Th17 master transcription factors RORα and RORγ regulate the expression of IL-17C, IL-17D and IL-17F in *Cynoglossus semilaevis*

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Abstract

The RAR-related orphan receptors (RORs) are members of the nuclear receptor family of intracellular transcription factors. In this study, we examined the regulatory properties of RORα (CsRORα) and RORγ (CsRORγ) in tongue sole (*Cynoglossus semilaevis*). CsRORα and CsRORγ expression was detected in major lymphoid organs and altered to significant extents after bacterial and viral infection. CsRORα enhanced the activities of CsIL-17C, CsIL-17D, and CsIL-17F promoters, which contain CsRORα and CsRORγ binding sites. CsRORγ also upregulated the promoter activities of CsIL-17D and CsIL-17F but not CsIL-17C. CsRORα and CsRORγ proteins were detected in the nucleus, and overexpression of CsRORα in tongue sole significantly increased the expression of CsIL-17C, CsIL-17D, and CsIL-17F, whereas overexpression of CsRORγ significantly increased the expression of CsIL-17C and CsIL-17F, but no CsIL-17D,. These results indicate that RORα and RORγ in teleost regulate the expression of IL-17 members in different manners.

Key words: RORα; RORγ; IL-17; promoter activity; *Cynoglossus semilaevis*

1. Introduction

The RAR-related orphan receptors (RORs) are members of the nuclear receptor family of intracellular transcription factors (Giguère et al., 1994; Hirose et al., 1994). There are three known forms of ROR: RORα, β, and γ, each is encoded by a separate gene (*RORA*, *RORB*, and *RORC* respectively). RORα is expressed in a variety of cell types and is involved in regulation of different inflammatory responses and lymphocyte development (Dussault et al., 1998). RORγ and its spliceosome RORγt differ in their N-terminal sequences encoded by alternative 5' exons within the *RORC* locus (Eberl et al., 2003); they are the key transcription factors that orchestrate the differentiation of T-helper (Th) 17-cell lineage. Recently, it is reported that the closely related RORα, RORγ and RORγt work in concert to regulate the expression of IL-17A and IL-17F, and that perturbation of these transcription factors could be a viable strategy for treating autoimmune pathologies linked to Th17 effector function in mammals. (Yang et al., 2008; Ruan et al., 2011).

In the immune system, naive $CD4^+$ T cells can be differentiated into Th1/Th2/Th17/Treg cells upon interaction with antigen presenting cells (APCs) depending on the local cytokine milieu. The differentiation requires the precise action of lineage-determining transcription factors T-box expressed in T cells (T-bet), GATA binding protein 3 (GATA-3), RORs (RORα, RORγ and RORγt), and forkhead box P3 (Foxp3) (Martins et al., 2005; Hwang et al., 2005; Schulz et al., 2008; Zhou et al., 2008). Th1 cells may secrete effector cytokines IL-12 and IFN-γ; Th2 cells secrete IL-4, IL-5 and IL-13; Th17 cells secrete IL-17A and IL-17F; Treg cells secrete IL-10 and TGF-β (Bevan et al., 2004; Harrington et al., 2005; Steinman et al., 2007; Stockinger et al., 2007; Zhu et al., 2008; Swain et al., 2012). In teleosts, RORα, RORγ, T-bet,

GATA-3, and the cytokines related to Th-cells have been identified in some species (Flores et al., 2007; Castro et al., 2011; Du et al., 2012; Monte et al., 2012; Zhu et al., 2012). However, unlike mammals, little is known about $CD4^+$ T-cell diversity and the nature of the initial signals that determine the T-cell response pattern in teleosts.

The IL-17 family is a subset of cytokines consisting of IL-17A (CTLA8), IL-17B, IL-17C, IL-17D, IL-17E (IL-25), and IL-17F (Gu et al., 2013). In teleost, IL-17 members have been identified in several fish species and are reported to play crucial roles in host defense against microbial organisms (Gunimaladevi et al., 2006; Wang et al., 2014; Korenaga et al., 2010; Kono et al., 2011). It has been reported that RORα and RORγ regulate the expression of IL-17A and IL-17F in mammals (Yang et al., 2008), yet no reports on lower vertebrates have been documented. Moreover, the effect of RORα and RORγ on the expression of other IL-17 family members also remains unknown in teleost species.

Half-smooth tongue sole *Cynoglossus semilaevis* is an economically favorable teleost species farmed in China. Genomic sequencing has revealed the existence of RORα (CsRORα), RORγ (CsRORγ) genes as well as three IL-17 members (CsIL-17C, CsIL-17D, and CsIL-17F) in this species (Chen et al., 2014). In this study, we examined the structure and regulatory property of CsRORα and CsRORγ. In addition, the effect of CsRORα and CsRORγ on the expression of CsIL-17C, CsIL-17D, and CsIL-17F was also analyzed.

2. Materials and methods

2.1 Fish

Half-smooth tongue sole were purchased from a commercial fish farm in Shandong Province, China and were maintained at 20^{o}C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before the experimental started. Six fish were randomly sampled for the examination of the presence of bacteria and megalocytivirus in blood, liver, kidney, and spleen as reported previously (Li et al., 2015a). No bacteria or virus were detected from the examined fish. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as reported previously (Zhang et al., 2015).

2.2. Sequence analysis

The cDNA and amino acid sequences of tongue sole RORαand RORγ (GenBank accession numbers. XP_008310012.1 and XP_008321277.1) were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI), the Expert Protein Analysis System, the ExPASy Molecular Biology server (http://us.expasy.org) and Pfamp (Combet et al., 2000). Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. Amino acid identity and similarity were calculated with the Matrix Global Alignment Tool (MatGAT) program v 2.0 (Campanella et al., 2003) using default parameters. A multiple sequence alignment was created using CLUSTALW, and MEGA version 4.1 (Tamura et al., 2007) was used to assess the similarities among the aligned sequences. A phylogenetic tree based was constructed using the neighbor-joining (NJ) algorithm, and the reliability of the branching was tested using bootstrap re-samplings with 1,000 pseudo-replicates. Identification of transcription factor-binding motifs was performed with TRANSFAC (Biobase International) (Heinemeyer et al., 1998) and MatInspector version

6.2 (Cartharius et al., 2005).

2.3 Quantitative real time reverse transcription-PCR (qRT-PCR) analysis of CsRORα and CsRORγ expression under normal physiological conditions

Spleen, heart, gill, brain, kidney, liver, muscle, and gut were obtained aseptically from five tongue sole (average 14.3 g) and used for total RNA extraction with the RNAprep Tissue Kit (Omega Bio-Tek, Norcross, GA USA). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed using the primers CsRORaRTF/CsRORaRTR, CsRORrRTF/CsRORrRTR (Table 1) and carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Zheng and sun, 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression levels of *CsRORα* and *CsRORγ* were analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with ACTB as the control. All data are given in terms of mRNA levels relative to that of beta actin (ACTB) as reported previously (Long et al., 2014) and expressed as means plus or minus standard errors of the means (SEM). The assay was performed three times.

2.4 qRT-PCR analysis of gene expression during pathogen infection

Bacterial infection was performed as reported previously (Dang et al., 2011). The fish bacterial pathogen *Vibrio harveyi* (Sun et al., 2009) was cultured in Luria-Bertani broth (LB) medium at 28^{o}C to an

OD_{600} of 0.8. The cells were washed with PBS and re-suspended in PBS to yield 1×10^6 colony forming units (CFU)/ml. The fish viral pathogen megalocytivirus RBIV-C1 (Zhang et al., 2014a) was suspended in PBS to 5×10^4 copies/ml. Tongue sole were divided randomly into three groups and injected intraperitoneally (i.p.) with 100 μl *V. harveyi*, megalocytivirus, or PBS. Fish (five at each time point) were euthanized at 6 h, 12 h, 24 h, and 48 h post-bacterial infection and at 1 d, 3 d, 5 d, and 7 d post-viral infection. Tissues were collected under aseptic conditions. Total RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above. 60S ribosomal protein L18a (for spleen) and ACTB (for kidney) were used as the internal controls for bacterial infection, and ACTB (for both spleen and kidney) was used as the internal control for viral infection (Long et al., 2014). The assay was performed three times.

