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Characterization of *CLCN5* and Expression Profile under Low-Salinity Stress in *Takifugu rubripes*

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JLJ and MGM designed the study. RTL, YQT, QQZ and JYC collected specimens. LY and JX conduct the research work. LY wrote the manuscript. JLJ and MGM revised the manuscript.

Key words
Low-salinity stress, mRNA expression, *Takifugu rubripes*, Voltage-gated chloride channel 5

ABSTRACT

Voltage-gated chloride channel 5 (CLCN5) transports hydrogen ions into the nucleolus and removes chloride ions, which helps the cell to maintain its pH value and osmotic pressure. To reveal the role of *CLCN5* under low-salinity stress, a cDNA sequence of *CLCN5* (*TrCLCN5*) was cloned from *Takifugu rubripes* using the reverse transcription PCR (RT-PCR) technique. The results showed that *TrCLCN5* cDNA contained a complete open reading frame of 2505 bp continuously encoding 834 amino acid residues. Sequence alignments revealed that the *TrCLCN5* cDNA is highly homologous (96.2% and 96.1%) with that of *Takifugu bimaculatus* and *Takifugu flavidus*. SignalP and TMHMM analysis showed that *Tr*CLCN5-deduced protein was a type III transmembrane protein and lacked a typical signal peptide. Conserved Domain analysis revealed that there was a Voltage_CLC domain and two CBS domains located in the *Tr*CLCN5 deduced protein. qPCR analysis showed that *TrCLCN5* was highly expressed in the intestine, kidney and liver, and up-regulated in gills under low-salinity stress in 3h to 6h, which indicates that *TrCLCN5* may play an important role in the responses to acute low-salinity stress and would be useful in further understanding low-salinity adaption in *T. rubripes*.

INTRODUCTION

The chloride ion is the most abundant anion in organisms and is an essential participant in various physiological processes (Ji *et al*., 2016). Chloride channels are important ways for chloride ions to pass through the cells. Chloride channels are a superfamily of poorly understood ion channels specific for chloride (Jentsch *et al*., 2002). Chloride channels are widely distributed on the organelle membrane and plasma membrane. Based on sequence homology, chloride channels can be subdivided into a number of groups including voltage gated chloride channels (CLCs), epithelial chloride channels (E-ClCs), chloride intracellular ion channels (CLICs), cystic fibrosis transmembrane conductance regulators (CFTRs), GABAA receptors (GABAARs), glycine receptors (GlyRs or GLRs), and calcium dependent chloride channel (Ca-ClC) proteins (or calcium-activated chloride channels (CaCCs) (Nilius and Droogmans, 2003; Zhang *et al*., 2004). CLCs display a variety of important physiological and cellular roles that include regulation of pH, volume homeostasis, organic solute transport, cell migration, and cell proliferation and differentiation, and are involved in many physiological processes, including stable membrane potential, signal transduction, and trans cellular transport, which have important physiological and pathological significance (Cromer *et al*., 2002). Voltage gated chloride channel 5 (CLCN5) is an important member of the CLC family. The CLCN5 protein is responsible for the transport of ions through the membrane structure in the cell. In particular, the CLCN5 protein can transport intracellular chloride ions out of the cell and transport extracellular hydrogen ions into the cell. Based on this function, CLCN5 is also known as an H^+/Cl^- exchanger (Scheel *et al*., 2005). CLCN5 proteins were initially found in the kidneys; especially in the renal tubule where lost nutrients, ions, and water were reabsorbed from the bloodstream, CLCN5 is expressed at a high level. The kidney reabsorbs the required material into the blood and drains other unwanted substances into the urine. In the renal tubule, the CLCN5 protein is entrapped in the nuclear endoplasmic complex. The nuclear endoplasmic complex is attached to the cell surface and is responsible for transporting proteins and

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other molecules (Zifarelli, 2015).

