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Expression of Bcl-2 genes in channel catfish after bacterial infection and hypoxia stress

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ABSTRACT

Bcl-2 proteins are of vital importance in regulation of apoptosis, and are involved in a number of biological processes such as carcinogenesis and immune responses. Bcl-2 genes have been well studied in mammals, while they are not well investigated in teleost fish including channel catfish, the major aquaculture species in the United States. In this study, we identified 34 bcl-2 genes from the channel catfish genome, and verified their identities by conducting phylogenetic and syntenic analyses. The expression profiles of the bcl-2 genes in response to bacterial infections (*Edwardsiella ictaluri* and *Flavobacterium columnare*) and hypoxia stress were determined by performing meta-analysis using the existing RNA-Seq datasets. Differential expressions of bcl-2 genes were observed after bacterial infections and hypoxia treatment, including 22 bcl-2 genes after *E. ictaluri* infection, 22 bcl-2 genes after *F. columnare* infection, and 19 bcl-2 genes after hypoxia stress. Overall, the expression of the pro-apoptotic bcl-2 genes were repressed after bacterial infection and hypoxia stress, indicating that bcl-2 genes are potentially involved in the stress response by reducing cell apoptosis. Some bcl-2 genes, such as bcl2b, mcl1a, bmf1, and bnip3, showed different expression pattern during the *E. ictaluri* and *F. columnare* infection, suggesting the difference in the pathogenicity of diseases. This work presented the first systematic identification and annotation of bcl-2 genes in catfish, providing essential genomic resources for further immune and physiological studies.

1. Introduction

Bcl-2 proteins are a family of regulatory proteins that regulate cell apoptosis by either inducing or repressing cell death. They are involved in the intrinsic pathway of caspase-dependent apoptosis (Kratz et al., 2006). Based on their functions and homology motifs, the bcl-2 family proteins are generally divided into three categories, including the pro-apoptotic BH3-only proteins, the multidomain anti-apoptotic proteins, and the multidomain pro-apoptotic proteins. The apoptotic stimuli such as stress activate the pro-apoptotic BH3-only proteins, which antagonize the multidomain anti-apoptotic proteins, preventing them from blocking the functions of the multidomain pro-apoptotic proteins (Willis and Adams, 2005). Moreover, the pro-apoptotic BH3-only proteins can directly activate the multidomain pro-apoptotic proteins by forming the heterodimers (Letai et al., 2002). Consequently, the activated multidomain pro-apoptotic proteins punch the outer membrane of the mitochondrial and release cytochrome C to trigger the downstream caspase cascades in the cell (Green and Kroemer, 2004). Remarkably, in some cells, a pro-apoptotic BH3-only protein "bid" can also be activated by the caspase-8 from the extrinsic pathway, making it a converging point for the extrinsic and intrinsic pathway (Yin et al., 1999).

Bcl-2 genes have been well studies in mammals because of their important roles in apoptosis, and their involvement in numerous cancer diseases (Gascoyne et al., 1997; Gobé et al., 2002; Han et al., 2002; Hardwick and Soane, 2013; Hindy et al., 2011; Ola et al., 2011). Various studies conducted in humans indicated that bcl-2 genes are actively involved in the host immune response after the bacterial infection by regulating the process of apoptosis in the organism (Casalino-Matsuda et al., 2015; Liston et al., 2003; Vazquez and Colombo, 2010). In addition, previous studies

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indicated that the hypoxia activated cardiac death through the pro-apoptotic bcl-2 gene (Kubasiak et al., 2002), meanwhile the anti apoptotic bcl-2 genes effectively retarded the chemical hypoxia-induced necrotic cell death (Shimizu et al., 1996). Therefore, it is highly likely that the bcl-2 genes play critical roles in the stress response by regulating the process of apoptosis. In teleost fish, the bcl-2 family genes have not been well studied, including species that whole genomes have been sequenced and annotated.

Catfish is the major aquaculture species in the United States (Liu et al., 2011). In recent years, the catfish industry has been greatly hindered by frequent pond stresses such as devastating diseases. The most significant diseases in channel catfish are the enteric septicemia of catfish (ESC) caused by *Edwardsiella ictaluri* (Hawke et al., 1981), and columnaris disease caused by *Flavobacterium columnare* (Shoemaker et al., 2008). The incidents of these diseases are increased by stress such as exposure to hypoxic conditions. As apoptosis is a universal response to all stressors, the study of apoptosis is of vital importance in understanding host stress response mechanisms after stress. Previous studies have reported some apoptosis related genes including HSP proteins (Weber and Janz, 2001; Song et al., 2014, 2016; Xie et al., 2015), tumor necrosis factor (Zou et al., 2003), FasL-like protein (Long et al., 2004) and tumor suppressor genes (Mu et al., 2015) in channel catfish. A previous study has observed that the apoptosis would decrease in the presence of confinement stress in channel catfish (Alford Iii et al., 1994). However, studies of bcl-2 genes in relationship with other stress such as bacterial infections and hypoxia remain unexplored in channel catfish. In this study, with the objective to identify and annotate bcl-2 genes in the channel catfish genome, and understand their roles in response to disease infections and hypoxia stress, we performed a comprehensive genome-wide identification and annotation of bcl-2 genes in channel catfish, and characterized their expression profiles after infections of *E. ictaluri* and *F. columnare*, and after hypoxia stresses.

2. Materials and methods

2.1. Database mining and sequence analysis

To identify the bcl-2 genes in channel catfish, based on the references (Eimon and Ashkenazi, 2010; Hardwick and Soane, 2013; Kratz et al., 2006; Ola et al., 2011; Siddiqui et al., 2015), all available bcl-2 protein sequences from human, mouse, dog, chicken, frog and teleosts including (*Astyanax mexicanus, Danio rerio, Esox lucius, Gadus morhua, Larimichthys crocea, Lepisosteus oculatus, Takifugu rubripes, Tetraodon nigroviridis, Poecilia formosa, Scleropages formosus*) were retrieved from public databases such as NCBI database (http://www.ncbi.nlm.nih.gov/) and ENSEMBL genome browser (http://www.ensembl.org/), and were used as queries to search against channel catfish transcriptome databases (Li et al., 2012; Sun et al., 2012; Liu et al., 2012) via TBLASTN, the E-value was set to 1e−5 to ensure the quality of the searching results. The BLASTN was used to verify the cDNA sequences through comparing the transcriptome sequence with genome sequences (Liu et al., 2016). The homologous sequences identified from the channel catfish were then extracted for further annotation. The open reading frames (ORFs) were predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), followed by further verification using BLASTP against NCBI non-redundant protein database.

2.2. Phylogenetic and syntenic analysis

Phylogenetic analyses were conducted using bcl-2 genes identified from channel catfish together with those from other species including human, mouse, chicken, frog and several teleost fish. Multiple alignments of protein sequences were conducted by MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004) with default parameters. The phylogenetic tree was constructed using MEGA5.2.2 software (Tamura et al., 2011) with the maximum likelihood method. The best model (Figures legend 1–4) was selected by Prottest 3.2.1 according to the alignment results (Darriba et al., 2011). The bootstrap of 1000 replications was conducted to evaluate the phylogenetic tree. To provide additional evidence of gene orthologies, syntenic analyses were conducted for bcl-2 members that are not well supported by the phylogenetic tree. The predicted channel catfish bcl-2 protein sequences were searched against the channel catfish genome assembly, followed by the extraction of the genomic scaffolds and linkage groups that the channel catfish bcl-2 genes are located. The gene prediction of the genomic sequences was conducted using the FGENESH program (Solovyev et al., 2006). The conserved syntenic blocks near these bcl-2 genes in other species were based on information from Genomicus (Louis et al., 2012, 2015), NCBI database (http://www.ncbi.nlm.nih.gov/) and ENSEMBL genome browser (http://www.ensembl.org/).

