Fish and Shellfish Immunology 94 (2019) 752–760

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Duplication of toll-like receptor 22 in teleost fishes

Delin Qi[a,*], Yan Chao[b], Cunfang Zhang[a], Zhenji Wang[c], Wei Wang[a], Qichang Chen[a], Ziqin Zheng[a,b], Zhao Zhang[a,b]

[a] State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, 810016, China
[b] Animal Science Department of Agriculture and Animal Husbandry College, Qinghai University, Xining, 810016, China
[c] Fishery Environmental Monitoring Station of Qinghai Province, Xining, 810012, China

ARTICLE INFO

Keywords:
Toll-like receptor 22
Duplication
Expression pattern
Gymnocypris eckloni
Teleost fishes

ABSTRACT

The TLRs of teleost fishes have distinct features and are highly diverse, but the duplication characteristics and expression patterns of the *tlr22* gene remain unclear. Here, we identified paralogous *tlr22* genes in 13 teleost fishes by screening available fish genomic resources and using molecular cloning. We then conducted comprehensive bioinformatics analyses and investigated spatiotemporal differences in the expression patterns of the *tlr22* genes in *G. eckloni*. The results indicated that more than three paralogous *tlr22* genes were possessed by some teleost fishes. Of these, *tlr22c* is specific to some subfamilies of the Cyprinidae (e.g., Barbinae, Cyprininae, Schizothoracinae, and Leuciscinae). Phylogenetic and syntenic analyses showed that the paralogous *tlr22* genes originated from two single-gene duplication events. Molecular clock calculations dated the two gene duplication events at 49.5 and 39.3 MYA, which is before the common carp-specific genome duplication event and well after the fish-specific genome duplication. Gene duplication of *tlr22* was followed by gene loss or pseudogene events in certain lineages. Spatiotemporal expression differences between the three duplicated *tlr22* genes from *G. eckloni* suggested that these genes diverged functionally after gene duplication.

1. Introduction

Proteins in the Toll-like receptor (TLR) family are essential, well-characterized pattern recognition receptors (PRRs) [1–3]. TLRs recognize a wide variety of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, bacterial DNA, lipoproteins, lipoteichoic acid, peptidoglycan, mannose, and double stranded RNA (dsRNA) [4–6]. TLRs are type I transmembrane proteins with extracellular leucine-rich repeat (LRR) motifs, a transmembrane domain, and an intracellular Toll/interleukin-1 (TIR) domain. TLRs recognize specific PAMP ligands via the interactions of these ligands with the LRRs; these interactions trigger the activation of signaling cascades [7,8] and mediate innate immune responses for host defense.

Although generally conserved in structure, TLRs exhibit high levels of inter- and intraspecific genetic variation in vertebrates [1,9–14]. To date, 10 TLRs have been identified and functionally characterized in humans and birds [1,3,11]. Teleost fishes represent approximately half of all vertebrate species, and hence form the largest and most diverse group of vertebrates. In addition, teleost fishes also have distinct features and highly diverse TLRs. Recent genomic studies and the availability of draft teleost genome sequences have greatly facilitated the identification of TLRs in teleost fishes. For example, phylogenetic analyses of complete genomes were used to identify 11 distinct TLR types in the pufferfish (*Takifugu rubripes*) [15], and 14 distinct TLR types in the zebrafish (*Danio rerio*) [16,17] and common carp (*Cyprinus carpio*) [18]. Interestingly, of the 14 fish TLRs, 3 were duplicated in the zebrafish (TLR4b, 5, and 8), and 9 were duplicated in common carp (TLR2, 3, 4, 7, 8, 18, 21, 22, and 25).

TLR22 belongs to fish-specific family of TLRs, which includes TLR19, 20, 21, 22, and 23 [7,19]. Although fish-specific *tlr22* genes have been identified in various teleost fishes [7,13,18,20,21], the functions of these genes remain poorly understood. Previous studies have proposed that TLR22 recognizes long dsRNAs on the cell surface, and that, upon activation with polyI:C, TLR22 recruits TICAM-1 and induces IFN expression in fish cells [22] as part of the antiviral immune response [20,23,24]. Duplicated *tlr22* genes were identified in Atlantic salmon (*Salmo salar*) [22], rainbow trout (*Oncorhynchus mykiss*) [21], and *Gymnocypris eckloni* [14]. However, *tlr22-3* or *tlr22c* are unique to the common carp (*Cyprinus carpio*) [18]. The newly available open-access genomic resources for fish enable us to screen the TLR gene family at genomic scale. In the present study, we identified *tlr22* paralogs by screening available fish genomic resources and using molecular

* Corresponding author.
E-mail address: delinqi@126.com (D. Qi).

https://doi.org/10.1016/j.fsi.2019.09.067
Received 10 July 2019; Received in revised form 23 September 2019; Accepted 29 September 2019
Available online 30 September 2019

cloning. We then conducted phylogenetic and syntenic analyses. Finally, we measured the expression levels of *tlr22* genes in *G. eckloni* embryos at representative stages during development and in the major tissues of adult *G. eckloni*. The aims of this study were to characterize the variation and origin of *tlr22* paralogs among fish lineages, and to determine the spatiotemporal divergence in *tlr22* gene expression among fish species.

2. Materials and methods

2.1. Identification of tlr22 genes in fish genomic resources

Previously published *tlr22* gene sequences from common carp (*tlr22a*, *tlr22b*, and t*lr22c*; Genbank accession nos. LC150775, LC150776, and LC150776, respectively) were used as BLAST queries (E-value cutoff: 1e-10) against the NCBI genomic databases of *Sinocyclocheilus grahami* (release 100), *Sinocyclocheilus anshuiensis* (release 100), *Pygocentrus nattereri* (release 100), *Astyanax mexicanus* (release 102), *Ictalurus punctatus* (release 100), *Salmo salar* (release 100), *Oncorhynchus mykiss* (release 100), *Carassius auratus* (release 100) and *Oxygymnocypris stewartii* (GCA_003573665.1). To ensure access to all the paralogous *tlr22* genes, we used the same queries to search against the (draft) genome assembly of each fish (ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/vertebrate_other/). The nucleotide sequences were extracted from the genome databases, assembled, and translated using Lasergene 7.0 [25].

2.2. Animals

Specimens of *G. eckloni* were obtained from the Native Fish Artificial Proliferation and Release Station of Xunhua in Qinghai Province, China. Grass carp (*Ctenopharyngodon idellus*) were purchased from a local market in Xining, Qinghai Province, China. All of the fish were transported to our laboratory and kept in aerated buckets at a temperature of 10–13 °C. Selected tissues were removed from each fish, shock-frozen in liquid nitrogen, and stored at −80 °C. Embryos of *G. eckloni* were collected at 2, 4, 6, 8, 10, 12, 18, 24, 48, 72, 96, 120, 144, 168, 192, and 216 h postfertilization (hpf) at 11 °C, and flash frozen in liquid nitrogen. All of the research involving animals in this study followed the guidelines of, and was conducted under the approval of, the Animal Care and Use Committee, Qinghai University, China.

2.3. RNA extraction and cloning of tlr22 genes from G. eckloni and C. idellus

Total RNA was isolated from each tissue and embryo sample using an RNAprep Pure Tissue Kit (Tiangen Biotech Co., Ltd., China), and was treated with RNase-free DNase I (Takara, Japan). cDNA was obtained from the total RNA using an oligo (dT) primer RNA PCR Kit (AMV) Ver. 3.0 (Takara, Japan). To clone the complete coding sequence of each *tlr22* gene in *G. eckloni* and *C. idellus*, primers were designed using Primer 5.0 (www.premierbiosoft.com) based on previously published*tlr22* gene sequences from common carp (*tlr22a*, *tlr22b*, and *tlr22c*; Genbank accession nos. LC150775, LC150776, and LC150776, respectively), and on sequences from *Carassius auratus* obtained in this study (Table 1). PCR cycling conditions were as follows: initial denaturation at 94 °C for 2 min; followed by 35 cycles of 98 °C for 10 s, 52–58 °C for 30 s, and 72 °C for 1 min; and, finally, extension at 72 °C for 5 min. The PCR products were purified on 1.0% agarose gels using agarose gel DNA Purification Kits Ver. 2.0 (Takara, Japan), subcloned into vector pMD19-T (Takara, Japan), and then sequenced (both strands) by a commercial sequencing service (Sangon Biotech Co., Ltd., China). The *tlr22* gene sequences obtained in this study have been deposited in Genbank (Accession nos. MN106008–MN106013).

