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Genetic characterization, cloning, and expression of Toll-like Receptor 1 mRNA Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Toll-like receptors (TLRs) are the most studied group of pathogen recognition receptor categories that detects infectious agents in vertebrates. Fish TLRs exhibit clear, distinct features, structure and a larger diversity than in other vertebrates. This study focused on identifying and detecting the structure of *Oreochromis niloticus* (Nile tilapia) Toll-like receptor-1 (TLR1|) as a model in freshwater bony fish. The full-length cDNA sequence of *Oreochromis niloticus* TLR1 mRNA was cloned. Cloning and sequence analysis revealed that the complete cDNA sequence of *Oreochromis niloticus* TLR1 consists of 2355 base pairs and encodes a polypeptide of 785 amino acids. The molecular analysis of the amino acid sequence indicated that *Oreochromis niloticus* TLR1 has the standard structural features and major components of amino acids of TLR family members, and is considered an orthologue to the vertebrate TLR1, not a paralogue. The translated amino acid analysis showed 96%, 88%, 85%, and 85% degrees of identity with Zebra Mbuna, Sea bass, Damsel fish, and Clownfish, respectively; and showed 66% identity t with electric eels and 61% with starlets. Phylogenetic analysis revealed that the Nile tilapia TLR1 is closely related to Larimichthys crocea, Epinephelus coioides, and Takifugu rubripes TLR1. *Oreochromis niloticus* TLR1 was expressed in the kidneys, brain, spleen, intestines, muscle, liver, gills, heart and skin. Quantitative RT-PCR showed differences in the expression levels between the tested tissues. In conclusion, this study is the first report (according to our knowledge) and provides a

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complete molecular and functional characterization of *Oreochromis niloticus* toll-like receptor 1, which is considered to be functionally orthologous to TLR1 in other species models.

Key words: *Oreochromis niloticus* (Nile tilapia); transmembrane structure; fish; Toll-like receptor; TLR1; fish innate immunity

Introduction

Immunology research mainly targets descriptions of the human and animal immune systems, while little is known about the immune systems of other species, especially fish (ABOUELMAATTI et al., 2013b; AKIRA and TAKEDA, 2004; AYOUB et al., 2019; SULTAN et al., 2019a). Salmon is the most studied fish model among bony fish species, as its defense mechanism responds in a distinct way in relation to other types of fish. This studyfocused on *Oreochromis niloticus* as a typical model of freshwater bony fish. *Oreochromis niloticus* is a major culture fish globally, but little is known about its immune system structure, especially pattern recognition receptors (PRRs) that include three main sectors: the Toll-Like Receptor (TLRs) group, RIG-Like Receptors (RLRs) group and NOD-Like Receptors (NLRs). Pathogen-associated molecular patterns (PAMPs) are pathogen molecules which can be identified and recognized by the host immune system. Pattern recognition receptors (PRRs), especially TLRs, recognize PAMPs in both humans and animals. PRRs recognize certain conserved molecules from the pathogenic microorganism, with the subsequent activation of the innate immune response, and then the total immune cascades. Every TLR type is associated with recognition of certain pattern molecules, for example, TLR-2 is specific for the teichoic acid of Gram-positive bacteria (BOYD et al., 2001; ELFEIL et al., 2011; FAURE et al., 2001; FUKU et al., 2001). TLR-3 is associated with identifying nucleic acid from RNA viruses (ABOUELMAATTI et al., 2013b; BROWNLIE and ALLAN, 2011; MIGGIN and O'NEILL, 2006; ROACH et al., 2005; EL SAYED et al., 2019). TLR-4 recognizes lipopolysaccharides in Gram-negative bacteria (ELFEIL et al., 2012; FAURE et al., 2001; KAISER, 2007; KOGUT et al., 2005; LEVEQUE et al., 2003; PAN et al., 2012; VINKLER et al., 2009; ZHANG et al., 2011). The flagellin protein in flagellated bacteria can be identified by TLR-5 (KOGUT et al., 2005). Toll-like receptors type-15 and type-21 in birds recognize the viral CpG components (ALCAIDE and EDVARDS, 2011; EID et al., 2016; ELFEIL et al., 2016; ELHADY et al., 2018; HE et al., 2012; LI et al., 2012; RAMASAMY et al., 2011; SEDEIK et al., 2018; WERLING et al., 2009; 6-EL SAYED et al., 2019; DIA et al., 2019; SULTAN et al., 2019b); TLRs-7/8 are associated with sensing single-stranded RNA viruses, and play a pivotal role in the antiviral immune response (CHEN et al., 2013; PALTI et al., 2010a). TLRs are composed of three main portions: 1- the transmembrane portion, 2- the leucine-rich repeat (LRR) portion, which is responsible for recognition of PAMPs, 3- and the Toll/IL 1 receptor (TIR) portion which is located intracellularly and functions in signal transportation (ABOUELMAATTI, 2013). TLRs genes may differ from one species to another, and various TLR genes have been identified and characterized in both fish and birds (ABOUELMAATTI et al., 2013a; ELFEIL et al., 2012; ELFEIL, 2012b; ELFEIL et al., 2016; ROACH et al., 2005). Only 13 TLRs (1-13) have been characterized in mammals so far (ELFEIL, 2012a; REBL et al., 2010). Moreover, there are differences in the evolutionary development of pattern recognition receptors inside vertebrates, whether bony fish or mammals. There are other types of TLRs (TLR type-5S, TLR type-14, TLR type-19, TLR type-20, TLR type-21, TLR type-22, TLR type-23, TLR type-24, TLR type-25 and TLR type-26) present in bony fish, but they have not been cloned or identified in mammals yet. In addition, some TLRs probably possess different functions in fish in comparison to their function in mammal models (LV et al., 2012; ENANY et al., 2018; EID et al., 2019; SULTAN et al., 2020; AOKI et al., 2013; LI et al., 2012; MATSUMOTO et al., 2004; QUINIOU et al., 2013; RAJENDRAN et al., 2012; REBL et al., 2010). To date, a large number