So far, studies of CLCN5 have been focused on mammals (Gunther *et al*., 1998; Gabriel *et al*., 2017; Satoh *et al*., 2017; Zhang *et al*., 2017), and there are only a few reports about CLCN5 in fish (Wang *et al*., 2015; Gibbons *et al*., 2017). Information available concerning the role of CLCN5 in fish is very limited. *Takifugu rubripes* is an important fish economically and can survive normally in hypoosmotic conditions with salinity levels as low as 4, but the underlying mechanism is still unclear. In our previous study, a *CLCN5* cDNA fragment was screened from the *T. rubripes* gills transcriptome under low-salinity stress (Jiang *et al*., 2020). To further study the role of *CLCN5* under low-salinity stress, a cDNA sequence with a complete open reading frame (ORF) was cloned and characterized in *T. rubripes* (designated as *TrCLCN5*). The expression patterns of *TrCLCN5* in various tissues were examined and the molecular regulations of *TrCLCN5* mRNA expressions under low-salinity stress were further defined.

MATERIALS AND METHODS

Fish maintenance and sample collection

Juveniles *T. rubripes* with body length of 7.18±0.34 cm and body weight of 11.34±2.13 g was obtained from the Dalian Tianzheng Industrial Co., LTD and kept in tanks with 1000-liter aerated seawater for at least one week at 25.03±0.42°C. Fish were fed twice daily (8:00 and 16:00) with fresh bait of 2%-4% body weight. Feeding was stopped when the experiment began.

Six fish were anesthetized with tricaine methanesulfonate-222 (MS-222) (50 mg/L) (Gelin fucheng Biotech, Beijing, China). Tissues including gill, kidney, intestine, muscle, brain and liver were collected and all tissue samples were rapidly frozen in liquid nitrogen and stored at -80°C.

RNA extraction and cDNA synthesis

Total RNA was isolated from the gills using Trizol® reagent according to the manufacturer's instructions, and contaminating genomic DNA was eliminated using RQ1 RNase-free DNase (Promega, USA) following the manufacturer's protocols. The RNA integrity was checked using 1.2% agarose electrophoresis and the concentration was measured using the Nanodrop NV3000 micro-spectrophotometer (Wastech Inc., USA). RNAs with OD260/OD280 ranging from 1.8 to 2.0 were further used. A total of 1 μg of RNA was used for reverse transcription to synthesize the first-strand cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China).

Cloning of the TrCLCN5 *cDNA*

The gene-specific primers were designed using Primer Premier 5.0 according to the known sequence of the *CLCN5* cDNA fragment from the transcriptome (Table I) and synthesized by Sangon Biotech Co. LTD. Based on the first-strand cDNA synthesis, the cDNA sequence of *TrCLCN5* was obtained using the RT-PCR technique. The PCR products were purified using the SanPrep Column DNA Gel Extraction Kit and cloned into the pMD18-T vector, propagated in *Escherichia coli* DH5α. The positive clones were identified with colony PCR with M13 primers (F: 5'-TGTAAAACGACGGCCAGT-3' and R: 5'-CAGGAAACAGCTATGACC-3'), and sequenced at Sangon Biotech Co. LTD.

Table I. Primers used for cDNA cloning and quantitative real-time PCR.

Gene name	Primer sequence (5'-3')	Purpose
TrCLCN5	F1: TTATCTGCTTTCGCTCGGAC	cDNA cloning
	R1: GTTGAAGTCCTCGTAGGTGCCCAG	
	F2: ACCGTGGTTACCTTTGGCAT	
	R2: TCAGTTGAAGAGAATGGAGTCG	
	qF: ACCGTGGTTACCTTTGGCAT	qPCR
	qR: ACCCTTTGAAGATGAGCCCG	
β-actin	qF: ATCCGTAAGGACCTGTATGC	qPCR
	qR: AGTATTTACGCTCAGGTGGG	

Sequence analysis

The nucleotide sequence of the *TrCLCN5* cDNA sequence and deduced amino acid sequence were analyzed in the BLAST program at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/blast/). Sequences were assembled using DNAMAN. The ORF was identified with ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The physical and chemical parameters of the deduced protein, and the computed parameters including the molecular weight and theoretical isoelectric points (pI) were analyzed with the DNAssist program version 2.2. Signal peptide and transmembrane domains are predicted using the Signal P (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) server. The functional domain and important sites of the protein were predicted by the Conserved Domain web server (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The genetic distance and genetic similarity of the CLCN5 was analyzed using DNAStar (Supplementary Table I). The phylogenetic tree was constructed according to amino acid sequences of the

selected CLCN5 using the neighbor-joining (NJ) method in MEGA 5.0 software and bootstrap analysis (1000 times) to evaluate the reliability of the phylogenetic tree (Saitou and Nei, 1987).

