Selection of Reference Genes for Quantitative
Real-Time PCR in Chrysoperla nipponensis (Neuroptera: 
Chrysoptidae) Under Tissues in Reproduction and 
Diapause

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Abstract

Chrysoperla nipponensis (Okamoto), which has the unique diapause phenotype distinguishable from nondiapause 
adult, is an ideal model organism for studying the mechanism of reproductive diapause. However, there is no 
reliable and effective reference genes used for the reproductive diapause study of C. nipponensis. Therefore, in 
this study, we evaluated the expression stability of 10 candidate reference genes (Tub1, ArpC5, EF1a, 128up, RpS5, 
RpS26e, GAPDH, Arp3, Actin, α-Tub) in adults under diapause and nondiapause induction conditions using four 
statistical algorithms including GeNorm, NormFinder, Bestkeeper, and ∆CT method. Results showed that Arp3 and 
Tub1 were the most stable reference genes in all samples and in the adult tissues group. Arp3 and RpS5 were the 
most stable reference genes in the development degree group. α-Tub and EF1a were unstable reference genes 
under the conditions of this study. Meanwhile, to verify the reliability of the reference genes, we evaluated the 
relative expression levels of Vg and VgR in different treatments. Significant upregulation and downregulation in 
expression level of two genes in response to diapause termination and diapause fat body tissue was, respectively, 
observed when using Arp3 as the reference gene but not when using an unstable reference gene. The reference 
genesis identified in this work provided not only the basis for future functional genomics research in diapause of 
C. nipponensis and will also identify reliable normalization factors for real-time quantitative real-time polymerase 
chain reaction data for other related insects.

Key words: Chrysoperla nipponensis (Okamoto), reference genes, qRT-PCR, reproduction, diapause

Due to the advantages of high sensitivity, rapidity, specificity, and accuracy (Bustin et al. 2005, Valasek et al. 2005, Vanguilder et al. 2008, Shakeel et al. 2018), quantitative real-time polymerase chain reaction (qRT-PCR) has been widely used in the study of animals, plants, and microorganisms (Roy et al. 2007, Jia et al. 2014, Zhang et al. 2014, Ding et al. 2017, Sun et al. 2019). qRT-PCR is the most commonly used method for the expression analysis of target 
genesis. However, the reliability of qRT-PCR results in different 
samples is determined by a variety of factors, among which the use of stably expressed reference genes is an important link for accurate detection of gene expression changes by qRT-PCR (Bustin et al. 2009). At present, several commonly used reference genes for data 
normalization include tubulin, actin, ribosomal protein, elongation factor 1α, glyceraldehyde-3-phosphate dehydrogenase, 18S ribo-

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algorithms, and the most suitable reference gene was selected for the target gene expression analysis (Xiao et al. 2015, Kang et al. 2017). *Chrysoperla nipponensis* (Okamoto), as one of the important predatory natural enemies of agricultural and forestry pests, prefers to eat aphids, thrips, and other pests (Okamoto 1914, Nie et al. 2012). Because of its characteristically wide geographical distribution and broad range of host prey (Niijima 1997, Syed et al. 2008), it has good prospects for widespread application in biological control (McEwen et al. 2001, Memon et al. 2016). Reproductive diapause is an important way for *C. nipponensis* adults to escape from adverse environments (Xu et al. 1999). At present, there have been many reports on the diapause of *C. nipponensis*. Xu et al. (2002) found that the body color of *C. nipponensis* was green during the reproductive period, but turned brown and yellow during the diapause period. *Chrysoperla nipponensis* belongs to the photoperiodic sensitive insect; the adult diapause was induced by short photoperiods (Xu et al. 2004). Chen et al. (2013) found that different photoperiods affected the material content (e.g., protein and glycogen) of *C. nipponensis*. Diapause induced by the short photoperiod was beneficial to the storage of *C. nipponensis* (Chen et al. 2017). We expect an exponential increase of diapause research on *C. nipponensis* at the molecular level in the near future. Thus, stable and reliable reference genes are important for accurately quantifying gene expression of *C. nipponensis*.

