

Sequence and Evolution Differences of *Oreochromis niloticus* CXC Contribute to the Diversification of Cellular Immune Responses in Tilapias with Treatment of *Streptococcus iniae*

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Abstract: Hitherto that whether piscine CXC can interact with more immune cells and if there is any molecular evidence supports such activations are unclear. In present study, the levels of tilapia IL-8 in kidney, spleen, liver, macrophages and lymphocytes were significantly up-regulated ($p < 0.05$) post infection or immunization with *S. iniae*. Notably, the dynamic variation of tIL-8 in four tilapia species with immunization but not infection of *S. iniae* was positively associated with their features of disease resistance. The putative tIL-8 contains a typical structure of the incomplete ELR motif (ELH). However, the piscine incomplete ELR⁺ CXC and the mammals or avian complete ELR⁺ CXC are fell into two separate evolutionary branches both are independent to the lamprey IL-8. The findings showed tIL-8 is involved in the functions of tilapia macrophages and lymphocytes and can be a reference marker for disease resistant breeding this may due to its sequence and evolutionary features showing the early jawless fish not other fishes might be the intermediate stage evolving from ELR⁻ CXC to ELR⁺ CXC to obtain its chemotactic ability on immune cells.

Key words: Tilapia, CXC-chemokine, Interleukin-8 (IL-8), *Streptococcus iniae*, fish

INTRODUCTION

Chemokine is a superfamily of chemotactic cytokines secreted by various cells and can be classified into four groups among which the CXC chemokines can be further subdivided into ELR⁺ CXC and ELR⁻ CXC subtypes based on the presence of ELR (glutamic, leucine and arginine) motif in sequence (Baggiolini *et al.*, 1997; Bizzarri *et al.*, 2006). Generally, in mammal cells, the ELR⁺CXC, interacts with their cell surface receptors CXCR1 and CXCR2 to recruit polymorphonuclear leucocytes into inflammation foci and elicit angiogenesis and is associated with the migration and activation of leukocytes (Bizzarri *et al.*, 2006). Contrastingly, the ELR⁻ CXC has chemotactic ability on lymphocytes and monocytes but poor on neutrophils (Strieter *et al.*, 2006; Laing and Secombes, 2004).

Recently, increasing piscine CXC (fish IL-8) genes have been identified and classified into the ELR⁺ CXC sub-group based on their high identity to mammalian IL-8 (Fujiki *et al.*, 1999; Najakshin *et al.*, 1999; Lee *et al.*, 2001; Laing *et al.*, 2002a, b; Inoue *et al.*, 2003a, b; Baoprasertkul *et al.*, 2004; Corripio-Miyar *et al.*, 2007; Zhonghua *et al.*, 2008; Harun *et al.*, 2008;

Abdelkhalek *et al.*, 2009; Qiu *et al.*, 2009; Oehlers *et al.*, 2010). However, diverse features to mammal IL-8 have also been revealed. Fish IL-8 remains the chemotactic function of ELR⁻ CXC on macrophages and lymphocytes whereas the mammalian IL-8 does not (Hebert *et al.*, 1991; Zhonghua *et al.*, 2008; Harun *et al.*, 2008; Cai *et al.*, 2009; Oehlers *et al.*, 2010). Additionally, fish IL-8 cannot induce chemotaxis of human and mouse neutrophils while human and mouse IL-8 can induce chemotaxis of fish neutrophils (Zhonghua *et al.*, 2008). The mutation of ELH to ELR on fish IL-8 does not significantly enhance its chemotactic ability to neutrophils (Cai *et al.*, 2009) while mutation of ELR to ALR, EAR or ELR on human IL-8 significantly reduces its conjunction on neutrophils (Hebert *et al.*, 1991) indicating that effects of ELR on chemotactic ability of fish IL-8 is far less important than that on mammalian IL-8. Consequently, Cai *et al.* (2009) believed that fish IL-8 is in the intermediate stage of evolution from ELR⁻ CXC to ELR⁺ CXC and that the functions of fish IL-8 on lymphocytes and macrophages may result in various immune inductions and regulations (Hebert *et al.*, 1991; Zhonghua *et al.*, 2008; Harun *et al.*, 2008; Abdelkhalek *et al.*, 2009; Cai *et al.*, 2009; Oehlers *et al.*, 2010).