2.5 Plasmid construction

To construct pCsRORα-RFP and pCsRORγ-RFP, which express CsRORα-TagRFP and CsRORγ-TagRFP fusion proteins respectively, the coding sequences of CsRORα and CsRORγ were amplified with primers CsRORaEcoRIF/CsRORaEcoRIR and CsRORrHindIIIF/CsRORrHindIIIR (Table 1), respectively, and the PCR products were inserted into pTagRFP-N (Evrogen, Moscow, Russia) at the EcoRI or HindIII site. To construct pCsRORα and pCsRORγ, which express His-tagged CsRORα and CsRORγ respectively, the coding sequences of CsRORα and CsRORγ were amplified with primers CsRORaF1/CsRORaR1 and CsRORrF1/CsRORrR1 respectively, and the PCR products were inserted into pCN3 (Li et al., 2015b) at the EcoRV site.

Genomic DNA was isolated from tongue sole spleen with the TIANNamp Marine Animals DNA kit

(Tiangen, Beijing, China). About 1200 bp of the 5' flanking region sequences of the CsIL-17C, CsIL-17D and CsIL-17F genes were obtained from the genomic DNA by PCR using the primers CsIL17CproF/CsIL17CproR, CsIL17DproF/CsIL17DproR, and CsIL17FproF/CsIL17FproR (Table 1), respectively, and the PCR products were inserted into pMetLuc-2 (Clontech, Mountain View, CA, USA) at the HindIII site. All plasmid DNA constructs were isolated using Endo-Free plasmid maxi kit (Omega Bio-Tek, Norcross, GA, USA).

2.6 Cell culture, transfection and reporter activity assay

The cell line FG-9307 was derived from the gill tissue of flounder *Paralichthys olivaceus*. The cells were maintained in Eagle's minimal essential medium (MEM) (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) at 22°C. Transfection was performed as reported previously (Zhang et al., 2014b). Briefly, FG cells were distributed into 24-well culture plates (2×10^5 cells/well) in MEM medium without FBS. Transfection of the cells with pCsRORα-RFP, pCsRORγ-RFP and pTagRFP-N was performed with Lipofectamine LTX and PLUS^{TM} (Invitrogen, Carlsbad, CA, USA) according to the instructions given by the manufacturer. After transfection for 24 h, the medium was removed and replaced with new medium containing 500 ng/ml lipopolysaccharides (LPS) (Sigma, St Louis, MO, USA). After incubation at 22^{o}C for 6 h, the cells were fixed with 4% formaldehyde for 0.5 h, and 4, 6-diamino-2-phenyl indole (DAPI) (Invitrogen) was used for nucleic acid staining according to manufacturer's instructions. The cells were observed with fluorescence microscope (Carl Zeiss Imager A2, Jena, Germany).

For reporter activity assay, the FG cells were re-suspended in MEM medium and seeded in 24-well

culture plates (2×10^5 cells/well). Transfection of the cells with different proportions of pCsRORα, pCsRORγ, pCN3 and reporter vectors was performed with Lipofectamine LTX and PLUS™ according to manufacturer's instructions. The pSEAP2 (Clontech, Mountain View, CA, USA) control vector for normalizing transfection efficiency was included in all assays. After transfection for 48 h, the culture mediums of the transfectants were analyzed for luciferase activity and SEAP activity using the Luciferase Assay Kit (Clontech) and the Great EscAPe™ SEAP Chemiluminescence Detection Kit (Clontech), respectively.

2.7 Overexpression of CsRORα and CsRORγ in vivo

Overexpression of CsRORα and CsRORγ *in vivo* was performed as reported previously (Zhou et al., 2014). Briefly, pCsRORα, pCsRORγ, and the control plasmid pCN3 were diluted in PBS to 200 μg/ml. Tongue sole were divided randomly into four groups and injected intramuscularly with 100 μl of pCsRORα, pCsRORγ, pCN3, or PBS. Tissues were taken from 5 fish at 5 days post-plasmid administration and used for examination of the presence of plasmids and the mRNA expression of *RORα, RORγ, IL-17C, IL-17D IL-17F, T-bet* and *GATA-3* (GenBank accession numbers: XP_008310012.1, XP_008321277.1, XP_008309677.1, XP_008326667.1, XP_008335392.1, XP_008312713.1, and XP_008314324.1 respectively). PCR detection of pCsRORα, pCsRORγ, and pCN3 was performed with the primers pF1/pR1 (Table 1). To examine expression of plasmid-derived *CsRORα* and *CsRORγ*, IL-17C, IL-17D IL-17F, T-bet and GATA-3, total RNA was extracted from the tissues as described above and used for RT-PCR with the primer pairs shown in Table 1. The experiment was repeated three times.

2.8. Statistical analysis

All statistical analyses were performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA), and statistical significance was defined as $P < 0.05$.

3. Results

3.1 Nucleotide and deduced amino acid sequences of CsRORα and CsRORγ

CsRORα and CsRORγ are composed of 468 amino acids (molecular mass of 53.0 kDa) and 469 amino acids (54.0 kDa), respectively. Secondary structure analysis using SOPMA software indicated that CsRORα and CsRORγ were comprised of α-helixes (47.44% and 44.56%) and random coils (32.26% and 29.0%) connected by extended strands (13.03% and 17.91%) and β-turns (7.26% and 8.53%). According to BLAST search, CsRORα and CsRORγ share 91-99.6% and 45.3-72.4%, respectively, overall sequence identities with the equivalent genes of other teleost species and humans (Fig. 1A and Fig. 2A). Sequence alignment revealed the presence of a conserved ZnF_C4 (C4 zinc finger in nuclear hormone receptors) and HOLI (Ligand binding domain of hormones) domains in CsRORα and CsRORγ (Fig. 1B and Fig. 2B). A phylogenetic tree based on multiple alignments of the ROR family genes from various vertebrates showed that the lineage sorting of the clusters corresponded to the sequence identities of the respective genes of ROR family. Three distinct branches were generated, namely RORα, RORβ, and RORγ. CsRORα and CsRORγ fell into the RORα and RORγ clades, respectively (Fig. 3).

3.2 Distribution of CsRORα and CsRORγ in fish tissues under normal conditions

As shown in Fig. 4, the *CsRORα* and *CsRORγ* genes were expressed in all the tissues analyzed. *CsRORα* was expressed, in increasing order, in the spleen, kidney, blood, liver, gill, heart, intestine, muscle, and brain (Fig. 4A), while *CsRORγ* was expressed, in increasing order, in the spleen, blood, liver, muscle, brain, intestine, kidney, gill, and heart (Fig. 4B).

3.3 Regulation of the expression of CsRORα and CsRORγ by bacterial and viral infection

The expression levels of *CsRORα* and *CsRORγ* following bacterial and megalocytivirus infection were examined in the spleen and kidney. When the fish were infected with the bacterial pathogen *V. harveyi*, the mRNA transcript of *CsRORα* was significantly upregulated in spleen and the maximum fold increase (5.27-fold) occurred at 12 h (Fig. 5A). In kidney, *CsRORα* expression was significantly increased at 12 h and 24 h post-infection, with a maximum of 7.87-fold increase at 24 h (Fig. 5B). The mRNA level of *CsRORγ* was significantly increased in spleen (7.00-fold) and kidney (17.29-fold) at 6 h post-infection (Fig. 5E and Fig. 5F). When the fish were infected with the viral pathogen megalocytivirus, the *CsRORα* expression was significantly upregulated in spleen (3.66-fold) and kidney (3.29-fold) at 7 d (Fig. 5C and Fig. 5D). For *CsRORγ*, the mRNA transcript in spleen was significantly decreased at 3 d (0.17-fold) and 5 d (0.12-fold) post-infection compared to the control (Fig. 5G); the same trend was observed in the kidney at 3 d (0.43-fold) and 7d (0.25-fold) post-infection (Fig. 5H).

3.4 Intracellular localization of CsRORα and CsRORγ

To examine the subcellular localization of CsRORα and CsRORγ, FG cells were transfected with pCsRORα-RFP and pCsRORγ-RFP, which express CsRORα and CsRORγ respectively, fused to red fluorescent protein (RFP). Microscopy showed that in the transfcetants, CsRORα and CsRORγ were observed to overlap with the nuclei (blue), whereas in the cells transfected with the control vector (pTagRFP-N), RFP was found to be expressed evenly in the cytoplasm (Fig. 6).

