Channel catfish CD8α and CD8β co-receptors: Characterization, expression and polymorphism

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In this study we report the identification and characterization of channel catfish, Ictalurus punctatus CD8α and CD8β genes. Both genes encode predicted proteins containing a leader, a immunoglobulin superfamily V domain, a stalk/hinge region, a transmembrane region and a positively charged cytoplasmic tail (CYT) containing the conserved teleost C-X-H motif. Catfish CD8α and CD8β are encoded as single copy genes and as in other vertebrates exhibit a conserved head to tail synteny; the CD8β gene is found 14.1 kb upstream of the CD8α gene. Both CD8α and CD8β transcripts showed a low degree of polymorphism. Finally, as determined by q-PCR both CD8α and CD8β are expressed in various catfish lymphoid tissues with the highest expression observed in thymus from 2 month old catfish-fry. In the future these results will provide the basis for evaluating the role of CD8+ CTL and other CD8-bearing cells in response to immunization or infection in the catfish.

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1. Introduction

In mammals, T cell development and T cell effector functions are dependent on T cell receptor (TCR)-mediated recognition of peptide antigens presented on major histocompatibility complex (MHC) molecules. This TCR-MHC interaction requires a co-receptor, either CD4 or CD8. T helper (TH) cells express the co-receptor CD4 and this molecule binds to the base of the β2 domain of MHC class II molecules, while CD8 is the TCR co-receptor expressed by γδ cytotoxic T lymphocytes (CTL) and it binds to the α2 domain of MHC class I molecules [1–4]. These co-receptors serve two functions 1) they stabilize the TCRβ-MHC interaction and 2) they initiate the T-cell signaling process by recruiting the Src family kinase p56lck that phosphorylates the T cell CD3 accessory signaling molecules. Mammalian CD4 molecules consist of four extracellular immunoglobulin (Ig) domains (D1, D2, D3, D4), a transmembrane (TM) region and a cytoplasmic tail (CYT) containing a p56lck binding site [5]. CD4 D1 and D2 domains are separated from the D3 and D4 domains by a flexible hinge region. Domains D1 and D3 are classified as Ig superfamily variable (IgSFv) domains, while D2 and D4 are IgSF constant (IgSFc2) domains. Mammalian CD8 molecules are expressed as disulfide-linked dimers of two chains and are usually found as heterodimers of CD8α and CD8β, although homodimers of CD8α are mostly expressed on NK cells and intraepithelial lymphocytes. Both CD8α and CD8β consist of a single IgSFv domain, followed by an extended glycosylated flexible stalk region or hinge, a TM and CYT. However, in mammals and birds, only CD8α has a p56lck site binding site in its CYT [6]. Like mammals, all teleosts, including channel catfish, Ictalurus punctatus, have functional T cells [7–9] and possess genes homologous to TRA, TRB TRC and TRD [10–22]. For example, studies in the catfish first demonstrated that surface IgM negative (slgM−) lymphocytes are the cells that responded in mixed leukocyte culture (MLC) and these together with B cells (slgM+) and macrophage (adherent accessory cells) were required for in vitro antibody responses to thymus dependent antigen [23,24]. Also catfish slgM+ cells were the cells that responded to concanavalin A (Con A), but not to lipopolysaccharide (LPS) [25]. However, the existence of catfish T cells was only formally established by the identification of TRA and TRB genes and isolation of clonal T cells [19,26,27]. As in mammals, teleost T cells can also be further classified by the presence of CD4 or CD8 message and genes encoding these molecules have been identified in several teleost species. Teleost CD4 molecules were first reported in the tiger pufferfish, Takifugu rubripes [28], rainbow trout, Oncorhynchus mykiss [29,30], catfish [31] and more recently in the Atlantic salmon, Salmo salar [32]. In each of these species, two CD4-like genes/cDNAs were found: CD4-1 and CD4-2 (or CD4REL).
The teleost CD4-1 cDNAs encoded mature proteins consisting of four extracellular Ig domains, TM, and a CYT containing the conserved aspartic acid ps62R binding site C-X-C. In contrast, CD4-2 cDNAs encoded proteins consisting of two or three Ig domains, a TM, and a C-X-C motif bearing CYT. CD4-2 genes/cDNAs encoding two Ig domains are found in rainbow trout, tiger pufferfish and Atlantic salmon [29,30,32], while a gene encoding a three Ig domain CD4-2 has only been described in catfish [31]. Notably both “CD4-types” exhibit homology with mammalian and chicken CD4 molecules. The first teleost CD8 homolog (CD8z) was identified in rainbow trout and was similar to mammalian CD8z, molecules, i.e. it consists of an extracellular IgSFV domain, a glycosylated hinge stalk-like region, a TM and CYT [33]. However, the rainbow trout CD8z does not contain the conserved ps65 binding C-X-C motif found in mammalian CD8x CYTs; instead a C-X-H motif is present. Subsequently, CD8z and DB8 genes/cDNAs were described in other teleosts, including Atlantic salmon; tiger pufferfish; gibelica crucian carp; Carassius auratus; common carp, Cyprinus carpio; sea bass; sea bream, Sparus aurata; and Atlantic halibut, Hippoglossus hippoglossus, and like rainbow trout CD8z, these teleost CD8z and DB8 molecules exhibit a typical mammalian CD8-like structure, e.g. IgSV, hinge, TM and CYT. However, it should be noted that in teleosts both DB8 and CD8z contain the C-X-H motif in their CYT [34-42].

