The Israeli Journal of Aquaculture - Bamidgeh, IJA_72.2020.119154, 14 pages
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Genome-wide identification and expression analysis of Bcl-2 gene family under low temperature stress in tilapia (*Oreochromis niloticus*)

Changgeng Yang[1, a], Meizi Wang[2, a], Hua Wen[2], Ming Jiang[2*], Juan Tian[2], Xing Lu[2]

[1] Life Science and Technology School, Lingnan Normal University, Zhanjiang, 524048, China

[2] Key Lab of Freshwater Biodiversity Conservation of Ministry of Agriculture and Rural Affairs, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan 430223, China

Key words: tilapia, *Bcl-2*, Phylogeny, low temperature stress, expression profile

Abstract

Low temperature stress can lead to variety of changes, including apoptosis in tilapia (*Oreochromis niloticus*). The B cell lymphoma-2 (*Bcl-2*) gene family plays an important role in the process of apoptosis. The present study conducted genome-wide characterization of the *Bcl-2* family genes in tilapia and their mRNA expression profiles were analyzed in different tissues of tilapia under the low temperature stress (10°C). Twenty-four *Bcl-2* family genes were identified, containing 2~8 exons. These genes were classified into two subfamilies (*Bcl-2* homologs and BH3-only) based on their conserved domains. Besides, these BCL-2 proteins in tilapia possess at least one of the four conserved BH domains. The phylogenetic analysis showed that the *Bcl-2* family genes did not aggregate by species, demonstrating sequence conservation of different types of *Bcl-2* family members. Real-time quantitative PCR (RT-qPCR) analysis showed that *Bcl-2* family genes were broadly expressed in different tissues of tilapia. When reared at 10 °C, the transcriptional expression levels of most of anti-apoptotic *Bcl-2* homologs subgroup members and other BH3-only subgroup members in most tissues of tilapia were higher than those at 30°C. However, most of other *Bcl-2* family members revealed a lower expression. The results suggested that hypothermia had significantly induced apoptotic in tilapia.

* Corresponding author. e-mail: jiangming@yfi.ac.cn (Ming Jiang)
[a] These authors contributed equally to the paper

Introduction

Apoptosis is a genetically regulated and programmed cell death, which plays an important role in the growth and development of multicellular organisms and the homeostasis of cell numbers (Renault and Chipuk, 2014). *Bcl-2* genes family is an important genes family, which functions as important regulators in the apoptotic pathway and participates in the regulation of cell proliferation and autophagy (Levine et al., 2008). It can determine whether the cell apoptosis occurs or not (Adams and Cory, 2018; Danial and Korsmeyer, 2004). The *Bcl-2* gene, a first discovered *Bcl-2* family gene, was discovered in human follicular B cell lymphomas more than 20 years ago (Tsujimoto et al., 1984), and its anti-apoptotic function was confirmed in 1989 (Tsujimoto, 1989). With the deepening of research, many *Bcl-2* genes family members were discovered and identified, and could be divided into two functionally opposing subsets: the anti-apoptotic *Bcl-2* genes and pro-apoptotic *Bcl-2* genes (Czabotar et al., 2014). All members in the *Bcl-2* genes family proteins have at least one of the four conserved BH domains, and one transmembrane region can be found at the C-terminal of most of BCL-2 family proteins (Christoph, 2003; Lanave et al., 2004). The BH3 domain is known as a death domain, and the detailed mechanism by which it interacts with pro-survival related molecules to regulate apoptosis has become a hot topic in recent years (Strasser et al., 2010; Suzanne et al., 2003). Three-dimensional structural studies have shown that the BH3 domain is usually required for the formation of dimers between pro-apoptotic proteins and anti-apoptotic proteins (Borner, 2003), and generally the BH1, BH2, and BH3 domain of anti-apoptotic proteins forms an elongated hydrophobic pocket. The BH3 domain of the pro-apoptotic protein binds to it as an amphipathic helix, stabilized by the BH4 domain of the anti-apoptotic protein, which allows the BH3 domain to be covered and thus unable to exert its apoptotic activity (Sattler et al., 1997). On the reports about the description and classification of *Bcl-2* family genes of Aouacheria(Aouacheria et al., 2013), *Bcl-2* family genes could be identified and further subdivided into three subfamilies, *Bcl-2* homologs, canonical BH3-only and other BH3-only, based on the composition of BH motifs. Moreover, the *Bcl-2* homologs subfamily had could be divided into three subgroups (pro-apoptotic *Bcl-2* homologs, anti-apoptotic *Bcl-2* homologs, and divergent *Bcl-2* homologs) based on their function and composition of BH motifs. *Bcl-2* homologs subfamily had one or more BH1-4 functional domains. BH3-only subfamily had only BH3 functional domains.

