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Na^+-K^+-ATPase and *nka* genes in spotted sea bass (*Lateolabrax maculatus*) and their involvement in salinity adaptation

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ABSTRACT

Euryhaline teleosts can survive in a wide salinity range via alteration of the molecular mechanisms to maintain internal ionic and osmotic balance in osmoregulatory organs such as gill , kidney and intestine. Na^+/K^+-ATPase (NKA), plays a crucial role in sustaining intracellular homeostasis and is characterized by association of multiple isoforms of *α*- and *β*-subunits. To gain insight into the potential function of *nka* genes in salinity adaptation, 5 *nkaa* genes (*nkaa1a*, *nkaa1b*, *nkaa2*, *nkaa3a*, *nkaa3b*) and 7 *nkaβ* genes (*nkaβ1a*, *nkaβ1b*, *nkaβ2a*, *nkaβ2b*, *nkaβ3a*, *nkaβ3b and nkaβ4*) were identified from transcriptomic and genomic databases of *Lateolabrax maculatus*. The annotation and evolutionary footprint of these *nka* genes was revealed via the analysis of phylogenetic tree, gene synteny, copy numbers, exon-intron structures and motif compositions. The expressions of 12 *nka* genes in spotted sea bass was tested in ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain) and 6 genes (*nkaa1a*, *nkaa1b*, *nkaa3a*, *nkaa3b*, *nkaβ1b* and *nkaβ2a*) showed high expression in osmoregulatory organs. Furthermore, the responses of NKA and potential salinity-sensitive *nka* genes were examined under different salinity treatment (0 ppt, 12 ppt, 30 ppt, 45 ppt). Results showed that the enzyme activity of NKA was highest in gill and exhibited salinity dependent variation, with the highest activity identified in 45 ppt. Different *nkaα/β*-isoforms showed their diverse responses to salinity changes and the expression of *nka* genes including *nkaa1a*, *nkaa3b*, *nkaβ1b* in gill, *nkaa3a* in kidney and *nkaβ2a* in intestine were transcriptionally regulated by altered salinity. Notably, the expression patterns of *nkaa1a* and *nkaβ1b* in gill showed similar variation trend with NKA activity, suggesting that *nkaa1a/β1b* could be the major function isoforms involved in primary ion transport during salinity adaptation. Our results provided insights into the roles of *nkas* in osmotic regulation and a theoretical basis for future studies that focus on detailed molecular mechanisms in salinity adaptation of euryhaline teleosts.

1. Introduction

The spotted sea bass, *Lateolabrax maculatus*, belonging to *Lateolabrax*, Serranidae, is a euryhaline teleost species, which can live in a wide salinity range from freshwater to seawater and hypersaline environments, and is able to maintain internal ionic and osmotic balance (Zhang et al., 2017). The robust hyper-osmoregulatory and hypo-osmoregulatory abilities are mainly achieved by a group of organs including the gills, kidneys and intestine (Yang et al., 2016). For hyper-osmoregulation, where fishes regulate their internal extracellular fluids at a higher salt concentration than that of the surrounding dilute environment, gills, intestine and kidneys are used for active ion uptake, dietary ion uptake and renal uptake, respectively (Edwards and Marshall, 2012). On the contrary, hypo-osmoregulation happens when the internal extracellular fluids below the salt concentration of the surrounding seawater (SW), fishes use gills for NaCl secretion, intestine for absorption of salts and water, and kidney for renal ion excretion (Edwards and Marshall, 2012).

In the whole salinity adaptation stage, a large number of enzymes are involved in the osmotic pressure recovery process of fish, among which the most important to osmotic regulation is Na^+-K^+-ATPase (NKA), drives indirectly a number of transport process and also known as Na^+/K^+ pump, a major source of ATP consumption in cells (Evans et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011). Based on accumulating evidence, the well-accepted model for NaCl secretion of euryhaline teleosts proposed that the basolateral-located NKA transports three Na^+ ions outward in exchange for two K^+ ions, creating low intracellular Na^+ and a highly negative charge within the cell; Na^+ is

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transported back outside the cells by a paracellular pathway, probably involving NKA and junctions between chloride cells and adjacent accessory cells (Evans et al., 2005; Hwang and Lee, 2007; Hwang et al., 2011; Hiroi and McCormick, 2012). In this process, the electric potential formed in the cells inside and outside, change the secondary membrane structure and open ion channels, maintain the environment in the steady state, and through the hydrolysis of ATP to provide all kinds of ion transport driver (Mccormick, 2001). Based on this theory, the levels of *nka* genes are supposed to be up-regulated by exposure to increased salinity. However, studies on variety of teleost species showed issues remain debatable or conflicting due to the fact that different isoforms of each gene may diverge in regulatory structures and osmoregulatory functions, difference in tissues, or the natural habitats salinity varied among different species (Hwang et al., 2018).

NKA is a membrane-spanning P-type ATPase consisting of an $(\alpha\beta)_2$ protein complex, which composed of a catalytic α-subunit with a molecular weight of about 100 kDa and a smaller glycosylated β-subunit with a molecular weight of approximately 55 kDa (Blanco and Mercer, 1998). As a primary active pump, NKA is important not only for sustaining intracellular homeostasis, but also for providing the driving force for ion-transporting systems within a variety of osmoregulatory epithelia (Lin et al., 2003). It has been widely reported that teleosts exhibit adaptive changes in NKA activity following salinity changes. Studies on salmon (*Salmo salar*), eel (*Anguilla anguilla*) and freshwater cichlids (*Sarotherodon mossambicus*) showed a positive correlation between environmental salinity and gill NKA activity, while this correlation does not hold universally because some species like flounder (*Platichthys flesus*), mullet (*Mugil cephalus*) and sea bream (*Sparus auratus*) showed no change or a reduction in NKA activity during adaptation to seawater (Hirose et al., 2003; Perry et al., 2003; Marshall and Bryson, 1998; Lin et al., 2003; Nilsen et al., 2007). Recent studies revealed that these distinct salinity-induced NKA responses were considered correlating with the natural habitats and/or life histories of different species (Hwang et al., 2011; Takei et al., 2014; Yang et al., 2016). Salinity-induced higher NKA responses were found in hyper-osmotic environment in several FW preference teleosts like medaka (*Oryzias latipes* and *Oryzias dancena*), tilapia (*Oreochromis niloticus*) and eel (Freire et al., 2008; Kang et al., 2008; Yang et al., 2016), while higher NKA responses were found in hypo-osmotic media in marine-resident species including killifish (*Fundulus heteroclitus*), milkfish (*Chanos chanos*) and sea bream (Marshall and Bryson, 1998; Lin et al., 2003; Hwang and Lee, 2007). Those studies suggest that fish may maintain a comparatively low NKA activity in their natural habitats (Lin et al., 2003).

NKA activity was reported to be regulated by the switching of NKAα- and/or β-isoform composition (Blanco, 2005; Yang et al., 2016). The α-subunit isoform contains the binding sites for Na^+, K^+ and ATPase, taking responsibility for the major catalytic and ion-transporting work of NKA, while β-subunit is important for stabilizing folding of the α-subunit (Sundh et al., 2014). Three isoforms of α-subunit (α1, α2, α3) have been identified in teleosts (Armesto et al., 2014; Yang et al., 2016). Distinct responses of NKA α-isoforms during salinity acclimation have been reported at mRNA and protein levels in a number of species (Richards et al., 2003; Tang et al., 2009; Yang et al., 2016), suggesting that alteration of expression of NKA α-isoforms might be the crucial mechanism for acclimation to various environmental salinities (Tang et al., 2009).

Spotted sea bass, as a euryhaline teleost that can survive in both hyper-osmoregulatory and hypo-osmoregulatory environments, is an ideal template for studying the mechanisms of responses to salinity changes. As an important osmotic regulator, the *nka* genes in the spotted sea bass genome and their involvements in response to salinity regulation are currently unexplored. To fill in the gap, in the present study, we characterized and confirmed the annotations of these *nka* genes by phylogenetic, syntenic and gene structure analysis. Moreover, the mRNA expression patterns of these genes in different tissues were determined and their expressions at different salinities were further examined to study the functions in salinity adaptation. In addition, the NKA enzyme activities in the kidney, gill or intestinal tissues after adapted to different salinities were examined respectively in spotted sea bass.

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 field studies did not involve endangered or protected species.

