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Expression levels of *vitellogenin receptor* (*Vtgr*) during ovarian development and association between its single nucleotide polymorphisms (SNPs) and reproduction-related parameters of the giant tiger shrimp *Penaeus monodon*

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ABSTRACT

Identification of molecular markers linked to phenotypic parameters of reproduction is important and can be applied to domestication and selective breeding programs of the giant tiger shrimp (*Penaeus monodon*). In this study, the full-length cDNA and expression levels of *P. monodon vitellogenin receptor* (*PmVtgr*) during ovarian development in wild shrimp and association between its single nucleotide polymorphisms (SNPs) and reproduction-related parameters in domesticated shrimp were examined. *PmVtgr* was 5990 bp in length composing of an open reading frame (ORF) of 5832 bp deducing to a polypeptide of 1943 amino acids. *PmVtgr* was only expressed in ovaries but not in other tissues of female broodstock. The expression level of *PmVtgr* in premature ovaries of juveniles was significantly lower than in those of broodstock ($P < 0.05$). In intact broodstock, *PmVtgr* mRNA significantly increased in stage IV (mature) relative to stage I (previtellogenic) ovaries ($P < 0.05$). However, *PmVtgr* was comparably expressed at all stages of ovarian development in eyestalk-ablated shrimp ($P > 0.05$). In addition, SNPs in the *PmVtgr* gene segment of 14-month-old *P. monodon* ($N = 64$) were indirectly examined by PCR-SSCP. Significant relationships between different SSCP patterns of *PmVtgr* and important reproduction-related phenotypes (i.e. gonadosomatic index, GSI and ovarian weight) of *P. monodon* were found. Results were consistent when further tested against non-related 19-month-old shrimp ($N = 54$, $P < 0.05$). Representative 14-month-old shrimp exhibiting different SSCP genotypes of *PmVtgr* were cloned and sequenced ($N = 10$ for each SSCP pattern). SNP positions 126 (C → T), 226 (T → A), 441 (T → A), 477 (T → A), 499 (A → C), 500 (T → C), 519 (A → G), 540 (A → G), 545 (G → A), 614 (T → C) and 678 (A → C) of *PmVtgr* were significantly associated with GSI values of examined shrimp ($N = 40$).

1. Introduction

Among commercial penaeid species listed by the Food and Agriculture Organization of the United Nations (FAO), the giant tiger shrimp (*Penaeus monodon*) is one of the economically important species mainly cultured in Southeast Asia (Bailey-Brock and Moss, 1992). The farming cycle of this species has yet to be completed as the seed of *P. monodon* used in the shrimp industry relies almost entirely from wild broodstock (Klinbunga et al., 2001). This results in heavy exploitation of male and female broodstock in natural populations leading to the lack of high quality broodstock required by the shrimp industry (Khamnamtong et al., 2009). This circumstance has probably caused the reduction of aquacultural production of *P. monodon* since the last several years (Limsuwan, 2004).

Reduced reproductive maturation of captive *P. monodon* females is found (Kenway et al., 2006; Klinbunga et al., 2009; Preechaphol et al., 2007). Accordingly, breeding of pond-reared *P. monodon* is extremely difficult and rarely produce enough quality of larvae required by the

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industry (Preechaphol et al., 2010). Eyestalk ablation is practically used to induce the maturation of penaeid shrimp but it does not give the same induction effects when applied to cultured shrimp (Withyachumnarnkul et al., 1998). Control of reproductive maturation of *P. monodon* without eyestalk ablation is the ultimate goal for the shrimp industry.

Domestication and selective breeding programs have been applied to select commercially important desired traits in several species (Argue et al., 2002; Goyard et al., 2001; Goyard et al., 2002). Basically, the effective selective breeding programs of aquaculture species would be gained if the establishment of high genetic diversity stocks is carried out (Allendorf and Ryman, 1987; Cock et al., 2009; Cruz et al., 2004; De Donato et al., 2005). Integration of population genetic knowledge (for estimation of genetic variation levels of selected stocks), molecular genetics (for identification of molecular markers linked to commercially important phenotypes) and quantitative genetics (for estimation of breeding values of brooders) would elevate the selection efficiency of *P. monodon*.

Nevertheless, genetic improvement of *P. monodon* is slow due to the lack of the basic information related with ovarian development and maturation in penaeid shrimp. Determining molecular mechanisms involving female broodstock quality (i.e. maturation potential, hatching rate, number of spawning, etc.) can be applied to promote reproductive performance of domesticated stocks. Accordingly, the use of genetic markers that allow selection of broodstock with a high potential for reproductive success would be useful for the shrimp industry.

The information on association between genotypic and phenotypic variations of penaeid shrimp is limited at present. Analysis of gene-based single nucleotide polymorphisms (SNPs) is one of the efficient approaches for discovery of genes that significantly contribute in production traits of commercially important species (Liu and Cordes, 2004; Tao and Boulding, 2003). This allows the possibility to locate major loci responsible for the difference in quantitative traits of *P. monodon* where this information is not available at present.

Recently, polymorphism of an ATP-dependent DNA helicase gene, *RuvB-like 2* (*PmRuvBL2*) in relation with the body weight of *P. monodon* was examined using single-strand conformational polymorphism (SSCP, Orita et al., 1989). Association between *PmRuvBL2* genotypes and the body weight of *P. monodon* was tested using commercially cultivated shrimp from the same pond (3 months old, average BW = 17.39 ± 4.36 g, N = 359). Disregarding sexes, the body weight of shrimp carrying genotypes A (average BW = 19.277 ± 3.640 g, N = 37) and B (average BW = 19.293 ± 4.548 g, N = 79) was significantly greater than that of shrimp carrying C (average BW = 16.528 ± 3.847 g, N = 93) and D (average BW = 16.365 ± 4.378 g, N = 124) genotypes. One exonic (G → A_{81}) and two intronic (A → T_{196} and G → T_{248}) SNPs corresponding to ATG, A[T/A]G, GAG and GAT for respective SSCP genotypes were found (Prasertlux et al., 2010).

Tiu et al. (2008) isolated the full-length cDNA of a putative *vitellogenin receptor* (*Vtgr*) in *P. monodon*. It was 6037 bp in length containing an ORF of 5823 bp corresponding to 1941 amino acids. The expression level of ovarian *Vtgr* mRNA examined by Northern blot analysis was low during early vitellogenesis and peaked in late vitellogenesis. Immunohistochemistry revealed the positive signal of the Vtgr protein in plasma membrane of developing oocytes. Injection of dsRNA of *VtgR* resulted in a decrease in Vtgr protein content in ovaries, but an increase of vitellogenin (Vtg) levels in the hemolymph.

Association between SNPs of functionally important genes and reproduction-related parameters (e.g. gonadosomatic index, GSI and/or ovarian weight) in penaeid shrimp has not been reported. In this study, the full-length cDNA and expression levels of *P. monodon Vtgr* (*PmVtgr*) during ovarian development in wild broodstock were examined. PCR-SSCP which is simple and cost-effective was initially applied to detect polymorphism of the *PmVtgr* gene segment. SNPs in cloned *PmVtgr* of multiple individuals exhibiting different SSCP patterns were examined. Association between SSCP/SNP genotypes and GSI values of examined shrimp is reported.

