Chinese Journal of Oceanology and Limnology
Vol. 31 No. 1, P. 106-117, 2013
http://dx.doi.org/10.1007/s00343-013-2018-1

Sequencing and expression analysis of CD3γ/δ and CD3ε chains in mandarin fish, *Siniperca chuatsi**

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Received Feb. 1, 2012; accepted in principle Feb. 18, 2012; accepted for publication May 7, 2012

Abstract The genomic and cDNA sequences of the CD3γ/δ and CD3ε homologues in the mandarin fish, *Siniperca chuatsi*, were determined. As in other vertebrate CD3 molecules, the deduced amino acid sequences of mandarin fish CD3γ/δ and CD3ε contained conserved residues and motifs, such as cysteine residues and CXXC and immunoreceptor tyrosine-based activation motifs. However, mandarin fish CD3γ/δ and CD3ε showed some differences to their mammalian counterparts, specifically the absence of a negatively charged residue in the transmembrane region of CD3γ/δ. Additionally, while an *N*-glycosylation site was present in CD3ε, the site was not observed in CD3γ/δ. The CD3γ/δ and CD3ε subunit sequences contain six and five exons, respectively, consistent with homologues from Atlantic salmon, *Salmo salar*. Phylogenetic analysis also revealed that CD3γ/δ and CD3ε in mandarin fish are closely related to their counterparts in Acanthopterygian fish. Real-time PCR showed CD3γ/δ and CD3ε were expressed mainly in the thymus and spleen in normal healthy fish and, to a lesser extent, in mucosal-associated lymphoid tissues, such as the intestine and gills. When lymphocytes isolated from head kidney were treated with the mitogens phytohemagglutinin, concanavalin, and polyriboinosinic polyribocytidylic acid, mRNA expression levels of CD3γ/δ and CD3ε were significantly elevated within 12 h of treatment. This indicated the presence of T lymphocytes in the head kidney of teleost fish, and also the recognition of mitogens by the lymphocytes. Mandarin fish infected with the bacterial pathogen *Flavobacterium columnare* also showed an increase in the expression of CD3γ/δ and CD3ε mRNA, indicating that CD3γ/δ and CD3ε lymphocytes are involved in the immune response of this species.

Keyword: CD3; CD3γ/δ; CD3ε; mandarin fish; mitogen; *Flavobacterium columnare*

1 INTRODUCTION

In mammals, T cell receptor (TCR), which includes clonotypic TCRα/β and TCRγ/δ heterodimers, associates with CD3, which possesses γ, δ, and ε chains, and ζ chain-containing homo- or heterodimers, to form a TCR complex in the activation of T lymphocytes. The function of TCR is to recognize antigens bound to major histocompatibility complex (MHC), while the CD3 components transduce signals after antigen binding (Davis and Chien, 2003). In the complex, CD3 subunits are associated with TCR through a set of conserved ionizable transmembrane residues in the transmembrane region (Call and Wucherpfennig, 2007).

Because of their similarity to immunoglobulins, CD3γ, δ, and ε molecules are included in the immunoglobulin superfamily (Gold et al., 1987). They contain a signal peptide (SP), a highly variable extracellular region (EX), a transmembrane region (TM), and a cytoplasmic tail (CY). Many residues and motifs are conserved in CD3 subunits, including four cysteine residues, an *N*-glycosylation site, a CXXC motif, a negative residue in the TM region,

* Supported by the National Natural Science Foundation of China (No. U0631010), the Government of Guangdong Province, and the National Basic Research Program of China (973 Program) (No. 2009CB118703)
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and an immunoreceptor tyrosine-based activation motif (ITAM) in the CY tail. All of these conserved features are important for the maturation, differentiation, and signal transduction of T cells (Dave, 2009). In humans, the CD3γ, δ, and ε genes are contiguously located on chromosome 11, and share the primordial SP-EX-TM-CY1-CY2 structure (Tunnacliffe et al., 1987). Based on the gene location on the chromosome and the molecular structure, it was hypothesized that the three genes are derived from one primodial gene by two evolutionary events from one primodium (Göbel and Dangy, 2000).

In 1991, the first non-mammalian CD3 homologue was found in chickens, and was named as CD3γ/δ (Göbel and Dangy, 2000). This was shown to be the precursor of CD3γ and CD3δ, as it harbored all features of CD3γ and CD3δ and was functionally similar to its mammalian counterparts (Bernot and Auffray, 1991). Since then, all CD3 subunits, γ/δ, ε, and ζ, have been identified in lower vertebrates, including fish, amphibians and reptiles. However, complete analysis of the subunits has only been reported for two fish species, Atlantic salmon (*Salmo salar*) (Liu et al., 2008) and Atlantic halibut (*Hippoglossus hippoglossus*) (Overgård et al., 2009). CD3γ/δ and CD3ε have been described in Japanese flounder (*Paralichthys olivaceus*) (Park et al., 2001, 2005), fugu (*Takifugu rupbripes*) (Araki et al., 2005), and Mexican axolotl (*Ambystoma mexicanum*) (André et al., 2011), whereas CD3γ/δ from common carp (*Cyprinus carpio*) (Shang et al., 2008), African frog (*Xenopus laevis*) (Dzialo and Cooper, 1997), and Spanish ribbed newt (*Pleurodeles waltl*) (Ropars et al., 2002) has been identified, as has CD3ε from sterlet (*Acipenser ruthenus*) (Alabyev et al., 2000). Surprisingly, some splice variants of CD3 have been reported in teleost fish (Alabyev et al., 2000; Araki et al., 2005; Overgård et al., 2009; Maisey and Imarai, 2011). These variants lack partial amino acids in the EX or TM regions, and their function in the immune system is unknown.

