Available online at www.buu.ac.th/BUUConference

Burapha University International Conference 2015

"Moving Forward to a Prosperous and Sustainable Community"

ST-P-026

Partial Cloning of *Na^+K^+ATPase* and *Na^-K^-Cl cotransporter* genes in the Asian Sea Bass (*Lates calcarifer)*

Preeraya Singkhaimuk[a], Chadapon Kidpipit[a], Sripapan Tharanat[a], Rachanimuk Hiransuchalert[a] and Napaporn Leadpratom[a] *

*[a]Faculty of Marine Technology, Burapha University, Chanthaburi Campus, 57 Moo 1, Kamong, Thamai, Chanthaburi 22170, Thailand

Abstract

This study investigates the partial sequences of *Na^+K^+ATPase* and *Na^-K^-Cl cotransporter* genes and their expression in various tissues of the Asian Sea Bass (*Lates calcarifer*). Primers were designed from the nucleotide sequences of *Na^+K^+ATPase* of the striped sea bass (*Morone saxatilis*) (ABF83853) and *Na-K-Cl cotransporter* of the European sea bass (*Dicentrarchus labrax*) (AY954108) from GenBank. RT-PCR was further examined using the cDNA of gills of *L. calcarifer*. The derived amplified products from which expected sizes were further cloned and sequenced. The nucleotide sequences of a 291 base pair sized are similar to *NA^+/K^+ATPase alpha 1b* in Mummichog (*Fundulus heterclitus*) (98% identity, e-value = 1e-35), whereas, the nucleotide sequences of a 360 base pair sized are similar to *putative sodium-potassium-chloride cotransporter* in European eel *(Anguilla anguilla*) (93% identity, e-value = 3e-52). According to the study of the expression pattern of genes in *L. calcarifer*, tissue distribution RT-PCR was examined in several organs such as blood, intestine, liver, gastric, kidney, heart, gills, brain and muscle. The results showed that the responsive levels of genes were expressed in all of examined tissues including non-specific products. Surprisingly, the highest expression levels of *Na^+K^+ATPase* and *NKCC* gene were found in blood and gills of *L. calcarifer*.

Keywords: Asian Sea bass; *Na^+ K^+ ATPase*; *Na-K-Cl cotransporter*; *gene*; *Lates calcarifer*

* Corresponding author. Tel: +6639310000; fax: +6639310128.
E-mail address: rachanimuk@buu.ac.th.

1. Introduction

The osmoregulation, involving energized ion pumps in the gills and in other osmoregulatory organs such as intestine and kidney, is a critically temperature sensitive process (Juriaan, 2003). Sodium-Potassium Adenosine Triphosphatase (Na^+K^+ATPase) and Na-K-Cl cotransporter (NKCC) are involving to osmotic regulation. Na^+K^+ATPase, also known as sodium-potassium pump and/or sodium pump, located in the plasma membrane of all animal cells. The functions of Na^+K^+ATPase are maintaining resting potential, effect transport, and regulate cellular volume. For neurons, the Na^+K^+ATPase can be responsible for up to 2/3 of the cell's energy expenditure (Howarth et al., 2012). For NKCC, it is a membrane transport protein that aids in the active transport of sodium (Na), potassium (K), and chloride (Cl) ion into and out of cells (Haas, 1994).

In the osmoregulatory organs, such as the gills, kidney, and intestine, the Na,K-ATPase is the major contributor to total ATPase activity (Venturini et al., 1992). The *Na^+K^+ATPase* in fish gills play a major role in the maintenance of ion balance (Zhichao et al., 2000). The salinity affects to the balance of ion in the body of *Myoxocephalus octodecemspinosus* and expression levels of *Na^+K^+ATPase alpha* and *NKCC1* transcripts (Kelly and David, 2009). In *Trichogaster microlepis*, *Na^+K^+ATPase* levels, the mortality, plasma osmolality, and Na^+ concentration were highest in gills in salinity of 10 g/L acclimated water, when compared to deionized water and fresh water acclimated fish (Chun et al., 2010). The *NKCC* determine the osmoregulatory role of this protein in different tissues during the ontogeny of the sea bass (Lorin-Nebel et al., 2006). NKCC was dominantly found in gills of the European eel (*Anguilla anguilla*) (Cutler and Cramb, 2002).

An initial step toward understanding molecular mechanisms of osmoregulation in *L. calcarifer* is the identification and characterization of genes involving to osmoregulation in this economically important species. The nucleotide sequences of *Na^+K^+ATPase* and *NKCC* was characterized. The expression patterns of *Na^+K^+ATPase* and *NKCC* in various tissues of *L. calcarifer* were examined.