Teleost CTL have been demonstrated at a functional level in catfish, gibelica crucian carp and common carp, and rainbow trout [43-47]. For example, in catfish two types of CTL effectors have been cloned from alloantigen-stimulated cultures initiated with PBL from alloantigen immunized catfish [26]. The catfish clonal CTL are granular and each clonal cell line expresses unique TCRz and TCRB rearrangements. However, even though morphologically similar, the two types of catfish CTL differ in their specificity to allogeneic targets and their killing pathways. Group I CTL exhibit specificity only for the immunizing allogeneic B cell line, 3B11. In contrast, Group II CTL have broader specificity and kill several allogeneic cell lines, including 3B11, as well as LPS blasts from allogeneic fish. Furthermore, alloantigen specific Group I CTL kill predominantly by the perforin granzyme secretory pathway, and Group II CTL kill by both the secretory pathway and an additional pathway that likely involves Fas-Fas ligand interactions [27]. Interestingly, the group II CTL express message for catfish CD4, which implies they represent the teleost equivalents of mammalian CD4+ CTL [31]. Similarly, in rainbow trout, Fischer et al. 2003 demonstrated that alloantigen specific cell mediated cytotoxicity can be induced by allogeneic skin grafts, immunization with allogeneic cell lines, and immunization with allogeneic or xenogeneic erythrocytes. This cytolytic activity occurred in the mlgM PBL population and correlated with an increase in TCRz and CD8z mRNA expression [48]. More recently, by using anti-gibelica crucian carp CD8z, CD4 and IgM mAbs, gibelica crucian carp were shown to express a CD8α - CTL population in their PBL [49].

Here we report the identification of channel catfish CD8z and DB8 genes and cDNA homologs, their characterization and examine their message expression in catfish lymphoid tissues.

2. Materials and methods

2.1. Experimental animals and isolation of PBL

Channel catfish were maintained under standard conditions at the USDA-ARS Catfish Genetics Research Unit, Stoneville, MS following the accepted standards of animal care and use approved by the Institutional Animal Care and Use Committee (IACUC) according to USDA-ARS policies and procedures. The fish used ranged from 2 months of age to adults as described below.

Leukocytes from catfish peripheral blood (PBL) were isolated from heparinized blood by centrifugation on a cushion of ficoll-hypaque (Lymphoprep, Accurate Chemical Corp.) as described previously from adult fish maintained the University of Mississippi Medical Center, Jackson MS [50,51].

2.2. RNA and cDNA preparation

Catfish tissues were rapidly excised from fish, flash frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA from pronephros (anterior kidney), mesonephros (posterior kidney), thymus, spleen, gill, liver and PBL was isolated using TRI-reagent or RNAzol RT (Molecular Research Center) according to the manufacturer’s protocol and quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). For cDNA synthesis, the RNA was first treated with DNase I (Ambion, Inc) and 1 μg was subsequently converted into cDNA using the iScript cDNA (Bio-Rad) synthesis kit according to manufacturer protocol. The synthesized cDNA was then quantified and diluted to 200 ng/μl for all samples.