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Tilapia is the major freshwater aquaculture species in the world. Recently, its farming has been threatened by infection of Streptococci (*Streptococcus agalactiae* and *Streptococcus iniae*), causing nearly \$400 million economic loss in China annually (Zhou *et al.*, 2008; Chen *et al.*, 2010, 2012). Vaccination and disease-resistant breeding are becoming more urgent for control of streptococcosis (Evans *et al.*, 2004; Pridgeon and Klesius, 2011). However, the lack of basic immunological and molecular knowledge of tilapia against infection of pathogens slowed down the vaccine development. For this reason, IL-8 from tilapia (tIL-8) was chosen because of its special functions described above. In present study, researchers raised the question that whether piscine CXC (fish IL-8) can interact with more immune cells that mammal IL-8 cannot and if there is any molecular evidence supports such activations. To address it, researchers investigated the bioactivities of tIL-8 on the chemotactic ability of immune cells when the tilapia was immunized or infected with *S. iniae* and subsequently tried to find out the relationship between its sequence diversification and bioactivities.

MATERIALS AND METHODS

Sequence assembling and analysis: A total of 28 Expressed Sequence Tags (ESTs), highly homologous to animal IL-8 cDNA were identified in a cDNA library constructed from mRNA *O. niloticus* in the previous researches (Chen *et al.*, 2010) by Blastn and Blastx Methods. Then, the 28 ESTs were spliced and assembled by using SeqMan Software. The obtained contigs were subsequently used to screen homologous sequences in Swissport and nr database by BLASTN and BLASTP Methods. The contigs were reassembled and extended after proof-reading and the longest final contig was analyzed using online softwares. The parameters of nucleotide and protein similarity, start and stop codon, protein characteristics and so on were analyzed online. The phylogenetic tree of IL-8 from different species such as mammalian, avian and piscine CXC ligands was constructed based on their full-length amino acid sequences using UPGMAN method in MEGA Version 5.0 Software.

Fish and bacteria strains: The four types of tilapia fishes, *O. niloticus* x *O. aureus*, *O. niloticus*, *O. aureus* and GIFT, each weighing 100±10 g were provided by the National Tilapia Seed Farm (Nanning, Guangxi, China) and raised in circular freshwater tanks with a filter and an aeration system in the laboratory. Fish were acclimated at 28°C for 3 weeks prior to experiments and fed twice 1 day with a

formulated diet (Tongwei Feed Company, Nanning, China). The concentration of dissolved oxygen (from 6.09-9.63 mg L⁻¹), pH (from 6.8-8.1) and ammonia-N (<0.017 mg L⁻¹) were strictly managed through out the experiments. Bacteria strain, CMS005 of *S. iniae*, previously described as a tilapia pathogen (Gan *et al.*, 2007) was used to infect fishes (LD₅₀ <1.0×10⁶ CFU) by intraperitoneal injection (i.p.). All animal experiments were performed according to the principles and procedures of the Laboratory Animal Management Ordinance of China.

Immunization and infection: Fishes were divided into 2 groups: immunization and infection group. In each group, 200 fishes including four types of tilapia fishes, *O. niloticus* x *O. aureus*, *O. niloticus*, *O. aureus* and GIFT were raised and 50 of each type of tilapia was given in each group. For immunization, the inactivated *S. iniae* vaccine (0.5×10⁹ cells/fish), prepared as reported by Evans *et al.* (2004) were injected (i.p.) to all tilapias. Three fishes of each type of tilapia were harvested at 0, 1, 3, 5 and 7 days post immunization, respectively. Liver, spleen and anterior kidney were collected and frozen in liquid nitrogen for real-time PCR analysis. Regard to infection group, fishes were injected (i.p.) with bacteria *S. iniae* (0.5×10⁸ CFU/fish). Three fishes of each type of tilapia were harvested at 0, 6, 12, 24 and 48 h post infection, respectively. Tissue samples were collected as described earlier.

Macrophage cultivation and stimulation: Briefly, five *O. niloticus* in immunization or infection group were injected (i.p.) with 250 mL squalene (Sigma, USA). Peritoneal macrophages were isolated as earlier reported by Klesius *et al.* (2007) 5 days post injection. Macrophages were then suspended in L-15 complete medium (GIBCO, USA) containing 10% tilapia serum, 5×10⁻⁵ μM β-mercaptoethanol, 100 IU mL⁻¹ ampicillin and 100 μg mL⁻¹ streptomycin sulfate. About 100 μL cells per well (1.0×10⁶ cells mL⁻¹) were cultured in a 96 well plate at 28°C in a 5% CO₂ incubator. Cells were continuously cultured for 48 h post the exclusion of unattached cells and treated with 200 L medium containing inactivated *S. iniae* (1.0×10⁵ cells). About 50 L supernatants were collected at 12, 24, 48 and 72 h post treatment and were prepared for Nitric Oxide (NO) measurement by using a NO assay kit (Promega, USA). Additionally, the phagocytic activity of macrophages was measured by using a macrophage phagocytic activity detection kit (Jiancheng, Nanjing, China). The expression level of tIL-8 in macrophages at different time points was also measured by real-time PCR.

