Identification and Expression of Vitellogenin Gene in Polychaetes (*Perinereis* sp.)

Khwanta Phoonsamran[1], Sataporn Direkbusarakom[1], Piyapong Chotipuntu[1], Ikuo Hirono[2], Sasimanas Unajak[3], Pijug Summpunn[1] and Suwit Wuthisuthimethavee[1*]

ABSTRACT

Polychaetes (*Perinereis* sp.) have been used extensively in shrimp broodstock maturation diets because of their nutritional value, being rich in protein, fat and minerals. The aims of this study were to identify the reproduction-related gene in polychaetes, and analyze gene expression levels in polychaetes. Messenger RNA (mRNA) was isolated from mature polychaete samples and used as the template for complementary DNA (cDNA) synthesis. The cDNA was then subjected to cloning and sequencing. However, no cDNA sequence was found to match the reproductive-related gene. As an alternative procedure, a set of cDNA was synthesized and sequenced by the pyrosequencing method. From 454 Pyrosequencing, 40,402 raw sequences were obtained, covering 14,747,824 nucleotides 200-300 bp in length and grouped into 609 isotigs, 712 contigs and 15,975 singletons. In addition, BLASTX analysis showed that 410 sequences (67.3%) matched known genes, whereas, 199 sequences (32.7%) showed no significant homology to known genes. A significant match was found with the vitellogenin structural genes' (yolk protein genes) family member (vit-1)-like gene, which was grouped as the reproduction-related gene in polychaetes. The gene was then used to analyze the expression levels in polychaetes aged 2, 3, 4, 5, 6, 7, and 8 months by real-time quantitative RT-PCR. The highest vitellogenin gene expression was found in 6 – month old polychaetes ($P<0.05$) and dramatically decreased after reaching 6 months. This result suggested that 6 – month old polychaetes will be suitable as the maturation diet for shrimp broodstock; however, the effect on shrimp reproductive development may need to be analyzed further.

Keywords: Polychaete, Vitellogenin gene, Real-time quantitative RT-PCR

INTRODUCTION

Penaeid shrimp have since been domesticated, and selective breeding programs have been developed to overcome problems arising from the use of wild shrimp or unknown performance as a source of fry in grow-out farms. The relevant parameters have been focused on ensuring the production of high-quality broodstock in terms of genetic improvement, optimum diet, and control of health. However, the limited and inconsistent availability of high-quality broodstock remains a constraint, because of very low success rates in raising penaeid shrimp to maturity. This limitation requires the condition and maturation of broodstock shrimp

[1] Research Center of Excellence for Shrimp, School of Agricultural Technology, Walailak University, Nakhon Si Thammarat 80161, Thailand
[2] Laboratory of Genome Science, Department of Marine Biosciences, Faculty of Marine Science, Tokyo University of Marine Science and Technology, Tokyo 108-8477, Japan
[3] Department of Biochemistry, Faculty of Science, Kasetsart University, Chatuchak, Bangkok 10900, Thailand
* Corresponding author, e-mail address: wsuwit@wu.ac.th

to stimulate gonadal development and induce mating, spawning, and, ultimately, the hatching of eggs to produce viable larvae. Broodstock diets are very important in the maturation process, especially in stimulating ovarian development in female shrimp (Harrison 1990, 1997). In captive conditions, shrimp reproduction generally relies on live feed such as squid, bivalves (mussel, clam and oyster), and polychaetes to ensure optimal reproductive output. However, these types of live feed are generally wild-caught marine organisms selected according to their availability and fed in mixed ratios to increase the likelihood of meeting the nutritional requirements of broodstock shrimp (Bray and Lawrence, 1992; Harrison, 1997; Pinon, 2000).

Due to their high nutritional composition, high unsaturated fatty acid content (e.g. arachidonic acid) and presence of reproductive hormones, polychaetes are extensively used as food for shrimp broodstock, especially when the polychaetes are in the reproductive stage (Wouters *et al*., 2001; Meunpol *et al*., 2005; Chimsung, 2014). Furthermore, hormones extracted from polychaetes, such as progesterone (Koskela *et al*., 1992), prostaglandin E2 (D, Croz *et al*., 1988), methyl farnesoate (Laufer *et al*., 1987), and 17α-hydroxyprogesterone, promote reproductive performance in shrimp. They influence both vitellogenesis and final maturation (Meunpol *et al*., 2007; Meunpol *et al*., 2010). In general, most frozen or live polychaetes used to enhance shrimp broodstock maturation are at the adult stage (reared 5 months from nectochaete). In this stage, some adults become epitokes (Poltana *et al*., 2007), which contain high levels of nutrition and reproductive hormones. To obtain high-quality polychaetes, culture methods have been developed using various propagation, feed, substrate, and water quality management conditions (Poltana *et al*., 2007; Brown *et al*., 2011; Palmer, 2011). While the domestication of specific-pathogen-free (SPF) polychaetes has been successful in ordinary farm facilities, to produce sufficient quantities as food for shrimp broodstock, issues of inconsistent nutrition and hormones need to be investigated further.

