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Structural Similarity and Expression Differences of Two *Pj-Vg* Genes from the Pandalus Shrimp *Pandalopsis japonica*

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Vitellogenin (*Vg*) is the precursor of vitellin (*Vn*), which is the major yolk protein in nearly all oviparous species, including fish, amphibians, reptiles, and most invertebrates. It is one of the most important factors during reproduction, and numerous studies have shown that *Vg* genes are markers of the reproductive cycle and effecter genes induced by endocrine-disrupting chemicals (EDCs). Previously, we isolated two distinct cDNAs encoding vitellogenin homologs *Pj-Vg1* and *Pj-Vg2* from Pandalus shrimp *Pandalopsis japonica*. In this study, full-length genomic sequences of *Pj-Vg1* and *Pj-Vg2* were determined using a PCR-based genome walking strategy. Isolated *Pj-Vg1* and *Pj-Vg2* genes were 11,910 and 11,850 bp long, respectively. Both *Pj-Vg* genes had 15 exons and 14 introns, and the splicing sites were also the same, suggesting that they arose via gene duplication. The similar structural characteristics of decapod *Vg* genes suggest that they are all orthologs that evolved from the same ancestral gene. Analysis of *Pj-Vg1* and *Pj-Vg2* expression revealed that the relative copy numbers of *Pj-Vg1* and *Pj-Vg2* were similar in the hepatopancreas, whereas *Pj-Vg2* transcripts were also detected in the ovary. Expression of both *Pj-Vg* genes was induced in hepatopancreas of mature individuals, whereas only *Pj-Vg2* transcripts were upregulated in the ovaries from mature animals, suggesting that both *Pj-Vgs* are important for oocyte development. A strong positive correlation was found between *Pj-Vg1* and *Pj-Vg2* transcripts in the same individual, indicating they are under the same control mechanisms. Additionally, a positive correlation was found between ovarian and hepatopancreatic *Pj-Vg2* transcripts, suggesting that its dual expression is regulated by similar physiological conditions. Knowledge of the similarities and differences between the two vitellogenin-like genes, *Pj-Vg1* and *Pj-Vg2*, would help us to understand their roles in reproduction and other physiological effects.

Key words: Arthropod, Crustacean, *Pandalopsis japonica*, Reproduction, Vitellogenin, Genomic structure

Introduction

During vitellogenesis in oviparous animals, nutrient-rich egg yolk composed mostly of lipids and proteins accumulates. Vitellin (*Vn*) is the major protein component in the yolk, and it is synthesized as a precursor protein, vitellogenin (*Vg*) (Gerber-Huber et al., 1987; Chen et al., 1997). *Vg* is one of the most important proteins during oocyte maturation, and numerous studies have examined its characteristics and roles during the reproductive cycle. Furthermore, the *Vg* genes have been studied as representative markers induced by endocrine-disrupting chemicals (EDCs).

Due to its possible industrial applications, such as in seed production, the *Vgs* from commercially important decapod crustaceans have been studied. To our knowledge, 19 crustacean *Vg* cDNAs have been isolated and characterized. Most are from the commercially important penaeid shrimps in the suborder of Dendrobranchiata, including *Marsupenaeus japonicus* (Tsutsui et al., 2000), *Penaeus monodon* (Tiu et al., 2006), *Litopenaeus vannamei* (Raviv et al., 2006), *Penaeus semisulcatus* (Avarre et al., 2003), *Fenneropenaeus chinensis* (Xie et al., 2009), *Fenneropenaeus merguiensis* (Phiriyangkul and Utarabhand, 2006), and *Pandalopsis japonica* (Jeon et al., 2010). Others are from brachyuran and

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astacidean species in the suborder Pleocyemata, including *Macrobrachium rosenbergii* (Okuno et al., 2002), *Pandalus hypsinotus* (Tsutsui et al., 2004), *Portunus trituberculatus* (Yang et al., 2005), *Cherax quadricarinatus* (Abdu et al., 2002), *Homarus americanus* (Tiu et al., 2009), *Upogebia major* (Kang et al., 2008), *Charybdis feriatus* (Mak et al., 2005), *Portunus trituberculatus* (Yang et al., 2005), and *Callinectes sapidus* (Zmora et al., 2007).

Compared with the *Vg* cDNA sequences, only a few genomic analysis of *Vg* genes from decapod crustaceans have been conducted, mainly due to the large gene size (>10 kb), which makes it difficult to isolate full-length *Vg* genes. However, recent advances in molecular techniques and the development of genomic analysis tools enabled us to obtain *Vg* cDNA sequences with less effort. The full-length crustacean *Vg* genes have been determined only in three species: *Metapenaeus ensis* (Tsang et al., 2003; Kung et al., 2004), *P. monodon* (Tiu et al., 2006), and *H. americanus* (Tiu et al., 2009).

The accumulated information on *Vg* in decapod crustaceans raised two major questions. First, how many copies of *Vg* genes do these animals possess? Until now, the full length of two distinct *Vg* genes has been determined only in *M. ensis* (Tsang et al., 2003; Kung et al., 2004). However, several studies support the idea that decapods possess multiple *Vg* genes (Mak et al., 2005; Tiu et al., 2009). Recently, we first isolated two distinct copies of *Vg* genes from a species belonging to the suborder Pleocyemata: the Pandalus shrimp, *P. japonica* (Jeon et al., 2010). It is necessary to analyze all known crustacean *Vgs* to determine structural and biological differences. Second, where is the major site of *Vg* production? Despite contradictory results, it is generally accepted that the major production site for *Vg* is the hepatopancreas, and the synthesized *Vg* is understood to be processed into *Vn*, circulated, and transported into the oocyte via receptor-mediated endocytosis. However, several studies have shown that the ovary is another source of *Vg* production, and the similarities and differences in the hepatopancreatic and ovarian expression of *Vg* need to be investigated.