Tissue expression of TrCLCN5

Total RNAs were extracted separately from tissues including gill, kidney, intestine, muscle, brain, and liver, and the first-strand cDNAs were synthesized and treated according to the method mentioned above. The relative levels of *TrCLCN5* mRNA transcripts in different *T. rubripes* tissues were analyzed with quantitative real-time PCR (qPCR) using the Applied Biosystems 7500 Real-time System (Applied Biosystems, USA). The qPCR procedure was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, respectively. Three independent biological replicates were carried out, and the dissociation curve of the amplicon was analyzed to confirm that there was only PCR product in each reaction. The gill was used as the control tissue. The *β-actin* gene was used as the internal reference gene (Sun *et al*., 2017). The primer sequences for *TrCLCN5* and *β-actin* are listed in Table I. After being normalized to the *β-actin* gene, the relative expressions of the *TrCLCN5* mRNA were calculated with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Experimental design of low-salinity stress

Before the low-salinity stress experiment, 12 identical tanks with 200-liter water were prepared for the low-salinity groups (salinity 16, 12, 8 and 4) with 3 tanks for each group, and 3 tanks were prepared for the control group (salinity 32). Low-salinity water was formulated from seawater and aerated tap water.

Experiment 1: First, fish were randomly divided into two groups. One group was reared under low-salinity conditions (salinity 4), while the other group was kept in water with salinity 32 as a control group. Both groups were kept in 200-liter water with 16 fish in each aquarium. The experiments were carried out with 3 replications. Fish (n=6) were randomly collected from three identical tanks and sampled at different time points (3h, 6 h, 12 h, 24 h, 48 h, 72 h). Gills were collected from each fish and rapidly frozen in liquid nitrogen and stored at -80°C.

Experiment 2: At first, fish were randomly divided into five groups. Four groups were reared in water with salinity of 16, 12, 8, and 4, respectively, while the other was kept in water with salinity 32 as a control group. The rearing conditions were the same as Experiment 1. According to the results of experiment 1, fish (n=6) were randomly collected from three parallel tanks and sampled at 3 h. Gills were collected and stored at -80°C as mentioned in Experiment 1.

Expression of TrCLCN5 *after low-salinity stress*

Total RNAs from gills collected in Exp1 and Exp2 were extracted. qPCR was carried out, and the mRNA expression of *TrCLCN5* after low-salinity stress was determined and calculated as described above.

RESULTS

TrCLCN5 *cDNA cloning and sequence analysis*

The nucleotide and deduced amino acid sequence of *TrCLCN5* cDNA are shown in Figure 1. The *TrCLCN5* cDNA sequence was submitted to GenBank (accession no. MH203229). The *TrCLCN5* cDNA was 2792 bp in length and contained a complete ORF of 2505 bp that encoded an 834-amino acid polypeptide (GenBank accession no. QAU56837.1) with a predicted molecular mass of 91 kDa and a theoretical pI of 6.2. The deduced amino acid sequence of *Tr*CLCN5 lacked a typical signal peptide as predicted by the SignalP Server. Results of the TMHMM web server analysis showed that the predicted protein had 10 transmembrane structures, indicating that it was a type III transmembrane protein (Fig. 2A). Conserved Domain web server analysis revealed that there was a Voltage_CLC domain located from position 232-637 amino acid, and two CBS domains located from position 681-731 amino acid and 772-821 amino acid, respectively (Fig. 2B).

The deduced amino acid sequence of *Tr*CLCN5 was highly homologous with those of other representative species including 23 teleosts, 2 amphibians, 2 aves, 2 reptiles and 3 mammals (Supplementary Table I). Searching for sequence similarities revealed that CLCN5 shared more than 70% similarity in all the matches. *Tr*CLCN5 presented the highest similarity with two other species of teleosts, sharing 96.2% and 96.1% similarity with *Takifugu bimaculatus* (TNM93685.1) and *Takifugu flavidus* (TWW63544.1), respectively (Supplementary Table I). To examine the relationship between various CLCN5 sequences, phylogenetic trees were generated using MEGA 5.0 NJ methods (Fig. 3). The resulting phylogenetic tree was composed of two major large clusters, including the five major groups: teleosts, mammals, reptiles, aves and amphibians. *Tr*CLCN5 was divided into the teleosts cluster.

