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Molecular characterization and expression analysis of T cell receptor (TCR) γ and δ genes in dojo loach (*Misgurnus anguillicaudatus*) in response to bacterial, parasitic and fungal challenge

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ABSTRACT

In mammalian, T-cell receptors (TCRs) play a key role in recognizing the presented antigen from external to protect organisms against environmental pathogens. To understand the potential roles of TCRγ and TCRδ in dojo loach (*Misgurnus anguillicaudatus*), *Ma*-TCRγ and *Ma*-TCRδ cDNAs were cloned and their gene expression profiles were investigated after bacterial, parasitic and fungal challenge. The open reading frame (ORF) of *Ma*-TCRγ and *Ma*-TCRδ cDNAs contained 948 and 867 bp, encoding 316 and 288 amino acid residues, respectively. Structurally, *Ma*-TCRγ and *Ma*-TCRδ were consisted of a signal peptide, a variable region, a constant region (IgC), a connecting peptide (CPS), a transmembrane region (TM) and a cytoplasmic domain (CYT), which were similar to those of other vertebrates. Multiple sequence alignment and phylogenetic analysis showed *Ma*-TCRγ and *Ma*-TCRδ were closely related to fish of Cyprinidae family. *Ma*-TCRγ and *Ma*-TCRδ were widely expressed in all tested organs/tissues, as the highest expressions of *Ma*-TCRγ and *Ma*-TCRδ were detected in kidney and gill, respectively. In addition, three infection models of dojo loach with bacteria (*F. columnare* G_4), parasite (*Ichthyophthirius multifiliis*) and fungus (*Saprolegnia sp.*) were constructed. The morphological changes of gills and skin after challenged with *F. columnare* G_4 and *Ichthyophthirius multifiliis* were investigated. Compared to *F. columnare* G_4 infection, mRNA expression of both TCRγ and TCRδ showed higher sensitivity in classical immune organs (kidney and spleen) and mucosal tissues (skin and gill) after challenge with *Ichthyophthirius multifiliis* and *Saprolegnia sp*. Our results first indicated that TCRγ and TCRδ of dojo loach might function differently in response to challenge with different pathogens.

1. Introduction

T cells function as both the effectors to directly kill infected/targeted cells and the coordinators in the responses of other immune cells, they are important players of adaptive immune responses in vertebrates, which are crucial in the body defense against various invading pathogens [1,2]. Importantly, T cells are characterized by the presence of T-cell receptors (TCRs), a hallmark of all T cell surfaces. In the conventional T cells, the TCR consists of an α (α) chain and a β (β) chain [3], while in the other tiny minority of T cells, it is made up of γ and δ (γ/δ) chains. **γδ TCRs polypeptide chains are derived from variable (V), diversity (D), joining (J), and constant (C) gene segments, during intrathymic T cell maturation genes somatically rearranged through the V-(D)-J recombination process to encoded TCRγ and TCRδ chain proteins [4]. The variable domains included CDR1, CDR2 and CDR3 loops, during rearrangement of receptors for a particular T cell lineage, CDR1 and CDR2 loops diversity comes from differences in the V gene segments were used within a species [5]. In addition, CDR3 loops usually play important roles in antibodies to recognize antigen. Thus, CDR3 loop diversity and length are often related to antigen recognition capacity [5].** αβ TCRs can specifically recognize fragments of antigen as peptides bound to major histocompatibility complex (MHC) molecules [6–9]. Contrary to αβ TCRs, γδ TCRs possess unique features, for example, they do not

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display MHC restriction, which is in accord with the absence of CD4 or CD8 expression in the majority [2,10]. **Compared to αβ T cells, the functions of γδ T cells were much less known. In teleost fish, γδ T cells were first confirming existed in 2016 and γδ T cells play crucial roles in IgZ (also named IgT) production, a prevalent Ig molecule in all fish mucosa-associated lymphoid tissues (MALTs), such as gut-associated lymphoid tissue, skin-associated lymphoid tissue, gill-associated lymphoid tissue and nasopharynx-associated lymphoid tissue in zebrafish, rainbow trout, and common carp which play an important role in adaptive immune response** [11–16]. In previous studies, the structure, locus and expression of γδ TCR molecules also have been investigated in almost all groups of vertebrates such as mammals [17–21], avian [22–24], reptiles [25], amphibians [26,27] and elasmobranches [28–30]. However, the studies of γδ TCRs in teleost are much less. γδ TCR chains in teleost were firstly identified in Japanese flounder in 2003 [31], followed by salmon, zebrafish and puffer [32–35]. Furthermore, the genomic organization of the TCR δ locus and the transcription of TCRδ was described in teleost such as *Tetraodon nigroviridis* [35], *Cyprinus carpio* [36], *Dicentrarchus labrax* [37], which were well expressed in gut-associated lymphoid tissue. It implies that γδ TCR may play a defensive role to protect fish against pathogen infection. **In addition, the bacterial pathogen of *Aeromonas hydrophila* has been reported that it could suppress the TCR signaling pathway in the large yellow croaker *Larimichthys crocea* [38], and the expression level of TCRγ after stimulation with *Flavobacterium columnare* was reported in mandarin fish** [39]. **However, by far very few study has been reported about the transcriptional changes of γδ TCR in teleost fish during infected with bacteria, parasite and fungus at the same time.**

Dojo loach (*Misgurnus anguillicaudatus*) remains one of the important commercial fish species in Eastern Asian counties including Korea, Japan and China, possessing high nutritional, medicinal and market value [40–42]. Nowadays, high stocking density and deteriorating aquaculture environments have resulted in frequently infectious diseases and finally great economic losses [43–45]. However, the immunological basis of dojo loach is little known so far. **TCRs reside at the surface of T cells, its function is recognize the antigen and play a crucial role in immune responses,** and αβ TCR in loach have been cloned and checked their roles during pathogenic infection in our previous study [46]. In this study, we firstly cloned the full-length cDNA sequences of *Ma*-TCRγ and *Ma*-TCRδ and analyzed their expression pattern in different tissues and during early embryonic development. Three infection models of dojo loach with *F. columnare* G_4, *I. multifiliis* and *Saprolegnia sp.* were constructed with obvious morphological changes of gills and skin. Moreover, the γδ TCR mRNA expressions in skin, gill, spleen and kidney of loach were assessed after bacterial, parasitic and fungal challenge. Combing with our previous results of αβ TCR in loach, the present study not only provided the information for the evolutional research of γδ TCR, but also helped to illustrate the role of T cell receptors in the immune response of dojo loach against different aquatic pathogenic infection.

2. Material and methods

2.1. Animals and sample collection

A total of 600 healthy dojo loaches (average weight of 5.0 ± 0.5 g) were purchased from a farm in Wuhan city (Hubei Province, China) and maintained in running aerated water at 22–25 °C for two weeks prior to experiment. Loaches were fed with commercial diets twice per day and a quarter of water in each tank was renewed daily.

For spatial gene expression analysis, eight fish were sacrificed after anesthetized with 100 mg/L tricaine methane sulfonate (MS-222). **In order to obtain the gill for RNA analysis, blood in the gills was first removed by perfusion with cold PBS-heparin through the heart until the gills were completely blanched.** Tissues including spleen, liver, kidney, skin, gills, intestine, eye, brain, blood, muscle, fin, fat and gonad were dissected, immediately frozen in liquid nitrogen and then stored at −80 °C before analysis. For gene expression analysis during ontogeny, mature male and female loaches were injected with mixed oxytocin to produce sperms and eggs as described by Zhou et al. [47]. Loach sperms were added to eggs and mixed well for fertilization. Zygote, blastodisc, 2 cells, 4 cells, 8 cells, 16 cells, 32 cells, 64 cells, gastrula, neurula, sarcomere, organogenesis, hatching, prelarva, 2 d, 4 d, 6 d larvae post hatching (dph) were separately frozen in liquid nitrogen and then stored at −80 °C prior to use.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from each sample using Trizol Reagent (Invitrogen Life Technologies, USA) following the manufacturer's instruction. The concentration of RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (IMPLEN, Germany), and the purity was checked by measuring the ratio of OD 260 nm/OD 280 nm. Reverse transcription was then conducted using the AccuRT Genomic DNA Removal Kit (Abm, Canada) following the manufacturer's instruction. The obtained cDNA was stored at −20 °C for gene cloning and qRT-PCR experiment.

2.3. Cloning of TCRγ and TCRδ genes from M. anguillicaudatus

Partial sequences of TCRγ and TCRδ were obtained from transcriptome database of *M. anguillicaudatus* (**unpublished**), which were confirmed using designed gene-specific primers. To obtain the full-length cDNA sequence of TCRγ and TCRδ, a rapid amplification of cDNA ends (RACE) approach was performed using SMART™ RACE

Table 1
PCR primers used in this study.

Name	Sequence(5'-3')	Application
Clo-TCRγ −F	GCTGGAAAACCCAAAATCG	Conserved region cloning
Clo-TCRγ −R	AGACTGGGTGCTTCATCCGT	
Clo-TCRδ −F	TTAACATTCGGAAAGCC	
Clo-TCRδ −R	TGTCACAAGTAGGGTCAT	
TCRγ5'-Race1	CATTGAATCTGCCTCGGAAA	RACE-PCR
TCRγ5'-Race2	GCCAATCTACCTTTTGTTCT	
TCRγ3'-Race1	GAAACCACGGATGAAGCACC	
TCRγ3'-Race2	CCCAGTCTGTATCTGTTTCTCTATGC	
TCRδ5'-Race1	TCTTGTCCATCGGGTTCTAC	
TCRδ5'-Race2	TGGGGTCTCCACTTCATTCAGC	
TCRδ3'-Race1	GTCCAACAAACGCTCCT	
TCRδ3'-Race2	TTCTGCTTGCTAAATGTG	
UMP-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
UMP-short	CTAATACGACTCACTATAGGGC	

Table 2
Real time PCR Primers used in this study.

Name	Sequence(5'-3')	Location of primers	Application
RTq-TCRγ −F	AAGCATACAAACCATCA	Amplification of Cαfragments using 470-632bp as template	qRT-PCR
RTq-TCRγ −R	CGTCCCTCTGTTCAATGA		
RTq-TCRδ −F	ATCCGTCTCACCGTCGT	Amplification of Cαfragments using 450-631bp as template	
RTq-TCRδ −R	ACAAGGCAGCATTTTCG		
18S−F	AGTTGGTGGAGCGATTTG		
18S-R	CTCGGCGAAGGGTAGACA		
EF1aF	TCAGCGCCTACATCAAGAAG		
EF1aR	TTACGCTCAACCTTCCATCC		

cDNA Amplification Kit (Clontech, USA) with specific primers. The PCR products were ligated into PMD19-T simple vector (TaKaRa, China) and sequenced (TSINGKE, China). All primers used for gene clone were listed in Table 1.

