Fish and Shellfish Immunology 94 (2019) 346–354

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Short communication

Identification and characterization of five Nk-lysins from *Pseudocrossocheilus bamaensis* and their diverse expression patterns in response to bacterial infection

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ARTICLE INFO

Keywords:
Pseudocrossocheilus bamaensis
Aeromonas hydrophila
Antimicrobial peptide
Nk-lysin
Bacterial infection

ABSTRACT

Nk-lysin is an effector protein of cytotoxic T lymphocytes and natural killer cells. It is known to possess antibacterial, anti-fungal, anti-viral, and anti-tumor activity. Here we describe five Nk-lysin genes (*PbNkla*, *PbNklb*, *PbNklc*, *PbNkld*, and *PbNkle*) from *Pseudocrossocheilus bamaensis*, a rare indigenous species distributed in Guangxi, China. The open reading frames (ORFs) consisted of 426 (*PbNkla*), 435 (*PbNklb*), 369 (*PbNklc*), 366 (*PbNkld*), and 339 (*PbNkle*) bp nucleic acids. The surfactant-associated protein B (SapB) domain and six conserved cysteine residues were identified in each *PbNkl* gene. Phylogenetic analysis revealed similar results to homology comparison that *PbNkla* and *PbNklb* consist of five exons and four introns and grouped together, whereas *PbNklc* and *PbNkld* each contain four exons and three introns and formed a separate clade. *PbNkle* had three exons and two introns and formed an independent clade separate from the four other *PbNkls*. qPCR analysis demonstrated that *PbNkla*, *PbNklc*, *PbNkld*, and *PbNkle* were ubiquitously expressed in all tissues examined, whereas *PbNklb* was expressed only after bacterial infection. *Aeromonas hydrophila* challenge significantly up- and down-regulated *PbNkls* at different time points post-injection and in different immune-related tissues. These results suggested that *PbNkls* were conserved immune molecules that may be involved in the immune response to pathogen invasion.

1. Introduction

Pseudocrossocheilus bamaensis belongs to the Labeoninae subfamily (Cypriniformes: Cyprinidae). It is a rare indigenous species that is distributed mainly in the upper reaches of the Xijiang River in Guangxi, China [1,2]. In Bama Yao Autonomous County, one of the world's top longevity townships, *P. bamaensis* is a delicacy famous for its high nutritional and economic value. Recently, *P. bamaensis* population sizes have declined dramatically due to overfishing, water pollution, and exotic species invasion [3].

In 2004, our team began researching the domestication and artificial breeding of *P. bamaensis*. However, during the breeding process, *P. bamaensis* failed to adapt to intensive farming due to the strict culture environment requirements of this species and to *Aeromonas hydrophila*-related disease outbreaks. This has greatly restricted the large-scale breeding of *P. bamaensis*. To date, antibiotics are still the main measure used to treat infections caused by a variety of bacterial pathogens in fish. However, frequent, improper or high dose use of antibiotics has resulted in the emergence of antibiotic-resistant bacteria [4]. Therefore, it is necessary and urgent to study the immune responses of fish to bacterial diseases and find better measures to control disease outbreaks.

Antimicrobial peptides (AMPs)[1] are short, cationic peptides with an amphipathic secondary structure [5]. Their broad-spectrum antimicrobial activities have attracted increasing interest as pharmaceuticals in medicine and aquaculture [6,7]. To date, more than 3,000 AMPs have been discovered from six kingdoms (http://aps.unmc.edu/AP/main.php). In fish, a large number of AMPs have been identified in recent years, including hepcidin, defensins, piscidin, and Nk-lysin [8].

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[1] $2^{-\Delta\Delta CT}$, comparative threshold cycle method; AMP, antimicrobial peptide; CFU, colony forming units; LC_{50}, median lethal dose; ORF, open reading frame; pI, isoelectric point; SapB, surfactant-associated protein B; SEM, standard error of the means; TSB, tryptic soy broth.

https://doi.org/10.1016/j.fsi.2019.09.014
Received 23 April 2019; Received in revised form 2 September 2019; Accepted 5 September 2019
Available online 06 September 2019
1050-4648/

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Primer application
PbNkla-F1	GATGCTCCGCAACATCTT	ORF and genomic amplification
PbNkla-R1	AAGGCTTTCACAGTAATCGT	
PbNklb-F1	GATCTGCCAAGATGCTCC	
PbNklb-R1	TCACTGTAATGCATACAG	
PbNklc-F1	ATGCTGCGGAGTATTGTC	
PbNklc-R1	CAGGATTCTTAAAAACGT	
PbNkld-F1	GAACAGACTCTTTTGCCA	
PbNkld-R1	AAGGCCATTAACTCCAT	
PbNkle-F1	GTTCAGGCATTTCAGATG	
PbNkle-R1	TAAGGTTGAAGCATTCAC	
PbNkla-F2	TTCGTGAAGTGGACTCTGCT	qPCR
PbNkla-R2	GGCGTTGAGTTAGGGGAGAT	
PbNklb-F2	TGCTGAGGACCAAGATCAAGA	
PbNklb-R2	GCGTTGGGTTAGCAGAGATT	
PbNklc-F2	ATTGTCCTGATCACCCTGCT	
PbNklc-R2	CCCAACACACTCCAGGTAGT	
PbNkld-F2	GGTCTGTGATGAGATCGGCT	
PbNkld-R2	ACACAGATGGTTTTGGCATCA	
PbNkle-F2	GAGGGAGCTGGAGTTGAGTT	
PbNkle-R2	GCTGCCGATTTACCTCATCC	
EF1α-F	ACCACCGGCCATTTGATCTA	
EF1α-R	CTTCAGTTTGTCCAGCACCC	

Table 2
Nk-lysin genes used in this study.