Expression of TrCLCN5 *in different tissues*

TrCLCN5 mRNA expressions were quantified in different tissues with qPCR with *β-actin* as an internal reference gene and expressed values with the mean of the gill set as 1 (Fig. 4). *TrCLCN5* mRNA was expressed in all the tissues examined, and the relative expression level

was from high to low in intestine > kidney > liver > brain > muscle > gill. The expression of *TrCLCN5* mRNA in gill was significantly lower than other tissues ($p < 0.05$).

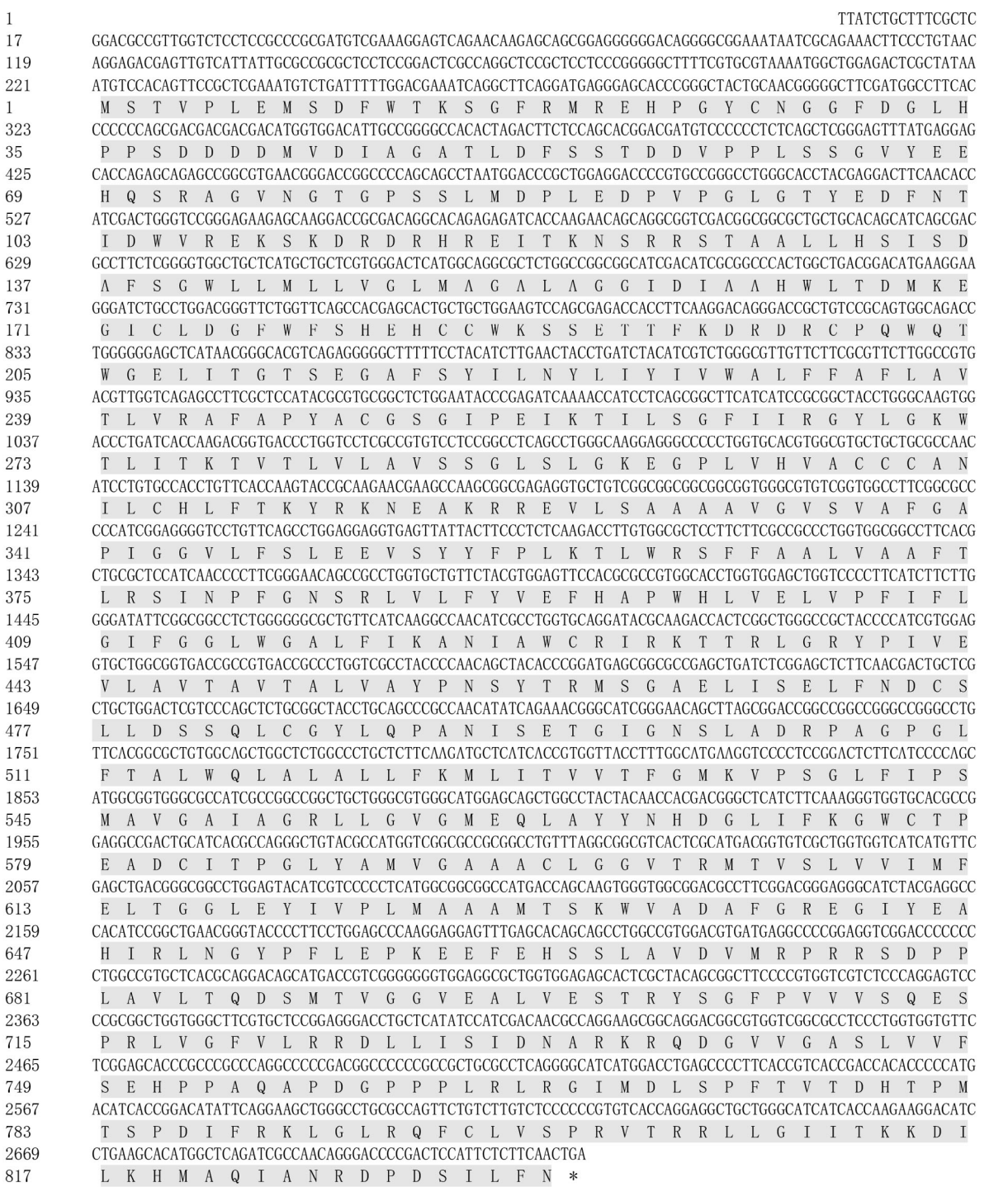

Fig. 1. Nucleotide sequence of the *TrCLCN5* gene and its deduced amino acid sequence from *Takifugu rubripes*.
The amino acid sequence is shown with one letter codes below the nucleotide sequence in shadow. The initiation codon is underlined, and the stop codon is indicated with an asterisk.

