Fish and Shellfish Immunology 84 (2019) 962–969

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Short communication

Cloning, expression prolife, and immune characterization of a novel *stat* family member (*stat5bl*) in Chinese tongue sole (*Cynoglossus semilaevis*)

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ARTICLE INFO

Keywords:
stat5b-like
Chinese tongue sole
Expression pattern
Immunity
Vibrio harveyi

ABSTRACT

STAT plays important roles in innate immunity during JAK/STAT signaling pathway, and STAT5 is particularly focused due to the existence of duplicated forms in fish and mammal. In Chinese tongue sole, *stat5bl* was suggested to be a candidate related to *Vibrio harveyi* resistance based on previous QTL screening. In this study, the full length of *stat5bl* cDNA was cloned and its expression patterns were analyzed. *stat5bl* was predominantly expressed in immune tissues, where the highest level was observed in liver, followed by skin and gill. Time course expression patterns were examined in six tissues (liver, skin, gill, kidney, intestine, spleen) after *V. harveyi* infection. *stat5bl* could be up-regulated by *V. harveyi* infection in all tissues except liver, despite the timepoints of peak were different. In contrast, *stat5bl* was significantly downregulated in liver. To elucidate the role of *stat5bl* in liver, *in vitro* RNAi were performed using primary liver cell culture. Knockdown of *stat5bl* could regulate the expression of genes closely related to JAK/STAT pathway. This study would enlarge our understanding of *stat5bl* in fish immunity.

1. Introduction

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway plays important roles in various biological processes, including innate immunity, cellular proliferation, differentiation, and apoptosis [1]. JAK/STAT pathway constitutes of three components: cellular pathogen recognition receptor (PRR), JAK kinase and STAT. In the immune response, JAK/STAT is initiated via pathogen binding on the cellular pathogen recognition receptor (PRR), which could activate JAK kinase and in turn phosphorylates the STAT. Phosphorylated STATs then dimerize and enter the nucleus to regulate a series of immune-related gene transcription [2,3]. To achieve the regulatory role, STAT need to interact with other transcription factors or coactivators, such as IRFs, Sp1, NF-κB, PCAF, GCN5 [4]. Until now, seven encoding gene members have been identified in mammals, e.g. *stat1*, *stat2*, *stat3*, *stat4*, *stat5* (*stat5a* and *stat5b*), and *stat6*. The encoded STAT proteins share a common structure, which consists of an N-terminal protein interaction domain, followed by alpha domain (coiled-coil), DNA-binding domain, and Src homology 2 (SH2) domain [5].

Amongst these *stat* members, *stat5* has duplicated forms, *stat5a* and *stat5b*, which exhibited high similarity at protein level. *stat5a* were firstly identified in lactating ovine mammary tissue in 1994, and the next year *stat5a* and its homologue *stat5b* were identified in mouse [6,7]. However, the duplicated forms of *stat5* only existed in mammal and fish, but not in birds and amphibians [8]. *stat5* members are involved in diverse biological processes, such as mammary gland development, body growth, and immune regulation [9,10]. Due to their versatile roles, they were extensively studied in mammals and especially in mouse great effort was made to generate knockout lines since 1997. Regarding the immune response, loss of *stat5* in mouse could result in developmental failure of T cells, B cells, and natural killer (NK) cells [11,12]. In human, patients with mutated *stat5* also showed immunological dysfunction, including decreased number of Tregs, NK cells, and T cells [13–16].

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https://doi.org/10.1016/j.fsi.2018.10.030
Received 29 July 2018; Received in revised form 29 September 2018; Accepted 10 October 2018
Available online 03 November 2018

In contrast to mammals, studies of *stat5* in fish mainly focused on the gene identification and evolutionary analysis. In pufferfish, only a single *stat5* was identified, showing 78% similarity to mouse *stat5b*. However, this *Tfstat5* could not induce the transcription of *β-casein* promoter via rat prolactin and Nb2 prolactin receptor [17]. Another research has identified two distinct *stat5* (*stat5.1* and *stat5.2*) homologues in zebrafish, where *stat5.1* was similar to mammalian *Stat5b*, but *stat5.*2 had no apparent homologue [18]. Recently, a systematic study in channels fish revealed eight *stat* members, e.g. *stat1a/1b*, *stat2*, *stat3*, *stat4*, *stat5a/5b*, *stat6* [19]. In turbot (*Scophthalmus maximus*), two stat family members (*stat2* and *stat3*) were cloned and significantly up-regulated in immune-related tissues (liver, spleen, kidney) after *Vibrio anguillarum* and lymphocystis disease virus challenge, suggesting that turbot Stat2 and Stat3 are involved in the immune defense process [20,21]. However, the role of *stat5* in fish is rarely investigated.

As an important economic marine fish, Chinese tongue sole (*Cynoglossus semilaevis*) is widely cultivated in Northeast Asia. However, outbreak of bacterial diseases in recent years caused severe economic loss and greatly limited the tongue sole industry. Based on the whole genome sequence and QTL screening data [22,23], *stat5bl* was identified as candidate gene that might function in the immune response to *Vibrio harveyi*. In this study, the full length of *stat5bl* cDNA was cloned and subjected to evolutionary analysis. *stat5bl* was highly expressed in several immune-related tissues, liver, skin and gill, whereas the expression patterns in these tissues were different after *V. harveyi* infection. Unlike the up-regulation after bacterial infection in most immune-related tissue, *stat5bl* was significantly down-regulated in liver. To further investigate the role of *stat5bl* in liver, primary liver cell culture was conducted and *in vitro* RNAi were performed. Knockdown of *stat5bl* exhibited similar effect on those genes. This study would enlarge our understanding of *stat5bl* in fish immunity.

