebrafish    56 SDLLMVLSLPFWALYAQGHYLKTDAMCKAMAGAYQVGFYSGIFFVTLMSVDRYLVIVHAVAVLGAKMLRYGIVASVIIWM
Medaka       64 SDLLLAVSLPLWIVKSHN-----IGLCKLVTGIYQLGFYSGTFFVTVMSVDRYLAIVHAVAAIRARALRYGIIVSVVIWI
Platyfish    77 SDLLMAVSLPVWVHVAQS-----LPSCKLATGFYQLGFYSGTFFVTMMSVDRYLAIVHAVAAMRKRTLRYGLIASVIIWV
Tetraodon    78 SDLIFAVSLPLWAFNFQI-----LALCKVMTAIYQVGFYSGTLFVTLMSLDRYVAIVHAVSSMRARTLHRGIIASISIWA

                             ECL2                                       TM5           ICL3
Trout-c1    157 ASISAALPEAIFVAVVRENDENSGTSCQLIYPENTEKTWKLLRNFGENGVGLVLCLPIVVFCYICILTVLQRLRNSKKDR
Trout-c2    157 ASIGAALPEAIFAAEVWEDDEDSGSSCQRIYPENTEKTWKLLRNFGENGVGLVLCLPIVVFCYISILTVLQRLRNSKKDR
Salmon-c1   160 ASISAALPEAIFAAVVRENDENSGTSCQRIYPEDTEKTWKLLRNLGENGVGLLLCLPIMVFCYISILTVLQRLRNSKKDR
Salmon-c2   154 ASISAALPEAIFVAVVRENDESSGTSCQRIYPEDTEKTWKLLRNFGENGVGLLLCLPIMVFCYISILTVLQRLRNSKKDR
Zebrafish   136 VSIGAALPEVIFAEVVKDSE---SNSCQRHYPDESARKWKLFRNFGENAVGLFISLPIIAYCYLRVLMVVKKTKNSKKNR
