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Grouper (*Epinephelus coioides*) *CXCR4* is expressed in response to pathogens infection and early stage of development

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Abstract

Chemokine (C-X-C motif) receptor 4 (CXCR4) from orange-spotted grouper (*Epinephelus coioides*) was identified and characterized in this study. gCXCR4 shared common features in protein sequence and predicted structure of CXCR4 family. This suggested that gCXCR4 is a member of G protein-coupled receptors with seven transmembrane domains. The expression patterns revealed that gCXCR4 may play a key role in early development of grouper. Furthermore, overexpression of gCXCR4-GFP for 48 h had significant effects on the GF-1 cell viability. gCXCR4 protein was mainly expressed in the marginal zone of head kidney and on the surface of intestinal villi. *gCXCR4* expression can be detected in all the examined tissues and significantly up-regulated in eye and brain, which are the main targets for nervous necrosis virus (NNV) infection and replication. *gCXCR4* gene expression can be induced in the spleen and eye by lipopolysaccharide and NNV, respectively. Our data suggested that gCXCR4 may not only play a role in the early immune response to microbial infection but also restrain to the immune system and central nervous system.

Keywords: *Epinephelus coioides*; CXCR4; G-protein-coupled receptors; chemokine

1. Introduction

The orange-spotted grouper, *Epinephelus coioides,* is a commercially important fish that is widely farmed in tropical waters of many countries. Considerable economic losses have been sustained in grouper aquaculture due to the infection of grouper by piscine nodavirus, i.e. nervous necrosis virus (NNV). The virus causes viral nervous necrosis (VNN) on grouper hatchery larvae and juvenilies, resulting in a high mortality rate (80-100%) (Kuo et al., 2011; Munday et al., 2002).

Chemokines are a group of small molecular weight (6–14 kDa) cytokines which play important roles not only in against microbial infection by guiding leukocyte migration but also in embryonic growth and development (Kim et al., 1999; Olson et al., 2002). Chemokines can be classified into four different kinds, CXC, CC, C and CX3C (Murphy et al., 2000), according to its cysteine motif in the N-terminal region. Chemokine receptors are a group of G protein-coupled receptors with seven transmembrane domains. Upon stimulation by chemokines, chemokine receptors trigger a series of intracellular signal transductions via interaction with the G proteins. To this day, different chemokine receptors have been found on various cells such as monocytes, T lymphocytes, B lymphocytes, natural killer cells, macrophages, endothelial cells and neuron cells in mammals (Horuk et al., 2009). To date, CXCR4 has been identified in several species including human (Federsppiel et al., 1993), mouse (Heesen et al., 1996) and dog (Tsuchida et al., 2007), but

56 less is known in fish (Daniels et al., 1999; Chong et al., 2001; Alabyev et al., 2000; Jia and
57 Zhang, 2009), and no functional characterization of CXCR4 in orange-spotted grouper has
58 been reported.

59 Chemokines and their receptors serve an important role in viral infections and among
60 the chemokine receptors, CXCR4 is also a co-receptor for human immunodeficiency virus
61 (HIV) entry into target cells (Feng et al., 1996; Berson et al., 1996). Nonetheless, CXCR4 is
62 not only involved in the pathogenesis of viral infections but also plays a critical role in
63 organogenesis and embryonic development-related vascularization, lymphopoiesis and
64 myelopoiesis (Tachibana et al., 1998). CXCR4 deficiency produces a lethal phenotype and
65 abnormal development of central nervous system, such as abnormal migration of granule
66 cells and an altered location of Purkinje cells in mice malformed cerebellum (Ma et al.,
67 1998). In fish, CXCR4 has been found in the early stage of zebrafish embryo, it can be
68 detected in the lateral mesoderm and posterior midbrain (Chong et al., 2001), moreover the
69 migration of lateral-line-primordium is impeded in CXCR4 homologue-mutated zebrafish
70 and is completely absent in SDF-1a defective zebrafish (Valentin et al., 2007). In addition,
71 CXCR4 plays a crucial role for tissue polarity (Haas and Gilmour, 2006). CXCR4
72 homologue deficiency leads to the random migration of cells and the loss of coordinated
73 motility within the posterior lateral line primordium in zebrafish (Haas and Gilmour, 2006).
74 These observations indicate that CXCR4 is multifunctional and plays crucial roles in

75 embryonic growth development and hematopoiesis.

76 However, no functional characterization of grouper CXCR4 has been reported.
77 Previously, a partial portion of grouper CXCR4 cDNA was identified by subtractive cDNA
78 hybridization from healthy and NNV-infected groupers (Chen et al., 2010). In the present
79 study, orange-spotted grouper CXCR4 (gCXCR4) was cloned and the expression profile
80 that response to lipopolysaccharide (LPS) and NNV infection was investigated. In addition,
81 we showed that cell proliferation was impeded after gCXCR4 overexpression for 48 h.

82

2. Materials and methods

2.1. Fish and challenge experiments

Fish weighing approximately 300g (150 days post-hatching) and different ages (1-40 days-old) of orange-spotted grouper (*E. coioides*) were collected from an indoor fish farm in Linyuan and maintained in 10L containers at 27 ± 1 °C. For challenge experiments, 15 300g-in-weight-fish were divided into five groups (n=3 per group) and challenged with 100 µl phosphate buffered saline (PBS) contained approximately 20 µg of purified *Escherichia coli* 0127:B8 LPS (Sigma-Aldrich, St. Louis, MO) per fish via intraperitoneal injection. Fish with 100 µl PBS injection was used as a control group. The fish were sacrificed and the spleens were collected at 0, 6, 24, 48 and 72 h post-injection. In the experiments of virus challenging, juvenile groupers (about 0.8 g in weight, 40–45 days post-hatching) were collected from Linyuan fish farms in southern Taiwan. Twelve juvenile groupers were divided into two groups, NNV infection group and control group. Each six fish were immersed into 500 ml of rearing water which contained 50 ml of a viral solution (10^6 $TCID_{50}$/0.1 ml) or saline for 2 h. The fish were transferred to a virus-free aquarium, which had been exposure to ultraviolet (UV) light for 24 h, and cultured at 28 °C. Real time PCR was then used to confirm the fish was infected by NNV after 72 h of challenging.

2.2. Total RNA extraction and cDNA synthesis

Eye or whole fish samples (n=3 per group) were used for total RNA extraction by

102 homogenized using a MagNALysis homogenizer (Roche, Basel, Switzerland) following the
103 manufacture's recommendations of TRIzol™ (Invitrogen) method.. cDNA was synthesized
104 with 2 µg RNA, 0.1 µM oligo dT primer, 12.5 µM dNTP (Bioman Scientific Co. Ltd.,
105 Taipei, Taiwan) and 50 units Molony Murine Leukemia Virus (MMLV) reverse transcriptase
106 (Promega, Madison, WI) at 42 ^{o}C for 1 h.

107 RNA and cDNA were quantified using an Ultrospec 3300 Pro spectrophotometer
108 (Amersham Biosciences, Piscataway, NJ, USA); nucleic acids were diluted using sheared
109 salmon sperm DNA (5 ng mL^{-1}) as a carrier.

110 *2.3 RNA gel electrophoresis*

111 To confirm the integrity of RNA samples, the extracted RNA was evaluated by RNA
112 electrophoresis. In brief, 2 µl RNA sample was mixed with 18 µl 1× reaction buffer (1×
113 MOPS, 20 % formaldehyde and 50 % formamide), 2 µl of 10× formaldehyde gel loading
114 buffer (50 % glycerol, 10 mM EDTA, pH8, 0.25% bromphenol blue and 0.25 % xylene
115 cyanol) and was visualized by using ethidium bromide staining

116 *2.4. Rapid amplification of cDNA ends (RACE)*

117 A 950 bp cDNA fragment obtained from our previous study by subtractive cDNA
118 hybridization from healthy and NNV infected groupers (Chen et al., 2010). The sequence
119 showed 75% similarity to CXCR4 from *Psetta maxima* by blasting
120 (http://www.ebi.ac.uk/blastall/). Full length cDNA was obtained by 5'/3' RACE which was