2. Materials and methods

2.1. Experimental animals

Clinically healthy Chinese tongue sole (average weight, 31.0 ± 6.5 g) were purchased from fish farm (Haiyang Huanghai Aquatic Ltd.) in Shandong Province, China, and cultured in circulating seawater at room temperature. The various tissues were collected from six untreated and anaesthetized fish, and immediately transferred to liquid nitrogen and stored at − 80 °C for RNA extraction. The sixty individuals were selected and cultured for *V. harveyi* infection as reported previously [24], and the final bacterium concentration of 1.0×10^4 colony-forming units (cfu) was used for intraperitoneal injection. The sampling time-points of *V. harveyi* treated group were set at 0, 12, 24, 48, and 72 h after injection, and the six tissues (liver, spleen, intestine, kidney, gill and skin) were collected at each time point after bacterial challenge for the total RNA extraction. Moreover, the fresh liver was collected from healthy tongue sole disinfected with 70% ethanol (weight 450 g), then washed three times with phosphate-buffered saline (PBS, pH 7.4) for primary culture. The handling of experimental animals in this study was approved by the Yellow Sea Fisheries Research Institute's animal care and use committee.

2.2. RACE PCR and sequence analysis

The total RNA extraction and cDNA synthesis were performed as described previously [24]. The 5′-RACE and 3′-RACE PCR were conducted to obtained the full-length cDNA sequence of *stat5bl* using a Smart RACE cDNA amplification kit (Clontech Inc., CA, USA) according to the manufacturer's instructions. The specific primers for RACE PCR amplification were listed in Table 1, and the conditions of RACE PCR amplification were performed as previously described [25].

The full-length cDNA sequence assembly and amino acid sequence prediction were conducted using DNAStar 7.0 software (DNAStar, Madison, WI, USA). The multiple sequence alignments and phylogenetic analysis were performed using MEGA v6.0 as previously reported [24]. The functional domains prediction and the genomic structure analysis were conducted using online software SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) and Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form), respectively.

2.3. Real-time quantitative PCR

The expression levels of *stat5bl* were determined using Quantitative real-time PCR (qRT-PCR) in a 7500Fast real time PCR system (Applied Biosystems, USA). The system and procedure of qRT-PCR amplification were described as before [25]. *β-actin* was used as endogenous control, and $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression level of *stat5bl*. Furthermore, the differences of expressional level were analyzed using one-way ANOVA with the SPSS 18.0 software as reported previously [25].

2.4. Primary culture and subculture of tongue sole liver cells

The fresh liver tissue was removed aseptically and minced into small pieces ($< 1\ mm^3$) by surgical scissors in complete Leibovitz's L-15 medium containing 20% foetal bovine serum (FBS), 400 IU/ml penicillin, 400 μg/ml streptomycin, and 5 ng/ml basic fibroblast growth factor (bFGF). After the medium was drained, small tissue pieces was transferred into 25 cm^2 tissue culture flasks and evenly coated on the bottom of culture flasks. It was cultured at 24 °C for 6 h with inverted placement, then added 3 ml of L-15 complete medium and cultured at 24 °C for 3–5 days with positive placement. Once a large number of cells migrating from the tissue pieces were observed with the microscope, the tissue pieces should be washed off with L-15 complete medium. After washing, 3 ml of fresh complete medium was added in culture flasks and cultured at 24 °C. When the primary cell cultures grew to 95% confluence, cells were subcultured at a ratio of 1:2 according to the previously described trypsinization method [26]. Confluent cells were trypsinized with trypsin–EDTA solution (0.25% Trypsin and 0.2% EDTA), and half of them were maintained in L-15 complete medium. One half of the medium was changed every 4 days for about 3 weeks.

2.5. In vitro RNAi silencing of stat5bl and expression analysis of Csglys, Csbcl2, Cssocs2 and Cscsf3r

The *stat5bl*-specific small interfering RNAs (siRNAs), si-*stat5bl* 001, si-*stat5bl* 002, si-*stat5bl* 003, and a nonspecific siRNA negative control (NC) were designed and synthesized by RayBiotech (Guangzhou, China). After the liver cells formed a confluent monolayer, they were transferred to six-well plates and cultured at 24 °C for 24 h to ensure complete attachment to the plates. The siRNA was transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The treated groups were transfected with siRNA (si-*stat5bl* 001, si-*stat5bl* 002, and si-*stat5bl* 003) at a final concentration of 20 nM, and the control groups were transfected with NC siRNA. The cells from the treated and control groups were cultured at 24 °C for 48 h, and the medium was discarded. The 300 μl TRIzol was added in each well of six-well plates and mixed thoroughly with cells, and then sucked into 1.5 ml RNase-free EP tubes for total RNA extraction. The primers of qRT-PCR for *stat5bl*, *Csglys*, *Csbcl2*, *Cssocs2*, and *Cscsf3r* expression were listed in Table 1. Three replicates were conducted for each group.

