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# Four lysozymes (one c-type and three g-type) in catfish are drastically but differentially induced after bacterial infection

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ABSTRACT

Lysozyme is an important component of the innate immune system. In this study, four lysozyme genes including one c-type lysozyme and three g-type lysozymes were identified from channel catfish (*Ictalurus punctatus*). The lysozyme genes are highly conserved in their structural features as compared to those from other species. Phylogenetic analyses were conducted allowing annotation of these genes. Additional analyses using conserved syntenies allowed determination of orthologies for the c-type lysozyme. Phylogenetic analysis indicated that the g-type lysozyme may have gone through species-specific gene duplications leading to multiple copies in some teleost species. Channel catfish possessed three copies of the g-type lysozyme genes. Expression analysis revealed that the catfish lysozyme genes were expressed in a broad range of tissues. The highest levels of expression were found in head kidney, liver, spleen, and trunk kidney, compatible with the immune functions of these tissues/organs. The c-type and g-type lysozymes were drastically induced after bacterial infection, but exhibited large differences in the extent of induction and the tissue with the highest level of induction, with the g-type lysozyme being most highly induced in the head kidney whereas the other three lysozymes being most highly induced in the liver, suggesting their cooperative actions in the immune responses but difference in their detailed functions.



## 1. Introduction

The innate immune defense system is the first line of defense against invading pathogens. In lower vertebrates such as teleost fish, the innate immunity is even more important in defense responses as it makes a difference between life and death before adaptive immunity get activated by the invasive agents, which often takes a couple of weeks or longer to be fully activated [1,2]. Many components make up the innate immune system including physical barriers, phagocytes, inflammation-related serum proteins, surface and phagocyte granule antimicrobial peptides and lyzosomal systems that degrade the invading agents [1,3].

Lysozymes are a group of enzymes that ubiquitously exist in major taxa of living organisms. They collectively serve as catalytic enzymes to hydrolyze 1, 4-beta-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid in peptidoglycan heteropolymers of bacterial cell walls, thereby serving as innate immune guards against bacterial invasions [3,4]. To date, six types of lysozymes have been reported based on their structures including chicken-type lysozyme (c-type lysozyme), goose-type lysozyme (g-type lysozyme), invertebrate-type lysozyme (i-type lysozyme), phage lysozyme, bacterial lysozyme and plant lysozyme [5]. Of the six types of lysozymes, three types, i.e., the c-type, g-type, and i-type lysozymes, have been identified from animals [6]. In teleost fish and related lower vertebrates, only c-type and g-type lysozymes have been reported [1,2]. Although the c-type and g-type lysozymes are similar in three-dimensional structures, they share little identity at the amino acid level of primary structures and differ in genetic organization, catalytic mechanisms and enzymatic properties [7,8]. It was reported that the function of the c-type lysozyme rely more on the structural factors than on its enzymatic activities [9,10], and that the c-type lysozyme destroyed the AIDS virus with its charge, rather than with its lytic activities [10].

Much research has been conducted with both c-type and g-type lysozymes in teleost fish because of their importance in innate immunity. The first fish c-type lysozyme was reported in rainbow trout [11]. Since then, c-type lysozyme has been identified from a number of fish species including *Danio rerio*, *Oryzias latipes*, *Cyprinus carpio*, *Oreochromis aureus*, *Perca flavescens*, *Takifugu rubripes*, *Scophthalmus rhombus*, *Scophthalmus maximus*,

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*Solea senegalensis*, *Paralichthys olivaceus*, *Salmo salar*, *Ctenopharyngodon idellus*, *Epinephelus coioides*, *Epinephelus bruneus*, and *Esox lucius* [12–16]. Similarly, the first fish g-type lysozyme was identified from *C. carpio*, and since then a large number of fish g-type lysozymes have been identified from several fish species [17–21]. In a number of cases, up-regulation of the c-type and g-type lysozymes after bacterial infection was reported. For instance, in half-smooth tongue sole, the g-type lysozyme was up-regulated in blood, head kidney, liver and spleen at 12 h after infection with *Vibrio anguillarum* [19]. Similarly, in Japanese flounders both the c-type and the g-type lysozymes were induced after infection with *Edwardsiella tarda*, in spite of some tissue specificity of the gene induction, with the c-type more induced in the head kidney, spleen and ovary while g-type more induced in intestine, heart and whole blood [12]. In grass carp, the g-type lysozyme was significantly up-regulated in the liver and spleen after infection with *Aeromonas hydrophila*, while the c-type lysozyme was up-regulated in the gill [15]. Channel catfish, *Ictalurus punctatus*, is the most important aquaculture species in the United States [22]. Its immune system is perhaps the best characterized among teleost fish [23–29]. In recent years, efforts have been devoted to characterize the innate immune genes such as chemokines [30,31], antimicrobial peptides [32–35], pathogen recognition receptors [36–41], lectins [42], and other innate immunity-related genes [43–47]. In this study, our goal was to characterize lysozyme genes and their expression after bacterial infection in catfish. Here we report four catfish lysozyme genes including one c-type and three g-type lysozyme genes, and their expression profiles in various healthy tissues and after infection with *Edwardsiella ictaluri*.

## 2. Materials and methods

### 2.1. Database mining and sequence analysis

In order to identify the lysozyme genes from catfish, the transcriptome database [48,49] and the draft whole genome sequence database of catfish (unpublished) were searched using all available zebrafish lysozymes as queries. The transcriptome database was generated from a transcriptome sequencing of a doubled haploid channel catfish which harbors two identical sets of chromosomes. Therefore, the assembly was efficiently and accurately assembled because of no allelic variations [49]. TBLASTN was used to obtain the initial pool of lysozyme gene sequences, with a cutoff E-value of $1e^{-5}$. Upon identification of the initial pool of catfish lysozyme-related sequences, sequences were aligned to delete the repeated entries and a unique set of sequences were subjected to further analysis. In order to further confirm the candidate cDNA sequences, the cDNA sequences obtained from RNA-seq database were confirmed by comparison with the draft catfish whole genome sequence database by BLASTN with cutoff E-value of $1e^{-10}$. The retrieved genome sequences were subjected to *ab initio* gene prediction by GENSCAN (http://genes.mit.edu/GENSCAN.html). The resulted protein sequences were further verified by BLASTP against NCBI nr database. Only the cDNA sequences which passed these two steps of validation were kept. The open reading frame (ORF) was identified by using ORF finder (http://www.ncbi.nlm.nih.gov). The predicted ORFs were verified by BLASTP against NCBI non-redundant (nr) protein sequence database.

