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Molecular characterization and expression analysis of Asian swamp eel (*Monopterus albus*) CXC chemokine receptor (CXCR) 1a, CXCR1b, CXCR2, CXCR3a, CXCR3b, and CXCR4 after bacteria and poly I:C challenge

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ABSTRACT

The CXC chemokine receptors (CXCRs) play critical roles in innate and adaptive immune systems. In this study, six Asian swamp eel (*Monopterus albus*) CXCRs (*Ma*CXCR1–4) were identified and their molecular characterization and expression patterns were analyzed. The open reading frames (ORFs) of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b, and *Ma*CXCR4 were 1074 bp (base pairs), 1080 bp, 1125 bp, 1146 bp, 1083 bp, and 1140 bp, and encoded proteins of 357 aa (amino acids), 359 aa, 374 aa, 381 aa, 360 aa, and 379 aa, respectively. All these CXCRs have seven conserved transmembrane domains and four cysteines (with the exception of *Ma*CXCR3b). Multiple sequence alignment revealed that the *Ma*CXCRs possess a typical G-protein receptor family 1 signature and a DRY motif. There are also one to four potential N-glycosylation sites in the extracellular regions of the *Ma*CXCRs, mainly distributed in the N-terminus and extracellular hydrophilic loop (ECL) 2 region. Phylogenetic analysis demonstrated that the *Ma*CXCRs were clustered together with homologous proteins from other fish. Taken together with the amino acid identity and similarity analysis, these results suggested that the *Ma*CXCRs are conserved with other homologous genes, in which CXCR4 is more conserved than CXCR1–3. The *Ma*CXCRs loci showed conserved synteny among teleost fish, and we found that human CXCR1 shares a common ancestor with fish CXCR1a. *Ma*CXCRs were constitutively expressed in a wide range of tissues (especially in immune-related tissues) with different expression levels, suggesting that the *Ma*CXCRs have different roles in un-stimulated tissues, and may play vital roles under normal conditions. *Ma*CXCRs showed different fold changes in the spleen after *Aeromonas veronii* and polyinosinic-polycytidylic acid (poly I:C) challenge, which suggested that *Ma*CXCR1a and *Ma*CXCR3a have longer antiviral activities compared with their antibacterial functions, and that *Ma*CXCR1b possesses stronger antiviral than antibacterial activity. *Ma*CXCR4 may play vital roles during bacterial and viral infection; however, *Ma*CXCR2 has relatively small effect in antibacterial and antiviral responses. The differential responses of these genes to bacteria and poly I:C implied the differences in the mechanisms of defense against viruses and bacteria.

1. Introduction

Leucocytes play critical roles in innate and adaptive immune systems. During this process, the movement of leucocytes is mediated primarily by the chemokine system, including the chemokine receptors, which interact with a group of peptide ligands and are indispensable for the coordination of migration in diverse physiological processes, such as development, angiogenesis, immune defense and neuroendocrine regulation [1–3]. Based on which ligand they bind, the chemokine receptors can be classified into four subfamilies, including CC chemokine

receptors (CCRs), CXCRs, XCRs, and CX₃CRs [4,5]. They belong to the largest rhodopsin family of G protein-coupled receptors (GPCRs) and inherited the majority of the repertoires of GPCRs [6]. Structurally, the CXCRs possess seven transmembrane domains (TM1–7), an extracellular N-terminal region, three extracellular hydrophilic loops (ECL1–3), three intracellular loops (ICL1–3) and an intracellular C-terminal region [7].

C-X-C chemokine receptor type 1 (CXCR1) and CXCR2, which mediate leukocyte migration, activation and regulation, have been well characterized in vertebrates [8,9]. CXCR1 primarily binds with CXCL6 and CXCL8 [10], while CXCR2 interacts with CXCL1–3 and CXCL5–8 [11]. Recent studies have shown that CXCR1 predominantly couples to GPK2, whereas CXCR2 interacts with GPK6 to negatively regulate receptor sensitization and trafficking to influence cell signaling and angiogenesis [12]. This can lead to functional differences. One CXCR2 and two CXCR1 (termed CXCR1a and CXCR1b) homologs have been identified in a wide range of teleost fish species, including common carp [13], rainbow trout [14] and zebrafish [15,16]. In mammals, a single CXCR3 has been identified; however, alternative splicing gives rise to two transcript variants [17]. CXCR3 is highly expressed on effector T cells and plays an important role in T cell trafficking and function [18]. CXCR3 can be activated by CXCL9–11 [19]. In teleost fish, two apparent CXCR3 genes are found, termed CXCR3a and CXCR3b [14,20,21]. Among the CXCRs, CXCR4 is the most well studied receptor because of its critical roles in development, and its association with health and disease [22,23]. CXCR4, as well as CXCR5 and CXCR6/Bonzo, has been reported as a coreceptor of HIV and SIV for entry into host cells [24–26]. CXCR4 can bind to CXCL12 to exert its various functions [27]. In recent years, CXCR4 has been characterized with respect to its innate immune role in certain fish species [28–32]. Despite reports of these CXCRs in teleost fish, the function of fish CXCR1–4 are largely unknown, especially in economically important fish species, such as the Asian swamp eel (*Monopterus albus*).

In the present study, Asian swamp eel CXCR1a (*Ma*CXCR1a), *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b, and *Ma*CXCR4 were identified from the Asian swamp eel genome. To explore the features and functions of these CXCRs, we focused on the identification and molecular characterization of *Ma*CXCRs, and expression analysis of the *Ma*CXCRs were conducted following immune stimulations.

2. Materials and methods

2.1. Fish

Asian swamp eels (30–35 g) were supplied by the Yangtze University Aquaculture Base (Jingzhou, Hubei, China). The fish were maintained with a flow-through water supply at 28 °C ± 1 °C for two weeks, and no clinical signs were observed during this period. The fish were fed twice daily with appropriate feed. Healthy fish were used for the experiments.

2.2. Preparation of tissues cDNA samples

Four healthy fish were sacrificed and nine tissues (muscle, liver, spleen, kidney, intestine, heart, brain, skin, and blood) were collected and homogenized in the Trizol reagent (Invitrogen, Carlsbad, CA, USA).

Total RNAs were extracted using the Trizol reagent and treated with RNase-free DNase I (Thermo Scientific, USA). The cDNA was synthesized using Oligo (dT)18 primers according to the instructions of the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA), as described previously [33,34]. The synthesized cDNA was diluted with Tris-EDTA (TE) buffer and kept at −80 °C for quantitative real-time PCR (qPCR) analysis.

Table 1
Primers used for this study.

Name	Sequence (5′-3′)	Usage
CXCR1a-tF	TGTGCAGAAGTCATGGAGTC	ORF cloning
CXCR1a-tR	GCCACCGAGGTTTCAGAAA	ORF cloning
CXCR1b-tF	CAGTGCTTGACGGAAAGAAGA	ORF cloning
CXCR1b-tR	GGTGGTCTCACATGAATGTAGAT	ORF cloning
CXCR2-tF	CAGATACTATGGTGCCCTGAAAT	ORF cloning
CXCR2-tR	TTCTCCCATTACAGCACAAACA	ORF cloning
CXCR3a-tF	AATGCCAGAGGATAATAGTTGGG	ORF cloning
CXCR3a-tR	TAATGCTTGTGTGTCTCTGTCA	ORF cloning
CXCR3b-tF	CTCAACGGAAGCCCTTTATGA	ORF cloning
CXCR3b-tR	GTTAACTTGCACCTGATGTGG	ORF cloning
CXCR4-tF	AGCGGTGAACATGGAGTATGA	ORF cloning
CXCR4-tR	CTGGAGTTGTTTGTTAGCTTGAC	ORF cloning
CXCR1a-F	TGGTGGTTGCCTTTCTGCTGTG	qPCR
CXCR1a-R	TCCTTGCCCTTCTGAGGTCTGAG	qPCR
CXCR1b-F	TGCTGCTGTCTGGTCTGTTGGA	qPCR
CXCR1b-R	CATCCGCACTGCTGGCATCATAT	qPCR
CXCR2-F	GCTGCTGCGACTTCTACATGCT	qPCR
CXCR2-R	ATGCCGTTGCCTACAATGCTGAA	qPCR
CXCR3a-F	GGAGAACGCCAAGACAGTTACCT	qPCR
CXCR3a-R	GCAGCCAAGAGACCTCAGGATG	qPCR
CXCR3b-F	TGGCTCAGAAGAGGCAGTATTGG	qPCR
CXCR3b-R	GATGATGGACAGGCAGCGATCT	qPCR
CXCR4-F	GCCACTAACAGCCAAGCCATAAG	qPCR
CXCR4-R	GCAGTAGCAGATGAGGATGACCA	qPCR
EF-1α-F	CGGTGTGAAGCAGCTCATCGT	qPCR
EF-1α-R	GCAGAGTGGTTCCAGTGGCATT	qPCR

2.3. Gene sequences

Identification of the homologous CXCR genes were performed using the Asian swamp eel genome (Accession No: AONE00000000.1). Specific primers (Table 1) were designed to clone CXCR ORFs (Open Reading Frames) to verify the full-length gene using PCR amplification. All PCR products were then ligated into pMD18-T (Takara, Japan) and sequenced. The sequences were further assembled using SeqMan Pro (version 7.1.0) software and aligned with CXCR genes.

All primers were designed using Primer Premier 5.0 (Table 1) according to the obtained sequences. The primers used for qPCR were pretested to ensure that each primer pair could amplify the cDNA.

2.4. Phylogenetic tree, sequence and gene synteny analysis

The deduced amino acid sequences were predicted using the web-based tool ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). A phylogenetic tree was constructed based on the deduced amino acid multiple alignment using the Neighbor-Joining method in the MEGA software package (version 7.0). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed, and the bootstrap values of the major branches are shown in the trees as percentages.

The putative amino acid similarity and identity were calculated using MatGAT 2.01 software [35]. The signal peptides were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane domains were analyzed using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The properties of the proteins were determined using various software programs: The theoretical pI (isoelectric point) and Mw (molecular weight) tool (https://web.expasy.org/compute_pi/); and ExPASy Prosite (https://prosite.expasy.org/) to identify N-glycosylation sites, G protein-coupled receptors family 1 signature, and DRY motifs. Multiple alignments of amino acids were carried out using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and decorated using the R software (version 3.5, https://www.r-project.org/) and Bioconductor (msa package: https://bioconductor.org/packages/release/bioc/html/msa.html) [36]. The decorated shading mode was set to be similar mode of amino acid

sequences, as described previously [37].