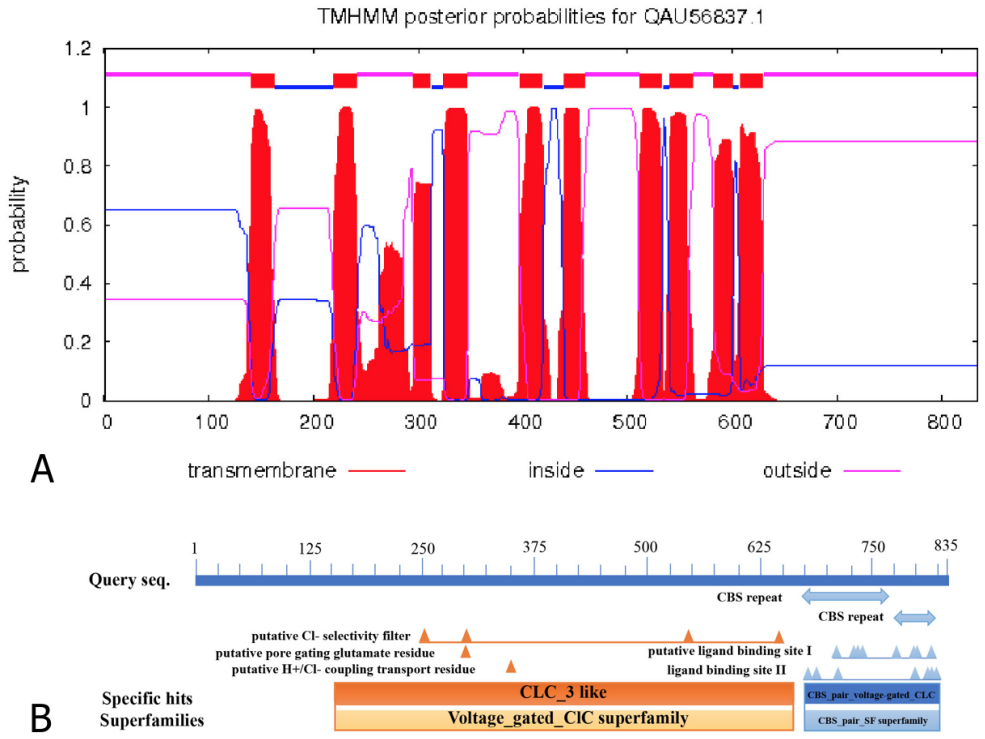

Fig. 2. Transmembrane structures and functional domains in *Tr*CLCN5. A: Transmembrane structures in *Tr*CLCN5 detected using the TMHMM web server; B: Functional domains in *Tr*CLCN5 detected using the conserved domain database web server.

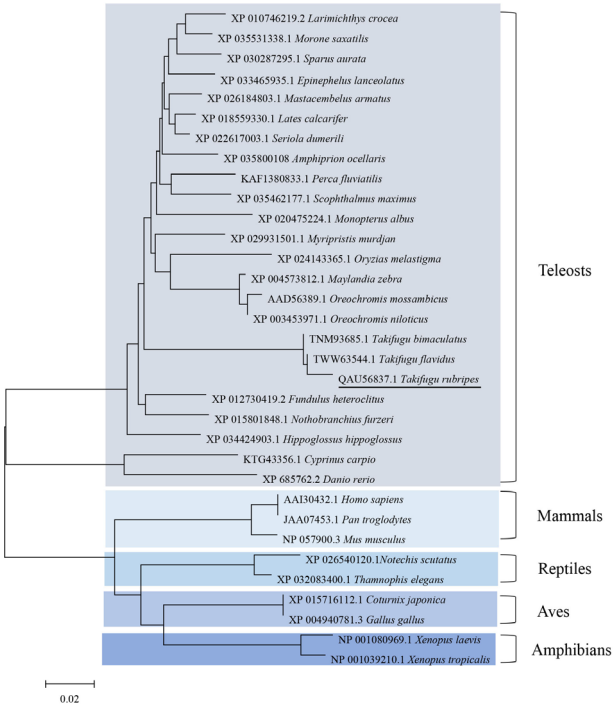

Fig. 3. Neighbor-joining phylogenetic tree of TrCLCN5 amino acid sequences and other representative species.