of studies have been carried out for characterization of TLRs in human and mouse models, but there have been only been a few studies concerned with the characterization of fish TLRs, especially in Nile tilapia (ABOUELMAATTI, 2013; ABOUELMAATTI et al., 2013b). It is equally important to the evolutionary biology of host-pathogen interactions. In contrast to mammals, the molecular structure of the fish immune system is very limited (ELFEIL et al., 2012; RAJENDRAN et al., 2012). As a result, it is necessary to establish a freshwater bony fish model to verify the universal accuracy and validity of the results obtained in human and mice, and *Oreochromis niloticus* was selected as a typical model of freshwater fish (HUANG et al., 2012; LI et al., 2012; PALTI, 2011; REBL et al., 2010; ZHANG et al., 2013). This study was planned for molecular characterization, cloning, and expression of Toll-like Receptor1 in *Oreochromis niloticus*.

Materials and methods

Sampling. Five mature *Oreochromis niloticus* (Nile tilapia) fish were brought from a private farm and kept under inspection for 5 days to guarantee their freedom from any clinical manifestations. Tissue samples, including internal organs (kidneys, intestines, muscle, liver, brain, spleen, gills and heart) and skin were collected.

Primer design. The degenerative primers were designed on the basis on conserved motifs using the iCODEHOP system to clone a short sequence of Nile tilapia TLR1. In previously identified TLR1 models, such as rainbow trout (Oncorhynchus mykiss) (accession #AAX68425); Takifugu rubripes (Japanese pufferfish) (AAW69373); Larimichthys crocea (large yellow croaker) (ADW79423); Paralichthys olivaceus (Japanese flounder) (BAM11216); and Epinephelus coioides (orange-spotted grouper): (AEX01718) the RACE system was used to obtain the full length sequence of Nile tilapia TLR1 for both 3' and 5' end directions using special RACE primers, designed at our lab, as shown in Table 1.

