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Molecular cloning, characterization and constitutive expression analysis of *TLR4* gene in *Pangasianodon hypophthalmus*

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ABSTRACT

Toll like receptor (TLR) signalling pathway plays a central role in inflammatory responses through the recognition of PAMPs and DAMPs. Toll-like receptor 4 (TLR4) is well known for the recognition of LPS of gram-negative bacteria leading to production of inflammatory cytokines. Modulation of a TLR pathway gene in different tissues indicates the functional importance of the tissues in innate immunity. The aim of this study was to clone and characterize TLR4 and evaluate its constitutive tissue distribution in *Pangasianodon hypophthalmus,* a commercially important fish species. The study was carried out at ICAR-CIFE, Mumbai during 2017. Partial nucleotide and amino acid sequences of TLR4 were identified in *P. hypophthalmus* and termed as *ph*TLR4. Bio-informatics analysis of partial sequence revealed four putative LRR domains characteristic of TLRs. Nucleotide and amino acid sequences showed varying degree of identity with TLR4 sequences from other fish species in BLASTn and BLASTp analysis. Phylogenetic analysis placed the *phTLR4* close to *Ictalurus punctatus.* TLR4 was found to be constitutively expressed in all the tissues studied. The significantly highest expression was recorded in intestine and spleen. The lowest expression was in muscle, heart and skin. House-keeping gene *ß-actin* was used as an internal control. The outcome of this research will be helpful for studying mRNA expression levels of *ph*TLR4 under different conditions in different tissues of *Pangasianodon hypophthalmus*.

Keywords: Fish, *Pangasianodon hypophthalmus*, *ph*TLR4, RT-PCR, Tissue distribution, TLR4

Differentiation between self and non-self-molecules by host immunity results in activation of a downstream signaling cascade (Arancibia *et al.* 2007). Pattern Recognition Receptors (PRRs) predominantly expressed on sentinel cells can detect both pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (Matzinger 2002, Kawai and Akira 2009). Four classes of PRR families which include transmembrane proteins such as toll-like receptors (TLRs) and C-type lectin receptors as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-1-like receptors (RLRs) and NOD- like receptors (Takeuchi and Akira 2010) are involved in recognition of a wide array of stimuli. Different PAMPs are recognized by different TLRs. Hence, the level of each TLR mRNA in different tissues indicates the natural load of each PAMP and ability of host tissue to resist the infection, and is helpful to understand the functional relationship of TLRs and PAMPs (Dhanasekaran *et al.* 2010, Pietretti and Wiegertjes 2014).

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TLR4 is an extensively studied mammalian receptor and important in the recognition of LPS of gram-negative bacteria (Kaisho and Akira 2000) and is thought to belong to ancestral genome common to mammals and fish (Oshiumi *et al.* 2003, Leos *et al.* 2022). First TLR4 was characterized in zebrafish (*D. rerio*) (Jault *et al.* 2004) and subsequently in a few more fish species**.** So far, 20 different TLRs have been identified in Channel catfish *(Ictalurus punctatus)* (Zhang *et al.* 2013). However, the repertoire of TLRs in *Pangasianodon hypophthalmus* which is a commercially important fish in many countries including India (Nguyen *et al.* 2009, Lakra and Singh 2010, FAO 2011, Sugunan *et al.* 2016) remains to be explored.

Very little information is available (Uma and Rebecca 2017, Ghode *et al.* 2018) about the nature of TLR4 and its tissue distribution in *P. hypophthalmus*. Hence, this study was undertaken with the objective to characterize TLR4 and to study the constitutive expression of TLR4 in different tissues by quantitative real-time PCR (qRT-PCR).

MATERIALS AND METHODS

Fish and tissue sampling: Fingerlings of apparently healthy, *P. hypophthalmus* with an average body weight of 30±5.0 g used in the experiment were collected from Indepesca Aquaculture Pvt. Ltd., Dhasai, Maharashtra,

India. The fishes were acclimatized in the wet laboratory of ICAR-CIFE, Mumbai for 15 days in 500 L capacity container at 28-30ºC. Fishes were fed twice daily at 3% body weight with a commercial pelleted diet. For cDNA cloning, kidney tissue was collected from fingerling fish and immediately processed for total RNA extraction. To study the constitutive expression of TLR4 by qRT-PCR, RNA was isolated from 10 different tissues of three separate fish. Tissues included brain, gills, heart, liver, kidney, spleen, skin, muscle, intestine and blood. Individual tissues from three fishes were pooled together and used for total RNA extraction for getting average values of constitutive expression as value from single fish may not represent constitutive expression for the species appropriately.