**Table 1**
Primers used in this study.

| Primer name | Primer sequence 5′–3′ |
|---|---|
| 18S-F | GAGAAACGGCTACCACATCC |
| 18S-R | GATACGCTCATTCCGATTACAG |
| Lyc-F | GATGGATCAACGGACTATG |
| Lyc-R | CTGTCTCACTATGGTCTTG |
| Lyg-F | CATCGGAAATAACAGCCAAG |
| Lyg-R | TCTCTGGATATAATGCCTGC |
| Lygl1-F | CACACAAGGCACTGAGATT |
| Lygl1-R | CATAATCATTTCCCGTGGTC |
| Lygl2-F | TCGCAGGTATCATATCCAG |
| Lygl2-R | GAAGCCGATTAGAATCTCTG |

### 2.2. Phylogenetic and structural analysis

The amino acid sequences of lysozyme genes from various organisms along the evolutionary spectrum were selected and retrieved from NCBI databases to conduct phylogenetic analysis including those from human, mouse, rat, chicken, goose, frog, and various fish species including zebrafish, tilapia, rainbow trout, Japanese flounder, medaka, fugu, common carp, grass carp, northern pike, sole, Atlantic salmon, orange-spotted grouper, brill, and turbot. The amino acid sequences were aligned using CLUSTALW2 [50]. Phylogenetic analyses were performed with MEGA 5.1 using the neighbor-joining method [40]. Bootstrapping with 10,000 replications were conducted to evaluate the phylogenetic tree [51].

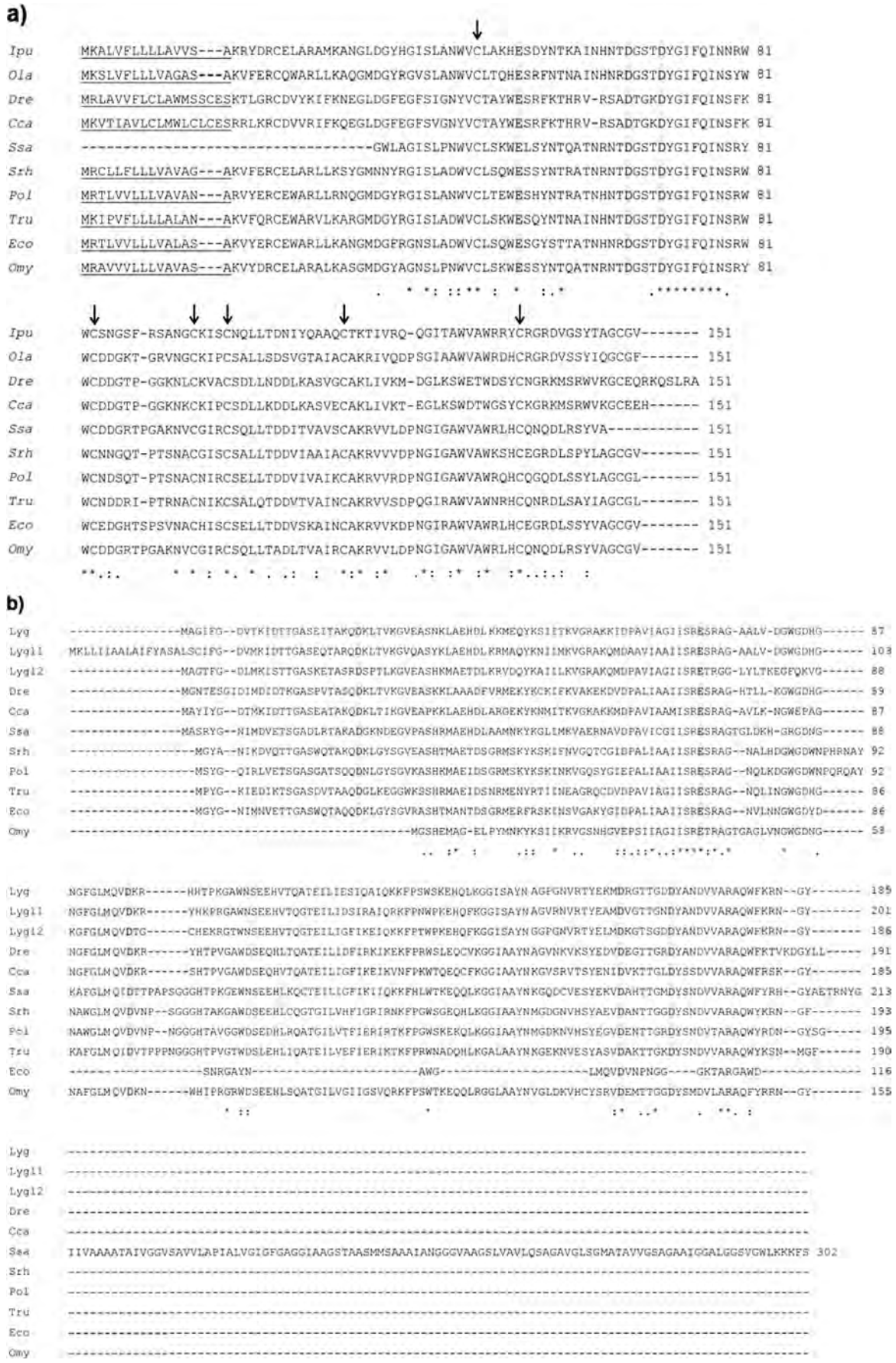
Signal peptides of the lysozymes were determined by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP). Expert Protein Analysis System (http://www.expasy.org) was used to identify N-glycosylation sites. The physical and chemical characteristics of lysozymes were analyzed with the Predict protein database (http://www.predictprotein.org/). The 3-dimensional and secondary structure models of the lysozymes were constructed by using 3D-JIGSAW (http://bmm.cancerresearchuk.org/w3djigsaw/) and Geneious 6.0.5 (http://www.geneious.com/), respectively.

### 2.3. Syntenic analysis

In order to provide additional evidence for gene identification using phylogenetic analysis, syntenic analysis was conducted by examination of neighboring genes in the same genomic location. Briefly, the deduced catfish c-type lysozyme amino acid sequences were used as query to search the draft catfish genome sequences to obtain the genomic scaffold containing the catfish lysozyme genes. The contig was retrieved and then used to build the scaffold with large insert mate paired reads. The scaffold containing the catfish

**Table 2**
Structure features of the *Ictalurus punctatus* c-type and g-type lysozymes.

| | g-Type lysozymes | | | c-Type lysozymes |
|---|---|---|---|---|
| | Lyg | Lygl1 | Lygl2 | Lyc |
| cDNA length (bp) | 944 | 833 | 985 | 719 |
| ORF length (bp) | 558 | 606 | 561 | 429 |
| 5′-UTR (bp) | 42 | 87 | 111 | 41 |
| 3′-UTR (bp) | 317 | 140 | 313 | 249 |
| Mature peptide AA | 185 | 201 | 186 | 142 |
| Mature peptide MW (KD) | 20.2 | 22.3 | 20.4 | 15.8 |
| Number of α-helices | 9 | 11 | 10 | 5 |
| Number of β-sheets | 11 | 12 | 14 | 9 |
| Number of γ-turns | 14 | 12 | 14 | 12 |
| Number of coils | 14 | 14 | 13 | 6 |
| Theoretical pI | 9.15 | 9.56 | 9.32 | 9.22 |
| Predicted disulfide bonds | 0 | 0 | 0 | 4 |
| Number of N-glycosylation sites | 4 (NTDG, NQLL, NVRT, NIYQ) | 4 (NIIM, NSEE, NAGV, NVRT) | 1 (NVRT) | 3 (NTDG, NQLL, NIYQ) |


