



Article

Comprehensive Screening and Validation of Stable Internal Reference Genes for Accurate qRT-PCR Analysis in *Holotrichia parallela* under Diverse Biological Conditions and Environmental Stresses

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Simple Summary: The dark black chafer, *Holotrichia parallela* Motschulsky (Coleoptera: Scarabaeidae), is an important subterranean insect in China due to its wide distribution and the high degree of damage it causes. Selecting stable reference genes is crucial for accurate quantitative polymerase chain reaction (qPCR) and gene expression analysis. This study evaluated the expression stability of 11 candidate reference genes in *H. parallela* under various biological conditions and environmental stresses. Our findings suggest that the optimum reference genes were as follows: *RPL18* and *RPL13a* for developmental stages and RNAi conditions, *RPL13a*, *RPL18*, and *RPL32* for female and male adults, *RPL13a* and *RPS3* for different tissues, *RPL32*, *RPL13a*, and *RPS3* for varying photoperiod conditions, and *Actin* and *RPL13a* for different temperatures. These discoveries will serve as a foundation for subsequent precise qPCR and gene expression studies in *H. parallela* and other closely related insect species.



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Abstract: *Holotrichia parallela* is among the world's most destructive pests. For accurate qPCR and gene expression studies, the selection of stable and appropriate reference genes is crucial. However, a thorough evaluation of potential reference genes for use in *H. parallela* research is lacking. In this study, 11 reference genes (*GAPDH*, *RPL32*, *RPL7A*, *RPS18*, *RPL13a*, *RPL18*, *Actin*, *RPS7*, *RPS3*, *VATB*, and *EF1A*) were evaluated under different biological conditions and environmental stresses. The stability of 11 potential reference gene transcripts was evaluated through various computational tools, including geNorm, BestKeeper, NormFinder, the Δ Ct method, and the RefFinder program. Under various developmental stages and RNAi conditions, *RPL18* and *RPL13a* exhibited the greatest stability. *RPL13a*, *RPL18*, and *RPL32* were the most stable genes in both male and female adults. Under differing tissue conditions, *RPL13a* and *RPS3* stood out as the most reliable. Moreover, under varying photoperiod conditions, *RPL13a*, *RPS3* and *RPL32* were the most stable genes. Lastly, *Actin* and *RPL13a* were the most stable genes across different temperatures. These findings offer essential criteria for selecting suitable reference genes across diverse experimental settings, thereby establishing a solid basis for accurate gene expression studies in *H. parallela* using RT-qPCR.

Keywords: *Holotrichia parallela*; qRT-PCR; reference gene screening; stability validation

1. Introduction

The reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is an efficient, dependable, and reproducible technique that facilitates the accurate measurement of gene expression during various biological processes [1]. A widely adopted strategy in

RT-qPCR for normalizing gene expression data involves the concurrent measurement of the expression of an reference gene within the same sample, ensuring the accuracy and reliability of the results [1]. However, it is important to note that the levels of expression for frequently used reference genes may exhibit considerable variation across diverse experimental settings [2–6]. Therefore, it is advisable to conduct a thorough and species-specific investigation to ascertain appropriate reference genes [3,7].

Holotrichia parallela is an important subterranean pest in China because of its wide distribution and the significant damage it causes, which is usually between 20 and 30%. In severe cases, losses may exceed 50% [8–10]. The larvae, referred to as white grubs, dwell underground and consume the roots of crops [11]. This makes the larvae susceptible to infection by pathogens present in the soil [12].

In recent times, the adoption of agricultural practices, such as no-till and reduced tillage methods, along with straw return, has fostered favorable conditions for the survival and proliferation of white grubs, resulting in a significant increase in the *H. parallela* population [13]. Although chemical insecticides have been widely utilized to effectively control white grub infestations, this approach has unfortunately resulted in soil and groundwater contamination, leading to significant environmental issues [14]. Furthermore, the indiscriminate and improper utilization of chemical pesticides has fostered the emergence of resistance in grubs, thereby contributing to the progressive intensification of pest infestations in certain regions on an annual basis [12].