Total RNA extraction: Total RNA was extracted individually from different tissues using Trizol™ reagent (Invitrogen, USA) following the manufacturer's instructions.

Evaluation of total RNA quality: Quality of total RNA after DNAseI treatment was assessed on denaturing agarose gel electrophoresis. The 28S:18S ratio was similar to 2:1 and thus indicated good integrity of total RNA. OD260/280 was found to be 1.99. This indicated that the RNA was highly pure and meets the requirements of RT-PCR.

DNAseI treatment and RT-PCR analysis: About 2 µg of total RNA was taken from each tissue and incubated separately with DNAseI (Thermo Scientific, USA) to remove genomic DNA, for 30 min at 37ºC. The reaction was stopped by adding 1 µl of 50 mM EDTA and incubating at 70ºC for 10 min. The purity of total RNA was checked by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA) at OD of 260/280. Uniform quantity of DNAseI treated RNA (1 µg) from each tissue was used for the synthesis of first-strand cDNA. RevertAid™ reverse transcription First Strand cDNA synthesis kit (Thermo Scientific, USA) and oligo-dT primers were used following the manufacturer's instructions. Synthesized cDNA was subjected to RT-PCR using gene-specific primers designed for TLR4. β-actin gene was used as reference gene. Primers were designed from highly conserved regions of sequences of closely related fish species, retrieved from the GenBank database (www.ncbi.nlm.gov.in). Primer sequences and temperature protocol used for RT-PCR of TLR4 and Beta-actin in this study are given in Table 1.

Constitutive expression of TLR4 in healthy fish: Real-time PCR amplification for analysing tissue specific distribution of TLR4 were carried out in LightCycler® 96 Real-Time System (Roche, Switzerland), in 96-well plate with a total volume of 10 µl containing 2× SYBR green super mix (Thermo Scientific, USA), diluted cDNA and primer pairs (10 pM final concentration). The temperature protocol for qPCR and primers used for qPCR in this study are given in Table 1. β-actin was used as a reference gene.

For quantification of TLR4 gene expression, the cycle of threshold (Ct) was determined. According to the ΔΔCt method (Schmittgen and Livak 2008), the relative expression ratio (RE) was calculated in relation to the expression of reference gene and was defined as relative expression of a TLR4 gene. First, the C_T of the TLR4 gene was normalized to that of the β-actin and the calibrator sample. Gill tissue was used as calibrator.

Molecular cloning of TLR4 gene: The desired DNA fragments (923 bp) were cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific, USA) following manufacturer's protocol. PCR product was mixed with the cloning vector in 3:1 proportion of molar ends in 1× ligase buffer. The ligation mixture was prepared by adding 6 µL of 5× Ligation buffer, 3 µL of pTZ57R/T vector, 4 µL of purified PCR fragment, 1 µL of T4 DNA Ligase (5 units) and making the final volume up to 30 µL and incubated at 16°C overnight. The vector used for the transformation was DH5α strain of *E. coli.* Competent cells (DH5α) were prepared according to the supplier's protocol described in the InsTAclone™ PCR Cloning Kit (Thermo Fisher Scientific Manual, USA). The cells were spread plated on the pre warmed X-gal-IPTG and Ampicillin added LB agar plates and incubated overnight at 37°C. Recombinant colonies were identified on the basis of blue –white selection and confirmed by colony PCR.

GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA) was used for plasmid extraction from recombinant clones of DH5α. Manufacturer's instruction were followed for the same. Briefly, harvested bacterial culture was lysed and centrifuged, and supernatant applied to silica column for selective DNA binding. The adsorbed

Table 1. Primer sequences and cycling temperatures used in this study

Target gene	Primer	Primer sequence (5'-3') and Cycling temperatures	Amplicon size (bp)
TLR4 for RT-PCR	F4TLR4 R4TLR4	TTG CTG ATG ACG CTT TCC ACA ATG GGG TAT GAA TGT TTG GCA TCC CTG One cycle of 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 sec, 54ºC for 30 sec and 72ºC for 45 sec, and final extension for 72ºC for 10 min	923
TLR4 for qPCR	F6TLR4 R6TLR4	TTATAGGGCATCCAGACAGCAGGAA TGTTAGCGTTGCCTTGCATCACTAGC One cycle of 95ºC for 30 sec followed by 45 cycles of 95ºC for 20 sec, 59ºC for 30 sec and 72ºC for 30 sec	280
β-actin	F Beta-actin R-Beta actin	GCC GAG AGG GAA ATT GTC CGT GAC TTG CCA ATG GTG ATG ACC TGT CCG One cycle of 95ºC for 30 sec followed by 45 cycles of 95ºC for 20 sec, 59ºC for 30 sec and 72ºC for 30 sec	143

DNA washed and eluted in elution buffer. The extracted plasmids were sequenced following Sanger sequencing using commercial services (Eurofins Genomics India Pvt. Ltd., Bengaluru, India).

Bioinformatics analysis: Sequence assembly was performed using online DNA Baser Sequence Assembly Software (www.dnabaser.com). Assembled sequence was subjected to SMART analysis (http://smart.embl-heidelberg.de/) for the identification of protein domains. Clustal Omega multiple sequence alignment program was used to align the sequence with the reference sequence (accession no. AEI59666.1). BLASTn and BLASTp analysis (www.ncbi.nlm.in) of *phTLR4* sequence were performed to confirm the identity and homology of the sequence. Online ExPasy translate tool (http://www.expasy.org/tools/) was used to deduce corresponding amino acid sequence. Phylogenetic analysis of deduced amino acid sequence was done with MEGA 7.0 software according to Neighbor-Joining method using the Poisson correction distance model.

Ethics statement: The research undertaken is in accordance with the present animal welfare laws in India. The study was approved by the Board of Studies and Authorities of the ICAR-Central Institute of Fisheries Education (Deemed University), Mumbai. As the experimental fish, *P. hypophthalmus* is not an endangered fish, the rule of the Govt of India's Wildlife Protection Act of 1972 is not applicable for experiments on this fish.

RESULTS AND DISCUSSION

PCR amplicons: RT-PCR using *P. hypophthalmus* cDNA and primers designed for cloning and tissue distribution analysis resulted in amplicons of expected sizes as indicated in Table 1.

Editing and assembly of cloned TLR4 sequence resulted in the 702 bp (Gene Bank Accession: MH166727). The nucleotide sequence and deduced amino acid sequence are represented in Fig.1.

