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Re-identification and characterization of grass carp *Ctenopharyngodon idella* TLR20

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ABSTRACT

Toll-like receptors (TLRs) play a crucial role in the recognition of microbial-associated molecular patterns in the innate immune system. Fish TLRs have undergone significant gene expansion to adapt to complex aquatic environments. Among them, TLR20 from the TLR11 family actively responds to viral and bacterial invasions. Previous studies have reported two TLR20s in grass carp (*Ctenopharyngodon idella*), and in this study, we revised this conclusion. Based on the latest grass carp genome, we identified a new TLR20 member. These three TLR20s are arranged in tandem on chromosome 9, indicating that they are generated by gene duplication events. They were renamed CiTLR20.1 to CiTLR20.3 based on their chromosomal positions. The CiTLR20s in *C. idella* exhibit higher similarities with those in *Danio rerio, Cyprinus carpio*, and *Megalobrama amblycephala*, and lower similarities with those in other distantly related fish species. Selective pressure analysis revealed low conservation and negative evolution of TLR20s during evolution. The 3D structures of the three TLR20s showed significant differences, reflecting functional variations and different downstream adaptor molecule recruitment. Transcriptome data revealed tissue distribution differences of TLR20s, with TLR20.1 showing relatively low expression levels in all the tissues, while TLR20.2 and TLR20.3 showed higher expression in the head kidney, spleen, and gill. Additionally, TLR20.2 and TLR20.3 actively responded to GCRV-II infection, with higher upregulation of TLR20.2 in response to *Aeromonas hydrophila* challenge. In conclusion, this study corrected the number of grass carp TLR20 members and analyzed TLR20 from an evolutionary and structural perspective, exploring its role in antiviral and antibacterial defense. This study provides reference for future research on fish TLR20.

1. Introduction

Toll-like receptors (TLRs) play a crucial role in the innate immune response against pathogen invasion. They belong to a class of pattern-recognition receptors (PRRs) that detect and respond to conserved motifs such as nucleic acids of viruses (double-stranded and single-stranded RNA), some specific DNA and RNA structures of bacteria, certain components of the bacterial cell wall (e.g. LPS and PGN) and flagellum, which are defined as microbe-associated molecular patterns (MAMPs) [11,19,44,46]. TLRs activate downstream signaling pathways and initiate the immune system response to combat the invasion of pathogenic microorganisms upon associating with their respective MAMPs [3,45].

TLRs are generally type I transmembrane proteins and consist of three distinct domains: a horseshoe-shaped extracellular domain (ECD) containing a high number of leucine-rich repeat sequences (LRRs), a transmembrane domain (TM) for dimerization, and an intracellular Toll/Interleukin-1 receptor (TIR) domain, which is implicated in downstream signal transduction [1]. The ECD comprises a series of 16 to 28 LRRs composing of 20–30 amino acids. These LRRs possess a conserved motif segment, LxxLxLxxN(Cx)xL, which play a vital role in facilitating TLR recognition of MAMPs [2,30]. When MAMPs bind to the ECD, the TIR domain of each TLR recruits its respective adapters. In mammals, there are a total of seven adapters involved, namely MyD88, MAL, TRIF, TRAM, SARM, BCAP, and SCIMP [22,27,32,36]. These adaptors then deliver signals downstream to activate either the NF-κB signaling pathway or the IFN signaling pathway [16,35,57].

Thirteen TLR members have been identified in mammals, with 12 TLRs (TLR1–9, TLR11–13) in mice (*Mus musculus*) and 10 TLRs (TLR1–10) in humans (*Homo sapiens*) [1,15]. However, the fish genome

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has undergone a significant expansion in the number of TLR genes owing to whole genome duplication (WGD) events [13,31,47], and more than 22 TLR genes have been identified in the fish genome [37]. In addition to the commonly found TLR1–5, TLR7–9 in both mammals and fishes, fishes also possess specific TLR18-TLR20 and TLR22-TLR28 and soluble TLR5S [29,51]. Furthermore, multiple copies of TLR3, TLR4, TLR5, TLR7, TLR8, TLR20, and TLR22 are present in the fish genome [37]. The ultimate fate of gene duplication is gene loss, silencing, subfunctionalization, or neofunctionalization [4,10]. In fish, TLR paralogous genes exhibit more complexity in terms of their functions. Both TLR5a and TLR5b in *Cyprinidae* can recognize flagellin, but the immune response to flagellin protein is only triggered by the heterodimer of TLR5a/b [25]. In grass carp (*Ctenopharyngodon idella*), TLR22a and TLR22b can both recognize dsRNA, but they exhibit functional antagonism [17].

With regard to TLR20, multiple partial sequences of TLR20 (TLR20a-f) were initially identified in the genome of zebrafish (*Danio rerio*) [34]. However, subsequent studies revealed that two copies of TLR20 (TLR20e and TLR20f) contain mutations in the open reading frame, resulting in premature termination and an inability to encode complete proteins. The remaining four TLR20 copies can encode full-length proteins but display relatively low expression levels [39]. Furthermore, TLR20s have also been reported in rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), channel catfish (*Ictalurus punctatus*), blunt snout bream (*Megalobrama amblycephala*), common carp (*Cyprinus carpio*) and grass carp [14,21,23,38,41]. TLR20s exhibit different functions in various fish species. In zebrafish, the expression of TLR20s does not significantly change upon infection with *Mycobacterium marinum* or *Streptococcus iniae* [12,49], but they have a strong response to infection by the blood parasite *Trypanosoma carassii* [39]. In common carp, TLR20 similarly responds to parasite invasion but does not respond to spring viremia of carp virus (SVCV) [9]. Channel catfish TLR20 demonstrates significant upregulation in response to invasion by *Edwardsiella ictaluri* [40]. Therefore, the functionality of TLR20 varies in different fish species and its response to specific pathogens differs as well.