Ribosomal proteins and ribosomal RNA have been used as reference genes in previous diapause studies. For example, Williams et al. (2006) used Ribosomal protein 49 (*RP49*) as a reference gene to study the natural variation of *Drosophila melanogaster* diapause (Williams et al. 2006), and Sim and Denlinger (2009) used Ribosomal protein large subunit 19 (*Rpl19*) as a reference gene in a study of ovarian development of *Culex pipiens* during overwintering diapause (Sim and Denlinger 2009). This indicates that under the same experimental conditions, the selected reference genes in different species research are also different.

In this research, 10 candidate reference genes were selected, including *Tub1*, *Arpc5*, *EF1α*, *12S rRNA*, *Rps5*, *Rps26e*, *GAPDH*, *Arp3*, *Actin*, and *α-Tub*, whose expression profiles were measured by the qRT-PCR. The stability was analyzed by four statistical algorithms (GeNorm, NormFinder, Bestkeeper, and ∆CT method) in different developmental stages (reproductive and diapause) of adults and among different tissues. The optimal reference genes under different conditions were determined, which contributed to the accurate expression of target genes for future research.

**Materials and Methods**

**Insect**

A stable population of *C. nipponensis* was maintained in an artificial climate chamber (RSZ intelligent artificial climate chamber, Changzhou Guohua, Jiangsu province) under the following conditions: 25 ± 1°C temperature, 70 ± 5% relative humidity (RH), and long photoperiod of 15: 9 (L:D) h in our laboratory. The eggs were produced from the artificial climate chamber. The adults used in this study were kept under the conditions of short photoperiod of 9:15 (L:D) h in all processes from eggs, larvae, pupae to adults, whereas the nondiapause adults were kept under the conditions of long photoperiod of 15: 9 (L:D) h.

**Sample Collection**

1. **Development degree.** Samples from individuals from varying developmental stages included female adults in the diapause induction period (5–10 d under the short photoperiod), the diapause maintenance period (20–30 d under the short photoperiod), the diapause termination period (60–70 d under the short photoperiod), and the reproduction period (10–15 d under the long photoperiod). Each sample, which included three to four females, was independently replicated three times as three biological replicates.

2. **Adult tissues (reproduction).** Seven different tissues were collected from reproductive adults, including ovarian, fat body, head, wings, antennae, front thorax, and epidermis. The tissues of reproductive adults under long photoperiod were collected on the 10th day after emergence. Each tissue required about 10–20 mg of material, and each tissue sample collection was independently replicated three times as three biological replicates.

3. **Adult tissues (diapause).** Seven different tissues were collected from diapause adults, including ovarian, fat body, head, wings, antennae, front thorax, and epidermis. The tissues of diapause adults under short photoperiod were collected on the 20th day after emergence. Each tissue required about 10–20 mg of material, and each tissue sample collection was independently replicated three times as three biological replicates.

4. **Adult tissues.** Samples from reproductive and diapause adult tissues.

5. **All samples.** Samples from group 1), 2), and 3).

All treatments were immediately frozen with liquid nitrogen and stored in an ultra-low temperature refrigerator at −80°C prior to RNA extraction.

**Total RNA Extraction and cDNA Synthesis**

In this study, total RNA was extracted using MiniBEST Universal RNA Extraction Kit (Takara, Japan), and DNase I was used for digestion of the membrane. RNA integrity was estimated by 1% agarose gel electrophoresis, RNA concentration, and purity were measured with a NanoDrop One spectrophotometer (Thermo Scientific). Then, 1-μg RNA was reverse-transcribed into the first-strand complementary DNA (cDNA) according to the HiScript II Q RT SuperMix for qPCR (+ gDNA wipers; Vazyme, Nanjing, China) instructions, and stored at −20°C. All cDNA was diluted 10-fold with DNase/rnase-free sterile water before use.