3.5 Effect of CsRORα and CsRORγ on the promoter activity of IL-17 cytokines

In a previous study, the CsIL-17C, CsIL-17D and CsIL-17F promoter reporter plasmids pLucCsIL-17C, pLucCsIL-17D, and pLucCsIL-17F, respectively, were created (Chi et al., manuscript submitted), in which the promoter activities were reflected by the activities of the luciferase reporter. The promoters contain ~1.2 kb 5'-flanking regions (5'-FRs) of CsIL-17C, CsIL-17D and CsIL-17F, which exhibit putative RORα and RORγ binding sites (ROREs) (Fig. S1). In the current study, we examined the potential effect of CsRORα and CsRORγ on the activity of the CsIL-17C, CsIL-17D and CsIL-17F promoters. For this purpose, FG cells were transfected with pCsRORα and pCsRORγ plus pLucCsIL-17C, pLucCsIL-17D, or pLucCsIL-17F, and the luciferase activities were determined. The results showed that in plucCsIL-17C transfectants, luciferase activity was significantly increased in the presence of pCsRORα (3.19-fold), but not in the presence of pCsRORγ (Fig. 7A). In plucCsIL-17D transfectants, luciferase activity was significantly increased in the presence of pCsRORα and pCsRORγ (3.64- and 2.58-fold respectively) (Fig. 7B). In the plucCsIL-17F transfectants, luciferase activity was also significantly

increased in the presence of pCsRORα and pCsRORγ (2.85- and 3.31-fold respectively) (Fig. 7C).

3.6 Biological effect of CsRORα and CsRORγ in tongue sole

In order to examine the *in vivo* biological effect of the CsRORα and CsRORγ, tongue sole were administered with pCsRORα, pCsRORγ, or the control vector pCN3. At 5 days post-plasmid administration, the presence of the plasmids and expression of the plasmid-derived *CsRORα* and *CsRORγ* were examined by PCR and RT-PCR respectively (Fig. S2). By PCR, pCsRORα, pCsRORγ, and pCN3 were all detected in the muscle, spleen, and kidney. RT-PCR showed that the expression of pCsRORα- and pCsRORγ-derived *CsRORα* and *CsRORγ* was found in the fish administered with pCsRORα and pCsRORγ respectively, but not in the control fish (Fig. S2).

The expression of IL-17C, IL-17D, IL-17F, T-bet, and GATA-3 genes in the kidney of pCsRORα- and pCsRORγ-administered fish was determined by qRT-PCR at 5 d post-plasmid injection. The results showed that compared to fish administered with the control plasmid pCN3, fish administered with pCsRORα exhibited significantly upregulated expression of IL-17C, IL-17D and IL-17F, significantly decreased expression of T-bet, and no significant change in the expression of GATA-3. pCsRORγ-injected fish exhibited significantly increased expression of IL-17C and IL-17F, significantly decreased expression of T-bet and GATA-3, and no significant change in the expression of IL-17D (Fig. 8).

4 Discussion

In this report, we studied the gene structure, expression profile, and transcriptional property of

CsRORα and CsRORγ from tongue sole. Multiple alignment analysis revealed that CsRORα and CsRORγ shared high degrees of identities with homologues of other teleost species and humans, suggesting that CsRORα and CsRORγ are highly conserved among lower and higher vertebrates, which is consistent with their fundamental roles in cells (Flores et al., 2007; Monte et al., 2012; Du et al., 2012). Both CsRORα and CsRORγ contain ZnF_C4 and HOLI domains, the former is a small DNA-binding peptide motif that can be used as modular building blocks for the construction of larger protein domains that recognize and bind to specific DNA sequences (Klug et al., 1999). HOLI is a ligand-binding domain that acts in response to ligand binding, causing a conformational change in the receptor to induce a response, thereby acting as a molecular switch to turn on transcriptional activity (Bledsoe et al., 2004). The presence of these structural features in CsRORα and CsRORγ suggests a conserved operational mechanism of RORα and RORγ in lower and higher vertebrate species.

In mammals, RORα and RORγ exhibit distinct tissue-specific expressions. RORα is expressed in a variety of tissues, including testis, kidney, liver, and particularly brain (Becker-Andre et al., 1993; Carlberg et al., 1994; Hamilton et al., 1996; Dussault et al., 1998). RORγ has been found to be highly expressed in the liver, skeletal muscle, and kidney of mammalian species (Eberl and Littman, 2003; Eberl and Littman, 2004; Jetten, 2004; Jetten and Joo, 2006). Similar to mammals, in tongue sole we found that the expression of CsRORα and CsRORγ occurred in multiple tissues. CsRORα was highly expressed in intestine, muscle and brain, while CsRORγ was highly expressed in kidney, gill, and heart. This is in consistence with the reports on grass carp and zebrafish (Du et al., 2012; Monte et al., 2012). It is known that the expression of RORα and RORγ in lymphoid organs is stimulated after bacterial infection or LPS stimulation (Du et al., 2012; Monte et al., 2012). Similarly, we found that the expression of CsRORα and CsRORγ was upregulated by experimental infection with the bacterial pathogen *V. harveyi*. However, after viral infection,

CsRORγ expression was inhibited, while CsRORα expression was enhanced. These results indicate that CsRORα and CsRORγ responded differently to different types of pathogens.

Previous studies have shown that RORs binds to a consensus core sequence and regulates the expression of IL-17 (Giguère et al., 1994; Carlberg et al., 1994; Medvedey et al., 1996; Ruan et al., 2011). In Atlantic salmon, the 5' flanking region of IL-17D contains some putative ROREs (Kumari et al., 2009). Likewise, we found that multiple ROREs are present in the 5'-flanking regions of the CsIL-17C, CsIL-17D and CsIL-17F genes. In mammals, IL-17C promotes Th17 cell responses and autoimmune disease via the IL-17 receptor E (Chang et al., 2011); IL-17F plays an important role in antitumor immunity in Th17 cell-dependent autoimmune disease, and the regulation of RORα and RORγ on IL-17F has been widely reported (Ivanov et al., 2006; Yang et al., 2008). In our study, co-transcriptional activity analysis showed that CsRORα increased the promoter activities of CsIL-17C, CsIL-17D and CsIL-17F, and that CsRORγ also upregulated the promoter activities of CsIL-17D and CsIL-17F but had no effect on CsIL-17C promoter activity. These results suggest that CsRORα and CsRORγ had different regulatory effects on IL-17 members. In agrement with these observations, subcellular distribution analysis showed that in FG cells transfected with pCsRORα-RFP and pCsRORγ-RFP, CsRORα and CsRORγ were detected in the nucleus, suggesting that CsRORα and CsRORγ were localized in the nucleus.

Transcription factors play a critical role during the differentiation of Th cells that may result in Th cell polarization. RORα overexpression has been shown to reduce the frequency of IFN-γ-producing cells (Th1) and IL-5-producing cells (Th2) in mice (Yang et al., 2008). RORγ may control Th1/Th2 cytokine balance during adaptive immune response, and it has been reported that IFN-γ production was markedly increased in the splenocytes of RORγ-deficient mice (Tilley et al., 2007). In our study, the expression levels of IL-17C, IL-17D and IL-17F in tongue sole increased after CsRORα overexpression, which is in line with

the *in vitro* observation that CsRORα overexpression upregulated the promoter activities of these IL-17 members. Fish injected with pCsRORγ exhibited upregulation of IL-17C and IL-17F, but not IL-17D, expression. These results indicate that the expressions of these three IL-17 members were regulated differently by CsRORα and CsRORγ overexpression *in vivo*. In mammals, T-bet and GATA-3 are master transcription factors involved in the process of Th1 and Th2 polarization respectively (Szabo et al., 2003; Ansel et al., 2006). In our study, the expression of T-bet was suppressed after CsRORα and CsRORγ overexpression. The expression of GATA-3 was also inhibited after CsRORγ overexpression but not after CsRORα overexpression. These results indicate a certain balance of the expressions of transcription factors, which could be the case if there exist in tongue sole Th1/Th2/Th17-like cells as reported in some mammals (Tilley et al., 2007; Yang et al., 2008). However, functional proofs must be presented before stating that fish possess mammalian-like Th cells.

