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EST-based identification of genes expressed in the branchiae of black tiger shrimp (*Penaeus monodon* Fabricius)

Suwit Wuthisuthimethavee[a,*], Prajuab Lumubol[b], Apichart Vanavichit[c], Somvong Tragoonrung[a]

[a] DNA Technology Laboratory, National Center for Genetic Engineering and Biotechnology, Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom 73140, Thailand.
[b] Marine Science Department, Fisheries Faculty, Kasetsart University, Bangkok 10900, Thailand.
[c] Rice Genes Discovery Unit, National Center for Genetic Engineering and Biotechnology, Kasetsart University, Kamphaengsaen Campus, Nakorn Pathom 73140, Thailand.

* Corresponding author, E-mail: suwit@dnatec.kps.ku.ac.th, suwitwuth@hotmail.com

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ABSTRACT: Expressed sequence tag (EST) analysis is a powerful tool for gene discovery. A preliminary study of genes expressed in branchiae of *Penaeus monodon* comprised constructing a cDNA library, cDNA sequencing and a homology search of public databases. The constructed cDNA library contained insert sizes ranging from 0.5-1.6 kb. From 200 randomly selected EST clones, 176 (88%) were successfully sequenced and gave average read-length after vector clipping of 325 bp. A total of 26 contigs were formed after clustering the 176 ESTs. The contigs contained a total of 72 EST sequences and 104 sequences remained as singleton ESTs. Bioinformatics analysis revealed that 42 from 104 transcripts matched known genes. These were categorized into 6 classes of genes involved in gene/protein expression (50%), metabolism (23.8%), cell/organism defense (9.6%), cell structure/motility (7.1%), and cell signaling and communication (2.4%). Genes lacking enough information to be classified constituted the remaining 7.1%. This study also found a transcript with high homology to an Na^+/K^+-ATPase gene reported to be involved in osmoregulation of crustaceans, and especially to the α-subunit Na^+/K^+-ATPase in *H. americanus*. Expression of this gene in branchiae of low salinity-stressed *P. monodon* was examined using RT-PCR analysis. The results showed that the mRNA abundance in stressed shrimps decreased over 7 days in contrast to a previously- described increase in the enzyme activity.

KEYWORDS: ESTs, *Penaeus monodon*, Branchiae, Osmoregulatory system.

INTRODUCTION

The black tiger shrimp *Penaeus monodon* is naturally distributed over a wide range of salinities during its life cycle. Fry and juveniles inhabit mangrove swamps and other estuarine environments, while adults are found in deeper water down to 150 m[1]. The habitat pattern of pre-reproduction migration in wild *P. monodon* is common throughout the genus. *P. monodon* is the most widely cultured shrimp species in the world. Successful culture to broodstock size has been reported in salinities ranging from brackish to oceanic. Several additional papers have documented pond rearing of *P. monodon* to adult size without reporting culture water salinity[2,3].

The effect of salinity on the osmotic and ionic properties of the hemolymph is well studied in *P. monodon* and other penaeid shrimp with more than ten species having been studied so far[4,5,6]. In general, *P. monodon* exhibits hyperosmotic regulation to seawater at salinities below isosmotic concentrations and hypoosmotic regulation to those above, with the isosmotic concentration being 20-30 ppt. Sodium and chloride account for about 75-85% of the total osmolytes in hemolymph. Calcium is accumulated while magnesium is greatly hyporegulated within the whole range of salinities (3-50 ppt) in which *P. monodon* can survive. However, there may be considerable differences between the osmoregulatory abilities of juveniles and adults[4].

There are no recent, significant reports regarding the molecular mechanisms of osmorelation in *P. monodon*. However, Lin et al.[7] reported that the antennal gland regulated sodium and potassium concentrations and body volume when the salinity decreased. On the other hand, the antennal gland did not appear to regulate osmolarity or the concentration of chloride, sodium, potassium and calcium ions as salinity increased.