This study aimed to identify the vitellogenin gene in polychaetes using the cDNA sequencing analysis and to determine the expression levels of vitellogenin gene at different ages of polychaete using real-time RT-PCR method.

MATERIALS AND METHODS

Pyrosequencing analysis

Polychaete samples

The polychaetes (*Perinereis* sp.) used in this study were obtained from culture condition before undergo epitoky. Due to difficult identification using morphological observation, polychaete samples were not sex discriminated. However, Darya *et al* (2016) reported that sex ratio of *Perinereis nuntia* was 1:1.

Total RNA extraction and GS-FLX sequencing

Total RNA was extracted from polychaetes (6-8 months old) using TriPure Isolation reagent. Messenger RNA (mRNA) was further purified using the PolyATtract® mRNA Isolation Systems III. The quantity and purity of eluted mRNA were determined by NanoDrop spectrophotometer. A cDNA library was constructed according to manufacturer's instructions (Roche Applied Science, Genome Sequencer FLX™ System). The expressed cDNA sequencing was performed at the Genome Institute (National Center for Genetic Engineering and Biotechnology, Thailand) with a GS-FLX sequencer.

Assembly, gene annotation, and gene ontology (GO) analysis

Newbler Assembler software was used in stage 1 of assembly, and gene prediction was carried out using Glimmer 3.02 (Delcher *et al*., 2007) with an E-value of 0.01. The assembled EST translations were aligned with the GenBank NR (non-redundant) amino acid sequence database using BLASTX. Stage 2 assembly was performed with the TGI clustering tool (TGICL, http://compbio.dfci.harvard.edu/tgi/software), which was used to check improvement of length and quality with whole contigs and singletons. Subsequently, the data was sorted according to read length, gene annotation, GenBank number, E-value, and the targeted closed species by its GenBank accession number. GO analysis was conducted using the Blast2GO automated sequence annotation tool (http://www.blast2go.org). GO analysis was employed to compare the similarity of the annotated sequences. GO terms from the biological process, molecular function, and cellular component GO categories were

assigned to the annotated transcripts (Conesa and Götz, 2008). In the present study, the results of the GO analysis are presented based on level 2 terms, which describe general functional categories. Unique sequences were assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using the online KEGG Automatic Annotation Server (KAAS) (http://www.genome.jp/kegg/kaas/) to search the KEGG GENES database.

Gene expression analysis: reproductive gene of polychaete

Total RNA extraction

Total RNA was extracted from the heads of polychaetes aged 2, 3, 4, 5, 6, 7, and 8 months (five samples per age group) using TriPure Isolation reagent. The quantity and quality of eluted total RNA were determined by NanoDrop spectrophotometer. The first-strand cDNA was generated according to Transcriptor High Fidelity cDNA Synthesis Kit protocol.

From the pyrosequencing results, specific primers were designed based on the cDNA hit to vitellogenin structural genes' (yolk protein genes) family member (vit-1)-like gene in polychaetes using the Primer 3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

Real-time Quantitative PCR

The cDNA of the polychaetes was subsequently amplified using gene-specific primer sets. Real-time quantitative PCR (qPCR) was carried out with the Applied Biosystems 7300 real-time PCR system. Amplification was performed in a mixture containing 2 × SsoFast EvaGreen Supermix, specific primer mix, and cDNA in a 96-well real-time qPCR plate. The 10 µL reaction volume contained 5 µL of 2× SsoFast EvaGreen Supermix, 25 ng of the first-strand cDNA template, and 0.25 µM each of forward (V3race-F1) and reverse (V5race-R1) gene-specific primers for the vitellogenin structural gene and the control gene (EF-1α). The process was conducted in triplicate for each sample. For the negative control, sterile water replaced the template. Ten-fold serially diluted cDNA (pooled) was included in the same real-time qPCR plate to calculate the efficiency of the gene-specific primers. The amplification conditions included a holding step of 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72 °C for 1 min. Dissociation melt-curve analysis was performed at completion. Relative expressions of a target gene were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) using elongation factor-1 alpha (EF-1α) as an internal control gene in a single sample of polychaetes of different ages. Moreover, the C_T values of polychaetes of different ages were normalized with control samples and presented as a relative expression ratio according to the equation.

Statistical analysis

The relative expression ratio data are presented as mean ± standard deviation and the levels of gene expression were determined using one-way ANOVA followed by Duncan's new multiple range test. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Pyrosequencing analysis

The comprehensive cDNA sequence results for the polychaete samples is summarized in Table 1. Using the pyrosequencing technique, 40,402 raw reads were obtained, covering a total of 14,747,824 nucleotides with an average read length of 200-300 bp.