The advent of RNA interference (RNAi) technology has revolutionized the field of functional genomics, emerging as a potent tool for deciphering gene function and validating genetic targets [15,16]. RNAi serves as a pivotal strategy in agricultural plant protection [17]. Gene expression analyses are crucial for understanding the molecular mechanisms of physiological, developmental and reproductive processes in coleopteran insects, particularly given the high effectiveness and systemic penetration of RNAi in these species [15]. To date, these research efforts have successfully identified several genes in *H. parallela* [18–21]. However, to further our understanding and ultimately develop effective control strategies for this pest, it is imperative to precisely ascertain the gene expression levels of this pest under diverse biological conditions and environmental stresses.

The aim of this study was to establish a set of stably expressed reference genes in *H. parallela* for RT-qPCR analysis under various conditions, including different developmental stages, tissues, sexes, temperature treatments, light treatments, and RNAi treatments. To accomplish this, 11 potential reference genes were selected from the transcriptomes of *H. parallela*. These genes were glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Vacuolar-type ATPase B (*VATB*), actin (*Actin*), elongation factor 1- α (*EF1A*), and ribosomal proteins (*L7a*, *L13a*, *L18*, *L32*, *S3*, *S7*, and *S18*). Many of these genes encode for proteins involved in essential cellular processes, such as glycolysis (*GAPDH*), energy transduction (*VATB*), cytoskeleton organization (*Actin*), protein synthesis (*EF1A*), and ribosome assembly (ribosomal proteins). All these genes are commonly used reference genes and were previously examined across other insect species [22–24]. The stability of these genes was then evaluated using four commonly used algorithms, namely BestKeeper, NormFinder, geNorm, and comparative Δ Ct, as well as a comprehensive RefFinder program. These findings will enhance gene expression studies and facilitate gene function research in *H. parallela*, benefiting future investigations.

2. Materials and Methods

2.1. Experimental Materials and Treatment Methods

The *H. parallela* specimens utilized in the study were captured under a black light or collected from elm trees near the Henan Research and Development Center for Modern Agriculture, located in Yuanyang County, Henan, China (35°00' N, 113°40' E). Laboratory-reared *H. parallela* beetles were maintained under controlled conditions of 25 °C \pm 1 °C temperature, 70% humidity, and a 16-hour light/8-hour dark photoperiod [25]. Samples were collected and dissected in accordance with rigorous experimental guidelines. The

study examined the developmental stages of *H. parallela*, ranging from eggs to third-instar larvae. Adult tissues, including the head, prothorax, legs, and fat body, were dissected. Sex-specific samples, including the head and ovary or testis, were dissected for female and male adults, respectively.

The female beetles, comprising a total of 5 groups with 20 individuals in each, were placed in culture boxes with dimensions of 16 × 24 cm. Each culture box contained a layer of 30 cm of sterile soil. For the photoperiod experiments, adults were treated under five different light conditions (24L:0D, 16L:8D, 12L:12D, 8L:16D, 0L:24D) for four days [26]. Randomly, three beetles were chosen from within each group and dissected to isolate their heads.

Third-instar larvae were selected for temperature effect assessment [27] and cleaned before being subjected to treatments at 4 and 30 °C for 2 h each, with consistent humidity and light conditions between treatments.

Female beetles were fed elm leaves soaked in RNAi solutions (ddH₂O, dsGFP, and dsFcp3C) for 11 days, as documented in prior research [20]. The ovaries of female beetles from each treatment were randomly selected, dissected, and transferred into sterile 1.5 mL centrifuge tubes.

The tissue dissection method for *H. parallela* was as follows. The samples to be dissected were fixed on paraffin dishes with clean insect needles and observed under a stereomicroscope. Chilled 1×PBS buffer solution was poured into the dish, and the tissues required for the experiment were dissected using dissecting forceps according to experimental requirements. Three biological replicates per sample group were frozen in liquid nitrogen and stored in 1.5 mL centrifuge tubes at −70 °C for future use.

2.2. RNA Isolation and Reverse Transcription

Total RNA was isolated from each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA purity and concentration were analyzed spectrophotometrically using a Thermo NanoDrop. Subsequently, cDNA synthesis was performed using the PrimeScript™ RT reagent kit (Takara, Dalian, China).