2.2. Salinity challenge experiment

The spotted sea bass fingerlings (120.66 ± 13.05 g) were obtained in Shuangying Aquatic Seedling Co., Ltd., lijin, Shandong, China. Prior to experiment, fish were maintained under a 14:10 h light–dark photoperiod in a 5 m*5 m*1 m cement pond for one week at a water temperature of 25.3 ± 0.7 °C, dissolved oxygen of 7.01 ± 0.45 mg/L, pH of 7.8 ± 0.5 and salinity of 30 ppt. After an initial acclimation period, fish were exposed to gradually changing salinity over 12 h until it reached to 0 ppt (FW, fresh water group), 12 ppt (IP, isotonic point group), 45 ppt (HS, high salinity group) and sea water group (SW, control group) was kept at 30 ppt. Experiments were conducted in 12 cuboid tanks (120 L capacity) (12 fish per tank) for one month and all treatment groups were triplicated. The fish were fed a commercial goldfish diet daily and the water was partially replaced once per day.

2.3. Fish sampling, plasma analysis and NKA activity assay

After 30 days breeding, 9 fish per tank were treated with tricaine methane sulfonate (MS 222, 200 mg/L) and sampled immediately. Fish blood was collected from the caudal vein. After centrifugation at 4000 r/min for 10 min, upper serum was collected and stored at −20 °C for osmolality analysis. Serum osmolality was measured by Wescor 5500 vapro osmometer (Wescor, Logan, UT). Samples (kidney, intestine and gill tissues) used for enzyme activity assays were milled in liquid nitrogen and added normal saline with 9 times the volume of the tissue mass, fully ground with a tissue grinder, then centrifuged, and the supernatant was absorbed for determination. NKA activity was determined by Na^+/K^+-ATPase assay kit (Nanjing Jiancheng Bioengineering institute) and the specific measurement method was carried out in reference to the instruction manual.

2.4. Identification analysis of nka genes

To identify *nka* genes in spotted sea bass, gene sequences of human (*Homo sapiens*), mouse (*Mus musculus*), chicken (*Gallus gallus*), catfish (*Ictalurus punctatus*), medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), pufferfish (*Takifugu rubripes*) and European sea bass (*Dicentrarchus labrax*) were retrieved from the Ensembl (http://www.ensembl.org) and NCBI (http://www.ncbi.nlm.nih.gov/) databases and were used as queries for TBLASTN ($1e^{-5}$) search against the transcriptome database (NCBI accession numbers: SRR4409341 and SRR4409397) (Zhang et al., 2017) and the reference genome database (Assembly: GCA_004028665.1 ASM402354v1) of the spotted sea bass. TBLASTN was used to obtain the initial pool of *nka* transcript sequences in the spotted sea bass, and 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 were validated using BLASTP against

NCBI non-redundant protein sequence database.

The lengths of mRNA and the number of amino acids of *nka* genes were obtained from the transcriptome database and genome database of spotted sea bass. Molecular weight (MW, kDa) and isoelectric point (pI) of each putative NKA proteins were calculated using ExPASyProtParam tool (https://web.expasy.org/protparam/). Subcellular localization of all putative NKA proteins in spotted sea bass was predicted by subcellular localization predictor (http://cello.life.nctu.edu.tw/) (Yu et al., 2006).

2.5. Phylogenetic and syntenic analysis of nka genes

To investigate the phylogenetic relationship and classification of *nka* genes in spotted sea bass, the amino acid sequences of these genes from several representative vertebrates including human, mouse, chicken, medaka, zebrafish, pufferfish and European sea bass were selected and retrieved from the NCBI non-redundant protein sequence database for phylogenetic analysis. Multiple protein sequences were aligned by ClustalX1.83 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/).To provide additional evidences for orthologues and identification, syntenic analysis was performed for five *nka* genes that were not well supported by phylogenetic analysis. Syntenic analysis was conducted by comparing the genomic regions around the *nka* genes between spotted sea bass and several other species. The neighboring genes of spotted sea bass *nka* genes were extracted from the whole genome annotations, whereas other species were obtained from Genomicus (Louis et al., 2015), NCBI database (http://www.ncbi.nlm.nih.gov) and Ensembl genome browser (http://www.ensembl.org/).

2.6. Sequence analysis of nka genes in spotted sea bass

The sizes of the exons and the positions of exon-intron were concluded by each predicted *nka* genes from the L. *maculatus* databases. Exon-intron structures of the *nka* genes were generated using the Gene Structure Display Server (Hu et al., 2015) (GSDS, http://gsds.cbi.pku.edu.cn/). The homologous domain architectures of the *nka* genes were generated by the SMART 7.0 program (Letunic et al., 2012) (http://smart.embl.de/smart/). Alignment analysis was conducted using DNAMAN V6.0 software. Potential phosphorylation sites of protein kinase C (PKC) and protein kinase A (PKA) were predicted via NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom et al., 2004). The binding sites of Na^+ and K^+ were predicted according to the homology modeling of the cation binding sites of human NKA (Ogawa and Toyoshima, 2002; Yang et al., 2019).The conserved motifs of NKA proteins were observed with MEME (Multiple Expectation Maximization for Motif Elicitation) software (http://meme-suite.org/tools/meme) (Bailey et al., 2009) and the parameters were set as follows: distribution of motif occurrences, zero or one per sequence; width of motifs ranged, 6 to 50 residues; other parameters, default. *Nka* genes were mapped on chromosomes by identifying their chromosomal position provided in L. *maculatus* databases. The distribution map of *nka* genes throughout L. *maculatus* genome was protracted using MapDraw V2.1 software (Liu and Meng, 2003).

2.7. RNA extraction and tissues distribution analysis of nka genes

3 fish maintained in natural sea water condition was desected to examine genes expression profiles in different tissues of L. *maculatus*. Ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain) per fish were collected respectively and stored at −80 °C for RNA extraction. Sample used for RNA extraction was placed into 1.5 mL RNAs-free tubes immediately and stored at −80 °C until processed.

Quantitative real-time PCR (qRT-PCR) was used to detect the mRNA expressions of *nka* genes among different tissues of spotted sea bass. Total RNAs were isolated from ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain) using TRIzol® reagent (Invitrogen, USA). The concentration and integrity of total RNAs were assessed using the Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing) and electrophoresis. Before first-strand cDNA was synthesized, gDNA was removed by using a PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara). Then, 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 supplementary Table 1. *18S* ribosomal RNA (*18S*) was used as the reference gene for qRT-PCR normalization (Wang et al., 2018). Each reaction for qRT-PCR consisted of a total volume of 20 μL containing 10 μL of SYBR® FAST qPCR Master Mix (2×) (Takara, Shiga, Japan), 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 used the following conditions: 95 °C for 5 s followed by 40 cycles of 95 °C for 5 s and 59 °C for 30 s with a final dissociation curve to verify the specificity of the 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 analysis the expression level of genes.

2.8. Expression analysis of nka genes after acclimation to different salinities

qRT-PCR was also used to detect the mRNA expression variation among different salinity treatment groups. Total RNAs were extracted from fish gills, intestines and kidneys in each salinity treatment group (0, 12, 30, 45 ppt) in the preceding experiment. 3 individual fish in the same salinity tank were pooled as one sample, and 3 replicated samples were made for each salinity treatment group. The reaction condition and statistical analysis for qRT-PCR were performed as previously described.

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

3. Results

3.1. Effect of salinity on osmolality in serum and Na^+/K^+-ATPase (NKA) activity of spotted sea bass

No fish died during the whole experimental period. Plasma osmolality was lowest in FW (0 ppt, 396.8 mOsm/kg), which increased to 398.5 mOsm/kg in BW (12 ppt), 413 mOsm/kg in SW (30 ppt) and arrived to the highest in HS (45 ppt, 416.5 mOsm/kg). There was no marked difference in plasma osmolality among FW, IP and SW groups, whereas the plasma osmolality in HS was significantly higher than FW and IP groups (Fig. 1 A).

NKA activities in gill, kidney and intestine in four salinity treatment groups were listed in Fig. 1B, and gill harbored the highest NKA activity among the three tissues. The highest NKA activity of three tissues was all found in HS group. In gill, the NKA activity exhibited U-shaped distribution with the increases of salinity, which ranged from the lowest 0.82 μmol $P{\cdot}mg^{-1}{\cdot}h^{-1}$ in IP to the highest 3.85 μmol $P{\cdot}mg^{-1}{\cdot}h^{-1}$ in HS. The variation tendency of NKA activities in kidney and intestine was similar that the NKA activities in FW and HS were greater than those in IP and SW (Fig. 1 B).