Table 1
Primers used in cloning and qRT-PCR for paralogous *tlr22* genes of *G. eckloni* and *C. idellus*.

Primer	5′-3′ Sequence	Application
TLR22a-1-408F	ATGGGAACACTGAAACAAATC	Cloning
TLR22a-1-408R	ATCCAGACGTAGCACCAGCAG	Cloning
TLR22a-305-1173F	GTGCCCTTGACAATCTTTC	Cloning
TLR22a-305-1173R	GTCGATGCTGTTTCTACTGA	Cloning
TLR22a-953-1805F	CTGCTAATGTGACTGCGTAT	Cloning
TLR22a-953-1805R	CGGTGATGCTGCTTTACT	Cloning
TLR22a-1657-2838F	AGAGTATTGGCCTTACACAGT	Cloning
TLR22a-1657-2838R	TCATGGAATTAAAAAATCTGAT	Cloning
TLR22b-1-372F	ATGTCTGTGAGAGAACATATTT	Cloning
TLR22b-1-372R	CCAGATTTAACTCCTGCAAAGCC	Cloning
TLR22b-317-1762F	GGGAAGTGGAGGAAGGAGC	Cloning
TLR22b-317-1762R	GGAGTATGAGTAAATGTATCTGGGTGT	Cloning
TLR22b-1574-2418F	GGACAATCTTGCTCAACCACCCT	Cloning
TLR22b-1574-2418R	TTCCAGCCCTGCTCGCCTTC	Cloning
TLR22b-2227-2805F	CTCTTTCACTTCCTCAAATGGCA	Cloning
TLR22b-2227-2805R	TTAGTTGACCCCTAAATCCCA	Cloning
TLR22c-1-739F	ATGAGGGAAAGTTCTGCATTTTTC	Cloning
TLR22c-1-739R	TCAGGAACGCCTTGTCTAGAAT	Cloning
TLR22c-593-1771F	ACATCCCATCGGCATTCTTTG	Cloning
TLR22c-593-1771R	TCTTGACATTAAGGTTCCCAGCA	Cloning
TLR22c-1628-2474F	AAAGTCCACCGTTCATCCTGTT	Cloning
TLR22c-1628-2474R	CTCAAGCCGTGTTGACCCTC	Cloning
TLR22c-2374-2869F	CTGGATTTCCAATATGACGCCT	Cloning
TLR22c-2374-2869R	TTAAATATGATCTTCGTCTTTC	Cloning
TLR22a real F	AGCCTTCCTTTACGACAGCA	qRT-PCR
TLR22a real R	CAGTCTCCAGCCCTGTTCTC	qRT-PCR
TLR22b real F	CTCCCAAACCTGAAAACACTTC	qRT-PCR
TLR22b real R	TTAAAGGGTGGTTGAGCAAGAT	qRT-PCR
TLR22c real F	CGGATCGTTGAATGGGATT	qRT-PCR
TLR22c real R	AAGTGTCGCAAGTGACTGGTT	qRT-PCR
β-actin real F	GCCAACAGGGAAAAGATGAC	qRT-PCR
β-actin real R	TTGCCAATGGTGATGACCTG	qRT-PCR

2.4. Sequence analysis

The complete coding sequences of the *tlr22* genes from *G. eckloni* and grass carp were confirmed by BLAST [26], and assembled with DNAMAN Version 6.0. (http://www.lynnon.com). The putative amino acid sequences were predicted using Lasergene [25]. The biochemical properties of the mature peptides were evaluated using ProtParam (http://ca.expasy.org/tools/protparam.html). The characteristic functional domains of the TLRs were identified using the SMART server (http://smart.embl-heidelberg.de/).

2.5. Phylogenetic and syntenic analyses

The deduced amino acid sequences from *G. eckloni* and the grass carp were aligned with putative homologs from other fish genomes using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). We then constructed a maximum-likelihood (ML) phylogeny of TLR22 paralogs based on this alignment using MEGA X [27], using bootstrap (BS) re-sampling (100 replicates) to assess support for individual nodes. The best-fitting models of sequence evolution were determined with 'Find Best Protein Models' supplemented in MEGA X. The phylogenetic analysis included the protein sequences of all the zebrafish TLRs, the TLR 25 sequences from the common carp and the previously-published TLR22 protein sequences (TLR22 and TLR22L from *O. mykiss* [21]; TLR22a and TLR22b from *S. salar* [22]). Considering the evolutionary conservation of TIR domain, we further constructed a ML phylogeny of TLR22 paralogs based on TIR domains of all the above-mentioned TLRs.

To determine the divergence time of the duplicated *tlr22* genes, we calculated the synonymous site divergence value (*Ks*) for paralogous pairs within species using the CodeML module (run mode −2) in the PAML package [28]. Using a clock-like evolutionary rate (3.51×10^{-9} substitutions per synonymous site per year [29]), the time of divergence was calculated using the formula T = *Ks*/2r, where *Ks* was the number

of substitutions per synonymous site per year and r was the substitution rate.

In the syntenic analysis, the orientations and chromosomal positions of the *tlr22* homologs and their adjacent genes were determined using Genomicus (http://genomicus.biologie.ens.fr/genomicus-95.01/cgi-bin/search.pl) and NCBI BLAST against the fish genomic resources; the *tlr*22 paralogs from *G. eckloni* were used as query sequences.

2.6. Expression of tlr22 gene paralogs in G. eckloni

Total RNA was extracted from each tissue and pooled embryo sample (each sample include three embryos) as described above, and converted into cDNA using a FastQuant RT Kit and Oligo (dT)18 primers. Quantitative real-time RT-PCR (qRT-PCR) experiments were performed in an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA) using SuperReal PreMix Plus (SYBR Green) (Tiangen Biotech Co., Ltd.). Primers for TLRs and *β-actin* were designed using an intron-spanning method (Table 1). Reactions were run in triplicate for each sample, using a reaction volume of 20.0 μl, containing 1.0 μl of diluted cDNA, 10.0 μl 2 × SuperReal PreMix Plus, 1.0 μl of 10 μmol/l each primer, and 7.0 μl ddH_2O. Quantitative analyses for embryo samples were performed using the Pfaffl method [30]. Significant differences between *tlr22* genes within the same tissue were analyzed by Student's t-test.

3. Results

3.1. Identification of tlr22 paralogs in teleost fishes

BLAST searches against the genomic databases indicated that teleost fishes possessed 1–5 *tlr22* paralogs. Like zebrafish, *S. salar*, *O. mykiss,* and *I. punctatus* each harbored a single copy of *tlr22*, while both *P. nattereri* and *A. mexicanus* possessed two *tlr22* paralogs (*tlr22a* and *tlr22b*). The remaining fishes in the Barbinae (*S. graham* and *S. anshuiensis*), Cyprininae (*C. auratus*), and Schizothoracinae (*O. stewartii*) possess 3–5 *tlr22* paralogs. For example, *S. graham* and *O. stewartii* each possessed three *tlr22* paralogs (*tlr22a*, *tlr22b*, and *tlr22c*); *C. auratus* had an additional *tlr22c* gene, thus which possessed four *tlr22* paralogs (*tlr22a*, *tlr22b*, *tlr22c1* and *tlr22c2*). It is worth noting that *S. anshuiensis* possessed five *tlr22* paralogs (*tlr22a1*, *tlr22a2*, *tlr22b*, *tlr22c1* and *tlr22c2*). The *tlr22a2* from *S. anshuiensis*, however, was much shorter than *tlr22a1*, and disrupted by stop codons and frame shifts. To investigate whether *tlr22* gene duplication has occurred in other teleost fishes, we designed gene-specific primers based on the complete *tlr22* gene sequences genes from the cyprinid fishes. We used these primers to clone the corresponding genes from *G. eckloni* (Schizothoracinae) and *C. idellus* (Leuciscinae). The results revealed that each of these species possessed three duplicated *tlr22* genes, although only partial sequence of *tlr22c* was obtained from *C. idellus* (Table 2). Details of the genomic sequences and coding sequences of the fishes examined in this study were summarized in Table 2.

