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Spotted Sea Bass (*Lateolabrax maculatus*) *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2*: Genome-Wide Identification, Characterization and Expression Analysis Under Salinity Stress

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Abstract The $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and the cystic fibrosis transmembrane conductance regulator (CFTR) proteins play crucial roles in the transportation of Na^+ and Cl^-. In this study, we identified *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* in spotted sea bass (*Lateolabrax maculatus*) genomic and transcriptomic databases. We also characterized these genes *via* phylogenetic and structural analyses. The results showed that both *cftr* and *nkcc* were highly conservative in *L. maculatus*. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis in ten tissues showed that *cftr*, *nkcc1a* and *nkcc2* highly express in osmoregulatory organs such as gill, kidney and intestine. Furthermore, the expressions of *cftr* and *nkcc1a* in gill as well as *nkcc2* in intestine were up-regulated by high salinity, indicating that these genes function potentially in osmoregulation. Our findings provided the insights into the *cftr* and *nkcc* functions in euryhaline teleost.

Key words *cftr*; *nkccs*; *Lateolabrax maculatus*; salinity; gene expression

1 Introduction

The spotted sea bass, *Lateolabrax maculatus*, belongs to *Lateolabrax*, Serranidae, and is a euryhaline teleost which can live in salinities ranging from freshwater to seawater and hypersaline environments. *L. maculatus* shows robust hyper-osmoregulatory and hypo-osmoregulatory abilities. To cope with the challenges of environmental salinities, euryhaline fishes have evolved sophisticated iono/osmoregulatory mechanisms. The gills, kidneys and intestine are important organs to maintain a stable internal body fluid homeostasis (Yang *et al*., 2016). Several other regulatory elements including Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and the cystic fibrosis transmembrane conductance regulator (CFTR) were also proved to be important for NaCl secretion in gill (Evans *et al*., 2005; Hwang and Lee, 2007; Hwang *et al*., 2011).

Following the electrochemical gradient energized by NKA, ions are driven by a number of different transmembrane proteins (Hirose *et al*., 2003). NKCC and CFTR are two major ion-transport proteins within ionocytes. Generally, *nkcc* has two main isoforms including *nkcc1* and *nkcc2*. NKCC1 is a member of cation-chloride cotransporter (CCC) family (Gamba, 2005), which is considered to be the secretory isoform. It participates in the simultaneous transport of Na^+, K^+ and Cl^- into cells and is expressed ubiquitously and especially in ion-epithelial cells (Gamba *et al*., 1994; Marshall *et al*., 2002; Hiroi *et al*., 2008). NKCC1 is thought to play a crucial role in ionocytes during seawater acclimation because the elevated mRNA and/or protein levels of NKCC1 in gills have been reported in a variety of teleost including European eel (*Anguilla anguilla*), Atlantic killifish (*Fundulus heteroclitus*), brackish medaka (*Oryzias melastigma*), European sea bass (*Dicentrarchus labrax*), green sturgeon (*Acipenser medirostris*) and brown trout (*Salmo trutta*) when they are exposed to higher salinity (Cutler and Cramb, 2002; Tang and Lee, 2007; Hiroi *et al*., 2008; Flemmer *et al*., 2010; Hiroi and McCormick, 2012). Conversely, NKCC2 is the absorptive isoform which expresses specifically in intestine and kidney (Gamba, 2005). Similar to NKCC1, CFTR belongs to the cyclic adenosine monophosphate (cAMP)-activated Cl^- channel (ClC), and also has an important role in NaCl secretion after salinity challenge (Hwang and Lee, 2007). Higher levels of CFTR protein and increased expression levels of *cftr* were identified in gills of different teleost after the fishes are transported from freshwater to seawater (McCormick *et al*., 2003; Scott *et al*., 2004; Nilsen *et al*., 2007; Shaw *et al*., 2008; Bodinier

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et al., 2009). The higher *cftr* expression is also found in seawater-adapted intestine of some species such as toadfish and killifish (Marshall *et al*., 2002; Ruhr *et al*., 2014).

L. maculatus is an ideal model for studying salinity adaptation as it can survive in both hyper-osmoregulatory and hypo-osmoregulatory environments. However, the gene features of *cftr* and *nkccs* and their functions in salinity regulation are currently unexplored. To fill in the gap, in this study, we reported the identification and characterization of one *cftr* and three *nkcc* in *L. maculatus*. Moreover, the expressions of these genes were determined in different tissues of *L. maculatus* under natural physiological condition and different salinities.

2 Materials and Methods

2.1 Ethics Statement

All animal experiments were conducted in accordance with the guidelines and approval of the respective Animal Research and Ethics Committees of Ocean University of China (Permit Number: 20141201. http://www.gov.cn/gongbao/content/2011/content_1860757.htm). The studies did not involve endangered or protected species.

2.2 Identification of *cftr* and *nkcc*

To identify *cftr* and *nkcc* in *L. maculatus*, gene sequences of human (*Homo sapiens*), zebrafish (*Danio rerio*), Atlantic salmon (*salmo salar*), large yellow croaker (*Larimichthys crocea*), barramundi perch (*Lates calcarifer*) and European seabass (*Dicentrarchus labrax*) were retrieved from GenBank and were used as queries for TBLASTN ($1e^{-5}$) search against the transcriptome database (accession numbers: SRR4409341 and SRR4409397) (Zhang *et al*., 2017) and the whole genome sequence database (unpublished data) of *L. maculatus*. TBLASTN was used to obtain the initial pool of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* transcript sequences in *L. maculatus*, and BLASTN was then used to verify the cDNA sequences through comparing the transcriptome sequences with the whole genome sequences. The ORFs (open reading frames) of those genes were searched from the retrieved transcript sequences by ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) and we are validated by using BLASTP against NCBI non-redundant protein sequence database.

The lengths of mRNA, 5'-untranslated region (5'-UTR), 3'-untranslated region (3'-UTR), and the number of amino acids translated by *cftr* and *nkcc* were obtained from the transcriptome database and genome database of *L. maculatus*. Molecular weight (MW, kDa) and isoelectric point (pI) of putative CFTR and NKCC proteins were calculated by using ExPASy Prot-Param tool (https://web.expasy.org/protparam/). Subcellular localization of all putative CFTR and NKCC proteins in *L. maculatus* was predicted by subcellular localization predictor (http://cello.life.nctu.edu.tw/) (Yu *et al*., 2006).

