

# Molecular Characterization and Expression of Vitellogenin and Vitellogenin Receptor of *Thitarodes pui* (Lepidoptera: Hepialidae), an Insect on the Tibetan Plateau

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## Abstract

Vitellogenin (Vg) and vitellogenin receptor (VgR) play important roles in the vitellogenesis of insects. In this study, we cloned and characterized the two corresponding genes (*TpVg* and *TpVgR*) in an economically important insect, *Thitarodes pui* (Lepidoptera: Hepialidae), from the Tibetan plateau. The full length of *TpVg* is 5566 bp with a 5373 bp open reading frame (ORF) encoding 1,790 amino acids. Sequence alignment revealed that *TpVg* has three conserved domains: a Vitellogenin\_N domain, a DUF1943 domain, and a von Willebrand factor type D domain (VWD). The full length of *TpVgR* is 5732 bp, with a 5397 bp ORF encoding 1798 amino acids. BLASTP showed that *TpVgR* belongs to the low-density lipoprotein receptor (LDLR) gene superfamily. Structural analysis revealed that *TpVgR* has a group of four structural domains: a ligand-binding domain (LBD), an epidermal growth factor (EGF)-precursor homology domain, a transmembrane (TM) domain, and a cytoplasmic domain. In addition, *TpVgR* has four cysteine-rich LDL repeats in the first ligand-binding site and seven in the second. Quantitative real-time polymerase chain reaction analysis revealed that the expression levels of *TpVg* and *TpVgR* are much higher in later pupa than in either the larval or adult stage, implying that the synthesis and uptake of Vg in *T. pui* occurs in the later pupal stage. These results will help us to understand the molecular mechanism of the reproductive capacity and will provide new insight into the mass rearing and utilization of *T. pui*.

**Key words:** vitellogenin, vitellogenin receptor, *Thitarodes pui*

Like other animals, insects require large amounts of vitellogenin (Vg) to meet the nutritional needs of egg development. Vg, the precursor of vitellin and the major yolk protein, is usually synthesized in the fat body, released into the hemolymph, and subsequently taken up by the vitellogenin receptor (VgR) located on the external surface of competent oocytes (Tufail and Takeda 2008, 2009). Vg has been studied extensively in many insects of different orders. The identification of insect Vgs revealed that these Vgs belong to a large family of lipid transfer genes and that they share similar structural motifs, including an N-terminal lipid binding domain (LPD\_N), an unknown functional region (DUF1943), and a von Willebrand factor type D similar domain (VWD) (Trewitt *et al.* 1992, Chen *et al.* 1994, Yano *et al.* 1994a, Veerana *et al.* 2014, Upadhyay *et al.* 2016, Zhao *et al.* 2016, Ibanez *et al.* 2017).

VgR, the key element in the process of Vg uptake, plays an important role during vitellogenesis. To date, the molecular characteristics of VgRs have been well documented in vertebrates and invertebrates including many orders of insects, such as Diptera (Schonbaum *et al.*

1995, Cho and Raikhel 2001), Lepidoptera (Shu *et al.* 2011, Qian *et al.* 2015, Zhang *et al.* 2016), Hemiptera (Upadhyay *et al.* 2016), Coleoptera (Roy-Zokan *et al.* 2015), Hymenoptera (Chen *et al.* 2004), and Blattaria (Tufail and Takeda 2007, 2008). The deduced amino acid (aa) sequences of these VgRs indicate that they are members of the low-density lipoprotein receptor (LDLR) family. In general, the LDLR family members have five distinct, conserved motifs, including a ligand-binding domain (LBD) containing LDLR Class A cysteine-rich repeats, an epidermal growth factor (EGF)-like domain containing LDLR Class B cysteine-rich repeats and YWXD repeats, an O-linked carbohydrate domain with a serine/threonine-rich track, a transmembrane (TM) domain, and a cytoplasmic tail (Tufail and Takeda 2007, 2009; Shu *et al.* 2011; Upadhyay *et al.* 2016; Zhang *et al.* 2016).

*Thitarodes pui* (Lepidoptera: Hepialidae) is naturally distributed in Segrila Mountain of the Tibetan Plateau, which is characterized by a harsh climate with low temperatures and hypobaric hypoxia. *T. pui* larvae are the hosts for the famous

traditional Chinese medicinal caterpillar fungus, the ascomycete *Ophiocordyceps sinensis* (Berk.) Sung, Sung, Hywel-Jones, and Spatafora (syn. *Cordyceps sinensis*).