2. Materials and Methods

2.1 Fish

To examine expression of genes involving to osmoregulation, domesticated fish (average size of 13.5 cm.) were acclimated at 28-30ºC and 15 ppt of seawater for 1 week.

2.2 RNA extraction and cDNA synthesis.

RT-PCR was examined using the cDNA of gills of *L. calcarifer*. For tissue distribution analysis, various tissues of juvenile *L. calcarifer* were collected immediately kept at −70 °C until needed. Total RNA were extracted by using TRI REAGENT® (Molecular Research Center). The first strand cDNAs of RT-PCR and tissue distribution were synthesized using an ImProm- II™ Reverse Transcription System Kit (Promega) and the Select cDNA Synthesis Kit (biorad), respectively.

2.3 Primers design

The putative amino acid sequences of *Na^+K^+ATPase* and *NKCC* was identified from striped sea bass (*Morone saxatilis*) (ABF83853) and European sea bass (*Dicentrarchus labrax*) (AY954108) obtained from Genbank. Primers were designed against conserved motifs of *Na^+K^+ATPase* and *NKCC* of related species using Primer Premier 5.0. (Table1).

2.4 RT-PCR and tissue distribution analysis

Expression of *Na^+K^+ATPase* and *NKCC* in gills and various tissues of *L. calcarifer* were analysed by RT-PCR. *EF-1α* was used as the positive control. RT-PCR and tissue distribution were carried out in 25 µl reaction composing of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 200 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM for RT-PCR and 0.3 µM for tissue distribution, 1 unit of Dynazyme™ DNA Polymerase (FINNZYMES). The thermal profile for quantitative RT-PCR was 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, 72°C for 30 seconds and the final extension at 72 C for 7 minutes. Five microlittres of the amplification product was electrophoretically analyzed though 1.5-2.0% agarose gels, stained with ethidium bromide and visualized under a UV transilluminator.

2.5 Sequencing of RT-PCR products

The desired RT-PCR fragments were isolated using NucleoSpin® Gel and PCR Clean-up. The DNA fragments were ligated to pGEM®-T Easy vector (Promega) in a 10 µl reaction volume and cloned into *Escherichia coli* JM109. Recombinant clones were selected by a lacZ′ system following standard protocols (Sambrook and Russel, 2001) and randomly selected by colony PCR. Recombinant clones carrying desired DNA fragments were selected. Plasmid DNA was extracted and subjected to unidirectional sequencing.

Table 1 Primer sequences and the expected size of the PCR products.

Primer pairs	Primer name	Forward sequence	Reverse sequence	Product size (bp.)
1	*Na^+K^+ATPase*	ATT gTg CTT TCT gCT gTC gTC	ggT TTC CAg Tgg gTT TTC ATT	318
2	*NKCC*	TgA ggA gAC TgC TgC TAA AgA A	gCA AAg CCC ACC ACA TAC A	360
3	*EF-1α*	ATT ggC ggT ATT ggA ACT gTC	TTg ggT ggg TCA TTC TTg CT	239

3. Results and Discussion

3.1 RT-PCR and tissue distribution analysis

Expression of expected targets using primers as described in Table 1 in gills and other tissues of juvenile *L. calcarifer* was analyzed by RT-PCR. For *Na^+K^+ATPase* and *NKCC* bands were obtained in gills (approximately 300 and 350 bp) (Fig. 1). The nucleotide sequences of the *Na^+K^+ATPase* and *NKCC* were showed in Fig. 3, with actual sizes of 291 and 360 bp, respectively. Moreover, tissue distribution was performed in various tissue of *L. calcarifer* using *Na^+K^+ATPase* and *NKCC* primers. The positive bands were expressed in all of examined tissues (Fig.2). Similarly, this corresponds in the *Fundulus heteroclitus*, the *Na^+K^+ATPase* was expressed in all organs (Semple et al., 2002). *Na^+K^+ATPase* was abundantly expressed in blood and gills, greater than other tissues of *L. calcarifer*. It was expressed with a low level in intestine liver, gastric, kidney, brain and muscle. *NKCC* was abundantly expressed in blood, heart and gills. It was expressed with a low level in gastric, kidney, brain and muscle (Table 2). *Na^+K^+ATPase α subunit* was highly detected in gills, intestine and kidney of eel (Cutler et al., 2000). Northern blot analysis showed that *Na^+K^+ATPase α subunit* were expressed in gills, kidneys and other organs in tilapia (Hwang et al., 1998).