The DNA and cDNA sequences were aligned using Clustal Omega to analyze the exon-intron structures of genes. The intron phase was calculated and labeled based on size. The gene synteny analysis was performed using Ensemble and NCBI database.

2.5. Expression variations of MaCXCRs during bacteria and poly I:C infection

Aeromonas veronii was cultivated and counted, and washed with phosphate-buffered saline (PBS) to eliminate bacterial secretory products before intraperitoneal injection into fish. Polyinosinic-polycytidylic acid (poly I:C; Sigma, USA) was dissolved in PBS. The fish were then stimulated with 100 μL A. *veronii* (1.83×10^6 colony forming units (cfu)/mL) and poly I:C (7 μg/g fish) *in vivo* for 4, 8, 24 and 48 h. Control groups were treated with an equal volume of PBS buffer. The fish were than sacrificed and their spleens were used for qPCR. Each group had four repetitions (n = 4). RNA extraction and cDNA synthesis were performed as previously described [33].

2.6. Quantitative real-time PCR (qPCR)

The expression levels of MaCXCR1–4 were quantified by qPCR using KAPA SYBR® FAST qPCR Master Mix (KAPA BIOSYSTEMS) and a Stepone Plus real-time PCR system (ABI). EF-1α (elongation factor 1 alpha) was included as a common reference gene. For comparison of the relative expression levels of different genes, a standard curve was established using a series of 10-fold dilutions of purified PCR products of each gene amplified from cDNA. A serial of dilution of the standard was run along with the cDNA samples in the same 96-well PCR plate and served as reference for quantification.

The primers used for qPCR analysis and the accession numbers for the identified MaCXCR1–4 are listed in Tables 1 and 2, respectively. The amplification reaction was performed in a volume of 20 μL, containing 4 μL of the corresponding cDNA, 10 μL of KAPA SYBR® FAST qPCR Master Mix, 0.5 μL of each target gene primer (10 μmoL/μL), and 5 μL of sterile water. The following thermocycling conditions were used to determine the expression profiles for each gene: 95 °C for 3 min; followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s; with subsequent incubations at 95 °C for 15 s and 60 °C for 1 min and 95 °C for 15 s to detect fluorescence.

2.7. Statistical analysis

The expression data of each genes was normalized to that of the reference gene EF-1α. The fold change in gene expression was obtained by comparing the normalized gene expression level of the treated groups with the corresponding untreated groups (defined as 1). SPSS 19.0 was used for statistical analysis. One way-analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test (when appropriate) was used to analyze the expression induction data,

Fig. 1. Neighbor-joining phylogenetic tree of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b, and *Ma*CXCR4. The tree was constructed using the deduced amino acid sequence multiple alignment and the neighbor-joining method within the MEGA 7.0 program, using the method based on a Poisson model. The percentage of the tree in which the associated taxa clustered together is shown next to the branches, based on 1000 bootstrap replications. The accession numbers of the sequences used are shown.

Table 2

Summary of sequences analysis of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b, and *Ma*CXCR4.

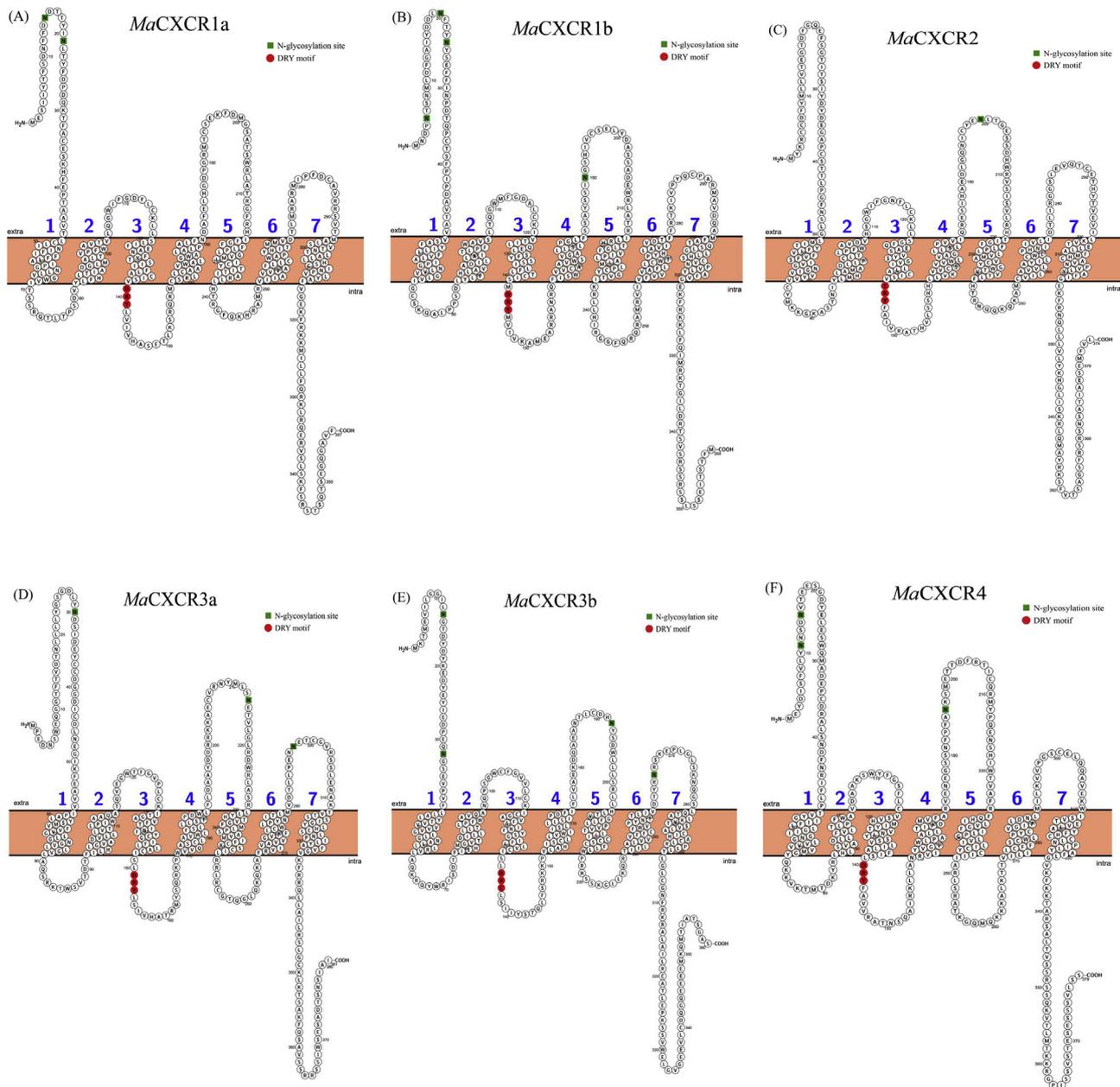| Sequence features	*Ma*CXCR1a	*Ma*CXCR1b	*Ma*CXCR2	*Ma*CXCR3a	*Ma*CXCR3b	*Ma*CXCR4
GenBank Accession NO.	MH706749	MH706750	MH706751	MH706752	MH706753	MH706754
ORF (bp)	1074	1080	1125	1146	1083	1140
Length of amino acids (aa)	357	359	374	381	360	379
Molecular weight (kDa)	40.58	40.17	41.99	42.47	40.41	42.85
Theoretical pI	9.06	8.79	7.55	8.12	6.59	8.86
Conserved cysteine	4	4	4	4	1	4
Transmembrane domain	7	7	7	7	7	7
Signal peptide (aa)	No	No	No	No	No	No
Number of introns	0	1	1	2	2	4
N-glycosylation site	2	4	1	3	4	3
Signature motif (aa)	128–144	131–147	132–148	140–156	123–139	129–145

Fig. 2.

Fig. 2. Deduced amino acid (aa) sequences of *Ma*CXCR1a (A), *Ma*CXCR1b (B), *Ma*CXCR2 (C), *Ma*CXCR3a (D), *Ma*CXCR3b (E), and *Ma*CXCR4 (F). The putative amino acid sequences are shown in circles. The N-glycosylation site and transmembrane domain are marked with a blue frame and in the membrane, respectively. The DRY motif is marked by a red cycle. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with $P < 0.05$, $P < 0.01$, or $P < 0.001$ between control and treatment groups being considered significant, as described previously [34].

3. Results

3.1. Sequences features of MaCXCR1–4

The full ORF of *Ma*CXCR1–4 were verified using PCR amplification. The sequence features of *Ma*CXCR1–4 are summarized in Table 2. The ORFs of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 were 1074 bp (base pairs), 1080 bp, 1125 bp, 1146 bp, 1083 bp and 1140 bp, and encoded putative proteins of 357 aa (amino acids), 359 aa, 374 aa, 381 aa, 360 aa, and 379 aa, respectively. The theoretical pI/molecular weights (kDa) of *Ma*CXCR1a, *Ma*CXCR1b,

*Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 were 9.06/40.58, 8.79/40.17, 7.55/41.99, 8.12/42.47, 6.59/40.41, and 8.86/42.85, respectively. No signal peptides were observed in these sequences.