2.3. Identification of catfish (lp) CD8z and CD8a homologs

A catfish DB8z fragment was obtained by using degenerate primers corresponding to conserved sequences in the framework (FR) 2 region of the CD8β immunoglobulin (IG)-like domain and the CYT of known CD8β sequences. The primers were designed to amplify a CD8β fragment estimated to be approximately 450-460 bp. The eight forward (sense) primers were 2304-fold degenerate and encoded the amino acid sequence Q/D/E/T/S-V-F/Y-W-Y-F-R found at the complementary determining region (CDR) framework (FR) 2 border, while the four reverse (antisense) primers were 512-fold degenerate and encoded the CYT amino acid sequence P-K-K-C-R-H-R/Q (see Supplementary Table 1). In a checkerboard design these primers were used to amplify cDNA synthesized from catfish thymus. RT-PCR conditions were as follows: 2 μl thymus cDNA, 500 nM of each sense and antisense primer, 2 μlProof Taq masterMix (Biorad), in a final reaction volume of 25 μl. Typical parameters for PCR reactions were: 3 min 98 °C, followed by 44 cycles of 98 °C 10 s, 50 °C 20 s, 72 °C 1 min then a final extension at 72 °C for 5 min. The resulting products were electrophoresed and visualized on a 1.6% agarose gel containing ethidium bromide. The PCR products were purified using a QIAquick gel extraction kit (QIAGEN GmbH), and subcloned into pCR4-blunt-TOPO vector (Invitrogen) according to the manufacturer’s instructions. The putative DB8z plasmids were purified using a standard alkaline lysis protocol (Qiagen) and sequenced with universal primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The CD8b-F1 and CD8b-R1 combination amplified a true DB8β fragment, which was used to design gene-specific primers DB8β-PCR-F and CD8b-PCR-R for PCR screening of the catfish CBCL1 bacterial artificial chromosome library (BAC; [52]). Once a DB8β positive BAC (GY003A07) was isolated, the complete DB8β genomic sequence was obtained by primer walking. The complete catfish DB8z cDNA sequence was subsequently isolated from thymus cDNA by PCR and then sequenced on both strands using gene specific primers and universal forward and reverse primers. The full-length CD8z cDNA was obtained using 5’ and 3’ RACE protocols (Table 1; SMART Race Kit; Clontech).
1. Specific reverse and forward primers were then designed to sequence the entire CD8α gene by primer walking. Finally a catfish full-length CD8α cDNA was obtained as described above.

2.4. In silico sequence analysis

The DNASTAR Software Suite (DNASTAR, Inc) was used for nucleotide and amino acid sequence analyses and assembly, CLUSTALW multiple alignments, and polymorphism analyses. Amino acid identities to the catfish CD8α and CD8β sequences were calculated from pairwise alignments. Similarity searches were performed using BLAST analysis [53] against the National Center for Biotechnology Information (NCBI) non-redundant database. The leader sequence, IgSF V domain, TM and CYT regions were identified and O-linked and N-linked glycosylation sites were based on predictions made using the CBS Prediction Servers available at http://www.cbs.dtu.dk/services/ (SignalP 3.0, TMHMM V2.0, NetOGlyc 3.1, NetNGlyc 1.0 Servers). CD8α and CD8β promoter analysis was performed using the Genomatix suite (Genomatix Software GmbH).