2.4. Sequence alignment and phylogenetic analysis

The nucleotide sequences of TCRγ and TCRδ was analyzed by ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The deduced amino acid sequences of TCRγ and TCRδ were both spliced and edited using the DNAstar software. Signal peptide prediction was performed by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/). The protein domain features and transmembrane domain were predicted by IMGT site (http://www.imgt.org/) and InterPro (http://www.ebi.ac.uk/interpro/). TCRγ and TCRδ cDNA sequences of different vertebrate species were obtained from GenBank databases. Multiple alignments analysis of amino acid sequences were performed using the ESPript (http://multalin.toulouse.inra.fr/multalin/). Besides, two phylogenetic trees were constructed using MEGA 6.0 with the neighbor-joining method based on the deduced amino acid sequences of TCRγ and TCRδ [48], respectively.

2.5. Pathogen challenge experiment

2.5.1. Bacterial challenge with F. columnare G_4

F. columnare G_4 was a gift from the Institute of Hydrobiology, Chinese Academy of Sciences (Hubei, China). *F. columnare* G_4 was recovered in Shieh medium plate at 28 °C for 24 h. Then a single colony was picked and inoculated in Shieh broth at 28 °C for 36 h to a final concentration of 1×10^5 CFU/ml. In the challenge test, 180 healthy fish were divided into 6 tanks including three control groups and three challenge groups. Loaches in challenge groups were separately bathed with 2000 ml of *F. columnare* G_4 at 1×10^5 CFU/ml, while loaches in the control group were bathed with normal Shieh broth. Six individuals from each group were randomly selected to be over-anesthetized at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d and 28 d post infection. In order to obtain the gill for histology analysis, blood in the gills was first removed by perfusion with cold PBS-heparin through the heart until the gills were completely blanched. Then part of gills (~0.01 g) and skin (~0.25 cm^2) were dissected and fixed in 4% neutral buffered paraformaldehyde for 24 h. Other tissues including kidney, spleen, skin and gills were harvested, immediately frozen in liquid nitrogen and stored at −80 °C before further analysis.

2.5.2. Parasitic challenge with I. multifiliis

I. multifiliis challenge experiment was conducted following the same method as reported before [49]. Briefly, mature theronts which left from the heavily infected rainbow trout were collected and transferred to glass beakers containing aerated tap water. 90 healthy fish were divided randomly into three tanks, each containing 400,000 theronts (with the ratio of 10,000 Ich theronts per fish). Samples were collected at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d and 28 d post infection after over-anesthetized. All sampling procedures are same as 2.5.1.

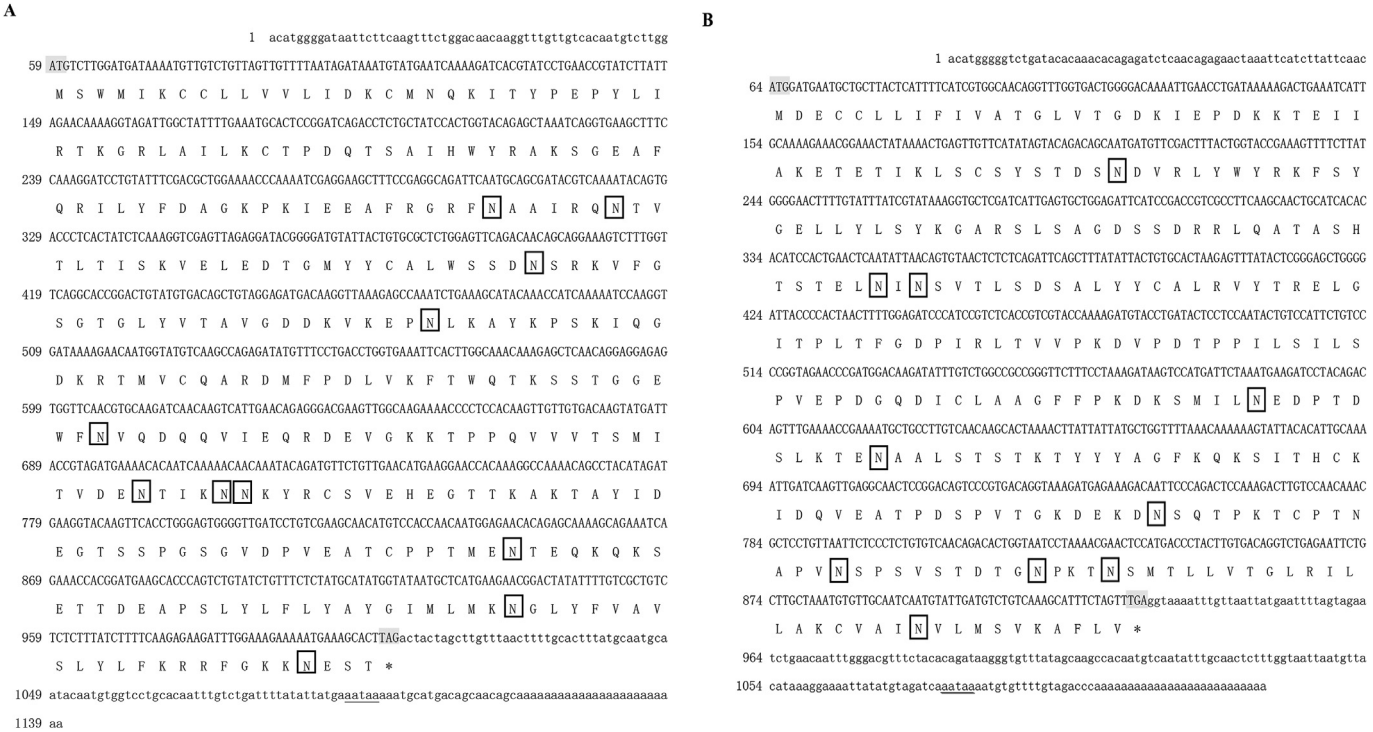

Fig. 1. The cDNA and deduced amino acid sequences of *M. anguillicaudatus* TCRγ (A) and TCRδ (B). Numbers on the left in each row represent the amino acid or nucleotide position. The initiation codon (ATG) and termination codon (TAG or TGA) are shadowed in gray. N-linked glycosylation sites are indicated by boxes. The stop codon of the open reading frame is indicated by an asterisk (*). The polyadenylation signal and mRNA instability motif underlined.

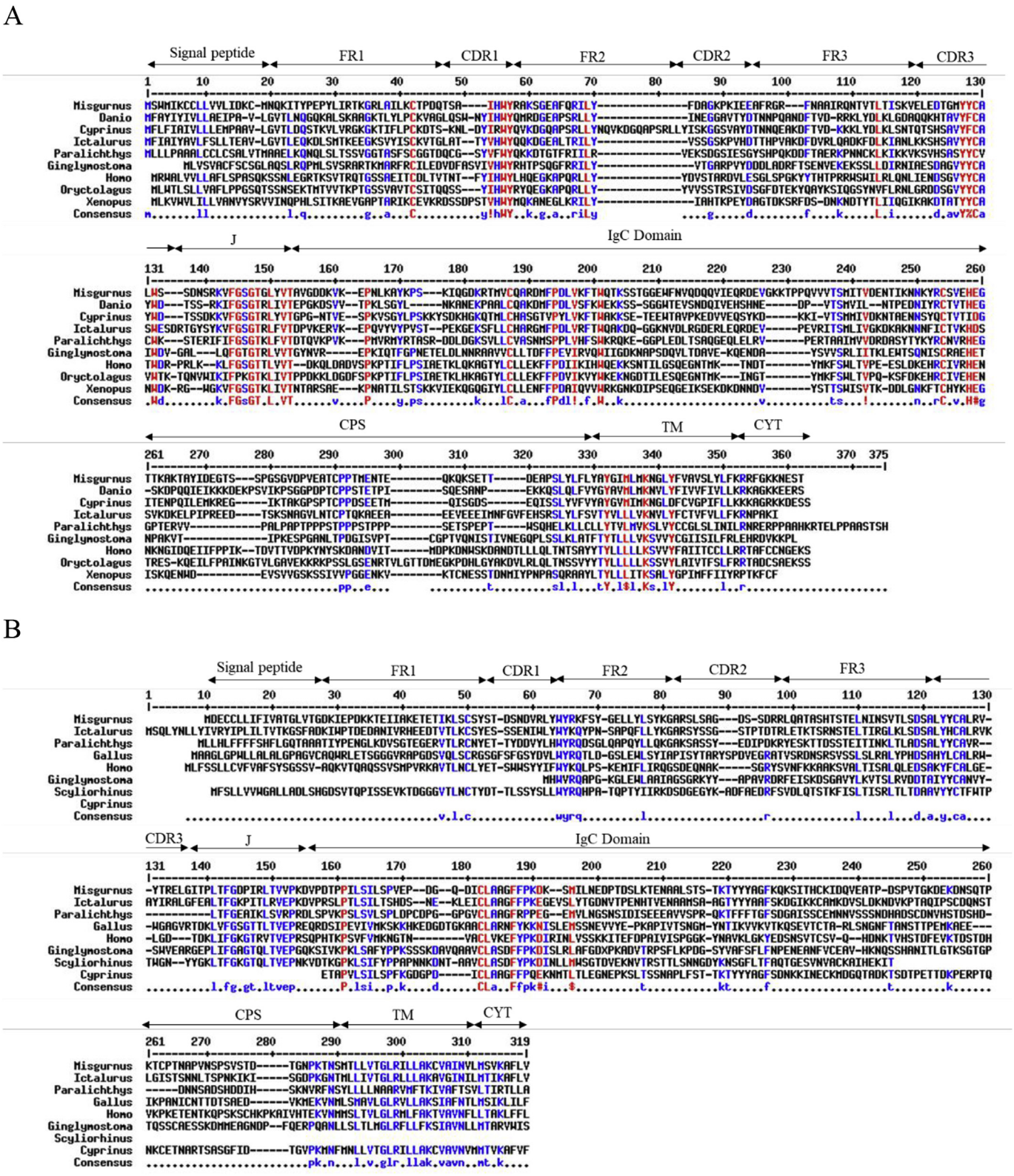

Fig. 2. Alignment of the amino acid sequences of *Ma*-TCRγ (A) and *Ma*-TCRδ (B) with that of other species. The accession numbers of these proteins from GenBank were as follows: (A) *Homo sapiens* (S01895), *Oryctolagus cuniculus* BAA07332, *Xenopus laevis* (AF440825), *Ginglymostoma cirratum* (ADW95913.1), *Danio rerio* (AY973921), *Cyprinus carpio* (DQ367842), *Ictalurus punctatus* (DQ435303), *Paralichthys olivaceus* (AB076073.1). (B) *Ictalurus punctatus* (HQ913590.1), *Paralichthys olivaceus* (AB076072.1), *Gallus gallus* (AAD51740.1), *Homo sapiens* (AY357942.1), *Ginglymostoma cirratum* (FJ513799.1), *Ginglymostoma cirratum* igc(AAA87016.1), *Cyprinus carpio* (B541473.2). The identical and similar amino acids were highlighted in red and blue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