Other molecules functionally related to CD3 have been characterized in fish, including TCR (Chen et al., 2009; Meeker et al., 2010; for reviews see Castro et al. (2011) and Laing and Hansen (2011)), CD4 and CD8 (Laing et al., 2006; Buonocore et al., 2008; Moore et al., 2009; Patel et al., 2009; Picchietti et al., 2009; Xu et al., 2010; Quiniou et al., 2011; for reviews see Castro et al., 2011 and Laing and Hansen, 2011), MHC (Xu et al., 2011; McClelland et al., 2011), lymphocyte-specific protein tyrosine kinase (Lck), and ZAP-70 (Øvergård et al., 2010). These molecules showed expression patterns similar to their mammalian counterparts (Shang et al., 2008; Øvergård et al., 2009), and CD4- and CD8-positive subsets of T lymphocytes were also recently characterized in ginbuna crucian carp, *Carassius auratus langsdorfii* (Shibasaki et al., 2010; Toda et al., 2009, 2011). These studies indicate that the T cell system in fish is similar to that in higher vertebrates (Toda et al., 2009, 2011; Castro et al., 2011; Laing and Hansen, 2011).

Fish are a diversified group of vertebrates, and some species are of high economic importance in aquaculture. Mandarin fish (*Siniperca chuatsi*), also known as Chinese perch, has a relatively high market value, and is widely cultured in China. However, information on the mandarin fish immune system is rather limited (Zhang et al., 2003; Chen et al., 2010; Tian et al., 2009a, in press; Guo and Nie, 2011). In this work, the cDNA and genomic sequences of CD3γ/δ and ε were characterized in mandarin fish. The expression of the two subunits was examined at the mrna level in different lymphoid organs and tissues. Additionally, the change in expression of CD3 mRNA was examined in head kidney lymphocytes following stimulation with three stimulants, the mitogens phytohaemagglutinin (PHA), concanavalin A (ConA), and polyriboinosinic polyribocytidylic acid (Poly I:C)(Sigma, St. Louis, Missouri, USA), and examined in mandarin fish infected with the bacterial pathogen *Flavobacterium columnare*.

2 MATERIAL AND METHOD

2.1 Fish

Mandarin fish, ranging from 0.6–0.8 kg in body weight, were obtained from a fish farm located at Liangzi Lake in Hubei Province, China. Fish were acclimatized in aerated tap water in an aquarium and fed with small cyprinid fish for at least 10 d prior to experimentation. Organs or tissues were dissected out following anaesthetization with MS222.

2.2 Amplification of cDNA and genomic sequences

To amplify the cDNA sequences of CD3γ/δ and CD3ε, total RNA was isolated from thymus using TRIzol reagent (Invitrogen, Carlsbad, California, USA). The first strand cDNA was synthesized from 3 μg total RNA with the SMART RACE cDNA amplification kit (Clontech, Mountain View, California, USA) for 3′ RACE and 5′ RACE according

Table 1 Oligonucleotide primers used in this study

Primer	Sequence (from 5′ to 3′)	Application
CD3-1	GAAGTT(T/C)CG(A/C)AC(G/C)TG(C/T)GA(T/C)AACTG	Cloning conserved region
CD3-2	CAG(C/A)GGCTGGTA(G/A)TG(A/G)TCGT	
CD3e-1	TG(C/T)G(A/C)(T/G/C)(G/A)ACTGCTTTGAG	
CD3e-2	GCG(A/G)GTGTG(A/C/G)(A/G/T)GGTTCAG	
CD3f1	TGGTGGCAACGACTGTGATAGGA	3′ RACE-PCR
CD3f2	TTGCGTCTCAGAATCGGAGTGGT	
CD3ef1	TGGACTTACTCCCGCTTCCAAAC	
CD3ef2	GCTTCCAAACCACCTGCTCGCTC	
CD3gsp1	ACCACTCCGATTCTGAGACGCAA	5′ RACE-PCR
CD3gsp2	GCCACTCCTATCACAGTCGTTGCCA	
CD3egsp1	GCTGGGCTTTTCTTCTTGGT	
CD3egsp2	TCACAAATGCTGTCCCGACCACG	
UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UPM-short	CTAATACGACTCACTATAGGGC	
CD3f	ATGAAATGTCAAGTAGTTTTGACTG	Cloning full genome
CD3d	CTATTTTCTGTTGGTCAGCACATCATAC	
CD3ef	ATGAACAGCATGGGTGTTCGGG	
CD3ed	CTATCCCATCCTGTTCACGCTAGAG	
RT-CD3f	GTGGTGGCAACGACTGTG	Real-time PCR
RT-CD3r	CCAGCCTTTGAGACACGG	
RT-CD3ef	CACCAAGAAGAAAAGCCCAGC	
RT-CD3er	GTAAGTATCCTGGGAGCGGGTG	
Actinf	GAGAGGGAAATCGTGCGTGA	
Actinr	CATACCGAGGAAGGAAGGCTG	

to the manufacturer's instructions. Based on the conserved sequences of CD3 homologues from other fish species, degenerate primers (CD3-1 and CD3-2 for CD3γ/δ sequence, CD3e-1 and CD3e-2 for CD3ε sequence) were designed to obtain partial sequences of mandarin fish CD3 molecules. Amplification was performed in a 50 μL reaction system containing 1×PCR buffer, 0.2 μmol/L of each dNTP, 0.024 U Ex *Taq* DNA polymerase (Takara, Japan), and 1 μL cDNA template. Thermal cycler conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, and finally 72°C for 5 min. To recover the full cDNA sequence, 3′ RACE and 5′ RACE were performed using gene-specific primers (CD3gsp1 and CD3gsp2 for CD3γ/δ sequence, CD3egsp1 and CD3egsp2 for CD3ε sequence) and adaptor primers (UPM). The thermal cycler conditions were 94°C for 5 min, 9 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 90 s, 29 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 90 s, followed by 72°C for 10 min. The fragments were purified using an agarose purification kit, then cloned into pMD18-T vector (Takara, Japan) following the manufacturer's instructions, and sequenced on an automatic DNA sequencer PRISM3730xL using a BigDye terminator v3.1 sequencing kit (ABI, New York, USA).