For the *tlr22* genes with complete coding sequence (CDS), the nucleotide sequences ranged from 2784 bp (*tlr22b* in *C. idellus*) to 2910 bp (*tlr22* in *O. mykiss*), encoding proteins 927–969 amino acids in length. The functional domain prediction showed that TLR22a and TLR22c possessed a signal peptide in the N-terminal, but that TLR22b did not. In addition, TLR22a and TLR22c each had a transmembrane domain close to the TIR domain, but TLR22b had a transmembrane domain close to the N-terminal. Finally, the number of the extracellular LRR motifs differs among TLR22a, TLR22b, and TLR22c (Fig. 1).

3.2. Phylogenetic and syntenic analyses of tlr22 genes in teleost fishes

The best-fit substitution model of molecular evolution for the entire data set obtained from MEGA X based on the Bayesian information criterion (BIC) was the LG + G + I model, which had the lowest score: 112212.96. This model was used for the construction of the ML phylogeny in MEGA X. An ML phylogeny ($lnL = -55563.51$) based on all of the TLR-family amino acid sequences from zebrafish, as well as the TLR22 paralogs obtained from other teleost fishes, recovered all of the TLR22s in a well-supported monophyletic clade, divided into three subclades corresponding to TLR22a, TLR22b, and TLR22c (Fig. 2). The TLR22a subclade included TLR22 from *D. rerio,* TLR22 from *I. punctatus*, and TLR22a homologs from other teleost fishes; the TLR22b subclade included TLR22 from *S. salar,* TLR22 from *O. mykiss*, and TLR22b homologs from other teleost fishes; and the TLR22c group included all of the TLR22c homologs from seven species of Barbinae, Cyprininae, Schizothoracinae, and Leuciscinae. To further investigate the phylogenetic relationships with the TLR22 clade, we constructed a phylogenetic tree of fish TLRs using previously-published sequences (TLR22 and TLR22L from *O. mykiss* [21]; TLR22a and TLR22b from *S. salar* [22]) and the sequences of the TLR22 paralogs obtained in this study (Fig. 3). We found that all of the TLR22 sequences from *O. mykiss* and *S. salar* formed a monophyletic clade with the TLR22b sequences from other teleost fishes, suggesting that the TLR22s from *O. mykiss* and *S. salar* were teleost TLR22b homologs. The phylogenetic analysis based on TIR domains recovered largely congruent tree topologies as described above, although the relationship between TLR22s from three species (*A. mexicanus*, *P. nattereri* and *I. punctatus*) and TLR22s from other species has not been properly solved (Fig. S1).

In five teleost fishes, at least three genes upstream of *tlr22a* and one gene downstream of *tlr22a*, as well as *tlr22* of *D. rerio,* exhibited conserved synteny (Fig. 3A). We conducted syntenic analyses of the *tlr22b* gene only in two species due to the fragmentary nature of the genome sequences. The results showed that at least 14 genes adjacent to *tlr22b* remained in the same order in *P. nattereri* and *A. mexicanus* (Fig. 3B). In addition, five genes downstream of *tlr22c* and eight genes upstream of *tlr22c* exhibited conserved synteny in *C. auratus* and *S. anshuiensis*. Although the *O. stewartii* genome was fragmented, one gene upstream of *tlr22c* was in the same order between *C. auratus* and *S. anshuiensis* (Fig. 3C). It is worth noting that no conserved synteny of the neighboring gene loci was found across the *tlr22a*, *tlr22b*, and *tlr22c* genes. Interestingly, four genes adjacent to *tlr22a1* and *tlr22a2*, and ten genes adjacent to *tlr22c1* and *tlr22c2* exhibited conserved synteny of *S. anshuiensis*.

3.3. Expression profiles of tlr22 genes in G. eckloni

Our qRT-PCR analyses indicated that *tlr22a*, *tlr22b,* and *tlr22c* had different expression patterns during the major stages of *G. eckloni* embryonic development, as well as in the muscle and primary immune tissues (liver, spleen, gill, and kidney) of adult *G. eckloni* (three years old). The mRNA levels of *tlr22b* and *tlr22c* genes were slightly fluctuated from 2 hpf to 216 hpf, which corresponded to the hatching stage. The gene expression of *tlr22a* increased gradually during the cleavage stage (6–12 hpf), then decreased slightly in the early blastula stage (18–24 hpf). From the later blastula stage (24 hpf) tothe gastrula stage (24–48 hpf), the neurula stage (72–96 hpf), the organogenetic stage (96–144 hpf), the muscular contraction stage (144–168 hpf), the heartbeat stage (168–192 hpf), and the hatching stage (192–216 hpf), the gene expression of *tlr22a* re-increased gradually and reached a peak at 216 hpf (Fig. 4A).

We investigated the *tlr22s* relative mRNA expression levels in the major tissues of adult *G. eckloni*, including muscle, liver, spleen, gill and kidney, by qRT-PCR. The results showed that the highest level of *tlr22c* mRNA expression was observed in muscle, followed by *tlr22b* and *tlr22a*. In liver, the mRNA expression of *tlr22c* significantly higher than that of *tlr22a* and *tlr22b*, whereas the expressions of *tlr22a* and *tlr22b* remained similar. In spleen and gill, the mRNA expression of *tlr22c* significantly higher than that of tlr22a, but the expressions of *tlr22a* and *tlr22b* remained similar. In kidney, the expressions of *tlr22a*, *tlr22b* and *tlr22c* were almost the same (Fig. 4B).

Table 2
Comparative analysis of *TLR22* genes in the teleost fishes.

Species	Gene	CDS (bp)	Amino acids (aa)	CDS status	Accession No.	Genome location
Cyprinus carpio	*tlr22a1*	2838	945	Complete	LC150775	LG35
	tlr22a2	2412		Partial	NW_017545300	Scaffold
	tlr22b	2805	934	Complete	LC150776	LG7
	tlr22c	2869	955	Complete	LC150777	Scaffold
Sinocyclocheilus grahami	*tlr22a*	2838	945	Complete	NW_015505270	Scaffold
	tlr22b	2799	932	Complete	NW_015514490	Scaffold
	tlr22c	2145	714	Partial	NW_015505664	Scaffold
Sinocyclocheilus anshuiensis	*tlr22a1*	2838	945	Complete	NW_015548175	Scaffold
	tlr22a2	1624		Partial	NW_015551045	Scaffold
	tlr22b	2805	934	Complete	NW_015542606	Scaffold
	tlr22c1	2865	954	Complete	NW_015552615	Scaffold
	tlr22c2	2868	955	Complete	NW_015558674	Scaffold
Pygocentrus nattereri	*tlr22a*	2898	965	Complete	NW_016243855	Scaffold
	tlr22b	2838	945	Complete	NW_016243918	Scaffold
Astyanax mexicanus	*tlr22a*	2754	918	Partial	NC_035910	LG14
	tlr22b	2874	957	Complete	NC_035908	LG12
Salmo salar	*tlr22*	2790	929	Complete	NC_027315	LG16
Oncorhynchus mykiss	*tlr22*	2910	969	Complete	NC_035094	LG15
Carassius auratus	*tlr22a*	2838	945	Complete	NW_020523497	Scaffold
	tlr22b	2805	934	Complete	NW_020529075	Scaffold
	tlr22c1	2859	952	Complete	NC_039248	LG6
	tlr22c2	2852	950	Partial	NC_039273	LG31
Oxygymnocypris stewartii	*tlr22a*	2853	950	Complete	QVTF01004493	ctg141
	tlr22b	2808	935	Complete	QVTF01002673	ctg1244
	tlr22c	2868	955	Complete	QVTF01019087	ctg3424
Ctenopharyngodon idellus	*tlr22a*	2865	954	Complete	MN106011[a]	unknown
	tlr22b	2784	927	Complete	MN106012[a]	unknown
	tlr22c	734	244	Partial	MN106013[a]	unknown
Gymnocypris eckloni	*tlr22a*	2853	950	Complete	MN106008[a]	unknown
	tlr22b	2808	935	Complete	MN106009[a]	unknown
	tlr22c	2868	955	Complete	MN106010[a]	unknown
Ictalurus punctatus	*tlr22*	2898	965	Complete	NC_030433.1	LG18

[a] Indicate *tlr22* gene sequences obtained in this study. Other sequences are from NCBI.