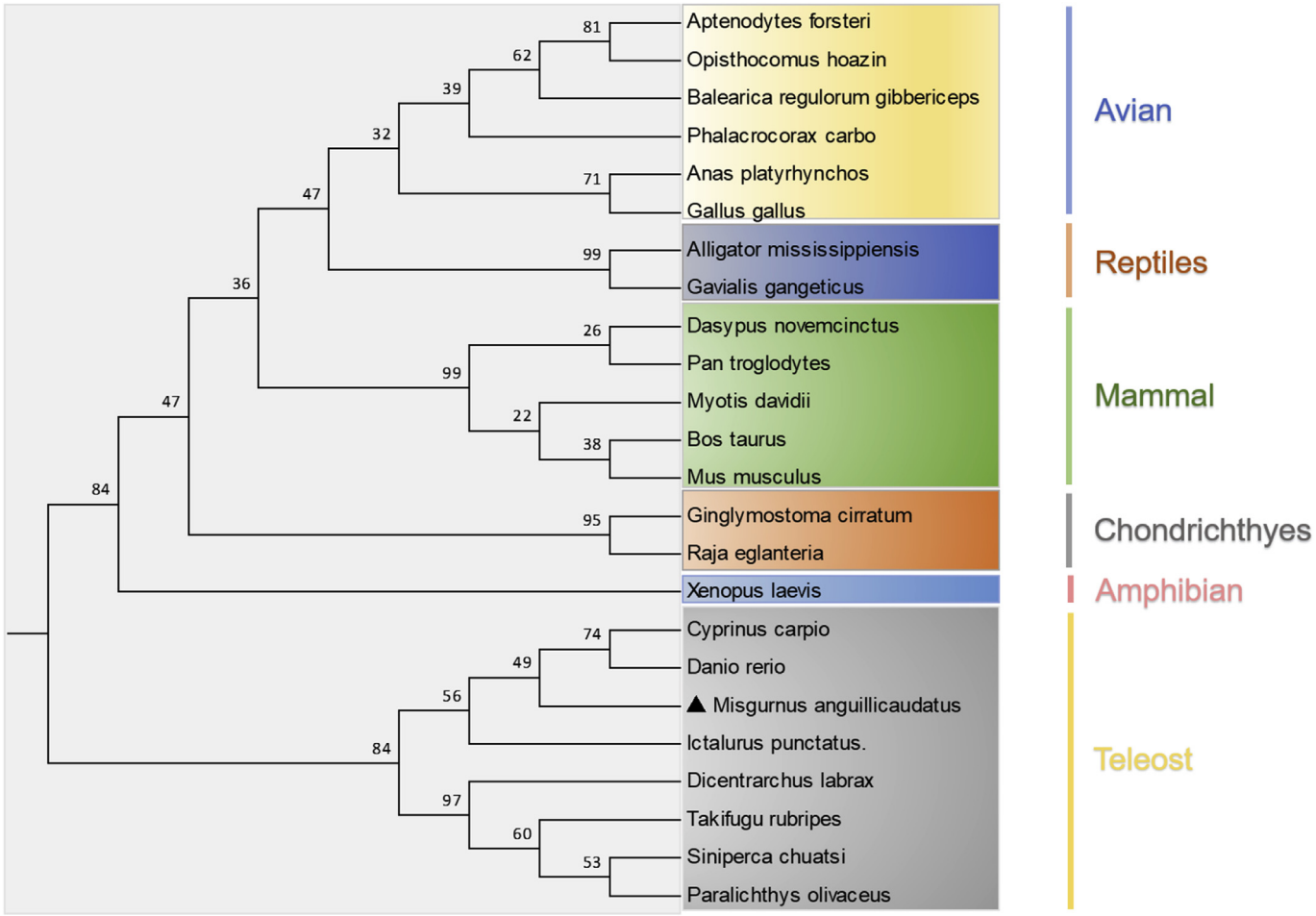

Fig. 3. Phylogenetic analysis of the T-cell antigen receptor (TCRγ) constant regions between loach and those of other vertebrates. The numbers indicate the percentage frequencies with which the phylogram topology represented here replicated for every 1000 bootstrap iterations. The sequences were aligned by CLUSTAL W program and the phylogenetic tree was constructed by neighbor-joining methods using MEGA software version 6.0. *Ma*-TCRγ was marked with a solid black triangle. GenBank accession numbers for TCRγ molecule used **in the analysis: Anas platyrhynchos (XP_021147366.1), *Gallus gallus* (AAA87009.1), *Alligator mississippiensis* (XP_019338877.1), *Gavialis gangeticus* (XP_019364263.1), *Phalacrocorax carbo* (KFW89802.1), *Balearica regulorum gibbericeps* (KFO11515.1), Aptenodytes forsteri (KFM03624.1), *Opisthocomus hoazin* (KFR17381.1), Dasypus novemcinctus (XP_023445745.1), *Bos taurus* (XP_024846900.1), Mus musculus (pir||D26420), Pan troglodytes (XP_016812778.1), *Myotis davidii* (ELK34092.1), *Ginglymostoma cirratum* (ADW95913.1), *Raja eglanteria* (AAB51498.1), *Xenopus laevis* (AAM21541.1), *Cyprinus carpio* (DQ367842), *Danio rerio* (AY973921), *Ictalurus punctatus* (DQ435303), *Dicentrarchus labrax* (CAY12508.2), *Takifugu rubripes* (BAE16941.1), *Siniperca chuatsi* (EF596787.1), *Paralichthys olivaceus* (AB076073.1).**

2.5.3. Fungal challenge with Saprolegnia sp

Mycelium of one *Saprolegnia sp.* strain was isolated from heavily infected rainbow trout in the fishery research base at Huazhong Agricultural University (Hubei, China). In this study, sporulation was induced as described before [50] with peptone-glu-cose agar (PGA) medium [51]. The mycelium was first grown in PGA medium which was covered with sterile rapeseeds for 3 d at 25 °C. Then the rapeseeds were taken from mycelium of the plate and incubated in sterile water for 2 days at 20 °C to induce sporulation. Zoospores were counted using a hemocytometer and then adjusted to 1×10^5 zoospore/ml water. 90 loaches were distributed in three same tanks (each tank contained ~ 1×10^8 zoospores) during the challenge experiment. The loach in the infected group and the control group were slightly scratched with a glass slide. All other sampling procedures including sampling tissues and sampling time are same as 2.5.1.

2.6. Histopathology analysis

Morphological changes in gill and skin were evaluated after bacterial, parasitic and fungal challenge [52]. Briefly, fixed tissues were taken out from 4% neutral buffered paraformaldehyde after 24 h, dehydrated in a graded ethanol series, cleared in xylene and embedded in paraffin for histological analysis. Sections of 5–7 μm were obtained with a rotary microtome (HM 325 Manual Microtome, MICROM International GmbH, Waldorf, Germany) and stained with conventional haematoxylin and eosin (H.E.) for routine histology examination. Then, the stain sections were examined under an inverted phase-contrast microscope (Olympus, BX53, Japan) using the Axiovision software.

2.7. Relative quantification of TCRγ and TCRδ genes by qRT-PCR

qRT-PCR was performed on qTOWER^{3}G Real-time PCR instrument (Analytik Jena, Germany) with a 96 well plate layout, using the EvaGreen 2 × qPCR Master mix (Abm, Canada). All samples were analyzed in triplicate, with following cycling conditions: 95 °C for 30 s, followed by 40 cycles of 94 °C 1 s, 58 °C 10 s. Melting curve was analyzed after thermal cycling to assess the specificity of qRT-PCR. EF1α and 18S were selected as the internal housekeeping gene and the expression levels of TCRγ and TCRδ were calculated using the $2^{-\Delta\Delta Ct}$ method [53]. All primers used in the present study were listed in Table 2.