The transcriptions of aligned nucleotides were compared with GenBank using BLASTX. The assembled transcripts were used to obtain reliable annotation. A total of 410 sequences (67.3%) matched known genes whereas 199 sequences (32.7%) had no significant homology to known genes. The sequences matched with known genes were characterized as follows: 16 (2.6%) with mitochondrial proteins, 71 (11.7%) with ribosomal proteins, 77 (12.6%) with hypothetical proteins, and 246 (40.4%) with other genes.

The size distribution of the isotigs is shown in Figure 1. The average isotig length was 819 bp; the longest and shortest isotig lengths were 3,972 bp

Table 1. Summary of cDNA sequencing by 454 Pyrosequencing

Number of Reads	40,402
Number of Bases	14,747,824
Number of Isotigs	609
Average Isotig Size	819 bp
N50 Isotig Size	837 bp
Largest Isotig Size	3972 bp
Number of Contigs	712
Number of Singleton	15975

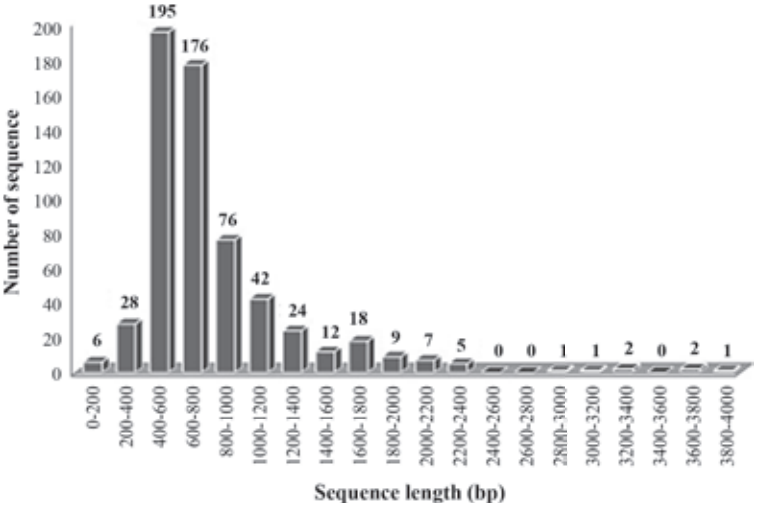
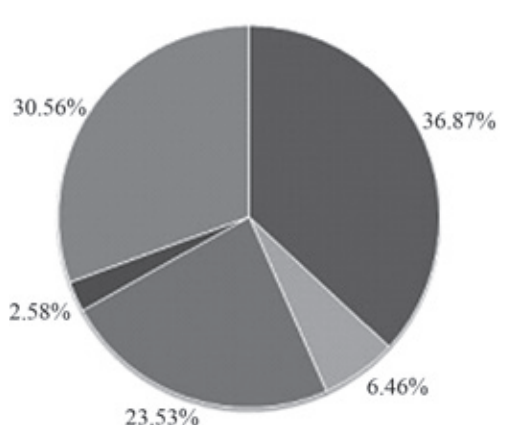


Figure 1. Length distribution of the assembled isotigs

and 80 bp, respectively. The 438 annotated sequences matched known sequences from 157 different species. The species with the highest percentages of sequence hits were *Saccoglossus kowalevskii* (5.71%), *Branchiostoma floridae* (4.79%), *Platynereis dumerilii* (4.11%), and *Nematostella vectensis* (2.74%). The *Perinereis* species with the highest percentages of sequence hits were *Perinereis aibuhitensis* (2.28%) and *Perinereis nuntia* (0.91%).

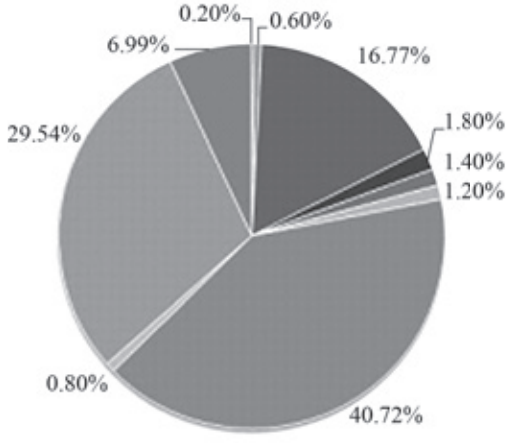
GO analysis and KEGG pathway analysis

Based on the GO analysis results, the sequences were sorted into five cellular component categories, 10 molecular function categories, and 15 biological process categories. The distribution of the annotated sequences in the three GO categories (level 2 terms) is shown in Figure 2. GO analysis for cellular components revealed that the majority of the sequences were encoded for proteins comprising cytoplasmic protein or cellular localization (36.87%), followed by genes encoded for proteins localized in the organelles (30.56%), macromolecular complex (23.53%), membrane-enclosed lumen (6.46%) and extracellular region (2.58%). This distribution pattern was expected, as most of the gene products are located in the cytoplasm and organelles, with the exception of specific individual proteins.