2.5. Southern blot analyses

Genomic DNA was prepared using erythrocytes from outbred and homozgyous gynogenetic [54] catfish as previously described [55] Briefly, genomic DNA (10 μg) was digested to completion with EcoRI or Pst I restriction enzyme, separated on 1% agarose gels and transfened by capillary action onto Hybond-N (Amersham Pharmacia Biotech AB) at 65 °C. Genomic DNA was prepared using erythrocytes from outbred and homozgyous gynogenetic [54] catfish as previously described [55] Briefly, genomic DNA (10 μg) was digested to completion with EcoRI or Pst I restriction enzyme, separated on 1% agarose gels and transferred by capillary action onto Hybond-N membranes (GE Healthcare Bio-Sciences AB) using standard techniques. Hybridizations were performed in Rapid-hyb buffer (GE Healthcare Bio-Sciences AB) at 65 °C according to manufacturer’s instructions and membranes were washed at high stringency (65 °C with 0.1X SSC, 0.1% SDS). The catfish CD8α probe encompassed the complete signal peptide plus the first half of the Ig domain and the CD8β probe consisted of the entire Ig domain plus the region directly 5’ of the TM (see Table 2 for primers used). Probes were amplified by PCR using IDPol DNA polymerase (ID Labs Biotechnology) according to the manufacturer’s protocol. PCR parameters were: 1 min 94 °C, followed by 29 cycles of 94 °C 30 s, 61 °C 30 s, 72 °C 1 min, then extension at 72 °C for 5 min. Both products were verified by sequencing and the probes were random primed labeled with [32P]-dCTP by Megaprime labeling (GE Healthcare Bio-Sciences AB) using the manufacturer’s protocol.

2.6. Single nucleotide polymorphism (SNP) determination

Fifteen two-month-old outbred catfish were selected and thymus cDNA was prepared as described above. RT-PCR was performed as described above using gene specific forward and reverse CD8α and CD8β primers that amplified full-length transcripts (see Table 2). However, annealing temperatures of 60 °C and 44 cycles was used for CD8α, and 56 °C and 44 cycles for CD8β. For each fish, eight plasmids each of CD8α and CD8β were sequenced and analyzed for SNPs using DNASTAR alignments.

2.7. q-PCR analysis

Total RNA from anterior kidney, posterior kidney, thymus, spleen, gill and liver was isolated from three one-year-old juvenile catfish as described above. In addition, total RNA from the thymus was also isolated from three 2-month-old fry and PBL RNA was isolated from three adult fish. cDNA was synthesized as described above and used in q-PCR to determine distribution and variation in CD8 mRNA levels in these tissues using the iCycler IQ™ Real Time PCR Detection System (Bio-Rad). Briefly, q-PCR was performed using a total of 200 ng of cDNA and the iQ SYBR green master mix reagent in a total volume of 25 ul, according to the manufacturer’s protocol. All reactions were performed in triplicates. The amplification profile was 95 °C for 3.5 min, followed by 45 cycles of 94 °C for 15 s and 63 °C for 20 s. Data analysis was performed as described in the ABI PRISM 7700 sequence detection bulletin #2 from Applied Biosystems, following the ΔCT method and all results were normalized to expression of catfish α-tubulin. Primers used for real-time q-PCR are indicated in Table 2.

