Identification of NF- κ B related genes in channel catfish and their expression profiles in mucosal tissues after columnaris bacterial infection

Xiaozhu Wang ^a, Shikai Liu ^a, Yujia Yang ^a, Qiang Fu ^a, Ash Abebe ^b, Zhanjiang Liu ^{a,§}

Abstract

Interactions of NF- κ B family, I κ B family and IKK complex are the key components of NF- κ B pathway that is essential for many biological processes including innate and adaptive immunity, and inflammation and stress responses. In spite of their importance, systematic analysis of these genes in fish has been lacking. Here we report a systematic study of the NF- κ B related genes in channel catfish. Five NF- κ B family genes, five I κ B family genes and three IKK complex genes were identified in the channel catfish genome. Annotation of these 13 NF- κ B related genes was further confirmed by phylogenetic and syntenic analysis. Negative selection was found to play a crucial role in the adaptive evolution of these genes. Expression profiles of NF- κ B related genes after *Flavobacterium columnare* (columnaris) infection were determined by analysis of the existing RNA-Seq dataset. The majority of NF- κ B related genes were significantly regulated in mucosal tissues of gill, skin and intestine after columnaris infection, indicating their potential involvement in host defense responses. Distinct expression patterns of NF- κ B related genes were observed in susceptible and resistant catfish in response to columnaris infection, suggesting that expression of these genes may contribute to the variations in disease resistance/susceptibility of catfish.

Keywords: NF- κ B, I κ B, IKK, Immune response, Gene expression, Fish

1. Introduction

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) are transcription factors that regulate innate and adaptive immune responses (Pahl, 1999). They are among the key regulators of inflammation after bacterial infection (Gerondakis et al., 1999). There are five NF- κ B family proteins in mammals: RelA (p65), RelB, c-Rel, p50 (p105/NF κ B1) and p52 (p100/NF κ B2), encoded by *RELA*, *RELB*, *REL*, *NFKB1*, and *NFKB2* gene, respectively (Lawrence, 2009). NF- κ B proteins are sequestered in cytoplasm by inhibitory molecules of I κ B family, which includes five proteins: I κ B α , I κ B β , I κ B ϵ , Bcl3 and I κ B ζ , encoded by *NFKBIA*, *NFKBIB*, *NFKBIE*, *BCL3* and *NFKBID* gene, respectively (Baldwin, 1996). However, p105 and p100, the precursor proteins of p50 and p52 respectively, can be assigned to both NF- κ B and I κ B families as they have the characteristics of both NF- κ B and I κ B proteins (Oeckinghaus and Ghosh, 2009). The activation of NF- κ B is mainly triggered by the upstream I κ B kinase (IKK) complex, which includes three major members: IKK α , IKK β and IKK γ , encoded by *CHUK*, *IKBKB*, and *IKBK* gene, respectively (Israel, 2010). NF- κ B family, along with its inhibitors (I κ B family) and activators (IKK complex), work together to exert important roles in pro- and anti-inflammatory responses after bacterial infection.

NF- κ B proteins are DNA sequence-specific transcription factors which are associated with each other to form functional complexes and regulate inflammatory and innate immune responses (Karin, 1999). They all share a conserved Rel homology domain (RHD), which is functionally important for dimerization, I κ B binding, and nuclear translocation (Baldwin, 1996). Although NF- κ B proteins were originally thought to be only produced in mature B cells and plasma cells, they are now known to be present in cytoplasm of most cells in an inactive form due to their binding with I κ Bs (Baeuerle and Baltimore, 1988). Activation of NF- κ B has been demonstrated to be achieved by degradation of I κ Bs through their phosphorylation by IKK complex, which allows free NF- κ B to enter the nucleus

and activate transcription of target genes (Israel, 2010). Expression of the genes regulated by NF- κ B is mostly responsive to environmental changes. Such genes include tumor necrosis factor alpha (TNF α), MHC pathway genes, granulocyte colony-stimulating factor (G-CSF), and inhibitor of apoptosis proteins (IAPs) (Oeckinghaus and Ghosh, 2009). In addition, post-translational modifications such as phosphorylation, ubiquitination, or acetylation can significantly affect the regulation of the NF- κ B pathway (Perkins, 2006).

I κ B proteins were originally known as inhibitors of NF- κ B with the function of cytoplasmic retention, while subsequent discoveries suggested that I κ B proteins act as complex regulators of NF- κ B (Grilli et al., 1993). I κ B proteins can be further divided into two groups: ‘cytoplasmic I κ Bs’ (I κ B α , I κ B β and I κ B ϵ) and ‘nuclear I κ Bs’ (Bcl3 and I κ B ζ) (Yamamoto and Takeda, 2008). They all contain five to seven Ankyrin repeats (ANK) which are essential for interactions with the RHD in NF- κ B proteins. The inhibitory function of I κ Bs is delivered through their binding to NF- κ B proteins to form dimers, thereby preventing NF- κ B to be transported into the nucleus. Upon IKK activation, I κ B α is rapidly degraded, leading to an oscillatory NF- κ B activation, while the degradation of I κ B β and I κ B ϵ is much slower, providing a long-term oscillatory response of NF- κ B (Hoffmann et al., 2002). After ubiquitination and proteasomal degradation of phosphorylated cytoplasmic I κ Bs, free NF- κ B dimers translocate from cytoplasm to nucleus, where nuclear I κ Bs can either activate or inhibit NF- κ B transcriptional activity by interacting with NF- κ B subunits (Oeckinghaus et al., 2011; Yamamoto and Takeda, 2008).

IKK complex, as a core element of NF- κ B activation, is composed of two catalytically active kinases (IKK α and IKK β) and a regulatory subunit (IKK γ) (Chen et al., 1996). There are two separate NF- κ B activation mechanisms depend on different IKK proteins: 1) the canonical pathway requires IKK β associated with IKK γ to rapidly degrade I κ B proteins in response to upstream TNF α and IL-1 signaling; and 2) the non-canonical pathway relies on activation of IKK α by upstream NF-

κ B-inducing kinase (NIK), and then IKK α specifically phosphorylates p52-RelB complexes (Pomerantz and Baltimore, 2002; Scheidereit, 2006; Senftleben et al., 2001).

