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# Gene expression of Na<sup>+</sup>/K<sup>+</sup>-ATPase $\alpha$ -isoforms and FXYD proteins and potential modulatory mechanisms in euryhaline milkfish kidneys upon hypoosmotic challenges

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Euryhaline milkfish (*Chanos chanos*) is an important economic species in Taiwan and is able to survive in both seawater (SW) and fresh water (FW). Upon salinity changes, euryhaline fish kidneys play a crucial role in osmoregulation. During this process, Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) mainly functions in providing the driving force for the other ion-transporting systems. In the milkfish, NKA activities have been widely studied, yet the isoforms and modulatory mechanisms of the NKA  $\alpha$ -subunits are unknown. Hence, this study was undertaken to reveal potential modulatory mechanisms, including the isoform composition of NKA  $\alpha$ -subunits and FXYD proteins (the modulators of NKA), of NKA expression/activity in milkfish kidneys in response to salinity changes. Four isoforms (CcNKA  $\alpha$ 1a,  $\alpha$ 1c,  $\alpha$ 2, and  $\alpha$ 3) of NKA  $\alpha$ -subunits with distinct tissue distribution were identified in milkfish. Among them, *Ccnka a1a* and *Ccfxyd2* were predominantly expressed in the kidney. Upon salinity challenge, parallel expression patterns were found in renal *Ccnka a1a* and *Ccfxyd2*, showing an increase in the FW-acclimated milkfish but not in the SW-acclimated group. After transfer, no matter from SW to FW or FW to SW, most of the renal *Ccnka* and *Ccfxyd* genes (particularly *Ccnka a1a* and *Ccfxyd2*) significantly changed. Overall, these results suggest that the NKA activity of milkfish kidneys may be modulated via CcNKA  $\alpha$ 1a and CcFXYD2 for osmoregulation in response to salinity challenges.

## 1. Introduction

Milkfish (*Chanos chanos*), a marine teleost that resides in tropical regions from the Pacific Ocean to the Indian Ocean, is a commercially important aquaculture species in Southeast Asian countries, including Taiwan (Martinez et al., 2006). Milkfish can survive in hypoosmotic (i.e., fresh water [FW]) or hyperosmotic (e.g., seawater [SW]) environments (Bagarinao, 1994; Martinez et al., 2006). In Taiwan, milkfish are cultured in ponds with water of various salinities including FW,

brackish water, or SW (Martinez et al., 2006). Hence, for elucidating the effects of environmental factors (such as salinity) on physiological mechanisms as the background knowledge to improve aquaculture practice of milkfish farm, further investigation of their molecular mechanisms of osmoregulation in response to ambient salinity changes is warranted (Lin et al., 2003). There is evidence that the milkfish is able to maintain internal homeostasis, such as muscle water content, osmolality of the plasma and urine, and concentrations of plasma Na<sup>+</sup> and Cl<sup>-</sup>, within narrow physiological ranges under different

*Abbreviations:* Cc, *Chanos chanos*; FW, freshwater/fresh water; NKA, Na<sup>+</sup>/K<sup>+</sup>-ATPase; PCR, polymerase chain reaction; PK, protein kinase; QPCR, quantitative real-time PCR; SE, standard error; SW, seawater; TM, transmembrane domain.

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environmental salinity conditions (Kang et al., 2015; Lin et al., 2003; Tang et al., 2010). Thus, milkfish is a good experimental species for studies on osmoregulation.

Teleosts maintain relatively stable internal environments by the activation of osmoregulatory organs, including the gill, kidney, and gut (Marshall and Grosell, 2006; Takei et al., 2014; Whittamore, 2012). Among these organs, the kidney plays an important role in osmoregulation in both SW and FW teleosts, performing completely different functions in these two environments (Madsen et al., 2015; Marshall and Grosell, 2006; Takei et al., 2014). In SW residents, the hypertonic environment results in passive loss of water and influx of excess ions. They thus drink SW to prevent dehydration and actively excrete ions through the kidneys and gills. In contrast, FW teleosts gain excess water loading and lose ions to their surrounding hypotonic environment. For compensation, the primary function of fish kidneys is to excrete excess water and reabsorb filtered solutes (Madsen et al., 2015; Marshall and Grosell, 2006). In these processes, renal  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) plays a crucial role in ionoregulation/osmoregulation for cellular homeostasis of ions and water (Marshall and Grosell, 2006; Whittamore, 2012).

NKA is a primary driving force for other ion-transporting systems in the osmoregulatory organs, including the kidneys (Hwang et al., 2011; Whittamore, 2012). It is a P-type ATPase and consists of catalytic  $\alpha$ -subunits and smaller glycosylated  $\beta$ -subunits (Morth et al. 2011; Toyoshima et al. 2011). Among them, the catalytic  $\alpha$ -subunit, containing the binding sites of  $\text{Na}^+$ ,  $\text{K}^+$ , and ATP, plays a crucial role in NKA function, including osmoregulation (Evans and Claiborne, 2009; Tang et al., 2009). In birds and mammals, four  $\alpha$ -isoforms ( $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 4$ ) of NKA with tissue-specific distribution have been reported (Blanco, 2005; Morth et al., 2011; Suhail, 2010). Among these isoforms,  $\alpha 1$  is expressed ubiquitously in transporting epithelia for maintaining osmotic balance and cell volume regulation. Meanwhile,  $\alpha 2$  is mainly found in the brain, muscle, and heart;  $\alpha 3$  is present primarily in the neurons and ovaries; and  $\alpha 4$  is expressed only in the testis. The switch of these  $\alpha$ -isoforms is considered to meet specific requirements for differentiated cell-specific functions of cation transport (Blanco, 2005; Blanco and Mercer, 1998; Suhail, 2010). In teleosts, only three  $\alpha$ -isoforms ( $\alpha 1$ ,  $\alpha 2$ , and  $\alpha 3$ ) but numerous paralogs have been identified (Armesto et al., 2014; Bollinger et al., 2016; Ching et al., 2015; Richards et al., 2003; Wong et al., 2016). As in mammals, tissue-specific expression of these teleostean  $\alpha$ -subunits has been demonstrated. Moreover, in the osmoregulatory organs, the expression (mRNA and/or protein) of the predominant NKA  $\alpha$ -isoforms (especially  $\alpha 1$  and its paralogs) is salinity-dependent, indicating their roles in fish osmoregulation (Armesto et al., 2014; Bollinger et al., 2016; Richards et al., 2003; Wong et al., 2016).

FXYP proteins identified in some teleosts are considered to be important modulatory mechanisms for NKA activity (Saito et al., 2010; Tipsmark, 2008; Yang et al., 2013). At present, at least 12 members of the FXYP protein family (i.e., FXYP1-12) have been reported in mammals and fish, and most FXYP proteins exhibit distinct tissue distribution (Geering, 2008; Hwang et al., 2011; Yang et al., 2013). The FXYP protein family has been more widely studied in mammals and elasmobranchs compared to teleosts (Crambert and Geering, 2003; Geering, 2008). Recently, salinity effects on expression of FXYP proteins were investigated in teleostean osmoregulatory organs (Tipsmark, 2008; Wang et al., 2017; Yang et al., 2018). The potential function of some FXYP proteins upon osmotic challenge has been demonstrated in a limited number of teleostean species (Chang et al., 2016; Saito et al., 2010; Wang et al., 2017; Yang et al., 2016b). To date, however, the roles of most FXYP proteins are not yet clear in teleosts.