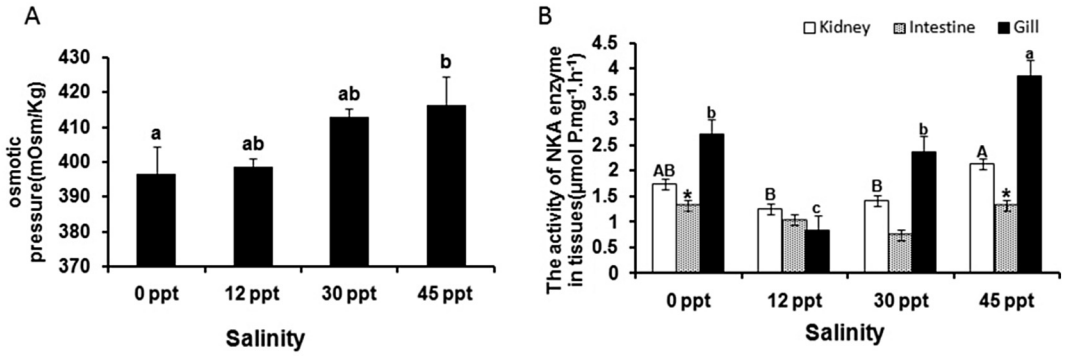

Fig. 1. (A) Effect of salinity on osmolality in serum of spotted sea bass. (B) Effects of salinity on the activity of Na^+-K^+-ATP in gill, kidney and intestine of spotted sea bass. The results are shown as the means ± standard error of mean (SEM). The significant differences ($P < 0.05$) in different tissues are represented by capital, lowercase letters and asterisks, respectively. The graphs were made with Excel 2010.

3.2. Identification of nka genes in spotted sea bass and gene copy number analysis

Twelve *nka* genes were identified in L. *maculatus* genome including *nkaα1a*, *nkaα1b*, *nkaα2*, *nkaα3a*, *nkaα3b*, *nkaβ1a*, *nkaβ1b*, *nkaβ2a*, *nkaβ2b*, *nkaβ3a*, *nkaβ3b* and *nkaβ4*. All the sequences for twelve genes have been submitted to Genbank, and their accession numbers and other protein characteristics were presented in Table 1. The complete encoding sequence was obtained for almost all *nka* genes except for the *nkaα3b* gene, which lack of 3′ end. The transcript lengths of the *nka* genes ranged from 840 bp (*nkaβ3b*) to 3348 bp (*nkaα1a*), the predicted amino acids of the twelve proteins ranged from 279 (*nkaβ3b*) to 1055 (*nkaα2*), the putative MWs ranged from 32.38 (*nkaβ3b*) to 116.76 kDa (*nkaα2*), and the theoretical pIs ranged from 5.15 (*nkaα3a*) to 8.64 (*nkaβ1b*). Subcellular location prediction for the putative NKA*α* proteins showed that most of them were localized in the cytoplasm (Cy) and inner membrane (Im), except for *nkaα3b*, which was only distributed in the Im. Subcellular location prediction for the putative NKA*β* proteins showed that they were mainly localized in the cytoplasm (Cy) or periplasm (Pp), except for *nkaβ2a*, which was distributed in the out membrane (Om) and extracellular space (Ec) (Table 1).

The copy number of the *nka* genes was investigated in spotted sea bass and other selected vertebrates (Table 2). Among these genes, five genes owned two copies in the spotted sea bass genome, including *nkaα1a, nkaα1b*, *nkaα3*a, *nkaα3b, nkaβ1a*, *nkaβ1b, nkaβ2a, nkaβ2b, nkaβ3a* and *nkaβ3b*. The remaining genes of spotted sea bass were present in a single copy, including *nkaα2* and *nkaβ4*.

The gene copy numbers of the *nka* genes varied significantly among different species. In general, 12 genes were detected in spotted sea bass, medaka and zebrafish; 11 in fugu; 9 in catfish; 7 in human, mouse and chicken (Table 2). For mammals and birds, each *nka* gene harbored only one copy. However, duplicates genes existed for several genes in the teleost species we analyzed. For instance, four genes (*nkaα1*, *nkaα3*, *nkaβ1* and *nkaβ3*) had two copies in fugu, medaka, spotted sea bass and zebrafish. Notably, two genes (*nkaα1* and *nkaα3*) had only one copy in human, mouse and chicken, but two copies existed in all the tested teleosts. In addition, the *nkaβ4* gene was only found in spotted sea bass and zebrafish with a single copy.

Table 2
Comparison of gene copy numbers of genes among selected vertebrate genomes.

Genes	Hsa	Mmu	Gga	Ipu	Ola	Tru	Dre	Lmu
nkaα1	1	1	1	2	2	2	2	2
nkaα2	1	1	1	1	1	1	1	1
nkaα3	1	1	1	2	2	2	2	2
nkaα4	1	1	1	0	0	0	0	0
nkaβ1	1	1	1	1	2	2	2	2
nkaβ2	1	1	1	2	2	2	2	2
nkaβ3	1	1	1	1	2	2	2	2
nkaβ4	0	0	0	0	1	0	1	1
Total	7	7	7	9	12	11	12	12

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

3.3. Phylogenetic and syntenic analysis of nka genes in spotted sea bass

The annotation of *nka* genes in spotted sea bass were further confirmed by phylogenetic analysis depending on the inclusion of *nka* genes from human, mouse, chicken, and several teleost species. For delineating the evolution history, the phylogenetic tree was constructed using the deduced amino acid sequences of L. *maculatus* and selected

Table 1
Summary of *nka* genes identified in the spotted sea bass genome.

Genes	mRNA length (bp)	Protein length (aa)	MW (kDa)	pI	Localization	Sequence integrity	Accession number
nkaα1a	3348	1023	112.43	5.32	Im, Cy	Complete	MH142151
nkaα1b	3078	1025	112.65	5.16	Im, Cy	Complete	MH142152
nkaα2	3168	1055	116.76	5.89	Im, Cy	Complete	MH142153
nkaα3a	3069	1022	112.73	5.15	Im, Cy	Complete	MH142154
nkaα3b	2967	988	109.21	5.44	Im	partial	MH142155
nkaβ1a	2884	301	34.35	8.64	Pp	Complete	MH142157
nkaβ1b	1526	301	34.51	6.75	Cy, Pp	Complete	MH142156
nkaβ2a	1475	288	33.04	5.82	Om, Ec	Complete	MH142158
nkaβ2b	855	284	32.77	6.62	Pp	Complete	MH142159
nkaβ3b	960	319	37.29	5.35	Cy	Complete	MH142160
nkaβ3a	840	279	32.38	7.49	Cy, Pp	Complete	MH142161
nkaβ4	1029	342	39.34	7.15	Cy, Pp	Complete	MH142162

Abbreviations: mRNA: messenger RNA; MW, molecular weight; pI, isoelectric point; bp, base pairs; Ec, extracellular space; Pp, periplasm; Cy, cytoplasm; Im, inner membrane; Om, out membrane.