2.3. Expression analysis of bcl-2 following stress challenges

Meta-analyses of RNA-Seq datasets were conducted to determine the expression profiles of bcl-2 genes in response to bacterial infections (*E. ictaluri* and *F. columnare*) and hypoxia stress. The stress challenges were conducted as previously described (Li et al., 2012; Sun et al., 2012; Feng et al., 2013). Briefly, the fish were challenged in six 30 L aquatia with 3 control and 3 treatment groups for each challenge. The bacterial were isolated form symptomatic fish and biochemically confirmed before being inoculated in Brain Heart Infusion broth (BHI) and incubated in the shaker incubator overnight. The concentrations of bacteria were determined by colony forming unit (CFU) per mL by plating 10 μL of 10-fold serial dilutions onto BHI agar plates. For ESC challenge, 200 ml *E. ictaluri* with a concentration of 4×10^8 CFU/mL was added into the treatment aquaria with the water flow turned off for 2 h. Control fish were exposed to the same routine as treatment fish, except were immersed in sterilized media alone. At each sampling time-point of 4 h, 24 h and 3 d after infection, 30 fish were collected from the control and treatment aquaria and anesthetized with MS-222 (300 mg/L). The entire intestinal tracts intestine from 10 fish (3 replicates of 10 fish each) were pooled together for each tissue, flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

Similarly, *F. columnare* challenges were conducted with six 30 L aquaria of which three aquaria were used as control and the other three were used as challenge groups. 200 ml *F. columnare* with a concentration of 3×10^6 CFU/mL were added into the treatment aquaria for 2 h. Gills of 18 fish were collected and randomly divided into three replicate pools (6 fish each) at each sampling time point:4 h, 24 h, 48 h. Samples were flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

For hypoxia challenge, 350 fish were acclimated in 90 × 60 × 30 cm (L × W × H) tanks with flow-through water for a week. After acclimation, 50 fish were taken to another tank as control group, the remaining fish were exposed to hypoxia stress. Dissolve oxygen levels in the challenge groups were reduced from 8.50 mg/L to 1.00 mg/L after bubbling nitrogen gas into the aquarium for 1.5 h. The oxygen level was measured every hour and the oxygen concentration was properly maintained. Starting at 1.5 h when first fish started to lose balance, samples were collected continuously until 45 fish were collected. At 5 h after low oxygen challenge, 45 tolerant fish were randomly collected. 15 fish were

collected for hypoxia resistance, hypoxia tolerant and control group and anesthetized with MS-222 (300 mg/L), brain, gill, head kidney, heart, liver, muscle, spleen, stomach, swim bladder and trunk kidney samples are collected and pooled for RNA-seq analysis.

The RNA-Seq datasets for ESC (NCBI SRA no. SRP009069) were generated from samples collected from the intestine 3 h, 24 h and 3 days after *E. ictaluri* infection (Li et al., 2012). The RNA-Seq datasets for columnaris (NCBI SRA no. SRP012586) were generated from samples collected from the gill 4 h, 24 h and 48 h after the *F. columnare* infection (Sun et al., 2012). The RNA-Seq datasets for hypoxia (NCBI SRA no. SRP039612) were generated from the samples collected from the gill of the channel catfish that were sensitive and resistant to hypoxia after hypoxia stress (Feng et al., 2013). Expression analyses using RNA-Seq datasets were conducted using CLC Genomics Workbench (v5.5.2). RNA-Seq reads were mapped to the reference transcripts with the parameters setting as at least 95% read length with a maximum of 2 mismatches. Then, Reads Per Kilobase of exon model (RPK) was calculated based on the number of total mapped reads of the bcl-2 genes and the housekeeping gene, beta-actin (actb). The expression value of each gene was determined based on the ratio of bcl-2 gene RPK to that of the actb in the same sample. Differential expression in fold-change was determined based on the ratio of the expression value of each bcl-2 gene between control and challenged groups and unchallenged groups. Based on the previous work, the genes with absolute expression fold change value $\geq$ 1.5 and total gene read $\geq$5 were considered as genes that were significantly altered in expression after stress treatments (Fu et al., 2016; Li et al., 2016; Xie et al., 2015).

3. Results

3.1. Identification of channel catfish bcl-2 genes

A total of 34 bcl-2 genes were identified from the channel catfish genome. Their characteristics are summarized in Table 1, including their mammalian homologs, gene abbreviations, open reading frames, their genomic location in linkage groups and their accessions. These 34 bcl-2 genes included 22 pro-apoptotic BH3-only proteins (bada, badb, bid, bik, bim, bmf1, bmf2, pmaip1, puma, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bcl2l15l, bcl2l13, bnip1a, bnip1b, bnip2a, bnip2b, bnip2c, bnip2l, bnip3, bnip3la, bnip3lb), 7 multidomain anti-apoptotic proteins (mcl1a, mcl1b, blp1, bcl2a, bcl2b, bcl2l10, bcl2l12), and 5 multidomain pro-apoptotic proteins (baxa, baxb, baxl, bok, bcl2l16). These bcl-2 genes shared a relatively high level of sequence similarity with their mammalian counterparts. All these sequences have been submitted to GenBank and their accession numbers are provided in Table 1. The bad, bmf, bcl2l14, bnip1, mcl and bcl-2 each possessed 2 copies, the bnip3 and bax each had three copies, whereas bnip2 had four copies (Table 1).

3.2. Phylogenetic analysis of channel catfish bcl-2 genes

Phylogenetic analysis was conducted to determine the identities of the channel catfish bcl-2 genes. Although the bcl-2 genes are divided into three major categories as mentioned above, in consistence with previous studies on phylogenomics of bcl-2 gene families (Aouacheria et al., 2005), we separated the bnips (bnip1, bnip2, bnip3) from the subfamily of the BH3-only proteins to

Table 1
Summary of Bcl-2 family genes identified from channel catfish genome.

Group	Mammalian bcl-2 genes	Catfish bcl-2 genes	Gene full name	Accession	ORF (#aa)	Linkage group
Pro-apoptotic BH3-only proteins	BAD	bada	bcl-2 antagonist of cell death a	JT341548	198	Lg13
		badb	bcl-2 antagonist of cell death b	JT418523	145	Lg5
	BID	bid	bcl-2 interacting domain death agonist	JT413841	199	Lg4
	BIK	bik	bcl-2 interacting killer	JT412108	149	Lg20
	BIM	bim	Bcl-2 interacting mediator of cell death	JT413427	221	Lg6
	BMF	bmf1	bcl-2 modifying factor 1	JT410268	152	Lg6
		bmf2	bcl-2 modifying factor 2	JT444349	164	Lg3
	PMAIP1	pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	JT347455	48	Lg25
	PUMA	puma	the p53 up regulated modulator of apoptosis	JT407711	180	Lg22
	BCL-G	bcl2l14 (1 of 2)	bcl-2-like protein 14 (1 of 2)	JT417930	208	Lg4
		bcl2l14 (2 of 2)	bcl-2-like protein 14 (2 of 2)	JT341698	265	Lg23
	BFK	bcl2l15l	bcl-2-like protein 15 like	JT340656	147	Lg21
	BCL-RAMBO	bcl2l13	bcl-2-like protein 13	JT416612	603	Lg4
	BNIP1	bnip1a	bcl2/adenovirus E1B 19 kDa interacting protein 1 a	JT417074	226	Lg18
		bnip1b	bcl2/adenovirus E1B 19 kDa interacting protein 1 b	JT470485	189	Lg13
	BNIP2	bnip2a	bcl2/adenovirus E1B 19 kDa interacting protein 2 a	JT418893	449	Lg18
		bnip2b	bcl2/adenovirus E1B 19 kDa interacting protein 2 b	JT212460	311	Lg14
		bnip2c	bcl2/adenovirus E1B 19 kDa interacting protein 2 c	JT407749	355	Lg2
		bnip2l	bcl2/adenovirus E1B 19 kDa interacting protein 2 like	JT411501	333	Lg7
	BNIP3	Bnip3	bcl2/adenovirus E1B 19 kDa interacting protein 3	JT218129	197	Lg5
		bnip3la	bcl2/adenovirus E1B 19 kDa interacting protein 3 like a	JT406133	237	Lg11
		bnip3lb	bcl2/adenovirus E1B 19 kDa interacting protein 3 like b	JT417754	211	Lg25
Multidomain anti-apoptotic proteins	MCL	mcl1a	myeloid cell leukemia 1 a	JT407366	258	Lg24
		mcl1b	myeloid cell leukemia 1 b	JT411974	253	Lg1
	BCL-XL	blp1	B-cell lymphoma-extra large 1	JT479645	242	Lg15
	BCL-2	bcl2a	B-cell lymphoma 2a	JT400297	204	Lg19
		bcl2b	B-cell lymphoma 2b	JT489706	204	Lg8
	BCL2L10	bcl2l10	bcl-2-like protein 10	JT315779	178	Lg4
	BCL2L12	bcl2l12	bcl-2-like protein 12	JT341893	363	Lg3
Multidomain pro-apoptotic proteins	BAX	baxa	bcl-2 associated X protein a	JT320960	112	Lg3
		baxb	bcl-2 associated X protein b	JT406892	203	Lg3
		baxl	bcl-2 associated X protein like	JT415726	202	Lg20
	BOK	bok	bcl-2 related ovarian killer	JT409073	214	Lg7
	Not present	1	bcl-2-like protein 16	JT410195	199	Lg11