**Candidate Reference Gene Selection and Primer Design**

According to several commonly used reference genes, 10 candidate reference gene sequences were obtained by screening from the existing transcriptome of *C. nipponensis* in the laboratory, namely, *Tub1*, *Arpc5*, *EF1α*, *12S rRNA*, *Rps5*, *Rps26e*, *GAPDH*, *Arp3*, *Actin*, and *α-Tub*. In order to ensure the predictive accuracy of the selected sequences, we conducted BLAST alignment. All primers were designed using Primer Premier 5 based on the following criteria: GC content 45–55%, annealing temperature 55–65°C and primers length 18–24 bp, and the specificity of each pair of amplicons was determined by qRT-PCR followed by 2% agarose gel electrophoresis and melting curve analysis. The amplification efficiency of the PCR was calculated by using the formula $E = (10^{(−1/\text{slope})})−1)*100%$. The slope was obtained by the standard curve, which was generated by qRT-PCR of a series of continuously diluted cDNA samples.
Real-Time qRT-PCR Analysis
The 20-µl total reaction volume were configured according to the protocol of ChamQ SYBR qPCR Master Mix (Vazyme), contained 10-µl 2 × ChamQ SYBR qPCR Master Mix, 0.4 µl (10 μM) of each gene specific primer, 1 µl of cDNA, and 8.2 µl of ddH2O. The amplification reaction program was set as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s. The melting curves were analyzed in the 60–95°C temperature range after amplification step. The reaction was performed on a Roche LightCycler96 instrument to obtain CT values, amplification curves, melting curves, and standard curves. All samples were carried out in four technical and three biological replicates, and the negative control (no template) was performed in parallel.

Data Analysis
CT values for all samples were exported into an excel spreadsheet and were used to analyze the stability of candidate reference gene expression by GeNorm, NormFinder, Bestkeeper, and ΔCT method. The comprehensive ranking were performed following methods adapted from (Xiao et al. 2015). The optimal number of genes was determined by the pairwise variation (Vn/n+1) between the normalization factors calculated by GeNorm. Among the four algorithms, GeNorm and NormFinder need to convert the original CT value according to the corresponding requirements before analysis. In GeNorm, the stability ranking of genes was determined by the expression stability value (M value). In BestKeeper, the stability ranking of genes was determined by the coefficient of variation (CV) and SD. In NormFinder, the stability ranking of genes was determined by the gene expression stability value (SV). In ΔCT method, the stability ranking of genes was ranked according to the SD of genes ΔCT values. In four statistical algorithms, genes with the lowest value were the most stably expressed.

Validation of Reference Genes
In most insects, vitellogenin (Vg) and vitellogenin receptor (VgR) play important roles in the reproductive process of female insects. Vg is taken up by developing oocytes through receptor-mediated endocytosis (RME), thereby promoting the development of oocytes and the formation of eggs. In this process, VgR is the main receptor mediating endocytosis. Previous studies have shown that reproductive diapause arrests development of oogenesis and vitellogenesis (Tatar and Yin 2001), and the expression levels of the Vg and VgR in nondiapause female were significantly higher than those in reproductive diapause female (Jiang et al. 2020). In order to evaluate the effectiveness of the selected reference genes, the expression levels of the target genes Vg and VgR were, respectively, detected by qRT-PCR in the different development degree and tissues of adults, and the most unstable reference gene was used for comparison in parallel. The reaction system and program were the same as for qRT-PCR of reference genes, and four technical and three biological replicates were performed for each treatment. The relative expression levels of Vg and VgR were, respectively, calculated in EXCEL using the 2^−∆∆CT method. The differences of target genes’ expression levels were analyzed by Tukey’s test using SPSS 22.0 software (SPSS Inc.) under different experimental conditions.

Results
Selection and Primer Performance of Candidate Reference Genes
The 10 candidate reference genes including Tub1, Arp3, EF1a, 128up, RpS5, RpS26e, GAPDH, Arp3, Actin, and α-Tub were selected to identify the normalization factors for qRT-PCR analysis, and the sequence information has been submitted to GenBank, and the accession numbers are shown in Supp Table 1 (online only). To determine the amplification specificity of the primers, agarose gel electrophoresis and melting curve analysis were performed. All primer pairs showed a single band and a single peak (Fig. 1). To obtain correlation coefficient (R^2) and amplification efficiency (E) of PCR, a standard curve was generated with the 10-fold dilution series of cDNA. The results showed that the amplification efficiency ranged from 92.90 to 109.91%, and the correlation coefficient varied from 0.9838 to 0.9983 (Supp Table 1 [online only]).