The presence of 21 TLRs in grass carp has been reported based on the previous grass carp genome [24,52], which contains two TLR20 (TLR20a or TLR20.2 and TLR20b or TLR20.1) [14,24]. Grass carp TLR20.2 can actively respond to stimulation from bacteria and viruses, and can activate downstream signaling pathways, indicating the importance of grass carp TLR20 in antiviral and antibacterial functions [14,60]. However, due to the limitation of second-generation sequencing technology, there is a possibility that the identification of certain TLRs was omitted. The identification of new TLRs can be facilitated by the development of third-generation of genome sequencing technology. To obtain more comprehensive sequences of grass carp TLR20 members, we retrieved the latest grass carp genome. Our results revealed the presence of a previously unreported TLR20, which is situated on chromosome 9 and located between TLR20a and TLR20b. Therefore, we renamed the three grass carp TLR20s as CiTLR20.1-CiTLR20.3 based on their positions on the chromosomes.

2. Materials and methods

2.1. Re-identification of TLR20 genes in grass carp

A total of nine TLR20 protein sequences from zebrafish (*D. rerio*), blunt snout bream (*M. amblycephala*), common carp (*C. carpio*), Atlantic salmon (*S. salar*), and channel catfish (*I. punctatus*) were used as query sequences to conduct a TBLASTN search in the most recent grass carp genome (PRJNA745929) using TBtools (v1.120) software with an e-value cut-off of 1×10^{-5} and the default parameters for intron size prediction [5,54]. To confirm the sequences of grass carp TLR20s, the gene structures of the obtained candidate sequences were predicted. The ultimate potential TLR20 proteins were found out after removing redundant sequences without a similar TLR domain. Then, the obtained genes were annotated based on the existing grass carp genome annotation results. To further verify the accuracy of the annotations, the candidate genes were compared to the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) non-redundant database (BLASTX).

2.2. Phylogenetic analysis

A phylogenetic tree was conducted based on the TLR gene sequences of grass carp, and the known TLR11 family members of six teleost fishes and one mammal, including *M. musculus* (TLR11, NP_991,388, TLR12, NP_991,392, TLR13, AAS37674), *C. idella* (TLR19, AUF71965.1; TLR20.1, XP_051761650.1; TLR20.2, OR437356; TLR20.3, AHN49762; TLR21, AGM21642; TLR22a, ADX97523; TLR22b, AVI26517.1), *D. rerio* (TLR19, XP_002664892, TLR20.1, XP_009303036.2, TLR20.2, NP_001170914.2, TLR20.3, XP_021334630.1, TLR20.4, XP_009303038.2, TLR21, NP_001186264, TLR22, NP_001122147), *C. carpio* (TLR19, BAU98390, TLR20, AHH85805, TLR21, BAU98391, TLR22, ADR66025), *M. amblycephala* (TLR19, APT35508.1, TLR20, APT35509.1, TLR21, XP_048007711.1, TLR22, XP_048021849.1), *S. salar* (TLR19, CDH93609, TLR20, CDH93610, TLR21, CDH93614, TLR22, CAJ80696) and *I. punctatus* (TLR19, AEI59675, TLR20.1 AEI59676, TLR20.2, AEI59677, TLR21, AEI59678, TLR22, AEI59679). The amino acid sequences of TLR11 family members were aligned using the Clustal W program in and a Maximum Likelihood was constructed with default parameters [20].

2.3. Collinearity analysis

The three CiTLR20s were matched with the chromosomes of grass carp on the basis of the genome annotations of grass carp. The TBtools was used for gene synteny analysis of orthologous and paralogs genes among *D. rerio, C. idella* and *C. carpio*, and the results were further visualized to collinearity analysis map.

2.4. Sequence similarity and Ka/Ks value analyses

Amino acid sequence similarity was performed by the blast module in NCBI and the results were visualized using Jalview software [53]. The genelogo of BB-loop sequence was plotted with the online website WEBLOGO (http://weblogo.berkeley.edu/logo.cgi) [7]. The Ka/Ks value represents the ratio between the rate of nonsynonymous substitutions (Ka) and the rate of synonymous substitutions (Ks) for two protein-coding genes to determine whether there is selection pressure acting on this protein-coding gene. We calculated Ka/Ks values using MEGA-X software to investigate the evolutionary direction of TLR20s. Each codon site of each protein was identified by online website SELECTON (http://selecton.tau.ac.il/) [43].

2.5. 3D protein structure

The 3D protein structure of three CiTLR20 proteins were estimated utilizing AlphaFold 2.0 software with default parameters [48]. The PyMOL software was used to annotate the structure of each protein model, where α-helix was annotated as green, β-fold as yellow, irregular curl as blue, and BB-loop as red.

2.6. mRNA expression profiles of CiTLR20 in healthy and challenged grass carp

RNA sequencing data from 12 tissue types (eye, brain, gill, skin, fin, swim bladder, head kidney, trunk kidney, liver, spleen, intestine, and blood) in grass carp were retrieved from the NCBI (SRR23719652 (eye), SRR23719660 (brain), SRR23719654 (gill), SRR23719661 (skin), SRR23719653 (fin), SRR23719659 (swim bladder), SRR8380240 (head kidney), SRR8380198 (trunk kidney), SRR23719657 (hepatopancreas),

SRR23719662 (spleen), SRR8380203 (intestine) and SRR8380241 (blood)). In addition, we collected transcriptome data libraries of spleen tissues in grass carp at different time points (0 h, 4 h, 8 h, 24 h, and 48 h) following challenge with *Aeromonas hydrophila* (SRR2086445, SRR2086468, SRR2086474, SRR2086456, and SRR2086471) and obtained transcriptome data libraries of spleen tissues in grass carp from challenged groups with GCRV II (SRX2451397, SRX2451398, SRX2451399, and SRX2451400) and control groups (SRX2451402, SRX2451403, SRX2451404, and SRX2451405) at different time points (D1, D3, D5, and D7). All these libraries were obtained from NCBI. All clean reads from the 25 different RNA-seq libraries were mapped onto the grass carp genome sequences using Bowtie2, and the number of mapped reads was normalized with the TPM (reads per kilobase per million) method. According to the TPM value of CiTLR20s in the respective samples, two heat maps indicating tissue-specific expression and differential expression of CiTLR20s in response to GCRV II were generated using Graph prism 8.0 software.