In the milkfish, the effects of salinity on NKA expression, including NKA activity (Kang et al., 2015; Lin et al., 2003; Tang et al., 2010) and potential isoform composition of NKA  $\alpha$ -subunits (protein levels only) (Tang et al., 2009; Yang et al., 2016a), have been investigated. Molecular characteristics and modulatory mechanisms of NKA  $\alpha$ -subunit isoforms, however, are not clear. Hence, this study focused on potential

modulatory mechanisms, including isoform composition of NKA  $\alpha$ -subunits and FXYP proteins, on NKA expression/activity in response to salinity changes. The goals of the present study were to (1) identify the isoforms of milkfish NKA  $\alpha$ -subunits; and (2) investigate the gene expression of NKA  $\alpha$ -subunits and FXYP protein in milkfish kidneys upon hypoosmotic challenge. This paper sheds a light on the molecular modulatory mechanisms of NKA expression of euryhaline teleosts following salinity challenge.

## 2. Materials and methods

### 2.1. Ethical approval

The protocols employed for the experimental fish were reviewed and approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 105-130), and were carried out in accordance with the approved guidelines.

### 2.2. Experimental animals and environments

Juvenile milkfish (*C. chanos*) with a standard length of 9–11 cm were purchased from the Da-Shun milkfish aquafarm in Tainan, Taiwan. Fish were transported to the laboratory, where they were acclimated to aerated dechlorinated local tap water (i.e., FW) or SW (35‰) at  $28 \pm 1^\circ\text{C}$  under a 12-h light: 12-h dark cycle. SW was prepared from FW by adding standardized amounts of synthetic sea salt (Blue Treasure Synthetic Sea Salt; Blue Treasure, Surry Hills, Australia). The details of water parameters were reported in our previous study (Yang et al., 2017). Water was continuously circulated through fabric-floss filters. Fish were fed twice a day with a diet of commercial pellets.

### 2.3. Acclimation and transfer experiments

For long-term acclimation, the milkfish were acclimated to SW or FW for at least 4 weeks. In the time-course experiments, after long-term acclimation, milkfish in the study groups were transferred directly from SW to FW, and vice versa. Moreover, fish in the control group were transferred from SW to SW or from FW to FW for their respective experiments. Milkfish were sampled at 1/2, 1, 2, 4, and 7 days post-transfer, based on previous studies (Hu et al., 2017; Tang et al., 2009). Before sampling, the fish were not fed for one day and were then anesthetized using 39.8  $\mu\text{M}$  phenoxethanol. The tissues and organs were dissected and directly placed into liquid nitrogen and then stored immediately at  $-80^\circ\text{C}$  for subsequent analysis.

### 2.4. Total RNA extraction and reverse transcription

Total RNA from various milkfish tissues/organs was extracted with Iso-RNA Lysis Reagent (2302700; 5 Prime, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. The integrity and concentration of extracted RNA was verified and measured by 1% agarose gel electrophoresis and a NanoDrop 2000 (Thermo, Wilmington, DE, USA), respectively. For reverse transcription, cDNAs were synthesized with the iScript™ cDNA Synthesis Kit (170–8891; Bio-Rad, Hercules, CA, USA) or SMART™ RACE cDNA Amplification Kit (634858; Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The RNA and cDNA were stored at  $-80^\circ\text{C}$  and  $-20^\circ\text{C}$ , respectively.

### 2.5. Sequencing and identification of milkfish NKA $\alpha$ -subunits

Genes coding for NKA  $\alpha$ -subunits were derived from the milkfish transcriptome database (Hu et al., 2017). They were confirmed through RT- (reverse transcription) or RACE- (rapid amplification of cDNA ends) PCR (polymerase chain reaction) and then sequenced by the Tri-Biotech (Taipei, Taiwan).

To characterize NKA proteins, the amino acid sequences were deduced from sequenced genes using the NCBI Open Reading Frame Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). Subsequently, the transmembrane domains were predicted using the MEMSAT-SVM provided by the PSIPRED protein structure prediction server (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Buchan et al., 2013), and comparisons were made with transmembrane domains of teleostean NKA including the Senegalese sole (*Solea senegalensis*; Armesto et al., 2014) and European sea bass (*Dicentrarchus labrax*; Blondeau-Bidet et al., 2016). Potential phosphorylation sites of protein kinase C (PKC) and A (PKA) were predicted via NetPhos 3.1 (<http://www.cbs.dtu.dk/services/NetPhos/>) (Blom et al., 2004). The binding sites of Na<sup>+</sup> and K<sup>+</sup> were predicted according to the homology modeling of the cation binding sites of human NKA (Ogawa and Toyoshima, 2002).

For identification of these protein sequences, multiple sequence alignments, phylogenetic analysis, and protein identity were performed. Amino acid sequences for NKA  $\alpha$ -subunits from different organisms (Tables S1 and S2) were aligned by MUSCLE (multiple sequence comparison by log-expectation) using MEGA X (Kumar et al., 2018) for the subsequent analyses. These aligned sequences were used to confirm the positions of  $\alpha$ 1a-like and  $\alpha$ 1b-like residues based on Blondeau-Bidet et al. (2016) in order to distinguish the  $\alpha$ 1 paralogs by comparison with sequences of other teleostean species (Fig. S1). A phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstraps using MEGA X. Moreover, amino acid identity among teleosts and mammals was assessed using BioEdit 7.2.5 with the Sequence Identity Matrix (Hall, 1999).

## 2.6. Quantitative real-time PCR (QPCR)

The methods, including PCR protocol and data calculation, were described previously (Chang et al., 2016; Hu et al., 2017). Expression levels of target genes were quantified using 2 $\times$  FastStart Universal SYBR Green Master (04913850001; Roche, Mannheim, Germany) with the MiniOpticon real-time PCR system (Bio-Rad). Then, mRNA values for the target genes were normalized using the expression of  $\beta$ -actin mRNA from the same cDNA sample (Tang et al., 2010). Primer information used is listed in Table S2. The amplification efficiency was similar for all primer pairs used in this study (range: 0.93–1.08). To confirm the specificity of these amplifications from each test sample, sequencing, melting curve analysis, and electrophoresis were carried out. For each unknown sample, relative *fxyd* gene expression was obtained using the formula  $2^{-[(Ct_{target\ genes, n} - Ct_{\beta-actin, n}) - (Ct_{target\ genes, c} - Ct_{\beta-actin, c})]}$ , where Ct corresponds to the threshold cycle number (Livak and Schmittgen, 2001). The letter “n” indicates each cDNA sample used in these experiments and “c” indicates the control mixed with cDNAs from many organs.

## 2.7. Statistical analysis

All values are shown as the mean  $\pm$  standard error (SE). The Shapiro-Wilk normality test was applied. Because some of the data were not normally distributed, non-parametric tests were used. Differences in the mRNA levels between groups were analyzed via the Mann-Whitney *U* test or Kruskal-Wallis test for pair-wise or multiple comparisons, respectively. All analyses were performed using SPSS 20 software (SPSS, Chicago, IL, USA), and *P* values of < 0.05 were considered to be significant.