Once infection occurs, the fungus grows within the body cavity of the insect larvae and finally kills and mummifies them in their underground burrows. A fruiting fungal body emerges from the front end of the caterpillar and protrudes from the ground in spring and early summer (Buenz *et al.* 2005). The medicinal activity is derived from the parasite complex of the mummified caterpillar and the fungal stromata (Zhang *et al.* 2009).

The complete life cycle of *T. pui* lasts at least 3 yr under natural conditions (Li *et al.* 2011). A better understanding of the molecular mechanisms regulating reproduction can indicate potential approaches for the mass rearing of these economically important insects. Vg and VgR are important molecular markers for insect fecundity. In this study, we cloned the full lengths of vitellogenin (*TpVg*) and the vitellogenin receptor (*TpVgR*) of *T. pui*, characterized the molecular structure of the corresponding deduced proteins, and analyzed their gene expression profiles at different developmental stages. Our results are expected to provide the theoretical basis for the conservation and utilization of this economically important insect.

## Materials and Methods

### Insect Rearing and Collection

*T. pui* samples were collected in the Tibetan Plateau Peculiar Bioresources Research Station of Sun Yat-sen University at Nyingchi in the Tibet Autonomous Region (4,156 m altitude, 29°36'N, 94°36'E) from June to August in 2014. The larvae were reared as previously described (Sun *et al.* 2011, Wu *et al.* 2015). The larvae of different instars (third through eighth), female pupae of different ages (1, 9, 17, and 33 d old), and 1-d-old adult females were used for the analysis of gene expression. All of the samples at different developmental stages were freshly frozen in a Sample Protector (Takara, Tokyo, Japan) at -80°C until RNA isolation.

### RNA Isolation and cDNA Synthesis

Total RNA was extracted from the whole body of *T. pui* using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and RNA purity and degradation were checked on 1% agarose gels and Bioanalyzer 2100 system (Agilent Technologies). For tissue-specific expression profiles, total RNAs were extracted from the thorax and abdomen parts of newly emerged female adults which were stored separately in a refrigerator at -80°C. The RNA sample was dissolved in 20 µl diethylpyrocarbonate (DEPC)-treated H<sub>2</sub>O and assessed by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, DE). A PrimeScript Reagent Kit with gDNA Eraser (TaKaRa, Tokyo, Japan) was used to synthesize first-strand complementary DNA (cDNA) with 1 µg total RNA in a 20 µl reaction mixture following the manufacturer's recommendations.

### Molecular Cloning of *TpVg* and *TpVgR*

Two partial sequences of both Vg and VgR were identified from the pupal transcriptome of *T. pui* (unpublished data). The fragment sequences of *TpVg* and *TpVgR* were further verified by polymerase chain reaction (PCR) amplification using the primers listed in Supplementary Table 1. The PCR program was performed as follows: one cycle of predenaturing at 95°C for 5 min; 32 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min; and then 72°C for 5 min for elongation. The PCR products were then gel purified, cloned into

the pGEM-T vector (Takara, Dalian, China), and transformed into *Escherichia coli* DH5α cells for amplification. Positive clones were assessed by PCR and sequenced. To obtain the full-length cDNA sequences of *TpVg* and *TpVgR*, a SMARTer RACE (rapid amplification of cDNA ends) cDNA Amplification Kit (Clontech) was used according to the manufacturer's instructions. The first round of RACE-PCR was performed using outer primer (Supplementary Fig. 1 and Supplementary Table 1) and a universal primer mix (UPM) with the following protocol: one cycle of predenaturing at 94°C for 5 min; 35 cycles of 30 s at 95°C, 30 s at 65°C, and 2 min at 72°C; and a final extension at 72°C for 5 min. The second round of RACE-PCR was performed using inner primers (Supplementary Fig. 1 and Supplementary Table 1) and a nested universal primer (NUP). The second RACE-PCR protocol was performed as follows: one cycle of predenaturing at 94°C for 5 min; 35 cycles of 30 s at 95°C, 30 s at 68°C, and 2 min at 72°C; and a final extension at 72°C for 5 min. The RACE products were gel purified and sequenced as described above.

