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# Divergent Toll-like receptors in catfish (*Ictalurus punctatus*): TLR5S, TLR20, TLR21

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**Abstract**

Toll-like receptors (TLR) mediate pathogen recognition in vertebrate species through detection of conserved microbial ligands. Families of TLR molecules have been described from the genomes of the teleost fish model species zebrafish and *Takifugu*, but much research remains to characterize the full length sequences and pathogen specificities of individual TLR members in fish. While the majority of these pathogen receptors are conserved among vertebrate species with clear orthologues present in fish for most mammalian TLRs, several interesting differences are present in the TLR repertoire of teleost fish when compared to that of mammals. A soluble form of TLR5 has been reported from salmonid fish and *Takifugu rubripes* which is not present in mammals, and a large group of TLRs (arbitrarily numbered 19–23) was identified from teleost genomes with no easily discernible orthologues in mammals. To better understand these teleost adaptations to the TLR family, we have isolated, sequenced, and characterized the full-length cDNA and gene sequences of TLR5S, TLR20, and TLR21 from catfish as well as studied their expression pattern in tissues. We also mapped these genes to bacterial artificial chromosome (BAC) clones for genome analysis. While TLR5S appeared to be common in teleost fish, and TLR21 is common to birds, amphibians and fish, TLR20 has only been identified in zebrafish and catfish. Phylogenetic analysis of catfish TLR20 indicated that it is closely related to murine TLR11 and TLR12, two divergent TLRs about which little is known. All three genes appear to exist in catfish as single copy genes.


*Keywords:* Catfish; Genome; TLR5; TLR20; TLR21; Toll-like receptor; Disease; Innate immunity

## 1. Introduction

Since their identification as pattern recognition receptors (PRRs) almost a decade ago [1], the Toll-like receptor (TLR) family has provided a complicated, yet crucial addition to our understanding of the innate and adaptive immune responses. Through recognition of pathogen-associated molecular patterns (PAMPs; [2]) such as lipopolysaccharide (LPS), flagellin, and dsRNA, TLRs trigger pro-inflammatory and antiviral signaling pathways that ultimately lead to elimination of invading pathogens (see [3] for a recent review).

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TLRs are characterized by leucine-rich repeats (LRR) in their extracellular domain that function in ligand binding, and a Toll/IL-1 receptor (TIR) domain in their cytosolic domain that binds to TIR-containing adaptor molecules which regulate downstream signaling [4]. Five such TIR-containing adaptor proteins have been identified from mammalian species [5]: a myeloid differentiation protein named MyD88; MAL (MyD88-adaptor-like protein, or TIRAP); TRIF (TIR-domain containing adaptor inducing INF-β) or TICAM-1 (TIR domain-containing adapter molecule-1); TRAM (TRIF-related adaptor protein) or TICAM-2; and SARM (sterile α and HEAT-Armadillo motifs). The TLR family itself continues to expand, and 13 TLR members have been identified to date in mammals, 10 of which are expressed in humans [6]. The ligand specificities of many of the mammalian TLRs are still the subject of investigation. For example, TLR11 was most recently found to recognize a protozoan profilin-like protein [7]. The best characterized ligand/receptor specificities for the TLR family are: lipoproteins (TLR2), dsRNA (TLR3), LPS (TLR4), and flagellin (TLR5).

Research on the TLR system in higher vertebrates quickly prompted interest in tracing the origins and divergences of this pathogen recognition system in lower vertebrates and invertebrates [8,9]. Many deuterostome invertebrates, such as the sea urchin, appear to have large, sophisticated TLR families based on preliminary *in silico* studies [8]. In teleost fish, genome surveys of TLR-like genes have been carried out in two model species, pufferfish *Takifugu rubripes* [10] and zebrafish *Danio rerio* [11,12]. Subsequent, in-depth studies of individual TLRs from several fish species have increased our knowledge of the pathogen specificity and expression patterns of teleost TLRs and their adaptors [13–25].

Overall, the TLR family appears to be highly conserved among vertebrate species with clear orthologues present in fish for most mammalian TLRs. However, several interesting differences are present in the TLR repertoire of teleost fish when compared to mammals, suggesting novel adaptations in pathogen recognition and inflammatory signaling. First, TLR4 is absent in the genomes of pufferfish, *Takifugu rubripes* and *Tetraodon nigroviridis*, but is present in zebrafish [12,26]. A recent search also failed to find TLR4 in the draft genome sequence of the stickleback *Gasterosteus aculeatus* (our unpublished data). Second, a soluble form of TLR5 has been reported from salmonid fish and *Takifugu rubripes* but is not present in mammals [20]. This inducible soluble TLR5 has been reported in rainbow trout to amplify membrane TLR5-mediated cellular responses in a positive feedback fashion [19]. Lastly, a group of fish TLRs has been identified with no easily discernible orthologues in mammals. These include the TLR21–23 members from *Tetraodon nigroviridis* and *Takifugu rubripes* [8,10], and a number of TLR genes (19–22) from *Danio rerio* [11,12]. This group has been suggested as a possible alternative receptor for LPS recognition (in the absence of TLR4), and may be related to divergent TLR11–13 genes in mouse about which little is presently known [8,10,27].