3. Results and discussion

3.1. Catfish CD8α and CD8β

The catfish CD8α and CD8β cDNA transcripts encode mature proteins consisting of an IgSF V domain, a stalk-like hinge region, a TM and a positively charged CYT. The catfish full-length CD8α cDNA, including the predicted leader sequence has a 672 nucleotide open reading frame (ORF) which encodes 223 amino acid residues. Nine potential O-linked glycosylation sites are predicted in the stalk/hinge region, and no N-linked glycosylation sites are found within the IgSF V domain or in the stalk/hinge region (Fig. 1). Similarly, the catfish full-length CD8β cDNA contains a 633 nucleotide ORF encoding 210 amino acid residues. A single N-linked and three potential O-linked glycosylation sites are encoded in the IgSF V domain and the stalk/hinge region, respectively (Fig. 2). This structurally conserved stalk/hinge-like region was first described in mammalian CD8 molecules and it is found between the Ig domain and TM region. For example, the stalk/hinge regions of both catfish CD8α and CD8β contain two cysteine residues, either of which could be used to form the interchain disulfide bond that holds the CD8 heterodimer (CD8α-CD8β or CD8α-CD8β) together. Also both CD8 stalk/hinge regions have O-linked glycosylation sites and are proline rich. This conservation of glycosylation, as noted by Hansen and Strassburger for rainbow trout CD8α is predicted to help maintain an extended CD structure allowing for MHC class I interaction and to prevent CD8 from protease degradation [33,56–59]. The alignment of CD8α chains shows that teleost have two conserved cysteines near or in CDR1, which are four amino acids apart. However, whether these cysteines can form intrachain disulphide bonds has not been determined. As expected, amino acid alignments show that like other teleosts, the catfish CD8α chain CYT contains a C-X-H motif instead of the C-X-C motif that is found in all mammalian and avian CD8α chains sequenced to date [33,35–37,41] Also, the finding of the C-X-H motif in a chondrichthyan, the shovelnose guitarfish (Rhinobatos productus) suggests that C-X-H may represent a primordial Lck binding site (see Fig. 1). Importantly, functional studies in mammals and chickens demonstrate that this motif is the binding site for the protein tyrosine kinase p56Lck (Shaw et al. 1990; Turner et al. 1990). In the catfish the C-X-H motif is found as part of a highly conserved stretch of 25 amino acids that begin near the TM-CYT border of all teleost CD8α chains sequenced to date (see Fig. 1). This C-X-H motif is also found in the CYT of all teleost CD8β sequences and like the CD8α motif, the CD8β C-X-H motif is part of a conserved stretch of 18 amino acids, which begins near the TM-CYT border and extends into the CYT (see Fig. 2). Also, here it should be emphasized that chicken and mammalian CD8β chains do not bind p56Lck [6] and CD8β cannot be expressed on a cell unless it is part of a CD8α-CD8β heterodimer [60]. When CD8β was first identified in the rainbow trout, the lack of a p56Lck binding site was puzzling since p56Lck is critical for the initiation of TCR signaling since it phosphorylates the C-X-H motif ([33,37,41]). It is also interesting to note
that the highly charged CYT of mouse CD8β (10 amino acids) was shown to enhance the p56Lck activity by 1) stabilizing the binding of Lck to CD8α and 2) increasing p56Lck enzymatic activity [61]. Later it was hypothesized that the cysteine and histidine amino acid residues of the teleost C-X-H motif might form a clasp structure, which would be able to bind the p56Lck kinase homolog in teleost as does the C-X-C zinc clasp of human CD8α and CD4 [36,62]. Importantly, Hayashi et al., recently confirmed by surface plasmon resonance that a rainbow trout CD8α peptide R-I-R-T-K-R-C-P-H-H-Y-K-R-Q-P-R is able to bind rainbow trout p56Lck in the presence of Zn2+ ions [63]. Since the R-I-R-T-K-R-C-P-H-H-Y-K-R-Q-P-R motif is highly conserved among the different teleost species, it is likely that the catfish CD8α will also bind p56Lck. However, in the same study the rainbow trout CD8β, which contains a similar highly charged motif R-L-P-K-K-C-R-H-Q-F-A-K-K-R-P-M was not observed to bind rainbow trout p56Lck. The catfish CD8β CYT, like most teleost CD8β sequences, also resembles rainbow trout in that an arginine residue is included in the binding motif, i.e. R-L-P-K-K-C-R-H-Q-F-A-K-K-Q. Since a catfish putative p56Lck homolog has been identified from catfish ESTs (S. Quiniou, unpublished) we anticipate that future studies using a combination of CD8α and p56Lck recombinant proteins and/or monoclonal antibody (mAb) reagents will be able to address these binding issues. As expected, the amino acid percent identities of the catfish CD8 homologs compared with the different teleost CD8 homologs (including leader sequences) are relatively high; for CD8α sequences the percent identities range from 31–48% and for CD8β sequences the percent identities range from 22–48%.

3.2. CD8 expression in catfish tissues

Both CD8α and CD8β expression is detected in catfish lymphoid tissues (Fig. 3), the anterior and posterior kidneys, spleen, thymus, and also in the gill, which in Atlantic salmon was demonstrated to be associated with intraepithelial cell aggregations (ILT) that express TCR message [64,65]. Additionally, through the use of polyclonal antibody the authors showed the aggregated gill associated tissue contained MHC class II positive cells. As expected, the catfish thymus expressed the highest levels of CD8 message compared with the other tissues (Fig. 3). Moreover, the greatest levels of CD8 expression were detected in the thymus tissues from 2-month-old catfish fry, i.e. CD8α and CD8β expression was 15–80 times higher than CD8α and CD8β expression in one-year-old catfish.
juvenile catfish. Whether the differences in catfish thymus CD8 message expression is due to thymic involution occurring between these two time points or the timing of CD4 and CD8T cell selection in the thymus cannot currently be resolved.

