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Immunoglobulin (Ig) heavy chain gene locus and immune responses upon parasitic, bacterial and fungal infection in loach, *Misgurnus anguillicaudatus*

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ABSTRACT

Teleost fish are the most primitive bony vertebrates that contain immunoglobulin (Ig). Although teleost Ig is known to be important during tetrapod evolution and comparative immunology, little is known about the genomic organization of the immunoglobulin heavy-chain (IgH) locus. Here, three Ig isotype classes, IgM, IgD and IgT, were firstly identified in dojo loach (*Misgurnus anguillicaudatus*), and the IgH locus covering τ, μ and δ genes was also illustrated. Variable (V) gene segments lie upstream of two tandem diversity (D), joining (J) and constant (C) clusters and the genomic organization of the IgH locus presented as V_n-D_n-J_n-C_τ-D_n-J_n-C_μ-C_δ, similar to some other teleost fish. However, unlike some other teleost fish, ten V_H, ten D and nine J genes were observed in this locus, which suggest teleost Igs might be conserved and diverse. Thus, it would be interesting to determine how Igs divide among themselves in immune response to different antigens. To address this hypothesis, we have developed three models by bath infection with parasitic, bacterial and fungal pathogens, respectively. We found that IgM, IgD and IgT were highly upregulated in the head kidney and spleen after infection with *Ichthyophthirius multifiliis* (Ich), suggesting that the three Igs might participate in the systemic immune responses to Ich. Moreover, the high expression of IgT in mucosal tissue, such as skin or gills, appeared after being infected with three different pathogens infection, respectively, in which the expression of IgT increased more rapidly in response to Ich infection. Interestingly, the expression of IgD showed a higher increase in spleen and head kidney being challenged with fungi, suggesting that IgD might play an important role in antifungal infection.

1. Introduction

The adaptive immunity system emerged in vertebrates more than 550 million years ago along with the appearance of the jawless vertebrates [1]. However, due to the lack of B and T cell receptors, only variable lymphocyte receptors can be used to recognize antigen in jawless vertebrates [2]. Comparatively, the jawed vertebrates exhibit adaptive immunity with immunoglobulin (Ig) involvement, which can recognize and initiate a protective response against potentially lethal pathogens in a process of immune exclusion [3].

As the major effector molecules of the adaptive immune system [4], under evolutionary selection pressure, Igs were diversified into several isotypes with special roles in species with different evolutionary statuses, exhibiting the rearrangement of V, D, J and C genes. Mammals have five Ig classes, namely IgM, IgD, IgG, IgA and IgE [5], and a new Ig isotype IgO, has been found in the duckbilled platypus (*Ornithorhynchus anatinus*) [6]. Human IgG is critical for systemic adaptive immunity to many infections [7,8], while IgA may play an important role in mucosal adaptive immunity [9,10]. Avians contain IgM, IgD, IgY and IgA [11,12], and reptiles contain four Ig heavy chain classes, IgM, IgY, IgA and IgD [13,14]. Differently, five isotypes, IgM, IgD, IgY, IgX and IgF, have so far been reported from anuran amphibians, and IgP has been identified in urodele amphibians [15]. Research shows that IgY as a functional homologue is similar to mammalian IgG [16], and IgX has been identified as an analogue of mammalian IgA [17]. By far, teleost fish represent the oldest living bony vertebrate with Ig and have an important evolutionary status, in that only three different Ig isotypes, IgM [18], IgD [19–21] and IgT/Z [22,23] have been identified.

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However, little is known about the genomic organization of the IgH locus.

Additionally, in teleosts, IgM has been found to be the most abundant class, which may play an important role in systemic immune responses [24–26]. While secreted IgD has been identified in plasma and gill mucus, its function in teleosts remains undetermined (except for coating a few types of bacteria in gill mucus) [27]. IgT, recently discovered in 2005, has been reported in many species [28–31], and it has been proved to be specialized in fish mucosal immune responses after parasitic infection. Despite this, teleost fish might endure strongly continuous stimulation from different pathogenic microorganisms in both wild and farmed conditions, including bacteria, parasites and fungi. Molecular evidence is absence on comparative analysis among three Igs in responses to different pathogens.

The dojo loach (*Misgurnus anguillicaudatus*) is an economically important freshwater fish species with high nutritional and medical value in Eastern Asian countries [32–34]. With the expansion of the cultivation scale, there have been frequent outbreaks of diseases caused by bacteria, parasites and fungi. Meanwhile, a large amount of mucus secretions appearing on the skin surface suggests a strong immune response during infection. Here, we firstly identified three Igs including IgM, IgT and IgD, and analyzed the genomic organization of the IgH locus. Moreover, we have developed three models by bath infection: parasitic, bacterial and fungal pathogens, respectively. Histopathological sections and Polymerase Chain Reaction (PCR) showed successful infection. Importantly, the expressions of *Ma*-IgM, *Ma*-IgD and *Ma*-IgT in systemic tissues (spleen, head kidney) and mucosal tissues (skin, gills) of the dojo loach were analyzed in responses to the infections with three different pathogens, respectively.

2. Materials and methods

2.1. Fish husbandry and sampling

Healthy loaches juvenile specimens (mean body weight: 7.0 ± 1.0 g, mean body length: 10 ± 3 cm) were obtained from a farm in Wuhan (Hubei Province, China) and kept in tanks with recirculation water at temperature always above 21 °C and below 26 °C, with continuous aeration and natural photoperiod. Fish were fed with UV-irradiated frozen red worm twice a day and acclimated for 2 weeks prior to treatments.

2.2. RNA extraction and cDNA synthesis

Total RNA from the different tissues was extracted using Trizol Reagent (Invitrogen Life Technologies) following manufacturer's protocol. The integrity of extracted RNA was checked in 1% agarose gel and quantified using Nanodrop 2000 (Thermo scientific, USA). Then the total RNA was used immediately for the cDNA synthesis, 1 μg of DNase treated RNA was reverse transcribed to cDNA using a 5 × All-In-One MasterMix with AccuRT Genomic DNA Removal Kit (Abm, Canada).

2.3. Molecular cloning and sequence analysis of Ma-Igs

The partial sequences of the *Ma*-Igs were identified through data mining of the transcriptomic database. To obtain the full-length cDNA of *Ma*-Igs, gene-specific primers were designed to amplify the internal region of *Ma*-Igs. PCR was performed using Takara PCR Amplification Kit (Takara, Japan). The PCR products were isolated using a Gel Extraction Kit (Sangon Biotech, China) and cloned in pGEMT-Easy vector (Promega, USA), followed by blue-white screening of the positive colonies, and then sequenced. Based on the partial nucleotide sequences of Igs, forward and reverse primers were designed for the amplification of cDNA ends using SMARTer RACE cDNA Amplification Kit (Clontech, USA). All primers used are showed in Table 1.

Based on the complete sequences, open reading frame was identified using the ORFfinder available at the NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). All sequences of nucleotide and amino acid were edited using the Editseq tool of DNAstar software. The structural features of protein sequence were predicted with SMART online website (http://smart.embl-heidelberg.de/). Different species sequences of Igs were retrieved from GenBank database and then multiple alignments of amino acid sequences were performed by the online software Multalin (http://multalin.toulouse.inra.fr/multalin/). The phylogenetic trees of Igs were constructed by using the Neighbor-Joining (NJ) method with MEGA6.0.

2.4. Annotation of IgH gene locus

The whole length of the loach immunoglobulin locus was obtained from transcriptome sequencing (unpublished). To deduce the amino acid sequence, the ExPASy translate tool (http://ca.expasy.org/tools/dna.html) was used. Gene structure prediction was done with GENSCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://linux1.softberry.com/all.htm). D and J segments were identified by pattern search with the program FUZZNUC, and with manual sequence analyses. The positions of C domains were determined by comparison with cDNA sequences.

2.5. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted on 7500 Real-time PCR system (Applied Biosystems, USA) using the EvaGreen 2 × qPCR Master mix (ABM, Canada). All samples were performed in triplet and the amplification reaction condition consisted of 95 °C for 30 s, 40 cycles of 95 °C for 1 s, 60 °C for 10 s and 72 °C for 10 s. The specificity of each primer pair was verified by dissociation curve to determine whether a specific-sized single amplicon. All the primer pairs used in this study were qualified, with the efficiencies between 90% and 105%. The quantification of the genes of interest was determined as being relative to housekeeping gene (EF1α) by using the $2^{-\triangle\triangle Ct}$ method.

2.6. Histology, light microscopy study

Morphological changes of skin were detected after challenged with different pathogens. Briefly, histological sections of the separated skin (~0.25 cm^2) were fixed in 4% neutral buffered paraformaldehyde for at least 24 h. Fixed tissues were dewatered in a graded ethanol series, cleaned in xylene and embedded in paraffin for histological analysis. Sections of 5–7 μm were acquired with a rotary microtome (HM 325 Manual Microtome, MICROM International GmbH, Waldorf, Germany). Then, stained with conventional HE (hematoxylin and eosin) and AB (alcian blue) for histological observation. The stain sections were examined under microscope (Olympus, BX53, Japan) using the Axiovision software.

2.7. Experimental design

Six healthy loaches from each trial were anesthetized with tricaine methanesulfonate (MS-222). Different tissues (kidney, spleen, gill, heart, blood, gut, muscle, fin ray, skin, liver and ovary) were rapidly excised, then frozen in liquid nitrogen, and stored at −80 °C.

F. columnare G_4 strain was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Hubei, China). The G_4 strain was incubated at 28 °C for 24 h on a Shieh Medium plate, single colonies were picked and inoculated into 10 ml Shieh broth at 28 °C for 48 h, then *F. columnare* G_4 were amplified in liquid medium and counted under a microscope (1×10^8 CFU/ml). At last, a final concentration of *F. columnare* G_4 with 1×10^6 CFU/ml was used to make infection. For the infection group, 60 loaches were divided into 3 replicate tanks, bathing with a dose of 20 ml F. *columnare* G_4 suspension

Table 1
Primer sequences and their designated application in this study.