a)
Ipu MKALVFLLLLAVVS---AKRYDRCELARAMKANGLDGYHGISLANWVCLAKHESDYNTKAINHNTDGSTDYGIFQINNRW 81
Ola MKSLVFLLLVAGAS---AKVFERCQWARLLKAQGMDGYRGVSLANWVCLTQHESRFNTNAINHNRDGSTDYGIFQINSYW 81
Dre MRLAVVFLCLAWMSSCESKTLGRCDVYKIFKNEGLDGFEGFSIGNYVCTAYWESRFKTHRV-RSADTGKDYGIFQINSFK 81
Cca MKVTIAVLCLMWLCLCESRRLKRCDVVRIFKQEGLDGFEGFSVGNYVCTAYWESRFKTHRV-RSADTGKDYGIFQINSFK 81
Ssa ----------GWLAGISLPNWVCLSKWELSYNTQATNRNTDGSTDYGIFQINSRY 81
Srh MRCLLFLLLVAVAG---AKVFERCELARLLKSYGMNNYRGISLADWVCLSQWESSYNTRATNRNTDGSTDYGIFQINSRW 81
Pol MRTLVVLLLVAVAN---ARVYERCEWARLLRNQGMDGYRGISLANWVCLTEWESHYNTRATNHNTDGSTDYGIFQINSRW 81
Tru MKIPVFLLLLALAN---AKVFQRCEWARVLKARGMDGYRGISLADWVCLSKWESQYNTNAINHNTDGSTDYGIFQINSRW 81
Eco MRTLVVLLLVALAS---AKVYERCEWARLLKANGMDGFRGNSLADWVCLSQWESGYSTTATNHNRDGSTDYGIFQINSRW 81
Omy MRAVVVLLLVAVAS---AKVYDRCELARALKASGMDGYAGNSLPNWVCLSKWESSYNTQATNRNTDGSTDYGIFQINSRY 81
WCSNGSF-RSANGCKISCNQLLTDNIYQAAQCTKTIVRQ-QGITAWVAWRRYCRGRDVGSYTAGCGV------- 151
WCDDGKT-GRVNGCKIPCSALLSDSVGTAIACAKRIVQDPSGIAAWVAWRDHCRGRDVSSYIQGCGF------- 151
WCDDGTP-GGKNLCKVACSDLLNDDLKASVGCAKLIVKM-DGLKSWETWDSYCNGRKMSRWVKGCEQRKQSLRA 151
WCDDGTP-GGKNKCKIPCSDLLKDDLKASVECAKLIVKT-EGLKSWDTWGSYCKGRKMSRWVKGCEEH------ 151
WCDDGRTPGAKNVCGIRCSQLLTDDITVAVSCAKRVVLDPNGIGAWVAWRLHCQNQDLRSYVA----------- 151
WCNNGQT-PTSNACGISCSALLTDDVIAAIACAKRVVVDPNGIGAWVAWKSHCEGRDLSPYLAGCGV------- 151
WCNDSQT-PTSNACNIRCSELLTDDVIVAIKCAKRVVRDPNGIGAWVAWRQHCQGQDLSSYLAGCGL------- 151
WCNDDRI-PTRNACNIKCSALQTDDVTVAINCAKRVVSDPQGIRAWVAWNRHCQNRDLSAYIAGCGL------- 151
WCEDGHTSPSVNACHISCSELLTDDVSKAINCAKRVVKDPNGIRAWVAWRLHCEGRDLSSYVAGCGV------- 151
WCDDGRTPGAKNVCGIRCSQLLTADLTVAIRCAKRVVLDPNGIGAWVAWRLHCQNQDLRSYVAGCGV------- 151
b)
Lyg ----------MAGIFG--DVTKIDTTGASEITAKQDKLTVKGVEASNKLAEHDLKKMEQYKSIITKVGRAKKIDPAVIAGIISRESRAG-AALV-DGWGDNG------ 87
Lygl1 MKLLIIAALAIFYASALSCIFG--DVMKIDTTGASEQTARQDKLTVKGVQASYKLAEHDLKRMAQYKNIIMKVGRAKQMDAAVIAAIISRESRAG-AALV-DGWGDNG------ 103
Lygl2 ----------MAGTFG--DLMKISTTGASKETASRDSPTLKGVEASHKMAETDLKRVDQYKAIILKVGRAKQMDPAVIAGIISRETRGG-LYLTKEGFQKVG------ 88
Dre ----------MGNTESGIDIMDIDTKGASPVTASQDKLTVKGVEASKKLAAADFVRMEKYKCKIFKVAKEKDVDPALIAAIISRESRAG-HTLL-KGWGDHG------ 89
Cca ----------MAYIYG--DTMKIDTTGASEATAKQDKLTIKGVEAPKKLAEHDLARGEKYKNMITKVGKAKKMDPAVIAAMISRESRAG-AVLK-NGWEPAG------ 87
Ssa ----------MASRYG--NIMDVETSGADLRTARADGKNDEGVPASHRMAEHDLAAMNKYKGLIMKVAERNAVDPAVICGIISRESRAGTGLDKH-GRGDNG------ 88
Srh ----------MGYA--NIKDVQTTGASWQTAKQDKLGYSGVEASHTMAETDSGRMSKYKSKIFNVGQTCGIDPALIAAIISRESRAG--NALHDGWGDWNPHRNAY 92
Pol ----------MSYG--QIRLVETSGASGATSQQDNLGYSGVKASHKMAEIDSGRMSKYKSKINKVGQSYGIEPALIAAIISRESRAG--NQLKDGWGDWNPQRQAY 92
Tru ----------MPYG--KIEDIKTSGASDVTAAQDGLKEGGWKSSHRMAEIDSNRMENYRTIINEAGRQCDVDPAVIAGIISRESRAG--NQLINGWGDNG------ 86
Eco ----------MGYG--NIMNVETTGASWQTAQQDKLGYSGVRASHTMANTDSGRMERFRSKINSVGAKYGIDPALIAAIISRESRAG--NVLNNGWGDYD------ 86
Omy ----------MGSHEMAG-ELPYMNKYKSIIKRVGSNHGVEPSIIAGIISRETRAGTGAGLVNGWGDNG------ 58
NGFGLMQVDKR------HHTPKGAWNSEEHVTQATEILIESIQAIQKKFPSWSKEHQLKGGISAYNAGPGNVRTYEKMDRGTTGDDYANDVVARAQWFKRN--GY------- 185
NGFGLMQVDKR------YHKPRGAWNSEEHVTQGTEILIDSIRAIQRKFPNWPKEHQFKGGISAYNAGVRNVRTYEAMDVGTTGNDYANDVVARAQWFKRN--GY------- 201
KGFGLMQVDTG------CHEKRGTWNSEEHVTQGTEILIGFIKEIQKKFPTWPKEHQFKGGISAYNGGPGNVRTYELMDKGTSGDDYANDVVARAQWFKRN--GY------- 186
NGFGLMQVDKR------YHTPVGAWDSEQHLTQATEILIDFIRKIKEKFPRWSLEQCVKGGIAAYNAGVNKVKSYEDVDEGTTGRDYANDVVARAQWFKTVKDGYLL----- 191
NGFGLMQVDKR------SHTPVGAWDSEQHVTQATEILIGFIKEIKVNFPKWTQEQCFKGGIAAYNKGVSRVTSYENIDVKTTGLDYSSDVVARAQWFRSK--GY------- 185
KAFGLMQIDTTPAPSGGGHTPKGEWNSEEHLKQCTEILIGFIKIIQKKFHLWTKEQQLKGGIAAYNKGQDCVESYEKVDAHTTGMDYSNDVVARAQWFYRH--GYAETRNYG 213
NAWGLMQVDVNP--SGGGHTAKGAWDSEEHLCQGTGILVHFIGRIRNKFPGWSGEQHLKGGIAAYNMGDGNVHSYAEVDANTTGGDYSNDVVARAQWYKRN--GF------- 193
NAWGLMQVDVNP--NGGGHTAVGGWDSEDHLRQATGILVTFIERIRTKFPGWSKEKQLKGGIAAYNMGDKNVHSYEGVDENTTGRDYSNDVTARAQWYRDN--GYSG----- 195
KAFGLMQIDVTPPPNGGGHTPVGTWDSLEHLIQATEILVEFIERIKTKFPRWNADQHLKGALAAYNKGEKNVESYASVDAKTTGKDYSNDVVARAQWYKSN--MGF------ 190
Eco ------------SNRGAYN----------AWG----------LMQVDVNPNGG----GKTARGAWD---------- 116
NAFGLMQVDKN------WHIPRGRWDSEENLSQATGILVGIIGSVQRKFPSWTKEQQLRGGLAAYNVGLDKVHCYSRVDEMTTGGDYSMDVLARAQFYRRN--GY------- 155
Lyg ----------
Lygl1 ----------
Lygl2 ----------
Dre ----------
Cca ----------
Ssa IIVAAAATAIVGGVSAVVLAPIALVGIGFGAGGIAAGSTAASMMSAAAIANGGGVAAGSLVAVLQSAGAVGLSGMATAVVGSAGAAIGGALGGSVGWLKKKFS 302
Srh ----------
Pol ----------
Tru ----------
Eco ----------
Omy ----------