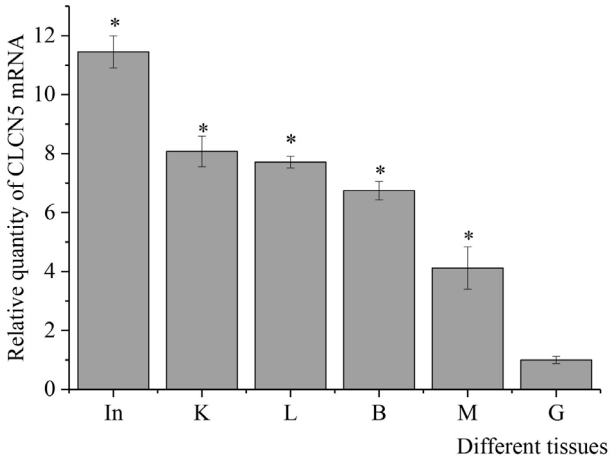

Fig. 4. Relative expression level of *CLCN5* mRNA in different tissues of *Takifugu rubripes* by quantitative real-time PCR analysis. The tissues include gill (G), liver (L), brain (B), muscle (M), intestine (In), and kidney (K). After normalization to *β-actin*, relative expressions of *TrCLCN5* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± SD. The * indicates significant differences ($p < 0.05$).

Expression of TrCLCN5 *mRNA under low-salinity stress*

In our previous study, a *CLCN5* cDNA fragment was

screened from the *T. rubripes* gill transcriptome under low-salinity stress (Jiang *et al*., 2020). In this study, *TrCLCN5* mRNA expression levels in gills that responded to low-salinity stress at different time points were further detected. When compared to the control group, *TrCLCN5* mRNA in the group cultured under salinity 4 was significantly up-regulated at 3 h, and lasted for 6 h, then down-regulated from 12 h to 72 h (Fig. 5).

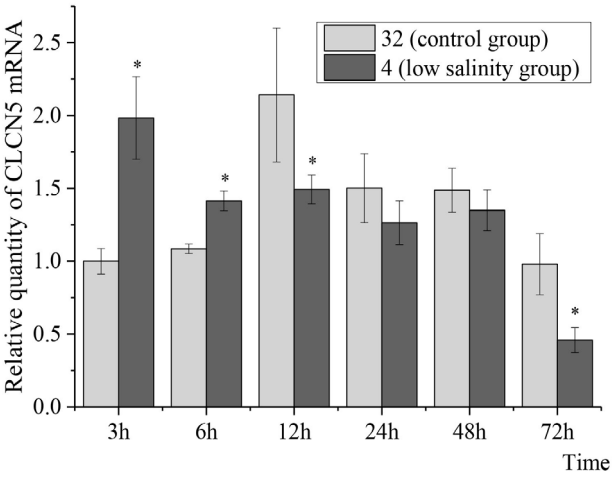

Fig. 5. Relative expression of *TrCLCN5* mRNA in *Takifugu rubripes* gills under low-salinity stress at different time points. After normalization to *β-actin*, relative expressions of *TrCLCN5* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± SD. The * indicates significant differences from the control group ($p < 0.05$).

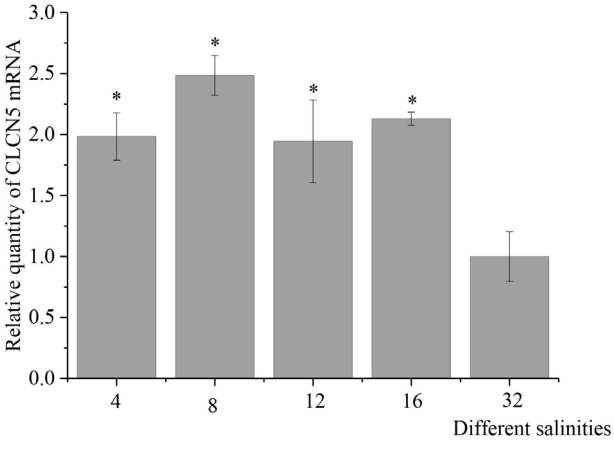

Fig. 6. Relative expression of *TrCLCN5* mRNA in *Takifugu rubripes* gills after 3 h of low-salinity stress. After normalization to *β-actin*, relative expressions of *TrCLCN5* mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. Bars represent the mean ± SD. The * indicates significant differences from the control group ($p < 0.05$).