3.3. Cloning and mapping of the catfish CD8 locus

Sequence analyses of BAC GY003A07 show that the CD8α and CD8β genes are separated by 14.1 kb and are in the same transcription orientation, arranged head to tail in the catfish genome. The CD8β gene is located 5’ of the CD8α gene (Fig. 4). This tandem gene arrangement of CD8β followed by CD8α is reminiscent of the CD8α gene synteny of zebra fish, Danio rerio (Zebra fish Zv8 Build), chicken, Gallus gallus (Chicken Build 2.1), the marsupial gray shortailed possum, Monodelphis domestica and human [66]. Also, Southern blot analyses using two different restriction enzymes (EcoRI and PstI) indicate that catfish CD8α and CD8β, like other vertebrate CD8α and CD8β genes are likely encoded by single copy genes since restriction digests from six fish show a single hybridizing bands (see sFig. 1). Furthermore, an assembly of genomic short sequencing reads at 25× depth coverage (C. Waldbieser, unpublished data) did not show any evidence of CD8α or CD8β gene duplications. The catfish CD8α gene spans 5082 nucleotides and consists of six exons (Fig. 4). Exon 1 encodes the first 44 nucleotides of the predicted leader sequence and the 5’ untranslated region (UTR) and exon 2 encodes the last 11 nucleotides of the leader and the entire IgSF V domain and 12 nucleotides encoding the first four amino acids of the predicted stalk-like hinge region. The remainder of the hinge and the TM are encoded by exon 4 and the CVT and 3’ UTR is encoded by exons 5 and 6. The intron-exon boundaries and UTR regions were determined by examining catfish CD8α cDNAs and 3’ and 5’ RACE products (sFig. 2). The catfish CD8β gene is slightly longer and spans 7741 nucleotides. Like CD8α, it is also encoded by 6 exons. Exon 1 encodes the first 46 nucleotides of the predicted Leader sequence and the 5’ UTR. Exon 2 encodes the last 8 nucleotides of the Leader and the entire IgSF V domain. Exon 3 encodes most of the predicted stalk-like hinge, while exon 4 encodes the last four amino acid residues of the hinge and the TM. The CD8β CVT and 3’ UTR are encoded by exons 5 and 6 (sFig. 3).

The 300 bp upstream of the start codon of the catfish CD8α and CD8β genes was analyzed for the presence of classic core promoter elements by manually examining the sequences for TATA, initiator element (Inr), TFIIB recognition element (BRE), and downstream promoter element (DPE) motifs as described by Smale [67]. Of these, only an Inr element could be identified and in both CD8α and CD8β the Inr sequence matched the mammalian Inr consensus sequence, T/C-T/C-A-A/C/G/T/-T/A-T/C-T/C (see Figs. 2 and 3). Inr elements are functionally similar to TATA boxes and they are able to support transcription on their own. The adenosine of the Inr is frequently the transcription initiation site (+1), although exceptions do occur. In this study, 5’ RACE showed that the longest catfish CD8α and CD8β transcripts began at +1 and +2, respectively.

3.4. Catfish CD8α and CD8β polymorphism

To examine the putative incidence of CD8α and CD8β polymorphisms, the full-length coding regions of CD8α and CD8β from four outbred catfish were amplified by RT-PCR using Pre-ATG and Post-Stop primers (sTable 2), and eight PCR products from each fish were sequenced. For CD8α, only one nucleotide substitution...
Fig. 3. Relative expression of CD8α and CD8β mRNA in catfish tissues. CD8α and CD8β gene expression levels were determined in tissue samples from three one-year-old fish and compared to CD8α and CD8β gene expression levels in PBL isolated from three adult fish, and thymus from three 2-month-old fish. Samples from the three one-year-old fish are: anterior kidney (AK-1, AK-2, AK-3), posterior kidney (PK-1, PK-2, PK-3), liver (Liv-1, Liv-2, Liv-3), spleen (Spl-1, Spl-2, Spl-3), thymus (Thy-1, Thy-2, Thy-3). Samples from the three adult fish are PBL-4, PBL-5, PBL-6. Samples from the three 2-month-old fish are Thy-7, Thy-8, Thy-9. Each bar represents data from an individual fish and each sample was normalized against catfish β-tubulin. The error bars represent the standard deviation. For Thy-7, Thy-8 and Thy-9, as those values are not to scale, standard deviations have been added or subtracted to the data values and indicated in parentheses.

