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Do the toll-like receptors and complement systems play equally important roles in freshwater adapted Dolly Varden char (*Salvelinus malma*)?

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ABSTRACT

Unlike the normal anadromous lifestyle, Chinese native Dolly Varden char (*Salvelinus malma*) is locked in land and lives in fresh water lifetime. To explore the effect of freshwater adaption on its immune system, we constructed a pooled cDNA library of hepatopancreas and spleen of Chinese freshwater Dolly Varden char (*S. malma*). A total of 27,829 unigenes were generated from 31,233 high-quality transcripts and 17,670 complete open reading frames (ORF) were identified. Totally 25,809 unigenes were successfully annotated and it classified more native than adaptive immunity-associated genes, and more genes involved in toll-like receptor signal pathway than those in complement and coagulation cascades (51 vs 3), implying the relative more important role of toll-like receptors than the complement system under bacterial injection for the freshwater Dolly Varden char. These huge different numbers of TLR and complement system identified in freshwater Dolly Varden char probably caused by distinct evolution pressure patterns between fish TLR and complement system, representative by *TLR3* and *TLR5* as well as *C4* and *C6*, respectively, which were under purifying and positively selecting pressure, respectively. Further seawater adaptation experiment and the comparison study with our library will no doubt be helpful to elucidate the effect of freshwater adaption of Chinese native Dolly Varden char on its immune system.

1. Introduction

The vertebrate immune system includes innate and adaptive immunity, both of which play important roles in distinguishing non-self, dangerous signals, pathogens and intracellular components released through injury or infection [1]. The innate immune system functions as the front line of antimicrobial host defense [2,3] and acts as the primary defense of fish against aquatic microbial pathogens [4]. For lower vertebrates, their adaptive immune system is not well developed comparing with higher vertebrates [3] and a series of astonishing innate immunity strategies were identified in fish through large-scale sequencing in recent years [5–7], showing the important role of innate immune system as the primary antimicrobial host defense for fish [8]. Among innate immunity, the complement system, well known for the ability of detecting infection, is a constituent part of immune system and a bridge with an immune regulatory role linking innate and acquired immune responses for the host [9]. It includes kinds of important immune molecules and is regulated by 40 distinct protein molecules through the accurate mechanism of protein cascade [10]. Studies on mice showed that the complement system was efficiently activated after lipopolysaccharide (LPS) injection, and insufficiency of the complement system resulted in the increasing rate for bacteria survival [11]. All the three pathways for activating complement system are present in many vertebrates including bony fish [12]. But fish complement components showed the structural and functional diversity than those of higher vertebrates, such as C3 for gilthead sea bream [13], common carp [14], rainbow trout [15], zebrafish [16], C7 for miiuy croaker [17], C1q for surfperch [18] and zebrafish [19].

The toll-like receptors (TLRs) are another group of innate immunity members found in various invertebrates and vertebrates and are one of the oldest innate immune components for playing a key role in combating bacterial invasion [20,21]. TLRs mainly function through the identification of structurally conserved pathogen-associated molecular patterns (PAMPs), such as peptidoglycan, lipoprotein, lipopolysaccharide, dsRNA, and flagellin, to activate downstream signaling cascades, eventually leading to the protection against infectious disease [20,22]. There were significant differences in immune recognition and activation between fish and mammalian TLR homologous genes though

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Table 1
The summary of unigenes annotated in eight main databases.

Annotated databases	Annotation number	300 < =length < 1000	length > =1000
COG	8660	1135	7525
GO	16,612	2271	14341
KEGG	16,917	2166	14751
KOG	18,149	2255	15894
Pfam	28,198	2722	25476
Swiss-Prot	16,861	2074	14787
eggNOG	24,709	3353	21356
nr	25,653	3615	22038
All	25,809	3709	22100

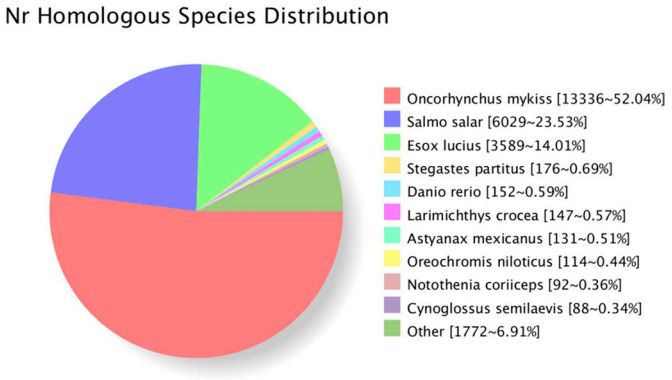


Fig. 1. Homologous species distribution of the Chinese native freshwater Dolly Varden char pooled liver and spleen cDNA library using BLASTx to the NCBI non-redundant protein sequences (Nr) database.

their sequences are conserved [23,24]. These diversified TLRs in fish may be due to the high numbers of prokaryotic cells and viruses in aquatic habitats which drove the co-evolutionary process of fish non-specific immunity [4], such as the two types of TLR4 and TLR8 genes in zebrafish [25], the soluble TLR5 in rainbow trout [26] and the two splicing isoforms of TLR9 in gilthead seabream [27] and large yellow croaker [28]. As important members of the innate immune systems, researches were focused either on the complement system or on TLR pathway. A comprehensive study is conductive to compare and understand these two vital members of innate immunity.

Dolly Varden chars (*Salvelinus malma*) are anadromous fish and have a pan-Pacific distribution [29]. While the populations in the southern part of the range below 44°N are often freshwater residents [30]. For those resident in the mountain streams of Northeast China, they are locked in land and live in fresh water lifetime. As a land-locked char, Chinese native freshwater Dolly Varden char showed a closer relationship with other two Asian Dolly Varden char subspecies than those in America according to the reconstructed phylogeny based on *Cytb* genes [31], indicating the no negligible differences among Dolly Varden char populations living in different salinities. For the delicate meat quality and plentiful nutrient substance, it was one of the best-selling high grade aquatic products in the international market [32,33]. Studies were focused on the growth and geographical distribution of *S. malma* [34,35] while little is known about the effect of freshwater ecological settlement on its immune system against bacterial infection for Chinese native freshwater Dolly Varden char (*S. malma*). To explore the effect of the adaption to freshwater on its immune system, especially the complement system and TLR pathway, we studied the cDNA library of pooled hepatopancreas and spleen of Chinese native freshwater Dolly Varden char (*S. malma*) under bacterial challenge.

2. Materials and methods

2.1. Ethics statement

All experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Ningbo University. This study was approved by the Institutional Animal Care and Use Ethics Committee of Ningbo University.

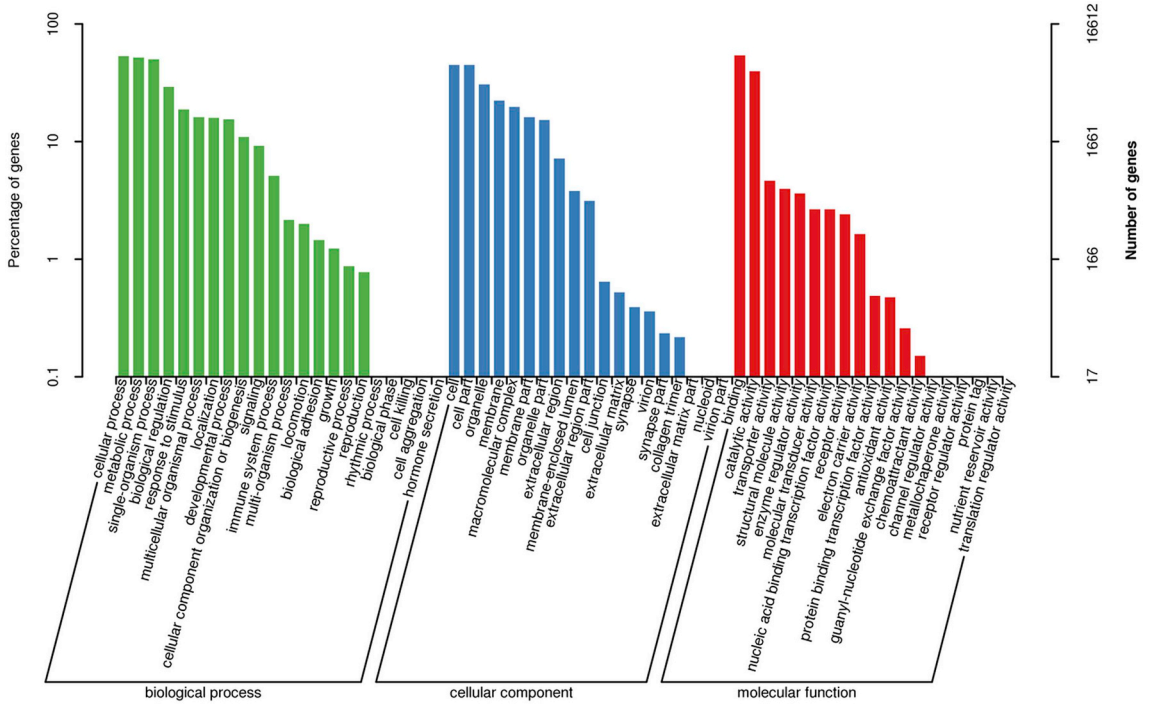


Fig. 2. GO (Gene ontology) classification of the assembled unigenes.

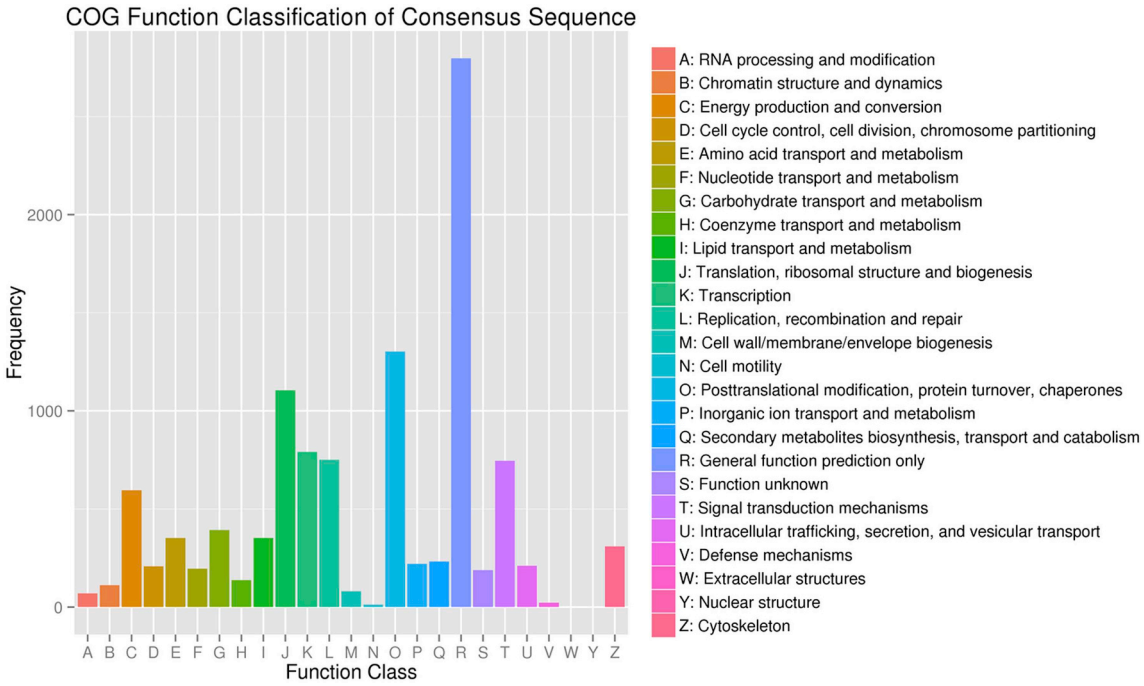


Fig. 3. COG (Clusters of Orthologous Groups) classification of the assembled unigenes.