Temperature is one of the most important environmental parameters affecting tilapia (*Oreochromis niloticus*) cultivation. The growth temperature for tilapia is between 16°C and 38°C (Wohlfarth and Hulata, 1981). If the temperature is lower than 13°C or drops rapidly, tilapia will experience the process of adaptation, motion imbalance, stress, coma, and death (Behrends and Smitherman, 2010; Kindle and Whitmore, 2010; Sun et al., 1992), indicating that tilapia has poor cold resistance (Potts et al., 1967). As reported, low-temperature stress can significantly affect the behavior and physiology of tilapia, including a decline in the immune system, changes in physiological characteristics, and potential death (Yang et al., 2015). With the decrease in temperature, the plasma osmotic pressure (Atwood et al., 2015; Sun et al., 1992), serum sodium concentration, and lymphocyte concentration of Nile tilapia decreased, while serum glucose concentration increased (Atwood et al., 2015; Renault and Chipuk, 2014). In addition, there appears to be a series of changes in cellular levels under low temperature conditions, such as changes in cell membrane fluidity and cell mass transport, which can lead to cell division, growth arrest, and apoptosis (Los and Murata, 2004; Yang et al., 2017). Moreover, when subjected to low temperature, the gene expression involved in apoptosis of tilapia is also changed (Yang et al., 2015; Zhou et al., 2018).

In this study, we focused on the apoptosis related gene family, *Bcl-2*, to reveal the connection between apoptosis and low-temperature stress. Firstly, we analyzed the gene structures, conserved domains, and phylogenetic relationships of tilapia *Bcl-2* family genes, which are closely related to apoptosis. Twenty-nine *Bcl-2* family genes were identified by searching tilapia genome-wide data using bioinformatics methods. The tissue-specific expression analysis of these genes in tilapia was carried out to understand the composition and expression characteristics of *Bcl-2* family genes. Secondly, the *Bcl-2* gene expression

profiles in tilapia reared under low temperature and normal temperature conditions were analyzed. The present study would provide insight into the effect of low-temperature stress on *Bcl-2* genes expression in tilapia, and lay a foundation for further research on the function of fish *Bcl-2* genes.

Materials and Methods

Declaration of Ethics Statement

Tilapia is cultivated widely in South China and is not listed as endangered or protected species. All the experimental animal programs involved in this study were approved by the Yangtze River Fisheries Research Institute's animal care and use committee, and followed the experimental basic principles. The field studies did not involve endangered or protected species.

Experimental fish and sample preparation

Nile tilapia were purchased from a fish hatchery in Guangxi Province of China, and then transported to the experimental base of the Yangtze River Fisheries Research Institute (Wuhan, Hubei Province, China). Their initial body weights were 100.0 ± 10.0 g. The fish were domesticated for two weeks in an indoor recirculating aquaculture system to allow for a substantial amount of time to accommodate to a new environment. The fish were grown for 1 week at 30°C, then subjected to low temperature of 10°C by cooling the water at a rate of 1°C per day (Li et al., 2002).Dissolved oxygen was maintained at>5 mg/L by an air compressor, water pH was 7.2-7.5, and the total ammonia nitrogen concentration was 0.26±0.10mg/L. One month later, three individuals in normal temperature (30°C) or low temperature (10°C) were anesthetized and samples of heart, liver, intestine, muscle, fin, brain, spleen, skin, and gill were collected and frozen in liquid nitrogen. Samples were stored at −80°C until use.

Genome-wide screening and classification of *Bcl-2* family genes in tilapia

The whole genome sequence of tilapia was downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/). All predicted candidate *Bcl-2* genes family protein sequences were scanned by PFAM (http://pfam.xfam.org/family/PF00125) and PROSITE (https://prosite.expasy.org/) software for known BCL-2 protein domain/motif. And the genes sequences of all predicted protein were also obtained. Then, using PCR, the genes of all predicted protein were validated. Meanwhile, the obtained genes are classified into subclass of BCL-2 family, according to their function base on original bioinformatics analysis (BLASTP search) in NCBI database. Additional protein domains/motifs may be added by searching conserved domains using Batch CD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpSB.cgi).

Molecular evolution analysis of *Bcl-2* family genes in tilapia

BLASTP searches the known BCL-2 family gene sequences of tilapia, zebrafish (*Danio rerio*), common carp (*Cyprinus carpio*) and house mouse (*Mus musculus*) in the UniProt protein database (e<1e-5). FASTA file containing zebrafish, common carp, house mouse and tilapia amino acid sequence of *Bcl-2* family genes were subjected to multiple sequence alignments using ClustalX software. MEGA7.0 software was used to construct a phylogenetic tree using Neighbor-Joining method with Bootstrap value of 1000. The resulting Newick phylogenetic tree file was visualized by iTOL software (https://itol.embl.de).

Tissue expression of *Bcl-2* family genes in tilapia

Total RNA was extracted from the heart, liver, small intestine, muscle, fin, brain, spleen, skin, and gill tissues of tilapia reared under low temperature and normal temperature conditions, using TRIzol Reagent (Tiangen Biotech, China), respectively. High-quality RNA determined by OD A260/A280 ratio (1.8-2.0) and electrophoresis were subjected to reverse transcription (500ng of total RNA per sample) using FastkinggDNA

Dispelling Quant RT Kit cDNA (Tiangen Biotech, China). cDNA was used as a template for fluorescence quantitative PCR detection.