Lymphocyte cultivation and stimulation: *O. niloticus* was used to isolate blood lymphocytes. Briefly, 3 mL blood was taken from tail vein and gently mixed with 8 mL HBSS (GIBCO, USA) containing 20 IU mL⁻¹ heparin (Sigma, USA) after anesthesia with MS-222 (Sigma, USA). Cells were resuspended in 6 mL HBSS following centrifugation at 300 g for 10 min and was slowly added onto the surface of 6 mL of 65% percoll solution (Sigma, USA). The cells were resuspended in L-15 complete medium and adjusted to concentration of 1.0×10⁶ cells mL⁻¹ after washes with 1×HBSS. The 100 L per well of lymphocytes were then cultured in 96 well plate with treatment of 10 L inactivated *S. iniae* (1.0×10⁵ cells) at 28°C in 5% CO₂ incubator. The lymphocytes were harvested for measurement of IL-8 expression level by real-time PCR.

Measurement of tIL-8 expression: The total RNA from liver, spleen, kidney, macrophages and lymphocytes were extracted by using Trizol reagent (Invitrogen, USA). The first strand cDNA was amplified by using SYBR[®] PrimeScript[™] RT-PCR kit (TaKaRa, Dalian, China) according to the manufacture's instruction. IL-8 mRNA levels in different tissues and cells were measured by real-time PCR. A 128 bp fragment of IL-8 was amplified using primers IL8F: 5'-GCACTGCCGCTGCATTAAG-3' and IL8R: 5'-GCAGTGGGAGTTGGGAAGAA-3'. A 134 bp fragment of β -actin was amplified with primers β -actinF: 5'-AACAAACCACACACCACACATTTC-3' and β -actinR: 5'-TGTCTCCTTCATCGTTCCAGTTT-3'. The amplification condition was as follows: 95°C for 10 sec followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Standard curves, amplification efficiency and specificity of primers for IL-8 and β -actin were measured by using the CT slope Method and melting curve, respectively. Relative expression level of IL-8 to β -actin was calculated by using 2^{- Δ} Method.

Statistical analysis: Data were analyzed by using SPSS Statistics 17.0 Software. The one-way ANOVA method was applied. p<0.05 was considered as statistical significance.

RESULTS AND DISCUSSION

Expression levels of tIL-8 in kidney, liver and spleen were significantly upregulated post immunization and infection with of *S. iniae*: To determine the expression level of tIL-8 in kidney, liver and spleen at different time courses post immunization and infection with *S. iniae*, the real-time PCR was performed according to manuscript's

instruction of SYBR[®] PrimeScript[™] RT-PCR kit. Results showed expression levels of tIL-8 either in kidney, liver or spleen in all GIFTs were lower than the other three tilapia fishes in which the *O. niloticus* x *O. aureus* expressed highest tIL-8 in kidney and liver but not spleen (at day 7). For GIFT, a lowest level of tIL-8 was seen in kidney instead of in liver and spleen and it peaked at day 1, 3 and 7 in kidney, liver and spleen, correspondingly. Regarding to *O. aureus* the highest level of tIL-8 was at day 3 in kidney, liver and spleen but it peaked at day 7 in *O. niloticus* liver and spleen but not kidney in which it peaked at day 3 (Fig. 1a-c).

Regards to fishes that infected with *S. iniae*, similar to that of immunization, the expression levels of tIL-8 in kidney, liver and spleen in GIFTs were lowest among these four tilapia fishes except that in kidney in which the *O. aureus* expressed lower level of tIL-8 than GIFT. The highest levels were found in *O. niloticus* x *O. aureus* in all tissues. Specifically, in kidney, the tIL-8 peaked at 24 h in all tilapia fishes except *O. aureus*, peaking at 48 h. Similarly, in liver, the tIL-8 peaked at 24 h in all tilapia fishes with exception of *O. niloticus* x *O. aureus* which peaked at 6 h. However, the trends of tIL-8 expression varied in spleen, it peaked at hour 6, 48, 12 and 24 in GIFT, *O. aureus*, *O. niloticus* and *O. niloticus*×*O. aureus*, correspondingly (Fig. 1d-f).