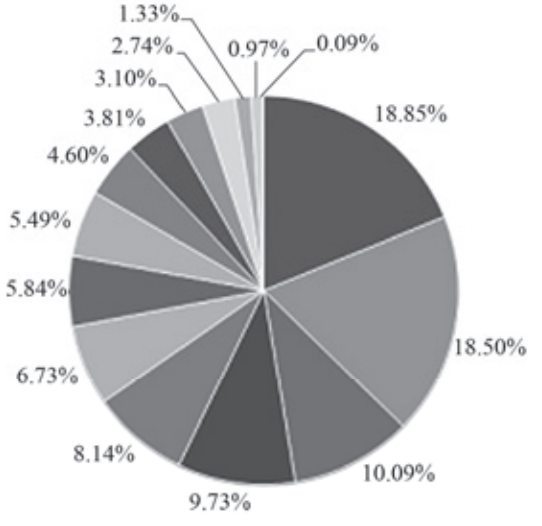
Cellular component

- cell 36.87%
- membrane-enclosed lumen 6.46%
- macromolecular complex 23.53%
- extracellular region 2.58%
- organelle 30.56%



Molecular function

- molecular transducer activity 0.20%
- transcription regulator activity 0.60%
- structural molecule activity 16.77%
- electron carrier activity 1.80%
- antioxidant activity 1.40%
- enzyme regulator activity 1.20%
- binding 40.72%
- translation regulator activity 0.80%
- catalytic activity 29.54%
- transporter activity 6.99%



Biological process

- metabolic process 18.85%
- cellular processes 18.50%
- localization 10.09%
- biological regulation 9.73%
- cellular component organization 8.14%
- respons to stimulus 6.73%
- developmental process 5.84%
- multicellular organismal process 5.49%
- reproduction 4.60%
- viral reproduction 3.81%
- signaling 3.10%
- death 2.74%
- growth 1.33%
- cell proliferation 0.97%
- multi-organism process 0.09%

Figure 2. Distribution of the annotated sequences in the three GO categories (Level 2): cellular component, molecular function, and biological process.

For the genes categorized by molecular function, the majority of the genes were encoded for proteins involved in binding (40.72%) and catalytic activity (29.54%), followed by proteins related to structural molecule activity (16.77%) and transporter activity (6.99%). These results suggest that most of the reads represent proteins that have a role in stable binding interaction and activity of enzymes (70.26%). Another category of interest is genes related to translation regulator activity (0.80%).

For the genes categorized by biological process, the highest percentages represented the metabolic process (18.85%), cellular processes (18.50%), localization (10.09%), biological regulation (9.73%), cellular component organization (8.14%), response to stimulus (6.73%), developmental process (5.84%), multicellular organismal process (5.49%), reproduction (4.60%), and viral reproduction (3.81%). The GO results suggest that a number of different gene families coordinate the diverse functions in the studied polychaetes.

The functional classification and pathway assignments of identified cDNA were determined by KEGG analysis. Among the 438 annotated sequences, 171 sequences were annotated with an enzyme code (EC) number and mapped into 52 KEGG pathways. For all sequences analyzed from mature polychaetes, a single gene was annotated as a reproduction-related gene, namely the vitellogenin structural genes' (yolk protein genes) family member (vit-1)-like gene. It was confirmed that the vitellogenin gene sequence hits the vitellogenin structural genes in *Saccoglossus* with 36% similarity.

In polychaetes, as in many invertebrates, reproduction is controlled by both environmental and endocrinal factors. In the marine polychaete *Nereis virens*, the yolk protein precursor vitellogenin (Vg) is synthesized in specialized coelomic cells called eleocytes during oogenesis (Garcı´a-Alonso *et al*., 2006; Fischer and Dhainaut, 1985; Fischer and Rabien, 1986; Baert and Slomianny, 1987; Hafer *et al*., 1992). During oogenesis, the oocytes of nereidids freely float in the coelomic fluid of female worms. The precursor is taken into the oocytes by receptor-mediated endocytosis and stored as vitellin, a yolk protein that is a nutritive amino acid and lipid resource for the developing embryo (Fischer *et al*., 1991; Fischer *et al*., 1996; Olive *et al*., 1998). During this stage, called the rapid growth phase (RGP), worms undergo a morphological transformation that includes a partial degradation of the longitudinal muscles. Muscles fragments (sarcolytes) are released into the coelomic cavity, where they are phagocytosed by eleocytes. In general, phagocytic eleocytes play a key role in the transfer of metabolites from somatic tissue to germ cells (Fischer and Hoeger, 1993; Lee *et al*., 2005). Environmental factors such as the lunar cycle, photoperiod, and temperature are known to regulate the reproductive process in polychaetes (Hardege *et al*., 1994). A decrease in photoperiod has been shown to accelerate the incorporation of Vg by *N. virens* oocytes (Rees and Olive, 1999).