construct the phylogenetic tree, because the bnip genes were an atypical subfamily in the pro-apoptotic BH3-only proteins. Bnips possess some of unique characteristics such as interaction with the adenovirus E1B 19 kDa protein, which is an anti-apoptotic protein (Boyd et al., 1994).

Phylogenetic trees were constructed for each of the four classes of bcl-2 genes (Figs. 1–4), in general, catfish bcl-2 genes within each family were clustered with their counterparts from human and zebrafish, respectively. As shown in Fig. 1, 7 pro-apoptotic BH3-only proteins (bada, bik, bim, bmf1, bmf2, pmaip1, puma) were clustered into the proper clades with their counterparts from zebrafish, bcl2l15 fell into the proper clade with its counterpart with bcl2l15l from cavefish. Of the 9 bnip proteins, 3 (bnip1a, bnip3la, bnip3lb) fell into the proper clade with their counterparts from zebrafish (Fig. 2). Of the 7 multidomain anti-apoptotic proteins, 4 (mcl1a, mcl1b, blp1, bcl2l10) of them fell into proper clades with their counterparts from zebrafish (Fig. 3). Of the 5 multidomain pro-apoptotic proteins, 3 (baxa, baxb, bok) fell into proper clades with their counter parts from zebrafish (Fig. 4). The putative channel catfish genes, including badb, bid, bcl2l13, bcl2l14s, bnip1b, bnip2s, bnip3, bcl2s, bcl2l12, one copy of bax on linkage group 3 and bcl2l16 did not form a well-defined cluster with that in zebrafish, and therefore the syntenic analysis requires to be conducted to verify their identities.

3.3. Syntenic analysis of channel catfish bcl-2 genes

Phylogenetic analysis alone did not allow concrete annotation for several of the bcl2 genes, and therefore, syntenic analysis was conducted to provide insights into orthologies. Syntenic analysis of several bcl-2 genes including badb, bid, bcl2l13, bcl2l14s, bnip1b, bnip2s, bnip3, bcl2s, bcl2l12, a copy of bax on linkage group 3 and bcl2l16 were conducted as shown in Fig. 5. Apparently, conserved synteny regions were found between channel catfish and other species for badb (Fig. 5A), bid (Fig. 5B), bcl2l13 (Fig. 5B), bcl2l14s (Fig. 5C), bnip1b (Fig. 5D), bnip2s (Fig. 5E), bnip3 (Fig. 5F), bcl2s (Fig. 5G), blc2l12 (Fig. 5H), and bcl2l16 (Fig. 5I). For badb, the zebrafish and channel catfish has the same group of neighboring

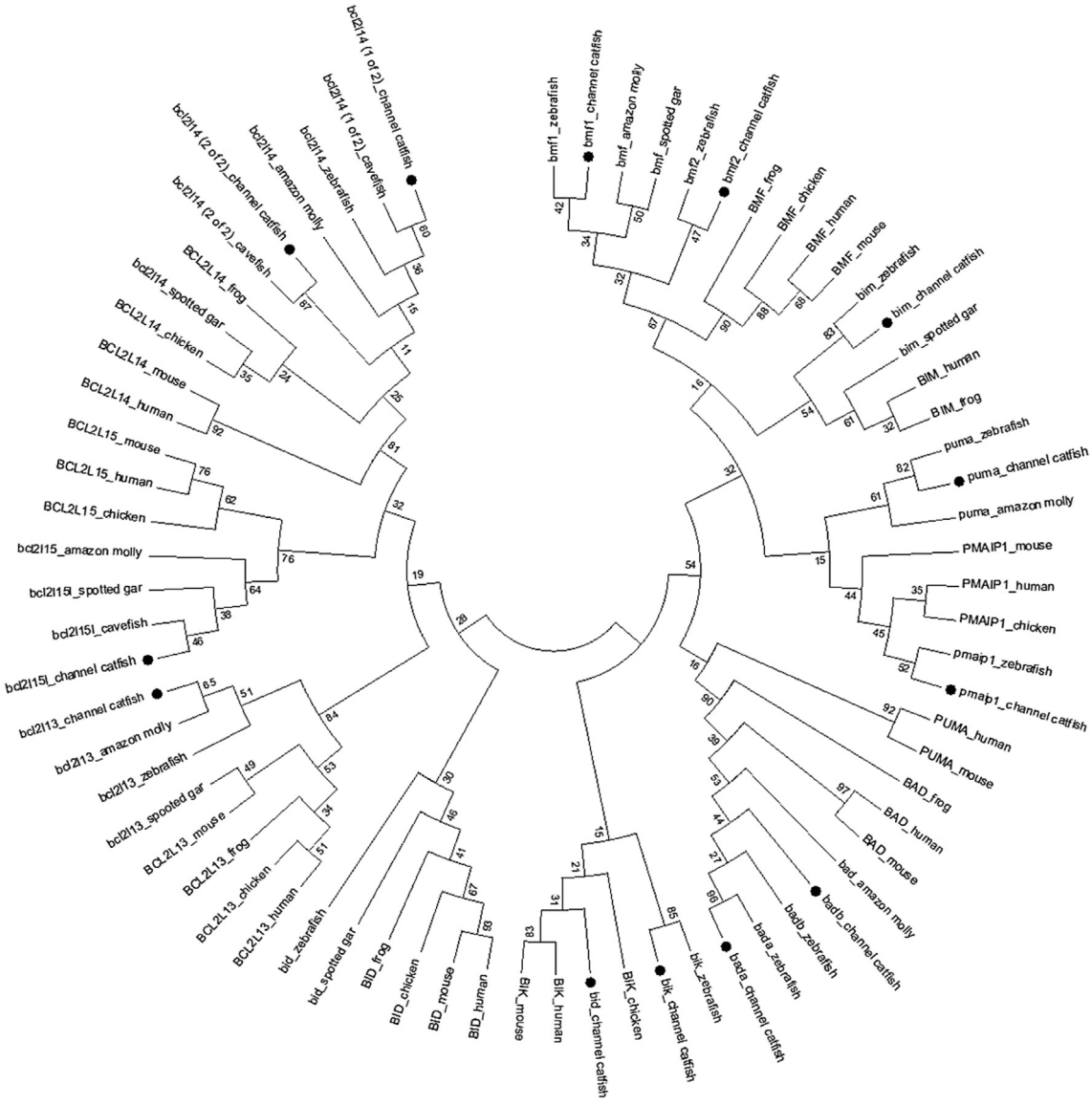

Fig. 1. Phylogenetic analysis of the pro-apoptotic BH3-only proteins. The phylogenetic tree was constructed using the maximum likelihood method with JTT + G + F model and 95% partial deletion method. The bootstrap values are shown at the nodes.