Medaka      139 VSVIMAAPQVVFASLEKE-D-FDTSHCHPVYPEETVEFWKKLRNFSENTVGIFVCLPIMIFCYVKILLVLSKSRNSKKDK
Platyfish   152 ASVVLATPHVVFASLESL-D-NESFQCHPIYPEETESSWKMQRNFTENVVALFLCLPVIIFCYVNILVVVSKSRNSKRDK
Tetraodon   153 VSIIIAAPQVKYASLEIDPE-NNLSQCQPLYPEDSMEFWKMRRNLSENIVALFVCLPIMIFCYVKILIVLSKSPNSKKDK

                      TM6                    ECL3                       TM7
Trout-c1    237 AMKLIFAIVGVFVVSWVPYNVVVFLQTLQMF-DIGNSCEASTQLDTAMEVTETIALAHCCVNPVIYAFVGEKFRKCLGTV
Trout-c2    237 AMKLIFAIVGVFVVSWVPYNIVVFLQTLQMF-DIGNSCEASTQLDKAMEVTETVALAHCCVNPVIYAFVGEKFGKCLGTV
Salmon-c1   240 AMKLIFAIVGVFVVSWVPYNVVVFLRTLQMF-DIGNSCEASTQVDRAMEVTETIALAHCCVNPVIYAFVGEKFRKCLGTA
Salmon-c2   234 AMKLIFAIVGVFVVSWVPYNVVVFLQTLQMF-DIGNSCEASTQLDAAMEVTETIALAHCCVNPVIYAFVGEKFRKCLGTV
Zebrafish   213 AIKLILGIVIMFVVFWVPYNVVVFLKTLHEF-DMLTSCEPYKIINMAMDVTETIALTHCCVNPFIYAFVGEKFRKYLASA
Medaka      217 VVKLIFTVVCVFVACWVPYNILVFLQTLEQL-EILDDCQLSNNINKAMHFTEIMALSHCCLNPIIYAFIGEKFRKSLGNA
Platyfish   230 AIKLIFIVVCLFVLCWVPYNIVVFLKTLQPMSEYLNNCESSQAIDAAMVFAEIIALSHCCVNPVIYAFVGEKFRKTLAKV