Based on the complete cDNA sequences of mandarin fish CD3, two pairs of primers (CD3f and CD3d, CD3ef and CD3ed) were designed to obtain the CD3 molecules DNA sequences. Genomic DNA was extracted from gills, and subjected to PCR using the following cycling conditions: 94°C for 5 min, 30 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 4 min, followed by 72°C for 10 min. Fragment purification, ligation, and sequencing were carried out as described above. All primers are listed in Table 1.

2.3 Sequence and phylogenetic analysis

The sequences were translated using the translation

tool on the ExPASy website (http://www.expasy.ch/tools/dna.html), and aligned with other known sequences using the MegAlign program in DNAstar to determine identities (http://www.dnastar.com/t-sub-products-lasergene-megalign.aspx). Multiple sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) (Thompson et al., 1994). Immunoglobulin-like domains were predicted using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) (Letunic et al., 2012). SP and TM region prediction were performed using the SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) programs (Sonnhammer et al., 1998), respectively. *N*-glycosylation sites were predicted using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) (Blom et al., 1999). The phylogenetic tree was constructed based on full-length amino acid sequences of CD3γ/δ and CD3ε from vertebrates using a neighbor-joining algorithm with bootstrap value of 1 000 in MEGA version 4.0 (Tamura et al., 2007).

2.4 Expression analysis of CD3γ/δ and CD3ε mRNA in lymphoid organs and tissues

Thymus, spleen, head kidney, liver, intestine, and gills were dissected from five healthy mandarin fish to determine the organ and tissue distribution of CD3γ/δ and ε transcripts. The cDNA was synthesized using a RevertAid first strand cDNA synthesis kit (Fermentas, Ottawa, Ontario, Canada) according to the manufacturer's instructions. The CD3 chain and β-actin cDNA fragments were generated by RT-PCR, and confirmed by sequencing. Amplicons were gel purified, and ten-fold serial dilutions of this DNA were used to generate a standard curve to determine the amplification efficiency of three primer pairs (RT-CD3f and RT-CD3d, RT-CD3ef and RT-CD3ed, actinf and actind). Amplifications were carried out in a final volume of 20 μL containing 10 μL SYBR mix (BIO-RAD, Mississauga, Ontario, Canada), 0.04 μmol/L of each primer, 1 μL template, and 7 μL double-distilled H_2O. The thermal cycler program was 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Each sample was run in triplicate wells on a 96-well qPCR plate. Dissociation curve analysis of amplification products was performed at the end of each reaction. PCR reactions confirmed the uniqueness of the PCR amplified product. Relative gene expression was normalized against cycle threshold (C_t) values for the housekeeping (β-actin) gene.

2.5 Lymphocyte preparation from head kidney and examination of CD3γ/δ and CD3ε expression at the mRNA level in lymphocytes following stimulation in vitro with PHA, ConA, and PolyI:C

Three mandarin fish were anaesthetized with tricaine methanesulfonate (MS-222) (Sigma, St Louis, Missouri, USA) and then exsanguinated by drawing venous blood. The head kidney was dissected from each fish and stored in L15 culture medium (Invitrogen, Carlsbad, California, USA). Cell suspensions were obtained by teasing and grinding with a frosted glass slide and passing through a 100 μm sieve (BD Falcon, San Jose, California, USA). Following centrifugation at 4°C for 5 min at 500×*g*, the cells were washed twice in ice-cold culture medium without fetal bovine serum (FBS) (Invitrogen, Carlsbad, California, USA). Cells were then resuspended in 10 mL tissue culture medium (TCM; L15 supplemented with 10% FBS and 1% penicillin/streptomycin; Sigma, St Louis, Missouri, USA). The cell suspension was layered on the surface of two layers of Percoll separating medium at densities of 51% (v/v) and 34% (v/v) (Pharmacia, Uppsala, Sweden). Following centrifugation at 4°C for 50 min at 500×*g*, the cell layer between the two different densities was collected and washed twice with TCM. The resultant cells were cultured in TCM at 28°C for 4 h to remove adherent cells, and non-adherent lymphocytes were carefully collected and re-cultured in TCM. The lymphocytes were adjusted to a cell concentration of 1.0×10^6 cell/mL, and 500 μL cell suspension was then added to each well of a 24-well plate, followed by treatment with either 20 μg/mL PHA, 10 μg/mL ConA, or 50 μg/mL Poly I:C. Untreated cells were used as controls. Cells were then collected at 4, 8, 12 and 24 h post-treatment (hpt) by centrifugation at 4°C for 10 min at 500×*g* to remove medium, before adding 1 mL TRIzol reagent to each centrifuge tube for total RNA extraction. All treatments were performed in triplicate. CD3 gene expression was analyzed by real-time PCR.

2.6 Expression of CD3γ/δ and CD3ε at the mRNA level in mandarin fish following infection with *Flavobacterium columnare*

Flavobacterium columnare was cultured in 50 mL of Shieh broth (Decostere et al., 1997) at 28°C for 36 h under continuous shaking at 200 r/min. Bacteria

were then harvested by centrifugation at 4°C for 10 min at 9 000×*g*. The cell pellets were washed twice in phosphate-buffered saline (PBS, 140 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L $Na_2HPO_4 \cdot 7H_2O$ and 1.5 mmol/L KH_2PO_4, pH 7.4), followed by resuspension in 20 mL PBS. The bacterial concentration was calculated by the gradient dilution-plate method (Shen et al., 1989).

Twenty-four mandarin fish were randomly divided into two groups of equal number. One group was injected intraperitoneally with *F. columnare* at a dose of 0.508×10^4 CFU/g body weight per fish, and the control group was injected with PBS (0.1 mol/L, pH 7.4). Thymus, spleen, and intestine from individuals from each group were collected at 1, 4, 7, and 14 d post-injection (dpi). Total RNA was isolated using TRIzol and expression analysis of CD3γ/δ and CD3ε was performed as described above.

