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Screening potential reference genes for reverse transcription-quantitative polymerase chain reaction normalization in *Atrijuglans hetaohei* (Lepidoptera: Gelechioidea)

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Abstract

Atrijuglans hetaohei Yang (Lepidoptera: Gelechioidea), is one of the major pests that seriously damage the walnut fruits. Although the morphology and physiology of *A. hetaohei* have been widely studied, suitable reference genes for normalizing target gene expression have not been identified. In this study, the expressions of eight candidate reference genes including *GAPDH*, *β-Tubulin*, TATA-binding protein (*TBP*), *Histone*, Ubiquitin-conjugating enzyme 9 (*UBC9*), arginine kinase (*AK*), *28S* and elongation factor 1 alpha (*EF1α*) in different developmental stages and various larval tissues of *A. hetaohei* were determined using reverse transcription-quantitative polymerase chain reaction. Moreover, the expression stability of the selected reference genes was evaluated by ΔCt method, BestKeeper, NormFinder, geNorm and ReFinder, a comprehensive software platform. These results demonstrated that the best reference genes were *GAPDH* and *28S* at different developmental stages of *A. hetaohei*; and the optimum references in various larval tissues of *A. hetaohei* were *28S*, *Histone* and *TBP*. Our study should be useful to analyze the expression profiles of target genes and form a solid foundation for future research on the understanding of the genes' biological functions in *A. hetaohei*.

Key words: *Atrijuglans hetaohei*, reference genes, Reverse transcription-quantitative polymerase chain reaction, stability evaluation

1 Introduction

With the development of high-throughput sequencing strategies, the genomes and transcriptomes of multiple insect species have been increasingly published, which provide a great deal of genetic information for studying the expression patterns of specific genes to understand their molecular mechanisms (Adams et al. 2000; Holt et al. 2002; Li et al. 2012). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) has become the most extensively used method for determining the relative transcription levels of target genes because of its outstanding advantages of speed, sensitivity, specificity, accuracy, and reliability (Bustin 2002; Vandesompele et al. 2002). The accuracy of RT-qPCR generated results depends on accurate transcript normalization using stably expressed reference genes under various treatments and experimental conditions (Dheda et al. 2005; Yang et al. 2015a), however, it can be dramatically changed in the expression pattern of a given target gene if unstable reference genes are used for normalization, and arise flaws in results (Ferguson et al. 2010; Kong et al. 2014; Yang et al. 2015b). Hence, reliable reference genes are essential for precisely interpreting the expression of target genes (Artico et al. 2010).

To date, genes encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin (*ACT*), elongation factor 1

alpha (*EF1α*), 18S ribosomal RNA (*18S*), and tubulin (*TUB*) have been frequently used as endogenous controls for RT-qPCR analyses in many different insect species (Bin et al. 2018; Goidin et al. 2001; Radonić et al. 2004; Thellin et al. 1999). Nevertheless, several studies have reported that the expression of universal reference genes can display great variation under different abiotic and biotic conditions. For example, the traditional reference gene *ACT* was regarded as the most stable reference gene at different developmental stages in *Aedes albopictus* (Diptera: Culicidae) (Dzaki et al. 2017) and *Diaphania caesalis* (Lepidoptera, Pyralidae), and *ACT* was also recommended as stable reference gene for different tissues of *D. caesalis* (Wang et al. 2020), yet, it was not suitable in *Heliconius numata* (Lepidoptera: Nymphalidae) (Prunier et al. 2016) and *Helopeltis theivora* (Waterhouse) (Hemiptera, Miridae) (Wang et al. 2019). *GAPDH* was determined to be the best reference gene suited for normalization in different developmental stages and larval tissues of *Thitarodes armoricanus* (Lepidoptera: Hepialidae), while it was unstable under the conditions of lower-temperature, fungal infections and dietary treatments (Liu et al. 2016). Besides, some studies have also pointed out the combination use of two or more reference genes selected in RT-qPCR analysis will help to achieve more accurate and reliable results in a wide variety of samples (Huang et al. 2015; Ma et al. 2016; Yang et al. 2015b). Therefore, to obtain accurate conclusions, it is crucial to evaluate the expression stability of reference genes under specific experimental conditions before normalizing target gene expression (Guenin et al. 2009).