Functions of NF- κ B related genes in immune system has been well studied in mammals (Baeuerle and Henkel, 1994; Yamamoto and Gaynor, 2001). Toll-like receptors (TLRs), as primary sensors, are the key pattern recognition receptors (PRRs) for pathogen associated molecular patterns (PAMPs) of bacterial pathogens (Philpott and Girardin, 2004). TLRs can active NF- κ B pathway, leading to the subsequent innate immune responses (Kawai and Akira, 2007). The roles of NF- κ B pathway in the production of inflammatory mediators against bacterial infection is well documented in mammalian species, but only a few studies in teleost fish were reported. For instance, three NF- κ B molecules (p65, c-rel, and p52) and two I κ B α orthologues (I κ B α a and I κ B α b) were found to be involved in notochord development in zebrafish (Correa et al., 2004; Pradhan et al., 2012). NF- κ B subunits and their activation by aryl hydrocarbon receptor agonists (AhR) were reported in marine fish (Schlezingner et al., 2000). In spite of these studies, systematic analysis of NF- κ B related genes in teleost fish are still lacking, especially their expression patterns after bacterial infection.

Channel catfish is the primary aquaculture species in the United States. The catfish industry is facing serious challenges of disease problems. Columnaris disease, caused by *Flavobacterium columnare*, is one of the most frequently occurring disease of catfish (Shoemaker et al., 2008; Zhao et al., 2015). To understand the immune responses after bacterial infection, a large number of studies have been conducted with immune related genes in catfish, including pathogen recognition receptors (Rajendran et al. 2012a; 2012b; Zhang et al., 2013; Sun et al., 2014), claudins (Sun et al., 2015), serpins (Li et al., 2015), chemokines (Bao et al., 2006; Peatman et al., 2006; Fu et al., 2017), complementary proteins (Jiang et al., 2015), tumor suppressor proteins (Mu et al., 2015), Rho GTPase genes (Tan et al., 2017), apolipoprotein genes (Yang et al., 2017), and a number of regulatory and response genes involved in disease responses (Yao et al., 2014; 2015; Yuan et al.,

2016). Suppression of NF- κ B pathway was reported in catfish gill tissue after columnaris infection (Sun et al., 2012). However, the immune response of NF- κ B related genes to columnaris disease at transcriptional level remains unclear in different mucosal tissues. In this study, we identified 13 NF- κ B related genes in the channel catfish genome, and determined their expression profiles in three mucosal tissues of gill, skin and intestine after *F. columnare* infection.

2. Material and method

2.1. Identification of channel catfish NF- κ B related genes

To identify the catfish sequences of NF- κ B related genes, we searched the channel catfish RNA-Seq dataset (Liu et al., 2012) using TBLASTN with amino acid sequences of NF- κ B related genes from human (*Homo sapiens*), mouse (*Mus musculus*), zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*) and stickleback (*Gasterosteus aculeatus*) as queries. For the most comprehensive BLAST, we included additional RNA-Seq datasets in our analysis (Li et al., 2012; Sun et al., 2012).

Duplicates in the initial sequence pool were eliminated by using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), and the resulting unique set of sequences were subjected to further analysis. The open reading frame (ORF) of each unique sequence was identified by ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and then verified by BLASTP against NCBI non-redundant protein sequence database. BLASTN was conducted against the catfish reference genome sequence (Liu et al., 2016) for validation and for the determination of copy numbers. The genes within the catfish reference genome were predicted by FGENESH program (Solovyev et al., 2006).

2.2. Sequence and evolutionary analysis of NF- κ B related genes

The gene structure was investigated based on each coding sequence (CDS) and corresponding genomic sequence by using the online Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/index.php>). The functional domain characteristics of NF- κ B related genes were identified using NCBI Conserved Domain Search (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). To provide insights into the evolutionary patterns of NF- κ B related genes, patterns of selection pressure were examined with maximum likelihood analysis by using the YN00 program in the PAML X package (Xu and Yang, 2013).

2.3. Phylogenetic and syntenic analysis

The full-length amino acid sequences of NF- κ B related genes from several representative vertebrates, including human, mouse, cow, lizard, frog, and several fish species such as zebrafish, fugu, tilapia, stickleback, Amazon molly, and Atlantic cod were retrieved from ENSEMBL genome browser (<http://www.ensembl.org/>) or NCBI database (<http://www.ncbi.nlm.nih.gov/>) for phylogenetic analysis. Multiple protein sequence alignments were conducted using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) with default parameters (Edgar, 2004). Phylogenetic analysis was conducted with maximum likelihood method using MEGA 6.0 software (Tamura et al., 2013). Bootstrap tests with 1,000 replicates were performed to evaluate the phylogenetic trees.

In cases where phylogenetic analysis was not sufficient to establish the orthologies (e.g., *NFKB2* and *BCL3*), syntenic analysis was also performed to properly annotate the genes. Conserved syntenic blocks of human and zebrafish were first identified from the Genomicus database version 84.01 (Louis et al., 2015; Muffato et al., 2010), and then compared to those of catfish.

2.4. Bacterial infection and sample collection

All procedures involved in the handling and treatment of fish during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation of the study. One-year old experimental fish were maintained in four 50-gallon tanks and acclimatized for 2 weeks at a temperature of 28°C. *F. columnare* bacteria were cultured from a single colony and re-isolated from a symptomatic fish. Challenge experiments were conducted by immersion exposure for 2 h at a final concentration 1×10^5 CFU/mL. Gill, skin, and intestine tissues were collected before challenge (0 h) and at 8 h after challenge. Equal amounts of tissue samples (50 mg) were collected from each fish within the three replicate pools (3 pools of 5 fish each) at each sampling time-point. Samples were flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

2.5. RNA extraction and quantitative real-time qPCR

Total RNA was extracted using RNeasy Mini kit (Qiagen, USA). RNA concentration and quality were checked using a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, USA). For real-time PCR, first strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, USA). All the cDNA products were diluted to 250 ng/μL for real-time PCR reaction using the PerfeCTa® SYBR® Green FastMix® Reaction Mixes (Bio-Rad, USA) on a CFX96 real-time PCR Detection System (Bio-Rad, USA). The primers used in quantitative real-time PCR were listed in Table 1. The 18S rRNA and 28S rRNA were used as the internal reference controls. The thermal cycling profile consisted of an initial denaturation at 95°C (for 30 s), followed by 40 cycles of denaturation at 94°C (5 s), an annealing/extension temperature at 60°C (5 s), and 72°C for 5 s, followed by dissociation curve analysis to verify the specificity of amplified products. Relative fold changes were calculated based on the expression levels of 18S rRNA and 28S rRNA using a randomization test in Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002).

Expression differences between groups were considered significant when $p\text{-value} < 0.05$. Three biological replicate RNA samples and three technical replicates were conducted to confirm expression patterns.