3. Results

3.1. Cloning of stat5bl cDNA and characterization analysis

The full length cDNA of the *stat5bl* was cloned using RACE PCR. As

Table 1
Primers and their sequences in this study.

Primer	Sequence (5′–3′)	Purpose	Product size/bp
stat5bl-F	ATGTCATAGACCTGGAGAACC	Partial fragment cloning	2135
stat5bl-R	TCCGTCTGTGTCCAACATC	Partial fragment cloning	2135
stat5bl-GSP5′	ATCATCCAGTATGATGGTCTGCTGCTTCC	5′RACE outer primer	
stat5bl-NGSP5′	TGATATTTCTGAGACATGCTG	5′RACE inner primer	
stat5bl-GSP3′	AACAAGCAGCAGGCTCAGGATATGTTGATG	3′RACE outer primer	
stat5bl-NGSP3′	CCAACGGCACCTTTCTCCTG	3′RACE inner primer	
Upm-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE	
Upm-short	CTAATACGACTCACTATAGGGC	RACE	
NUP	AAGCAGTGGTAACAACGCAGAGT	RACE	
stat5bl-RT-F	ACCGCTGTCCTCTGGAGTTG	qRT-PCR	116
stat5bl-RT-R	CATGCTGTCCATCATTCCACC	qRT-PCR	116
CsGLys-F	TCTTATTGCTGCCATCATCTCC	qRT-PCR	
CsGLys-R	GTCTTTTTGGGCTCCAGTCTCC	qRT-PCR	
CSBCL2-F	CAAGTGGAGCAGGTCGTGG	qRT-PCR	172
CSBCL2-R	GTGCAGCCAGACCAAAGACG	qRT-PCR	172
CSSOCS2-F	GAGCTGTAGAATCTGATGAGAGCC	qRT-PCR	147
CSSOCS2-R	AGCTGTCTCGTACCAGGAAG	qRT-PCR	147
CSCSF3R-F	CAAATATCCAGACCAACGACTCC	qRT-PCR	202
CSCSF3R-R	CTGTGGTTGAAGCTGGGTATG	qRT-PCR	202
actinF	GCTGTGCTGTCCCTGTA	Internal reference	185
actinR	GAGTAGCCACGCTCTGTC	Internal reference	185

shown in Fig. 1B, the *stat5bl* cDNA was 2799 bp containing 2361 bp open reading frame (ORF), 205 bp 5′ UTR and 233 bp 3′ UTR. At the end of the sequence, there was a 20 bp polyA tail and a typical polyA acidification signal (AATAAA) was localized 106 bp upstream of the polyA tail. To further illustrate the genomic structures of *stat5bl*, the ORF sequence was mapped on the genomic sequence (Gene ID: 103393172). The *stat5bl* gene constituted of 18 exons (1–18) that were separated by 17 introns (A-Q) as shown in Fig. 1A. The ORF region encoded a 785 amino acid (AA) protein with molecular weight 89.9 kDa and the isoelectric point 6.4. As indicated in Fig. 2, Stat5bl contained four conserved domains: protein interaction domain, alpha domain, DNA binding domain, and Src Homology 2 domain.

To analyze the evolutionary relationship of Stat5bl among species, phylogenetic tree was conducted by using the protein sequences of 21 different species. As shown in Fig. 3, mammalian Stat5bl grouped into one cluster, while Stat5bl from teleost accumulated into another cluster, where the tongue sole Stat5bl showed a close evolutionary relationship to that of Japanese flounder (*Paralichthys olivaceus*). It was worth noting that although Stat5bl proteins were annotated in teleost but not in mammal, they were not clearly separated from Stat5b according to the phylogenetic analysis.

3.2. Expression patterns of stat5bl in different tissues

To evaluate *stat5bl* transcription under normal cultivating condition, qRT-PCR was performed in 11 tissues (gonad, liver, brain, gill, skin, muscle, intestine, spleen, kidney, heart, and stomach). As shown in Fig. 4, the highest transcription was observed in liver, followed by skin and gill, while in other tissues only low transcription levels were detected.

3.3. Expression patterns of stat5bl in different tissues after V. harveyi infection

Regarding the relative high expression of *stat5bl* in immune related tissues (liver, skin, gill), we speculated its possible participation in the immune response. Thus, the expression patterns of *stat5bl* were examined in 6 tissues (gill, intestine, skin, spleen, liver and kidney) after *V. harveyi* infection. Except no significant change of expression level in spleen, the *V. harveyi* challenge could either up-regulate or down-

Table 2
List of species used in this study.