RT-qPCR was performed on a QuantStudio 6 Flex Quantitative PCR instrument (Life Technologies, USA). The 20μLqRT-PCR reaction system consisted of 10μL of 2 × SuperRealPreMix Plus (Tiangen Biotech, China), 6.8μL of RNase-free ddH2O, 1.0 μL of cDNA template (500ng/μL), and 0.6μL of forward and reverse primer (10μmol/L). qRT-PCR reaction conditions are as follows, 95°C denaturation 5 min; 28 cycles include 95°C 30 s, 60°C 30 s, 72°C 30 s; 72°C 5 min. Each sample is duplicated 3 times. The relative gene expression was normalized to 18S rRNA (NCBI accession number: JF69868318S) levels using $2^{-\triangle\triangle CT}$ method. The gene-specific primers were designed using the Primer premier software 5 and are listed in **Table 1**.

Table 1 Primers used for qRT-PCR.

Gene	Sequence	
	Forward Primer (5 '→3')	Reverse Primer (5 '→3')
Bcl2l1a (Bcl2 like1a)	GCATACAATGGCATAGAGGCTTT	GATTCCATCCCTGAACAACTCCT
Bcl2l1b (Bcl2 like1b)	AATGAGTTCGAGCTGCGATACG	GGAACACCTCGTCCATCACGT
Mcl1 (Bcl2 family apoptosis regulator)	ATGACAGAACACAACCACTCGG	CTACAGGCCCTCAAACTCGTAC
Bcl2a (Bcl2, apoptosis regulator)	TAACTGACCCTCCACCGACTTT	TCTATCACCTCGGCGAACCTC
Bcl2b (Bcl2, apoptosis regulator)	AGCGACTTCACGGCAACGAG	GTGACGATGCGTCCCCAGTT
NR13 (Bcl2l10)	GCTGGTTGGAGATGGACACTTG	TGGCACTGCGGGAGAACTTAC
Baxa (Bcl2 associated x, apoptosis regulator)	AAGATGGCAATAAAGCAGTGACG	TGCAAAGTAGAACAGGGCAACC
Baxb (Bcl2 associated x, apoptosis regulator)	TGCAGCATGACCAGGAGTTTC	AAGTTCACTGCCCAGGTAAGGAC
Baxc (Bcl2 associated x, apoptosis regulator)	GGAGCGGTTGTCTTTAGAGGGT	TCTGAAGTTCAGCATTCCGATTT
Boka (Bok, Bcl2 family apoptosis regulator)	ACTGTGTCCGCCATGGTCAT	TCCACCCAGCCTCCTCTCTT
Bokb (Bok, Bcl2 family apoptosis regulator)	ATTTTTCTCCCTCGAACGCAG	ACATGGACACCACCTTACCCC
Bcl2l12 (Bcl2 like 12)	CACCACAAGTACAGTTCCAACCC	TGTCTTGCCCTTCCTGCCC
bcl2l13 (Bcl2 like 13)	CACCTGTCAGCAGCCGTACAC	AATACTTCACCCCATCCCCC
Bcl2l14 (Bcl2 like 14)	TGAGGTTCAGATTCAGAGGCAGAC	ACCCAAGCCGAGTGGTTCTC
Bcl2l15 (Bcl2 like 15)	AGAACGCTGACATTGCTCCAG	AACTTTACTTACCCATCCACCTTTC
Bid (BH3 interacting domain death agonist)	TCAACAGCAATGGGCACGAC	TCAGGTTCCTGGTAGCTTCAGTC
Bad (Bcl2 associated agonist of cell death)	CTTGCCCTTCCTGTAATCAAAAC	GTCAAACTCGTCACTCATCCGTC
Bcl2l11 (Bcl2 like 11)	CCCTCTAGGACGTGGCAGC	CATCGCCCAATCAGTATCAGC
Bmfa (Bcl2 modifying factor)	CAGCCTTGCCTACCGTTTCAT	CTGCCACGCTGTCAATGTCTT
Bmfb (Bcl2 modifying factor)	TCACAGAGCGAGGCGACAAG	TCAAACAGAAGGCTGAGAAGGG

Bnip3a (Bcl2 interacting protein 3)	AAATGCTGACTGGATCTGGGAC	GTATGTGGGAAATGATAAATGATGG
Bnip3b (Bcl2 protein-interacting protein 3)	GACCCACCAGCGAGCACG	CCAGCAGCGAGGGAACAAG
Bnip3lb (Bcl2 protein-interacting protein 3-like)	GCCATCGTCCTCCTCTATCCAC	AGTCAGCAACCCAGTCTACTTCTTT
Bnip3la (Bcl2 protein-interacting protein 3-like)	GGGTGGAGTTGGAGATGAACAG	GGATTGAAGAAGACGATGGGAC
18S (18S ribosomal RNA)	GGACACGGAAAGGATTGACAG	GTTCGTTATCGGAATTAACCAGAC