## 3. Results

### 3.1. Characterization of milkfish NKA $\alpha$ -isoforms

Four NKA  $\alpha$ -isoforms, CcNKA  $\alpha$ 1a,  $\alpha$ 1c,  $\alpha$ 2, and  $\alpha$ 3, were identified in milkfish (Fig. 1; Table S2). Among them, CcNKA  $\alpha$ 2 was a partial sequence because only 952 deduced amino acids were obtained. These

amino acid sequences of four CcNKA  $\alpha$ -isoforms shared similar characteristic features, including transmembrane domains (TMs), phosphorylation sites of PKC and PKA binding sites, binding sites for Na<sup>+</sup> and K<sup>+</sup>, and a conserved C-terminus (Fig. 1). For further confirmation, a multiple sequence alignment of teleostean NKA  $\alpha$ 1-isoforms was carried out (Fig. S1). The results revealed that CcNKA  $\alpha$ 1a belongs to one of the teleostean NKA  $\alpha$ 1a groups, because it has more  $\alpha$ 1a-like residues (blue triangles) as opposed to  $\alpha$ 1b-like residues (green triangles). On the other hand, in the phylogenetic analysis, amino acid sequences of NKA  $\alpha$ -subunit paralogs from teleosts, chicken, and human, with the sea urchin as outgroup, were aligned and a phylogenetic tree was constructed (Fig. 2). These proteins were divided into four groups ( $\alpha$ 1a/b,  $\alpha$ 1c,  $\alpha$ 2, and  $\alpha$ 3), and all CcNKA  $\alpha$ -isoforms of milkfish were classified into their respective groups. Among these clades, moreover, close relationships were noted among the  $\alpha$ 1a/b and  $\alpha$ 1c of teleosts and the  $\alpha$ 1 of human and chicken. Additionally, higher identities of both cDNA and amino acid sequences were found between CcNKA  $\alpha$ 1a or  $\alpha$ 1c and  $\alpha$ 3 rather than  $\alpha$ 2 (Table 1). Moreover, the sequence identities among all CcNKA  $\alpha$ -isoforms were higher for protein than for cDNA (Table 1). The comparisons of amino acid sequences of CcNKA  $\alpha$ 1a,  $\alpha$ 1c (Table 2),  $\alpha$ 2, and  $\alpha$ 3 (Table 3) individually with the homologues from other animal species showed that they shared a higher degree of homology with teleosts than with bird and mammal. Among the sequences of NKA  $\alpha$ -isoforms, CcNKA  $\alpha$ 2 revealed a lower identity with other teleostean NKA  $\alpha$ 2 (83.5–85.6%; Table 3).

### 3.2. Tissue distribution of milkfish *Ccnka* $\alpha$ genes

The QPCR results revealed tissue-specific expression for most of the *Ccnka*  $\alpha$  genes (Fig. 3). *Ccnka*  $\alpha$ 1a was present predominantly in the intestine and kidney, meanwhile *Ccnka*  $\alpha$ 1c was mainly expressed in the brain, heart, and liver. *Ccnka*  $\alpha$ 2 was expressed pervasively in all detected organs, while *Ccnka*  $\alpha$ 3 was primarily found in the brain and liver.

### 3.3. Salinity effects on *Ccnka* $\alpha$ and *Ccfxyd* genes in kidneys of SW and FW milkfish

In milkfish kidneys, *Ccnka*  $\alpha$ 1a was most predominantly expressed among *Ccnka*  $\alpha$  genes at both salinities, followed by *Ccnka*  $\alpha$ 1c (Fig. 4A). Meanwhile, renal *Ccfxyd*2 mRNA abundance was the highest in both SW and FW groups, followed by *Ccfxyd*8 and *Ccfxyd*9 (Fig. 4B). Low mRNA levels of *Ccnka*  $\alpha$ 2, *Ccnka*  $\alpha$ 3, *Ccfxyd*7a, *Ccfxyd*7b, and *Ccfxyd*11 were observed in milkfish kidneys. Moreover, the expression of *Ccnka*  $\alpha$ 1a, *Ccfxyd*2, *Ccfxyd*7b, and *Ccfxyd*9 genes in milkfish kidneys was salinity-dependent (Fig. 4). Renal mRNA levels of *Ccnka*  $\alpha$ 1a, *Ccfxyd*2, and *Ccfxyd*9 were significantly higher in the FW group than in the SW group. In contrast, a higher mRNA abundance of renal *Ccfxyd*7b was found in the SW-acclimated milkfish.

### 3.4. Time-course changes in the *Ccnka* $\alpha$ and *Ccfxyd* mRNA levels of milkfish kidneys upon salinity challenge

In milkfish kidneys, after transfer from SW to FW, the mRNA profiles of *Ccnka*  $\alpha$  and *Ccfxyd* genes were distinct, divided roughly into three patterns (Fig. 5). (1) mRNA expression of *Ccnka*  $\alpha$ 1a, *Ccfxyd*2, and *Ccfxyd*8 first changed (increased/decreased) and then recovered, compared to either the initial time-point (day 0) or the control group at the same time-point (Fig. 5A, C, D). (2) mRNA levels of *Ccfxyd*9 quickly increased and then slightly decreased. Expression of *Ccfxyd*9 mRNA was not significantly different from the initial time-point but was significantly higher than that of the control group at 7 days post-transfer (Fig. 5E). (3) No difference was observed in *Ccnka*  $\alpha$ 1c mRNA expression among different time-points or between the study and control group of each time-point after transfer (Fig. 5B). Among these profiles, most of the peak values were observed within 2 days post-transfer.