121 performed by using 5'/3' RACE Kit. Gene-specific primers (Table 1) for 5' and 3' RACE
122 were designed based on partial sequence of gCXCR4 For 5' RACE, mRNA was transcribed
123 by MMLV reverse transcriptase (Sigma-Aldrich) with primer gCXCR4-5SP1and
124 gCXCR4-5SP2 and gCXCR4-5SP3 were used for PCR and nested PCR. 3' RACE was
125 performed by using primers gCXCR4-3SP1 and gCXCR4-3SP2 for PCR and nested PCR,
126 respectively. PCR condition was one cycle of 3 min at 95 ^{o}C, followed by 35 cycles each at
127 95 ^{o}C for 30 s, 58 ^{o}C for 30 s, 72 ^{o}C for 30s and a final extension at 72 ^{o}C for 10 min. The
128 primers gCXCR-F and gCXCR-R were used to amplify the gCXCR4 cDNA fragment.
129 *2.5 Bioinformatic analyses of gCXCR4*
130 The transmembrane domains, extracellular domains and cytoplasmic domains of CXCR4
131 were identified by the TMHMM Server 2.0 program
132 (http://www.cbs.dtu.dk/services/TMHMM/). The protein sequences of different CXCR4
133 species were obtained from GenBank and were aligned using Vector NTI 10 software. A
134 phylogenetic tree of CXCR4 was constructed by the neighbor-joining method using
135 MEGA4.0. The reliability of the tree was established by bootstrap analysis, based on 1,000
136 bootstrap replications.
137 *2.6. RT-PCR*
138 The tissue distribution of gCXCR4 gene expressions was investigated by RT-PCR. Total
139 RNA was extracted from different tissues of grouper such as eye, fin, gill, muscle, head

kidney, heart, spleen, intestine and brain. PCR condition was one cycle of 3 min at 95 ^{o}C, followed by 35 cycles each at 95 ^{o}C for 30 s, 58 ^{o}C for 30 s, 72 ^{o}C for 30 s and a final extension at 72 ^{o}C for 10 min. To detect the expression of gCXCR4 in different developmental stages of grouper larvae, total RNA were prepared from pooled larvae that contained 20 fish fry in each group at 1, 2, 4, 6, 8, 10, 14, 18 and 20-days post hatch (dph), and 3 fish larvae were pooled at 24, 26, 28, 30, 32, 34, 38 and 40 dph.

2.6. Real Time-quantitative PCR

Real-time quantitative PCR was performed by StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA, USA). 1ul of cDNA (from 100 ng RNA) was mixed with 12.5 µl 2×SYBR® Green Master Mix (Applied Biosystems) and 1 µl of each 10 µM specific primer (Table 1). The thermal profile for real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and a final stage at 95 °C for 15 s, 60 °C for 1 min, 95°C for 15 s. The results of real-time PCR were analyzed with StepOne Software v2.1.

2.7 Statistical analysis

The C_T for gCXCR4 and *β*-actin were determined for each sample. *β-actin* was used as internal control. $\triangle C_T$ (Differences between gCXCR4 and *β*-actin) was calculated to normalize the differences in the efficiency of reverse transcription reactions. The $\triangle C_T$ for each sample was subtracted from the $\triangle C_T$ of the calibrator, and the difference was

159 designated as the $\triangle\triangle C_T$ value. The relative expression level of gCXCR4 could be
160 calculated by $2^{-\triangle\triangle CT}$. All real-time PCR data were subjected to analysis of t-test and are
161 presented as the mean ± S.E. of the relative mRNA expression. *P*-values of < 0.05 were
162 considered significantly different.

163 *2.8 Plasmid construction*

164 To prepare anti-gCXCR4 antiserum, extracellular domain I and III of gCXCR4 were
165 constructed into pET29b expression vector (Novagen, USA) by PCR using primers. Primers
166 gCSCR4-EXI-F (BamHI) and gCSCR4-EXI-R (SalI) were used for extracellular domain I
167 and gCXCR4-EXIII-F (SalI) and gCXCR4-EXIII-R (XhoI) were used for amplifying
168 extracellular domain III (181-215 a.a.). This recombinant plasmid was named
169 pET29b-gCXCR4-EXI-EXIII which can express a fusion protein of gCXCR4 extracellular
170 domains I and III along with a 6×His tag.

171 The gCXCR4 overexpression vector, pcDNA3.1-gCXCR4-GFP, was constructed by
172 PCR amplifying gCXCR4 using primers gCXCR-F and gCXCR4-GFP-R (Table 1) and the
173 PCR products were then cloned into the pcDNA3.1-CT-GFP-TOPO expression vector
174 (Invitrogen). The inserted DNA fragments of each clone were confirmed by sequencing
175 (Mission Biotech Co., Ltd., Taipei, Taiwan).

176 *2.9 Recombinant protein and anti-gCXCR4 antiserum preparation*

177 The gCXCR4-EXI-EXIII-His recombinant protein was expressed by transforming

178 pET-29b-gCXCR4-EXI-EXIII intoBL21(DE3) cells (Novagen)and induced by adding
179 isopropyl-beta-D-thiogalactopyranoside (IPTG; MDBIO, Frederick, MD) to a final
180 concentration of 0.1 mM. Protein purification was performed using a HisTrap HP 1 ml
181 column (Amersham Biosciences, Piscataway, NJ). Antisera against gCXCR4 was obtained
182 by immunizing (injection of 1 mg/ml protein which was mixed with Freund's complete
183 adjuvant [Sigma-Aldrich] on days 1, 14 and 28) a New Zealand White rabbit (Taiwan
184 Livestock Research Institute, Tainan, Taiwan) with recombinant gCXCR4-extracellular
185 domain I-extracellular domain III fusion protein. The antiserum collected at day 0 (before
186 treatment) was used as control. The blood samples were incubated at 37℃for 1 h and left
187 overnight at 4 ℃. The supernatant (containing rabbit anti-gCXCR4 antiserum) was
188 collected after centrifuging at 900 ×g for 10 min at 4 °C. The rabbit anti-gCXCR4
189 antiserum was stored at –20 °C.
190 *2.10 Immunofluorescence staining*
191 The head kidney and intestines were obtained from healthy groupers and treated with 30 %
192 sucrose at 4 ℃ overnight. The different tissue blocks were covered with an optimal cutting
193 temperature compound (Tissue-Tek®; Sakura Finetek, Tokyo, Japan), and the samples were
194 slowly placed into liquid nitrogen. The frozen tissue block was transferred into a cryotome
195 cryostat and 5 μm-thick sections were cut. Each slide was fixed with 3% paraformaldehyde
196 (Kanto Chemical, Tokyo, Japan) and incubated at room temperature for 30 min. The

197 samples were then washed with 1× PBST (0.1% Tween 20, 1× PBS) and blocked with 5 %
198 skim milk. Rabbit anti-gCXCR4 antisera (1:200 dilution) were added and subsequence
199 Alexa Fluor® 594 goat anti-rabbit IgG (1:200 dilution) (H+L) (Invitrogen) secondary
200 antibody was added. The nuclei were stained with Hoechst 33342 (Invitrogen) at room
201 temperature for 20 min, then washed extensively with 1×PBS and mounted on a coverslip
202 with mounting medium.

203 *2.11 Cell proliferation assay*

204 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay was performed
205 using Cell Proliferation Kit I（Roche）to analyze the effects of gCXCR4-GFP
206 overexpression. 5×10^4 *Epinephelus coicoides* fin cells (GF-1, BCRC 960094) were seeded
207 in 24-well plate and grown in a humidified incubator operating at 28°C in an antibiotic-free
208 L15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 5% v/v
209 heat-inactivated fetal bovine serum (FBS) (Chen et al., 2008). After 24hr, when cells
210 attached completely, pcDNA3.1-CT-GFP-CXCR4 or pcDNA3.1-CT-GFP vector was
211 transfected using Lipofectamine 2000 (Invitrogen). Then 20μl of MTT labeling reagent was
212 added to each well and cultured for 4 hours in incubator. To dissolve formazan crystals,
213 200μl solubilization solution was added to each well. Covered with tinfoil and agitate cells
214 on orbital shaker for 10 min. Read absorbance at 570 nm.

3. Results

3.1. Characterization of full-length gCXCR4

Orange-spotted grouper CXCR4 (gCXCR4) contained an open reading frame (ORF) of 1,104 nucleotides encoding a 367 a.a. protein with a predicted molecular weight of 40.37 kDa (Fig. 1). The structure of the protein was predicted to have seven-transmembrane domains, four extracellular domains and four cytoplasmic domains. The DRY motif was found at the second intracellular loop. A conserved cysteine residue on each of the four extracellular domains located at positions Cys^{33}, Cys^{118}, Cys^{198} and Cys^{292}. The C-terminal is a region rich in serine and threonine residues.

The results of different species CXCR4 alignment (Fig.2) showed that gCXCR4 not only similar to fish (zebrafish [58%], carp [54%], rainbow trout [53%] and turbot [52%]) but also to mammalian (human [51%) and mouse [50%)) and the conserved regions appeared in seven putative transmembrane domains (Fig. 2). The sequence of extracellular domains showed very diverse in different species. gCXCR4 has 22.1%, 8.5% , 28.8%, 32.2%, 33.6% and 29.3% similarity to human, mice, common carp, zebrafish, rainbow trout and turbot, respectively (Table 2).