Atrijuglans hetaohei Yang (Lepidoptera: Gelechioidea), is one of the major pests that can reduce the quality and yield of walnuts and seriously damage the walnut forest leading to harvest loss (Wang et al. 2016). So far, the morphology and physiology of *A. hetaohei* have been widely studied (Nan et al. 2017; Tian et al. 2010; Wang et al. 2007), however, the studies on the molecular biology of *A. hetaohei*, such as the functional study of target genes and the mechanism of its fundamental events such as development, reproduction and behavior, are remain unclear. This is largely due to the lack of the stably expressed reference genes for the accurate normalization for RT-qPCR analysis. The aim of this study is to determine the expression and evaluate the stability of eight commonly used reference genes including *GAPDH*, TATA-binding protein (*TBP*), *Histone*, Ubiquitin-conjugating enzyme 9 (*UBC9*), β-tubulin (*β-TUB*), 28S ribosomal RNA (*28S*), *EF1α* and arginine kinase (*AK*) under different developmental stages and various larval tissues of *A. hetaohei*. In the present study, five different algorithms, ΔCt method, GeNorm, NormFinder, BestKeeper and RefFinder, were used to normalize the RT-qPCR data to identify the most suitable reference genes. Our results could provide a solid foundation for further studies of the molecular mechanism of *A. hetaohei*.

2 Material and Methods

2.1 Insects

Larvae of *A. hetaohei* were obtained from the Walnut Demonstration Garden in the Walnut Experimental Station associated with Northwest Agriculture and Forestry University in Shangluo city, Shaanxi province (33°2′30″-34°24′40″N, 108°34′20″-111°1′25″E). In late August 2016, the walnut fruits bored by *A. hetaohei* larvae were collected and placed on the soil surface. The pest overwintered in old larvae from the beginning of September 2016 to April 2017. The cocoons were screened out and reared in a temperature-controlled basin with soil at 25 ± 1°C, 75%–80% relative humidity (RH) with a photoperiod cycle of 14hL/10hD. A month later, the pupae were collected after dissecting a portion of cocoons, the remainder were reared until incubation. The newly-emerge adults were transferred into a plastic box (60×60×60 cm) according to sex, and supplied with a 10% (w/v) honey solution per day.

2.2 Sample collection

2.2.1 Development stages

Six samples including female larva (two individuals), male larva (n = 2), female pupa (n = 2), male pupa (n = 2), female adults (n = 2), and male adults (n = 2), were separately collected for stability evaluation of reference genes. No mortality was observed during the sample collection.

2.2.2 Tissues

Five tissue samples (fat body, silk gland, Malpighian tube, digestive tract and residual body) were dissected from female or male old larva using sterilized scalpel and tweezers. Tissue sample of fat body was collected from eight individuals, silk gland was collected from eight individuals, Malpighian tube was collected from 30 individuals, digestive tract was collected from 20 individuals and residual body was collected from eight individuals.

All the samples were flash-frozen in liquid nitrogen and stored at −80°C until used. Triplicates were carried out for each sample.

2.3 Total RNA extraction and cDNA synthesis

Total RNA was isolated with a RNAisoPlus Total RNA Isolation Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. The integrity and purity of total RNA in all samples were verified by agarose gel electrophoresis (1%) and spectrophotometric measurements (Quawell, Beijing, China), respectively. The first-strand cDNA was synthesized with a PrimeScript RT reagent Kit

with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions using 1 μg total RNA in a 20 μL final reaction volume. The synthesized cDNA was stored at −20°C.

2.4 RT-qPCR analysis

We obtained the candidate reference genes (including *GAPDH*, *TBP*, *Histone*, *UBC9*, *β-TUB*, *28S*, *AK* and *EF1α*) from our transcriptome database (NCBI accession NO. SRP226553). For each gene, specific primers were designed with Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Sangon Biotech (Shanghai, China) (shown in Table 1). Each pair of primers was validated by calculating standard curves generated as serial 2-fold dilutions of each amplicon (range from 2^0 to 2^{-5} copies) using *A. hetaohei* silk gland cDNA (50 ng/μL) as a template. To obtain the Ct values of the selected reference genes for different samples, the cDNA of each sample was distilled to 25 ng/μL as a working concentration. RT-qPCR was performed in 20 μL reaction volumes containing 10 μL SYBR Premix Ex Taq II (TaKaRa, Dalian, China), 0.8 μL of each gene-specific primer (0.4 μM each), 1 μL of cDNA template, 7.4 μL of RNase Free H_2O. Reactions were conducted in Bio-rad IQ5 Thermol System (CWBIO, Beijing, China). The Cycling conditions were as follows: 95°C for 30 s (pre-incubation), 40 cycles of 95°C for 0.05 s, 60°C for 30 s, 72°C for 30 s, followed by melting analysis to investigate the specificity of primers: 65–95°C held for 0.05 s for 0.5°C. For each RT-qPCR run, the negative control sample (without cDNA) was included. Each treatment included three replicates, and each reaction was run in triplicate.