Scientific name	Gene Name	Code name	Accession number
Cyprinus carpio	*Nkl1*	*CcNkl1*	XP_018976518.1
	Nkl2	*CcNkl2*	AOT80800.1
	Nkl3	*CcNkl3*	XP_018926630.1
	Nkl4	*CcNkl4*	XP_018947975.1
	Nkl5	*CcNkl5*	XP_018952410.1
	Nkl6	*CcNkl6*	XP_018970060.1
	Nkl7	*CcNkl7*	XP_018926066.1
Danio rerio	*Nkla*	*DrNkla*	AKT74321.1
	Nklb	*DrNklb*	AKT74322.1
	Nklc	*DrNklc*	AKT74323.1
	Nkld	*DrNkld*	AKT74324.1
Ictalurus punctatus	*Nkl1*	*IpNkl1*	AAY16122.1
	Nkl2	*IpNkl2*	ABC17994.1
	Nkl3	*IpNkl3*	ABC17995.1
Megalobrama amblycephala	*Nkla*	*MaNkla*	[33]
	Nklb	*MaNklb*	[33]
Anabarilius grahami	*Nkla*	*AgNkla*	ROJ78830.1
	Nklb	*AgNklb*	ROJ78828.1
Acipenser dabryanus	*Nkl*	*AdNkl*	AZL56981.1
Coturnix japonica	*Nkl*	*CjNkl*	BAR91522.1
Gallus gallus	*Nkl*	*GgNkl*	AMY26518.1
Chelonia mydas	*Nkl*	*CmNkl*	XP_027685485.1
Alligator mississippiensis	*Nkl*	*AmNkl*	XP_019340852.1
Sus scrofa	*Nkl*	*SsNkl*	NP_001265684.1
Ovis aries	*Nkl*	*OaNkl*	AFT64210.1
Equus caballus	*Nkl*	*EcNkl*	NP_001075398.2
Bos Taurus	*Nkl*	*BtNkl*	XP_010808403.2
Homo sapiens	*Gnl*	*HsGnl*	NP_006424.2

Nk-lysin is a type of cationic AMP produced by cytotoxic T lymphocytes and natural killer cells. It was first isolated from porcine small intestinal tissue and found to have antimicrobial properties [9]. Structural analysis in different species showed that the Nk-lysin peptide is characterized by a surfactant-associated protein B (SapB) domain and six conserved cysteine residues that form three intrachain disulfide bonds [10]. The activity of the peptide is dependent on intact disulfide linkages; when Nk-lysin is pretreated with dithiothreitol, both its bactericidal and cytolytic effects are inhibited [11]. Extensive research has confirmed that Nk-lysin exhibits potent antibacterial activity against various microorganisms [12–16] and can kill cancer cells [17,18]. Because of its notable antibacterial activities, Nk-lysin may be a promising antibiotic substitute to combat drug-resistant pathogens.

In the present study, we provide the first characterization of five Nk-lysin genes from *P. bamaensis*. The *PbNkl* genes were cloned and analyzed for their structures and homology with other vertebrates. Expression patterns of the *PbNkls* in different tissues and their potential roles during *A. hydrophila* infection were investigated. This study may contribute to further explorations of the functions of Nk-lysin genes and shed light on new methods for combatting bacterial diseases.

2. Material and methods

2.1. Fish collection and bacterial challenge experiments

Wild adult *P. bamaensis* (25.50 ± 4.20 g, 98.42 ± 3.28 mm) were obtained from Bama County, Guangxi, China (24°12′47.05″N, 107°07′13.00″E). Fish were acclimatized in a 6,000 L tank containing aerated freshwater at 28 ± 2 °C for 2 weeks prior to starting the experiment. Five healthy individuals were randomly selected and anesthetized using MS-222 (Sigma-Aldrich, USA) for collection of brain, gill, heart, intestine, liver, skin, spleen, trunk kidney, head kidney, and muscle tissues. Tissues were immediately stored in RNAstore (Qiagen, Germany) and transferred to −80 °C until use.

The *A. hydrophila* strain used in the challenge experiment was isolated and purified from diseased *P. bamaensis*. This strain was previously found to be highly pathogenic to *P. bamaensis* (unpublished results). A total of 80 healthy fish were randomly assigned to the bacterial challenge experiment. *A. hydrophila* was activated in tryptic soy broth (TSB) medium at 30 °C for 24 h and subcultured twice in TSB medium for a further 24 h. Bacteria were then harvested, washed three times, and suspended in 0.85% normal saline. A 100 μL suspension containing 3.25×10^4 colony forming units (CFU)/mL, the median lethal dose (LC_{50}), of *A. hydrophila* was intraperitoneally injected into the fish. At 0, 3, 6, 12, 24, 48, and 72 h post-injection, gill, liver, spleen, and head kidney tissues were collected from five fish and placed on ice until storage at −80 °C. Fish in the control group were injected with 0.85% normal saline and sampled as described above.

2.2. Total RNA isolation, DNA isolation and cDNA synthesis

Frozen tissues were transferred to a mortar and ground to a fine powder. Total RNA was isolated from each tissue using TRIzol reagent (TaKaRa, Japan). Genomic DNA was extracted from muscle using a Genomic DNA Isolation Kit (Qiagen) following the manufacturer's instructions. The cDNA synthesis was performed using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA) using 1 μg of total RNA.

2.3. Complete open reading frame and DNA cloning of P. bamaensis Nk-lysin genes

Five Nk-lysin unigenes were identified based on gene annotation using the transcriptome database and designated as *PbNkla, PbNklb, PbNklc, PbNkld*, and *PbNkle*. Based on the initial sequence of *PbNkl* genes, specific primers were designed to verify the open reading frame (ORF) sequences and to clone the entire DNA sequences. PCR was performed in 50 μL reactions containing 25 μL 2 × *Ex Taq* Buffer, 1 μL dNTP Mixture (10 mM), 1 μL *Ex Taq* (TaKaRa), 5 μL first strand cDNA or DNA, and 1.5 μL each primer (10 μM). The PCR amplification was performed under the following conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30–55 s, and 72 °C for 1–2 min; followed by 72 °C for 10 min. All PCR products were gel-purified using a DNA Extraction Kit (Omega, USA) and sequenced by Sangon Biotech (Shanghai, China). All primers are listed in Table 1.

2.4. Characterization and phylogenetic analysis of P. bamaensis Nk-lysin genes

Gene intron-exon structure information and conserved domains were identified using BLAST and SMART viewer (http://smart.embl-heidelberg.

Table 3
Sequence features of *PbNkls*.