Expression Profiling of Candidate Reference Genes
To evaluate expression levels of the 10 candidate reference genes, the cycle threshold (CT) values under different groups were obtained by qRT-PCR and represented by box-plot (Fig. 2). The results indicated that CT values of all candidate reference genes were different under different conditions and also varied under the same condition. Overall, CT values ranged from 14.42 to 35.46. Among them, the genes with higher expression abundance were Tub1 (15.62–18.54), RpS26e (16.18–19.91), and Actin (14.42–21.5), followed by 128up (20.32–24.07), Arp3 (22.92–24.71), Arp5 (22.02–25.91), RpS5 (22.41–26.29), and EF1a (20.65–29.22). The genes with lower expression abundance were α-Tub (26.22–35.46) and GAPDH (30.94–34.07). According to the CT value range of each candidate reference gene, the genes with relatively stable expression were Tub1 and Arp3, whereas the most unstable genes were EF1a, Actin, and α-Tub.

Expression Stability of Candidate Reference Genes Under Different Conditions
In this study, four statistical algorithms were used to analyze the expression stability of the candidate reference genes under different conditions, including GeNorm, NormFinder, Bestkeeper, and ΔCT method, as different statistical algorithms would generate different ranking patterns, the comprehensive ranking of genes was finally determined through the geometric mean of sequencing. Bestkeeper, the original CT values were used for analysis, evaluated the stabilities of the candidate reference genes according to the CV and SD of the CT values. ΔCT method, the difference values of original CT values were used for analysis, performed stability ordering according to the mean SD of ΔCT value. GeNorm and NormFinder, the original CT values were converted for analysis, sequenced the candidate reference genes according to the stability values. The lower the stability value, the more stable the gene expression.

Development Degree of Adults
The expression stability of the 10 candidate reference genes at different periods of reproductive and diapause female showed that the top four ranked genes identified by the GeNorm, Bestkeeper, NormFinder, and ΔCT method were similar, but the rank order was slightly different. Arp3 and RpS5 were the first and second stably expressed genes in the four statistical algorithms (Supp Table 2 [online only]) and comprehensive ranking analysis (Fig. 3). As for the third and fourth ranked gene, Tub1 and Actin, identified by comprehensive ranking analysis were the same as those generated by GeNorm and NormFinder. While Bestkeeper selected Arp5 and Tub1, ΔCT method selected Actin and RpS26e (Supp Table 2 [online only]). α-Tub was ranked by GeNorm, Bestkeeper, Normfinder, and ΔCT method as the least stable gene among the 10 candidate reference...
Adult Tissues

The expression stability ranking of 10 candidate reference genes in different tissues of reproductive and diapause females was varied according to the four statistical algorithms. In different tissues of reproductive females, the top four genes were RpS5, Arp3, Arpc5, and Tub1, respectively (Fig. 3). But the rank order of the four genes was significantly different among different statistical algorithms. RpS5 was ranked first by GeNorm and ∆CT method, and was ranked third and fourth by Normfinder and Bestkeeper, respectively. Arp3 was ranked first by Bestkeeper, and was ranked second, third, and fifth by Normfinder, ∆CT method, and GeNorm, respectively. Arpc5 was ranked first and second by GeNorm and ∆CT method, and was ranked fourth and fifth by Normfinder and Bestkeeper, respectively. Tub1 was ranked first by Normfinder and Bestkeeper, and was ranked fifth and sixth by ∆CT method and GeNorm, respectively (Supp Table 2 [online only]). However, the four statistical algorithms found that EF1a was ranked as the least stable gene in the tissues from reproductive females (Supp Table 2 [online only]).