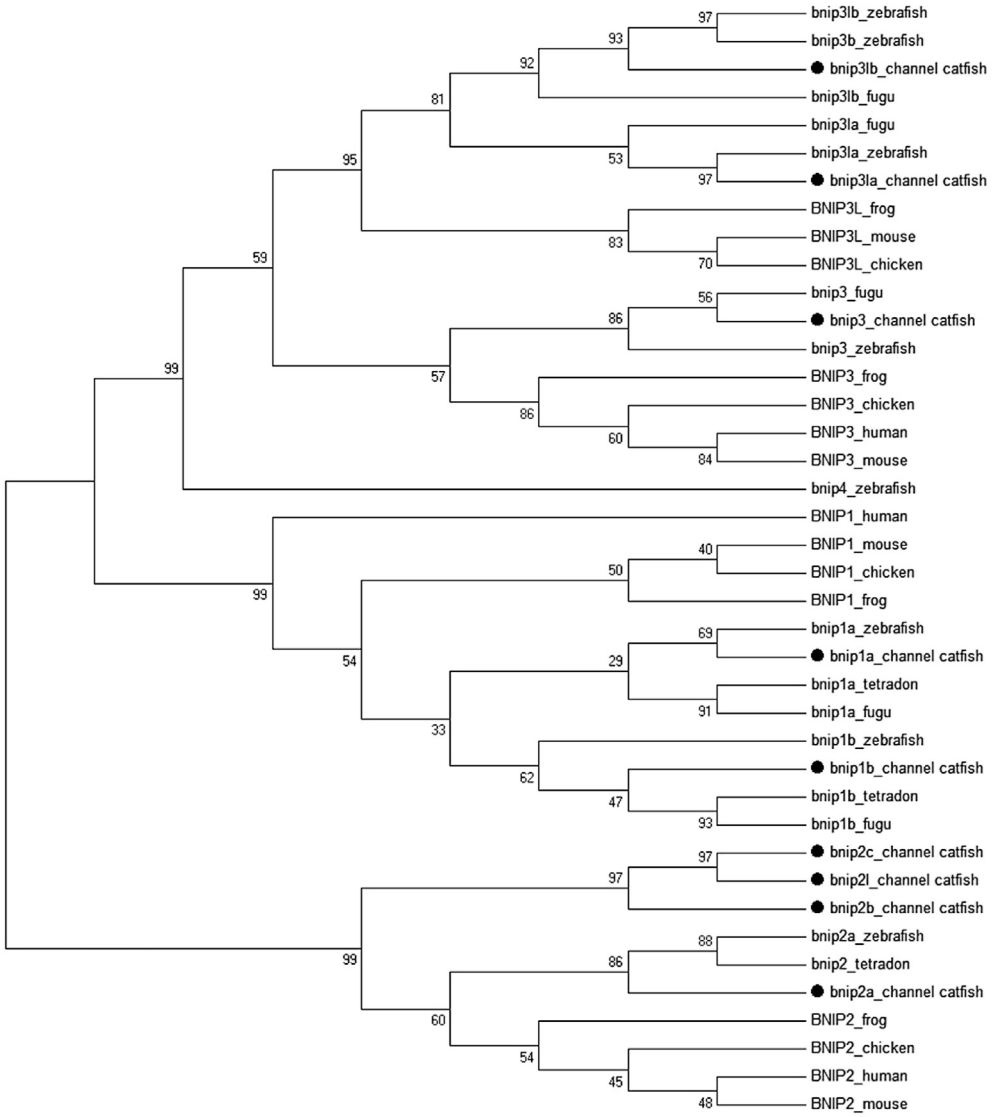

Fig. 2. Phylogenetic analysis of the bnip subfamily. The phylogenetic tree was constructed using the maximum likelihood method with JTT + G + F model and 95% partial deletion. The bootstrap numbers at the nodes indicts the bootstrap values.

genes such as b3gat3, arl2, ppp2r5b gpha2, exoc6b and cyp26b1.

For bid, bcl2l13 genes were neighboring genes in human, zebrafish and channel catfish while they were located between cftr and rapsn in zebrafish and channel catfish. Two copies of the bcl2l14 genes were identified in cavefish and channel catfish, and they shared the conserved neighboring genes with that of zebrafish, respectively. The bcl2l14 (1 of 2) in channel catfish and cavefish shared the neighboring genes of tbc1d15, chrf8, and has3, the bcl2l14(2 of 2) in channel catfish and cavefish shared the neighboring genes of rassf3 and dgki (Fig. 5C). The bnip1b in channel catfish and zebrafish shared conserved neighboring genes of crebrf, stc2b and bod1 (Fig. 5D). The copies of bnip2 on LG18, LG14, LG2 shared at least one of the neighboring genes including dnaja4, otud7a, klf13, trpm1 and cib with those in zebrafish. For one copy of bnip2 on scaffold jcf7180014891172, the neighboring genes were not conserved between channel catfish and zebrafish. Due to the lack of evidence for conserved synteny, we named the channel catfish bnip2 gene on LG7 as “bnip2-like”. Meanwhile, the bnip3 gene in channel catfish shared the gene of dpysl4 and jakmip3 with that in zebrafish (Fig. 5F). The bcl2a gene in channel catfish and zebrafish has the same neighboring genes of fbxo, prkag2, hs6st1b and ek1. In addition, channel catfish bcl2b is located between vps4b, kdsr and prkag2 in the genomes, which was consistent with that of zebrafish (Fig. 5G). The channel catfish bcl2l12 gene shared the same neighboring genes with that in zebrafish such as pax, tspan4b, unc119.2 and srrm2 (Fig. 5H). The bcl2l16 gene in channel catfish on LG8 shared the neighboring genes of nr6a1a, nr5a1a, adgrd2 and tubb2b with that in zebrafish. (Fig. 5I). Moreover, we were not able to identify conserved synteny blocks of the bax on LG3 in catfish with that in zebrafish, thus we named it as baxl. Taken together, the syntenic analysis provided sufficient evidence for the annotation and nomenclature of the bcl-2 genes in channel catfish.

3.4. Expression of bcl-2 genes after bacterial infection

Expression profiles of bcl-2 genes after bacterial infections with two pathogens, *E. ictaluri* and *F. columnare* were determined using the available RNA-Seq datasets. Following the meta-analysis, the bcl-2 genes that potentially exhibited differential expression were