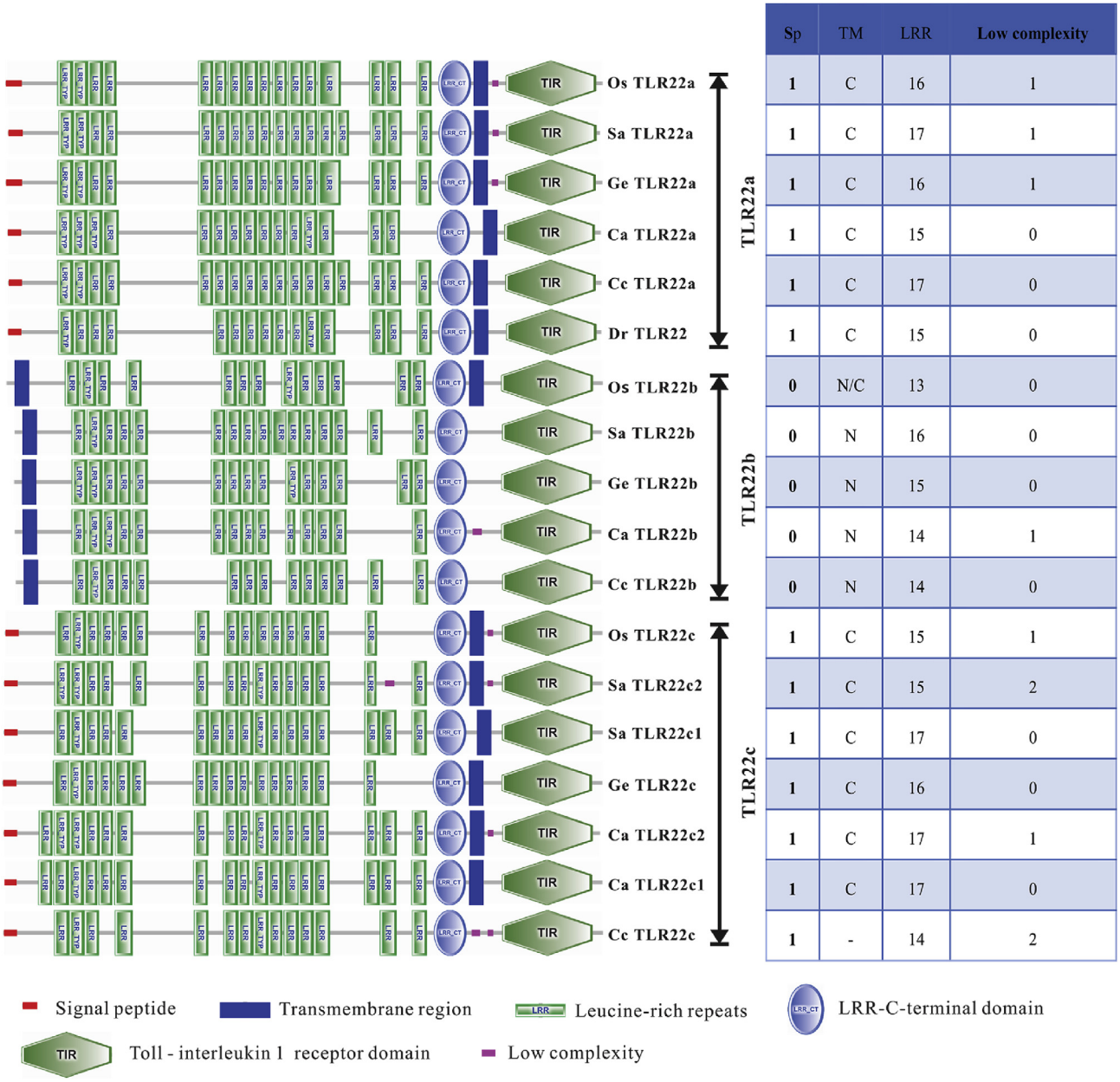
4. Discussion

Duplicated genes are common in *tlrs*, similar to other immune genes in teleost fishes [18,31–33]. Duplications of the genes encoding TLR3, 4, 5, 8, 20, 22, and 25 have been described in several fish taxa [16,18,21,34–37], but three duplicated *tlr22* genes were previously only known from the common carp [18]. In the present study, we investigated duplicated *tlr22* genes in 13 teleost fishes by screening available fish genomic resources and using molecular cloning. We found that four species (*D. rerio*, *S. salar*, *O. mykiss,* and *I. punctatus*) possess a single copy of *tlr22*, two species (*P. nattereri* and *A. mexicanus*) possess two *tlr22* paralogs, fivespecies (*C. carpio*, *S. graham*, *O. stewartii*, *C. idellus*, *G. eckloni*) possess three *tlr22* paralogs, while one species (*C. auratus*) possesses four *tlr22* paralogs, and one species (*S. anshuiensis*) possess five *tlr22* paralogs. Phylogenetic analysis indicated that the TLR22 paralogs identified in this study formed three distinct groups (TLR22a, TLR22b, and TLR22c). This suggested that TLR22 is encoded by at least three distinct paralogous genes in some fish species, as has been previously shown in the common carp [18]. This result was consistent with our functional domain predictions. That is, although all of the TLR22 paralogs had typical TLR characteristics, each was distinct. That is, TLR22b differs from TLR22a and TLR22c with respect to the locations of the transmembrane domain and the absence of signal peptide, while TLR22a and TLR22c have different numbers of the extracellular LRR motifs. Thus, our results indicated that the *tlr22* gene has been duplicated in some teleost fishes, and that the resulting TLR22 paralogs have evolved novel functional properties. The transmembrane domain, together with the TIR domain, play important role in enabling the downstream signal transmission of TLR signaling pathway [38]. For TLR22b, however, further studies are needed to clarify the roles of distinct structure features (e.g., the absence of signal peptide and the transmembrane domain located at the beginning of the protein rather than close to the TIR) in signaling cascades.

The phylogenetic tree, together with the syntenic analysis, showed that the *tlr22a* gene from species of Barbinae, Cyprininae, Schizothoracinae, Leuciscinae, and Serrasalmidae was orthologus to the *tlr22* gene from *D. rerio*, while the *tlr22b* gene from species from Barbinae, Cyprininae, Schizothoracinae, Leuciscinae, and Serrasalmidae was orthologus to the *tlr22* gene in the Salmonidae. The *tlr22c* gene is specific to some subfamilies of Cyprinidae (e.g., Barbinae, Cyprininae, Schizothoracinae, and Leuciscinae). Phylogenetic analysis showed that all of the TLR22s formed a monophyletic clade, but TLR22a clade was phylogenetically closer to TLR22c clade compared to TLR22b clade, suggesting that TLR22a and TLR22c clades have the recent common origin.