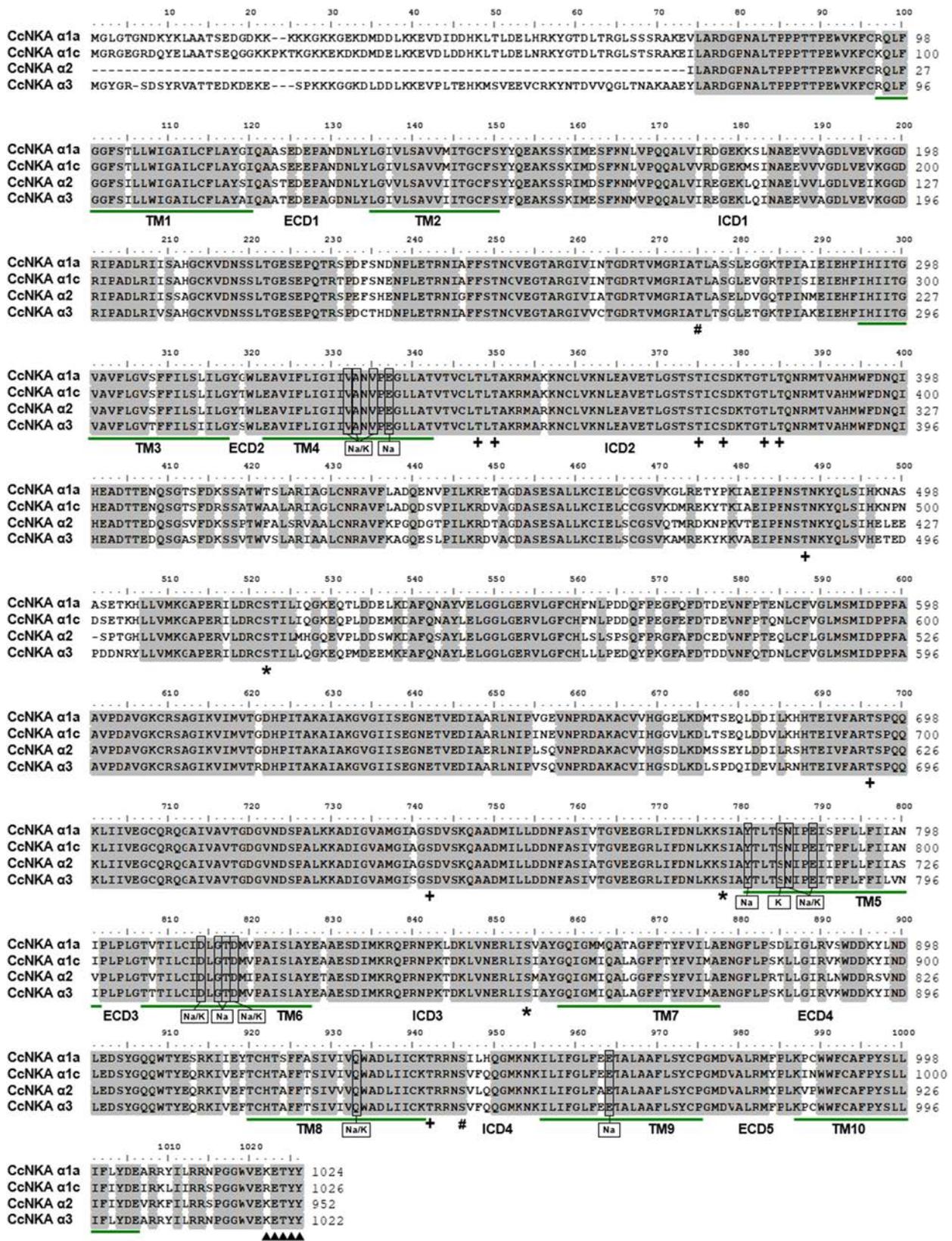
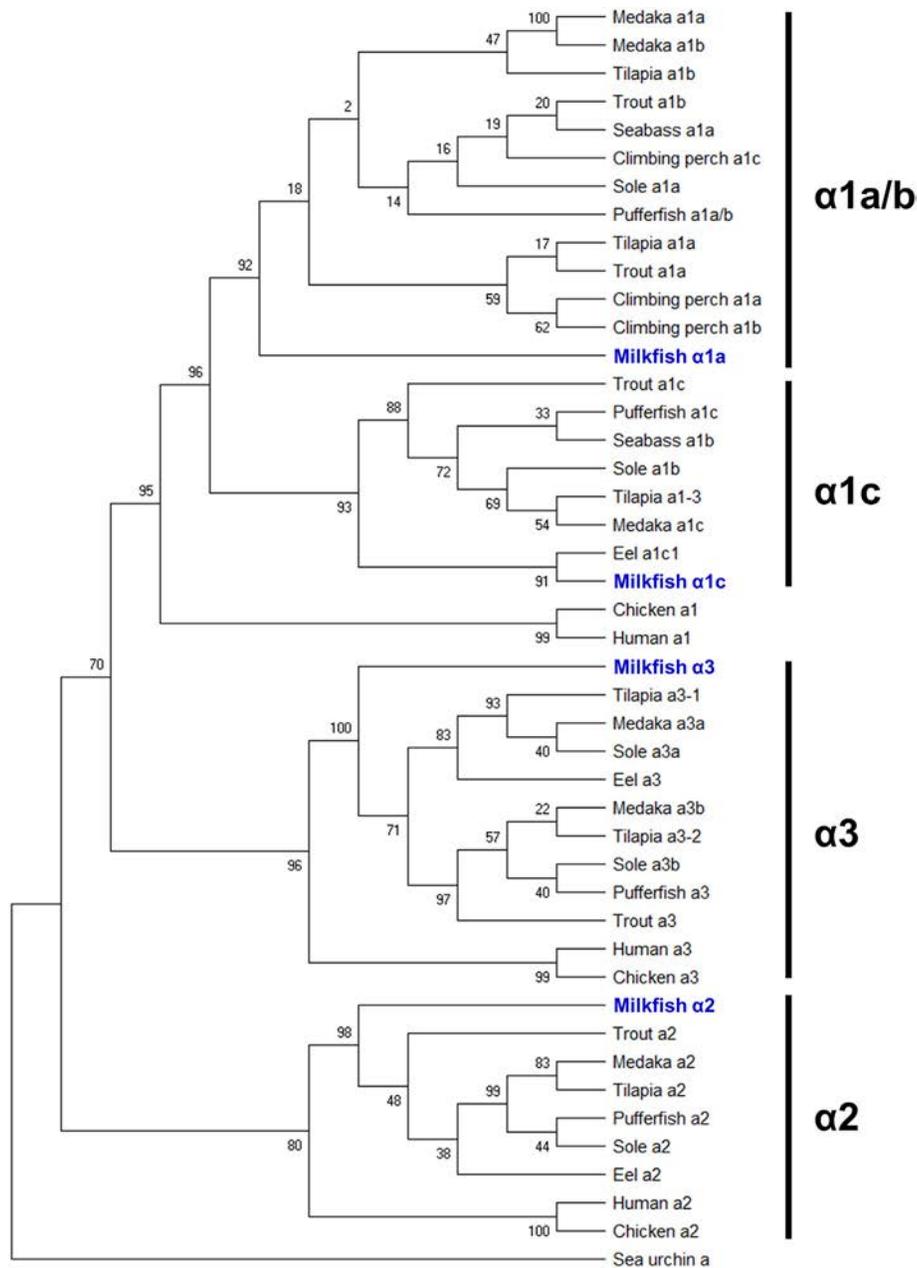


Fig. 1. Alignment of putative amino acid sequences of milkfish NKA (CcNKA)  $\alpha$ -isoforms. Dashes represent gaps, and the predicted transmembrane domains (TMs) are shown in green underlines. Vertical boxes represent coordinating residues for  $\text{Na}^+$  (Na) or  $\text{K}^+$  (K) binding and for either  $\text{Na}^+$  or  $\text{K}^+$  binding (Na/K). Potential phosphorylation sites are shown with crosses (+), asterisks (\*), and hash tags (#) for different kinases, including protein kinase C, protein kinase A, and both, respectively. Triangles indicate the KETYY motif. ECD, extracellular domain; ICD, intracellular domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Phylogenetic analysis of putative amino acid sequences of NKA  $\alpha$ -isoforms using the maximum likelihood method. The numbers at the forks indicate the bootstrap values (%). Sea urchin NKA  $\alpha$  was used as the outgroup for the root tree. Accession numbers are listed in Tables S1 and S2.

**Table 1**

Percentage of cDNA (open reading frame only; above diagonal) and amino acid (below diagonal) sequence identity among milkfish  $\text{Na}^+/\text{K}^+$ -ATPase (CcNKA)  $\alpha$ -subunits.

|                   | CcNKA $\alpha$ 1a (%) | CcNKA $\alpha$ 1c (%) | CcNKA $\alpha$ 2 (%) | CcNKA $\alpha$ 3 (%) |
|-------------------|-----------------------|-----------------------|----------------------|----------------------|
| CcNKA $\alpha$ 1a | –                     | 82.0                  | 72.4                 | 75.5                 |
| CcNKA $\alpha$ 1c | 91.4                  | –                     | 70.6                 | 78.4                 |
| CcNKA $\alpha$ 2  | 79.9                  | 81.1                  | –                    | 72.8                 |
| CcNKA $\alpha$ 3  | 85.4                  | 86.0                  | 80.8                 | –                    |

Accession numbers are listed in Table S2.

On the other hand, after transfer from FW to SW, mRNA levels of renal *Ccnka a1a* were clearly downregulated (Fig. 6A), while mRNA expression of renal *Ccnka a1c* and *Ccfxyd2* was first changed (increased/decreased) and then recovered (Fig. 6B, C). In renal *Ccfxyd8*

mRNA abundance, a significant change was found between 1 and 2 days post-transfer, and there was no difference among the other time-points (Fig. 6D). Among these profiles, mRNA levels of renal *Ccnka a1a* and *Ccfxyd2* clearly decreased at 1 day post-transfer, and the peak value of *Ccnka a1c* mRNA was observed at 4 days post-transfer. Moreover, no difference was observed in *Ccfxyd9* mRNA expression of milkfish kidneys among time-points or between the study and control group of each time-point after transfer (Fig. 6E).

Meanwhile, in both control groups (transfer from SW to SW and from FW to FW), no difference was found among time-points after transfer.