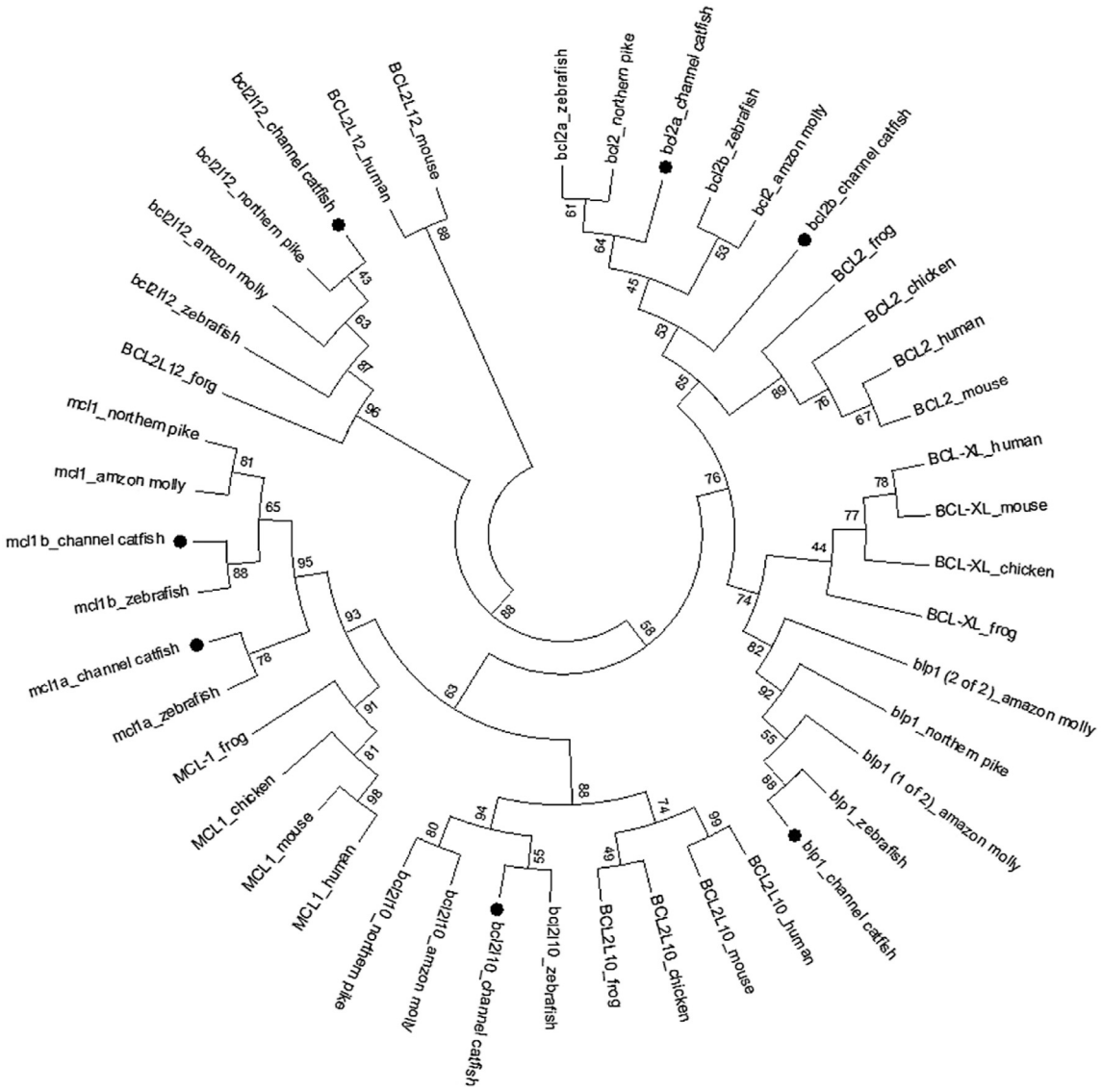

Fig. 3. Phylogenetic analysis of the multidomain anti-apoptotic proteins. The phylogenetic tree was constructed using the maximum likelihood with JTT + G + F model and 95% partial deletion method. The bootstrap values are shown at the nodes.

identified. Of the 34 bcl-2 genes, 29 were differentially expressed after bacterial infections, with 22 genes being differentially expressed after *E. ictaluri* infection and 22 genes being differentially expressed after *F. columnare* infection. As shown in Fig. 6, after *E. ictaluri* infection, 13 genes (bada, badb, bik, pmaip1, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bcl2l13, bcl2l15l, bnip1b, mcl1a, bcl2l10, baxa, baxl) were down-regulated and 9 genes (bmf1, bnip2a, bnip2b, bnip2c, bnip3, bnip3la, bcl2a, bcl2b, baxb) were up-regulated. The numbers of down-regulated genes increased during the infection progress, from 6 genes at 3 h (badb, bik, bcl2l14(1of 2), bcl2l14(2 of 2), mcl1a, baxl) to 12 genes at 72 h (bada, badb, bik, pmaip1, bcl2l14(1 of 2), bcl2l14(2 of 2), bcl2l15l, bcl2l13, bnip1b, bcl2l10, baxa, baxl) after infection. The number of up-regulated genes decreased from 7 to 5 and to 4 through the period of 3 h (bmf1, bnip2a, bnip2c, bnip3, bnip3la, bcl2a, baxb), 24 h (bmf1, bnip2b, bnip3la, bcl2a, bcl2b) and 72 h (bnip2b, bnip2c, bnip3la, bcl2a) after infection.

The expression of bcl-2 genes exhibited a different pattern after *F. columnare* infection (Fig. 7). Among the 16 constantly down-regulated genes (bada, bik, bmf1, puma, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bcl2l13, bnip3, bnip3lb, bnip3la, bcl2b, bcl2l10, bcl2l12, baxa, baxl, bcl2l16), 15 (bada, bik, bmf1, puma, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bnip3, bnip3lb, bnip3la, bcl2b, bcl2l10, bcl2l12, baxa, baxl, bcl2l16) were repressed 24 h after infection. The expression of up-regulated genes showed a more modest pattern, with three genes (blp1, mcl1a, baxb) up-regulated 4 h after infection, bnip1a were up-regulated 24 h while badb and bid are up-regulated 48 h after infection.

3.5. Expression of bcl-2 genes after hypoxia challenge

The expression of bcl-2 genes in response to hypoxia stress was conducted among groups of fish with different levels of hypoxia tolerance. Pairwise comparison was conducted among groups with different hypoxia tolerance, i.e., tolerant vs control, sensitive vs control, and tolerant vs sensitive. A total of 19 genes were significantly differentially expressed after hypoxia stress, including 14 genes that were significantly regulated in the tolerant group as compared with the control group. Among them, eight genes (badb, pmaip1, bcl2l13, bnip1b, bnip2b, blp1, bcl2l10, baxl) were induced,

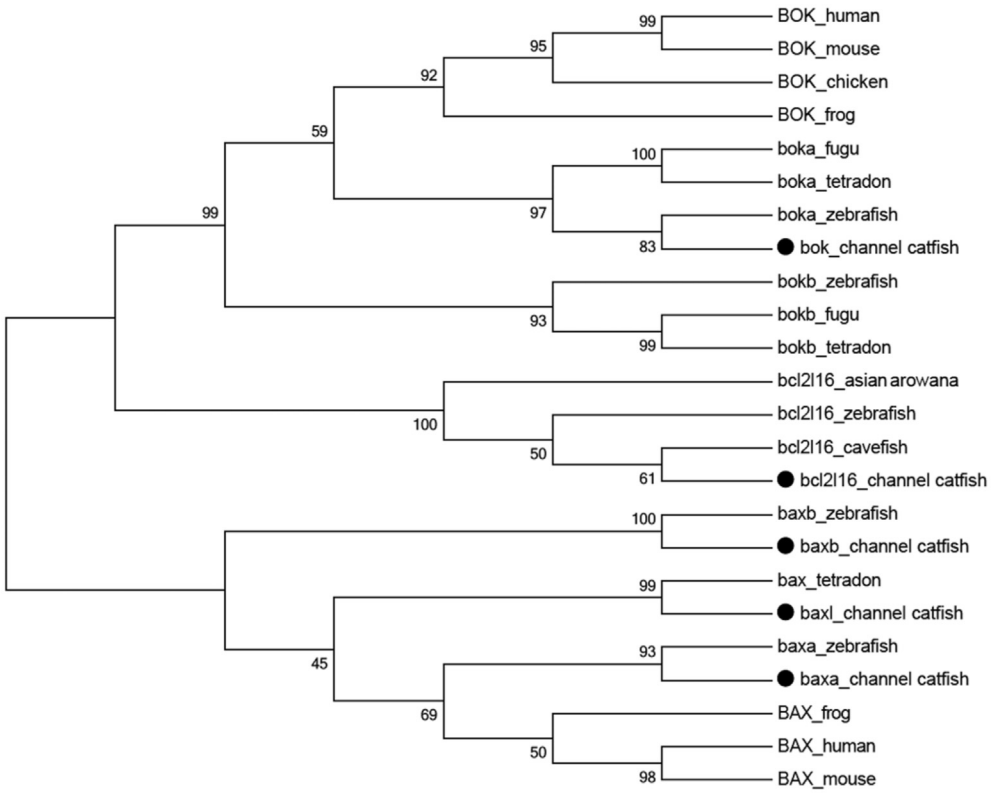

Fig. 4. Phylogenetic analysis of the multidomain pro-apoptotic proteins. The phylogenetic tree was constructed using the maximum likelihood method with JTT + I + G + F model and 95% partial deletion. The bootstrap values are shown at the nodes.

while six genes (bik, bnip1a, bnip2a, bcl2a, baxa, bok) were suppressed under hypoxia conditions. The comparison between sensitive and control group revealed that pmaip1, mcl1a and bcl2l10 were up-regulated at higher level in sensitive than in control group, and bid, bik, bmf1, puma, bnip2b and bnip2c were expressed in lower levels in sensitive group than those in control group (Fig. 8).