3. Results

3.1. A new CiTLR20 was identified in grass carp

To identify TLR20 in grass carp, we used nine TLR20 sequences from five fishes as query sequences and blasted them with the most recent genome of grass carp separately [54]. A total of 27 sequences were obtained by intersection of the results. Further, the gene structures of these 27 sequences were predicted, and 14 of them were defined as candidate sequences for containing typical TLR domain. Then, three grass carp TLR20 sequences were identified based on grass carp genome annotation and the results of matching with non-redundant databases (Fig. 1). One of them was a new unreported sequence, while the other two have been previously described as TLR20a (or TLR20.2) and TLR20b, respectively [14,24].

3.2. Three TLR20s arise through tandem replication events

To analyze the phylogenetic relationship of the fourteen candidate genes from grass carp and TLR11 family among different species, a Maximum Likelihood phylogenetic tree was constructed based on the alignments of 41 full-length TLR family protein sequences from *M. musculus* (3), *C. idella* (14), *D. rerio* (7), *C. carpio* (4), *M. amblycephala* (4), *S. salar* (4) and *I. punctatus* (5) (Fig. 2A). Major clades had bootstrap values greater than 50% (1000 replicates). In the phylogenetic tree, all sequences were divided into five groups, and the three CiTLR20s of grass carp were assigned to the same branch as TLR20.

Conservation of synteny was investigated by comparing the genomic regions immediately up- and downstream of zebrafish TLR20s on chromosome 9, common carp TLR20 on chromosome A9 and grass carp CiTLR20s on the chromosome 9. Syntenic analysis revealed that TLR20 loci are highly conserved in zebrafish, common carp and grass carp, with the presence of multiple copies TLR20 genes located between solute carrier family 10 member 2 (SLC10A2) and nucleolus and neural progenitor protein (NEPRO), except in common carp which has only one TLR20 (Fig. 2B). In addition, two tandem TLR20s (TLR20e and TLR20f) are also present in zebrafish at another locus on chromosome 9. To provide a reference for subsequent studies, we standardized the nomenclature of the three CiTLR20s in grass carp according to their loci on the chromosome. They are CiTLR20.1, CiTLR20.2, CiTLR20.3, where CiTLR20.1 and CiTLR20.3 were referred to as TLR20b and TLR20a (or TLR20.2) in previous reports [14,24]. We deposited the new CiTLR20.2 nucleotide sequence in GenBank (GenBank accession number: OR437356). The three CiTLR20s were located in the same intergenic region and the phylogenetic tree revealed their close evolutionary relationship, suggesting that they arose through tandem replication

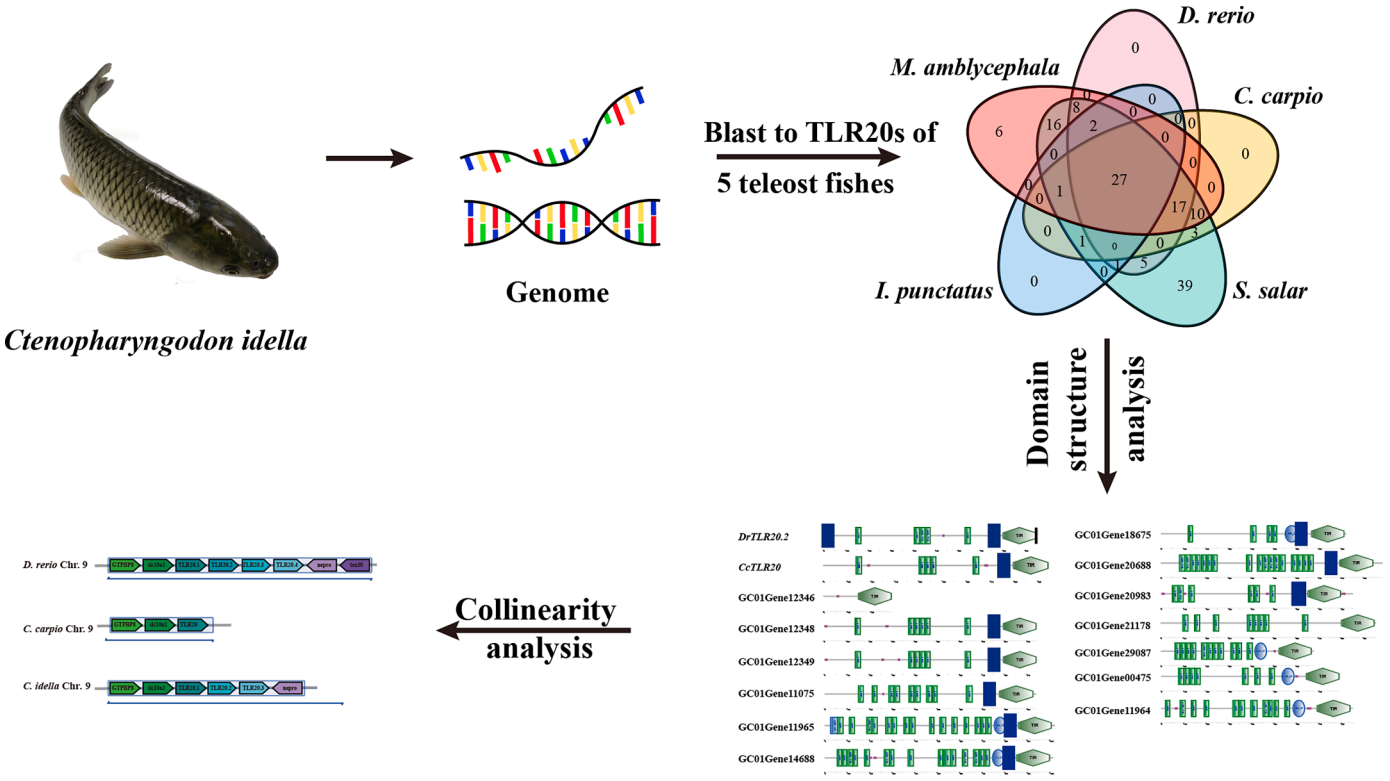

Fig. 1. The three CiTLR20s (CiTLR20.1, CiTLR20.2 and CiTLR20.3) of grass carp were identified from the most recent genome. The nine TLR20s from five teleost fishes was blasted to the most recent genome of grass carp and obtained intersection of the results. Thereafter, sequences containing typical TLR domain were designated as candidate sequences by structure prediction. Then, the three grass carp TLR20 sequences were identified based on grass carp genome annotation and the results of matching with non-redundant databases. Finally, the names of the three TLR20s were specified according to their location on the chromosome.