Results

Identification and classification of the *Bcl-2* family genes in tilapia genome

A total of twenty-four *Bcl-2* genes with conserved BH domains were identified from the tilapia genome. Meanwhile, we also searched and analyzed the *Bcl-2* family genes in other two teleost; zebrafish (*D. rerio*) and common carp (*C. carpio*), and a mammal, house mouse (*M. musculus*), with 27, 34 and 35 genes respectively (**Table 2 and 3**). Like the description in Abdel (Aouacheria et al., 2013), the *Bcl-2* family genes were divided into two subfamilies based on the BH domains, including *Bcl-2* homologs and BH3-only. Moreover, the *Bcl-2* homologs can be further divided into three subgroups, including pro-apoptotic *Bcl-2* homologs, anti-apoptotic *Bcl-2* homologs, and divergent Bcl-2 homologs. Among them, Anti-apoptotic *Bcl-2* homologs have seven members, and their protein structures contain four short conserved BH domains (BH1-BH4). Pro-apoptotic *Bcl-2* homologs have five members, and their protein structures contain three to four short conserved BH domains (BH1-BH4). Divergent *Bcl-2* homologs have five members with multiple BH domains. Moreover, the BH3-only subfamily has eight members, and this subfamily member only contained one BH3 domain or a C-terminal hydrophobic tail structure, i.e., transmembrane (TM) function domain. Among them, canonical BH3-only subgroups have 4 members, and these subfamily members only contained one BH3 domain. The other BH3-only subgroups have 4 members who had one BH3 domain and a TM function domain. The results showed that the longest BCL-2 protein (Bcl2l13) contained 554 amino acids and the shortest one (Bcl2l14) only contained 127 amino acids (**Table 4**).

Table 2 In Silico identification of *Bcl-2* family genes in tilapia genome (*Oreochromis niloticus* genome assembly).

Subgroups		Gene Name assigned	Gene ID (NCBI/EMBL)	Chromesome location	Genomic Position	Exon Number	**Protein_ID**	**Length (aa)[1]**
Bcl-2 homologs	Anti-apoptotic Bcl-2 homologs	Bcl2l1a	100704382	LG20	31801604..31835473	3	XP_003457009.1	232
		Bcl2l1b	100700270	LG5	31247734..31249724	3	XP_003442785.1	197
		Mcl1	100695229	LG11	31138143..31140131	3	XP_003450317.1	272
		Bcl2a	100710191	LG18	16802357..16828801	2	XP_003437950.1	228
		Bcl2b	102079353	LG9	2983686..2991228	3	XP_005461925.1	198
		Bcl2L10 (NR13)	100699619	LG1	28554301..28559035	3	XP_003437747.1	231
	Pro-apoptotic	Baxa	100706164	LG1	4175914..4184884	6	XP_003456606.1	203

BH3-only	Bcl-2 homologs	Baxb	100705633	LG4	36738199..36743862	4	XP_003456932.3	192
		Baxc	109201890	LG4	25766522..25769663	6	XP_019213295.1	192
		Boka	100689837	LG23	32302318..32314996	5	XP_003444315.1	210
		Bokb	100707238	LG18	16036504..16047466	5	XP_005476355.1	213
		Bcl-2l12	106097209	LG4	6538804..6548808	8	XP_019211481.1	359
	Divergent Bcl-2 homologs	Bcl2l13	100692352	LG7	61926215..61945421	7	XP_003447161.1	554
		Bcl2l14	102080759	LG7	1962348..1964434	7	XP_005470268.1	127
		Bcl2l15[2]	I3KT992/112846943	LG5	12614027..12615836	3	I3KT99/XP_025763155.1	162
	Canonical BH3-only[1]	Bad	100712130	LG3	10197446..10202008	4	XP_003452465.1	158
		Bid	100699927	LG7	61923921..61926616	4	XP_003447106.1	194
		Bcl-2l11	102081903	LG13	9890853..9921002	4	XP_005474458.1	223
		Bmfa	102081386	LG19	9103359..9115260	4	XP_005453360.1	163
		Bmfb	100690873	LG15	8978141..8989886	4	XP_003455660.1	179
	Other BH3-only[1]	Bnip3a	100697476	LG13	14388730..14392585	6	XP_003438323.1	189
		Bnip3b	100705876	LG7	16871909..16878512	6	XP_005456274.1	181
		Bnip3lb	100711031	LG12	12383733..12387976	6	XP_003445727.1	236
		Bnip3la	100689723	LG7	29889572..29901785	6	XP_013123966.1	217

[1] Canonical: the protein functional domains of these genes had only a canonical BH3 domain. Other: the protein functional domains of these genes had BH3 domain and TM domain.
[2]This gene was identified via protein search (it was totally missed by NCBI annotation pipeline).