Table 2
Main immunity-relevant genes identified in the pooled cDNA library of liver and spleen from Chinese native Dolly Varden (*Salvelinus malma*).

Gene Annotation	Accession Number	Matched species	Matched species NCBI No.	E-value
Alpha-2-macroglobulin	MH784954	*Esox lucius*	XP_010866339.1	1.91E-158
B-cell lymphoma 6 protein	MH883285	*Oncorhynchus mykiss*	CDQ61280.1	0.00E+00
Beta-1,3-glucosyltransferase	MH883249	*Esox lucius*	XP_010895107.1	0.00E+00
Cathepsin D	MH784960	*Oncorhynchus mykiss*	CDQ95751.1	1.03E-16
C-C motif chemokine 19 precursor	MH883271	*Phalacrocorax carbo*	XP_009509546.1	2.22E-104
CCAAT/enhancer-binding protein beta	MH784955	*Esox lucius*	XP_010875195.1	1.45E-101
Chondroitin sulfate synthase 1	MH883254	*Larimichthys crocea*	XP_010746285.1	4.17E-12
Complement component 4	MG490977	*Oreochromis niloticus*	XP_005453830.1	0.00E+00
Complement component 6	MH784953	*Salvelinus alpinus*	XP_023843376.1	0.00E+00
Complement factor B/C2 precursor	MH883257	*Esox lucius*	XP_010900324.1	0.00E+00
DLA class II histocompatibility antigen	MH883287	*Danio rerio*	BAC82613.1	1.02E-19
Glutathione S-transferase kappa 1-like	MH883244	*Oncorhynchus mykiss*	CDQ78546.1	0.00E+00
Glutathione S-transferase omega 1	MH883243	*Oncorhynchus mykiss*	CDQ67358.1	1.41E-18
Granulocyte colony-stimulating factor receptor	MH883274	*Esox lucius*	XP_010886439.1	0.00E+00
Interferon-gamma receptor alpha chain	MH883283	*Esox lucius*	XP_010886613.1	0.00E+00
Interleukin-1 beta	MH883272	*Oncorhynchus mykiss*	CDQ56835.1	0.00E+00
Interleukin-1 receptor type 1-like	MH883275	*Salmo salar*	NP_001158782.1	3.62E-36
Interleukin-1 receptor-associated kinase 3	MH883276	*Esox lucius*	XP_010875189.1	0.00E+00
Interleukin-1 receptor-like protein	MH784961	*Oncorhynchus mykiss*	CDQ74839.1	6.11E-164
Interleukin-11	MH883279	*Oncorhynchus mykiss*	CDQ58690.1	2.17E-161
Interleukin-11 receptor subunit alpha	MH883278	*Esox lucius*	XP_010897883.1	0.00E+00
Interleukin-17 receptor A	MH883281	*Oncorhynchus mykiss*	CDQ84575.1	5.69E-24
Interleukin-17 receptor E-like	MH883282	*Pundamilia nyererei*	XP_005747081.1	1.51E-53
Interleukin-6 receptor subunit beta-like	MH883273	*Oncorhynchus mykiss*	CDQ66473.1	4.28E-15
Liver-expressed antimicrobial peptide 2B	MH883238	*Esox lucius*	XP_010885117.1	0.00E+00
Lysozyme g	MH883263	*Esox lucius*	XP_010880422.1	1.17E-98
Macrophage mannose receptor 1	MH883264	*Oncorhynchus mykiss*	NP_001118027.1	2.19E-28
Macrophage mannose receptor 1-like	MH883265	*Esox lucius*	XP_010874532.1	2.60E-93
N-acetylgalactosaminyltransferase 7 isoform X1	MH883256	*Danio rerio*	XP_009289397.1	0.00E+00
NF-kappa-B inhibitor alpha	MH883267	*Oncorhynchus mykiss*	CDQ91733.1	3.09E-142
NF-kappa-B inhibitor epsilon	MH883268	*Esox lucius*	XP_010903075.1	8.97E-36
Polypeptide N-acetylgalactosaminyltransferase 14	MH883255	*Oncorhynchus mykiss*	CDQ78152.1	2.73E-09
P-selectin precursor	MH784956	*Oncorhynchus mykiss*	CDQ56596.1	2.90E-12
Thrombospondin-1-like	MH883258	*Oncorhynchus mykiss*	CDQ57643.1	7.11E-95
TNFAIP3-interacting protein 1	MH784963	*Oncorhynchus mykiss*	CDQ83157.1	0.00E+00
Toll-like receptor 3	MH883239	*Oncorhynchus mykiss*	CDQ83090.1	8.14E-144
Toll-like receptor 5	MG490980	*Oncorhynchus mykiss*	CDQ72687.1	3.15E-35
Tumor necrosis factor alpha-induced protein 3	MH883284	*Oncorhynchus mykiss*	CDQ89726.1	5.72E-12

Table 3
PCR primers used in this study.

Primer	Sequences(5′–3′)	Application
For *S.m-C4* gene		
S.m-C4–F1	ATCACTGCTCCGAACATAGT	For cloning 70–1375 bp
S.m-C4-R1	TAGACTGGACTGCTAACACTGT	
S.m-C4-F2	AACCAATCTACAATCCAGGAGAG	For cloning 422–1749 bp
S.m-C4-R2	CACCACAGTCACCAAGTCAG	
S.m-C4-F3	CCACGACCAGCCTACATCTA	For cloning 1474–2975 bp
S.m-C4-R3	ACTCCATCCAGGTTCAGACAG	
S.m-C4-F4	CATCCACGCCATGAGATACCT	For cloning 3039–4142 bp
S.m-C4-R4	TCCACCGAGACATTGAGACTG	
S.m-C4-F5	TGAGCAGAGGCAGTATGGAG	For cloning 3813–5011 bp
S.m-C4-R5	TGTGGTCAGTGGTGTCAGAG	
S.m-C4-qF1	CTTCCACGGCTCAGACCAAACTC	For *S.m-C4* qPCR
S.m-C4-qR1	TCCTCCATACTGCCTCTGCTCAG	
For *S.m-TLR5*gene		
S.m-TLR5-F1	CTCTGGTATGGTAACAACTTC	For cloning 1404–1953 bp
S.m-TLR5-R1	TCTCGTCATCCTCCTCACAC	
S.m-TLR5-qF1	CCTCGCACATTCACCACTTCTTACG	For *S.m-TLR5* qPCR
S.m-TLR5-qR1	ATGGACGAAGGACATAGGGCTGAG	
For β-actin gene		
β-actin-F1	AGGGTGTGATGGTCGGTATGGG	For β-actin qPCR
β-actin-R1	CGCAGCTCGTTGTAGAAGGTGT	

2.2. Experimental animals and sample collection

Fish (184.6 ± 23.4 g in weight) were collected from tributary of upper Yalu River, Tonghua City, China and were fed twice every day and held in net cages (2.0 × 2.0 × 1.0 m, L: W: H) anchored in an outdoor pond for one-month acclimatization. Then they were randomly divided into two groups and anesthetized with 100 mg/L tricaine methanesulfonate (MS-222), then intraperitoneally challenged with approximately 1.2×10^7 colony forming units *Aeromonas hydrophila* [36,37] or corresponding volume of 0.9% saline. At the time points just after injection (0 h) and 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, and 96 h-post-injection, three fish from each group anesthetized by MS-222 were sacrificed at each time point. The liver and spleen tissues were immediately frozen in liquid nitrogen and then preserved in −80 °C until RNA isolation. Furthermore, at 24 h-post-injection, three more fish from the group of bacterial injection were sacrificed and the tissue samples of liver and spleen were collected for cDNA library construction.

2.3. RNA isolation, library preparation and sequencing

Total RNA was isolated using RNAiso Plus (Takara Bio Inc, Shiga, Japan) according to the RNA extraction protocols and treated with RNase-free DNase I (Takara Bio Inc, Shiga, Japan) at 37 °C for 1 h to remove the DNA contaminants. RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). Its concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Flurometer (Life Technologies, CA, USA). Sequencing libraries were generated as described in Ref. [38]. Briefly speaking, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads and was reversely transcripted into the first strand cDNA using random hexamer primer. Then DNA Polymerase I and RNase H were used for the second strand cDNA synthesis. After ends-blunting and adenylation of 3' ends, the DNA fragments were ligated with sequencing adaptors and those between 150 and 200 bp in length were enriched by PCR amplification. The cDNA library was sequenced on a PacBio RS II platform (Biomarker Technologies, Beijing, China).

2.4. De Novo assembly and gene function annotation

Raw data were processed by removing adapter and ambiguous sequences before assembly. These clean reads of the library were assembled into transcripts by Trinity software base on *de novo* results compared with different assemblers [39]. After assembly, the transcripts were clustered and the redundant ones were removed and the remaining sequences were defined as unigenes. These unigenes were annotated using BLASTx programs with NCBI non-redundant protein sequences (Nr), NCBI non-redundant nucleotide sequences (Nt), SwissProt [40], Pfam [41], Kyoto Encyclopedia of Genes and Genomes (KEGG) [42], Gene Ontology (GO) [43], Clusters of Orthologous Groups (COG) [44], evolutionary genealogy of genes: non-supervised orthologous groups (eggNOG) [45], and euKaryotic Orthologous Groups (KOG) [46]. GO and their protein function annotations were produced using Blast2GO [47] and Blastn to the Nr database, respectively.