Catfish (*Ictalurus* sp.), the major aquaculture species in the United States, has served as a model for immunological studies in lower vertebrates for decades. Systematic studies of its adaptive and innate immune components [28,29] have led to important advances in comparative immunology and will guide future genetic improvement in catfish strains. Sensitive pathogen recognition systems are crucial for species in the microbe-rich aquatic environment. Our previous studies of catfish TLR2, TLR3, and the TICAM adaptor have indicated: (1) that teleost TLR ligand specificities may differ from those seen in mammals, and (2) that TLRs may be linked to observed differences in susceptibility to fish pathogens between channel (*I. punctatus*) and blue (*I. furcatus*) catfish [24,25]. Additional characterization of TLR expression patterns in catfish during development and following disease challenge have been carried out previously using partial transcripts of TLR3 and TLR5 [15,16,30]. To better understand teleost adaptations to the TLR family, we have identified and characterized three TLR members not present in mammalian species, TLR5S, TLR20, and TLR21. Here we present molecular analysis of their full-length sequences, gene structure, orthologies, gene copy numbers, and expression patterns in catfish.

## 2. Material and methods

### 2.1. Identification, sequencing, and sequence analysis of catfish TLR5S, TLR20 and TLR21 cDNAs

Three catfish TLR sequences were initially identified from BLAST analysis of expressed sequence tags from catfish [31–34]. BLASTX searches using the three ESTs as queries indicated that the highest identities were to teleost fish TLR5S, TLR20 and TLR21, respectively. Sequencing of the EST clones indicated that the clones contained only partial cDNAs for the three genes. In each case, the 5′ end of the cDNA was missing in the EST clone. Therefore, 5′ RLM-RACE (RNA ligase-mediated rapid amplification of 5′ cDNA ends) was conducted for TLR5S, TLR20, and

TLR21 using the GeneRACER™ Kit with SuperScript™ III RT from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions, as we previously described [25]. Primers and probes used throughout this study are shown in Table 1. The purified PCR products were cloned into TOPO TA cloning® kit vector (Invitrogen) and sequenced using an ABI 3130XL automated DNA sequencer (Applied Biosystems, Foster City, CA).

### 2.2. BAC library screening, genomic sequencing, and sequence analysis

The cDNA sequences of three catfish TLRs were used to design overlapping oligonucleotide probes or overgos for screening the channel catfish CHORI-212 BAC library (http://bacpac.chori.org/library.php?id=103). In each case, two 24-base-long oligonucleotide primers were synthesized such that they had an 8-bp complementary region at their 3′ end. Upon mixing the two primers, they base pair to form a complex structure that can be filled-in with radioactively labeled nucleotides. High-density BAC filters containing CHORI-212 BAC library clones were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Overgo hybridization was conducted according to methods of a web protocol (http://www.tree.caltech.edu/) with modifications [29,35,36]. Briefly, overgo primers were purchased from Sigma Genosys (Woodlands, TX), then labelled with [$^{32}$P]dATP and [$^{32}$P]dCTP (Amersham, Piscataway, NJ) in 10 mg ml$^{-1}$ bovine serum albumin, overgo labeling reaction 1× buffer [37], and incubated for 1 h at room temperature with Klenow polymerase (Invitrogen). Sephadex G50 spin columns were used to remove unincorporated nucleotides. Probes were denatured at 95 °C for 10 min and added into hybridization tubes that had been under pre-hybridization for 2 h with the hybridization solution. The filters were hybridized at 50 °C for 18 h in 50 ml hybridization solution (1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 M sodium phosphate, pH 7.2). The filters were washed at room temperature and exposed to X-ray film at −80 °C for 24 h. Positive clones were identified according to the clone distribution pattern from CHORI. Positive clones were picked and cultured in 2×YT medium. After overnight culture, BAC DNA was isolated using the Perfectprep® BAC 96 BAC DNA isolation kit (Brinkmann Instruments Inc., Westbury, NY).

Table 1
Primers used for this study

| Gene | Primer name | Primer sequences (5′ to 3′) |
|---|---|---|
| TLR5S | RT–PCR U | ATTAGCACGCCTTCCACAGC |
| | RT–PCR L | AGAGGTTCTGCAAGCCGGTC |
| | Overgo A | TGCAGAACCTCTGGTTACAAGGAC |
| | Overgo B | AACATGTCGAGACACCGTCCTTGT |
| | cDNA probe U | ACATACATTAGCACGCCTTCC |
| | cDNA probe L | CCTCGCTGCTGTTCGGGATACG |
| | 5′ RACE PCR L | ACTTGTAGGTCTTTGTTGTATGCCAGAT |
| | 5′ RACE nested PCR L | CTCAAGACAAGTCGTCCTTCCTGTTGCT |
| TLR20 | RT–PCR U | TGGTAGTGCTGCTCCATCAG |
| | RT–PCR L | TAGGTTGCAGTTTCAGGTGC |
| | Overgo A | TGGGACTGGTGTTCTTCATGCTGG |
| | Overgo B | TGATGGAGCAGCACTACCAGCATG |
| | cDNA probe U | GATTGTCTTGTGAAGAGTGGGCA |
| | cDNA probe L | TAACCAAGAAGTCCCTTCAGC |
| | 5′ RACE PCR L | GCTATGTGAAAGAAAGCAAGCAGATAAT |
| | 5′ RACE nested PCR L | CAGTCCCAGAGAGGTGGAGGCAAAAAG |
| TLR21 | RT–PCR U | GCCTACTTGGATCGAAAAGCAC |
| | RT–PCR L | ACTTGCAGTGTGCGATGCAG |
| | Overgo A | GCTTGTTACACTTCGCCTGGACAA |
| | Overgo B | ATCAGACAGAAGGTTGTTGTCCAG |
| | cDNA probe U | ACTACGTCTTCCGCTCTTGGT |
| | cDNA probe L | TAAGTCTCATGCCAGCACAAG |
| | 5′ RACE PCR L | TATTGTAGATTAGCAAAGGCATCATTGT |
| | 5′ RACE nested PCR L | GCAGTAAGAGATTGAAATGTTTGTT |
| β-actin | RT–PCR U | AGAGAGAAATTGTCCGTGACATC |
| | RT–PCR L | CTCCGATCCAGACAGAGTATTTG |