3.2. Phylogenetic analysis, amino acid characterization, and sequence alignment

To analyze the evolutionary relationships of the newly identified *Ma*CXCR1–4, a phylogenetic tree was established using the putative amino acid sequences (Fig. 1). The tree showed that *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b, and *Ma*CXCR4 were clustered together with homologous proteins from other fish. It was clear that all vertebrate CXCR1 and CXCR2 molecules form a clade that is separate from CXCR3 and CXCR4. The CXCR1/2 clade was further
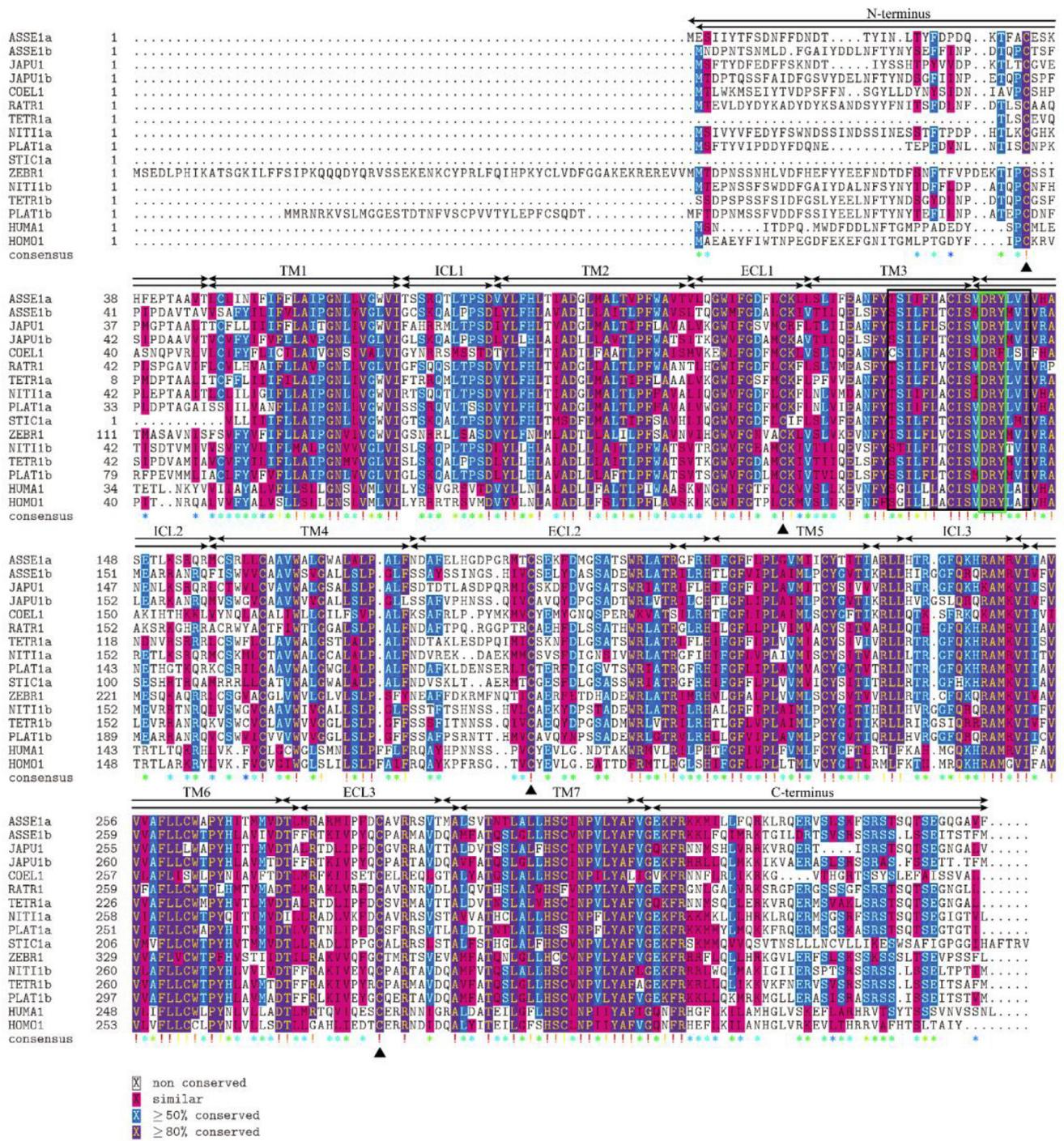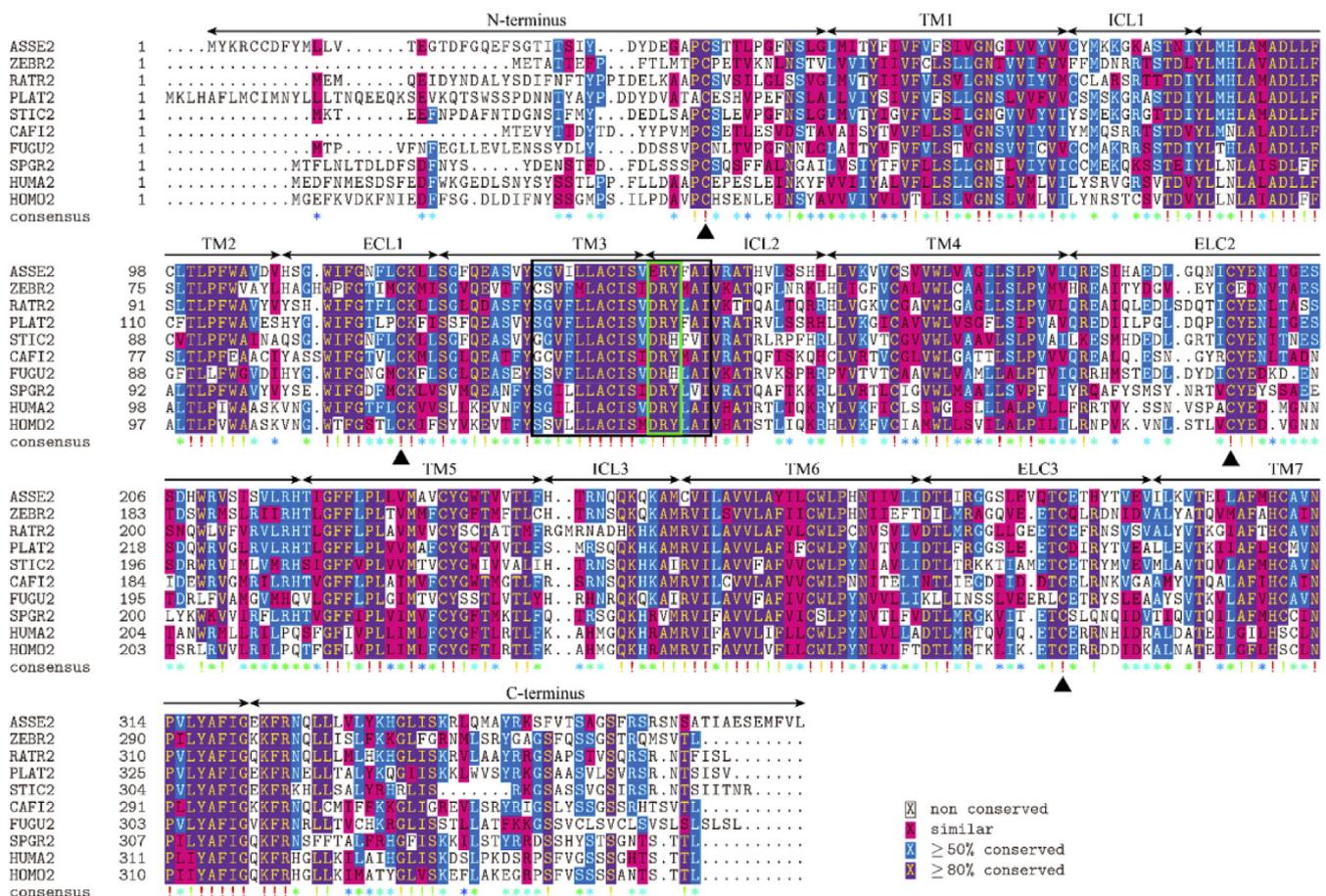
Fig. 3. Multiple alignment of *Ma*CXCR1 with CXCR1 sequences from other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter, representing a sequence position in which all the sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The N-terminus, seven transmembrane domains (TM1–7), the three intracellular (ICL1–3) and extracellular loops (ECL1–3), and the C-terminus are marked above the alignment. The first line with arrowheads represents the *Ma*CXCR1a, and the second line with arrowheads represents *Ma*CXCR1b. The four conserved cysteine residues in each extracellular domain are indicated by black arrows. The G-protein coupled receptors family 1 signature and DRY motif are marked with black and green frames, respectively. The abbreviations are Asian swamp eel (ASSE), Japanese pufferfish (JAPU), Coelacanth (COEL), Rainbow trout (RATR), Tetraodon (TETR), Nile tilapia (NITI), Platyfish (PLAT), Stickleback (STIC), Zebrafish (ZEBR), Human (HUMA) and House mouse (HOMO). The "1", "1a" and "1b" after the species represent CXCR1, CXCR1a and CXCR1b, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

divided into three subclades: CXCR1a, CXCR1b, and CXCR2. The CXCR3 clade was also split into two subclades: CXCR3a and CXCR3b.

All six receptors have seven conserved transmembrane (TM1–7) domains (Fig. 2 A–E). There are also one to four potential N-

glycosylation sites present in the extracellular regions, which are mainly distributed in the N-terminus and ECL2 region (Fig. 2). The DRY motifs of *Ma*CXCR1–4 were also observed in ICL2. This motif appears mainly in the first few amino acids of ICL2 (Fig. 2). Sequence

Fig. 4. Multiple alignment of *Ma*CXCR2 with CXCR2 sequences from other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter, representing a sequence position in which all the sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The N-terminus, seven transmembrane domains (TM1–7), the three intracellular (ICL1–3) and extracellular loops (ECL1–3), and the C-terminus are marked above the alignment. The first line with arrowheads represented the *Ma*CXCR1a, and second line with arrowheads represented *Ma*CXCR1b. The four conserved cysteine residues in each extracellular domain were indicated by black arrow. The G-protein coupled receptors family 1 signature and DRY motif were marked with black and green frame, respectively. The abbreviations were Asian swamp eel (ASSE), Zebrafish (ZEBR), Rainbow trout (RATR), Platyfish (PLAT), Stickleback (STIC), Cave fish (CAFI), Fugu (FUGU), Spotted gar (SPGA), Human (HUMA) and House mouse (HOMO). The "2" after the species represented CXCR2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alignments suggested that fish CXCR1a and CXCR1b both have four conserved cysteine (Fig. 3). The first conserved cysteine is in the N-terminus and the other three are in ECL1–3, respectively. The same phenomenon was observed in *Ma*CXCR2 (Fig. 4), *Ma*CXCR3a (Fig. 5), and *Ma*CXCR4 (Fig. 6). To our surprise, the *Ma*CXCR3b seemed to possess only one conserved cysteine in the ECL1 region (Fig. 5). The multiple alignment showed that *Ma*CXCR1–4 have a typical G protein-coupled receptor family 1 signature sequence, and the DRY motif of corresponding *Ma*CXCR was mainly within this signature (Figs. 3–6).