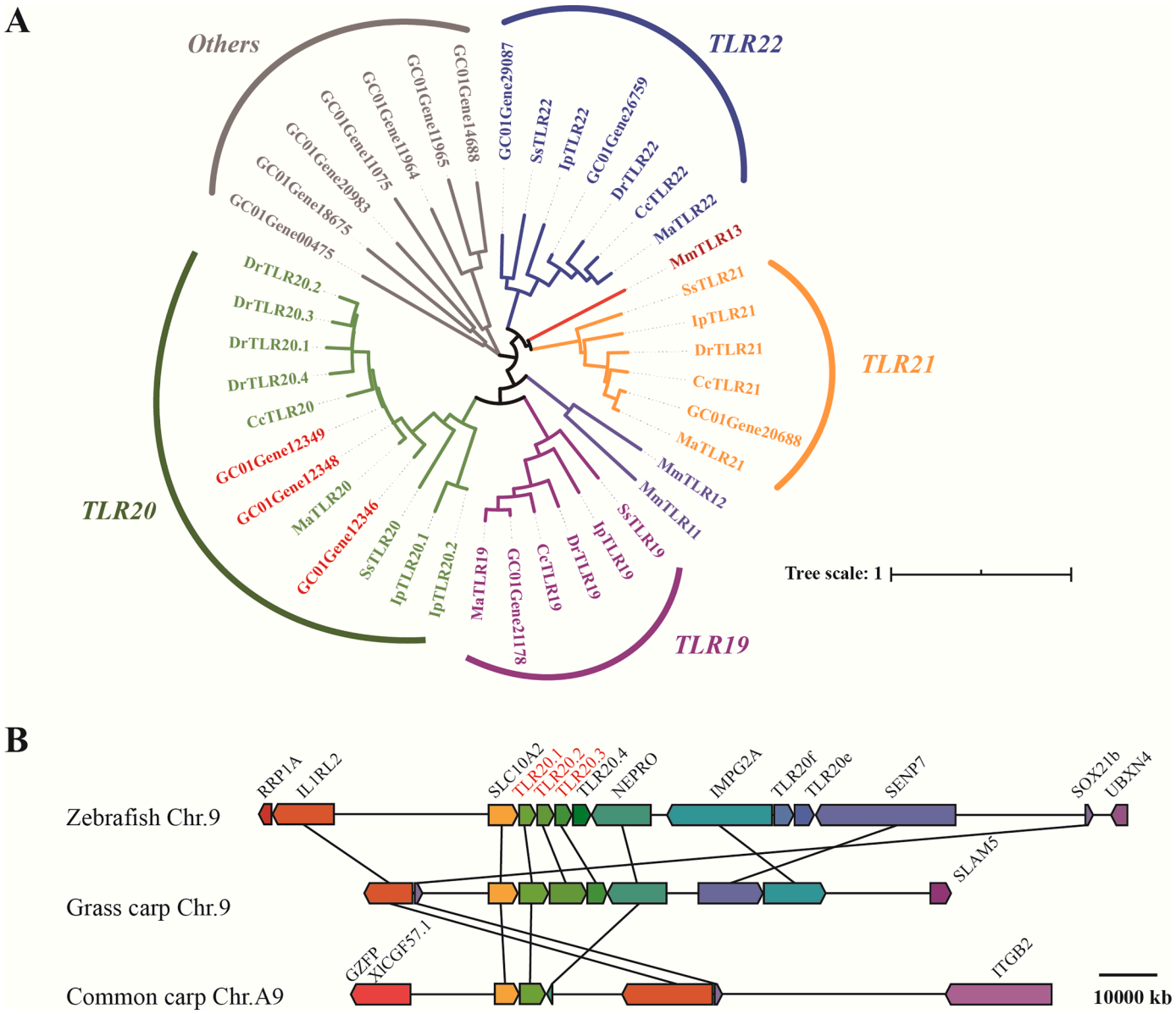

Fig. 2. The phylogenetic relationship of the TLR11 family and synteny analyze of TLR20s. (A) The maximum likelihood phylogeny tree of 41 full-length TLR11 family protein sequences. The three grass carp TLR20s tagged in red were clustered into a taxon with other TLR20s. Phylogenetic tree was reconstructed using MEGA-X. Major clades had bootstrap values greater than 50% (1000 replicates). (B) Genomic organization of genes surrounding TLR20s in D. rerio, C. idella and C. carpio. Arrows for genes represent the coding strand. The genomes are aligned to the TLR20.1 start site.

events. In addition, phylogenetic analysis and collinearity analysis conjointly demonstrated that TLR20s in fish had produced a large divergence in evolution.

3.3. Multiple sites in the extracellular region of TLR20 undergo positive selection, while intracellular region undergoes strong purifying selection

In general, TLR20 exhibits a high degree of similarity (>60%) among cyprinid fish species, with the closest sequence similarity observed between grass carp and zebrafish, but the similarity was lower comparing the sequences of grass carp and channel catfish or Atlantic salmon (Fig. 3A). Within the same species, different subtypes show a high level of similarity. For instance, the similarity between grass carp CiTLR20.2 and CiTLR20.3 is 93.68%, while the similarity among the four TLR20 subtypes in zebrafish is also above 75%. Comparative analysis was performed on the amino acid sequences of grass carp CiTLR20.1-CiTLR20.3, zebrafish DrTLR20.1-DrTLR20.4, and common carp CcTLR20. The analysis confirmed the conservation of structural features in TLR20, particularly in the TIR domain. Amongst the grass carp sequences, CiTLR20.2 and CiTLR20.3 exhibited complete extracellular, transmembrane, and TIR domains. CiTLR20.2 and CiTLR20.3 contained a total of 26 LRR motifs. However, CiTLR20.1 exhibited a noticeable truncation in its extracellular domain as it possessed only three LRRs motifs and lacked a signal peptide (Supplementary Fig. 1, Table 1).