Expression levels of tIL-8 in macrophages and lymphocytes were significantly upregulated with treatment of *S. iniae*: Then, to figure out the expression level of tIL-8 in immune cells, its level in macrophages and lymphocytes was detected by real-time PCR. Results showed tIL-8 expression in macrophage with treatment of *S. iniae* significantly (p<0.05) increased at 24, 48 and 72 h with the highest level at 48 h, compared to that in medium control. Similar trends were seen in lymphocytes except the expression of tIL-8 at 12 h was significantly upregulated in lymphocytes (p<0.05) instead of in macrophages (Fig. 2a and b).

Concentration of NO released by macrophages significantly increased post treatment of *S. iniae*: NO plays an important role on pathogen killing. In present study, the concentration of NO was measured by NO assay kit. Results showed the concentrations of NO significantly increased at 24, 48 and 72 h post stimulation of *S. iniae* with comparison to the medium control, correspondingly (p<0.05). Specifically, it increased from 24 h, peaked at 48 h (p<0.05) and then decreased at 72 h post stimulation of *S. iniae* (Fig. 3a).

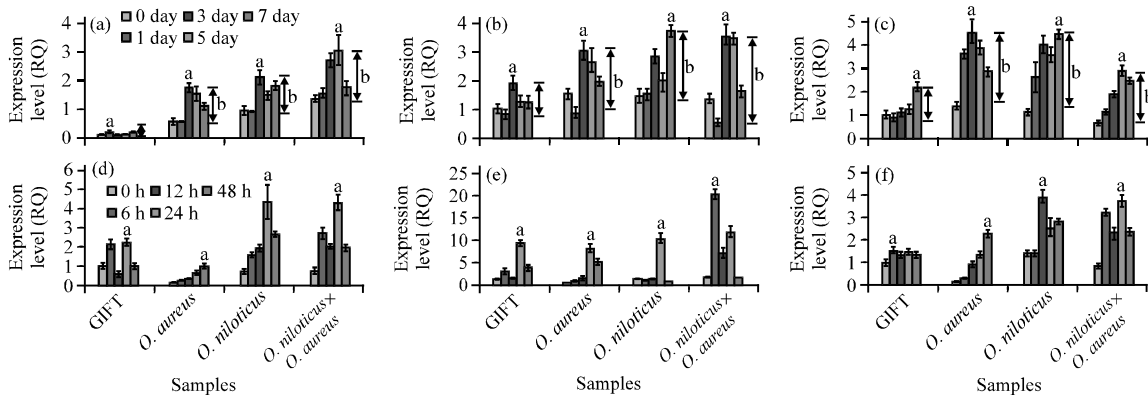


Fig. 1: The expression level of tIL-8 in different tissues of four tilapia species post immunization or infection with *S. iniae*. Four species of tilapia (*O. niloticus* x *O. aureus*, *O. niloticus*, *O. aureus* and GIFT) were classified into 2 groups: immunization and infection groups. Fishes in immunization group were vaccinated with inactivated *S. iniae* (i.p., 0.5×10^9 cells/fish). Fishes in the infection group were inoculated with *S. iniae* (i.p., 0.5×10^8 CFU/fish); a-c) the IL-8 expression level in anterior kidney, liver and spleen in immunization group, respectively; d-f) the IL-8 expression level in anterior kidney, liver and spleen in infection group, respectively. a represents $p < 0.05$ when fishes were immunized with inactivated *S. iniae* compared to that without vaccination (0 day). b represents statistics difference is < 0.05 between the *O. niloticus* x *O. aureus*, *O. niloticus*, *O. aureu* and GIFT, respectively when the subtraction (top value-bottom value of tIL-8) was analyzed

The phagocytic activity of macrophage increased earlier than the release of NO post treatment of *S. iniae*: Then, the phagocytic activity was also measured by a macrophage phagocytic activity detection kit. Results showed that the phagocytic activity increased as early as at 12 h post treatment of *S. iniae* and peaked at 24 h followed by a significant decrease at 48 h (Fig. 3b). Interestingly, the time point that NO released by macrophages which was at 24 h post treatment of *S. iniae* was 12 h earlier than the time point that the phagocytic activity of macrophage increased (Fig. 3a and b).