2.5. Quantitative real-time PCR

The quantitative real-time PCR (qPCR) was carried out to explore the expression patterns of immune-related genes under bacterial challenge. Primer sets were designed based on the coding sequences of identified genes in this pooled cDNA library and specific PCR products of each gene were observed on the 1% agarose gels. Briefly, 1000 ng of total RNA was reverse transcripted into cDNA by using TransScript All-in-One Reverse Transcriptase Kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The reaction was performed in a reaction mixture of 10 μL, containing 2 μL 5 × TransScript® All-in-One SuperMix for qPCR, 0.5 μL gDNA Remover, 1 mg total RNA, and RNase-free water add up to 10 μL, and followed by 1 cycles of 15 min at 42 °C, 5 sec at 85 °C. The fold change was calculated as the average expression level of target gene in bacterial challenge group comparing with that of the control sample (0 h). The qPCR results of the relative mRNA levels were measured by $2^{-\Delta\Delta CT}$ method [48]. The data was

Table 4
Identity and similarity analysis *S.m-C4* gene with human and other seven fish homologs.

Organism (Gene)		Identity %								
		1	2	3	4	5	6	7	8	9
Similarity %	1. *Salvelinus malma*		94.4	52	61.8	51.5	50.9	49.7	51.3	52.4
	2. *Salmo salar*	88.3		52.2	62.3	51.3	50.2	49.3	51.3	52.8
	3. *Oncorhynchus mykiss*	50.5	50.5		51.1	62.7	62.4	65	67.2	49.7
	4. *Danio rerio*	57	57.2	48.8		51.1	50.1	50.4	51.3	49.6
	5. *Cyprinus carpio*	49.1	48.9	58.2	49		57.2	57.2	60.1	48.6
	6. *Takifugu rubripes*	49.1	48.2	57.8	47.6	53.8		70.8	73.9	48.8
	7. *Larimichthys crocea*	47.9	47.6	60.4	48.5	53.5	65.2		82.8	48.1
	8. *Oplegnathus fasciatus*	49.1	49.2	62.2	49.2	55.6	67.6	77		48.7
	9. *Homo sapiens*	50.8	51.1	48.9	47.7	47.6	48	47.4	47.9	

represented as means ± standard deviation (means ± SD), with a significant level at 0.05. The differences of expression between the control and experimental group were detected by Student's *t*-test and those among different time points in the control or experimental group were calculated by the one-way ANOVA (analysis of variance).

2.6. Molecular characteristic analysis and phylogeny reconstruction

The re-sequenced and verified immune-related genes were translated under the standard genetic code. The signal peptide was predicted on signalP software (http://www.cbs.dtu.dk/services/SignalP/) [49] and the theoretical MW and isoelectric point (pI) were determined by the ExPASy ProtParam online tool (http://web.expasy.org/protparam/)

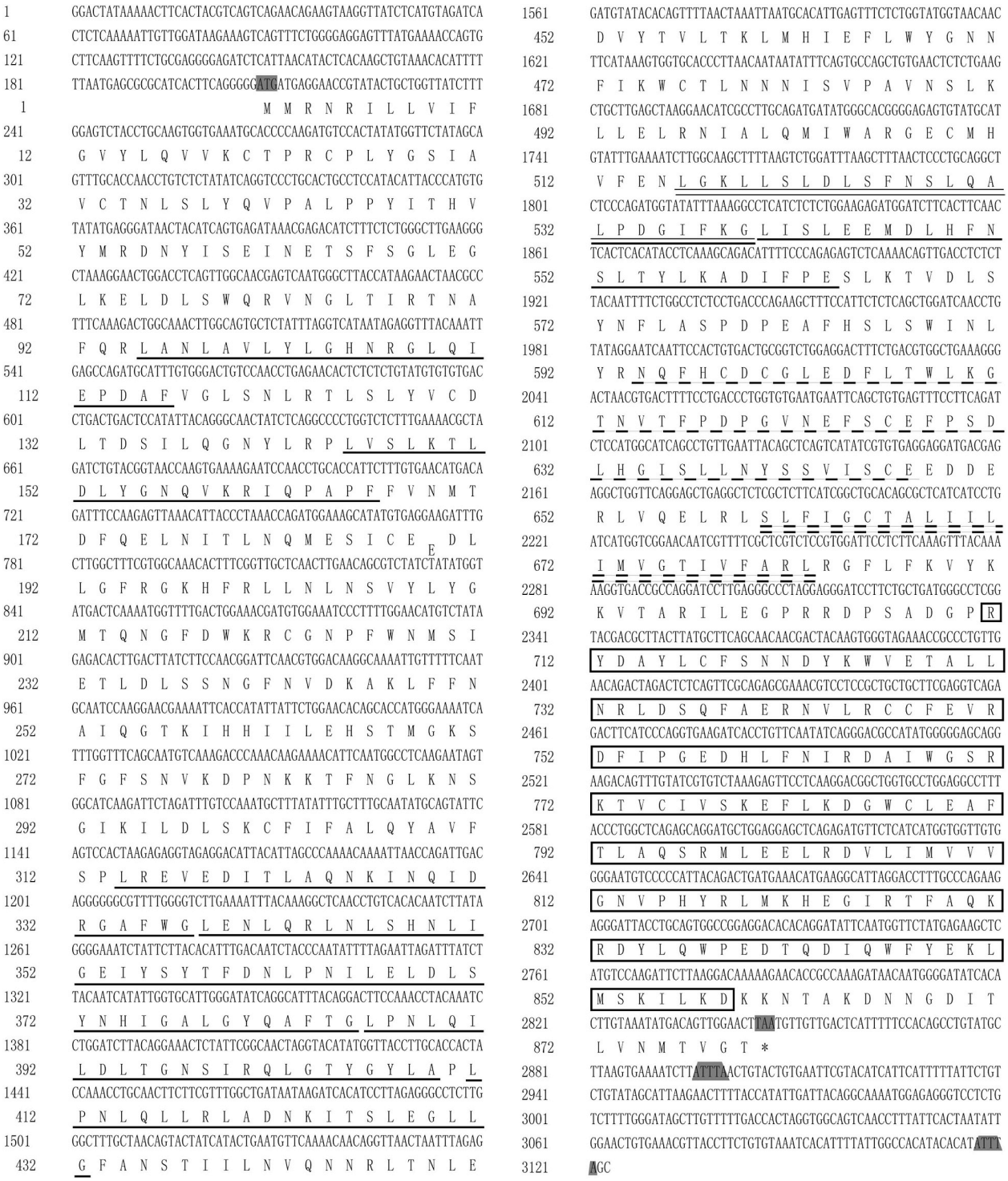
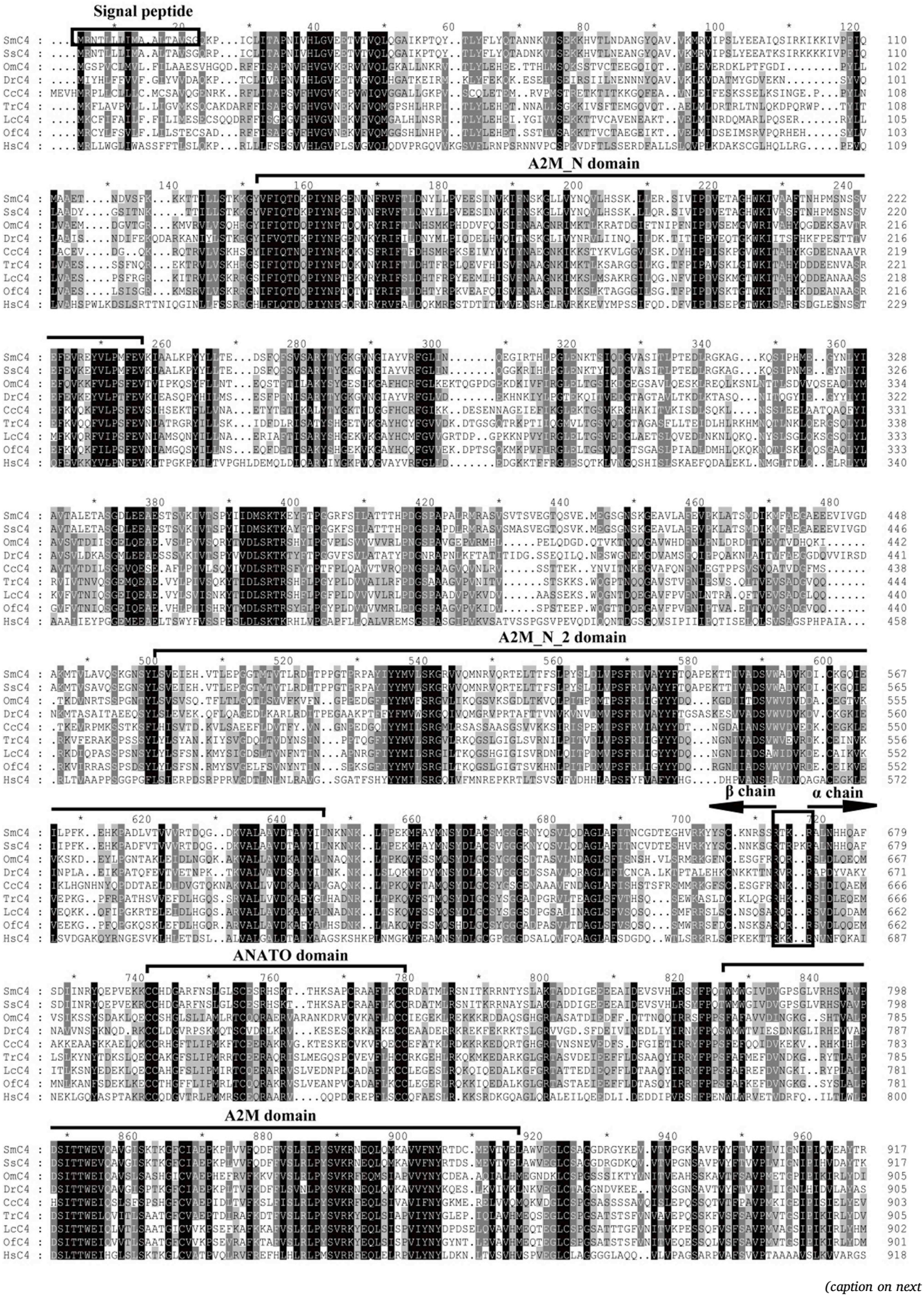


Fig. 4. Nucleotide and deduced amino acid sequence of *S.m-TLR5*.
The shadow part represented initiation code (ATG) and the termination code (TAA). The LRRs, LRR_CT, and transmembrane domain were showed by the underline, the broken line, and double broken line, respectively, and the TIR domain was shown by boxed sequences. The motif associated with mRNA instability (ATTTA) is marked by trapezoid shadow.