2.3 Phylogenetic Analysis of *cftr* and *nkcc*

To investigate the phylogenetic relationship and classification of *cftr*, *nkcc* genes in *L. maculatus*, the amino acid sequences of these genes of several representative vertebrates including *Mus musculus*, *H. sapiens*, *D. rerio*, *S. salar*, *L. crocea*, *L. calcarifer* and *D. labrax* were selected and retrieved from the NCBI non-redundant protein sequence database for phylogenetic analysis. Multiple protein sequences were aligned by Clustal X1.83 Omega program (Goujon *et al*., 2010). Phylogenetic analyses were conducted using MEGA 7 with bootstrapping values taken from 1000 replicates by neighbor-joining method (Darriba *et al*., 2011; Kumar *et al*., 2016). The tree was displayed with Interactive Tree of Life (iTOL, http://itol.embl.de/).

2.4 Sequence Analysis of *cftr* and *nkcc* of *L. maculatus*

Multiple protein sequence alignments of CFTR, NKCC were performed using DNAMAN 6.0 (Lynnon Biosoft, USA) with the default parameters. The species selected for CFTR sequence alignment included *L. maculatus*, *H. sapiens*, *M. musculus*, *D. rerio*, *L. calcarifer* and *D. labrax*. The predicted potential transmembrane (TM) domains of CFTR and NKCC were determined with Split 4.0 Server (http://split.pmsft.hr/split/4/). To confirm the presence of the conserved domains, all *L. maculatus* CFTR and NKCC proteins were analyzed with the Simple Modular Architecture Research Tool (SMART) (http:// smart.embl-heidelberg.de/). The DNA and cDNA sequences corresponding to each predicted *cftr and nkcc* genes in *L. maculatus* genome and transcriptome databases were used to determine the sizes of the exons and the positions of exon-intron boundaries. Exon-intron structure schematic diagrams of the *cftr and nkcc* genes were generated using the Gene Structure Display Server (GSDS, http://gsds.cbi. pku.edu.cn/). Conserved motifs among the CFTR and NKCC proteins in *L. maculatus* were predicted with MEME (Multiple Expectation Maximization for Motif Elicitation) software (http://memesuite.org/tools/meme) (Bailey *et al*., 2009). The parameters were set as follows: distribution of motif occurrences was zero or one per sequence; sizes of motifs were 6 to 50 residues; other parameters were default. The three-dimensional (3D) structures of CFTR and NKCC proteins were built by Swiss-Model (http://swissmodel.expasy.org/) and corrected by NCBI structure (https://www.ncbi.nlm.nih.gov/structure). The images were generated by PyMOL 2.2 (W.L. DeLano, The PyMOL Molecular Graphics System, 2002.)

2.5 Chromosomal Location Analysis of *cftr* and *nkcc* Genes

cftr, *nkcc* genes were mapped on chromosomes by identifying their chromosomal positions provided in *L. maculatus* genome database. The distribution map of *cftr*, *nkcc* genes throughout *L. maculatus* genome was protracted using MapDraw V2.1 software (Liu and Meng, 2003).

2.6 Salinity Challenge and Sample Collection

The *L. maculatus* fingerlings (120.66 g ± 13.05 g) were

obtained from Shuangying Aquaculture Farms (Dongying, Shandong Province, China). Prior to the experiment, fish were maintained under a 14 h:10 h light–dark photoperiod in a 5 m×5 m×1 m cement pond for one week. The water temperature was 25.3 ± 0.7℃; the concentration of dissolved oxygen was 7.01 ± 0.45 mg L^{-1}; pH was 7.8 ± 0.5; and the salinity was 30. After an initial acclimation period, fish were exposed to gradually changing salinity over 12 h until it reached 0 (FW, fresh water group), 12 (IP, isotonic point group), 45 (HS, high salinity group). In control group (SW, sea water group) the salinity was kept at 30. Experiments were conducted in 12 cuboid tanks (120 L) with 12 fish per tank for one month and all treatment were conducted with triplicate. The fish were fed a commercial pellet and the water was replaced 50% once per day.

After 30 days of rearing, 3 fish in sea water group (SW) were collected to observe genes expression profiles in ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain). At the same time, three fish per tank of each salinity treatment group were treated with tricainemethane sulfonate (MS 222, 200 mg L^{-1}) and the kidney, intestine and gill were sampled immediately. Then the samples were placed into 1.5 mL RNase-free tubes immediately and were quickly frozen by liquid nitrogen. Finally, they were stored at −80℃ for RNA extraction and further analyses.

2.7 Total RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Quantitative real-time PCR (qRT-PCR) was used to detect the mRNA expressions of *cftr* and *nkcc* genes in different tissues of *L. maculatus* with different salinity treatments. Total RNA was isolated using TRIzol® reagent (Invitrogen, USA). The concentration and integrity of total RNA were assessed using the Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing). Any potential gDNA contamination was removed by using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Beijing, China) and the first-strand cDNA was synthesized using random primers and Reverse Transcriptase M-MLV (TaKaRa) according to the manufacturer's instructions. Gene-specific primers were listed in Table 1. 18S ribosomal RNA (*18S rRNA*) was used as the reference gene for qRT-PCR normalization as described in previous studies (Aitken, 2006). Each reaction for qRT-PCR consisted of a total volume of 20 μL containing 10 μL of SYBR® FAST qPCR Master Mix (2X), 0.4 μL of ROX, 2 μL of template cDNA, 0.4 μL of each primer and 6.8 μL of nuclease-free water. The PCR amplification was in a 96-well optical plate at 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s, 60℃ for 30 s, and finally followed by a dissociation curve to verify the specificity of amplified products. qRT-PCR was performed using the StepOne Plus Real-Time PCR system (Applied Biosystems) and $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of genes.

Table 1 Primers of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* and *18S rRNA* genes for qRT-PCR

Gene	Primer (5'–3')	Amplification efficiency (%)
cftr	F: GATTGTTACGGGTGCCATCT R: TTGAGTTGCTGTCCAGTTCG	100.9
nkcc1a	F: ACGCTCATCACCTGGAAGTC R: TCAGCGCTCAGTCACAAAC	98.6
nkcc1b	F: CGCAGAGACCGTCGTTGAGATG R: CAGCAGGATGAACAGGAGGACAATC	109.5
nkcc2	F: ATCCTACGCCAAGTCTCCAG R: GCAGCCCACCAGTTGATAA	104.2
18S	F: GGGTCCGAAGCGTTTACT R: TCACCTCTAGCGGCACAA	103.1

2.8 Statistical Analysis

The data were further analyzed statistically using one-way ANOVA and Ducan's multiple range tests with SPSS 19.0 software (SPSS, Chicago, IL, USA). The values are presented as mean ± SEM (standard error of mean). Difference was considered significant at $P<0.05$.