Sequence and evolution analysis: Since, the data showed tIL-8 increased not only in kidney, liver, spleen but also in macrophage and lymphocytes in tilapia fishes and it can increase the NO and phagocytosis ability of macrophages, this was in part, accordant with earlier studies showing the CXC chemokine from Black Sea bream (BS CXC) can increase the phagocytosis ability of piscine neutrophils and Head Kidney (HK) macrophages in both black sea bream and common carp (Cai *et al.*, 2009), researchers speculated this difference may due to the gene structure diverse of tIL-8 to piscine CXC and other known CXC. Then, researchers analyzed the assembled sequence, tIL-8 by online BLASTn and BLASTx programs. This cDNA is highly identical to a 570 bp partial tilapia IL-8 cDNA (GenBank access number GQ355864) which includes a 164 bp 5' non-coding region (UTR), a 282 bp complete Open Reading Frame (ORF) and

a 124 bp incomplete 3' UTR. It encodes a 93 amino acids peptide with 10410.35 Da molecular weight and 7.8 isoelectric point. Additionally, cysteine residues 21 and 88, a chemokine CXC domain from cysteines 27-90 and a SCY domain from cysteines 27-88 are found in tIL-8 sequence by the motif scan and conservative amino acid analysis. Moreover, tIL-8 contains a typical structure of the chemokine CXC ligand, a motif consisting of an arginine with two cysteines at both sides (Fig. 4).

Furthermore, the amino acid sequence of tIL-8 and other known CXCs were used to construct the phylogenetic tree by using the UPGMA program. As shown in Fig. 5, the lamprey (jawless fish) IL-8, without ELR motif, falls into an independent clade which was firstly evolved from the ancestral ELR⁻ CXC (the clade includes huam CXCL10-14, *Oncorhynchus mykiss* CLCL10 and *Salmo salar* CXCL10). Subsequently, the completed mammalian and avian ELR⁺ CXC and incomplete piscine ELR⁺ CXC share sister-taxon relationships on the evolutionary tree. In the mammalian ELR⁺ CXC clade, the orthologous genes of *IL-8* with the orthologous genes of *CXCL 1* and *3* formed sister branches on the evolutionary tree. All the piscine incomplete ELR⁺ CXC chemokines which diverged from lamprey IL-8 and are orthologous genes representing independent lineages, formed one clade. In this clade, *Triakis scyllium* IL-8 of the cartilaginous fish is in an independent branch while the teleost fish IL-8s are in the other independent branch.

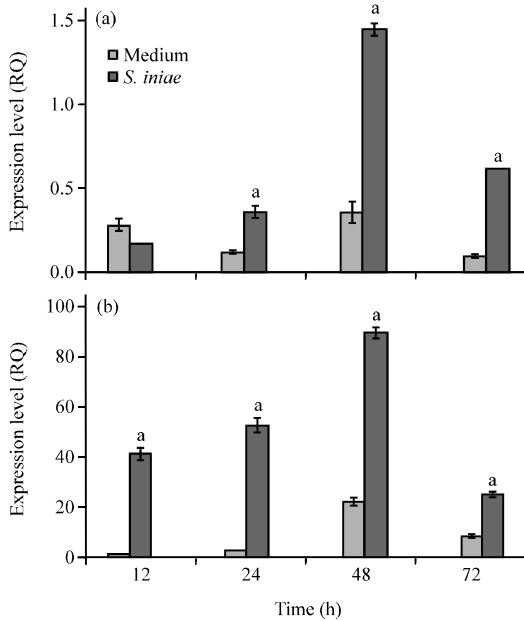


Fig. 2: tIL-8 expression levels of macrophages and lymphocytes post treatment with inactivated *S. iniae in vitro*. Cultured peritoneal macrophages (1.0×10^5 cells/well) and peripheral lymphocytes (1.0×10^5 cells/well) were treated with inactivated *S. iniae* (1.0×10^5 cells/well). Levels of tIL-8 were measured by real-time PCR; a) Expression level of tIL-8 in macrophages; b) Expression level of tIL-8 in lymphocytes. a represents $p < 0.01$ when compared to medium control. b indicate represents $p < 0.05$ when compared to medium control

It has been speculated that fish IL-8s like mammalian ELR⁺ CXC chemokines, exert their physiological functions by specifically binding to CXCR1 and CXCR2 receptor on neutrophils (Secombes *et al.*, 2001; Yoshie *et al.*, 2001; Laing and Secombes, 2004; Bizzarri *et al.*, 2006; Strieter *et al.*, 2006; Abdelkhalek *et al.*, 2009). Additionally, earlier studies showed the CXC chemokine from Black Sea bream (BS CXC) can increase the phagocytosis ability of piscine neutrophils and Head Kidney (HK) macrophages in both black sea bream and common carp (Cai *et al.*, 2009). However that whether fish IL-8 can interact with more immune cells and if there is any molecular evidence supports such activations are unclear.