2. Materials and methods

2.1. Experimental samples

For gene expression analysis, female broodstock were wild-caught from the Andaman Sea (west of peninsular Thailand) and acclimated under farm conditions for 2–3 days. Ovaries were dissected out from each intact shrimp and weighted. For the eyestalk ablation group, wild shrimp were acclimated for 7 days before unilateral eyestalk ablation. Ovaries of eyestalk-ablated shrimp were collected at 2–7 days after the ablation. The ovarian developmental stages of *P. monodon* were classified according to the gonadosomatic index (GSI, ovarian weight / body weight × 100) to previtellogenic (stage I, GSI < 1.5%, N = 4 and 7 for intact and eyestalk-ablated broodstock, respectively), vitellogenic (stage II, GSI = 2–4%, N = 11 and 12), late vitellogenic (stage III, GSI = 4–6%, N = 5 and 10) and mature (stage IV, GSI > 6%, N = 6 and 5) ovaries, respectively. The ovarian stage of each shrimp was further confirmed by a conventional histology (Hiransuchalert et al., 2013; Qiu et al., 2005). Premature ovaries of cultured juvenile *P. monodon* (4-month-old) maintained at the Broodstock Multiplication Center (BMC), Burapha University, Chanthaburi Campus, (N = 5) were also included in the experiments.

For RT-PCR and tissue distribution analysis, ovaries and testes of commercially cultured juveniles (N = 5 for each sex) and wild broodstock (N = 11 for each sex) were collected. In addition, various tissues of female broodstock and testes of male broodstock (N = 3 for each group) were collected. Shrimp tissues were immediately placed in liquid N_2 and kept at −80 °C until needed.

For identification of SNPs, domesticated female *P. monodon*: 14-month-old (average body weight = 87.32 ± 12.47 g and average ovarian weight = 0.84 ± 0.34 g, N = 66) and 19-month-old (average body weight = 60.56 ± 13.21 g and average ovarian weight = 0.44 ± 0.20 g, N = 55) which were established from stocks originally from different locations, were collected from the BMC. Pleopods were dissected out from each shrimp and kept at −20 °C. The GSI of each shrimp was calculated.

2.2. Genomic DNA and total RNA extraction

Genomic DNA was extracted from a piece of the pleopod of *P. monodon* adults using a phenol-chloroform-proteinase K method (Klinbunga et al., 2001). The concentration of extracted DNA was spectrophotometrically estimated. DNA was stored at 4 °C until used.

Total RNA was extracted from ovaries and other tissues of *P. monodon* using TRI Reagent (Molecular Research Center). The concentration of extracted total RNA was spectrophotometrically measured. Total RNA was kept in the absolute ethanol at −80 °C prior to reverse transcription.

2.3. Isolation of the full-length cDNA of PmVtgr

The partial cDNA sequence of *PmVtgr* was identified from the ovarian cDNA library of *P. monodon* (EST-OV-0395; Preechaphol et al., 2007). Initially, *PmVtgr* was further characterized using rapid amplification of cDNA end-polymerase chain reaction (RACE-PCR). Messenger (m) RNA was purified from total RNA using a QuickPrep Micro mRNA Purification Kit (GE Healthcare). Primers for RACE-PCR (5′Vtgr-GSP1-1: 5′-GACCCTGAGGACGCTTTCTGTCGTT-3′ for 5′RACE-PCR and 3′Vtgr-GSP2: 5′-GTGTGGTGACCTGGCAGTGGATGA-3′ for 3′RACE-PCR; positions 4470–4495 and 4820–4844 according to the full-length cDNA of *PmVtgr*, respectively) were designed. The purified mRNA was reverse-transcribed and 5′ and 3′ RACE-PCRs were carried out using a SMART RACE cDNA amplification kit following the protocol recommended by

the manufacturer (BD Bioscience Clontech). The remaining 5′ portion of the *PmVtgr* open reading frame (ORF) was isolated by conventional PCR [primers Vtgr-primer walking (PW) 1-F/R, positions 1868–1887 + 2665–2683, Vtgr-PW2-F/Rpositions 1564–1583 + 1828–1848 and Vtgr-PW3-F/R positions 214–234 + 1602–1622] designed from *Vtgr* previously described by Tiu et al. (2008). The 5′ end of *PmVtgr* was further isolated by RACE-PCR using a primer 5′Vtgr-GSP1-2 (5′-CTACTGCT TGTAACTCTGGTCG-3′, positions 160–180). The gel-eluted product was ligated with pGEM-T Easy vector and transformed into *Escherichia coli* JM109. Nucleotide sequences of the positive clones were sequenced in both directions. The sequences obtained were assembled with the original EST sequence and then compared to previously deposited sequences in GenBank using Blast*N* and Blast*X* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). The p*I* value and molecular weight of the deduced PmVtgr protein were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The protein domains and signal peptide in the deduced PmVCP protein were predicted using SMART (http://smart.embl-heidelberg.de). Potential *N*-liked glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.4. RT-PCR and tissue distribution analysis

One and a half microgram of DNase I-treated total RNA (0.5 U/μg cDNA for 30 min at 37 °C) was reverse-transcribed using an Improm-II™ Reverse Transcription System (Promega). Expression levels of $PmVtgr_{256}$ (primers Vtgr-RT-F: 5′-CGCCCGATGCCCATCATT TC-3′ and Vtgr-RT-R: 5′-TGTCGCCCTCCATCCCAC-3′; positions 1818–1838 and 2055–2073 of the full-length cDNA of *PmVtgr*) in ovaries and testes of wild broodstock ($N = 11$ for each sex) and testes of cultured juveniles ($N = 5$ for each sex) were analyzed by RT-PCR (Sittikankaew et al., 2010). $EF\text{-}1\alpha_{500}$ (F: 5′-ATGGTTGTCAACTT TGCCCC-3′ and R: 5′-TTGACCTCCTTGATCACACC-3′) amplified from the same template was included as the positive control. The thermal profiles were predenaturation at 94 °C for 3 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 30 s with the final extension at 72 °C for 7 min. An amplification product sample (5 μl) was analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining (0.5 μg/ml) with visualization under a UV transilluminator (Sambrook and Russell, 2001). Expression of *PmVtgr* mRNA in various tissues of females and in testes of males was carried out using the same conditions.

2.5. Quantitative real-time PCR

Expression levels of $PmVtgr_{143}$ (primers PmVtgr-F: 5′-CATCTGGGTC CATCCTGAATATGT-3′ and PmVtgr-R: 5′-CCGATAACTCCATGGGCTTTGT CA-3′; positions 4291–4315 and 4409–4433 of the full-length cDNA of *PmVtgr*) in cultured juveniles and different stages in wild intact and eyestalk-ablated broodstock of *P. monodon*, were examined. Quantitative real-time PCR was carried out in a 10 μl reaction volume containing 5 μl of 2× LightCycler 480 SYBR Green I Master (Roche), 50 ng of the first strand cDNA template, and 0.2 μM each of PmVtgr-F/R primers. The thermal profile for quantitative real-time PCR was 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 45 s and 72 °C for 45 s. Real-time PCR of each specimen was carried out in duplicate using a LightCycler® 480 (Roche). $EF\text{-}1\alpha_{214}$ (F: 5′-GTCTTCCCCTTCAG GACGTC-3′ and R: 5′-CTTTACAGACACGTTCTTCACGTTG-3′) amplified from the same template was included as the positive control. The average quantification of $PmVtgr_{143}$ and $EF\text{-}1\alpha_{214}$ mRNA of each sample was evaluated from the respective standard curves generated from 10^3 to 10^8 copies of recombinant plasmids of the target or reference sequences. The relative expression level (copy number of $PmVtgr_{143}$ and that of $EF\text{-}1\alpha_{214}$) between different groups of intact and eyestalk-ablated broodstock possessing different stages of ovarian development was statistically tested using one way analysis of variance (ANOVA) followed by Duncan's new multiple range test ($P < 0.05$).