Molecular cloning of Nile tilapia TLR1. RNA extraction. TRIzol® (Invitrogen, USA) was used for total RNA extraction according to the manufacturer's manual, from fish tissues including kidney, brain, spleen, intestine, muscle, liver, gills, heart, and skin. Each organ was considered as a separate sample to detect the presence of

Table 1. Different primers sets used in the experiment

Primer name	Sequence of oligonucleotide (5′ → 3′)	Aim
Degenerative TLR-1 F	5'- CCCTCTTGTATGATGGGATGtgygarwsnat-3'	Gene Cloning
Degenerative TLR-1 R	5'- GAAATGAAGGCGTGGAACTGytcnarrcaraa-3'	Gene Cloning
5-end-F	AATTACTGTTGACCACTGAGCTCTTGA	5′ End-RACE
5-End-R	CAGGCAAGCAGGATTATGGACCAC	
3-end-F	ACCACATGCATAGCTAGGTAGCATGC	3′End-RACE
3-end-R	ATGCTGTGCATTGAACTACATGAG	
Universal Primer A Mix	Long: CTAATACGACTCACTATAGGGCAA GCAGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC	Race Package
Nested Universal Primer A	AAGCAGTGGTATCAACGCAGAGT	Race Package
qTLR1-F	TGGAGAGGACAAACAGCGTC	q-PCR
qTLR1-R	GTTAGATCGGTACCATGCAG	
qTLR1-P	CCGCTTCAGCAGTCTTTCCT	
GADPH -F	5'- GATAATGGCAAACTTGTCGTCG-3'	Housekeeping
GADPH -R	5'- ACATTGGAGCATCGGGTGAG -3'	Housekeeping

TLR1 in different fish tissues. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

cDNA synthesis and PCR reaction. cDNA was synthesized from RNA samples in a 10-µL reaction mixture using a BioRT cDNA first strand synthesis kit (Hangzhou Boiler, China), according to the manufacturer's instructions.

PCR was performed to amplify the target gene using specific primers, where 25 µL PCR mixture contained 2 µL cDNA template, 200 µM of each dNTPs mix, 50 pmol from each forward and reverse primer, and 2.5 U Ex Taq polymerase (Takara bio Dalian, China) in 1× Ex buffer. The amplification conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 35 s, annealing at 56-60 °C for 35 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The PCR amplicons were applied to 1.2% agarose gel electrophoresis in TBE buffer using ethidium bromide. The relative expression for the tilapia TLR1 was evaluated by Quantitative RT-PCR, using a applied biosystem 7500 engine (Thermo-Fisher, USA) using specific primers designed at our lab (Table 1). The previously standardized GADPH primers were used as the housekeeping gene (YANG et al., 2013), using a PrimeScript High Fidelity RT-PCR Kit according to the manufacturer's instructions (Takara Bio, Japan).

TLR1 sequencing. The PCR DNA products were ligated to the PMD18-T simple vector (Takara Bio, China) according to the manufacturer's instructions; they were then transformed to competent DH5α *E. coli,* as previously described by (ABOUETMAATTI, 2013; ELFEIL et al., 2012). The DH5α *E. coli* with PCR products were sent for partial gene sequencing to obtain a Tilapia TLR1 partial sequence based on the degenerative primers. The RACE primers were designed on the basis of the partial tilapia TLR1 sequence obtained and used with SMARTer RACE cDNA amplification kits (Clontech, USA) to get the full length toward the 3'end and 5' end, according to the manufacturer's instructions. The RACE products (toward 3'end and toward 5' end reactions) were ligated to PMD18-T simple vector (Takara Bio, China) then transformed to the competent DH5α *E. coli* as previously described (ABOUELMAATTI, 2013; ELFEIL et al., 2012).The DH5α *E. coli* with PCR products were sent for full TLR1gene sequencing.

Sequence analysis. The blast tool in GenBank was used to confirm the identity of the partial TLR1 sequence based on the degenerative primers, then the complete TLR1 mRNA sequence was obtained following the RACE system with other orthologue sequences. The sequence was aligned using Geneious® 8.1.4 software (Invitrogen Corporation, USA). Maximum Likelihood, Bootstrap method, with 500 replicate numbers based on the Jones-Taylor-Thornton (JTT) mode, using MEGA X software (KUMAR et al., 2018). MEGA7 software was used to construct a phylogenetic tree for the cloned sequence, and other available TLR1 sequences in databases (ABOUELMAATTI et al., 2013a; TAMURA et al., 2011) The transmembrane structure of the tilapia TLR-1 protein sequences was predicted with the analysis tools provided on the website (http://smart.embl-heidelberg.de and http://split.pmfst.hr).