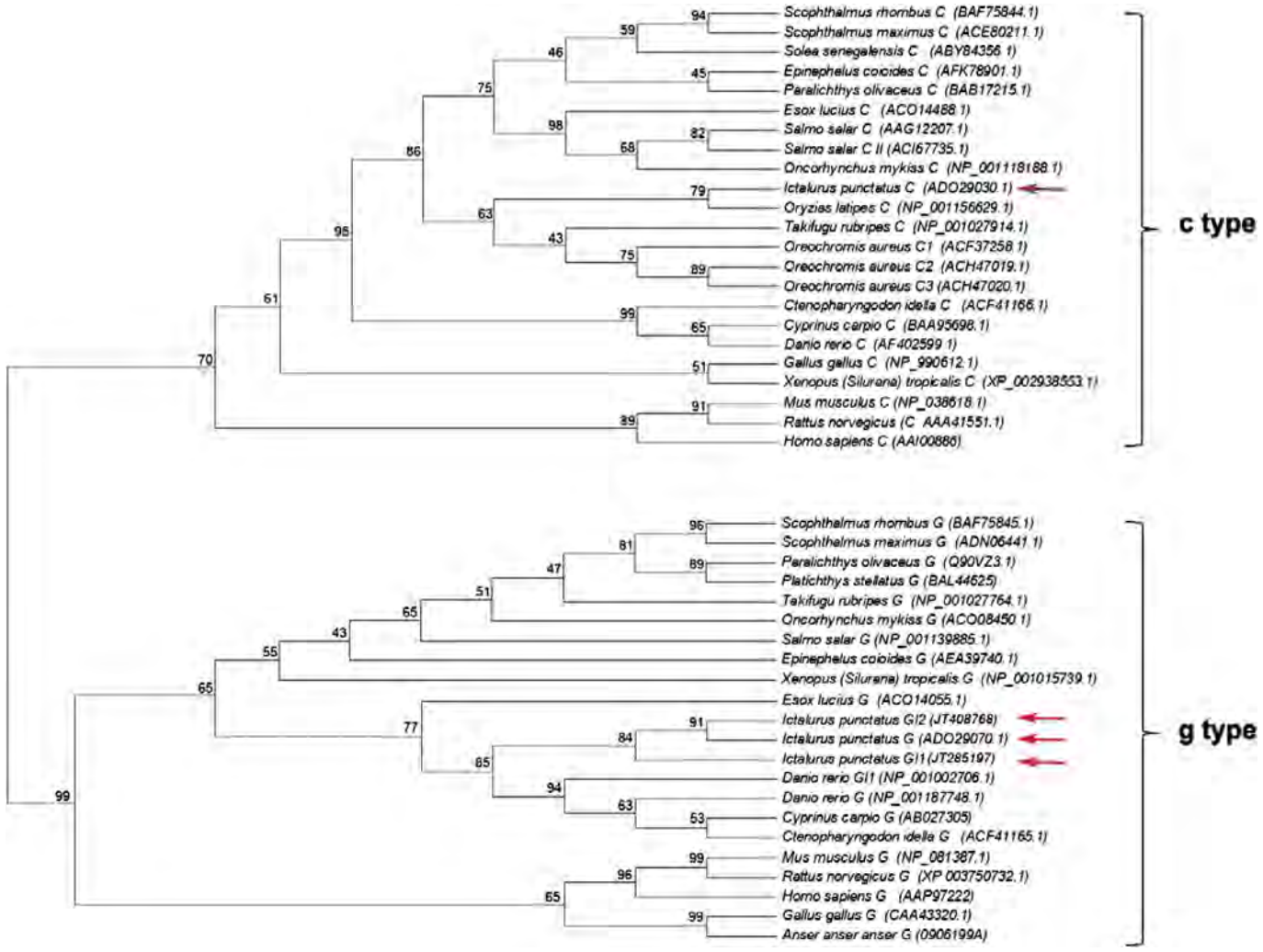



**Fig. 2.** Phylogenetic tree for the c-type and g-type lysozyme genes. The phylogenetic tree was constructed based on the amino acid sequences of c-type and g-type lysozymes from selected species of fish, birds, and mammals, using the neighbor-joining method in MEGA 5.1. The accession numbers of the protein sequences followed the name of the species. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor-joining tree was evaluated by 10,000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes.

c-type lysozyme gene is approximately 30 Kb. After the assembly of the scaffold, the neighboring genes were identified by GENESCAN (http://genes.mit.edu/GENSCAN.html) and BLASTP. The order of these neighboring genes was compared with those from other fish species including medaka, fugu, zebrafish, stickleback and tilapia.