Pandalopsis japonica is a commercially important species in the coastal regions of eastern Asia, including Korea and Japan. Recently, its production has been declining due to a combination of factors, including climate change, overfishing, and pollution. To restore its population, it is important to develop a seed-production technique. However, little is known about the endocrinology and molecular mechanisms of crustacean reproduction. Previously, we isolated two distinct *Vg* cDNAs, *Pj-Vg1* and *Pj-Vg2*, from *P. japonica* and found that their expression was downregulated by the major component of insecticides, endosulfan (Jeon et al., 2010). In this study, we isolated and analyzed the two full-length *Pj-Vg1* and *Pj-Vg2* genes. To estimate their roles in crustacean reproduction, we also analyzed the relationship of the expression of the two *Pj-Vgs* to the reproductive stage and production sites.

Materials and Methods

Experimental animals

Live female *P. japonica* were purchased from a local seafood market. The live shrimp were dissected quickly after sacrifice. Isolated tissues were deep-frozen by immersing them in liquid nitrogen. The frozen tissues were stored at -70°C until use. The individual gonad-somatic index (GSI=gonad weight/body weight) was also determined for further study.

Identification of the two *Pj-Vg* gene structures

The structures of the two *Pj-Vg* genes were determined using a PCR-based strategy. First, genomic DNA was purified from muscle tissue using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea). Then, 200 ng of genomic DNA were used for each PCR reaction. Given the expected large size of each *Pj-Vg* gene, several primers were designed to amplify products smaller than 2 kb to avoid landing on an exon-intron boundary (Table 1). Some PCR products that were too large to be cloned into T-vector were digested with *Sau*3AI, mixed with *Taq* polymerase and dNTPs, and incubated at 72°C for 10 min. The smaller fragments were ligated into pGEM T-easy vector (Promega, USA). The full lengths of the *Pj-Vg1* and two genomic sequences were determined by combining the fragment sequences. The resulting contig sequences were then confirmed by PCR using two primer pairs (PJ-gVG1F1 and PJ-gVG1R16; PJ-gVG2F1 and PJ-gVG2R17), which land on both the 5' and 3' ends (Tables 1 and 2). Exons and introns were determined by comparing the cDNA sequences with the obtained genomic DNA sequences.

Expression analysis of *Pj-Vgs*

To understand the expression of the two *Pj-Vgs*, quantitative PCR analysis was carried out. Fourteen females with various GSI indices were sacrificed to obtain the ovary and hepatopancreas. Total RNA was purified using TRIzol reagent according to the