Results

The identity results of Nile tilapia TLR-1. The cloned full sequence of Nile tilapia TLR1 mRNA, composed of 2355 nucleotides, was deposited in GenBank under accession number (JQ809458) and the translated amino acids were given accession number AFP44841. The translated amino acids analysis of Nile tilapia TLR1 highlighted that is an orthologue to other fish TLR1 with a degree of identity of 96%, 88%, 85%, and 85% with Zebra Mbuna, Sea bass, Damsel fish, and Clownfish, respectively. It also showed 66% identity with electric eels and 61% with starlets, as shown in Table 2. These data confirmed that the new sequence is probably a homologue of fish TLR1. *Oreochromis niloticus* The TLR1 mRNA sequence encodes 785 amino acids where it begins with common starting codon (ATG), then Methionine, similar to other fish TLR1 sequences. The amino acid compositions of the encoded polypeptide are described in Fig. 1.

Transmembrane and domain structure. The transmembrane structure of the cloned and sequenced mRNA of *Oreochromis niloticus* TLR1

Table 2. Translated Amino acid Identity matrix among available fish TLR-1

	Nile Tilapia	Zebra Mbuna	Damsel fish	Clownfish	Sea bass	Rice fish	Roho labeo	Goldfish	Shark	Electrical Eel	Sterlet
Nile Tilapia		96.688	85.032	85.287	88.061	79.833	67.554	66.094	67.949	66.41	61.154
Zebra Mbuna	96.688		86.178	86.433	89.464	80.603	68.561	67.274	68.846	67.564	61.795
Damsel fish	85.032	86.178		93.503	88.954	78.164	67.698	66.881	67.692	67.051	61.026
Clownfish	85.287	86.433	93.503		89.592	78.421	67.986	67.143	68.846	67.821	60.897
Sea bass	88.061	89.464	88.954	89.592		82.622	68.79	68.136	70.462	70.334	63.017
Rice fish	79.833	80.603	78.164	78.421	82.622		67.554	65.832	66.483	64.942	60
Roho labeo	67.554	68.561	67.698	67.986	68.79	67.554		89.065	77.338	77.194	64.011
Goldfish	66.094	67.274	66.881	67.143	68.136	65.832	89.065		78.414	77.628	63.394
Shark	67.949	68.846	67.692	68.846	70.462	66.483	77.338	78.414		87.692	64.487
Eel	66.41	67.564	67.051	67.821	70.334	64.942	77.194	77.628	87.692		64.359
Starlet	61.154	61.795	61.026	60.897	63.017	60	64.011	63.394	64.487	64.359	

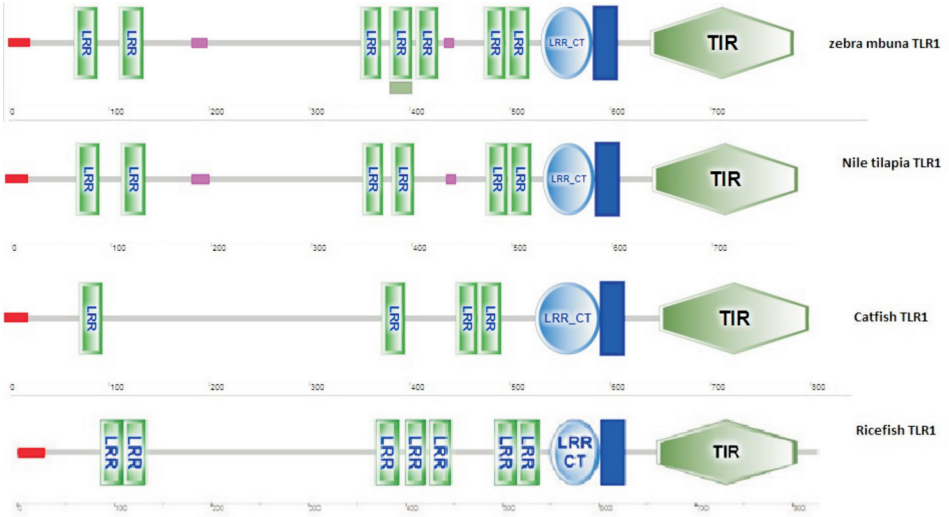

Fig. 1. Distribution of the motifs, repeat and domains in catfish, Nile tilapia, and Japanese medaka TLR1 models