No.	Species name	Accession ID	Source
1	*Cynoglossus semilaevis*	XM_008330036; XP_008328258	Obtained in this study
2	*Maylandia zebra*	XM_014408089; XP_014263575	GenBank
3	*Pundamilia nyererei*	XM_005726080; XP_005726137	
4	*Oreochromis niloticus*	XM_005468847	
5	*Acanthochromis polyacanthus*	XM_022206218	
6	*Stegastes partitus*	XM_008296405	
7	*Nothobranchius furzeri*	XM_015948650	
8	*Kryptolebias marmoratus*	XM_017429488	
9	*Austrofundulus limnaeus*	XM_014023159	
10	*Paralichthys olivaceus*	XM_020094512; XP_019950071	
11	*Hippocampus comes*	XM_019866457	
12	*Salmo salar*	XM_014192796; XP_014048271	
13	*Oncorhynchus mykiss*	XM_021624838	
14	*Oncorhynchus kisutch*	XM_020486504	
15	*Mus musculus*	XM_017314403; XP_017169892	
16	*Homo sapiens*	XM_017024977; NP_036580	
17	*Canis lupus familiaris*	XM_548092	
18	*Sus scrofa*	XM_021066239	
19	*Ovis aries*	XM_012186114	
20	*Bos taurus*	XM_005220673; XP_005220730	

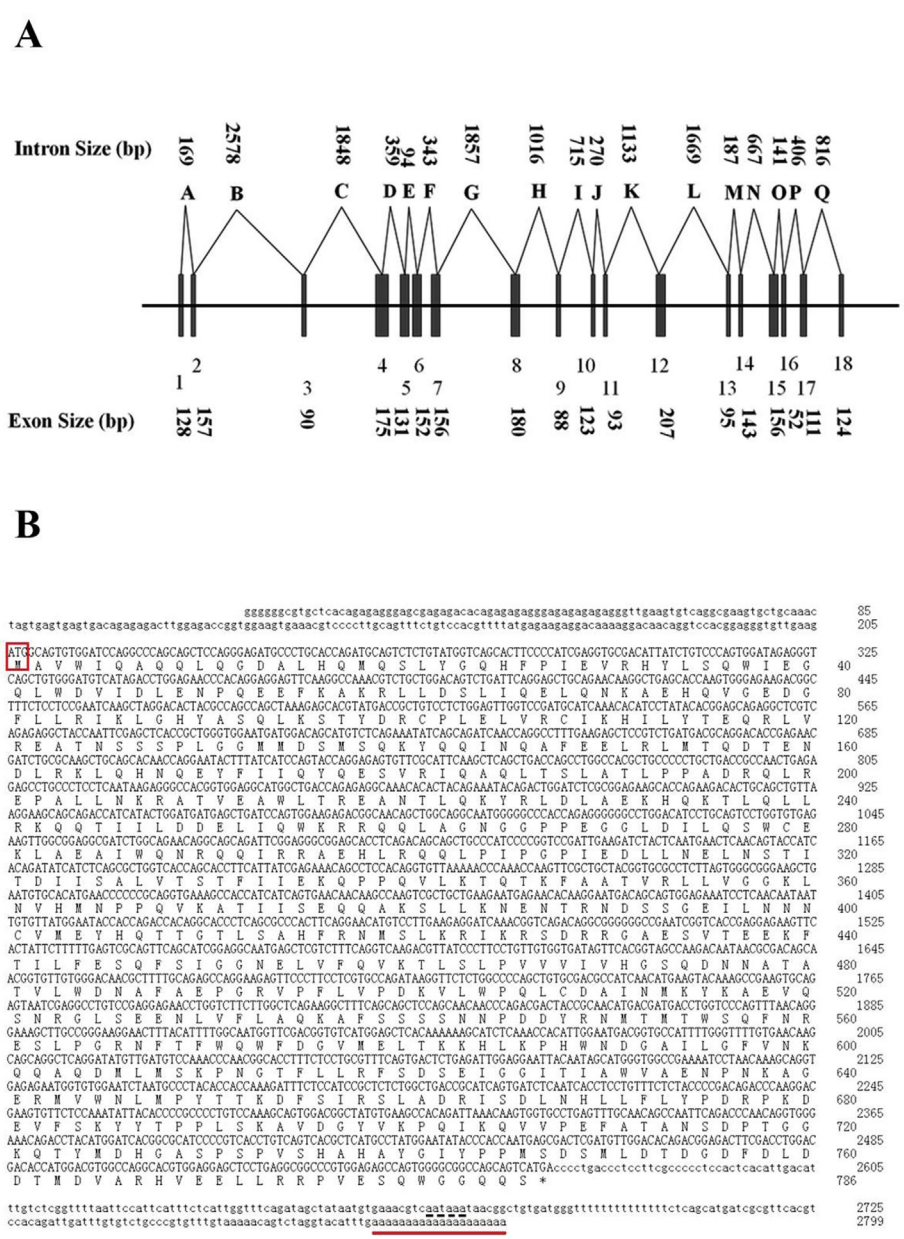

Fig. 1. The schematic representation of genomic structure and the full-length cDNA sequence of *stat5bl*. **(A)** Schematic representation of the *C. semilaevis stat5bl* genomic structure. The capital letters (A–Q) above the line represent 17 introns, and the solid bodies (1–18) represent 18 exons. **(B)** The full-length cDNA and deduced amino acid sequence of *stat5bl*. The start codon was boxed by a red frame, and the stop codon was labeled with asterisk. PolyA acidification signal was indicated by black dash line. PolyA tail was indicated by a red solid line. The noncoding region was in lowercase, and ORF region was in uppercase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

regulate the *stat5bl* transcription in skin, intestine, gill, kidney, and liver, although the time points were different (Fig. 5). The *stat5bl* transcription was significantly elevated at 12 h in skin but obviously induced at 24 and 48 h in intestine. In gill and kidney, the up-regulations were observed at 48 h. However, bacterial challenge could induce obvious down-regulation in liver at 12 h and not recovered until 72 h.