2.6. In situ hybridization

Pieces of ovaries were dissected out from wild *P. monodon* broodstock and fixed overnight at 4 °C in 4% paraformaldehyde prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.2). The fixed ovarian tissue was washed four times with PBS at room temperature and stored in 70% ethanol at −20 °C until used. Tissues were histologically prepared and embedded in paraffin. Conventional paraffin sections (5 μm) were carried out. The sense and anti-sense cRNA probes: primers $PmVtgr_{1058}$-T7/F, 5′-TAATACGACTCACTATAGGGACCATAAAGTCGCTTG TAGAGATGG-3′ and $PmVtgr_{1058}$-SP6/R, 5′-*ATTTAGGTGACACTATAG AA*CAGCATAAACGAAATTATGGGACTC-3′ containing T7 (underlined) and SP6 (italicized and underlined) promoter sequences, were synthesized by using DIG RNA labeling mix (Roche). Tissue sections and hybridization were carried out as described in Sittikankaew et al., 2010. The bound probes were detected with a DIG Wash and Block Buffer Kit (Roche) (Qiu and Yamano, 2005).

2.7. Single-strand conformational polymorphism (SSCP) analysis of the PmVtgr gene segment

A pair of primers for amplification of the *PmVtgr* gene segment was designed (F: 5′-ACCCGGGTATACTCTTAGACCTGAC-3′ and R: 5′-TATC AAGCCATTATCCACAACTTCA-3′; positions 4215–4239 and 4551–4575 of the full-length cDNA of *PmVtgr*). The *PmVtgr* gene segment was amplified using genomic DNA of 14- ($N = 64$) and 19-month-old ($N = 54$) *P. monodon* as the template. The thermal profiles were 94 °C for 3 min followed by 35 cycles of predenaturation at 94 °C for 45 s; annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final extension was carried out at 72 °C for 7 min. The amplification product was analyzed by SSCP (Orita et al., 1989) using PROTEAN II xi cells (Bio-Rad). The PCR product (6 μl) was mixed with four volumes of the loading dye (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol, and 10 mM NaOH), denatured in a boiling bath for 5 min, and immediately cooled on ice for 3 min. The denatured products of *PmVtgr* were electrophoretically analyzed (native 12.5% PAGE; 37.5:1 crosslink) at 250 V for 16 h at 4 °C. SSCP bands were visualized by silver staining. Association between the frequencies of identified SSCP genotypes and reproduction-related parameters (GSI and ovarian weight) of shrimp was statistically analyzed using ANOVA followed by Duncan's new multiple range test ($P < 0.05$).

2.8. Identification of SNPs in PmVtgr by PCR-cloning and sequencing

The *PmVtgr* gene segment of 14-month-old *P. monodon* ($N = 10$ for each pattern) was amplified, electrophoretically analyzed and eluted from the gel. The resulting product was cloned into pGEM-T Easy vector. One-tenth volume of each ligation reaction was transformed to *E. coli* JM109. Recombinant clones were selected by a *lacZ* system following standard protocols (Sambrook and Russell, 2001). Nucleotide sequence of each insert was examined in both directions and searched against previously deposited data in GenBank using Blast*N* (Altschul et al., 1990; available at http://ncbi.nlm.nih.gov). Nucleotide sequences of fragments representing different SSCP genotypes of each shrimp were multiple-aligned using Clustal W (Thompson et al., 1994). Statistical analysis was carried out to determine whether shrimp carrying different genotypes of each SNP possessed significantly different average GSI and/or ovarian weight using an independent *t*-test (for bi-allelic SNPs with two genotypes) or ANOVA followed by Duncan's new multiple range test (for bi-allelic SNPs with three genotypes). Significant differences were considered if $P < 0.05$.

3. Results

3.1. Isolation and characterization of the full-length cDNA of PmVtgr

The full-length cDNA of *PmVtgr* was 5990 bp in length composing of an ORF of 5832 bp deducing to a polypeptide of 1943 amino acids with 5′- and 3′UTRs of 39 and 119 bp (excluding the poly A tail, GenBank accession no. KJ601728). This sequence exhibited the closest similarity to *vitellogenin receptor* of *P. monodon* (*E*-value = 0.00). The predicted molecular mass and isoelectric point (p*I*) of the deduced PmVtgr protein were 213.35 kDa and 5.18, respectively.

The deduced PmVtgr protein contained the signal peptide (positions 1–20), five low-density lipoprotein-receptor class A domains (LDLa; positions 79–117, 118–156, 161–198, 202–239, 241–281), an epidermal growth factor-like domain (EGF, positions 282–316), a calcium-binding EGF-like domain (EGF-CA, positions 317–357), eight low-density lipoprotein-receptor YWTD domains (LY, positions 384–424, 426–468, 469–510, 511–553, 554–594, 734–776, 778–819 and 820–864) intervened and followed by two EGF domains (positions 623–661 and 941–979), eight LDLa domains (positions 982–1020, 1023–1062, 1064–1101, 1102–1139, 1140–1179, 1185–1227, 1228–1267 and 1290–1328), two EGF domains (positions 1329–1365 and 1369–1405), three LY domains (positions 1473–1514, 1585–1627 and 1628–1668), two EGF domains (positions 1695–1733 and 1737–1771) and a single transmembrane domain (positions 1786–1808) in order (Figs. 1 and 2). PmVtgr consists of 13 predicted *N*-linked glycosylation sites (NXS/T). Two putative internalization signals (FXNPXY/F) were found at positions 1836–1841 (FANPGF) and 1876–1881 (FENPFF) of the deduced PmVtgr protein.

3.2. RT-PCR and tissue distribution analysis

PmVtgr was specifically expressed in ovaries but not the testes of juveniles ($N = 5$ for each sex) and broodstock ($N = 11$ for each sex) of *P. monodon* (Fig. 3A and C). Tissue distribution analysis further confirmed its specific expression in ovaries but not in other tissues (heart, hemocytes, lymphoid organs, intestine, gills, pleopods, thoracic ganglion, stomach, eyestalk and hepatopancreas) of female broodstock and testes of male broodstock (Fig. 3E).

3.3. Expression profiles of PmVtgr during ovarian development of P. monodon

The expression level of *PmVtgr* in premature ovaries of juveniles was significantly lower than in ovaries of either intact or eyestalk-ablated broodstock ($P < 0.05$). In intact adults, *PmVtgr* significantly increased in stage IV relative to stage I (previtellogenic) ovaries ($P < 0.05$). However, *PmVtgr* was comparably expressed at all stages of ovarian development in eyestalk-ablated shrimp ($P > 0.05$). Its expression in each ovarian developmental stage of intact mature broodstock was not significantly different from that in the same stage of unilateral eyestalk-ablated broodstock ($P > 0.05$, Fig. 4).

3.4. Localization of PmVtgr transcript

In situ hybridization revealed that the *PmPmgr* transcript was clearly localized in the ooplasm of previtellogenic oocytes in both intact and eyestalk-ablated broodstock. No signal was found with the sense cRNA probe (Fig. 5). The positive hybridization signal was not observed in more mature (vitellogenic, cortical rod and mature) oocytes, oogonia, follicular cells, and follicular layers in both intact and eyestalk-ablated broodstock.

3.5. Association between SSCP patterns of PmVtgr and GSI of domesticated broodstock

The amplified 720 bp fragment of *PmVtgr* gene segment was cloned and sequenced. Two introns (223 and 136 bp) were found in the amplified region along with two partial exons (49 and 161 bp) at the 5′ and 3′ portions and one complete exon (151 bp) within the amplified fragment (Fig. 6). SNP by SSCP analysis was applied for examination of polymorphism of *PmVtgr* in 14-month-old shrimp. Four SSCP patterns (A, B, C, D) were observed (Fig. 7A) . Association between SSCP patterns and GSI values was statistically analyzed. Shrimp carrying SSCP pattern A ($1.10 \pm 0.31\%$) had a significantly greater average GSI value than those carrying SSCP pattern D ($0.80 \pm 0.30\%$, $P < 0.05$). Although results from analysis of relationships between SSCP patterns and ovarian weight of shrimp exhibiting different genotypes were not significant, those possessing SSCP pattern A showed a trend of higher ovarian weight than shrimp carrying SSCP pattern D (Table 1).