Gene duplication is one of the important incidents of gene expansion and contributes to functional diversity in the evolutionary process [28]. To investigate whether TLR20 gene duplication led to divergence, we calculated the ratio of non-synonymous (Ka) and synonymous (Ks) substitution rates (Ka/Ks) using the CDS of TLR20s in representative fishes. The ratios of Ka/Ks for all TLR20 paralogous pairs in various fishes were <1 (Fig. 3B), suggesting that the TLR20 genes had undergone purifying selection pressure in fishes. To investigate the evolutionary conservation of each amino acid site of TLR20 proteins, we analyzed the selection pressure on TLR20s using SELECTON. We found

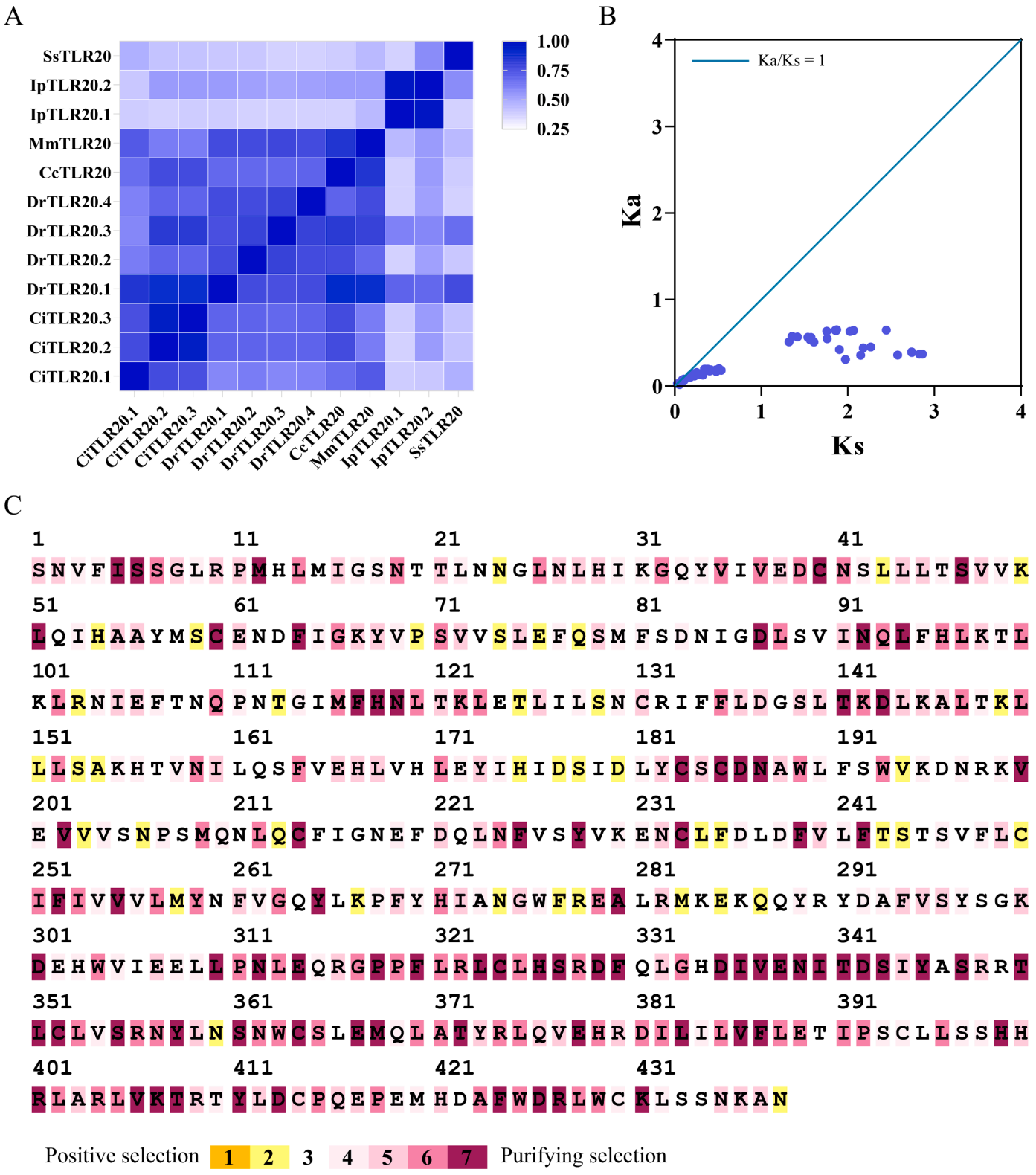

Fig. 3. The analyses of amino acid similarity and selection pressure of TLR20 genes. (A) Amino acid sequence similarity was performed by the blast module in NCBI. (B) Calculation of selection pressure for TLR20 genes in fishes. The value of Ka/Ks less than 1 indicates that TLR20 in fish has undergone purifying selection during evolution. (C) Selection pressure analysis of the TLR20 proteins in fishes. The red shades represent $\omega < 1$ (purifying selection). The TIR domain of TLR20 protein is undergoing strong purifying selection and multiple sites in the extracellular region are undergoing positive selection.

Table 1
Molecular characteristics of representative teleost TLR20s.

Gene name	amino acid length	Signal peptide length	Number of LRR	GenBank accession no.
CiTLR20.1	290	–	3	XP_051761650.1
CiTLR20.2	944	21	26	OR437356*
CiTLR20.3	944	21	26	AHN49762
DrTLR20.1	951	27	26	XP_009303036.2
DrTLR20.2	950	26	26	NP_001170914.2
DrTLR20.3	951	27	26	XP_021334630.1
DrTLR20.4	951	27	26	XP_009303038.2
CcTLR20.1	946	20	26	AHH85805

Note:List of open reading frame (aa length), signal peptide, number of LRR in grass carp, common carp and zebrafish. * indicates the sequence was deposited in GenBank by ourselves.

that the TIR domain of TLR20 protein is undergoing strong purifying selection (Fig. 3C). Similarly, we found multiple sites in the extracellular region that were under positive selection, implying that the extracellular region of TLR20 may have undergone functional divergence, that is, different recognition of ligands.