**Table 2**Amino acid identity (%) among NKA  $\alpha 1$ -isoforms.

| Classification | Species                      | Milkfish        |                 |
|----------------|------------------------------|-----------------|-----------------|
|                |                              | $\alpha 1a$ (%) | $\alpha 1c$ (%) |
| Teleost        | Killifish $\alpha 1a$        | 92.2            | 90.7            |
|                | European seabass $\alpha 1a$ | 91.5            | 90.4            |
|                | European seabass $\alpha 1b$ | 89.2            | 92.5            |
|                | Senegalese sole $\alpha 1a$  | 91.9            | 90.5            |
|                | Senegalese sole $\alpha 1b$  | 89.6            | 93.1            |
|                | Stickleback $\alpha 1a$      | 89.1            | 88.2            |
|                | Stickleback $\alpha 1b$      | 88.6            | 91.8            |
|                | Japanese medaka $\alpha 1a$  | 88.5            | 87.5            |
|                | Japanese medaka $\alpha 1b$  | 90.1            | 88.9            |
|                | Japanese medaka $\alpha 1c$  | 88.8            | 92.3            |
|                | Rainbow trout $\alpha 1a$    | 82.7            | 82.9            |
|                | Rainbow trout $\alpha 1b$    | 90.5            | 88.7            |
|                | Rainbow trout $\alpha 1c$    | 87.7            | 91.8            |
|                | Climbing perch $\alpha 1a$   | 81.2            | 80.2            |
|                | Climbing perch $\alpha 1b$   | 86.0            | 85.9            |
|                | Climbing perch $\alpha 1c$   | 90.5            | 89.8            |
|                | Electric eel $\alpha 1c1$    | 91.0            | 96.0            |
| Bird           | Chicken $\alpha 1$           | 87.5            | 88.6            |
| Mammal         | Human $\alpha 1$             | 88.5            | 89.4            |

Accession numbers are listed in Tables S1 and S2.

**Table 3**Amino acid identity (%) among NKA  $\alpha 2$ - and  $\alpha 3$ -isoforms.

| Classification | Species                     | Milkfish           |                |      |
|----------------|-----------------------------|--------------------|----------------|------|
|                |                             | $\alpha 2$ (%)     | $\alpha 3$ (%) |      |
| Teleost        | Killifish $\alpha 2$        | 85.6               | 81.9           |      |
|                | Killifish $\alpha 3$        | 81.1               | 94.1           |      |
|                | Senegalese sole $\alpha 2$  | 85.1               | 81.4           |      |
|                | Senegalese sole $\alpha 3a$ | 81.1               | 93.7           |      |
|                | Senegalese sole $\alpha 3b$ | 80.9               | 90.6           |      |
|                | Japanese medaka $\alpha 2$  | 83.5               | 81.1           |      |
|                | Japanese medaka $\alpha 3a$ | 80.9               | 93.2           |      |
|                | Japanese medaka $\alpha 3b$ | 80.2               | 91.3           |      |
|                | Rainbow trout $\alpha 2$    | 84.0               | 82.0           |      |
|                | Rainbow trout $\alpha 3$    | 79.7               | 89.0           |      |
|                | Electric eel $\alpha 2$     | 83.8               | 80.4           |      |
|                | Electric eel $\alpha 3$     | 81.2               | 94.8           |      |
|                | Bird                        | Chicken $\alpha 2$ | 83.8           | 84.2 |
|                |                             | Chicken $\alpha 3$ | 82.8           | 89.5 |
| Mammal         | Human $\alpha 2$            | 83.1               | 84.3           |      |
|                | Human $\alpha 3$            | 82.9               | 88.9           |      |

Accession numbers are listed in Tables S1 and S2.

## 4. Discussion

### 4.1. Milkfish NKA $\alpha$ -isoforms

To further verify the modulatory mechanisms and molecular responses of milkfish NKA in response to salinity changes, we identified the cDNA sequences of *nka*  $\alpha$ -subunits of milkfish for the first time. Four isoforms of the NKA  $\alpha$ -subunits in milkfish, i.e., CcNKA  $\alpha 1a$ ,  $\alpha 1c$ ,  $\alpha 2$ , and  $\alpha 3$ , were identified. Among them, the amino acid sequences of CcNKA  $\alpha 1a$  and  $\alpha 1c$  were the most similar compared to  $\alpha 2$  and  $\alpha 3$ . Similar results (highly similar  $\alpha 1$  paralogs) were found in other teleosts (Armesto et al., 2014; Richards et al., 2003). A previous study indicated that fish  $\alpha 1$  paralogs are derived from an ancestral  $\alpha 1$ . The ancestral  $\alpha 1$  was first duplicated into  $\alpha 1a/b$  and  $\alpha 1c$ , and then  $\alpha 1a/b$  was duplicated into  $\alpha 1a$  and  $\alpha 1b$  (Wong et al., 2016). The grouping of  $\alpha 1a$  and  $\alpha 1b$  in the phylogenetic trees of previous studies was sometimes confusing because of high similarities between their sequences (Bollinger et al., 2016; Ip et al., 2012; Wong et al., 2016). In this study, only one gene of the  $\alpha 1a/b$  paralogs was cloned from milkfish. However, it cannot be clearly identified from  $\alpha 1a$  or  $\alpha 1b$  via phylogenetic analysis

and protein identity as compared with other homologues. Therefore, according to Blondeau-Bidet et al. (2016), a multiple sequence alignment was performed. Subsequently, the gene was named *Ccnka a1a* because of its higher similarity of amino acid sequences with other teleostean NKA  $\alpha 1a$ . In addition to CcNKA  $\alpha 1a$ , other CcNKA  $\alpha$ -subunits could be named via the results of phylogenetic analysis and protein identity, referring to the nomenclature from previous studies (Armesto et al., 2014; Blondeau-Bidet et al., 2016).

Previous studies indicated that amino acid sequences of NKA  $\alpha$ -subunits are highly conserved across animal species (Armesto et al., 2014; Martin, 2005). In this study, higher similarities (over 80 and 90% for  $\alpha 2$  and others, respectively) of amino acid sequences among CcNKA  $\alpha$ -isoforms and their respective homologues from other animal species were obtained. Likewise, similar results were found in other fish, including the Senegalese sole (Armesto et al., 2014), electric eel (*Electrophorus electricus*; Ching et al., 2015), and European seabass (Blondeau-Bidet et al., 2016). However, in the climbing perch (*Anabas testudineus*), NKA  $\alpha 1a$  showed 70–80% homology to most NKA  $\alpha 1$ -isoforms from other teleosts (Ip et al., 2012). These results revealed that there might still be certain differences among NKA  $\alpha$  homologues. On the other hand, in a protein structure analysis, four CcNKA  $\alpha$ -isoforms showed the typical topology of P-type ATPase, including 10 TMs with ion-binding sites, five extracellular domains, and four intracellular domains containing phosphorylation sites of PKC and PKA, as seen in mammalian NKA  $\alpha$ -subunits (Blanco, 2005; Martin, 2005). These characteristic features have also been found in other teleosts (Armesto et al., 2014; Blondeau-Bidet et al., 2016; Ching et al., 2015). Moreover, the lysine (K)-rich sequences in the N-terminus of CcNKA  $\alpha$ -isoforms (except CcNKA  $\alpha 2$ ) present in many teleostean NKA  $\alpha$ -subunits are considered to play a key role in forming a cation-selective gate via an excess of positively charged amino acids during certain stages of the transport process (Blanco and Mercer, 1998; Daly et al., 1994; Vasilets and Schwarz, 1993) and/or in interacting with other proteins (Toyoshima et al., 2011). The KETYY motif in the tails of CcNKA  $\alpha$ -isoforms is well-conserved except for lysine (K), indicating that it is crucial for  $\text{Na}^+$  binding (Morth et al., 2011). The conserved degrees of KETYY motif differ among teleostean NKA  $\alpha$ -subunits, and these differences might cause a difference in  $\text{Na}^+$  affinity (Blondeau-Bidet et al., 2016; Ching et al., 2015). Taken together, these conserved motifs and domains of the CcNKA  $\alpha$ -isoforms play an important role in membrane localization and in ionoregulation via phosphorylation and dephosphorylation, as do other NKA  $\alpha$ -subunits.