### 2.4. Bacterial challenge and sample collection

Enteric septicemia of catfish (ESC) bacterial challenge was conducted as previously described [44]. Briefly, the bacteria were isolated from a single colony and cultured in Brain Heart Infusion broth (BHI) and incubated in a shaker incubator at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 μl of 10-fold serial dilutions onto BHI agar plates. The bacterial culture was diluted with PBS (pH 7.4), and $1 \times 10^5$ CFU of bacteria in 100 μl PBS were injected intraperitoneally into the channel catfish. The same volume of PBS was injected into the fish as the control group and samples were collected at 4 h, 24 h, 48 h and 4 day after treatment under anesthesia using tricaine methanesulphonate (MS 222) at 100 mg/L. Nine tissues including brain, head kidney, trunk kidney, liver, spleen, gill, skin, intestine and heart were collected from 45 fish (three pools of 15 fish per pool) of both the challenged group and the control group at each time point. During the challenge, symptomatic treatment fish and control fish of each species were collected and confirmed to be infected with *E. ictaluri* and pathogen-free, respectively, at the Fish Disease Diagnostic

**Fig. 1.** Alignments of the deduced amino acid sequences of channel catfish c-type (a) and g-type (b) lysozymes with those from other fish species. Dashes represent amino acid deletions. The predicted signal peptides were underlined. The catalytic residues were shaded, and conserved cysteine residues were indicated by arrows. Asterisks indicate identical amino acids; colons indicate similar amino acids and empty spaces represent absence or low level of similarities. Abbreviations were used for sequences from various species: *Ipu*: *Ictalurus punctatus*; *Ola*: *Oryzias latipes*; *Dre*: *Danio rerio*; *Cca*: *Cyprinus carpio*; *Ssa*: *Salmo salar*; *Srh*: *Scophthalmus rhombus*; *Pol*: *Paralichthys olivaceus*; *Tru*: *Takifugu rubripes*; *Eco*: *Epinephelus coioides*; and *Omy*: *Oncorhynchus mykiss*; Lyg: g-type lysozyme g; Lygl1: g-type lysozyme g-like 1; Lygl2: g-type lysozyme g-like 2 from channel catfish.

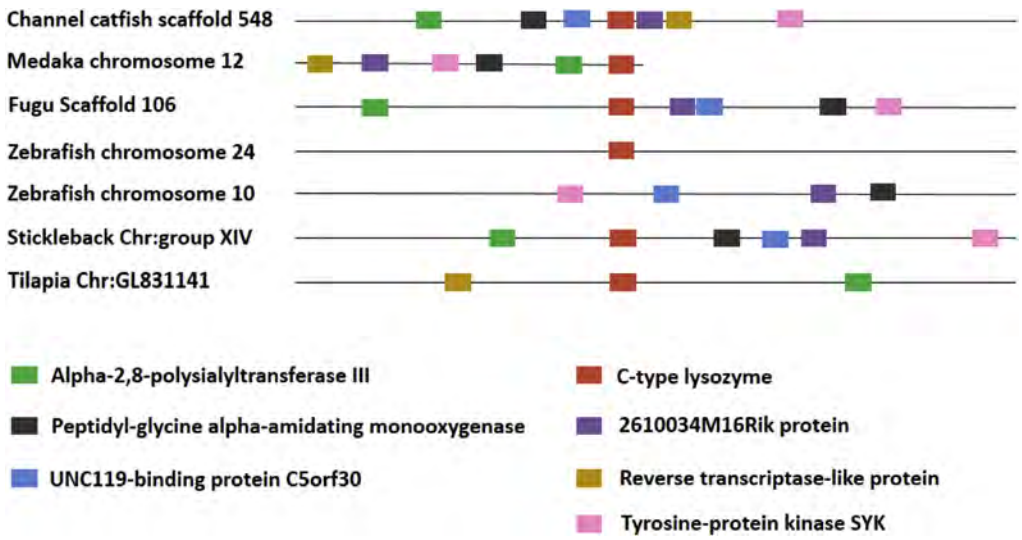



**Fig. 3.** Schematic representation of the results from syntenic analysis of c-type lysozyme genes from various fish with species names and their chromosomes or scaffolds indicated on the left margin. Gene names are color-coded and are self-explanatory.

Laboratory, Auburn University. In order to determine gene expression patterns in healthy catfish tissues, samples of nine tissues including brain, gill, heart, head kidney, trunk kidney, intestine, liver, skin and spleen with three pools (15 fish in each pool) for each tissue were collected from healthy channel catfish. Samples were flash-frozen in liquid nitrogen during collection and stored at −80 °C until RNA extraction.

### 2.5. RNA extraction

Tissue samples were homogenized with a mortar and pestle in the presence of liquid nitrogen. Total RNA was then extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's instructions. The cDNA samples were subsequently used for determination of lysozyme gene expression by quantitative real-time RT-PCR.

### 2.6. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed to determine lysozyme mRNA expression in different tissues and following bacterial challenge. All the cDNA products were first diluted to 250 ng/μl and utilized for quantitative real-time PCR using the SsoFast™ EvaGreen® Supermix on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primers used in quantitative real-time RT-PCR are listed in Table 1. The thermal cycling profile consisted of an initial denaturation at 95 °C (for 30 s), followed by 40 cycles of denaturation at 94 °C (5 s), an appropriate annealing/extension temperature (58 °C, 5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C. The housekeeping gene 18S was set as the reference gene according to the previous catfish immune gene expression studies [22,39–41,44,46–48]. Relative fold changes were calculated in the Relative Expression Software Tool version 2009 based on the cycle threshold (Ct) values (Supplementary Table 1) generated by q-RT-PCR [52]. For the expression analysis in healthy tissues, Ct values of each gene in brain were set as control group in REST, the relative expression of each lysozyme gene in healthy tissues were obtained by comparing expression in other tissues to expression in the brain. Expression levels of both the c-type and g-type lysozyme genes in channel catfish after ESC bacterial infection were calculated by comparing treatment group with control group in each time point and tissue. Expression levels among healthy tissues were then compared by using ANOVA (SPSS 14.0 package, SPSS Inc., New York, USA). Differences were considered significant at $p \leq 0.05$.

## 3. Results

### 3.1. Identification of catfish lysozyme cDNAs

One c-type lysozyme cDNA sequence and three distinct g-type lysozyme cDNAs were identified from the catfish transcriptome and confirmed with the draft genome database. The complete cDNA sequences have been submitted to GenBank with accession number of ADO29030 for c-type lysozyme and ADO29070, JT285197, and JT408768 for g-type lysozyme, g-type lysozyme like 1, and g-type lysozyme like 2, respectively. The c-type lysozyme cDNA was 719 bp long including an ORF of 429 bp encoding 142

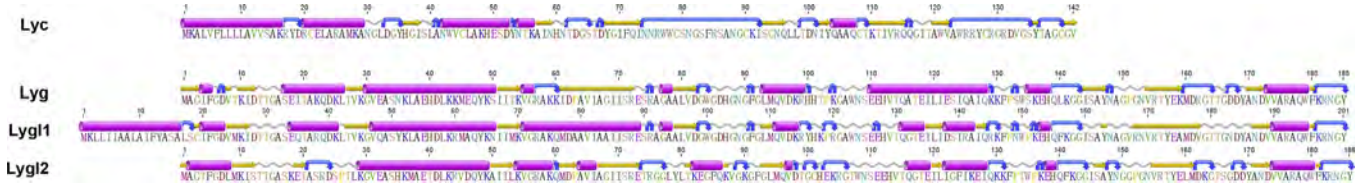



**Fig. 4.** Secondary structure of c-type and three g-type lysozymes of channel catfish constructed by Geneious 6.0.5 program. Blue stands for γ-turns, gray stands for coils, yellow stands for β-sheets, and magenta stands for α-helices.