3.4. *In vitro* RNAi of stat5bl in liver cells and expression analysis of immune-related genes

To elucidate the knockdown effect of *stat5bl* in liver, *in vitro* RNAi of *stat5bl* were performed in liver cells. At the fifth day of culture, when liver cells were observed to migrate and stay surrounding the tissue, the subculture was conducted (Figs. S1A and B). The cells exhibited good vitality at the passage 3rd, and they were used for RNAi experiments. Three siRNA (si-*stat5bl* 001, si-*stat5bl* 002, and si-*stat5bl* 003) were transfected into cell and si-*stat5bl* 001 was selected for further knockdown experiment due to its most obvious effect (55%) (Fig. S1C).

After RNAi, the expression profiles of four immune-related genes were evaluated, including goose-type lysozyme gene (*Csglys*), B-cell lymphoma-2 gene (*Csbcl2*, also known as apoptosis suppressor gene), suppressor of cytokine signaling 2 gene (*Cssocs2*) and colony-stimulating factor 3 receptor gene (*Cscsf3r*). Compared to the negative control group, the expression of *Cssocs2* and *Cscsf3r* were significantly up-regulated to approximate 2-fold and 10-fold after RNAi (shown in Fig. 6), respectively. The expression levels of *Csglys* were significantly down-regulated (12%). For *Csbcl2*, it showed strong declined tendency (decreased to 76%), despite the difference was not significant.

4. Discussion

There are 18 exons found in *stat5bl*, indicating it belongs to the gene categories with rather complex genomic structure. Four classic STAT domains were predicted in the deduced protein sequence as the mammalian STAT, interaction domain, alpha domain, DNA binding domain, and Src homology 2 domain. However, sequence alignment showed that there were still differences among teleost and mammal, suggesting that *stat5bl* might exhibit divergent functions in teleost.

Based on the sequence similarity to mammalian *stat5b*, *stat5b* or *stat5bl* (*stat5b-like*) was annotated [27]. Nevertheless, phylogenetic