### Sequence Comparisons and Phylogenetic Analysis

The sequence similarities were analyzed using the online BLASP program on the NCBI website (<https://blast.ncbi.nlm.nih.gov/>). The open reading frames (ORFs) of *TpVg* and *TpVgR* were predicted using the NCBI ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The molecular weights and isoelectric points (pIs) of the deduced protein sequences were predicted using the ExPASy proteomics server (<http://www.expasy.org>). The domain architecture and the conserved domains were analyzed using the online servers of Scan-Prosite (<http://prosite.expasy.org/scanprosite/>), SMART (<http://smart.embl-heidelberg.de/>), and InterProScan (<http://ebi.ac.uk/Tools/pfa/iprscan/>). The signal peptide was predicted by using the SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>). The potential O-linked glycosylation sites were evaluated using the GPP Prediction Server (<http://comp.chem.nottingham.ac.uk/glyco/>), and the TM regions were predicted using the TMHMM server v2.0 (<http://cbs.dtu.dk/services/TMHMM/>). All the computational programs were run with the recommended default values set up by the programs. The phylogenetic tree was constructed with MEGA 6.0 by using the neighbor-joining method with a bootstrap of 1,000 replicates.

### Developmental Expression Profile of *TpVg* and *TpVgR*

Equal quantities of cDNA (1 µg) from different developmental stages were used for a relative expression analysis of *TpVg* and *TpVgR* messenger RNA (mRNA) using a real-time PCR machine (LightCycler 480 real-time PCR machine, Roche Applied Science, Switzerland) with SYBR Green (Invitrogen). β-actin was used as an internal control based on a previous study (Sun *et al.* 2011). Primers used for quantitative PCR (qPCR) are listed in Supplementary Table 1. The qPCR amplifications were conducted in a total volume of 10 µl, including 1 µg of cDNA, 5 µl of SYBR Green I Master mix, 0.5 µl of each primer (10 µM), and a certain amount of ddH<sub>2</sub>O. The qPCR program included one cycle of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 20 s. To ensure reliability, each sample included three biological replicates. The melt curve genotyping analysis was performed to confirm the homogeneity of the PCR products. Reactions were performed in triplicate for each sample, and gene expression levels were normalized against *T. pui* β-actin according to the 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen 2001).

## Results

### Sequence and Structural Analysis of *TpVg* and *TpVgR*

The full sequence of *TpVg* was 5566 bp, including an 83 bp 5' untranslated region (UTR), a 5373 bp ORF encoding 1,790 aa residues, and a 110 bp 3' UTR (GenBank accession no. MF622538). The theoretical molecular weight was 204.43 kDa, and the pI was 6.51. A BLASTP analysis showed that the aa sequence of *TpVg* had a 30 to 40% similarity with other insects from different orders. *TpVg* showed a 34% and 33% similarity with *Operophtera brumata* and *Actias selene*, respectively. As with other insects, *TpVg* in *T. pui* also contained the typical conserved domains of the Vg protein.

Domain architecture analysis by SMART showed that the *TpVg* aa sequence has three conserved domains (Fig. 1): the vitellogenin\_N domain, the DUF1943 domain, and the VWD. A signal peptide with 18 amino acids (MRTFVLVALLATAFCANS) was found in the analysis of the predicted aa sequence of *TpVg*. In addition, the deduced aa sequences of *TpVg* included four N-glycosylation sites (NXS/T) and seven K/RXXR cleavage sites.

The full sequence of *TpVgR* was 5732 bp, including a 74 bp 5' UTR, a 5397 bp ORF encoding 1,798 aa residues, and a 261 bp 3' UTR (GenBank accession no. MF622539). The theoretical molecular weight was 200.47 kDa, and the pI was 5.46. The analysis of the predicted aa sequence revealed that a signal peptide with 27 amino acids (MGHADLLHIALIIVNVFLKWKMAGA) was located at the N-terminal of *TpVgR*.

The protein sequence analysis indicated that *TpVgR* contained the characteristic features of the LDLR family (Fig. 1). *TpVgR* showed two LBDs with four class A (LDLa) cysteine-rich repeats in the first domain and eight repeats in the second domain. Each repeat had six cysteine residues, and each LDLa was followed by an EGF-like domain with/without a calcium-binding region. The EGF-like domain contained repeats of the LDLR YWTD motif. After the second EGF-like domain, a TM domain spanning amino acids 1674–1691 and a cytoplasmic domain spanning amino acids 1692–1798 were predicted by the TMHMM server v. 2.0.