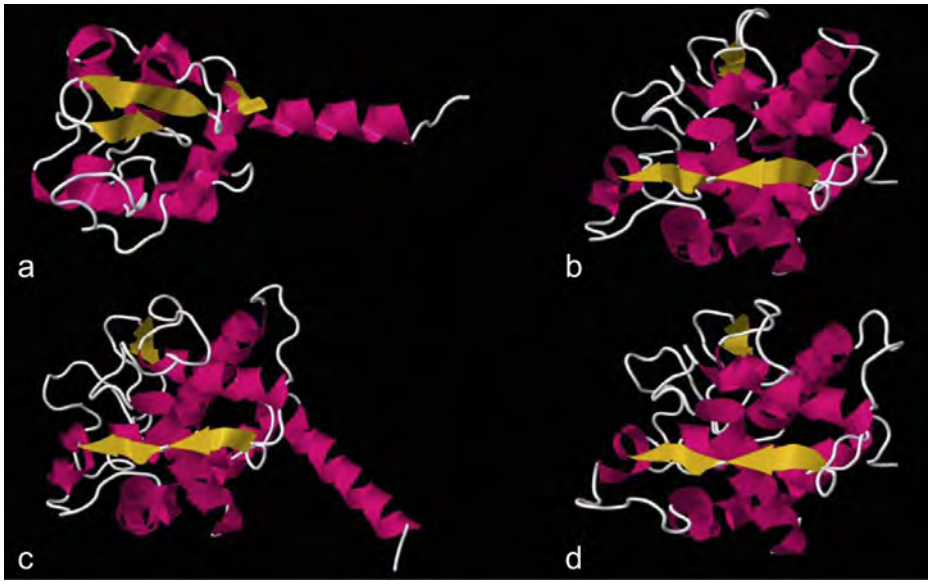



**Fig. 5.** Three-dimensional structures of channel catfish lysozymes constructed by 3D-JIGSAW modeling program for c-type lysozyme (Lyc) of channel catfish (a), g-type lysozyme (Lyg) of channel catfish (b), g-type lysozyme like 1 (Lygl1) of channel catfish (c), and g-type lysozyme like 2 (Lygl2) of channel catfish (d). Alpha helices are colored in magenta, beta sheets are colored in yellow, other regions of the structure (the loops) are shown in gray.

amino acids, and a 5′-UTR and a 3′-UTR of 41 bp and 249 bp, respectively. The deduced c-type lysozyme protein included a predicted 18 amino acid signal peptide at the N-terminus. The calculated molecular mass of the mature peptide was approximately 15.8 kD, with a pI of 9.2. The general structural features of the g-type lysozymes are similar, but without a signal peptide. The features of the g-type lysozymes are summarized in Table 2.

The deduced amino acid sequences of c-type lysozyme in the catfish shared 35–66% identity with those from other fish species. It appeared that the catfish c-type lysozyme shared the highest identity (66%) with that of medaka, and lowest identity (35%) with that of zebrafish. Sequence alignments indicated the presence of conserved structural motifs such as aspartic acid (D) and glutamic acid (E) residues in both the c-type and g-type lysozymes (Fig. 1a and b), and the six conserved cysteine residues required for the secondary structures in the c-type lysozymes (Fig. 1a).

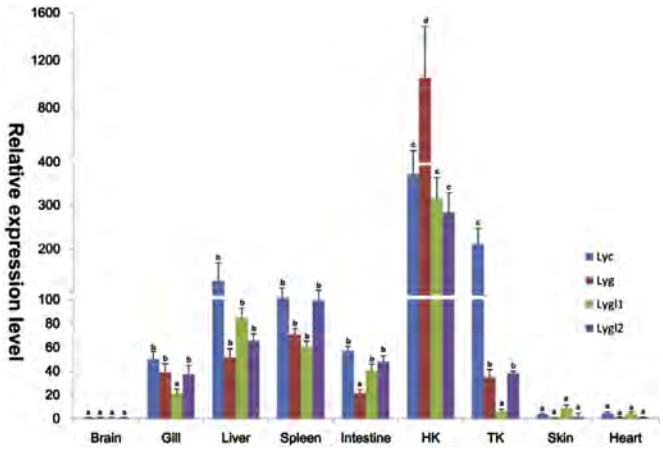



**Fig. 6.** Tissue expression of the c-type and g-type lysozymes in channel catfish. Expression values were normalized to that of the 18S rRNA. Data are expressed as the mean fold change from the calibrator group (brain). The abbreviations are as the following: HK for head kidney and TK for trunk kidney. Bars with different letters indicate statistical differences ($p \leq 0.05$).

The deduced amino acid sequence of g-type lysozymes of channel catfish shared 49–81% identities with those from other fish species. The highest identity (81%) was found between channel catfish and zebrafish, while the lowest identity (49%) was found between channel catfish and orange-spotted grouper. The amino acid sequences of the three g-type lysozymes: g-type lysozyme, g-type lysozyme like 1, and g-type lysozyme like 2 are highly conserved (71–83%). They all possessed 14 conserved catalytic residues in mature peptide, including Asp7, Asp24, Asp41, Asp62, Asp97, Asp161, Asp168, Glu39, Glu73, Glu110, Glu111, Glu118, Glu137 and Glu158 (Fig. 1b).

### 3.2. Phylogenetic analyses

A phylogenetic tree was constructed to determine the identities of the catfish lysozyme genes (Fig. 2). Clearly c-type lysozymes and g-type lysozymes each formed their own clade as expected. However, the phylogenetic analysis of the c-type lysozymes did not provide a clear relationship of orthologies. Three separate clades formed among the teleost c-type lysozyme genes. The first clade included c-type lysozymes from turbot, brill, senegalese sole, Japanese flounder, orange-spotted grouper, Atlantic salmon and rainbow trout; the second clade included channel catfish, medaka, fugu and tilapia, and third clade included c-type lysozymes mainly from the carps (common carp, grass carp, and the zebrafish). Such relationships were not consistent with the phylogenies of these fish species. For instance, the catfish is phylogenetically closer to the carps than to medaka, but its c-type lysozyme gene is most similar to that of medaka, suggesting that these genes may not be orthologous.