In summary, we have compared the expression and regulatory functions of RORα and RORγ in tongue sole. We found for the first time that teleost RORα and RORγ are involved in the regulation of the IL-17C, IL-17D and IL-17F expression, and that the regulation patterns of RORα and RORγ differ in some aspects.

Acknowledgements

This work was funded by the grants of National Natural Science Foundation of China (31402326), the National Basic Research Program of China (2012CB114406), and the Taishan Scholar Program of Shandong Province.

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Tables

Table 1. List of primers and their designated applications.

Primer	Sequence (5’-3’)	Use
CsRORaEcoRIF	cgaattctggccaccatggatgatgtattttgtgat	Plasmid construction
CsRORaEcoRIR	cgaattctgcccgtcaacgggcatggactg	Plasmid construction
CsRORrHindIIIF	aagcttgccaccatggatggaatatgcagaccct	Plasmid construction
CsRORrHindIIIR	aagcttatgagtggtccccggcagcag	Plasmid construction
CsIL17CproF	agctcaagcttctatcttcttggataaacg	Plasmid construction
CsIL17CproR	attcgaagcttcttctcctactcctaaact	Plasmid construction
CsIL17DproF	agctcaagctttgtttttggttgccttcag	Plasmid construction
CsIL17DproR	attcgaagcttctccgtgcgttttctggag	Plasmid construction
CsIL17FproF	agctcaagcttgctgtcgttcttcgggttt	Plasmid construction
CsIL17FproR	attcgaagctttagcagagttgtcaacaac	Plasmid construction
CsRORaF1	cccggggccaccatggatgatgtattttgtgatttca	Plasmid construction
CsRORaR1	cccgggcccgtcaacgggcatggactg	Plasmid construction
CsRORrF1	cccggggccaccatggatggaatatgcagaccctga	Plasmid construction
CsRORrR1	cccgggatgagtggtccccggcagcag	Plasmid construction
pF1	cttgcgtttctgataggcaccta	RT-PCR
pR1	tgcgggcctcttcgctatt	RT-PCR
CsRORaRTF	atgtggcagctgtgtgctat	qRT-PCR
CsRORaRTR	atcgggtccggcatatttcc	qRT-PCR
CsRORrRTF	tttgcaaaacgcatcccagg	qRT-PCR
CsRORrRTR	agcttcagcgtacacaggtc	qRT-PCR
CsIL17CRTF	atcggtgtctccctggacat	qRT-PCR
CsIL17CRTR	gatggtacttcgatccgccg	qRT-PCR
CsIL17DRTF	gcaggtcgacactcctacac	qRT-PCR
CsIL17DRTR	tcctcgtgtgtccagctttg	qRT-PCR
CsIL17FRTF	tctctgtcaccgtggacgta	qRT-PCR
CsIL17FRTR	tttgtgcaggaccagcatct	qRT-PCR
CsGATA3RTF	ccggtcactcaagtcctcac	qRT-PCR
CsGATA3RTR	cgactccagcttcatgctct	qRT-PCR
CsT-betRTF	tggaaccaaccgctcactac	qRT-PCR
CsT-betRTR	ttgttggtgctccccttgtt	qRT-PCR

Figure legends

Figure 1. Multiple sequence alignments of known teleost RORα (A) and schematic domain structure of CsRORα (B). ZnF_C4, C4 zinc finger in nuclear hormone receptors; HOLI, ligand binding domain of hormone receptors. Pink represents low complexity domain.

Figure 2. Multiple sequence alignments of known teleost RORγ (A) and schematic domain structure of CsRORγ (B). ZnF_C4, C4 zinc finger in nuclear hormone receptors; HOLI, ligand binding domain of hormone receptors. Pink represents low complexity domain.

Figure 3. Phylogenetic analysis of CsRORα and CsRORγ. The phylogram was constructed with MEGA 4.0 software using the neighbor-joining method. Numbers beside the internal branches indicate bootstrap values based on 10,000 replications. The 0.05 scale indicates the genetic distance. The GenBank accession numbers of the sequences used for the analysis are: RORα: CsRORα: XP_008310012; *Oreochromis niloticus*: XP_005470779.1; *Poecilia formosa*: XP_007556823.1; *Pundamilia nyererei*: XP_005730049.1; *Danio rerio*: NP_001103637.1; *Ctenopharyngodon idella*: AFC34772.1; *Oryzias latipe*: XP_004069686.1; *Takifugu rubripe*s: XP_003967486.1; *Gallus gallus*: NP_001276816.1; *Homo sapiens*: NP_599024.1; *Mus musculus*: NP_001276845.1. RORβ: *Cynoglossus semilaevis*: XP_008333883.1; *Danio rerio*: NP_001076325.1; *Oreochromis niloticus*: XP_005473204.1; *Solea senegalensis*: BAN42605.1; *Mus musculus*: NP_001036819.1; *Gallus gallus*: NP_990424.1; *Homo sapiens:* BAH02286.1. RORγ: CsRORγ: XP_008321277.1; *Oncorhynchus mykiss*: NP_001186755.1; *Ctenopharyngodon idella*: AFC34773.1; *Clupea harengus*: XP_012684660.1; *Poecilia reticulate*: XP_008429898.1; *Oryzias latipes*: XP_011483568.1; *Mus musculus*: NP_035411.2; *Homo sapiens*: NP_005051.2.

Figure 4. CsRORα and CsRORγ expression in fish tissues under normal physiological condition. CsRORα and CsRORγ expression in the spleen, kidney, blood, liver, gill, heart, intestine, muscle, and brain of tongue

sole was determined by quantitative real time RT-PCR. For comparison, the expression levels of CsRORα and CsRORγ in spleen (the lowest expression levels) were set as 1. Data are the means of three independent experiments and shown as means ± SEM.

Figure 5. Expression of CsRORα and CsRORγ in response to bacterial and viral infection. Tongue sole were infected with *Vibrio harveyi* or megalocytivirus. The control fish were mock infected with PBS. CsRORα (A to D) and CsRORγ (E to H) expression in kidney and spleen was determined by quantitative real time RT-PCR at various time points. In each case, the expression level of the control fish was set as 1. Data are the means of three independent experiments and shown as means ± SEM. $^{**}P < 0.01$; $^{*}P < 0.05$.

Figure 6. Subcellular localization of recombinant CsRORα and CsRORγ in FG cells. FG cells were transfected with pCsRORα-RFP, pCsRORγ-RFP, or the control vector pTagRFP-N. The cells were stained with DAPI and examined with a fluorescence microscope. In all cases, the right panels are merges of the left and middle panels. Arrows indicate some representative transfectants. Bar = 10 μm.