### Phylogenetic Analyses of *TpVg* and *TpVgR*

A phylogenetic tree was constructed based on the distances of aa sequences of *TpVg* and *TpVgR*. The phylogenetic tree showed that *TpVg* and Vgs of other Lepidoptera species clustered together (Fig. 2). Vgs from the same insect order also clustered in the same group, implying the conservation of Vgs and the closer relationships within the same taxonomic status. A similar phylogenetic tree was constructed by comparing *VgR* aa sequences from different groups of insects (Fig. 3). *TpVgR* and other *VgRs* of moth species formed a

cluster. Other *VgRs* from the same insect orders were also clustered. In the phylogenetic tree of *VgRs*, Lepidoptera *VgRs* formed a single branch and was separated from other species (Fig. 3). However, the dendrogram of Vgs showed that the Vgs of Diptera and Lepidoptera species were close together (Fig. 2), suggesting that the Vgs of those two insect orders share a closer ancestry than other insects.

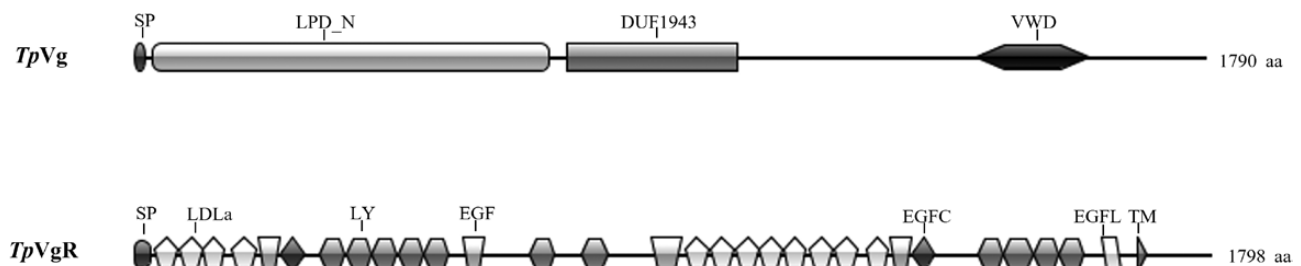
### Expression Profile of *TpVg* and *TpVgR*

The expression analysis of *TpVg* and *TpVgR* was conducted during the larval, pupal, and adult stages by real-time PCR (Fig. 4). The expression level of *TpVg* was normalized with a reference gene,  $\beta$ -actin of *T. pui*. The results showed that *TpVg* had no detectable expression in the larval stage, but it had a high expression during the pupae stage, decreasing significantly in adult females (Fig. 4). The expression of *TpVg* began to increase in the pupal stage and reached its peak in the 25 and 33-d-old pupae, i.e., the final stage of pupae. Then, the expression level of *TpVg* decreased significantly in the adult stage. *TpVgR* had an expression profile similar to that of *TpVg*. *TpVgR* was found to have lower expression in the early pupal stage and then a remarkably high expression in the 17-d-old pupae, subsequently reaching a maximum expression level in the later pupae stage. Then, a lower expression level was maintained in the adult period. In addition, the tissue-specific expression profile revealed that *TpVg* and *TpVgR* were highly expressed in the abdomen which contained the reproductive organ-ovary (Fig. 5).