2.6. Expression analysis of NF- κ B related genes using RNA-Seq dataset

Expression of the NF- κ B related genes after disease infection were determined using existing RNA-Seq dataset. The Illumina-based RNA-Seq dataset was retrieved from the SRA database (SRA Accession SRP017689). These RNA-Seq short reads were generated from gill samples after *F. columnare* infection. The details of bacterial challenge, RNA extraction, library construction and transcriptome sequencing were described by Peatman et al. (2013). Trimmed reads of the RNA-Seq dataset were analyzed using CLC Genomics Workbench (v5.5.2). The trimmed RNA-Seq reads were aligned with all genes identified in channel catfish genome along with all cDNA sequences of channel catfish NF- κ B related genes. Mapping parameters were set to allow reads identity $\geq 95\%$ and mismatches ≤ 2 . The number of total mapped reads for each NF- κ B related gene was determined and RPKM (Reads Per Kilobase of the transcript per Million mapped reads) was calculated. The expression fold-change of each NF- κ B related gene was calculated based on the normalized RPKM using proportions-based Kal's test. NF- κ B related genes with absolute expression fold change values ≥ 1.5 , total read number ≥ 5 and $p\text{-value} \leq 0.05$ were regarded as differentially expressed genes.

3. Results

3.1. Identification of NF- κ B related genes in channel catfish

Thirteen NF- κ B related genes were identified in channel catfish including: five genes in NF- κ B family (*RELA*, *RELB*, *REL*, *NFKB1*, *NFKB2*), five genes in I κ B family (*NFKBIAA*, *NFKBIAB*,

NFKBIB, *NFKBIE*, *BCL3*), and three genes in IKK complex (*CHUK*, *IKBKB*, *IKBKG*). *NFKBID* in IκB family was not found in the channel catfish genome. The characteristics of these genes, including transcript length, coding sequences, conserved domains, chromosomal locations, and accession numbers, are listed in Table 2. Gene length of all NF-κB related genes ranged from 1,392 to 3,776 bp in channel catfish. Three genes (*REL*, *NFKB1* and *CHUK*) were located in linkage group (LG) 6, and two genes (*NFKBIAA* and *NFKBIE*) were located in LG28; the remaining genes were each in various linkage groups. Members of NF-κB family shared the IPT and RHD domains, while IκB proteins contained 4 to 6 ANK repeats. *CHUK* and *IKBKB* shared the PKc domain, whereas *IKBKG* had two unique domains UBAN and NEMO. In all cases, except *NFKBIA* from IκB family, where gene duplications were observed in teleost fish, catfish had just one copy of all NF-κB related genes (Table 3). It is interesting that *NFKBIB* was only identified in zebrafish and catfish, while *NFKBID* was represented in all other analyzed teleost species but not in zebrafish and catfish.

To further investigate the structural diversity of NF-κB related genes in channel catfish, we searched exon/intron organization within each family. As shown in Fig. 1, NF-κB family members had 7-22 introns, and *RELA* had a relatively long intron length. IκB family members had 5-9 introns, and *BCL3* had a much longer intron than other IκB members. IKK complex members had 12-20 introns which were considerably longer than that in any members of the other two families. To measure the selective pressure on NF-κB related genes, pairwise estimation of d_N/d_S values was obtained by the method of maximum likelihood with genes from human, mouse, cow, frog, zebrafish and channel catfish. All d_N/d_S values were much smaller than 1, indicating that negative selection is the major selection pressure in the evolution of the NF-κB related genes (Supplemental Table 1).

3.2. Phylogenetic and syntenic analysis of catfish NF-κB related genes

The channel catfish NF- κ B related genes were annotated through phylogenetic and syntenic analysis. Phylogenetic trees were constructed for each of the three families. In cases where phylogenetic analysis alone failed to provide sufficient evidence for the gene annotation, syntenic analysis was also conducted with the sequences of channel catfish, human and zebrafish.

Phylogenetic analysis of NF- κ B family genes is shown in Fig. 2. Catfish *RELA*, *RELB*, *REL* and *NFKB1* genes were grouped with their respective counterparts in other species, and most closely related with the corresponding zebrafish genes. However, catfish *NFKB2* gene was not clustered with zebrafish *NFKB2* gene, requiring additional analysis for its annotation. Syntenic analysis revealed conservation of the syntenic blocks, suggesting proper annotation of the *NFKB2* gene (Fig. 5A).

Phylogenetic analysis of I κ B family genes was grouped into five clades as expected from the phylogenetic relationship with various organisms (Fig. 3). All catfish I κ B family genes except *BCL3* were most closely grouped with their respective counterparts in zebrafish. Further, syntenic analysis was conducted to provide additional evidence for the annotation of *BCL3*. As shown in Fig. 5B, the catfish *BCL3* was located within the conserved syntenic block as that of the zebrafish *BCL3* gene, suggesting its proper annotation.

As shown in Fig. 4, phylogenetic analysis placed every IKK complex gene into its expected phylogenetic cluster with other organisms. Catfish *CHUK*, *IKBKB* and *IKBKG* were most closely associated with their respective zebrafish genes.

3.3.Expression of NF- κ B related genes after *F. columnare* infection

The expression profiles of catfish NF- κ B related genes were determined in three mucosal tissues of gill, skin and intestine after *F. columnare* infection. The levels of gene expression were determined of all 13 NF- κ B related genes at 0 h and 8 h post-infection (Fig. 6). In the gill at 8 h after *F.*

columnare infection, significant down-regulation was observed in three members of NF- κ B family (*RELA*, *RELB* and *NFKB1*) and one member of IKK complex (*IKBKB*); while significant up-regulation was observed in three members of I κ B family (*NFKBIAA*, *NFKBIAB* and *BCL3*) (Fig. 6A). The extent of expression differences of these genes was between 1.2 to 3.1-fold changes. Surprisingly, most of NF- κ B related genes were significantly induced in the skin at 8 h after *F. columnare* infection including four members of NF- κ B family (*RELA*, *RELB*, *NFKB1* and *NFKB2*), three members of I κ B family (*NFKBIB*, *NFKBIE* and *BCL3*), and one member of IKK complex (*IKBKB*) (Fig. 6B). Two genes (*BCL3* and *IKBKB*) were observed to be dramatically induced with greater than 5-fold changes at 8 h post-infection, while the remaining genes were induced 2 - 4 folds. In the intestine at 8 h after bacterial infection, four members of NF- κ B family (*RELB*, *REL*, *NFKB1* and *NFKB2*), three members of I κ B family (*NFKBIAA*, *NFKBIAB* and *NFKBIE*), and two members of IKK complex (*CHUK* and *IKBKG*) were significantly induced with 2- to 5-fold changes (Fig. 6C).