Name	Sequence(5′–3′)	Application
Clo-IgM-F	CCAAAATGAAAAGCAAATAC	Conserved region cloning
Clo-IgM-R	AAGCAACAGGCTACAAACA	
Clo-IgD-F	GACCTGCTTGGTTATTGGT	
Clo-IgD-R	AAAGAACGAGAAAGGCACA	
Clo-IgT-F	CTACTTCAGCTACCCAAACG	
Clo-IgT-R	GTAATGCAAAGCCTGTCTC	
IgM5′-Race1	TGAATCTGCCTTGAACAGCACTG	RACE-PCR
IgM5′-Race2	CAATGATAGCAACAAAATCCAGTCC	
IgD5′-Race1	TCACATCACTGAGGGAGGGACCTA	
IgD5′-Race2	TGAGGGAGGGACCTAGTAAATAGAAGTA	
IgT5′-Race1	AAGGGTTTGTGCTGTCACTTTGTTGT	
IgT5′-Race2	CACTGGGTTTCTTTGAATCTCCTT	
IgM3′-Race1	GACGAAATGAGCAGCAATA	
IgM3′-Race2	CATCATCAGCAAGCCAAGA	
IgD3′-Race1	TTTGGAGRACTTGTATGCTTCGTT	
IgD3′-Race2	CTTCGTTGAYCTACTCTGCTCCAT	
IgT3′-Race1	GATATATTCCTCTTCGGTGACGT	
IgT3′-Race2	AGGTCAGCAGCCCTCAACTTGGT	
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
NUP	CTAATACGACTCACTATAGGGC	
IgM-F	CTCATTTCGTCCCTCCATCCT	qRT-PCR
IgM-R	GTTCCATTGCTCCATTCTTCA	
IgD-F	ACTGACATTTCTTTGACCTGCTT	
IgD-R	ATGCTTTGAGTCCCGTTGG	
IgT-F	CTTAGTTTCAAATGGACCGA	
IgT-R	GTTATTACAGGGACCAGGC	
EF1α-F	TCAGCGCCTACATCAAGAAG	
EF1α-R	TTACGCTCAACCTTCCATCC	
β-actin-F	TTACCCACACCGTGCCCATCTAC	
β-actin-R	TACCGCAAGACTCCATACCCA	

(1×10^8 CFU/ml) in 2000 ml aeration water for 3 h. Then, loaches were removed to fresh water. Six loaches were randomly sampled at 6 h, 24 h, 60 h, 4 d, 7 d, 14 d, 21 d and 28 d after challenged, respectively. Blood in the gills was first removed by perfusion with cold PBS-heparin through the heart until the gills were completely blanched. Four immunologically relevant tissues, namely head kidney, spleen, skin and gill were chosen for study. For the control group, immersing with a dose of 20 ml sterile Shieh broth in 2000 ml aeration water for 3 h. The subsequent treatment was the same as the infection group. All tissue samples were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C until total RNA isolation was performed.

I. multifiliis (Ich) was isolated from heavily infected loach in the fishery research base at Huazhong Agricultural University (Hubei, China). A pre-experiment was conducted to obtain the optimal dose of infection with the ratio of 10,000 Ich larvae per fish. For infected group, 60 healthy loaches were co-cultured in tanks containing 600,000 theronts. For control group, 60 loaches were reared in tanks with normal water supply. Samples were collected at 6 h, 24 h, 60 h, 4 d, 7 d, 14 d, 21 d and 28 d after treatment. Blood in the gills was also removed by perfusion with PBS-heparin through the heart for the histology analysis of gill, as described above. Tissues including head kidney, spleen, skin and gill were collected, immediately frozen with liquid nitrogen and then stored at −80 °C.

To investigate *Ma*-Igs mRNA response against fungi stimulation, healthy loaches were treated with the *Saprolegnia* antigen. Test fish were divided into 3 replicate tanks with *Saprolegnia* (1×10^5 zoospores/cysts milliliter^{-1}). Mycelium of *Saprolegnia* was isolated from heavily infected loach in the fishery research base at Huazhong Agricultural University (Hubei, China). Control fish were cultured in tanks with circulating water. The stimulated fish were incubated for 6 h, 24 h, 60 h, 4 d, 7 d, 14 d, 21 d and 28 d, after which the tissues (head kidney, spleen, skin and gill) to be examined were harvested in liquid nitrogen, stored at −80 °C and processed for expression studies.

2.8. Statistics

All data obtained from qRT-PCR experiments were presented as mean ± standard deviation of the mean (SD). Significance differences were evaluated using the Student's *t*-test. $P \leq 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Cloning and characteristics of Igs gene from M. anguillicaudatus

The complete cDNA sequence of the Igs gene from *M. anguillicaudatus* was cloned using the 5′ and 3′ rapid amplification of cDNA end (RACE) method. The full-length cDNA of *Ma*-IgM had 1963 base pairs (bp), contained an ORF of 1746 bp, and encoded a predicted protein of 581 amino acids composed of one variable domain and four constant domains (Fig. 1A). *Ma*-IgD had an ORF of 2970 bp, encoded a predicted protein of 989 amino acids, and had a structure of VH-μ1-δ1-δ2-δ3-δ4-δ5-δ6-δ7-TM-UTR (Fig. 1B). The cloned loach IgT cDNA sequence had 1826 bp, contained an ORF of 1635 bp, and encoded a predicted protein of 544 amino acids composed of VH, CH1, CH2, CH3 and CH4 (Fig. 1C).

3.2. Phylogenetic analysis

To further clarify the evolutionary relationships among Igs (IgM, IgD and IgT/Z) genes, amino acid sequences of Igs in other teleost species downloaded from GenBank, as well as the deduced amino acid sequences of *Ma*-Igs obtained from this study, were used to build a phylogenetic tree by using the neighbor-joining (NJ) method. The result showed that *Ma*-IgM was grouped with IgM of *Elops saurus*, *Ma*-IgD clustered together with IgD of *Paralichthys olivaceus* and *Scophthalmus maximus*, and *Ma*-IgT was grouped with IgZ of *Ctenopharyngodon idella*. As predicted, IgM and IgD were more closely related than IgT, which was the first cluster (Fig. 2).