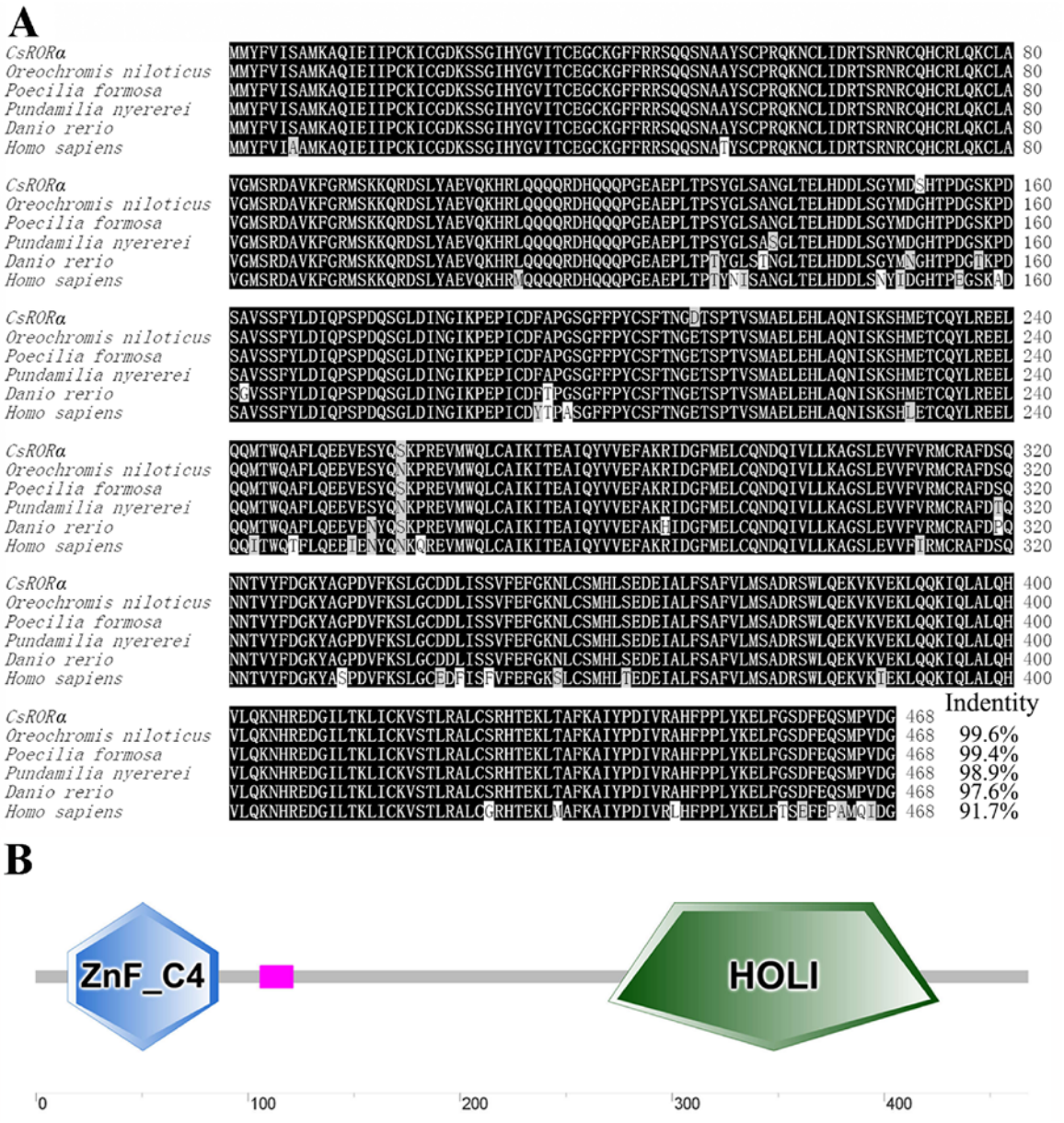
Figure 7. Effect of CsRORα and CsRORγ on CsIL-17C (A), CsIL-17D (B), and CsIL-17F (C) promoter activity. FG cells were transfected with pLucCsIL-17C, pLucCsIL-17D, pLucCsIL-17F, pCsRORα, pCsRORγ, pMetLuc2, pSeap-Control, or pCN3 in different combinations and concentrations. The luciferase activity of the transfectants was subsequently determined. Data are the means of three independent experiments and shown as means ± SEM. Bars labeled with different small letters are significantly different ($P < 0.05$).

Figure 8. Gene expression in fish overexpressing CsRORα and CsRORγ. Tongue sole were injected with pCsRORα, pCsRORγ, or the control vector pCN3, and the expression of IL-17C, IL-17D, IL-17F, T-bet, and GATA-3 in kidney was determined by quantitative real time RT-PCR at 5 days post-injection. The expression levels of the control fish were set as 1. Data are the means of three independent experiments and

shown as means ± SEM. ∗∗ $P < 0.01$, ∗ $P < 0.05$.

542

543 **Fig. 1.**



544

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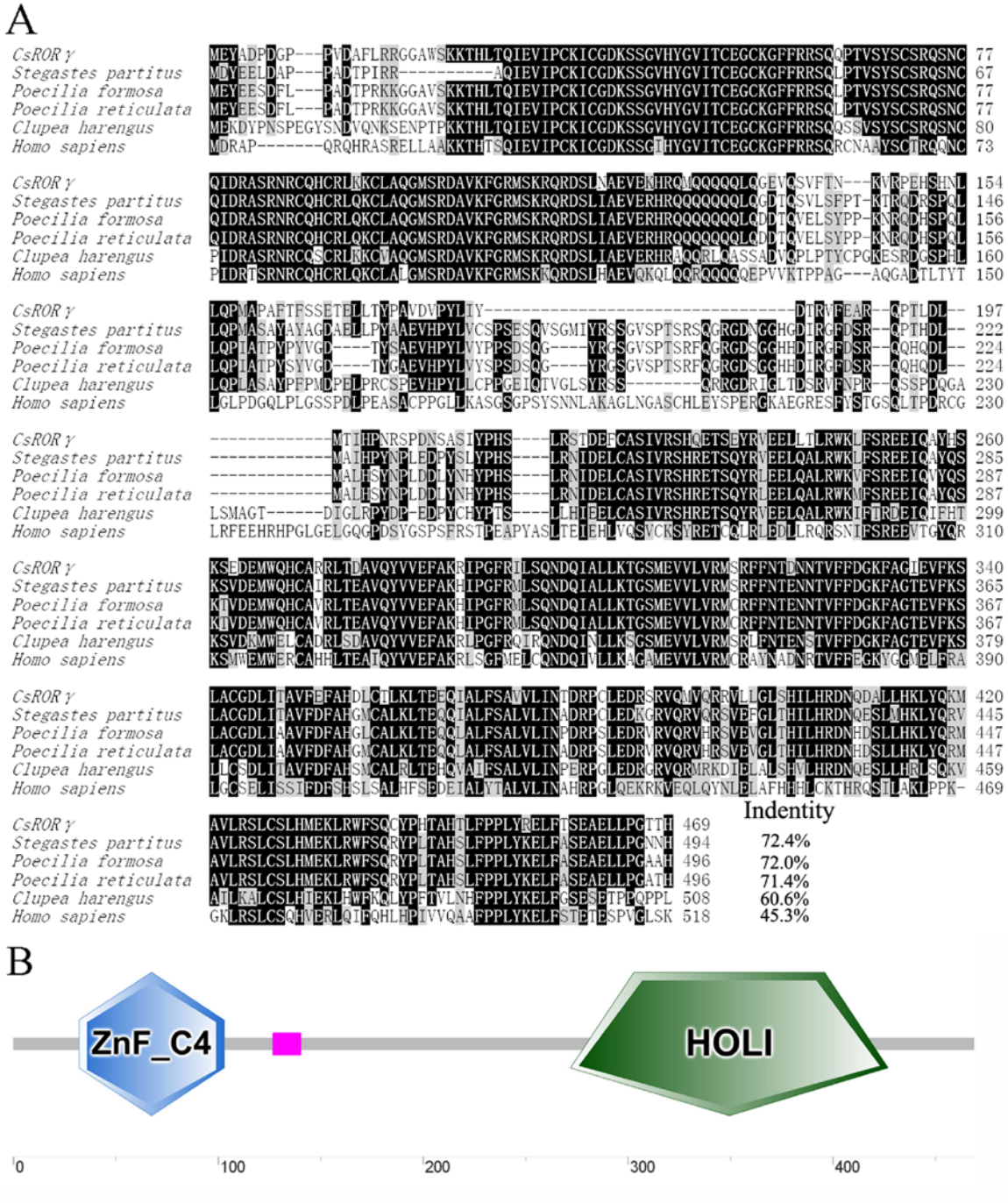
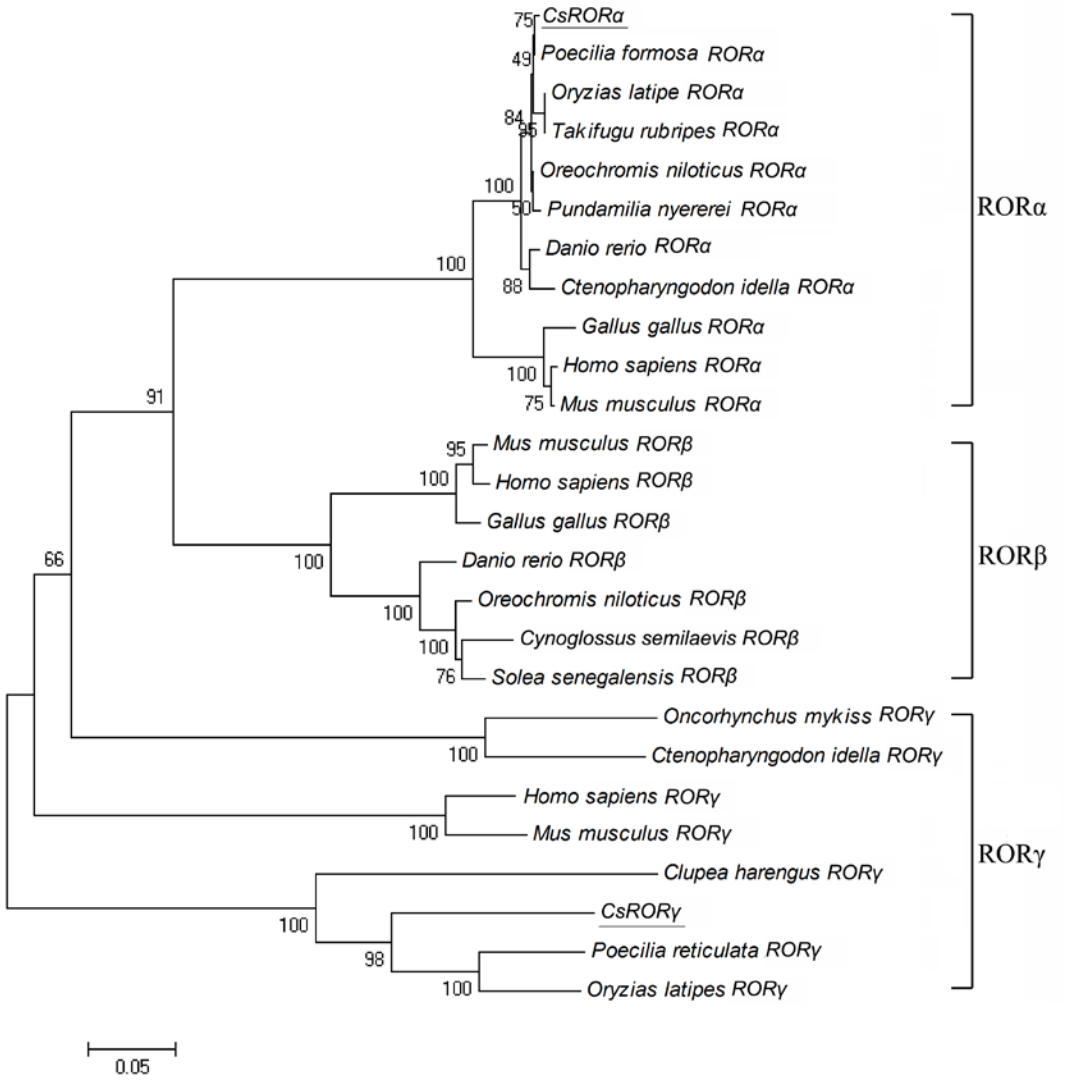
Fig. 2.