2.5 Data analysis

All technical and biological replicates were used to calculate the average Ct value. The stability of candidate reference genes was evaluated using the comparative ΔCt method (Silver et al. 2006) and three software programs BestKeeper, geNorm, and NormFinder. For BestKeeper, the average Ct values were used to calculate the coefficient of variance (CV), Correlation coefficient (r) and the standard deviation (SD), with the lowest SD representing the highest stability (Pfaffl et al. 2004). For GeNorm and NormFinder, the Ct values were transformed into relative quantities using the formula $Q = 2^{-\Delta Ct}$, in which ΔCt = each corresponding Ct value − minimum Ct value (Yang et al. 2015b). The GeNorm algorithm calculates the expression stability value (M) and pairwise variation value (V). Gene expression is considered stable when the M value is below 1.5, and the lower the M values, the more stable the expression. Besides, the pairwise variation (V_n/V_{n+1}) is used to determine the optimal number of reference genes. The threshold of V_n/V_{n+1} is 0.15. The V_n/V_{n+1} value below 0.15 suggested that an extra reference gene is not required for normalization (Vandesompele et al. 2002). The NormFinder algorithm generates the stability value of each single reference gene based on the evaluation of both intra- and inter-group variations and a separate analysis of the sample subgroups in expression levels (Andersen

Table 1 Details of eight candidate reference genes used for RT-qPCR analysis

Gene	Primer sequences 5′ to 3′	Amplicon size (bp)	Efficiency (%)	R^2	slope
AK	F:CCCTCAACACTCCTGTCTCCGATAC R:CACAGCCACCCATTTTCACAGATAC	179	114%	0.9942	−3.0274
β-TUB	F:GTTTCCAGATGACCCACTCCC R:GCACCACCTCGCTCACCTTAG	144	92.8%	0.9891	−3.5063
EF1α	F:GCTCCCGACTGGCAGGTTGACTACG R:GTGGTGGGTCCAAGGTTCAGGTTTA	175	103.4%	0.9917	−3.2422
GAPDH	F:GAGCCACCATTGTTCCACCCA R:AGCAGGGTCTTTTACATTGGGC	165	96.9%	0.9816	−3.3983
Histone	F: TAAACCACGAAGGCGAAGTCAACAG R:TTCAGGCTTGGACGGGTGCT	125	95.1%	0.982	−3.4448
TBP	F:ATCCTGTTCATTCCTTGCTCCA R:CTTAACGCCTATGGCTAGTGGG	244	112.4%	0.9903	−3.0562
28S	F:ATGACTGGCACCTGGCACGGATTAG R:TTAGTTGGAGACACCGCTCGCCTGA	107	113.1%	0.996	−3.0429
UBC9	F:CATCCATTTGGTTTCGTCGCT R:TGGGTGGAACAATGGTGGC	195	112.8%	0.995	−3.0495

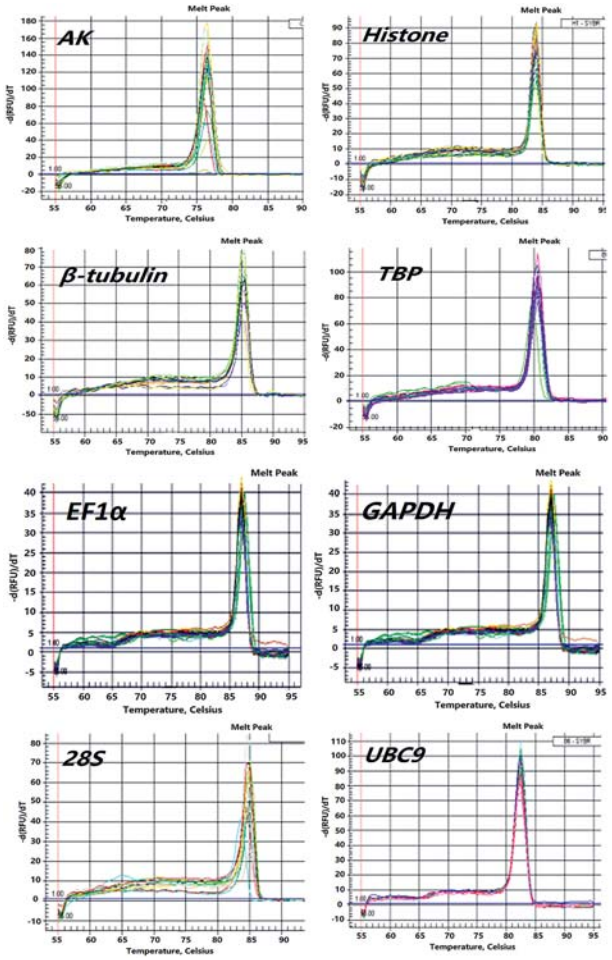

Figure 1 The melting curve analysis of eight candidate reference genes.

et al. 2004). Finally, we used a web-based analysis tool, RefFinder (http://150.216.56.64/referencegene.php#) (Meng et al. 2019) to produce the comprehensive ranking by integrating the results from the ΔCt values, BestKeeper (CV and SD), GeNorm (M values) and NormFinder (stability values).