(TTG → TTC) was observed and it occurred in the leader (Table 1). This substitution was non-synonymous and resulted in a Leu → Phe amino acid change. This allelic difference was equally represented in the fish population examined and likely does not disrupt leader function. For CD8β, three nucleotide changes were found in three different positions, two were synonymous (GTG → GTT; GCG → GCA) and the third (AAA → AAC) resulted in a Lys → Asn amino acid change. This change from a positively charged amino acid to a polar amino acid occurs in the predicted FR3 of catfish CD8β and likely does not affect CD8β binding to MHC class I (Fig. 2).

Also, the observed combination of the CD8β nucleotide substitutions in the fifteen outbred fish indicate that these SNPs represent four alleles. Therefore it appears that catfish CD8β molecules exhibit very little polymorphism, at least within their coding regions. A low level of CD8α polymorphism was also found in rainbow trout where three nucleotide changes in the CDR2 resulted in two amino acid changes [33]. These levels of polymorphism are similar to the polymorphism levels observed within the coding regions of CD8α and CD8β in mouse strains. Four SNPs occur in mouse CD8α. Two are nonsynonymous and are at amino positions #46 in the IgSF V domain and at position #199 in the CYT; the two synonymous SNPs occur at position #46 in the the IgSF domain and #191 in the CYT.

The two SNPs for mouse CD8β are nonsynonymous, one is found at amino acid position #98 in the IgSF V domain and the other at position #206 in the CYT (http://www.informatics.jax.org/marker key 875 and 876). Currently, the chicken is the only vertebrate where extensive CD8 polymorphism has been observed. In chickens eight CD8α alleles have been described and among these identified alleles there are 13 amino acid substitutions in the coding region and nine of these are found in the CDR (three in CDR1, four in CDR2, and two in CDR3) [68,69]. In comparison only two amino acid substitutions have been identified in the coding region of the chicken CD8β [70]. Furthermore, sequence analyses indicate that this unusually high degree of polymorphism in the chicken CD8a is the result of gene conversion mechanisms which exchange blocks of nucleotides originating from a set of 29 CD8α-like genes that are located immediately upstream of CD8α. These CD8α-like genes constitute a distinct multigene family of immune receptors with unique CYT, but highly conserved IgSF V domain and TMs (nucleotide identities of leader-intron-IgSF V exon can be as high as 85%). In addition, phylogenetic analyses demonstrate that these 29 CD8α-like sequences most closely resemble the teleost novel-immune type receptors (NITRs).

Using the same primers designed to analyze catfish CD8 SNPs (CD8α and CD8β pre-ATG and post-Stop primer pairs), cDNA from various catfish tissues were examined for the presence of CD8 alternatively spliced transcripts encoding possibly truncated CD8 molecules since several CD8 alternative splice forms have been

**Table 1**

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<td>AAC</td>
<td>Asn</td>
<td>GCA</td>
<td>Ala</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(nt: nucleotide; AA: Amino Acid).
These transcripts encode for CD8αβ, CD8αδ, and CD8αε chains, which are expressed in different cell populations depending on their developmental stage and function. Alternative splicing at the level of exon V, which encodes for the transmembrane (TM) domain, results in the generation of multiple splice variants of CD8α. The TM domain is critical for the function of CD8 cells, as it mediates homodimerization and clustering, which is essential for T-cell activation and cytokine production. The existence of CD8α splice variants allows for the generation of CD8α dimers that exhibit different effector functions, providing a mechanism for the fine-tuning of immune responses.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.fsi.2011.01.011.

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