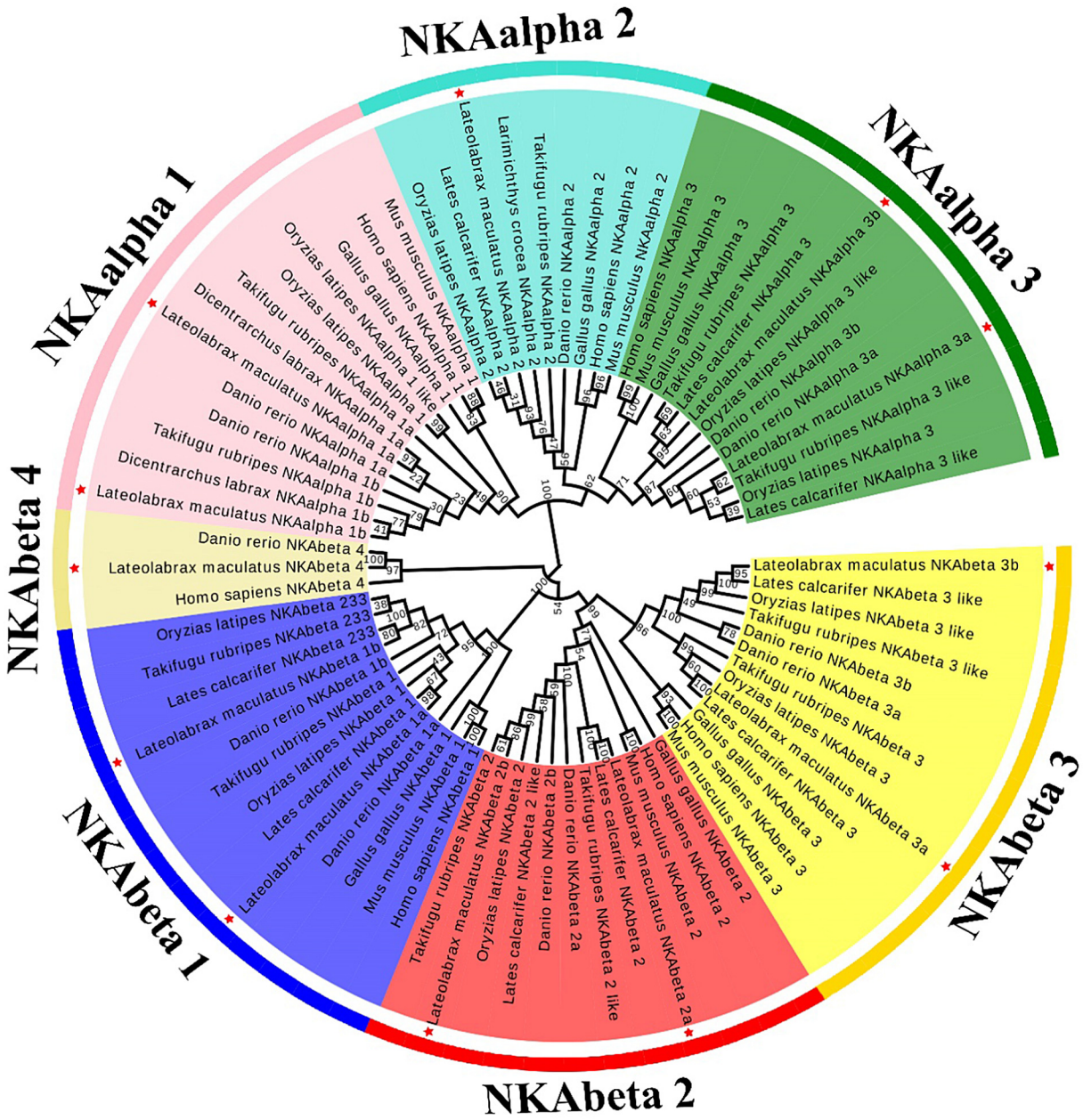

Fig. 2. Phylogenetic analysis of *nka* genes. The phylogenetic tree was constructed by the amino acid sequences from several representative vertebrates with 1000 bootstrap replications in MEGA 7, ClustalX1.83 and iTOL online software. *nka* genes of spotted sea bass were labeled with red star.

species. As shown in Fig. 2, the *nkaα1a, nkaα1b,* and *nkaα2* genes of spotted sea bass were categorized into the same clades as those of the other tested species and had the closest relationships with teleost sequences. The annotations for the remaining genes had multiple copies needed further analysis.

Syntenic analysis was performed to provide additional evidence for the annotation of ambiguous *nka* genes, including *nkaα3* (2 copies), *nkaβ1* (2 copies), *nkaβ2* (2 copies), *nkaβ3* (2 copies) and *nkaβ4*. Gene abbreviations and full names were listed in Supplementary abbreviation. As shown in Fig. 3, a conserved synteny was identified between spotted sea bass and zebrafish that the *nkaα3a* gene were surrounded with *zbtb22b*, *kifc1* and *bmp8a* (Fig. 3A). Another copy gene of *nkaα3*, the *nkaα3b* gene, shared similar neighbor genes with that of zebrafish, including *tubb5*, *vars*, *mrl51*, *grik5*, *pafah1b3*, *cblc* and *mrpl17* (Fig. 3A).

As shown in Fig. 3B, the *nkaβ1a* genes of spotted sea bass had the similar neighbor genes with human *nkaβ1*, including *nme7*, *barhl2*, *tgfbr3* and *brdt*. Moreover, the arrangement of surrounding genes of *nkaβ1a* in spotted sea bass was highly conserved with that of zebrafish, which confirmed the annotation of *nkaβ1a*. Similarity, the annotation for *nkaβ1b* gene was supported by the conserved synteny between spotted sea bass and zebrafish (Fig. 3B). The spotted sea bass *nkaβ2a* gene had similar neighboring genes to those of the *nkaβ2a* gene in zebrafish, including *cnga1*, *nfxl1*, *corin*, *pfn1* and *hmgb2b* (Fig. 3C). The *nkaβ2b* gene in spotted sea bass was located between *gc3*, *slc12a9*, *pld2*, *chrne* and *rnf167* in the chromosome 12, which was in accordance with the location of zebrafish *nkaβ2b*. The spotted sea bass *nkaβ3a* gene had similar neighboring genes to those in zebrafish, including *gk5*, *imp3*, *tsen15*, *eif4a2* and *hfm1*. Moreover, for another copy of the *nkaβ3* gene, the annotation for *nkaβ3b* gene was supported by the conserved synteny between spotted sea bass and zebrafish (Fig. 3D). The *nkaβ4* gene had the same neighboring genes, including *upf3b*, *ndufa1*, *zbtb33*, *cal4b*, *mcts1*, *galt1c1* and *clic2* (Fig. 3E).

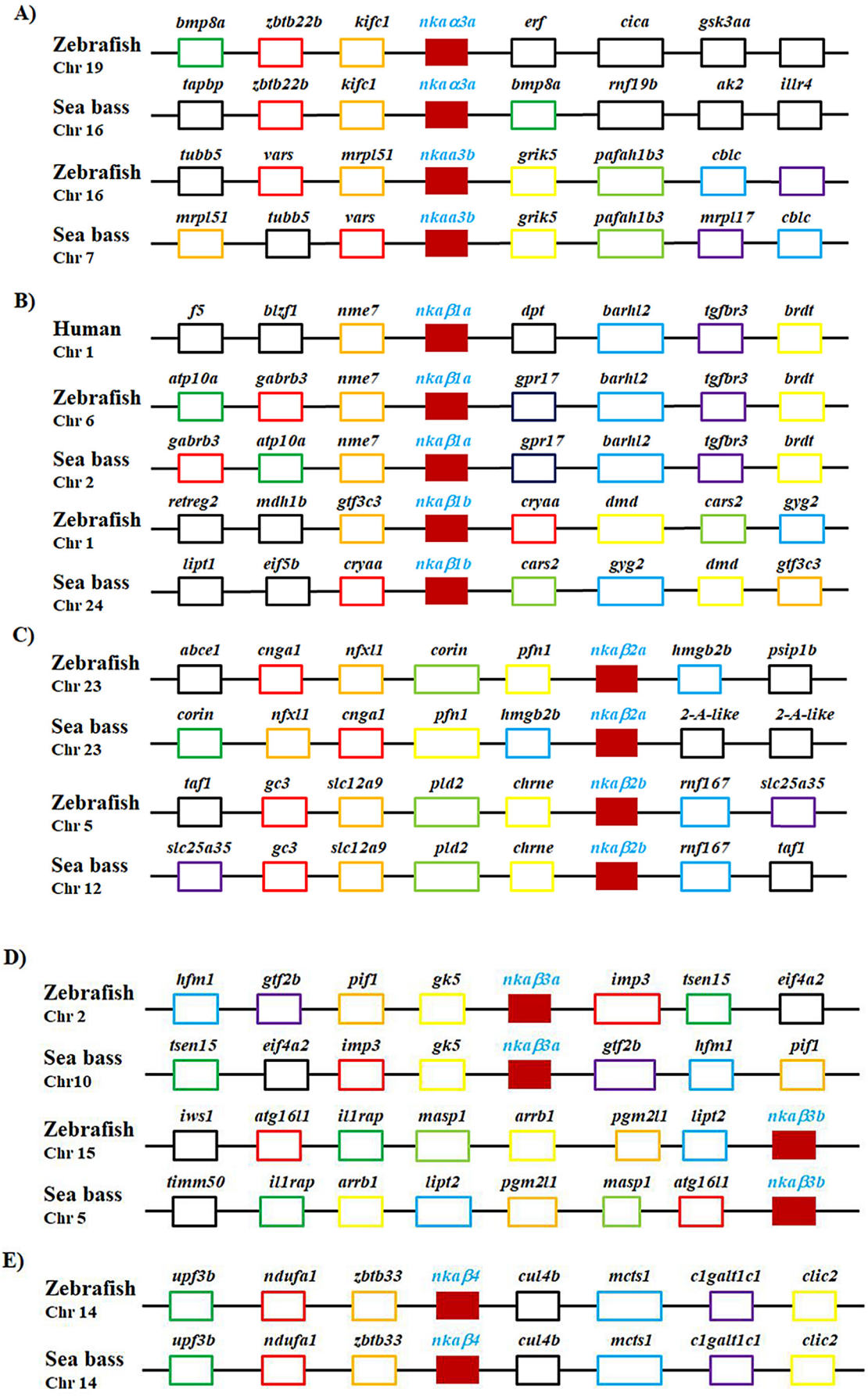

Fig. 3. Syntenic analysis of spotted sea bass *nka* genes. (A) *nkaα3* (B) *nkaβ1* (C) *nkaβ2* (D) *nkaβ3* and (E) *nkaβ4*. These syntenies were generated with the information obtained from NCBI and Ensembl.