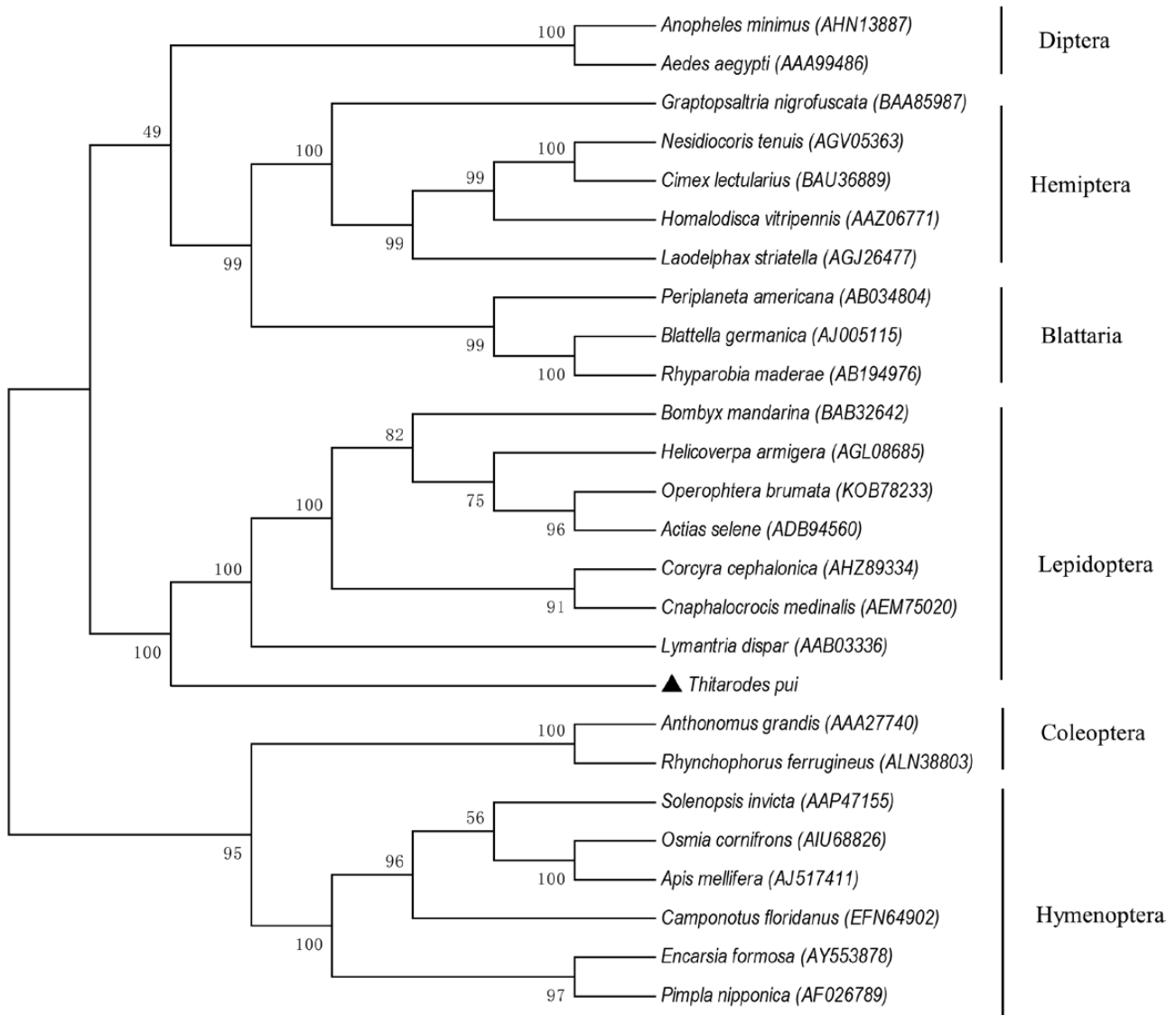
## Discussion

*T. pui* is a holometabolous insect with a long life history. The complete life cycle includes 41–47 d egg stage, 990–1,350 d larval stage, 35–41 d pupal stage, and 3–8 d adult stage (Li *et al.* 2011). The reproductive success of all species, including insects, relies on Vg biosynthesis and its uptake by *VgR* in the developing oocytes. As an important host of traditional Chinese medicine caterpillar fungus, it is of great significance to study the Vg and *VgR* in *T. pui*.

In this study, the full-length cDNA corresponding to the *TpVg* and *TpVgR* genes was obtained, and the molecular characteristics of these two genes were also analyzed. The GL/ICG, DGXR, and K/RXXR motifs, considered to be the conserved domains, have been reported in many insect Vg proteins (Sappington and Raikhel 1998, Tufail and Takeda 2008, Upadhyay *et al.* 2016). In earlier studies, the GL/ICG motif was assumed to be essential for the proper function of Vg during embryogenesis (Tufail *et al.* 2001). As is the case with other insects, a GLCG motif was found in the aa sequence of *TpVg* (Supplementary Fig. 2). Apart from the class Insecta, the GLCG motif was also present in Arachnoidea species, such as *Tetranychus urticae* (Kawakami *et al.* 2009), *Panonychus citri*



**Fig. 1.** Conserved domain architecture of vitellogenin (*TpVg*) and vitellogenin receptor (*TpVgR*) in *T. pui*. SP, signal peptide; LPD-N, lipoprotein N-terminal domain; DUF1943, domains of unknown function protein families; VWD, von Willebrand factor type D domain; LDLa, low-density lipoprotein receptor domain class A; EGF, epidermal growth factor-like domain; EGFC, calcium-binding EGF-like domain; LY, low-density lipoprotein receptor YWTD protein; EGFL, epidermal growth factor-like domain.