It is worth noting that the phylogenetic tree showed that the TLR22a, TLR22b, and TLR22c subclades were clustered close to TLR19, TLR20, and TLR21 from *D. rerio*. This suggested that the TLRs from *D. rerio* belonged to the TLR11 subfamily, consistent with the previous studies [4,7,14,16,18,19]. Syntenic analysis identified no conserved synteny of the neighboring gene loci across the *tlr22a*, *tlr22b,* and *tlr22c* genes, suggesting that the paralogous *tlr22* genes in teleosts did not arise via a large-scale genomic duplication, but via two single-gene duplication events. Based on our phylogeny, *tlr22a* and *tlr22b* differentiated first, followed by *tlr22c* and *tlr22a*. Although TLR22a clade was phylogenetically closer to TLR22c clade, syntenic analysis indicated that *tlr22a* and *tlr22c* genes were localized on different chromosomes/linkage groups, suggesting they did not originate the local gene duplication. However, the conserved synteny found in *tlr22a1* and *tlr22a2*, as well as in *tlr22c1* and *tlr22c2* in *S. anshuiensis*, supported their origins of local gene duplication.

Genome and gene duplications considerably increase gene diversification [33,39–42]. It has been proposed that multiple whole genome duplication events have occurred in vertebrate lineages [32]. The first genome duplication (1R) is thought to have occurred before the divergence of jawed and jawless vertebrates, followed by a second



Sp	TM	LRR	Low complexity
1	C	16	1
1	C	17	1
1	C	16	1
1	C	15	0
1	C	17	0
1	C	15	0
0	N/C	13	0
0	N	16	0
0	N	15	0
0	N	14	1
0	N	14	0
1	C	15	1
1	C	15	2
1	C	17	0
1	C	16	0
1	C	17	1
1	C	17	0
1	-	14	2

Fig. 1. Structural features of the TLRs homologs. The schematic structures of the TLRs domains were predicted by the SMART online server (http://smart.embl-heidelberg.de/). The features of protein sequences were summerized in right panel. N and C in right panel meant the transmembrane domain was near the N- and C-terminal of protein, respectively. - referred to no transmembrane domain. Sp referred to signal peptide. The species abbreviations were as follows: Os, *O. stewartii*; Sa, *S. anshuiensis*; Ge, *G. eckloni*; Ca, *C. auratus*; Cc, *C. carpio*; Dr, *D. rerio*.

genome duplication (2R) immediately after the divergence of the lamprey lineage, 500–800 million years ago (MYA) [31,43]. An analysis of more than 20 vertebrate genomes indicated that many gene families, including engrailed-related homeobox protein (EN), MHC class III complement factor (MHCIII), polyadenylate binding protein (PAB), insulin-like growth factor (Insulin-IGF), and peroxisome proliferator-activated receptor (PPAR), have experienced gene duplication during these two genome duplication events [31]. It has also been suggested that, after the two whole genome duplication events during early vertebrate evolution, a third genome duplication even occurred in most teleost fish species 250–350 MYA [33,42,44,45]. This so-called fish-specific genome duplication (FSGD) event is thought to explain the vast biodiversity and varied environmental adaptations of the teleost fishes, in conjunction with duplications in other important, environmentally sensitive genes [33,42,46,47]. For example, seven *hox* gene clusters were identified in the genomes of zebrafish and other ray-fin fishes, but only four *hox* clusters are known in mammals; the greater number of *hox* genes in fishes may be a result of a fish specific whole-genome duplication [44]. Examples of other such duplicated genes in teleost fishes include those involved in pigment cell development, such as *sox*, *mitf*, *kit*, *csf1r,* and *pomc* [48–51], and globin genes, which are involved in oxygen transport and storage [42,46,52]. The common carp is a known allotetraploid and experienced an additional whole genome duplication event 8–12 MYA [29,53], with the segmental duplications occurring between 2.3 and 6.8 MYA [29]. Using a clock-like evolutionary rate with 3.51×10^{-9} substitutions per synonymous site per year [53], molecular clock calculations date the divergence of *tlr22a* + *tlr22c* and *tlr22b* approximately at 46.5–53.4 MYA (an average of 49.5 MYA), and the divergence of *tlr22a* and *tlr22c* approximately at 26.2–40.1 MYA (an average of 39.3 MYA). Thus, our results suggested

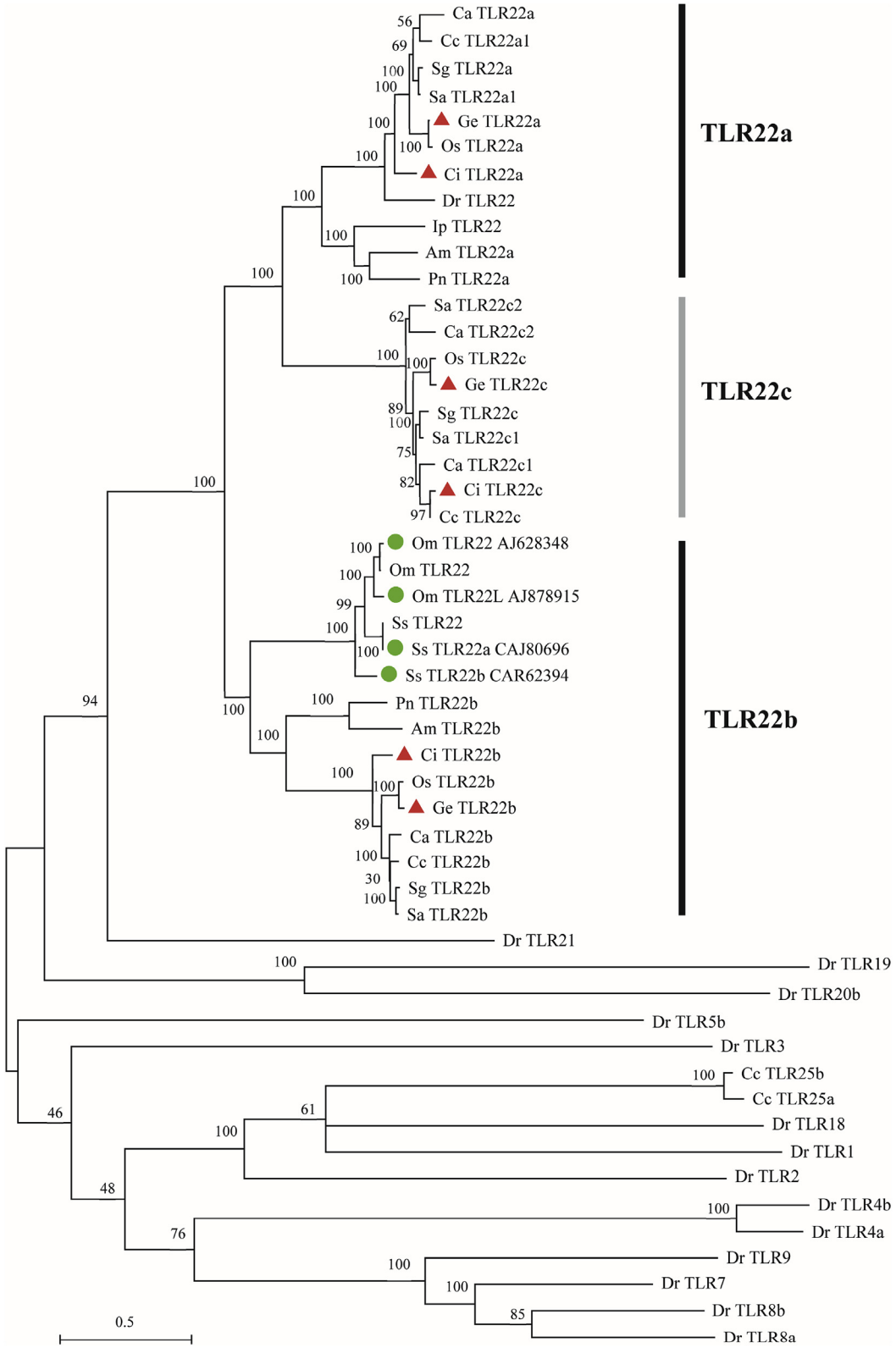
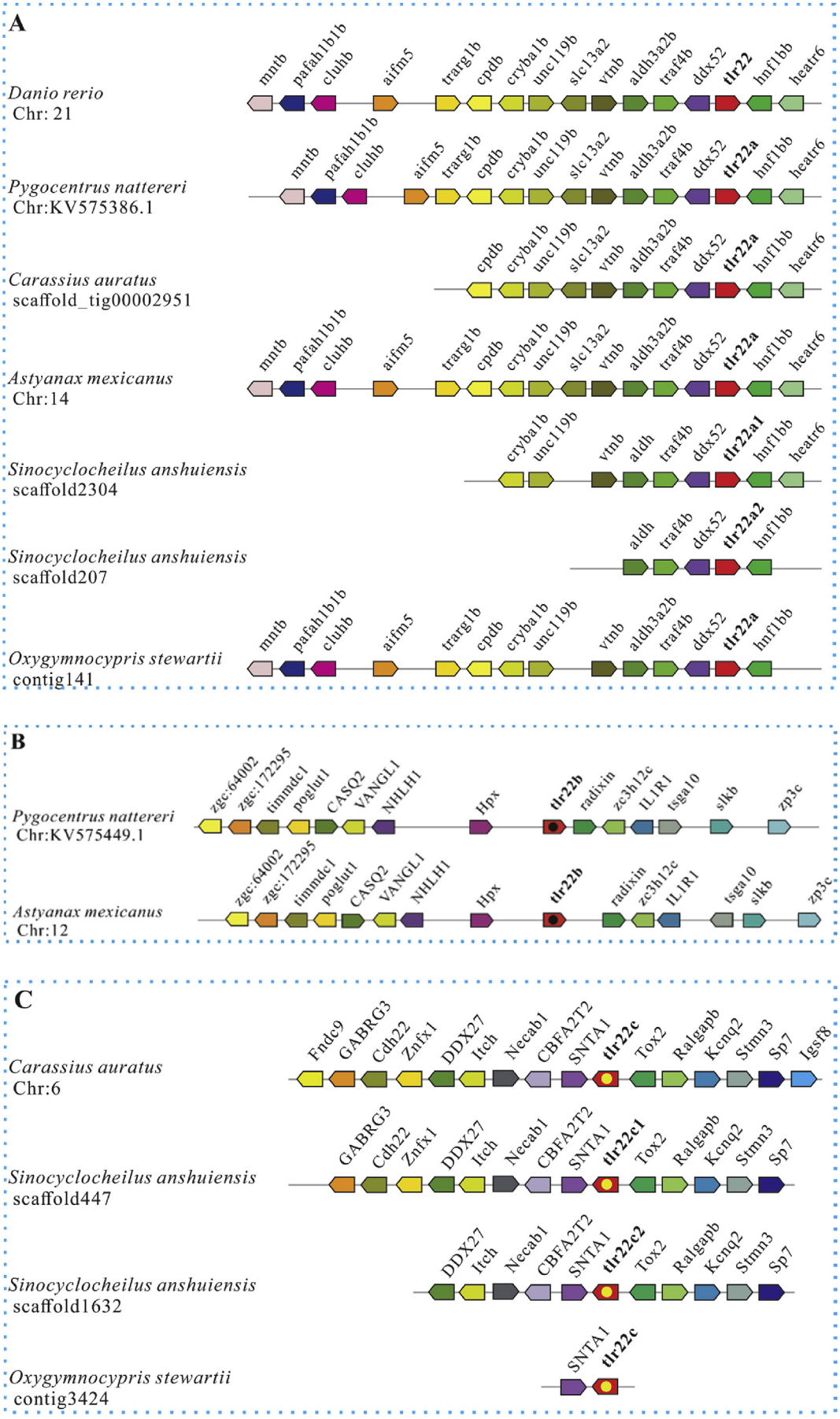