3.2 Isolation of the nucleotide sequences.

The nucleotide sequence identities were determined by comparing each sequence with the sequences in the GenBank mirror site using the BLAST*X* program (Altschul et al., 1990; available at http://www.ncbi.nih.gov).

The derives nucleotide sequence from *Na^+K^+ATPase* and *NKCC* primers were matched to *NA^+/K^+ATPase* alpha 1b (*Fundulus heterclitus*, evalue = 1e-35) and *putative sodium-potassium-chloride cotransporter (Anguilla anguilla*, e-value = 3e-52), respectively (Table 3). The nucleotide sequences of of *Na^+K^+ATPase* and *Na-K-Cl cotransporter* of gills of *L. calcarifer* were considered as similar to the sequences in GenBank and were characterized because in this gene in *L. calcarifer* has not been reported previously.

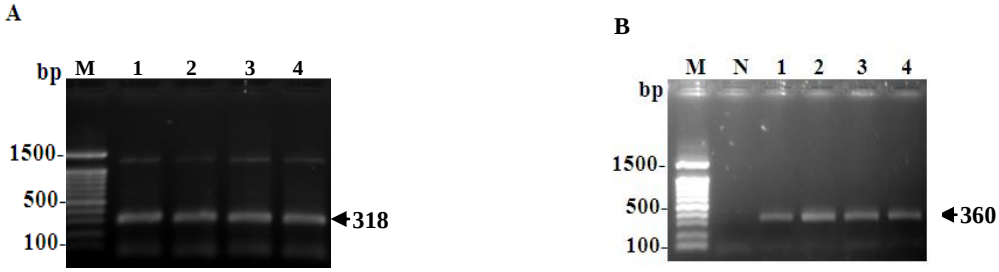

Fig. 1 A 1.5% ethidium bromide-stained agarose gel showing RT-PCR results using primers *Na^+K^+ATPase* (A) and *NKCC* (B) transcripts using the cDNA template from gills of a sea bass *L. calcarifer* (Lanes 1-4). Lane M represents DNA marker. Distilled water was used as the template in the negative control (N).