2.7 Statistical analyses

Quantitative data were expressed as mean±SD. For stimulated expression, statistical analyses were carried out directly on the fold change data using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). Statistical significance of differences were calculated in Statistica 10.0 (StatSoft, Tulsa, Oklahoma, USA) using two-way ANOVA and *t*-tests when appropriate. A probability level of $P<0.05$ was used to indicate significant difference.

3 RESULT

3.1 Characterization of CD3γ/δ and CD3ε cDNA and genomic sequences in mandarin fish

The full cDNA sequence of CD3γ/δ in mandarin fish is 1 186 bp, encoding 182 amino acids (aa), and including a short 5′ untranslated region (UTR) of 135 bp, an open reading frame (ORF) of 549 bp, and a 3′ UTR of 502 bp with a polyadenylation motif of AATAAA, and a potential instability motif of ATTTA (GenBank accession No. DQ367842). The putative primary structure of the CD3γ/δ polypeptide deduced from the cDNA sequence contains a SP region of 23 aa, an EX region of 81 aa, a TM region of 26 aa, and a CY tail of 52 aa.

The full cDNA sequence of CD3ε in mandarin fish is 1 050 bp, containing a 5′ UTR of 151 bp, an ORF of 537 bp coding for 179 aa, and a 3′ UTR of 362 bp including a polyadenylation motif of AATAAA and a potential instability motif of ATTTA (GU550708). The putative amino acid sequence is composed of a SP region of 22 aa, an EX region of 82 aa, a TM region of 26 aa, and a CY tail of 50 aa.

Multiple sequence alignment between mandarin fish CD3γ/δ, CD3ε sequences and CD3γ/δ in other vertebrates, CD3γ and CD3δ in mammals, and CD3ε in vertebrates revealed that the most conserved regions are the last 13 aa of the EX region, including the CXXC motif, the transmembrane domain, and the core of the CY tail (Fig.1a, b). Four cysteine residues present in previously determined CD3γ/δ and ε sequences were also found in CD3γ/δ and CD3ε of mandarin fish.

Interestingly, the presence of an *N*-glycosylation site differs between CD3γ/δ or CD3γ and CD3δ in vertebrates. This site is present in the EX region of CD3γ/δ of Atlantic salmon, Atlantic halibut, amphibians, reptiles, and birds, and also in CD3γ and CD3δ of mammals, but is absent in mandarin fish, Japanese flounder, common carp, and fugu (Park et al., 2001; Araki et al., 2005; Shang et al., 2008) (Fig.1a). The negatively charged residue is also absent in the TM region of CD3γ/δ in mandarin fish, common carp, and fugu. In the CY tail, the ITAM sequence (D/E-$(X)_{(5-7)}$-D/E-$(X)_{(1-4)}$-Y-$(X)_2$-L/I-$X_{(6-10)}$-Y-$(X)_2$-L/I), which is present in teleost CD3γ/δ sequences, shows a common structure with the ITAM sequence (D/E-$(X)_7$-D/E-$(X)_2$-Y-$(X)_2$-L/I-$(X)_7$-Y-$(X)_2$-L/I) that is found in CD3γ/δ sequences in amphibians, reptiles, and birds, and CD3γ and CD3δ sequences in mammals. However, there is a difference in the number (X) of amino acids between the two Y-$(X)_2$-L/I repeats in the ITAM regions, with 6, 7, 8, 9, 10, and 10 aa in common carp, mandarin fish, fugu, Atlantic salmon, Atlantic halibut, and Japanese flounder, respectively, and 7 aa in tetrapods. The *N*-glycosylation site exists in the EX region of CD3ε in mandarin fish and Japanese flounder. The number (X) of amino acids between the two Y-$(X)_2$-L/I repeats in the ITAM motif of CD3ε is 9 aa in teleost fish, with the exception of 7 aa in sterlet, as observed in the ITAM motif of CD3ε in tetrapods (Fig.1b).

Mandarin fish CD3γ/δ and CD3ε molecules are composed of six and five exons, respectively, spanning 2.4 and 2.2 kb, respectively. The mandarin fish contains a short exon in the CD3γ/δ EX region that encodes seven amino acids (Fig.2).

3.2 Phylogenetic analysis

The mandarin fish CD3γ/δ sequence shows 56.0% amino acid identity to the fugu sequence, and 51.5%, 50.3%, 36.8%, 32.7%, 31.0%, 26.4%, and 23.7%