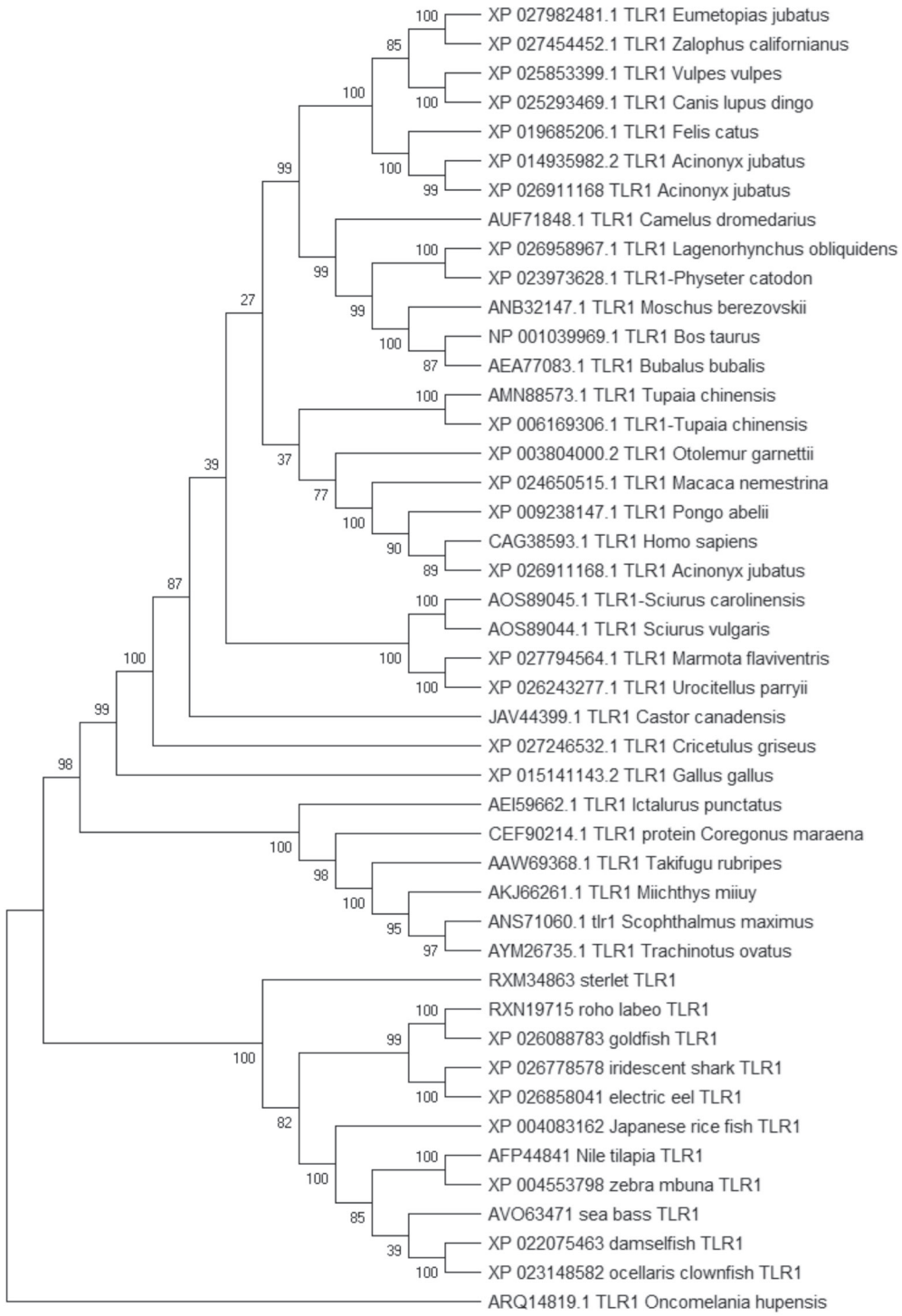

Fig. 2. Phylogenetic analysis of Nile tilapia TLR1 against available vertebrates TLR1 models

The tree with the highest log likelihood (-30287.03) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value; Evolutionary analyses were conducted in MEGA X.