Fig. 2. Phylogenetic tree of TLRs based on amino acid sequences. The tree was constructed using the maximum-likelihood method in MEGA X, with the LG + G + I model and 100 bootstrap replicates. The species abbreviations were as follows: Dr, *D. rerio*; Cc, *C. carpio*; Sa, *S. anshuiensis*; Sg, *S. grahami*; Ca, *C. auratus*; Ge, *G. eckloni*; Os, *O. stewartii*; Ci, *C. idellus*; Am, *A. mexicanus*; Pn, *P. nattereri*; Ss, *S. salar*; Om, *O. mykiss*; Ip, *I. punctatus*.

that the divergence of the *tlr22a* and the *tlr22b* genes resulted from a single-gene duplication event in teleost fishes, followed by another single-gene duplication event specific to some cyprinids (e.g., the Barbinae, Cyprininae, Schizothoracinae, and Leuciscinae), which produced the *tlr22c* gene. In any case, these gene duplication events pre-date the common carp-specific genome duplication event, and occurred much more recently than the fish-specific genome duplication. In the present study, duplicated genes of *tlr22a* and *tlr22c* were identified only in *C.*



(caption on next page)

Fig. 3. Syntenic analyses of the *tlr22a* (A), *tlr22b* (B), and *tlr22c* (C) genes in selected teleosts, constructed using the Ensembl Genome Browser (http://www. ensembl. org) and BLAST searches against fish genomes. Gene symbols are given based on the Ensembl database. Bar lengths are not proportional to distances between genes. The direction of the each arrow indicates the orientation of the corresponding gene.

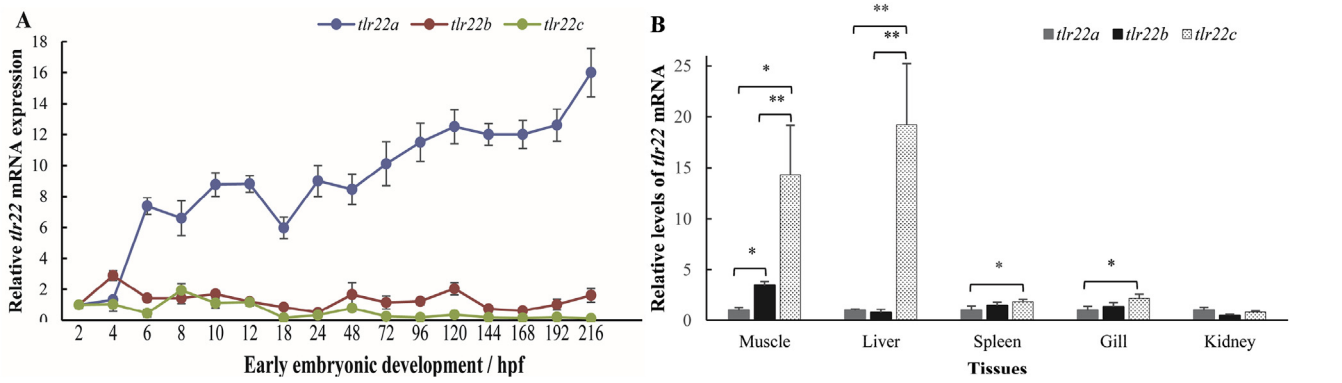


Fig. 4. Expression patterns of the three duplicated *tlr22* genes in *G. eckloni* during the major stages of embryonic development (A) and in the primary tissues of adults (B). Expression levels were normalized to *β-actin* mRNA expression. Values represent means ± SEM (n = 3). Significant differences between *tlr22* genes within the same tissue were analyzed by Student's t-test. Asterisks (*$P < 0.05$, **$P < 0.01$) indicate significant difference.

carpio, *C. auratus* and *S. anshuiensis* instead of all the cyprinid fishes. This findings together with the absence of duplicated genes of *tlr22b* in cyprinid fishes suggested gene loss events might have occurred in certain lineages during the evolutionary history of *tlr22* gene family. In addition, we speculated that *tlr22a2* from *C. carpio* and *S. anshuiensis* might be a pseudogene due to premature stop codons and disrupted reading frames. Gene loss events and pseudogenes in TLR family have been reported in vertebrates, further studies, however, are required to fully understand the dynamics of *tlr22* gene loss and pseudogene event in fishes.