Table 1. Primer list for *Pj-Vg1* genomic DNA sequence

Name	Sequence (5'-3')	Description (base pair numbers from the start codon)
PJ-gVG1F1	ATGACCTCCTCCACGGCACTCTTCGTGTTG	Forward primer from 1bp to 30 bp
PJ-gVG1R1	AAGAAGCTTGGCTTCAGGAGAACCTGC	Reverse primer from 321 to295 bp
PJ-gVG1F2	GCAGGTTCTCCTGAAGCCAAGC	Forward primer from 295 to 316 bp
PJ-gVG1R2	GCAGCAGTTTCTTCGACCGCC	Reverse primer from 965 to 945 bp
PJ-gVG1F3	CCAAACAGGCGGTCGAAGAAACTGCTGC	Forward primer from 938 to 961 bp
PJ-gVG1R3	CCTCGAACTTGTGTGTTTTGTTCCACAG	Reverse primer from 1,748 to 1,721 bp
PJ-gVG1F4	GTGCACACCATCAGAAGCTGAGG	Forward primer from 1,391 to 1,413 bp
PJ-gVG1R4	GGAATCCAAATTAACATTGGCTGCTC	Reverse primer from 2,421 to 2,396 bp
PJ-gVG1F5	GGTGTTGGAGCTGGTATGGAATCCAATG	Forward primer from 1,909 to 1,936 bp
PJ-gVG1R5	CCTGTTGTCCCTTTAGTGCCTTCACGAG	Reverse primer from 2,791 to 2,764 bp
PJ-gVG1F6	GATTCACATATTGCTCGAGTGGGAG	Forward primer from 2,605 to 2,629 bp
PJ-gVG1R6	CATGATCTTGGTCATATCTTGGTCACG	Reverse primer from 3,133 to 3,106 bp
PJ-gVG1F7	CATGAGGGCATTAGAGCCAATGCTTGG	Forward primer from 2,847 to 2,873 bp
PJ-gVG1R7	GAGTGTTAGGTCTCGGTATGAGCTTCC	Reverse primer from 3,600 to 3,574 bp
PJ-gVG1F8	GTTTGTGCGACAGGCAGGTGG	Forward primer from 3,474 to 3,494 bp
PJ-gVG1R8	CCCATGCAAACTCAACTTGTGCCTTC	Reverse primer from 4,297 to 4,274 bp
PJ-gVG1F9	GAGCGTCAATATGCTTGTGATGCC	Forward primer from 4,191 to 4,214 bp
PJ-gVG1R9	CAAGTCATATCAGCTGTGGTGGTCAG	Reverse primer from 4,682 to 4,657 bp
PJ-gVG1F10	CTGTGGAGTGGCTTGGAGGC	Forward primer from 4,622 to 4,641 bp
PJ-gVG1R10	GGAGAGTACTTAACTGCACCTTCCATTGTTCG	Reverse primer from 5,092 to 5,061 bp
PJ-gVG1F11	GCGAAGCTACCTACCTCTTCCAGTACC	Forward primer from 5,018 to 5,044 bp
PJ-gVG1R11	CTGCCACTTCCGTGTTTCTTTGTG	Reverse primer from 5,774 to 5,751 bp
PJ-gVG1F12	GCCAAGACTCACTCATCTGTGATTGGC	Forward primer from 5,674 to 5,700 bp
PJ-gVG1R12	GTTCTGAAAATGGCAGTCTCCCTCATCTG	Reverse primer from 6,416 to 6,388 bp
PJ-gVG1F13	AGCCAGATGAGGGAGACTGCCATTT	Forward primer from 6,385 to 6,409 bp
PJ-gVG1R13	ATTGCACTTGCACGTCATTGCCTTCC	Reverse primer from 6,700 to 6,675 bp
PJ-gVG1F14	ACGACACAACCTCATGCAACACAGCA	Forward primer from 6,528 to 6,553 bp
PJ-gVG1R14	GGCAGTGCGATTGTAAGGTGGAACCAAA	Reverse primer from 6,897 to 6,870 bp
PJ-gVG1F15	TTCGAGGCTCTTATGCCTACTCCAGT	Forward primer from 6,818 to 6,843 bp
PJ-gVG1R15	CTTTCAGGACCTCTGATTGGTTGTCCA	Reverse primer from 7,127 to 7,101 bp
PJ-gVG1F16	GCTTCAGGAGTTACAGTAGAAGTCAAACC	Forward primer from 7,051 to 7,079 bp
PJ-gVG1R16	TCAGCATCCTAATGGGAAGACCTCCTTCACTC	Reverse primer from 7,602 to 7,571 bp
PJVG1RT-F	ACGACACAACCTCATGCAACACAGCA	FWD primer for *Pj-Vg1* expression
PJVG1RT-R	GGCAGTGCGATTGTAAGGTGGAACCAAA	RVS primer for *Pj-Vg1* expression
18SrRNA-F	AAGTCTGGTGCCAGCASCCGCGGT	FWD primer for 18SrDNA expression
18SrRNA-R	AAGGGCATYACAGACCTGTTATTG	RVS primer for 18SrDNA expression

manufacturer's instructions (Invitrogen, USA), quantified using the absorbance at 260 nm (NanoDrop Technologies, USA), and stored at -70°C. Before cDNA synthesis, the total RNA was pretreated with DNase I (Takara, Japan) for 20 min at 37°C to remove any genomic DNA contamination. Then, the cDNA was synthesized in a reaction (12 μL) containing 1 μg of total RNA, 1 μL of 20 μM random hexamer, and 4 μL of dNTPs (2.5 mM each). The mixture was heated to 70°C for 5 min and then chilled on ice for 2 min. First-strand buffer (5×, 4 μL), 2 μL DTT (0.1 M), and 1 μL RNaseOUT (Invitrogen, USA) were added, and the reactant was incubated at 37°C for 2 min. Finally, 1 μL MMLV reverse transcriptase (Invitrogen, USA) was added, and the mixture was incubated at 37°C for 50 min. The synthesized cDNA was quantified and stored at -20°C before being used for PCR.

Quantitative PCR was carried out using the DNA Engine Opticon 3 Real-Time PCR Detection System (Bio-Rad, USA) and SYBR Green premix Ex *Taq* II (Takara Bio, Japan) to measure the mRNA transcripts. Sequence-specific primers for each target gene were designed using PrimerQuest (http://www.idtdna.com). The reaction mixture (20 μL) contained 1 μL cDNA (100 ng), 2 μL 4 μM sequence-specific primers, which are shown in Tables 1 and 2 (PJVG1 RT-F and PJVG1 RT-R for *Pj-Vg1*, PJVG2 RT-F and PJVG2 RT-R for *Pj-Vg2*), and autoclaved deionized water to a volume of 20 μL. The PCR conditions were 1 min at 94°C followed by 40 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s. Standard curves were constructed to confirm the efficiency of the primers and to quantify copy numbers, as described previously (Kim et al., 2005). The *Pj-Vg* copy numbers were normalized to the 18S rRNA copy number using to the following equation: (actual copy numbers of *Pj-Vg1* or *Pj-Vg2* / actual copy number of