3.2. Phylogenetic analysis of gCXCR4

There are six groups of CXCRs on the phylogenetic tree. Interestingly, within the group of CXCR4, mammalian and fish origins of CXCR4s was clear separated except for gCXCR4

234 and *Petromyzon marinus* CXCR4 (Fig. 3). This indicated that gCXCR4 might be a common

235 ancestor to other CXCR4 proteins.

236 *3.3. in vitro and in vivo expression of gCXCR4*

237 To clarify the role of gCXCR4 in grouper, the expression of gCXCR4 in different

238 growth stages of grouper was measured by real-time PCR. *gCXCR4* can be detected in all

239 examined fish samples (from 1dph to 40 dph). The expression levels of gCXCR4 was up

240 regulated and fluctuated in the period 1-4 dph and 6-8 dph , the *gCXCR4* expressions are <

241 50. Two higher expression peaks (>100, $p < 0.05$) were observed at 18 dph and 38 dph (Fig.

242 4).

243 To evaluate the effect of gCXCR4 overexpression on cell proliferation, GF-1 cells

244 were transfected by pcDNA3.1-gCXCR4-GFP or pcDNA3.1-GFP overexpression vector.

245 The results showed that overexpression of gCXCR4-GFP for 12 h, 24 h, and 36 h had no

246 significant effects on cell viability (Fig. 5) but significantly repressed after 48 h ($p < 0.05$)

247 (Fig. 5) (n=5 per group).

248 *3.4. Expression patterns of gCXCR4 on head kidney and intestine*

249 gCXCR4 was mainly expressed in the head kidney (Fig. 6A) and on the surface of intestinal

250 villi of intestine (Fig. 6B).

251 *3.5 The expressions of gCXCR4 in different organs of grouper*

252 *gCXCR4* was highly expressed in eye, gill, brain and important immune organs such as

spleen and head kidney (Fig. 7). Higher levels of expression were detected in eye, gill, spleen, brain and head kidney tissues. Lower levels of expression were detected in fin, muscle and heart tissues. Barely any gCXCR4 transcript was detected in the intestine (Fig. 7B).

3.6 The expressions of gCXCR4 after LPS or NNV challenge

The expression level of *gCXCR4* in spleen was significantly increased after 6 h post-injection of LPS ($p < 0.05$) and decreased at 24 h and 48 h post-injection of LPS ($p < 0.05$). At 72 h post-injection, *gCXCR4* had returned to the base level as the control (Fig. 8A).

Forty-eight h after NNV infection, the juvenile groupers exhibited abnormal behaviors such as loss of equilibrium and spiral swimming pattern and NNV can be detected at 72 h. *gCXCR4* expression was also up-regulated at the time point which was 72 h post-NNV infection in eyes ($p < 0.05$) (Fig. 8B).

4. Discussion

The chemokine system has an important role in the host immune response against microbial pathogens and provides a link between innate and adaptive immunity (Murphy et al., 2000). The similar structure of gCXCR4 to other species (Alabyev et al., 2000; Tsuchida et al., 2007; Jia and Zhang, 2009) contains seven transmembrane regions, four extracellular regions and four intracellular regions, and a conserved DRY motif (Fig. 2). The predicted function of the gCXCR4 DRY motif was supported by the results of amino acid sequence alignments of gCXCR4 and CXCR4 of other species, which function had been demonstrated as important to G protein coupling (Doranz et al., 1999). The transmembrane regions as well as the cysteine residue positions in the extracellular regions appear to be highly conserved in CXCR4 evolution (Federsppiel et al., 1993; Heesen et al., 1996; Alabyev et al., 2000; Tsuchida et al., 2007; Jia and Zhang, 2009). The posttranslational modification, i.e. the tyrosine residues of the N terminus are sulfated in Golgi, of human CXCR4 plays a crucial role on the infective ability of HIV (Farzan et al., 2002). However, these tyrosine residues were not conserved in gCXCR4 (Figs.1 and 2), suggesting that the posttranslational modification of gCXCR4 N-terminus is different. In addition, many serine and threonine residues were identified in the C-terminus of gCXCR4 and might have the modifications, i.e. phosphorylated as a prerequisite of signal transfer (Berson et al., 1996), like other protein in CXCR4 family.

286 CXCR4 is expressed mainly in immune organs and central nervous system: thymus
287 and spleen of mouse (Heesen et al., 1996), chicken bursa (Liang et al., 2001), primate
288 (*Macaca mulatta*) brain (Federsppiel et al., 1993) and cattle locus coeruleus, cerebellum and
289 pons (Rimland et al., 1991). In grouper, *gCXCR4* was highly expressed in NNV major target
290 organs, such as eyes and brain, and major lymphoid organs, such as gill, spleen and head
291 kidney. This also been reported in other fish species (Daniels et al., 1999; Jia and Zhang,
292 2009) in which CXCR4 expressed in central nervous system and immune system.
293 Interestingly, the expression of *gCXCR4* in eye other than the immune related organs or
294 central nervous system has never been reported which raised the other possible function of
295 gCXCR4. Accordingly the grouper major lymphoid organs such as spleen, head kidney, gill
296 and mucosa-associated tissues appeared to be regions of gCXCR4 overproduction (Press
297 and Evensen., 1999). Furthermore, CXC chemokine system originates from the central
298 nervous system and may participate in central nervous system development (Huising et al.,
299 2003).

300 SDF1/CXCR4 signaling plays a critical role in embryonic development and is essential
301 for development of cardiovascular, central nervous system, bone marrow colonization and
302 hematopoiesis in mice (Ma et al., 1998; Tachibana et al., 1998). In fish, CXCR4 has been
303 found in the early stage of zebrafish embryo and related to tissue polarity (Chong et al.,
304 2001; Haas and Gilmour, 2006). The gene expression of gCXCR4 was highly expressed in

305 the period day14-day20 and day34-day40 larva that is coincided with dorsal spine formation
306 and pigmentation (Katsutoshi and Hiroshi, 2009). The results implied that chemokine
307 system exist in early developmental stage and play a key role in grouper development.

308 Immunohistofluorescence staining suggested that the protein gCXCR4 is expressed in
309 lymphoid organs (Fig. 6A) and mainly on the surface of intestinal villi. This may be due to
310 eyes, gills and surface of intestinal villi are continuously exposed to an environment which
311 may have potentially pathogenic microbes. LPS is an endotoxin constituent of the outer
312 membrane of Gram-negative bacteria which can induce immune responses and
313 inflammation (Raetz et al., 2008). In fish, it has been demonstrated that LPS can stimulate
314 the proliferation of neutrophils, monocytes, B lymphocytes and macrophages in a response
315 against LPS-induced inflammation (Swain et al., 2008). The results shown that LPS can
316 up-regulate the expression of *gCXCR4* in the spleen and this also been showed in head
317 kidney and spleen of turbot after challenging with *Vibrio harveyi* (Jia et al., 2009).

318 gCXCR4 mRNA was up-regulated at 3 days post-infection and was significantly
319 increased in the eyes (Fig. 8B), suggesting that gCXCR4 is not only involved in the
320 response to bacteria invasion, but also have response to NNV infection. Although the eye
321 has been known to express chemokine receptors, such as CXCR1 and CXCR2 in mammals
322 (Goczalik et al., 2008), our detection of abundant g*CXCR4* in the organs and significantly
323 up-regulated in NNV-infected fish was unexpected. Interestingly, grouper eye is one of the

main organs for NNV replication (Munday et al., 2002) and the immune response of NNV infection involving macrophage-like cells and lymphocytes migrate to the eyes (Grotmol et al., 1997; Nilsen et al., 2001; Munday et al., 2002). Taking those results together, we hypothesized that the gCXCR4 expression is related to NNV infection and which may cause by the immune related cells migration. However, too much gCXCR4 in cell could result in significant growth obstruction which might due to the other functions of CXCR4 (Bleul et al., 1996; Ganju et al., 1998).

In summary, our data indicated that the expression of grouper CXCR4 is regulated by LPS or NNV challenge, and is expressed during embryogenesis, speculating its importance in both immune and early developmental stage. The characterization of gCXCR4 between a teleost fish and mammalians has provided valuable information for future functional analysis of the gene.

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451

Legends of figures

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the open reading frame of *Epinephelus coioides* cDNA. The bold letters represent the start codon (ATG) and the stop codon (TAG). The regions of seven-transmembrane domains are at amino acids 50 –72, 85–104, 119–141, 161–180, 216–238, 259–281 and 301–323. The Genbank accession number of gCXCR4 is HQ185191.