3.4. *CiTLR20s may recruit different adaptors*

The three-dimensional structures of the three CiTLR20 proteins were modeled using AlphaFold 2.0 software. The overall 3D structures of CiTLR20.2 and CiTLR20.3 are extremely comparable. However, compared to CiTLR20.2, CiTLR20.3 forms an α-helix spanning amino acids 190 to 193, and the specific location of the disparity between the two is denoted by the red arrow (Fig. 4A). The biological consequence of these slight differences is uncertain. CiTLR20.1 was totally different from the other two, missing most of the extracellular region, and the structural of TIR domain was also distinct. In recent studies, the BB-loop of the TIR domain in TLRs was demonstrated to interact with adaptors, and the proline residue in the BB-loop could bind to MyD88, while valine could bind to TRIF. We compared the sequences of the BB-loop of grass carp TLR20, and CiTLR20.2 and CiTLR20.3 have leucine rather than proline or valine on the BB-loop, unlike CiTLR19, CiTLR22a and CiTLR22b (Fig. 4B and C), suggesting that the adaptors of CiTLR20.2 and CiTLR20.3 might not be MyD88 or TRIF. The CiTLR20.1 had a valine residue, which may mean it can employ TRIF as an adaptor (Fig. 4D).

3.5. *CiTLR20.2 and CiTLR20.3 responds to viral and bacterial infections*

To obtain the expression patterns of CiTLR20 genes in different tissues, we performed expression patterns analysis of the CiTLR20 genes with the RNA-Seq database in NCBI. The expression profiles of the three CiTLR20s showed different patterns of tissue-specific expression (Fig. 5A). Generally, CiTLR20.2 and CiTLR20.3 are expressed in most tissues and more strongly in immune tissues. Both CiTLR20.2 and CiTLR20.3 displayed strong expression in the swim bladder. CiTLR20.3 was also highly expressed in the skin and eye. However, CiTLR20.1 hardly expressed in most tissues, except for lower expression in eye, fin and skin. To determine the immune responses of CiTLR20s, we obtained RNA-Seq data from the spleen of grass carp challenged with GCRV-II and *A. hydrophila* (Fig. 5B and C). The results demonstrated that the expression levels of CiTLR20.2 and CiTLR20.3 increased as the duration of GCRV II infection progressed, peaking at 5d, and subsequently declining to lower levels by 7d Following the injection of *A. hydrophila*, there was a notable increase in the expression level of CiTLR20.2, reaching its peak at 48 h, whereas the expression level of CiTLR20.3

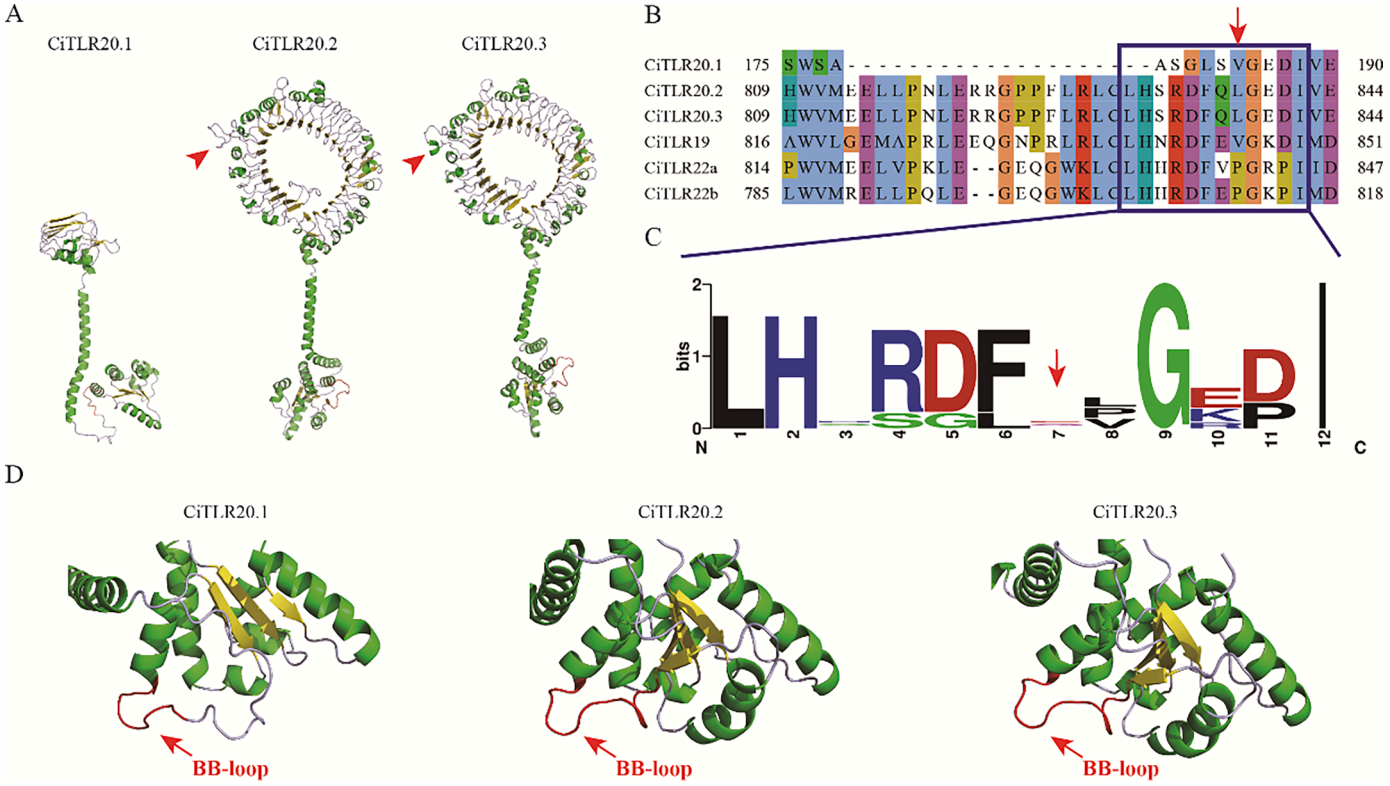

Fig. 4. The three-dimensional structures of the three CiTLR20 proteins. (A) The protein structure of grass carp CiTLR20s was predicted with AlphaFold 2.0. CiTLR20.1 lacks a signal peptide but retains three LRR motifs, transmembrane region, and TIR domain. CiTLR20.2 and CiTLR20.3 containe typical signal peptide, LRR domain, transmembrane region and TIR domain. (B) The amino acid comparison of BB-loop. CiTLR20.1 has a non-conserved BB-loop. CiTLR20.2 and CiTLR20.3 replace proline or valine with leucine. (C) Genelogo of BB-loop. (D) Protein structure of the BB-loop protein of CiTLR20s. The difference in structure among CiTLR20.1, CiTLR20.2 and CiTLR 20.3 on the BB-loop predicted that CiTLR20s may recruit different adapters.