Features	*PbNkla*	*PbNklb*	*PbNklc*	*PbNkld*	*PbNkle*
Accession number	MH910093	MH916607	MH916605	MK263337	MH916606
ORF length	426	435	369	366	339
Amino acid residues	141	144	122	121	112
Signal peptide	17	17	17	17	0
Mature peptide	124	127	105	104	112
SapB domain	75	75	75	75	73
Molecular weight	14.35	14.70	12.00	11.91	13.17
Theoretical pI	7.79	7.08	6.90	7.74	9.99
Number of exons	5	5	4	4	3
Number of introns	4	4	3	3	2
Number of alpha helixes	7	8	6	5	5
Number of coils	8	9	7	6	6

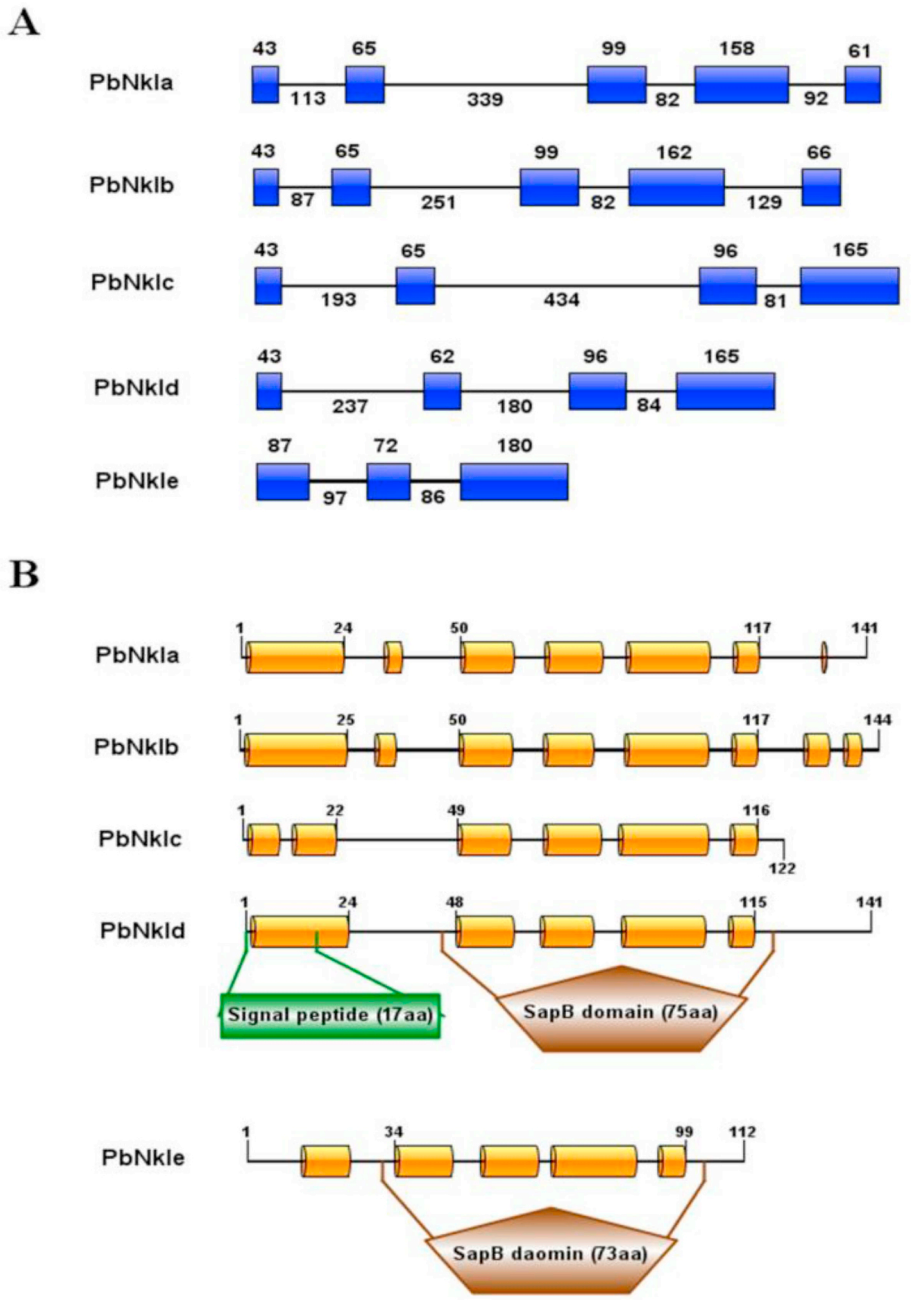

Fig. 1. Sequence and structure analysis of PbNkls. (A) Genomic structure of *PbNkls*. Exons and introns are presented by blue boxes and full lines, respectively. (B) Secondary structure and domain organization of PbNkls predicted by PSIPRED and SMART program. The orange cylinders indicate alpha helixes, the full lines represent coils. Signal peptide and SapB domain are shown as green box and brown pentagon. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

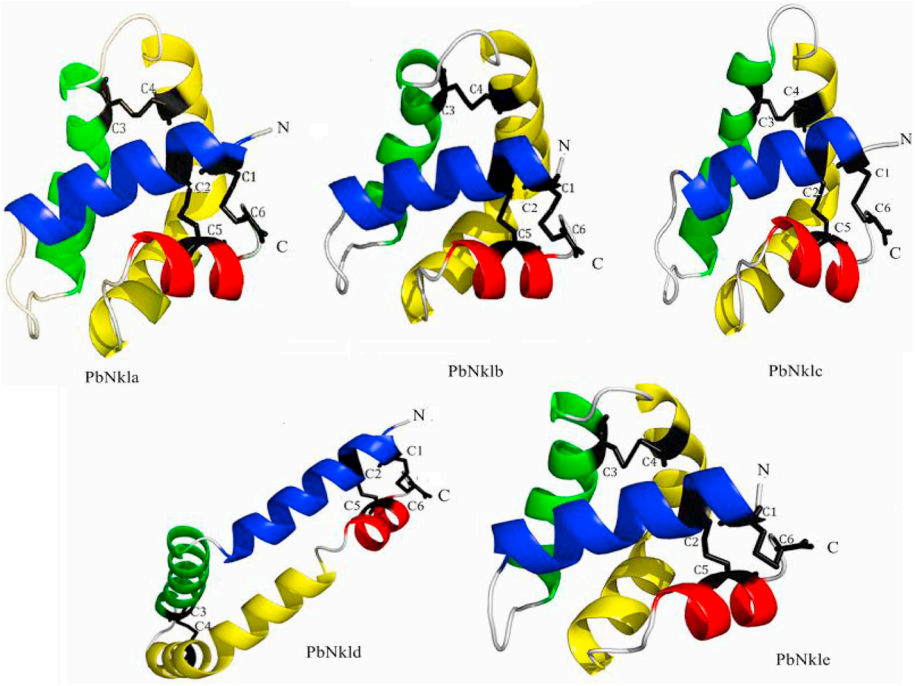

Fig. 2. Potential tertiary structure of PbNkls predicted by SWISS-MODEL program. Four helixes are depicted in blue, green, yellow, and red, respectively. Three disulfide bridges are indicated as the stick model in black. C1–C6 are the six conserved cysteines involved in forming disulfide bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

de/) and displayed in the IBS program (http://ibs.biocuckoo.org/download.php). Secondary structures were predicted using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). Signal peptide characteristics were predicted using SignalP 5.0 (http://www.cbs.dtu.dk/services/SignalP/). Molecular weight and isoelectric point (pI) prediction was performed using ProtParam tool (https://web.expasy.org/protparam/). 3D structures were predicted using the SWISS-MODEL program (https://www.swissmodel.expasy.org) and visualized using the PyMOL program [19]. Multiple sequence alignment was conducted using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and analyzed in ESPript 3 (http://espript.ibcp.fr/ESPript/ESPript/). Homology analysis was conducted with BLAST program. Phylogenetic tree of the PbNkls and Nk-lysins from other species were constructed using MEGA 6.06 using the neighbor-joining method with 10,000 bootstrap replicates. All sequences used in this section were retrieved from the GenBank database or reference sequence (Table 2).