To confirm the association between SSCP patterns of *PmVtgr* and reproduction-related parameters of domesticated *P. monodon*, SSCP patterns of 19-month-old shrimp which were established from a different stock origin were examined. A total of 4 SSCP patterns (designed as E, F, G and H) were observed (Fig. 7B). Similarly, relationships between SSCP patterns of *PmVtgr* and the average GSI values were found where that of shrimp possessing pattern E ($0.68 \pm 0.34\%$) was significantly greater than that of shrimp possessing patterns F ($0.43 \pm 0.22\%$), G ($0.29 \pm 0.16\%$) and H ($0.35 \pm 0.19\%$) ($P < 0.05$) (Table 1). Although the average ovarian weight of these shrimp was also statistically different, results should be interpreted with caution as the average body weight of shrimp carrying different SSCP patterns was also significantly different ($P < 0.05$).

3.6. Identification of SNPs and their association with reproduction-related parameters of P. monodon

After multiple alignments, 57 substitutions were found from sequencing of cloned *PmVtgr* gene segment of 14-month-old shrimp ($N = 10$ for each SSCP pattern). Considering polymorphic sites found in at least 10% of

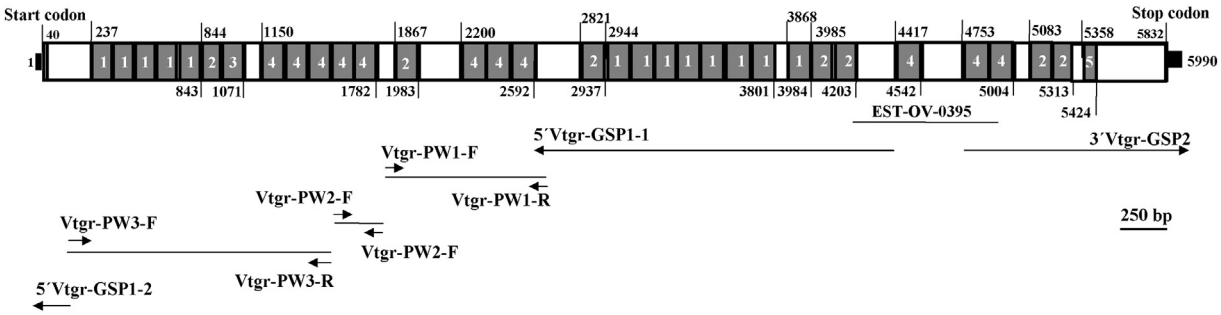

Fig. 1. Schematic diagram of the *PmVtgr* cDNA. Complete cDNA was obtained by RACE-PCR and from amplification of overlapping PCR fragments. Predicted functional domains in the deduced PmVtgr protein are illustrated by gray-boxes (1 = LDLa domain, 2 = EGF domain, 3 = EGF_CA domain, 4 = LY domain and 5 = transmembrane domain). Nucleotide positions and names of primers are shown.

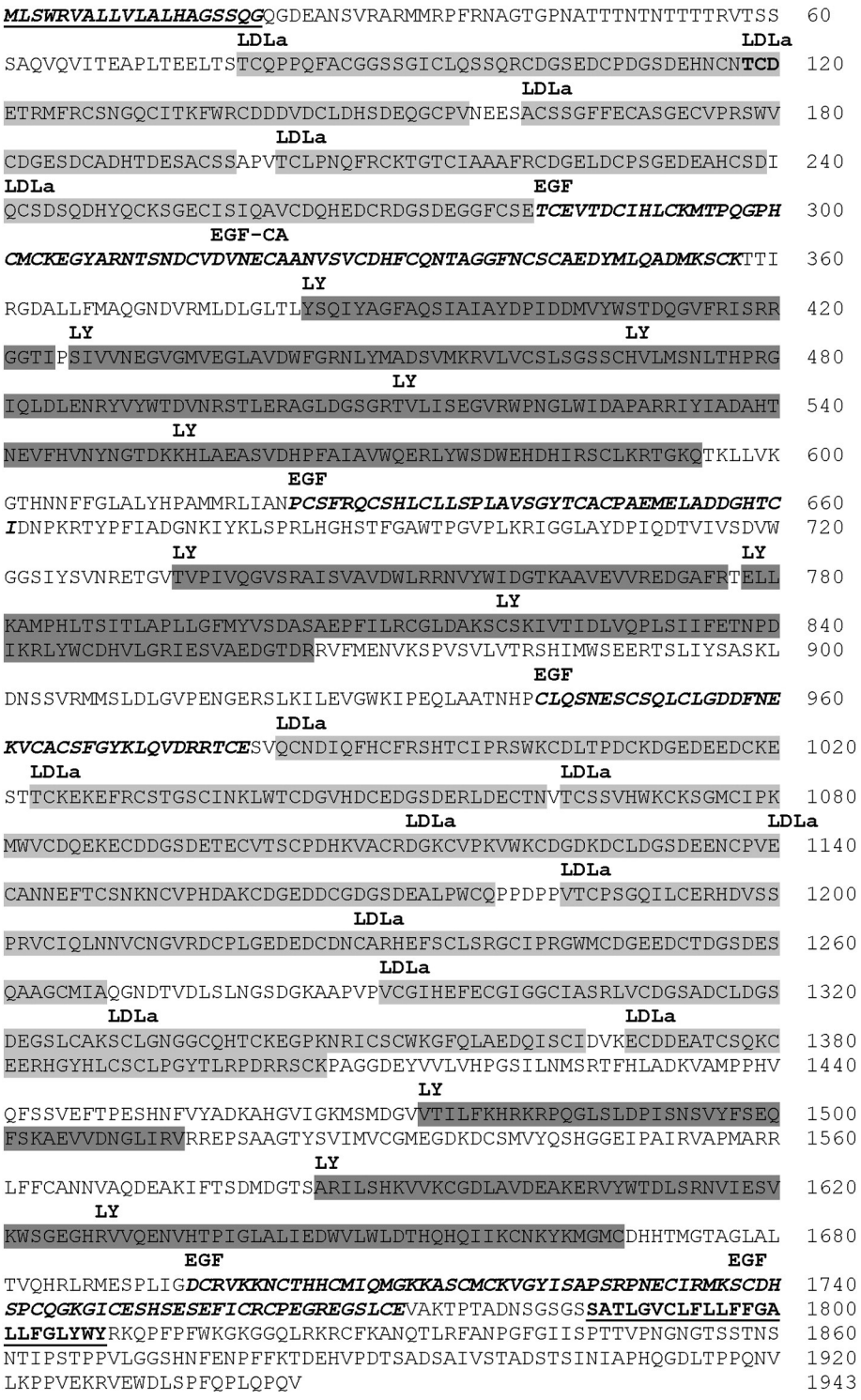

Fig. 2. The deduced amino acid sequence of PmVtgr. The predicted signal peptide (positions 1–20) is bold-italicized and underlined. LDLa and LY domains are highlighted with light and dark gray. The EGF and EGF-CA domains are illustrated in boldface and italicized. The transmembrane domain is boldfaced and underlined.