To determine the correlation between expression of NF- κ B related genes in response to *F. columnare* infection and catfish resistance against the disease, expression of the 13 NF- κ B genes was compared between resistant and susceptible catfish. In general, the NF- κ B family and the IKK family of genes were expressed at higher levels in resistant fish than in susceptible fish, whereas the I κ B family (except *BCL3*) of genes were expressed at higher levels in susceptible fish than in resistant fish (Fig. 7). However, the level and timing of expression differences cannot be generalized. For example, at 1 h post-infection, *NFKB2* and *BCL3* were induced approximately two-fold in resistant catfish; at 2 h post-infection, three members of NF- κ B family (*RELA*, *NFKB1* and *NFKB2*) and one member of I κ B family (*BCL3*) were induced in resistant catfish; at 8 h post-infection, two members of NF- κ B family (*RELA* and *NFKB1*) were induced approximately two-fold in resistant catfish. Taken together, these results suggested that higher expression of NF- κ B and IKK genes may

be correlated with disease resistance, while higher expression of I κ B genes (except *BCL3*) may be correlated with disease susceptibility.

4. Discussion

Innate immunity is the first line of defense against invading pathogens. After TLRs detect the microbial components, they recruit adaptor proteins to activate the NF- κ B pathway (Kawai and Akira, 2007). The interaction of NF- κ B, I κ B and IKK subunits is a negative feedback-containing signal-transduction pathway, which regulates the expression of an array of inflammatory cytokine genes (Baeuerle and Henkel, 1994; Pahl, 1999). Given the close interaction of NF- κ B, I κ B and IKK families, this study included genes from all three families to have a better understanding of their immune functions in catfish. We identified a total of 13 NF- κ B related genes in the channel catfish genome including 5 NF- κ B family genes, 5 I κ B family genes, and 3 IKK complex genes. Similar exon/intron structural features were shared by members within each family. Negative selection patterns were identified in these genes for maintaining their conserved functions. Distinct expression patterns of NF- κ B related genes were observed after *F. columnare* infection in three mucosal tissues, as well as in resistant and susceptible catfish, indicating their involvement in immune response to bacterial infection and complex function mechanisms in host defense system. As the first systematic work of analyzing NF- κ B related genes in catfish, we provided useful genomic resources, evolutionary patterns and expression profiles for future research in catfish and in other teleost fish.

The annotation of NF- κ B related genes was relatively straightforward due to their conservancy during gene evolution. All mammalian NF- κ B related genes were identified in channel catfish except for *NFKBID* that is also missing in the zebrafish genome. It is highly likely that *NFKBID* was not present in the channel catfish genome as well (Blomme et al., 2006). Interestingly, both catfish and zebrafish missed *NFKBID*, but they both harbor *NFKBIB* that are not present in any other teleost fish

species. This may suggest that *NFKBIB* in catfish and zebrafish may play similar roles as *NFKBID* in other teleost fish species.

Phylogenetic analysis provided sufficient evidence for the identities of most catfish NF- κ B related genes as they group with their respective counterparts in higher vertebrates and other fish species. Two genes (*NFKB2* and *BCL3*) required additional syntenic analysis, and their conserved syntenies confirmed our proper annotations.

Copy numbers of NF- κ B related genes were compared between catfish and other tetrapods. For the majority of NF- κ B related genes, only one copy exists in various tetrapods except for *NFKB1* and *NFKBIE*, which are not found in lizard, and *NFKBIB*, which is not found in frog. All NF- κ B related genes in fish species have only a single copy except for *NFKBIA*. Two copies of *NFKBIA* (*NFKBIAA* and *NFKBIAB*) exist in teleost fish, indicating teleost-specific whole genome duplication (Hoegg et al., 2004; Thornton and DeSalle, 2000). It is interesting that most of the NF- κ B related genes derived from the whole genome duplication were lost, leading to the current single copy genes. This may suggest that gene dosage of these genes is crucially important such that most of them were lost after whole genome duplication.

Identification of selection pressure of NF- κ B related genes during the evolution was conducted using maximum likelihood model to compare nonsynonymous (d_N) and synonymous (d_S) substitutions rates (Yang and Bielawski, 2000). The ratio d_N/d_S of each NF- κ B related gene was significantly less than 1 and very close to zero in some cases, indicating that these genes were globally strongly constrained by purifying or negative selection. Negative selection is believed to be crucial to maintain the functional stability and improved structures of gene family by removing deleterious mutations (Charlesworth et al., 1995). Because of the essential roles of NF- κ B related genes in various biological processes, negative selection eliminated less-adapted variants of these genes to ensure their proper functions.

Conserved domains were identified for understanding the functional structures of NF- κ B related genes. All catfish NF- κ B family proteins shared two same domains IPT and RHD, while NF κ B1 and NF κ B2 contained two extra domains ANK and DD. RHD is crucial for NF- κ B family because of its function in DNA-binding, dimerization, nuclear localization signal and interaction with I κ Bs (Ghosh et al., 1995; Muller et al., 1995). While NF κ B1 and NF κ B2 can act as inhibitors like I κ Bs by combining with other NF- κ B proteins due to the presence of ANK repeat domains (Baldwin, 1996). All catfish I κ Bs contained ANK repeats and their inner helices that were reported to be involved in the interaction of I κ Bs with NF- κ B proteins (Mosavi et al., 2004). For catfish IKK complex, IKK α and IKK β shared PKc domain for their function as catalytically active kinases, while the regulatory subunit IKK γ contained unique domain NEMO for binding with IKK β , as reviewed by Oeckinghaus and Ghosh (2009).

NF- κ B related genes have been widely reported to play key roles in regulating the immune response to bacterial infection in mammals (Johannessen et al., 2013; Le Negrate, 2012). To determine if these genes play a similar role in catfish immune response, we analyzed the expression profiles of all thirteen NF- κ B related genes after *F. columnare* infection in three mucosal tissues. Dramatic expression pattern differences of the NF- κ B related genes were observed among three mucosal tissues at 8 h post-infection. In gill, all members of NF- κ B family and IKK family were generally suppressed, while most members of I κ B family were generally induced after bacterial infection (Fig. 6A). This may suggest the inhibition of NF- κ B pathway in gill by *F. columnare* invasion, which validates our finding in previous RNA-Seq study (Sun et al., 2012). In skin, *IKBKB* and *BCL3* were extremely induced along with the upregulation of several members of NF- κ B family and I κ B family (Fig. 6B). As the significant upregulation of most members of NF- κ B family and IKK family were detected, it seems that NF- κ B pathway was activated in the skin after *F. columnare* infection. Although some members of I κ B family were also induced, this may be caused by the NF-

κ B-induced feedback regulation (Vallabhapurapu and Karin, 2009). In intestine, most members of NF- κ B family and IKK family were extremely induced after *F. columnare* infection, while members of I κ B family were also observed to be induced due to the NF- κ B-induced feedback regulation as the situation in skin (Fig. 6C). Notably, different NF- κ B related genes were significantly regulated in three mucosal tissues, which may suggest potential tissue-specific regulation preference of different NF- κ B related genes in response to bacterial infection.