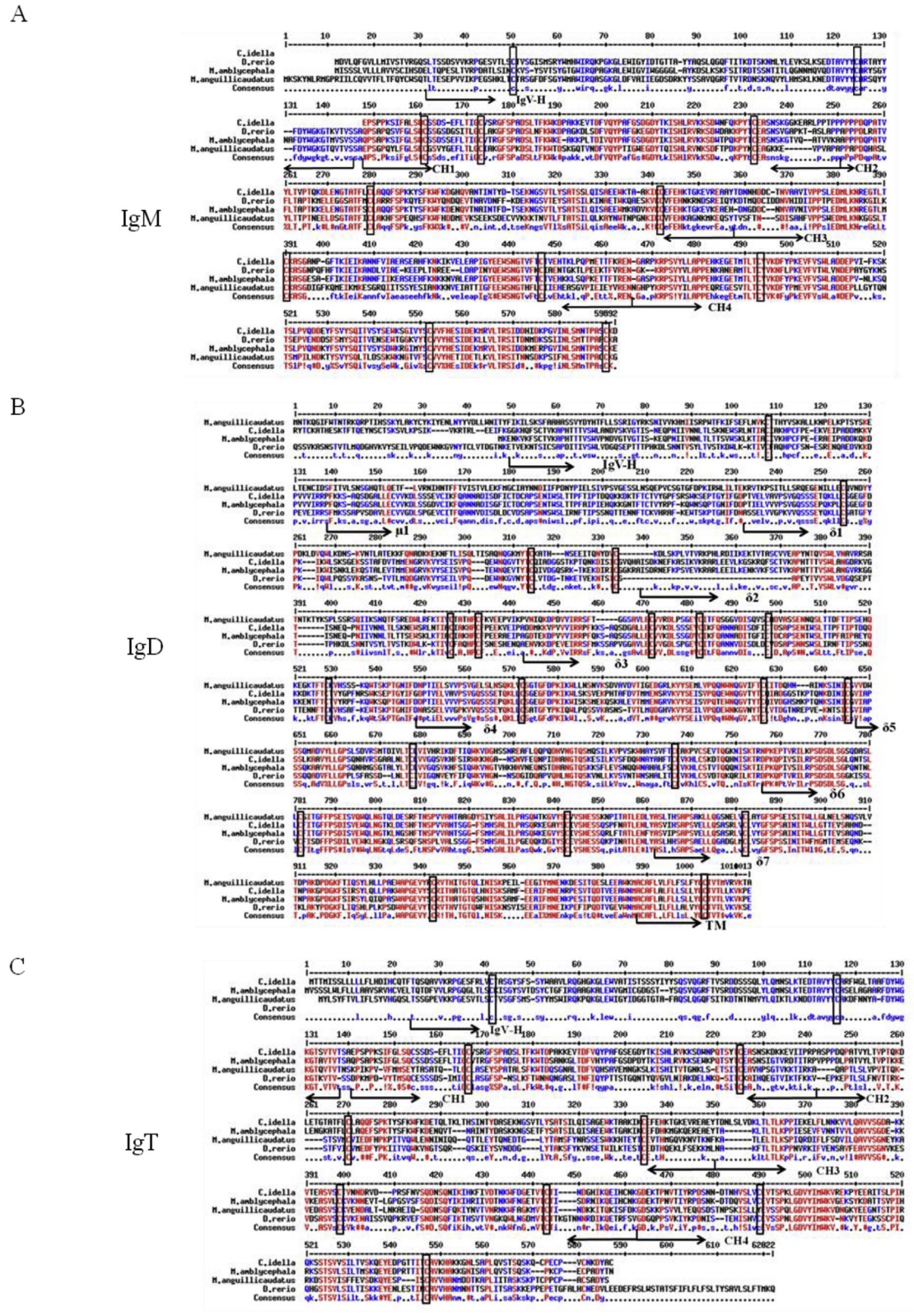

Fig. 1. Multiple sequence alignment of deduced amino acid sequences of *Ma*-IgM (A), *Ma*-IgD (B) and *Ma*-IgT (C) with the corresponding sequences of other species, respectively. Cysteine residues are marked with black frame. (A) The sequence of *Ma*-IgM was divided into VH and four CH domains (CH1−CH4). (B) The sequence of *Ma*-IgD was divided into VH, Cμ1, seven Cδ domains (Cδ1-Cδ7) and one transmembrane domain (TM) on the basis of sequence comparisons with the IgD heavy chains of other fish species. (C) The sequence of *Ma*-IgT was divided into VH and four CH domains (CH1−CH4). GeneBank accession no.: C*tenopharyngodon idella*-IgM (ACV21057.1); *Danio rerio*-IgM (AAH91644.1); *Megalobrama amblycephala*-IgM (AGR34023.1); *Ctenopharyngodon idella*-IgD (ACV21058.1); *Danio rerio*-IgD (XP_021330467.1); *Megalobrama amblycephala*-IgD (AGR34025.1); *Ctenopharyngodon idella*-IgT (ADD82655.1); *Danio rerio*-IgT (ACH92959.1); *Megalobrama amblycephala*-IgT (AGR34024.1).

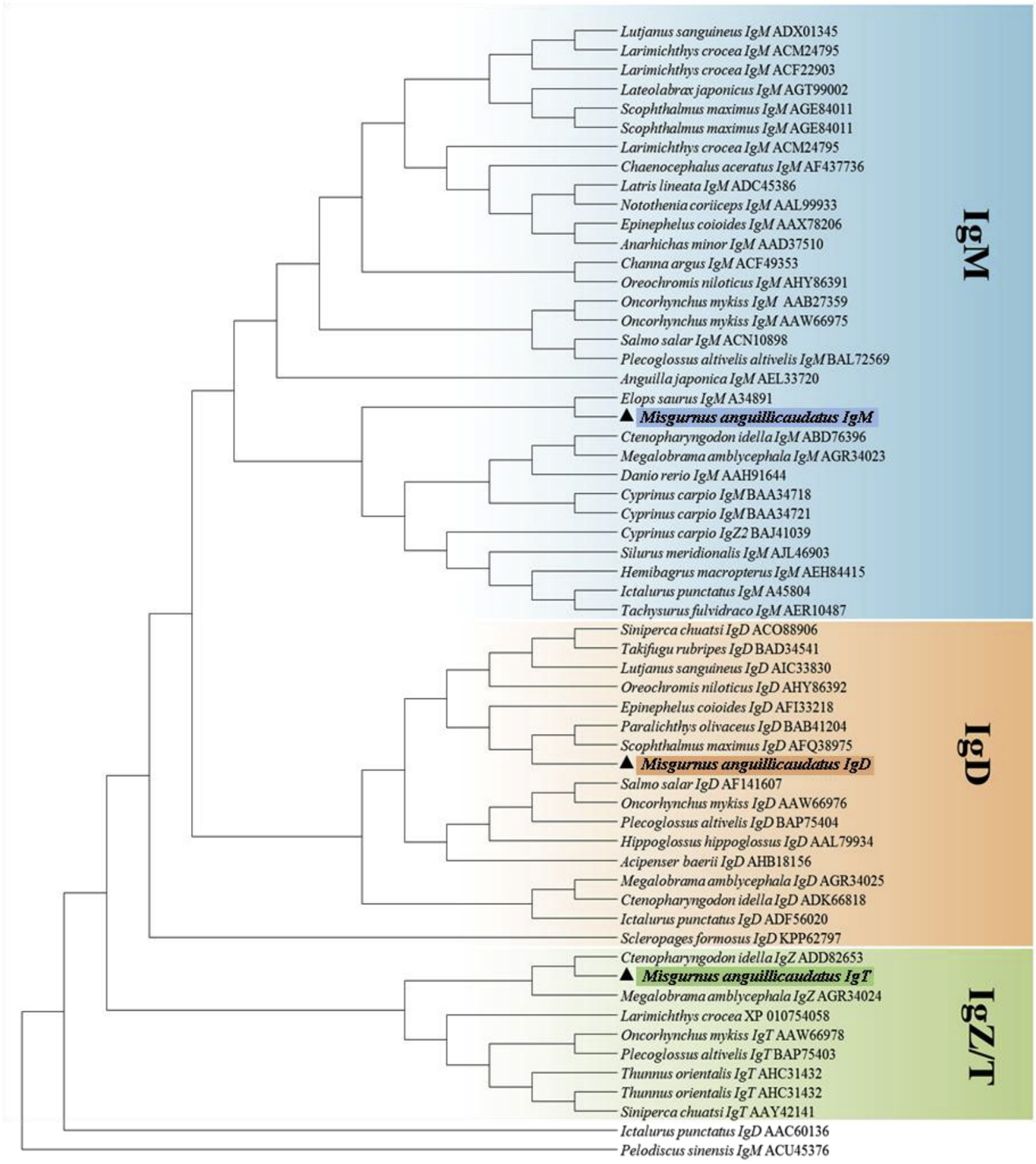

Fig. 2. Phylogenetic analyses of *Ma*-Igs with other vertebrates using neighboring-joining (NJ).

3.3. Loach IgH locus

The IgH locus sequence was from the genomic sequence of *M. anguillicaudatus* (unpublished), which covers the V, D, J and C genes and is 330.378 kilobases (kb) long (Fig. 3). The V, D_τ and J_τ segments are located upstream of the C_τ segments, which are, in turn, located upstream of the D_μ, J_μ, C_μ and C_δ segments. The genomic organization of the loach was summarized as follows: V_{10}-D_6-J_4-C_τ-D_4-J_5-C_μ-C_δ. Here, IgT, IgM and IgD were encoded by the τ, μ and δ genes, respectively. The τ gene was first in the loach IgH locus, and the position of the loach δ gene was immediately downstream of the μ gene. At the 5' end of the IgH locus, a segment of 59 kb contained 10 V_H gene segments. A C_τ gene, flanked upstream by four J and six D gene segments, was located about 99 kb downstream of the nearest V_H. However, the C_μ gene was located about 195 kb downstream of the nearest V_H, which was embedded between five J segments and five C_δ segments. The C_μ gene and

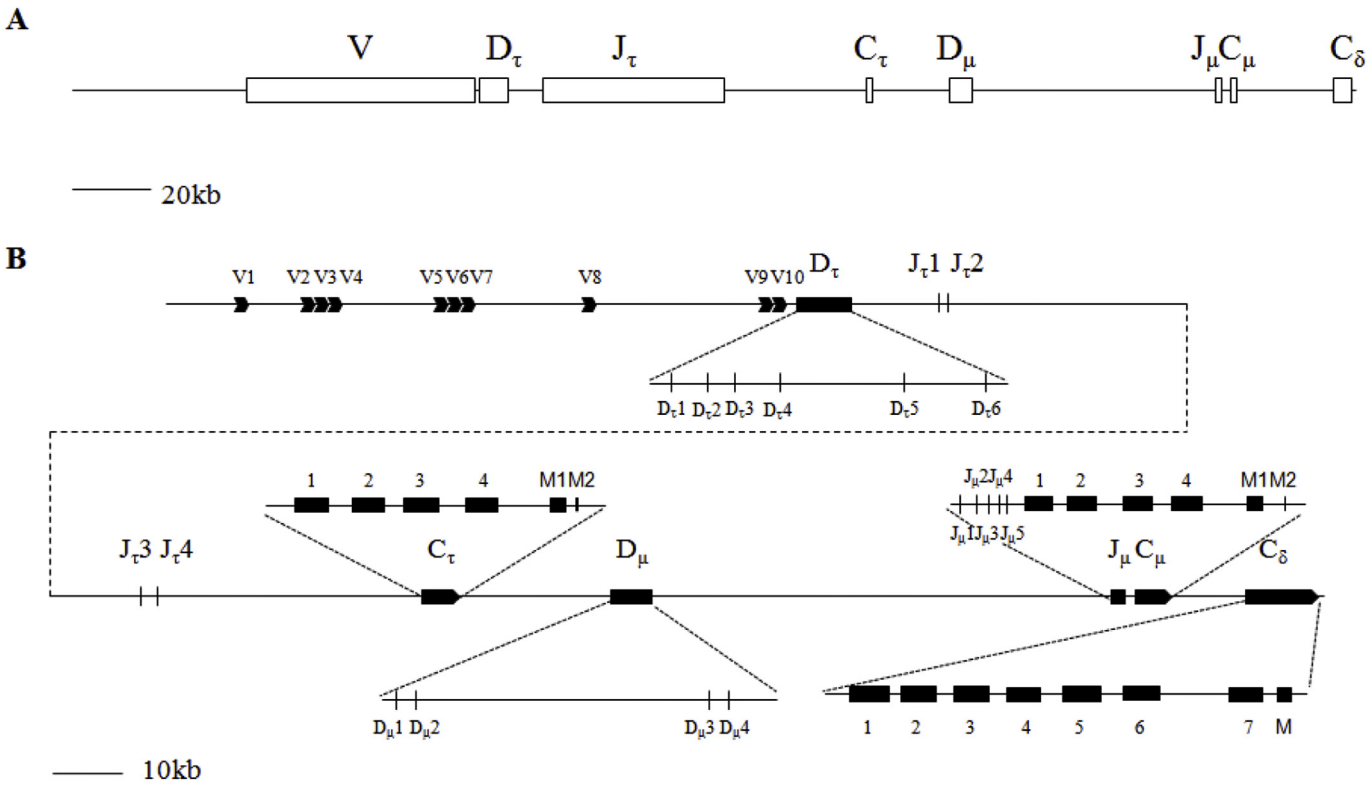

Fig. 3. Organization of IgH locus in loach. V, variable gene segments; D, diversity gene segments; J, joining gene segments; C, constant gene segments; M, transmembrane domain. (A) Immunoglobulin gene segments, to scale. (B) Organization of locus, to scale. Exons, black boxes. Arrowheads indicate direction of transcription.

its downstream gene C_δ were separated by a 26-kb spacer. In the IgT genomic sequence, there were four C segments and two transmembrane exons, TM1 and TM2. IgM was similar to IgT. In the IgD genomic sequence, there were seven δ exons and one TM exon.