Fig. 2. Protein alignment and analysis of gCXCR4 with homologues from other species. The other species used for comparison were *Homo sapiens* (GenBank accession number CAA12166), *Mus muluscus* (GenBank accession number AAH31665), *Cyprinus carpio*, *Oncorhynchus mykiss* (GenBank accession number CAA04493) and *Psetta maxima* (GenBank accession number ABP48751). The black letters denote the consensus sequence of CXCR4 of the different species. The bars represent transmembrane region of gCXCR4 determined using the TMHMMM program in the ExPASy Proteomics Server database. The DRY motif is boxed. The asterisk represents conserved cysteine residue on each of the four extracellular domains that located at positions 33aa, 118aa, 198aa and 292aa.

Fig. 3. Phylogenetic analysis of CXCR4 protein family members. The amino acids of the different *CXCR4* species obtained from the NCBI GenBank were aligned using ClustalW. The Neighbor-Joining tree was created by MEGA4.0 software with a bootstrap value of 1,000. Accession numbers of chemokine receptors amino acid sequences obtained from

GenBank were: *Homo sapiens* CXCR1 NP_000625; *Mus musculus* CXCR1 NP_839972; *Cyprinus carpio* CXCR1 BAA31458; *Takifugu rubripes* CXCR1 NP_001072110; *Homo sapiens* CXCR2 NP_001161770; *Mus musculus* CXCR2 NP_034039; *Bos taurus* CXCR2 ABC59060; *Homo sapiens* CXCR3 EAX05283; *Danio rerio* CXCR3a NP_001082899; *Ctenopharyngodon idella* CXCR3 AAW69766; *Petromyzon marinus* CXCR4 AAO21209; *Epinephelus coioides* CXCR4 HQ185191; *Homo sapiens* CXCR4 CAA12166; *Mus musculus* CXCR4 AAH98322; *Bos taurus* CXCR4 NP_776726; *Sus scrofa* CXCR4 AAZ32767; *Cyprinus carpio* CXCR4 BAA32797; *Oncorhynchus mykiss* CXCR4 CAA04493; *Salmo salar* CXCR4 BT060355; *Danio rerio* CXCR4 AAF1756; *Ictalurus punctatus* CXCR4 ACS45337; *Acipenser ruthenus* CXCR4 CAB60252; *Xenopus laevis* CXCR4 AAI10722; *Oryzias latipes* CXCR4 ABC41565; *Psetta maxima* CXCR4 ABP48751; *Homo sapiens* CXCR5 AAI10353; *Mus musculus* CXCR5 AAH64059; *Ctenopharyngodon idella* CXCR5 ACZ06880; *Mus musculus* CXCR6 NP_109637; *Homo sapiens* CXCR6 *NP_006555*; *Bos taurus* CXCR6 NP_001014859; *Homo sapiens* CXCR7 NP_064707; *Mus musculus* CXCR7 NP_031748 and *Xenopus laevis* CXCR7 NP_001082236.

Fig. 4. Gene expression profile of *gCXCR4* was examined in different development stages of *Epinephelus coioides*. The total RNA was isolated from different stages and gene expression of gCXCR4 was determined by real-time PCR.

Fig. 5. Effects of overexpression of gCXCR4 on GF-1 cell proliferation. The cells proliferation was quantified by measuring MTT absorbance at 570 nm. Vertical bars indicate the mean ± S.E (N=3). $^{**}p < 0.01$. The Blank was the spontaneous proliferation of GF-1 cells without treating any plasmid; the GFP was the group transfected with the same backbone of the plasmid to the gCXCR4-GFP group without inserting the gCXCR4.

Fig. 6. Expression of gCXCR4 in (A) head kidney and (B) intestine of healthy grouper using immunohistofluorescence staining. (a) and (d): Nucleus was detected using Hoechst 33342 (blue). (b) Control experiments were carried out with control rabbit antiserum as the primary antiserum, and visualized with Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (red). (e) The expression of gCXCR4 was detected using rabbit anti-gCXCR4 antiserum and visualized with Alexa Fluor® 594 goat anti-rabbit IgG (H+L). (c) Merged image from figures (a) and (b). (f) Merged image from figures (d) and (e). Bars = 1 mm (A) and 50 μm (B).

Fig. 7. Gene expression of *gCXCR4* in different tissues. (A) RT-PCR and (B) real-time PCR analysis of *gCXCR4* gene expression in different tissues including eye, fin, gill, muscle, head kidney, heart, spleen ,intestine and brain of healthy adult grouper. *β-actin* amplification was used as an internal control. Vertical bars indicate the mean ± S.E (N=3). $^{*}p < 0.05$.

Fig. 8. Expression level of gCXCR4 mRNA in grouper after challenge with LPS (A)

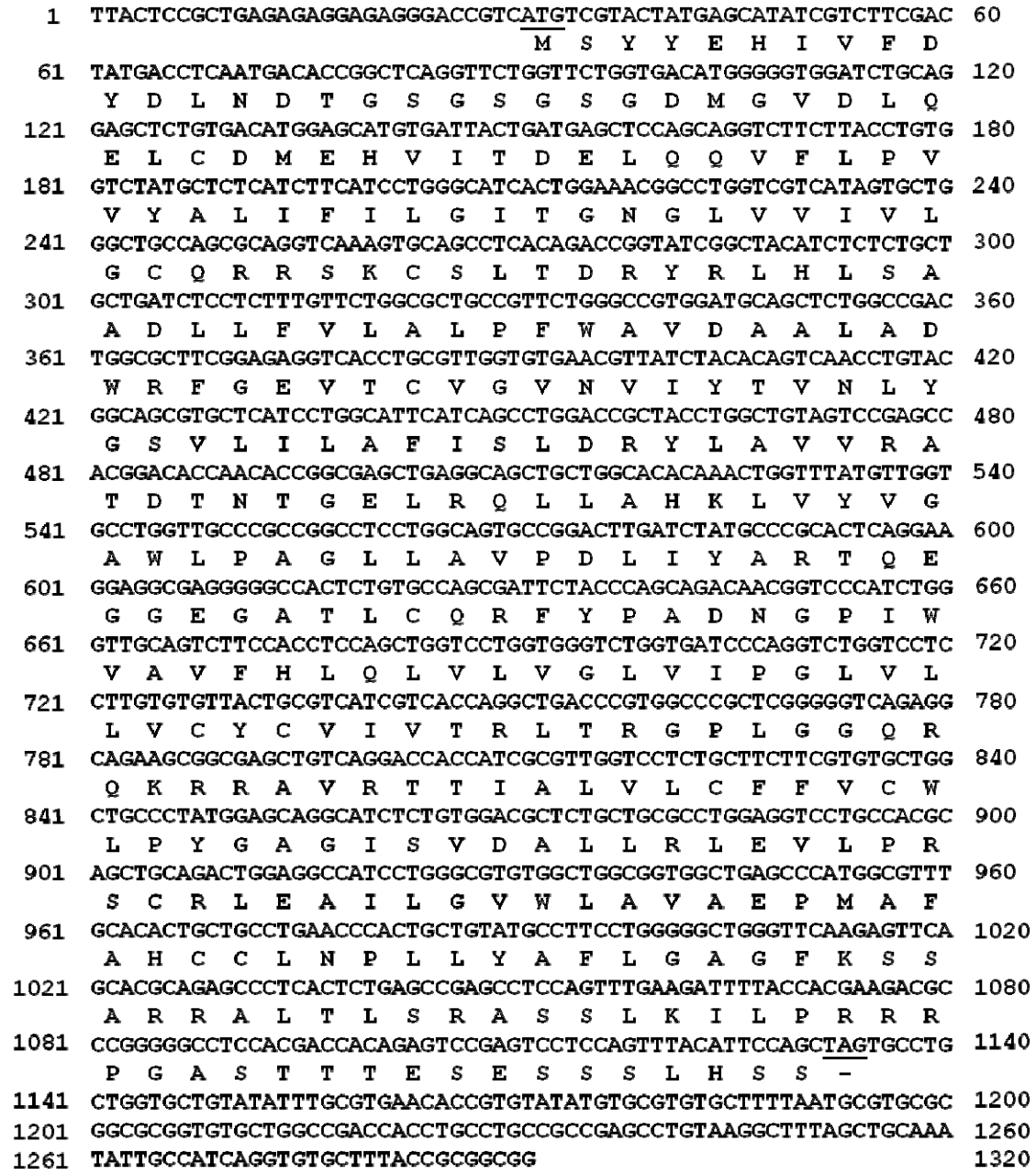
509 **and NNV (B)**. (A) Relative expression level of gCXCR4 mRNA in spleen of grouper after

510 challenge with LPS or PBS. (B) Analysis of expression of *gCXCR4* gene in control or NNV

511 infected juvenile grouper or eye of juvenile grouper groups by real-time RT-PCR. gCXCR4

512 mRNA levels (relative to *β-actin* mRNA) between different time were compared by the