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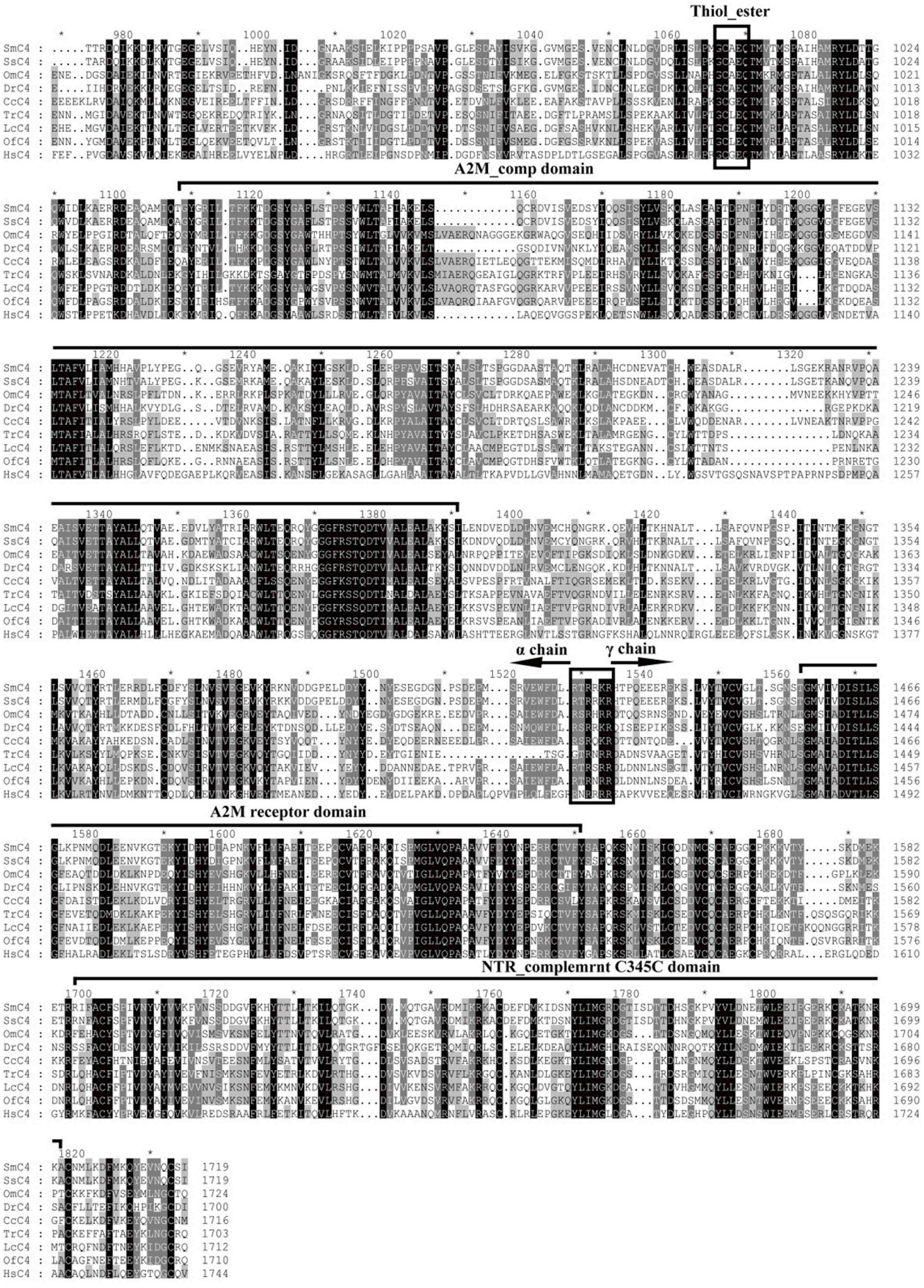

Fig. 5. (*continued*)

Fig. 5. Multiple sequence alignment of Chinese native freshwater Dolly Varden char C4 amino acid sequences with other species.
The C4 or C4-like amino acids sequences of *Salmo salar* (Ss), *Oncorhynchus mykiss* (Om), *Danio rerio* (Dr), *Cyprinus carpio* (Cc), *Takifugu rubripes* (Tr), *Larimichthys crocea* (Lc), *Oplegnathus fasciatus* (Of), and *Homo sapiens* (Hs) were retrieved from public database. The conserved and identical residues were represented by black shading, and conservative substitutions were represented by grey shading. The conserved seven motifs of C4 were shown above the alignment by bidirectional line. The signal peptide, α-β junction, α-γ junction, thioester site was showed by line-box.

[50]. Domain analysis was carried out by Simple Modular Architecture Research Tools (SMART) (http://smart.embl-heidelberg.de/) [51]. The homology sequences were retrieved from NCBI website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were performed with MEGA5.1 [52]. The amino acid identity and similarity between homologues were evaluated by MatGAT program [53]. The best-fit nucleotide substitution model was predicted by jModelTest software [54] and was then used Bayesian method using MrBayes3.1 [55] with 5,000,000 generations and a 25% burn-in.

2.7. Molecular evolutionary analysis

Then the reconstructed phylogeny was used for molecular evolutionary analysis by PAML4 software [56] to explorer the evolutionary process of the immune-related genes in teleost. The nonsynonymous (dN) and synonymous (dS) nucleotide substitution rates (ω) is calculated by CODEML [57]. And $\omega < 1$, $\omega = 1$ and $\omega > 1$ are used as criterion of judgment for purifying selection, neutral evolution and positive selection, respectively. The site models were used to identify the overall selective pressure on these immune-related genes. While the branch-site models were used to detect the positive selection signal on the branches leading to the present or ancestral lineages with particular characters. Then the posterior probability (PP) [56] was calculated for those positive selection signals detected by the site or branch-site models.

3. Results

3.1. Characteristics of cDNA library

The cDNA library was sequenced from pooled liver and spleen of Chinese native freshwater Dolly Varden char (*Salvelinus malma*) under bacterial injection. After eliminating adaptor sequences and low-quality sequences (< 50 bp), more than 14.88 gigabase (Gb) of cleandata, with 7,728,364 in total number, were generated in this library. Among these cleandata, there were 4,565,697, 2,087,033 and 1,075,634 reads with length ranging in 0–2 kilo base pair (kb), 2–3 kb, and 3–6 kb, respectively. After quality control, data filtering and removing redundant reads, a total of 27,829 unigenes were generated from 31,233 high-quality transcripts, using *de novo* assembly. Totally, 24,541 open reading frames (ORF) were identified, in which 17,670 were complete.

3.2. Functional annotation and classification of unigenes

All unigenes from the library were used for function enrichment and classification analysis using the BLASTx tools against the major databases, finally making 25,809 unigenes annotated (see Table 1). The top hit species distribution based on the NCBI-Nr database showed that unigenes matched sequences from a range of species (Fig. 1). Among the 25,653 (92.18%) unigenes matched in Nr database, 52.04% unigenes were similar to *Oncorhynchus mykiss*, followed by *Salmo salar* (23.53%) and *Esox lucius* (14.01%), the remaining species matched accounted for less than 10%.

Totally, 16,612 (59.69%) unigenes were successfully annotated by GO and were classified into three major categories: “biology process”, “molecular function”, and “cellular component” (Fig. 2). Among the unigenes categorized as cellular components, cell (7461 unigenes), cell part (7461 unigenes), organelle (5110 unigenes), and membrane (3713 unigenes) represented the majorities of this category. For the molecular function category, binding (8982 unigenes) and catalytic activity (6564 unigenes) represented a high percentage of this category. Moreover, cellular process (8883 unigenes), metabolic process (8604 unigenes), single-organism process (8,342), and biological regulation (4856 unigenes) represented the majorities of the biology process (Fig. 2). Moreover, two immune-relevant subcategories, “response to stimulus” (3124 unigenes) and “immune system process” (851 unigenes) were also enriched (Fig. 2).

Furthermore, the unigenes were aligned to the COG database to further annotate their functional orthologous genes. Together, 8660 (31.12%) unigenes were annotated in COG and grouped into 25 COG classifications (Fig. 3). The largest cluster was the “General function prediction only” category, which meant that most of the gene functions were predicted by informatics methods and had not been confirmed by experimentation, and then followed by “Posttranslational modification, protein turnover, chaperones”, “Translation, ribosomal structure and biogenesis”, “Transcription”, and “Replication, recombination and repair” categories (Fig. 3), reflecting the activation of gene transcription, translation and repair during bacterial injection.

Then the unigenes were aligned to the KEGG database to further classify the biological pathways. Totally, 16,917 unigenes were assigned to 271 pathways. Immune-relevant KEGG pathways included cytokine-cytokine receptor interaction (106 genes), toll-like receptor signal pathway (51 genes), intestinal immune network for IgA production (20 genes), cytokine signaling pathway (7 genes), T cell receptor signaling pathway (3 genes), B cell receptor signaling pathway (4 genes), antigen processing and presentation (4 genes), and complement and coagulation cascades (3 genes). Furthermore, other relevant pathways were concentrated on regulation of autophagy (16 genes), lysosome (58 genes), peroxisome (59 genes), and apoptosis (45 genes). The main genes involved in immunity were summarized in Table 2. Both as important members of the native immune system, the huge different numbers of genes identified in TLR signal pathway and complement system cascade (51 vs 3, see Supplementary Figs. 2 and 3) indicating the possible distinct involvement patterns between these two native immune system against acute bacterial challenge.

3.3. Validation and characterization of immune-related gene sequence

To validate the gene sequences identified from our cDNA library, two immune-related genes were selected for resequencing, namely the complement component 4 gene (*S.m-C4*) and Toll-like receptor 5 (*S.m-TLR5*) as the representatives for the complement system and TLR pathway, respectively, of Chinese native freshwater Dolly Varden char (*Salvelinus malma*) (GenBank accession MG490977 and MG490980, respectively). The resequencing had confirmed the complete ORF of *S.m-C4* by primers in Table 3 and it was 5160 bp in length, encoding a protein with 1719 amino acid (aa) residues. The first seventeen amino acids were predicted to be the signal peptide, which was presumed to facilitate the extracellular localization. The mature peptide, without the signal peptide, was predicted to be 189.4 kDa for MW and 6.05 for pI. The pairwise amino acid sequence comparison showed the highest percent identity (94.4%) and similarity (88.3%) between *Salmo salar* and Chinese native freshwater Dolly Varden char (Table 4). The domain research predicted an A2M_N domain (residues 134–226), α2-macroglobulin family N-terminal region (A2M_N_2) (residues 464–604), the anaphylatoxin-like domain (ANATO) (residues 694–730), the α2-macroglobulin family domain (A2M) (residues 777–867), the α-macroglobulin complement component (A2M_comp) (residues 1043–1298),