A
CsRORγ
Stegastes partitus
Poecilia formosa
Poecilia reticulata
Clupea harengus
Homo sapiens
Indentity
72.4%
72.0%
71.4%
60.6%
45.3%
B
ZnF_C4
HOLI
0
100
200
300
400

549

550 **Fig. 3.**



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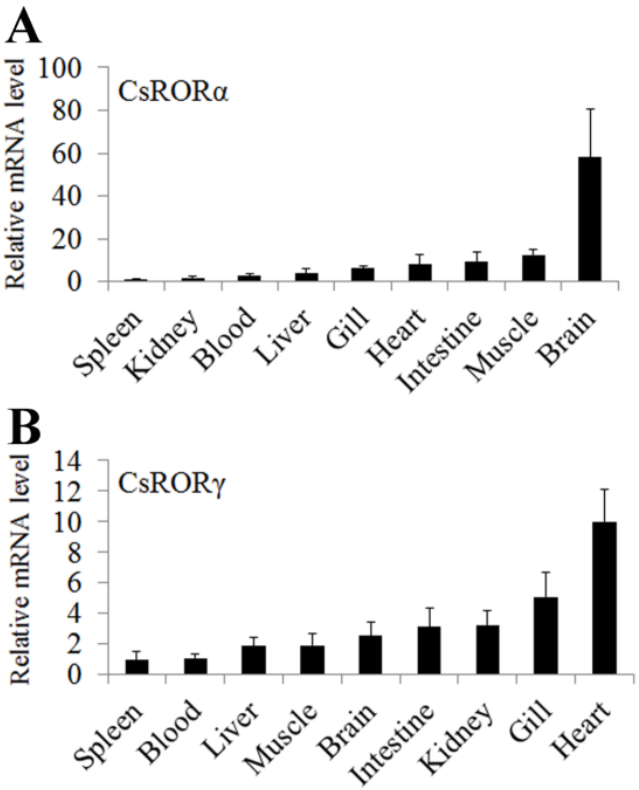
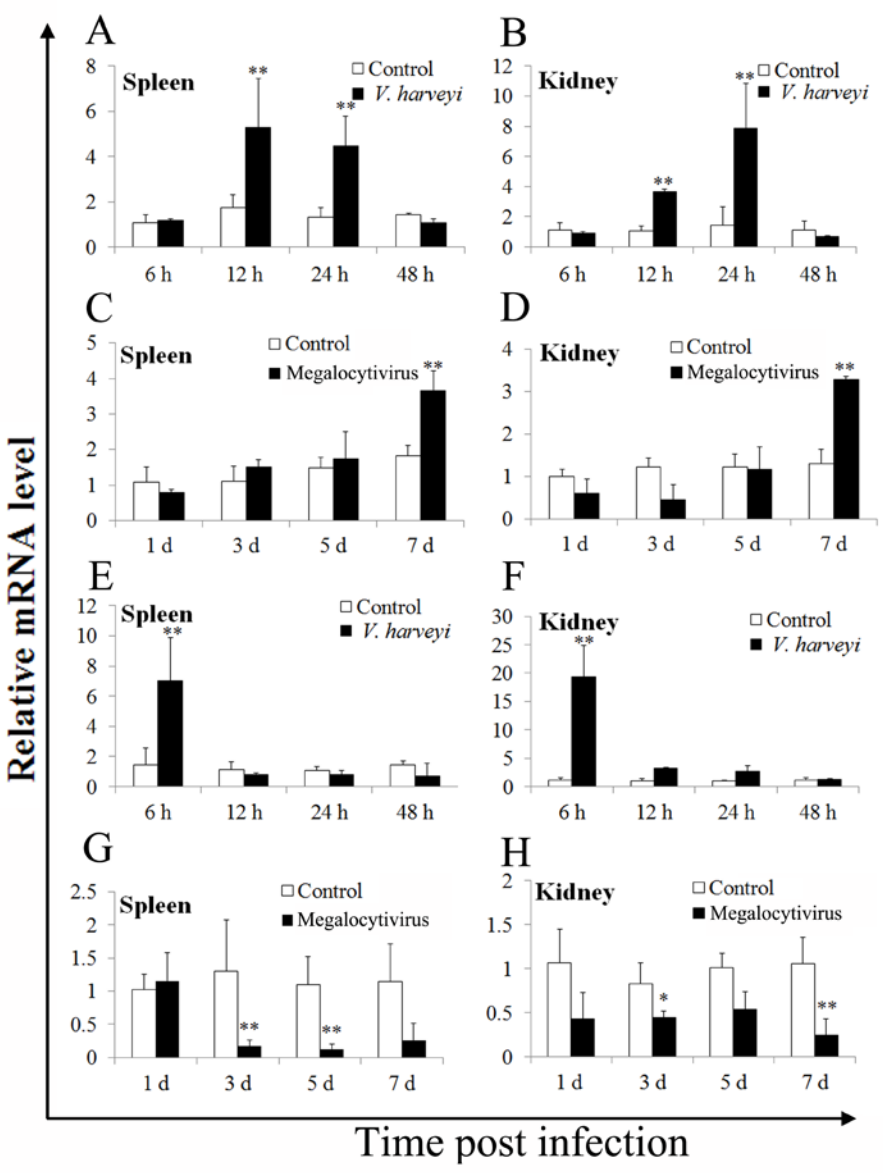
552

Fig. 4.