513 t-test. Vertical bars indicate the mean ± S.E (N=3). *$p < 0.05$.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M S Y Y E H I V F D
61 TATGACCTCAATGACACCGGCTCAGGTTCTGGTTCTGGTGACATGGGGGTGGATCTGCAG 120
Y D L N D T G S G S G S G D M G V D L Q
121 GAGCTCTGTGACATGGAGCATGTGATTACTGATGAGCTCCAGCAGGTCTTCTTACCTGTG 180
E L C D M E H V I T D E L Q Q V F L P V
181 GTCTATGCTCTCATCTTCATCCTGGGCATCACTGGAAACGGCCTGGTCGTCATAGTGCTG 240
V Y A L I F I L G I T G N G L V V I V L
241 GGCTGCCAGCGCAGGTCAAAGTGCAGCCTCACAGACCGGTATCGGCTACATCTCTCTGCT 300
G C Q R R S K C S L T D R Y R L H L S A
301 GCTGATCTCCTCTTTGTTCTGGCGCTGCCGTTCTGGGCCGTGGATGCAGCTCTGGCCGAC 360
A D L L F V L A L P F W A V D A A L A D
361 TGGCGCTTCGGAGAGGTCACCTGCGTTGGTGTGAACGTTATCTACACAGTCAACCTGTAC 420
W R F G E V T C V G V N V I Y T V N L Y
421 GGCAGCGTGCTCATCCTGGCATTCATCAGCCTGGACCGCTACCTGGCTGTAGTCCGAGCC 480
G S V L I L A F I S L D R Y L A V V R A
481 ACGGACACCAACACCGGCGAGCTGAGGCAGCTGCTGGCACACAAACTGGTTTATGTTGGT 540
T D T N T G E L R Q L L A H K L V Y V G
541 GCCTGGTTGCCCGCCGGCCTCCTGGCAGTGCCGGACTTGATCTATGCCCGCACTCAGGAA 600
A W L P A G L L A V P D L I Y A R T Q E
601 GGAGGCGAGGGGGCCACTCTGTGCCAGCGATTCTACCCAGCAGACAACGGTCCCATCTGG 660
G G E G A T L C Q R F Y P A D N G P I W
661 GTTGCAGTCTTCCACCTCCAGCTGGTCCTGGTGGGTCTGGTGATCCCAGGTCTGGTCCTC 720
V A V F H L Q L V L V G L V I P G L V L
721 CTTGTGTGTTACTGCGTCATCGTCACCAGGCTGACCCGTGGCCCGCTCGGGGGTCAGAGG 780
L V C Y C V I V T R L T R G P L G G Q R
781 CAGAAGCGGCGAGCTGTCAGGACCACCATCGCGTTGGTCCTCTGCTTCTTCGTGTGCTGG 840
Q K R R A V R T T I A L V L C F F V C W
841 CTGCCCTATGGAGCAGGCATCTCTGTGGACGCTCTGCTGCGCCTGGAGGTCCTGCCACGC 900
L P Y G A G I S V D A L L R L E V L P R
901 AGCTGCAGACTGGAGGCCATCCTGGGCGTGTGGCTGGCGGTGGCTGAGCCCATGGCGTTT 960
S C R L E A I L G V W L A V A E P M A F
961 GCACACTGCTGCCTGAACCCACTGCTGTATGCCTTCCTGGGGGCTGGGTTCAAGAGTTCA 1020
A H C C L N P L L Y A F L G A G F K S S
1021 GCACGCAGAGCCCTCACTCTGAGCCGAGCCTCCAGTTTGAAGATTTTACCACGAAGACGC 1080
A R R A L T L S R A S S L K I L P R R R
1081 CCGGGGGCCTCCACGACCACAGAGTCCGAGTCCTCCAGTTTACATTCCAGCTAGTGCCTG 1140
P G A S T T T E S E S S S L H S S -
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Figure 1

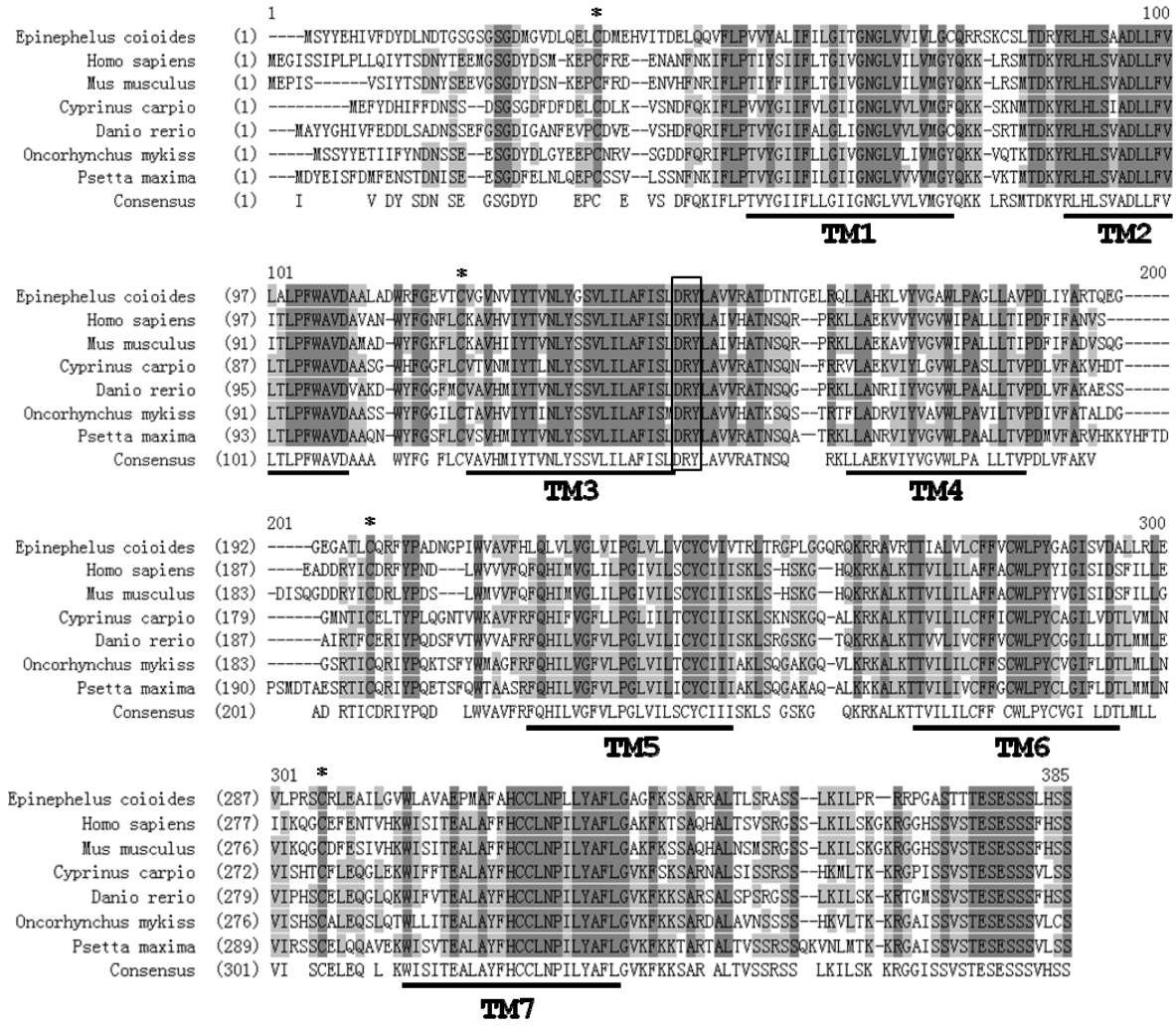
Epinephelus coioides
Homo sapiens
Mus musculus
Cyprinus carpio
Danio rerio
Oncorhynchus mykiss
Psetta maxima
Consensus
TM1
TM2
TM3
TM4
TM5
TM6
TM7