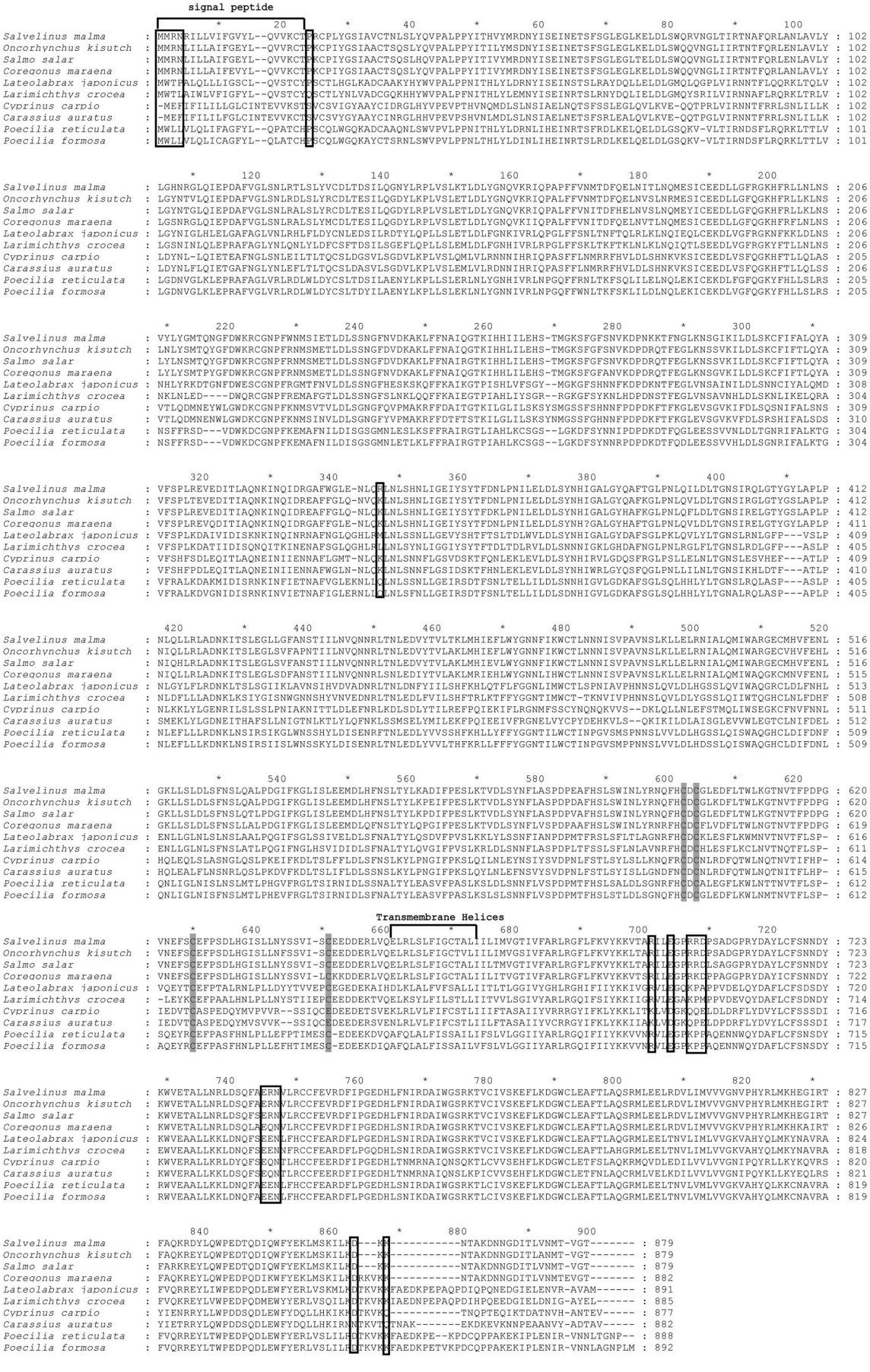

Fig. 6. Multiple sequence alignment of the deduced amino acid sequences of S.m-TLR5 as well as other nine fish TLR5.
The protein binding region was showed by box, the four conserved cysteine residues in the LRR-CT were shadowed by grey.

Table 5
Comparison of Chinese native freshwater Dolly Varden char (*Salvelinus malma*) TLR5 amino acid sequences with those of other teleost.

Species	No. of amino acids	No. of leucine-rich repeats	*S.m-TLR5*	
			Similarity (%)	Identity (%)
S. malma	879	10	–	–
O. kisutch	879	13	96.0	91.5
S. salar	879	10	95.6	91.7
C. maraena	882	10	95.0	89.6
L. japonicus	891	11	72.2	54.5
L. crocea	885	12	71.1	54.4
C. carpio	877	15	67.8	48.1
C. auratus	882	14	68.6	48.8
P. reticulata	888	13	69.7	51.4
P. formosa	892	12	70.2	52.1

the α-macroglobulin receptor (A2M_recep) (residues 1455–1544), and the Netrin C-terminal domain (C345C) (residues 1586–1701) which was a characteristic of the complement C3, C4 and C5 protein family (Supplemental Fig. 1).

The resequencing had also confirmed the cDNA sequence of Chinese native freshwater Dolly Varden char's *TLR5* gene (*S.m-TLR5*) by primers in Table 3. It was 3123 bp in length, including a 207-bp 5′-untranslated region (UTR), a 3′-UTR with 279 bp in length, and an open reading frame (ORF) of 2640 bp encoding 879 amino acids (Fig. 4). There were two mRNA instability signals (ATTTA) in the 3′-UTR (Fig. 4). The theoretical molecular weight of S.m-TLR5 protein was 100.6 kDa. The deduced S.m-TLR5 protein possessed typical structural domains of the TLRs family, including 8 leucine-rich repeat (LRR) domains (residues 95–116, 145–166, 314–337, 338–364, 362–385, 386–409, 411–432, and 540–563, respectively), a leucine-rich repeat C-terminal (LRR-CT) domain (residues 594–647), a leucine-rich repeats-typical subfamily (LRR-TYP) domain (residues 516–539), a transmembrane domain (residues 660–682) and a cytoplasmic Toll-interleukin-1 receptor (TIR) domain with 148 amino acids in length at the C-terminal (residues 711–858) (Fig. 4).

3.4. Multiple sequence alignment

Multiple sequence alignment showed the highly conserved amino acids in teleost and human. The deduced S.m-C4 protein had the α-β linker sequence RXXR (at residues 668–671) and α-γ linker sequence RRXR (residues 1426–1429) where C4 amino acid chain was incised into three chains (α, β and γ chain). In addition, the conserved thioester (GCGEQ) site was also positioned at residues 1001 to 1005 (Fig. 5). For TLR5, a putative signal peptide comprising the first 21 amino acids and a transmembrane helix made up of 14 amino acids were predicted. The four conserved cysteine residues in the LRR-CT of the membrane-bound form of TLR5 were found, furthermore, there were nine protein binding region (Fig. 6). The identities were also explored among *S.m-TLR5* and other nine teleost *TLR5* genes. It showed higher degree of identities for S.m-TLR5 protein with those of *Oncorhynchus kisutch* (91.5%), *Salmo salar* (91.7%) and *Coregonus maraena* (89.6%), but lower values with *Cyprinus carpio* (48.1%) and *Carassius auratusis* (48.8%) (Table 5).

3.5. Phylogeny reconstruction

To explorer the phylogeny of these two immune genes in fish, Chinese native freshwater Dolly Varden char, together with 23 other teleost, two sharks, and one Coelacanth, were used for *C4* genes (Table 6). The four Salmoniformes fish grouped together with high posterior probability (PP) in the reconstructed Bayesian phylogeny (Fig. 7). And three Cypriniformes fish and two Characiformes fish were also grouped together with 1.00 PP, respectively, forming the Ostariophysi clade. All five Cyprinodontiformes fish were groups together

Table 6
Taxonomy and accession numbers of fish *C4* genes used in this study.

Taxonomy	Common name	Species name	Accession Number
Chondrichthyes			
Chimaeriformes	Elephant shark	*Callorhinchus milii*	XP_007908084.1
Orectolobiformes	Whale shark	*Rhincodon typus*	XP_020382737.1
Osteichthyes			
Coelacanthiformes			
Coelacanth	Coelacanth	*Latimeria chalumnae*	XP_014347018.1
Protacanthopterygii			
Salmoniformes	Atlantic salmon	*Salmo salar*	XP_014069949.1
Salmoniformes	Rainbow trout	*Oncorhynchus mykiss*	XP_021438059.1
Salmoniformes	Coho salmon	*Oncorhynchus kisutch*	XP_020363611.1
Salmoniformes	Dolly Varden char	*Salvelinus malma*	MG490977
Ostariophsi			
Cypriniform	Zebrafish	*Danio rerio*	XP_005157429.1
Cypriniform	Bony fishes	*Sinocyclocheilus grahami*	XP_016098199.1
Cypriniform	Bony fishes	*Sinocyclocheilus rhinocerous*	XP_016428773.1
Characiformes	Mexican tetra	*Astyanax mexicanus*	XP_022524517.1
Characiformes	Red-bellied piranha	*Pygocentrus nattereri*	XP_017564087.1
Acanthopteygii			
Perciformes	Greater amberjack	*Seriola dumerili*	XP_022624171.1
Perciformes	Barramundi perch	*Lates calcarifer*	XP_018543722.1
Perciformes	Ballan wrasse	*Labrus bergylta*	XP_020493080.1
Perciformes	Large yellow croaker	*Larimichthys crocea*	XP_010733975.2
Perciformes	Miiuy croaker	*Miichthys miiuy*	KJ563220.1
Perciformes	Bicolor damselfish	*Stegastes partitus*	XP_008294739.1
Perciformes	Spiny chromis	*Acanthochromis polyacanthus*	XP_022072882.1
Perciformes	Nile tilapia	*Oreochromis niloticus*	XP_005472532.2
Perciformes	Bony fishes	*Pundamilia nyererei*	XP_005738483.1
Perciformes	Zebra mbuna	*Maylandia zebra*	XP_004541586.1
Cyprinodontiformes	Mummichog	*Fundulus heteroclitus*	XP_012718030.1
Cyprinodontiformes	Guppy	*Poecilia reticulata*	XP_008430678.1
Cyprinodontiformes	Amazon molly	*Poecilia formosa*	XP_007570077.1
Cyprinodontiformes	Turquoise killifish	*Nothobranchius furzeri*	XP_017282054.1
Cyprinodontiformes	Mangrove rivulus	*Kryptolebias marmoratus*	XP_015796075.1