examined samples, 15 SNP positions were found. Of these, 11 SNPs were located in introns where those at positions 109 (A/A and A/T), 126 (T/T, T/C and C/C), 226 (T/T and T/A) and 264 (T/T and T/C) were located in the first intron and those at positions 441 (T/T and T/C), 477 (T/T and T/A), 499 (C/C, C/A and A/A), 500 (C/C, C/T and T/T), 519 (A/A, A/G and G/G), 540 (A/A and A/G) and 545 (G/G and G/A) were located in the second intron. Four SNPs located at positions 589 (A/A and A/G), 614 (T/T and T/C), 618 (G/G, G/A) and 678 (A/A, A/C) of the examined sequence, were located in the same exon. The composite SNP genotypes of shrimp in this study were constructed from combination of a single SNP in order, as a result, those for SSCP patterns A–D were $A/A_{109}C/C_{126}T/T_{226}T/T_{264}T/T_{441}T/T_{477}A/A_{499}T/T_{500}A/A_{519}A/G_{540}G/G_{545}A/G_{589}T/T_{614}G/G_{618}A/A_{678}$, $A/A_{109}C/T_{126}T/A_{226}T/C_{264}T/A_{441}T/A_{477}A/C_{499}T/C_{500}A/G_{519}A/A_{540}G/A_{545}A/G_{589}T/C_{614}$ $G/G_{618}A/C_{678}$, $A/T_{109}C/T_{126}T/T_{226}T/C_{264}T/T_{441}T/T_{477}A/C_{499}T/C_{500}A/G_{519}A/G_{540}G/G_{545}A/A_{589}$ $T/T_{614}G/A_{618}A/A_{678}$ and $A/T_{109}T/T_{126}T/A_{226}T/C_{264}T/A_{441}T/A_{477}C/C_{499}C/C_{500}G/G_{519}A/A_{540}$ $G/A_{545}A/A_{589}T/C_{614}G/A_{618}A/C_{678}$, respectively. These composite SNPs differentiated four SSCP patterns of *PmVtgr* unambiguously.

Among SNPs exhibiting significant association with GSI values of 14-month-old shrimp ($P < 0.05$, Table 2), those with two genotypes (one

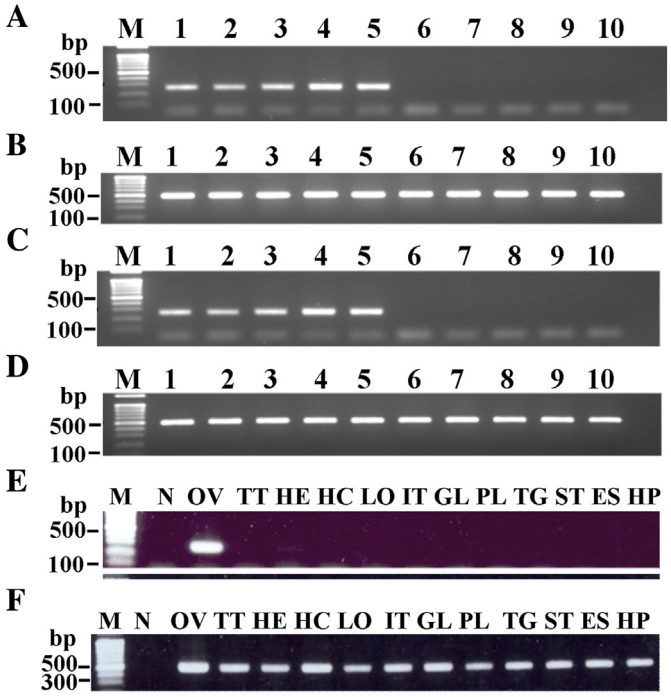

Fig. 3. 1.5% ethidium bromide-stained agarose gels showing results from RT-PCR and tissue distribution analysis of *PmVtgr* using the first strand cDNA of ovaries (lanes 1–5) and testes (lanes 6–10) of juvenile (A) and broodstock (C) and various tissues of wild *P. monodon* broodstock (E). *EF-1α* was successfully amplified from the same template (lanes 1–10, B, D and F). Lanes M and N are a 100 bp DNA marker and the negative control, respectively. OV = ovaries, TT = testes, HE = heart, HC = hemocytes, LO = lymphoid organs, IT = intestine, GL = gill, PL = pleopods, TG = thoracic ganglion, ST = stomach, ES = eyestalk and HP = hepatopancreas.

homozygote and one heterozygote) were found at positions 226 (T → A), 441 (T → A), 477 (T → A), 540 (A → G), 545 (G → A), 614 (T → C) and 678 (A → C) when homozygotes at these positions except A → G_{540} possessed a greater average GSI value than heterozygotes. SNPs with three genotypes were observed at positions 126 (C → T), 499 (A → C), 500 T → C) and 519 (A → G) where shrimp with a homozygous genotype found in SSCP pattern A showed a greater average GSI than those with an alternative homozygous genotype found in SSCP pattern D ($P < 0.05$). However, the average GSI value of shrimp possessing these alleles was not significantly different from that of shrimp carrying the heterozygous SNP genotype of a particular position ($P > 0.05$) (Table 2).

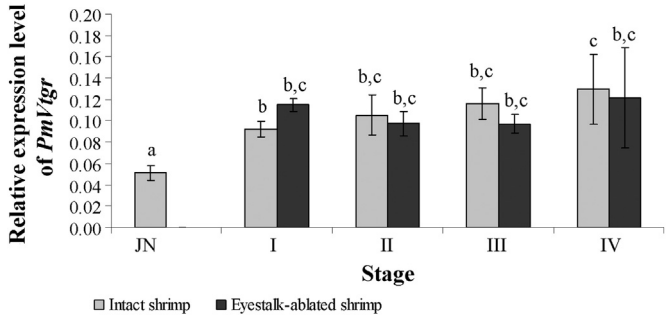

Fig. 4. Mean relative expression levels of *PmVtgr* mRNA during ovarian development of intact and unilateral eyestalk-ablated *P. monodon* broodstock. The expression level was measured as an absolute copy number of $PmVtgr_{143}$ and normalized by that of $EF\text{-}1\alpha_{214}$ from the same shrimp. Each histogram corresponds to a particular ovarian stage. The same letters above bars indicate that the expression levels were not significantly different ($P > 0.05$). JN = juvenile ovaries; I–IV = previtellogenic, vitellogenic, late vitellogenic, and mature ovaries of intact or eyestalk-ablated broodstock, respectively.

4. Discussion

4.1. Isolation and primary structure of PmVtgr

Isolation and characterization of genes specifically expressed in ovaries are necessary for understanding ovarian development processes. Ovarian maturation of *P. monodon* results from rapid synthesis and accumulation of a major yolk protein, vitellin (Vt) which is derived from its precursor, vitellogenin (Vtg). In *P. monodon*, the *Vtg* transcript is abundantly synthesized in hepatopancreas and follicular cells surrounding various stages of oocytes. In addition, it is also expressed at a low level in oocytes (Hiransuchalert et al., 2013). It has been proposed that an extraovarian Vtg protein is transported through hemolymph and enter oocytes by receptor-mediated endocytosis (Tiu et al., 2008).

In the mud crab (*Scylla serrata*), the Vtgr protein was purified by gel filtration. The Vtgr band was visualized by ligand blotting. Levels of intracellular Ca^{2+} affected the interactions of Vtgr and vitellogenin and the binding was inhibited by suramin. Results suggested that *S. serrata* Vtgr was similar to low density lipoprotein receptor (LDLR) superfamily of the receptor proteins (Warrier and Subramoniam, 2002).

In this study, the full-length cDNA of *PmVtgr* was characterized and its expression profiles during ovarian development and maturation in intact and eyestalk-ablated broodstock of wild *P. monodon* were examined. The *P. monodon Vtgr* was first characterized by Tiu et al. (2008) and the organization of its functional domains is similar to *PmVtgr* reported here. Our results further confirmed that the deduced single transmembrane PmVtgr protein contained two ligand-binding domains (LBD) which consisted of five and eight LDLa (class-A cysteine-rich repeats) functional domains as in *Drosophila melanogaster* (Schonbaum et al., 1995), *Aedes aegypti* (Cheon et al., 2001) and other insect (Chen et al., 2004) Vtgrs previously characterized so far. Each LBD was followed by an EGF homology domain which contains two types of motif; EGF/EGF_CA (class-B repeats with six-cysteine residues) and LY functional domains. The EGF_CA is a Ca^{2+}- binding domain (David, 1990). Two internalization signals (FANPGF and FENPFF) which control the entry of Vtg–Vtgr complex were also found in the deduced PmVtgr protein. On the basis of the primary structure, the PmVtgr protein should be involved in shrimp reproduction.