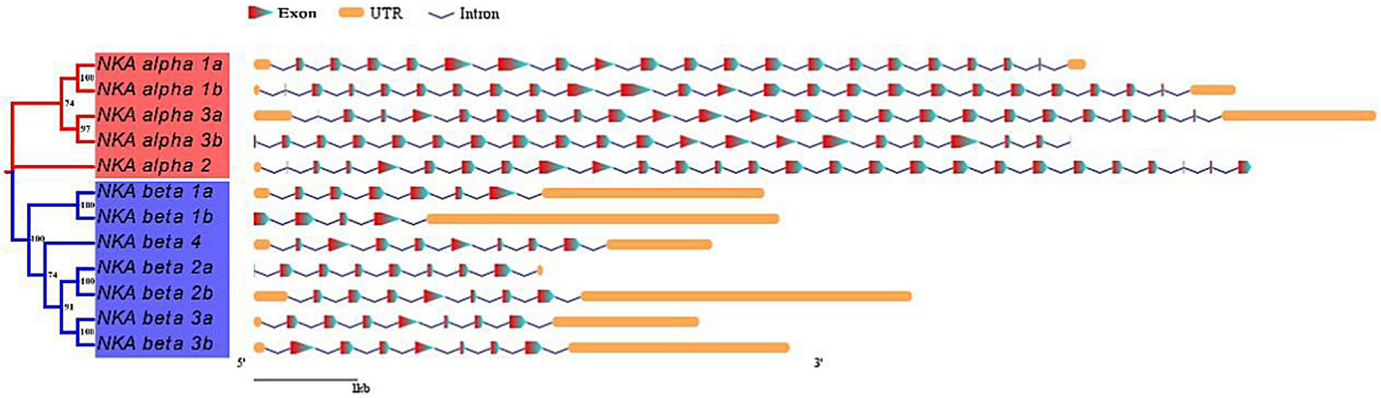

Fig. 4. Gene structure of spotted sea bass *nka* genes and proteins. Exon-intron structure analyses were performed using the Gene Structure Display Server database. The red-green boxes indicate exons, the blue lines indicate introns.

3.4. Gene structure, conserved domains and motif analysis of the nka genes

The analysis of exon-intron structural could provide additional insights into the evolution of gene families. In Fig. 4, the exon numbers of the 12 *nka* genes varied from 4 to 26, and the *nkaβ1b* genes and *nkaα2* separately owned the minimum and maximum value. Compared to *nkaα* genes, these *nkaβ* paralog genes (*nkaβ2a* & *nkaβ2b*, *nkaβ3*a & *nkaβ3b*) harbored the same exon numbers, while the *nkaα* genes showed the different exon numbers. The diverse exon-intron structures of *nkas* may relate to their distinct biological functions.

One smart domain (Cation_ATPase_N), three Pfam domains (E1-E2_ATPase, Cation_ATPase, Cation_ATPase_C) and one transmembrane region was found in all the NKAα proteins and a few component divergences presented across different members of NKAα proteins (Fig. 5 A). The low complexity region was detected in NKAα1a, NKAα3a, NKAα3b and NKAα2, except the NKAα1b protein. NKAα3b protein contained two transmembrane regions. In addition, another Pfam domain (Hydrolase) was detected in NKAα1a, NKAα1b and NKAα3a proteins. Only one Pfam domain (Na_K-ATPase) was found in NKAβ proteins and the conserved domains of NKAβ proteins show higher identity with slight differences detecting in NKAβ4 protein, which had a low complexity region.

Furthermore, a total of twelve conserved motifs were identified in the predicted spotted sea bass NKA proteins by MEME software (Fig. 5B). The length of these motifs varied from 21 to 50 amino acids. All NKAα proteins are highly conserved, harboring 11 motifs in common. As shown in supplementary Fig. 1A, potential phosphorylation sites of PKC binding sites were located in motif1, motif2 and motif6, while PKA binding sites were shown in motif7, motif8 and motif11. Na^+ (Na) or K^+ (K) binding and for either Na^+ or K^+ binding (Na/K) were detected in motif2 (Na/K), motif7 (Na, Na/K), motif8 (Na, K, Na/K) and motif11 (Na, Na/K). Besides, all the predicted NKAβ proteins were also highly conserved, containing the same motif. Two PKA binding sites were found in motif 10 (supplementary Fig. 1B).

3.5. Chromosomal location of nka genes

The twelve *nka* genes were located on nine out of twenty-four

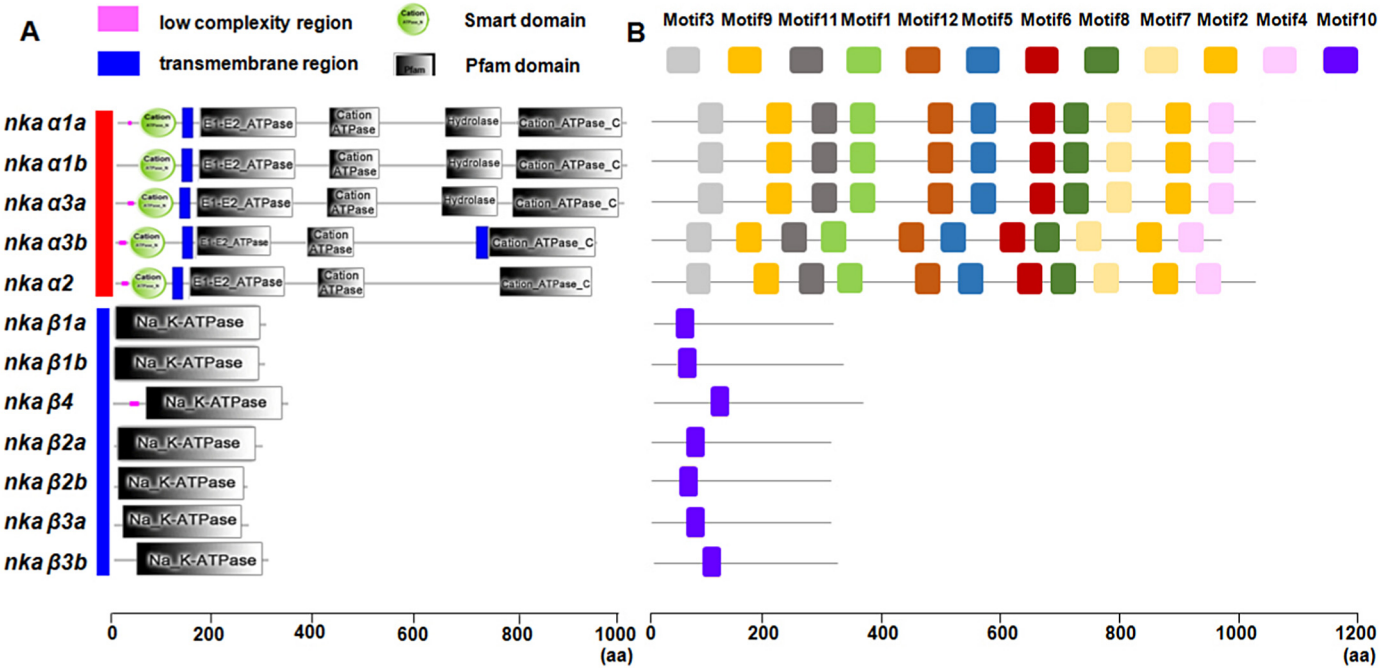

Fig. 5. Motifs and homeodomain analyses of NKA proteins in spotted sea bass. (A) The domain analysis of NKA proteins was performed by SMART analyses service. The low complexity domain and transmembrane region were represented in pink and blue, respectively. (B) Twelve motifs were identified by the MEME software in the amino acid sequences of the NKA proteins. The width of each motif ranged from 6 to 50 amino acids. The different colored blocks represent the different motifs.