In present study, tIL-8 expression could be enhanced not only in macrophages but also in lymphocytes when stimulated with *S. iniae in vitro* (Fig. 2a and b) indicating that except macrophages and neutrophils, more immune cells, at least lymphocytes can be involved in piscine IL-8-related immune responses. Meanwhile, the phagocytostic activity and NO released by macrophages

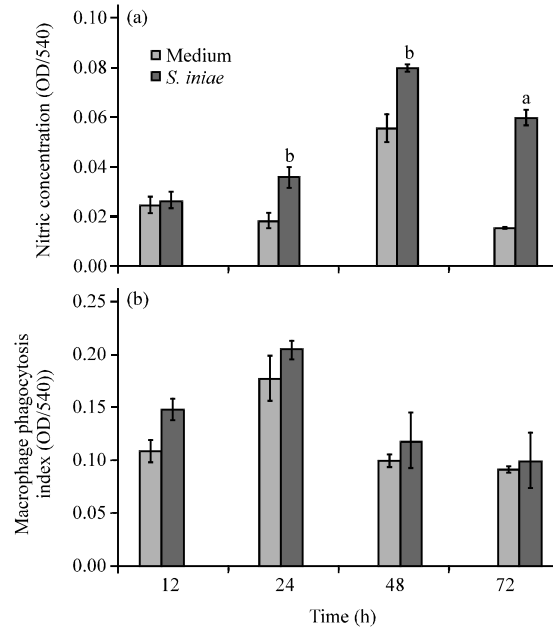


Fig. 3: The concentration of NO and macrophage activity when macrophages were treated with inactivated *S. iniae in vitro*. Cultured peritoneal macrophages (1.0×10^5 cells/well) and peripheral lymphocytes (1.0×10^5 cells/well) were treated with inactivated *S. iniae* (1.0×10^5 cells/well); a) Concentration of Nitric Oxide (NO) released by macrophage; b) Macrophage activity. a represents $p < 0.01$ when compared to medium control. b indicate represents $p < 0.05$ when compared to medium control

were also upregulated (Fig. 3a and b). Notably, the trends of tIL-8 expression and phagocytostic activity of macrophages had a positive time-dependent correlation, suggesting the increase of tIL-8 may result in the increase of phagocytostic activity of macrophages. However, an asymmetry between IL-8 expression and NO release of macrophages had also been found. The tIL-8 and NO peaked at 48 h post stimulation while the phagocytostic activity peaked at 24 h post infection, indicating the pathogen killing by NO occurred within the cells post the phagocytose of macrophages (Ding *et al.*, 1990). In addition, based on the current data that IL-8 expression in anterior kidney, liver and spleen peaked at 3~5 days post vaccination (Fig. 1a-c), together with the findings showing that the numbers of monocytes and lymphocytes also peaked at 3~5 days post vaccination, researchers speculate that tIL-8 plays an important role in immune function of lymphocytes and macrophage during the early stage of vaccination and infection and its expression may associate with more host cellular immunities.

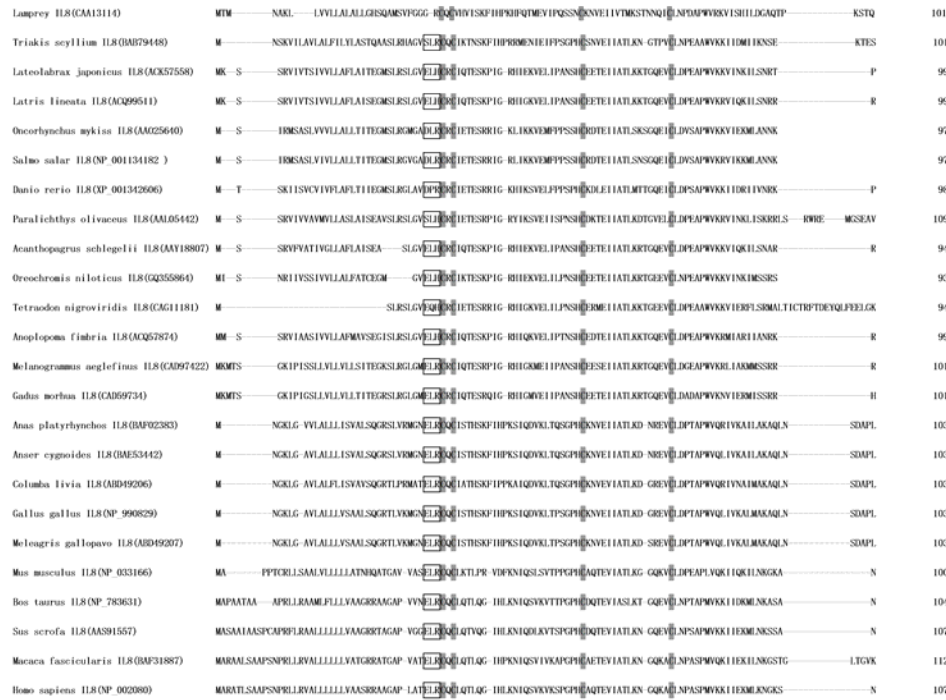


Fig. 4: Amino acid sequence alignment of *O. niloticus* IL-8 with other known CXCL8. The alignment was performed with the Clustal W Program. The conserved cysteines were highlighted and the ELR motif was boxed