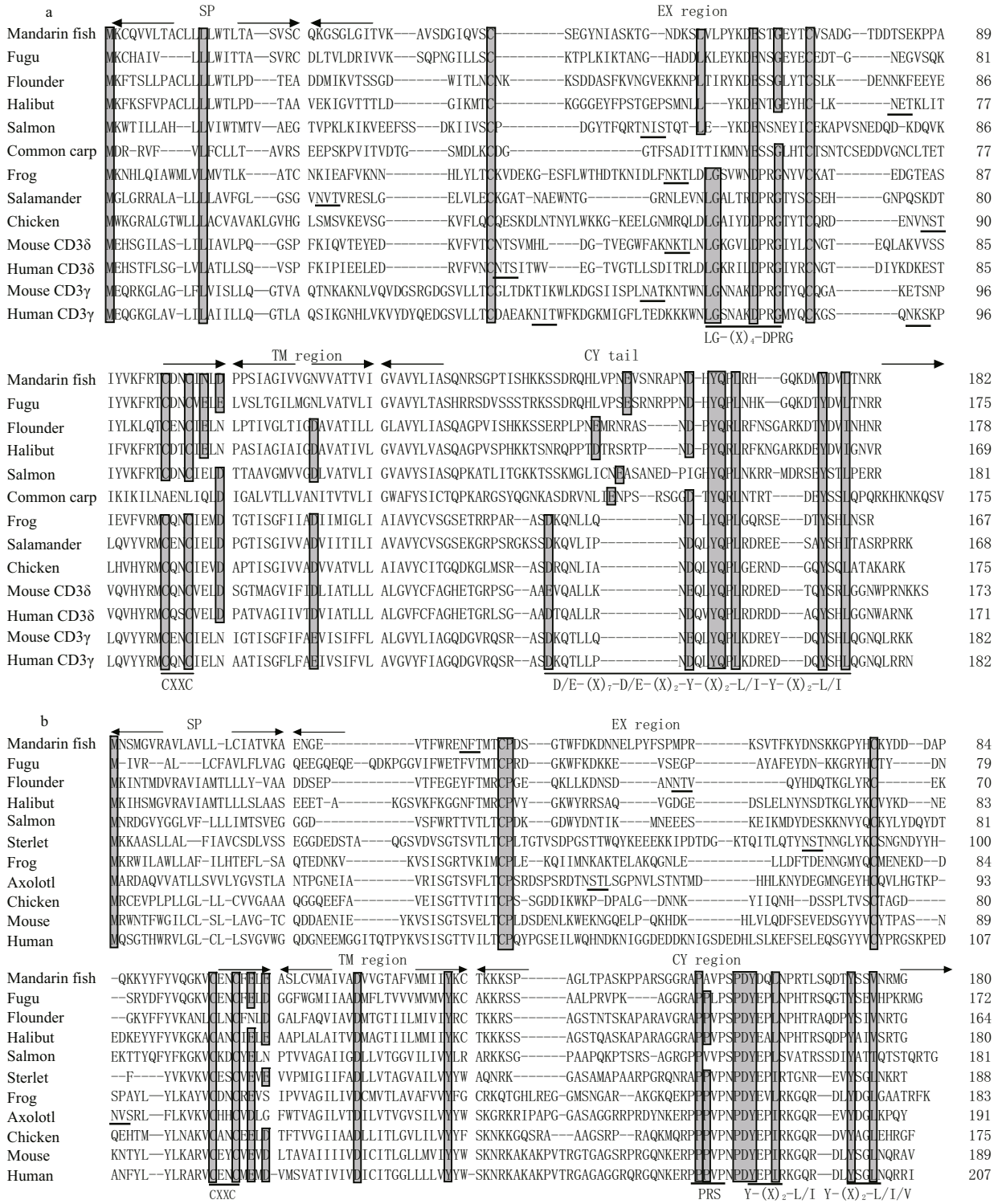

Fig.1 Alignment of CD3γ/δ and CD3ε with their vertebrate counterparts

Gaps are indicated by hyphens. Conserved amino acids and regions are indicated in shadow, with conserved motifs indicated with asterisks, followed with the motif. *N*-glycosylation sites are underlined. CD3γ/δ sequences are from mandarin fish, *Siniperca chuatsi*, GenBank accession ID DQ367842; fugu, *Takifugu rubripes*, AB166800; Japanese flounder, *Paralichthys olivaceus*, AB054068; Atlantic halibut, *Hippoglossus hippoglossus*, FJ769818; Atlantic salmon, *Salmon salar*, EF421416; common carp, *Cyprinus carpio*, DQ340867; African frog, *Xenopus laevis*, U78290; salamander, *Pleurodeles waltl*, AF397406; chicken, *Gallus gallus*, M59925; rat, *Rattus norvegicus* CD3γ, S79711 and CD3δ, NM_013169; and human, *Homo sapiens* CD3γ, X04145 and CD3δ, NM_000732. CD3ε sequences are from mandarin fish, *Siniperca chuatsi*; fugu, *Takifugu rubripes*, AB166798; Japanese flounder, *Paralichthys olivaceus*; AB081751; Atlantic halibut, *Hippoglossus hippoglossus*, FJ769815; Atlantic salmon, *Salmon salar*, EF421420; sterlet, *Acipencer ruthenus*, CAB46434, African frog, *Xenopus laevis*, AAI53710; Mexican axolotl, *Ambystoma mexicanum*, AY212509; chicken, *Gallus gallus*, Y08918; mouse, *Mus musculus*, J02990; and human, *Homo sapiens*, X03884.

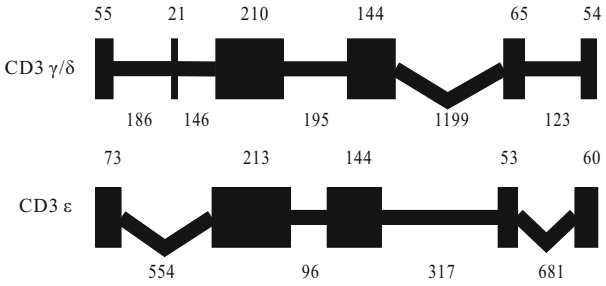

Fig.2 Genomic structure of CD3γ/δ and CD3ε in mandarin fish

Exons and introns are shown as black boxes and lines, respectively. Exon and intron sizes, in bp, are indicated above the boxes and below the lines, respectively. Exons and introns are shown in proportion to their bp sizes, but concave-lines are non-proportional.