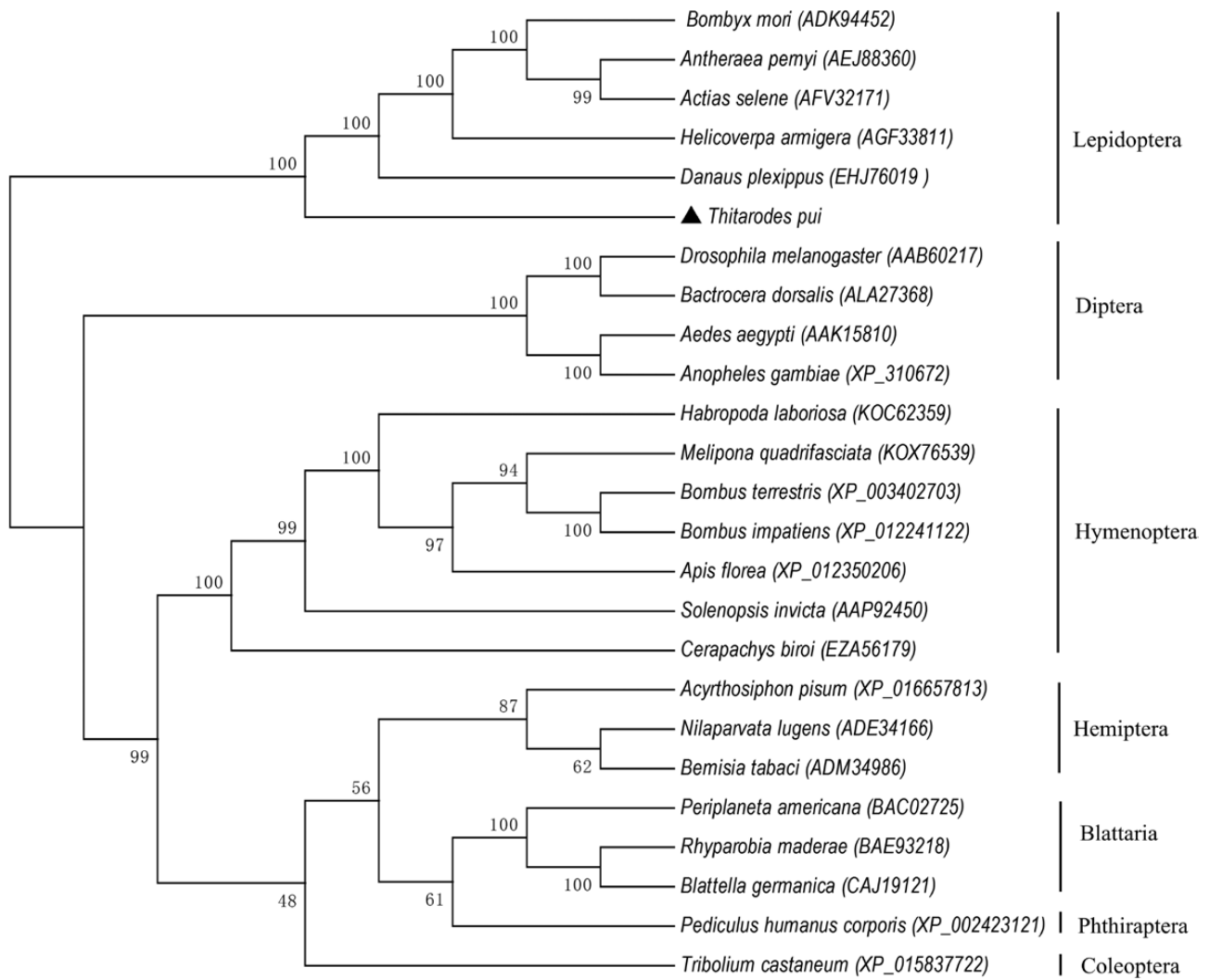
**Fig. 2.** Phylogenetic analysis of vitellogenin in *T. pui* with that of insects from different orders. The phylogenetic tree was constructed by Mega 6 with a statistical method of neighbor joining. Numbers indicate bootstrap support values (%) based on 1,000 replicates. The numbers in the brackets indicate the GenBank accession for each insect.

(Zhong *et al.* 2015), and *Ornithodoros moubata* (Horigane *et al.* 2010). A DGXR motif is usually located 17–19 residues upstream of the GL/ICG motif in most insect Vg sequences (Tufail and Takeda 2008), but that motif was not found in *Rhyparobia maderae* or in *T. pui* in this study (Supplementary Fig. 2). In addition, some aa residues, such as E (acidic), G, T and P (hydrophobic), and Y (hydrophilic) were also highly conserved at the C-terminus of all the tested Vgs (Supplementary Fig. 3). However, the specific roles of these conservative sites remain unclear.