The difference in expression patterns of NF- κ B related genes between resistant and susceptible catfish was determined by analyzing an existing RNA-Seq dataset after *F. columnare* infection. *RELA*, *NFKB1* and *NFKB2* from NF- κ B family were identified to be significantly induced in resistant catfish, but not so in susceptible catfish. These genes may contribute to the successful defense against *F. columnare* by activating NF- κ B pathway and its downstream TLR pathway in the resistant catfish (Yamamoto and Takeda, 2008). Notably, *BCL3* from I κ B family was also significantly induced in resistant catfish. Unlike other members of I κ B family, *BCL3* can either positively or negatively modulate nuclear NF- κ B activity through the exclusive interaction with p50 and p52 homodimers (Palmer and Chen, 2008; Zhang et al., 1994). For instance, *BCL3* was reported to positively regulate the NF- κ B activity in response to HIV in human (Franzoso et al., 1992) and *Klebsiella pneumoniae* infection in mice (Pène et al., 2011). On the other hand, *BCL3* was considered to be a negative regulator to NF- κ B by Carmody et al. (2007). Given the success of the immune response of the resistant catfish against *F. columnare* invasion, *BCL3* may respond positively by promoting the NF- κ B activity to defend the host against *F. columnare* in resistant catfish.

In most cases, regulation of NF- κ B, I κ B and IKK family members has been believed at the translational levels. Although expressed in most cells, NF- κ B dimers are kept inactivated by binding with I κ B proteins until the activation of IKKs (Oeckinghaus and Ghosh, 2009). However, RelB

protein level was reported to be transcriptionally regulated in a RelA dependent manner, leading to its delay in response to stimuli in human cells (Bren et al., 2001). Our results suggest that NF- κ B related genes are also regulated at the transcriptional level with the continued bacterial infection. Clearly, the regulation of NF- κ B related genes could be quite complex regarding the tissue specificity, species susceptibility to diseases, and specific genes under study.

In conclusion, 5 NF- κ B protein genes, 5 I κ B protein genes, and 3 IKK complex genes were identified and characterized in channel catfish. The conservancy in copy numbers and functional domains from fish to human indicated evolutionary conservancy of these NF- κ B related genes. Negative selection may play a key role in maintaining the conserved structure and function of these genes. Expression of most of NF- κ B related genes were significantly regulated in three mucosal tissues after *F. columnare* infection, indicating their involvement in immune response to bacterial infection. Expression patterns of NF- κ B related genes appeared to be dramatically different between susceptible catfish and resistant catfish after *F. columnare* infection, further suggesting their contribution to the variations in disease resistance/susceptibility of catfish. As a core component of NF- κ B pathway, the interaction of NF- κ B proteins, I κ B proteins, and IKK complexes still require further study for a more detailed understanding of how they respond to bacterial infections and their roles in the host defense.

Acknowledgements

This work was supported by USDA National Institute of Food and Agriculture through a grant from Animal Disease Program (2015-67015-22975), and from Agriculture and Food Research Initiative Animal Genomics, Genetics and Breeding Program (2015-67015-22907). The authors wish to thank Milla Kaltenboeck, Xiaoyan Xu and Honggang Zhao for their technical assistance. We are grateful to

Dr. Eric Peatman for his generation of the RNA-Seq dataset from catfish infected with columnaris bacterial pathogen.

References

- Baeuerle, P.A., Baltimore, D., 1988. Activation of DNA-Binding Activity in an Apparently Cytoplasmic Precursor of the NF-Kappa-B Transcription Factor. *Cell* 53, 211-217.
- Baeuerle, P.A., Henkel, T., 1994. Function and activation of NF-kappaB in the immune system. *Annu. Rev. Immunol.* 12, 141-179.
- Baldwin, A.S., 1996. The NF-kappa B and I kappa B proteins: New discoveries and insights. *Annu. Rev. Immunol.* 14, 649-683.
- Bao, B., Peatman, E., Peng, X., Baoprasertkul, P., Wang, G. and Liu, Z., 2006. Characterization of 23 CC chemokine genes and analysis of their expression in channel catfish (*Ictalurus punctatus*). *Dev. Comp. Immunol.* 30, 783-796.
- Blomme, T., Vandepoele, K., De Bodt, S., Simillion, C., Maere, S., Van de Peer, Y., 2006. The gain and loss of genes during 600 million years of vertebrate evolution. *Genome Biol.* 7, R43.
- Bren, G.D., Solan, N.J., Miyoshi, H., Pennington, K.N., Pobst, L.J., Paya, C.V., 2001. Transcription of the RelB gene is regulated by NF-kappa B. *Oncogene* 20, 7722-7733.
- Carmody, R.J., Ruan, Q., Palmer, S., Hilliard, B., Chen, Y.H., 2007. Negative regulation of toll-like receptor signaling by NF- κ B p50 ubiquitination blockade. *Science* 317, 675-678.
- Charlesworth, D., Charlesworth, B., Morgan, M.T., 1995. The Pattern of Neutral Molecular Variation under the Background Selection Model. *Genetics* 141, 1619-1632.
- Chen, Z.J., Parent, L., Maniatis, T., 1996. Site-specific phosphorylation of I κ B α by a novel ubiquitination-dependent protein kinase activity. *Cell* 84, 853-862.
- Correa, R.G., Tergaonkar, V., Ng, J.K., Dubova, I., Izpisua-Belmonte, J.C., Verma, I.M., 2004. Characterization of NF- κ B/I κ B proteins in zebrafish and their involvement in notochord development. *Mol. Cell. Biol.* 24, 5257-5268.
- Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792-1797.
- Franzoso, G., Bours, V., Park, S., Tomfta-Yamaguchi, M., Kelly, K., Siebenlist, U., 1992. The candidate oncoprotein Bcl-3 is an antagonist of p50/NF- κ B-mediated inhibition. *Nature* 359, 339-342.
- Fu, Q., Zeng, Q., Li, Y., Yang, Y., Li, C., Liu, S., Zhou, T., Li, N., Yao, J., Jiang, C. and Li, D., 2017. The chemokine superfamily in channel catfish: I. CXC subfamily and their involvement in disease defense and hypoxia responses. *Fish Shellfish Immunol.* 60, 380-390.
- Gerondakis, S., Grossmann, M., Nakamura, Y., Pohl, T., Grumont, R., 1999. Genetic approaches in mice to understand Rel/NF- κ B and I κ B function: transgenics and knockouts. *Oncogene* 18, 6888-6895.
- Ghosh, G., Vanduyne, G., Ghosh, S., Sigler, P.B., 1995. Structure of NF-Kappa-B P50 Homodimer Bound to a Kappa-B Site. *Nature* 373, 303-310.
- Grilli, M., Chiu, J.J.-S., Lenardo, M.J., 1993. I κ B and Rel: Participants in a Multifunctional Transcriptional Regulatory System. *Int. Rev. Cytol.* 143, 1-62.
- Hoegg, S., Brinkmann, H., Taylor, J.S., Meyer, A., 2004. Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. *J. Mol. Evol.* 59, 190-203.
- Hoffmann, A., Levchenko, A., Scott, M.L., Baltimore, D., 2002. The I κ B-NF- κ B signaling module: temporal control and selective gene activation. *Science* 298, 1241-1245.
- Israel, A., 2010. The IKK Complex, a Central Regulator of NF-kappa B Activation. *Cold Spring Harb. Perspect Biol.* 2, a000158.