Tetraodon   232 AIRLIFAIVCVFVMCWVPYNVTVFLQTLQIF-EILVSCSASRSISLTMSFAEIIALSHCCLNPIIYAFAGEKFRKSL---

                       C-terminus
Trout-c1    316 LSRYPLCKKLGKHAMVSSRGSENETSNTPV----
Trout-c2    316 LSRYPLCKKLSKHAMVSSRGSENETSNTAV----
Salmon-c1   319 LSRYPLCKKLSKHAMVSSRGSENETSNTPV----
Salmon-c2   313 LSRYPLCKKLSKHAMVSSRGSENETSNTPV----
Zebrafish   292 FSKYLRCLKTYQSTPSQSRISENDTSNTAIFSTS
Medaka      296 L-KNHFC---------------------------
Platyfish   310 LSKYFRWNYQS-----TSQTTDNETSNTPVRSDY
Tetraodon       ----------------------------------
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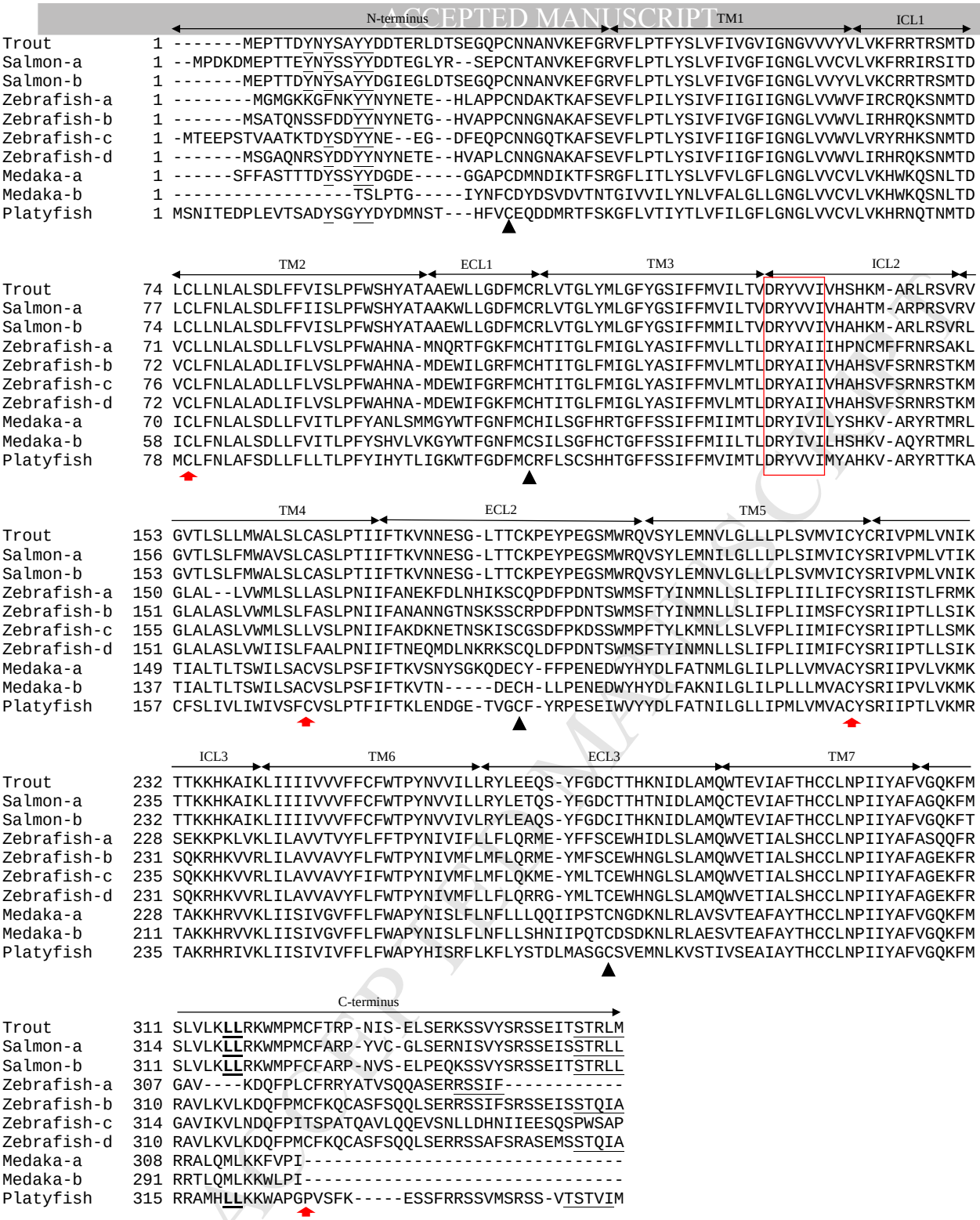
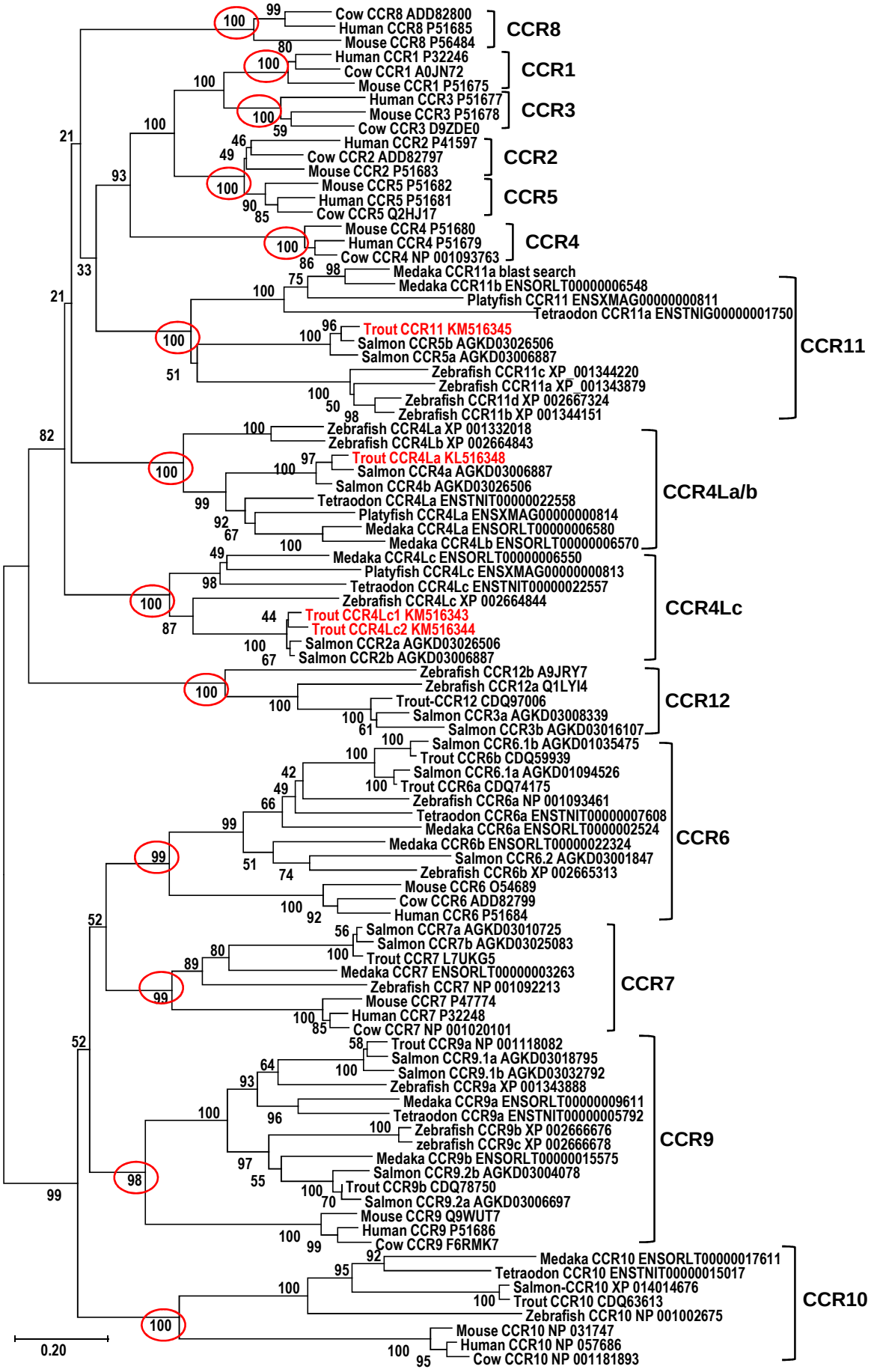
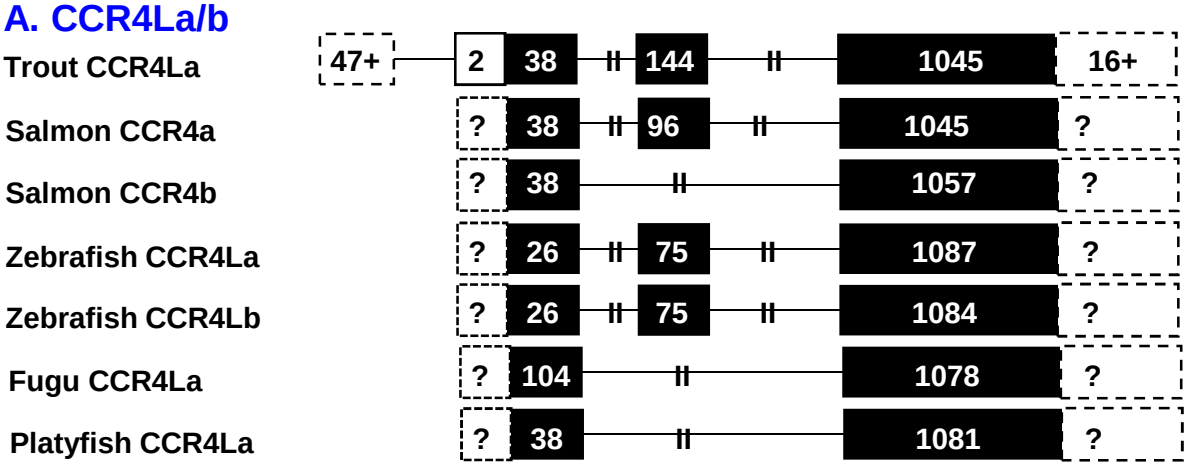
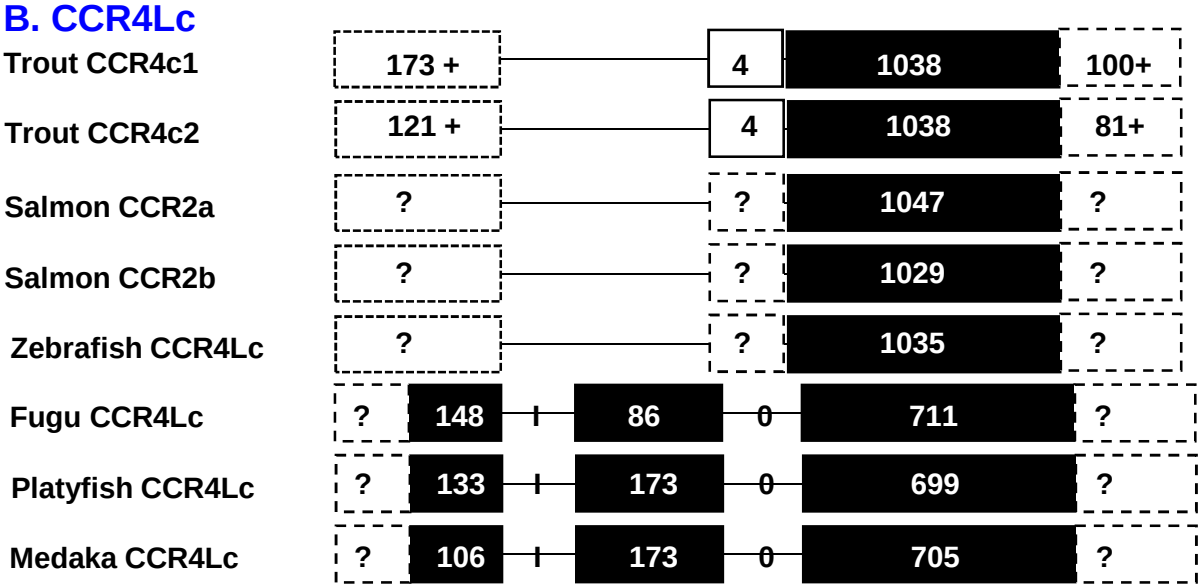




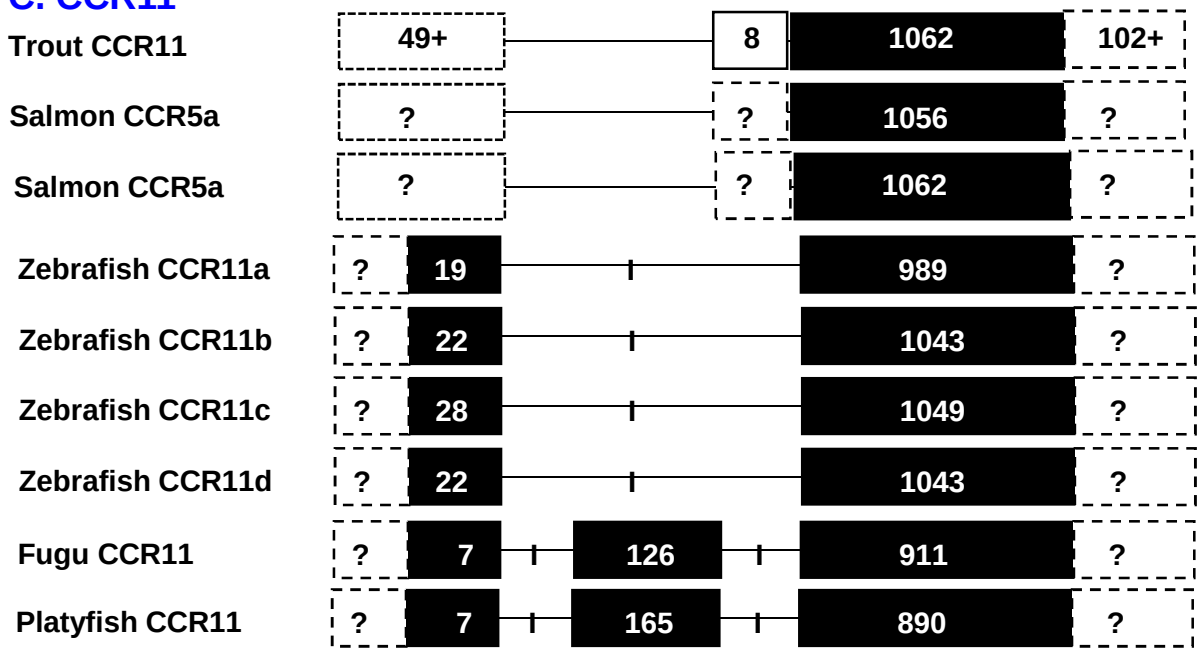
A. CCR4La/b

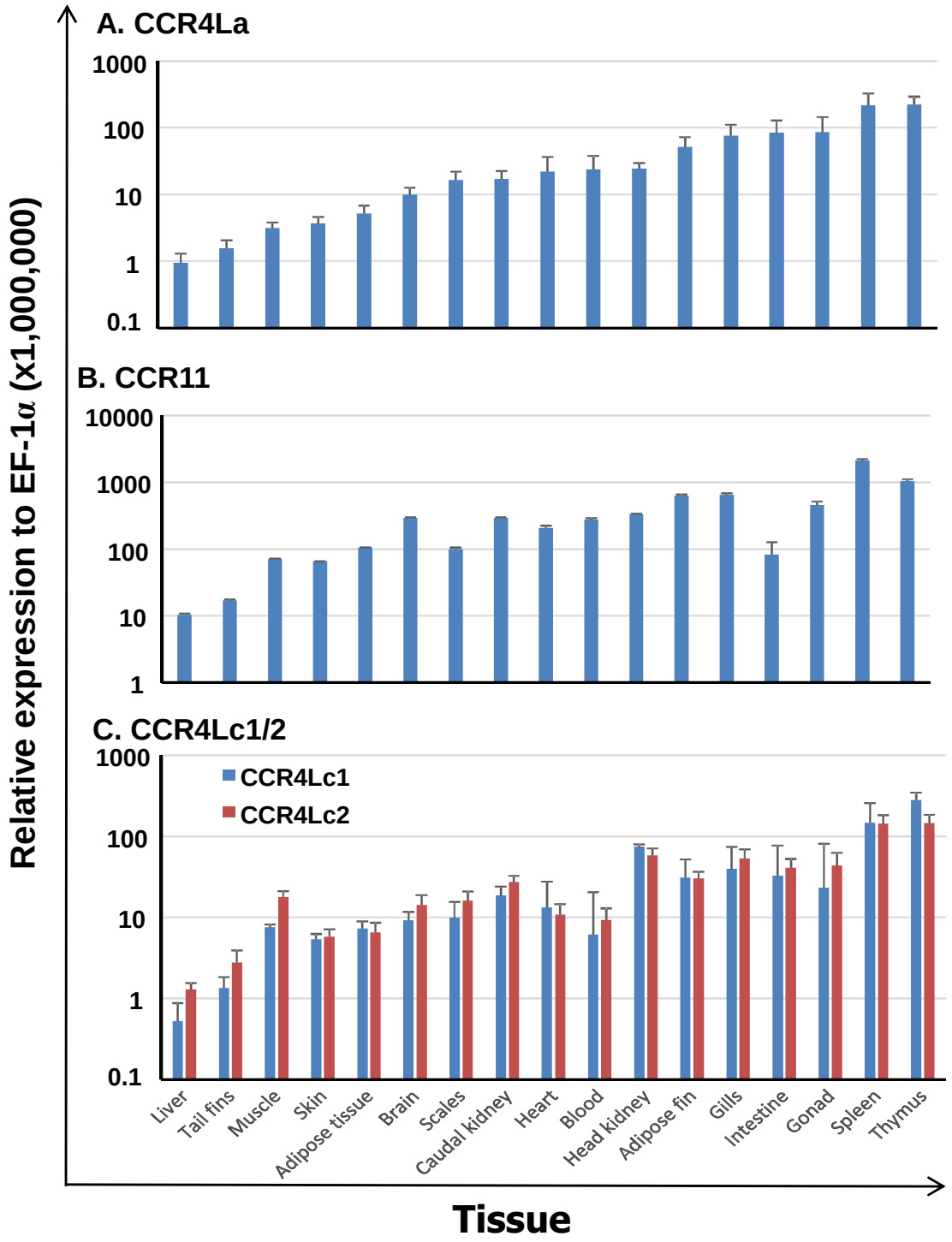


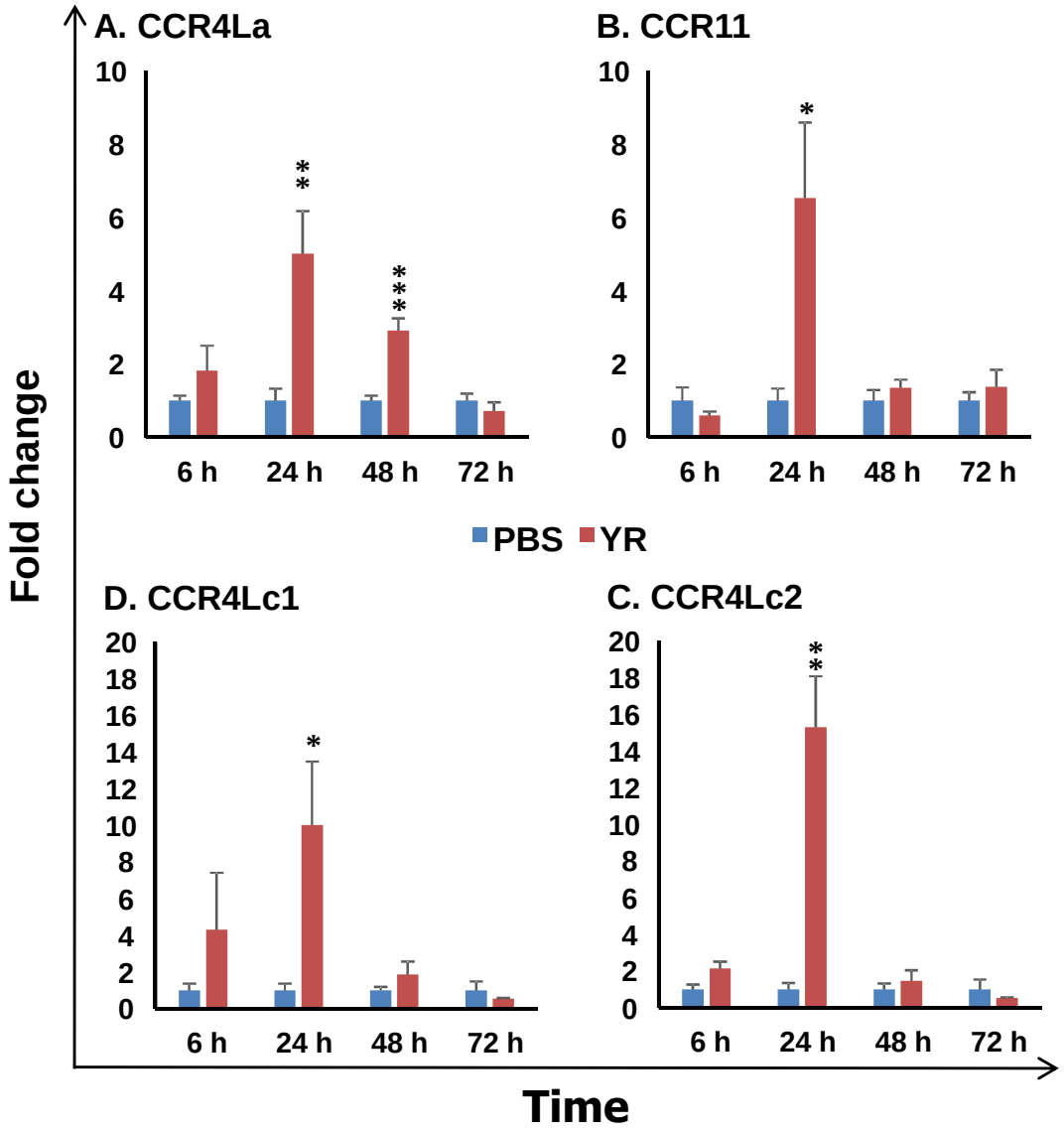
B. CCR4Lc



C. CCR11




A. CCR4La
1000
100
10
1
0.1
B. CCR11
10000
1000
100
10
1
C. CCR4Lc1/2