The comparison between tolerant group and sensitive group revealed that four genes were significantly differentially expressed between the groups (Fig. 8). After hypoxia challenge, pmaip1 and bcl2l10 were expressed at higher levels in hypoxia tolerant group than those in sensitive group. Similarly, the expression of bik decreased less in hypoxia tolerant group than that in sensitive group; and the expression of bnip2b decreased in hypoxia sensitive fish but increased in hypoxia tolerant fish.

4. Discussion

The bcl-2 genes are important in the regulation of apoptosis. Although bcl-2 have been observed to be expressed in a multitude of tissue types in channel catfish (Chen et al., 2010; Zeng et al., 2016), a comprehensive analysis of bcl-2 genes in channel catfish is lacking. In this study, we conducted systematic analysis of channel catfish bcl-2 gene family and identified a total of 34 bcl-2 genes in channel catfish genome. The analysis phylogeny and synteny allowed annotation of these bcl-2 genes. The 34 bcl-2 genes in channel catfish include 22 pro-apoptotic BH3-only proteins, seven multidomain anti-apoptotic proteins and five multidomain pro-apoptotic proteins. Furthermore, for the first time, the expression profiling of 34 bcl-2 genes after bacterial infection and hypoxia challenge were determined to provide insight into their involvement in the stress response. A number of bcl-2 genes differentially expressed after stress treatments were identified; and different expression patterns were observed between bacterial infections and hypoxia stress, and between the two different bacterial infections.

The phylogenetic and syntenic analysis verified the gene identities, and facilitated the annotation of duplicated genes. The analysis of copy numbers of bcl-2 genes in teleost fish and several other model species suggested that the copy numbers of bcl-2 genes were generally conserved across species (Table 1). However, teleost fish such as channel catfish, zebrafish and cavefish had more copies of several bcl-2 genes than those in the reptile, bird and mammals. Syntenic analysis indicated that expansion of these genes could be related to fish-specific whole genome duplication (Meyer and Van de Peer, 2005). For the duplicated gene bax, two copies of bax genes in channel catfish were both on scaffold jcf7180014892032, which is consistent with the observation in zebrafish in which the baxa and baxb were both on chromosome 3. Besides the teleost-specific duplication of bcl-2 genes, we identified bcl2l16, which involves the controlling of cell migration during gastrulation (Prudent et al., 2014). Bcl2l16 is unique to channel catfish, zebrafish and cavefish. The loss or duplication of genes during the fish-specific whole genome duplication 226–350 Myr ago (Hurley et al., 2007) may account for the various gene copies in different species in the subgroup of bnips, the loss of bcl2l15 in zebrafish and the loss of bim and pmaip1 in cavefish.

The channel catfish production has been severely impacted by bacterial diseases such as the enteric septicemia of catfish (ESC) (Hawke et al., 1998), columnaris disease (Bader and Starliper, 2002) and low oxygen stress. In the present study, we determined the expression profile of bcl-2 genes after *E. ictaluri* and *F. columnare* infection, and hypoxia challenges. As for *E. ictaluri* challenge, 15 pro-apoptotic BH3-only proteins (bada, badb, bik, bmf1, pmaip1, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bcl2l15l, bcl2l13, bnip1b, bnip2a,

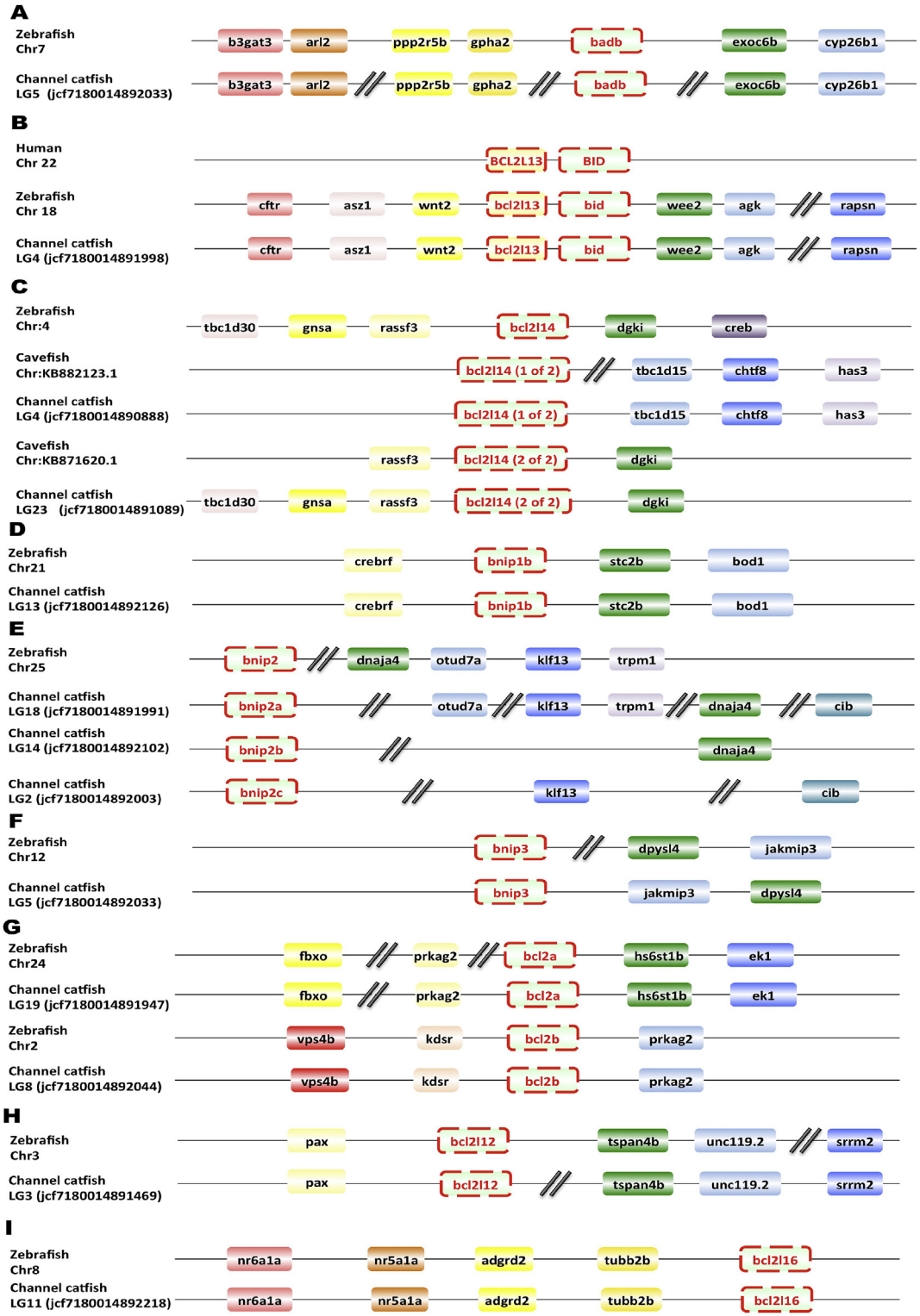

Fig. 5. Syntenic analysis of selected bcl-2 genes. (A) badb, (B) bcl213, bid, (C) bcl2l14s, (D) bnip1b, (E) bnip2s, (F) bnip3. (G) bcl2s, (H) blc2l12, (I) bcl2l16. Full gene names were provided in Supplemental Table 1.