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  1 S  L  H  K  L  Q  R  L  V  L  V  D  I  G  L  L  S  L  N  V
  1 TCATTACATAAATTACAGAGATTAGTTCTCGTGGACATTGGCCTGCTGTCTTTAAATGTG
 21 Q  F  N  N  L  T  K  L  Q  E  L  K  A  G  T  N  K  I  Q  T
 61 CAGTTCAACAATCTCACCAAGCTTCAGGAACTAAAAGCTGGAACTAACAAGATTCAAACA
 41 I  A  L  P  P  F  M  I  N  F  K  D  F  C  V  L  D  L  H  A
121 ATTGCTCTTCCACCATTCATGATCAACTTTAAAGACTTCTGCGTACTGGACCTGCATGCA
 61 N  N  I  S  R  I  K  V  N  H  T  A  V  L  R  E  M  R  G  N
181 AATAACATATCCAGAATAAAAGTAAATCACACGGCTGTACTGCGAGAGATGAGGGGAAAT
 81 I  T  L  I  P  S  S  N  P  I  L  H  I  E  P  G  V  F  K  D
241 ATCACTCTAATACCCTCCAGCAACCCAATATTACATATCGAACCAGGAGTATTTAAGGAC
101 I  Y  L  K  E  L  N  I  Q  S  A  F  V  S  F  D  A  M  R  Y
301 ATTTACCTAAAAGAGCTTAACATACAGAGTGCTTTCGTTTCATTTGATGCAATGAGGTAT
121 G  L  K  A  L  S  G  L  N  V  G  K  L  I  I  G  N  Y  I  I
361 GGTCTAAAAGCTCTCAGTGGTCTTAATGTTGGAAAACTCATTATAGGAAACTACATAATA
141 D  K  N  I  K  I  S  D  A  D  F  L  D  G  L  C  L  I  N  F
421 GACAAGAATATAAAGATATCAGATGCTGATTTTCTTGACGGTCTCTGTTTGATCAATTTT
161 N  E  L  Y  F  F  Q  K  Q  H  S  D  Y  E  I  N  V  F  H  C
481 AATGAATTATATTTTTTTCAAAAACAACATTCAGATTATGAAATCAATGTGTTCCACTGC
181 M  I  N  A  T  K  I  T  L  K  E  V  H  I  G  A  I  Q  H  V
541 ATGATCAATGCAACAAAAATCACTCTGAAAGAAGTCCATATAGGAGCCATTCAGCATGTC
201 S  F  R  Q  L  K  E  L  Y  M  Q  H  N  Q  L  P  L  I  L  E
601 TCATTCCGTCAGCTTAAAGAACTTTACATGCAGCATAACCAATTGCCCCTCATACTGGAA
221 I  S  H  L  H  L  L  E  K  L  V  V  V  T
661 ATTTCACATCTACACTTGTTAGAAAAACTAGTGGTGGTTACA
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Fig. 1. Nucleotide and deduced amino acid sequence of TLR4 cDNA of *Pangasianodon hypophthalmus*. Upper case black letters indicate nucleotide sequence, blue letters indicate deduced amino acids.

The occurrence of TLR4 gene in fish species has been considered to be very limited (Roach *et al.* 2005). With the first report from zebrafish (Jault *et al.* 2004), TLR4 has been subsequently identified and characterized in a very few species such as rare minnow (Su *et al.* 2009), common carp (Kongchum *et al.* 2010), grass carp (Huang *et al.* 2012), channel catfish (Quiniou *et al.* 2013) and blunt snout bream (Lai *et al.* 2016, Tang *et al.* 2016), Samanta *et al.* (2017) in rohu (*Labeo rohita*) and recently by Chen *et al.* (2020) in Yellowfin catfish. So, the identification of TLR4 in *P. hypophthalmus* in the present study indicates the possibility of a wider distribution of TLR4 in fishes. Uma and Rebecca (2017) had identified a partial sequence of TIR domain of TLR4 from *P. hypophthalmus*. However, TIR is highly conserved across TLRs and also present in adaptor molecules (Zhang *et al.* 2003, Jault *et al.* 2004, Meijer *et al.* 2004). Further, TLRs are distinguished on the basis of variations in sequence composition and length of consensus sequence of LRRs. Hence, cloning and characterization of partial TLR4 gene sequence in this study from variable LRR domain further confirms the presence of TLR4 in *P. hypophthalmus*. The results will be helpful for characterization of complete TLR4 gene sequence in *P. hypophthalmus.*

Molecular characterization of partial cDNA of phTLR4: Amplified partial cDNA of 702 bp towards 5' end of the coding region was translated into 234 amino acid residues all contained in an open reading frame. The homology search using BLASTn showed that *phTLR4* shares 90% identity with TLR4-1 and TLR4-2 of *Ictalurus punctatus.* BLASTp showed the homology of 86%, 85%, with TLR4-1 and TLR4-2 sequences of *I. punctatus* respectively and 61%, 60%, 58% homology with TLR4b of *Cyprinus carpio*, TLR4.4 of *Ctenopharyngodon idella,* and TLR4b of *Danio rerio* respectively. It also revealed 56% and 55% homology with TLR4 and TLR4a respectively of *Megalobrama amblycephala.*

Four LRR motifs were predicted in the BLASTp analysis. LRR motifs were at following locations 5-27aa residues (LQRLVLVDIGLLSLNVQFNNLTK), 28-52aa residues (LQELKAGTNKIQTIALPPFMINFKD), 53-79aa residues (FCVLDLHANNISRIKVNHTAVLREMRG) and 79-101aa residues (NITLIPSSNPILHIEPGVFKDI).