Table 2. Primer list for *Pj-Vg2* genomic DNA sequence

Name	Sequence (5'-3')	Description(base pair numbers from the start codon)
PJ-gVG2F1	ATGACCTCCTCAACTGCACTCCTTGTGC	Forward primer from 1 to 28 bp
PJ-gVG2R1	CTGATGCAATACCCTTTTTAAGGTTGATG	Reverse primer from 430 to 402 bp
PJ-gVG2F2	GCTACATGCCTGCAGCCACC	Forward primer from 363 to 382 bp
PJ-gVG2R2	CTGTAGAGTTCGCGGTATCTCCTCCTCG	Reverse primer from 1035 to 1,008 bp
PJ-gVG2F3	CTATGGTGTCCAAGCTAGATGAAGTAC	Forward primer from 890 to 916 bp
PJ-gVG2R3	CGTTTCCAGGTGGAGAGCACTG	Reverse primer from 1,410 to 1,389b p
PJ-gVG2F4	GCATCCATGGTAAACACTTACTGCAGG	Forward primer from 1,294 to 1,320 bp
PJ-gVG2R4	GGTGCACTAGACTCTTGGATGTTCAG	Reverse primer from 1,793 to 1,768 bp
PJ-gVG2F5	CAATGAAATGCGCTGATCGCG	Forward primer from 1,670 to 1,690 bp
PJ-gVG2R5	CGCCATACAGTTTGTCGAAGAGGC	Reverse primer from 2,222 to 2,199 bp
PJ-gVG2F6	CCCGGATCATTCCTTCCTCGTTCAG	Forward primer from 1,948 to 1,972 bp
PJ-gVG2R6	CCAAGCATTGGCTCTAATGCCATCATAC	Reverse primer from 2,885 to 2,858 bp
PJ-gVG2F7	GAGCGAAACTTATCTTGTGAAGGAAGTAG	Forward primer from 2,762 to 2,790 bp
PJ-gVG2R7	CTCTTGATTGTGGCTAAGGTTCGG	Reverse primer from 3,359 to 3,336 bp
PJ-gVG2F8	GAAATCAAGGTTGAAACAGAAGCCCAAGG	Forward primer from 3,046 to 3,074 bp
PJ-gVG2R8	CCAAGAAGTTCATTTTGTAGGTCTCCCCAG	Reverse primer from 3,790 to 3,761 bp
PJ-gVG2F9	CCAATGGATGCAGAGTGGGCTG	Forward primer from 3,538 to 3,559 bp
PJ-gVG2R9	CTACTGCCATCTTGCCTTGGGCAG	Reverse primer from 4,042 to 4,019 bp
PJ-gVG2F10	GCGTAGTCAACCATTCACTGTTTCAACATC	Forward primer from 3,981 to 4,010 bp
PJ-gVG2R10	CCTTTTCGACATTCAATGTAGTCTCAAGCC	Reverse primer from 4,555 to 4,526 bp
PJ-gVG2F11	CTTCCACCCCTCAACAGACTATTGTTCA	Forward primer from 4,367 to 4,394 bp
PJ-gVG2R11	CTCCCTCCATTTTACGAGATGGAGAGTG	Reverse primer from 5,122 to 5,095 bp
PJ-gVG2F12	CTTGAAACAAAACACGAGAAGACTCCCC	Forward primer from 4,726 to 4,753 bp
PJ-gVG2R12	GCAATCATGGTTGACTCCAAAGTTCCAG	Reverse primer from 5,405 to 5,378 bp
PJ-gVG2F13	GAGGGAGAGGAAGATGGAAGTGATG	Forward primer from 5,280 to 5,304 bp
PJ-gVG2R13	GCAGGTGCGCAGAGCTCAGAG	Reverse primer from 5,756 to 5,736 bp
PJ-gVG2F14	GCAATGGACTGAAGATATCTGCTGTAGC	Forward primer from 5,675 to 5,702 bp
PJ-gVG2R14	CGTCCCATTTCTTCAGGCATTTCTCC	Reverse primer from 6,389 to 6,364 bp
PJ-gVG2F15	GGAGAAATGCCTGAAGAAATGGGACG	Forward primer from 6,364 to 6,389 bp
PJ-gVG2R15	ACGAAGAGTCACGGCTTCATACAATCGC	Reverse primer from 6,825 to 6,798 bp
PJ-gVG2F16	GGTTGTCCAGATGTCTCTCCTCTCTTCTGTCC	Forward primer from 6,650 to 6,681 bp
PJ-gVG2R16	GACAGGACTTTGATCTCAGCTGAAG	Reverse primer from 7,193 to 7,169 bp
PJ-gVG2F17	CATCATCCTCTCCACAGGTCACCC	Forward primer from 7,034 to 7,057 bp
PJ-gVG2R17	TTAACATGTGAGAGGAAACACATCCTTTACACC	Reverse primer from 7,614 to 7,582 bp
PJVG2RT-F	GACATCGAGACAATTCGCGAAGACCTCA	FWD primer for *Pj-Vg2* expression
PJVG2RT-R	ACGAAGAGTCACGGCTTCATACAATCGC	RVS primer for *Pj-Vg2* expression

18S rRNA)×10,000.

Data analysis and statistics

Multiple amino-acid sequence analysis was performed using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and presented using GeneDoc software (http://www.nrbsc.org/gfx/genedoc/index.html). The *Pj-Vg* expression was analyzed statistically by the independent *t*-test using the Statistical Package for the Social Sciences (SPSS) (version 10.1.3). The results were considered significant at $P<0.05$.