It had been also found that *tIL-8* gene, like *ICER* gene (Chen *et al.*, 2010) could be used as reference marker for tilapia disease-resistant breeding. As known, significant different disease resistance prosperities were found in four current chosen tilapias. The *O. niloticus* x *O. aureus* has the strongest disease resistance followed by the *O. niloticus* and *O. aureus* which have better resistance to *S. imiae* than the GIFT. In present study, the overall expression levels of IL-8 in anterior kidney, spleen and liver from GIFT was far less than that in other three tilapias and kept line with the abilities of disease resistance among all chosen tilapias (Fig. 3), suggesting the role of *tIL-8* on disease-resistant breeding.

Next, experiments try to figure out the reasons why *tIL-8*, unlike the BS CXC and mammal IL-8 had the chemotaxis not only on macrophages but also lymphocytes. Researchers analyzed the amino acids and found that *tIL-8* contains five cysteine residues (at sites 21, 30, 32, 56 and 73) among which one cysteine residue (at site 21) is extra compared to other animal IL-8. Furthermore, an ELH motif (an arginine residue is bracketed by two cysteines at sites 30 and 32) is found in *tIL-8* sequence, indicating an incomplete ELR⁺ CXC motif exists in *tIL-8*. Additionally, two cysteine residues in fish, bird and mammalian are highly conserved, suggesting conserved biological functions. However, the variable sections were also found among fish, bird and mammalian.

In bird, NGK at the N-terminals and SDAPL at the C-terminals are conserved. Similarly, mammalian IL-8 also has conserved amino acid residues in the N-terminals. However, in all fish IL-8, there are no conserved amino acid residues in the N-terminal and C-terminal. Only two conserved cysteine residues were found but it is separated by arginine not the glutamine which isolates the same cysteine residues in bird and mammalian (Fig. 4). All these residue differences might result in the function diversifications.

Then, experiments tried to find evidence on their evolutionary tree. The phylogenetic tree showed that the incomplete ELR⁺ CXC of piscine and the complete ELR⁺ CXC of bird and mammals are classified into two independent evolutionary clades (Fig. 5). The lampetra IL-8, without ELR motif, firstly evolved from ELR⁻ CXC is in an independent clade earlier than bird and mammal IL-8. This classification in part, differs to that made by Cai *et al.* (2009) showing that taking fish IL-8-like chemokine as one member of ELR⁺ CXC was not accurate (Zhonghua *et al.*, 2008; Cai *et al.*, 2009) base on the evidence that most fish IL-8 sequences do not contain complete ELR motif, except those from Haddock and Atlantic cod (Corripio-Miyar *et al.*, 2007; Cai *et al.*, 2009). On the other hand, most identified fish IL-8s contain ELR-like motif whereas lamprey IL-8 does not contain any ELR-like or ELR motif. Moreover, there are two