The TLR4 sequence elucidated in the present study showed similarity to TLR4.1 gene isoform of channel catfish. Orthologs have been found to be conserved across species with respect to DNA and protein sequences; however, there can be differences in functional essentiality (Liao and Zhang 2008). Basu *et al* (2016) indicated involvement of TLR4 in stress related inflammatory response in catla fish. This assumes importance in fish as fish TLR4 is hypothesised to be incapable to recognise LPS molecule of gram-negative bacteria which is a prominent ligand of TLR4 in mammals (Sullivan *et al.* 2009). But recent study on zebrafish (Loes *et al.* 2021) has indicated possibility of existence of TLR4/Md-2 complex in zebrafish with low

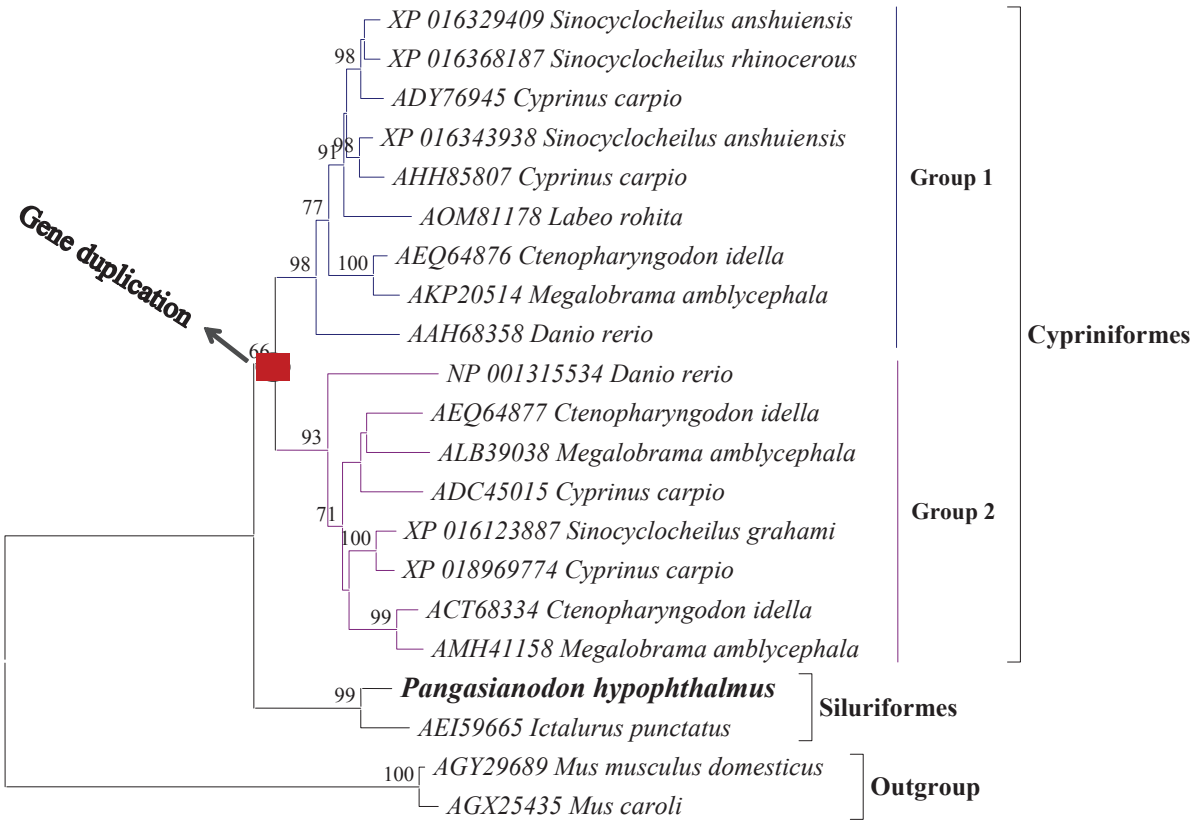

Fig. 2. Phylogenetic tree inferred from the amino acid sequence data of TLR4-1 depicting the phylogenetic placement of *P. hypophthalmus*. The tree also showed the evidence of gene duplication within cypriniform fishes. Mus TLR-4 sequences were used as outgroup. MEGA 7.0.25 reconstructed the phylogenetic tree according to Neighbor-Joining method using the Poisson correction distance model. Bootstrap values (> 60) are shown at the base of the nodes. Species from which amino acid sequences are used are indicated in the diagram.