There were 10 V_H gene segments in the loach, each with an octamer sequence, a TATA box and a downstream recombination signal sequence (RSS) (Supplementary Table 1). The RSS was composed of conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) sequences, and the interval between them was 12 or 23 bp. In addition, not all RSSs had the same heptamer and nonamer sequences, differing by several nucleotides. Ten D segments were found in the IgH locus of the loach. Six D segments were found between the V_H and J_H segments upstream of C_τ, and the other four were found between the C_τ and J_H segments upstream of C_μ. Each D segment had 12 RSSs on both sides, and most of them were GC-rich motifs (Supplementary Table 2). As shown in Fig. 3, nine J_H segments were found; four J_H segments were located upstream of C_τ, and five J_H segments were located upstream of C_μ. All J_H segments had heptamer and nonamer sequences, which were separated by a spacer of either 22 or 23 bp in length (Supplementary Table 3).

3.4. Basal expression of Ma-Igs in early developmental stages and different tissues

To research the level of *Ma*-Igs expression during early developmental stages, samples were collected from egg to day 4 post-hatching and analyzed by qRT-PCR assay (Fig. 4A). In the ovum period, *Ma*-Igs had a higher expression and gradually decreased during the cleavage stage. From gastrula to organogenesis, the expression of both IgM and IgT showed a significant increase, while IgD expression was fairly stable. After hatching, the expression of *Ma*-Igs continued to increase.

Ma-Igs expression in loach tissues was analyzed by qRT-PCR. The results showed that the Igs gene was constitutively expressed in all tested loach tissues (Fig. 4B), which had similar expression patterns. The highest Igs expression was detected in the kidney followed by the spleen. High expression of IgT was found in the skin, fins, heart, ovary, blood and gut, but lower levels were found in the gills, liver and muscle. Higher expression of IgM was found in the skin, gills, blood, fins and heart, followed by the gut, liver, ovary and muscle. However, higher levels of IgD were observed in the blood, gills, skin, fins, heart and gut, and the lowest expression was found in the liver, ovary and muscle.

3.5. Expression of Ma-Igs post infection

3.5.1. Expression analysis of Ma-Igs against F. columnare infection

To assess the ability of Igs to fight against bacterial challenge, we developed the *F. columnare* G_4 bath infection. Some distant phenotype appeared on the surface of gill, skin and fins at 24 h post infection (Fig. 5A) and morphological analysis showed the skin mucus cells increased significantly after 24 h infection (Fig. 5B–C). By PCR and bacterial culture, a large number *F. columnare* bacteria were detected in the gill, fin and skin, while a little in spleen and head kidney (Fig. 5D–E). Moreover, the mRNA expression of Igs in loach was evaluated in four tissues (skin, gills, spleen and head kidney) by qRT-PCR at 6 h, 24 h, 60 h, 4 d, 7 d, 14 d, 21 d and 28 d post bacterial infection (Fig. 5F). The result was shown in Fig. 5F–G, the highest IgT and IgM expression in the skin (~26.75-fold) and spleen (~16.08-fold) at 7 days post-infection, respectively (Fig. 5G). However, the highest expression levels of IgD were observed in the gills at 60 h (~3.08-fold). In the skin, IgT and IgM expression was increased significantly and with a similar dynamic tendency that steadily increased and decreased and then increased and decreased again. However, IgD expression was downregulated at all the sampling points after infection. In the gills, the strongest IgT expression was noticed at 60 h (~5.06-fold), and greater expression was found at 6 h (~3.95-fold) and 21 days (~4-fold). The peak of IgM expression emerged at day 7 (~2.27-fold). Whereas IgD expression almost reached

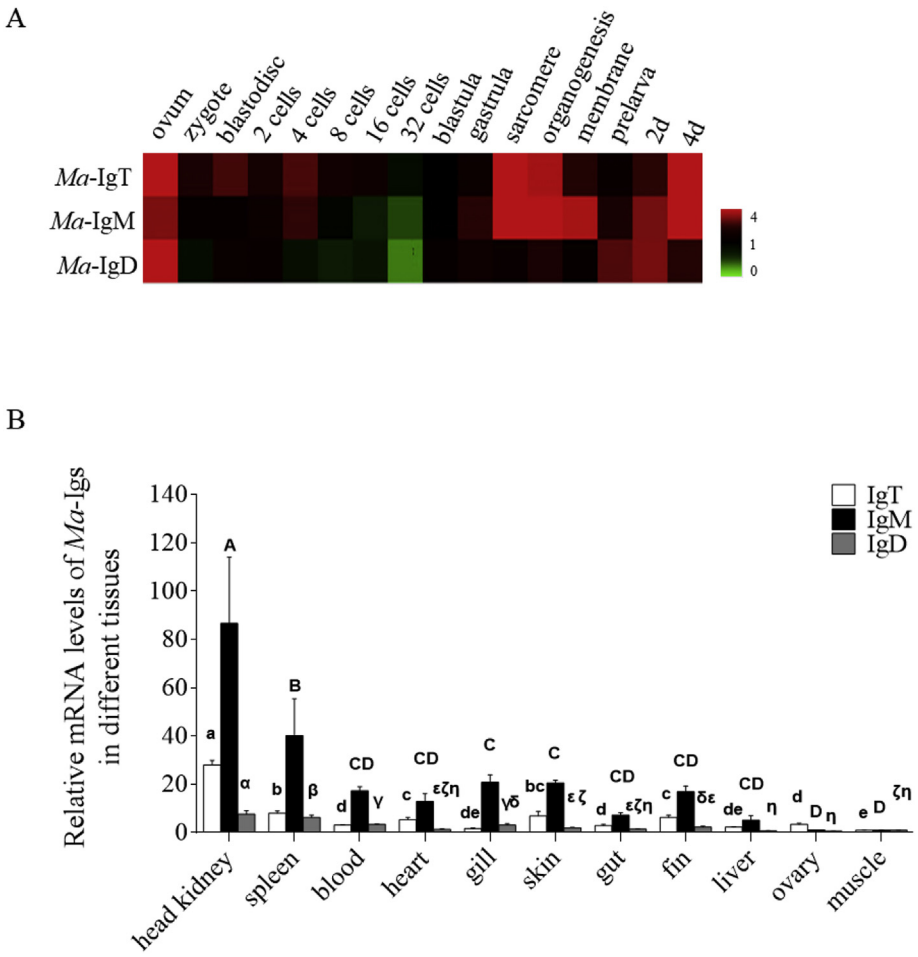

Fig. 4. Basal expression of *Ma*-Igs in early developmental stages and different tissues. (A) The level of *Ma*-Igs expression during early developmental stages. (B) The level of *Ma*-Igs mRNA in initial developmental stages of *M. anguillicaudatus*. Different letters above bars represented significant difference at the levels of $P < 0.05$, and same letters above bars indicated no significant difference.

a threefold upregulation at 60 h, a slight upregulation of IgD was detected at days 1 (~2.1-fold), 7 (~2.45-fold), and 14 (~2.28-fold) after stimulation. Compared to the control, no significant difference in IgD existed in the spleen or head kidney, while IgT expression rose slightly in the spleen at days 4 (~2.95-fold) and 7 (~3.12-fold) post-infection and then dropped sharply to near-baseline level. IgM expression in the spleen reached a higher value at day 1 (~8.32 fold) after being challenged with bacteria. In the head kidney, IgT expression was noted at days 4 (~5.57-fold) and 7 (~4.76-fold). In addition, the expression of IgM increased rapidly to its peak at day 4 (~10.74-fold) and then decreased.

3.5.2. Expression analysis of Ma-Igs against Ich infection

Some small white points appear on the surface of the fin at day 7 post-infection with Ich (Fig. 6A). Histology analysis of skin based on H & E showed that Ich start appearing at 2 d post infection and gradually grows up at epidermis of skin (Fig. 6B). At 7 d post Ich infection, the expression of 18S rRNA of Ich parasite was high in skin and gill, but low in spleen and head kidney (Fig. 6C–D). qRT-PCR assays were conducted to investigate the expression of Igs in parasitic infection (Fig. 6E–F). Of note, the upregulation of IgT was the most prominent in the gills and skin after the infection of Ich; the strongest expression was detected at day 1 (~12.78-fold) and 6 h (~18.70-fold), respectively. Moreover, marginal expression was noticed at day 7 (~1.59-fold) in the skin. In the skin, IgM expression was downregulated clearly at days 7 (~0.35-fold) and 14 (~0.4-fold) but moderately increased at day 21 (~2.79-fold) post-infection, while IgD showed the lowest expression at 60 h (~0.47-fold) and its expression difference was not obvious at other time points compared to the control. In the gills, IgM and IgD expression showed no significant changes until day 14 post-challenge, but higher expression was observed from day 21 onward. In the spleen, the highest Igs (IgT, IgM, and IgD) expression was discovered at day 1 (~22.19-fold, ~23.85-fold, and ~33.27-fold, respectively) post-challenge. Higher IgT expression was observed at 60 h (~20.46-fold) and then decreased to non-significant at day 4 (~0.98-fold) after being challenged with Ich. Similar to IgT, IgM expression was evidently upregulated at 60 h (~23.23-fold), and its expression rose again at day 7 (~12.55-fold). IgD expression reached the peak at day 1 post-infection and then reduced rapidly. Interestingly, Ig expression trends were similar in the head kidney from 6 h to 7 days post-infection.