Many studies have shown that gills are the primary organs for salt transport in crabs[8,9,10] and lobsters[11]. They utilize apical membrane branchial Na^+/H^+ and Cl^-

/HCO_3^- exchange powered by a basal membrane Na^+/K^+-ATPase. The HCO_3^- is provided by carbonic anhydrase facilitating CO_2 excretion while NH_4^+ can substitute for K^+ in the basal ATPase and for H^+ in the apical exchange. Aquatic hyper-regulators also release neuroamines from pericardial organs, including dopamine and 5-hydroxytryptamine (5-HT), which via a cAMP-mediated phosphorylation stimulate Na^+/K^+-ATPase activity and NaCl uptake[10,12].

Since these molecules are well-documented in other closely-related crustaceans but not in *P. monodon*, we aimed to investigate genes that control osmoregulation in *P. monodon* by analyzing cDNA from branchial organs.

Materials and Methods

Total RNA and Messenger RNA Preparation

Total RNA was extracted from the branchiae of broodstock-sized *P. monodon* and snap frozen in liquid nitrogen under RNAse-free conditions. Poly(A) mRNA was directly captured from the total RNA using the PolyATract System 1000 (Promega Corp, Madison, WI, USA) according to the manufacturer's instruction.

cDNA Synthesis and Branchiae cDNA Library Construction

Five micrograms of branchiae mRNA from *P. monodon* was used to synthesize double-stranded complementary DNA using a Time Saver cDNA Synthesis Kit (Amersham Biosciences, Little Wamply, UK). Blunt-ended double-stranded cDNA was directly cloned into dephosphorylated, *Eco*RV-digested pBluescript II vector. ELECTROMAX DH10B competent cells (Invitrogen Inc., Carlsbad, CA, USA) were transformed with the resultant recombinants.

EST Sequencing, Analysis and Annotation

Randomly selected colonies were picked and cultured overnight in LB containing ampicillin and screened for insert size by polymerase chain reaction (PCR) using M13 universal primers to cut off clones containing inserts smaller than 500 bp. Plasmid DNA was then purified from the selected cDNA clones and subjected to DNA sequencing. Sequencing was carried out using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 377 with T7 sequencing primer. All EST sequences were analyzed with the Vector screen program at the National Center for Biotechnological Information (NCBI, http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) to eliminate the vector sequences, then submitted to EST Assembly program (http://www.ifom-firct.it) for contig construction. The contigs and singletons > 200 bp in length[15] were compared with nucleotide and protein sequence databases of NCBI with BLAST, (available at http://www.ncbi.nlm.nih.gov/BLAST)[13].

For identification of *P. monodon* cDNA clone homologues, the cutoff was set at an expected value (*E*) < 10^{-4}. Identified clones generally shared a sequence identity >65% over a relatively long range >150 bp with the most similar sequence for BLASTN[14] and a minimum amino acid sequence identity of 35% for BLASTX[15]. ESTs that matched known or predicted genes were categorized into different groups according to functions, as described by Hwang et al.[16].

Na^+/K^+-ATPase Gene Characterization and Expression

A cDNA sequence of *P. monodon* that matched an Na^+/K^+-ATPase gene of *Homarus americanus* was characterized by comparison with the full-length cDNA and amino acid sequences of the Na^+/K^+-ATPase gene of *Homarus americanus*.

The abundance of Na^+/K^+-ATPase mRNA in the branchiae of salinity-stressed shrimps was analyzed by RT-PCR. Shrimp samples from rearing ponds were maintained in 500-liter tanks at 30 ppt salinity for 2 weeks and transferred into 3 ppt for 7 days. The control samples were maintained at 30 ppt. The branchial tissue was removed individually from stressed and controlled shrimp at 24, 48, 72 hours and 7 days after beginning the stress and kept at –80 °C. Total RNA was purified from the branchiae using the High Pure Total RNA Isolation Kit (Roche, Basel, switzerland) and quantified by agarose gel electrophoresis.