3 Results

3.1 Expression profiles of candidate reference genes

The concentration of total RNA isolated from all investigated samples was sufficient for reverse transcription and RT-qPCR analyses with the A260/A280 ratio ranged between 1.8 and 2.2. The eight candidate reference genes were amplified as single bands of the predicted size in 1% agarose gels (data not shown). Gene-specific amplification was confirmed by a single peak in melting-curve analysis (Fig. 1). Moreover, the amplification efficiency of the eight genes ranged from 92% to 115% with correlation coefficients (R^2) ranged from 0.9800 to 0.9960 and slope between −3 and − 3.5 (Table 1).

The Ct values of eight candidate reference genes of all samples are shown in Figure 2. In developmental stages of *A. hetaohei*, *β-TUB* had the highest level of expression, with a mean Ct of 18.95; *EF1α* showed the lowest expression level, with a mean Ct of 26.94. The reference genes *AK* (21.63) and *Histone* (21.71) displayed a moderately abundant expression level (Fig. 2A). In larval tissues of *A. hetaohei*, the mean Ct values of all the candidate reference genes ranged from 20.30 to 29.74. Out of eight reference genes, *EF1α* (20.30) was the most abundant transcripts, reaching the threshold

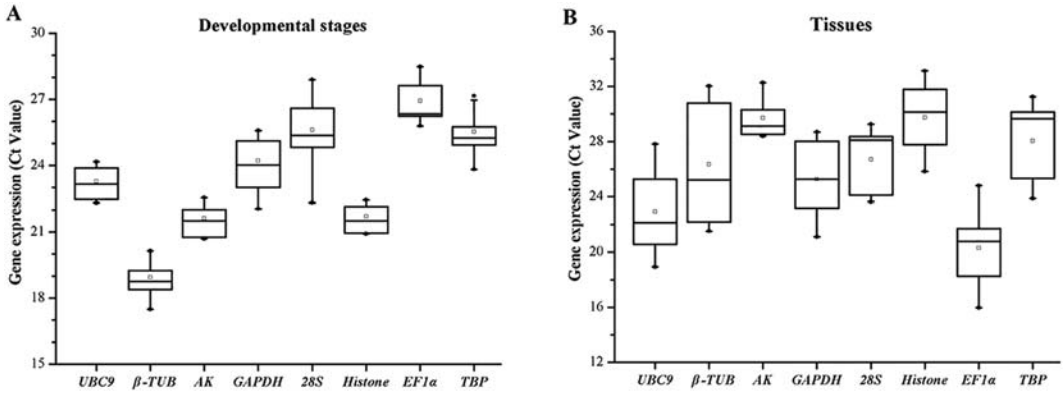

Figure 2 The expression profiles of the eight selected reference genes. (A) Different developmental stages of *A. hetaohei*; (B) Different larval tissues of *A. hetaohei*. Distribution of Ct values for each gene is represented using box plots. The square in the box indicates Ct mean value; the box represents the 25th - 75th percentiles, middle line across the box indicates the median; the whisker (dashed lines) on each box refers to the distribution of the Ct values, and the dark spots represent the extreme outliers.

Table 2 Stability ranking of eight candidate reference genes based on ΔCt method

Reference gene	Different developmental stages		Different tissues	
	Standard deviation (SD)	Rank	Standard deviation (SD)	Rank
AK	0.856	5	1.445	1
β-TUB	1.597	8	4.331	8
EF1α	1.190	7	3.019	6
GAPDH	0.674	3	2.859	4
Histone	0.596	1	2.644	3
TBP	0.788	4	2.905	5
28S	0.636	2	2.346	2
UBC9	1.029	6	3.213	7

fluorescence peak after 20 cycles. *Histone* (29.74) and *AK* (29.73) were expressed at the lowest levels (Fig. 2B).

3.2 Stability analysis of candidate reference genes by ΔCt method

The ΔCt method identified *Histone* (SD = 0.596) *and 28S* (0.636) as the most stable reference genes among different developmental stages of *A. hetaohei*, and *β-TUB* (1.597) as the least stable gene (Table 2). In different larval tissues of *A. hetaohei*, *AK* (1.445) was the most stable reference gene, followed by *28S* (2.346) and *Histone* (2.644), and *β-TUB* (4.331) exhibited the highest SD and therefore was the least stable gene (Table 2).