The phylogenetic tree of the g-type lysozymes was also constructed (Fig. 2, lower panel) that reflected the phylogenies of the related species under study. For instance, the g-type lysozyme genes of zebrafish clustered with those from the common carp and grass carp, and then next to those from the catfish, consistent with their phylogenetic relationships. However, the multiple genes in each species were placed into its own clade, suggesting lineage-specific gene duplications. Therefore, we named the three g-type lysozyme genes from the catfish as g-type lysozyme, g-type

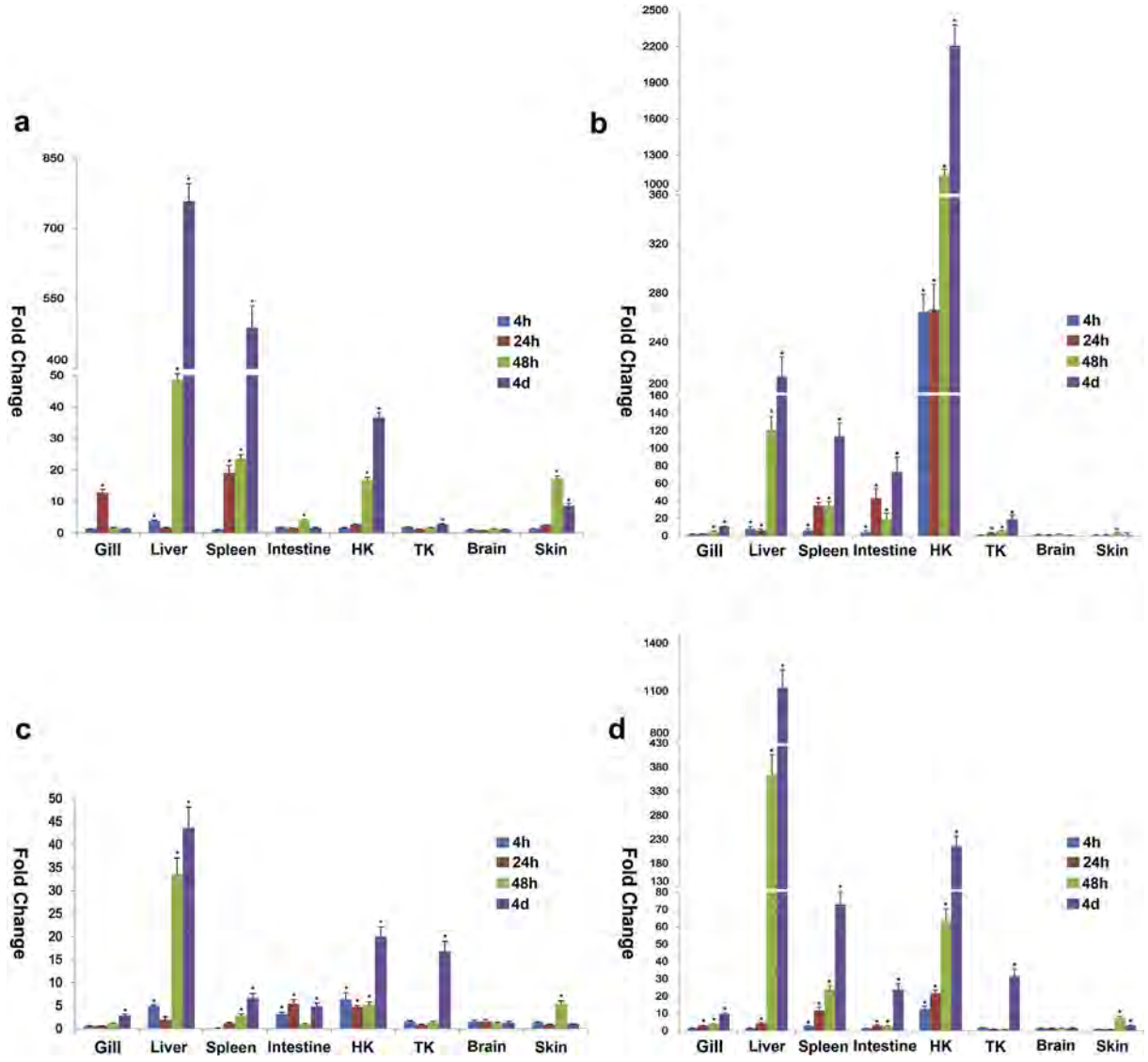



**Fig. 7.** Quantitative real-time RT-PCR analysis for the expression of the catfish c-type lysozyme (a), g-type lysozyme g (b), g-type lysozyme g-like 1 (c), and g-type lysozyme g-like 2 (d), in various tissues including gill, liver, spleen, intestine, head kidney (HK), trunk kidney (TK), brain, and skin, at various times after challenge with *Edwardsiella ictaluri* including 4 h, 24 h, 48 h, and 4 days. Fold changes are expressed as the ratio of gene expression after *E. ictaluri* challenge to the control group at same time point as normalized with 18S rRNA. Error bars indicate standard error and asterisks indicate statistical significance ($p \leq 0.05$).

lysozyme like 1, and g-type lysozyme like 2, following zebrafish genome nomenclature.

### 3.3. Syntenic analysis of c-type lysozyme gene

As phylogenetic analysis provided inconclusive results concerning the gene identity of the c-type lysozyme, we conducted additional syntenic analysis to provide clues of the orthologies. As the catfish reference genome is not available yet, we first constructed the scaffold that contained the catfish c-type lysozyme gene, and identified the genes surrounding the c-type lysozyme gene. As shown in Fig. 3 (top row), the genomic neighborhood in the catfish genome included Alpha-2,8-polysialyltransferase III, Peptidyl-glycine alpha-amidating monooxygenase, UNC119-binding protein C5orf30, c-type lysozyme, 2610034M16Rik protein, Reverse transcriptase-like protein, and tyrosine-protein kinase SYK. Comparison of this genomic segment in the catfish genome with those of medaka, fugu, zebrafish, stickleback and tilapia apparently confirmed the orthology of the catfish c-type lysozyme gene with that in fugu, medaka, stickleback and tilapia, as suggested by the phylogenetic analysis (Fig. 2). However, the catfish c-type lysozyme gene indeed was not orthologous to the zebrafish c-type lysozyme gene. The zebrafish c-type lysozyme gene is located on chromosome 24, but its genomic neighborhood does not contain any genes in the genomic neighborhood containing the catfish c-type lysozyme gene (Fig. 3). Therefore, this syntenic analysis supported the results from the phylogenetic analysis.

### 3.4. Predicted protein structures

To provide insight into second and tertiary structures of the catfish c-type and g-type lysozymes, computer software packages were used to predict their structural features. As shown in Fig. 4, the secondary structures of the c-type and g-type lysozymes are distinct between them, but high levels of secondary structure similarities were predicted among the three g-type lysozymes (Fig. 4), with conserved structures of alpha-helices, beta sheets, γ-

turns, and coils. Such secondary structural similarities allowed extension of the similarities in tertiary structure (Fig. 5).

### 3.5. Expression of the lysozyme genes in healthy individuals

Tissue expression of g-type and c-type lysozymes was determined by real-time quantitative PCR in nine tissues: brain, gill, liver, spleen, intestine, head kidney, trunk kidney, skin, and heart. As shown in Fig. 6, the c-type and g-type lysozymes were expressed most highly in head kidney, followed by liver and spleen. Expression in the head kidney was 5–10 times higher than those in other tissues with exception of the c-type lysozyme expression in the trunk kidney. The four genes exhibited different expression profiles in different tissues. For instance, in the head kidney, g-type lysozyme was expressed at highest level, but in trunk kidney, the c-type lysozyme was expressed at the highest level (Fig. 6).