Specific primers were designed at each end of the Na^+/K^+-ATPase cDNA sequence of *P. monodon* with sequences of PmNAK-F 5'-TGT GGT TCG ACA ACA CCA T-3' and PmNAK-R 5'-TTG ATG TAG ATG GTG GAG CA-3'. Multiplex RT-PCR reactions were carried out with the Titan One-Tube RT-PCR System (Roche) and contained the primers for Na^+/K^+-ATPase mRNA and the primers for elongation factor II mRNA (PmEft2-F 5'-GTC TTC GTG CAG GAG AAA CTC G-3' PmEft2-R 5'-CGA CGA GGG CAC CAT CAG TTA C-3')[17] as the internal standard. The conditions for RT-PCR were 50 °C for 60 min, then 94 °C for 2 min and 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, followed by an extension at 72 °C for 5 min and storage at 4 °C. PCR products were separated electrophoretically on 1% agarose gels and visualized by ethidium bromide staining. The fluorescence intensity of the gene-specific bands was quantified with GeneTools v. 3.04 (SynGene). Prior to the statistical analysis, the intensity of Na^+/K^+-ATPase gene amplified from shrimps under five experimental conditions was normalized with that of elongation factor II. Data were then analyzed by one-way ANOVA), with the least significant difference (LSD) test for post-hoc analysis using the SPSS-PC$^+$ statistical

package (SPSS Inc). Statistical significance was defined as $\rho < 0.05$.

Results

cDNA Sequencing

An EST library was successfully constructed to survey osmoregulatory-related genes expressed in branchiae of *P. monodon*. The number of clones in the library was approximately 9.6 x 10^4 cfu. The cDNA library had average insert sizes of 500-1600 bp. A total of 200 randomly selected clones with insert sizes larger than 500 bp were subjected to single-pass DNA sequencing, resulting in 176 (88%) EST sequences, while 24 clones failed. The average read-length after vector clipping was 325 bp.

Table 1. General characterization of *P. monodon* 's branchiae ESTs.

Total number of cDNA sequenced	200
Total number of cDNA analyzed	176
Average insert size (range; bp)	500-1600
Average ESTs length (bp)	325
EST contigs	26
Singletons	104
Redundancy (%)	40.9

Cluster Assembling

A total of 26 contigs were formed after assembly of the 176 ESTs (Table 1). Each contig consisted of at least two ESTs that were considered to be derived from the same gene, taking sequence ambiguities into account. The contigs included a total of 72 sequences, whereas 104 sequences remained as singleton ESTs. This corresponded to a redundancy of 40.9%. The number of sequences in a given contig ranged between two (18 contigs) and 12 (1 contig). Totally, 104 transcripts (19 contigs and 85 singletons) >200 bp in length were analyzed in homology searches via NCBI.

Bioinformatic analysis

Based on our subjective criteria for gene identification at a minimum amino acid sequence similarity of 35%, 42 (40.4%) *P. monodon* transcripts showed homology to previously described genes from *P. monodon* and other species, including shrimp (Table 2).

Table 2. Summary of ESTs >200 bp from the *P. monodon*'s branchiae.

Unmatched-novel	**62 (59.6%)**
ESTs matching to known sequences	
Matched to known genes	21 (20.2 %)
Mitochondrial DNA	8 (7.7%)
Ribosomal proteins & RNA	13 (12.5%)
Total	104(100%)

ESTs matching known genes were categorized into seven categories on the basis of general functions (cell division, cell signaling/communication, cell structure/ motility, cell/organism defense, gene/ protein expression, metabolism, and unclassified).[16] In total, 42 known genes were represented and the percentage of transcripts in each category was calculated. The largest class of genes comprised those involved in gene/ protein expression (50%). This class was followed by genes involved in metabolism (23.8%), cell/organism defense (9.6%), cell structure/motility (7.1%) and cell signaling and communication (2.4%). No transcripts matched cell division gene records. Genes lacking enough information to be classified constituted the remaining 7.1% (Table 3).

Table 3. Number and percentage of *P. monodon* ESTs matching known genes by functional category.

Functional Category	Number in Category (%)
Cell signaling/communication	1 (2.4%)
Cell structure/motility	3 (7.1%)
Cell/organism defense	4 (9.6%)
Gene/protein expression	21 (50%)
Metabolism	10 (23.8%)
Unclassified	3 (7.1%)
Total	42 (100%)

Several transcripts had high similarity to proteins of known function, including tubulin alpha chain, Na^+/K^+-ATPase, cyclophilin isoform 5, and peptidylprolyl isomerase (cyp1). Several transcripts matched previously-reported known proteins of *P. monodon*, including 6 mitochondrial genes (i.e. cytochrome b, cytochrome c oxidase, ATP synthase), clottable protein, and translationally controlled tumor protein (TCTP) (Table 4). Mitochondrial genes like, cytochrome b, cytochrome c oxidase corresponding to mitochondrial transcripts are likely of physiological relevance, because branchiae have a high oxidative metabolic rate. In this study, the only osmoregulatory gene found was a Na^+/K^+-ATPase gene with high similarity to a gene from *Homarus americanus*.