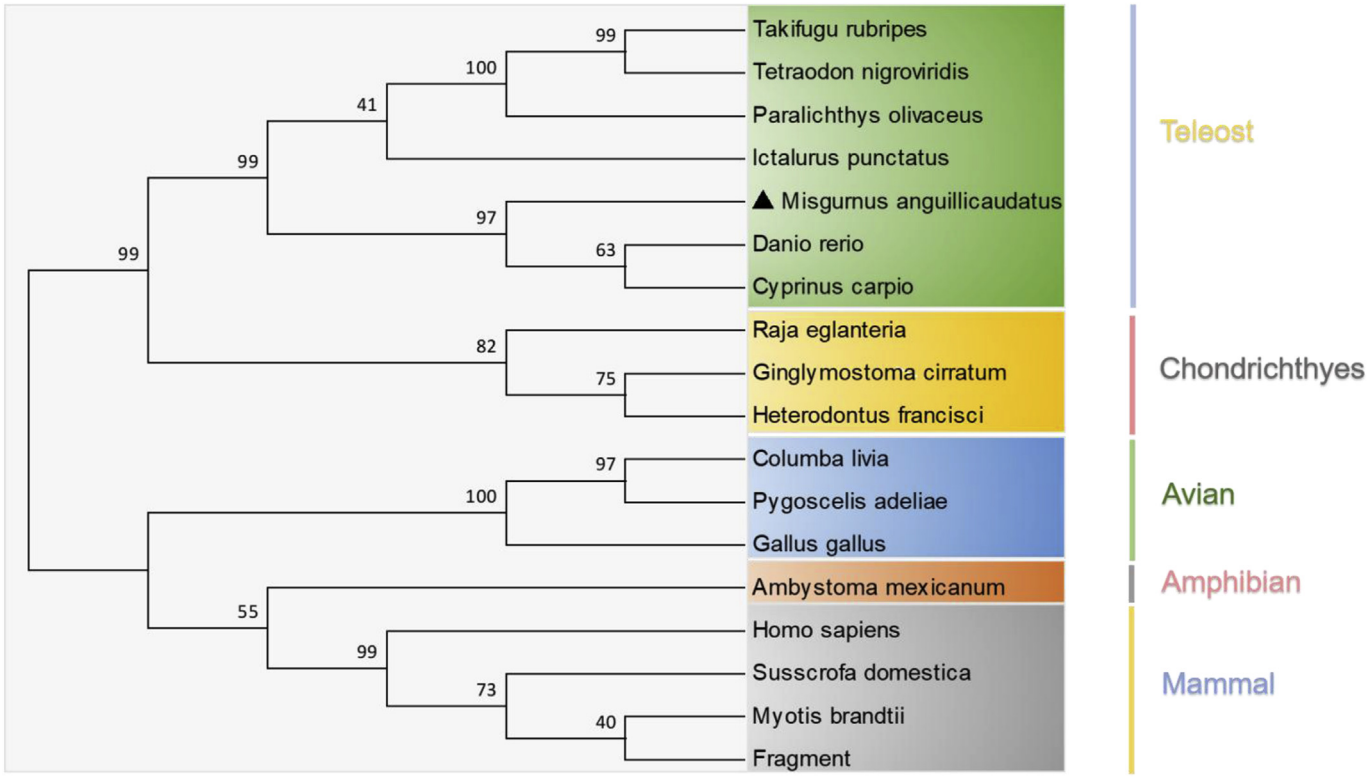

Fig. 4. Phylogenetic analysis of the T-cell antigen receptor (TCRγ) constant regions between loach and those of other vertebrates. The numbers indicate the percentage frequencies with which the phylogram topology represented here replicated for every 1000 bootstrap iterations. The sequences were aligned by CLUSTAL W program and the phylogenetic tree was constructed by neighbor-joining methods using MEGA software version 6.0. *Ma*-TCRγ was marked with a solid black triangle. GenBank accession numbers for TCRγ molecule used **in the analysis: *Danio rerio* (KX009743.1), *Cyprinus carpio* (AB541473.2), *Ictalurus punctatus* (HQ913590.1), *Paralichthys olivaceus* (AB076072.1), *Takifugu rubripes* (BAE16866.1), *Tetraodon nigroviridis* (CAC84647.1), *Ginglymostoma cirratum* (FJ513799.1), *Heterodontus francisci* (AAA87016.1), *Myotis brandtii* (EPQ17658.1), *Homo sapiens* (AY357942.1), *Susscrofa domestica* (L14602.1), *Fragment* (pir||D49054), *Gallus gallus* (AAD51740.1), *Columba livia* (XP_021147366.1), *Pygoscelis adeliae* (KFW68195.1), *Ambystoma mexicanum* (AAK33006.1).**

2.8. Statistical analysis

Data points were analyzed from at least three independent experiments and were reported as mean ± SD. Statistical analysis of the results was conducted using one-way analysis of variance (ANOVA) with Graphpad Prism 6 (GraphPad Software Inc, USA). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Molecular cloning of TCRγ and TCRδ cDNAs

Full-length of *Ma*-TCRγ and *Ma*-TCRδ cDNA sequences were 1131 bp and 1128 bp, respectively. The *Ma*-TCRγ contained an open reading frame (ORF) of 942bp encoding 317 amino acids, a 5'-untranslated region (UTR) of 58 bp and a 3′-UTR of 131 bp (Fig. 1A). The *Ma*-TCRδ is constituted by an ORF of 867 bp encoding a protein of 288 amino acids, a 5′ UTR of 63 bp and a 3′-UTR of 198 bp. (Fig. 1B). 3′-UTRs of both *Ma*-TCRγ and *Ma*-TCRδ contained a polyadenylation signal (AATAAA).

3.2. Multiple sequence alignment and phylogenetic analysis of Ma-TCR γ and Ma-TCRδ

The deduced *Ma*-TCRγ protein had a putative signal peptide of 19 amino acids, two Ig domains containing a 109 amino acids variable region contain (FR1, FR2, FR3 CDR1, CDR2, CDR3, J segment) and a 102 amino acids (IgC), a 53 amino acids connecting peptide (CPS), a 21 amino acids transmembrane region (TM) and a 12 amino acids cytoplasmic (CYT) (Fig. 2A). *Ma*-TCRδ protein also had a putative 18 amino acids signal peptide, two Ig domains containing a 122 amino acids variable region (FR1, FR2, FR3, CDR1, CDR2, CDR3, J segment) and a 95 amino acids (IgC), a 23 amino acids connecting peptide (CPS), a 22 amino acids transmembrane region (TM) and a 8 amino acids cytoplasmic (CYT) (Fig. 2B). *M. anguillicaudatus* TCRγ shared 33.9% amino acid identity with the *Danio rerio* TCRγ, and 32.0%, 30.4%, 21.7%, 20.4%, 16.4%, 15.2%, 15.3%, 14.8% and 13.2% with the *Cyprinus carpio*, *Ictalurus punctatus*, *Dicentrarchus labrax*, T*akifugu rubripes*, *Ginglymostoma cirratum*, *Xenopus laevis*, *Gavialis gangeticus*, *Bos taurus* and *Phalacrocorax carbo* TCRγs (Fig. 3). On the other hand, *M. anguillicaudatus* TCRδ shared 48.3% amino acid identity with the *Cyprinus carpio* TCRδ, and 43.6%, 34.2%, 24.5%, 21.5%, 20.3% and 20.1% with the *Danio rerio*, *Ictalurus punctatus*, *Paralichthys olivaceus*, *Gallus gallus*, *Homo sapiens* and *Heterodontus francisci* TCRδs (Fig. 4). The phylogenetic trees were constructed based on the constant region amino acid sequences of *M. anguillicaudatus*. According to the phylogenetic trees, the examined species of *Ma*-TCRγ and *Ma*-TCRδ could be divided into six discrete clusters: avian, reptiles, mammal, Chondrichthyes, amphibian, teleost. *Ma*-TCRγ and *Ma*-TCRδ were clustered with fish TCRγ and TCRδ groups, and they were highly related to that of *Cyprinus carpio* and *Danio rerio* (Figs. 3 and 4).

3.3. Haematoxylin-eosin staining of gill and skin

The morphological changes in loach skin and gill after infected with *F. columnare* G_4 and *I. multifiliis* were evaluated. Loach in the control group showed normal appearance of skin and gills. Nevertheless, the amount of mucous-secreting cells in skin significantly increased at 4 d and 21 d after infection (Figs. 6 and 7). Gill morphology also showed

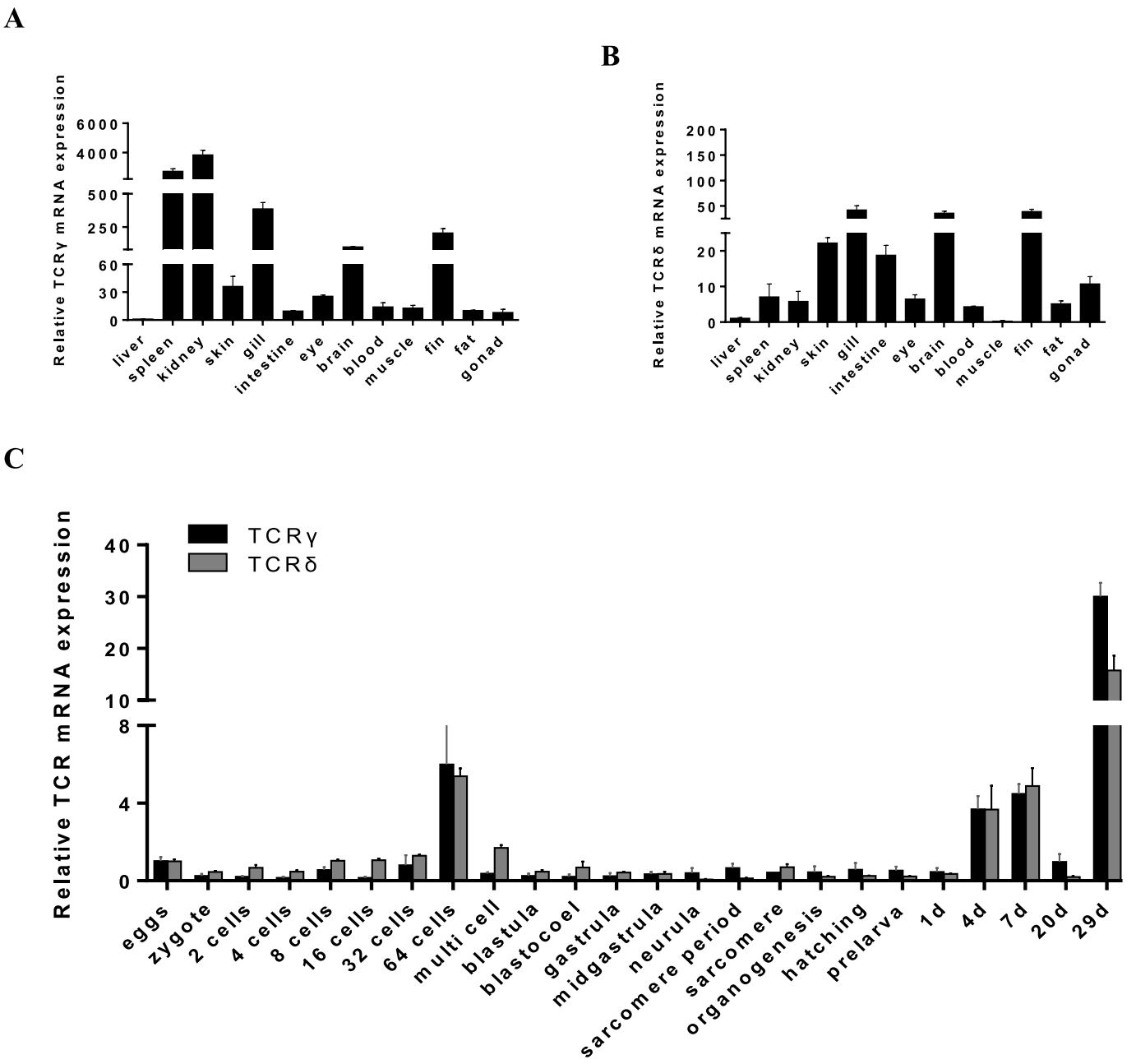

Fig. 5. The mRNA expression profiles of *Ma*-TCRγ and *Ma*-TCRδ expression in different tissues and embryonic developmental stages. mRNA expression profiles of *Ma*-TCRγ and *Ma*-TCRδ (n = 8) (A and B). The tissues included liver, spleen, kidney, skin, gill, intestine, eye, brain, blood, muscle, fin, fat, gonad. Relative expression of *Ma*-TCRγ and *Ma*-TCRδ was detected in different embryonic developmental stages (n = 4) (C).

significant changes as the length-width ratio of the secondary lamellae (SL) was much smaller than control fish at day 4 and 21 post infection. (Figs. 6 and 7).