3 Results

3.1 Identification and Copy Numbers of *cftr* and *nkcc* Genes

One *cftr* and three *nkcc* genes were identified in *L. maculatus* genome including *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2*. Their obtained amino acid sequences were named according to the BLASTP results based on several common fish species, such as *D. rerio*, *S. salar*, *L. crocea*, *L. calcarifer* and *D. labrax*. The cDNA sequences of the four genes have been submitted to GenBank. Their accession numbers and other sequence characteristics were presented in Table 2. The complete encoding sequences were obtained for *cftr*, *nkcc1a* and *nkcc2* genes, and only partial sequence was generated for *nkcc1b* gene (Table 2). The transcript lengths of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes were from 2442 bp (*nkcc1b*) to 4868 bp (*cftr*), the number of amino acids in the predicted four proteins were from 813 (NKCC1B) to 1507 (CFTR), the putative MWs were from 87.64 (NKCC1B) to 169.70 kDa (CFTR), and the theoretical pI values were from 5.68 (NKCC1A) to 8.32 (CFTR) (Table 2). Subcellular location prediction indicated putative proteins of CFTR, NKCC1A, NKCC1B and NKCC2 were localized in the inner membrane (Im) (Table 2).

Table 2 Characteristics of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes in spotted sea bass

Gene	mRNA length (bp)	5'-UTR (bp)	3'-UTR (bp)	Protein length (aa)	MW (kDa)	pI	Localization	ORF integrity	Accession no.
cftr	4864	250	90	1507	169.70	8.32	Im	Complete	MH142147
nkcc1a	4165	442	246	1158	126.05	5.68	Im	Complete	MH142148
nkcc1b	2442	–	–	813	87.64	5.87	Im	Partial	MH142149
nkcc2	3144	21	–	1040	114.54	6.63	Im	Complete	MH142150

Notes: ORF, open reading frame; MW, molecular weight; pI, isoelectric point; UTR, untranslated region; Im, innermembrane. –, not detected.

The copy numbers of *cftr*, *nkcc* genes were investigated in *L. maculatus* and other vertebrates (Table 3). Among these genes, *nkcc1* gene had two copies in *L. maculatus* genome, while both *cftr* and *nkcc2* genes showed a single copy. The identities and copy numbers of *cftr*, *nkcc1* and *nkcc2* genes were relatively conservative among different species (Table 3). For *cftr* and *nkcc2* genes, only one copy was identified in all tested species. However, *nkcc1* gene had two copies in all the tested teleost including catfish (Ipu), medaka (Ola), fugu (Tru), zebrafish (Dre) and spotted sea bass (Lmu). It had a single copy in higher vertebrates including human (Hsa), mouse (Mmu) and chicken (Gga).

Table 3 Comparison of copy numbers of genes in vertebrate genomes

Gene	Hsa	Mmu	Gga	Ipu	Ola	Tru	Dre	Lmu
cftr	1	1	1	1	1	1	1	1
nkcc1	1	1	1	2	2	2	2	2
nkcc2	1	1	1	1	1	1	1	1
Total	3	3	3	4	4	4	4	4

Notes: human (Hsa), mouse (Mmu), chicken (Gga), catfish (Ipu), medaka (Ola), fugu (Tru), zebrafish (Dre) and spotted sea bass (Lmu).

3.2 Phylogenetic Analyses of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* Genes of *L. maculatus*

To further confirm the annotations of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes in *L. maculatus*, phylogenetic analyses were conducted based on the amino acid sequences of several vertebrates including *H. sapiens*, *M. musculus*, *G. gallus*, *D. rerio*, *S. salar*, *L. calcarifer*, *O. niloticus*, *L. crocea*, *C. semilaevis*, *T. rubripes* and *D. labrax*. As shown in Fig.1, these genes of *L. maculatus* were clustered with respective counterparts consistent with their annotation. Additionally, the phylogenetic tree was divided into three groups (*nkcc1*, *nkcc2*, *cftr*) marked with covered lines, suggesting the conservation of the three genes in evolution. The annotations were further confirmed by the sizes of amino acid sequences out of this phylogenetic tree.