A total of 62 transcripts did not share significant sequence homology with any known sequence in the databases. Most of them are expected to be derived from hitherto uncharacterized or novel genes. Some may have had too low sequence similarity to detect homology in the region that was sequenced.

Table 4. List of identified *Penaeus monodon* branchial ESTs.

Penaeus monodon branchial ESTs				Matching sequences			
Clone ID	Sequence length (bp)	dbEST Accession no.	Gene	Species with closest homology	Accession no.	E-value	Identity
Cell signaling/ communication							
1-4B	387	22969577	gelsolin	*Homarus americanus*	S41391	2e-57	104/128 (81%)
Cell structure/motility							
Br11E	589	22969588	alpha-tubulin	*Oncorhynchus keta*	S25004	1e-102	178/196 (90%)
Br7F	205	22969599	alpha-III tubulin	*Homarus americanus*	AAB08889	3e-19	44/44 (100%)
Br1-6E	243	22969605	myosin heavy chain	*Drosophila melanogaster*	A36014	4e-36	71/80 (88%)
Cell/organism defense							
Br2G	304	22969606	Putative antimicrobial peptide	*Litopenaeus setiferus*	AAL36897	5e-08	22/27 (81%)
Br9F2-F1	232	AAF19002*	clottable protein	*Penaeus monodon*	AAF19002	4e-37	76/76 (100%)
Br2H	309	22969607	glutathione S-transferase	*Schistosoma mansoni*	AAA29888	1e-12	41/101 (40%)
Br10D	578	22969608	iron superoxide dismutase	*Salmonella typhimurium LT2*	AAL20353	2e-53	99/173 (57%)
Gene/protein expression							
BR6E	474	22969609	peptidylprolyl isomerase (EC 5.2.1.8) (cyclophilin) cyp-1	*Drosophila melanogaster*	B38388	3e-55	101/129 (78%)
Br10B	717	22969610	cyclophilin isoform 5	*Caenorhabditis elegans*	NP_493624	7e-60	118/171 (69%)
Br9D2-F1	286	22969578	elongation factor 1-alpha	*Litopenaeus stylirostris*	AAM90311	2e-15	38/39 (97%)
Br1A	265	22969579	H2-K region expressed gene 2	*Mus musculus*	NP_034515	2e-20	48/88 (54%)
BrCol43	465	22969580	ubiquitin B precursor polyubiquitin B	*Homo sapiens*	NP_061828	3e-47	96/98 (97%)
Br12C	220	22969581	splicing factor hPRP17	*Homo sapiens*	AAC25166	3e-24	48/71 (67%)
5Br7B	377	22969582	Threonyl-tRNA synthetase	*Homo sapiens*	AAH10578	8e-43	74/104 (71%)
BrPC110	530	22969583	translation initiation factor eIF-2 gamma chain	*Homo sapiens*	A53048	8e-41	83/97 (85%)
CONTIG11	434	22969584	40S ribosomal protein S5	*Rattus norvegicus*	XP_341789	5e-67	119/128 (92%)
BrPC99	379	22969585	40S ribosomal protein S8	*Xenopus laevis*	CAA50399	2e-47	94/121 (77%)
CONTIG17	498	22969586	40S ribosomal protein S9	*Homo sapiens*	NP_001004	5e-74	137/157 (87%)
CONTIG12	353	22969587	40S ribosomal protein S17	*Gallus gallus*	CAA30244	8e-38	76/84 (90%)
Br4D-2	417	22969589	40S ribosomal protein S23	*Spodoptera frugiperda*	AAK92191	1e-71	128/138 (92%)
CONTIG7	364	22969590	40S ribosomal protein S25	*Drosophila melanogaster*	P48588	4e-27	59/78 (75%)
CONTIG10	416	22969591	acidic ribosomal protein P1	*Artemia sp.*	R6SSP2	1e-19	51/113 (45%)
5Br-8G	370	22969592	Acidic ribosomal phosphoprotein P2	*Equus caballus*	AAP78699	3e-14	41/65 (63%)
CONTIG23	435	22969593	ribosomal protein L4	*Pagrus major*	AAP20200	4e-18	43/89 (48%)
Br10E	658	22969594	60S ribosomal protein L12	*Rattus norvegicus*	R7RT12	2e-67	121/163 (74%)
CONTIG8	397	22969595	ribosomal protein L28	*Homo sapiens*	S55915	3e-16	40/84 (47%)
Br7E-2F2	406	22969596	ribosomal protein L30	*Argopecten irradians*	AAN05584	1e-46	92/111 (82%)
5Br-12D	389	22969597	60S ribosomal protein L36	*Branchiostoma belcheri*	AAN52381	4e-27	57/83 (68%)
Metabolism							
CONTIG9	753	AAF43372*	ATP synthase F0 subunit 6	*Penaeus monodon*	AAF43372	2e-78	162/231 (70%)