3.4. Expression patterns of Ma-TCRγ and Ma-TCRδ in different tissues and during embryonic development

The expression levels of TCRγ and TCRδ in several tissues of healthy loaches were detected by qRT-PCR. Both *Ma*-TCRγ and *Ma*-TCRδ genes were widely expressed in all the analyzed tissues, including liver, spleen, kidney, skin, gill, intestine, eye, brain, blood, muscle, fin, fat and gonad (Fig. 5A). The highest relative expression of *Ma*-TCRγ was detected in kidney, followed by spleen, gill, fin, brain and skin, while the lowest expression level was observed in the liver. On the other hand, the highest relative expression of *Ma*-TCRδ was detected in the gill while the lowest expression level was observed in muscle (Fig. 5B).

The expression levels of *Ma*-TCRγ and *Ma*-TCRδ during embryonic development were also analyzed by qRT-PCR. The results showed that *Ma*-TCRγ and *Ma*-TCRδ could be detected in every embryonic development stages since fertilized eggs. The highest expression level of *Ma*-TCRγ and *Ma*-TCRδ was observed at the stage of 29 d, followed by 64 cells, 7 d, and 4 d (Fig. 5C).

3.5. Expression patterns of Ma-TCRγ and Ma-TCRδ during pathogenic challenge

3.5.1. F. columnare G_4 challenge

The expression levels of *Ma*-TCRγ and *Ma*-TCRδ in skin and gill, kidney, spleen after *F. columnare* G_4 infection was evaluated by qRT-PCR (Fig. 6B and C). The expression level of *Ma*-TCRγ in the skin was

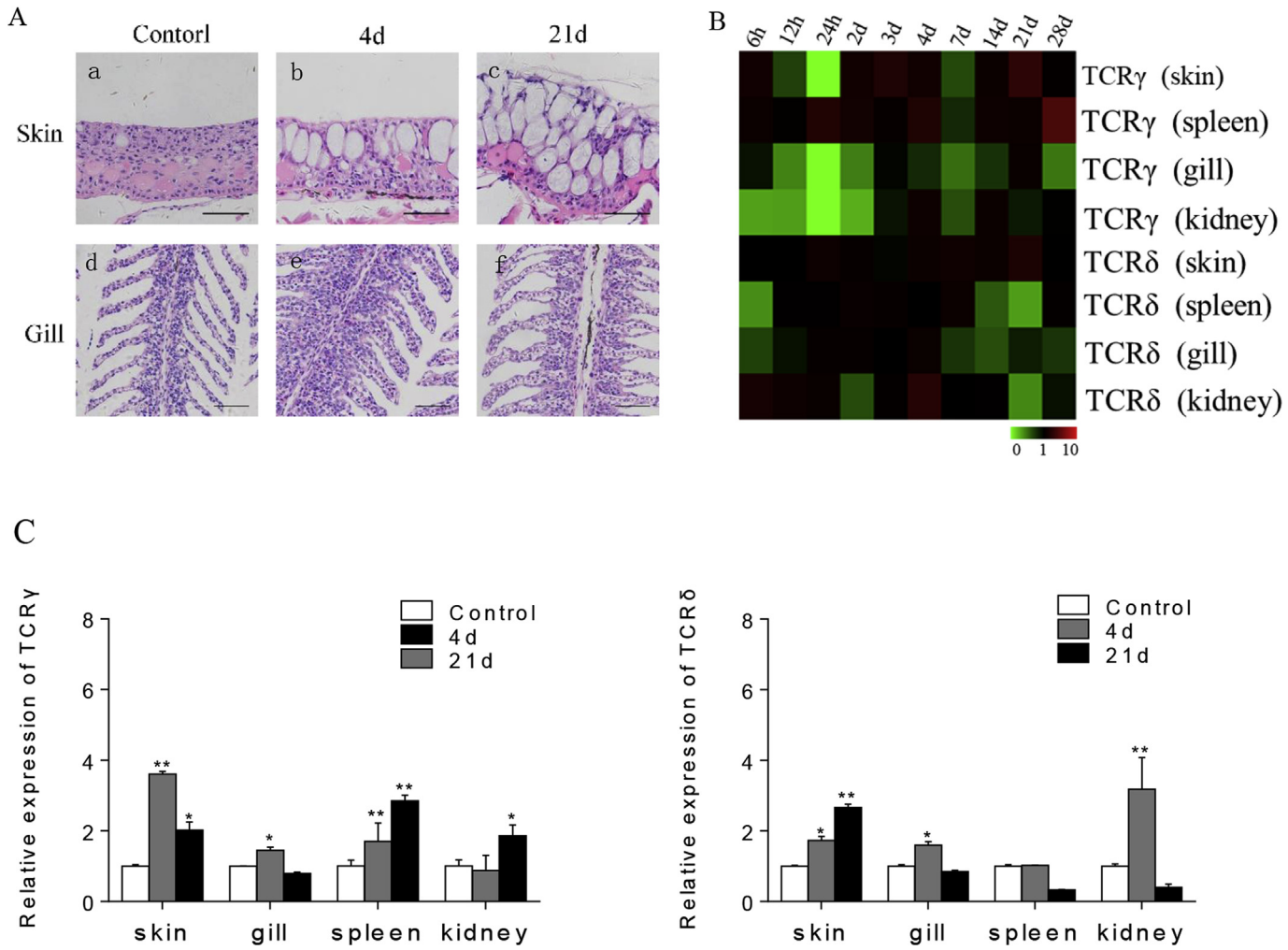

Fig. 6. Morphological changes in skin and gills (A) and the transcription levels of *Ma*-TCRγ and *Ma*-TCRδ in different tissues responses to *F. cloumnare* G_4 (B and C) of *M. anguillicaudatus*. (a–c) Haematoxylin/eosin staining on loach skin from uninfected fish (a), 4 d and 21 d infected fish (b–c), respectively. (d–f) Haematoxylin/eosin staining on loach gills from uninfected fish (d), 4 d and 21 d infected fish (e–f), respectively. Arrowheads indicate mucus cells. Scal bar, 100 μm. (B) Heat map illustrates results from real-time PCR of *Ma*-TCRγ and *Ma*-TCRδ mRNA expression in *F. cloumnare* G_4 infected fish versus control fish measured at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d, 28 d post infection (n = 6) in the skin, gill, spleen and kidney. (C) Temporal expression profiles of *Ma*-TCRγ and *Ma*-TCRδ in skin, gill, kidney, spleen at 4 d and 21 d after *F. cloumnare* G_4 challenge. Vertical bars were mean ± SD of three technical replicates, and the asterisks above the bars represented statistically significant differences from the control samples. $^*P < 0.05$, $^{**}P < 0.01$.

up-regulated from 2 d to 21 d post challenge with *F. columnare* G_4 and reached a peak value at 21 d (3.69-fold). In the spleen, the expression of *Ma*-TCRγ mRNA level increased at 24 h (2.96-fold), and then reached the highest value at 28 d (5.14-fold). Furthermore, there was no significant change after *F. columnare* G_4 challenge in the gills. Meanwhile, expressions of *Ma*-TCRδ in the skin, gills and spleen were not significant changed after *F. columnare* G_4 challenge. In the kidney, the mRNA level showed a peak at 4 d (4.1-fold), after challenge with *F. columnare* G_4.

3.5.2. *I. multifiliis challenge*

The mRNA expression levels of *Ma*-TCRγ and *Ma*-TCRδ genes were determined using qRT-PCR in skin, spleen, gill, and kidney at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d and 28 d after *I. multifiliis* challenge. When compared with the control fish, skin and spleen of loaches challenged with *I. multifiliis* showed increased in *Ma*-TCRγ expression up at 6 h post infection, then increased to 13.11-fold, 5.94-fold and 12.15-fold at 21 d and peak at this time point. On the other hand, in the spleen, the expression of *Ma*-TCRδ mRNA level increased at 24 h, and then reached a peak at 28 d (9.79-fold).

However, expressions of *Ma*-TCRδ in the skin, gill and kidney were not significantly up-regulated from 6 h to 21 d after *I. multifiliis* challenge. In addition, the higher expression of *Ma*-TCRδ in the skin, spleen, gill, and kidney appeared at the same time point of day 28 response to *I. multifiliis* challenge (Fig. 7B and C).

3.5.3. *Saprolegnia sp. challenge*

M. anguillicaudatus showed significant appearance changes, with lots of grayish-white cotton flocs covered on the loach skin at day 1 post infected with *Saprolegnia sp.* (Fig. 8A). The level of *Ma*-TCRγ mRNA transcript of skin and gill showed the most significant increase in 14 d and peak at this time point. In the spleen and kidney, the expression of *Ma*-TCRγ mRNA level increased at 7 d and steadied till 28 d when compared with the control fish. On the other hand, the expression level of *Ma*-TCRδ in the spleen increased at 12 h, and then reached a peak at 7 d. In gills, the expression of *Ma*-TCRδ mRNA level increased at 4 d (6.05-fold), and then reached a peak at 28 d (20.6-fold). In conclusion, the higher expression of *Ma*-TCRγ and *Ma*-TCRδ in the skin, spleen, gill, and kidney most appeared after 7 d in response to *Saprolegnia sp.* infection (Fig. 8B and C).