3.5.3. Expression analysis of Ma-Igs against Saprolegnia infection

In order to investigate the expression of Igs during *Saprolegnia* infection, the loach was co-cultured with water mold spores. At 24 h post fungal infection, mycelium was observed on the surface of skin (Fig. 7A–B) and skin mucus cells showed a significant increase (Fig. 7C–D). Igs gene transcripts in tissues were analyzed by qRT-PCR assay after stimulation with spores (Fig. 7E). In the skin, the expression of IgT and IgM reached the peak at 60 h (~12.97-fold and ~14.62-fold, respectively) post-infection (Fig. 7F). However, IgD expression was mildly upregulated at 6 h (~1.81-fold), then downregulated until day 4 (~3.65-fold), and again downregulated. In the gills, IgD expression was dominant in the three Igs, and the highest expression was recorded at day 4 (~4.01-fold). IgT expression was downregulated at all sampling

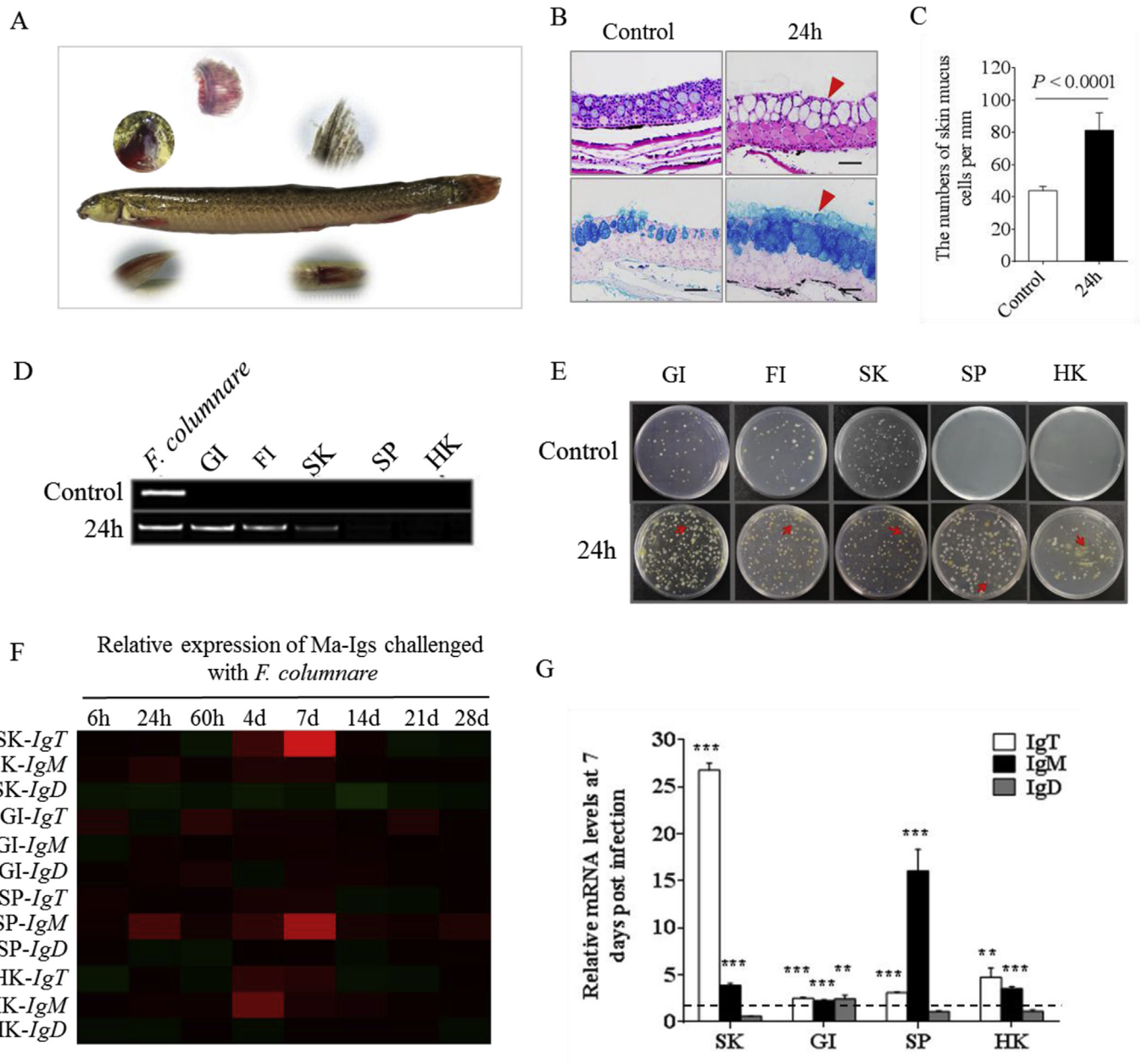

Fig. 5. *F. columnare* G_4 infection model of loach was successfully constructed. (A) Phenotypic characteristics of loach after infection with *F. columnare* G_4. (B) Histological examination (hematoxylin/eosin and alcian blue stain) of skin from loach infected with *F. columnare* G_4 after 24 h and control fish (n = 6 fish per group). Scale bars, 50 μm. (C) The numbers of skin mucus cells per millimetre after 24 h and control fish. (D) The picture of agarose gel electrophoresis. (E) The picture of flat coating. (F) Relative expression of *Ma*-Igs challenged with *F. columnare* G_4. (G) Relative mRNA levels of *Ma*-Igs at 7 days post infection. GI, gill; SK, skin; SP, spleen; HK, head kidney. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's t-test). The difference of expression (dotted line at y = 2) was ignored. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

points, and its expression was the lowest at day 28 (~0.08-fold). Only induction of IgM was noted on days 4 (~2.11-fold) and 7 (~2.02-fold) post-stimulation. In the spleen, IgT failed to prominently upregulate. In contrast, the expression of IgM was significantly enhanced at day 1 (~9.85-fold), whereas notable elevation of IgD was only detected at day 21 (~8.69-fold). In the head kidney, the strongest expression of IgT was observed at day 7 (~10.06-fold), IgM was found at day 4 (~6.25-fold), and IgD was discovered at day 21 (~5.88-fold).

4. Discussion

Teleost fish represent the oldest living bony vertebrate containing adaptive immune with Ig. It was noted that the important evolutionary significance of Ig would depend on the genetic structure characteristics. Although three different Ig isotypes, IgM, IgD and IgT, have been identified in some teleost fish, there are few studies reported on IgH locus. In the present study, the IgH genes and their locus have been characterized in the loach, an economically important freshwater fish species in aquaculture.

The structure of the loach IgH locus is similar to that of the loci reported from the zebrafish, rainbow trout, fugu and grass carp, with the following pattern: V_n-D_n-J_n-C_ζ/C_τ/C_H-D_n-J_n-C_μ-C_δ [22,23,35,36]. In the loach, the D and J segments upstream of C_τ are different from the D and J segments upstream of C_μ, similar to the zebrafish, rainbow trout and fugu [22,23,35], indicating that the loach IgH locus is analogous to the loci in the zebrafish, rainbow trout and fugu. In the channel catfish,