The amino acid homology of CXCRs was analyzed using MatGAT 2.01 (Tables 3–6). *Ma*CXCR1a shares high aa identity/similarity (%) with proteins from other species, ranging from 42.9/61.6 to 71.3/84.1, While *Ma*CXCR1b shares higher aa identity/similarity (%) with that of other fish, ranging from 68.3/78.6 to 75.8/88.9 (Table 3). Among the *Ma*CXCR1s, their aa identity/similarity (48.4%/69.1%) is low. *Ma*CXCR1a and *Ma*CXCR1b have relatively low aa identity (39.4% and 41.3%, respectively) and similarity (59.7% and 63.5%, respectively) with human CXCR1. *Ma*CXCR2 shares 44.8–62.3% aa identity and 67.1–77.5% aa similarity with its counterparts from teleost fish, and relatively low aa identity (41.0%) and similarity (64.2%) were observed when *Ma*CXCR2 was compared with human CXCR2 (Table 4). *Ma*CXCR3a has high aa identity and similarity with those of teleost fish, ranging from 50.0 to 65.4% aa identity and 70.1–77.7% aa similarity,

while *Ma*CXCR3b has lower aa identity (27.5–34.5%) and similarity (49.7–57.5%) (Table 5). Among *Ma*CXCR3s, their aa identity/similarity (42.4%/59.1%) is low. *Ma*CXCR3a and *Ma*CXCR3b have relatively low aa identity (42.8% and 33.5% respectively) and similarity (61.2% and 54.6%) with human CXCR3. *Ma*CXCR4 have higher aa identity and similarity with that of other fish, ranging from 60.9 to 83.5% aa identity and 75.5–92.3% aa similarity (Table 6). Compared with its human counterpart, *Ma*CXCR4 possesses 61.1% aa identity and 76.0% aa similarity, which were higher than those of *Ma*CXCR1–3.

3.3. Exon-intron structure and gene synteny analysis

The DNA and cDNA sequences were aligned using Clustal Omega to analyze the exon-intron structures of the genes (Fig. 7). No intron was observed in *Ma*CXCR1a, while *Ma*CXCR1b possesses one intron. *Ma*CXCR2 and *Ma*CXCR4 also have two exons/one intron. *Ma*CXCR3a and *Ma*CXCR3b possess three exons/two introns. These intron-containing CXCRs were separated by one to two phase 0 introns.

To help clarify the evolutionary relationship of these molecules, synteny analysis of the genomic loci was carried out using Ensemble and NCBI database. The human CXCR1 and CXCR2 are tandemly linked on chromosome 2 (Fig. 8). In teleost fish, however, two loci for CXCR1 (CXCR1a and CXCR1b) were observed on different chromosomes
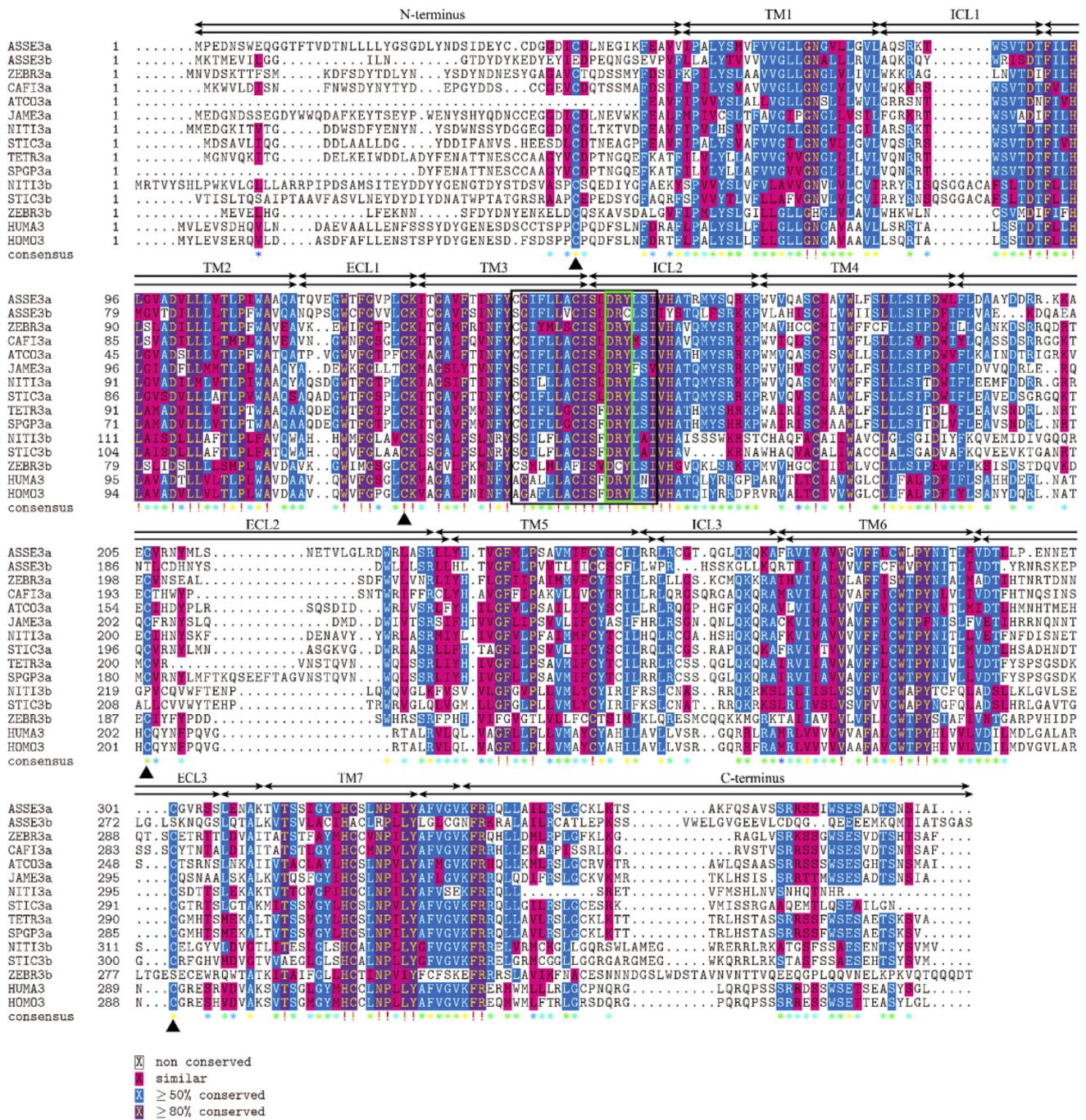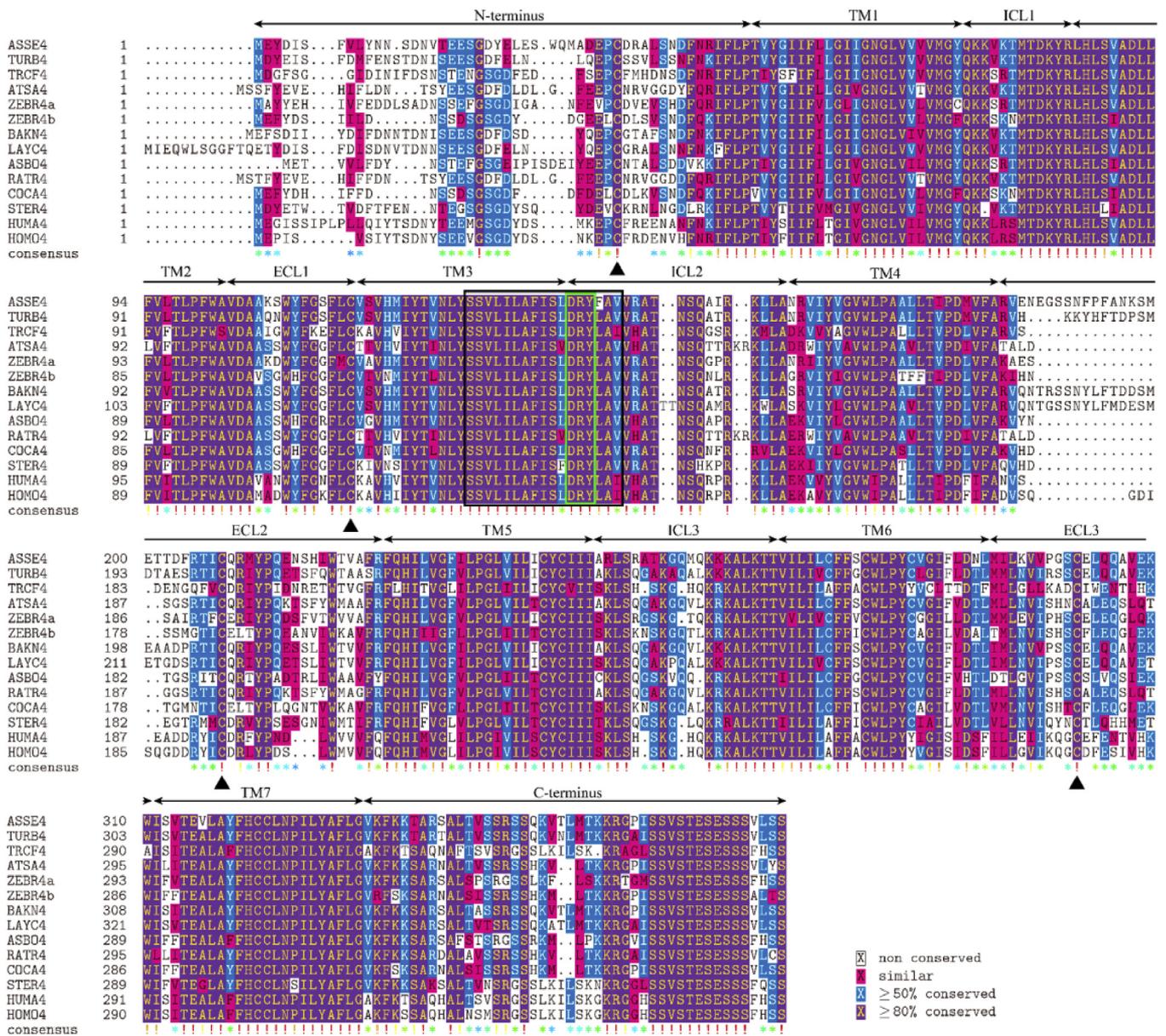
Fig. 5. Multiple alignment of *Ma*CXCR3 with CXCR3 sequences from other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter, representing a sequence position in which all the sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The N-terminus, seven transmembrane domains (TM1–7), the three intracellular (ICL1–3) and extracellular loops (ECL1–3), and the C-terminus are marked above the alignment. The first line with arrowheads represented the *Ma*CXCR1a, and second line with arrowheads represented *Ma*CXCR1b. The four conserved cysteine residues in each extracellular domain were indicated by black arrow. The G-protein coupled receptors family 1 signature and DRY motif were marked with black and green frame, respectively. The abbreviations were Asian swamp eel (ASSE), Zebrafish (ZEBR), Cave fish (CAFI), Atlantic cod (ATCO), Japanese medaka (JAME), Nile tilapia (NITI), Stickleback (STIC), Tetraodon (TETR), Spotted green pufferfish (SPGP). The "3", "3a" and "3b" after the species represented CXCR3, CXCR3a and CXCR3b, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 8). Only CXCR1b was linked to CXCR2. The downstream gene of CXCR1a is a conserved SLC19a1, while the downstream gene of CXCR1b is COL18a1. The fish CXCR2 was in the upstream of CXCR1b, while zebrafish CXCR2 was downstream of CXCR1, which is the same as that for human CXCR2. The *Ma*CXCR3a and *Ma*CXCR3b loci were syntenically conserved among fish species (Fig. 9). CXCR3b always appears downstream of CXCR3a, and also has conserved CNFN,

PAFAH1B3, and TLR genes. *Ma*CXCR4 was syntenically conserved with other teleost fish (Fig. 10). The conserved TMBIM2, DRTS and MCM6 genes were observed in the downstream region of CXCR4.