* dbEST accession numbers from previous reports.

Table 4. *Cont'd.*

Penaeus monodon branchial ESTs				Matching sequences			
Clone ID	Sequence length (bp)	dbEST Accession no.	Gene	Species with closest homology	Accession no.	*E*-value	Identity
Br8D	402	22969598	ATP synthase subunit e	*Drosophila melanogaster*	BAA02423	4e-06	27/62 (43%)
Br9F	401	AAF36100*	cytochrome b	*Penaeus monodon*	AAF36100	3e-52	104/130 (80%)
CONTIG5	608	AAF43374*	cytochrome c oxidase subunit I	*Penaeus monodon*	AAF43374	4e-89	170/191 (89%)
BrCol54	179	AAF43375*	cytochrome c oxidase subunit II	*Penaeus monodon*	AAF43375	6e-17	40/42 (95%)
Br1D	264	AAF43376*	cytochrome c oxidase subunit III	*Penaeus monodon*	AAF43376	6e-39	80/87 (91%)
Br10A	380	22969600	cytochrome c oxidase subunit VIc precursor	*Thunnus obesus*	AAQ14272	4e-14	38/76 (50%)
Br1A-2F2	237	AAF43380*	NADH dehydrogenase subunit 3	*Penaeus monodon*	AAF43380	1e-18	49/68 (72%)
BR179	428	22969601	putative Na+/K+-ATPase alpha subunit	*Homarus americanus*	AAN17736	1e-74	133/142 (93%)
5Br-12E	722	22969602	Trypsin	*Pacifastacus leniusculus*	CAA10915	9e-07	31/67 (46%)
Unclassified							
6B-2	417	22969603	cysteine-rich intestinal protein	*Hirudo medicinalis*	AAN73075	6e-22	41/56 (73%)
Br6G-2F2	205	22969604	immediate early response3 interacting protein 1	*Homo sapiens*	AAK53816	2e-07	26/44 (59%)
Br3G	245	AAO61938*	translationally controlled tumor protein	*Penaeus monodon*	AAO61938	2e-37	77/79 (97%)

Penaeus monodon Na^+/K^+-ATPase Gene Characterization and Expression

A 428-bp Na^+/K^+-ATPase cDNA sequence was obtained from a cDNA library representing genes expressed in branchiae of *P. monodon*. Alignment of that sequence with a 4159-bp sequence of the Na^+/K^+-ATPase α-subunit gene of *H. americanus* revealed that very high sequence identity (364/428 bp) was found, starting from nucleotide positions 1510-1937 of *H. americanus* (Fig. 1). This alignment also corresponded to the amino acid sequence comparison (Table 4, Fig. 2).

Structural analysis of the Na^+/K^+-ATPase amino acid sequence of *H. americanus* using the NCBI Conserved

Fig 1. Alignment of the Na^+/K^+-ATPase cDNA sequences of *P. monodon* and *H. americanus*.