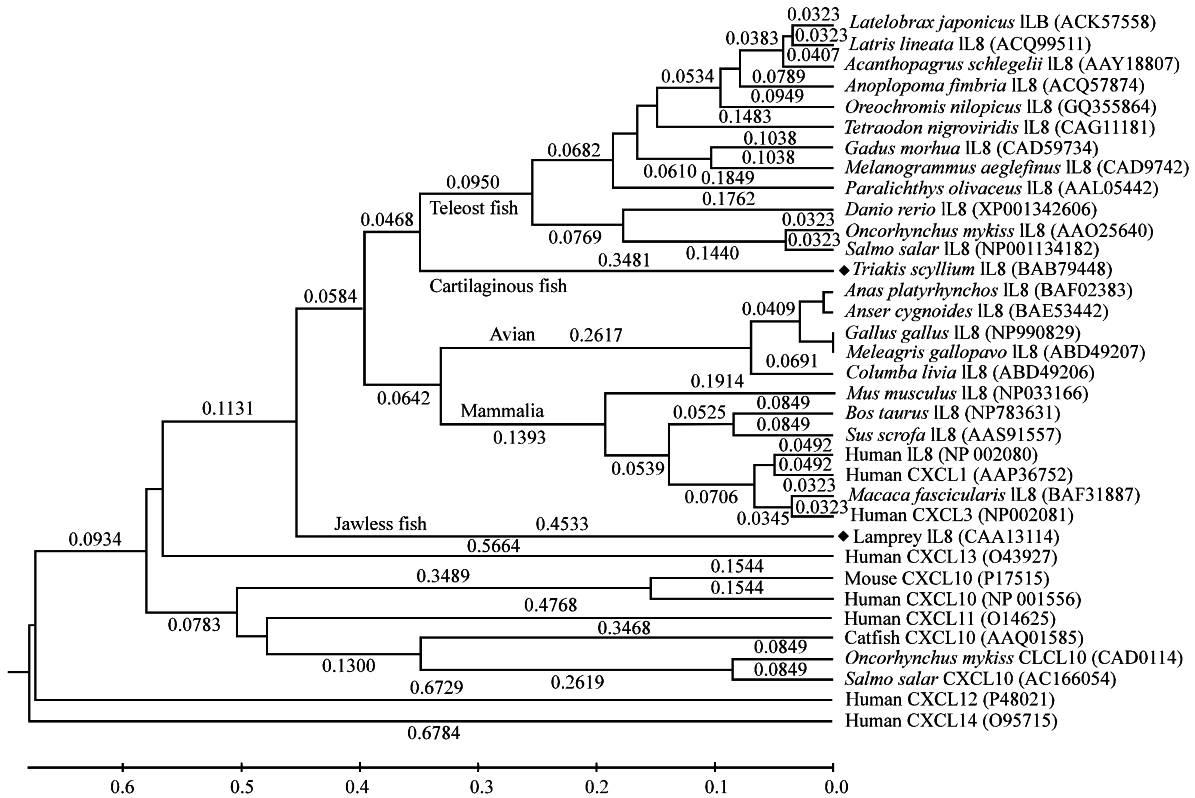


Fig. 5: Phylogenetic analysis: the sequences were aligned by the Clustal W Program and the phylogenetic tree was constructed by UPGMA Method using MEGA Version 5.0 Software. The phylogenetic tree showed the relationship among the full-length amino acid sequences of the mature *O. niloticus* IL-8 peptide with other representative CXC sequences. All amino acid sequences are given a unique Genebank access number. Numbers at branch nodes represent the branch length of the evolution distance

major IL-8-like CXC-chemokines in carp, one has ELR motif while the other has not (Fujiki *et al.*, 1999; Abdelkhalek *et al.*, 2009). Actually, Cai *et al.* (2009) speculated that incomplete ELR⁺ CXC might be the intermediate stage evolving from ELR⁻ CXC to ELR⁺ CXC (Zhonghua *et al.*, 2008; Cai *et al.*, 2009). This is not in part, consistent with the findings. Researchers speculated CXC without ELR (such as IL-8 from Lampetra one of the first primitive and vertebrate appeared in the world) not the all incomplete ELR⁺ CXC fishes was likely to be the intermediate phase evolving from ELR⁻ CXC to ELR⁺ CXC based on the phylogenetic tree analysis and function differences between incomplete/complete ELR⁺ CXC. It has been indicated that other elements of ELR⁻ CXC other than ELR motif allowed CXC to be chemotactic to neutrophils when ELR⁻ CXC evolved to incomplete/complete ELR⁺ CXC (Huising *et al.*, 2003; Wiens *et al.*, 2006; Zhonghua *et al.*, 2008; Abdelkhalek *et al.*, 2009; Cai *et al.*, 2009). However, that how could ELR⁻ CXC evolve to incomplete and complete

ELR⁺ CXC to obtain its chemotactic ability on neutrophils without ELR participation is not yet clear. Although, both hypotheses are partly inconformity both emphasized that the fish incomplete ELR⁺ CXC plays broader immune functions based on the facts that it can not only interact with neutrophils but also macrophages and other non-neutrophil immune cells (Cai *et al.*, 2009).

CONCLUSION

Taken together, the findings exhibited that tIL-8 could interact with tilapia macrophages and lymphocytes and could be upregulated against early vaccination and infection with *S. iniae* thus might be used as a reference marker for disease-resistant breeding. Sequence and phylogenetic tree analysis indicated this may due to its sequence and evolutionary features showing the early jawless fish, instead of the entire fishes, might be the intermediate stage evolving from ELR⁻ CXC to ELR⁺ CXC to obtain its chemotactic ability on immune cells.

ACKNOWLEDGEMENTS

This study was supported by the Guangxi Scientific and Technological Developmental Program (1123006-4), Tilapia Industrial Technology System of China (CARS-49) and Guangxi (GXYB-03) and Public Welfare Industry (Agriculture) Special Funds for Scientific Research Projects (201303054).

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