- Jiang, C., Zhang, J.R., Yao, J., Liu, S.K., Li, Y., Song, L., Li, C., Wang, X.Z., Liu, Z.J., 2015. Complement regulatory protein genes in channel catfish and their involvement in disease defense response. *Dev. Comp. Immunol.* 53, 33-41.
- Johannessen, M., Askarian, F., Sangvik, M., Sollid, J.E., 2013. Bacterial interference with canonical NF κ B signalling. *Microbiology* 159, 2001-2013.
- Karin, M., 1999. How NF- κ B is activated: the role of the I κ B kinase (IKK) complex. *Oncogene* 18.
- Kawai, T., Akira, S., 2007. Signaling to NF- κ B by Toll-like receptors. *Trends Mol. Med.* 13, 460-469.
- Lawrence, T., 2009. The nuclear factor NF- κ B pathway in inflammation. *Cold Spring Harb. Perspect Biol.* 1, a001651.
- Le Negrate, G., 2012. Subversion of innate immune responses by bacterial hindrance of NF- κ B pathway. *Cell. Microbiol.* 14, 155-167.
- Li, C., Zhang, Y., Wang, R.J., Lu, J.G., Nandi, S., Mohanty, S., Terhune, J., Liu, Z.J., Peatman, E., 2012. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. *Fish Shellfish Immunol.* 32, 816-827.
- Li, Y., Liu, S.K., Qin, Z.K., Yao, J., Jiang, C., Song, L., Dunham, R., Liu, Z.J., 2015. The serpin superfamily in channel catfish: Identification, phylogenetic analysis and expression profiling in mucosal tissues after bacterial infections. *Dev. Comp. Immunol.* 49, 267-277.
- Liu, S., Zhang, Y., Zhou, Z., Waldbieser, G., Sun, F., Lu, J., Zhang, J., Jiang, Y., Zhang, H., Wang, X., 2012. Efficient assembly and annotation of the transcriptome of catfish by RNA-Seq analysis of a doubled haploid homozygote. *BMC genomics* 13, 595.
- Liu, Z.J., Liu, S.K., Yao, J., Bao, L.S., Zhang, J.R., Li, Y., Jiang, C., Sun, L.Y., Wang, R.J., Zhang, Y., 2016. The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. *Nat. Commun.* 7, 11757.
- Louis, A., Nguyen, N.T.T., Muffato, M., Crollius, H.R., 2015. Genomicus update 2015: KaryoView and MatrixView provide a genome-wide perspective to multispecies comparative genomics. *Nucleic Acids Res.* 43, D682-D689.
- Mosavi, L.K., Cammett, T.J., Desrosiers, D.C., Peng, Z.Y., 2004. The ankyrin repeat as molecular architecture for protein recognition. *Prot. Sci.* 13, 1435-1448.
- Mu, W., Yao, J., Zhang, J., Liu, S., Wen, H., Feng, J. and Liu, Z., 2015. Expression of tumor suppressor genes in channel catfish after bacterial infections. *Dev. Comp. Immunol.* 48, 171-177.
- Muffato, M., Louis, A., Poisnel, C.E., Crollius, H.R., 2010. Genomicus: a database and a browser to study gene synteny in modern and ancestral genomes. *Bioinformatics* 26, 1119-1121.
- Muller, C.W., Rey, F.A., Sodeoka, M., Verdine, G.L., Harrison, S.C., 1995. Structure of the Nf-Kappa-B P50 Homodimer Bound to DNA. *Nature* 373, 311-317.
- Oeckinghaus, A., Ghosh, S., 2009. The NF-kappa B Family of Transcription Factors and Its Regulation. *Cold Spring Harb. Perspect Biol.* 1, a000034.
- Oeckinghaus, A., Hayden, M.S., Ghosh, S., 2011. Crosstalk in NF- κ B signaling pathways. *Nature Immunol.* 12, 695-708.
- Pahl, H.L., 1999. Activators and target genes of Rel/NF- κ B transcription factors. *Oncogene* 18, 6853-6866.
- Palmer, S., Chen, Y.H.H., 2008. Bcl-3, a multifaceted modulator of NF-kappa B-mediated gene transcription. *Immunol. Res.* 42, 210-218.