Expression of vitellogenin gene at different ages of polychaetes

Domesticated polychaetes (*Perinereis* sp.) at 2, 3, 4, 5, 6, 7 and 8 months (cultured from nectochaete) were used to analyze differential expressions of a vitellogenin gene. During the study, the growth rate of polychaetes was measured by taking the average body weight of polychaetes aged 2 to 8 months. The results showed that the average body weight was related to the age of polychaetes which ranged from 0.04 – 0.45g for 2 to 8 months (Table 2). It is similar to the study of Sukcharoen *et al*. (2011) which reported that the growth rate of polychaetes collected from the Gulf of Thailand and the Andaman Sea at ages 2, 3, 4, 5, 6, 7, 8, 9, and 10 months were 0.05±0.04, 0.21 ±0.14, 0.52±0.43, 0.62±0.47, 0.74±0.55, 0.79±0.59, 0.75±0.54, 0.78±0.57 and 0.81±0.56 g, respectively.

In this study, the body weight of polychaetes increased rapidly during months 2 and 3 and became stable around months 5 to 6, which is the optimal age for harvesting. In this study, growth of the polychaetes was initially recorded at month 2 because it was impossible to sample them earlier due to their extremely small size. Sampling prior to two months would have likely resulted in low survival rates as well as low growth in the following months.

In Nereidae, the age at which individuals enter reproduction and proceed to maturity and spawning is dependent on the growth rate (Olive *et al*., 1986).

The present study further indicated that polychaetes at 5, 6, 7 and 8 months (the ages at which they are generally used as feed for shrimp maturation), there was no significant difference in weights based on age (Figure 3). This assumes that polychaetes normally mature at 6 months.

From the pyrosequencing results, the vitellogenin structural genes' (yolk protein genes) family member (vit-1)-like gene was determined to be the reproductive-related gene in polychaetes (*Perinereis* sp.). Its expression from development through maturation was further studied. Specific primers were designed from the cDNA hit to vitellogenin structural genes' (yolk protein genes) family member (vit-1)-like gene in polychaetes using the Primer 3 Plus program.

The gene-specific primers designed for the vitellogenin structural gene were tested using reverse transcription PCR (RT-PCR), and the results showed a product of 504 bp for the vitellogenin gene (VTG) and a product of 148 bp for EF-1α (an internal control) (Figure 4). To detect the expression level of the vitellogenin structural gene, real-time quantitative RT-PCR (qRT-PCR) was used to analyze the RNA extracted from the heads of polychaetes at 2, 3, 4, 5, 6, 7, and 8 months of age.

Table 2. Size distribution of polychaete samples at different ages (n=10)

Age (month)	Size variation of polychaete samples	
	Weight (g)	Length (cm)
2	0.04 ± 0.02^{a}	3.20 ± 0.42^{a}
3	0.14 ± 0.05^{ab}	4.35 ± 0.67^{b}
4	0.20 ± 0.08^{b}	5.45 ± 0.83^{c}
5	0.33 ± 0.13^{c}	6.95 ± 1.61^{d}
6	0.37 ± 0.10^{c}	6.80 ± 0.79^{d}
7	0.38 ± 0.02^{c}	7.60 ± 1.26^{d}
8	0.45 ± 0.28^{c}	7.90 ± 1.78^{d}
P-value	0.000	0.000

The data are presented as mean ± standard deviation. Means in the same column with different superscript are significantly different from each other (P<0.05).

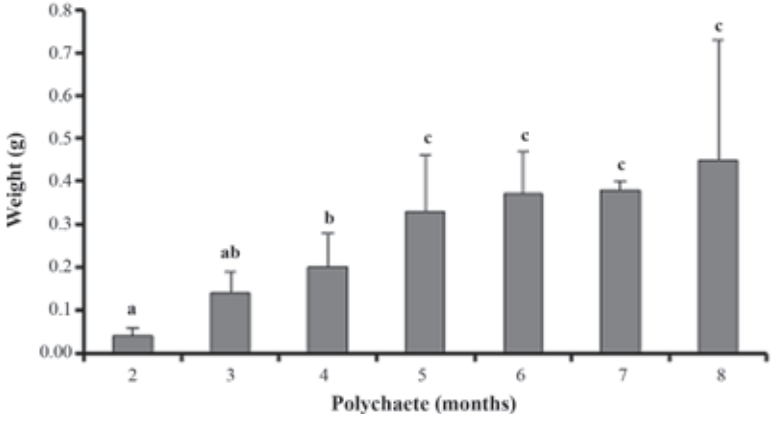


Figure 3. Weight (g) of sampled polychaetes at different ages. The data are presented as means ± SD and the same letters above the histograms reveals non-significant differences between groups of sample (P>0.05).