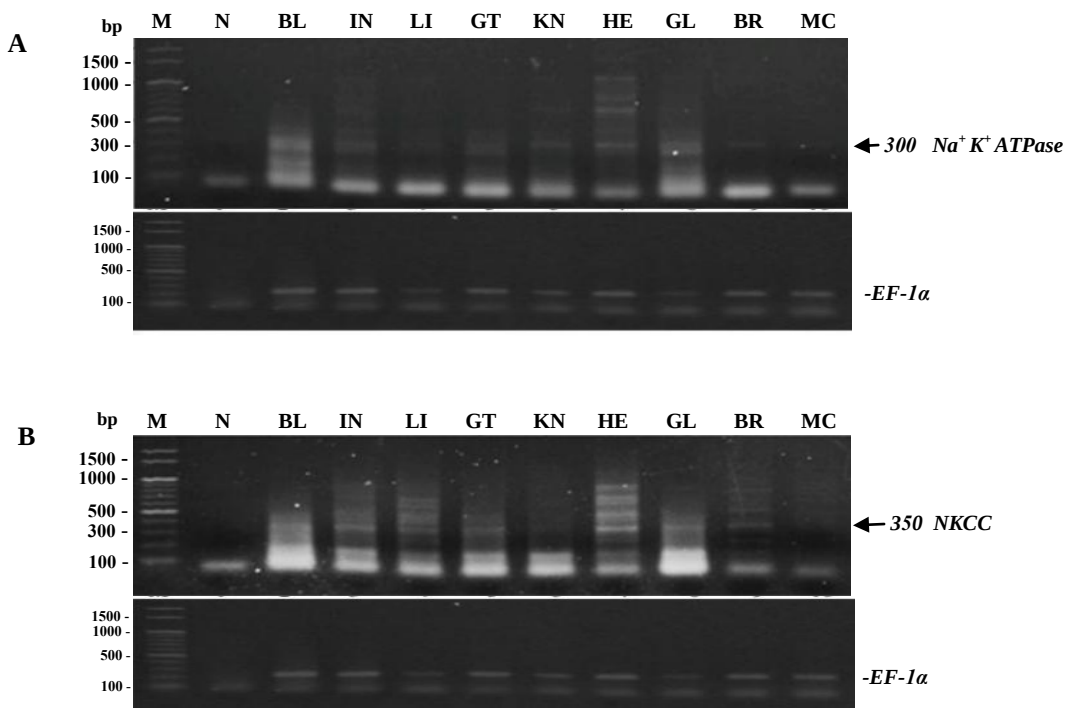

Fig. 2 A 1.5% ethidium bromide-stained agarose gel showing results from tissue distribution analysis of *Na^+K^+ATPase (A) and NKCC* (B) transcripts using the cDNA template from blood (BL), intestine (IN), liver (LI), gastric (GT), kidney (KN), heart (HE), gills (GL), brain (BR) and muscle (MC) of *L. calcarifer*. *EF-1α* was included as the positive control and successfully amplified from the same template. Lane M represents DNA marker. Distilled water was used as the template in the negative control (N).

Table 2 Tissue expression analysis of *Na^+K^+ATPase* and *NKCC* transcripts in *L. calcarifer*.

Gene	Tissue expression analysis								
	Blood	intestine	liver	gastric	kidney	heart	gills	brain	muscle
Na^+K^+ ATPase	+++	+	+	+	+	++	+++	+	+
NKCC	+++	++	++	+	+	+++	+++	+	+

+++ = high expression level, ++ = moderate expression levels, + = low expression levels

Table 3 Summary of the nucleotide sequence blast results comparing with the nucleotide sequences submitted in Genbank.

Primer pairs	organs	Product sizes (bp)	Blast*X*	
			Similar transcripts	Species (% Identities. e-value,)
Na^+K^+ ATPase	gills	291	*NA+/K+ATPase alpha 1b*	*Fundulus heterclitus* (98%, 1e-35)
NKCC	gills	360	*putative sodium-potassium-chloride cotransporter*	*Anguilla anguilla* (93%, 3e-52)

A.

```
GGCGTTGGATTAGCGTCTACCACAGCTTCGACAGCCTTGGAGTCAGTGTGCACTGCACGG      60
TACGGTATGAACCCCACTGAAAACCACTCCGTGACGGACAAGAAGAAGAGCATCAACGCT      120
GAGGAGGTGGTGGTCGGAGATTTGGTGGAGGTGAAAGGTGGAGACAGGATCCCAGCTGAT      180
CTGCGAATCATCTCTGCCCACGGCTGCAAGGTGGACAACTCCTCTCTGACTGGTGAATCA      240
GAGCCTCAGACTCGTACTCCAGACTTCTCCAATGAAAACCCACTGGAAACC               291
```

B.

```
TGAGGAGACTGCTGCTAAAGAAGCAGCTGAATCCAAAGGAGTGGTGAAGTTTGGCTGGAT      60
TAAGGGGGTGCTGGTCCGCTGCATGTTGAACATTTGGGGTGTCATGCTCTTCATCCGCAT      120
GTCATGGATCGTTGGTCAGGCTGGGATTGTTCTCTCCTGTGTGATTGTTGCCATGGCTAC      180
CGTAGTGACGACCATCACCGGCCTCTCCACCTCTGCCATTGCCACCAATGGATTTGCACG      240
AGGAGGTGGAGCATATTACTTAATTTCAAGGAGTCTGGGCCCAGAGTTTGGAGGCTCTAT      300
TGGTCTGATATTTGCCTTTGCCAATGCAGTGGCTGTAGCCATGTATGTGGTGGGCTTTGC      360
```

Fig.3 The nucleotide sequences of *Na^+K^+ATPase* (A) and *NKCC* (B) transcripts of *L. calcarifer*.

4. Conclusions

Partial cloning of *$Na^+K^+ATPase$* and *Na^-K^-Cl cotransporter* genes from the Asian Sea Bass (*Lates calcarifer*) was carried out. The nucleotide sequences of a 291 base pair sized are similar to *$NA^+/K^+ATPase$ alpha 1b* in Mummichog (*Fundulus heterclitus*) (98% identity, e-value = 1e-35), whereas, the nucleotide sequences of a 360 base pair sized are similar to *putative sodium-potassium-chloride cotransporter* in European eel *(Anguilla anguilla*) (93% identity, e-value = 3e-52). The tissue expression patterns of *$Na^+K^+ATPase$* and *NKCC* were examined. These transcripts were differentially expressed in tissues of *L. calcarifer*. The highest expression levels of *$Na^+K^+ATPase$* and *NKCC* transcripts were found in gills of this economically important species.

5. Acknowledgements

A travel grant is supported by Faculty of Marine Technology, Burapha University, Chanthaburi Campus.