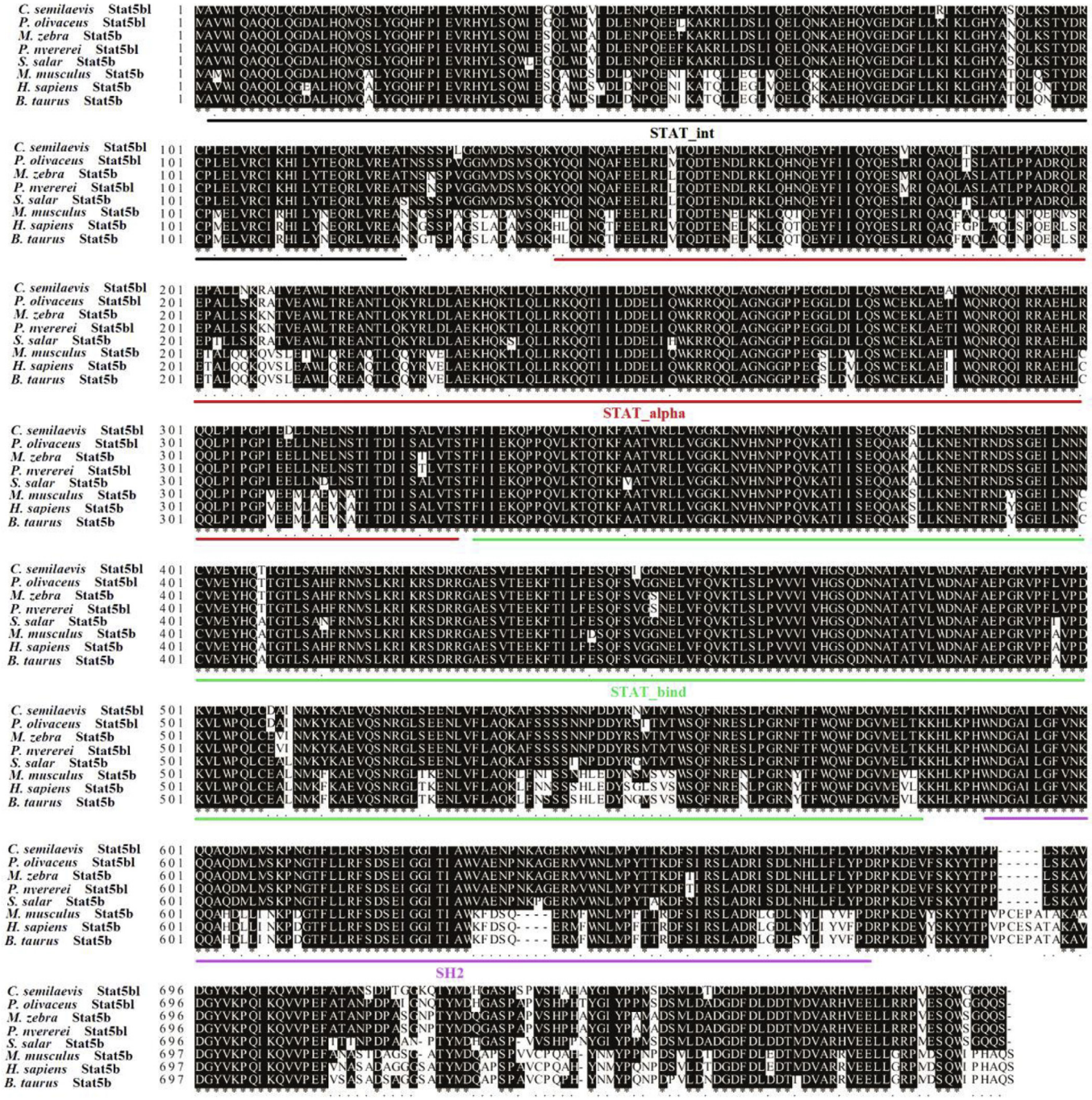

Fig. 2. Multiple sequence alignment of Stat5b and Stat5bl protein sequences from *C. semilaevis* and other vertebrates (Teleosts and mammals). Sequences were aligned using MEGA (Version 6.0) and the identical or similar amino acids were shaded by BOXSHADE. Conserved domains were underlined with different colors: STAT_int, protein interaction domain of STAT protein (black solid line); STAT_alpha, alpha domain of STAT protein (red solid line); STAT_bind, DNA binding domain of STAT protein (green solid line); SH2, Src Homology 2 domain (purple solid line). The abbreviated species names and their GenBank accession numbers were listed in Table 2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analysis found *stat5bl* and *stat5b* in teleost group into the same cluster. In addition, the amino acid sequence similarity of *stat5bl* and *stat5b* in teleost was very high (Fig. 2). Therefore, we tend to speculate that *stat5bl* and *stat5b* may be the same gene in teleost. Despite *stat5* duplication was observed both in mammal and fish, the members of *stat5* in fish seemed more variable. For example, only one *stat5* was found in pufferfish genome [17], and in zebrafish two *stat5* members (*stat5.1* and *stat5.2*) were identified [18]. In Chinese tongue sole, two *stat5bl* were annotated on NCBI but no *stat5a* was found. These data might suggest that *stat5* in mammal and fish might employ different strategies during the duplication process.

Our previous work has found that *stat5bl* was localized a QTL region related to bacterial infection [23]. In this study, *stat5bl* mRNA was evaluated in 11 tissues and relatively high expression was observed in liver, gill and skin, which were all immune related tissues. Therefore, we speculated that *stat5bl* may be involved in immune response. In order to verify the role in immunity, time-course expression of *stat5bl* was examined in immune related tissues after *V. harveyi* infection. The results showed that *stat5bl* mRNA was up-regulated in skin, gill, kidney, and intestine. Skin and gill are served as the barrier to resist pathogens,

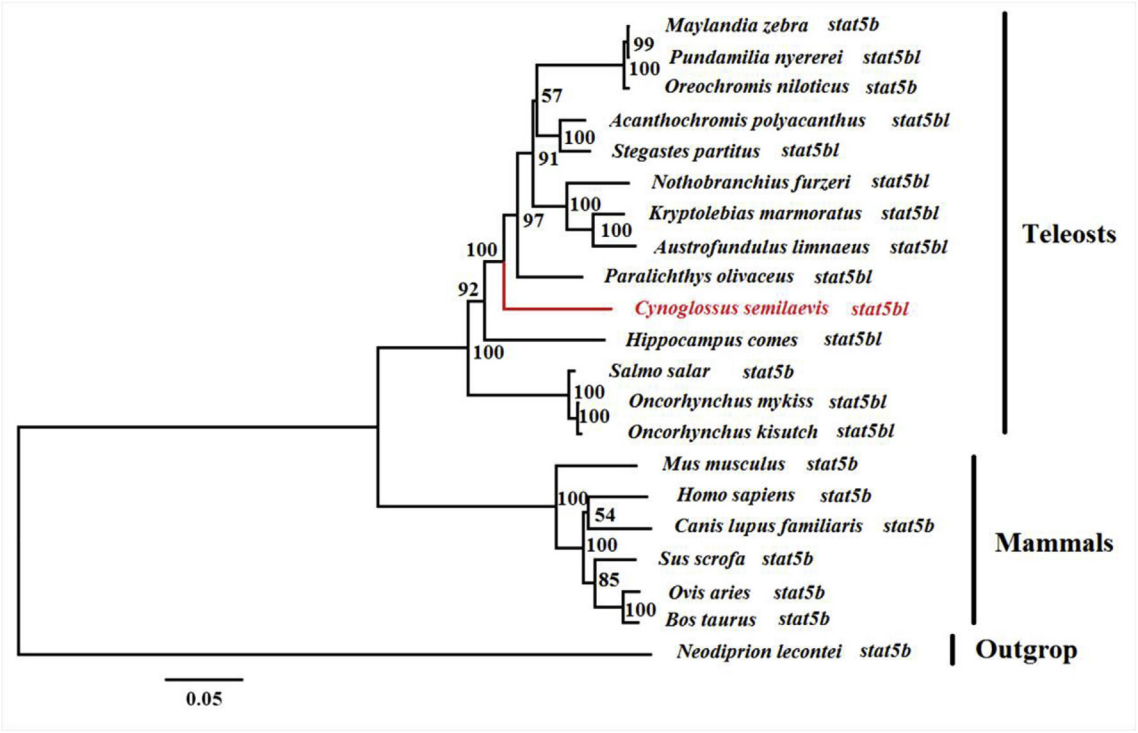

Fig. 3. Phylogenetic tree of *stat5bl* reconstructed by Neighbor-joining (NJ) statistical method. *Neodiprion lecontei* was used as outgroup in this phylogenetic analysis. Bootstrap values present at nodes. The GenBank accession numbers of the nucleotide sequences used in phylogenetic analysis were listed in Table 2.