Results and Discussion

Analysis of *Pj-Vgs* gene structure

The full-length *Pj-Vg1* and *Pj-Vg2* genes were obtained using a PCR-based strategy. The full-sized genomic DNA sequences assembled from the PCR products were confirmed by determining the nucleotide sequence of the large PCR products (-11 kb) generated using sequence-specific 5'- and 3'-end primers (Tables 1 and 2). The *Pj-Vg1* and -*Vg2* genes were 11,910 and 11,850 bp long, respectively, which is similar to the *Vg* genes from other decapod crustaceans (Fig. 1). To our knowledge, only three genomic structures of decapod *Vg* genes are known: *P. monodon* (Tiu et al., 2006), *M. ensis* (Tsang et al., 2003; Kung et al., 2004), and *H. americanus* (Tiu et al., 2009). Of these, the structures of two copies of the *Vg* gene were determined only in *M. ensis*, and only single gene structures were determined in the other two species. Our study is the first report on the genomic structure of two copies of *Vg* genes in the suborder Pleocyemata.

Structural characteristics of the *Pj-Vg* genes were analyzed, revealing that all of the exon-intron boundaries of the *Pj-Vgs* followed the general

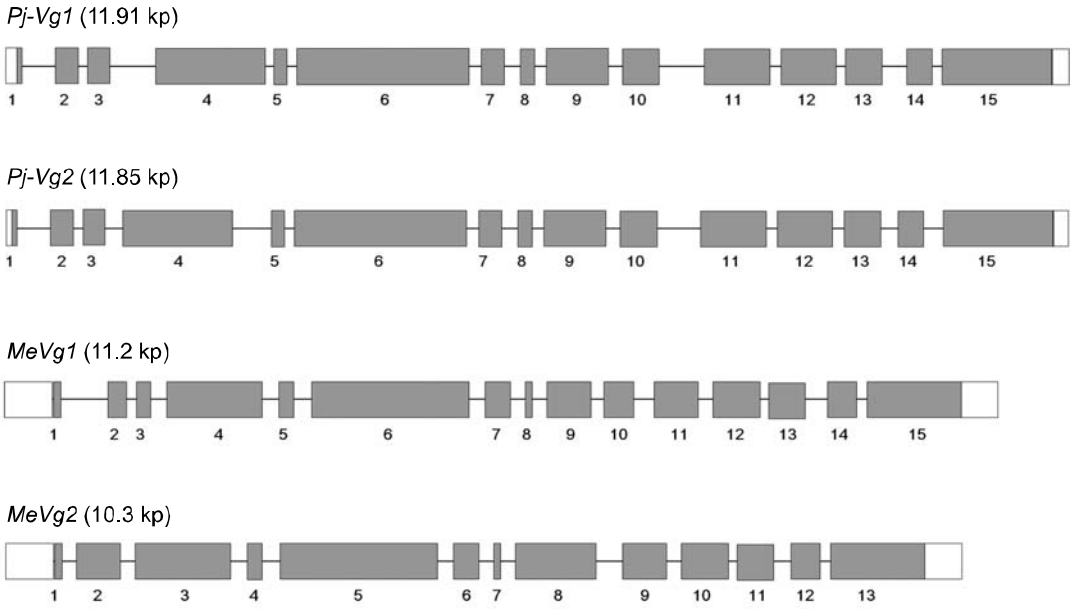

Fig. 1. Comparison of genomic organization between *Pj-Vgs* and *MeVgs*.
The gray boxes represent the exons, and numbers underneath the gray boxes indicate corresponding exon number. Untranslated regions are shown as white boxes. The exons are linked by introns (solid lines). *Pj-Vg1* and *Pj-Vg2* are isolated from *Pandalopsis japonica* and *MeVg1* and *MeVg2* are vitellogenin genes from *Metapenaeus ensis*.

Table 3. Organization of *Pj-Vg1* and *Pj-Vg2* gene

Pj-Vg1	Position (length)	*Pj-Vg2*	Position (length)
Exon 1	1 - 157 (157 bp)	Exon 1	1 - 118 (118 bp)
Intron 1	158 - 835 (678 bp)	Intron 1	119 - 805 (687 bp)
Exon 2	836 - 1,078 (243 bp)	Exon 2	806 - 1,048 (243 bp)
Intron 2	1,079 - 1,197 (119 bp)	Intron 2	1,049 - 1,170 (122 bp)
Exon 3	1,198 - 1,388 (191 bp)	Exon 3	1,171 - 1,361 (191 bp)
Intron 3	1,389 - 2,115 (727 bp)	Intron 3	1,362 - 1,616 (255 bp)
Exon 4	2,116 - 3,210 (1095 bp)	Exon 4	1,617 - 2,711 (1095 bp)
Intron 4	3,211 - 3,347 (137 bp)	Intron 4	2,712 - 3,480 (769 bp)
Exon5	3,348 - 3,504 (157 bp)	Exon5	3,481 - 3,637 (157 bp)
Intron 5	3,505 - 3,633 (129 bp)	Intron 5	3,638 - 3,760 (123 bp)
Exon 6	3,633 - 5,262 (1629 bp)	Exon 6	3,761 - 5,401 (1641 bp)
Intron 6	5,263 - 5,414 (152 bp)	Intron 6	5,402 - 5,558 (157 bp)
Exon 7	5,415 - 5,660 (246 bp)	Exon 7	5,559 - 5,804 (246 bp)
Intron 7	5,661 - 6,067 (407 bp)	Intron 7	5,805 - 6,198 (394 bp)
Exon 8	6,068 - 6,231 (164 bp)	Exon 8	6,199 - 6,359 (161 bp)
Intron 8	6,232 - 6,346 (115 bp)	Intron 8	6,360 - 6,469 (110 bp)
Exon 9	6,347 - 6,945 (599 bp)	Exon 9	6,470 - 7,074 (605 bp)
Intron 9	6,946 - 7,142 (197 bp)	Intron 9	7,075 - 7,225 (151 bp)
Exon 10	7,143 - 7,524 (382 bp)	Exon 10	7,226 - 7,607 (382 bp)
Intron 10	7,525 - 8,136 (612 bp)	Intron 10	7,608 - 8,156 (549 bp)
Exon 11	8,137 - 8,799 (663 bp)	Exon 11	8,157 - 8,825 (669 bp)
Intron 11	8,800 - 8,931 (132 bp)	Intron 11	8,826 - 8,944 (119 bp)
Exon 12	8,932 - 9,459 (528 bp)	Exon 12	8,945 - 9,472 (528 bp)
Intron 12	9,460 - 9,587 (128 bp)	Intron 12	9,473 - 9,653 (181 bp)
Exon 13	9,588 - 9,954 (367 bp)	Exon 13	9,654 - 10,011 (358 bp)
Intron 13	9,955 - 10,292 (338 bp)	Intron 13	10,011 - 10,172 (161 bp)
Exon 14	10,293 - 10,570 (278 bp)	Exon 14	10,173 - 10,450 (278 bp)
Intron 14	10,571 - 10,725 (155 bp)	Intron 14	10,451 - 10,730 (280 bp)
Exon15	10,726 - 11,910 (1185 bp)	Exon15	10,731 - 11,850 (1120 bp)