In NKA  $\alpha$ -subunits, the form and number of isoforms vary among teleostean species. Some teleosts, including zebrafish (*Danio rerio*; Liao et al., 2009), rainbow trout (*Oncorhynchus mykiss*; Richards et al., 2003), Senegalese sole (Armesto et al., 2014), Japanese medaka (*Oryzias latipes*; Bollinger et al., 2016), and Japanese eel (*Anguilla japonica*; Wong et al., 2016), have more NKA  $\alpha$ -subunit isoforms than milkfish. In teleosts, moreover, most of these subunits belong to the highly diversified  $\alpha 1$ -isoforms, with tissue-specific distribution (Armesto et al., 2014; Wong et al., 2016). These two findings were also observed in milkfish, which showed two  $\alpha 1$ -isoforms and distinct tissue distribution patterns of four *Ccnka a* genes. As in other teleosts, *Ccnka a1a* is present primarily in the osmoregulatory organs, while *Ccnka a1c* is expressed ubiquitously in milkfish. However, their distribution patterns and expression levels vary by species (Armesto et al., 2014; Blondeau-Bidet et al., 2016; Ip et al., 2012; Richards et al., 2003; Wong et al., 2016). In the Senegalese sole, the *nka a1a* is mainly expressed in the gill, kidney, and intestine (Armesto et al., 2014), while in the rainbow trout *nka a1a* is present primarily in the gill (Richards et al., 2003). On the other hand, *Ccnka a2* mRNA are expressed ubiquitously in all detected organs, yet their abundance is unexpectedly low as compared to that of other *Ccnka a* genes. In mammals and other teleosts, *nka a2* is expressed predominantly in the muscle (Armesto et al., 2014; Bollinger et al., 2016; Richards et al., 2003; Suhail, 2010). Similar to results found in other animal species, *Ccnka a2* mRNA was present primarily in milkfish

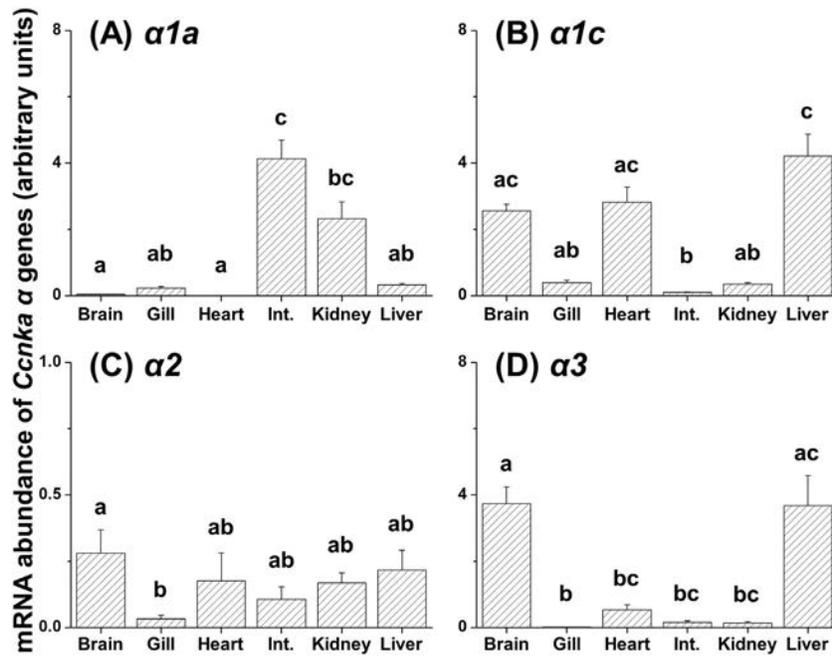


Fig. 3. Tissue distribution of four milkfish NKA (*Ccnka*)  $\alpha$  genes. The values are means  $\pm$  SE ( $N = 8$  or  $6$  for gill/kidney or other organs; combined with equal sample size from all salinity groups). Different letters indicate significant differences among *Ccnka*  $\alpha$  genes (Kruskal-Wallis test;  $P < .05$ ). Int., intestine.

muscle in our preliminary study (data not shown). In particular, the tissue distribution patterns of *Ccnka*  $\alpha3$ , which was found mainly in the brain and liver, were different from those of other teleosts. Some teleostean *nka*  $\alpha3$  are expressed predominantly in the brain alone, as in the Senegalese sole (both  $\alpha3a$  and  $\alpha3b$ ; Armesto et al., 2014). Other teleostean *nka*  $\alpha3$  are ubiquitously expressed in various organs, as in the Mozambique tilapia (*Oreochromis mossambicus*;  $\alpha3$ ; Feng et al., 2002) and rainbow trout ( $\alpha3$ ; Richards et al., 2003). Meanwhile, in the swamp eel (*Monopterus albus*),  $\alpha3a$  is found only in the brain and  $\alpha3b$  is widely distributed in various organs (Chen et al., 2013). Overall, these distinct tissue distribution patterns of four *Ccnka*  $\alpha$  genes might reveal that they play different roles in diverse organs as other NKA  $\alpha$ -subunits in mammals and other teleosts.

#### 4.2. Salinity-dependent expression of renal *Ccnka* $\alpha$ and *Ccfxyd* genes in milkfish

Both switching of NKA  $\alpha$ -subunits (the catalytic subunit) and FXYP proteins are modulatory mechanisms for NKA activity (Geering, 2008; Toyoshima et al., 2011). Recently, the relative abundance of multiple NKA  $\alpha$ -isoforms and FXYP proteins was found to be altered with ambient salinity changes in several species of teleosts (Hwang et al., 2011; Wong et al., 2016; Yang et al., 2018). Most of these studies, however, focused on the gills. Moreover, no study investigated the potential modulatory mechanisms for NKA expression/activity by analyzing

isoform compositions of NKA  $\alpha$ -subunits and FXYP proteins simultaneously in teleostean kidneys. In milkfish kidneys, *Ccnka*  $\alpha1a$  and *Ccfxyd2* might play major roles, since they were highly expressed. Conversely, the low levels of renal *Ccnka*  $\alpha2$ ,  $\alpha3$ , *Ccfxyd7a*, *Ccfxyd7b*, and *Ccfxyd11* implied that they might be functional in other organs, like those of mammals and other teleosts (Blanco, 2005; Geering, 2008; Hwang et al., 2011; Yang et al., 2013). In mammalian NKA  $\alpha$ -subunits, NKA  $\alpha$ -isoforms possessed different affinities for substrate ions, ouabain, and ATP (Blanco, 2005). Furthermore, in salmonid gills, NKA  $\alpha1a$  and  $\alpha1b$  were considered to be the FW- and SW-type isoforms, respectively (Dalziel et al., 2014; McCormick et al., 2009; Richards et al., 2003). In the kidneys of SW or FW milkfish, the mRNA abundance of *Ccnka*  $\alpha1a$  was higher than that of other *Ccnka*  $\alpha$  genes, indicating that there might be no switching of the NKA  $\alpha$ -isoforms, unlike the results in fish gills.