### 3.6. Expression of the lysozyme genes after ESC bacterial infection

Expression levels of both the c-type and g-type lysozyme genes were up-regulated in channel catfish after ESC bacterial infection (Fig. 7). In general, all four genes were up-regulated in all tested tissues except the brain. However, the four genes were regulated differentially in different tissues. The c-type lysozyme and the g-type lysozyme like 1 and g-type like 2 were most highly induced in the liver, spleen, and head kidney, whereas the g-type lysozyme was most highly induced in the head kidney, followed by in the liver, spleen, and intestine. In addition, the extent to which the four genes were induced were drastically different, with the g-type lysozyme being induced at highest levels up to over 2100-fold in the head kidney, followed by the g-type lysozyme g-like 2 (up over 1000 fold), the c-type lysozyme (up over 700 fold), and the induction of the g-type lysozyme like 1 was much less dramatic (up 40 times) in the liver.

## 4. Discussion

As the first barrier against pathogen infections, the innate immune system plays critical roles in all the living organisms. Lysozymes are very important and ubiquitous members of the innate immune system. Two types of lysozymes, the c-type and the g-type, have been characterized among vertebrates and invertebrates, with different molecular weights, amino acid compositions, and enzymatic properties [53–55]. These two types of lysozymes are regarded as different lysozymes because the amino acid sequence identities around the putative active site of these two enzymes are low, although their three-dimensional (3D) structures are similar [56,57].

In this study, we identified one c-type and three g-type lysozymes, conducted phylogenetic and syntenic analysis, and expression analysis of the four lysozymes in order to provide insight into their gene identities, orthologies, expression, and their involvement in the innate immune responses in catfish after bacterial infection.

Phylogenetic analysis provided reasonably high confidence in the gene identities as initially revealed by BLAST searches. This is particularly true with the g-type lysozymes (Fig. 2). With the c-type lysozymes, however, phylogenetic analysis placed the catfish c-type lysozyme into a clade with fugu, medaka, and tilapia, but not in the same clade with zebrafish and the carps. This suggested that the catfish gene is not orthologous with the zebrafish gene because the catfish is phylogenetically closer to the carps and zebrafish than to fugu, medaka, and tilapia. We therefore conducted additional analysis using syntenic approaches. As shown in Fig. 3, the catfish gene indeed shared the syntenic relationship with fugu, medaka and tilapia, but the zebrafish gene is on a different chromosome, chromosome 24, that is not orthologous with the same genomic region harboring the catfish c-type lysozyme gene. Apparently, this could indicate that 1) the c-type lysozyme gene was evolutionarily duplicated, then the orthologous copies were lost in zebrafish and the catfish; and/or 2) the second copy of the c-type lysozyme has not been found in the catfish and zebrafish genomes yet. With regard to the carps, as their genomes are not yet sequenced, the genes may have not been discovered. In addition, common carp is generally regarded as a tetraploidy fish, and therefore, perhaps multiple copies of its c-type lysozyme genes have yet to be found.

In spite of the correct placement of the g-type lysozymes in the phylogenetic tree, the g-type lysozymes are apparently duplicated in various fish lineages. For instance, three g-type lysozyme genes are here identified from channel catfish; two from zebrafish, but only one from the common carp and grass carp. Once again, the genomes of the carps have not been fully sequenced, and the lack of multiple copies may only indicate that they are to be discovered. However, the multiple copies of the g-type lysozyme genes in channel catfish and zebrafish are all paralogous, perhaps derived from species-specific gene duplications. Even in the catfish genome, among the three identified g-type lysozyme genes, the g-type lysozyme and g-type lysozyme like 2 are most related, suggesting that they are the most recent duplicates, while g-type lysozyme like 1 was a more ancient gene duplicate.

Tissue expression patterns of the c-type and g-type lysozymes reflected their functions in innate immunity as the high levels of expression were found in head kidney, liver, spleen, gill, intestine, and trunk kidney, all important organs/tissues involved in immune responses [38–54,58,59]. When comparing the expression of the c-type and g-type lysozymes, the expression level of c-type lysozyme in most tissues was higher than that of g-type lysozyme in healthy fish. Nonetheless, both types of lysozymes were widely expressed in all tested tissues.

The tissue expression patterns of the c-type and g-type lysozymes are consistent with those found in other teleost fish species such as carps, flounders, groupers and Atlantic cod [12,17,49]. However, expression patterns of the c-type and g-type lysozymes in teleost fish are quite different from those in birds and mammals. In healthy chicken egg whites, g-type lysozyme is only expressed in the cells of bone marrow and lung, similar expression pattern was found with goose [60]. The g-type lysozymes are expressed at low levels in the adult kidney of mammals [50], suggesting functional differentiation during evolution with kidney in mammals apparently specialized in functions other than immunity.

The drastically induced expression of the c-type and g-type lysozymes after bacterial infection with *E. ictaluri* reflected their functions in innate immunity. The levels of their induction were quite dramatic, from 30 to 40-fold to over 2100-fold induction after infection (Fig. 7). However, caution need to be exercised when interpreting the fold of expression changes. Although real time PCR is widely accepted as an accurate way of assessing quantitative expression, the assumption of equal amplification efficiency may not be warranted. In this regard, PCR efficiency could have been in favor of the samples with higher levels of target transcripts (e.g., after infection), thereby signifying the level of induction due to technical reasons. However, we do realize that ESC bacterium is an intracellular bacterium and high levels of bacterial load in the kidney tissue could potentially demand very high levels of expression of lysozymes.

The induced expression was most dramatic in the liver for all lysozyme genes except the g-type lysozyme that is most dramatically induced in head kidney. Liver synthesis many of the acute phase immune response genes and has been shown to exhibit high levels of gene induction after infection in the liver e.g., [22,42].

The timing of the induced expression is also very interesting. For instance, the highest induced expression occurred faster in the gill and skin, but most dramatically in the head kidney, liver, and spleen. In different tissues, the c-type lysozyme and g-type lysozyme like 1 and g-type lysozyme like 2 exhibited a similar pattern of induction whereas the g-type lysozyme behaved differently, with the highest induced expression in the head kidney. Such expression pattern apparently suggested the "collaborative" functions of the lysozymes in different tissues.

Expression patterns of g-type lysozyme after bacteria challenge differs among fish species. With orange-spotted grouper injected with *Vibrio alginolyticus*, g-type lysozyme was significantly induced in stomach, spleen, anterior kidney, posterior kidney, heart, brain and leucocytes [17]. For brill injected with *Pasteurella damselae subsp*. *Piscicida*, g-type lysozyme was significantly increased in head kidney [5] but the c-type lysozyme was not induced. With Japanese flounder injected with *E. tarda* [12], the mRNA level of g-type lysozyme was significantly increased in the intestine, heart and blood, but the mRNA level of c-type lysozyme was increased in the head kidney, spleen and ovary. For grass carp challenged with *A. hydrophila*, g-type lysozyme was significantly increased in liver and spleen, while c-type lysozyme was significantly expressed in the gill. Clearly, such differences in expression patterns after bacterial infection reflected the differences in pathogenesis of these bacterial diseases. It is reasonable to assume that the lysozyme gene expression may have followed the route and loads of bacteria after infection.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.04.014.

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