4.2. Tissue distribution analysis, expression profiles and localization of PmVtgr mRNA during ovarian development of P. monodon

Tissue-specific transcription is important during the development and maturation of specific cell types in adults (Grimes, 2004). *PmVtgr* was expressed only in ovaries but not in testes of both *P. monodon* juveniles and broodstock. Ovary-specific transcription of *PmVtgr* suggests its essential role in ovarian but not in testicular development in *P. monodon*. In addition, our tissue expression analysis further suggested that this transcript contributes mainly to female germ cell development in *P. monodon*.

Hiransuchalert et al. (2013) reported that *P. monodon Vtg1* mRNA was expressed in various ovarian stages of broodstock but not in juvenile ovaries. It was also abundantly expressed in hepatopancreas but not in other tissues of female broodstock and testes of male broodstock. In addition, the expression patterns of a transcript that significantly matched the polehole precursor (called *ovary-specific transcript 1*, *PmOST1*) and *adipose differentiation-related protein* (*PmADRP*) were examined and they were only expressed in ovaries but not in testes of male juveniles and broodstock and other tissues of female broodstock of *P. monodon* (Klinbunga et al., 2009; Sittikankaew et al., 2010). Like *PmOST1* and *PmADRP*, tissue-specific expression of *PmVtgr* transcript further indicated that it should contribute in ovarian development and maturation of *P. monodon*.

The expression levels of *PmVtgr* in ovaries were significantly higher in broodstock than in juveniles ($P < 0.05$) and it was up-regulated during the final stage of ovarian development in wild intact broodstock. The

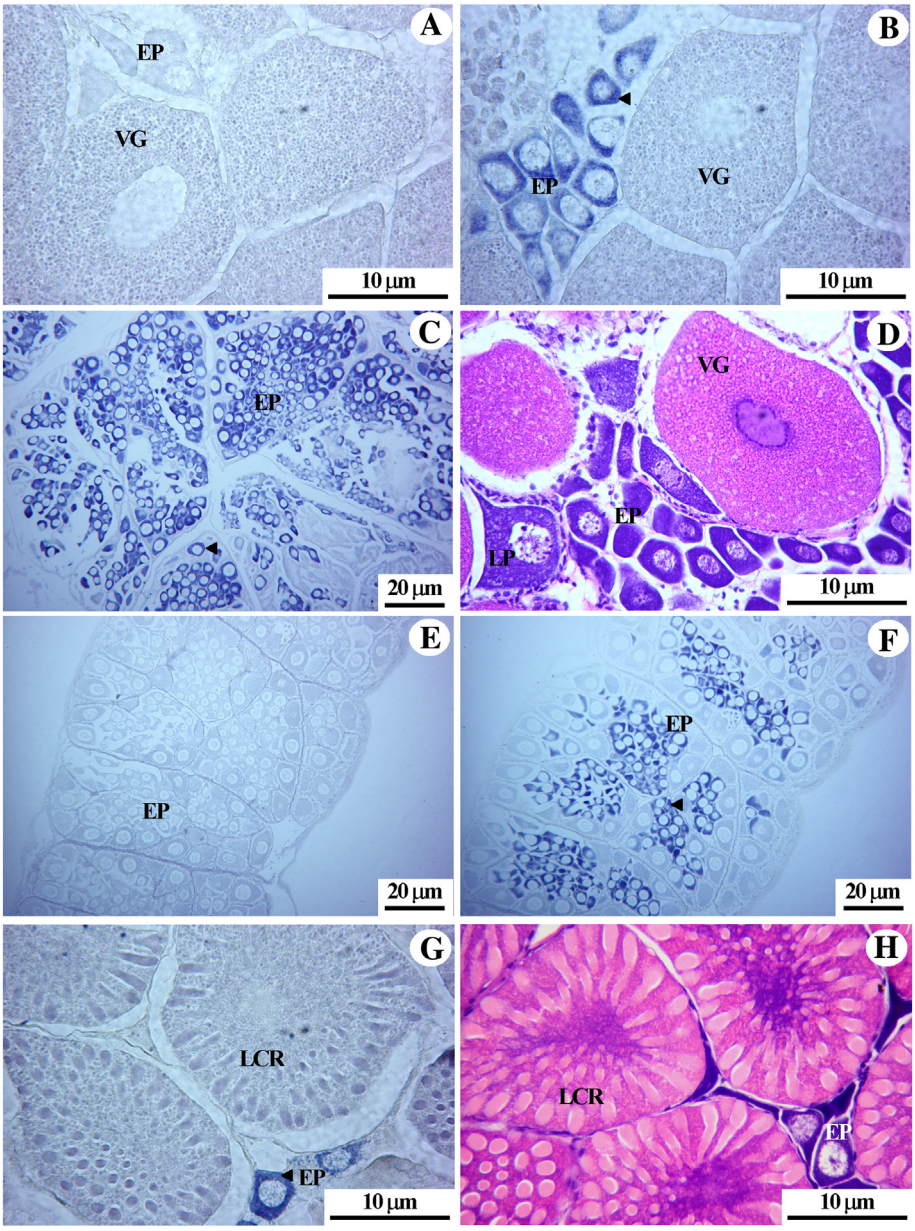

Fig. 5. Localization of *PmVtgr* mRNA during ovarian development in intact (A–D) and eyestalk-ablated (E–H) *P. monodon* broodstock, visualized by *in situ* hybridization using sense (A, E) and antisense (B-C, F-G) *PmVtgr* probes. Oocyte stages were classified by a conventional hematoxylin/eosin staining (D, H). EP = early previtellogenic; LP = late previtellogenic; VG = vitellogenic; LCR = late cortical rod oocytes. Arrowheads indicate positive hybridization signals.

expression profile of *PmVtgr* mRNA in this study was consonant with that previously reported by Tiu et al. (2008) based on Northern blot analysis. This further suggested that *PmVtgr* is involved in oogenesis. Our previous study indicated that the relative expression level of *PmVtg1* mRNA in hepatopancreas was abundantly expressed in wild broodstock possessing stages I and II ovaries. Its expression in

ACCCGGGTATACTCTTAGACCTGACAGACGCTCTTGCAAACCAGCAGGT*AAACTGTAAA*
AAATAATAAATAAATAAAAAACAATAAATCCAGTCCTAAAACACAACTTAAAAGTAAAA
AAGCAGCCACACCATTGACACTGTGGTTAATGGACAGTCACGACATTTAATAAACTTTC
TGTGCAAGCATAAATAGCCAATATTTTGCTAGTTAGTATAGAAGAAGATAACGTTTTGT
*AAATACACTATCATATTGTATTTCTTCTTTCCAGGT*GGCGACGAATATGTGGTCTTGGT
GCATCCTGGGTCCATCCTGAATATGTCCCGCACCTTCCATCTTGCTGACAAAGTGGCGA
TGCCCCCTCATGTTCAGTTTTCGTCTGTTGAGTTTGCGCCCGAGTCCCATCGTTTCGTT
TATGCTGACA*GTAAGAAAATGCTTTTATGCGAGATCGATACATTGCAAAACTACAGTGC*
TTGGTCCCCTATTAGTATGTAAAATAATCAGATTTGTGTTGAATGAATCAAATTACATT
*ATCACTAAACTGAGATTCCTATTTCTAG*AAGCCCATGGAGTTATCGGGAAGATGAGCAT
GGACGGCGTAGTGACAATACTCTTTAAGCACAGAAAGCGTCCTCAGGGTCTCTCCTTGG
ACCCCATTAGCAACAGCGTTTATTTCTCAGAACAGTTCAGTAAAGCTGAAGTTGTGGAT
AATGGCTTGATA

Fig. 6. Nucleotide sequence of the amplified *PmVtgr* gene fragment. Exons are illustrated in boldface. Introns are italicized. Primers are highlighted.