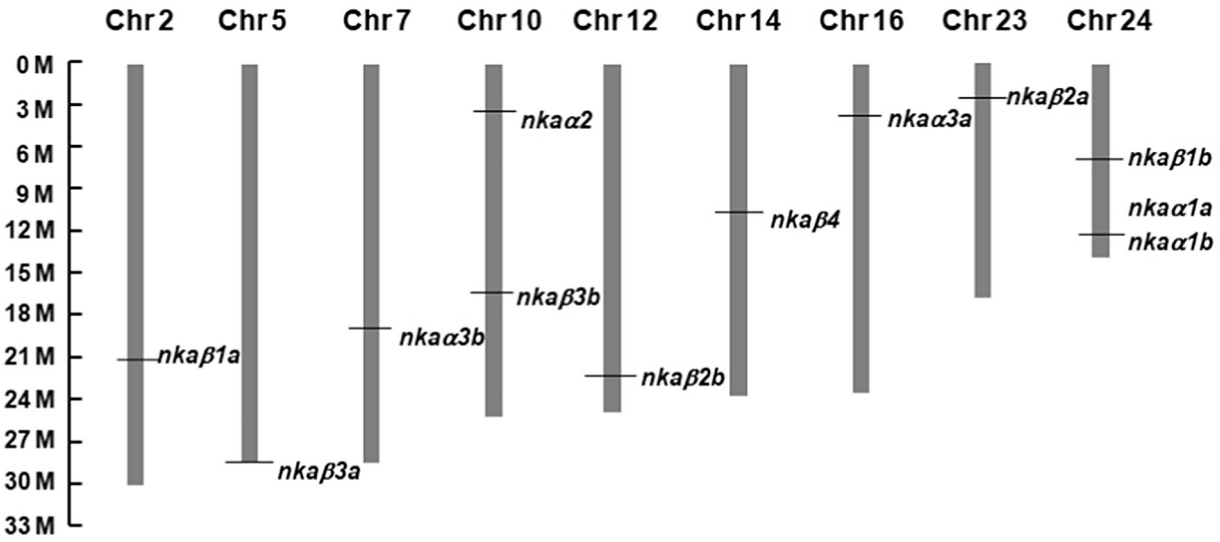

Fig. 6. Chromosomal locations of *nka* genes on different chromosomes in spotted sea bass. The chromosome number is shown at the top of each chromosome bar.

chromosomes of spotted sea bass (Fig. 6). Three *nka* genes, *nkaα1a*, *nkaα1b* and *nkaβ1b* were distributed on the same chromosome (chromosome 24). Two *nka* genes, *nkaα2* and *nkaβ3b,* were distributed on the chromosome 10. The remaining *nka* genes were located on separate chromosomes; the *nkaβ1a* gene was on chromosome 2, the *nkaβ3a* gene was on chromosome 5, the *nkaα3b* gene was on chromosome 7, the *nkaβ2b* gene was on chromosome 12, the *nkaβ*4 gene was on chromosome 14, and the *nkaα3a* gene was on chromosome 16.

3.6. Tissues distribution analysis of nka genes

qRT-PCR analysis was performed to detect the expression profile of *nka* genes in ten tissues (kidney, gonad, stomach, intestine, gill, muscle, heart, spleen, liver and brain) of L. *maculatus* under nature seawater (30 ppt) conditions. The expression of genes (*nkaα1a*, *nkaα1b*, *nkaα3a*, *nkaα3b*, *nkaβ1b and nkaβ2a*) with higher expression in osmoregulatory organs (kidney, gill or intestinal) were listed in Fig. 7. Results showed that *nkaα1a* strongly expressed in gill (Fig. 7 A), while *nkaα1b* expressed in all examined tissues especially highly in heart (Fig. 7 B). *nkaα3a* transcripts were significant enriched in kidney (Fig. 7 C). The most highly expression of *nkaα3b* was found in gill and heart, followed by brain and liver (Fig. 7 D). *nkaβ1b* was detected in all examined tissues with higher transcripts in the gill, liver and brain (Fig. 7 E). *nkaβ2a* strongly expressed in brain, followed by gonad, kidney and gill (Fig. 7 F). However, only trace amount of expression of *nkaα2*, *nkaβ2b*, *nkaβ3a* and *nkaβ3b* were detected in examined tissues, except that of *nkaα2* in muscle, *nkaβ3a* in muscle and brain, *nkaβ2b* and *nkaβ3b* in brain (supplementary Fig. 2).

3.7. Expression profile of nka genes at different salinities

According to the tissue distribution analysis results above, the expressions of selected genes (*nkaα1a*, *nkaα1b*, *nkaα3a*, *nkaα3a*, *nkaβ3b and nkaβ2a*) in three osmoregulatory organs (kidney, gill and intestinal) at different salinities were examined by qRT-PCR (Fig. 8). The *nkaα1a* mRNA level in gill exhibited U-shaped distribution with the increase of salinity, which reached to the highest in HS. Meanwhile, the expression of *nkaα1a* gene in kidney and intestine was dramatically lower than that in gill (Fig. 8 A). The overall expression level of *nkaα1b* was similar in all three tested tissues and change slightly with variation of salinities (Fig. 8 B). The expression level of *nkaα3a* in kidney was significantly higher than that in gill or intestine, with the highest expression found in SW and the lowest expression identified in FW. In addition, the *nkaα3a* mRNA level in intestine was obviously increased with increasing salinity but the opposite result was showed in gill which the highest expression was detected in FW (Fig. 8 C). *nkaα3b* strongly expressed in gill and the expression patterns of *nkaα3b* showed an increasing trend with the increase in salinity, reaching to the highest in SW (Fig. 8 D). The expression of *nkaβ1b* showed the similar expression profile to *nkaα1a* in the gill, with the highest expression found in HS, follows by FW (Fig. 8 E). The highest *nkaβ2a* mRNA level was identified in intestine of HS and the mRNA level in gill was significantly higher in FW and IP than in SW and HS (Fig. 8 F).

4. Discussion

Salinity adaptation is a complex process involving a set of physiological responses, while euryhaline teleosts must regulate their internal water and ionic concentrations to maintain ionic homeostasis (Lin et al., 2003). In the present study, no significant differences of plasma osmolality were found among fish in FW, BW and SW. Similar results were reported in *C. chanos* (Lin et al., 2003) and *Trachinotus marginatus* (Anni et al., 2016), that no significant changes in plasma osmolality were observed when adapted to various salinities. Euryhaline species are able to tolerate a much broader range of salinities, of which osmotic pressure is usually maintained at a stable level (Evans et al., 2005; Edwards and Marshall, 2012). Thus, it could be speculated that the spotted sea bass can maintain a relatively stable osmotic pressure when salinity does not exceed the natural seawater. However, high salinity can break that balance and cause great changes in the internal environment of fish, just as the result shows in Fig. 1A.

As the unique organ for aquatic animals, fish gills are the major site not only for gas exchange but also for ion transport, aid-base regulation and ammonia excretion (Marshall and Grosell, 2006; Edwards and Marshall, 2012). The gills carry out the majority of iono/osmoregulation mechanisms through the specified gill ionocytes named mitochondrion-rich (MR) cells (formerly called chloride cells), which are the major cells that express specific ion transporters (or enzymes)(Guh et al., 2015). The directions of ion transport in gills are reversed during acclimation to different salinities, thus they provide an excellent model to study the regulatory mechanisms of ion transport (Hwang and Lee, 2007). However, It is widely documented that the organs which are responsible for maintaining osmolality and ionic balance are accomplished not only by gill, but also the kidney and intestine (Whittamore, 2012; Yang et al., 2016). Therefore, Changes in the mRNA levels of *nka* genes and enzyme activities of NKA in gill, kidney and intestine of the