2.5. The qPCR analysis of PbNkl gene expression

The qPCR was performed on a CFX Connect™ system (Bio-Rad, USA) using the Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). The qPCR primers designed for *PbNkls* are listed in Table 1. Melting curve analysis was performed at the end of each qPCR program to confirm the uniqueness of the product. The amplification program was as follows: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s; followed by 75 °C for 5 s. Expression levels of *PbNkls* were analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with *EF1α* as the internal control. All samples were analyzed in triplicate, and data shown are means ± standard error of the means (SEM) of mRNA levels relative to *EF1α*.

3. Results

3.1. Characterization and sequence analysis of PbNkla, PbNklb, PbNklc, PbNkld, and PbNkle genes

Based on RNA-seq data, five *P. bamaensis* Nk-lysin genes (*PbNkla, PbNklb, PbNklc, PbNkld*, and *PbNkle*) were identified. The nucleotide sequences have been submitted to the NCBI GenBank database under accession numbers MH910093, MH916607, MH916605, MK263337, and MH916606. ORFs were confirmed by PCR, and consisted of 426 (*PbNkla)*, 435 (*PbNklb*), 369 (*PbNklc*), 366 (*PbNkld*), and 339 (*PbNkle*) bp nucleic acids, which encode proteins of 141, 144, 122, 121, and 112 amino acids, respctively (Supplementary Material 1). The signal peptides were all located in residues 1–17 with the exception of *PbNkle*, and a SapB domain was identified in all genes. The corresponding molecular weights and pI values were 14.35 kDa/7.79, 14.70 kDa/7.08, 12.00 kDa/6.90, 11.91 kDa/7.74, and 13.17 kDa/9.99 (Table 3).

The genomic DNA sequences of the ORFs of *PbNkla* and *PbNklb* were 1,052 and 984 bp, respectively, and both consisted of five exons and four introns. The genomic DNA sequences of *PbNklc* and *PbNkld* were 1,077 and 867 bp, respectively, and consisted of four exons and three introns. The genomic DNA sequence of *PbNkle* was 522 bp and consisted of three exons and two introns (Fig. 1A). The secondary structure and domain organization of the PbNkls were predicted using the PSIPRED and SMART programs. The characteristic SapB domain was identified in all five PbNkls; however, no signal peptide was detected in *PbNkle* (Fig. 1B). The hypothetical 3D structures of these peptides were established based on the 2rb3.1.A, 2z9a.1.A, 2r1q.1.A, 5u85.1.A, and 3s63.2.A templates. Tertiary structure observations revealed the existence of four helix structures and three disulfide bonds in all PbNkls (Fig. 2).

3.2. Homology and phylogenetic analysis

Six conserved cysteine residues were identified from each PbNkl via multiple alignments with other vertebrate Nk-lysins (Fig. 3). Homology analysis revealed that 1) PbNklc and PbNkld had the highest identity and similarity among the *P. bamaensis* Nk-lysins (82.8%/91.8%), followed by PbNkla and PbNklb (73.2%/78.3%), whereas PbNkle shared the lowest value with other PbNkls (28.6%–32.2%/50.0%–57.4%); 2) When the deduced PbNkls amino acid sequence compared with other cyprinid Nk-lysins, PbNklc and PbNkld were more homologous to *Cyprinus carpio* Nkl2, Nkl3, and Nkl7 (76.9%–84.3%/85.2%–93.5%). PbNkla and PbNklb were more related to *C. carpio* Nkl1 and Nkl5, in which, PbNklb showed the higher values with CcNkl1 (77.5%/85.5%) and CcNkl5 (65.0%/73.9%). Moreover, PbNkle showed the highest homology to *C. carpio* Nkl6 (70.5%/85.7%) (Table 4).

Phylogenetic tree showed that the PbNkls clustered with other fish Nk-lysins but were separated from those of mammals and birds (Fig. 4).