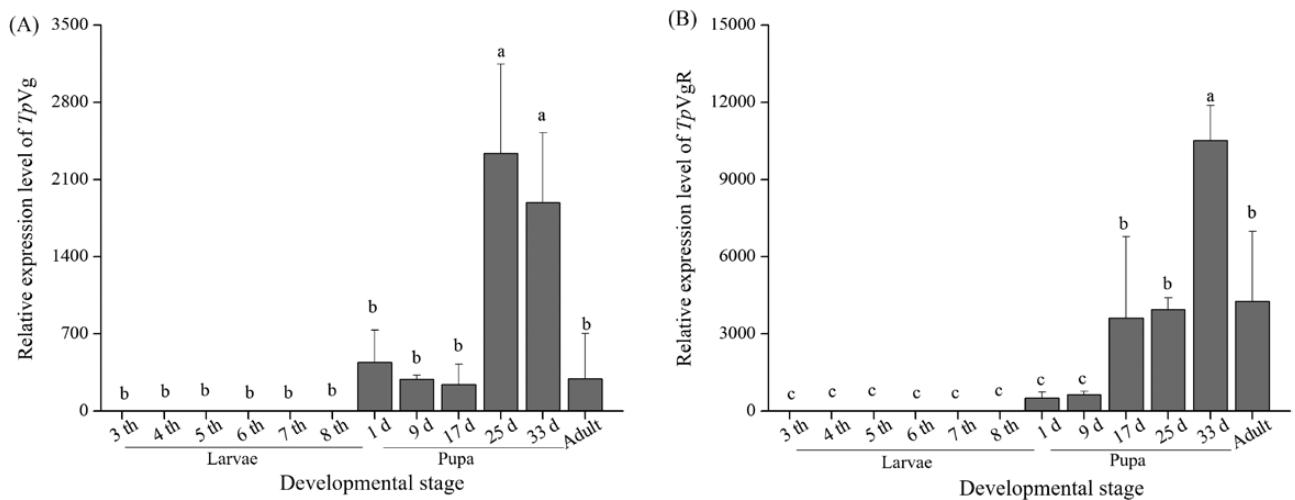
*TpVgR* also showed sequence similarities with other insect VgRs. The phylogenetic analysis indicated that *TpVgR* shares a homology with other Lepidopteran VgRs, such as *Bombyx mori*, *A. selene*, *Helicoverpa armigera*, *Danaus plexippus*, and *Antheraea pernyi* (Fig. 3). An analysis of the domain conservation of *TpVgR* suggests that *TpVgR* is a member of the LDLR family and shares several modular elements with other insect Vgs. *TpVgR* was shown to contain the classical LBDs, EGF-like domains, and a TM domain. *TpVgR* was found to have two ligand-binding sites that were characterized by cysteine-rich LDLa repeats. Most insect Vgs have two to

four LDLa repeats in the first ligand-binding site and seven to eight in the second (Tufail and Takeda 2009, Lu *et al.* 2015). There were four LDLa repeats found in the first ligand-binding site of *T. pui*, which is a typical feature in Lepidoptera species (Shu *et al.* 2011, Lin *et al.* 2013, Zhang *et al.* 2016). However, *TpVgR* bore eight LDLa repeats in the second ligand-binding site, which differs from other Vgs of Lepidoptera species; they generally have seven LDLa repeats. There are also eight LDLa repeats in the second ligand-binding site of the VgRs in other insects, including species of Hymenoptera, Coleoptera, Diptera, Hemiptera, Blattaria, which are also found in Acarina species (Mitchell *et al.* 2007, Boldbaatar *et al.* 2010, Zhong *et al.* 2015).