1000
100
10
1
0.1
CCR4Lc1
CCR4Lc2
Liver
Tail fins
Muscle
Skin
Adipose tissue
Brain
Scales
Caudal kidney
Heart
Blood
Head kidney
Adipose fin
Gills
Intestine
Gonad
Spleen
Thymus
Relative expression to EF-1α (x1,000,000)
Tissue


A. CCR4La
B. CCR11
D. CCR4Lc1
C. CCR4Lc2
Fold change
Time
PBS
YR
6 h
24 h
48 h
72 h
0
2
4
6
8
10
12
14
16
18
20
*
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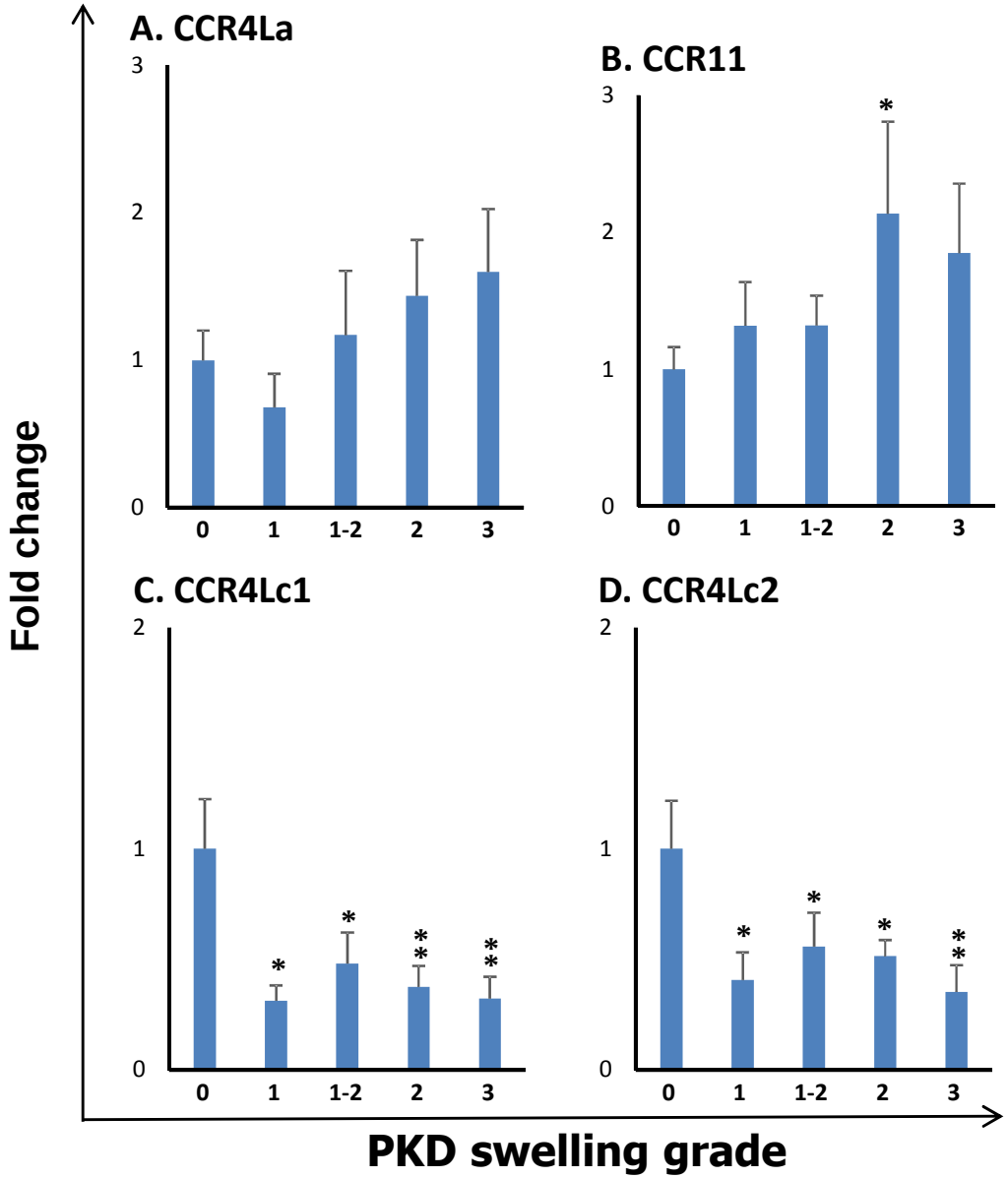
A. CCR4La
B. CCR11
C. CCR4Lc1
D. CCR4Lc2
Fold change
PKD swelling grade
0
1
1-2
2
3

Highlights

- Four fish-specific CCRs, namely CCR4La, CCR4Lc1, CCR4Lc2 and CCR11, have been characterized in rainbow trout.
- The gene organization of fish-specific CCRs has diversified in different fish species.
- Fish-specific CCRs are highly expressed in immune tissues, thymus, spleen, gills and HK.
- The CCR expression can be modulated *in vivo* by bacterial and parasitic infection.
- The CCR expression can be modulated *in vitro* by PAMPs and pro-inflammatory cytokines.