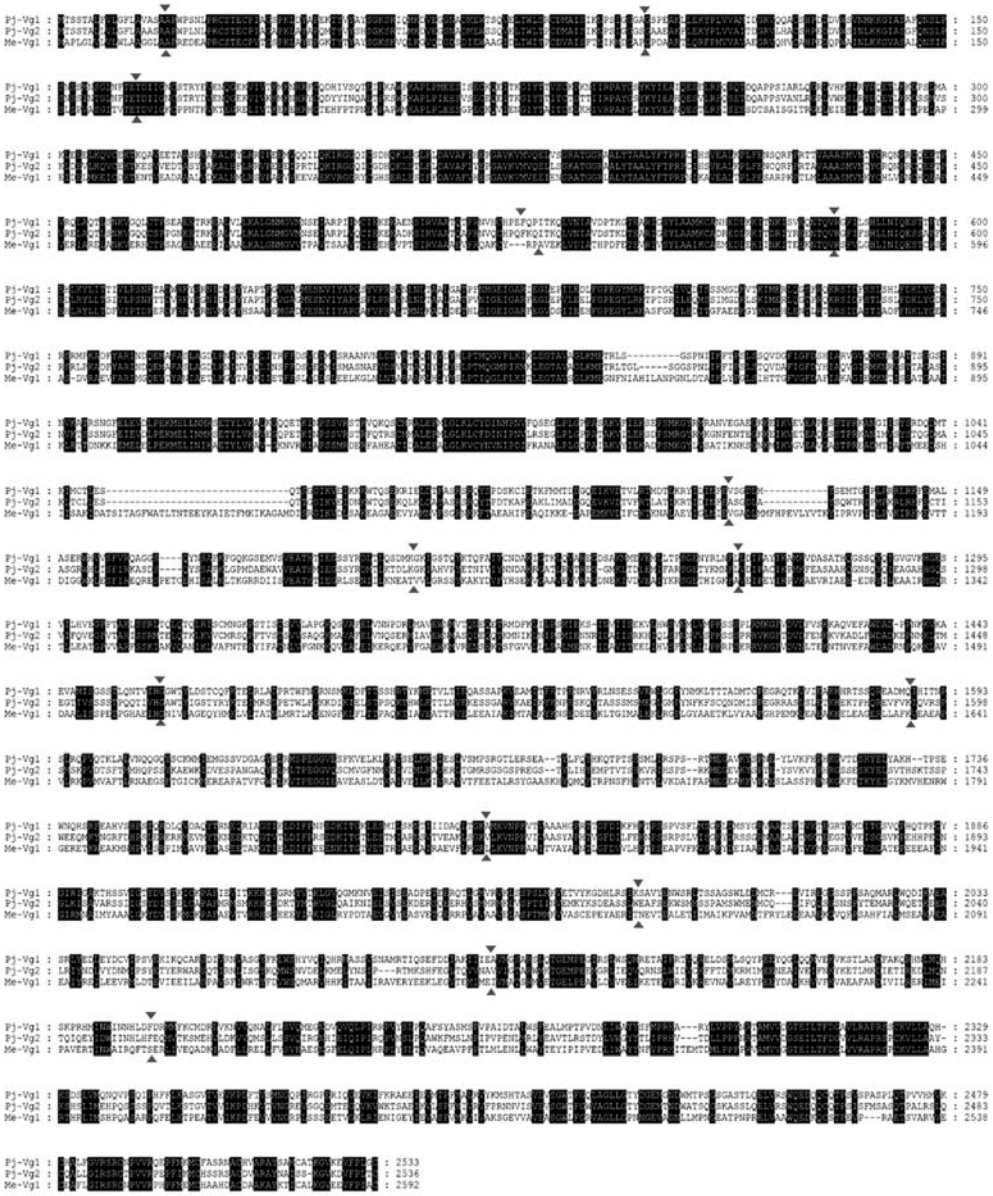

Fig. 2. Multiple alignment of deduced amino acid sequences of *Pj-Vgs* and *MeVg1*.
Amino acid residues with similar properties are shaded. Gaps were shown as broken lines. Arrows indicate the splicing sites.