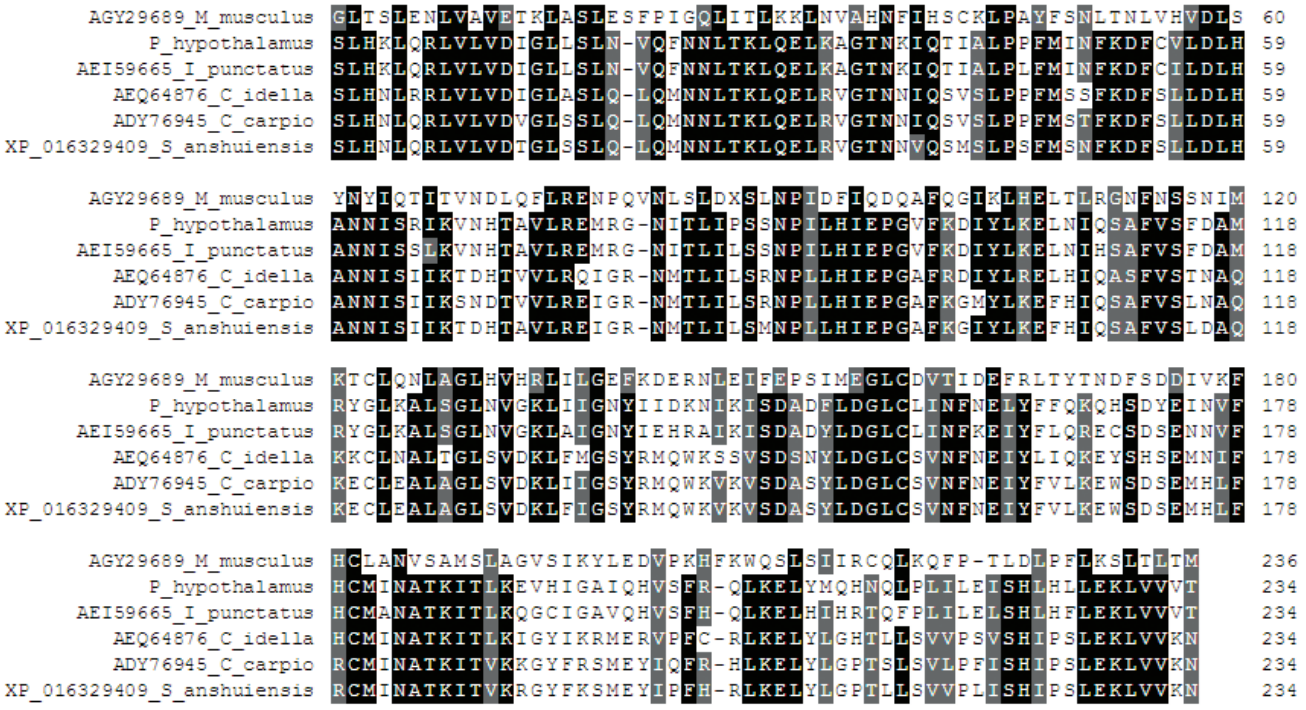

Fig. 3. Multiple amino acid alignment of *phTLR4* with selected fish and mice TLR4 sequences.

level sensitivity to LPS. This indicates possibility of fish species possessing ancestral TLR4. One to four isoforms of TLR4 gene have been identified in different fish species such as grass carp, mrigala, rare minnow Jault *et al.* 2004 zebrafish, channel catfish, blunt snout bream and rohu (Su *et al.* 2009, Huang *et al.* 2012, Basu *et al.* 2013, Zhang *et al.* 2013, Lai *et al.* 2016, Tang *et al.* 2016, Samanta *et al.* 2017, Chen *et al.* 2020). So, a complete characterization and detailed study of the *ph*TLR4 sequence will be helpful in identifying ligand specificities of each isoform.