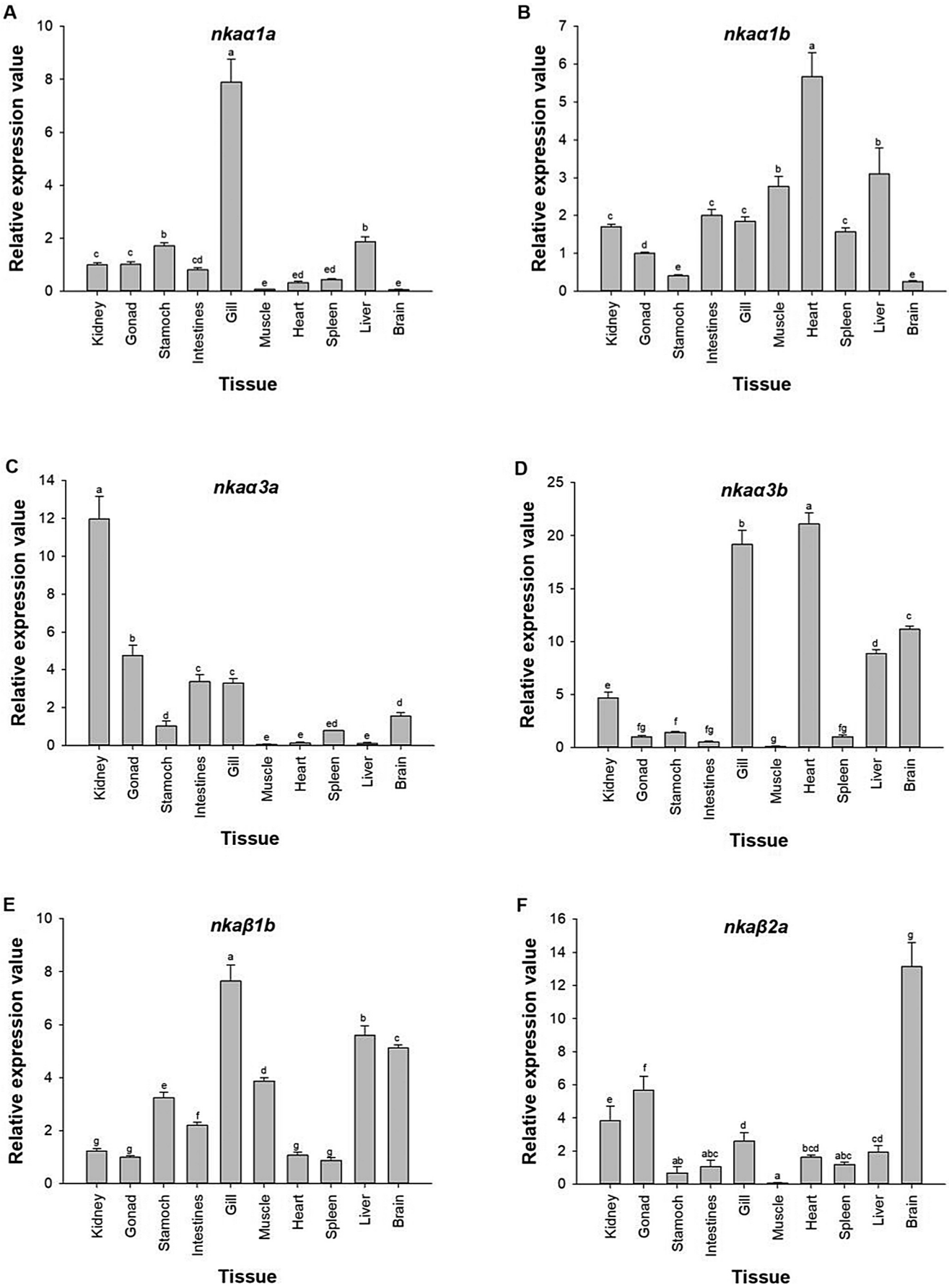

Fig. 7. The tissue distribution analysis of the *nka* genes with higher expression in kidney, gill or intestinal tissues. The results are shown as the means ± standard error of mean (SEM). Different letters in the different tissues indicate significant differences ($P < 0.05$).

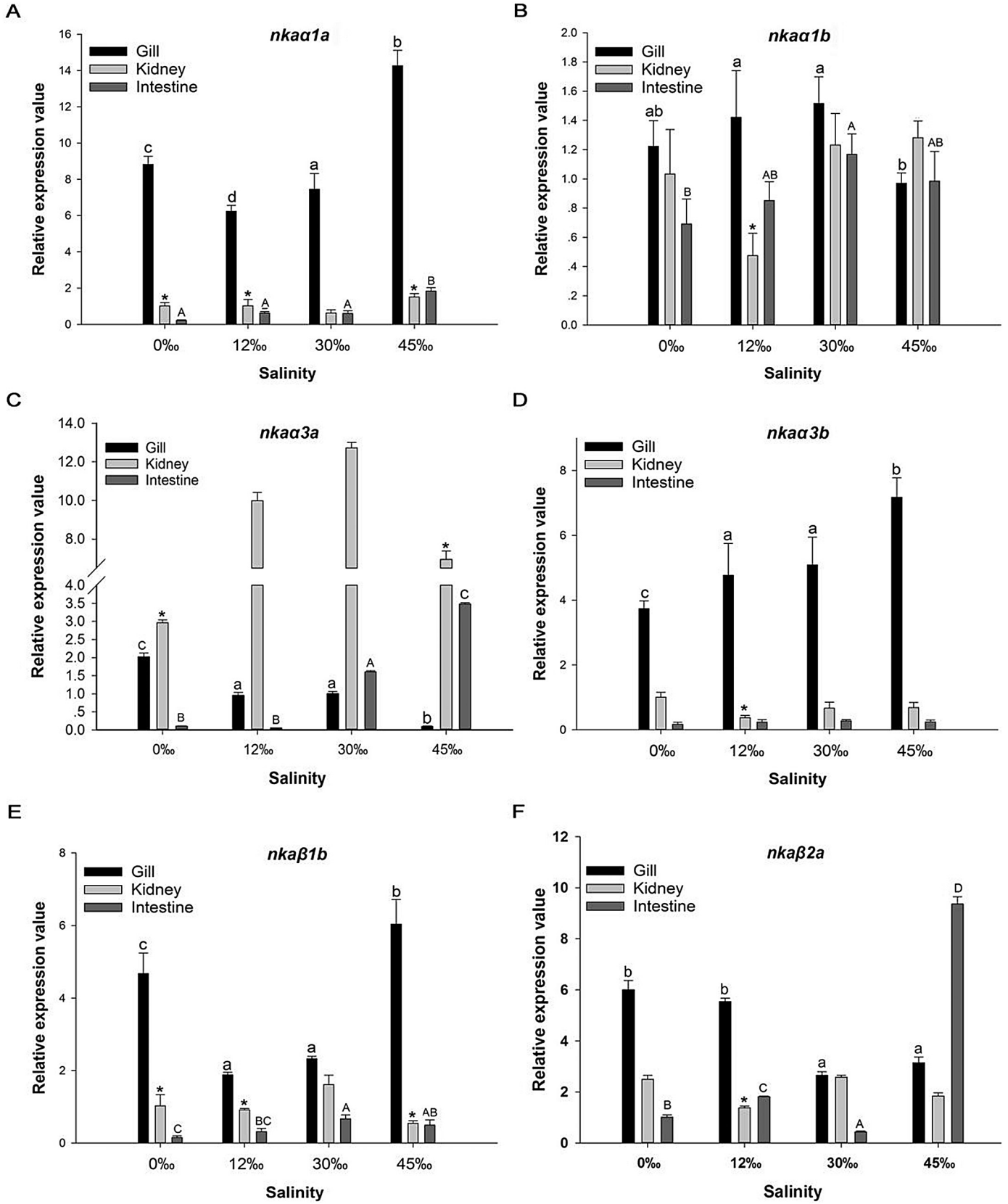

Fig. 8. The relative expression value of selected salinity responsive genes A) *nkaα1a*, B) *nkaα1b*, C) *nkaα3a*, D) *nkaα3b*, E) *nkaβ1b* and F) *nkaβ2a* in gill, kidney and intestinal tissues of spotted sea bass under different salinity groups (0 ppt, 12 ppt, 30 ppt, 45 ppt). The significant differences ($P < .05$) in intestine, gill and kidney among different salinity treatment groups are represented by capital, lowercase letters and asterisks respectively. For kidney, the 30 ppt group is considered as the control.

spotted sea bass during salinity acclimation were explored in our study.

As an important fundamental enzyme responsible for ion movement, Na^+/K^+-ATPase is a membrane-spanning protein that transports three Na^+ ions outward in exchange for two K^+ ions, creating low intracellular Na^+ and a highly negative charge within the cell (Richards et al., 2003). Indeed, cells involved with transport processes are large consumers of ATP. As a primary active pump, NKA is important not only for sustaining intracellular homeostasis, but also for providing the driving force for ion-transporting systems within a variety of osmoregulatory epithelia (Lin et al., 2003). In gills of euryhaline teleosts, NKA creates an electrochemical gradient to transport Na^+ ions and Cl^- ions in both secretory (SW) and absorptive (FW) modes. It have been widely reported that euryhaline teleosts exhibit adaptive changes in NKA activity following salinity changes (Hirose et al., 2003; Perry et al., 2003), however, conflicting opinions exist due to differences in species. In most euryhaline teleosts, NKA is up-regulated by an increase in environmental salinity, such as *O. latipes* (Bollinger et al., 2016), *Solea senegalensis* (Ruiz-Jarabo et al., 2016), but the opposite effect was found in a few species (such as *C. chanos* and *T. marginatus*) with the enzyme activity decreases by increasing salinity (Lin et al., 2003; Anni et al., 2016). It's worth noting that, those results may underscore the importance of the enzyme at both hyper- and hyposmotic stress (Evans et al., 2005; Marshall and Grosell, 2006; Edwards and Marshall, 2012). In our trial, the greatest volatility of NKA activity appeared in gill, the most important osmoregulatory organ in fish, and the greatest value was found in the HW group (Fig. 1B). Extreme salinities associated with higher NKA activity suggested the importance of the enzyme in salinity adaptation, meanwhile lower level of NKA in BW inferred that there is a threshold of acclimation of the spotted sea bass to a hypotonic environment. A U-shape curve of NKA activity with increasing salinity was also found in the gill of *Synechogobius ommaturus* (Shui et al., 2018), *D. labrax* (Jensen et al., 1998) and *Sparus aurata* (Laiz-Carrion et al., 2005a, 2005b). It suggested that fish may increase the activation of gill NKA to maintain the homeostasis of ions and osmolality under the stress of salinity environment.