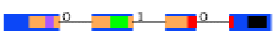
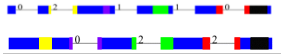
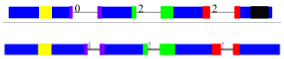
Table 3 Classification of *Bcl-2* family genes in 4 vertebrate genomes (modified from HGNC).

Subsets		Approved Symbol	Description	ON[1]	DR[1]	CC[1]	MM[1]
Bcl-2 homologs	Pro-apoptotic *Bcl-2* homologs	Bax	BCL2 associated X, apoptosis regulator	3(4)*	3	7	2
		Bak1	BCL2 antagonist/killer 1	0	0	0	1
		Bok	BOK, BCL2 family apoptosis regulator	2	2	3	1
		Bcl2L16[2]	BCL2 like 16	0	1	1	0
	Anti-apoptotic *Bcl-2* homologs	Bcl2	BCL2, apoptosis regulator	2	2	2	2
		Bcl2l1	BCL2 like 1	2	2	1	2
		Bcl2l2	BCL2 like 2	0	0	0	1

	Divergent *Bcl-2* homologs	Mcl1	BCL2 family apoptosis regulator	1(5)*	2	3	1
		Bcl2A1	BCL2 related protein A1	0	0	0	1
		Bcl2l10（NR13）	BCL2 like 10	1	1	1	1
		Bcl2l12	BCL2 like 12	1	1	2	1
		Bcl2l13	BCL2 like 13	1	1	1	1
		Bcl2l14	BCL2 like 14	1	1	0	1
		Bcl2l15	BCL2 like 15	1	0	0	2
BH3-only	Canonical BH3-only	Bad	BCL2 associated agonist of cell death	1	2	4	2
		Bid	BH3 interacting domain death agonist	1	1	0	1
		BBC3	BCL2 binding component 3	0	1	1	1
		Bcl2l11	BCL2 like 11	1	1	3	5
		Bik	BCL2 interacting killer	0	1	1	3
		Bmf	Bcl2 modifying factor	2	2	1	3
		PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	0	1	0	1
		HRK	Harakiri, BCL2 Interacting Protein	0	0	0	1
		C22orf29	C22orf29 chromosome 22 open reading frame 29	0	0	0	0
	Other BH3-only	Bnip3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	4	2	3	1
Total				24(29)*	27	34	35

[1]The numbers of genes found in ON: Tilapia (*Oreochromis niloticus*); DR: Zebra fish (*Danio rerio*); CC: Common carp (*Cyprinus carpio*); MM: House mouse (*Mus musculus*).
[2]Bcl-WAV, also named as Bcl2L16, was found in fish first.
*The number within parenthesis is from original bioinformatics analysis; the number outside parenthesis is based on PCR validation results.

Table 4 Structure and functional domains analysis of *Bcl-2* genes family protein in tilapia.

Subgroups		Gene Name assigned	Gene Structure and protein functional domains Legend: CDS Intron bcl-2_bad BH1 BH2 BH3 BH4 BID BMF SEC14 TM 0 1 2: intron phase	Predicted BH1/2/3/4
Bcl-2 homologs	Anti-apoptotic *Bcl-2* homologs	Bcl2l1a		BH1/2/3
		Bcl2l1b		BH1/2/3/4
		Mcl1		BH1/2/3/4
		Bcl2a		BH1/2/3
		Bcl2b		BH2/4
		BCL2L10(NR13)		BH1/2/3/4
	Pro-apoptotic *Bcl-2* homologs	Baxa		BH1/2/3/4
		Baxb		BH1/2/3
		Baxc		BH1/2/3/4
		Boka		BH1/2/3/4
		Bokb		BH1/2/3/4
		Bcl2l12		BH2

BH3-only	Divergent *Bcl-2* homologs	Bcl2l13		*
		Bcl2l14		*
		Bcl2l15		*
	Canonical BH3-only	Bad		BH3
		Bid		BH3
		Bcl2l11		BH3
		Bmfa		BH3
		Bmfb		BH3
	Other BH3-only	Bnip3		BH3
		Bnip3b		BH3
		Bnip3lb		BH3
		Bnip3la		BH3

*Not specifically for each BH domain. But combined domain search shows it contains the BCL-2-like region spanning BH1-4.

Chromosomal location and intron analysis of *Bcl-2* family genes in tilapia

Base on the genomic distribution analysis of *Bcl-2* family genes, the results showed that the genes of the 24 family members were widely distributed on 16 different chromosomes, and their distribution appeared to be uneven (**Table 2**).

The analysis of intron number showed that the number of introns in the same subgroup members of *Bcl-2* family had little difference, while the number of introns in different subgroup had obvious difference. Among them, the divergent *Bcl-2* homologs subgroup members and pro-apoptotic subgroup members had more the average number of introns, was 4.8 and 4.2, respectively. Moreover, the number of introns in divergent subgroup members is quite different. The average number of introns in anti-apoptotic subgroup, canonical BH3-only subgroup and the other BH3-only subgroup was 1.8, 3, and 2.2, respectively (**Table 2**).