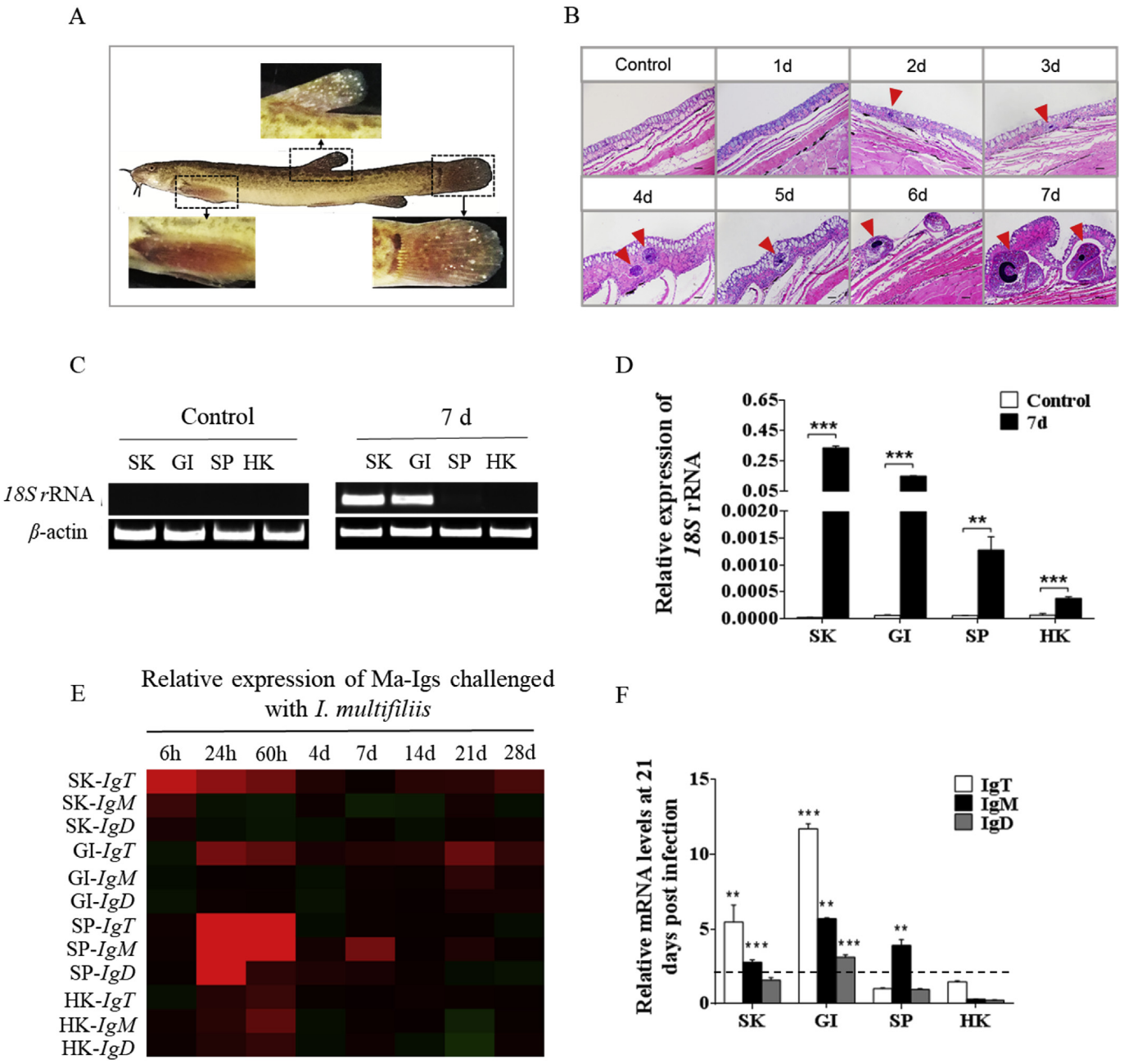

Fig. 6. *I. multifiliis* infection model of loach was successfully constructed. (A) Phenotypic characteristics of loach at 7 day after infection with *I. multifiliis*. (B) Histological examination (hematoxylin/eosin stain) of skin from loach infected with *I. multifiliis* after 1 d, 2 d, 3 d, 4 d, 5 d, 6 d, 7 d, and control fish (n = 6 fish per group). Scale bars, 50 μm. (C) Agarose gel electrophoresis was used to test the abundance of *I. multifiliis* in skin, gill, spleen and head kidney at 7 day after infection. (D) Relative expression of 18S rRNA of *I. multifiliis* was shown at 7 day after infection. (E) Relative expression of *Ma*-Igs challenged with *I. multifiliis*. (F) Relative mRNA levels of *Ma*-Igs at 21 days post infection. GI, gill; SK, skin; SP, spleen; HK, head kidney. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's t-test). The difference of expression (dotted line at y = 2) was ignored.

only two Ig isotypes, μ and δ genes, have been reported, and there is no evidence in the region sequenced of genes that could encode an Ig class similar to the ζ/τ [37,38]. The catfish IgH locus has caused an internal duplication of a 52.4-kb block of V_H genes, which makes it different from that in the zebrafish, rainbow trout, fugu, grass carp and loach. This indicates that the organization of the IgH locus in different species of teleost fish might be different. V_H genes in the IgH loci have only been reported in the zebrafish, grass carp, channel catfish and three-spined stickleback, with 47, 13, 55 and 49 V_H genes identified, respectively [22,36,37,39]. Thus, in different fish species, the number of V_H genes may vary. In the loach IgH locus, a segment of 59 kb contains 10 V_H genes. When comparing V_H genes among other fish species, it appears likely that the full composition of the V_H gene of the loach may need some further research. In addition, Ig δ2-δ3-δ4 exon duplications occur in some teleosts. There is a double duplication in the grass carp, a triplicate duplication in the channel catfish, and a quadruple duplication in the zebrafish [22,36,37]. However, in the loach, there is no duplication. In addition, qRT-PCR was performed to illustrate the expression patterns of *Ma*-Igs in early embryonic development and in different tissues. Results showed that *Ma*-Igs were highly expressed during ovum stage, may be due to maternal inheritance. The results of tissue-specific expression analysis showed that the highest values of *Ma*-Ig expression were detected in kidney, generally followed by spleen, due to the abundant B cells content in these organs, which was similar

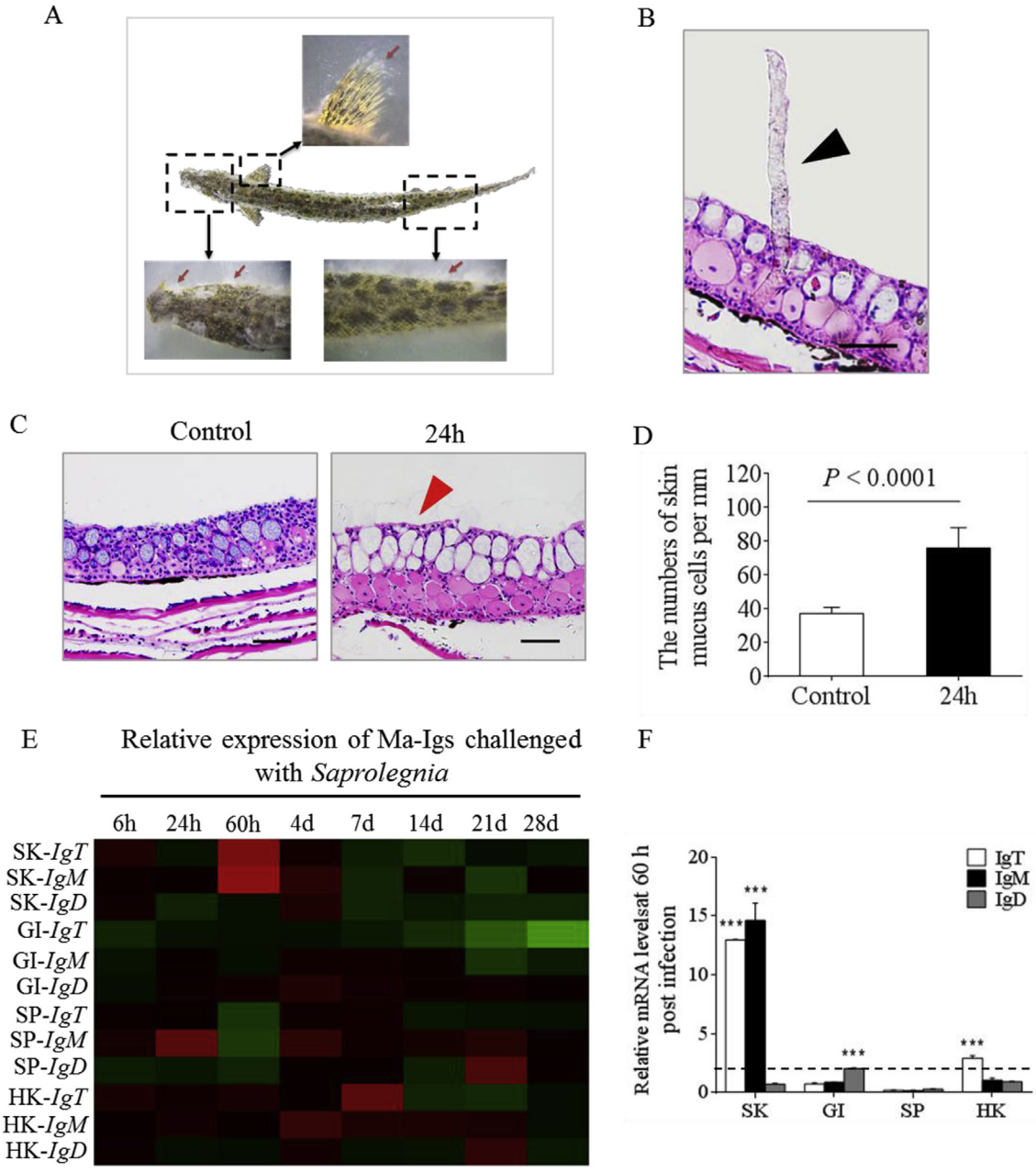

Fig. 7. *Saprolegnia* infection model of loach was successfully constructed. (A) Phenotypic characteristics of loach after infection with *Saprolegnia*. (B) Histological examination (hematoxylin/eosin stain) of skin from loach infected with *Saprolegnia*. Black arrow represents fungus silk. Scale bars, 50 μm. (C) Histological examination of skin from loach infected with *Saprolegnia* after 24 h and control fish. Red arrow represents skin mucus cells. Scale bars, 50 μm. (D) The numbers of skin mucus cells per millimetre after 24 h and control fish, counted from C. (E) Relative expression of *Ma*-Igs challenged with *Saprolegnia*. (F) Relative mRNA levels of *Ma*-Igs at 60 h post infection. SK, skin; GI, gill; SP, spleen; HK, head kidney. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's t-test). The difference of expression (dotted line at y = 2) was ignored. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to the findings in *Sparus aurata* [29].

Additionally, recent studies have been conducted on fish Igs responses [40–46]. Molecular-level research of rohu indicated the significant upregulation of IgM in head kidney and early upregulation of IgD in the skin after infection with *Argulus siamensis*, which is similar to our results of infection by Ich. In contrast to our results of up-regulation of IgM in the loach skin at 6 h after infection with parasites, Kar et al. found the transcript of IgM was below detectable range at all time-points [31]. Another study showed that the titers of IgT and IgM in serum of gilthead seabream were increased after long-term *Enteromyxum leei* infected and that *Photobacterium damselae* subsp. *piscicida* induced the increase of IgM expression in serum and a local IgT response in skin mucus by bath challenge [29]. These results suggest that different Igs may play different roles depending on the type of pathogen and infective route. In our study, the method of soaking infection was adopted, which simulates infection under natural conditions.