Figure 2

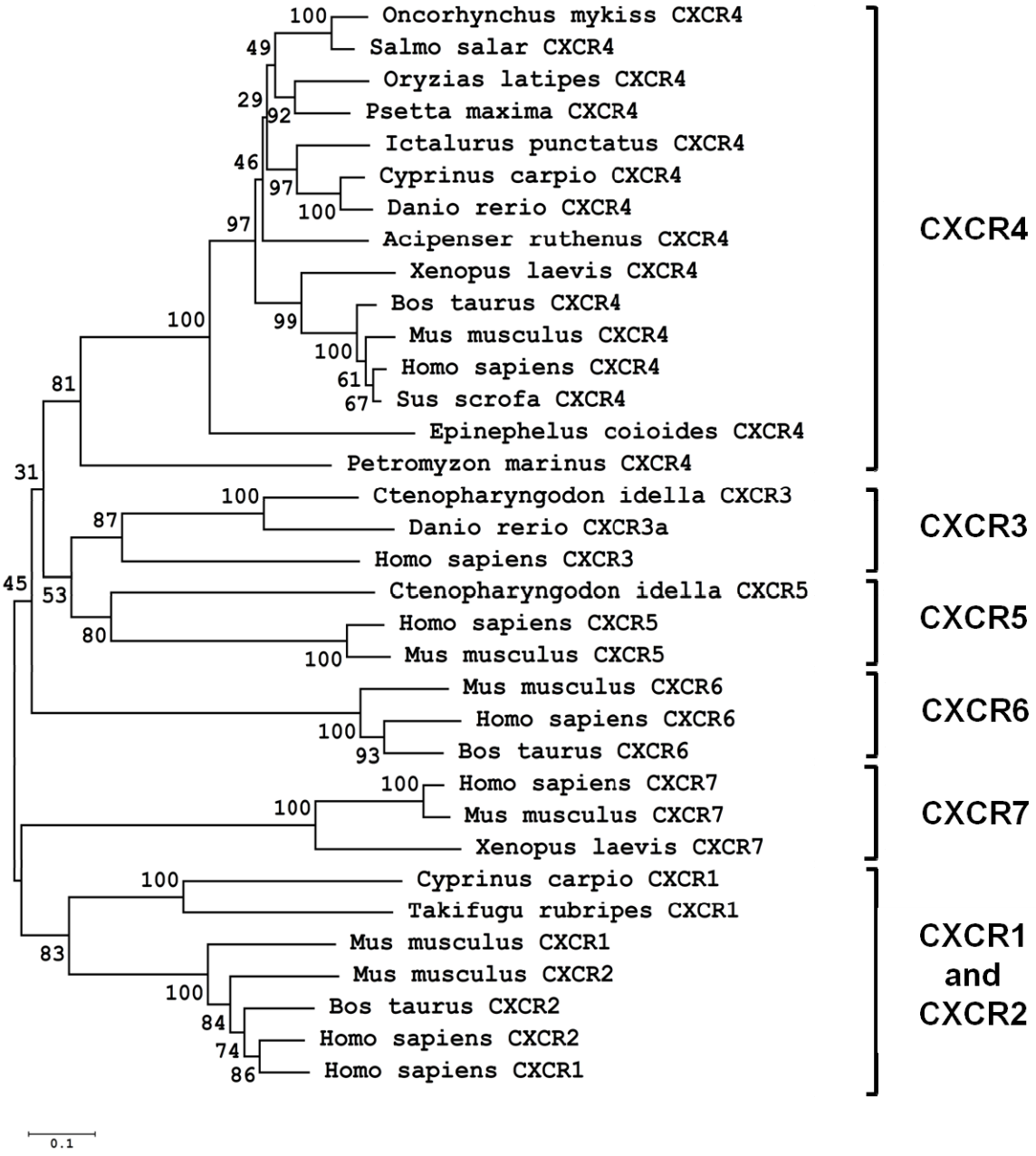
Oncorhynchus mykiss CXCR4
Salmo salar CXCR4
Oryzias latipes CXCR4
Psetta maxima CXCR4
Ictalurus punctatus CXCR4
Cyprinus carpio CXCR4
Danio rerio CXCR4
Acipenser ruthenus CXCR4
Xenopus laevis CXCR4
Bos taurus CXCR4
Mus musculus CXCR4
Homo sapiens CXCR4
Sus scrofa CXCR4
Epinephelus coioides CXCR4
Petromyzon marinus CXCR4
Ctenopharyngodon idella CXCR3
Danio rerio CXCR3a
Homo sapiens CXCR3
Ctenopharyngodon idella CXCR5
Homo sapiens CXCR5
Mus musculus CXCR5
Mus musculus CXCR6
Homo sapiens CXCR6
Bos taurus CXCR6
Homo sapiens CXCR7
Mus musculus CXCR7
Xenopus laevis CXCR7
Cyprinus carpio CXCR1
Takifugu rubripes CXCR1
Mus musculus CXCR1
Mus musculus CXCR2
Bos taurus CXCR2
Homo sapiens CXCR2
Homo sapiens CXCR1
CXCR4
CXCR3
CXCR5
CXCR6
CXCR7
CXCR1 and CXCR2
0.1

Figure 3

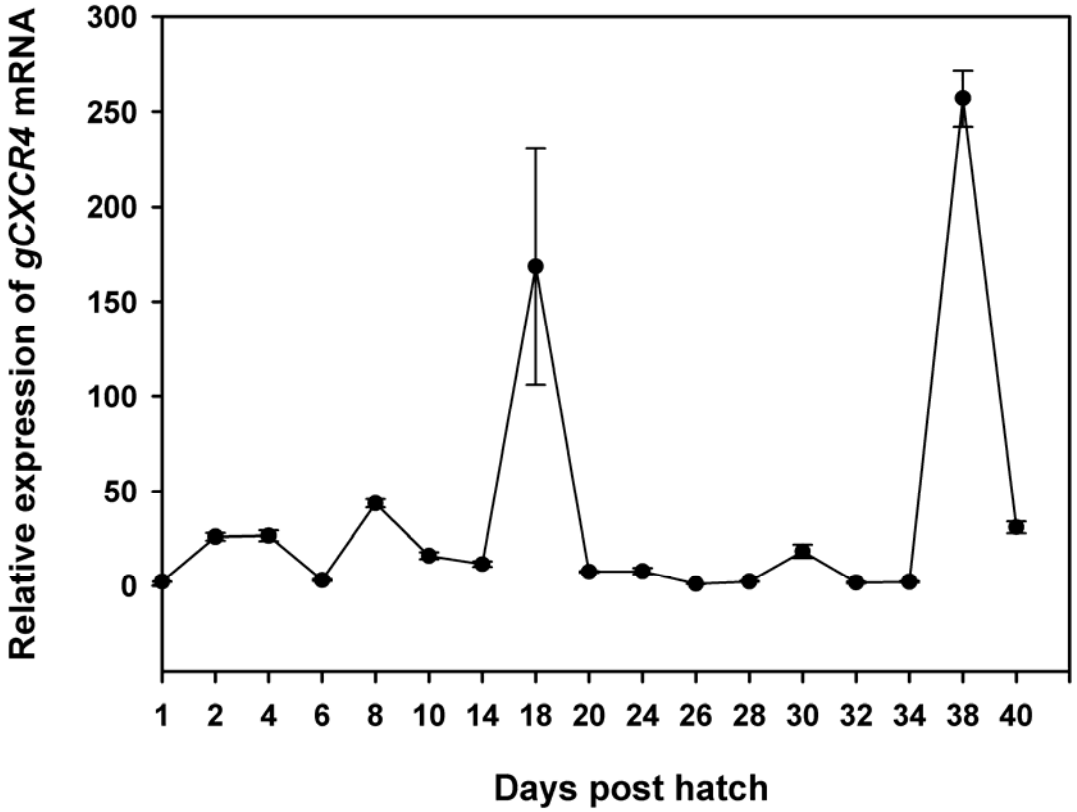
Relative expression of gCXCR4 mRNA
300
250
200
150
100
50
0
1
2
4
6
8
10
14
18
20
24
26
28
30
32
34
38
40
Days post hatch