(tTLR1) was predicted by the SMART website tool. The obtained structure showed a typical toll-like receptor mRNA structure, as it started with a signal peptide (26 amino acids) motif from 1-26 then six repeats from leucine-rich repeat motifs (6 LRR) domains(scattered on the residues, range 65-519) and a single C-terminal LRR domain segment (LRR-CT, residues 531-582) in the extracellular region, and a TIR domain (residues 640-785) in the cytoplasmic region, as shown in Fig. 1. The encoded polypeptide (mRNA) consists of 20 different amino acids. The highest amino acid encoded is Leucine while the lowest is methionine.

Phylogenetic analysis. The phylogenetic tree was constructed using Neighbor-joining and maximum parsimony methods, on the basis of the amino acids of TLR1 downloaded from GenBank. The phylogenetic analysis was performed using the translated *Oreochromis niloticus* amino acid sequence, with almost all known amino acid sequences in the GenBank. Both phylogenetic methods showed that the Nile tilapia TLR1 is closely related to Larimichthys crocea, Epinephelus coioides, and Takifugu rubripes TLRs1, as shown in Fig. 2.

Expression of the Oreochromis niloticus TLR1. The transcription of *Oreochromis niloticus* TLR1 was evaluated in the kidneys, brain, spleen, intestines, muscle, liver, gills, heart, and skin (Fig. 3). Quantitative RT-PCR showed differences in the expression levels between the tested tissues, where the highest level was observed in the spleen, kidney, and intestines, while a moderate expression level was detected in muscle and liver, as shown in Fig. 3.

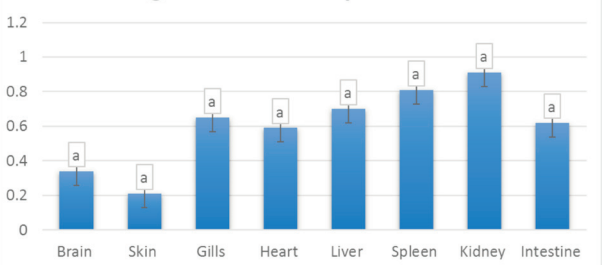

Fig. 3. Relative expression level of TLR1 in different Nile tilapia tissues

Discussion

Toll-like receptors are considered one of the main components of pattern recognition with a major role in the immune response. TLR1 is associated with recognition of tri-acylated lipoproteins and mycobacterial molecules by binding to TLR2 to form a heterodimer (JIN et al., 2007; WU et al., 2008). The findings obtained from this study are considered to be the first report (according to our knowledge) to characterize the *Oreochromis niloticus* TLR1, as most studies have focused on non-fish vertebrates. Tilapia TLR1 is considered a homologue to catfish, Japanese medaka, orange-spotted grouper, zebra fish, and other vertebrate TLR1. The structure analysis showed that the Nile tilapia TLR1 is very close to other fish TLR1 with a degree of identity of 96% with Zebra Mbuna, 88% with Sea bass, 85% with Damsel fish and Clownfish, while it showed 66% identity with electric eels, and 61% with starlets, as described in Table 2. These data are in agreement with FINK et al., 2016; HAN et al., 2018; HE et al., 2016; and LI et al., 2016. Also, these data are in accordance with a previous report regarding the Nile tilapia TLR3 (ABOUELMAATTI et al., 2013b), which showed a similar degree of identity between the same fish types when compared using the *Oreochromis niloticus* TLR3 sequence. The transmembrane structure analysis of the *Oreochromis niloticus* TLR1 showed a similar main transmembrane as in other fish models, while it has two additional leucin rich repeats (LRR) in comparison to catfish (ZHAO et al., 2013). Furthermore, the TLR1 structure of the Japanese medaka (rice fish) has one more LRR motif than Tilapia (LI et al., 2016). Tilapia TLR1 shares the same signal peptide at the beginning of the gene with Zebra Mbuna, catfish, and rice fish. (Fig. 1). These results match the model structures obtained in previous studies (LI et al., 2016; ZHAO et al., 2018). Furthermore, they are in agreement with previous reports regarding the similarity between the tilapia TLR and other bony fish models (ABOUETMAATTI, 2013; ABOUETMAATTI et al., 2013b). The Nile tilapia TIR domain consists of 143 amino acids, while the TIR domain in catfish consists of 139 amino acids (ZHAO et al., 2013), which might be due to unequal