3.4. Constitutive expression of MaCXCRs

To further explore the baseline expression patterns of *Ma*CXCR1–4,

Fig. 6. Multiple alignment of *Ma*CXCR4 with CXCR4 sequences from other species. The accession numbers for sequences used in this alignment are given in Fig. 1. The exclamation mark '!' in the consensus stands for a conserved letter, representing a sequence position in which all the sequences agree, whereas an asterisk '*' stands for positions in which there is a majority of sequences agreeing. Positions in which the sequences disagree were left blank in the consensus sequence. The N-terminus, seven transmembrane domains (TM1–7), the three intracellular (ICL1–3) and extracellular loops (ECL1–3), and the C-terminus are marked above the alignment. The first line with arrowheads represented the *Ma*CXCR1a, and second line with arrowheads represented *Ma*CXCR1b. The four conserved cysteine residues in each extracellular domain were indicated by black arrow. The G-protein coupled receptors family 1 signature and DRY motif were marked with black and green frame, respectively. The abbreviations were Asian swamp eel (ASSE), Turbot (TURB), Tropical clawed frog (TRCF), Atlantic salmon (ATSA), Zebrafish (ZEBR), Barred knifejaw (BAKN), Large yellow croaker (LAYC), Asian bonytongue (ASBO), Rainbow trout (RATR), Common carp (COCA), Sterlet (STER), Human (HUMA) and House mouse (HOMO). The "4", "4a" and "4b" after the species represented CXCR4, CXCR4a and CXCR4b, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tissue samples were taken from the muscle, liver, spleen, kidney, intestine, heart, brain, skin and blood of healthy Asian swamp eels. These genes were constitutively expressed in a wide range of tissues examined, but at different expression levels. *Ma*CXCR1a, *Ma*CXCR1b and *Ma*CXCR4 showed their highest expression in the spleen, and *Ma*CXCR2 showed its highest mRNA expression in skin. The expression levels of *Ma*CXCR3a and *Ma*CXCR3b were highest in the muscle and liver, respectively. The baseline expression levels of *Ma*CXCR3a and *Ma*CXCR4 were higher than those of the other *Ma*CXCRs in the nine tested tissues (Fig. 11).

3.5. Expression variation of MaCXCRs in response to A. veronii and poly I:C challenge in vivo

To understand how *Ma*CXCR1–4 are regulated in response to *A. veronii* and poly I:C, fish were challenged with 100 μL *A. veronii* (1.83×10^6 cfu/mL) or poly I:C (7 μg/g fish) for 4, 8, 24 and 48 h *in vivo*. After *A. veronii* administration, the expression of *Ma*CXCR1–4 were all upregulated (Fig. 12). *Ma*CXCR1a, *Ma*CXCR3a, and *Ma*CXCR3b were significantly upregulated from 4 to 24 h ($P < 0.05$), and reached their highest fold-change at 8 h (4.40-fold), 24 h (4.24-fold), and 24 h (5.60-fold), respectively. *Ma*CXCR1b was strikingly upregulated from 8 to

Table 3
Amino acid identity (top right) and similarity (bottom left) of *Ma*CXCR1a and *Ma*CXCR1b putative peptides compared with those of from other vertebrates.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Monopterus albus* CXCR1a		48.4	66.4	47.8	43.4	59.9	63.6	71.3	69.2	58.8	42.9	47.5	47.7	44.1	39.4	40.0
2. *Monopterus albus* CXCR1b	69.1		45.0	75.8	42.9	48.9	44.6	45.5	48.3	42.7	45.4	74.4	75.3	68.3	41.3	37.2
3. *Takifugu rubripes* CXCR1	79.6	69.1		44.0	41.2	55.5	75.0	62.0	61.9	52.2	40.4	44.3	44.8	40.1	36.0	38.6
4. *Takifugu rubripes* CXCR1b	69.9	88.9	69.1		43.8	49.4	45.4	46.0	47.1	40.2	44.1	73.1	81.4	67.3	41.4	38.1
5. *Latimeria chalumnae* CXCR1	65.0	65.7	63.2	65.5		42.1	41.6	40.1	41.2	37.0	35.0	41.6	43.5	40.1	42.2	39.7
6. *Oncorhynchus mykiss* CXCR1	77.4	68.8	72.1	68.8	64.1		55.1	58.1	57.5	49.3	45.0	49.4	48.6	46.3	39.2	39.3
7. *Tetraodon nigroviridis* CXCR1a	77.0	64.9	84.4	65.2	61.5	68.5		60.7	62.1	58.4	39.8	42.9	46.7	39.9	37.5	37.4
8. *Oreochromis niloticus* CXCR1a	84.1	66.6	79.1	69.4	61.8	73.3	75.8		66.3	53.6	40.8	44.2	45.3	43.2	37.2	36.9
9. *Xiphophorus maculatus* CXCR1a	82.9	68.5	80.4	68.8	65.4	71.0	77.2	80.5		54.9	41.6	46.7	45.2	44.9	40.2	38.0
10. *Gasterosteus aculeatus* CXCR1a	70.3	63.0	68.5	61.6	58.6	63.2	74.2	68.2	68.9		37.0	40.4	41.4	37.7	33.1	35.2
11. *Danio rerio* CXCR1	61.6	65.3	59.1	63.0	57.2	62.3	55.8	60.5	58.8	53.5		45.2	45.5	47.9	35.1	32.6
12. *Oreochromis niloticus* CXCR1b	69.2	87.5	69.2	86.7	63.6	68.9	63.1	67.5	68.3	60.8	63.0		72.6	64.5	41.0	39.5
13. *Tetraodon nigroviridis* CXCR1b	70.3	87.2	68.9	92.2	65.6	67.5	65.3	67.2	66.1	61.4	63.3	85.6		66.0	41.2	37.3
14. *Xiphophorus maculatus* CXCR1b	63.7	78.6	61.2	78.6	61.7	64.7	57.9	62.7	61.5	55.9	68.8	78.1	77.8		37.5	35.7
15. *Homo sapiens* CXCR1	59.7	63.5	57.4	63.8	64.3	58.2	58.6	59.6	59.5	54.9	53.0	62.2	62.5	59.7		64.7
16. *Mus musculus* CXCR1	62.7	62.7	59.7	60.2	64.3	58.8	58.7	59.1	59.5	57.3	52.3	60.8	59.7	57.9	77.5	

The accession numbers of these sequences are detailed in Fig. 1.

Table 4
Amino acid identity (top right) and similarity (bottom left) of *Ma*CXCR2 putative peptides compared with those of from other vertebrates.

	1	2	3	4	5	6	7	8	9	10
1. *Monopterus albus* CXCR2		46.9	57.8	61.4	62.3	50.1	52.3	44.8	41.0	39.9
2. *Danio rerio* CXCR2	67.1		51.5	47.4	46.2	58.8	43.7	45.7	42.6	43.9
3. *Oncorhynchus mykiss* CXCR2	73.5	71.3		56.2	56.3	55.2	50.5	50.1	45.7	43.9
4. *Xiphophorus maculatus* CXCR2	77.5	64.7	72.4		56.6	48.7	50.9	46.0	43.8	41.4
5. *Gasterosteus aculeatus* CXCR2	75.7	68.1	72.7	72.4		49.9	53.3	46.9	42.1	40.3
6. *Astyanax mexicanus* CXCR2	67.4	80.1	74.3	66.8	68.1		47.1	49.7	44.2	44.8
7. *Takifugu rubripes* CXCR2	69.3	65.0	70.2	67.4	69.2	63.3		41.8	41.4	41.7
8. *Lepisosteus oculatus* CXCR2	70.3	69.1	74.0	70.6	70.2	72.2	66.4		50.5	48.1
9. *Homo sapiens* CXCR2	64.2	66.4	68.8	65.0	63.6	65.3	60.6	70.3		71.1
10. *Mus musculus* CXCR2	64.4	66.0	69.9	62.9	62.7	66.6	63.8	67.4	85.8	

The accession numbers of these sequences are detailed in Fig. 1.