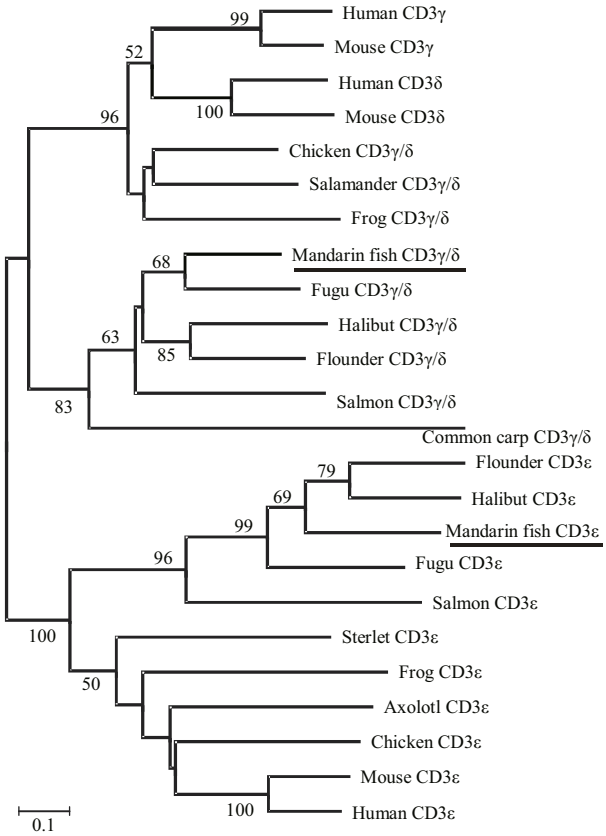

Fig.3 Phylogenetic tree showing the relationship of mandarin fish CD3γ/δ and CD3ε to their counterparts in other vertebrates based on amino acid sequences

The tree was constructed by neighbor-joining methods using MEGA version 4.0 with a bootstrap of 1 000.

identity to Atlantic halibut, Japanese flounder, Atlantic salmon, salamander, chicken, African frog, and common carp sequences, respectively. The CD3ε sequence shows 47.2% identity to the flounder

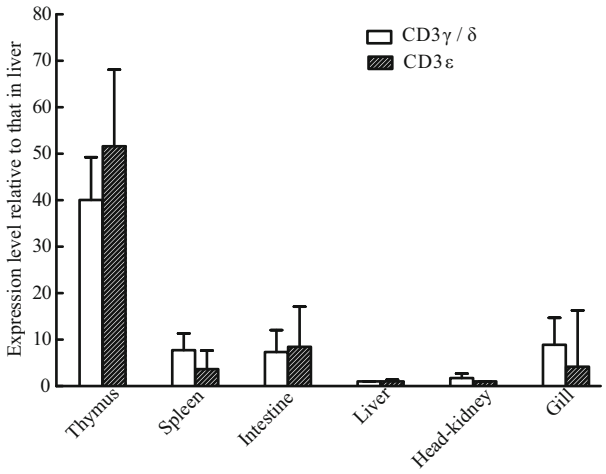

Fig.4 Expression of CD3γ/δ and CD3ε genes at the mRNA level in different organs and tissues of healthy mandarin fish

Expression levels of CD3 subunit genes are expressed as a ratio relative to β-actin, and as fold change based on the level in liver. Data are the mean (±SD).

sequence and 46.0%, 39.4%, 39.4%, 28.3%, 27.5%, and 23.8% identity to the sequences from fugu, Atlantic halibut, Atlantic salmon, chicken, sterlet, and mouse, respectively. The phylogenetic tree constructed on the basis of the amino acid sequences of CD3γ/δ and CD3ε indicated that CD3γ/δ in mandarin fish is closely related to that of fugu, and also shows similarity to a group containing Atlantic halibut and Japanese flounder. CD3ε was closely related to the homologues from Atlantic halibut and Japanese flounder, followed by fugu (Fig.3).

3.3 Expression of CD3γ/δ and CD3ε in healthy fish

The mRNA expression levels of CD3γ/δ and CD3ε in lymphoid organs and mucosal-associated lymphoid tissues (MALTs) is shown in Fig.4. The highest level of CD3γ/δ and CD3ε expression was in thymus, followed by spleen and intestine, with relatively low expression in head kidney and gills.

3.4 expression of CD3γ/δ and CD3ε following stimulation with PolyI:C, PHA, and ConA, and infection with *F. columnare*

The three stimulants, PHA, ConA, and PolyI:C, were all able to induce the expression of CD3γ/δ and CD3ε in lymphocytes isolated from head kidney of mandarin fish (Fig.5a, b). In general, the expression of CD3γ/δ and CD3ε showed a similar pattern following the stimulation. The expression of both CD3γ/δ and CD3ε increased significantly (two-way ANOVA,

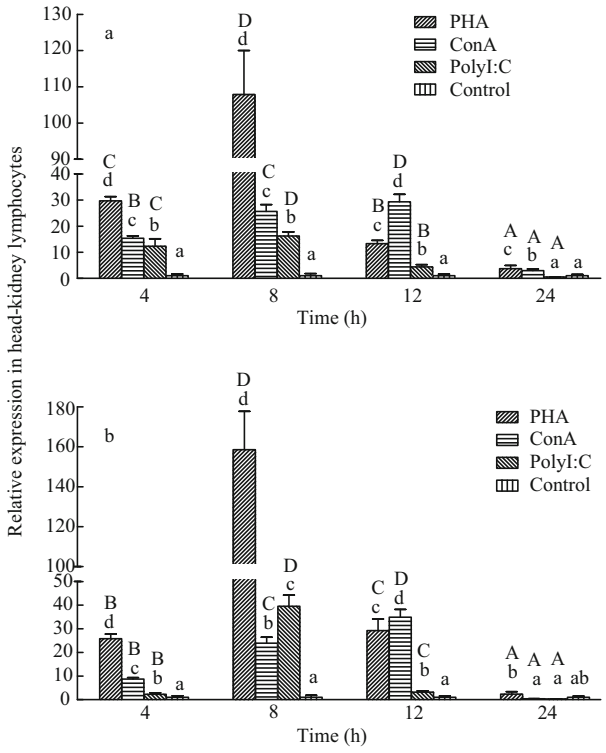

Fig.5 Expression of CD3γ/δ (a) and CD3ε (b) genes at the mRNA level in head kidney lymphocytes following stimulation with PHA, ConA, or PolyI:C

Data represent the mean (±SD) of ratios relative to β-actin, normalized against control. Lower case letters above bars, (a, b, c, and d), indicate the significant difference (P<0.05) in expression among the four treatments, i.e. the three experimental and one control groups, at each separate sampling occasion. Upper case letters above bars, (A, B, C, and D), indicate the significant difference (P<0.05) for the time-related change of each treatment. However, columns indicated with the same letters showed no significant difference (P>0.05). A, B, C, D or a, b, c, d indicate the order of expression from lowest to highest.