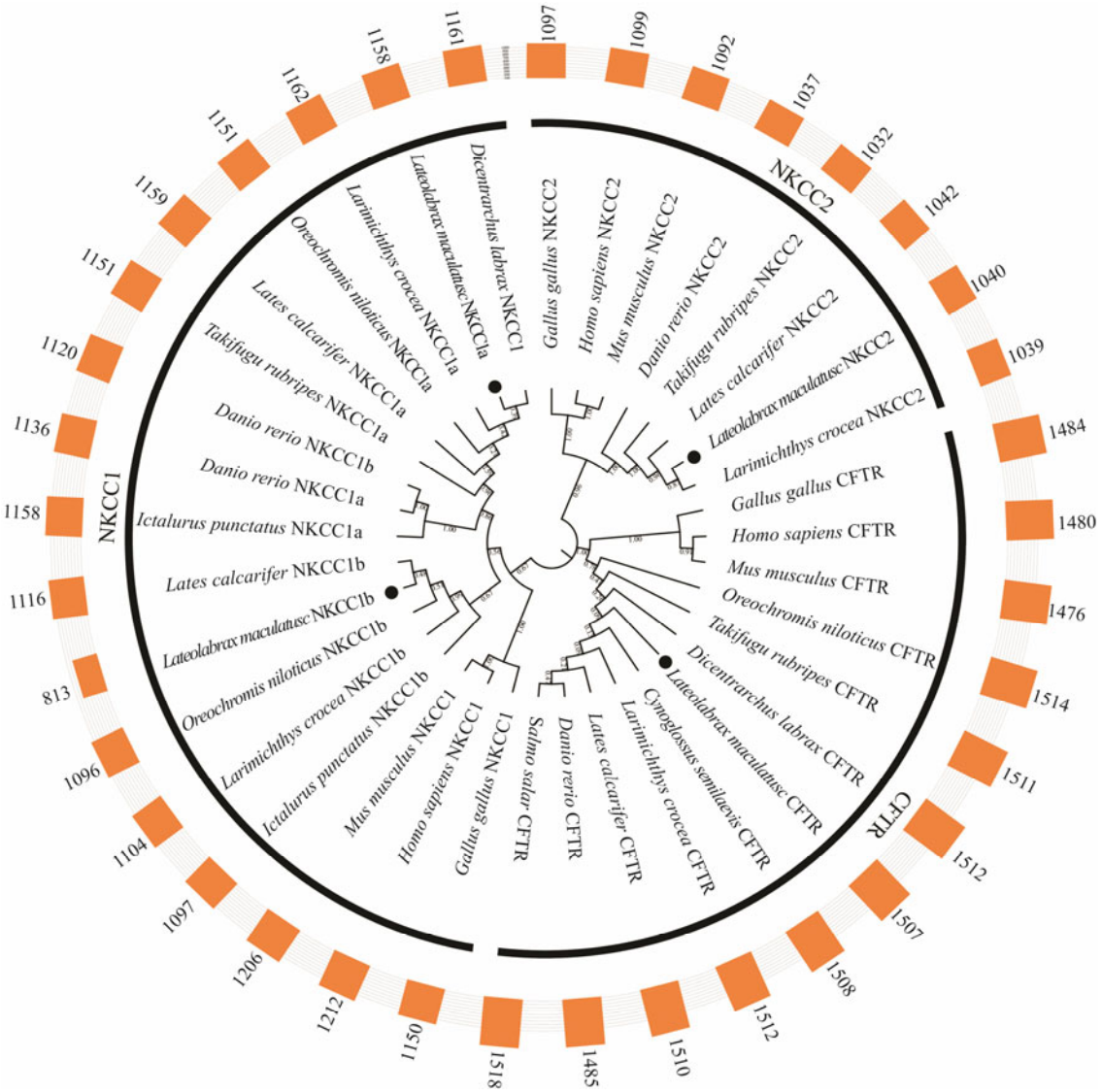

Fig.1 Phylogenetic analyses of *nkcc1*, *nkcc2* and *cftr* genes of *L. maculatus*. The phylogenetic tree was constructed by the amino acid sequences from several representative mammals and teleost with 1000 bootstrap replications in MEGA 7, ClustalX1.83 and iTOL online software. These genes of phylogenetic tree were marked with black dot. The phylogenetic tree was divided into three groups with covered lines. The simple bars outside phylogenetic tree stood for the sizes of amino acid sequences of these genes.

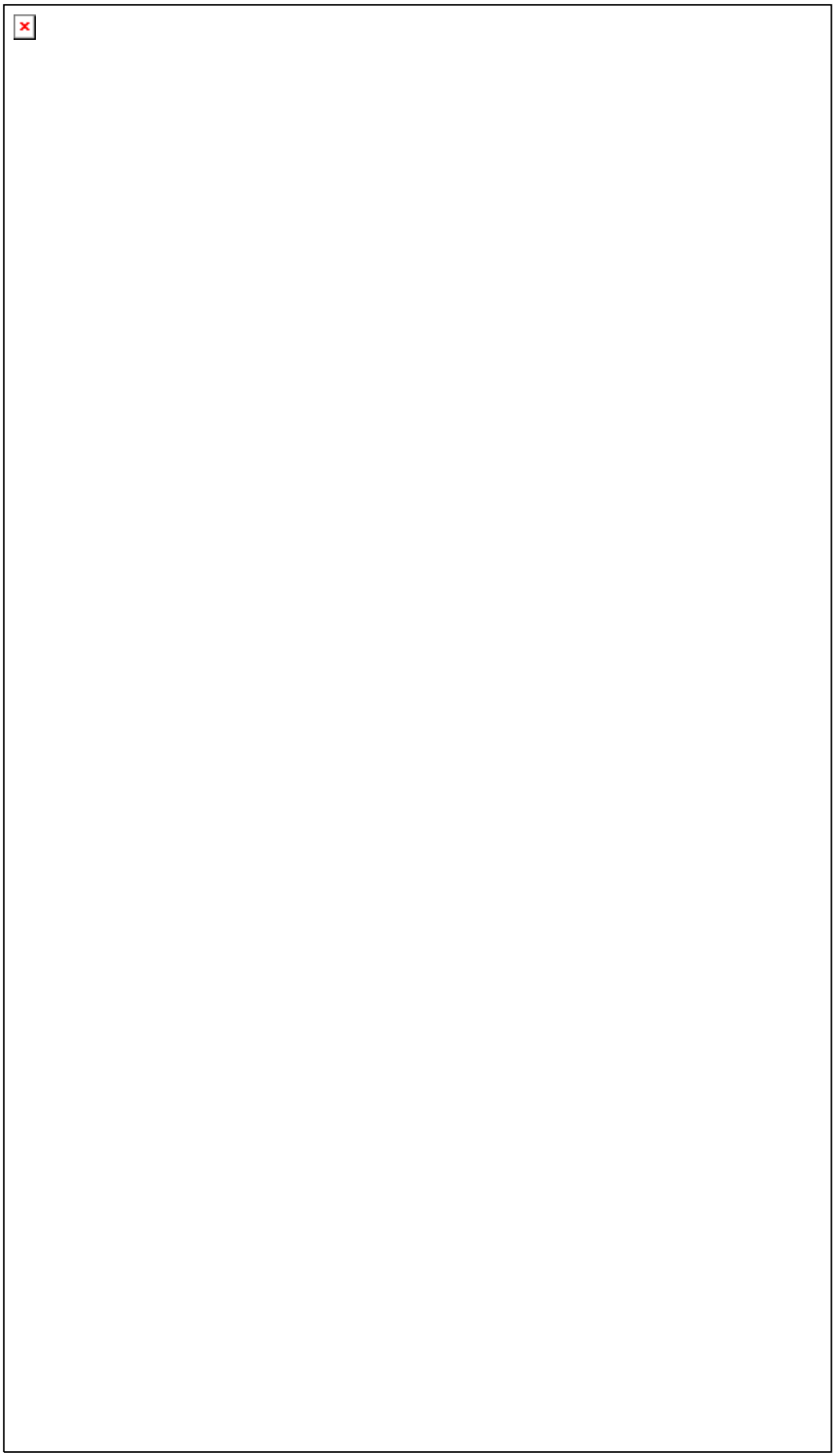

Fig.2 Sequence alignment of CFTR protein of spotted sea bass, zebrafish, human, European sea bass, mouse, chicken and barramundi. Identical amino acid residues are shaded in black, while relatively conserved residues are shaded in pink and blue. The twelve transmembrane (TM) helices of the CFTR protein are marked with black lines.

3.3 Multiple Alignments of Predicted CFTR Proteins

The predicted amino acid sequences of CFTR in *L. maculatus* showed high similarity to CFTR proteins of teleost species (Fig.2): *D. labrax* (95.30%), *L. calcarifer* (91.60%), *D. rerio* (71.80%). The homology of CFTR to mammals and bird was relatively low: *H. sapiens* (human) (59.33%), *G. gallus* (chicken) (60.05%), *M. musculus* (mouse) (56.16%). The results suggested that the twelve predicted TM domains of CFTR protein were identified.

3.4 Multiple Alignments of NKCC1A, NKCC1B and NKCC2 Proteins of *L. maculatus*

The predicted amino acid sequences showed a lower similarity among NKCC proteins in *L. maculatus* (Fig.3). Alignments of the deduced amino acid sequences of the NKCC proteins demonstrated that the similarity of NKCC proteins in *L. maculatus* was less than 55% to each other, while the similarity between NKCC1A and NKCC1B was 51.85%; the similarity between NKCC1A and NKCC2 was 52.09%; and the similarity between NKCC1B and NKCC2 was 41.29%. In addition, the eleven predicted TM domains (TM1-7, TM9-12) of NKCC proteins were identified. Compared with NKCC proteins, the amino acid sequences in the C-terminal were found to be highly homologous while the similarity was lower at N-terminal.