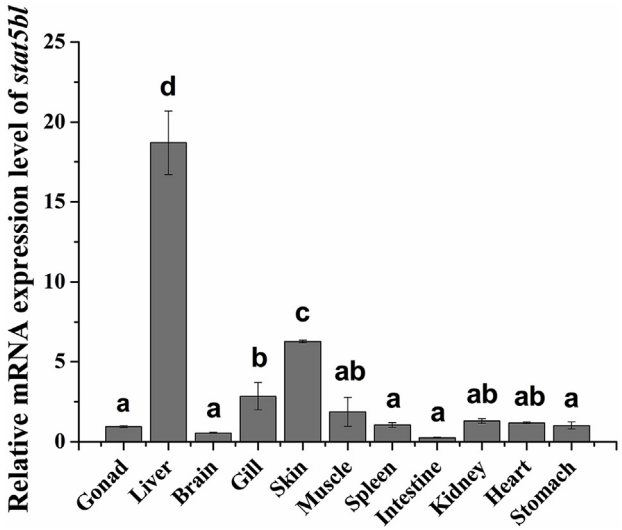

Fig. 4. Relative mRNA expression of *stat5bl* in various tissues. Values are indicated as means ± SE (N = 3). The expression levels with the same letters are not significantly different ($P < 0.05$).

which were reported to participate in innate and adaptive immunity [28,29]. Additionally, the kidney (especially the head kidney) is also an important fish immune tissue [30,31]. Studies of STAT5 have been mostly in mammals with only few being performed in fish. In mammals, STAT5a was firstly cloned and identified as a mammary gland factor, and it can regulate milk production via binding to the promoter of the *β-casein* [7]. STAT5b was identified in mouse mammary tissue and shared about 95% amino acid identities with STAT5a [6]. Only a single *stat5* was identified in pufferfish, which could not induce the transcription of *β-casein* promoter via rat prolactin and Nb2 prolactin receptor [17]. Two distinct *stat5* (*stat5.1* and *stat5.2*) homologues were identified in zebrafish, where *stat5.1* was similar to mammalian *Stat5b*, but the generation of *stat5.2* paralogue resulted from the duplication of *stat5* gene [18]. In the immune response, JAK kinase is activated via pathogen binding on the cellular pathogen recognition receptor (PRR) and in turn phosphorylates the STATs, the phosphorylated STAT5 then dimerize and enter the nucleus to regulate a series of immune-related gene transcription [2,3]. Moveover, the STAT5 can be activated by many cytokines including IL-2, IL-3, IL-5, IL-7, IL-15, granulocyte/macrophage colony stimulating factor, and so on [9]. Therefore, the up-regulation of *stat5bl* mRNA in these immune tissues after *V. harveyi* challenge indicated that *stat5bl* might be involved in immune response, but the specific mechanism needs further investigation.

RNAi was conducted in liver and four genes related to JAK-STAT pathway was assessed (Fig. 6). Lysozyme is the ubiquitous enzyme in the innate immune system of living organisms, which can breakdown of bacteria via hydrolyzing peptidoglycan heteropolymers of bacterial cell walls [32,33]. Besides, *Csglys* could act as an innate defense molecule against bacterial infection [34]. Anti-apoptotic B cell lymphoma 2 (BCL2) is key player in the regulation of intrinsic apoptosis [35]. In the mammals, the BCL2 has been reported to be the downstream effectors of JAK/STAT signaling pathway (Pathway Text Search Entry: map04630) [36]. Therefore, *Csglys* and *Csbcl2* were regarded as downstream effectors that were involved in innate immunity by promoting bacteriolysis or cell apoptosis [32,36]. *Cssocs2* is the suppressor of cytokine signaling and *Cscsf3r* is served as activator of JAK/STAT signal pathway, both of which should be upstream of *stat5bl* [37–39]. After *stat5bl* RNAi, *Csglys* and *Csbcl2* genes were down-regulated probably due to knockdown of *stat5bl* that is a key component of JAK/STAT signaling pathway. In other words, it indicates that *stat5bl* could positively regulate transcription of *Csglys* and *Csbcl2* to involve in antibacterial immune response and apoptosis process via JAK/STAT signaling pathway. In contrast, both *Cssocs2* and *Cscsf3r* were up-regulated, whether this is a feedback effect needs further investigation.