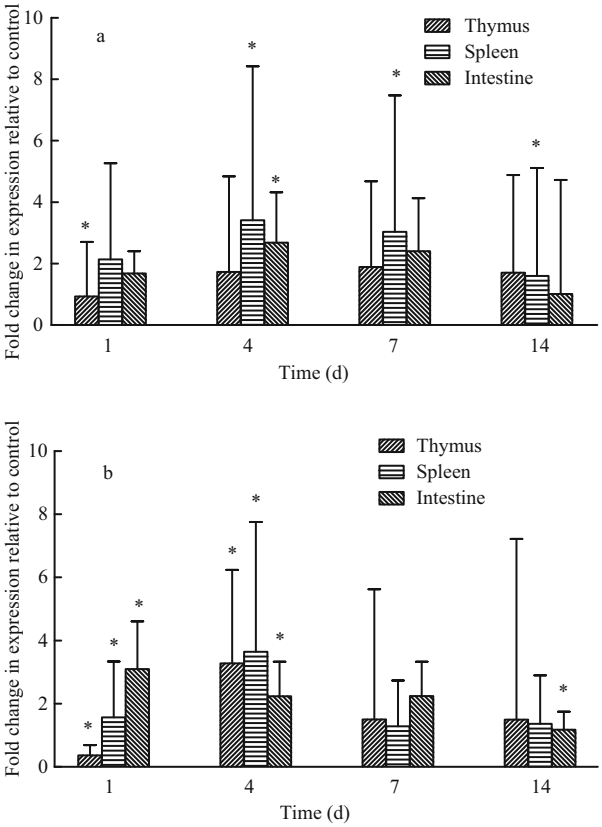

Fig.6 Expression of CD3γ/δ (a) and CD3ε (b) genes at the mRNA level in mandarin fish infected with *Flavobacterium columnare*

Data represent the mean (±SD) of ratios relative to β-actin, and normalized against the control, with asterisks indicating P<0.05.

P<0.05) at 4 hpt when compared with the control, and also showed significant variation over the duration of the experiment (two-way ANOVA, P<0.05). The expression level remained high at 8 and 12 hpt, followed by a decrease at 24 hpt. The highest level of expression of CD3γ/δ and CD3ε in lymphocytes stimulated with PHA and PolyI:C was observed at 8 hpt, with the highest expression level of CD3γ/δ and CD3ε at 12 hpt when stimulated with ConA (Fig.5a, b).

Bacterial infection also induced the expression of CD3γ/δ and CD3ε mRNA, as detected in thymus, spleen, and intestine of mandarin fish infected with *F. columnare* (Fig.6a, b). The highest levels of CD3γ/δ and CD3ε expression were observed at 4 and 7 dpi, and spleen had the most significant increase in CD3γ/δ and CD3ε mRNA expression following bacterial infection (t-test, P<0.05). CD3γ/δ expression was also significantly increased in the thymus at 1 dpi, and CD3ε expression significantly increased following the infection (t-test, P<0.05), except at 7 dpi when a non-significant expression value was observed (Fig.6b).

4 DISCUSSION

In the present study, we identified, cloned, and sequenced the two CD3 subunits, CD3γ/δ and CD3ε, from the mandarin fish. The expression of CD3γ/δ and CD3ε was examined at the mRNA level in head kidney lymphocytes following stimulation with the mitogens PHA and ConA, and also PolyI:C, which structurally mimics double-stranded RNA virus, and in fish infected with bacterial pathogen, *F. columnare*.

Conserved motifs or sequences in CD3γ/δ and CD3ε from mandarin fish, their exonic structure, and their phylogenetic relationship with counterparts in other vertebrates all confirmed the identity of these two subunits. In mammals, CD3γ and CD3δ are separate units; but in chickens and amphibians a CD3

subunit was found to be equally homogeneous to CD3γ and CD3δ in mammals, and was therefore designated CD3γ/δ (Bernot and Auffray, 1991; Dzialo and Cooper, 1997). CD3γ/δ has been reported in other teleost fish, e.g. Atlantic salmon (Liu et al., 2008), and our findings in the present study are congruent with previous studies. In all subunits of CD3, four cysteine residues are conserved in the EX region, two of which are involved in the formation of covalently linked heterodimers among CD3γ, CD3δ, and CD3ε chains. The remaining two residues are used to construct an inter-chain disulfide bond in Ig domains in mammals (Minami et al., 1987), which has been verified experimentally in CD3γ/δ and CD3ε heterodimers in chickens (Göbel and Dangy, 2000). It is likely that these conserved cysteine residues function similarly in teleost fish as they do in their counterparts in mammals and chickens. The ITAM motif, which is conserved in CD3 molecules, is present in CD3γ/δ and CD3ε of mandarin fish, and is reported to be the main component for signaling in adaptive immunity when the TCR-CD3 complex connects with the MHC-antigen complex (Cambier, 1995). The PRS in CD3ε is conserved in vertebrates, and is involved in CD3ε phosphorylation and CD8+cell functioning (Tailor et al., 2008). These conserved motifs in CD3γ/δ and CD3ε of mandarin fish indicate that these CD3 subunits may be functionally similar to their mammalian counterparts.