bnip2b, bnip2c, bnip3, bnip3la), four multidomain anti-apoptotic proteins (bcl2a, bcl2b, mcl1a, bcl2l10) and three multidomain pro-apoptotic proteins (baxa, baxb, baxl) were significantly changed in the expression after *E. ictaluri* challenge (Fig. 6). Thirteen pro-apoptotic BH3-only proteins (bada, badb, bid, bik, bmf1, puma, bcl2l14 (1 of 2), bcl2l14 (2 of 2), bcl2l13, bnip1a, bnip3, bnip3la, bnip3lb), five multidomain anti-apoptotic proteins (blp1, bcl2b, mcl1a, bcl2l10, bcl2l12) and four multidomain pro-apoptotic proteins (baxa, baxb, baxl, bcl2l16) were significantly differently expressed after *F. columnare* infection (Fig. 7). The expression of multidomain pro-apoptotic proteins and pro-apoptotic BH3-only proteins were generally decreased after both *E. ictaluri* and

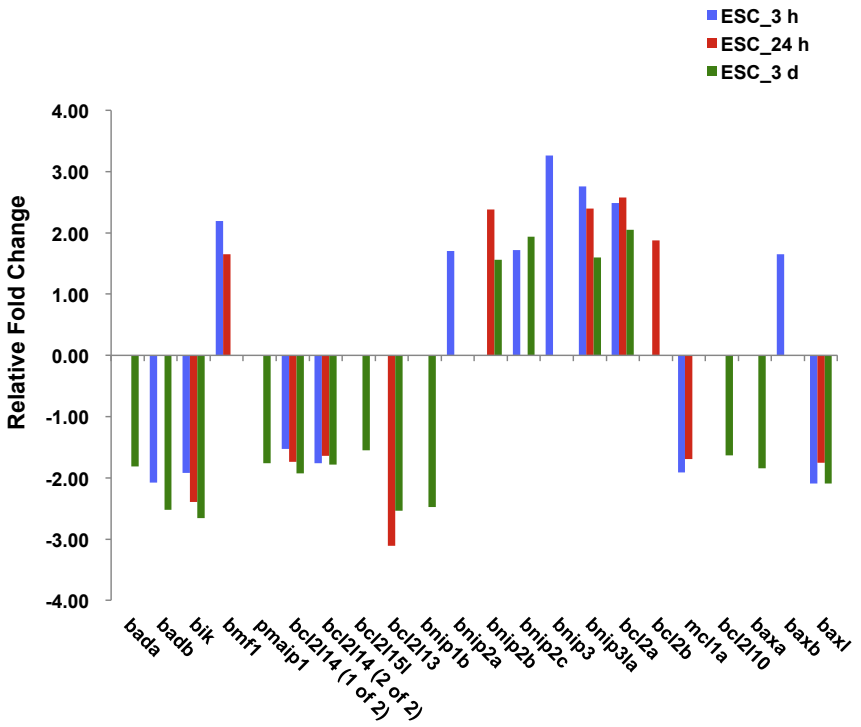

Fig. 6. Differentially expressed bcl-2 genes in intestine at 3h, 24h and 72h after *E. ictaluri* infection. The expression profiles were determined by meta-analysis of the RNA-Seq datasets. Gene expressions were presented as fold change, only the significantly expressed genes (|fold change| ≥1.5, with the total reads ≥ 5) were presented.

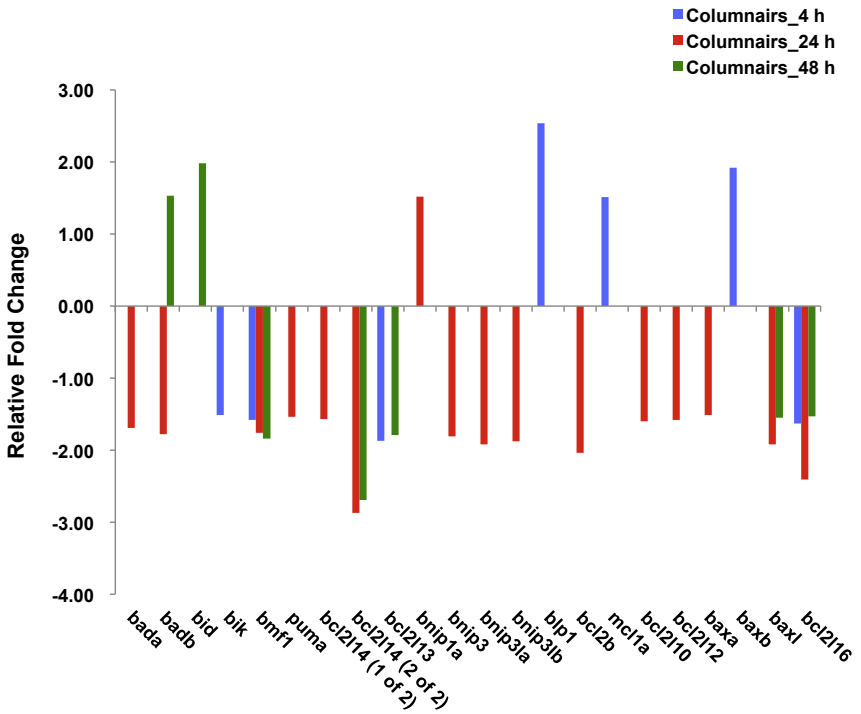

Fig. 7. Expression profile of channel catfish bcl-2 genes in gill after *F. columnare* infection at 4h, 24h and 48h. The expression profiles were determined by meta-analysis of the RNA-Seq datasets. Gene expressions were presented as fold change, only the significantly expressed genes (|fold change| ≥1.5, with the total reads ≥ 5) were presented.

F. columnare infection, suggesting the bacterial infection, like confinement stress (Alford Iii et al., 1994), triggered regulated expression of the bcl-2 family genes in channel catfish to decrease the apoptosis rate of cells.

Different patterns of expression of bcl-2 genes were observed between the two bacterial infections. After *F. columnare* infection, most of the differentially expressed genes showed a down-regulation observed at the 24 h after infection. However, after the *E. ictaluri* infection, the differential expression of most genes was observed at the 72 h after infection. This discrepancy could be

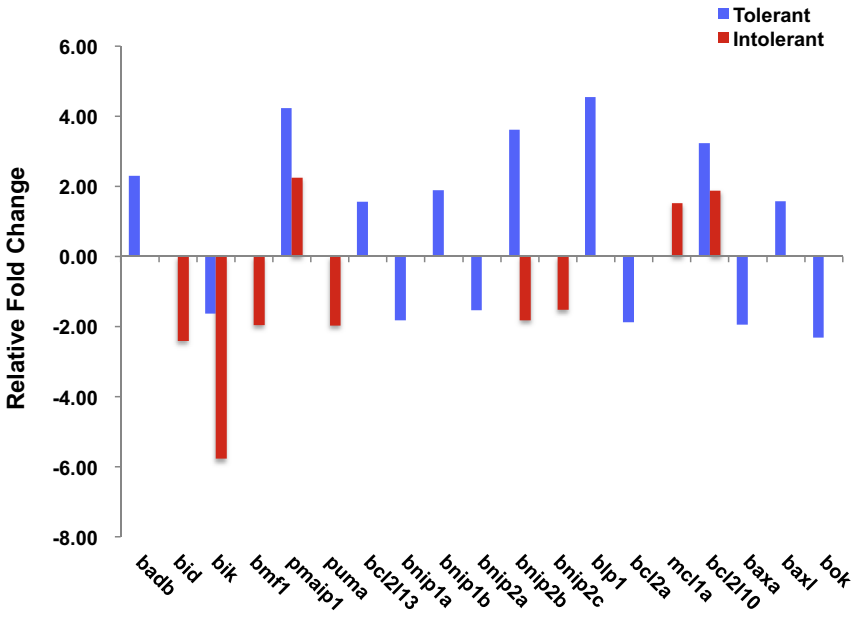

Fig. 8. Expression profile of channel catfish bcl-2 genes in gill after hypoxia challenge in hypoxia tolerant and hypoxia sensitive fish. The expression levels were determined by meta-analysis of the RNA-Seq datasets. Gene expressions were presented as fold change, only the significantly expressed genes (|fold change| ≥1.5, with the total reads ≥ 5) were presented.

attributed to the differences in pathogenicity of the *E. ictaluri* and the *F. columnare*.