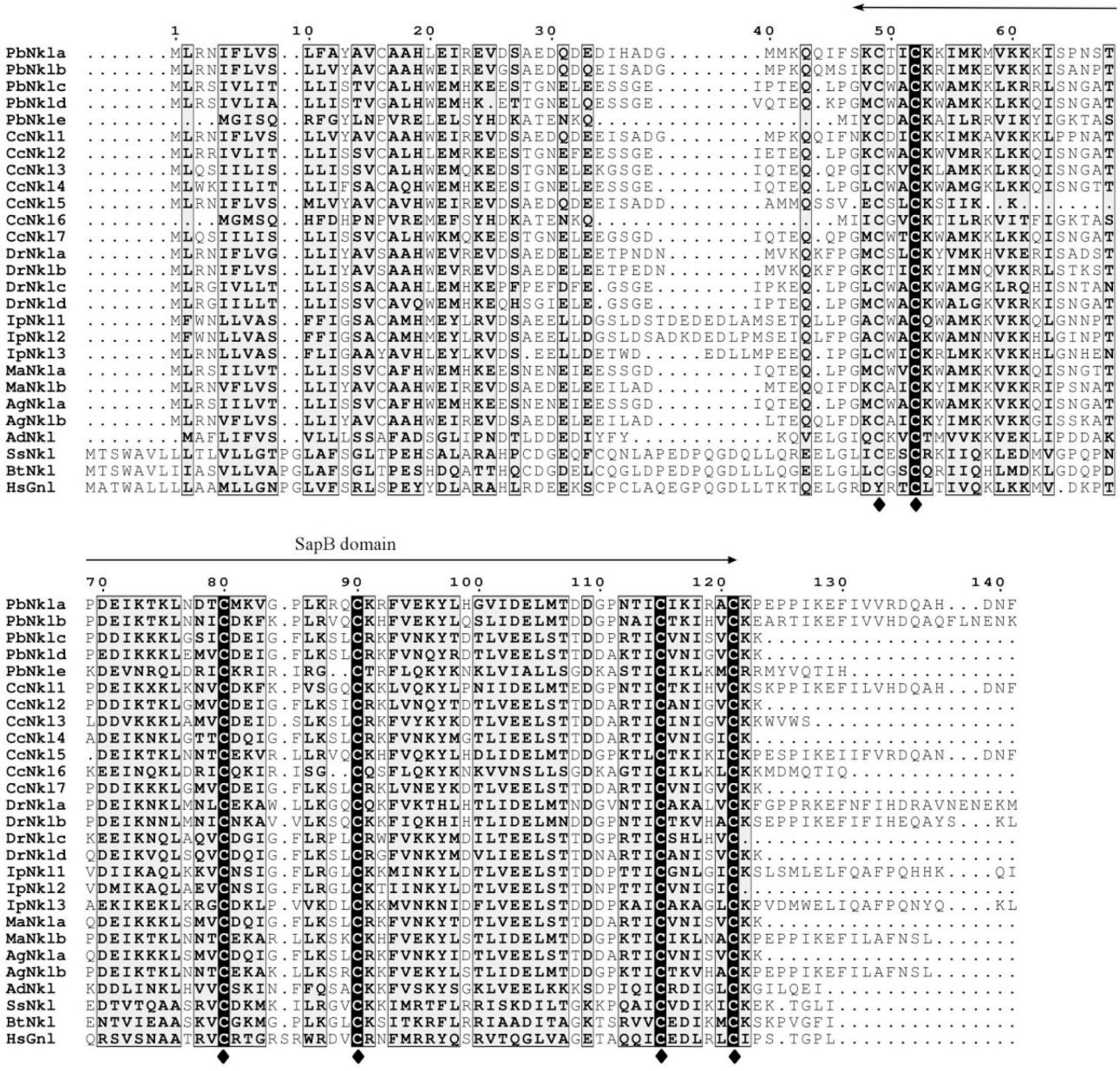

Fig. 3. Nk-lysin sequence alignment. Multiple alignment of Nk-lysin amino acid sequences from *P. bamaensis* and other vertebrates. The putative SapB domain is indicated with double-headed arrow. Six highly conserved cysteine residues are marked with rhombuses. The same amino acid residues are shown in white on a black background, while conserved residues are displayed in black font on a gray background.

PbNklc and PbNkld grouped together with *C. carpio* Nkl2, Nkl3, and Nkl7, PbNkla and PbNklb formed another clade with *C. carpio* Nkl1 and Nkl5. However, PbNkle was separated from the four others PbNkls and formed an independent clade with *C. carpio* Nkl6 (Fig. 4).

3.3. Distribution of PbNkl genes in different tissues

The distribution of *PbNkl* genes in the ten different *P. bamaensis* tissues was assessed using qPCR. The relative expression levels of the five *PbNkls* are shown in Fig. 5. *PbNkla*, *PbNklc*, *PbNkld*, and *PbNkle* were ubiquitously expressed in every examined tissue of healthy fish, although expression levels differed. Expression of *PbNkla* was highest in intestine, whereas the four other genes were expressed lower than *PbNkla*. *PbNklc* showed the highest expression level in skin, whereas expression levels in other tissues were relatively low. Expression levels of *PbNkld* were highest in brain, followed by spleen, skin, and muscle, and were higher than the four other genes. The highest expression of *PbNkle* was found in gill tissue, followed by spleen and head kidney. *PbNklb* was not detected in any tissue from healthy fish.

3.4. Temporal expression profiles of PbNkl genes in immune-related tissues after A. hydrophila infection

To understand the possible biological role of *PbNkls* in response to *A. hydrophila* challenge, the relative expression levels of the five *PbNkls* were measured in four immune tissues (head kidney, liver, spleen, and gill) using qPCR. All five genes were induced by bacterial challenge (Fig. 6). The expression pattern of *PbNkla* in head kidney and liver was similar, showing a rough “M” tendency. Specifically, *PbNkla* mRNA first appeared a significant increase at 3 h post-injection in head kidney and 6 h post-injection in liver. Then its expression level decreased at 12 h, increased again at 24 h and followed by downregulation but sitll kept a high level in both tissues, whereas only a clear upregulation showed at 24 h in gill. In spleen, *PbNkla* expression was significantly downregulated

Table 4
Homology analysis of Nk-lysin genes between *P. bamaensis* and other cyprinids. The identity matrix appeared at the right above column, whereas the similarity matrix was on the left below.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *P. bamaensis* Nkla		73.2	39.3	36.9	28.6	74.5	41.0	39.8	39.3	64.5	35.1	38.9	57.7	62.6	36.9	38.4	43.4	73.7	43.4	71.4
2. *P. bamaensis* Nklb	78.3		43.1	39.8	32.2	77.5	41.8	40.4	40.2	65.0	33.3	40.7	56.9	62.0	41.3	40.5	45.5	66.9	45.5	67.6
3. *P. bamaensis* Nklc	61.5	65.0		82.8	30.1	43.4	82.8	76.9	77.7	37.2	32.3	83.3	44.3	44.3	62.5	73.0	81.1	46.3	82.0	48.0
4. *P. bamaensis* Nkld	59.6	61.8	91.8		29.8	38.5	80.3	77.8	73.6	33.1	33.3	84.3	44.3	40.2	58.8	68.0	75.4	43.0	77.0	44.6
5. *P. bamaensis* Nkle	50.0	55.2	53.8	57.4		33.3	30.7	33.7	31.2	28.9	70.5	30.9	27.2	30.4	28.4	34.7	32.0	35.4	34.7	31.7
6. *C. carpio* Nkl1	83.0	85.5	63.9	58.2	59.3		42.6	40.7	38.5	61.7	35.1	42.6	58.4	63.3	38.0	39.2	45.1	67.6	45.9	67.6
7. *C. carpio* Nkl2	63.1	63.9	89.3	87.7	54.7	63.9		76.0	76.0	34.7	31.5	84.3	44.3	41.0	60.8	68.9	77.9	44.6	78.7	47.1
8. *C. carpio* Nkl3	58.3	61.5	85.2	88.9	53.7	59.3	83.3		71.9	35.5	36.2	84.3	42.6	41.0	55.8	66.4	76.2	45.5	76.2	46.3
9. *C. carpio* Nkl4	59.8	59.8	86.8	84.3	49.5	58.2	83.5	80.2		37.2	29.2	77.6	43.4	42.6	62.5	82.7	80.2	44.6	80.2	47.1
10. *C. carpio* Nkl5	73.8	73.9	57.9	55.4	54.4	71.6	57.9	58.7	55.4		33.8	33.6	52.5	54.0	33.3	34.7	39.7	65.2	39.7	64.4
11. *C. carpio* Nkl6	59.5	59.0	53.8	58.1	85.7	59.5	51.1	57.4	48.1	60.8		33.3	28.3	30.4	29.9	35.5	36.8	40.5	36.8	35.4
12. *C. carpio* Nkl7	59.3	60.2	90.7	93.5	53.2	60.2	90.7	91.7	90.7	53.3	55.9		44.3	41.0	55.8	68.9	82.0	44.6	82.8	46.3
13. *D. rerio* Nkla	72.3	72.2	64.8	64.8	56.5	73.0	63.1	64.8	62.3	65.2	55.4	64.8		73.7	37.2	41.0	48.4	64.6	48.4	64.6
14. *D. rerio* Nklb	75.5	75.2	66.4	64.8	52.2	77.0	64.0	64.8	63.1	68.6	53.3	64.8	83.9		36.4	38.5	45.1	68.2	45.9	71.2
15. *D. rerio* Nklc	54.9	60.3	74.2	69.7	48.4	59.5	71.7	68.3	71.7	53.3	48.5	70.0	57.9	60.3		68.1	60.8	37.5	62.5	39.2
16. *D. rerio* Nkld	55.2	58.7	86.9	82.8	56.0	56.0	82.0	80.3	81.0	53.7	56.6	83.6	58.2	59.8	80.7		76.2	41.3	77.9	43.0
17. *M. amblycephala* Nkla	63.1	65.0	91.8	85.2	57.3	62.3	86.1	87.7	86.0	58.7	59.2	89.3	65.6	65.6	73.3	85.2		47.9	97.5	50.4
18. *M. amblycephala* Nklb	82.0	80.1	69.4	65.3	57.3	80.9	67.0	66.9	65.3	75.0	63.5	67.0	76.9	80.3	60.0	62.8	69.4		48.8	91.9
19. *A. grahami* Nkla	63.1	65.0	92.6	86.9	60.0	63.1	86.9	87.7	86.8	58.7	59.2	90.2	65.6	65.6	75.8	87.7	97.5	70.2		51.2
20. *A. grahami* Nklb	81.2	79.4	68.6	65.3	57.3	80.1	67.8	66.9	65.3	75.0	60.0	67.0	76.9	82.6	61.7	62.0	68.6	96.3	69.4	