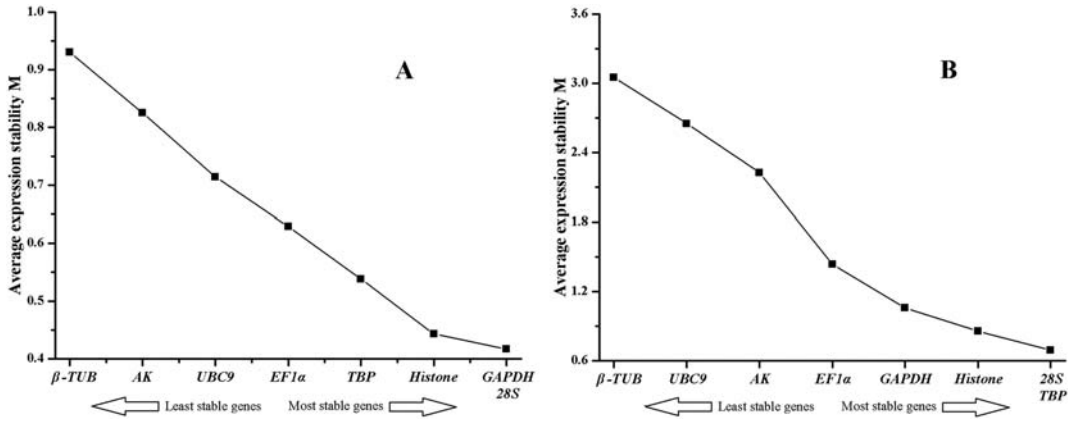

Figure 3 Results obtained by the GeNorm algorithm (M: stability value for all genes studied. (A) Different developmental stages of *A. hetaohei*; (B) Different larval tissues of *A. hetaohei*).

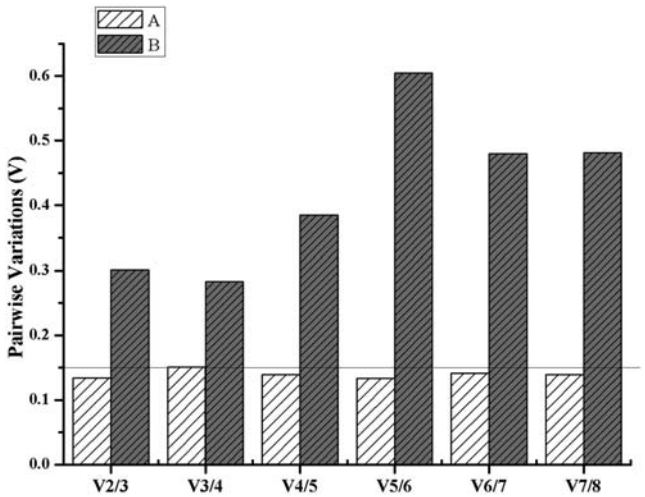

Figure 4 Determination of the optimal number of reference genes for normalization using GeNorm algorithm ((A) Different developmental stages of *A. hetaohei*; (B) Different larval tissues of *A. hetaohei*. Pairwise variation (Vn /Vn+1) value which does not exceed the threshold of V = 0.15 means n should be the optimal number of reference gene during RT-qPCR analysis).