One positive BAC clone for each catfish TLR, i.e. TLR5S (83-D18), TLR20 (5-M09) and TLR21 (86-O09), was sequenced with gene-specific primers using primer-walking methods. Fourteen picomoles of each primer were used for sequencing reactions. BAC sequencing was performed in a 10 μl reaction using the BigDye Terminator v3.0 Ready Reaction kit (Applied Biosystems) following manufacturer's instructions [38]. Sequences were analyzed on an ABI Prism 3130XL sequencer. BLAST searches were conducted to determine gene identities. The DNASTAR software package was used for sequence analysis. The MegAlign program of the DNASTAR package was used for sequence alignments using ClustalW [39]. NCBI's Spidey program was used for alignment of genomic and cDNA sequences. Receptor structure was characterized based on amino acid sequence using the simple modular architecture research tool (SMART) ([40]; http://smart.embl-heidelberg.de/).

### 2.3. Southern blot analysis

In order to determine the gene copy number of catfish TLR5S, TLR20, and TLR21, Southern blot analysis were conducted using both genomic DNA and BAC clones identified to contain the genes. Genomic DNA isolated from three channel catfish individuals was completely digested by EcoRI, HindIII and PstI. BAC DNA was digested with EcoRI and/or HindIII separately. After restriction digestion, DNA was electrophoresed on a 0.8% agarose gel. The DNA was transferred to a piece of Immobilon nylon membrane (Millipore, Bedford, MA) by capillary transfer with 0.4 M NaOH overnight. DNA was fixed to the membrane by UV cross linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto crosslink function. The membrane was washed in 0.5% SDS (w/v) at 65 °C for 15 min and then pre-hybridized in 50% formamide, 5× SSC [41], 0.1% SDS (w/v), 5× Denhardt's and 100 μg $ml^{-1}$ sonicated and denatured Atlantic salmon sperm DNA (100 μg $ml^{-1}$) overnight. Hybridization was conducted overnight at 42 °C in the same solution with appropriate probes added. A portion of the cDNAs amplified using the primers listed in Table 1 was used as the probe. The probe was prepared using the random primer labeling method [41] with a labelling kit from Roche Diagnostics (Indianapolis, IN) and [$^{32}$P]dCTP. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography.

### 2.4. Phylogenetic analysis

The relevant sequences were retrieved from GenBank for multiple amino acid sequence alignments using ClustalW. Phylogenetic trees were drawn by the neighbour-joining method [42] within the Molecular Evolutionary Genetics Analysis (MEGA 3.1) package [43]. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10,000 bootstrapping replications.

### 2.5. Fish collection and RNA extraction

Experimental fish were reared at the Auburn University Fish Genetics Research Unit Hatchery. Eleven tissues were collected from healthy channel catfish including head kidney, spleen, intestine, stomach, skin, muscle, liver, trunk kidney, ovary, brain and gill. The experimental fish were euthanized with tricaine methanesulphonate (MS 222) at 100 mg per litre before tissues were collected and stored at −80 °C; subsequently tissue samples were homogenized in Trizol Reagent (Invitrogen) and total RNA isolation was completed following the manufacturer's protocol. Extracted RNA was stored at −80 °C until it was used as the template for RT–PCR.

### 2.6. RT–PCR

RT–PCR reactions were conducted by two-step RT–PCR using M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). RT reactions were conducted in 40 μl reactions containing 4 μg DNase I-treated RNA, 4 μl (40 μM) oligo dT primers, 8 μl (2.5 mM each) dNTPs, 1 μl RNase inhibitor, 1× RT reaction buffer, and 200 units of RT. Detailed procedures followed the instructions of the manufacturer. After the RT reaction, 1 μl of the RT products was used as template for PCR using JumpStart Taq polymerase (Sigma, St. Louis, MO). The reactions also included the gene specific primers and primers of β-actin serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: denaturation at 94 °C for 2 min followed by 38 cycles of 94 °C for 30 s, 62 °C (for TLR5S) or 60 °C (for TLR20 and 21) for 30 s, 72 °C for 1 min. Upon the completion of PCR, the

reaction was incubated at 72 °C for an additional 10 min. The RT–PCR products were analyzed by electrophoresis on a 1.0% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

## 3. Results

### 3.1. Identification and sequencing of catfish TLR5S, TLR20 and TLR21 cDNAs

Clones containing putative TLR5S, TLR20, and TLR21 were obtained by EST analysis. Complete sequencing of the EST clones indicated that the clones contained only partial cDNA sequences. Complete cDNAs were then obtained by 5′ RACE, and they were completely sequenced by primer walking.

The complete cDNA sequence of TLR5S contains 2185 bp with a 5′-untranslated region (UTR) of 74 bp and a 3′-UTR of 167 bp (GenBank accession DQ529273). Translation of the TLR5S nucleotide sequence predicted a peptide sequence of 647 amino acids. BLASTX searches with the complete TLR5S cDNA sequence confirmed that it was most similar to TLR5 sequences from other teleost fish.

The complete cDNA sequence of TLR20 consists of 1853 bp with a 5′-UTR of 40 bp and a 3′-UTR of 757 bp (GenBank accession DQ529275). Translation of the TLR20 open reading frame predicted a peptide sequence of 351 amino acids. BLASTX searches with the complete TLR20 cDNA sequence confirmed that it was most similar to TLR20 sequences from *Danio rerio*, the only species from which a TLR20 sequence has been reported.