from 6–24 h post-injection. *PbNklb* mRNA was detected only after 48 h post *A. hydrophila* infection. *PbNklb* showed a significant increase in head kidney and liver at 72 h post-injection. In spleen and gill, its expression was significantly upregulated at 48 h post-injection and then decreased at 72 h. *PbNklc* gene expression was significantly upregulated at 6 h post-injection in head kidney. However, in spleen and gill, its expression was significantly downregulated from 6–72 h post-injection. *PbNkld* had several significantly descent trends from 3–72 h post-injection in spleen and gill, whereas only a few downregulations occurred in head kidney (24 h post-injection) and liver (24 and 72 h post-injection). *PbNkle* gene expression was significantly upregulated at 3 h post-injection in liver and 6 h post-injection in head kidney, whereas the expression was significantly downregulated at 3, 6, 24, 48, and 72 h post-injection in spleen.

4. Discussion

The present work represents the first characterization and expression study of the Nk-lysin genes of *P. bamaensis*. To date, only a few taxonomic studies [1,2] and mitochondrial genome information [20] were reported for this species. Furthermore, characterization of *P. bamaensis* AMPs and their immune responses at the molecular level is limited. In our study, five Nk-lysin genes were identified, and their sequences, structures, and expression profiles in healthy fish and in response to bacterial infection were analyzed. Although the biological function of Nk-lysins has been well-documented in higher vertebrates and other teleost species, studies on their role in immune responses in teleosts remain insufficient, especially in wild fish.

Similar to those found in higher vertebrate, the main characteristics of Nk-lysin genes in lower vertebrates are six conserved cysteine residues and a SapB domain. The six cysteine residues form three intrachain disulfide linkages, which are essential for the antimicrobial activity of Nk-lysin [11]. The SapB domain has been shown to participate in the degradation of sphingolipid, which is closely related to the cytolytic actions of Nk-lysin [21]. The conserved sequence domain suggests a similar biological function for PbNkls and other Nk-lysins. Similar to the five *PbNkls* analyzed in this study, Nk-lysin genes in teleosts can be divided into three groups according to their gene structure [22]. In the present study, genes with the same structure showed high sequence homology, and were organized together in the same clade in phylogenetic analysis. Prior to this study, the only known Nk-lysin gene with three exons and two introns was the common carp *Nkl6* [22], which we showed was closely phylogenetically related to *PbNkle*.

In healthy *P. bamaensis*, most *PbNkl* genes are constitutively expressed in various tissues, with the exception of *PbNklb*. *PbNkla* was mainly detected in intestine, gill, and spleen, whereas *PbNkle* was expressed in gill, spleen, and head kidney. This is similar to observations in most other teleosts, such as zebrafish, Japanese flounder, channel catfish, common carp, and large yellow croaker, whose Nk-lysins are primarily expressed in gill, head kidney, spleen, and intestine [22–26]. In the current study, *PbNklc* showed the highest expression levels in skin, whereas *PbNkld* expression was highest in brain, followed by spleen, skin, and muscle. However, Nk-lysins are not usually detected in the brain, skin, stomach, or muscle of some fishes [23,24]. In our study, the tissues in which *PbNkl* expression levels were highest were consistent with the major lymphoid tissues of teleost fishes, including the kidney, thymus, spleen, and mucosa-associated lymphoid tissues such as the skin and gill [27]. These results are similar to those in porcine, equine, and bovine species, where relatively high levels of Nk-lysin transcripts have been reported in lymphoid tissues and cells [28–31]. The diverse expression patterns observed in the different tissues of *P. bamaensis* suggested different functions for the various *PbNkl* genes.