Phylogenetic analysis of BCL-2 family protein in tilapia and other selected Animals

We further constructed *Bcl-2* gene phylogenetic trees among the tilapia, two other selected teleost (zebrafish (*D. rerio*) and common carp (*C. carpio*)) and mammal (house mouse (*M. musculus*)) using Neighbor-Joining method (**Figure 1**). All *Bcl-2* genes were grouped into seven groups in the phylogenetic tree, which was like the classification results based on conservative domain. The anti-apoptotic subgroup and pro-apoptotic subgroup of *Bcl-2* homologs genes subfamily were clustered on the same branches, respectively. Interestingly, the member of divergent subgroup of *Bcl-2* homologs genes subfamily and BH3-only subfamily had a closer evolution relationship. Most of the BH3-only subfamily members were grouped into the same branch. However, a small number of BH3-only genes were clustered into branches of the other *Bcl-2* subfamilies, and these genes were from multiple vertebrate species.

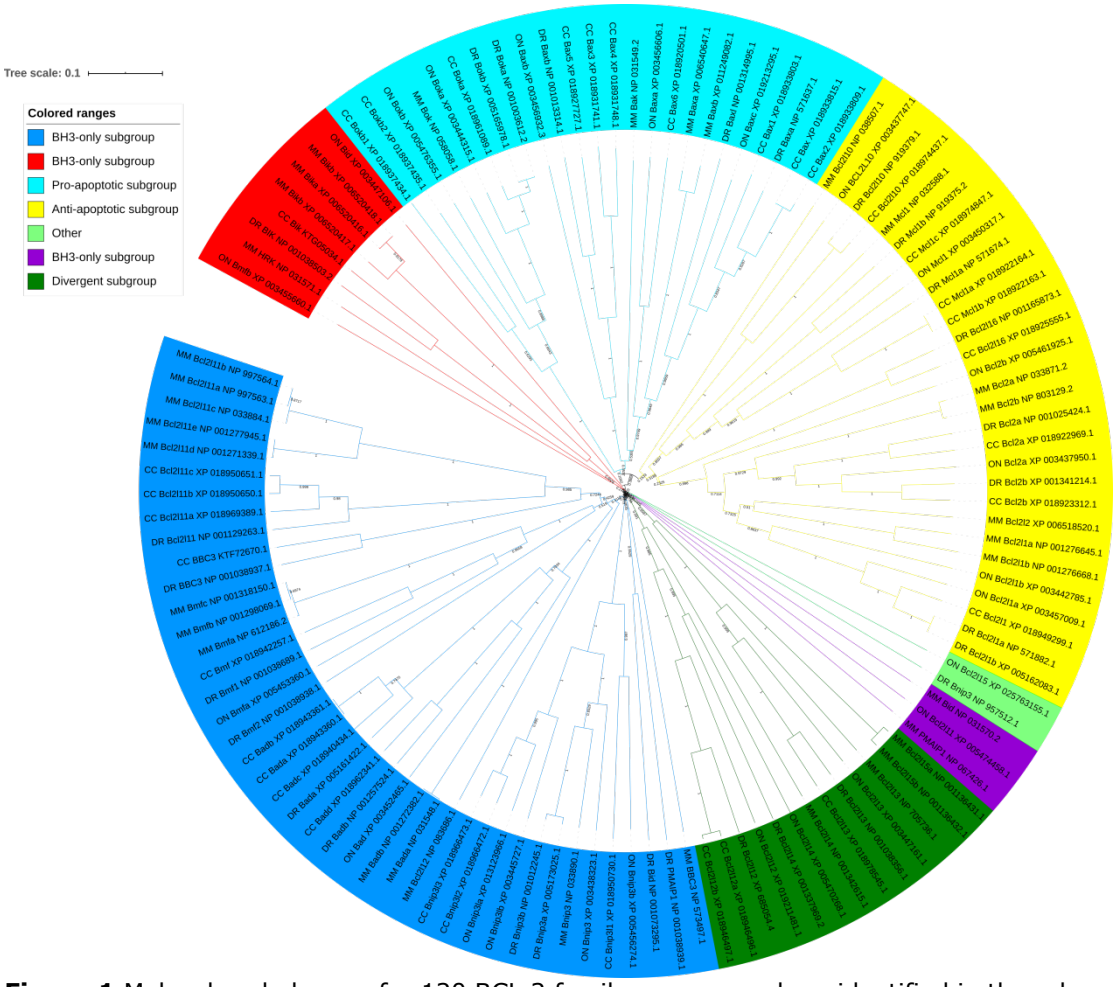


Figure 1 Molecular phylogeny for 120 BCL-2 family genes members identified in three bony fish genomes and *Mus musculus* genomes in silico. The bootstrap scores with 1000 trials are shown on branches. ON: Tilapia (*Oreochromis niloticus*); DR: Zebra fish (*Danio rerio*); CC: Common carp (*Cyprinus carpio*); MM: House mouse (*Mus musculus*)