The complete cDNA sequence of TLR21 contains 3409 bp with a 5′-UTR of 225 bp and a 3′-UTR of 223 bp (GenBank accession DQ529277) and a deduced peptide of 986 amino acids. BLASTX searches with the complete TLR21 cDNA sequence confirmed that it was most similar to TLR21 sequences from other teleost fish.

### 3.2. Sequence and structure of catfish TLR5S, TLR20, and TLR21 genes

The complete cDNA sequences of three catfish TLRs were used to design overgo probes for screening the channel catfish CHORI-212 BAC library. Positive BAC clones for each probe are listed in Table 2. One positive BAC clone for each gene was randomly selected and sequenced with gene-specific primers using the primer-walking method. BAC clones were sequenced upstream and downstream of the coding regions to obtain genomic sequences of 4182 bp (TLR5, GenBank accession DQ529272), 3928 bp (TLR20, GenBank accession DQ529274), and 7232 bp (TLR21, GenBank accession DQ529276).

The genomic structures of the three catfish TLR genes were generally conserved with their orthologues in other species. Genomic structures of the three catfish genes were determined by comparing their cDNA and genomic sequences. As illustrated in Fig. 1, catfish TLR5S has an intron within its 5′-UTR, similar to that seen in rainbow trout [19], but differing from *Takifugu* where an intron is found within the coding region. The rainbow trout 3′ UTR is also notably longer than that found in catfish TLR5S. Catfish TLR20 is an intronless gene. As there are no demonstrated gene structures for TLR20 genes from other species, a direct structural comparison is not possible. However, based on the computer predicted genes of the zebrafish genome, the TLR20 gene structures also appear to be conserved. The catfish TLR21 gene harbors an intron in its 5′-UTR. Its single long ORF appears conserved with that of *Takifugu* TLR21, for which a complete cDNA sequence has not been reported (Fig. 1).

Both catfish TLR5S and TLR20 have atypical domain structures for Toll-like receptors. Catfish TLR5S lacks a TIR domain and a transmembrane domain, as do soluble TLR5 genes from other fish species [23]. Comparison of catfish TLR5S with these genes from *Takifugu*, trout, and salmon revealed 21 well-conserved LRR repeats as outlined by Tsoi

Table 2
Location of BAC clones positive for three catfish TLR genes from high density BAC filters containing 10× genome coverage of the CHORI-212 BAC library

| Gene | Location in 384-well plates |
|---|---|
| TLR5S | 62-C11, **83-D18**, 96-O15, 111-A04, 138-M14, 141-B15, 148-H17, 164-F20, 187-L14 |
| TLR20 | **5-M09**, 47-M17, 50-F17, 70-O03, 93-C12, 105-H12, 136-C18 |
| TLR21 | 6-G18, 9-B01, 25-I17, 75-M09, **86-O09**, 92-G10, 104-E15, 108-D01, 160-L20, 161-L15, 164-I21, 176-P12, 192-P06 |

Clones in bold were used for genomic sequencing.

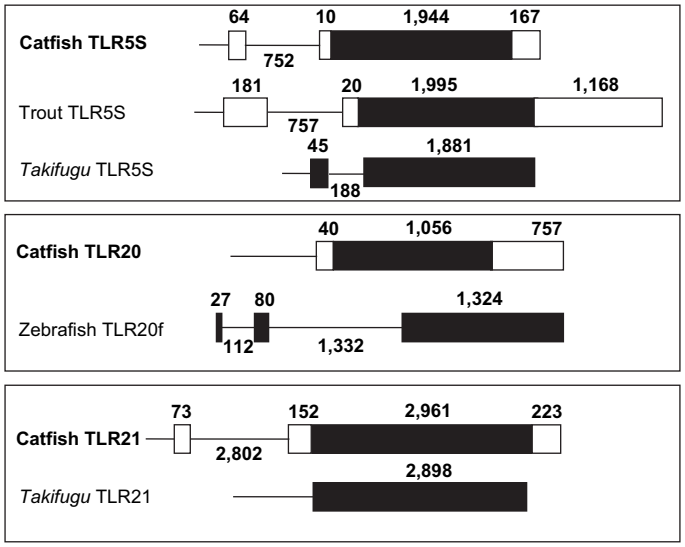



Fig. 1. Schematic representation of TLR5S, TLR20, and TLR21 gene structure and organization from catfish and similar sequences in zebrafish, trout and *Takifugu*. Exons are represented by boxes; solid boxes represent coding regions of the gene; open boxes represent 5′-UTR and 3′-UTR, when available. Exon sizes in base pairs are shown on the top of the boxes. Introns are represented by a line and the size (bp) is shown below the line.

et al. [23]. Interestingly, the LRR C-terminal domain (LRR-CT) in catfish differs from the three other fish soluble TLR5 genes in that all its four cysteine residues are conserved with the mammalian consensus, rather than only two in other fish species. Bell et al. [44] found that all four cysteines were needed in mammalian TLR5 to hold the extracellular domain close to the membrane. Catfish TLR20 by manual and computer-based inspection (SMART) was predicted to have only three LRR repeats upstream of LRR-CT, the transmembrane domain, and a well-conserved TIR domain. TLR genes described to date from mammals contain 19–25 LRR repeats [45]. In contrast to TLR5S and TLR20, the domain structure of catfish TLR21 is more typical of TLRs, with 23 LRR repeats, a LRR-CT domain, the transmembrane domain, and a TIR domain. These features are also conserved in TLR21 sequences from *Takifugu* and one predicted from the genome sequence of *Danio rerio*.