To better understand the expression profiles of *PbNkls* after *A. hydrophila* infection, three typical lymphoid tissues (head kidney, spleen, and gill) together with liver, an important immune organ in fish [25,32], were selected to evaluate relative expression levels from 0–72 h. The five *PbNkl* genes were induced during bacterial infection and responded differently to pathogen invasion. In particular, the expression of *PbNkla* in head kidney and liver was significantly upregulated at earlier time points (3 and 6 h post-injection) than that in gill (24 h). A similar situation was found in both *PbNklc* and *PbNkle* gene, in which, the significant increase of *PbNklc* appeared at 6 h post-injection in head kidney, as well as *PbNkle* appeared at 3 and 6 h post-injection in liver and head kidney, respectively. The early rapid rise of three *PbNkls* observed in the present study, which is very similar with the case in half-smooth tongue sole and blunt snout bream [12,33], indicating their immediate reaction during bacterial infection. Interestingly, *PbNklb* exhibited a unique expression pattern that its expression was not detected in healthy fish, but was initiated only after 48 h post-injection. Few previous studies have yielded similar results. For example, two epidermal antimicrobial peptides (cathelicidin and mBD-2) were not expression in normal tissues, but expression was significantly increased in the skin of atopic dermatitis-model mice [34]. Thus, it is possible that *PbNklb*, in contrast to other constitutively expressed genes, may only be expressed after bacterial invasion. Once bacteria invade, PbNklb may be synthesized and released to participate in the host immune response against *A. hydrophila*, which implied a vital

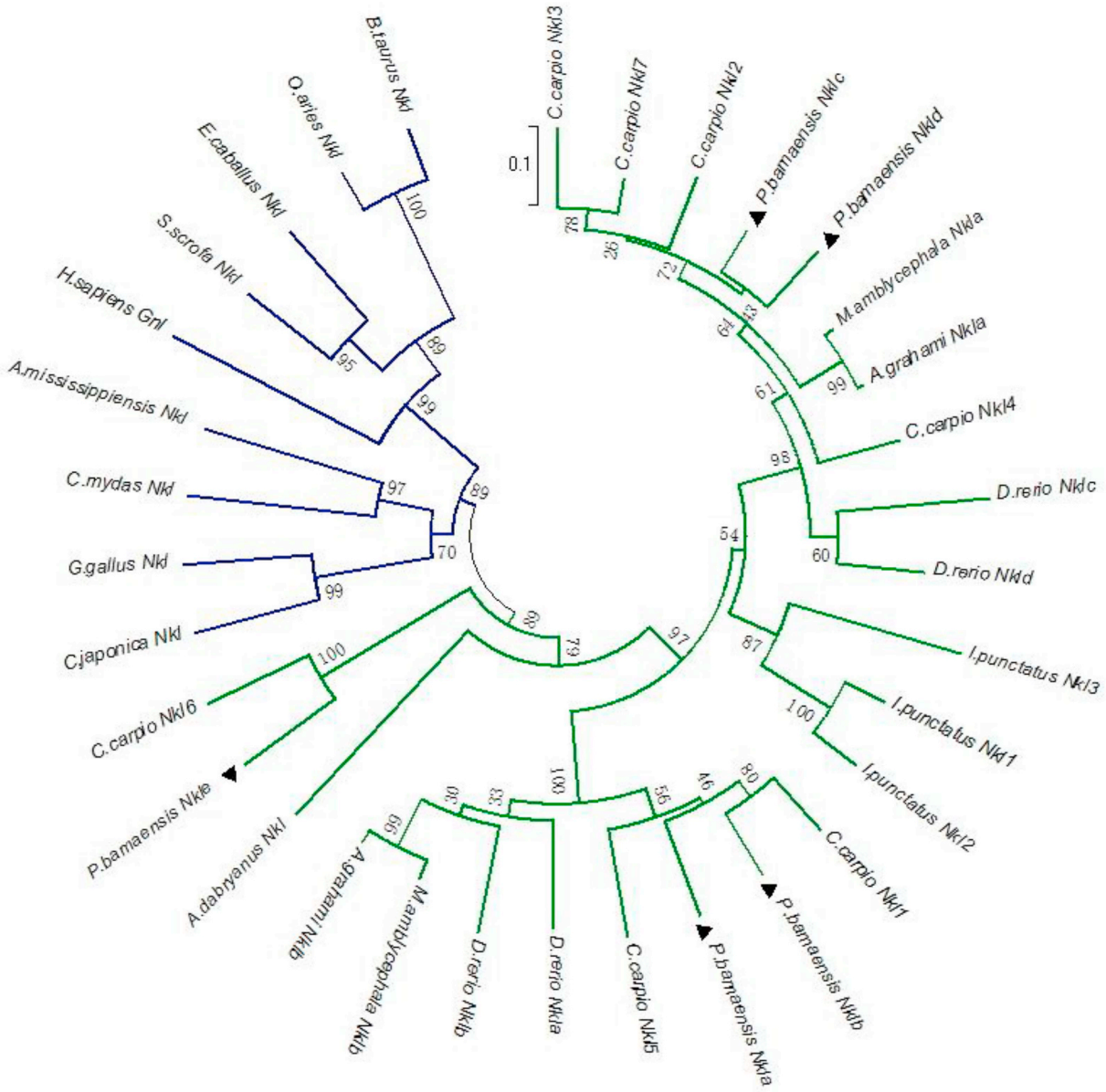

Fig. 4. Nk-lysins phylogenetic analysis. Phylogenetic tree of Nk-lysin amino acid sequences constructed with neighbor-joining method using MEGA 6.06. Node values represent the bootstrap confidence from 10,000 replicates. The reptile, avian, and mammalian Nk-lysins are marked with blue clades. Fish Nk-lysins are marked with green clades. Triangles indicate five PbNkls reported in this study. Accession numbers of Nk-lysins in the other species are listed in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

immune function for PbNklb during this period. Otherwise, a downward trend in *PbNkld* expression was observed in all four immune organs, showing a distinct expression profile and probably due to the altered transcription rate or immune cell migration [35]. Taken together, these results suggested that *PbNkls* may be involved in the host immune response and play different roles during bacterial infection. To fully understand the functions of PbNkls in the defense against pathogens, further study of the antimicrobial activity of recombinant or synthetic PbNkl peptides is needed.

In conclusion, our work represents the first report of five novel Nk-lysin genes from wild *P. bamaensis* fish. The results of our study demonstrated that PbNkls were saposin-like proteins that retained conserved features of Nk-lysin genes, including six conserved cysteine residues and a SapB domain. Phylogenetic analysis revealed that *PbNkla* and *PbNklb*, *PbNklc* and *PbNkld,* and *PbNkle* separated into three clades that were consistent with gene structure analysis and homology alignment results. *PbNkla*, *PbNklc*, *PbNkld*, and *PbNkle* were ubiquitously expressed in all examined tissues, whereas *PbNklb* was expressed only after bacterial infection. The up- and down-regulation of these *PbNkls* after *A. hydrophila* challenged at different time points post-injection and in different immune tissues suggested they were involved in immune defense against bacterial pathogens.