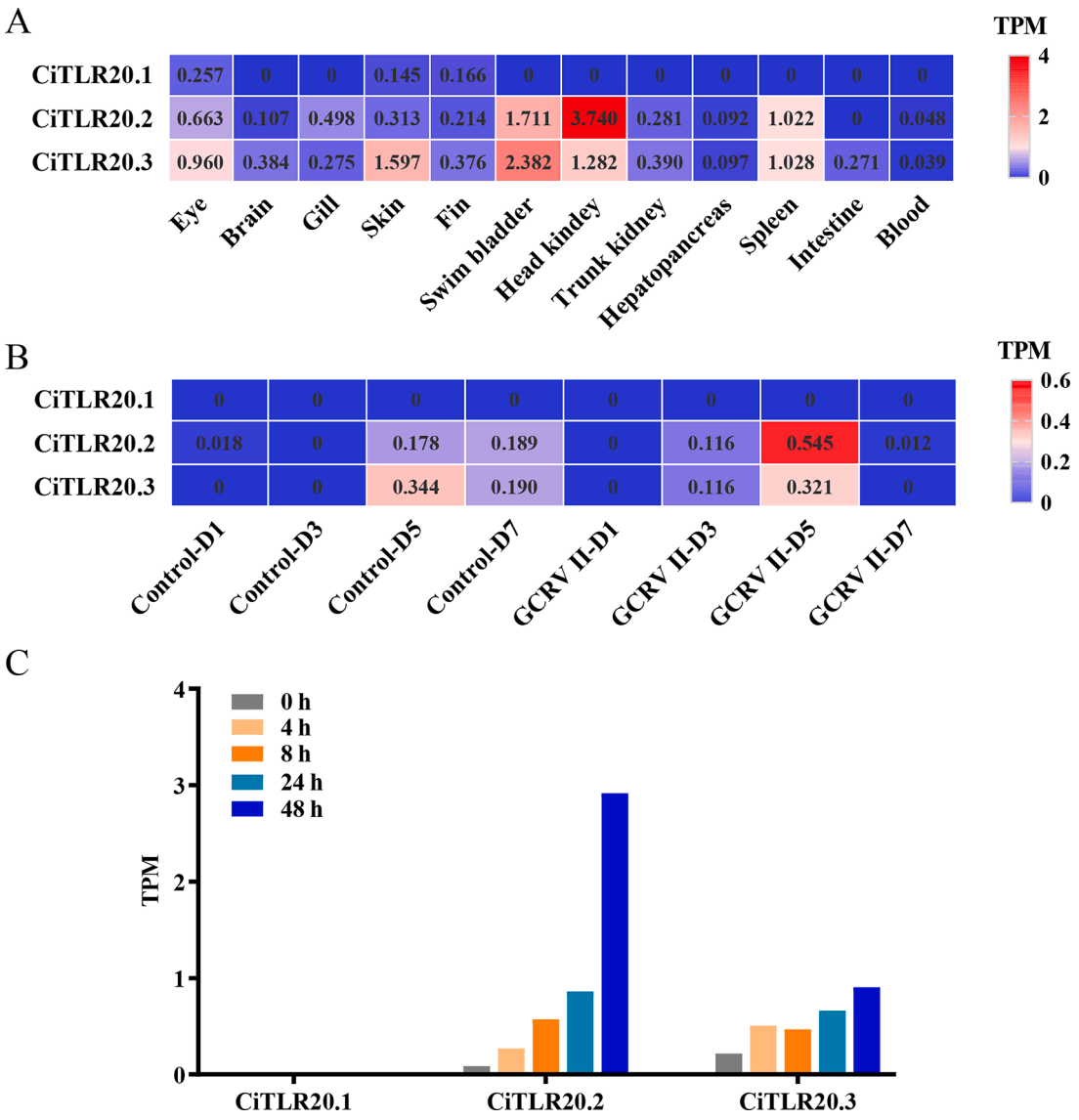

Fig. 5. mRNA expression profiles of CiTLR20s. (A) Tissue distribution of CiTLR20.1, CiTLR20.2 and CiTLR20.3. CiTLR20.2 and CiTLR20.3 express in most tissues and more strongly in immune tissues. CiTLR20.1 only minimally expresses in few tissues. The expression patterns of CiTLR20s in spleen tissues under the challenge of GCRV-II (B) and A. hydrophila (C).

displayed a slower and gradual increase. In contrast, CiTLR20.1 displayed consistently low expression levels, with minimal detectable expression, under both GCRV-II and *A. hydrophila* infection.

4. Discussion

Since the discovery of the first fish TLR in rainbow trout [42], the continuous development of sequencing technology has led to the identification of a large number of TLRs from the genome. In this study, we aimed to characterize the gene members of CiTLR20 from the most recent grass carp genome in order to enhance our understanding of the grass carp TLR family. Here, we successfully identified a novel TLR20 paralog, which shares a high similarity with previously reported TLR20a and TLR20b [24], and they are renamed based on their positions on the chromosome, CiTLR20.1 (previously TLR20b), CiTLR20.2 (newly discovered), CiTLR20.3 (previously TLR20a or TLR20.2).