Upon salinity challenge, in this study, parallel expression patterns were found in renal *Ccnka*  $\alpha1a$  and *Ccfxyd2*, showing an increase in FW-acclimated milkfish. Their expression profiles were similar to those found in previous studies focused on NKA expression of milkfish kidneys, including mRNA (pan- $\alpha1$ ), protein (both pan- $\alpha$ - and pan- $\beta$ -subunits), and enzyme activity (Tang et al., 2010; Yang et al., 2016a). A previous study also reported that in milkfish kidneys, protein abundance of the NKA  $\alpha1$ -isoform was increased in the FW-acclimated group, while no difference was found in NKA  $\alpha2$ - and  $\alpha3$ -isoforms between the SW and FW groups (Yang et al., 2016a), which is in

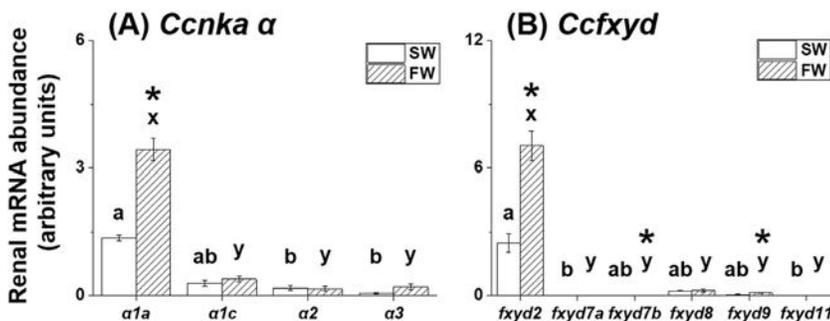
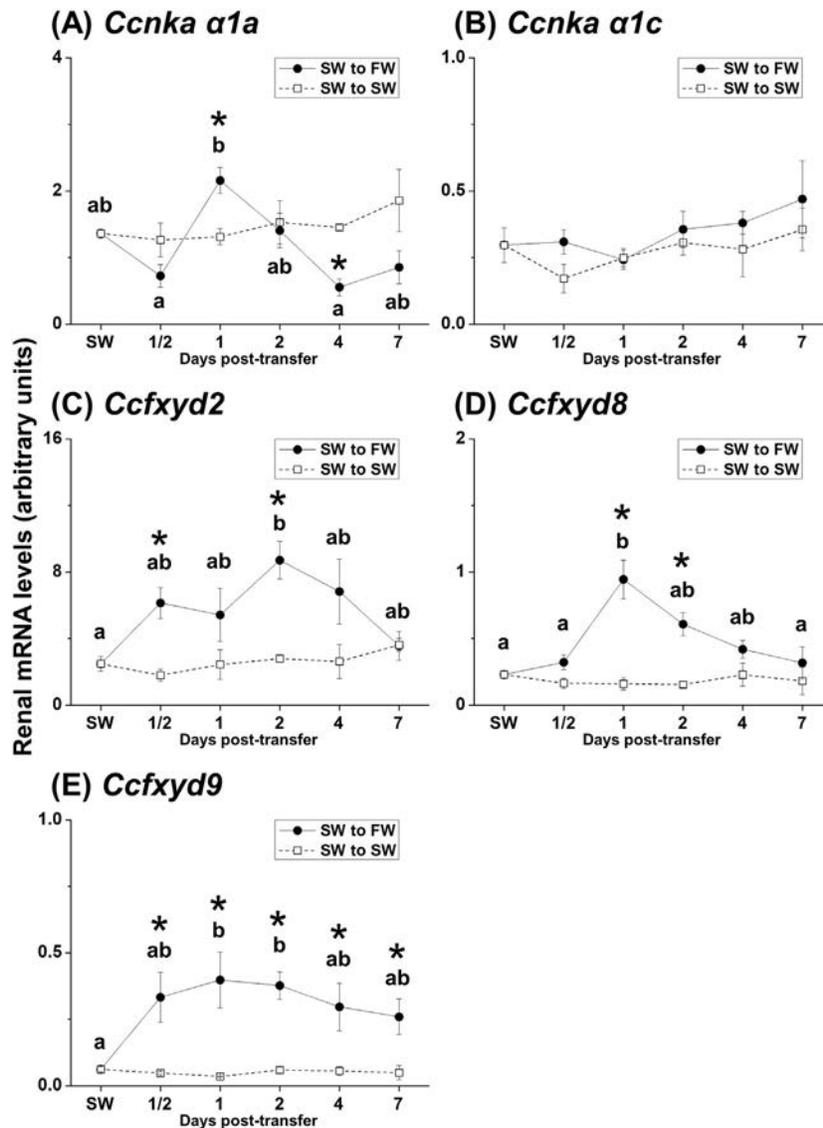


Fig. 4. Comparisons of renal *Ccnka*  $\alpha$  mRNA abundance in the milkfish. The values are means  $\pm$  SE ( $N = 4$ ). Different letters indicate significant differences among *Ccnka*  $\alpha$  genes in sea water (SW; letter: a, b) or freshwater groups (FW; letter: x, y) (Kruskal-Wallis test;  $P < .05$ ). The asterisks (\*) indicate significant differences between the SW and FW groups for the same gene (Mann-Whitney  $U$  test;  $P < .05$ ).