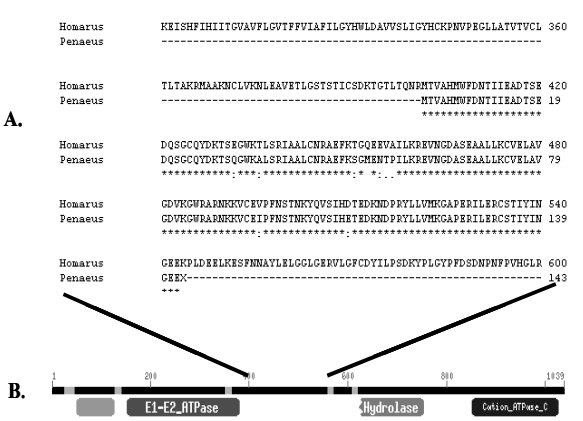

Fig 2. A) Alignment of the deduced amino acid sequences of Na^+/K^+-ATPase of *P. monodon* and *H. americanus*. B) Structural analysis of the Na^+/K^+-ATPase amino acid sequence of *H. americanus*.

Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/) revealed that there were four principle domains, consisting of N-terminal cation transporter/ATPase (49-128), E1-E2 ATPase (151-382), Hydrolase

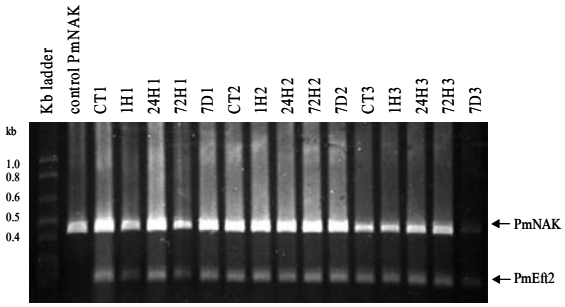

Fig 3. RT-PCR analysis of Na^+/K^+-ATPase gene expression in *Penaeus monodon* stressed by salinity change. The control shrimp (CT1-3) were maintained at 30 ppt, while 1H1, 24H1, 72H1 and 7D1 samples were placed at 3 ppt for 1, 24, 72 hours and 7 days, respectively. The RT-PCR products at different template concentrations (1, 2 and 3) show the expression of the Na^+/K^+-ATPase gene (PmNAK) and *Penaeus monodon* elongation factor II gene (PmEft2) as the internal standard.

(621-754), and C-terminal cation transporting ATPase (850-1028) domains. A 139-deduced amino acid sequence of Na^+/K^+-ATPase from *P. monodon* (PmNAK) matched the low complexity region located between the E1-E2 ATPase and hydrolase domains of the Na^+/K^+-ATPase of *H. americanus* (Fig. 3).

In order to determine whether gene activation might be responsible for the observed differences in enzymatic activity, the abundance of Na^+/K^+-ATPase mRNA was measured using semi-quantitative RT-PCR. Multiplex RT-PCR of shrimp total RNA samples showed 400-bp of PmNAK and 250-bp of PmEft2 PCR products. Using *P. monodon* elongation factor II mRNA as an internal standard for normalization, the significance of differences in Na^+/K^+-ATPase mRNA abundance in shrimps in the control (CT), 1, 24, 72 h and 7 days after initiation of low-salinity stress was analyzed with the single factor Analysis of Variance (ANOVA) test. However, the level of Na^+/K^+-ATPase gene expression detected and in this study decreased with time under the stress condition, though it was not significant by ANOVA ($\rho < 0.05$) (Fig 4).

Discussion

The generation of ESTs has proven to be a useful and rapid means to identify and isolate large numbers of expressed sequences[16,18]. Approximately 1,000 ESTs of *P. monodon* have been recently submitted to the GenBank (http://www.ncbi.nlm.nih.gov/) from various tissues, such as the cephalothorax, eyestalks,

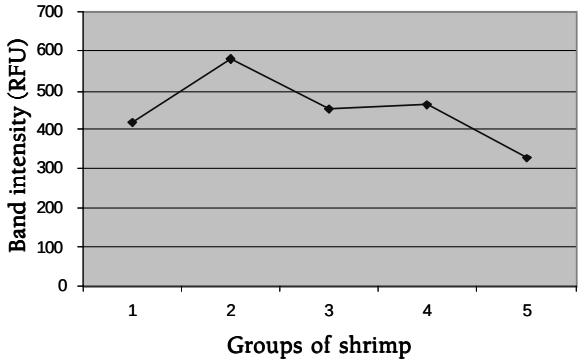

Fig 4. Differential Relative Fluorescent Units (RFU) from semiquantitative RT-PCR analysis of the Na^+/K^+-ATPase gene in *Penaeus monodon* stressed by salinity change. (1 = control, 2 = 1h, 3 = 24h, 4 = 72h and 5 = 7 days after stress induction).

pleopods[19] and hemocytes[20]. This number is relatively low compared with ESTs from *Litopenaeus vanamei* (> 6,000 ESTs), zebrafish (> 100,000 ESTs) and other organisms used for genomic studies.