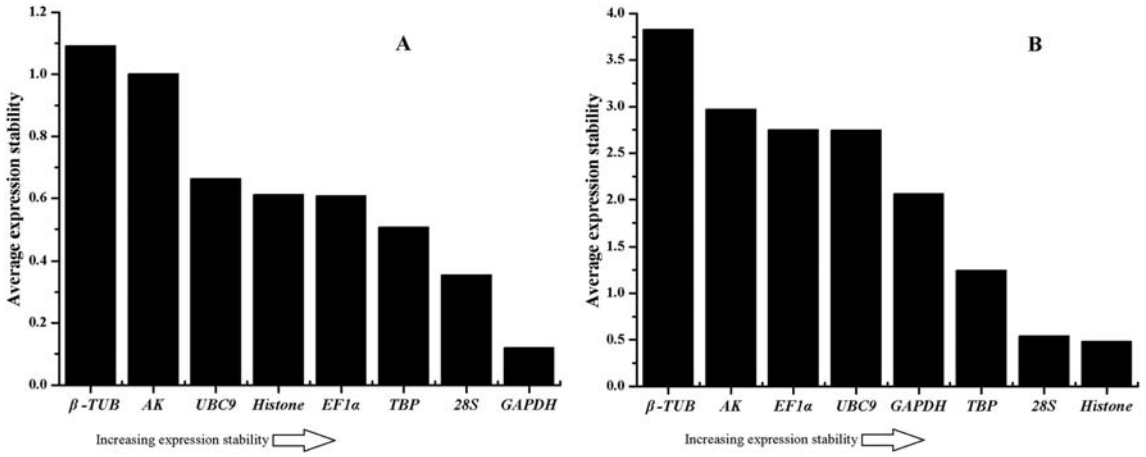

Figure 5 The expression stability values of candidate reference genes calculated by NormFinder algorithm ((A) Different developmental stages of *A. hetaohei*; (B) Different larval tissues of *A. hetaohei*).

3.3 Stability analysis of candidate reference genes by GeNorm algorithm

Analysis by the GeNorm algorithm revealed that the M value of all the candidate genes was below 1.5, *28S* and *GAPDH* genes (M value = 0.405) had the best stability of gene expression among different developmental stages of *A. hetaohei*, and *β-TUB* (0.920) was the least stable reference gene (Fig. 3A). In various larval tissues of *A. hetaohei*, the most stable reference genes were *TBP* and *28S*, with an *M* value of 0.690, followed by *Histone* (0.857), *GAPDH* (1.058) and *EF1α* (1.434). The least stable one was *β-TUB* (3.051) (Fig. 3B).

Pairwise variation (V_n/V_{n+1}) was calculated based on normalization factor values (NF_n and NF_{n+1}) by the GeNorm software to determine the optimal number of reference genes included in the RT-qPCR analysis (shown in Fig. 4). GeNorm analysis of *A. hetaohei* at different life stages showed a V value of 0.134 (V2/3), below the threshold of 0.15 (Fig. 4A), suggesting that two reference genes were needed for reliable normalization which are *28S* and *GAPDH*, while the V values in different larval tissues of *A. hetaohei* that all exceeded 0.15 (Fig. 4B), indicating that three reference genes were required for reliable normalization, which are top ranked *TBP*, *28S* and *Histone*.

3.4 Stability analysis of candidate reference genes by NormFinder algorithm

Similarly to GeNorm, analysis by the NormFinder algorithm at different developmental stages of *A. hetaohei* pointed out that *GAPDH* and *28S* were the most stable genes (stability value = 0.121 and 0.355, respectively), whereas the lowest stability (1.092) was recorded for *β-TUB* (Fig. 4A). With respect to different larval tissues of *A. hetaohei*, the most stable gene was *Histone* (0.485), followed by *28S* (0.485) and *TBP* (1.245), while *β-TUB* (3.829) was determined unsuitable as a reference gene (Fig. 4B).

Table 3 Determination of the stability of eight candidate reference genes using BestKeeper algorithm

Gene name	Different developmental stages			Different tissues		
	Correlation coefficient(r)	Standard deviation (SD)	Covariance (CV/%)	Correlation coefficient(r)	Standard deviation (SD)	Covariance (CV)
AK	0.451	0.76	2.84	0.160	1.26	4.23
β-TUB	0.938	1.21	4.73	0.750	4.05	15.37
EF1α	0.944	1.06	4.37	0.663	2.56	12.61
GAPDH	0.947	0.60	2.59	0.783	2.48	9.83
Histone	0.717	0.56	2.59	0.975	2.34	7.88
TBP	0.830	0.62	3.27	0.921	2.76	9.86
28S	0.885	0.56	2.60	0.926	2.26	8.48
UBC9	0.815	0.82	3.19	0.722	2.88	12.54

Table 4 Stability analysis of the eight candidate reference genes by RefFinder algorithm

Reference gene	Different developmental stages		Different tissues	
	Stability value	Rank	Stability value	Rank
AK	6.44	7	4.14	5
β-TUB	8.00	8	8.00	8
EF1α	5.14	5	5.23	6
GAPDH	1.32	1	4.00	4
Histone	3.31	3	2.06	2
TBP	3.46	4	2.71	3
28S	1.41	2	1.41	1
UBC9	6.00	6	6.19	7

3.5 Stability analysis of candidate reference genes by BestKeeper algorithm

The expression stability of eight candidate reference genes was also ranked by BestKeeper program based on r (correlation coefficient), CV (covariance) and SD (standard deviation) (Table 3). The most stable reference genes that showed the least overall variation at different developmental stages of *A. hetaohei* were *28S* and *Histone* (SD = 0.56), while the expression of *β-TUB* (1.21) should be classified as a clearly unstable reference gene and, therefore, unsuitable for normalization. Furthermore, the best correlation was observed for *GAPDH* (r = 0.947), thus identifying it as the most reliable reference gene for normalization. In different larval tissues of *A. hetaohei*, BestKeeper algorithm identified *AK* (SD = 1.26) as the most stable reference gene, followed by *28S* (2.26), *Histone* (2.34) and *GAPDH* (2.48), while the least stable one was *β-TUB* (4.05). In addition, *Histone* (r = 0.975) was considered as the most reliable reference gene for normalizing target gene expressions.