Here we found that the highest values of Igs basal expression were detected in the kidney, followed by the spleen, in terms of the abundance of Ig-producing B cells in these tissues. In bacterial expose experiment, we found that IgM and IgT showed high expression by *F. columnare* G_4 stimulation, suggesting that they might play a crucial role in response to bacterial pathogen, but significant changes in IgD mRNA levels did not appear in the gills or head kidney, which was analogous

to the result of Kato et al. [47]. Therefore, IgD might hardly affect immersion with bacteria. In addition, the high expression of both IgM and IgT were also found in all examined tissues at days 4 and 7 after being challenged with *F. columnare* G_4. IgM expression was increased remarkably in the spleen at day 1 after being challenged with *F. columnare* G_4. In addition, at day 4, the highest expression of IgM was found in the head kidney, followed by the spleen, while IgT expression reached the peak in the skin, followed by the spleen. Combined, these results indicate that IgM might be involved in systemic responses, while IgT might not only play a role in mucosal immunity, but have a certain function in systematic immunity by stimulating with bacterial pathogen. Similar result was found in mammals, bacterial infection could elicit strong adaptive immune responses in mucosal and systemic tissues with the IgA and IgG increasing, respectively [48–50]. However, ELISA results displayed that the level of IgM against *Mycobacterium tuberculosis* (Mtb) did not change, but the levels of IgA and IgG against Mtb were significantly higher in pulmonary tuberculosis patients [49]. Moreover, in Ich infection, our analysis indicates that the three Ig isotypes of fish have a certain role in resisting parasitic infection. On the first day of infection, IgT, IgM and IgD expression reached the peak in the spleen, and high IgT levels were noted in the skin and gills, which indicated that three Igs might participate in immune responses during parasites infection. Differently, the IgG and IgE of mammals are the main immunoglobulins involved in the immune responses after parasitic infection [51–53]. Calcagno et al. discovered that IgG and IgE are useful in the detection of total antibodies during the early diagnosis of *Trichinella spiralis* [51]. In fungal infections, IgG, IgA and IgE are very important in mammals [54–56], and IgM, IgX and IgY play a key role in amphibians [57]. Interestingly, our results of challenged with fungi showed that IgM and IgD with high expression, indicating that both of them might play a vital role in loach. In *Saprolegnia* infection, the time points of high Igs expression were similar to those in infection with *F. columnare* G_4. At day 4 post-infection, the highest IgD levels were discovered in the skin and gills, while the expression of IgM was observably increased in the spleen and head kidney. The highest expression of IgD was in the spleen at day 21 after being challenged, followed by the head kidney. The findings showed that IgD may play a greater role in specific immunity after infection with *Saprolegnia*. Interestingly, during three different infection models, the high expression of IgT appeared in mucosal tissues, such as skin or gills, in which the expression of IgT increased more rapidly in response to fungal infection. These results suggest that IgT might be involved in mucosal immune response during bacterial, parasitic and fungal infection. Although some studies have been reported IgT is the specialized Ig in mucosal tissues after parasitic and bacterial infection [24,25,27], we firstly provide the molecular evidence that IgT might play an important role in teleosts after fungal infection.

In conclusion, our research is the first time to describe the full sequences of the Igs and IgH loci of the loach and their expression under different pathogens infection conditions. Importantly, three infection models were established upon challenge with different pathogens (bacteria, parasites and fungi) by immersion. Moreover, we confirmed the important role of IgM in systemic responses under the three infection models and key role of IgD in Ich and *Saprolegnia* infection. In addition, our report shows that the expression of IgT could be strongly induced in mucosal tissues, which is similar to the findings of previous IgT studies [29,44]. All these results have important significance in evolution but also in practice to resist to pathogens infection in teleosts fish.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.064.

References

[1] M.D. Cooper, M.N. Alder, The evolution of adaptive immune systems, Cell 124 (4) (2006) 815–822.
[2] M.N. Alder, B.R. Herrin, A. Sadlonova, C.R. Stockard, W.E. Grizzle, L.A. Gartland, G.L. Gartland, J.A. Boydston, T.C. Jr, M.D. Cooper, Antibody responses of variable lymphocyte receptors in the lamprey, Nat. Immunol. 9 (3) (2008) 319.
[3] C. Kelly, F. Takizawa, J. Sunyer, I. Salinas, Rainbow trout (*Oncorhynchus mykiss*) secretory component binds to commensal bacteria and pathogens, Sci. Rep. 7 (2017).
[4] B. Patel, R. Banerjee, M. Basu, S. Lenka, M. Samanta, S. Das, Molecular cloning of IgZ heavy chain isotype in *Catla catla* and comparative expression profile of IgZ and IgM following pathogenic infection, Microbiol. Immunol. 60 (8) (2016) 561–567.
[5] R. Savan, A. Aman, M. Nakao, H. Watanuki, M. Sakai, Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L.), Immunogenetics 57 (6) (2005) 458–463.
[6] Y. Zhao, H.C. Cui, *Ornithorhynchus anatinus* (platypus) links the evolution of immunoglobulin genes in eutherian mammals and nonmammalian tetrapods, J. Immunol. 183 (5) (2009) 3285–3293.
[7] C. Dechavanne, S. Dechavanne, I. Sadissou, A.G. Lokossou, F. Alvarado, M. Dambrun, K. Moutairou, D. Courtin, G. Nuel, A. Garcia, Associations between an IgG3 polymorphism in the binding domain for FcRn, transplacental transfer of malaria-specific IgG3, and protection against Plasmodium falciparum malaria during infancy: a birth cohort study in Benin, PLoS Med. 14 (10) (2017) e1002403.
[8] F.R. Pastrana, J. Neef, D.G.A.M. Koedijk, D.D. Graaf, J. Duipmans, M.F. Jonkman, S. Engelmann, J.M.V. Dijl, G. Buist, Human antibody responses against non-covalently cell wall-bound *Staphylococcus aureus* proteins, Sci. Rep. 8 (1) (2018).
[9] M.F. Flajnik, Comparative analyses of immunoglobulin genes: surprises and portents, Nat. Rev. Immunol. 2 (9) (2002) 688–698.
[10] A. Cerutti, M. Rescigno, The biology of intestinal immunoglobulin a responses, Immunity 28 (6) (2008) 740–750.
[11] M.L. Lundqvist, D.L. Middleton, C. Radford, G.W. Warr, K.E. Magor, Immunoglobulins of the non-galliform birds: antibody expression and repertoire in the duck, Dev. Comp. Immunol. 30 (1) (2006) 93–100.
[12] B. Han, H. Yuan, T. Wang, B. Li, L. Ma, S. Yu, T. Huang, Y. Li, D. Fang, X. Chen, Multiple IgH isotypes including IgD, subclasses of IgM, and IgY are expressed in the common ancestors of modern birds, J. Immunol. 196 (12) (2016) 5138–5147.
[13] Z. Xu, G.L. Wang, P. Nie, IgM, IgD and IgY and their expression pattern in the Chinese soft-shelled turtle *Pelodiscus sinensis*, Mol. Immunol. 46 (10) (2009) 2124–2132.
[14] F.G. Deza, C.S. Espinel, J.V. Beneitez, A novel IgA-like immunoglobulin in the reptile, Dev. Comp. Immunol. 31 (6) (2007) 596–605.
[15] B. Schaerlinger, M. Bascove, J.P. Frippiat, A new isotype of immunoglobulin heavy chain in the urodele amphibian *Pleurodeles waltl* predominantly expressed in larvae, Mol. Immunol. 45 (3) (2008) 776–786.
[16] G.W. Warr, K.E. Magor, D.A. Higgins, IgY: clues to the origins of modern antibodies, Immunol. Today 16 (8) (1995) 392–398.
[17] M. Rainer, D.P. Louis, H. Ellen, Is *Xenopus* IgX an analog of IgA? Eur. J. Immunol. 26 (12) (2010) 2823–2830.
[18] G.W. Warr, The immunoglobulin genes of fish, Dev. Comp. Immunol. 19 (1) (1995) 1–12.
[19] M. Wilson, E. Bengtén, N.W. Miller, L.W. Clem, P.L. Du, G.W. Warr, A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD, Proc. Natl. Acad. Sci. U. S. A 94 (9) (1997) 4593–4597.
[20] I. Hordvik, J. Thevarajan, I. Samdal, N. Bastani, B. Krossoy, Molecular cloning and phylogenetic analysis of the Atlantic salmon immunoglobulin D gene, Scand. J. Immunol. 50 (1999) 202–210.
[21] I. Hirono, B.H. Nam, J. Enomoto, K. Uchino, T. Aoki, Cloning and characterisation of a cDNA encoding Japanese flounder *Paralichthys olivaceus* IgD, Fish Shellfish Immunol. 15 (1) (2003) 63–70.
[22] N. Danilova, J. Bussmann, K. Jekosch, L.A. Steiner, The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z, Nat. Immunol. 6 (3) (2005) 295–302.
[23] J.D. Hansen, E.D. Landis, R.B. Phillips, Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish, Proc. Natl. Acad. Sci. U. S. A 102 (19) (2005) 6919–6924.
[24] Y. Zhang, I. Salinas, J. Li, D. Parra, S. Bjork, Z. Xu, S.E. LaPatra, J. Bartholomew, J.O. Sunyer, IgT, a primitive immunoglobulin class specialized in mucosal immunity, Nat. Immunol. 11 (9) (2010) 827.
[25] Z. Xu, D. Parra, D. Gómez, I. Salinas, Y.A. Zhang, G.J.L. Von, R.D. Heinecke, K. Buchmann, S. Lapatra, J.O. Sunyer, Teleost skin, an ancient mucosal surface that elicits gut-like immune responses, Proc. Natl. Acad. Sci. U. S. A 110 (32) (2013) 13097–13102.
[26] L. Tacchi, R. Musharrafieh, E.T. Larragoite, K. Crossey, E.B. Erhardt, S.A. Martin, S.E. Lapatra, I. Salinas, Nasal immunity is an ancient arm of the mucosal immune system of vertebrates, Nat. Commun. 5 (08) (2014) 5205.