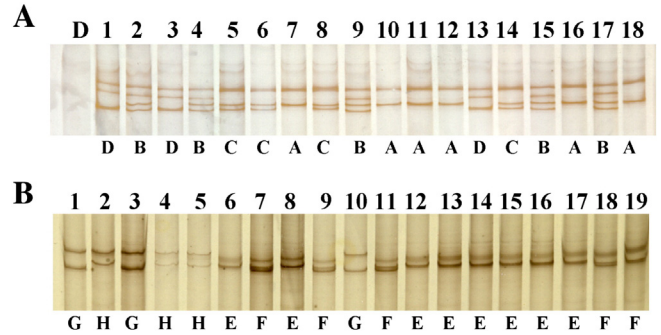

Fig. 7. SSCP patterns of the *PmVtgr* gene segment of 14- (lanes 1–18, A) and 19-month-old (lanes 1–19, B) *P. monodon* broodstock. Lane D is non-denatured PCR product (double stranded DNA control). Four polymorphic patterns (A–D and E–G) were observed in each sample group.

hepatopancreas was significantly reduced following the progression of ovarian development in shrimp having stages III and IV ovaries (Hiransuchalert et al., 2013). This circumstance suggested that a greater level of *PmVtgr* should compensate a lower level of *PmVtg1* during the final stages of ovarian development. The steady amounts of *PmVtgr* mRNA in eyestalk-ablated shrimp suggested that its expression level should be sufficient to maintain the translational product throughout oogenesis.

Immunohistochemistry revealed that the *P. monodon* Vtgr protein was localized in the cytoplasm of small oocytes. The immunoreactive signals were detected strongly in the oocyte membranes and weakly in the cytoplasm of the larger oocytes in early vitellogenic ovaries (Tiu et al., 2008). In this study, localization of *PmVtgr* mRNA was examined using *in situ* hybridization. While *PmVtg1* mRNA was localized in follicular cells surrounding different stages of oocytes (Hiransuchalert et al., 2013), the positive hybridization signals of *PmVtgr* mRNA were only observed in the cytoplasm of previtellogenic oocytes. No localization of *PmVtgr* mRNA was observed in vitellogenic, cortical rod and mature oocytes, oogonia and follicular cells. This suggested its important roles during meiotic rather than mitotic cell cycles in *P. monodon*. The disappearance of the hybridization signals of *PmVtgr* mRNA in more mature (vitellogenic and mature) stages of oocytes should be confirmed by a more sensitive technique like fluorescence *in situ* hybridization (FISH).

Notably, stages of developmental oocytes within shrimp ovaries are not synchronous even within a single lobe of ovaries (Medina et al., 1996). Therefore, a particular ovarian stage was determined based on the predominant oocyte type in each shrimp. Contradictory results from quantitative real-time PCR (on a greater expression level in broodstock than that in juveniles) and *in situ* hybridization (on the appearance of positive hybridization signals only in previtellogenic oocytes) may have resulted from the use of cDNA template from mixed stages of oocytes in the ovarian tissue for quantification of *PmVtgr1* mRNA expression profiles while *in situ* hybridization revealed subcellular localization of the *PmVtgr1* transcript in oogonia and various stages of oocytes. In addition, real-time PCR detects the expression of genes with much higher sensitivity than *in situ* hybridization (Klinbunga et al., 2009).

Table 1
Association between SSCP genotypes and reproduction-related parameters (GSI and ovarian weight) of the *PmVtgr* gene segment against non-related domesticated 14- ($N = 64$) and 19-month-old ($N = 54$) *P. monodon*.

SSCP pattern	*N*	%GSI ± SD	Ovarian weight ± SD (g)	Body weight ± SD (g)
14-month-*old shrimp*				
A	16	1.10 ± 0.31^{a}	0.94 ± 0.28^{a}	86.94 ± 14.65^{a}
B	13	$0.95 \pm 0.43^{a,b}$	0.85 ± 0.37^{a}	89.83 ± 11.23^{a}
C	21	$0.99 \pm 0.36^{a,b}$	0.87 ± 0.38^{a}	86.49 ± 13.41^{a}
D	14	0.80 ± 0.30^{b}	0.70 ± 0.32^{a}	85.88 ± 10.60^{a}
19-month-*old shrimp*				
E	14	0.68 ± 0.34^{a}	0.91 ± 0.31^{a}	70.25 ± 17.79^{a}
F	18	0.43 ± 0.22^{b}	0.68 ± 0.27^{b}	$61.34 \pm 10.90^{a,b}$
G	12	0.29 ± 0.16^{b}	0.53 ± 0.23^{b}	52.25 ± 9.46^{b}
H	10	0.35 ± 0.19^{b}	0.62 ± 0.25^{b}	54.14 ± 8.61^{b}

The same superscripts indicate non-significant difference between groups of samples ($P > 0.05$).

Table 2
Association between shrimp having different SNP genotypes of the *PmVtgr* gene segment and phenotypic parameters of 14-month-old *P. monodon* ($N = 40$).

Position*	SNP genotype	*N*	%GSI ± SD	OW ± SD (g)	BW ± SD (g)
109 (I)	A/A	20	0.97 ± 0.35^{a}	0.84 ± 0.32^{a}	87.38 ± 14.82^{a}
	A/T	20	0.91 ± 0.39^{a}	0.78 ± 0.37^{a}	84.23 ± 10.36^{a}
126 (I)	C/C	10	1.10 ± 0.32^{a}	0.91 ± 0.30^{a}	82.95 ± 16.51^{a}
	C/T	20	$0.94 \pm 0.41^{a,b}$	0.83 ± 0.39^{a}	87.82 ± 12.40^{a}
	T/T	10	0.79 ± 0.26^{b}	0.67 ± 0.30^{a}	84.62 ± 9.18^{a}
226 (I)	T/T	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	T/A	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
264 (I)	T/T	20	1.10 ± 0.32^{a}	0.91 ± 0.30^{a}	82.95 ± 16.51^{a}
	T/C	20	0.89 ± 0.37^{a}	0.77 ± 0.35^{a}	86.76 ± 11.37^{a}
441 (I)	T/T	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	T/A	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
477 (I)	T/T	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	T/A	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
499 (I)	A/A	10	1.10 ± 0.32^{a}	0.91 ± 0.30^{a}	82.95 ± 16.51^{a}
	A/C	20	$0.94 \pm 0.41^{a,b}$	0.83 ± 0.39^{a}	87.82 ± 12.40^{a}
	C/C	10	0.79 ± 0.26^{b}	0.67 ± 0.30^{a}	84.62 ± 9.18^{a}
500 (I)	T/T	10	1.10 ± 0.32^{a}	0.91 ± 0.30^{a}	82.95 ± 16.51^{a}
	T/C	20	$0.94 \pm 0.41^{a,b}$	0.83 ± 0.39^{a}	87.82 ± 12.40^{a}
	C/C	10	0.79 ± 0.26^{b}	0.67 ± 0.30^{a}	84.62 ± 9.18^{a}
519 (I)	A/A	10	1.10 ± 0.32^{a}	0.91 ± 0.30^{a}	82.95 ± 16.51^{a}
	A/G	20	$0.94 \pm 0.41^{a,b}$	0.83 ± 0.39^{a}	87.82 ± 12.40^{a}
	G/G	10	0.79 ± 0.26^{b}	0.67 ± 0.30^{a}	84.62 ± 9.18^{a}
540 (I)	A/G	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	A/A	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
545 (I)	G/G	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	G/A	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
589 (E)	A/G	20	0.97 ± 0.35^{a}	0.84 ± 0.32^{a}	87.38 ± 14.82^{a}
	A/A	20	0.91 ± 0.39^{a}	0.78 ± 0.37^{a}	84.23 ± 10.36^{a}
614 (E)	T/T	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	T/C	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}
618 (E)	G/G	20	0.97 ± 0.35^{a}	0.84 ± 0.32^{a}	87.38 ± 14.82^{a}
	G/A	20	0.91 ± 0.39^{a}	0.78 ± 0.37^{a}	84.23 ± 10.36^{a}
678 (E)	A/A	20	1.07 ± 0.38^{a}	0.90 ± 0.37^{a}	83.39 ± 14.02^{a}
	A/C	20	0.81 ± 0.30^{b}	0.72 ± 0.29^{a}	88.22 ± 11.11^{a}

The same superscripts indicated non-significant difference ($P > 0.05$). (I) = intron, (E) = exon; GSI = average gonadosomatic index; OW = average ovarian weight; BW = average body weight.
* Position on the *PmVtgr* gene segment after multiple alignments.