In some insect VgRs, the O-linked sugar domain (OLSD), which is rich in S and/or T residue, is always between the last EGF precursor domain and the TM domain (Tufail and Takeda 2007). No OLSD was found in *TpVgR*, which is consistent with VgRs of *Solenopsis invicta*, *H. armigera*, and *Drosophila melanogaster* but not with *B. mori*, *A. pernyi*, and *Nilaparvata lugens* (Chen *et al.* 2004, 2010; Lu *et al.* 2015; Zhang *et al.* 2016), implying that the

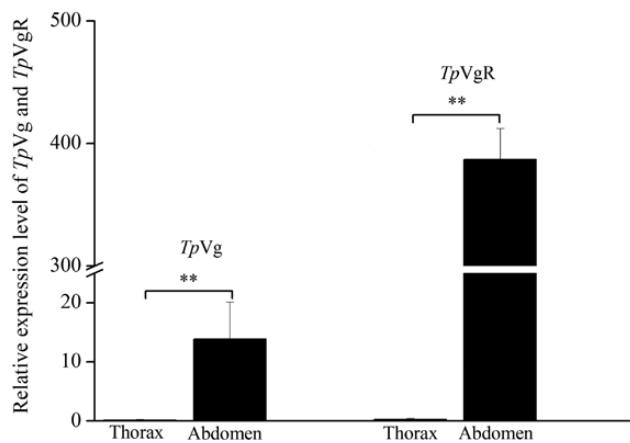


**Fig. 3.** Phylogenetic analysis of the vitellogenin receptor in *T. pui* with that of insects from different orders. The phylogenetic tree was constructed by Mega 6 with a statistical method of neighbor joining. Numbers indicate bootstrap support values (%) based on 1,000 replicates. The numbers in the brackets indicate the GenBank accession for each insect.



**Fig. 4.** Relative expression of *TpVg* and *TpVgR* at different developmental stages. Samples include cDNA from third, fourth, fifth, sixth, seventh, and eighth instar larvae, 1-d-old pupae, 9-d-old pupae, 17-d-old pupae, 25-d-old pupae, 33-d-old pupae, and 1-d-old female adults. The mRNA levels were normalized using  $\beta$ -actin as a reference gene. Different letters indicate statistically significant differences at  $P < 0.05$  (One-way ANOVA followed by LSD multiple comparison tests). The bars represent SD ( $n = 3$ ).





**Fig. 5.** Relative expression of *TpVg* and *TpVgR* in the thorax and abdomen of *T. pui*. The mRNA levels were normalized using  $\beta$ -actin as a reference gene. Double asterisks indicate statistically significant differences at  $P < 0.01$  (Student's *t*-test). The bars represent SD ( $n = 3$ ).

motif is not a typical feature in insect VgRs. OLSDs have also been shown to be absent in vertebrate VgRs (Prat *et al.* 1998, Hiramatsu *et al.* 2004).

In the current study, *TpVg* and *TpVgR* had similar expression patterns (Fig. 4). The transcription profiles of *TpVg* and *TpVgR* showed that the mRNA levels of these two genes began to increase in the pupae stage and reached their peak during that stage; then expression decreased after emergence. The difference in transcription lay in the fact that *TpVg* reached its peak in the 25-d-old pupae, whereas *TpVgR* reached its peak in the 33-d-old pupae, demonstrating that the maximum expression of *TpVgR* occurs later than that of *TpVg*. Despite the different expression profiles in different species, the expression peaks of Vgs and VgRs of insects primarily occur in the pupae or adult stage. For example, *B. mori* Vg first appeared in the sixth instar larvae and ended in the late pupal stage (Yano *et al.* 1994a,b). In *Spodoptera litura*, the Vg mRNA was first detected in late pupal stage and reached its maximum expression in adult females 2 d after emergence (Shu *et al.* 2009).

The expression of VgR is closely related to ovarian development, which has been investigated in many species. In *Periplaneta americana*, *Leucophaea maderae*, *N. lugens*, and *S. litura*, the VgR transcript level was low until it increased during the early ovarian development, reaching its peak during the vitellogenic period, after which expression was subsequently low again (Tufail and Takeda, 2005; Tufail and Takeda 2007, Shu *et al.* 2011, Lu *et al.* 2015). The expression of *TpVgR* mRNA appeared to peak on the 33rd d of the pupae, and it distinctly declined in the adults (Fig. 4), indicating that the vitellogenic period of *T. pui* occurs in the late pupae stage before emergence.

## Conclusion

In conclusion, we identified the cDNAs and analyzed the molecular characteristics of *TpVg* and *TpVgR*. Those two genes were structurally conserved when compared with the analogous genes of other species. The development expression profiles indicated that the synthesis and uptake of Vg in *T. pui* occur in the late pupal stage. Our study will provide insights for the molecular regulation of high fecundity in *T. pui*. In the future, we will focus on how *TpVg* interacts with *TpVgR* to regulate oocyte maturation.

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## Conflict of interest

These authors have no conflict of interest to declare.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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