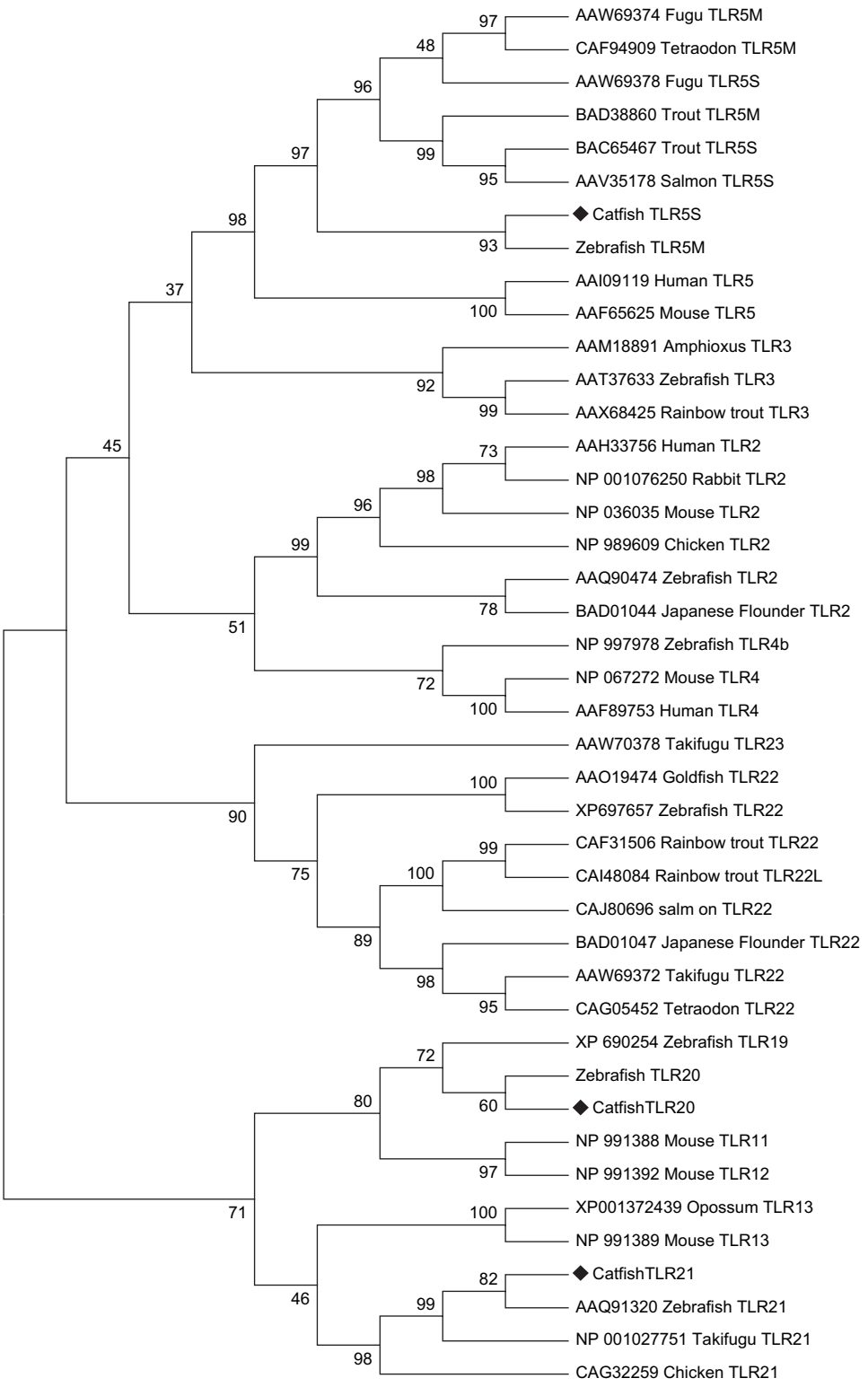
### 3.3. Phylogenetic analysis

In order to analyze the three catfish TLR genes in the larger context of vertebrate TLRs, we conducted phylogenetic analysis using the neighbour-joining method. Catfish TLR5S was grouped into the family of TLR5 genes within the teleost subclade that included both TLR5S and TLR5M genes (Fig. 2). The sequence was most similar to a zebrafish TLR5M predicted from the genome. Interestingly, a TLR5S gene is not presently apparent in the zebrafish genome, and all sequenced zebrafish ESTs code for a TIR domain. The full-length TLR5S sequence from catfish was also compared to a previously published partial sequence from catfish (AAU95563; [15]). The two sequences shared great level of sequence identities with just a few single nucleotide polymorphisms. It is likely that these represented the same gene, but different allelic variations.

The catfish TLR20 and TLR21 fell into a major clade containing a group of similar non-mammalian TLR19–21 sequences and the murine TLR11–13 which may be related to this group [8]. The catfish TLR20 was most similar to the zebrafish TLR20, and the annotation of zebrafish was followed, but they are both highly related to murine TLR11 and TLR12. Catfish TLR21 was included in the subclade that included only TLR21 genes from zebrafish, *Takifugu* and chicken (Fig. 2).

### 3.4. Analysis of gene locus number of TLR5S, TLR20, and TLR21

In order to determine the locus number of the three TLR genes in the channel catfish genome, genomic DNA isolated from three individuals were digested with EcoRI, HindIII, and PstI and subjected to Southern blot analysis. As