In different tissues of diapause females, the top four genes were different from those of different tissues of reproductive females. Tub1 was ranked first by the comprehensive ranking, followed by RpS26e, Arp3, and 128up (Fig. 3). Through analysis, it was found that ∆CT method and Normfinder displayed the same rankings for expression stability of candidate reference genes under this condition (Supp Table 2 [online only]). Tub1 was identified as the most stably expressed gene by GeNorm, ∆CT method and Normfinder, although it was ranked fifth by Bestkeeper. RpS26e, ranked steadily among the four statistical algorithms, was ranked second by ∆CT method and Normfinder, whereas it was ranked first and third by GeNorm and Bestkeeper, respectively. Arp3 was ranked first by Bestkeeper with the smallest coefficient of variation, whereas it was ranked third by ∆CT method and Normfinder. In addition to being ranked third by GeNorm, 128up was ranked fourth by Bestkeeper, ∆CT method, and Normfinder (Supp Table 2 [online only]). However, in tissue from diapause females, Actin was consistently identified as the gene with the most unstable expression by the four statistical algorithms (Supp Table 2 [online only]).

In the reproductive and diapause female tissues, the expression stability of the candidate reference genes was different. In order to accurately determine the expression of the target genes, the expression stability of candidate reference genes under the two conditions was analyzed. According to the comprehensive ranking,
Tub1 and EF1a were the most stable and unstable genes, respectively, whereas Arp3, 128up, and Arpc5 were ranked second, third, and fourth, respectively (Fig. 3). Tub1 was the best candidate reference gene identified by Normfinder and ∆CT method, and was ranked third and sixth by Bestkeeper and GeNorm, respectively. Arp3 was the most suitable candidate reference gene selected by Bestkeeper, and was ranked second by Normfinder and ∆CT method, and fifth by GeNorm. 128up and Arpc5 were the most stable candidate reference genes identified by GeNorm, whereas they were ranked separately fifth and sixth by Normfinder and ∆CT method, and seventh and fifth by Bestkeeper, respectively (Supp Table 2 [online only]). EF1a was identified as the least stable gene by GeNorm, NormFinder and ∆CT method, although BestKeeper selected Actin as the least stable gene (Supp Table 2 [online only]).

All Samples
In order to determine the best reference gene suitable for the different conditions of adults, the stability of the ten candidate reference genes was ranked for all samples. Arp3 was identified as the most stable gene by the comprehensive ranking, followed by Tub1, Arpc5, and RpS3, whereas EF1a was identified as the least stable gene (Fig. 3).
But, the most and least stable genes identified by different statistical algorithms were slightly different. Arp3 was selected as the most stable reference gene by BestKeeper and ∆CT method, although Tub1 and Arpc5 were selected as the most stable reference gene by NormFinder and GeNorm, respectively. EF1α was selected as the least stable reference gene by GeNorm and ∆CT method, despite it being ranked ninth by BestKeeper and NormFinder (Supp Table 2 [online only]).

The Best Combination of Candidate Reference Genes Under Different Conditions

According to the pairwise variation ($V_{n/n+1}$) between the normalization factors and cut-off value (0.150) calculated by GeNorm, the number of reference genes required for optimum normalization in each experimental condition was determined. The cut-off value of $V_{n/n+1} < 0.150$ suggested that $n$ reference genes were enough to make gene expression normalization. Otherwise, $n + 1$ reference genes were needed. The analysis results showed that all $V_{2/3}$ were <0.150, indicating that the optimal number of reference genes under each condition was two (Fig. 4). More specifically, Arp3 and RpS5 were the most stable gene combinations under adult developmental stage and reproductive adult tissues conditions, Tub1 and RpS26e were the most stable gene combinations under adult diapause tissues conditions, and Arp3 and Tub1 were the most stable gene combinations under adult tissues and all samples groups (Table 1).

Validation of Reference Genes

The stability of reference gene is very important for the analysis of expression level of target gene. Vg and VgR, which are important for insect reproduction, were selected to verify the applicability of the selected reference gene. We examined the relative expression levels of target genes Vg and VgR (GenBank MT308983, MT522179) in the whole adult and from tissues of reproductive and diapause adults, respectively. When the most stable reference genes (Arp3 and/or RpS5) were used as normalization factors at different periods of reproduction and diapause, the expression patterns of the target genes Vg and VgR were consistent, with low expression in the diapause period and rich expression in the late diapause and reproduction period. However, when the most unstable reference gene (α-Tub) was used as a normalization factor, neither the target gene Vg nor VgR showed a consistent expression pattern (Fig. 5). Under different tissue conditions, when the most stable reference genes (Tub1 and/or Arp3) were used as the normalization factors, the expression level of the target gene Vg in the fat body of the reproductive female was significantly higher than that in the ovary of the reproductive female. The expression level of the target gene VgR in the fat body of the reproductive female was lower than the ovary of the diapause female. While when the most unstable reference gene (EF1α) was used as the normalization factor, the expression pattern differed with normalization by Tub1 and Tub1+Arp3 (Fig. 5). In general, when the most stable reference genes were used as the normalization factors, the accurate expression pattern of the target gene could be obtained.