A
Relative mRNA level
100
80
60
40
20
0
CsRORα
Spleen
Kidney
Blood
Liver
Gill
Heart
Intestine
Muscle
Brain
B
Relative mRNA level
14
12
10
8
6
4
2
0
CsRORγ
Spleen
Blood
Liver
Muscle
Brain
Intestine
Kidney
Gill
Heart

556 **Fig. 5.**

557



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560 **Fig. 6.**

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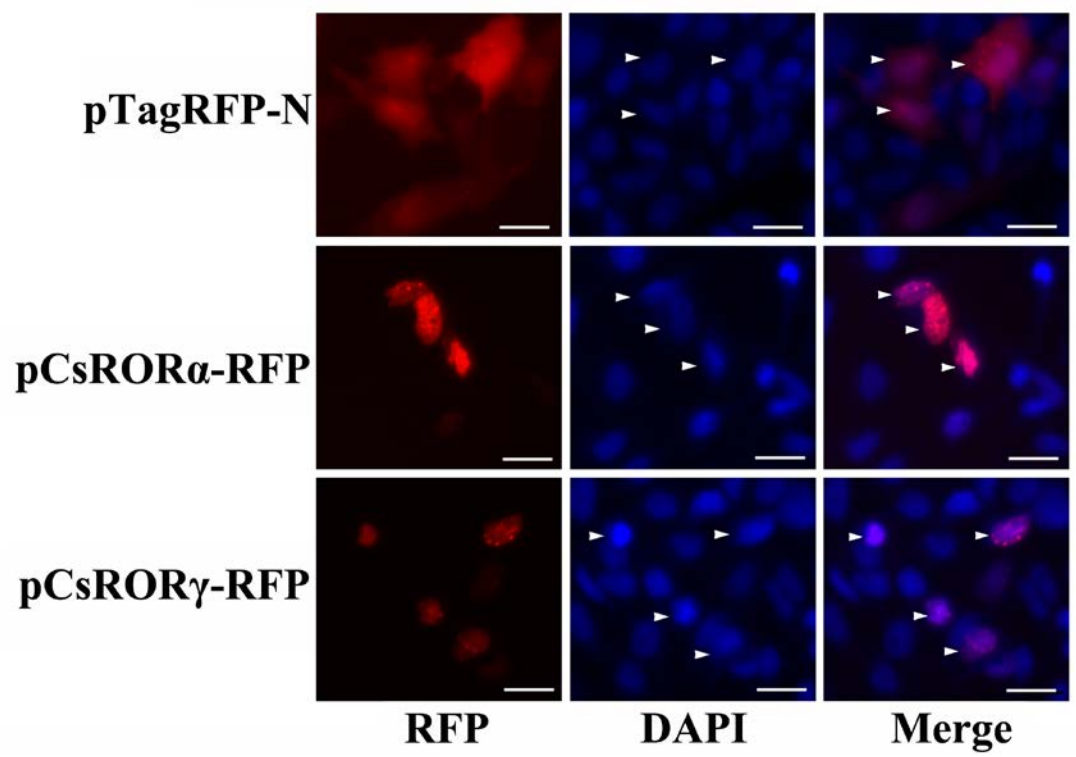

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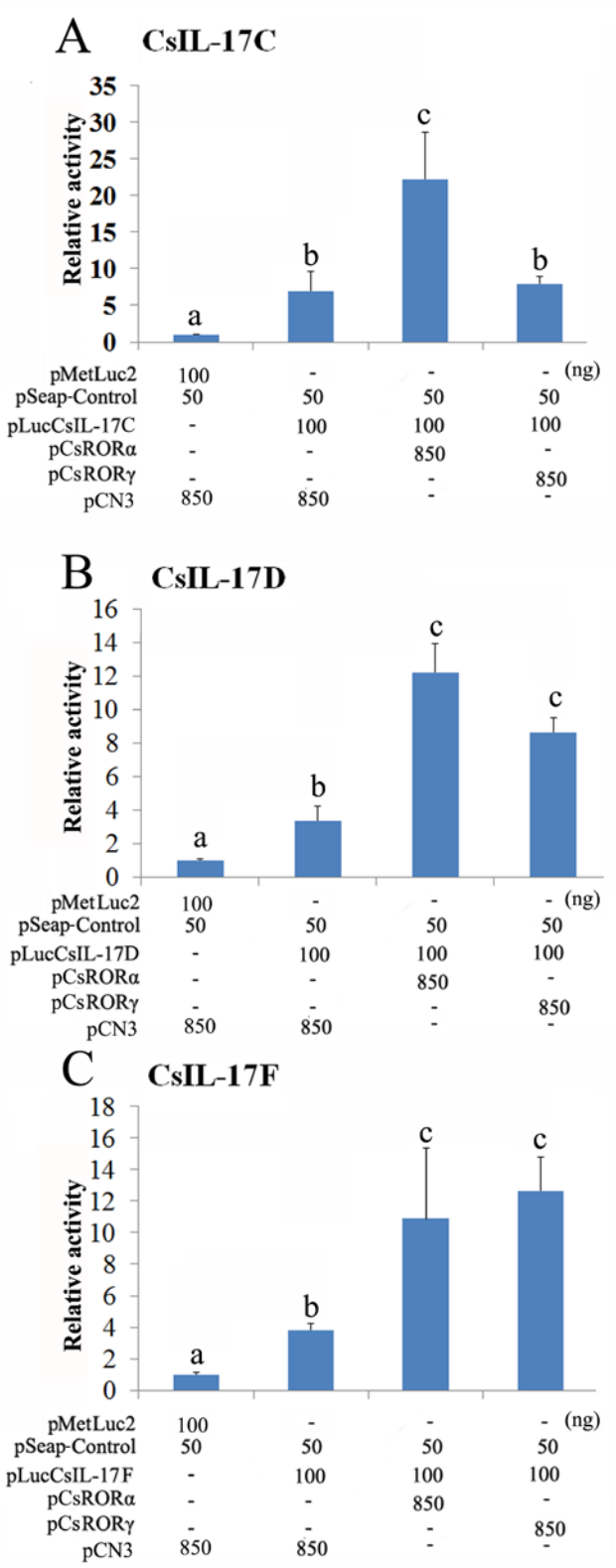
564 **Fig. 7.**



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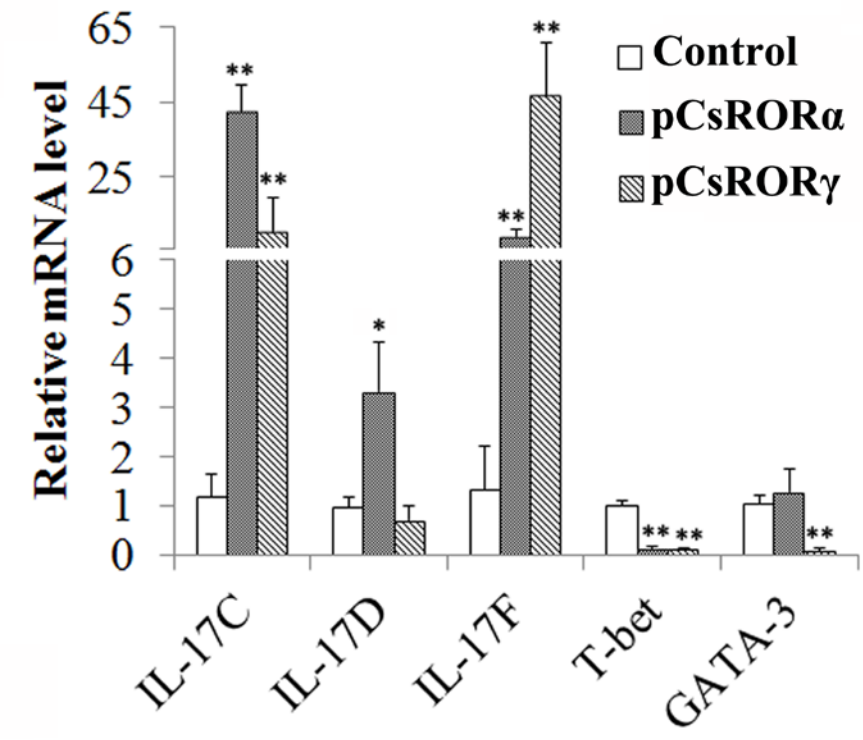
568 **Fig. 8.**