TLR4 is represented by three domains viz. extracellular ectodomain, transmembrane region and cytoplasmic TIR domain (Samanta *et al.* 2017, Chen *et al.* 2020). The *ph*TLR4 sequence in this study represents extracellular LRR domains wherein four putative LRR domains have been identified in protein blast of NCBI.

Phylogenetic analysis: Phylogenetic analysis was conducted using ingroup protein sequences of Cypriniformes, and Siluriformes. *Mus musculus domesticus* and *Mus caroli* were outgroup protein sequences. *phTLR4* was clearly placed closer to siluriform, *I. punctatus*. Among Cypriniformes, gene duplication event has also been depicted. The phylogenetic tree as inferred above is shown in Fig. 2. Multiple amino acid alignment of *phTLR4* with selected fish and mice TLR4 sequences showed considerable conservation as shown in Fig. 3**.**

Phylogenetic analysis and multiple alignments are important bioinformatics tools for identification of naive sequences (Roach *et al.* 2005). In the present study, phylogenetic analysis clearly placed *ph*TLR4 close to siluriformes TLR4.1 gene isoform of channel catfish, whereas TLR4 sequences from cypriniformes were placed in different clad and mammalian sequences were represented as outgroup sequences. Multiple alignments also showed considerable conservation with TLR4 sequences of other fish species. Thus, cloned sequence from *P. hypophthalmus* can be said to similar to TLR4-1 isoform. However, functional characterization is also important as sequence homology may not predict functions adequately (Sullivan *et al.* 2009).

Constitutive expression of phTLR4 in healthy P. hypophthalmus: TLR4 gene was found to be constitutively expressed in all studied tissues with a differential expression pattern (Fig. 4).

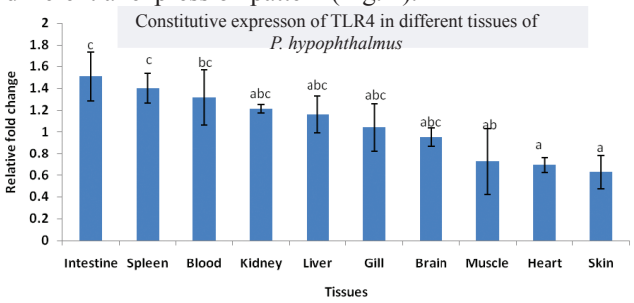

Fig. 4. Relative expression levels of TLR4 mRNA in healthy *P. hypophthalmus*. Each bar represents the mean value from three determinations with the standard error (SE). Data (mean±SE) with different letters significantly differ (Duncan test, $p<0.05$).

The highest expression was found in intestine (1.5 fold) relative to calibrator gill (1.04), followed by spleen (1.4 fold), blood (1.31 fold), kidney (1.2), and liver (1.16). Expression in brain (0.95), muscle (0.72), heart (0.67) and skin (0.62) was low as compared to the calibrator. Thus, constitutive expression levels of TLR4 were significantly higher in immune related tissues. Thus, the pattern of high constitutive expression of *ph*TLR4 in immune related and digestive organ and general low expression in muscle as depicted in this study is in agreement with earlier studies (Jault *et al.* 2004, Su *et al.* 2009, Huang *et al.* 2012, Basu *et al.* 2013, Swati *et al.* 2013, Basu *et al.* 2016, Samanta *et al.* 2017, Chen *et al.* 2020, Loes *et al.* 2021). Swati *et al*. (2013) reported highest expression of TLR4 in immune tissues such as liver, spleen and kidney of *Catla catla*. Pei *et al.* (2015) have reported high expression of TLR4.1 in heart, brain and liver, and low in kidney and intestine. Lai *et al.* (2016) has reported high levels of TLR4α in blood, skin, heart and skin, and low levels in head and trunk kidney and intestine of Wuchang bream. These differences in expression pattern might be indicating species-specific differences in tissue distribution of TLR4.

Partial sequence of TLR4 of *Pangasianodon hypophthalmus* designated as *phTLR4* was successfully cloned and sequenced. The present study also measures a basal mRNA expression of TLR4 gene in different tissues. The results of this study will be useful for full characterization and annotation of TLR4 in the pangasius species which can be utilized to find out ligand specificities of TLR4 and to device strategies to prevent infections due to gram-negative bacteria.

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