TrCLCN5 mRNA expression levels in gills exposed to different salinities for 3 h were tested (Fig. 6). Results showed that *TrCLCN5* mRNA was up-regulated in all four low-salinity groups ($p<0.05$).

DISCUSSION

In recent years, the desalination aquaculture of marine fish has become a hot topic in aquaculture. Studies of marine fish responses to low-salinity stress and the underlying mechanism of these responses can provide a theoretical basis for desalination aquaculture. Due to the compact size of the genome, and the similar number of genes with mammals, *T. rubripes* became a good model organism in comparative genomics research (Lee *et al*., 2006; Mochioka, 2012; Rakshambikai *et al*., 2012). In this study, a cDNA sequence of the *CLCN5* gene from the gill of *T. rubripes* was obtained using the RT-PCR technique, the nucleotide and deduced amino acid sequences were analyzed using various bioinformatics tools, and the expression patterns of *TrCLCN5* in different tissues and under low-salinity stress were determined using the qPCR technique. This study is the first time the expression of *CLCN5* has been investigated under low-salinity stress in *T. rubripes*.

The cDNA of *TrCLCN5* was 2792 bp in length, including a complete ORF of 2505 bp, which encoded an 834-amino acid polypeptide with a predicted molecular mass of 91 kDa and a theoretical pI of 6.2. A Voltage_CLC domain and two CBS domains were found in the predicted *Tr*CLCN5 protein, which primarily suggested that the sequence obtained was the *CLCN5* of *T. rubripes*. Results of Blast searches showed that *Tr*CLCN5 had high similarity (over 70%) with CLCN5 of other species; especially when compared with other species of teleosts, *Tr*CLCN5 shared 96.2% and 96.1% similarity with that of *Takifugu bimaculatus* (TNM93685.1) and *Takifugu flavidus* (TWW63544.1), respectively. The above results further confirmed that the cDNA sequence obtained was *CLCN5* of *T. rubripes*, and the *CLCN5* was conserved among species. A phylogenetic tree constructed based on the CLCN5 amino acid sequences showed that *Tr*CLCN5 formed a large cluster with 23 teleosts, whereas the CLCN5 of 3 mammals formed the other cluster with 3 reptiles, 2 aves and 2 amphibians. These results show the evolutionary relationships among different animals and suggest that CLCN5 is an evolutionarily conserved gene that has conserved functions in vertebrates.

TrCLCN5 mRNA was detected in various tissues, and results showed that *TrCLCN5* mRNA was expressed in all the tissues examined; the expression level was relatively high in intestine, kidney, and liver, whereas the expression

in gill was significantly lower than other tissues (Fig. 4). The results were similar in previous studies in mammals. It was found that CLCN5 was mainly expressed in kidney, small intestine, and liver in humans (Zhang *et al*., 2004; Zifarelli, 2015). In rat, CLCN5 was highly expressed in the kidneys and colon, and less expressed in the brain, heart, lung, and testis (Gunther *et al*., 1998). Another study in mice also showed that CLCN5 was abundantly expressed in the kidneys, mainly in the intercalated cells of the B-type aggregate tubule (Sakamoto *et al*., 1999). Because CLCN5 is closely related to cell endocytosis and regulation of cell osmotic pressure and pH value (Christensen *et al*., 2003; Claveriemartín *et al*., 2011), *TrCLCN5* mRNA may be highly expressed in *T. rubripes* intestine because *Tr*CLCN5 protein can help intestinal cells absorb nutrition from digested food (Jentsch, 2007). *TrCLCN5* mRNA is also highly expressed in *T. rubripes* kidney. In human kidney, CLCN5 is mainly expressed in the epithelial cells of the proximal tubule and collecting duct, and the expressed density was highest below the phagocytic cells of the brush border, where CLCN5 and H^+-ATPase were coexpressed in apical vesicles. The epithelial cell phagocytose proteins entered renal the capsule and transferred ions. Absence of *CLCN5* leads to a hereditary disease, Dent's disease (Wang *et al*., 2000; Neild *et al*., 2005). The main symptoms of the disease are high levels of proteinuria, kidney stones, etc. (Gabriel *et al*., 2017; Satoh *et al*., 2017; Zhang *et al*., 2017). Some researchers found the *CLCN5* gene knockout mice have the phenomenon of teeth and bone growth abnormalities (Wang *et al*., 2000). Studies in mice also suggest mutations of the *CLCN5* gene may cause acidification of inclusion bodies, which may then affect the cell endocytosis and cause abnormal phosphorus metabolism in the organisms (Shen *et al*., 2017). In fish, renal tubules played an important role in the process of reabsorption of nutrients, ions, and water from the blood. The high expression of *TrCLCN5* in kidney may be due to its important role in these processes. Gills are the main place for gas exchange and ion transport, but not for exchange of protein molecules in normal conditions in fish, which may be the reason why *TrCLCN5* is less expressed in the gills.