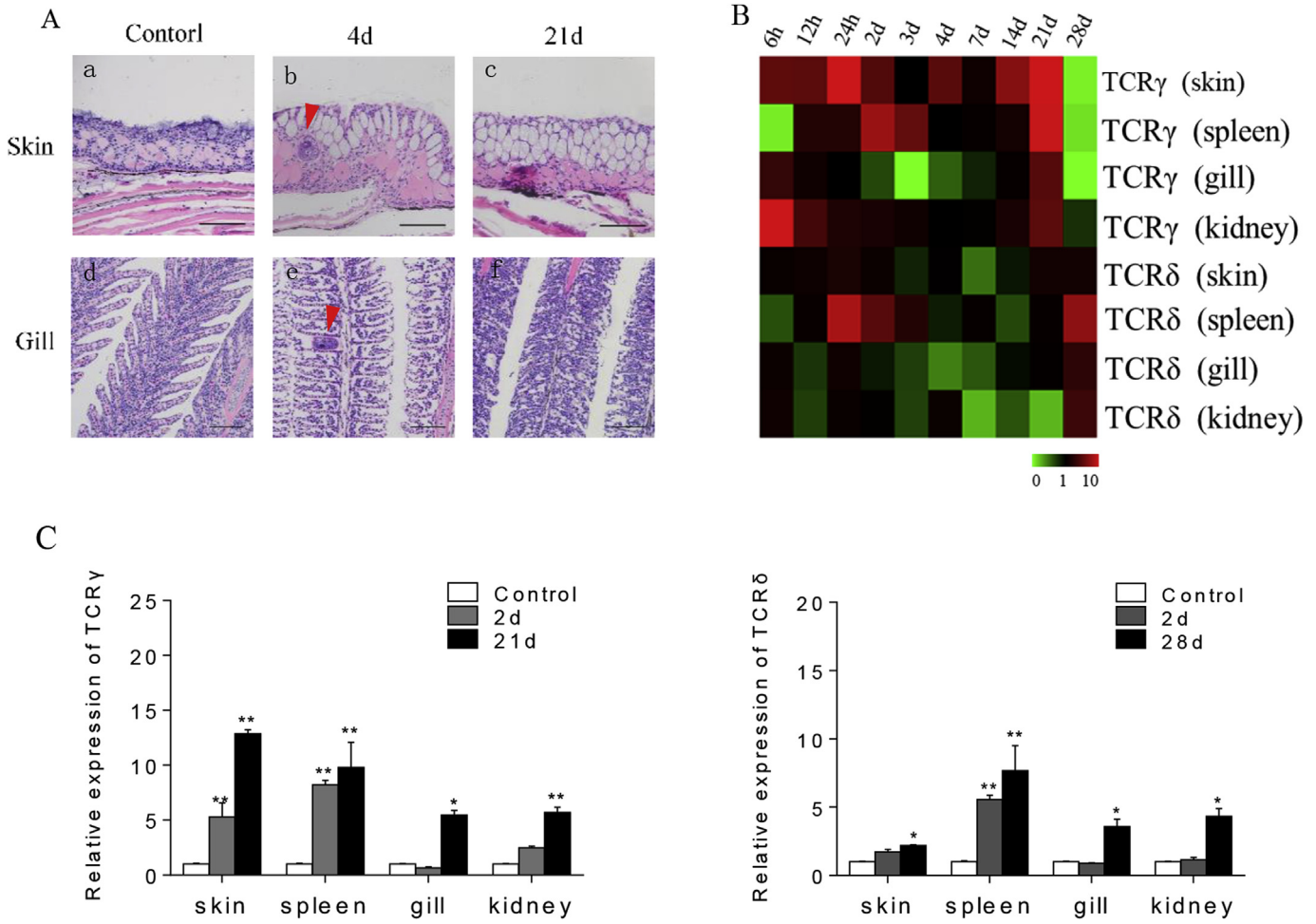

Fig. 7. Morphological changes in skin and gills (A) and the transcription levels of *Ma*-TCRγ and *Ma*-TCRδ in different tissues responses to *I. multifiliis* (B and C) of *M. anguillicaudatus*. (a–c). Haematoxylin/eosin staining on loach skin from uninfected fish (a), 4 d and 21 d infected fish (b–c), respectively. (d–f) Haematoxylin/eosin staining on loach gills from uninfected fish (d), 4 d and 21 d infected fish (e–f), respectively. Arrowheads indicate mucus cells. Scal bar, 100 μm. (B) Heat map illustrates results from real-time PCR of *Ma*-TCRγ and *Ma*-TCRδ mRNA expression in *I. multifiliis* infected fish versus control fish measured at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d, 28 d post infection (n = 6) in the skin, gill, spleen and kidney. (C) Temporal expression profiles of *Ma*-TCRγ and *Ma*-TCRδ in skin, gill, kidney, spleen at 2 d, 21 d and 28 d after *I. multifiliis* challenge. Vertical bars were mean ± SD of three technical replicates, and the asterisks above the bars represented statistically significant differences from the control samples. $^{*}P < 0.05$, $^{**}P < 0.01$.

4. Discussion

TCRs are a hallmark of all T cell surfaces and can specifically recognize fragments of antigen, functioning as immune repertoire to stimulate T cell activation and proliferation. **Similar to the performance of B cells in teleost fish, γδ T cells of zebrafish also showed a potent phagocytic ability, they can act as an antigen-presenting cell to induce B cell proliferation and IgM production initiation adaptive immunity** [2,3]. Compared to αβ TCR functioning in recognition of antigen bound on MHC, γδ TCR were much less known about their features and function [8], especially in teleost. In the current study, the full-length cDNA sequences of TCRγ and TCRδ were identified and characterized from *M. anguillicaudatus*. The expression patterns of these two genes in different tissues and during early embryonic development were also assayed. Then the expression profiles of these two TCRs during challenge with *F. columnare* G_4, *I. multifiliis* and *Saprolegnia sp.* were examined, which will help to illustrate the separate function of TCRγ and TCRδ in mucosal and systemic immunity of *M. anguillicaudatus*.

Sequence analysis showed that both *Ma*-TCRγ and *Ma*-TCRδ genes shared high identity with other known fish including *Danio rerio* and *Cyprinus carpio*. Gene structural analysis demonstrated that the deduced amino acid sequence of both *Ma*-TCRγ and *Ma*-TCRδ were similar with other fish species such as common carp [36], Nile tilapia [54,55]. The phylogenetic tree shows that the *Ma*-TCRγ and *Ma*-TCRδ could be clustered with *Danio rerio* and *Cyprinus carpio*, which may **insinuate** the close relationship between *M. anguillicaudatus* and *Cyprinidae* fish as they all belong to Cypriniformes. qRT-PCR was conducted to illustrate the expression patterns of *Ma*-TCRγ and *Ma*-TCRδ during ontogeny and relative expression levels in different tissues. Result showed that *Ma*-TCRγ and *Ma*-TCRδ were widely expressed in all checked tissues. **In this study, *Ma*-TCRγ mRNA was highest expressed in systematic tissues such as kidney and spleen, it intimate that *Ma*-TCRγ may play an important role in systemic immunity, similar to previous results in mandarin fish** [39]. **Differently, our previous study showed that the highest expression of αβ TCR localization in two mucosal-associated tissues (skin and gill) of loach** [46]. **Interestingly, *Ma*-TCRδ mRNA highest expressed in mucosal tissues such as gill, skin and intestine, it intimate that *Ma*-TCRδ may play an important role in mucosal immune, which was similar to earlier reports on TCRδ of zebrafish** [2]. **Importantly, similar with TCRα and TCRβ in loach, the low expression of TCRγ and TCRδ also could be detected in liver. Overall, like in the mammals, our results indicated that the expression distribution of γδ TCRs in all tested tissues is less common than αβ T cells in teleost** [46]. These results might suggest that *Ma*-TCRγ and *Ma*-TCRδ may play a different

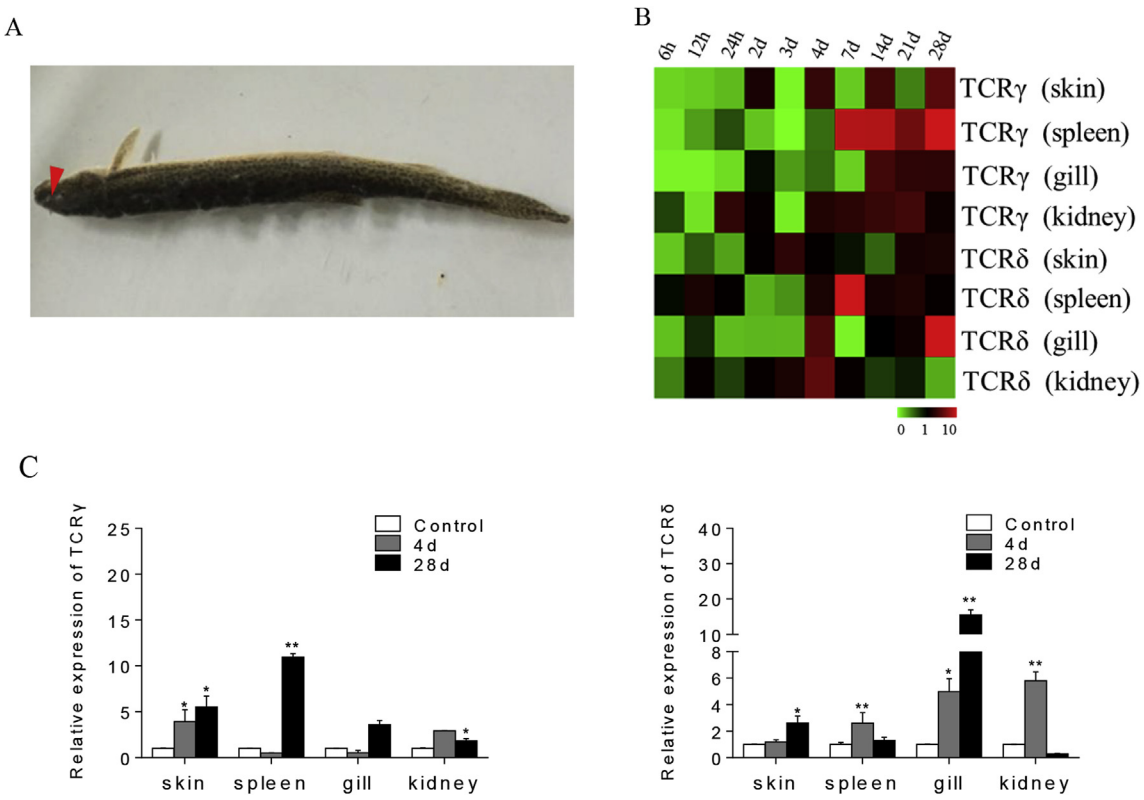

Fig. 8. Heat map illustrates results from real-time PCR of *Ma*-TCRγ and *Ma*-TCRδ mRNA expression in *Saprolegnia* infected fish versus control fish measured at 6 h, 12 h, 24 h, 2 d, 3 d, 4 d, 7 d, 14 d, 21 d, 28 d post infection (n = 6) in the skin, gill, spleen and kidney. (C) Temporal expression profiles of *Ma*-TCRγ and *Ma*-TCRδ in skin, gill, kidney, spleen at 4 d and 28 d after *Saprolegnia* challenge. Vertical bars were mean ± SD of three technical replicates, and the asterisks above the bars represented statistically significant differences from the control samples. $^{*}P < 0.05$, $^{**}P < 0.01$.

role in immune tissues. However, the specific antigens which could be recognized by *Ma*-TCRγ or *Ma*-TCRδ were still unknown. In addition, mRNA expression levels of *Ma*-TCRγ and *Ma*-TCRδ during early embryonic development stages were also checked. Notably, both *Ma*-TCRγ and *Ma*-TCRδ mRNA showed highest expression levels at 29 d post hatching, followed by 64 cells and 4 d, 7 d post hatching. These results might suggest TCRs expression increased accompanied with the maturation of most organs including immune organs during prolonged developmental period [12]. *Ma*-TCRγ and *Ma*-TCRδ also showed higher expression at 64 cell periods, which may prepare for cell differentiation in the gut stage. However, more studies are needed to explain the inner mechanism.