- Peatman, E., Bao, B.L., Peng, X., Baoprasertkul, P., Brady, Y., Liu, Z.J., 2006. Catfish CC chemokines: genomic clustering, duplications, and expression after bacterial infection with *Edwardsiella ictaluri*. *Mol. Genet. Genomics* 275, 297-309.
- Peatman, E., Li, C., Peterson, B.C., Straus, D.L., Farmer, B.D., Beck, B.H., 2013. Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*). *Mol. Immunol.* 56, 317-327.
- Pène, F., Paun, A., Sønder, S.U., Rikhi, N., Wang, H., Claudio, E., Siebenlist, U., 2011. The I κ B family member Bcl-3 coordinates the pulmonary defense against *Klebsiella pneumoniae* infection. *J. Immunol.* 186, 2412-2421.
- Perkins, N.D., 2006. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* 25, 6717-6730.
- Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30, e36.
- Philpott, D.J., Girardin, S.E., 2004. The role of Toll-like receptors and Nod proteins in bacterial infection. *Mol. Immunol.* 41, 1099-1108.
- Pomerantz, J.L., Baltimore, D., 2002. Two pathways to NF-kappa B. *Mol. Cell* 10, 693-695.
- Pradhan, A., Khalaf, H., Ochsner, S.A., Sreenivasan, R., Koskinen, J., Karlsson, M., Karlsson, J., McKenna, N.J., Orbán, L., Olsson, P.-E., 2012. Activation of NF- κ B protein prevents the transition from juvenile ovary to testis and promotes ovarian development in zebrafish. *J. Biol. Chem.* 287, 37926-37938.
- Rajendran, K.V., Zhang, J., Liu, S., Peatman, E., Kucuktas, H., Wang, X., Liu, H., Wood, T., Terhune, J. and Liu, Z., 2012a. Pathogen recognition receptors in channel catfish: II. Identification, phylogeny and expression of retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). *Dev. Comp. Immunol.* 37, 381-389.
- Rajendran, K.V., Zhang, J., Liu, S., Kucuktas, H., Wang, X., Liu, H., Sha, Z., Terhune, J., Peatman, E. and Liu, Z., 2012b. Pathogen recognition receptors in channel catfish: I. Identification, phylogeny and expression of NOD-like receptors. *Dev. Comp. Immunol.* 37, 77-86.
- Scheidereit, C., 2006. I κ B kinase complexes: gateways to NF- κ B activation and transcription. *Oncogene* 25, 6685-6705.
- Schleizinger, J.J., Blickarz, C.E., Mann, K.K., Doerre, S., Stegeman, J.J., 2000. Identification of NF- κ B in the marine fish *Stenotomus chrysops* and examination of its activation by aryl hydrocarbon receptor agonists. *Chem. Biol. Interact.* 126, 137-157.
- Senftleben, U., Cao, Y., Xiao, G., Greten, F.R., Krähn, G., Bonizzi, G., Chen, Y., Hu, Y., Fong, A., Sun, S.-C., 2001. Activation by IKK α of a second, evolutionary conserved, NF- κ B signaling pathway. *Science* 293, 1495-1499.
- Shoemaker, C.A., Olivares-Fuster, O., Arias, C.R., Klesius, P.H., 2008. *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*). *Vet. Microbiol.* 127, 353-359.
- Solovyev, V., Kosarev, P., Seledsov, I., Vorobyev, D., 2006. Automatic annotation of eukaryotic genes, pseudogenes and promoters. *Genome Biol.* 7, S10.
- Sun, F.Y., Peatman, E., Li, C., Liu, S.K., Jiang, Y.L., Zhou, Z.C., Liu, Z.J., 2012. Transcriptomic signatures of attachment, NF-kappa B suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. *Dev. Comp. Immunol.* 38, 169-180.

- Sun, L.Y., Liu, S.K., Bao, L.S., Li, Y., Feng, J.B., Liu, Z.J., 2015. Claudin multigene family in channel catfish and their expression profiles in response to bacterial infection and hypoxia as revealed by meta-analysis of RNA-Seq datasets. *Comp. Biochem. Physiol. D* 13, 60-69.
- Sun, L., Liu, S., Wang, R., Li, C., Zhang, J. and Liu, Z., 2014. Pathogen recognition receptors in channel catfish: IV. Identification, phylogeny and expression analysis of peptidoglycan recognition proteins. *Dev. Comp. Immunol.* 46, 291-299.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725-2729.
- Tan, S., Yao, J., Zhou, T., Liu, S., Yuan, Z., Tian, C., Li, Q. and Liu, Z., 2017. Identification, annotation and expression analysis of 29 Rho GTPase genes from channel catfish (*Ictalurus punctatus*) after bacterial infections. *Dev. Comp. Immunol.* 67, 445-451.
- Thornton, J.W., DeSalle, R., 2000. Gene family evolution and homology: genomics meets phylogenetics. *Annu. Rev. Genomics Hum. Genet.* 1, 41-73.
- Vallabhapurapu, S., Karin, M., 2009. Regulation and Function of NF-kappa B Transcription Factors in the Immune System. *Annu Rev Immunol* 27, 693-733.
- Xu, B., Yang, Z., 2013. PAMLX: a graphical user interface for PAML. *Mol. Biol. Evol.* 30, 2723-2724.
- Yamamoto, M., Takeda, K., 2008. Role of nuclear IκB proteins in the regulation of host immune responses. *J. Infect. Chemother.* 14, 265-269.
- Yamamoto, Y., Gaynor, R.B., 2001. Role of the NF-κB pathway in the pathogenesis of human disease states. *Curr. Mol. Med.* 1, 287-296.
- Yang, Y., Fu, Q., Zhou, T., Li, Y., Liu, S., Zeng, Q., Wang, X., Jin, Y., Tian, C., Qin, Z., 2017. Analysis of apolipoprotein genes and their involvement in disease response of channel catfish after bacterial infection. *Dev. Comp. Immunol.* 67, 464-470.
- Yang, Z.H., Bielawski, J.P., 2000. Statistical methods for detecting molecular adaptation. *Trends Ecol. Evol.* 15, 496-503.
- Yao, J., Li, C., Zhang, J., Liu, S., Feng, J., Wang, R., Li, Y., Jiang, C., Song, L., Chen, A. and Liu, Z., 2014. Expression of nitric oxide synthase (NOS) genes in channel catfish is highly regulated and time dependent after bacterial challenges. *Dev. Comp. Immunol.* 45, 74-86.
- Yao, J., Mu, W., Liu, S., Zhang, J., Wen, H. and Liu, Z., 2015. Identification, phylogeny and expression analysis of suppressors of cytokine signaling in channel catfish. *Mol. immunol.* 64, 276-284.
- Yuan, Z., Liu, S., Yao, J., Zeng, Q., Tan, S. and Liu, Z., 2016. Expression of Bcl-2 genes in channel catfish after bacterial infection and hypoxia stress. *Dev. Comp. Immunol.* 65, 79-90.
- Zhang, J., Liu, S., Rajendran, K.V., Sun, L., Zhang, Y., Sun, F., Kucuktas, H., Liu, H. and Liu, Z., 2013. Pathogen recognition receptors in channel catfish: III Phylogeny and expression analysis of Toll-like receptors. *Dev. Comp. Immunol.* 40, 185-194.
- Zhang, Q., Didonato, J.A., Karin, M., McKeithan, T.W., 1994. BCL3 encodes a nuclear protein which can alter the subcellular location of NF-kappa B proteins. *Mol. Cell. Biol.* 14, 3915-3926.
- Zhao, H.G., Li, C., Beck, B.H., Zhang, R., Thongda, W., Davis, D.A., Pearnan, E., 2015. Impact of feed additives on surface mucosal health and columnaris susceptibility in channel catfish fingerlings, *Ictalurus punctatus*. *Fish Shellfish Immunol.* 46, 624-637.

Figure legends

Fig. 1. Structural analysis of NF- κ B related genes. The exons are represented by red round-cornered rectangles. The untranslated regions (UTRs) are represented by blue rectangles. The black lines connecting two exons represent introns.

Fig. 2. Phylogenetic analysis of NF- κ B family genes. The phylogenetic tree was constructed with amino acid sequences from selected fish and tetrapod species using maximum likelihood method with MEGA 6.0 software. Bootstrap values (≥ 50) were indicated by numbers at the nodes. The accession numbers were listed following the species names. Black dots indicated zebrafish NF- κ B family genes, while red dots indicated channel catfish NF- κ B family genes.