Teleost fish have undergone multiple rounds of WGD. In addition to the first two rounds experienced by early vertebrates, teleost fish have also experienced a unique third round of WGD [13,47]. Furthermore, species such as Salmonidae, *Sinocyclocheilus, Carassius auratus*, and *C. carpio* have undergone a fourth round of WGD [6,26,55,58]. Current research suggests that WGD and gene loss may contribute to variations in gene copy numbers in different fish species. In mammals, there are only 13 TLRs, and each of them is present as a single copy gene [18]. However, in fish, there are more than 20 TLRs, and multiple TLRs exist as duplicates and multiple paralogous genes such as TLR3a/b (zebrafish), TLR4–1/2/3/4 (grass carp), TLR5M/S (fugu, rainbow trout), TLR7a/b (common carp), TLR8a/b (zebrafish, grass carp), TLR20.1/2/3/4 (zebrafish), and TLR22a/b (grass carp, Atlantic salmon) [37]. Phylogenetic analysis and collinearity analysis indicated that the three CiTLR20 genes in grass carp are generated by tandem duplication rather than genome duplication. Furthermore, continuous mutations during the evolutionary process have contributed to the formation of the current three CiTLR20 genes. Zebrafish have six TLR20s, of which four encode proteins and two pseudogenes [34], and grass carp have three CiTLR20s, all of which encode proteins. In common carp, however, there was only one functional TLR20 [39], indicating that the number of TLR20 varied greatly among species. The TLR20 genes demonstrates a substantial degree of similarity (>60%) across various Cyprinid fishes, and they have experienced purifying selection pressure (Ka/Ks<1),

particularly in the TIR region. LRRs are essential for recognizing MAMPs, but the LRRs in fish TLR20 display notable variations, which are subject to positive selective pressure, implying that different fish TLR20s may recognize different targets. Conversely, the high conservation of the TIR domain suggests the conservation of downstream signaling pathways in TLR20.

The advent of AlphaFold 2.0 has revolutionized protein 3D structure prediction, making it significantly more convenient and accurate [48]. Using this advanced tool, we have successfully predicted the 3D structure of CiTLR20s from grass carp. Similar to other TLRs, CiTLR20.2 and CiTLR20.3 feature a distinctive horseshoe-shaped extracellular region. Indeed, it is worth noting that despite the similarity in the general structure of the extracellular domains of CiTLR20.2 and CiTLR20.3, there may still be subtle differences between them. The precise biological consequences of these differences are currently uncertain. It is possible that these variations could result in distinct ligand recognition or binding capabilities. Further research and experimentation will be necessary to elucidate the exact functional implications of these subtle variances in the extracellular domains of CiTLR20.2 and CiTLR20.3. Furthermore, the deletion of the CiTLR20.1 gene structure results in the noticeable truncation in its extracellular domain. Consequently, it may no longer be capable of recognizing ligands. Its biological functions remain uncertain. Further research is necessary to elucidate the roles and significance of CiTLR20.1 within the immune system. The BB-loop of the TIR domain is demonstrated to interact with adaptors [8,17,56]. With the exception of TLR3 and TLR19, which recruits TRIF and have a valine residue in the BB-loop, the BB-loop of the remaining TLRs contains a conserved proline residue that assists in binding MyD88. Following the substitution of the valine residue in the BB-loop of TLR3 with a proline residue, TLR3 shifts its signaling pathway from TRIF-mediated to MyD88-mediated [50]. We investigated the BB-loop of CiTLR20s and observed that TLR20.2 and TLR20.3 lack the conserved proline or valine residue, instead having leucine residues. This implies that they are likely to recruit alternative adaptors for signal transduction or it is not conserved in fishes. In the case of CiTLR20.1, it contains the valine residue, suggesting that it may transmit signals downstream through TRIF.

To explore the tissue distribution of CiTLR20s, we conducted an analysis of their expression patterns in various tissues using RNA-seq data obtained from NCBI. Consistent with previous reports, CiTLR20.3 exhibits broad expression across different tissues, with the highest expression observed in immune organs such as the head kidney and spleen [14]. The expression of CiTLR20.2 is significantly upregulated in the spleen and head kidney after GCRV-II infection, and it is involved in the inhibition of GCRV-II replication [60]. Similarly, our newly discovered CiTLR20.2 displayed a similar expression profile. Interestingly, both CiTLR20.2 and CiTLR20.3 exhibited significant expression in the swim bladder. Recent studies indicated the presence of diffuse mucosal-associated lymphoid tissue in the swim bladder mucosa, which plays a role in mucosal immunity [59]. The noteworthy expression of CiTLR20.2 and CiTLR20.3 suggests their involvement in the defense against pathogenic microorganisms through mucosal immune responses in the swim bladder. The expression of CiTLR20.3 can be induced by stimulation with LPS and poly(I:C), indicating its active response to bacterial and viral stimuli [14]. We investigated the expression changes in grass carp spleen tissue following infection with *A. hydrophila* and GCRV-II. The results demonstrated that both CiTLR20.3 and CiTLR20.2 exhibited increased expression levels in response to bacterial and viral infections. Specifically, CiTLR20.2 exhibited a highly significant upregulation in expression in response to *A. hydrophila* infection. In contrast, CiTLR20.1 displayed low expression levels across all the tissues and remained insensitive to viral and bacterial infections. This could be attributed to the fact that CiTLR20.1 has only three LRR motifs in its extracellular domain, which may not be sufficient to form the corresponding structure for ligand recognition. The subcellular localization of TLRs is crucial for their function. TLRs are located on the cell membrane in humans primarily recognize bacterial and fungal cell surface components. TLRs localize in endosomes or lysosomes, on the other hand, recognize pathogens' nucleic acids [33]. In zebrafish and common carp, TLR20 does not localize on the cell membrane but instead resides in the cytoplasm, indicating the significant potential of TLR20 in recognizing pathogenic microbial nucleic acids [39]. Although subcellular localization has not been studied for grass carp TLR20, based on the similarities in structure and sequence among zebrafish, common carp, and grass carp TLR20, it can be inferred that grass carp TLR20 is also cytoplasmic. This suggests that CiTLR20s may recognize specific nucleic acid components.

In summary, we identified a new member of the TLR20 family in grass carp, CiTLR20.2, based on the latest genome. These three CiTLR20s, resulting from tandem duplications, display a high degree of similarity and have undergone strong purification selection in the intracellular region. Multiple sites in the extracellular domain have undergone positive selection, indicating potential functional differentiation in the extracellular region of TLR20. The differences in the BB loop sequences suggest that CiTLR20.1 may be able to use TRIF as an adapter, while CiTLR20.2 and CiTLR20.3 may not. Additionally, the positive responses of CiTLR20.2 and CiTLR20.3 to viral and bacterial infections suggest their significant roles in pathogen recognition. These findings provide meaningful insights for further exploration of the immunoregulatory role played by grass carp TLR20.

Declaration of Competing Interest

The authors have no competing financial interests to declare.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100119.

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