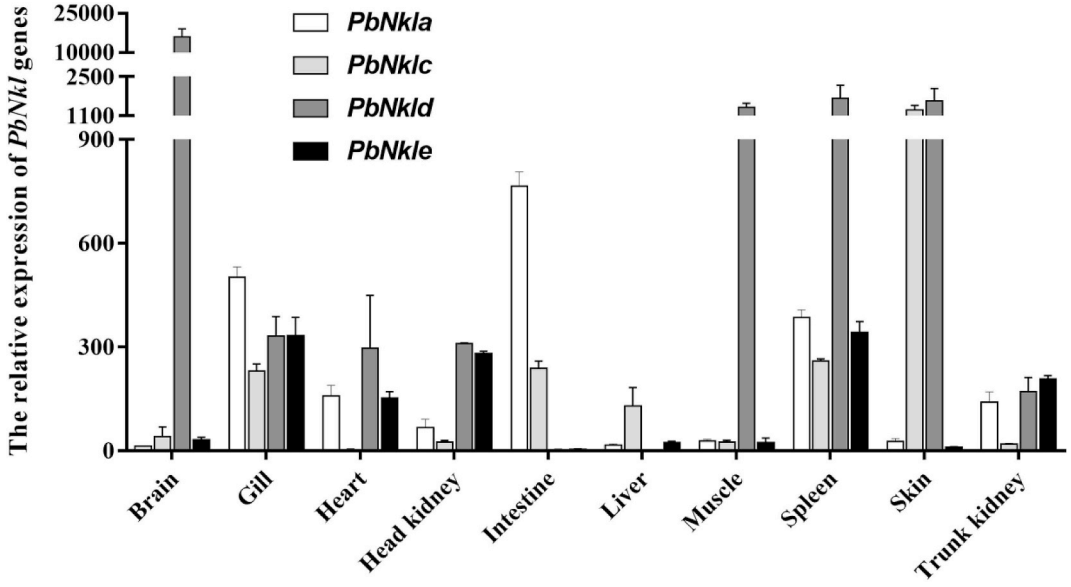

Fig. 5. *PbNkls* tissue distribution patterns. The relative expression levels of *PbNkla, PbNklc*, *PbNkld*, and *PbNkle* in ten tissues of healthy *P. bamaensis*. Statistical analysis was done by ANOVA with the Turkey's test using SPSS 20.0 software. The mean expression level is expressed as mean ± SEM (n = 5).

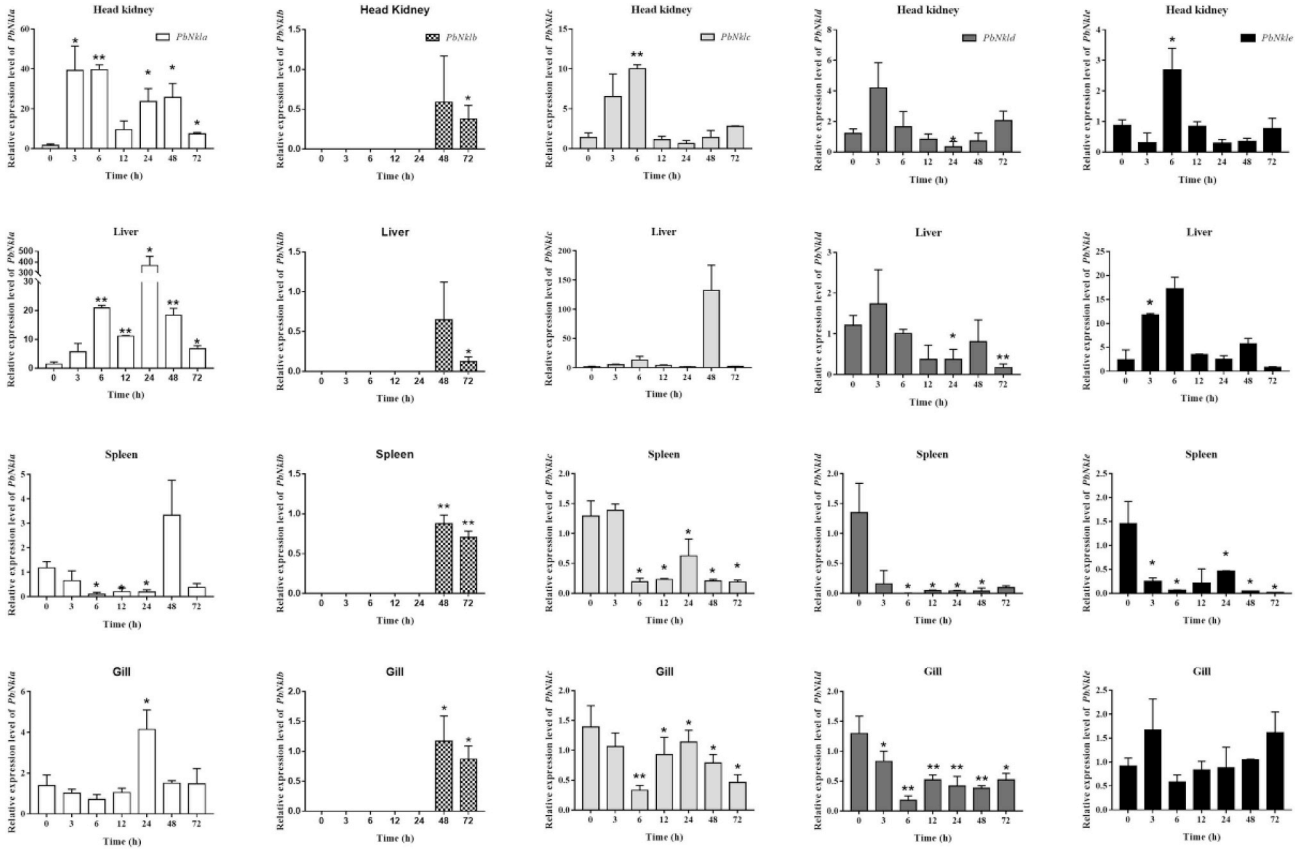

Fig. 6. *PbNkls* expression profile after *A. hydrophila* infection. The relative expression levels of *PbNkl* genes in head kidney, liver, spleen and gill after stimulation with *A. hydrophila* at different time points. Data are present as mean ± SEM (n = 5), with * indicating $p < 0.05$, ** indicating $p < 0.01$.

Acknowledgement

This work has been funded by the Natural Science Foundation of Guangxi (2017GXNSFAA198044), the Innovation Driven Development Foundation of Guangxi (AA17204088-3), the project to enhance the basic ability of young teachers of Guangxi (KY2016YB021), and the Doctoral Research Startup Fund of the Fourth Institute of Oceanography, Ministry of Natural Resources (No.201806).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.014.

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