AAW69374 Fugu TLR5M
CAF94909 Tetraodon TLR5M
AAW69378 Fugu TLR5S
BAD38860 Trout TLR5M
BAC65467 Trout TLR5S
AAV35178 Salmon TLR5S
Catfish TLR5S
Zebrafish TLR5M
AAI09119 Human TLR5
AAF65625 Mouse TLR5
AAM18891 Amphioxus TLR3
AAT37633 Zebrafish TLR3
AAX68425 Rainbow trout TLR3
AAH33756 Human TLR2
NP 001076250 Rabbit TLR2
NP 036035 Mouse TLR2
NP 989609 Chicken TLR2
AAQ90474 Zebrafish TLR2
BAD01044 Japanese Flounder TLR2
NP 997978 Zebrafish TLR4b
NP 067272 Mouse TLR4
AAF89753 Human TLR4
AAW70378 Takifugu TLR23
AAO19474 Goldfish TLR22
XP697657 Zebrafish TLR22
CAF31506 Rainbow trout TLR22
CAI48084 Rainbow trout TLR22L
CAJ80696 salm on TLR22
BAD01047 Japanese Flounder TLR22
AAW69372 Takifugu TLR22
CAG05452 Tetraodon TLR22
XP 690254 Zebrafish TLR19
Zebrafish TLR20
CatfishTLR20
NP 991388 Mouse TLR11
NP 991392 Mouse TLR12
XP001372439 Opossum TLR13
NP 991389 Mouse TLR13
CatfishTLR21
AAQ91320 Zebrafish TLR21
NP 001027751 Takifugu TLR21
CAG32259 Chicken TLR21

shown in Fig. 3, multiple bands were observed for TLR5. Checking of the genomic sequences of TLR5 gene revealed the presence of restriction sites within the probe area, but the internal restriction sites were not enough to explain all the bands. For instance, two EcoRI sites exist in the TLR5 gene that should result in three positive bands, but five bands were present. Careful examination of the gel suggested that a subset of the bands exhibit stronger intensities while the remaining bands were relatively weak (Fig. 3A), suggesting the presence of related sequences cross-hybridizing with the probe. It is possible that a related gene of TLR5S (e.g., TLR5M was identified in rainbow trout in addition to TLR5S) was involved in the hybridization patterns. In order to determine the copy number of the TLR5S gene, we took a second approach using BAC-based fingerprinting followed by Southern analysis. BAC DNA from the positive BAC clones (Table 2) was isolated and subjected to Southern blot analysis after digestion with EcoRI and/or HindIII. As we previously reported, the BAC-based Southern blot is highly sensitive for the determination of multi-loci located in different chromosomal regions as the number of loci is reflected as different restriction patterns after Southern blot analysis using the BAC DNA positive for the gene of interest. Each unique restriction pattern suggests the presence of a distinct locus in the genome [46,47]. The BAC-based Southern blot analysis indicated that TLR5S was present as a single copy gene as all nine BAC clones shared the same fingerprinting patterns after Southern blot hybridization (Fig. 3B). Despite the observation of slightly different sub-patterns, all BAC clones generated restriction digestion patterns belonging to the ''total pattern'' of the restriction enzyme. For instance, BAC DNA in lane 9 produced four EcoRI bands that are a part of the total pattern of five bands (e.g., lane 8). This result indicated the presence of only one locus of the TLR5S gene in the catfish genome. The partial restriction pattern for some BAC clones indicated truncation of part of the locus containing TLR5S gene(s) at the end of the BAC inserts [46,47].

The catfish TLR20 and TLR21 were both single-copy genes. Southern blot analysis using three individuals and three restriction enzymes all produced a single band (Figs. 4 and 5) except the first fish using EcoRI, in which two bands were observed for TLR20. We believe that this was caused by allelic variations as the other two fish had only one band. The same fish also exhibited some polymorphism in Southern blot analysis using HindIII with the TLR5S gene. These results were confirmed by BAC-based Southern blot analysis (data not shown).

### 3.5. Expression of catfish TLR5S, TLR20, and TLR21 in tissues

The mRNA expression patterns of the three catfish TLR genes were measured in eleven tissues by RT–PCR (Fig. 6). Expression of catfish TLR5S was detected predominantly in the head kidney, spleen, skin, liver, ovary, brain, and gill. This result overlaps with a similar tissue expression pattern reported previously for a partial TLR5 transcript from catfish [15]. A similar pattern of expression was also found in *Takifugu* tissues [10] with the exception that strong expression was detected in the digestive organs of that species. In contrast, rainbow trout TLR5S was expressed only in liver [19]. Catfish TLR20 was expressed predominantly in head kidney, spleen, intestine, stomach, liver, trunk kidney, brain, and gill. Expression of zebrafish TLR20 or TLR21 in healthy tissues was not reported. Catfish TLR21 was expressed in all tested tissues except muscle. Similarly, *Takifugu* TLR21 was expressed in liver, spleen, kidney, skin, heart, and gill [10], with very low expression in muscle.

## 4. Discussion

In this work, we have identified, cloned and characterized three TLR members of catfish not present in mammalian species, TLR5S, TLR20, and TLR21. Their gene copy numbers were determined, and they have been mapped to BACs allowing comparative mapping analysis using the BAC library resources [48] and the BAC-based contigs [49]. The discovery that a family of Toll-like receptors mediated a general mechanism of pathogen recognition for the vertebrate innate immune system represented a tremendous breakthrough in the understanding of immunity. It

Fig. 2. Phylogenetic analysis of catfish TLR5S, TLR20 and TLR21 with related vertebrate TLR sequences. The phylogenetic tree was drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbour-joining method within the MEGA 3.1 package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbour joining trees was evaluated by 10,000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. GenBank accession numbers are provided on the tree where available. The zebrafish TLR20 and zebrafish TLR21 sequences were extracted from its draft genome sequence. Multiple, highly similar zebrafish TLR20 sequences exist within its genome, of which a representative sequence was used for the phylogenetic analysis. Catfish TLR5S, TLR20 and TLR21 are marked with black diamonds.