force distribution during evolution (NIE et al., 2018). This is similar to a previous report regarding *Oreochromis niloticus* TLR3 (ABOUELMAATTI, 2013; ABOUELMAATTI et al., 2013b). Also, the *Oreochromis niloticus* TLR1 showed a unique LRR domain at the position 128-150, which is absent in rainbow trout and orange-spotted grouper structurea (LI et al., 2016), as shown in Fig. 1.; this variation may be associated with its role in the differences in the immune response against pathogens between *Oreochromis niloticus* and other fish species (NIE et al., 2018). The molecular and phylogenetic structure of Nile tilapia TLR1 in comparison to other fish TLR1 and other vertebrate TLR1 available in GenBank revealed that Tilapia TLR1 is closely related to Zebra Mbuna fish, rice fish, damselfish and sea bass, as illustrated in Fig. 2. These results matched with the studies by FINK et al., 2016 regarding carp, ZHAO et al., 2013 regarding channel catfish; WANG et al., 2013 regarding yellow croaker, and PALTI YNIV et al., 2010b regarding rainbow trout. The TLR1 mRNA expression varies among different tilapia tissues, as shown in Fig. 3, where it is highly expressed in the kidney, spleen, and intestines, and these results matching with previous data regarding the level of TLR1 expression in different fish tissues, like in zebra mbuna, yellow croaker (WANG et al., 2013), common carp (FINK et al., 2016), trout (PALTI et al., 2010b), catfish (ZHAO et al., 2013), orange-spotted grouper (LI et al., 2016), sea perch (LI et al., 2018), grass carp (HE et al., 2016) . The expression level in the kidneys, brain, spleen, intestines, liver, and muscles matched the expression of other TLRs in different animal models, such as rabbits, duck and geese (ABOUELMAATTI, 2013; ABOUELMAATTI et al., 2013b; ELFEIL et al., 2012; ELFEIL et al., 2016). In conclusion, this study provided the first report which characterizes the structure, tissue distribution and the expression level of TLR1 in *Oreochromis niloticus*. In addition, the investigation of TLR1 transmembrane structure and amino acid composition. *Oreochromis niloticus* TLR1 showed that it is considered to be completely functionally orthologous to that of other vertebrates.

Conflict of interest

All authors have no conflict of interest to declare

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SAŽETAK

Toll-like receptori (TLR) najviše su istraživana skupina receptora za prepoznavanje uzročnika bolesti u kralježnjaka. TLR u riba pokazuju jasna razlikovna svojstva, strukturu i veliku raznolikost u odnosu na druge kralježnjake. Ovo je istraživanje usredotočeno na identifikaciju i otkrivanje Toll-like receptora 1 (TLR1) u nilske

tilapije (*Oreochromis niloticus)* kao predstavnika slatkovodnih riba. Klonirana je puna sekvencija cDNA TLR1 mRNA. Utvrđeno je da se kompletna sekvencija cDNA TLR1 nilske tilapije sastoji od 2355 baznih parova i kodira polipeptid od 785 aminokiselina. Molekularna analiza sekvencija aminokiselina upućuje na to da TLR1 nilske tilapije ima standardna strukturna svojstva i glavne komponente porodice TLR receptora i smatra se ortologom, ne paralogom TLR1 kralježnjaka. Analiza prevedenih aminokiselina pokazala je stupanj identičnost od 96 % s mbuna zebrom, 88 % s lubinom, 85 % s damsel ribom i 85 % s ribom klaun, dok je stupanj identičnosti s električnom jeguljom bio 66 %, a s ribom starlet 61 %. Filogenetska analiza pokazala je da je TLR1 nilske tilapije usko povezan s TLR1 vrsta *Larimichthys crocea*, *Epinephelus coioides* i *Takifugu rubripes*. TLR1 nilske tilapije bio je izražen u bubrezima, mozgu, slezeni, crijevima, mišiću, jetri, škrgama, srcu i na koži. Kvantitativni RT-PCR pokazao je razlike u razini ekspresije među testiranim tkivima. Prema našim podacima ovo je istraživanje prvo koje donosi kompletnu molekularnu i funkcionalnu karakterizaciju Toll-like receptora 1 nilske tilapije, te se smatra funkcionalnim ortologom TLR1 u drugih vrsta.

Ključne riječi: nilska tilapija (*Oreochromis niloticus*)*;* transmembranska struktura; riba; Toll-like receptor; TLR1; urođena imunost riba