[27] X. Zhen, F. Takizawa, D. Parra, D. Gómez, L.V.G. Jørgensen, S.E. Lapatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nat. Commun. 7 (2016) 10728.
[28] M. Basu, S.S. Lenka, M. Paichha, B. Swain, B. Patel, R. Banerjee, P. Jayasankar, S. Das, M. Samanta, Immunoglobulin (Ig) D in *Labeo rohita* is widely expressed and differentially modulated in viral, bacterial and parasitic antigenic challenges, Vet. Immunol. Immunopathol. 179 (2016) 77–84.
[29] M.C. Piazzon, J. Galindovillegas, P. Pereiro, I. Estensoro, J.A. Calduchginer, E. Gómezcasado, B. Novoa, V. Mulero, A. Sitjàbobadilla, J. Pérezsánchez, Differential modulation of IgT and IgM upon parasitic, bacterial, viral, and dietary challenges in a perciform fish, Front. Immunol. 7 (637) (2016).
[30] R. Banerjee, B. Patel, M. Basu, S.S. Lenka, M. Paicha, M. Samanta, S. Das, Molecular cloning, characterization and expression of immunoglobulin D (IgD) on pathogen challenge and PAMPs stimulation in freshwater carp, *Catla catla*, Microbiol. Immunol. 61 (10) (2017).
[31] B. Kar, A. Mohapatra, J. Mohanty, P.K. Sahoo, Transcriptional changes in three immunoglobulin isotypes of rohu, *Labeo rohita* in response to *Argulus siamensis* infection, Fish Shellfish Immunol. 47 (1) (2015) 28–33.
[32] C. Qin, K. Huang, H. Xu, Protective effect of polysaccharide from the loach on the in vitro and in vivo peroxidative damage of hepatocyte, JNB (J. Nutr. Biochem.) 13 (10) (2002) 592–597.
[33] X. Xu, E. Sivaramasamy, S. Jin, F. Li, J. Xiang, Establishment and characterization of a skin epidermal cell line from mud loach, *Misgurnus anguillicaudatus*, (MASE) and its interaction with three bacterial pathogens, Fish Shellfish Immunol. 55 (2016) 444–451.
[34] M. Zhu, X.R. Wang, J. Li, G.Y. Li, Z.P. Liu, Z.L. Mo, Identification and virulence properties of *Aeromonas veronii* bv. sobria isolates causing an ulcerative syndrome of loach *Misgurnus anguillicaudatus*, J. Fish. Dis. 39 (6) (2016) 777.
[35] S. Ram, A. Azumi, S. Kenji, Y. Ryogi, S. Masahiro, Discovery of a new class of immunoglobulin heavy chain from fugu, Eur. J. Immunol. 35 (11) (2010) 3320–3331.
[36] F.S. Xiao, Y.P. Wang, W. Yan, M.X. Chang, W.J. Yao, Q.Q. Xu, X.X. Wang, Q. Gao, P. Nie, Ig heavy chain genes and their locus in grass carp *Ctenopharyngodon idella*, Fish Shellfish Immunol. 29 (4) (2010) 594–599.
[37] B. E, Q. S, H. J, W. G, W. GW, M. NW, W. M, Structure of the catfish IGH locus: analysis of the region including the single functional IGHM gene, Immunogenetics 58 (10) (2006) 831–844.
[38] E. Bengtén, L.W. Clem, N.W. Miller, G.W. Warr, M. Wilson, Channel catfish immunoglobulins: repertoire and expression, Dev. Comp. Immunol. 30 (1) (2006) 77–92.
[39] F. Gambóndeza, C. Sánchezespinel, S. Magadánmompó, Presence of an unique IgT on the IGH locus in three-spined stickleback fish (*Gasterosteus aculeatus*) and the very recent generation of a repertoire of VH genes, Dev. Comp. Immunol. 34 (2) (2010) 114–122.
[40] Y. Gao, Y. Yi, H. Wu, Q. Wang, J. Qu, Y. Zhang, Molecular cloning and characterization of secretory and membrane-bound IgM of turbot, Fish Shellfish Immunol. 40 (2) (2014) 354–361.
[41] Z.P. Wang, Y.Q. Wu, Q.L. Hu, Y.L. Li, Differences on the biological function of three Ig isotypes in zebrafish: a gene expression profile, Fish Shellfish Immunol. 44 (1) (2015) 283–286.
[42] H. Xia, K. Wu, W. Liu, W. Wang, X. Zhang, Spatio-temporal expression of blunt snout bream (*Megalobrama amblycephala*) mIgD and its immune response to*Aeromonas hydrophila*, Cent.-Eur. J. Immunol. 40 (2) (2015) 132–141.
[43] B. Wang, P. Wang, Z.H. Wu, Y.S. Lu, Z.L. Wang, J.C. Jian, Molecular cloning and expression analysis of IgD in nile Tilapia (*Oreochromis niloticus*) in response to *Streptococcus agalactiae* stimulus, Int. J. Mol. Sci. 17 (3) (2016) 348.
[44] D. Yang, T. Xiaoqian, Z. Wenbin, X. Jing, S. Xiuzhen, Immunoglobulin Tau heavy chain (IgT) in flounder, *Paralichthys olivaceus*: molecular cloning, characterization, and expression analyses, Int. J. Mol. Sci. 17 (9) (2016).
[45] N. Zhang, X.J. Zhang, D.D. Chen, J.O. Sunyer, Y.A. Zhang, Molecular characterization and expression analysis of three subclasses of IgT in rainbow trout (*Oncorhynchus mykiss*), Dev. Comp. Immunol. 70 (2017) 94.
[46] F. Buonocore, V. Stocchi, N. Nunezortiz, E. Randelli, M. Gerdol, A. Pallavicini, A. Facchiano, C. Bernini, L. Guerra, G. Scapigliati, Immunoglobulin T from sea bass (*Dicentrarchus labrax* L.): molecular characterization, tissue localization and expression after nodavirus infection, BMC Mol. Biol. 18 (1) (2017) 8.
[47] G. Kato, T. Takano, T. Sakai, T. Matsuyama, N. Sano, C. Nakayasu, Cloning and expression analyses of a unique IgT in ayu *Plecoglossus altivelis*, Fish. Sci. 81 (1) (2015) 29–36.
[48] Y. Zhang, C. Dominguezmedina, N.J. Cumley, J.N. Heath, S.J. Essex, S. Bobat, A. Schager, M. Goodall, S. Kracker, C.D. Buckley, IgG1 is required for optimal protection after immunization with the purified porin OmpD from *Salmonella* Typhimurium, J. Immunol. 199 (12) (2017) ji1700952.
[49] F. Abebe, M. Belay, M. Legesse, F.L.M.C. K, T. Ottenhoff, IgA and IgG against Mycobacterium tuberculosis Rv2031 discriminate between pulmonary tuberculosis patients, *Mycobacterium tuberculosis*-infected and non-infected individuals, PLoS One 13 (1) (2018) e0190989.
[50] F.R. Lima, I. Takenami, M.A. Cavalcanti, L.W. Riley, S. Arruda, ELISA-based assay of immunoglobulin G antibodies against mammalian cell entry 1A (Mce1A) protein: a novel diagnostic approach for leprosy, Memórias Do Instituto Oswaldo Cruz 112 (12) (2017) 844–849.
[51] M.A. Calcagno, M.A. Forastiero, M.P. Saracino, C.C. Vila, S.M. Venturiello, Serum IgE and IgG4 against muscle larva excretory-secretory products during the early and late phases of human trichinellosis, Parasitol. Res. 116 (11) (2017) 1–7.
[52] X. Liu, K. Zhou, D. Yu, X. Cai, Y. Hua, H. Zhou, C. Wang, A delayed diagnosis of X-linked hyper IgM syndrome complicated with toxoplasmic encephalitis in a child: a case report and literature review, Medicine 96 (49) (2017) e8989.
[53] H. Nahouli, A.N. El, E. Chalhoub, E. Anastadiadis, H.H. El, Seroprevalence of anti-*Toxoplasma gondii* antibodies among Lebanese pregnant women, Vector Borne Zoonotic Dis. 17 (12) (2017).
[54] A. Taylor, I. Peters, N.K. Dhand, J. Whitney, L.R. Johnson, J.A. Beatty, V.R. Barrs, Evaluation of serum Aspergillus-specific immunoglobulin a by indirect ELISA for diagnosis of feline upper respiratory Tract aspergillosis, J. Vet. Intern. Med. 30 (5) (2016) 1708–1714.
[55] J. Vitte, T. Romain, A. Carsin, M. Gouitaa, N. Stremler-Le Bel, M. Baravalle-Einaudi, I. Cleach, M. Reynaud-Gaubert, J.C. Dubus, J.L. Mège, *Aspergillus fumigatus* components distinguish IgE but not IgG4 profiles between fungal sensitization and allergic broncho-pulmonary aspergillosis, Allergy 71 (11) (2016) 1640–1643.
[56] N.A. Chow, M.D. Lindsley, O.Z. Mccotter, D. Kangiser, R.D. Wohrle, W.R. Clifford, H.D. Yaglom, L.E. Adams, K. Komatsu, M.M. Durkin, Development of an enzyme immunoassay for detection of antibodies against *Coccidioides* in dogs and other mammalian species, PLoS One 12 (4) (2017) e0175081.
[57] J.P. Ramsey, L.K. Reinert, L.K. Harper, D.C. Woodhams, L.A. Rollinssmith, Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*, Infect. Immun. 78 (9) (2010) 3981–3992.