Three infection models of dojo loach with bacteria (*F. columnare* G_4), parasite (and *I. multifiliis*) and fungus (*Saprolegnia sp.*) were constructed to evaluate the immune functions of *Ma*-TCRγ and *Ma*-TCRδ in systematic and mucosal tissues during different pathogenic infection. Firstly, the morphological changes of gill and skin in *M. anguillicaudatus* were examined, which remains one of the important evidence to prove the successful infection [56]. Results suggested that the numbers of mucous-secreting cells of loach skin significantly increased at 4 d and 21 d after challenge with *F. columnare* G_4 and *I. multifiliis,* compared to control fish. Moreover, the gills showed obvious hyperplasia as many interlamellar spaces became obliterated and the length of gills secondary lamellae (SL) in the infected loach turned to be much shorter, which was similar to earlier reports on loach when challenge with *A. hydrophila* [57,58]. These data proved that we have succeeded to construct a bacterial and parasite infection model of *M. anguillicaudatus* with *F. columnare* G_4 and *I. multifiliis*. Recent study has reported the up-regulation of TCRγ in mandarin fish after *F.* pathogenic infection [39]. In our study, *I. multifiliis* challenge induced the most obvious up-regulation of *Ma*-TCRγ and *Ma*-TCRδ in both systematic and mucosal immune organs, followed by *Saprolegnia sp.* challenge, and the up-regulation of both TCRs during *F. columnare* G_4 challenge was weakest, even cause *Ma*-TCRγ and *Ma*-TCRδ down-regulation expression in most tissues and at most time points. Which may suggest that the mRNA expression of *Ma*-TCRγ and *Ma*-TCRδ more sensitive against *I. multifiliis* infection. In previous study, human γδ T cells was proved that it produced γ-interferon, a major mediator of host resistance to against *Toxoplasma gondii* [59], it suggests that human γδ T cells might play a role in protection against *Toxoplasma gondii*. In addition, mouse γδ T cells also have been reported to play an important role in protection against *Toxoplasma* infection [60]. In our study, signi*f*icantly increased expression levels of both *Ma*-TCRγ and *Ma*-TCRδ were detected in all checked tissues during *I. multifiliis* challenge. Similar research has detected significantly increased γδ T cells in human during acute *Plasmodium falciparum* infection, it suggest that T cells may participate in the immune response against *Plasmodium falciparum* [61]. Highest expression levels of *Ma*-TCRγ in skin and gill were detected at 21 d, which may indicate its participation in adaptive immunity. On the other hand, the expression of *Ma*-TCRγ also showed increased expression of classical immune organs including the spleen and kidney. *Ma*-TCRγ in spleen showed much higher expression than kidney after *I. multifiliis* challenge, and significantly increased with prolonged infection period and showed highest expression levels at 21 d. *Ma*-TCRγ in kidney showed highest expression at 6 h after *I. multifiliis* challenge, and significantly decreased with prolonged infection period then increased again in 21 d. The up-regulation of *Ma*-TCRγ in skin and gill at early period post *I. multifiliis* challenge might be due to the fact that *I. multifiliis* directly stimulate mucosal tissues such as skin and gills by bath infection. *Ma*-TCRδ also showed significantly increased expression in four tissues after *I. multifiliis* challenge, although the expression of *Ma*-TCRδ was much lower than *Ma*-TCRγ. *Ma*-TCRγ and *Ma*-TCRδ mRNA expressions also showed significant increase during challenge with *Saprolegnia sp*. As we expected, *Ma*-TCRγ and *Ma*-TCRδ expression

increased with prolonged challenge time and mostly showed a significant increase from 7 d to 28 d post challenge, which may indicate its participation in adaptive immunity. Especially, *Ma*-TCRγ and *Ma*-TCRδ expression in spleen showed much higher increase than other tissues after *Saprolegnia sp.* challenge, starting from 7 d and *Ma*-TCRγ expression in spleen kept high expression levels from 7 d to 28 d after *Saprolegnia sp.* challenge. Other tissues also showed increased expression of *Ma*-TCRγ and *Ma*-TCRδ after challenge with *Saprolegnia sp.*, for example, *Ma*-TCRγ in gill showed high expression level post infection. The up-regulation of *Ma*-TCRγ and *Ma*-TCRδ mRNA levels during challenge with *F. columnare* G_4 were much lower than *I. multifiliis* and *Saprolegnia sp.* *Ma*-TCRδ mRNA levels showed a relatively high level at early time post *F. columnare* G_4 infection, while *Ma*-TCRγ mRNA levels during challenge with *F. columnare* G_4 showed relatively high levels at 4 d and 21 d post infection. *Ma*-TCRγ showed highest expression level in skin at 4 d post *F. columnare* G_4 infection, while showed highest expression level in spleen at 21 d, which is similar with earlier findings in mandarin fish as TCRγ significantly increased in some tissues during *F. columnare* G_4 challenge [39]. So the modulation patterns of *Ma*-TCRγ and *Ma*-TCRδ were different when stimulated by *F. columnare* G_4, *I. multifiliis* or *Saprolegnia sp.* infection, which caused their different sensitivity to different pathogen. Furthermore, the expression was differently modulated by *F. columnare* G_4, *I. multifiliis* and *Saprolegnia sp.* in kidney, spleen, skin and gill, which suggested that they might have different functions in these four immune tissues. It seemed that *Ma*-TCRγ and *Ma*-TCRδ were more sensitive in fungal infection rather than bacterial and parasite infection in kidney, spleen, skin and gill. Taken together, transcriptional changes in our results insinuate that our results suggest that TCRγ and TCRδ may play an important role in kidney, spleen, skin and gill immunity to protect the loach against among *F. columnare* G_4, *I. multifiliis* and *Saprolegnia sp.*.

In summary, we firstly identified and characterized the full-length cDNAs of TCRγ and TCRδ from *M. anguillicaudatus*. The deduced amino acid sequences of *Ma*-TCRγ and *Ma*-TCRδ shared similar characteristics with other fish species. *Ma*-TCRγ and *Ma*-TCRδ were expressed in various tissues, but with different tissue-specific expression profiles. The present study also constructed three infection models of *M. anguillicaudatus* with bacteria (*F. columnare* G_4), parasite (*I.multifiliis*) and fungus (*Saprolegnia sp.*) for the first time to explore the functions of *Ma*-TCRγ and *Ma*-TCRδ in immune tissues. The results of *F. columnare* G_4, *I.multifiliis* and *Saprolegnia sp.* challenge experiments showed that *Ma*-TCRγ and *Ma*-TCRδ were unregulated not only in kidney and spleen, but also in skin and gills. These findings will increase our knowledge of immunity and help exploit new strategies to resist bacterial, parasite and fungal infection in fish.

Acknowledgments

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References

[1] X. Niu, C. Wu, M. Li, Q. Zhao, S.N. Meydani, J. Wang, D. Wu, Naringenin is an inhibitor of T cell effector functions, Nutritional Biochemistry 58 (2018) 71–79.
[2] F. Wan, C. Hu, J. Ma, K. Gao, L. Xiang, J. Shao, Characterization of γδ T cells from zebrafish provides insights into their important role in adaptive humoral immunity, Front. Immunol. 7 (3) (2016).
[3] A.C. Hayday, γδ cells: a right time and a right place for a conserved third way of protection, Annu. Rev. Immunol. 18 (1) (1999) 975–1026.
[4] M.P. Lefranc, G. Lefranc, The T Cell Receptor FactsBook, Academic Press, 2001.
[5] C.C. Castro, A.M. Luoma, E.J. Adams, Coevolution of T-cell receptors with MHC and non-MHC ligands, Immunol. Rev. 267 (1) (2015) 30–55.
[6] M. Krogsgaard, M.M. Davis, How T cells 'see' antigen, Nat. Immunol. 6 (3) (2005) 239–245.
[7] S.T. Reddy, Immunology: the patterns of T-cell target recognition, Nature 547 (7661) (2017) 36–38.
[8] J.P. Scott-Browne, F. Crawford, M.H. Young, J.W. Kappler, P. Marrack, L. Gapin, Evolutionarily conserved features contribute to αβ T cell receptor specificity, Immunity 35 (4) (2011) 526–535.
[9] Y. Feng, E.L. Reinherz, M.J. Lang, Aβ T cell receptor mechanosensing forces out serial engagement, Trends Immunol. 39 (8) (2018) 596–609.
[10] H.R. Mirzaei, H. Mirzaei, S.Y. Lee, J. Hadjati, B.G. Till, Prospects for chimeric antigen receptor (CAR) γδ T cells: a potential game changer for adoptive T cell cancer immunotherapy, Cancer Lett. 380 (2) (2016) 413–423.
[11] 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.
[12] 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.
[13] S. Ryo, R.H. Wijdeven, A. Tyagi, T. Hermsen, T. Kono, I. Karunasagar, J.H. Rombout, M. Sakai, B.M. Verburg-van Kemenade, R. Savan, Common carp have two subclasses of bonyfish specific antibody IgZ showing differential expression in response to infection, Dev. Comp. Immunol. 34 (11) (2010) 1183–1190.
[14] 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.
[15] Z. Xu, 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.
[16] 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.
[17] G. Zhang, J. Wang, Comparative analysis of bat genomes provides insight into the evolution of flight and immunity, Science 339 (6118) (2013) 456–460.
[18] Y. Fan, Z.Y. Huang, C.C. Cao, C.S. Chen, Y.X. Chen, D.D. Fan, J. He, H.L. Hou, L. Hu, X.T. Hu, Genome of the Chinese tree shrew, Nat. Commun. 4 (1426) (2013) 1426.
[19] R.D. Garman, P.J. Doherty, D.H. Raulet, Diversity, rearrangement, and expression of murine T cell γ genes, Cell 45 (5) (1986) 733–742.
[20] I. Seim, X. Fang, Z. Xiong, A.V. Lobanov, Z. Huang, S. Ma, Y. Feng, A.A. Turanov, Y. Zhu, T.L. Lenz, Genome analysis reveals insights into physiology and longevity of the Brandt's bat *Myotis brandtii*, Nat. Commun. 4 (2013) 2212-2212.
[21] C.D. Wolfgang, M. Essand, J.J. Vincent, B. Lee, I. Pastan, TARP: a nuclear protein expressed in prostate and breast cancer cells derived from an alternate reading frame of the T cell receptor γ chain locus, Proc. Natl. Acad. Sci. U. S. A. 97 (17) (2000) 9437–9442.
[22] J.P. Rast, M.K. Anderson, S.J. Strong, C. Luer, R.T. Litman, G.W. Litman, α, β, γ, and δ T cell antigen receptor genes arose early in vertebrate phylogeny, Immunity 6 (1) (1997) 1–11.
[23] T. Kubota, J. Wang, T.W. Göbel, R.D. Hockett, M.D. Cooper, C.H. Chen, Characterization of an avian (*Gallus gallus domesticus*) TCR α/δ gene locus, Immunology 163 (7) (1999) 3858.
[24] A. Six, J.P. Rast, W.T. Mccormack, D. Dunon, D. Courtois, Y. Li, M.D. Cooper, Characterization of avian T-cell receptor γ genes, Proc. Natl. Acad. Sci. U. S. A. 93 (26) (1996) 15329–15334.
[25] X. Wang, P. Wang, R. Wang, C. Wang, J. Bai, C. Ke, D. Yu, K. Li, Y. Ma, H. Han, Analysis of TCRβ and TCRγ genes in Chinese alligator provides insights into the evolution of TCR genes in jawed vertebrates, Dev. Comp. Immunol. 85 (2018).
[26] R.N. Haire, M.K. Kitzan Haindfield, J.B. Turpen, G.W. Litman, Structure and diversity of T-lymphocyte antigen receptors α and γ in *Xenopus*, Immunogenetics 54 (6) (2002) 431–438.
[27] Z.E. Parra, Y. Ohta, M.F. Criscitiello, M.F. Flajnik, R.D. Miller, The dynamic TCRδ: TCRδ chains in the amphibian Xenopus tropicalis utilize antibody-like V genes, European Immunology 40 (8) (2010) 2319–2329.
[28] M.F. Criscitiello, Y. Ohta, M. Saltis, E.C. Mckinney, M.F. Flajnik, Evolutionarily conserved TCR binding sites, identification of T cells in primary lymphoid tissues, and surprising trans-rearrangements in nurse shark, Immunology 184 (12) (2010) 6950.
[29] R. Pettinello, A.K. Redmond, C.J. Secombes, D.J. Macqueen, H. Dooley, Evolutionary history of the T cell receptor complex as revealed by small-spotted catshark (*Scyliorhinus canicula*), Dev. Comp. Immunol. 74 (2017) 125–135.
[30] H. Chen, S. Kshirsagar, I. Jensen, K. Lau, R. Covarrubias, S.F. Schluter, J.J. Marchalonis, Characterization of arrangement and expression of the T cell receptor γ locus in the sandbar shark, Proc. Natl. Acad. Sci. U. S. A. 106 (21) (2009) 8591–8596.
[31] B.H. Nam, I. Hirono, T. Aoki, The four TCR genes of teleost fish: the cDNA and genomic DNA analysis of Japanese flounder (Paralichthys olivaceus) TCR α, β, γ, and δ chains, Immunology 170 (6) (2003) 3081–3090.
[32] R. Yazawa, G.A. Cooper, M. Beetz-Sargent, A. Robb, L. McKinnel, W.S. Davidson, B.F. Koop, Functional adaptive diversity of the Atlantic salmon T-cell receptor γ locus, Mol. Immunol. 45 (8) (2008) 2150–2157.
[33] R. Yazawa, G.A. Cooper, P. Hunt, M. Beetz-Sargent, A. Robb, M. Conrad, L. McKinnel, S. So, S. Jantzen, R.B. Phillips, W.S. Davidson, B.F. Koop, Striking antigen recognition diversity in the Atlantic salmon T-cell receptor α/δ locus, Dev. Comp. Immunol. 32 (3) (2008) 204–212.
[34] S.L. Seelye, P.L. Chen, T.C. Deiss, M.F. Criscitiello, Genomic organization of the zebrafish (*Danio rerio*) T cell receptor α/δ locus and analysis of expressed products, Immunogenetics 68 (5) (2016) 365–379.
[35] C. Fischer, L. Bouneau, C. Ozouf-Costaz, T. Crnogorac-Jurcevic, J. Weissenbach,