Table 5
Amino acid identity (top right) and similarity (bottom left) of *Ma*CXCR3a and *Ma*CXCR3b putative peptides compared with those of from other vertebrates.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *Monopterus albus* CXCR3a		42.8	50.0	51.3	57.6	56.2	58.0	65.4	63.7	62.1	32.9	34.3	31.9	42.8	42.4
2. *Monopterus albus* CXCR3b	59.1		37.8	40.8	40.7	36.0	40.9	43.9	41.3	40.5	27.7	27.5	34.5	33.5	34.0
3. *Danio rerio* CXCR3a	70.1	57.2		61.1	47.9	46.3	46.8	46.8	50.0	46.5	32.1	32.3	37.9	41.6	41.6
4. *Astyanax mexicanus* CXCR3a	70.6	60.2	77.9		50.0	46.8	48.7	50.5	51.7	49.5	33.8	32.9	38.5	45.0	44.7
5. *Gadus morhua* CXCR3a	70.3	57.8	67.0	70.4		48.8	49.6	52.3	52.1	53.4	29.3	33.1	34.9	38.0	37.8
6. *Oryzias latipes* CXCR3a	74.5	58.2	69.7	69.2	67.0		51.1	52.1	54.1	51.3	30.7	31.0	31.6	37.3	37.9
7. *Oreochromis niloticus* CXCR3a	73.5	61.7	66.5	71.0	68.2	68.9		56.5	52.5	50.8	30.2	30.6	33.1	38.5	37.6
8. *Gasterosteus aculeatus* CXCR3a	77.7	60.5	68.1	70.1	69.3	70.8	74.0		58.4	56.0	34.0	33.8	35.7	43.6	43.2
9. *Tetraodon nigroviridis* CXCR3a	77.2	58.3	68.8	72.1	69.4	72.4	70.7	75.9		90.6	35.3	35.2	31.9	42.4	42.4
10. *Tetraodon nigroviridis* CXCR3	76.9	57.9	67.3	71.9	71.3	70.8	70.0	74.0	94.3		32.7	33.7	30.6	40.1	40.6
11. *Oreochromis niloticus* CXCR3b	54.8	50.3	54.0	55.3	50.0	54.8	54.3	54.0	54.5	52.0		67.3	27.9	37.9	37.5
12. *Gasterosteus aculeatus* CXCR3b	54.2	49.7	54.4	54.4	50.5	53.6	55.5	55.5	56.3	55.2	80.3		27.2	39.5	39.4
13. *Danio rerio* CXCR3b	55.6	57.5	58.8	59.1	55.6	56.1	57.0	58.0	56.1	53.5	48.7	47.7		32.2	29.7
14. *Homo sapiens* CXCR3	61.2	54.6	62.8	64.9	58.4	61.4	61.7	64.1	64.0	61.4	54.0	55.5	52.1		86.4
15. *Mus musculus* CXCR3	60.6	56.9	63.5	65.1	58.0	61.7	61.9	63.2	63.1	61.3	55.8	56.8	51.1	92.7	

The accession numbers of these sequences are detailed in Fig. 1.

24 h, and had its highest fold-change at 24 h (6.64-fold, $P < 0.05$). *Ma*CXCR2 was only upregulated at 24 h with a relatively low fold-change (2.14-fold), and was significantly downregulated at 48 h *Ma*CXCR4 was prominently expressed from 4 to 48 h, and reached its highest fold-change at 24 h (3.15-fold). The expression levels of *Ma*CXCR1s and *Ma*CXCR3s decreased at 48 h to the level of the un-treated groups. After poly I:C treatment (Fig. 13), the expression levels of *Ma*CXCR1a and *Ma*CXCR3a were dramatically upregulated at 4–48 h, and both reached their highest fold-change at 8 h (3.50-fold and 7.13-fold, respectively). *Ma*CXCR1b expression was significantly upregulated at 24–48 h, with its highest expression at 48 h (13.49-fold). *Ma*CXCR2 was only upregulated at 24 h with a 3.47-fold change. *Ma*CXCR3b and

*Ma*CXCR4 were upregulated from 4 to 24 h, with the highest fold change observed at 4 and 8 h (4.77-fold and 2.99-fold, respectively). *Ma*CXCR2, *Ma*CXCR3b, and *Ma*CXCR4 expression levels were restored to normal levels at 48 h.

4. Discussion

Chemokines, or chemoattractant cytokines, are a family of cytokines that regulate immune cell migration under both inflammatory and normal physiological conditions [4]. CXCRs play important roles in the chemokine system [38]. Each CXCR protein may have distinct roles in the immune response. The Asian swamp eel is a commercially

Table 6

Amino acid identity (top right) and similarity (bottom left) of *Ma*CXCR4 putative peptides compared with those of from other vertebrates.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *Monopterus albus* CXCR4		81.1	60.9	70.0	69.1	67.8	83.5	79.4	67.4	69.7	66.0	63.4	61.1	62.7
2. *Scophthalmus maximus* CXCR4	88.9		61.7	73.8	71.2	68.0	84.4	82.3	69.4	74.1	69.4	65.2	61.3	61.0
3. *Xenopus tropicalis* CXCR4	75.5	77.4		63.6	67.0	60.8	63.3	61.2	65.6	63.3	63.6	64.2	73.6	72.0
4. *Salmo salar* CXCR4	82.6	85.8	76.2		71.4	71.8	73.5	69.6	69.4	96.4	73.3	68.3	62.2	63.5
5. *Danio rerio* CXCR4a	82.3	82.5	79.7	82.9		72.6	69.9	67.3	72.2	71.0	72.9	68.6	66.3	66.2
6. *Danio rerio* CXCR4b	81.0	82.0	77.1	83.1	83.9		70.3	66.7	70.8	70.7	89.2	65.8	61.9	61.5
7. *Oplegnathus fasciatus* CXCR4	92.3	92.6	77.7	83.6	82.8	83.3		86.4	71.6	73.3	71.1	65.3	64.0	63.6
8. *Larimichthys crocea* CXCR4	88.7	87.9	73.6	81.0	78.7	79.5	90.5		68.7	69.9	67.9	64.2	60.0	60.0
9. *Scleropages formosus* CXCR4	78.9	80.1	77.7	80.9	83.3	82.9	81.2	77.2		70.3	73.0	69.3	64.7	65.7
10. *Oncorhynchus mykiss* CXCR4	81.8	85.2	76.0	98.3	81.5	82.9	83.3	80.8	80.9		72.7	67.4	62.0	63.5
11. *Cyprinus carpio* CXCR4	81.0	82.8	77.7	85.6	83.6	95.2	83.0	79.7	83.4	85.1		68.6	61.9	61.5
12. *Acipenser ruthenus* CXCR4	77.8	78.2	80.7	82.6	82.8	82.1	78.0	76.2	82.4	81.5	83.2		64.9	64.8
13. *Homo sapiens* CXCR4	76.0	78.0	85.6	78.2	80.6	78.9	78.2	72.6	78.1	77.6	78.3	82.2		88.2
14. *Mus musculus* CXCR4	75.7	76.3	84.7	78.2	80.3	78.3	77.2	72.3	78.6	77.3	78.3	81.3	95.0	

The accession numbers of these sequences are detailed in Fig. 1.
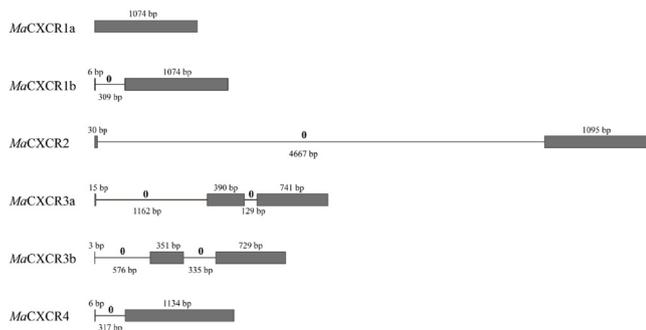
Fig. 7. Schematic diagrams of the exon-intron structure of *Ma*CXCR1–4 genes. Boxes represent exons and horizontal lines connecting exons represent introns. The numbers above the boxes and under the lines represent the nucleotide length (base pairs) of the exons or introns, respectively. The intron phase is indicated above the lines in boldface.

important cultured freshwater fish in China and other Asian countries [39,40]. A better understanding of the eel's immune responses may help to develop strategies for disease management, potentially improving yields and mitigating losses. Hence, in the current study, *Ma*CXCR1-4 were identified and their expression patterns were analyzed to provide further insights into the functions of CXCRs in the Asian swamp eel.

### 4.1. Sequences fuatures and phylogenetic analysis of MaCXCR1-4

In the present study, *Ma*CXCR1s (*Ma*CXCR1a/1b) and *Ma*CXCR3s (*Ma*CXCR3a/3b) were identified from the Asian swamp eel genome, which contrasts with mammals, which only have one CXCR1 and CXCR3 [41]. Despite some fish species having CXCR4a and CXCR4b, only one CXCR4 gene is found in most fish [42], including the Asian swamp eel. None of the eel CXCRs contain a signal peptide, which is similar to CXCRs reported in previous studies [43–46]. Our phylogenetic analysis showed that *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 were clustered together with the homologous proteins from other fish (Fig. 1), and all vertebrate CXCR1 and CXCR2 molecules formed a clade separate from the CXCR3 and CXCR4. The CXCR1/2 clade was divided into three subclades: CXCR1a, CXCR1b and CXCR2; and the CXCR3 clade was split into two subclades: CXCR3a and CXCR3b. This analysis illustrated that *Ma*CXCR1–2 and *Ma*CXCR4 are homologous to their human counterparts. Rainbow trout CXCR1-4 formed a clade with homologous human CXCRs, in which the bootstrap value of the CXCR3 clade was lower than that for CXCR1-2 or CXCR4 between fish and human CXCRs [28]. Despite the *Ma*CXCR3s being clustered together with human and house mouse CXCR3, no bootstrap value of the major branches was observed.

We speculated that *Ma*CXCR3 were also homologous to human CXCR3 (Fig. 1). The ayu (*Plecoglossus altivelis*) CXCR3.1 and CXCR3.2 were also placed in the same clade as human CXCR3 [44].

The amino acid homology analysis of *Ma*CXCR1–4 showed that *Ma*CXCR1a shared high aa identity/similarity with CXCR1s from other species, while *Ma*CXCR1b shared higher aa identity/similarity with those of other fish (Table 3). The sequence identity/similarity between *Ma*CXCR1a and *Ma*CXCR1b was low, which combined with the phylogenetic tree analysis, demonstrating that these *Ma*CXCRs are two different genes, termed CXCR1a and CXCR1b. The *Ma*CXCR1a and *Ma*CXCR1b have relatively low aa identity and similarity with human CXCR1. The mandarin fish CXCR1 shares high aa identity (49.6–64.9%), and low identity to the human CXCR1 (37.1%) [46]. Therefore, *Ma*CXCR1s have a closer relationship with their human counterpart than with that of mandarin fish. The *Ma*CXCR2 shared relatively high aa identity/similarity with its counterpart from teleost fish, but relatively lower aa identity/similarity compared with human CXCR2 (Table 4). The aa identity and similarity of *Ma*CXCR1s were higher that *Ma*CXCR2, which suggested that the CXCR1s were more conserved than CXCR2 among fish species. The *Ma*CXCR3a have a high aa identity/similarity with those of teleost fish, while *Ma*CXCR3b have lower aa identity/similarity (Table 5). This suggested that *Ma*CXCR3a is more conserved than *Ma*CXCR3b. This result was higher than that for rainbow trout CXCR3s [14]. The *Ma*CXCR3s have relatively low aa identity/similarity with human CXCR3. *Ma*CXCR4 has high aa identity/similarity with that of other fish (Table 6), and showed relatively higher aa identity/similarity with its human counterpart than *Ma*CXCR1–3, suggesting that CXCR4 is more conserved than CXCR1–3.