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Supplementary data

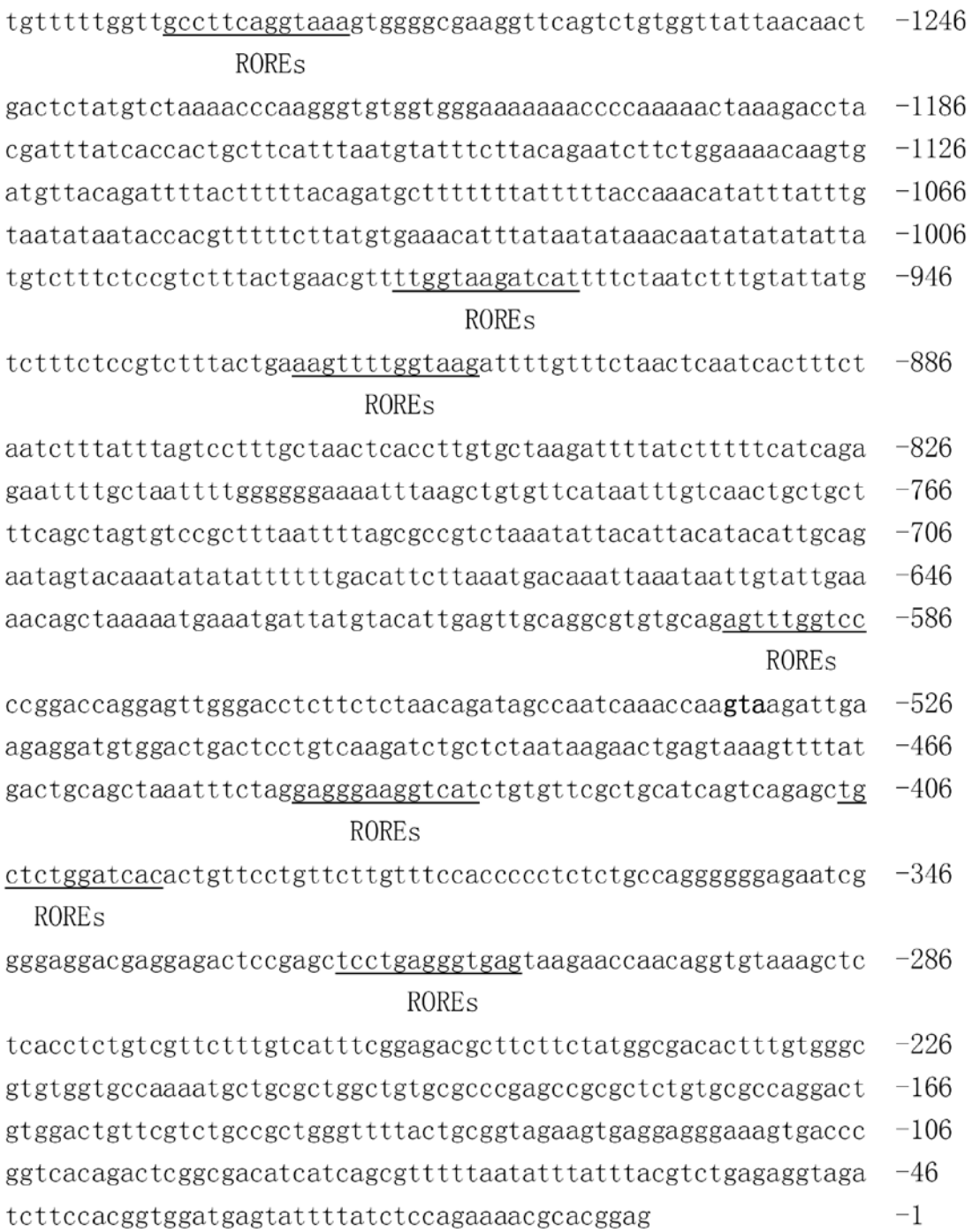
Figure S1. 5’-flanking regions of CsIL-17C (A), CsIL-17D (B), and CsIL-17F (C). The Nucleotide before translation initiation site is designated as -1. The predicted ROR response element sites (ROREs) are underlined.

A.

ctatcttcttggataaacgtgtttttttagtgtaactcaggtgtgatctgagtttgcta −1113
acctttagccgttgtttaaggtatctgttagctgttctggttcttgggtaactagttttt −1053
ttttcttagctaacttttagctaccacattatttaatgttacgactactcttacacatct −993
aatgctgtcacagtcttctctggttgccgttaacctttggctaatgtttacgcttaaag −933
attaacatttcatcgtctttgttgtcttgtgaggaattttaattgactgtaattgtgtgt −873
gtgcgcgtgtgtgtgtatgtgtgtgtgtgtgccaccacagagaacaatgacatcccat −813
cattacatcatcacacagctccggtctctaacaacatcattcactttctcttcactcact −753
tttaaacataaacaatcagtgatgatgtcatcgcagcagcggaggaatttcagtaggaac −693
RORE
agaaaataaataaatgtattacaagggaggaaaaggaaacaatttgtttttcatttcata −633
aaatcaaaacaaagatttttatttcacagaagtttgtttacattaataaaatctcatgtt −573
gaaagatgactttcataaaaaaatgtttggaatgaaacttttttctatttgtgtaaacatt −513
taaattgtgtaaaaattgaagatttgaaatccacgcaaaacgtgtgacaggtatgtgtgt −453
gtgtgtatgtgtgtaagtgtgctgcattccactgacagcacgtaattagcacacacacac −393
acacacacacacacacacacactcagggagaaagagcattgtttcctcctcctgcagc −333
agcttctggttttttcccaggtgaccttcataaaaggaggagaggagcgacagtcagacac −273
RORE
agtcagagcagcaacagtcagacacacagtcagagcagcaacagtcacagagaagagaca −213
acatggacatgacactggtgagtgaacactgaggaggagaggggtcctgatggaggacca −153
RORE RORE
ctgaggagaggatgaggaggagagcacttagtttgggctgacaggaggggggaggagaaa −93
ggggagtccaggtggacacaggtgaatctctgaggagagcagggaaacaggtgaaaatag −33
aggggagaaaaaaagtttaggagtaggagaag −1

578

579 **B.**



580

581

C.


ctcagtgcctcacttttttttttttttttgctgtcgttcttcgggttttagttaattcc -1098
tgcttccgtgtatcgtgaacgattgcacaacaatcgagaaatacagtgataaaggaatca -1038
cttattaaaaaatcgaatcaaatcaacactaaaatgtctcaccttccaaacggaagacag -978
aaaaccctgaactttgaccttgtcaacccccgcgaaaccatttgtatcatatttgctaca -918
caacattcagactggaaaactctgttttcaaaatgttacaggcaaaactcgtttttattt -858
ctaaatttttcagcaaaaagttgccaaaaaaaatgctgaaggtgtcaaatgctcacaaaca -798
RORE
aaaaatttttttttttcgtttgtttcgttttcgtatggttaatcaacatatctttta -738
aaaaaaggttcttacgtgtcaggctttacagcacgtgtcaaattcaaggcccgcgggct -678
RORE
aaatgcggtccgccatgtcactgtatttggcccgcgggagattaaaatgcatttatttct -618
RORE
tatcataaagttcatgttgctaagtttcttaacctgataaatatatatttttttaaaatta -558
RORE
tacagagcaattaaataatattattgataataatcaattccagaaacattcatgaacacc -498
ctatggagtagctacatttgtaccgtctttgatctacaaacctttcaaggggaacttctc -438
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RORE
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tgttgacaactctgcta -1

Figure S2. Detection of pCsRORα, pCsRORγ and pCN3 plasmids (A) and expression of *CsRORα* and *CsRORγ* (B and C) in kidney. A. Tongue sole were administered with pCsRORα, pCsRORγ, pCN3, or PBS (lanes 1 to 4 respectively), at 5 days post-administration DNA was extracted from kidney and used for PCR with primers specific to the common backbone of pCsRORα, pCsRORγ, and pCN3. B. Tongue sole were administered with pCsRORα (lane 1), pCsRORγ (lane 3), and pCN3 (lanes 2 and 4), at 5 days post-administration, RNA was extracted from the kidney of the fish and used for RT-PCR with primers targeting pCsRORα-derived *CsRORα* (lanes 1 and 2) and pCsRORγ-derived *CsRORγ* (lanes 3 and 4). C. The samples in (B) were used for RT-PCR with primers specific to β-actin (internal reference). M, DNA markers.