3.6 Stability analysis of reference genes by RefFinder algorithm

According to the results of RefFinder analysis, as shown in Table 4, the stability order of eight reference genes among different developmental stages of *A. hetaohei* was *GAPDH* > *28S* > *Histone* > *TBP* > *EF1α* > *UBC9* > *AK* > *β-TUB*, while the stability in different larval tissues of *A. hetaohei* can be ranked from the most stable to the least stable as follows: *28S* > *Histone* > *TBP* > *GAPDH* > *AK* > *EF1α* > *UBC9* > *β-TUB*. Collectively, the general consensus from all the above programs indicated that *GAPDH* and *28S* could be used as the most suitable reference genes among different developmental stages of *A. hetaohei*, while *28S*, *Histone* and *TBP* were selected as the best reference genes for normalizing target gene expression in different tissues of *A. hetaohei*.

4 Discussion

Reverse transcription-qPCR is a sensitive, efficient and accurate molecular technique to determine the changes in mRNA expressions and has also been used for better understanding the molecular mechanisms of numerous biological processes (Ma et al. 2016). Reliable RT-qPCR analyses depend on using stable endogenous controls referred as reference genes for normalization of target gene expression (Yang et al. 2015a; Zhu & Dong 2006). However, considerable variations in the expression of many universal reference genes (such as *18S*, *Actin*, *GAPDH* and *TUB*) have been found across different tissues, developmental stages and under different experimental treatments (Kang et al. 2017; Tang et al. 2019; Wang et al. 2020; Yu et al. 2018). Therefore, stable reference genes are crucial prerequisites in any experiment for obtaining reliable gene expression results.

As the RT-qPCR technology gradually improved, currently, in the field of entomological studies, the identification and evaluation of reference genes have been conducted in various insect species, such as *Drosophila melanogaster* (Diptera: Drosophilidae) (Ponton et al. 2011), the honey bee, *Apis mellifera* (Hymenoptera: Apidae) (Scharlaken et al. 2008), the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae) (Peng et al. 2012), *Plutella xylostella* (Lepidoptera: Plutellidae) (Fu et al. 2012), the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae) (Yuan et al. 2014), *Chilo suppressalis* (Lepidoptera, Pyralidae) (Xu et al. 2017), and so on. Despite one of the most important economic crop pests, however, studies on the selection of stable reference genes in *A. hetaohei* have not been reported, which has greatly hindered the further studies of this species at molecular level. Thereby, we decided to screen the most suitable reference genes for normalizing gene expression in *A. hetaohei*, and we selected eight commonly used reference genes (*GAPDH*, *TBP*, *Histone*, *UBC9*, *β-TUB*, *28S*, *AK* and *EF1α*) based on the previous studies and literature data (Bai et al. 2014; Radonić et al. 2004; Shi & Zhang 2016).

In the present study, we used five analysis systems, namely ΔCt method, GeNorm, NormFinder, BestKeeper and RefFinder, to evaluate the stability of the selected reference genes among different developmental stages and various larval tissues of *A. hetaohei*. Both GeNorm and NormFinder use relative quantities transformed from Ct values for stability calculation, while ΔCt method and BestKeeper use Ct values directly. This might explain why the best reference genes recommended by different algorithms were not exactly the same (Scharlaken et al. 2008). However, in this work, our results demonstrated that those four computational methods gave rise to similar stability rankings for the eight candidate reference genes under the same experiment conditions. For

example, ΔCt method, GeNorm and BestKeeper determined *GAPDH*, *28S* and *Histone* as the top three stable reference genes for different developmental stages of *A. hetaohei*, and *β-TUB* was determined to be the most unstably expressed gene. *28S*, *TBP* and *Histone* were ranked as the top three stable reference genes by the GeNorm and NormFinder algorithms across various larval tissues of *A. hetaohei*, and again *β-TUB* was the most unstable one. Furthermore, RefFinder integrates the results from the four algorithms to rank the stability of the candidate genes, indicating that *GAPDH* was the most stable reference gene for *A. hetaohei* at different developmental stages, followed by *28S*. In different larval tissues of *A. hetaohei*, *28S* was considered as the most stable reference gene, followed by *Histone* and *TBP*.