Figure 4

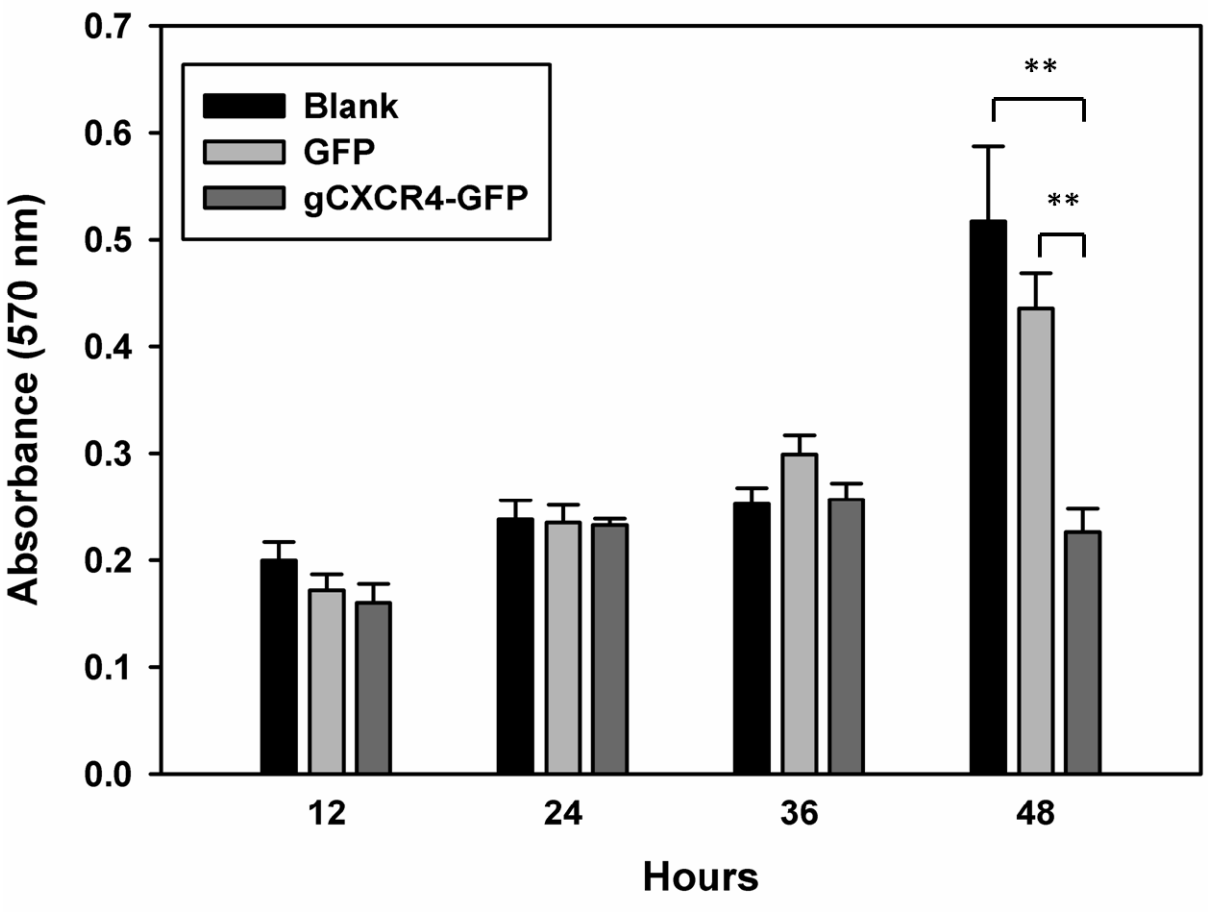
Blank
GFP
gCXCR4-GFP
Absorbance (570 nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
12
24
36
48
Hours
**
**

Figure 5

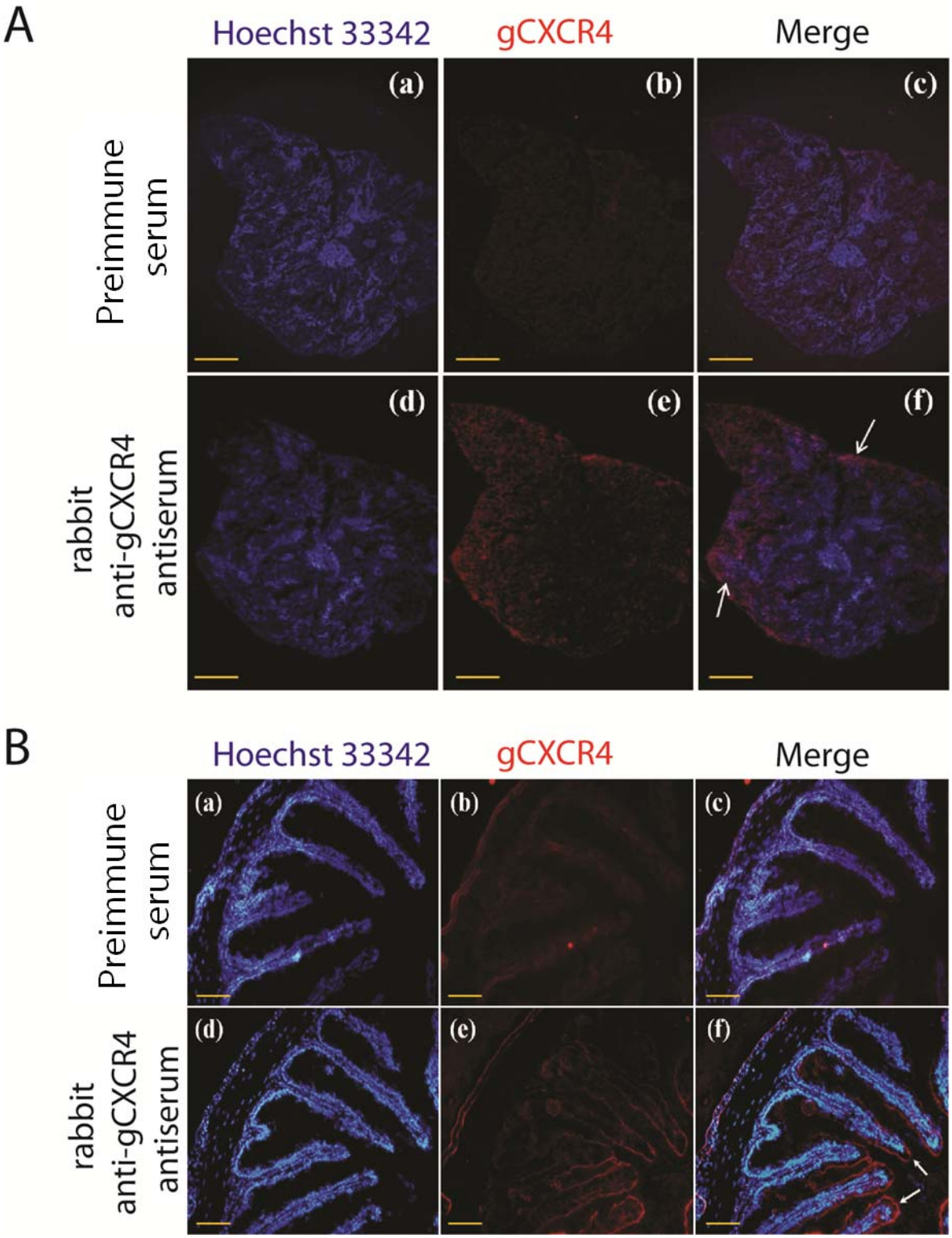
A
Hoechst 33342
gCXCR4
Merge
Preimmune serum
rabbit anti-gCXCR4 antiserum
(a)
(b)
(c)
(d)
(e)
(f)
B
Hoechst 33342
gCXCR4
Merge
Preimmune serum
rabbit anti-gCXCR4 antiserum
(a)
(b)
(c)
(d)
(e)
(f)

Figure 6

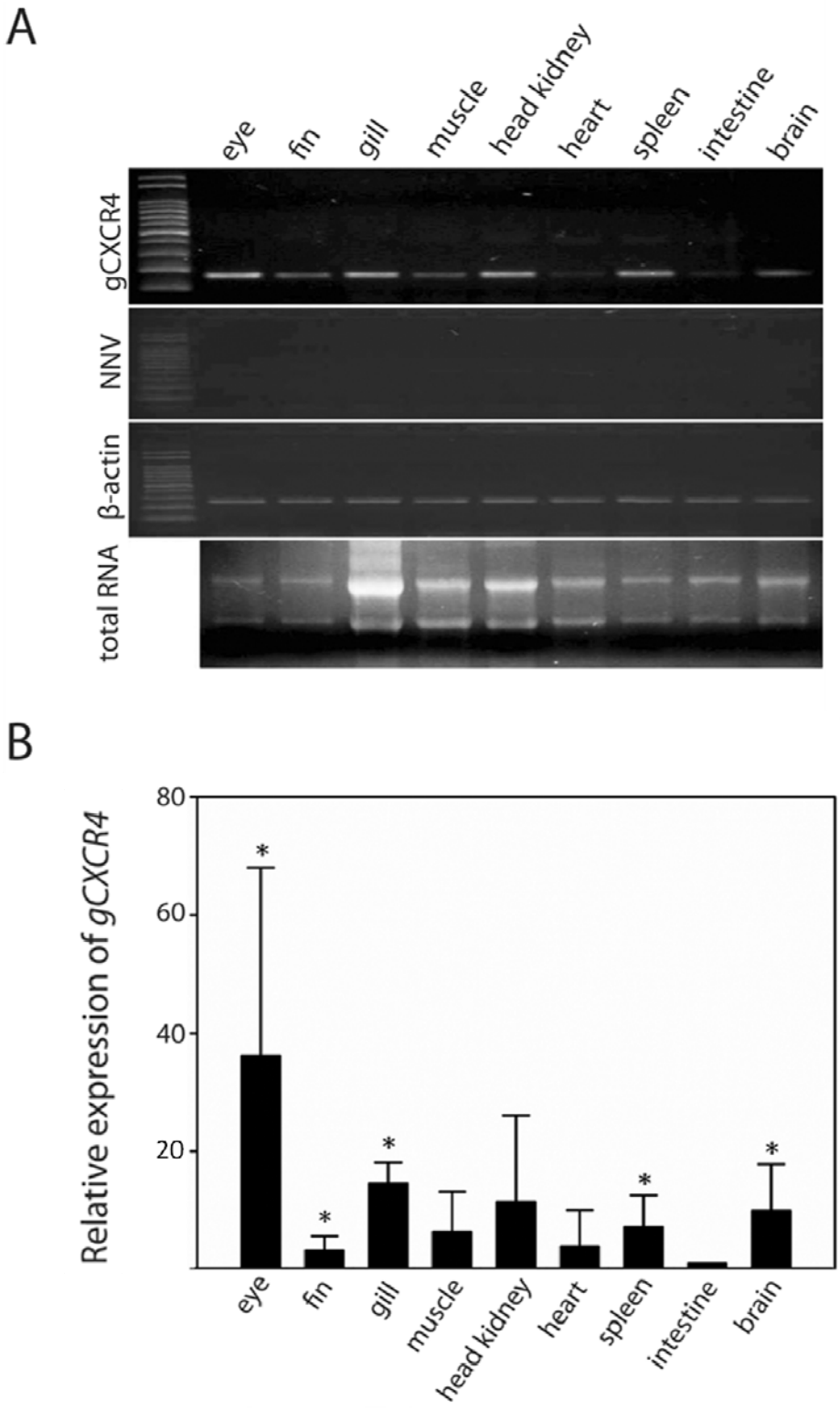
A
eye
fin
gill
muscle
head kidney
heart
spleen
intestine
brain
gCXCR4
NNV
β-actin
total RNA
B
Relative expression of gCXCR4
80
60
40
20
*
eye
fin
gill
muscle
head kidney
heart
spleen
intestine
brain