CD3γ/δ and CD3ε n teleost fish have six and five exons, respectively, despite the fact that the CD3γ/δ and CD3ε genes in mandarin fish are smaller than in Atlantic salmon (4.6 and 2.7 kb, respectively) and Japanese flounder (both 2.8 kb) (Park et al., 2005; Liu et al., 2008). Furthermore, CD3 subunits in vertebrates all maintain the primordial structure (L-EX-TM-CY1-CY2) (Tunnacliffe et al., 1987; Saito et al., 1987; Göbel and Dangy, 2000), and the phylogenetic tree shows that CD3γ/δ and CD3ε in mandarin fish are closely related to their counterparts in teleost fish.

Conversely, the LG-$(X)_4$-DPRG motif, which is conserved in CD3γ/δ or CD3γ and CD3δ in vertebrates from amphibian to mammals, showed some degree of variation in teleost fish (Fig.3a). This motif creates a binding site for CD3ε in mammals (Dietrich et al., 1996), but whether this function is pertinent in teleost fish is unknown. Other CD3γ/δ and CD3ε characteristics of teleost fish may also exhibit a certain level of variation. For example, in mammals, negatively charged residues, which are reported in CD3γε, CD3δε heterodimers, and the CD3ζζ homodimer (Call et al., 2005; Call et al., 2007), are not found in the TM region of CD3γ/δ in mandarin fish, common carp, and fugu (Araki et al., 2005; Shang et al., 2008), but are observed in Japanese flounder, Atlantic halibut, and Atlantic salmon (Park et al., 2001; Liu et al., 2008; Overgård et al., 2009). The *N*-glycosylation site, which is important for antigen recognition, signal transduction, and TCR-CD3 complex assembly in birds and mammals (Rudd et al., 2001), exists strictly in the CD3γ, CD3δ, and CD3γ/δ sequences (Göbel and Dangy, 2000). This site was not found in CD3γ/δ, but it is present in CD3ε in mandarin fish, and was also reported in Japanese flounder (Park et al., 2005). It is thus unclear how CD3 subunits are linked together, and how the glycosylation site influences the immune response in teleost fish.

The thymus is the primary immune organ for the production and maturation of T lymphocytes in fish (Jósefsson et al., 1993; Matsunaga et al., 2001). The spleen and anterior kidney, as well as intestines and gills, are secondary immune organs where T cells are functional (Zapata and Cooper, 1990; Zapata et al., 1996). CD3γ/δ and CD3ε expression was observed in lymphoid organs, thymus and spleen, and in MALTs, intestines and gills, of healthy mandarin fish. A similar pattern of expression of CD3γ/δ and CD3ε has been reported in other teleost fish, such as in fugu, Atlantic salmon, and Atlantic halibut (Araki et al., 2005; Liu et al., 2008; Øvergård et al., 2009). CD3 mRNA expression is expected in the thymus, as indicated above for other teleost fish, because it is a primary lymphoid organ. The secondary lymphoid organ, the spleen, also contains lymphocytes (Zapata et al., 1996). Intestines and gills are considered to be MALTs in fish (Dalmo et al., 1997; Rombout et al., 2005). It has been reported that there is a high percentage of T cells in the intestine (Romano et al., 2007; Picchietti et al., 2011; Rombout et al., 2011), and expression of CD3 in the intestines and gills may indicate that T cells are present and functional in these two tissues, as observed in other teleost species (Abelli et al., 1997; McMillan and Secombes, 1997; Bernard et al., 2006; Picchietti et al., 2011; Rombout et al., 2011). A relatively low level of CD3γ/δ and CD3ε expression was observed in liver and head kidney, indicating that liver may in fact not be a reservoir of T lymphocytes in normal fish. Head kidney is an important lymphoid organ, though not as important as thymus, for the production of T lymphocytes. This fact may account for the observed low levels of CD3γ/δ and CD3ε expression, which

were similarly observed in other teleost fish including Atlantic salmon (Liu et al., 2008).

However, it may not be possible to isolate an adequate number of clean cells from thymus for examining gene expression following in-vitro treatment, mainly because the organ is relatively small and taxonomically super-facial. Lymphocytes from head kidney were isolated in the present study and the mitogens PHA and ConA were used as stimulants. These mitogens have been proven to be TCR-binding and act on different populations of T lymphocytes, with PHA acting on all T lymphocytes and ConA mainly affecting suppressor T lymphocytes in mammals (Miller et al., 1975; Miyara and Sakaguchi, 2007). PHA and ConA both had significant effects on the expression of CD3γ/δ and CD3ε in lymphocytes isolated from head kidney. PolyI:C may need to be recognized by certain pattern recognition receptors, such as TLR (Mäkelä et al., 2011), before it can elicit an immune response. Nevertheless, the increase in expression of CD3γ/δ and CD3ε in head kidney lymphocytes may also indicate the presence of T lymphocytes in head kidney of teleost fish, which may be of some interest for further investigation.

The bacterial pathogen *F. columnare* causes columnaris disease in many freshwater fish species (Schneck and Caslake, 2006). In China, this bacterium is widely distributed and infects a variety of freshwater fish, resulting in relatively high mortality of some important aquaculture species (Wang et al., 2010). *F. columnare* can increase the expression level of immune molecules in channel catfish, *Ictalurus punctatus* (Pridgeon and Klesius, 2010), and the expression of IgM, IgD, and IgZ, and also TCRs, in mandarin fish (Tian et al., 2009b, in press). The increase in the expression of CD3γ/δ and CD3ε in thymus, spleen, and intestine in mandarin fish infected with *F. columnare* may indicate an immune response involving T lymphocytes, but a clear pattern of response by T cell subtypes needs to be clarified.

In conclusion, the identification of two subunits of CD3, CD3γ/δ and CD3ε, together with other recent reports on CD4, CD8, and TCRs α, β, and γ (Guo and Nie, 2011, in press; Tian et al., in press) in mandarin fish, confirms the presence of a TCR complex and TCR co-receptors, which are necessary for the activation of T lymphocytes in mammals (Davis and Chien, 2003), although CD3ζ has not been reported in mandarin fish. Further research should be devoted to examining the functional aspects of these molecules and to identifying the cells that express them.

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