spliceosome sequence rule (GT-AG, Shapiro and Senapathy, 1987). The overall sizes of the exons and introns were also analyzed (Table 3). The sizes of each intron in the *Pj-Vgs* were similar, except for the third and fourth introns (Fig. 1). The average size of the introns in *Pj-Vg1* and *Pj-Vg2* was 287 and 289 bp, respectively. These relatively small intron sizes appear to be common characteristics of decapod crustacean *Vg* genes, in which the average intron size ranges from 100 to 300 bp (Kung et al., 2004; Tiu. et al., 2006). Both *Pj-Vg1* and *Pj-Vg2* have 15 exons and 14 introns (Fig. 1). All reported *Vg* genes from the three other decapod crustaceans have the same numbers of exons and introns as the *Pj-Vg* genes, except *MeVg2*, which has 13 exons as a result of the fusion of exons 2 and 3 and of exons 9 and 10 (Kung et al., 2004). The exon–intron boundaries of *Pj-Vg1*, *Pj-Vg2*, and *MeVg1* were also compared (Fig. 2). Interestingly, all of the splicing sites were conserved in all three *Vg* genes. This suggests that the three *Vg*

genes are orthologs and likely have evolved from the same ancestral gene. *MeVg2* and the other genomic sequences were not used in the analysis because they are not yet publicly available.

In this study, we isolated and characterized two distinct *Vg* genes for the first time from a species in the suborder Pleocyemata: *P. japonica*. Multiple copies of *Vg* genes have also been identified in *M. ensis* in the suborder Dendrobranchiata. Although the full-length gene has not been isolated, data suggest that multiple copies of *Vg* genes are not limited to those species. In the infraorder Astacidea, several fragment *Vg* sequences subcloned from *H. americanus* differed slightly, supporting the existence of multiple *Vg* genes (Tiu et al., 2009). In brachyuran species, several sequences had distinct sequences, suggesting that there are two copies of the *Vg* genes (Mak et al., 2005). Two *Vg* cDNA sequences obtained from the penaeid shrimp *M. japonicus* differed at 47 nucleotide positions. The author proposed that this arose from individual variation and provided no experimental evidence of two distinct genes (Tsutsui et al., 2005). However, our finding of two copies of *Vg* genes in the suborder Pleocyemata leads us to postulate that the presence of two copies of the *Vg* gene is a general characteristic of decapod crustaceans.

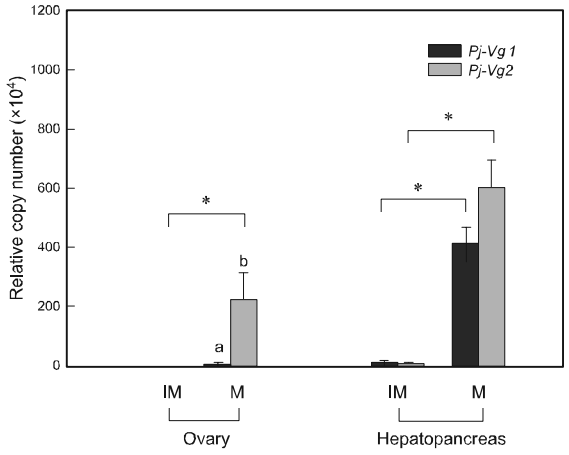

Fig. 3. Dual expression of *Pj-Vg* genes by different reproductive stages. Relative copy numbers were normalized by 18s rRNA transcript numbers. Mean bars are shown with standard error. Black and grey bars represent *Pj-Vg1* and *Pj-Vg2*, respectively. The asterisks indicate a significant difference between different maturating stages ($P<0.05$). Statistical significance between two *Pj-Vgs* is represented alphabetically ($P<0.05$). IM: immature stage (GSI; 0.2 to 1.3), M; mature stage (GSI; 2.1 to 9.8).

Expression analysis

Quantitative PCR was used to examine the relationship between the expression of *Pj-Vgs* and the reproductive cycle. Individual maturation was determined using the GSI, as described in Kim et al. (2006). The expression of both *Pj-Vg1* and *Pj-Vg2* transcripts was at a basal level in the hepatopancreas and ovary of immature individuals (GSI 0.2-1.3). Although their expression levels were extremely low in immature animals, both shrimp *Vg* transcript copy numbers were significantly higher in the hepatopancreas than in the ovary (200-fold for *Pj-Vg1* and 50-fold for *Pj-Vg2*). The expression of both *Pj-Vgs* in mature individuals (GSI 2.1-9.8) was also significantly higher in the hepatopancreas than in the ovary. The expression of *Pj-Vg1* and *Pj-Vg2* transcripts was 97- and 2.7-fold higher, respectively, in the hepatopancreas than in the ovary, suggesting that the major production site for both *Pj-Vgs* is the hepatopancreas regardless of the stage in the reproductive cycle. The high copy numbers of *Pj-Vg2* in the ovaries from mature animals were noteworthy. The induction of transcripts of both *Pj-Vg1* (34.6-fold) and *Pj-Vg2* (104.1-fold) was greater in the hepatopancreas from mature individuals compared with immature animals.