4.3. Association between SSCP/SNP genotypes and reproduction-related parameters of *P. monodon*

Domestication and selective breeding programs aim to increase commercially important traits in selected populations (Argue et al., 2002; Goyard et al., 2001; Goyard et al., 2002). Determining the relative effects between male and female broodstock quality on reproductive performance parameters will enhance the domestication of this species (Menasveta et al., 1993). The domestication and genetic selection program of *P. monodon* in Thailand is still at the beginning stage. The most crucial limitation of selective breeding programs in *P. monodon* is the reduced degrees of maturation and spawning potential in captivity. Although phenotypic improvement can be accomplished through conventional breeding programs, molecular markers linked to important traits is more efficiently applied for artificial selection processes based on the gene-assisted selection, GAS) approach (Liu and Cordes, 2004). Nevertheless, no validated marker linked to reproductive traits has been identified in penaeid shrimp up to date.

In the Japanese flounder (*Paralichthys olivaceus*), SNPs in the *CYP19a* gene were identified by PCR-SSCP and the genetic effect of observed SNPs on reproductive traits (i.e. serum testosterone, 17β-estradiol, hepatosomatic index, HSI and gonadosomatic index, GSI) was found

(He et al., 2008a). In addition, He et al. (2008b) further examined polymorphism of *estrogen receptor alpha* (*ER-α*) in *P. olivaceus* by the same approach. Twelve SNPs within eight exons and 3′UTR of *ER-α* and their association with reproductive traits were found. Fish carrying the diplotype (composite SNP) D1 had significantly higher HIS (females) and 17β-estradiol (E_2; males) than those carrying other eight diplotypes ($P < 0.05$). SNP P1 could be a potential quantitative trait marker for reproduction in this species. In addition, associations between SNPs in *estrogen receptor beta* (*ER-β*) and a putative *winged helix/forkhead transcription factor* (*FOXL2*) of *P. olivaceus* and serum estradiol levels and/or GSI were also illustrated (Shi et al., 2009a,b).

In this study, SSCP analysis was performed to examine polymorphism in the amplified fragment of *PmVtgr* (720 bp). Penaeid shrimp are diploid with the chromosome numbers of 2 $N = 88$–92 where *P. monodon* possesses 2 $N = 88$ (Benzie, 1998). No morphologically differentiated sex chromosomes are observed in penaeid shrimp. Nevertheless, Staelens et al. (2008) identified six sex-linked AFLP markers inherited from the female parents in each of the three mapping families evidencing a female heterogamy in *P. monodon*. In addition, a PCR-based allele-specific assay of a sex-linked EO6M45M347.0 marker was successfully developed and demonstrated a WZ-ZZ sex determination system in *P. monodon*. There has been no information whether *PmVtgr* presents singly in a haploid genome. Due to several point mutations found in the amplified gene segment (see below), SSCP patterns of *PmVtgr* in 14- and 19 month-old shrimp did not exhibit clear codominant segregation fashions. Therefore, genotype frequencies rather than allele frequencies were subjected to statistical analysis.

One of the disadvantages for association studies in *P. monodon* is that genetic diversity of different groups of natural shrimp was extremely high (Khamnamtong et al., 2009) and domestication of this species is still at the early stage. This reflected by non-overlapping SSCP patterns of *PmVtgr* established from different groups of domesticated samples (i.e. 14- and 19-month-old shrimp).

For association analysis, different SSCP patterns of *PmVtgr* were statistically tested against reproduction-related parameters (GSI and ovarian weight) of examined shrimp. Typically, GSI values are sufficiently accurate for the evaluation of ovarian developmental stages in shrimp (Marsden et al., 2007). In female *P. monodon*, different developmental stages of ovaries could simply be inferred from the GSI values (e.g. <1.5, 2–4, 4–6 and >6% for stages I–IV ovaries, respectively) (Preechaphol et al., 2010). However, captive 14- and 19-month-old *P. monodon* have the GSI value between 0.2–1.0% reflecting extremely low degrees of reproductive maturation under the culture conditions. Unilateral eyestalk ablation is used in practice to induce ovarian maturation in both wild and captive penaeid shrimp (Benzie, 1998; Okumura, 2004). Nevertheless, the induction effect is not equally effective in domesticated broodstock. The identification of phenotypic-related SNPs allows the selection of captive shrimp having higher GSI values of stage I ovaries resulting in more successful induction of ovarian and spawning without the sacrifice of domesticated *P. monodon* broodstock.

In this study, shrimp carrying different SSCP patterns/composite SNPs of *PmVtgr* (i.e. between patterns A and D) possessed significantly different GSI values in domesticated 14-month-old *P. monodon* ($N = 64$, $P < 0.05$). Results were also significant in 19-month-old shrimp where those exhibiting different SSCP genotypes of *PmVtgr* (i.e. between patterns E and F-H) also showed significant differences in both GSI values and ovarian weight ($N = 55$, $P < 0.05$). This critically indicated that *PmVtgr* is functional related with reproductive maturation and its polymorphism was associated with GSI values of *P. monodon*.

The amplified gene segment from 10 individuals for each genotype of *PmVtgr* found in 14-month-old shrimp were cloned and sequenced. Results revealed relatively high polymorphism in the amplified *PmVtgr* gene segment (57 substitutions with 15 SNPs). Association between SNP genotypes and phenotypic parameters were tested ($N = 40$). Shrimp exhibiting different SNP genotypes observed at positions 126 (C → T), 226 (T → A), 441 (T → A), 477 (T → A), 499 (A → C), 500 (T → C), 519 (A → G), 540 (A → G) and 545 (G → A) located in the intron and positions 614, (T → C) and 678 (A → C) located in the exon regions of the *PmVtgr* gene segment were significantly related with the average GSI values of 14-month-old domesticated shrimp. Nevertheless, correlations between SSCP/SNP genotypes and expression levels of ovarian *PmVtgr* mRNA in domesticated shrimp could not be assessed as its expression level was dramatically reduced compared with that in wild broodstock (data not shown). Additional experiments, for example, on the stability of mutations across a few generations and the affinity of wild-type and mutated PmVtgr protein (when non-synonymous SNPs are found) and vitellogenin, should be further carried out before SSCP/SNP genotypes of *PmVtgr* will be applied as a potential markers linked with reproduction-related phenotypes in *P. monodon*.

Genetic improvement and related biotechnological applications are crucial for the future development of the shrimp industry. Sustainable shrimp industry can be promoted through applications of molecular markers, particularly those related to economically important traits, to assist domestication and selective breeding programs of *P. monodon*. In this study, SSCP and SNP genotypes of *PmVtgr* significantly related with reproduction-related parameters of captive *P. monodon* were illustrated. Based on the fact that domestication and selective breeding programs of *P. monodon* in Thailand is still at the early stage, the availability of appropriate families of domesticated *P. monodon* are required for validation of SNP markers in this study. This will allow the direct application of phenotype-associated SNPs to assist genetic selection of this economically important species.

Acknowledgments

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