Discussion

qRT-PCR has become an important means to explore gene expression level due to its high sensitivity, rapidity, specificity, and accuracy, and was widely used in physiology studies that investigated insect diapause such as Drosophila melanogaster (Williams 6).
et al. 2006), Culex pipiens (Sim and Denlinger 2008, 2009, 2013), Leptinotarsa decemlineata (Lehmann et al. 2014), Chrysopa septempunctata (Liu et al. 2015), and Pieris melete (Wu et al. 2018). However, the selection of appropriate reference genes was the key to accurately analyze the gene expression level. For example, under conditions of injury, heat-stressed and experimentally varied diets, the best reference gene was different in Drosophila melanogaster (Ponton et al. 2011); Under biotic factors and abiotic stress, inappropriate selection of the reference genes in Locusta migratoria resulted in significant differences in the expression level of the target gene chitin synthase 1 (CHS1) (Yang et al. 2014). Diapause of most insects was mainly affected by photoperiod and temperature. In past studies, the screening of reference genes of Drosophila melanogaster (Ponton et al. 2011), Leptinotarsa decemlineata (Shi et al. 2013), Helicoverpa armigera (Zhang et al. 2015), Bombyx mori (Guo et al. 2016), and Harmonia axyridis (Qu et al. 2018) under main environmental factors was completed by GeNorm, NormFinder, Bestkeeper, and ∆CT method. In this study, the expression profiles of 10 candidate reference genes of C. nipponensis were analyzed under different conditions by the same four statistical algorithms, GeNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), Bestkeeper (Pfaffl 2004), and ∆CT method (Silver et al. 2006).

Different algorithms produced different stability rankings. In order to obtain statistically consistent and accurate results, we finally ranked the gene based on their stabilities determined by

Table 1. Recommendation for the best combination of reference genes based on the GeNorm and comprehensive rankings under various experimental conditions

| Group                        | Reference gene | Most       | Least      |
|------------------------------|----------------|------------|------------|
| Development degree           | Arp3           | RpS5       | α-Tub      |
| Adult tissues (reproduction) | RpS5           | Arp3       | EF1α       |
| Adult tissues (diapause)     | Tub1           | RpS26e     | Actin      |
| Adult tissues                | Tub1           | Arp3       | EF1α       |
| All samples                  | Arp3           | Tub1       | EF1α       |