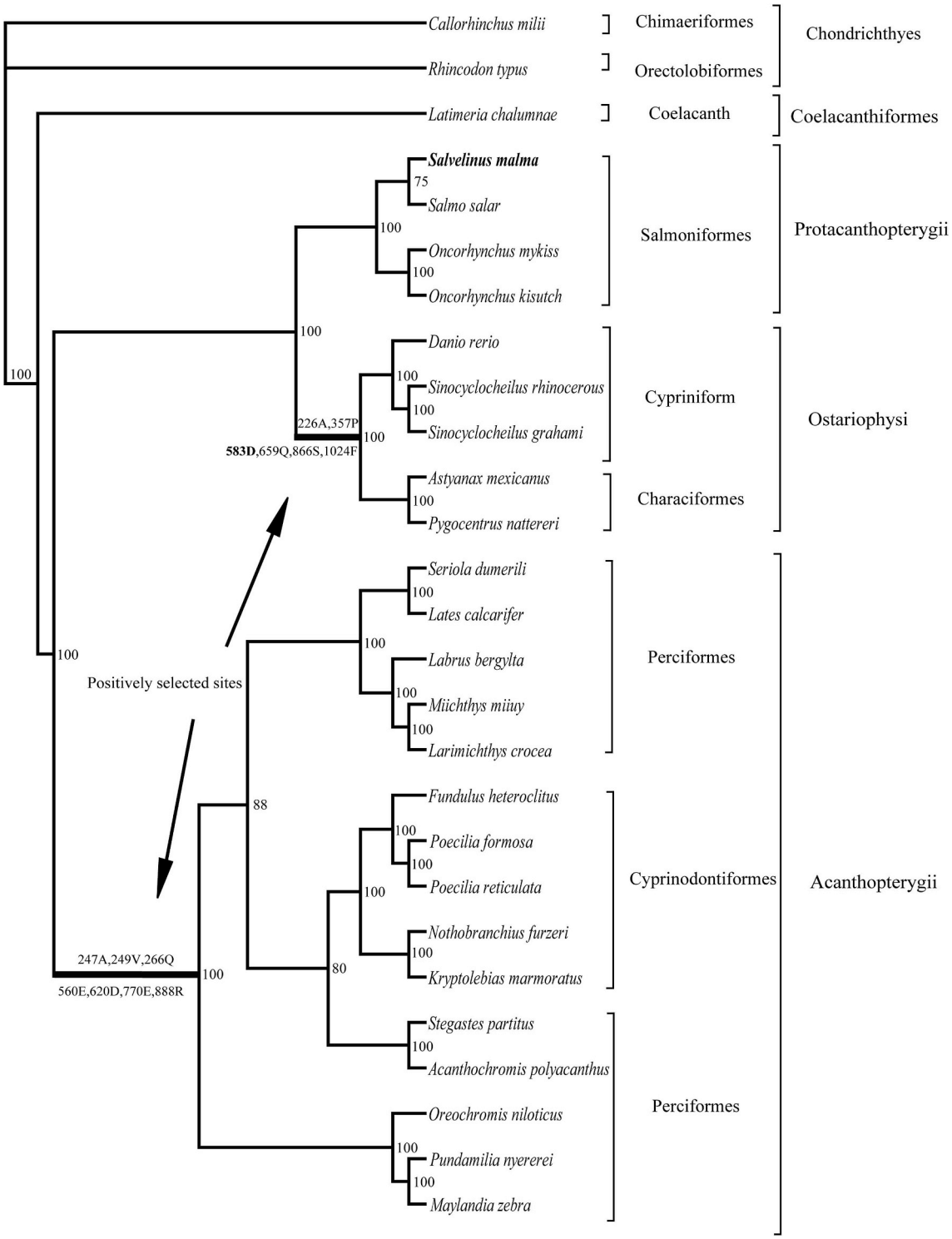

Fig. 7. The reconstructed Bayesian phylogeny of fish *C4* genes.
There were six and seven positively selected sites detected by branch-site models on the ancestral lineages leading to Ostariophysi and Acanthopterygii, respectively. Only the positively selected sites with posterior probabilities larger than 0.95 (PP > 0.99 in bold) were shown. The lineage of Chinese native freshwater Dolly Varden char was marked in bold.

with 1.00 PP, and then grouped with other ten Perciformes fishes, forming the Acanthopterygii clade, with 1.00 PP. This result was consistent with traditional taxonomy and phylogeny. The teleost fish was grouped into Coelacanthiformes, Protacanthopterygii, Ostariophysi, and Acanthopterygii, four clades, respectively. The two sharks were as the out-groups.

For fish *TLR5* genes, the *S.m-TLR5* gene together with other 24 teleost *TLR5* genes retrieved from public database (Table 7) were used for phylogeny reconstruction under Bayesian method. The tree showed that Cyprinodontiformes and Perciformes grouped together, then with Salmoniformes and Cypriniformes, respectively, with high posterior probability (PP) at all nodes. And *S.m-TLR5* together with three other salmonid fish were grouped into Salmoniformes with high PP values (Fig. 8).

Table 7
Taxonomy and accession numbers of fish *TLR5* genes used in this study.

Taxonomy	Common name	Species name	Accession Number
Holostei			
Semionotiformes	spotted gar	*Lepisosteus oculatus*	XM_006625986.2
Osteoglossomorpha			
Osteoglossiformes	Asian bonytongue	*Scleropages formosus*	XM_018743877.1
Protacanthopterygii			
Salmoniformes	Dolly Varden char	*Salvelinus malma*	MG490980
Salmoniformes	coho salmon	*Oncorhynchus kisutch*	XM_020496637.1
Salmoniformes	Atlantic salmon	*Salmo salar*	NM_001123691.1
Salmoniformes	Maraena whitefish	*Coregonus maraena*	LN610593.1
Ostariophsi			
Cypriniformes	common carp	*Cyprinus carpio*	LC150765.1
Cypriniformes	goldfish	*Carassius auratus*	KX759644.1
Cypriniformes	Wuchang bream	*Megalobrama amblycephala*	KX196271.1
Cypriniformes	snow trout	*Schizothorax richardsonii*	KF742778.3
Acanthopteygii			
Perciformes	suzuki	*Lateolabrax japonicus*	KY883372.1
Perciformes	large yellow croaker	*Larimichthys crocea*	XM_019267725.1
Perciformes	great blue-spotted mudskipper	*Boleophthalmus pectinirostris*	XM_020923598.1
Perciformes	black rockcod	*Notothenia coriiceps*	XM_010781754.1
Perciformes	Nile tilapia	*Oreochromis niloticus*	XM_019353524.1
Perciformes	Burton's mouthbrooder	*Haplochromis burtoni*	XM_005949079.2
Perciformes	zebra mbuna	*Maylandia zebra*	XM_004542335.3
Perciformes	suzuki	*Lateolabrax japonicus*	KY883372.1
Perciformes	large yellow croaker	*Larimichthys crocea*	XM_019267725.1
Perciformes	black rockcod	*Notothenia coriiceps*	XM_010781754.1
Cyprinodontiformes	guppy	*Poecilia reticulata*	XM_017302029.1
Cyprinodontiformes	Amazon molly	*Poecilia formosa*	XM_007567745.2
Cyprinodontiformes	turquoise killifish	*Nothobranchius furzeri*	XM_015948334.1
Cyprinodontiformes		*Nothobranchius korthause*	HAEB01016556.1
Cyprinodontiformes	sailfin molly	*Poecilia latipinna*	XM_015028358.1

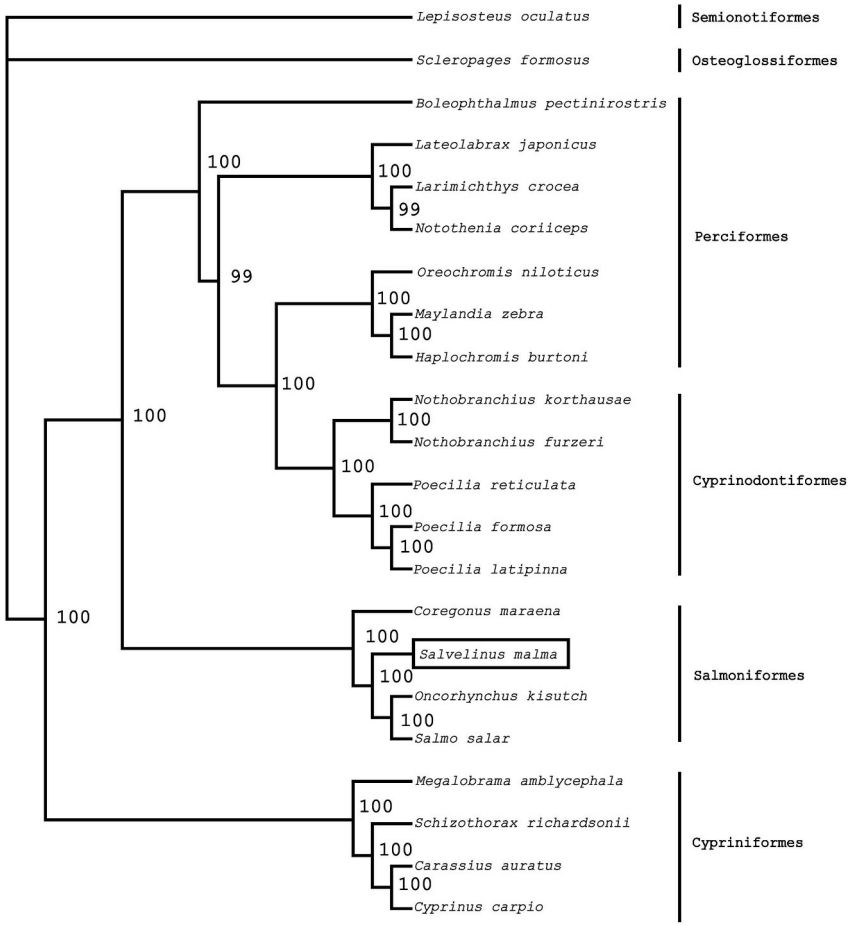

Fig. 8. The reconstructed Bayesian phylogeny of fish *TLR5* genes.
The posterior probabilities were shown after the lines, respectively. The taxonomy were listed in Table 7.

Table 8
The site, branch, and branch-site model tests on fish *C4* and *C6* genes.

Model	np[a]	-ln*L*[b]	Model comparison	LRT[c]	*P*-value[d]	Positive selected sites[e]
***C4* genes**						
Site-model						
M0	53	73468				
M1a	54	71981				
M2a	56	71942	M2a vs M1a	77	**0.0**	46S,**54S**,80L,86K,**87V**, 171Q,**523S**,596F,**1529K**
M3	57	71495	M3 vs M0	3945	**0.0**	None
M7	54	71491				
M8	56	71438	M8 vs M7	107	**0.0**	46S,**54S**,80L,86K,**87V**,171Q,523S,596F,1048W,1529K
Branch-model						
A:One-ratio	53	73468				
B:Free-ratio	103	73217	B VS A	500	**0.0**	Not analyzed
Branch-site model						
1:Null- Protacanthopterygii	55	71980				
2:Protacanthopterygii	56	71981	1 VS 2	12	**0.0**	None
3:Null-Ostariophysi	55	71981				
4:Ostariophysi	56	71958	3 VS 4	45	**0.0**	226A,357P,**583D**,659Q,866S,1024F
5:Null-Acanthopterygii	55	71962				
6:Acanthopterygii	56	71933	5 VS 6	58	**0.0**	247A,249V,266Q,560E,620D,770E,888R
***C6* genes**						
Site-model						
M0	34	22978				
M1a	35	22567				
M2a	37	22567	M2a vs M1a	0.0	1.0	Not allowed
M3	38	22385	M3 vs M0	1185	**0.0**	None
M7	35	22395				
M8	37	22383	M8 vs M7	24	**0.0**	266G
Branch-site model						
1:Null- *S. malma*	36	22565				
2:*S. malma*	37	22564	1 VS 2	0.235	0.313	Not allowed
3:Null-Cichlidae	36	22566				
4: Cichlidae	37	22563	3 VS 4	5.581	**0.009**	None
5:Null-Salmoninae	36	22567				
6: Salmoninae	37	22567	5 VS 6	0.0	1.0	Not allowed

Note:
[a] Number of parameters.
[b] Minus In [likelihood] value.
[c] Twice the difference of In[likelihood] between the two models compared.
[d] The *P*-value < 0.05 were shown in boldface.
[e] Only the sites with the posterior probabilities (PP) > 0.95 were shown and those with PP > 0.99 were in bold. The positively selected amino acids were shown with *Callorhinchus milii* as template.