There are inherent difficulties in using raw EST sequences to identify genes because they are incomplete, highly redundant, and error prone. These problems can be alleviated by grouping ESTs into consensus sequences[21]. The redundancy is beneficial in generation of assemblies of overlapping ESTs from a single gene that can be aligned and combined to generate a consensus sequence. Another potential use of redundancy is to identify highly expressed genes that may represent particularly important biological pathways for the organism[21].

After vector sequence elimination, the EST sequences < 200 bp were discarded before homology searches because analysis of the results showed that no algorithm worked well when the length of the query sequence was less than 200 bp[15]. The high abundance of the unmatched genes (62 ESTs, 59.6%) may be specific to *P. monodon* branchiae and some may be involved in osmoregulation.

Genes involving cell/organism defense, such as clottable protein and antimicrobial peptide, probably came from the hemocytes in the tissue preparation steps. Despite molecular studies, ion transportation in *P. monodon* remains unclear. At least 2 genes including, Na^+/K^+-ATPase and carbonic anhydrase localized in brachial epithelial cells have been confirmed to play a key role in crustacean osmoregulation[12,22]. The putative Na^+/K^+-ATPase gene reported in this study is the alpha subunit, which is responsible for the catalytic function of the Na^+/K^+-ATPase, binding and hydrolyzing ATP and itself becoming phosphorylated during the transport cycle. This gene of *P. monodon* has relatively high similarity to the Na^+/K^+-ATPase alpha subunit of *H.*

americanus with amino acid sequence identity of 93% (133/142). The multiple alignment of Na^+/K^+-ATPase amino acid sequence among crustaceans also reveals extensive regions of highly conserved amino acid sequence (data not shown).

The activity of Na^+/K^+-ATPase in crustacean branchiae has been demonstrated to increase when euryhaline crustaceans are subjected to osmoregulatory stress following transfer from seawater to dilute salinities, however, the Na^+/K^+-ATPase mRNA levels were not found to increase in relative proportions. In this study, we found that the Na^+/K^+-ATPase mRNA levels varied among individuals in each group and the average expression levels decreased over time during low-salinity stress. The mRNA abundance of Na^+/K^+-ATPase a subunit may not be a suitable marker for Na^+/K^+-ATPase quantification due to its functional composition which includes α, β, and γ subunits. It remains unclear whether the measured increases in activity result from *de novo* synthesis of Na^+/K^+-ATPase mRNA and/or protein or from post-translational processes such as subunit assembly, membrane trafficking and cell signaling[12].

This study does not clarify the mechanisms for Na^+/K^+-ATPase activity in *P. monodon* as well as the reports in other crustaceans[9,11,23]. In the method for determination of gene expression, RT-PCR, PCR band intensity reflects relative transcript abundance in the experimental RNA samples. However, this method may be less sensitive for discrimination of small differences in mRNA among samples and the detection depends upon post-PCR analysis that is, in principle, not accurate for relative comparison between amplified PCR products of two different starting samples. It is recommended to observe such differences with a more sensitive and accurate detector such as real-time PCR. To ensure RT-PCR accuracy, a multiplex RT-PCR method was described for the simultaneous detection of PmNAK in combination with PmEft2 (a gene encoding a protein involved in basic cellular processes) mRNA. The abundance of PmEft2 was used as the internal standard for normalizing the effectiveness of the RNA extraction and RT-PCR.

Future studies are therefore necessary to construct full-length cDNA clones of *P. monodon* Na^+/K^+-ATPase α-subunit gene, carbonic anhydrase gene and other genes and investigate the expression of those genes to environmentally salinity changes.

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