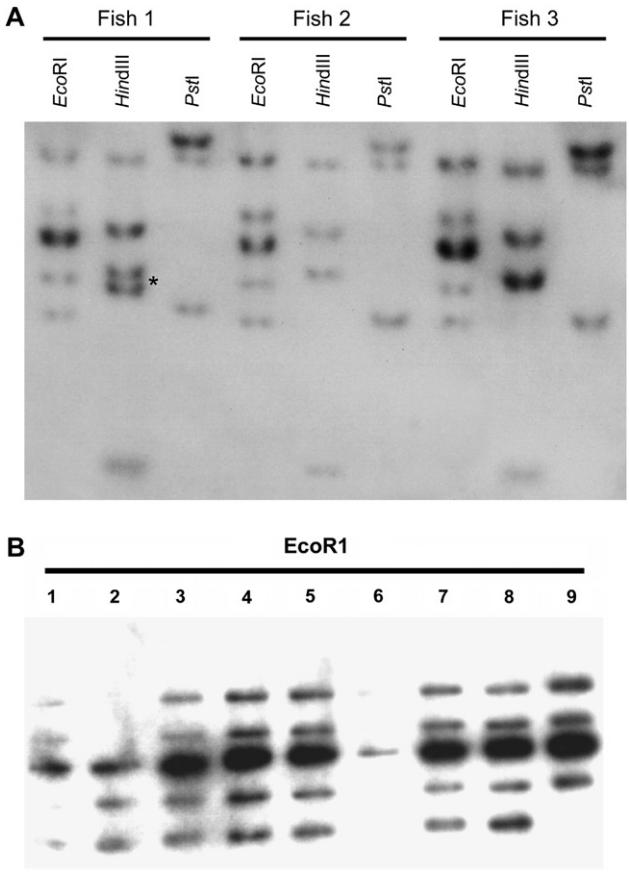



Fig. 3. Determination of genomic copy numbers of the catfish TLR5S gene. (A) Southern blot analysis using genomic DNA of three catfish individuals as labelled. Genomic DNA was digested with EcoRI, HindIII or PstI, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the TLR5S cDNA probes (see Materials and methods for details). Note that some bands are stronger than others. (B) Southern analysis of BAC DNA fingerprints. DNA of TLR5S-positive BAC clones were digested with EcoRI, transferred to Nylon membrane, and hybridized to TLR5S cDNA probe. Note the similar fingerprint patterns from all BAC clones.

has served to connect many of the previously disjointed elements of innate immune system into coherent pathways and helped to explain the complex outcomes of the inflammatory response [50]. In catfish, a model species for immune studies in lower vertebrates, we have systematically characterized many innate immune components, including chemokines [29,35,51–56], cytokines [47], and antimicrobial peptides [36,46,57–59]. A better understanding of the

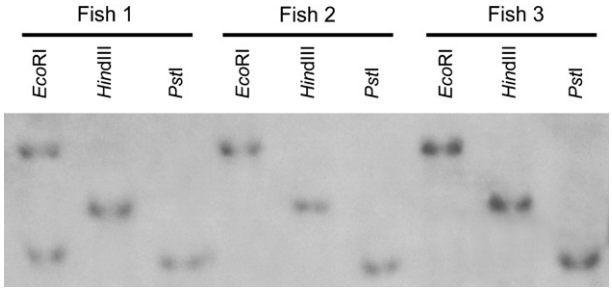



Fig. 4. Determination of genomic copy numbers of the catfish TLR20 gene. Southern blot analysis of the catfish TLR20 was conducted by using genomic DNA of three catfish individuals as labelled. Genomic DNA was digested with EcoRI, HindIII or PstI, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the TLR20 cDNA probes. The blot was processed as described in Section 2, and exposed to a piece of X-ray film for autoradiography.

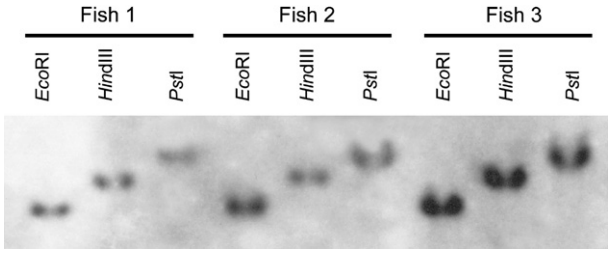



Fig. 5. Determination of genomic copy numbers of the catfish TLR21 gene. Southern blot analysis of the catfish TLR21 was conducted by using genomic DNA of three catfish individuals as labelled. Genomic DNA was digested with EcoRI, HindIII or PstI, electrophoresed through a 0.8% agarose gel, transferred to nylon membrane, and hybridized to the TLR21 cDNA probes. The blot was processed as described in Section 2, and exposed to a piece of X-ray film for autoradigraphy.

status and microbial ligand specificities of TLRs in teleost fish is necessary in order to progress from the study of individual immune genes to the study of the immune pathways relevant to specific diseases. Such information will also be valuable in advancing our overall knowledge of innate immunity in fish [60] and in the development of targeted immunostimulants [61]. Towards this end, we describe here three TLRs from catfish, TLR5S, TLR20, and TLR21, which are not found in the mammalian TLR repertoire. Complete cDNA sequences of TLR20 and TLR21 have not been published previously and little is known about their expression patterns. While the majority of mammalian TLRs have clear orthologues in teleost fish, these three catfish TLRs represent potentially divergent TLRs that may mediate novel pathogen recognition mechanisms.

Catfish TLR5S, TLR20, and TLR21 genes were all found to be coded by a continuous open reading frame uninterrupted by introns. This trait is shared with mammalian TLRs 1, 2, 4D, 5, 6, and 10, as well as by murine TLR11–13 [9]. Although intronless LRR-containing genes were found to be numerous in the sea urchin *Strongylocentrotus purpuratus* genome, they are fairly rare in vertebrate genomes. Intronless genes have been suggested as ideal gene templates for the rapid generation of diverse germ-line encoded receptors [9]. The three catfish TLRs we have identified fit well with this theory in consideration of the fact that they are not present in mammals.