4.2. Amino acid characterization and domains

All six receptors have seven conserved transmembrane (TM1–7) domains, which is the typical characteristic of chemokine receptors [1]. They also have one to four potential N-glycosylation sites in their extracellular regions, and mainly distributed in N-terminus and ECL2 region (Fig. 2). An N-glycosylation site was also found in ECL3. By comparison, rainbow trout CXCR1–3 possess one to three N-glycosylation sites [14]. The glycosylation sites perhaps influence protein conformation through the prevention of disulfide bond formation [47]. N-linked glycosylation of CXCR4 results in the loss of coreceptor activity for isolated HIV-1 that are dominant during the early stages of HIV-1 disease, as well as in virus transmission to an uninfected individual [48]. The distinct glycosylation sites of CXCRs may result in functional differences among different species, which should be further studied. The DRY motif of *Ma*CXCR1–4 was also observed, mainly in the first few amino acids of in ICL2 (in the G protein-coupled receptor family 1 signature sequences which was between TM2 and ICL2). The DRY motif
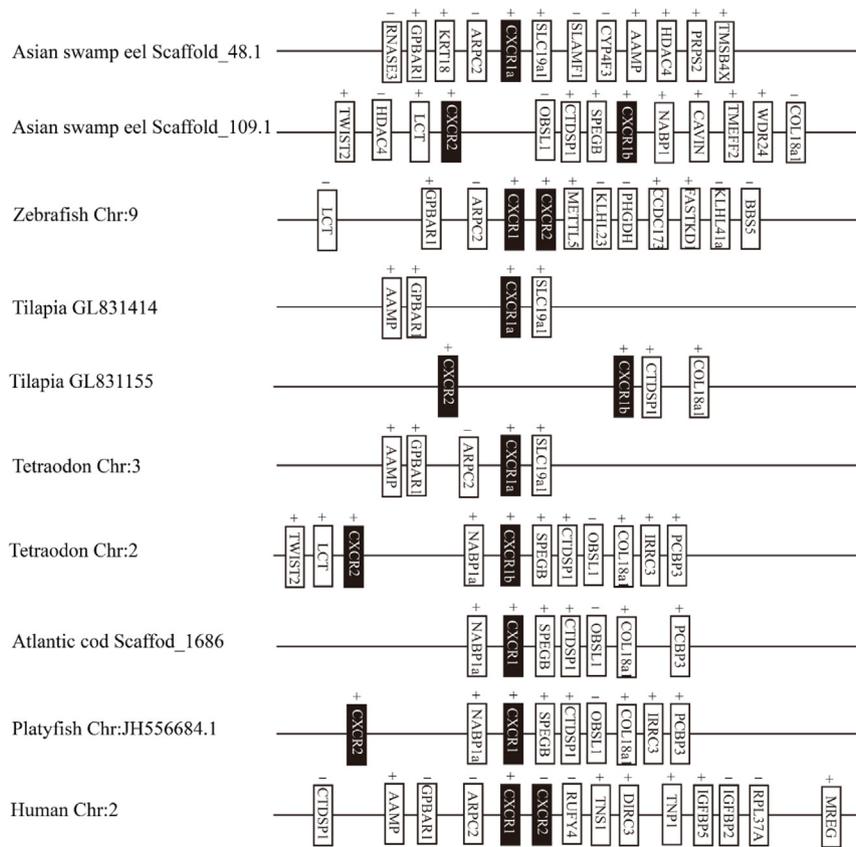
Fig. 8. Gene synteny analysis at the CXCR1/CXCR2 loci in vertebrates. The "+" and "-" above the genes indicate the transcriptional orientation. The abbreviations of these genes are taken from the Ensembl and NCBI database. The CXCR1a, CXCR1b and CXCR2 genes are marked with black frames.
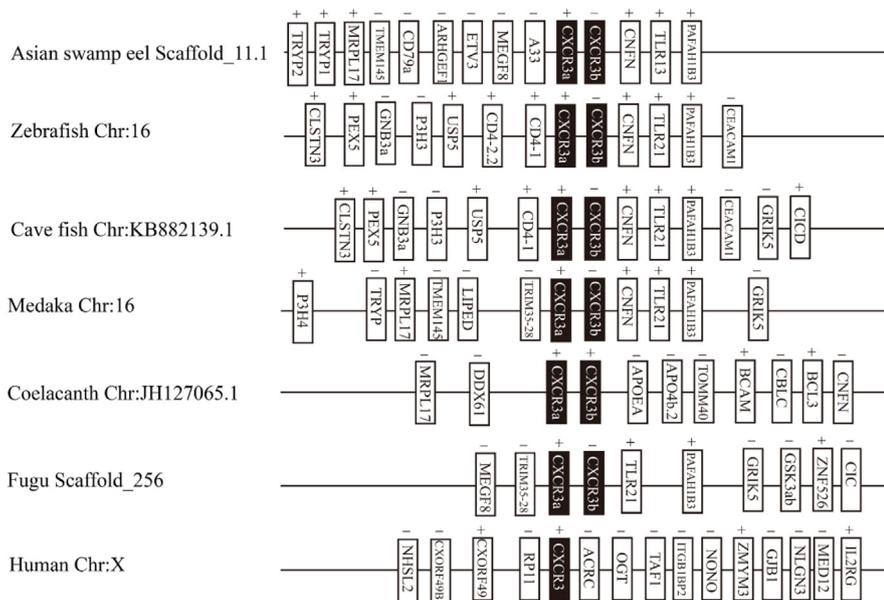
Fig. 9. Gene synteny analysis at the CXCR3a and CXCR3b loci in vertebrates. Boxes represent exons and horizontal lines connecting exons represent introns. The numbers above the boxes and under the lines represent the nucleotide length (base pairs) of exons or introns, respectively. The intron phase is indicated above the lines in boldface.

is essential for CXCRs, and is involved in coupling to G proteins [1].

Fish CXCR1a and CXCR1b have four conserved cysteine, one in the N-terminus and three in the ECL1-3, respectively (Fig. 3). This phenomenon was observed in *Ma*CXCR2, *Ma*CXCR3a and *Ma*CXCR4. Cysteines are conserved in most eukaryotic GPCRs and are linked by disulfide bonds [49]. A heterodimeric receptor complex can be formed by two members of the chemokine receptor family or with distantly related GPCRs [50]. Disulfide-linkage formation is one of the mechanisms implicated in dimerization of several GPCRs, e.g. dimerization of

the δ opioid receptor and heterodimerization of κ and δ opioid receptors [51,52]. Therefore, the conserved cysteines play critical roles in CXCRs' functions. The *Ma*CXCR3b only has one conserved cysteines in the ECL1 region (Fig. 5), which suggested a distinct function of *Ma*CXCR3b compared with that of other teleost fish CXCR3b, which requires further study.
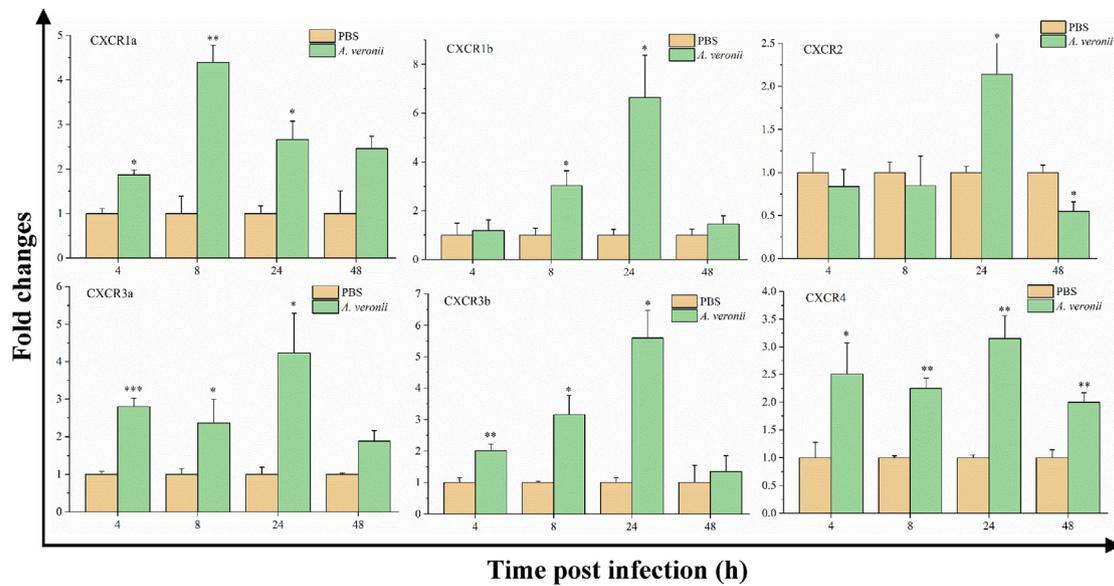
Fig. 10. Gene synteny analysis at the CXCR4 loci in vertebrates. Boxes represent exons and horizontal lines connecting exons represent introns. The numbers above the boxes and under the lines represented the nucleotide length (base pairs) of exons or introns, respectively. The intron phase is indicated above the lines in boldface.
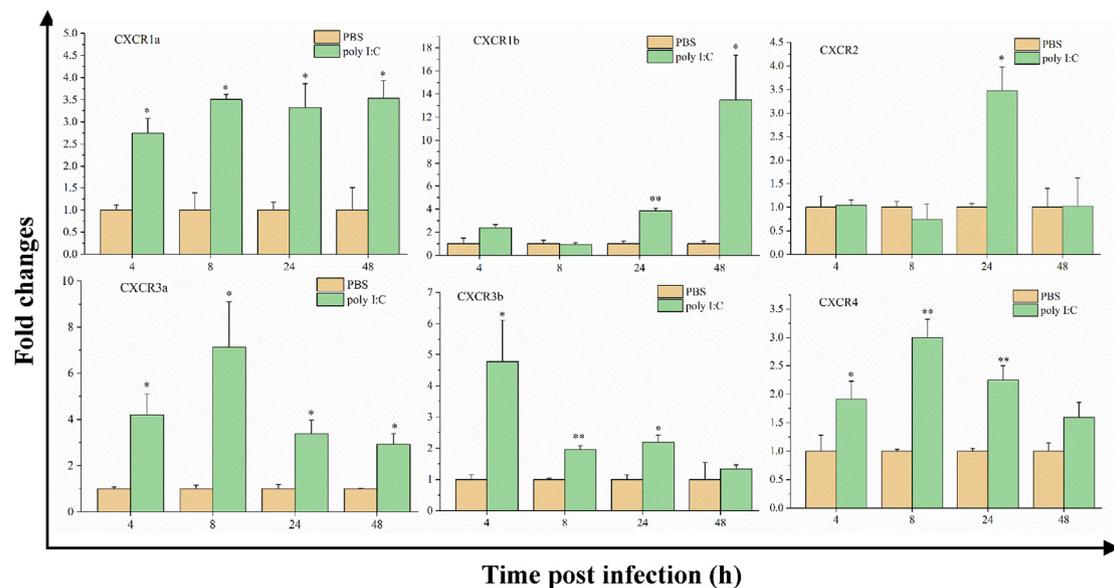

Fig. 11. Tissue distribution of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 mRNA. Muscle, M. Liver, L. Spleen, SP. Kidney, K. Intestine, I. Heart, H. Brain, BR. Skin, SK. Blood, BL. The data analysis was performed using quantitative real-time PCR, where gene expression was normalized to that of the elongation factor 1 alpha (EF-1α). Data are presented as the mean ± SE (n = 4).