As two classical reference genes, *GAPDH* and *TBP* have been widely used as stable internal controls in most of expression researches such as *Spodoptera litura* (Lepidoptera: Noctuidae) (Lu et al. 2013), *Harmonia axyridis* (Coleoptera: Coccinellidae) (Yang et al. 2018), *Aphis glycines* (Homoptera: Aphididae) (Bansal et al. 2012), *Bradysia odoriphaga* (Diptera: Sciaridae) (Tang et al. 2019) and *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Bin et al. 2018). Similarly, our results demonstrated that *GAPDH* was consistently stably expressed under developmental stages, and *TBP* exhibited the stable expression under larval tissues in *A. hetaohei*. Previous results indicated that *Histone* was found to be an ideal reference gene in *Euplotes octocarinatus* (Zhang 2010), *Ananas comosus var.bracteatus* (Li et al. 2017) and *Akebia trifoliate (Thunb.) Koidz* (Yang et al. 2016). In this study, *Histone* was the second optimal reference gene across various larval tissues of *A. hetaohei*. In addition, our data suggested that *28S* was evaluated as the most stable reference gene in various larval tissues of *A. hetaohei*. *28S* is a type of ribosomal RNA and high conserved in eukaryotes, which plays critical roles in maintaining basic cellular metabolism and intracellular functions (Yeap et al. 2014). *28S* has been reported to show the most stable expression in *Pandora neoaphidis* (Xue et al. 2014) and insect cells infected by viruses (Xue et al. 2010)

EF1α, a principal transcriptional elongation factor, has been regarded as an ideal internal reference in RT-qPCR analysis in normalization gene expression across different developmental stages, tissues and strains of *Plodia interpunctella* (Lepidoptera: Pyralidae) (Tang et al. 2016), in different sexes of *D. caesalis* (Wang et al. 2020) and in *P. xylostella* after exposure to Bt toxins (Fu et al. 2012). In contrast, the stability of *EF1α* was relatively poor in most cases of this study. These results were similar with previous studies in *N. lugens* infected by virus (Maroniche et al. 2011). A striking finding of this study was that the classical reference gene *β-TUB* was measured as the least stable reference gene in all treatments. *β-TUB*, an important cytoskeletal structure protein, involves in multiple physiological processes of organisms such as intracellular transportation (Shi et al. 2016). Although *β-TUB* was identified as unstable reference gene in some insects such as in different tissues of *Spodopt eraexigua* (Lepidoptera: Noctuidae) (Zhu et al. 2014) and the Sweet potato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae) (Li et al. 2013), it was still proven to be the stable reference gene across a range of experiment conditions such as at different stages of *Lucilia sericata* (Diptera: Calliphoridae), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Shi et al. 2016), and in *Empoasca pirisuga* Matumura (Hemiptera: Cicadellidae) nymphs exposed to different temperatures (Yu et al. 2018). Therefore, all these results further confirm that the stability of reference genes is varied in different organisms and experimental treatments. The selected reference gene should be pre-tested across more species and in response to different treatments before running a RT-qPCR experiment.

Previous studies have shown that a single reference gene is inadequate to precisely normalize the expression of target genes, and may lead to incorrect consequences, thereby, the understanding of gene function (Radonić et al. 2004). Recently, the use of multiple reference genes for normalization in RT-qPCR was proposed, which can raise the accuracy of quantification of target genes (Nailis et al. 2006). In this study, GeNorm algorithm is adopted to select the optimal number of reference genes, our results indicated that two reference genes should be chosen at different developmental stages of *A. hetaohei* and the best references were *28S* and *GAPDH*. This result is consistent with a previous study that revealed that mRNA expression of *28S* and *GAPDH* were the most stable in the posterior silk gland of B. mori (Wu et al. 2013). For tissue samples of *A. hetaohei*, three reference genes were recommended and the best references were *28S*, *Histone* and *TBP*.

In summary, five software programs (ΔCt, GeNorm, NormFinder, BestKeeper and RefFinder) were utilized for the first time to systematically evaluate the expression stability of eight candidate reference genes (*GAPDH*, *TBP*, *Histone*, *UBC9*, *β-TUB*, *28S*, *AK* and *EF1α*) at different developmental stages and in various larval tissues of *A. hetaohei*. The suitable reference genes and optimal combinations of reference genes were identified for normalizing target gene expression in *A. hetaohei* based on different experimental conditions. Our findings provide a theoretical basis for accurate normalizing RT-qPCR data and further research on the functions of the target genes in *A. hetaohei*.

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