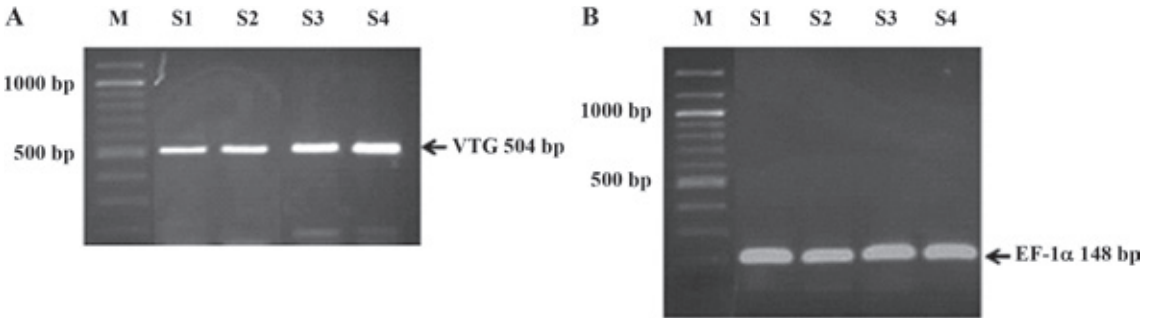


Figure 4. 1.5% agarose gel electrophoresis showing the amplification products of vitellogenin (VTG) (A) and elongation factor 1 (EF-1α) (B) by RT-PCR method: 100 bp DNA ladder (M) and S1-S4 were cDNA of polychaete samples. Amplicon sizes are indicated on the right.

Real-time qRT-PCR analysis

The cDNA of polychaetes (*Perinereis* sp.) at 2, 3, 4, 5, 6, 7, and 8 months of age was subsequently synthesized using gene-specific primers for the vitellogenin gene (VTG). The expression level of vitellogenin mRNA of polychaete at 6 months was significantly higher than in polychaetes of different ages ($P<0.05$), but there was no significant difference in the expression level of this gene at 4 and 5 months of age ($P>0.05$). The expression level of vitellogenin mRNA changed consistently with increasing age and then reduced gradually when maximum body weight was obtained (at 7 to 8 months). In addition, some sexually mature polychaetes (the epitokes) were observed when the ages reached 6-8 months. It might be suggested that the vitelogenin gene related to vitellogenesis which highly developed prior to epitoky. Hence, the expression level of vitellogenin mRNA in polychaetes increased rapidly between 3 and 5 months of age, with a higher level in polychaetes aged about 6 months. The expression level of the vitellogenin gene of polychaetes at different ages is shown in Figure 5.

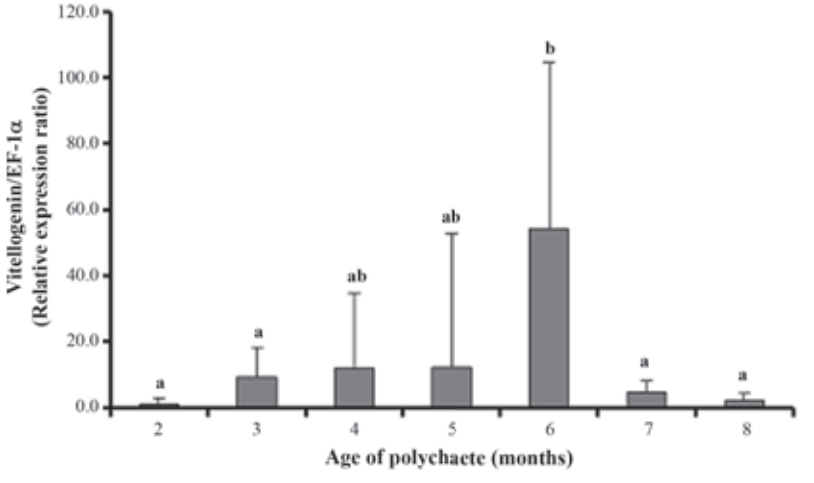


Figure 5. Real-time quantitative PCR of vitellogenin gene transcripts in polychaetes at 2 to 8 months and the data are expressed as mean ± SD calculated from five individual polychaetes. Significant differences in relative expression levels between each group ($p<0.05$) are indicated by different letters.

The vitellogenin gene expression in this study is supported by previous work on hormonal control in Nereidae, a marine polychaete. Specifically, gonadotrophic hormone (a neurohormone) was found to be present in mature females and to promote oocyte growth (Lawrence and Soame, 2009). Furthermore, vitellogenesis, the accumulation of vitellin (yolk protein) within the developing oocyte, also occurs in mature females (Dhainaut, 1984). Further, the ratio of arachidonic acid: eicosapentaenoic acid: docosahexaenoic acid (ARA: EPA: DHA) is similar in farm-raised *P. nuntia* at 4, 6, and 8 months of age (Lawrence and Soame, 2009).