Expression profiling of BCL-2 family genes in tilapia under normal temperature and low temperature stress

The heatmap was constructed to cluster tilapia *Bcl-2* family genes with same expression pattern under different temperature conditions (**Figure 2**). Genes from the same subfamily revealed different expression patterns at different temperatures, and no correlation could be identified among expression patterns of genes from different subfamilies. Notably, except in the muscle, at 10°C, the transcriptional expression levels of almost all canonical BH3-only subgroup genes were lower than 30°C. On the contrary, in every tissue, at 10°C, the transcriptional expression levels of almost all other BH3-only subgroup members were higher than 30°C. At 10°C, the transcriptional expression levels of most of anti-apoptotic *Bcl-2* homologs subgroup members were higher than 30°C, except in liver and fin. At 10°C, the transcriptional expression levels of most of pro-apoptotic *Bcl-2* homologs subgroup members were lower than 30°C, except in intestine, muscle, brain and heart. At 10°C, the transcriptional expression levels of most of divergent *Bcl-2* homologs subgroup members were lower than 30°C, except in muscle and brain.

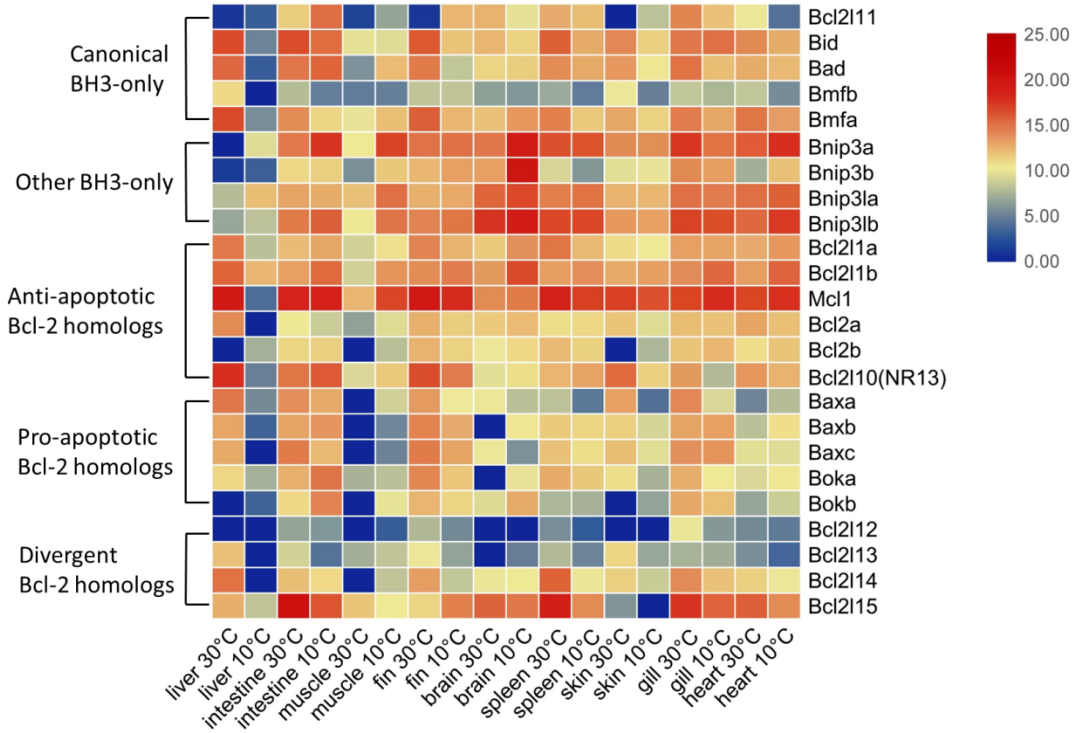


Figure 2 The expression profile of spleen BCL-2 family genes at normal temperature (30 ℃) and low temperature (10 ℃) stresses conditions. Control with the Bcl2l11 expression in liver at 30 ℃, then the $\log_2$ values of all genes relative to its expression were used for the heatmap.