3.5 Structure, Conserved Domains and Motif Analysis of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* Genes

The *nkcc1a* gene has twenty-one exons and twenty-two introns, while the *nkcc1b* gene has eighteen exons and seventeen introns. The *nkcc2* gene has twenty-six exons and twenty-six introns and the *cftr* gene has twenty-five exons and twenty-six introns (Fig.4A). The conserved homeodomain of Pfam:AA_permease and Pfam:SLC12 was detected in each of the NKCC proteins. The low complexity, Pfam:AA_permease_N, Pfam:AA_permease and Pfam:SLC12 conserved homeodomain were detected in NKCC1A and NKCC1B proteins. The NKCC2 had Pfam:AA_permease_N, Pfam:AA_permease, Pfam:AA_permease_N and Pfam:SLC12 domains. In addition, the CFTR protein had four domains including Pfam:ABC_ membrane, low complexity, AAA and Pfam:CFTR_R (Fig.4B). To explore the structural diversity and predict the functions of CFTR, NKCC1A, NKCC1B and NKCC2 proteins, only one motif in CFTR protein was identified by MEME software.

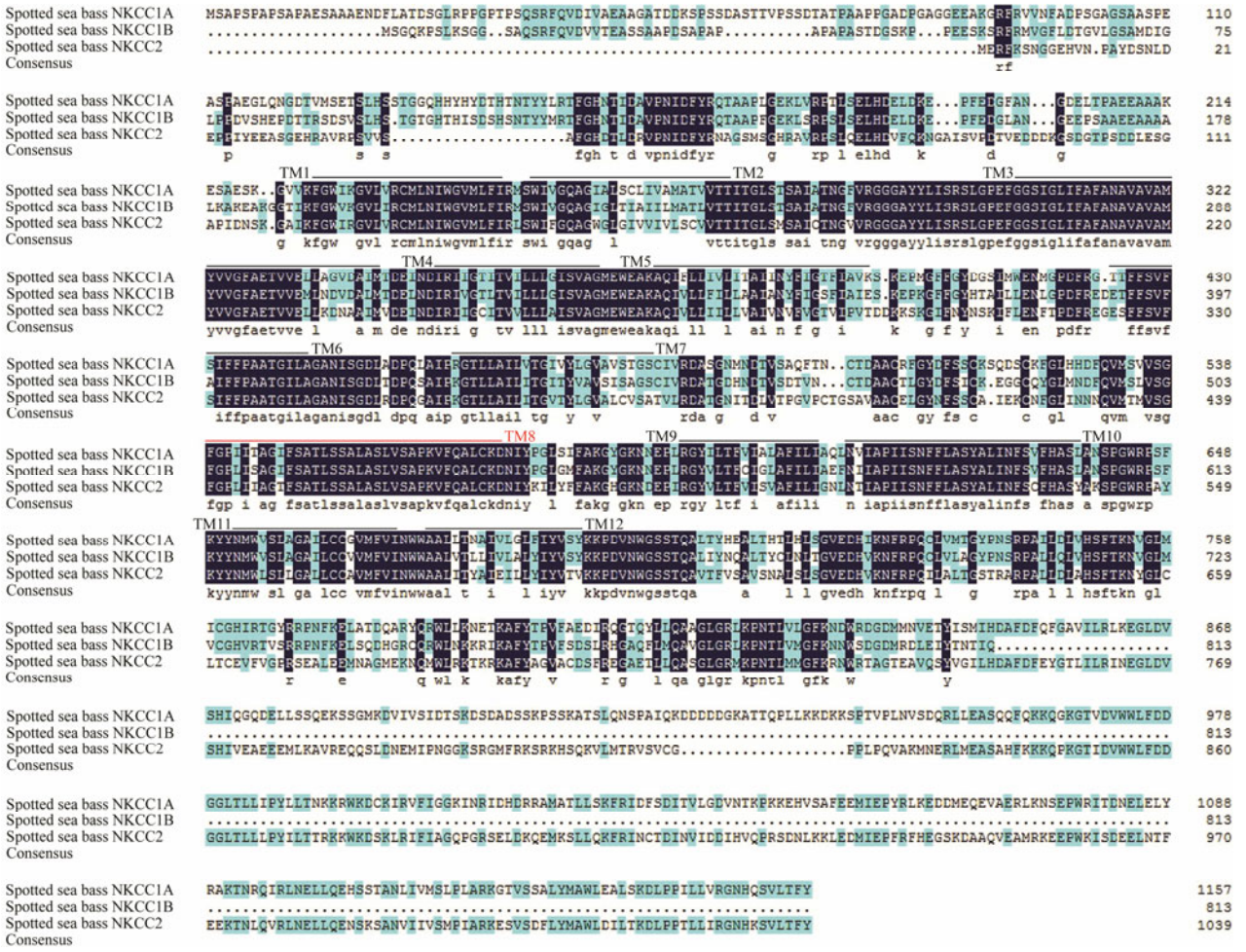

Fig.3 Sequence alignment of spotted sea bass NKCC proteins. Identical amino acid residues are shaded in black while relatively conserved residues are shaded in pink and blue. The eleven transmembrane (TM) helices (TM1-7, TM9-12) of the NKCC proteins are marked with black lines. Compared with three-dimensional structure analysis, an unpredictable transmembrane helix (TM8) is marked with red line.