3.6. Molecular evolutionary analysis

Firstly, the site model was used to explore whether there were positively selected sites among fish *C4* genes for their distinct taxonomic status and living environments. Among the three pairs of models, only M1a-M2a and M7-M8 pair were able to detect the positively selected sites among the species studied. The M7-M8 pair detected ten positively selected sites with PP > 0.95, nine of which were also found by the M1a-M2a pair (Table 8), indicating the positive selection on *C4* genes among fish. Secondly, the branch model was used to explore whether these positive selection events happened on particular fish lineage leading to the present or ancestral fish. The ω value of the one-ratio model which assumed of a uniform ω for all branches was estimated to be 0.322 which was significantly smaller than 1, indicating an overall strong purifying selection on fish *C4* gene. The free-ratio model that allowed different ω for each lineage showed a better fit than the one-ratio model, indicating a heterogeneous pressure among fish. Finally, the branch-site models were used to detect such sites among particular present and ancestral lineage (Table 8). There were six (226A, 357P, 583D, 659Q, 866S, and 1024F) and seven (247A, 249V, 266Q, 560E, 620D, 770E, and 888R) positively selected sites detected among the ancestral lineages leading to Ostariophysi and Acanthopterygii, respectively (Table 8). For *C6* genes, only one site was detected under positively selecting pressure by M7-M8 pair while none positive sites were detected by branch-site among Dolly Varden or other cold-water fish (Table 8).

Similarly, the molecular evolutionary analysis was carried out on fish *TLR3* and *TLR5* genes. Neither the site model nor the branch-site model detected any signals of positively selecting pressure. None of the two pairs (M1a-M2a and M7-M8) which were able to detect positive selection signals detected any sites under positive selection (Table 9), showing that transition to a land-locked life style for Chinese native Dolly Varden char did not result in the natural selection pressure for its *TLR3* and *TLR5* genes. For the lineages leading to present Salmoniformes, Perciformes, Cypriniformes, or Cyprinodontiformes, there were also no signals of positive selection pressure (Table 9), also indicating the strong purifying selection on *TLR3* and *TLR5* genes among fish.

3.7. Relative expression

To investigate the immune response of these two immune genes under bacterial challenge, the quantitative real-time PCR (qPCR) was carried out to explore the relative gene expression in liver and spleen within 96 h post injection. For the saline-injection group, namely the control group, *S.m-C4* gene showed no significant difference in liver at all the time points studied (Fig. 9A). For the liver of *A. hydrophila-*

Table 9
The site and branch-site model tests on fish *TLR3* and *TLR5* genes.

Model	np[a]	-ln*L*[b]	Model comparison	LRT[c]	*P*-value[d]	Positive selected sites[e]
***TLR3* genes**						
Site-model						
M0	51	30531				
M1a	52	29914				
M2a	54	29914	M2a and M1a	0.0	1.0	Not allowed
M3	47	28909	M3 and M0	1731.765	**0.0**	None
M7	52	29664				
M8	54	29663	M8 and M7	2.45	0.2056	Not allowed
Branch-site model						
1. Null-*S. malma*	53	37881				
2. *S. malma*	54	37633	1 VS 2	495.174	**0.0**	None
3. Null-Perciformes	53	37554				
4. Perciformes	54	36073	3 VS 4	2962.012	**0.0**	None
5.Null-Cypriniformes	53	38128				
6. Cypriniformes	54	38087	5 VS 6	83.321	**0.0**	None
7.Null-Cyprinodontiformes	53	38154				
8.Cyprinodontiformes	54	39410	7 VS 8	2513.134	**0.0**	None
***TLR5* genes**						
Site-model						
M0	43	29879				
M1a	44	29073				
M2a	46	29073	M2a and M1a	0.0	1.0	Not allowed
M3	47	28908	M3 and M0	1942.013	**0.0**	None
M7	44	28901				
M8	46	28888	M8 and M7	25.829	**2.0E-6**	None
Branch-site model						
1. Null-*S. malma*	45	35180				
2. *S. malma*	46	35180	1 VS 2	0.0997	0.752	Not allowed
3.Null-Salmoniformes	45	35181				
4. Salmoniformes	46	35181	3 VS 4	0.0	1.0	Not allowed
5. Null-Perciformes	45	35181				
6. Perciformes	46	35181	5 VS 6	0.0	1.0	Not allowed
7.Null-Cypriniformes	45	35178				
8. Cypriniformes	46	35176	7 VS 8	3.15	0.0759	Not allowed
9.Null-Cyprinodontiformes	45	35181				
10.Cyprinodontiformes	46	35181	9 VS 10	3.0E-6	0.999	Not allowed

Note: parameters are the same as those in Table 7.

injection group, namely the experimental group, showed a significant increase at 24 h ($p = 0.04$) and two peaks at 12 h and 72 h with marginal significant *p*-value 0.16 and 0.19, respectively. And the experimental group showed significant differences with the control group at 6 h, 12 h, 24 h, and 72 h, with *p*-value of 0.032, 0.028, 0.006, and 0.035, respectively. In spleen, the relative expression of *S.m-C4* gene in the control group just fluctuated up and down, showing no significant differences among the eight time points while for the experimental group, *S.m-C4* gene also showed no difference along time points except for 72 h, having a 59.6% decrease comparing with 0 h of the bacterial injection group (Fig. 9B).

For *S.m-TLR5* gene, there was also no change in liver for *TLR5* expression at all time points checked of the control group while for *A. hydrophila*-injection group, the relative expression of *S.m-TLR5* just fluctuated at the first day and then climaxed at 36 h and reached rock bottom at 72 h, respectively. In addition, there were significant differences between the control and experimental group at 36 h, 48 h and 72h (Fig. 10A). Furthermore, there was also no significant change in spleen, just as those of the liver tissues, for the control group. But it showed a huge difference for the bacterial injection group. It significantly increased at 6 h and then rapidly climaxed at 12 h. From 24 h post injection, the relative expression of *S.m-TLR5* became more and more lower comparing with 12 h in the spleen. Finally, the *S.m-TLR5* even dropped beneath the baseline at 96 h (Fig. 10B).

4. Discussion

The Chinese native Dolly Varden chars (*Salvelinus malma*) are locked in land and live in fresh water lifetime. As a result, these land-locked freshwater Dolly Varden chars showed a distinct relationship with other anadromous Dolly Varden chars [31], indicating the adaptions to different salinities among Dolly Varden char populations. To explore the effect of freshwater adaption on its immune system, we studied the cDNA library of pooled hepatopancreas and spleen of Chinese native freshwater Dolly Varden char (*S. malma*) under bacterial challenge. To reduce the redundancy rate and to increase the representation of lower abundance transcripts, the full-length normalized cDNA library is an efficient method for discovering genes with complete coding sequences. It had been applied in fish, such as yellow catfish [58], shark [59], Indian catfish [60], and large yellow croaker [61]. In the present study, a normalized full-length cDNA library was constructed from Chinese native freshwater Dolly Varden char (*S. malma*) under bacterial challenge. After quality control, a total of 27,829 unigenes were generated from 31,233 high-quality transcripts and identified 24,541 open reading frames (ORF), in which 17,670 were complete.

All of the 27,829 unigenes were used for function enrichment and classification analysis, resulting 25,809 unigenes annotated. The top hit species distribution based on the NCBI-Nr database showed that 52.04% unigenes were similar to *Oncorhynchus mykiss*, followed by *Salmo salar* (23.53%), indicating the high similarities with other Salmonidae fish for Chinese native freshwater Dolly Varden char.

The alignment in KEGG database successfully classified 16,917 unigenes into 271 biological pathways. For the immune-relevant KEGG pathways, 106 genes were found to be involved in cytokine-cytokine receptor interaction, and 51 genes in toll-like receptor signal pathway (see Supplemental Fig. 2). As another important member of native immune system, only three genes were identified in complement and coagulation cascades (see Supplemental Fig. 3). Other relevant

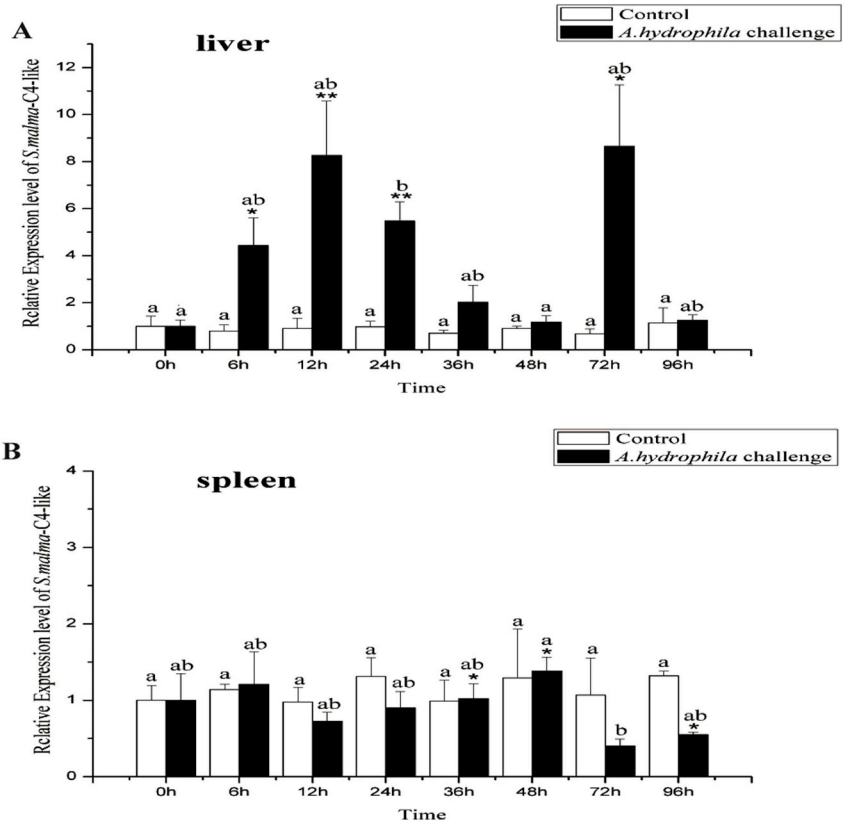

Fig. 9. Relative expression of *S.m-C4* gene in liver (A) and spleen (B) under *Aeromonas hydrophila* or saline challenge. Samples were collected at 0, 6, 12, 24, 36, 48, 72, 96 h after challenge. Relative expression, with β-actin as internal control, was showed as fold change over those of the corresponding 0 h time point. Bars represented the mean ± SD for each sample. Significant ($P < 0.05$) and extremely significant($P < 0.01$) differences, calculated by student's *t*-test, between the challenged group and the control group at the same sampling time were indicated by one or two asterisks above the bars, respectively. Significant differences ($P < 0.05$) calculated by ANOVA between the 0 h and those sampled at other time points were indicated by lowercase letters in *A. hydrophila*-challenged group or the control group, respectively.

pathways were concentrated on regulation of autophagy (16 genes), lysosome (58 genes), peroxisome (59 genes), and apoptosis (45 genes). Furthermore, genes involved in adaptive immunity were also identified, such as intestinal immune network for IgA production (20 genes), T cell receptor signaling pathway (3 genes), B cell receptor signaling pathway (4 genes), and antigen processing and presentation (4 genes), indicating the relative more important role of native immunity for fish liver and spleen under acute bacterial challenging.