6. Conflict of interest: none.

7. References

Alfonso, B. O., Carlos, S., Esther, P., Blanca, R., 2014. Na/K pump Regulation of Cardiac Repolarization: Insights from a Systems Approach, Journal of Physiology 446(2), p. 183−193.

Altschul, S. F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool, Journal of Molecular and Biology 215(3), p. 403-410.

Chun,Y. H.,Pe, L. C., Hui, C. L., 2010 · Na^+/K^+-ATPase and Vacuolar-type H^+-ATPase in the Gills of the Aquatic Air-breathing fish *Trichogaster microlepis* in Response to Salinity Variation, Journal of Molecular and Integrative Physiology 155(3), p. 309–318.

Cutler, C. P., Cramb, G., 2002. Branchial Expression of an Aquaporin 3 (AQP-3) Homologue is Down regulated in the European Eel (*Anguilla anguilla*) Following Sea Water a Acclimation, Journal of Experimental Biology 205, p. 2643-2651.

Cutler, C. P., Brezillon, S., Bekir, S., Sanders, I., Hazon, N., Cramb, G., 2000. Expression of a duplicate Na,K-ATPase beta(1)-Isoform in the European Eel (*Anguilla anguilla*), Journal of physiology Regulatory, Integrative and Comparative Physiology 279(1), p. 222-229.

Haas, M., 1994. The Na-K-Cl Cotransporters, Journal of Physiology 267(4), p. 869-885.

Howarth, C., Gleeson, P., Attwell, D., 2012. Updated Energy Budgets for Neural Computation in the Neocortex and Cerebellum, Journal of Cerebral Blood Flow and Metabolism 32(7), p. 1222–1232.

Hwang, P. P., Fang, M. J., Tsai, J. C., Huang, C. J., Chen, S. T., 1998. Expression of mRNA and protein of Na+-K+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*), Journal of Fish Physiology and Biochemistry 18, p. 363-373.

Kelly, A. H., David, H. E., 2009. Short-Term Low-Salinity Tolerance by the Longhorn Sculpin, *Myoxocephalus octodecimspinosus*, Journal of Experimental Zoology 311A, p. 45–56.

Lorin Nebel, C., Boulo, V., Bodinier, C., Charmantier, G., 2006. The Na+/K+/2Cl- Cotransporter in the Sea Bass *dicentrarchus labrax* during Ontogeny: Involvement in Osmoregulation, Journal of Experimental biology 209(24), p. 4908-4922.

Juriaan, R. M., Erwin, H. B., Sjoerd E. W. B., Gert, F., 2003. Regulation of Branchial Na^+/K^+-ATPase in Common Carp *Cyprinus Carpio* L. Aacclimated to Different Temperatures, Journal of Experimental Biology 206, p. 2273-2280.

Venturini, G., Cataldi, E., Marino, G., Pucci, P., Garibaldi, L., Bronzi, P., Cataudella, S. 1992. Serum Ions Concentration and ATPase activity in Gills, Kidney and Oesophagus of European Sea-Bass (*Dicentrarchus labrax*, Pisces, Perciformes) during Acclimation trials to Fresh Water, Journal of Comparative Biochemistry and Physiology 103A, p. 451–454.

Zhichao, D., Robert, A. C. L., Gert, F.,Wendelaar, B.S.E, 2000. Na+/K+-ATPase Immunoreactivity in Branchial Chloride Cells of *Oreochromis Mossambicus* Exposed to Copper, Journal of Experimental Biology 203, p. 379–387.

Available online at www.buu.ac.th/BUUConference

Burapha University International Conference 2015

"Moving Forward to a Prosperous and Sustainable Community"

ST-P-027

Dengue model in the flooding area

Puntani Pongsumpun

Department of Mathematics, Faculty of Science,
King Mongkut's institute of Technology Ladkrabang,
Chalongkrung road, Ladkrabang, Bangkok 10520, Thailand
corresponding author: kppuntan@kmitl.ac.th

Abstract

Dengue transmission is found around the world. Dengue fever, Dengue hemorrhagic fever and dengue shock syndrome are three types of this disease. This disease is transmitted between human by biting of infected female *Aedes aegypti* vector. The endemic of this disease is depending on many factors. Mosquitoes are usually found in the flooding area. We describe the transmission of this disease by formulating the mathematical model and then we analyze this model by standard dynamical modeling method. The factor of flooding areas is considered in this study. We simulate the transmission of dengue virus with the different transmission rates in the different areas to see the behaviors of each population group.

Keywords: states dengue; flooding area; model; stability; equilibrium.