CONCLUSION

Using next-generation sequencing (NGS) analysis, a total of 40,402 raw reads was obtained. The de novo assembly generated 609 sequences were subsequently compared with nucleotide and protein sequences of the NCBI database. 410 from 609 sequences (67.3%) matched known genes. Among 410 genes, vitellogenin related to reproductive gene was found in polychaetes.

Using the real-time quantitative RT-PCR (qRT-PCR), vitellogenin gene expression was observed at its highest in 6 – month old polychaetes ($P<0.05$). This result suggested that the 6 – month old polychaetes will be suitable as the maturation diet for shrimp broodstock. However, the effect on shrimp reproductive development may need to be further analyzed.

ACKNOWLEDGEMENT

This research was partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office (PERDO), Commission on Higher Education, Ministry of Education, Thailand, and the Research Center of Excellence for Shrimp at Walailak University, which also provided the experimental facilities.

LITERATURE CITED

Baert, J. L. and M. C. Slomianny. 1987. Heterosynthetic origin of the major yolk protein vitellin, in a nereid *Perinereis cultrifera*. **Comparative Biochemistry and Physiology** Part B 88: 1191-1199.

Bray, W. A. and A. L. Lawrence. 1992. Reproduction of *Penaeus* Species in Captivity. In: **Marine Shrimp Culture: Principle and Practices,** A.W. Fast and L.J. Lester, editors. (pp. 93-170). Amsterdam, The Netherlands: Elsevier.

Brown, N., S. Eddy and S. Plaud. 2011. Utilization of waste from a marine recirculating fish culture system as a feed source for the polychaete worm, *Nereis virens*. **Aquaculture** 322-323: 177-183.

Chimsung, N. 2014. Maturation diets for black tiger shrimp (*Penaeus monodon*) broodstock: a review. **Songklanakarin Journal of Science and Technology** 36 (3): 265-273.

Conesa, A. and S. Götz. 2008. Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. **International Journal of Plant Genomics:** 1-13.

Darya, M., M. Sajjadi and I. Sourinejad. 2016. Determination of fecundity, weight–fecundity relationship, oocyte diameter and sex ratio of polychaete *Perinereis nuntia* in the coastal waters of Persian Gulf (Bandar Abbas, Iran). **Journal of Aquaculture Development,** 10 (3):0-0. URL: http://aqudev.liau.ac.ir/article-1-408-en.html

D, Croz, L., L. Wong, G. Justines and M. Gupta. 1988. Prostaglandins and related compounds from the Polychaete Worm *Americonuphis reesei* Fauchald (Onuphidae) as possible inducer of gonad maturation in penaeid shrimps. **Revista de Biologia Tropical** 36 (1): 331-332.

Delcher, A. L., K. A. Bratke, E. C. Powers and S. L. Salzberg. 2007. Identifying bacterial genes and endosymbiont DNA with Glimmer. **Bioinformatics** 23 (6): 673-679.

Dhainaut, A. 1984. Oogenesis in polychaetes. Ultrastructural differentiation and metabolism of nereid oocytes. **Fortschr. Zool.** 29: 183-204.

Fischer, A. and A. Dhainaut. 1985. The origin of yolk in the oocytes of *Nereis virens* (Annelida Polychaeta). **Cell and Tissue Research** 240: 67-76.

Fischer, A. and H. Rabien. 1986. Molecular and cellular functions driving oocyte growth in nereid. **Advanced Invertebrate Reproduction** 4: 195-205.

Fischer, A., H. Rabien and A. E. Heacox. 1991. Specific, concentration-dependent uptake of vitellin by the oocytes of *Nereis virens* (Annelida Polychaeta) *in vitro*. **Journal of Experimental Zoology** 260: 106-115.

Fischer, A. and U. Hoeger. 1993. Metabolic links between somatic sexual maturation and oogenesis in nereid annelids–a brief review. **Invertebrate Reproduction and Development** 23: 131-138.

Fischer, A., A. W. C. Dorresteijn and U. Hoeger. 1996. Metabolism of oocyte construction and the generation of histospecificity in the cleaving egg. Lessons from nereid annelids. **The International Journal of Developmental Biology** 40: 421-430.

Garcı´a-Alonso, J., U. Hoeger and N. Rebscher. 2006. Regulation of vitellogenesis in *Nereis virens* (Annelida: Polychaeta): Effect of estradiol-17h on eleocyte **Comparative Biochemistry and Physiology** Part A 143: 55-61.

Hafer, J., A. Fischer and H. J. Ferenz. 1992. Identification of the yolk receptor protein in oocytes of *Nereis virens* (Annelida, Polychaeta) and comparison with the locust vitellogenin receptor. **Journal of Comparative Physiology B** 162: 148-152.