So next, we chose two native immune genes in this cDNA library as the representative genes, namely the complement system (*S.m-C4* gene) and toll-like receptor (*S.m-TLR5* gene), respectively. Complement is one important part of the innate immune system, well known for the ability of detecting infection. C4 is an important part of C3 and C5 converting enzymes in the classical and lectin pathway [62]. The *S.m-C4* cDNA sequence was predicted to have a 5160-bp-in-length ORF, encoding 1719 amino acid residues, sharing conserved structural characteristics with other C4 proteins, including the thioester site GCGEQ, α-β linker sequence RXXR and the α-γ linker sequence RRXR (Fig. 5), from where mammalian C4 protein precursors were sliced into three chains before secretion [63,64]. Furthermore, a phylogenetic tree was constructed based on nucleotide sequences of teleost *C4* genes (Fig. 7), and it showed high posteriori probability (PP). The overall topology of tree was consistent with traditional taxonomy and phylogenetic transition. This consistency on the structure and phylogeny suggested that S.m-C4 proteins probably possessed the basic biochemical function during evolution.

Previous studies showed that *C4* and *C3* genes of fish had the highest baseline expression in tissues associated with adaptive immunity, such as liver and spleen, in *Oncorhynchus mykiss* [65,66], *Gadus morhua* [67] and *Hippoglossus hippoglossus* [68]. So the liver and spleen were used to explore immune response of *S.m-C4* gene during *A. hydrophila* injection for being important immune organs [69]. When challenged by *A. hydrophila*, *S.m-C4* gene up-regulated in liver and climaxed at 12 h post injection and then decreased to the baseline at 48 h post injection while it only decreased at 72 h post injection in spleen. It showed similar *C4* responses against bacterial injection as those observed in *Miichthys miiuy* [70] and *Larimichthys crocea* [71]. For the control group, *S.m-C4* gene just fluctuated along the baseline in both liver and spleen tissues. These results supported that liver was the major site of complement C4 synthesis and that S.m-C4 preserved its role against acute bacterial infection [65,72].

Considering the special status of fish during the evolutionary process of their immunity, it was meaningful to compare their innate immune defense mechanisms. Therefore, molecular evolutionary analysis was conducted to detect the possibly positive selection pressure on fish considering their distinct different living environments and taxonomic status. Except for *S.m-C4* sequence, the coding sequences of *C4* gene of other twenty-six fishes, including two cartilaginous fishes and twenty-four teleost fish species, were retrieved from public database. Firstly, the M0 in site model demonstrated that fish *C4* gene had been predominantly subject to purifying selection with low estimate of ω ($\omega = 0.322$) for all branches. Secondly, the free-ratio model showed a significantly better fit to our data than the one-ratio model ($p < 0.001$, Table 8), indicated that some lineages were under heterogeneous selective pressure. Thirdly, in site model, nine and ten sites were detected under positive selection pressure with PP values larger than 0.95 by M2a and M8, respectively. Fourthly, the branch-site model was used to detect the possible positive selection that affected particular lineage, and the signature of positive selection was detected in the lineage leading to Ostariophysi and Acanthopterygii with six and seven positive selection sites (Table 8), respectively, indicating the positive selection pressure on these two groups of fish in the early evolutionary history, of which are mainly distributed in fresh water and sea water, respectively. The detected positive sites were concentrated in the A2M_N_2 and ANATO domains of Ostariophysi and in the A2M_N_2 and A2M domains of Acanthopterygii ancestral branch, respectively. A2M is involved in physical entrapment of the target protein [73] by covalently binding its reactive internal thiol ester bond to the target protease [74]. When

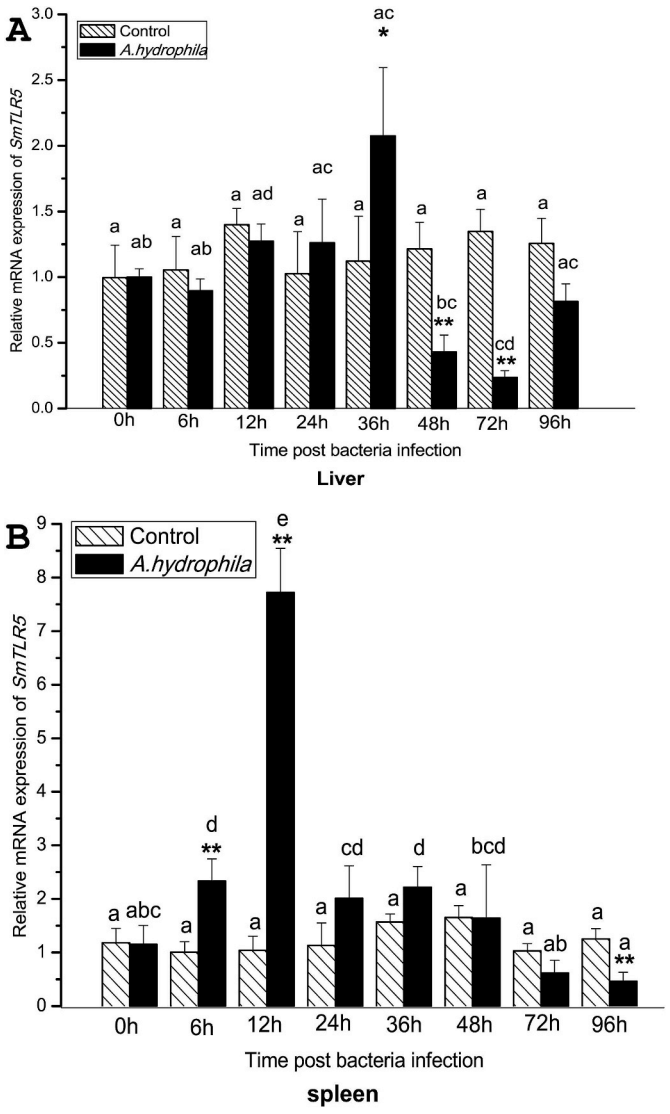

Fig. 10. Relative expression of *S.m-TLR5* in liver (A) and spleen (B) under *Aeromonas hydrophila* or saline challenge. More details see Fig. 9.

infection occurs locally, the cleavage products of complement can enhance capillary permeability, thus causing leukocytes to reach the inflammatory site, C3a, C4a and C5a had the role of inflammatory mediators which eventually increased inflammatory response [75]. Therefore, it was speculated that the A2M domain of C4 molecule was adapted to the environment in Ostariophysi and Acanthopterygii fishes. And those positively selected sites detected in A2M and A2M_N_2 domains might affect the teleost *C4* gene and cause the change in bonding ability to the target molecule, which with no doubt needs more protein experimental evidence to make further explore of the functions of this gene and its participate in selective mechanism. Similarly, we also checked another complement system gene, namely *C6* gene, the site model only detected one site (266G) under positively selecting pressure (Table 8), indicating the possible nature selecting pressure on fish complement component 6 as those on fish *C4* genes. For 266G, it located between LDLα domain (140–171) and MACPF (membrane-attack complex/perforin, 317–521) domain and in a segment of sequences with low complexity. It needs further investigation to explorer its detailed function in fish.

Then we screened and verified the *TLR5* sequence (*S.m-TLR5*) from Chinese native freshwater Dolly Varden char. Its ORF was 2637-bp in length, encoding 879 amino acids. The amino acid sequences contained the conserved typical structure of TLR family: extracellular LRR domain, TM domain and intracellular TIR domain, and there were four conserved cysteine residues in the LRR-CT. These conservation in domain structure implied the conservation of S.m-TLR5 protein which is a bacterial ligand sensor who used its extracellular LRR domains for identifying pathogens components [76]. The qPCR experiment showed no significant difference of *S.m-TLR5* expression in the control group, indicating no effect on *TLR5* expression for saline. But for bacterial injection, liver tissues showed an increase until 36 h but with no significant difference, followed by a sharply decrease at 48 h and 72 h. In spleen tissues, it showed the same pattern, but the increase and decrease appeared 24 h earlier than those in liver, indicating that the spleen involved earlier in immune response against bacterial invasion than liver for Chinese native freshwater Dolly Varden char in terms of *TLR5* gene. This pattern of expression is similar to *Cirrhinus mrigala* [77] and *Lateolabrax japonicas* [78], showing the important role of liver and spleen against acute bacterial invasion. While neither the site nor the branch-site model detected sites under positively selecting pressure, showing strong purifying pressure among teleost *TLR3* and *TLR5* genes. This indicated that the huge difference in taxonomy and living environment did not cause the positive evolution pressure on fish *TLR3* and *TLR5* genes (Table 9), but a strong pressure to maintain the function of TLR3 and TLR5 proteins for recognizing bacterial flagella [76]. To understand the detailed molecular evolutionary patterns of fish toll-like receptors and complement components, it needs a more comprehensive investigation on fish TLR and complement component genes.

In summary, we reported a pooled hepatopancreas and spleen cDNA library under bacterial challenge for Chinese native freshwater Dolly Varden char (*Salvelinus malma*). It successfully annotated and classified more native than adaptive immunity-associated genes and more genes involved in toll-like receptor signal pathway than in complement and coagulation cascades, implying the relative more important role of toll-like receptors than the complement system under acute bacterial injection for the Chinese native freshwater Dolly Varden char. It is a valuable resource for future studies of the land-locked Chinese native Dolly Varden char genomics, as well as genetic variation and molecular assisted selective breeding in the future, and will also benefit researches in other closely related cold-water fish with significantly aquaculture importance.

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Appendix A. Supplementary data

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