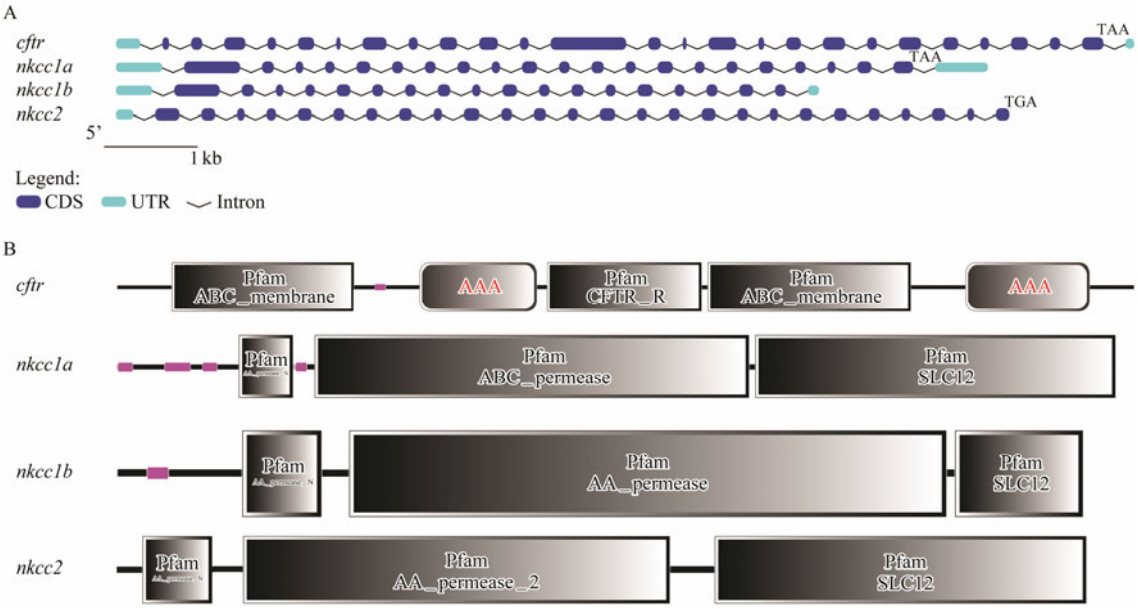

Fig.4 Gene structure and homeodomain analyses of spotted sea bass *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes. (A) Exon-intron structure analyses were performed using the Gene Structure Display Server database. The blue boxes indicate exons; the black lines indicate introns. The light blue boxes indicate UTR. The red arrows indicate the position of start and stop codons and black letters indicate the type of stop codon. (B) The domain analysis of CFTR, NKCC1A, NKCC1B and NKCC2 proteins was performed by the SMART analyses service. The low complexity domain was represented in pink.

3.6 Chromosomal Location Analysis of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* Genes

Four genes, *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2*, were located on four different chromosomes of *L. maculatus* (Fig.5). The *cftr* gene was on chromosome 6, the *nkcc1a* gene was on chromosome 17, the *nkcc1b* gene was on chromosome 8 and the *nkcc2* gene was on chromosome 1.

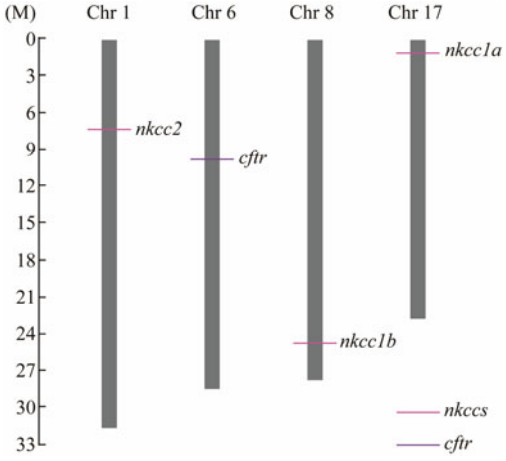

Fig.5 Chromosomal locations of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes on different chromosomes of spotted sea bass. *cftr* and *nkcc* genes were localized to an exact position on a particular chromosome by different colors in the lower right corner, which can be calculated using the scale on the left. The chromosome number is shown at the top of each chromosome bar.

3.7 Three-Dimensional Structure Analysis of Deduced CFTR and NKCC Proteins

The protein structure of CFTR, NKCC1A, NKCC1B and NKCC2 were predicted by homology modeling methods. The conserved 3D structure of *L. maculatus* CFTR contained two membrane-spanning domains (MSDs) including 6 TM helices in each MSDs (Fig.6A). The results are consistent with those of sequence alignment (Fig.2). Additionally, it contained two intracellular loops (ICLs) including 10 inner helices and β-sheets (Fig.6A). NKCC1A, NKCC1B and NKCC2 share highly conserved structures consisting of 12 TM helices (Figs.6B, C and D).

3.8 Tissue Distribution of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* Gene Transcripts

qRT-PCR analysis was performed to analyze the expression of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes in ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain) of *L. maculatus* under nature seawater conditions. Results showed that *cftr* has the highest transcription level in heart, followed by brain, gill and intestine. The highest expression level of *nkcc1a* gene was detected in gill, which was at least 5-fold higher than in other tissues. Gene *nkcc1b* was primarily expressed in brain, while low level or no transcription was detected in other tissues. Gene *nkcc2* showed the highest expression level in intestine, which was more than 10 times higher than in other tissues (Fig.7).

3.9 Expression Profiles of *cftr*, *nkcc1a* and *nkcc2* Genes at Different Salinities

According to the tissue distribution analysis, relatively high expression levels of *cftr*, *nkcc1a* and *nkcc2* genes were detected in osmoregulatory tissues including gill, kidney and intestine. To further examine their potential involvement in osmo-regulation and salinity adaptation, their expressions in these three tissues after treatments with different salinities were examined by qRT-PCR (Fig.8).

The mRNA levels of *cftr* in gill and intestine were higher in SW and HS groups, while the highest expression level was found in HS group. The expression level of *nkcc1a* in gill was significantly higher than those in kidney and intestine, which was consistent with the above results about its tissue distribution. There was obviously increasing trend of *nkcc1a* mRNA level in gill with the increasing salinity. Similarly, the mRNA level of *nkcc2* gene in the intestine was significantly higher than those in gill and kidney, and increased with the increasing salinity, indicating its potential involvement in salinity regulation in *L. maculatus* (Fig.8).

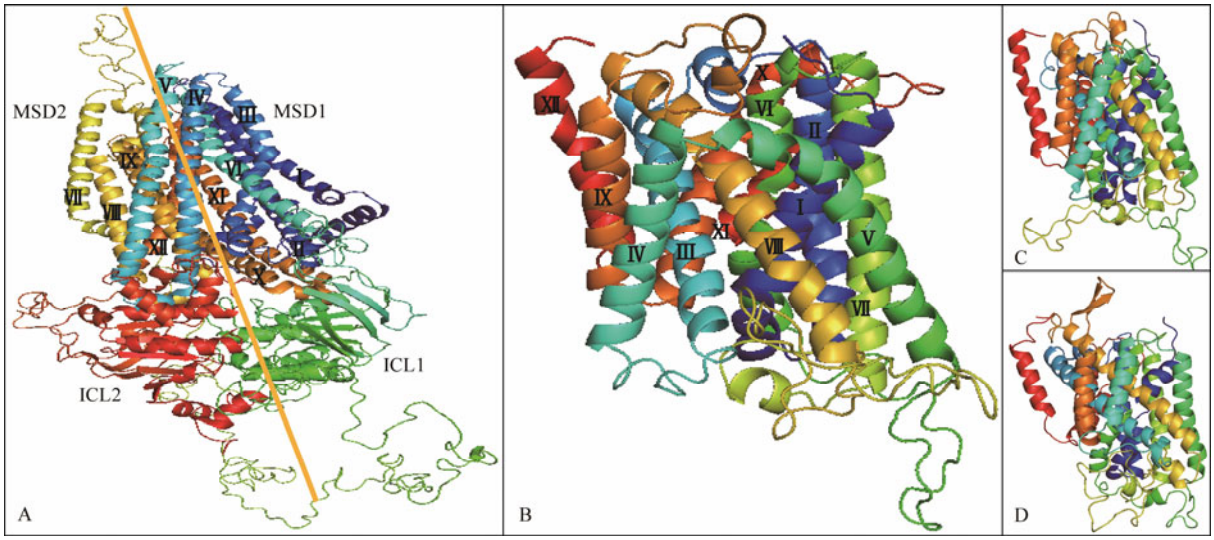

Fig.6 The tertiary structures of CFTR, NKCC1A, NKCC1B and NKCC2 proteins from spotted sea bass. A) CFTR; B) NKCC1A; C) NKCC1B; D) NKCC2. The 12 TM helices are represented by Roman (I-XII).