Discussion

As reported, the *Bcl-2* gene family has been discovered and grouped into three categories according to their functions (Suzanne and Adams, 2002). (1) The anti-apoptotic protein subfamily, including BCL-xL (SW et al., 1996), BCL-2 (Petros et al., 2001), BCL-W (also known as Bcl-2l10) (Denisov et al., 2003; Hinds et al., 2014), MCL-1 (myeloid cell leukemia sequence 1) (Day et al., 2005), and Al (also known as BFLI) (Herman et al., 2008). These genes contain 4-short conserved BH domains (BH1-BH4) (Roy et al., 2014) and a C-terminal hydrophobic tail structure, ie, transmembrane (TM) function domain. The TM junction domain allows them to localize in the mitochondrial outer membrane, and occasionally to the surface of the endoplasmic reticulum. The transmembrane structure of the protein is oriented toward the cytoplasm (Akao et al., 1994; Krajewski et al., 1993; Nguyen et al., 1993). These members can block apoptosis by inhibiting their pro-apoptotic counterparts (Adams and Cory, 2007); (2) the pro-apoptotic protein subfamily, including BAX (BCL-2 homologous antagonist/killer protein), BAK (BCL-2-associated X protein), and BOK containing multi-domains and directly facilitate MOMP (Czabotar et al., 2014; Youle and Strasser, 2008). They are structurally similar to the former members and contain all domains except BH4 (Tsujimoto et al., 1985); (3) The BH3-only protein subfamily, a special pro-apoptotic protein group that only has BH3 domain. It includes BIM (BCL-2 interacting mediator of cell death), BID, BAD (BCL-2-associated death promoter protein), PUMA (p53 up-regulated modulator of apoptosis protein) and NoxA. They act as initiators in response to discrete cellular apoptotic stimuli (such as growth factor withdrawal, DNA damage, and anoikis) (Czabotar et al., 2014; Yoshihide, 2010).

So far, there had been no report on the identification and analysis of *Bcl-2* family genes at the genome-wide level in teleosts. However, the tilapia genome and transcriptome data sets facilitated the structural and functional analysis of *Bcl-2* family at the genome-wide level. In this study, a total of 24 *Bcl-2* family genes were identified in the tilapia genome. And referring to the aforementioned classification, these *Bcl-2* family genes of tilapia were divided into two subfamilies, *Bcl-2* homologs and BH3-only, based on the composition of BH motifs. The subfamily of *Bcl-2* homologs contained anti-apoptotic Bcl-2 subgroup, Pro-

apoptotic *Bcl-2* subgroup and Divergent *Bcl-2* subgroup. BH3-only subfamily contained a canonical BH3-only subgroup and another BH3-only subgroup.

The phylogenetic analysis of the homologous sequences of *Bcl-2* family genes of tilapia and other species (two other teleost and a mammal species) suggested that the *Bcl-2* family genes did not aggregate by species, demonstrating sequence conservation of different types of *Bcl-2* family members. Moreover, the Anti-apoptotic, Pro-apoptotic, and Divergent subgroup from *Bcl-2* homologs subfamily genes were respectively clustered as three branches, and Divergent *Bcl-2* homologs subgroup and BH3-only subfamily had revealed a closer evolutionary relationship. According to this result and referring to the domain analysis of *Bcl-2* family proteins, we reclassified *Bcl-2* family genes into two subfamilies, *Bcl-2* homologs subfamily (including Anti-apoptotic *Bcl-2* homologs and Pro-apoptotic *Bcl-2* homologs), and other *Bcl-2* subfamily. The above mentioned results have further indicated that there was a clear differentiation between these two subfamilies, which evolved independently of each other. In addition, the results of domain analysis of *Bcl-2* gene family indicated that genes from the same subfamily was highly conserved among different species.

Gene expression profiling showed that upon low temperature conditions (10°C), the transcriptional expression of most of the anti-apoptotic *Bcl-2* homologs subgroup genes in most tissues of tilapia (except in liver and fin), was higher than those at normal temperature conditions (30°C). As for two types of typical apoptosis-promoting genes including pro-apoptotic and canonical BH3-only genes, the transcriptional expression of most of typical apoptosis-promoting genes at low temperature conditions (10°C) was lower when comparing with those at normal temperature conditions (30°C). The results indicated that low temperature had a significant effect on tilapia, overexpression of anti-apoptotic genes in tilapia under low temperature stress conditions formed apoptosis resistance (Mcdonnell et al., 1989). Explanation for this may be that anti-apoptotic proteins can binds to pro-apoptotic proteins. Meanwhile, the body can resist the low-temperature stress and maintain the body function, by increasing the expression of anti-apoptosis, organism inhibited apoptosis (Chipuk and Green, 2008; Dewson et al., 2012; Dewson et al., 2008; Green and Levine, 2014; Haiming et al., 2011).In addition, apoptosis-promoting genes also include other BH3-only genes, the transcriptional expression of the other BH3-only genes in every organization of tilapia at low temperature conditions (10°C) was higher than those at 30°C, the reason may be that the apoptosis promoting protein BNIP3la, BNIP3a, BNIP3b and BNIP3lb and the anti-apoptotic BCL-2 protein molecules could form a heterodimer to promote apoptosis (Kelekar and Thompson, 1998). Comparative analysis also revealed that hypothermia had a significant induced effect on apoptotic.

Conclusion

In the present study, we identified and characterized 24 *Bcl-2* family genes in tilapia. A phylogenetic analysis of the tilapia *Bcl-2* family genes indicated evolutionary conservation and diversification. Large scale-based expression profiling showed that low temperature stress has a greater impact on the expression of *Bcl-2* family genes in the brain, muscle, heart, and gill than those in the intestine and other tested tissues of tilapia. The information generated in this study will facilitate further research on *Bcl-2* genes and other gene families in tilapia.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31402290) and the China Agriculture Research System (Grant No. CARS-46).

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