Fig. 5. Validation of selected reference genes under different periods (A) and tissues (B) of reproductive and diapause female in C. nipponensis. Relative expression levels of the Vg and VgR in different samples using different normalization factors (the most and least stable genes). Asterisks indicate significant differences in the expression levels of the Vg and VgR. R: reproduction period, D1: the diapause induction period, D2: the diapause maintenance period, D3: the diapause termination period.
comprehensive analysis method (Xiao et al. 2015), and selected the most stable reference genes under each condition. As far as we know, actin, which played an important role in cell contraction and cytoskeletal maintenance, was found in virtually all eukaryotic cells and was considered as an ideal reference gene for many organisms (Søren-Castillo et al. 2012, Shakeel et al. 2015). For example, Actin was used as a reference gene for normalization in the determination of genes related to reproductive and nutritional signaling, such as vitellogenin of Chrysopa septempunctata (Liu et al. 2015). However, in this study, three genes related to actin were selected for analysis, among which Actin was 96% similar to Actin of C. septempunctata which was also a member of the Neuroptera. In our study, Actin was the most unstable gene in the diapause female tissues, but the actin-related protein, Arp3, which was structurally homologous with actin, showed better stability. Arp3 was selected as the most stable reference gene in adults of different developmental levels and all samples, while it was the second most stable gene in the reproductive adult tissues and all adult tissues. Although Arp3 was ranked as the third most stable gene in the diapause adult tissues, it showed relatively stable expression in the expression profile. Tubulin, which played an essential role in maintaining cell shape, movement and intracellular material transport, was also often used as a reference gene, but different types of tubulin have different stability. For example, in the study of Helicoverpa armigera, the expression of β-Tub was relatively stable compared with that of α-Tub under almost all conditions (Zhang et al. 2015). Similarly, in this study, α-Tub showed unstable expression and was the least stably expressed gene in adults of different developmental stages, whereas tub1 was considered to be the most stably expressed reference gene in diapausing adult tissues and all adult tissues, and was the second most stable gene in all samples. Ribosomal protein (RP), widely distributed in various tissues, played an important role in protein biosynthesis and was widely used as a reference gene in many insects (Lu et al. 2013, Koyama et al. 2014, Sun et al. 2015). In this study, RpS5 was considered to be stable in the tissues of reproductive females, and ranked second among different developmental stages of adults. Elongation factors (EF) was a protein factor, which promoted polypeptide chain to extension during the translation of mRNA, and was recommended as the ideal reference gene under different conditions of a variety of insects (Chapuis et al. 2011, Ponton et al. 2011). However, some studies showed that EF1a was one of the most unstable genes under certain conditions (Fu et al. 2013). In our study, EF1a was found to be the most unstable gene in the reproductive adult tissues, all tissues and all samples, and the second least stable gene in the adults of different developmental stages and diapause adult tissues; therefore, it was not suitable for the study of C. nipponensis.

Recently, an increasing number of studies have demonstrated the importance of using multiple stably expressed reference genes for the accuracy of qRT-PCR analysis (Ling and Salvaterra 2011, Yuan et al. 2014, Kang et al. 2017). However, this does not mean that the more reference genes increase the reliability of the results. The study has indicated that either too few or too many reference genes may detriment the accuracy of the target gene expression (Ling and Salvaterra 2011). Thus, how many reference genes are sufficient for data normalization varies on a case-by-case basis, and accurate data normalization requires validation of candidate reference genes for a particular sample set. In short, when the optimal number of reference genes recommended by GeNorm was too high or even exceeded the total number of candidate reference genes (i.e., all the value of \( \frac{V_n}{(n+1)} > 0.150 \)), we recommend validating or replacing candidate reference genes.

In this study, all V2/3 values were <0.150, therefore, the optimal combination of reference genes composed of two genes was obtained under each condition. At the same time, in order to verify the reliability of the results of this study, we examined the expression patterns of \( \text{Vg} \) and \( \text{VgR} \) among different developmental stages and tissues of adults, and the two most stable reference genes, individually or in combination, were used for data normalization, with the most unstable genes as the control. The two most stable reference genes showed consistent expression profiles, both individually and in combination, but the \( \text{Vg} \) and \( \text{VgR} \) expression profiles changed when the most unstable reference genes were used. Therefore, these results indicated that it was important for normalization to use different reference genes in different species and conditions, and it was meaningful to select and verify the reference genes of C. nipponensis in this study. This study only focused on different conditions of the female adults of C. nipponensis, thus if researchers experience limitation in experiments (e.g., limited samples, time, and funds) for the screening of the most suitable reference genes, then screened reference genes in all samples group can be preferentially considered as optimal reference genes.

In conclusion, this study systematically evaluated the expression stability of 10 candidate reference genes under different conditions in adults, and finally provided reliable results for the normalization of qRT-PCR. This study suggests that the most stable reference gene Arp3 can ensure the accuracy of the results of the target gene in different developmental stages of adults. In tissue studies, when the expression level of the target gene is abundant, Tub1 could be used as the reference gene, however when the expression level is moderate, Arp3 could be selected as the reference gene. The results of this work will contribute to future studies on the regulatory mechanism of reproduction and diapause of C. nipponensis.

Supplementary Data

Supplementary data are available at Journal of Insect Science online.

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