TLR5 is known to recognize bacterial flagellin in mammals. Similar ligand specificity is found in rainbow trout, albeit with the added complexity of distinct membrane and soluble TLR5 forms. Bacterial flagellin interacts first with trout TLR5M, facilitating the expression of TLR5S which then serves to amplify TLR5M cellular responses by positive feedback [19]. Although TLR5M has not been isolated from catfish yet, our Southern blot analysis using genomic DNA suggested that another TLR5-related gene exists in the catfish genome. This assessment is made with two lines of evidence: (1) the restriction sites within the TLR5S could not account for all the detected bands, suggesting some

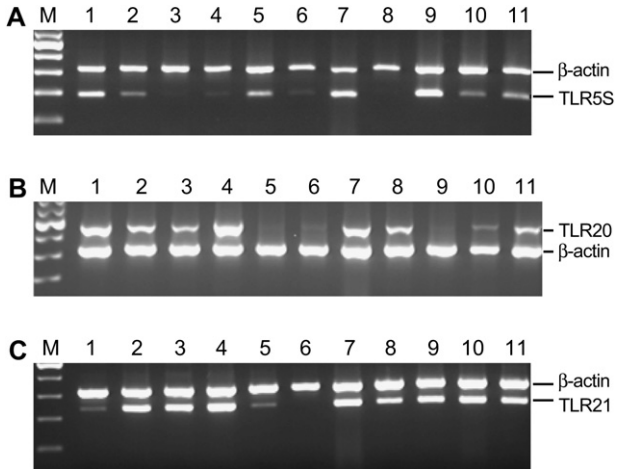



Fig. 6. RT–PCR analysis of catfish TLR5S (A), TLR20 (B) and TLR21 (C) expression in various tissues. RT–PCR products were analyzed on an agarose gel. M, 100 bp molecular weight; 1, head kidney; 2, spleen; 3, intestine; 4, stomach; 5, skin; 6, muscle; 7, liver; 8, trunk kidney; 9, ovary; 10, brain; 11, gill. The positions of the RT–PCR amplified bands of beta-actin and TLR5S, TLR20 and TLR21 are indicated on the right margin.

bands were from another related gene; and (2) some of the bands were weaker in intensities, suggesting lower homologies to the TLR5S probes. In addition, using the short overgo probes apparently did not identify the TLR5M gene from the BAC library, suggesting that the oligo probe was specific allowing only TLR5S to be hybridized, while the cDNA probe may have hybridized to both genes. Concrete evidence awaits the characterization of the TLR5M or its related gene from catfish in the future.

The identification in catfish of a short, divergent Toll-like receptor with few leucine-rich repeat regions caused us to investigate a group of orthologous TLR20 genes found in zebrafish. Zebrafish has a group of poorly-defined TLR20-like sequences coded in its genome. Some of these genes were previously described and annotated as TLR20a-f [12] or TLR21.3-.5 [11] and partial sequences containing the conserved TIR domain were submitted to GenBank. In addition, several TLR20 proteins have been predicted from the zebrafish genome (XP_690197, XP_690267, XP_701273, XP_700062). Unfortunately, no TLR20 zebrafish ESTs exist to support gene predictions, and some of these predictions appear incorrect or incomplete in light of our current knowledge. Our examination of the zebrafish genome revealed that highly-related TLR20-like sequences were present on chromosomes 5, 9, and 20 as well as on two unassembled contigs (WGA18205 and WGA12079). Two tandem TLR20 genes have been annotated on chromosome 5 (XP_690197 and XP_690267) with lengths of 266 and 393 amino acids, respectively. However, by inspection of the neighbouring genome sequence, sequence translation, and BLAST searches it was apparent that two much larger genes are likely encoded in this region. Two putative TLR20 sequences of 950 and 951 amino acids were predicted that contain long LRR stretches. These predicted TLR20 genes are comparable in length to TLR21 sequences from zebrafish and TLR11–13 from mouse. These two zebrafish TLR20 sequences share 76% amino acid identities. Similar pairs of long TLR20 sequences appear to exist on chromosome 9 (WGA729) and chromosome 20 (WGA1515). However, in these cases, sequence prediction is complicated by stretches of ''N's'', possible introns, and/or pseudogene remnants. A shorter TLR20 form, similar to the one described here from catfish, does appear to be encoded by the zebrafish genome. An unassembled contig (DrUn_WGA18205) had been previously predicted to code for a TLR20 sequence of 326 amino acids (XP_701273). Aligning the translated genome sequence from this region with our 351 amino acid TLR21 revealed that the sequences shared high levels of similarity upstream of the previously predicted start codon. Using a farther upstream start codon for the zebrafish TLR20 sequence yields a 359 amino acid that shares 80–90% identities with the longer zebrafish TLR20 forms. To summarize, TLR20 appears to exist as a highly duplicated TLR subfamily in zebrafish with long (>900 amino acids) and short members (~350 amino acids). We have identified a TLR20 gene in catfish with high sequence and structural similarity to the short zebrafish TLR20. TLR20-like sequences have not been found in the genomes of other fish species. However, TLR20 appears to be more closely related to murine TLR11 and TLR12 than to the TLR21–23 members which are well-represented in several fish species (Fig. 2). TLR11 was recently reported to recognize a protozoan profilin-like protein [7,27]. Future studies investigating the ligand specificity of the TLR20 genes and the roles of the long and short TLR20 forms should provide a better understanding of pathogen recognition mechanisms across vertebrate species.

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