Studies have shown that duplicated genes may be subjected to neo- and/or subfunctionalization, or they may even be eliminated from the genome during evolution [54–56]. Surviving paralogs might exhibit functional divergences not only with respect to functional domains, but also with respect to expression patterns. Interestingly, *tlr22a* was highly expressed from the cleavage stage to the hatching stage (6–216 dpf) in *G. eckloni*. However, no significant fluctuations in the expression levels of either *tlr22b* or *tlr22c* were observed throughout the embryonic development. This suggested that *tlr22a* might play an important role in the embryonic immunity of *G. eckloni* from 6 hpf. The expression patterns of the *tlr22* genes in the *G. eckloni* tissues indicated that *tlr22c* was highly expressed in most tissues compared to *tlr22a*, while *tlr22b* was highly expressed only in muscle compared to *tlr22a*. The observed spatiotemporal differences in the expression levels of the three duplicated *tlr22* genes in *G. eckloni* might imply that these genes had functionally diverged after gene duplication. However, further studies are required to fully understand the role of three duplicated *tlr22* genes in other embryonic, larval, and adult teleost fishes.

In conclusion, we first identified duplicated *tlr22* genes in 13 teleost fishes by screening available fish genomic resources and by molecular cloning. Different numbers of duplicated *tlr22* genes were identified in teleost fishes, with *tlr22c* being specific to some subfamilies of the Cyprinidae (e.g., Barbinae, Cyprininae, Schizothoracinae, and Leuciscinae). The duplicated *tlr22* genes originated from two single-gene duplication events (at 49.5 and 39.3 MYA). This was prior to the common carp-specific genome duplication event, and much later than the fish-specific genome duplication. The duplicated *tlr22* genes from *G. eckloni* differentiated not only with respect to protein function, but also with respect to expression pattern.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (31960127). We thank LetPub for its linguistic assistance during the preparation of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.067.

References

[1] J.C. Roach, G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood, A. Aderem, The evolution of vertebrate Toll-like receptors, Proc. Natl. Acad. Sci. U. S. A. 102 (27) (2005) 9577–9582.
[2] N.W. Palm, R. Medzhitov, Pattern recognition receptors and control of adaptive immunity, Immunol. Rev. 227 (1) (2009) 221–233.
[3] H. Velova, M.W. Gutowska-Ding, D.W. Burt, M. Vinkler, Toll-like receptor evolution in birds: gene duplication, pseudogenisation and diversifying selection, Mol. Biol. Evol. (2018) 2170–2184.
[4] S. Akira, K. Takeda, Toll-like receptor signalling, Nat. Rev. Immunol. 4 (7) (2004) 499–511.
[5] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (4) (2006) 783–801.
[6] J. Wang, Z. Zhang, J. Liu, J. Zhao, D. Yin, Ectodomain architecture affects sequence and functional evolution of vertebrate toll-like receptors, Sci. Rep. 6 (2016) 26705.
[7] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 1263–1272.
[8] A. Iwasaki, R. Medzhitov, Control of adaptive immunity by the innate immune system, Nat. Immunol. 16 (4) (2015) 343–353.
[9] R.L. Modlin, Mammalian toll-like receptors, Ann. Allergy Asthma Immunol. 88 (6) (2002) 543–547 quiz 548-50, 583.
[10] M.K. Purcell, K.D. Smith, L. Hood, J.R. Winton, J.C. Roach, Conservation of toll-like receptor signaling pathways in teleost fish, Comp. Biochem. Physiol. Genom. Proteonom. 1 (1) (2006) 77–88.
[11] N.D. Temperley, S. Berlin, I.R. Paton, D.K. Griffin, D.W. Burt, Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss, BMC Genomics 9 (2008) 62.
[12] D. Pietretti, M. Scheer, I.R. Fink, N. Taverne, H.F. Savelkoul, H.P. Spaink, M. Forlenza, G.F. Wiegertjes, Identification and functional characterization of nonmammalian Toll-like receptor 20, Immunogenetics 66 (2) (2014) 123–141.
[13] C. Tong, Y. Lin, C. Zhang, J. Shi, H. Qi, K. Zhao, Transcriptome-wide identification, molecular evolution and expression analysis of Toll-like receptor family in a Tibet fish, *Gymnocypris przewalskii*, Fish Shellfish Immunol. 46 (2) (2015) 334–345.
[14] D. Qi, M. Xia, Y. Chao, Y. Zhao, R. Wu, Identification, molecular evolution of toll-like receptors in a Tibetan schizothoracine fish (*Gymnocypris eckloni*) and their expression profiles in response to acute hypoxia, Fish Shellfish Immunol. 68 (2017) 102–113.
[15] H. Oshiumi, T. Tsujita, K. Shida, M. Matsumoto, K. Ikeo, T. Seya, Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome, Immunogenetics 54 (11) (2003) 791–800.
[16] C. Jault, L. Pichon, J. Chluba, Toll-like receptor gene family and TIR-domain adapters in Danio rerio, Mol. Immunol. 40 (11) (2004) 759–771.
[17] A.H. Meijer, S.F. Gabby Krens, I.A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, H.P. Spaink, Expression analysis of the Toll-like receptor and TIR