Under 72 h of low-salinity stress (salinity 4), *TrCLCN5* mRNA was significantly up-regulated at 3 h, lasted for 6 h, and was then down-regulated from 12 h to 72 h (Fig. 5). When transferred from natural seawater (salinity 32) to low-salinity water (salinity 4), the ambient environment changed from hyperosmotic to hypoosmotic. Studies have shown that changes in osmotic pressure in the external environment may affect membrane potential in fish (Linhart *et al*., 1999). Krasznai *et al*. (1995) suggested that the hypoosmotic solution changed the membrane potential of sperm cells by opening the voltage-gated K^+ channel. After that, along with the rearrangement of the cell membrane structure, the cell membrane changed from depolarization to polarization (Krasznai *et al*., 2003). CLCN5 has been thought to be a Cl^- channel, in particular because it mediates plasma membrane Cl^- currents upon heterologous expression (Steinmeyer *et al*., 1995; Friedrich *et al*., 1999). Recent research indicates that CLCN5 belongs to the family of voltage-gated chloride channel genes (Picollo and Pusch, 2005). It is an antiporter of H^+/Cl^- (Picollo and Pusch, 2005). *TrCLCN5* mRNA was significantly up-regulated at 3 h. When abruptly transferred from water with salinity 32 to water with salinity 4, changes in the osmotic pressure of the surrounding water environment led to changes in the membrane potential in gill cells. The *Tr*CLCN5 channel may be activated quickly, and the cells needed a large amount of *Tr*CLCN5, thus the expression of *TrCLCN5* was up-regulated in 3 h. Over time, *Tr*CLCN5 continuously transported Cl^- out of the cells, and transported H^+ into cells. At the same time, changes in osmotic pressure would also initiate some other membrane ion channels, such as the Na^+ channel, the cystic fibrosis transmembrane conductance regulator CFTR (Richards *et al*., 2003; Bodinier *et al*., 2009). Under the actions of various ion channels, cell membrane potential gradually returned to normal. After 12 h, cells no longer needed to exclude a large amount of Cl^-. Therefore, the quantity of *Tr*CLCN5 needed began to decrease, and the transcriptional level of *TrCLCN5* also decreased.

TrCLCN5 mRNA was up-regulated at 3 h in all four low-salinity groups (Fig. 6). As mentioned above, changes in the osmotic pressure of the surrounding water environment led to changes in the membrane potential in gill cells, which activated the *Tr*CLCN5 channel to transport Cl^- out of the cells, so as to maintain the stability of the membrane potential. The fish cannot complete this process within 3 h because the time is insufficient, so the fish still need to express a lot of *Tr*CLCN5.

CONCLUSION

In summary, a cDNA sequence with a complete ORF of *CLCN5* from *T. rubripes* was successfully cloned and characterized. The *TrCLCN5* cloned in this study displayed high sequence homology with its known counterparts in other species. *TrCLCN5* was highly expressed in intestines, kidney, and liver, and quickly up-regulated in gills under low-salinity stress in 3-6 h, which indicates that *TrCLCN5* may play an important role in the responses to acute low-salinity stress in *T. rubripes*. To further study the role of CLCN5 in the response to low-salinity stress, it is necessary to construct a suitable vector to express the

*Tr*CLCN5 protein and study the protein expression/subcellular localization under low-salinity stress. This study lays a foundation for follow-up research.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20200828090859

Statement of conflict of interest

The authors have declared no conflict of interest.

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