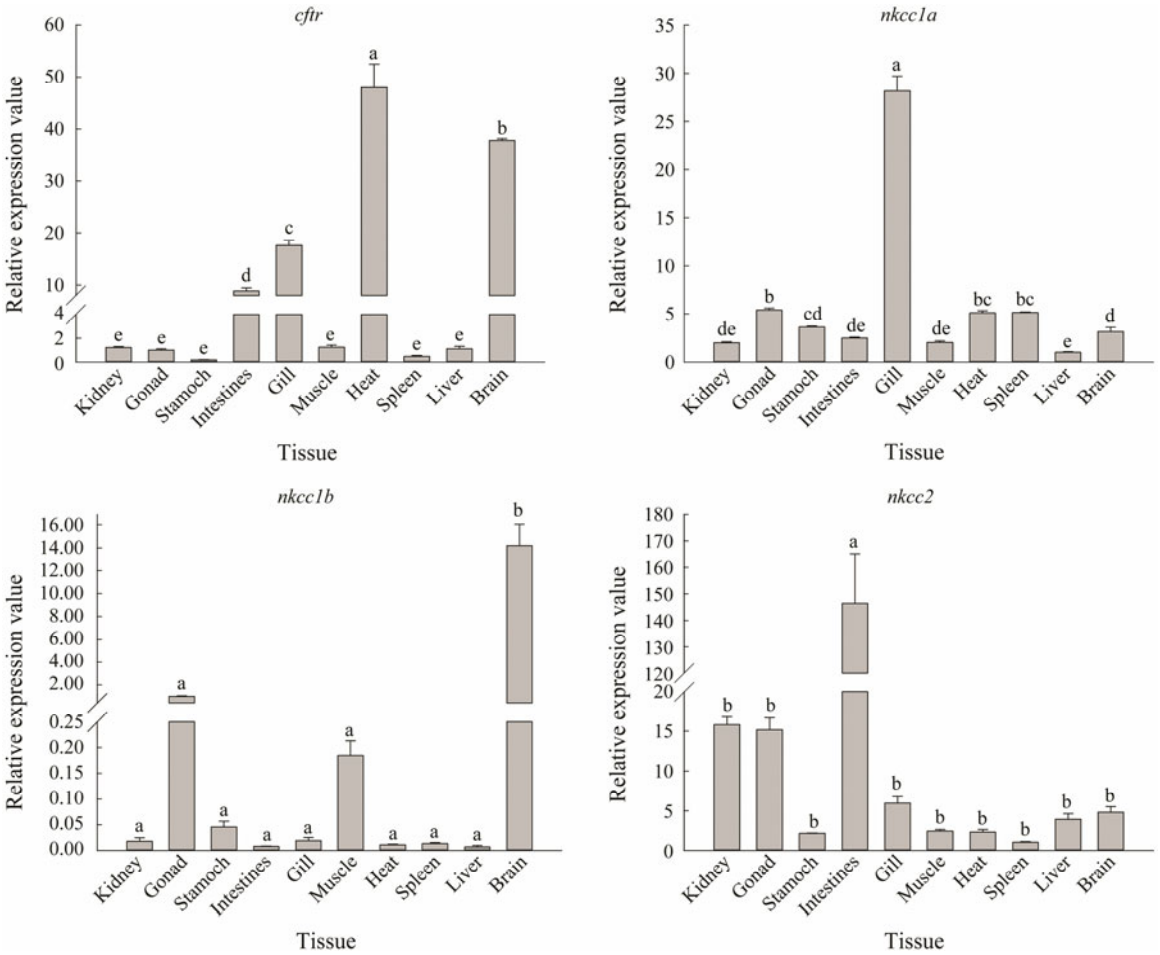

Fig.7 The tissue distribution of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* gene transcripts. The results are shown as the means ± standard error of mean (SEM). Different letters in the different tissues indicate significant differences ($P < 0.05$). The graphs were made with SigmaPlot 14.0.