Figure 7

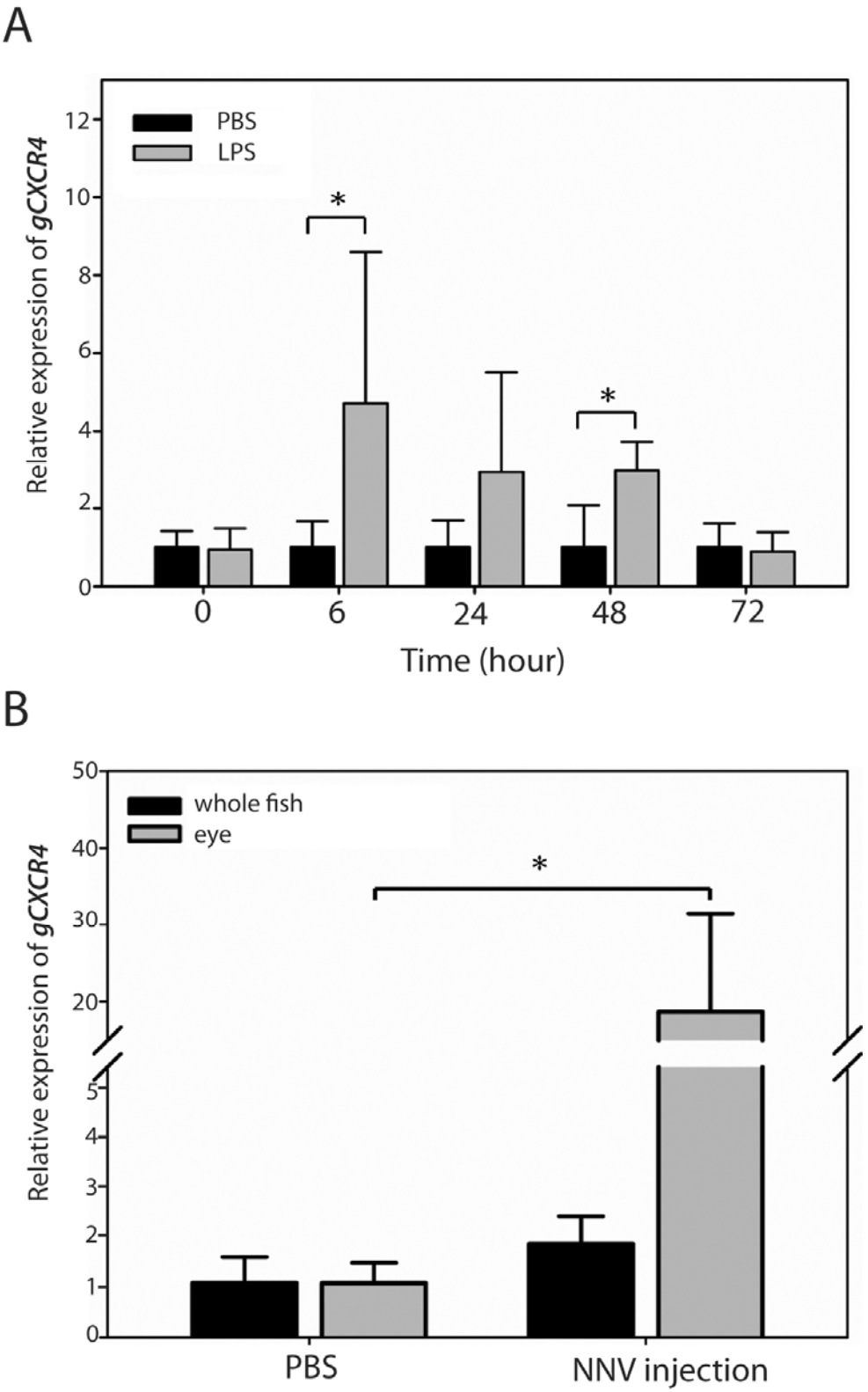
A
PBS
LPS
Relative expression of gCXCR4
12
10
8
6
4
2
0
*
*
0
6
24
48
72
Time (hour)
B
whole fish
eye
Relative expression of gCXCR4
50
40
30
20
5
4
3
2
1
0
*
PBS
NNV injection

Figure 8

Table1. Primers used in this study

Name	Sequence
gCXCR4-5SP1	CAGTAACACACAAGGAGGACCAG
gCXCR4-5SP2	GCTGATGAATGCCAGGATGAGCACACT
gCXCR4-5SP3	AGCAGCAGAGAGATGTAGCCG
gCXCR4-3SP1	CGCTACCTGGCTGTAGTCCGA
gCXCR4-3SP2	CTTCGTGTGCTGGCTGCCGTATGG
gCXCR-F	ATGTCGTACTATGAGCATATCGTCTTCG
gCXCR-R	CTAGCTGGAATGTAAACTGGAGGACTC
gCXCR4-GFP-R	GGCTGGAATGTAAACTGGAGGACTC
gCXCR4-EXI-F	ATCGTAGGATCCGATGTCGTACTATGAGCAT
gCXCR4-EXI-R	ACGATGTCGACAGGTAAGAAGACCTGCTGGAG
gCXCR4-EXIII-F	ATCGTAGTCGACCCGGACTTGATCTATGCCC
gCXCR4-EXIII-R	ACGATCTCGAGGTGGAAGACTGCAACCCAG
Q-gCXCR4-F	CACACTGCTGCCTGAACCCACTGCT
Q-gCXCR4-R	CTAGCTGGAATGTAAACTGGAGGACTC
β-actin-F	TGCCTCTGGTCGTACCACTGGTATTGTC
β-actin-R	GGCAGCAGTGCCCATCTCCTGCTCGA

Table 2. Protein sequences similarity* of CXCR4 from different species

	E. coioides	*H. sapiens*	*M. musculus*	*C. carpio*	*D. rerio*	*O. mykiss*	*P. maxima*
				Extracellular domains			
E. coioides	100	22.1	8.5	28.8	32.2	33.6	29.3
H. sapiens		100	15.9	30.3	36.3	31.9	30.3
M. musculus			100	13.7	16.2	14.6	14.9
C. carpio				100	75.5	43.4	33.1
D. rerio					100	51.4	37.3
O. mykiss						100	46.6
P. maxima							100
				Transmembrane domains			
E. coioides	100	57.7	32.9	59.0	59.3	59.1	62.3
H. sapiens		100	35.4	71.6	70.7	75.0	75.2
M. musculus			100	37.9	36.0	37.3	37.9
C. carpio				100	92.3	80.1	84.0
D. rerio					100	77.8	82.7
O. mykiss						100	84.8
P. maxima							100
				Intracellular domains			
E. coioides	100	43.6	15.8	44.0	45.4	41.4	38.8
H. sapiens		100	24.8	64.4	62.7	58.4	59.4
M. musculus			100	20.2	19.8	22.2	21.4
C. carpio				100	89.8	73.5	74
D. rerio					100	71.4	73.5
O. mykiss						100	76
P. maxima							100

*the number was showed in percentage