Conflicts of interest

The authors have declared that no competing financial interests

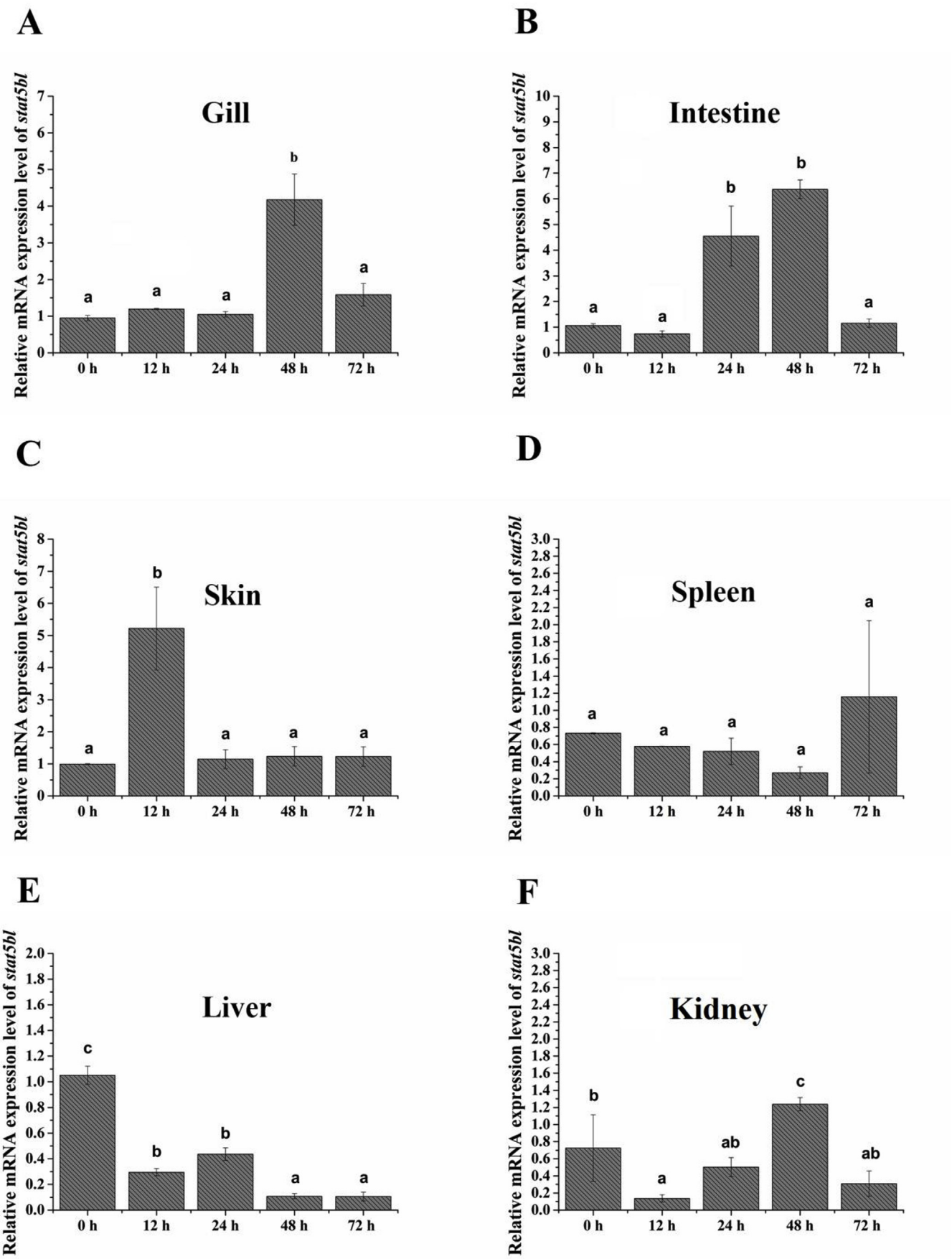

Fig. 5. Expression levels of *stat5l* in the gill (A), intestine (B), skin (C), spleen (D), liver (E) and kidney (F) at different time points after *V. harveyi* challenge. Values are indicated as means ± SE (N = 3). The expression levels with the same letters are not significantly different ($P < 0.05$).

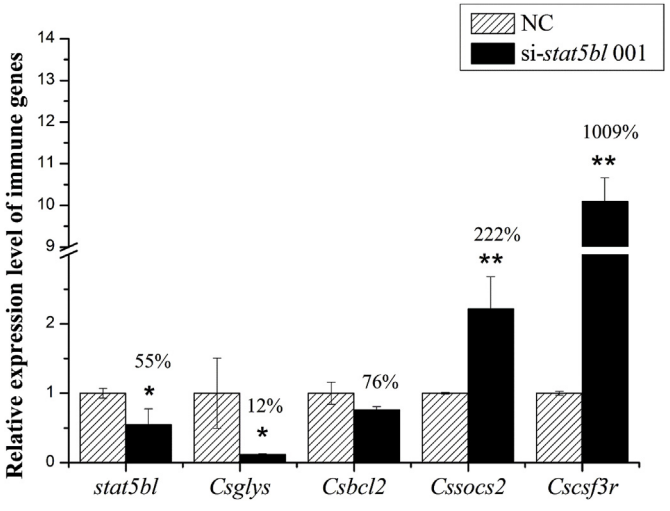

Fig. 6. The relative expression levels of immune-related genes in liver cells after RNAi treatment in *stat5bl*. *β-actin* was used as internal control. NC and si-*stat5bl* 001 represented the cells transfected with the siRNAs of the negative control and si-*stat5bl* 001, respectively. The significant and extremely significant difference between the treated and control group were marked with single and double asterisks, respectively.

exist.

Acknowledgements

This work was supported by the National Nature Science Foundation (31530078, 31730099); the Taishan Scholar Climbing Project Fund of Shandong, China; AoShan Talents Cultivation Program Supported by Qingdao National Laboratory for Marine Science and Technology (No.2017ASTCP-OS15); Central Public-interest Scientific Institution Basal Research Fund, CAFS (2016HY-ZD0201).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.030.

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