Fig. 3. Phylogenetic analysis of I κ B family genes. The phylogenetic tree was constructed with amino acid sequences from selected fish and tetrapod species using maximum likelihood method with MEGA 6.0 software. Bootstrap values (≥ 50) were indicated by numbers at the nodes. The accession numbers were listed following the species names. Black dots indicated zebrafish I κ B family genes, while red dots indicated channel catfish I κ B family genes.

Fig. 4. Phylogenetic analysis of IKK family genes. The phylogenetic tree was constructed with amino acid sequences from selected fish and tetrapod species using maximum likelihood method with MEGA 6.0 software. Bootstrap values (≥ 50) were indicated by numbers at the nodes. The accession numbers were listed following the species names. Black dots indicated zebrafish IKK family genes, while red dots indicated channel catfish IKK family genes.

Fig. 5. Syntenic analysis of (A) *NFKB2* and (B) *BCL3* genes in human, zebrafish and channel catfish. Syntenies were constructed based on the genomic information from Genomicus. Gene abbreviations are the following: *ascc3* (activating signal cointegrator 1 complex subunit 3), *cblc* (Cbl proto-oncogene C), *fam35a* (family with sequence similarity 35, member A), *glud1* (glutamate dehydrogenase 1), *glud1a* (glutamate dehydrogenase 1 a), *grik2* (Glutamate Receptor, Ionotropic, Kainate 2), *hnRNPH3* (heterogeneous nuclear ribonucleoprotein H3), *irgq1* (immunity-related GTPase family, q1), *prss59.2* (protease, serine, 59, tandem duplicate 2), *psd* (pleckstrin and Sec7 domain containing), *rufy1* (RUN and FYVE domain containing 1), *rufy2* (RUN and FYVE domain containing 2), *sncg* (synuclein, gamma), *sncga* (synuclein, gamma a).

Fig. 6. Expression of NF- κ B related genes following *Flavobacterium columnare* infection.

Quantitative real-time PCR was used for the expression analysis in three mucosal tissues: (A) gill, (B) skin and (C) intestine at 8 h post-infection. Fold change of expression as compared with control fish is presented on the Y-axis, 18S rRNA and 28S rRNA were used as internal reference. The results were expressed as mean $\pm$ standard error (bars). Significant differences ($p < 0.05$) among controls and various treatments were indicated by asterisks (*).

Fig. 7. Expression of NF- κ B related genes following *Flavobacterium columnare* infection in the gill of channel catfish with different susceptibility (resistant vs susceptible) to *F. columnare* at different time points. Asterisks (*) indicated significant differences (p -value < 0.05 , expression fold change > 1.5).

Table 1 Primers of catfish NF- κ B related genes used in this study.

Gene name	Primer name	Sequences (5' - 3')	Position
rela	rela-F	GAGCAGGCGGCGAGAAAC	1582-1599
	rela-R	CAACATGGACATGAGGCGATC	1741-1761
relb	relb-F	ACGGCACGCCTGAAATAGC	29-47
	relb-R	GGCCACGAGGTGATGTAAGC	237-256
rel	rel-F	ACCTGGTGGGGAAAGACTGC	173-192
	rel-R	AGGTCGTATTCCTCCGTCTGC	354-374
nfkb1	nfkb1-F	ATCAATGCCTTCCGCCTCA	1237-1255
	nfkb1-R	GCCTACTGTGGTCAGATTTTCGT	1409-1431
nfkb2	nfkb2-F	AGCGAAGCAGCCTGGATACC	495-514
	nfkb2-R	GCTGTCTAGATGGGGTTGG	662-681
nfkb1aa	nfkb1aa-F	CAGCGGCATGGACTCGTTA	96-114
	nfkb1aa-R	TGTCTGTTGAGGAATGGGTCG	306-326
nfkb1ab	nfkb1ab-F	AGGACGACACGGAGGATTACTG	122-143
	nfkb1ab-R	GTTTGTTCAGGAACGGGTGC	315-334
nfkb1b	nfkb1b-F	GGTTTGGGCTCGGATAAGCT	103-122
	nfkb1b-R	CTCGTCGTTGATCGAATCCC	299-318
nfkb1e	nfkb1e-F	GGAGGGAACGAGTGGGAAAA	768-787
	nfkb1e-R	TGGTGATGGCGGTGTCTTG	913-931
bcl3	bcl3-F	CTACCCTTTCCCCTGCGATAA	158-178
	bcl3-R	CTGCTGCACTTGGGCACTTT	412-431
chuk	chuk-F	AGTGTAGTTGCGGTTTTTCGTCC	641-662
	chuk-R	CAGGGTGTATTATTCCTCCCCTC	861-883
ikbkb	ikbkb-F	CCTCTGCCCTCACATACCTTCA	389-410
	ikbkb-R	CTCCTTAGCATAGCCCAAGTCAA	494-516
ikbkg	ikbkg-F	AGAACGGGCTGTCATTGTGG	1137-1156
	ikbkg-R	GGTCTCCTGTAAGGTCCTCTGCT	1344-1377

Table 2

Characteristics of NF- κ B related genes identified in channel catfish. LG: linkage group.

Family name	Genes	mRNA (bp)	CDS (aa)	Conserved Domain	Location	Accession NO.
NF- κ B Family	RELA	2372	601	IPT, RHD	LG29	JT417958
	RELB	2861	557	IPT, RHD	LG22	JT407102
	REL	2237	580	IPT, RHD	LG6	JT317830
	NFKB1	3776	507	IPT, RHD, ANK, DD	LG6	JT316856
	NFKB2	3383	909	IPT, RHD, ANK, DD	LG3	JT319661
	NFKBIAA	1392	308	ANK	LG28	JT419244
I κ B Family	NFKBIAB	1541	320	ANK	LG16	JT408574
	NFKBIB	2304	397	ANK	LG14	JT416051
	NFKBIE	1525	360	ANK	LG28	JT413485
	BCL3	2489	631	ANK	LG1	JT463618
IKK complex	CHUK	3706	756	PKc	LG6	JT406644
	IKBKB	3129	776	PKc, S_TKc	LG11	JT411949
	IKBKG	2816	568	UBAN, NEMO	LG15	JT417502

The abbreviations of the functional domains were immunoglobulin-like fold, plexins, transcription factors (IPT); Rel homology domain (RHD); Ankyrin repeats (ANK); death domain (DD); protein kinases, catalytic domain (PKc); Serine/Threonine protein kinases, catalytic domain (S_TKc); NF-Kappa-B essential modulator (NEMO); ubiquitin binding in ABIN and NEMO (UBAN).

Copy numbers of NF- κ B related genes in several representative vertebrate genomes.

[illegible]

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

A)

B)

Fig. 6

Fig. 7