A. Bernot, Conservation of the T-cell receptor α/δ linkage in the teleost fish Tetraodon nigroviridis, Genomics 79 (2) (2002) 241–248.
[36] N. Shang, X.F. Sun, W. Hu, Y.P. Wang, Q.L. Guo, Molecular cloning and characterization of common carp (*Cyprinus carpio* L.) TCRγ and CD3γ/δ chains, Fish Shellfish Immunol. 24 (4) (2008) 412–425.
[37] S. Picchietti, L. Guerra, F. Bertoni, E. Randelli, M.C. Belardinelli, F. Buonocore, A.M. Fausto, J.H. Rombout, G. Scapigliati, L. Abelli, Intestinal T cells of *Dicentrarchus labrax* (L.): gene expression and functional studies, Fish Shellfish Immunol. 30 (2) (2011) 609–617.
[38] Y. Mu, F. Ding, P. Cui, J. Ao, S. Hu, X. Chen, Transcriptome and expression profiling analysis revealed changes of multiple signaling pathways involved in immunity in large yellow croaker during *Aeromonas hydrophila* infection, BMC Genomics 11 (1) (2010) 1–14.
[39] J.Y. Tian, Z.T. Qi, N. Wu, M.X. Chang, P. Nie, Complementary DNA sequences of the constant regions of T-cell antigen receptors α, β and γ in Mandarin fish, Siniperca chuatsi Basilewsky, and their transcriptional changes after stimulation with *Flavobacterium columnare*, Fish Diseases 37 (2) (2014) 89–101.
[40] C. Qin, K. Huang, H. Xu, Protective effect of polysaccharide from the loach on the in vitro and in vivo peroxidative damage of hepatocyte, Nutritional Biochemistry 13 (10) (2002) 592–597.
[41] L.J. You, M.M. Zhao, J.M. Regenstein, J.Y. Ren, Changes in the antioxidant activity of loach (Misgurnus anguillicaudatus) protein hydrolysates during a simulated gastrointestinal digestion, Food Chem. 120 (3) (2010) 810–816.
[42] 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.
[43] W. Huang, X.X. Zhang, L.I. Wen-Jian, Culture and Common Disease Control of Loach in Net Cage, Hunan Environment-biological Polytechnic, 2004.
[44] 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, Fish Diseases 39 (6) (2016) 777.
[45] L. Qin, M. Zhu, J. Xu, First report of *Shewanella, sp.* and *Listonella, sp.* infection in freshwater cultured loach, *Misgurnus anguillicaudatus*, Aquacult. Res. 45 (4) (2014) 602–608.
[46] W. Yu, Y. Luo, Y. Yu, S. Dong, Y. Yin, Z. Huang, Z. Xu, T cell receptor (TCR) α and β genes of loach (*Misgurnus anguillicaudatus*): molecular cloning and expression analysis in response to bacterial, parasitic and fungal challenges, Developmental & Comparative Immunology, 2018.
[47] X.Y. Zhou, M.Y. Li, K. Abbas, Z.X. Gao, W.M. Wang, Comparison of ploidy level screening methods in Chinese dojo loach (*Misgurnus anguillicaudatus*), Applied Ichthyology 24 (6) (2008) 664–669.
[48] G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: Molecular Evolutionary Genetics Analysis, (2013).
[49] J. Sigh, T. Lindenstrøm, K. Buchmann, The parasitic ciliate *Ichthyophthirius multifiliis* induces expression of immune relevant genes in rainbow trout, Oncorhynchus mykiss (Walbaum), Fish Diseases 27 (7) (2010) 409–417.
[50] J. Diéguez-Uribeondo, L. Cerenius, K. Söderhäll, Repeated zoospore emergence in Saprolegnia parasitica, Mycol. Res. 98 (7) (1994) 810–815.
[51] T. Unestam, Studies on the crayfish plague fungus aphanomyces astaci I, Some Factors Affecting Growth in vitro 18 (2) (1965) 483–505.
[52] A.R. Oguz, Histological changes in the gill epithelium of endemic Lake Van Fish (*Chalcalburnus tarichi*) during migration from alkaline water to freshwater, North-Western Zoology 11 (2) (2015) 136–139.
[53] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method, Methods 25 (4) (2001) 402–408.
[54] N. Nithikulworawong, A. Yakupitiyage, S. Rakshit, P. Srisapoome, Molecular characterization and increased expression of the Nile tilapia, *Oreochromis niloticus* (L.), T-cell receptor β chain in response to *Streptococcus agalactiae* infection, Fish Diseases 35 (5) (2012) 343–358.
[55] M.F. Criscitiello, S.M. Kamper, E.C. McKinney, Allelic polymorphism of TCRα chain constant domain genes in the bicolor damselfish, Dev. Comp. Immunol. 28 (7–8) (2004) 781–792.
[56] J. Bhawan, P. Grimes, A.G. Pandya, M. Keady, H.R. Byers, I.L. Guevara, L.E. Colón, L.A. Johnson, R. Gottschalk, A histological examination for skin atrophy after 6 months of treatment with fluocinolone acetonide 0.01%, hydroquinone 4%, and tretinoin 0.05% cream, Am. J. Dermatopathol. 31 (8) (2009) 794–798.
[57] Y. Yu, Y. Liu, H. Li, S. Dong, Q. Wang, Z. Huang, W. Kong, X. Zhang, Y. Xu, X. Chen, Z. Xu, Polymeric immunoglobulin receptor in dojo loach (*Misgurnus anguillicaudatus*): molecular characterization and expression analysis in response to bacterial and parasitic challenge, Fish Shellfish Immunol. 73 (2018) 175–184.
[58] Y. Xu, Y. Yu, X. Zhang, Z. Huang, H. Li, S. Dong, Y. Liu, F. Dong, Z. Xu, Molecular characterization and expression analysis of complement component 3 in dojo loach (*Misgurnus anguillicaudatus*), Fish Shellfish Immunol. 72 (2018) 484–493.
[59] T. Hara, S. Ohashi, Y. Yamashita, T. Abe, H. Hisaeda, K. Himeno, R.A. Good, K. Takeshita, Human V δ 2+ γ δ T-cell tolerance to foreign antigens of *Toxoplasma gondii*, Proc. Natl. Acad. Sci. U. S. A. 93 (10) (1996) 5136.
[60] H. Hisaeda, H. Nagasawa, K. Maeda, Y. Maekawa, H. Ishikawa, Y. Ito, R.A. Good, K. Himeno, γδ T cells play an important role in hsp65 expression and in acquiring protective immune responses against infection with *Toxoplasma gondii*, Immunology 155 (1) (1995) 244.
[61] M. Ho, H.K. Webster, P. Tongtawe, K. Pattanapanyasat, W.P. Weidanz, Increased γδ T cells in acute *Plasmodium falciparum* malaria, Immunol. Lett. 25 (1–3) (1990) 139–141.