It is generally accepted that the expression of *Vg* genes is upregulated in the hepatopancreas during the mature stages. The induction of *Vg* genes in the ovary has also been detected in various species, including *P. monodon* (Tiu et al., 2006), *M. ensis* (Tsang et al., 2003), *Callinectes sapidus* (Zmora et al., 2007), and *Upogebia major* (Kang et al., 2008). In our study, there was a 1972-fold induction of *Pj-Vg2* transcripts in the mature ovary, whereas no significant increase in *Pj-Vg1* was seen. To our knowledge, this is to first report on the different expression patterns of two *Vg* genes. The existence of two mechanisms regulating *Vg* genes during the reproductive cycle is of interest. Further study is needed to elucidate the *cis*- and *trans*-elements that are responsible for regulating this and their biological roles. There was no statistical difference between *Pj-Vg1* and *Pj-Vg2* in the mature hepatopancreas.

To examine the correlation between the expression of the two *Pj-Vgs* within the same individual, a simple regression analysis was carried out. The expression of *Pj-Vg1* and that of *Pj-Vg2* were strongly correlated in both the gonad and hepatopancreas (Fig. 4). The relative copy numbers of *Pj-Vg2* in the hepatopancreas were also correlated with those in the ovary, suggesting that the dual expression of *Pj-Vg2* in the hepatopancreas and ovary is under

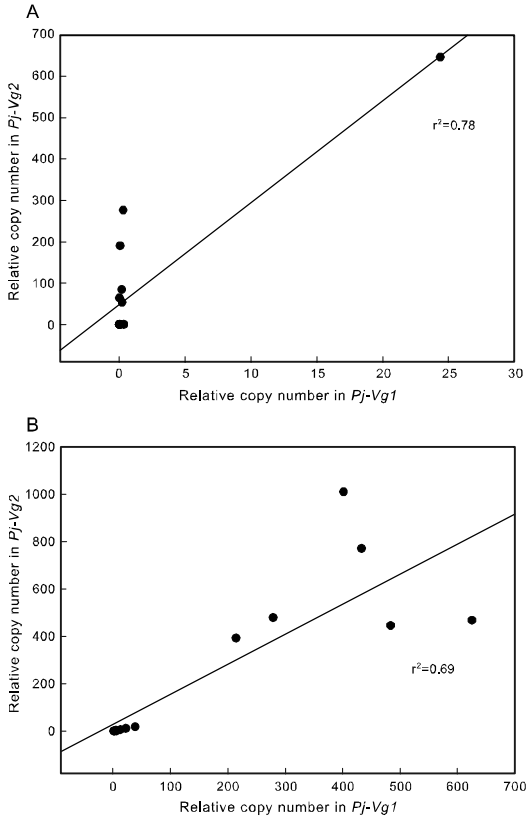

Fig. 4. Correlation graphs between *Pj-Vg1* and *Pj-Vg2* transcript numbers in ovary and hepatopancreas. A) Correlation of relative copy numbers between *Pj-Vgs* in ovary B) Correlation of relative copy numbers between *Pj-Vgs* in hepatopancreas.

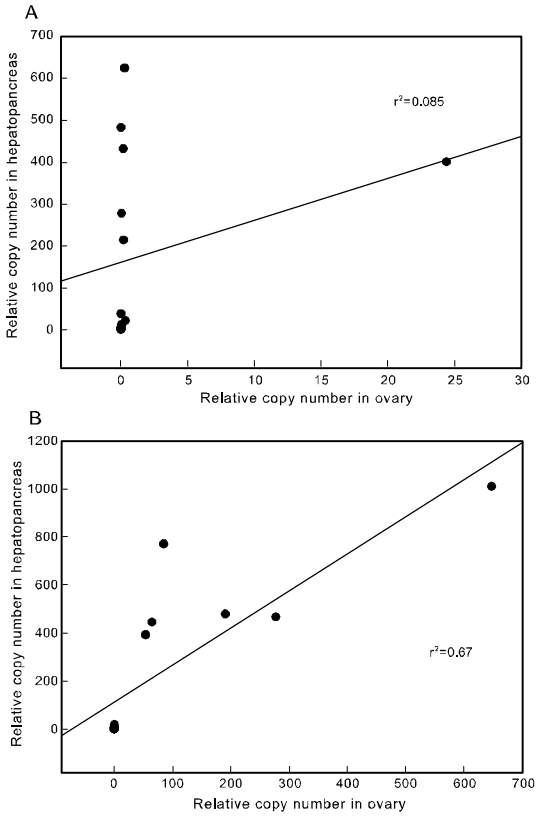

Fig. 5. Correlation graphs of two *Pj-Vg* transcripts between ovary and hepatopancreas
A) Correlation of *Pj-Vg1* copy numbers between in ovary and hepatopancreas. B) Correlation of *Pj-Vg2* copy numbers between in ovary and hepatopancreas.

the same regulatory mechanism during the reproductive cycle (Fig. 5). As no *Pj-Vg1* was expressed in the immature hepatopancreas, we could not examine this relationship for *Pj-Vg1*. We conclude that the expression of *Pj-Vg1* and that of *Pj-Vg2* were strongly correlated in the hepatopancreas, and the induction of *Pj-Vg2* in both the hepato-pancreas and ovary was synchronized. The meaning of the absence of *Pj-Vg1* in the ovary is not clear, but our findings constitute important data for under-standing the roles of multiple vitellogenin genes in the reproductive mechanism of decapod crustaceans.

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