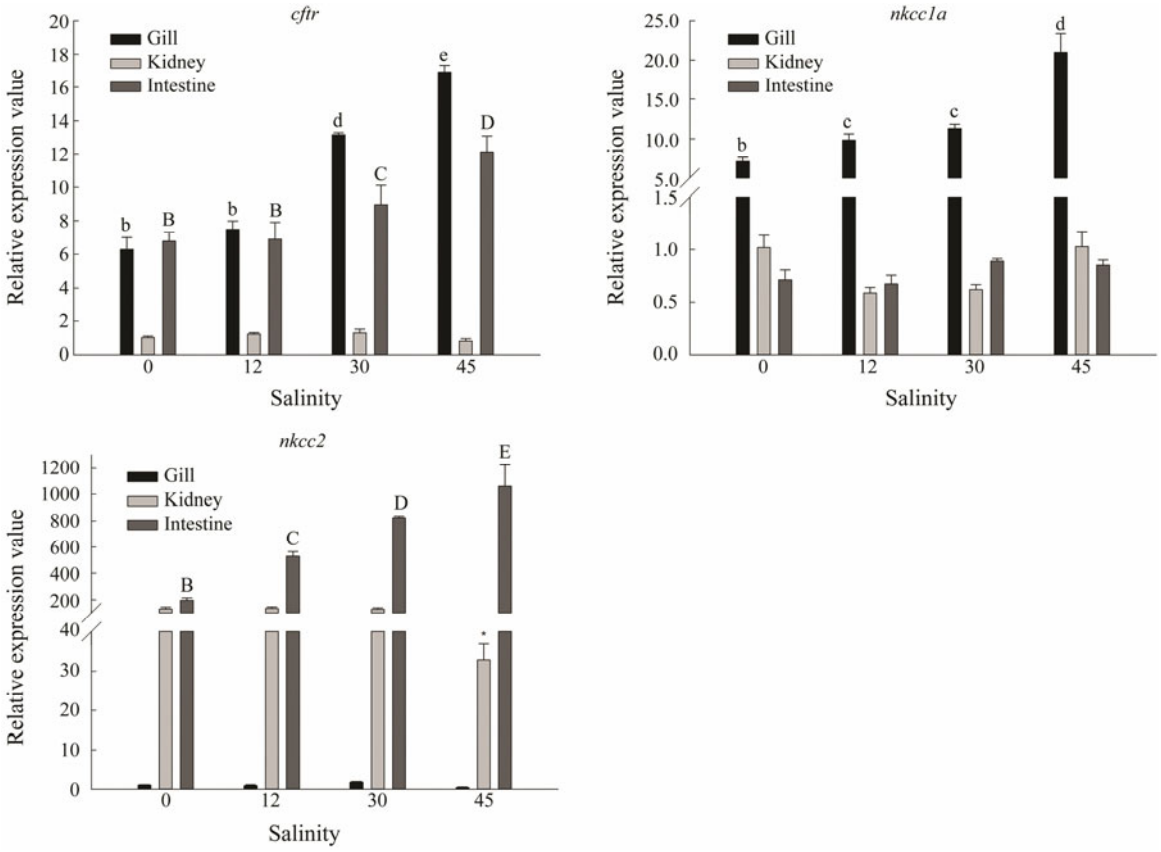

Fig.8 The relative expression levels of *cftr*, *nkcc1a* and *nkcc2* genes in gill, kidney and intestine of spotted sea bass under different salinity treatments. The significant differences ($P<0.05$) in different tissues are represented by capital, lowercase letters and asterisks, respectively. The graphs were made with SigmaPlot 14.0.

4 Discussion

To cope with the challenges exerted by environmental salinities, NKCC and CFTR are proved to be the important proteins for osmoregulation and salinity adaptation in teleost. CFTR is a member of the ATP-binding cassette (ABC) transporter family of an ion channel (Gadsby *et al*., 2006; Cant *et al*., 2014), which is found in the apical membranes of epithelial cells (Anderson *et al*., 1992). NKCC is a member of the cation-chloride cotransporter family, localizing on the basolateral or apical plasma membranes of epithelial cells and associating with facilitation of the particular ion transporting activities (McCormick *et al*., 2003; Hiroi *et al*., 2008). In this paper, the identification and characterization of *cftr* and *nkcc* genes were reported in *L. maculatus* for the first time. Furthermore, its biological functions responding to salinity challenges were evaluated by their expression patterns.

We identified the correct annotations of one *cftr* gene and three *nkcc* genes (*nkcc1a*, *nkcc1b* and *nkcc2*) from the data of *L. maculatus* transcriptome and genome. The copy numbers of *cftr* and *nkcc* genes were relatively conserved in vertebrates, while some differences in the copy numbers of *cftr* gene were observed in teleost. Two copies of *cftr* gene, including *cftra* and *cftrb* (or *cftrI* and *cftrII*), are identified in *Anguilla japonica* (Wong *et al*., 2016) and *Salmo salar* (Nilsen *et al*., 2007). In addition, sequence analysis of CFTR and NKCC proteins showed that CFTR and NKCC proteins in *L. maculatus* shared the conserved domains with twelve predicted TM helices, consisting with previous results (Park and Saier Jr., 1996; Callebaut *et al*., 2016; Liu *et al*., 2017; Tordai *et al*., 2017; Callebaut *et al*., 2018). Therefore, the conserved domains and 3D structure further support the results of the phylogenetic analyses.

CFTR and NKCC proteins are involved in chloride secretion in teleost during salinity challenge (McCormick *et al*., 2003; Hiroi and McCormick, 2007; Tse *et al*., 2007; Yan *et al*., 2013). Wong *et al.* (2016) find that *cftra* is mainly expressed in intestine and kidney with decreased expression level when fish were transferred to SW. It was also reported that *cftrb* is the dominant isoform expressed in the gill and the higher expression is observed under osmotic stress (Wong *et al*., 2016). In our study, high mRNA expression levels of *cftr* were detected in gill and intestine. In addition, up-regulated mRNA expressions of *cftr* in gill and intestine were also observed with increased environmental salinity, suggesting it might play a role during salinity adaption. Similar results are reported in many teleost species, such as *D. labrax* (Bodinier *et al*., 2009), *Fundulus heteroclitus* (Scott *et al*., 2004), *Juvenile anadromous* and *S. salar* (Nilsen *et al*., 2007), *Anabas testudineus* (Ip *et al*., 2012), and *O. latipes* (Hsu *et al*., 2014). All these studies support the crucial function of *cftr* gene during NaCl secretion, suggesting a potential association between *cftr* and salinity adaption.

The *nkcc1* gene is considered to be the secretory iso-

form with two copies (*nkcc1a* and *nkcc1b*). Indeed, *nkcc1a* is the main expressed isoform in gill of many teleost species including *Anguilla Anguilla* (Cutler and Cramb, 2002), *Oreochromis mossambicus* (Hiroi *et al*., 2008), *Oryzias dancena* (Kang *et al*., 2010). Similarly, high level of *nkcc1a* mRNA was detected in gill of *L. maculatus* in this study. There was obviously increasing trend of *nkcc1a* mRNA expression in gill with the increased salinity. Moreover, the expression level of *nkcc1a* gene in HS was the highest. These results strongly emphasized the important role of *nkcc1a* in ion/osmotic regulation. In contrast to the gill and kidney, the expression level of *nkcc2* gene was the highest in intestine and significantly increased with increasing environmental salinity. The same results indicate that the expression of *nkcc2* gene in the intestine is significantly increased from fresh water to seawater (Cutler and Cramb, 2008; Tresguerres *et al*., 2010). Moreover, previous studies also find that the expressions of *nkcc* genes in intestine are salinity-dependent in fish (Kalujnaia *et al*., 2007; Gregório *et al*., 2013; Li *et al*., 2014). All these results indicate that *nkcc1a* and *nkcc2* might play importance roles in ion/osmotic regulation in gill and intestine.

In summary, the present study firstly reported the existence of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes in *L. maculatus*, including their identification, sequence analysis, expression profiles in tissues and direct responses to environmental salinity changes in essential osmoregulatory organs including gill, kidney and intestine. Our findings suggest that the expression patterns of *cftr*, *nkcc1a*, *nkcc1b* and *nkcc2* genes are tissue-specific. In addition, expression profiles of *cftr*, *nkcc1a* and *nkcc2* genes after salinity challenges showed that the expressions of *cftr* and *nkcc1a* in gill and the mRNA level of *nkcc2* in intestine were induced by high salinity, indicating their potential involvement in response to salinity stress.

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