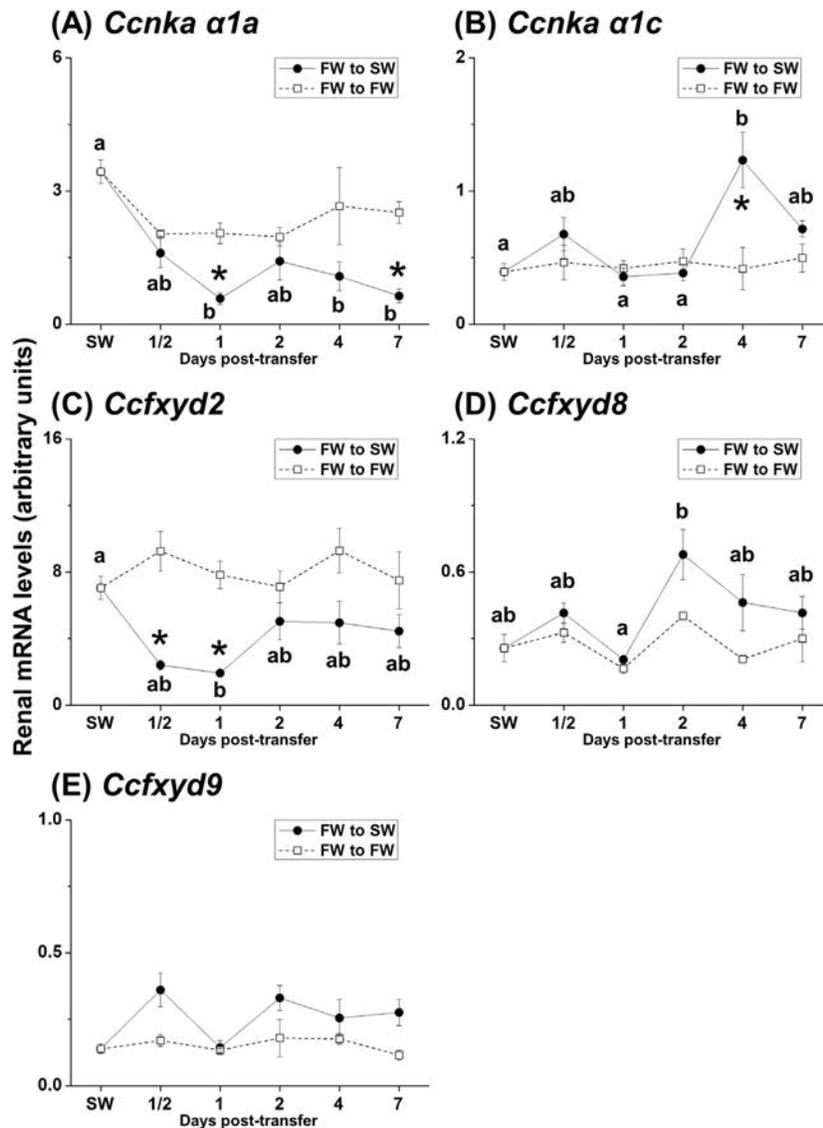


**Fig. 5.** Time-course changes in mRNA expression of renal *Ccnka*  $\alpha$  genes in the milkfish after transfer from seawater (SW) to fresh water (FW) (the study group) or to SW (the control group). The values are means  $\pm$  SE (N = 4 or 6 in the initial time-point or others, respectively). Different letters indicate significant differences among time-points in the study group (from SW to FW; Kruskal-Wallis test;  $P < .05$ ). No difference was found among time-points in the control group (from SW to SW). The asterisks (\*) indicate significant differences between the study and control groups at the same time-point post transfer (Mann-Whitney  $U$  test;  $P < .05$ ).

accordance with our data. Moreover, there was no difference in *Ccnka*  $\alpha 1c$  mRNA levels between FW and SW. Therefore, in FW-acclimated milkfish, induction of renal *Ccnka*  $\alpha 1a$  transcripts rather than *Ccnka*  $\alpha 1c$  might contribute to increased protein abundance of the NKA  $\alpha 1$ -isoform and then enhance NKA activity. On the other hand, a few studies have investigated the renal FXYD2 of teleosts, including the Atlantic salmon (*Salmo salar*; Tipsmark, 2008) and zebrafish (Saito et al., 2010). In kidneys of Atlantic salmon, *fxyd2* mRNA levels were higher in FW fish than in the SW group (Tipsmark, 2008). Although some studies have focused on other FXYD proteins of teleostean kidneys in response to salinity changes (Hu et al., 2014; Liang et al., 2017; Tang et al., 2012; Wang et al., 2017; Yang et al., 2016b), the role of renal FXYD2 in salinity challenge is not yet clear. Mammalian FXYD2 is also expressed in the kidney and inhibits NKA activity via binding with NKA (Arystarkhova, 2016; Geering, 2008). In the present study, however, we have no evidence regarding the potential inhibitory role of CcFXYD2 on NKA activity. In summary, when milkfish were acclimated to FW, high levels of renal *Ccnka*  $\alpha 1a$  and *Ccfxyd2* might cooperate to maintain a balance of high NKA activity for ionoregulation. More studies should be

performed in the future to clarify their roles in osmoregulation.

To further investigate the potential modulatory mechanisms, time-course experiments were performed. The levels of renal *Ccnka*  $\alpha 1a$  and *Ccfxyd2*, the predominant participants, changed quickly within 1 day after both transfer treatments. Similar results (quick changes) were also found in the gill NKA expression (both mRNA and protein of pan- $\alpha 1$ ) of milkfish since 1 day after transfer from SW to FW (Hu et al., 2017). Unlike the gill, changes in renal *Ccnka*  $\alpha 1a$  and *Ccfxyd2* expression were recovered subsequently. Similar patterns (recovered or unchanged expression) were found in renal *Ccnka*  $\alpha 1c$ , *Ccfxyd8, and *Ccfxyd9* after transfer. Furthermore, in milkfish kidneys, at 7 days post-transfer, significant changes in mRNA abundance were only observed in *Ccnka*  $\alpha 1a$  (from FW to SW) and *Ccfxyd9* (from SW to FW). These findings of recovered and stable expression in *nka*  $\alpha$  genes have also been reported in kidneys of other teleosts (Blondeau-Bidet et al., 2016; Wong et al., 2016; Zhu et al., 2018). In kidneys of the European seabass, mRNA levels of both *nka*  $\alpha 1a$  and  $\alpha 1b$  increased quickly and then recovered after transfer from SW to FW (Blondeau-Bidet et al., 2016). However, no difference in expression was found in most renal *nka*  $\alpha$  genes of the*



**Fig. 6.** Time-course changes in mRNA expression of renal *Ccnka α* genes in the milkfish after transfer from fresh water (FW) to seawater (SW) (the study group) or to FW (the control group). The values are means  $\pm$  SE (N = 4 or 6 in the initial time-point or others, respectively). Different letters indicate significant differences among time-points in the study group (from FW to SW; Kruskal-Wallis test;  $P < .05$ ). No difference was found among time-points in the control group (from FW to FW). The asterisks (\*) indicate significant differences between the study and control groups at the same time-point post transfer (Mann-Whitney *U* test;  $P < .05$ ).

Japanese eel after transfer from FW to SW (Wong et al., 2016). Previous studies reported that renal *nka α* expression might be modulated in a short time (Blondeau-Bidet et al., 2016) and that expression patterns of temporal changes might vary with species (Wong et al., 2016; Zhu et al., 2018). Moreover, the osmoregulatory capabilities of teleostean kidneys might be correlated with their evolutionary histories or natural habitats (Yang et al., 2016a,b). In the milkfish, which has excellent osmoregulatory capacity (Tang et al., 2010; Yang et al., 2016a), our results showed that genes expressed at the kidney level can be effectively regulated. The findings also revealed that the regulatory process might be divided into two periods, a short-term rapid modulation period (within 7 days) and a subsequent moderate regulation period, in response to salinity challenge. Other modulatory mechanisms of NKA  $\alpha$ -subunits might be involved during this period, such as endocrine regulation and protein phosphorylation (Crambert and Geering, 2003; Toyoshima et al., 2011). Notably, when the SW-residing milkfish were transferred to a hypoosmotic environment (FW), changes in renal *Ccnka* and *Ccfxyd* genes were more dramatic and more irregular compared to those in the group transferred from FW to SW, particularly for *Ccnka*

*α1a* and *Ccfxyd2*. Expression of these proteins was not assessed in this study and thus remains to be elucidated.

## 5. Conclusions

This study revealed the potential modulatory mechanisms for NKA expression from isoform compositions of NKA  $\alpha$ -subunits and FXYD proteins in kidneys of the milkfish upon hypoosmotic (i.e. FW) challenges. Four isoforms of milkfish NKA  $\alpha$ -subunits with tissue-specific distribution were identified: CcNKA  $\alpha1a$ ,  $\alpha1c$ ,  $\alpha2$ , and  $\alpha3$ . The highly conserved amino acid sequences and similar characteristic features to homologues from other animal species revealed their potential function. Notably, in milkfish kidneys, CcNKA  $\alpha1a$  and CcFXYD2 were predominantly expressed. Increases in renal *Ccnka α1a* and *Ccfxyd2* genes were found after exposure of milkfish to FW. After transfer, most renal *Ccnka* and *Ccfxyd* genes (particularly *Ccnka α1a* and *Ccfxyd2*) clearly changed their expression, although most of these changes were recovered subsequently. Our results suggest that the NKA activity of milkfish kidneys might be modulated via CcNKA  $\alpha1a$  and CcFXYD2 for

osmoregulation in response to salinity challenge. To our knowledge, this study provides the first evidence of the potential modulatory mechanisms for NKA expression/activity from isoform compositions of NKA  $\alpha$ -subunits and FXYP proteins in kidneys of euryhaline teleosts. Further studies are necessary to illustrate more regulatory mechanisms of milkfish upon salinity challenge and the results are expected to benefit and improve its aquaculture practice.

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## Conflict of interest

The authors have no competing interests to declare.

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