Hardege, J. D., H. D. Bartels-Hardege, Y. Yang, B. L. Wu, M. Y. Zhu and E. Zeeck. 1994. Environmental control of reproduction in *Perinereis nuntia* Var. brevicirrus. **Journal of the Marine Biological Association UK** 74: 903-918.

Harrison, K. 1990. The role of nutrition in maturation, reproduction and embryonic development of decapods crustaceans, a review. **Journal of Shellfish Research** 9: 1-28.

Harrison, K. 1997. Broodstock nutrition and maturation diets. In Crustacean Nutrition, Advances in World Aquaculture, L.R. D'Abramo, D.E. Conklin, and D.M. Akiyama, editors, **World Aquaculture Society,** (pp. 390-408). Louisiana, U.S.A., BatonRouge.

Koskela, R. W., J. G. Greenwood and P. C. Rothlisberg. 1992. The influence of prostaglandins E2 and the steroid hormones, 17α–hydroxyprogesteron development the tiger prawn, *Penaeus esculentus*. **Comparative Biochemistry and Physiology** 101(A): 295-299.

Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. **Methods** 25: 402-408.

Laufer, H., D. Borst, F. C. Baker, C. Carrasco, M. Sinkus, C. C. Reuter, … D. S. Schooley. 1987. Identification-of a juvenile hormone-like compound in a crustacean. **Science** 235 (4785): 202-205.

Lawrence, A. J. and J. M. Soame. 2009. The endocrine control of reproduction in Nereidae: a new multi-hormonal model with implications for their functional role in a changing environment. **Philosophical Transactions of the Royal Society B** 364: 3363-3376.

Lee, R., A. Walker and D. Reish. 2005. Characterization of lipovitellin in eggs of the polychaete *Neanthes arenaceodentata*. **Comparative Biochemistry and Physiology Part B** 140: 381-386.

Meunpol, O., P. Meejing and S. Piyatiratitivorakul. 2005. Maturation diet based on fatty acid content for male *Penaeus monodon* (Fabricius) broodstock. **Aquaculture Research** 36: 1216 -1225.

Meunpol, O., S. Iam-Pai, W. Suthikrai and S. Piyatiratitivorakul. 2007. Identification of progesterone and 17α-hydroxyprogesterone in polychaetes (*Perinereis* sp.) and the effects of hormone extracts on penaeid oocyte development *in vitro*. **Aquaculture** 270: 485-492.

Meunpol, O., E. Dangjai, R. Yoonpum and S. Piyatiratitivorakul. 2010. Detection of prostaglandin E2 in polychaete *Perinereis* sp. And its effect on *Penaeus monodon* oocyte development *in vitro*. **Fisheries Science** 76: 281-286.

Olive, P. J. W., A. Grant and P. D. Cowin. 1986. Decision and choice in Polychaete reproductive cycles. In Porchet M., Andries J.-C., Dhainaut A. (Eds), **Advances in invertebrate reproduction** (4th ed., pp. 371-378). Amsterdam, The Netherlands: Elsevier.

Olive, P. J. W., S. W. Rees and A. Djunaedi. 1998. Influence of photoperiod and temperature on oocyte growth in the semelparous polychaete *Nereis* (*Neanthes*) *virens*. **Marine Ecology Progress Series** 172: 169-183.

Palmer, P. J. 2011. Commercial application of *polychaete* sand filters for wastewater remediation and broodstock feeds. In **Technical Report** (p1-34). Queensland, Australia: Bribie Island Research Centre.

Pinon, E. 2000. Producing ragworms for shrimp broodstock maturation. **Global Aquaculture Advocate** 3: 82-84.

Poltana, P., T. Lerkitkul, P. Pongtippatee-Taweepreda, S. Asuvapongpattana, K. Wongprasert, S. Sriurairatana, J. Chavadej, P. Sobhon, P.J.W. Olive and B. Withyachumnarnkul. 2007. Culture and development of polychaete *Perinereis* cf. *nuntia*. **Invertebrate Reproduction and Development** 50: 13-20.

Rees, S. W. and P. J. W. Olive. 1999. Photoperiodic changes influence the incorporation of vitellin yolk protein by oocytes of the semelparous polychaete *Nereis* (*Neanthes*) *virens*. **Comparative Biochemistry and Physiology Part A** 123: 213-220.

Sukcharoen, K., J. Chiayvareesajja and P. Poltana. 2011. Growth rate of polychaete (*Perinereis nuntia*). **Valaya Alongkorn Review** 1 (2): 93-101.

Wouters, R., P. Lavens, J. Nieto and P. Sorgeloos. 2001. Penaeid shrimp broodstock nutrition: an updated review on research and development. **Aquaculture** 202: 1-21