Previous studies have revealed that three *nkaα*-subunit isoform (*α1*, *α2* and *α3*) and four β-isoforms (*β*1, *β*2, *β*3 and *β*4) were identified in teleosts (Armesto et al., 2014; Canfield et al., 2002; Richards et al., 2003; Gharbi et al., 2005; Armesto et al., 2015), while four *nka* α-isoforms (*α1*, *α2*, *α3*, *α4*) and three *nka* β-isoforms (*β*1, *β*2 and *β*3) have been identified in mammalian cells (Takeyasu et al., 1990; Blanco and Mercer, 1998; Blanco, 2005; Suhail, 2010; Yang et al., 2016). In this study, five *nkaα*-subunit coding genes (*nkaα1a*, *nkaα1b*, *nkaα2*, *nkaα3a* and *nkaα3b*) and seven β-subunit coding genes (*nkaβ1a*, *nkaβ1b*, *nkaβ2a*, *nkaβ2b*, *nkaβ3a*, *nkaβ3b* and *nkaβ4*) were identified from transcriptome and genome database of spotted sea bass and their annotations were confirmed by phylogenetic and syntenic analysis. In addition, we performed the sequence structure and conserved motif analyses of predicted NKA proteins. The sequence structures of NKAs proteins were highly conserved. NKAα proteins consisted of one smart domain (Cation_ATPase_N), three Pfam domains (E1-E2_ATPase, Cation_ATPase, Cation_ATPase_C) and one transmembrane regions, while only one Pfam domain (Na_K-ATPase) were found in all NKAβ proteins. The domains specific to the NKA proteins of L. *maculatus* may be considered as a classification standard for unknown NKA targets (Armesto et al., 2014).

The α-subunit isoform contains the binding sites for Na^+, K^+ and ATPase, taking responsibility for the major catalytic and ion-transporting work of *nka* while β-subunit is important for stabilizing folding of the α-subunit (Sundh et al., 2014). There was accumulating evidence indicating that multiple *nka* α-isoforms expressed in euryhaline teleosts osmoregulatory organs and the expression levels is strongly influenced by salinity (Bystriansky et al., 2006; Urbina et al., 2013; Blondeau-Bidet et al., 2016; Bollinger et al., 2016). In addition, different NKA isoforms plays distinct roles in osmoregulation and salinity adaptation process. In our salinity acclimation experiment, extreme salinity (HS) resulted in higher expression of two *nka* isoforms (*nkaα1a* and *nkaα3b*) in the gill, highlighting the importance of these gill *nka* in response to stress caused by salinity. In most studies, gill *nkaα1* appear to be essential to ionocyte function because of its salinity-dependent expression (Chew et al., 2014; Madsen et al., 2014; Hu et al., 2017). In our results, the similar salinity-dependent expression pattern of *nkaα1a* mRNA with enzyme activity of NKA (Fig. 8A and Fig. 1B) suggesting *nkaα1a* may be the major isoform affecting enzyme activity. Although the expression of *nkaα1b* in gill was not changed a lot in spotted sea bass, however, together with *nkaα1a*, *nkaα1b* was also the salinity-sensitive isoform which strongly influenced by salinity in several species including *O. latipes* (Rebecca et al., 2016), *O. mossambicus* (Feng et al., 2002; Tipsmark et al., 2011), *S. salar* and *Salvelinus alpinus* (Bystriansky et al., 2006) and *Galaxias maculatus* (Urbina et al., 2013).

Only a few study reported the expression changes of *α3* isoforms under salinity challenge conditions in teleosts, and *nkaα3* was considered as less important than *nkaα1* isoforms in salinity acclimation (Blanco and Mercer, 1998; Crambert et al., 2000; Nilsen et al., 2007). However, our results, showed that the spotted sea bass *nkaα3* expression is highly salinity sensitive, especially the *nkaα3a* in kidney (Fig. 8C) and the *nkaα3b* in gill (Fig. 8D). Present findings were found that the *nkaα3b* mRNA level in gill of spotted sea bass was up-regulated with an increase environmental salinity, which is consistent with findings in *O. mossmabicus* with gill *nkaα3* amounts increased with the level of environmentl salinity (Feng et al., 2002). Nevertheless, the *nkaα3* mRNA levels in gill of *O. mykiss* (Richards et al., 2003) and *S. salar* (Nilsen et al., 2007) showed no significant changes when transfer from FW to SW. In addition, the expression level of *nkaα3a* in kidney of spotted sea bass was higher than that in gill or intestine, which was the highest in SW (Fig. 8C). Similarity, renal *α3*-isoform in seawater acclimated *O. mossambicus* was significantly higher than in the freshwater group, whereas no difference was found in *C. chanos*. However, whether the salinity-induced *nkaα3* in *O. mossambicus* was *nkaα3a* or *nkaα3b* is still questionable and requires further sequencing information (Yang et al., 2016). Taken together, different responses of *nka* α-subunit to salinity adaptation among euryhaline teleosts could revealed that fish employ divergent mechanisms for maintaining osmotic balance with different isoform preference (Feng et al., 2002; Bollinger et al., 2016).

Membrane-spanning NKA β-subunit could be considered a specific chaperone assessing the correct folding of NKA α-subunit to the plasma membrane and assembly with β subunits is necessary for the catalytic α subunit to acquiring the pumping function and transport activity of NKA (Geering, 2001; Mcdonough et al., 1990; Hu et al., 2017; Armesto et al., 2015). However, compared to *α*-isoforms, information about *nka β* subunit in fish was limited. Studies in *S. salar*, *O. mykiss*, *C. chanos* and *S. senegalensis* have shown that *nka β*-subunits are transcriptionally regulated by altered salinity (Nilsen et al., 2007; Bystriansky and Schulte, 2011; Armesto et al., 2015; Hu et al., 2017). The potential function of the *nkaβ1* subunit involved in osmoregulation associated with the *nkaα1* subunit, which were proved by parallel increases in mRNA and protein expression of *nkaα1* and *nkaβ1* subunits in gills of *C. chanos* when transferred to FW from SW (Hu et al., 2017). Similarity, after exposing soles to high salinity environment, the NKA activity, and the mRNA expression of *α1/β1*- subunit were all activated in the gill of *S. senegalensis* (Armesto et al., 2015). In our study, *nkaβ1b* showed the highest mRNA levels in gill, and notably, the expression of *nkaβ1b* showed the similar expression profile with *nkaα1a* in the gill, which exhibited U-shaped distribution with the increase of salinity and reached to the highest in HS. Furthermore, the NKA activity in the gill of the spotted sea bass also showed the similar pattern. All these results indicate that α1a/β1b could be the major function isoenzyme involved in primary ion transport under salinity stress in spotted sea bass. Those findings suggested that different *nkaα/β*-isoforms showed their diverse responses to salinity changes and the protein is the function executor of a specific gene (Feng et al., 2002; Armesto et al., 2014).

5. Conclusion

In conclusion, a complete set of 12 *nka* genes, including 5 *nkaα* genes and 7 *nkaβ* genes were identified in spotted sea bass. Phylogenetic, syntenic and gene structure analyses were conducted and provided sufficient evidences for the annotation and orthologies of these genes. 6 genes showed highly mRNA expression levels in osmoregulatory organs and exhibited distinct tissue-specific responses to salinity changes. It was worth noting that the expression patterns of *nkaα1a* and *nkaβ1b* in gill showed similar variation trend with NKA activity, indicating *nkaα1a/β1b* could be the major function isoforms involved in primary ion transport during salinity adaptation of spotted sea bass.

Acknowledgements

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.05.017.

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