Interestingly, several genes exhibited an opposite expression pattern between the *E. ictaluri* infection and the *F. columnare* infection. These included bnip3, bcl2b, mcl1a and bmf1 (Figs. 6 and 7). The bnip3 and bcl2b were up-regulated after *E. ictaluri* infection while they were down-regulated after *F. columnare* infection. The altered expression of bnip3 was observed in tumor like prostatic carcinoma, lung cancer, endometrium cancer and breast cancer (Li and Sheng, 2014). The low expression of the pro-apoptotic bnip3 may contribute to the decreased expression of multidomain pro-apoptotic proteins like baxs and bcl2l16 during the *F. columnare* infection. However, a study in neuron cells indicated that bnip3 was significantly up-regulated after ischemia (Li et al., 2013). Since the *E. ictaluri* infection can cause ischemia in channel catfish, the up-regulation of the bnip3 after *E. ictaluri* infection may be related to the ischemia. Bcl-2 is a well-studied oncogene. Overexpression of bcl-2 gene prevents neurons from apoptosis in the ischemia mice (Martinou et al., 1994). Similar to the expression pattern of bnip3, the high expression of the bcl2b after *E. ictaluri* infection may also be associated with the hemorrhages in intestine. The down-regulated expression of mc11a was observed after *E. ictaluri* infection. In humans, mcl1 is critical for the survival of macrophages to battle against hemorrhages and inflation (Liu et al., 2006). Since a typical symptom of ESC disease is the serious hemorrhages and inflation, it's expected an increased expression of mc11a, which was opposite to the observation of this work. The mechanism of down-regulated mc11a expression after *E. ictaluri* infection in our study remains to be explained. The bmf1 was highly expressed after *E. ictaluri* infection, while its expression was suppressed during the *F. columnare* infection. Bmf was reported to trigger apoptosis (Frenzel et al., 2010). The mechanisms underlying the opposite expression of bmf after *E. ictaluri* and *F. columnare* deserves further investigation. Some in vitro systems such as cell lines can be used in the future to determine the roles of bcl-2 family members in the process of apoptosis. Channel catfish is the only fish species where clonal functionally distinct lymphocyte lines have been established (Clem et al., 1990, 1996; Miller et al., 1998; Shen et al., 2002). Along with these lymphocyte cell lines, several other cell lines have been established from various tissues of channel catfish including ovaries (Bowser and Plumb, 1980), leukocytes (Miller et al., 1994), monocytelike cells (Vallejo et al., 1991), kidney (Zeng et al., 2009) and lymphoid cells (Majji et al., 2009). Future investigation with these cell lines would allow determination of tissue specificity as well as the involvement of bcl-2 genes in apoptosis under various physiological conditions.

After hypoxia stress, 12 pro-apoptotic BH3-only proteins (badb, bid, bik, bmf1, pmaip1, puma, bcl2l13, bnip1a, bnip1b, bnip2a, bnip2b, bnip2c), four multidomain anti-apoptotic proteins (blp1, bcl2a, mcl1a, bcl2l10), three multidomain pro-apoptotic proteins (baxa, baxl, bok) were found to be significantly differentially expressed after hypoxia challenge. Among the differentially expressed genes, the expression of 2 out of 3 multidomain pro-apoptotic bcl-2 genes were down-regulated, 3 out of 4 multidomain anti-apoptotic bcl-2 genes were induced, 7 out of 12 pro-apoptotic BH3-only bcl-2 genes were suppressed. In general, the increased expression of most multidomain anti-apoptotic bcl-2 genes and decreased expression of most pro-apoptotic BH3-only bcl-2 genes could be reason for the decreased expression of the multidomain pro-apoptotic bcl-2 genes, and thus repress the apoptosis process in channel catfish after stress (Alford Iii et al., 1994).

The differentially expressed bcl-2 genes between the hypoxia tolerant and sensitive groups are of particular interest. The genes showing great differential expression may suggest their association with the hypoxia resistance in channel catfish. Two genes, blp1 (6.04 fold change) and bnip2b (6.57 fold change), were drastically higher expressed in hypoxia tolerant fish than those in the hypoxia sensitive fish. Although no previous study suggested any interactions between these two genes, protein interaction prediction by program GeneMANIA revealed the interactive relationship between the bnip2 and bcl2l1 (blp1) through bcl-2 (ENSDARG00000089109) (Fig. 9). Interestingly, from the predicted interaction pattern, blp1 and bnip2 also interact with other differentially expressed genes during the hypoxia challenge, including bcl2l13, mcl1, bcl2l10, baxa and bok, indicating a potential

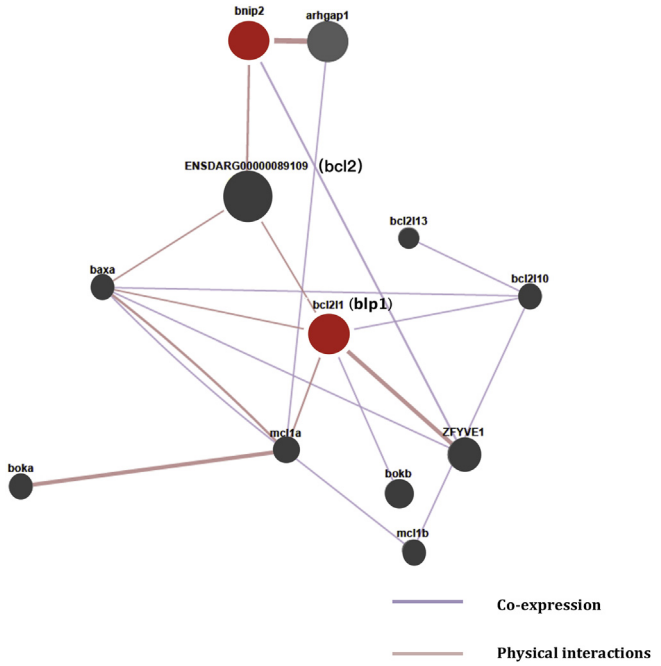

Fig. 9. GeneMANIA predicts the relationship between bnip2 and blp1. bnip2 and blp1 have been predicted to physically interact with each other through multidomain anti-apoptotic protein bcl2 (ENSDARG00000089109), and they also interact with the genes that are significantly changed in expression during the hypoxia stress, including bcl2l13, mcl1a, bcl2l10, baxa, bok.

interactive networks formed by those bcl-2 genes that regulate the apoptosis process in response to hypoxia stress in channel catfish.

In summary, we, for the first time, identified and annotate a set of 34 bcl-2 genes in channel catfish. We further determined their expression profiles to gain insights into their putative roles in response to bacterial infection and hypoxia stress. After stress, most anti-apoptotic bcl-2 genes were significantly up-regulated, while most pro-apoptotic bcl-2 genes were significantly down-regulated, suggesting their involvement in the host response to stress by suppressing apoptosis process. Different expression pattern of bcl-2 genes during pathogenesis of two bacterial pathogens indicated the pathogen-specific pattern of regulation. However, the bcl-2 family contains a number of genes, which may interact and complement with each other to function and form a balanced regulation network of apoptosis in the cell.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.06.018.

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