4.3. Exon-intron structure and gene synteny analysis

Intronless and intron-containing CXCR1s were found in Asian swamp eel (Fig. 7). *Ma*CXCR1a has no introns, while *Ma*CXCR1b has one intron. *Ma*CXCR2 and *Ma*CXCR4 also have two exons and one intron. The human and mouse CXCR1 and CXCR2 genes do not have introns in the ORF region, while rock bream CXCR1 has also lost its intron, and rock bream CXCR2 and CXCR4 contain one intron [53]. *Ma*CXCR3a and *Ma*CXCR3b have three exons and two introns, which is the same as human CXCR3. All these intron-containing *Ma*CXCRs were separated by one to two phase 0 introns. It was hypothesized that a retroposition event might have occurred in teleost fish, resulting in the generation of intronless CXCRs in mammals [54]. By means of retroposition, the intron-containing CXCRs have partially lost their introns to form the intronless CXCRs in mammals. All *Ma*CXCRs introns possessed the classic 5′ GT/AG 3′ intron spice motifs.

Human CXCR1 and CXCR2 are tandemly linked on chromosome 2. In teleost fish, however, two loci for CXCR1s (CXCR1a and CXCR1b) were observed on different chromosomes (Fig. 8), suggesting that the presence of two CXCR1 genes in teleost resulted from chromosomal duplication or teleost fish-wide whole genome duplication (WGD) [55].

Only CXCR1b was linked to CXCR2, and CXCR1a was on anther chromosome. The downstream region of CXCR1a has conserved gene SLC19a1, while the downstream region of CXCR1b has COL18a1. Fish CXCR2 is in the upstream region of CXCR1b, while zebrafish CXCR2 is downstream of its CXCR1 gene, which is the same as the human CXCR2. The CXCR1 and CXCR2 loci are syntenically well conserved among teleost fish, while the differential evolutionary directions appear in these species (Fig. 8). The ARPC and GPBAR1 genes appear in the upstream regions of fish CXCR1a and human CXCR1, suggesting that the human CXCR1 might have evolved from fish CXCR1a. This result is supported by the observation of the intronless fish and human CXCR1 genes mentioned above. The *Ma*CXCR3a and *Ma*CXCR3b loci were syntenically conserved among fish species (Fig. 9). The CXCR3b genes always appears downstream of CXCR3a, and this locus has conserved CNFN, PAFAH1B3 and TLR genes. This demonstrated that the evolution of CXCR3 was more conserved than that of CXCR1/2. The *Ma*CXCR4 locus is syntenically conserved with other teleost fish (Fig. 10). The conserved TMBIM2, DRTS and MCM6 genes were observed downstream of CXCR4. The DARS and MCM6 genes appear downstream of the human CXCR4, while these two genes were separated in different fish CXCR4 loci. We speculated that human CXCR4 evolved from two

Fig. 12. *A. veronii*-induced expression patterns of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 in vivo. Gene expression levels were normalized to that of the elongation factor 1 alpha (EF-1α), and are presented as the fold-change compared with the respective control group (which was set to 1). Data are presented as the group means ± SE (n = 4) of four independent fish. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

Fig. 13. Poly I:C-induced expression patterns of *Ma*CXCR1a, *Ma*CXCR1b, *Ma*CXCR2, *Ma*CXCR3a, *Ma*CXCR3b and *Ma*CXCR4 in vivo. Gene expression levels were normalized to that of the elongation factor 1 alpha (EF-1α), and are presented as the fold-change compared with the respective control group (which was set to 1). Data are presented as the group means ± SE (n = 4) of four independent wells of cells from four fish. Statistical comparison of the mRNA levels detected at different time points was carried out by one way-analysis of variance (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

types of teleost CXCR4, which should be further analyzed.

4.4. Expression patterns of MaCXCR1-4

*Ma*CXCRs were constitutively expressed in a wide range of tissues examined, but at different levels (Fig. 11). *Ma*CXCR1a, *Ma*CXCR1b and *Ma*CXCR4 showed their highest expression in the spleen, and *Ma*CXCR2 was highly expressed in skin. The expression levels of *Ma*CXCR3a and *Ma*CXCR3b were highest in the muscle and liver, respectively. All these genes were highly expressed in immune-related tissues, especially in the spleen, skin, and kidney, which supported their involvement in immune system. They were also expressed in non-immune tissues. These results suggested that MaCXCRs are required for homeostasis of

phagocytes [15,16,46]. In zebrafish, CXCR4 is required for muscle formation [56], and human CXCR3 is involved proliferation of vascular smooth muscle cells [57]. The high expression of *Ma*CXCR3a in muscle suggested that *Ma*CXCR3a might have the vital functions in Asian swamp eel muscles. CXCR3 is constitutively expressed by endothelial cells located in medium and large caliber blood vessels, but not in small vessels from different organs [58]. The liver contains abundant blood vessels; therefore, the high mRNA level of *Ma*CXCR3b in the liver suggested that *Ma*CXCR3b might play important roles in blood vessels of the Asian swamp ell liver. The baseline expression of *Ma*CXCR3a and *Ma*CXCR4 was higher than those of the other *Ma*CXCRs in the nine tested tissues. These results suggested that the *Ma*CXCRs might have potentially different roles in un-stimulated tissues, and play vital roles

in normal conditions.

Several previous studies have demonstrated that CXCR expression could be stimulated by poly I:C and bacteria [29,42–44,59,60]. Poly I:C, an extracellular dsRNA, mainly activates interferon (IFN) α/β via the TLR3 (Toll-like receptor 3) pathway, finally stimulating the host antiviral defense through the JAK/STAT pathway [61]. *A. veronii* is a gramnegative, rod-shaped, facultatively anaerobic and non-spore forming bacteria, which is distributed widely in soil water, and human or animal related environments [62,63]. Hence, we examined the mRNA levels of *Ma*CXCRs *in vivo* using spleen tissue to analyze their expression patterns in response to *A. veronii* and poly I:C challenge.

After *A. veronii* administration, all *Ma*CXCR1–4 were upregulated, and the expression levels of all *Ma*CXCR1s and *Ma*CXCR3s decreased at 48 h to the level of the untreated groups (Fig. 12). This result suggested that the *Ma*CXCR1a, *Ma*CXCR3s and *Ma*CXCR4 had a faster antibacterial response than *Ma*CXCR1b and *Ma*CXCR2, and that *Ma*CXCR4 had longest effect. The *Ma*CXCR2 showed slight antibacterial response, and the downregulation *Ma*CXCR2 might be regulated by other cytokines as a protective mechanism. Despite *Ma*CXCR1b's response to *A. veronii* at 8–24 h, its fold change was higher than other *Ma*CXCRs, suggested that the *Ma*CXCR1b had shorter but stronger antibacterial response. Large yellow croaker CXCR2–4 were upregulated at 12, 6, and 12 h in spleen, respectively, when challenged with *Vibrio anguillarum* [59]. Rainbow trout CXCR1–2 and CXCR3a responded to bacterial infection, while CXCR3b was not upregulated after bacterial infection [14]. Thus, we speculated that different CXCRs have distinctive antibacterial functions according to their species or the different bacteria they encounter. Poly I:C treatment (Fig. 13) dramatically upregulated *Ma*CXCR1a and *Ma*CXCR3a expression at 4–48 h, and *Ma*CXCR1b and *Ma*CXCR2 expression upregulated at 24–48 h and 24 h, respectively. *Ma*CXCR3b and *Ma*CXCR4 expression were upregulated from 4 to 24 h. The *Ma*CXCR2, *Ma*CXCR3b and *Ma*CXCR4 were both restored to normal condition at 48 h. These results demonstrated that *Ma*CXCR1a and *Ma*CXCR3a have longer antiviral responses than the other *Ma*CXCRs, while the antiviral effect of *Ma*CXCR3a was stronger than that of *Ma*CXCR1a (Fig. 13). *Ma*CXCR1a, *Ma*CXCR3a, *Ma*CXCR3b, that *Ma*CXCR4 has a fast antiviral response, and that *Ma*CXCR2 has slight antiviral function. *Ma*CXCR1b mainly exert its antiviral functions in the later stage, while its antiviral activity was strongest among the *Ma*CXCRs. Large yellow croaker CXCR2-3, but not CXCR4, were not upregulated after poly I:C challenge in spleens [59]. Big-belly seahorse CXCR3 and CXCR4 have a fast response to poly I:C [43], and rock bream CXCR1 and CXCR2 were also upregulated after poly I:C administration [53]. Thus, we speculated that the function of CXCRs might be different among teleosts. Comprehensive analysis of *Ma*CXCRs showed that *Ma*CXCR1a and *Ma*CXCR3a have longer antiviral activities compared with their antibacterial functions, and *Ma*CXCR1b possesses a stronger antiviral response compared with its antibacterial activity. *Ma*CXCR4 might play vital roles both in bacterial and viral infection, and *Ma*CXCR2 may have a relatively small effect in this process.

In summary, our findings identified six *Ma*CXCRs and suggested that the *Ma*CXCRs have important, but different functions in antiviral and antibacterial immune responses. How these proteins exert their functions and the signaling pathways involved remain to be determined in a future study.

Acknowledgments

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