domain adaptor families of zebrafish, Mol. Immunol. 40 (11) (2004) 773–783.
[18] Y. Gong, S. Feng, S. Li, Y. Zhang, Z. Zhao, M. Hu, P. Xu, Y. Jiang, Genome-wide characterization of Toll-like receptor gene family in common carp (*Cyprinus carpio*) and their involvement in host immune response to Aeromonas hydrophila infection, Comp. Biochem. Physiol. Genom. Proteonom. 24 (2017) 89–98.
[19] A. Rebl, T. Goldammer, H.M. Seyfert, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (3–4) (2010) 139–150.
[20] X. Ding, D.Q. Lu, Q.H. Hou, S.S. Li, X.C. Liu, Y. Zhang, H.R. Lin, Orange-spotted grouper (*Epinephelus coioides*) toll-like receptor 22: molecular characterization, expression pattern and pertinent signaling pathways, Fish Shellfish Immunol. 33 (3) (2012) 494–503.
[21] A. Rebl, E. Siegl, B. Kollner, U. Fischer, H.M. Seyfert, Characterization of twin toll-like receptors from rainbow trout (*Oncorhynchus mykiss*): evolutionary relationship and induced expression by Aeromonas salmonicida salmonicida, Dev. Comp. Immunol. 31 (5) (2007) 499–510.
[22] A. Matsuo, H. Oshiumi, T. Tsujita, H. Mitani, H. Kasai, M. Yoshimizu, M. Matsumoto, T. Seya, Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses, J. Immunol. 181 (5) (2008) 3474–3485.
[23] X. Xiao, Q. Qin, X. Chen, Molecular characterization of a Toll-like receptor 22 homologue in large yellow croaker (*Pseudosciaena crocea*) and promoter activity analysis of its 5'-flanking sequence, Fish Shellfish Immunol. 30 (1) (2011) 224–233.
[24] R.H. Wang, W. Li, Y.D. Fan, Q.L. Liu, L.B. Zeng, T.Y. Xiao, Tlr22 structure and expression characteristic of barbel chub, Squaliobarbus curriculus provides insights into antiviral immunity against infection with grass carp reovirus, Fish Shellfish Immunol. 66 (2017) 120–128.
[25] T.G. Burland, DNASTAR's Lasergene sequence analysis software, Methods Mol. Biol. 132 (2000) 71–91.
[26] M. Johnson, I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis, T.L. Madden, NCBI BLAST: a better web interface, Nucleic Acids Res. 36 (Web Server issue) (2008) W5–W9.
[27] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, MEGA X: molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (6) (2018) 1547–1549.
[28] Z. Yang, PAML 4: phylogenetic analysis by maximum likelihood, Mol. Biol. Evol. 24 (8) (2007) 1586–1591.
[29] L. David, S. Blum, M.W. Feldman, U. Lavi, J. Hillel, Recent duplication of the common carp (*Cyprinus carpio* L.) genome as revealed by analyses of microsatellite loci, Mol. Biol. Evol. 20 (9) (2003) 1425–1434.
[30] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (9) (2001) e45.
[31] H. Escriva, L. Manzon, J. Youson, V. Laudet, Analysis of lamprey and hagfish genes reveals a complex history of gene duplications during early vertebrate evolution, Mol. Biol. Evol. 19 (9) (2002) 1440–1450.
[32] O. Jaillon, J.M. Aury, F. Brunet, J.L. Petit, N. Stange-Thomann, E. Mauceli, L. Bouneau, C. Fischer, C. Ozouf-Costaz, A. Bernot, S. Nicaud, D. Jaffe, S. Fisher, G. Lutfalla, C. Dossat, B. Segurens, C. Dasilva, M. Salanoubat, M. Levy, N. Boudet, S. Castellano, V. Anthouard, C. Jubin, V. Castelli, M. Katinka, B. Vacherie, C. Biemont, Z. Skalli, L. Cattolico, J. Poulain, V. De Berardinis, C. Cruaud, S. Duprat, P. Brottier, J.P. Coutanceau, J. Gouzy, G. Parra, G. Lardier, C. Chapple, K.J. McKernan, P. McEwan, S. Bosak, M. Kellis, J.N. Volff, R. Guigo, M.C. Zody, J. Mesirov, K. Lindblad-Toh, B. Birren, C. Nusbaum, D. Kahn, M. Robinson-Rechavi, V. Laudet, V. Schachter, F. Quetier, W. Saurin, C. Scarpelli, P. Wincker, E.S. Lander, J. Weissenbach, H. Roest Crollius, Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype, Nature 431 (7011) (2004) 946–957.
[33] B.C. Guo, A. Wagner, S.P. He, Duplicated gene evolution following whole genome duplication in teleost fish, Gene Duplic (2011) 27–36.
[34] P. Kongchum, Y. Palti, E.M. Hallerman, G. Hulata, L. David, SNP discovery and development of genetic markers for mapping innate immune response genes in common carp (*Cyprinus carpio*), Fish Shellfish Immunol. 29 (2) (2010) 356–361.
[35] C. Sullivan, J. Charette, J. Catchen, C.R. Lage, G. Giasson, J.H. Postlethwait, P.J. Millard, C.H. Kim, The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions, J. Immunol. 183 (9) (2009) 5896–5908.
[36] T. Tsujita, H. Tsukada, M. Nakao, H. Oshiumi, M. Matsumoto, T. Seya, Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*), J. Biol. Chem. 279 (47) (2004) 48588–48597.
[37] Y. Palti, S.A. Gahr, M.K. Purcell, S. Hadidi, C.E. Rexroad 3rd, G.D. Wiens, Identification, characterization and genetic mapping of TLR7, TLR8a1 and TLR8a2 genes in rainbow trout (*Oncorhynchus mykiss*), Dev. Comp. Immunol. 34 (2) (2010) 219–233.
[38] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (5) (2010) 373–384.
[39] Z. Gu, A. Cavalcanti, F.C. Chen, P. Bouman, W.H. Li, Extent of gene duplication in the genomes of Drosophila, nematode, and yeast, Mol. Biol. Evol. 19 (3) (2002) 256–262.
[40] P.W. Holland, J. Garcia-Fernandez, N.A. Williams, A. Sidow, Gene duplications and the origins of vertebrate development, Dev Suppl (1994) 125–133.
[41] A. Sidow, Gen(om)e duplications in the evolution of early vertebrates, Curr. Opin. Genet. Dev. 6 (6) (1996) 715–722.
[42] J.C. Opazo, G.T. Butts, M.F. Nery, J.F. Storz, F.G. Hoffmann, Whole-genome duplication and the functional diversification of teleost fish hemoglobins, Mol. Biol. Evol. 30 (1) (2013) 140–153.
[43] K.H. Wolfe, Yesterday's polyploids and the mystery of diploidization, Nat. Rev. Genet. 2 (5) (2001) 333–341.
[44] A. Amores, T. Suzuki, Y.L. Yan, J. Pomeroy, A. Singer, C. Amemiya, J.H. Postlethwait, Developmental roles of pufferfish Hox clusters and genome evolution in ray-fin fish, Genome Res. 14 (1) (2004) 1–10.
[45] A. Meyer, Y. Van de Peer, From 2R to 3R: evidence for a fish-specific genome duplication (FSGD), Bioessays 27 (9) (2005) 937–945.
[46] Z.X. Zhao, P. Xu, D.C. Cao, Y.Y. Kuang, H.X. Deng, Y. Zhang, L.M. Xu, J.T. Li, J. Xu, X.W. Sun, Duplication and differentiation of common carp (*Cyprinus carpio*) myoglobin genes revealed by BAC analysis, Gene 548 (2) (2014) 210–216.
[47] I. Braasch, M. Schartl, J.N. Volff, Evolution of pigment synthesis pathways by gene and genome duplication in fish, BMC Evol. Biol. 7 (2007) 74.
[48] M. Lang, T. Miyake, I. Braasch, D. Tinnemore, N. Siegel, W. Salzburger, C.T. Amemiya, A. Meyer, A BAC library of the East African haplochromine cichlid fish *Astatotilapia burtoni*, J. Exp. Zool. B Mol. Dev. Evol. 306 (1) (2006) 35–44.
[49] J.A. Lister, J. Close, D.W. Raible, Duplicate mitf genes in zebrafish: complementary expression and conservation of melanogenic potential, Dev. Biol. 237 (2) (2001) 333–344.
[50] E.M. Mellgren, S.L. Johnson, kitb, A second zebrafish ortholog of mouse Kit, Dev. Genes Evol. 215 (9) (2005) 470–477.
[51] F.S. de Souza, V.F. Bumaschny, M.J. Low, M. Rubinstein, Subfunctionalization of expression and peptide domains following the ancient duplication of the proopiomelanocortin gene in teleost fishes, Mol. Biol. Evol. 22 (12) (2005) 2417–2427.
[52] C. Fuchs, A. Luckhardt, F. Gerlach, T. Burmester, T. Hankeln, Duplicated cytoglobin genes in teleost fishes, Biochem. Biophys. Res. Commun. 337 (1) (2005) 216–223.
[53] P. Xu, X. Zhang, X. Wang, J. Li, G. Liu, Y. Kuang, J. Xu, X. Zheng, L. Ren, G. Wang, Y. Zhang, L. Huo, Z. Zhao, D. Cao, C. Lu, C. Li, Y. Zhou, Z. Liu, Z. Fan, G. Shan, X. Li, S. Wu, L. Song, G. Hou, Y. Jiang, Z. Jeney, D. Yu, L. Wang, C. Shao, L. Song, J. Sun, P. Ji, J. Wang, Q. Li, L. Xu, F. Sun, J. Feng, C. Wang, S. Wang, B. Wang, Y. Li, Y. Zhu, W. Xue, L. Zhao, J. Wang, Y. Gu, W. Lv, K. Wu, J. Xiao, J. Wu, Z. Zhang, J. Yu, X. Sun, Genome sequence and genetic diversity of the common carp, Cyprinus carpio, Nat. Genet. 46 (11) (2014) 1212–1219.
[54] W. Engel, J.O. Hof, U. Wolf, [Gene duplication by polyploid evolution: the isoenzyme of the sorbitol dehydrogenase in herring- and salmon-like fishes (Isospondyli)], Humangenetik 9 (2) (1970) 157–163.
[55] X. Gu, Z. Zhang, W. Huang, Rapid evolution of expression and regulatory divergences after yeast gene duplication, Proc. Natl. Acad. Sci. U. S. A. 102 (3) (2005) 707–712.
[56] S. Rastogi, D.A. Liberles, Subfunctionalization of duplicated genes as a transition state to neofunctionalization, BMC Evol. Biol. 5 (2005) 28.