2.3. Selection of Reference Genes and Primer Formulation

The sequences of 11 candidate reference genes commonly used in insect studies, including *GAPDH*, *RPL32*, *RPL7A*, *RPS18*, *RPL13a*, *RPL18*, *Actin*, *RPS7*, *RPS3*, *VATB*, and *EF1A*, were obtained from the transcriptome data for *H. parallela* [20]. Primer design and evaluation were conducted using DNAMAN 8.0 software and Primer3 (v. 0.4.0) software (accessible at <http://bioinfo.ut.ee/primer3/>, accessed on 10 June 2022) following the principles for designing qRT-PCR primers.

2.4. Fluorescent Quantitative PCR Reaction Conditions

RT-qPCR was performed on the CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green® Premix Ex Taq™ II kit (Takara, Dalian, China). Primer sequences are detailed in Table 1. Each 10 µL reaction contained 5 µL of TB Green Premix, 0.4 µL of each primer, 0.8 µL of cDNA, and 3.4 µL of sterile water. The thermal cycling conditions included an initial denaturation step followed by 40 cycles of denaturation, annealing, and extension, and a final melting curve analysis. Melting curve and standard curve were conducted to ensure specificity and assess amplification efficiency. A standard curve was constructed using serial 10-fold dilutions of cDNA, which yielded the following five concentration gradients of cDNA templates: 10⁰ (500 ng/µL), 10^{−1}, 10^{−2}, 10^{−3}, and 10^{−4}. The amplification efficiency (E) value was then calculated for all primers as a percentage, as follows: $E = (10^{[-1/\text{slope}]} - 1) \times 100\%$.

Table 1. The primer sequences for the candidate reference genes and the two target genes utilized in this study.

Gene	Primer Sequences (5'-3', F/R)	Efficiency (E) (%)	Regression Coefficient (R ²)
GAPDH	AGTCGCCGTAATGATCCCT CGTCGACTGTGCCATTGAAT	110.68	0.9971
RPL32	GCAAAAACCCGTCATATGCT TGATACGCCATGTGCAATTT	110.17	0.998
RPL7A	TGCAAATACAATCCGAGCTG GGCAGGAAGAGCACAAGTTC	107.71	0.9916
RPS18	CGTGCTGGAGAATGTTCTGA GCTGGCTATATTTGCCATCAA	105.82	0.9972
RPL13a	GGCGTACCTCCACCTTATGA CAGAAATTTGCGACCAGGTTT	106.76	0.9978
RPL18	CATTACGCTCACCAACAGGA CTGGAGCAGGACCAAAGTGT	111.19	0.998
Actin	TGTCCTGTATGCCTCTGGT TACCAGCCAAATCCAAACGC	109.67	0.9978
RPS7	CGCGAGCTTGAGAAGAAGTT AGAACGTGGACGCTTCTGTT	109.18	0.9994
RPS3	ATCCACTCAGGTGACCCTTG AACGGCCTCTTAGGTCCAAT	114.35	0.9956
VATB	GGTCTACCGCACAACGAAAT ACCTAGCGGTTTCCATGTTG	126.92	0.9933
EF1A	GCCAGAAGCTGTACCTGGAG TGTCACCGGCTACATAACCA	106.76	0.9994
Target gene			
VgR	TGGCGAAGACGAGAAAAACT TCGTCCGACAAATCGTAACA	-	-
FoxL 2	CAGCAGCCTATACGCAACAA AGGAGGCCAATAAGCTGGAT	-	-

2.5. Assessment of Reference Gene Stability

To assess the stability of candidate reference genes, this study employed a range of algorithms, including geNorm [1], NormFinder [28], BestKeeper [29], and the ΔCt method [30], providing a comprehensive evaluation. After this thorough assessment, the RefFinder [31] was employed to compare and rank the candidate genes, facilitating their prioritization.

2.6. Validation of Selected Reference Genes

The forkhead transcription factor *FoxL2* gene and the vitellogenin receptor (*VgR*) gene are associated with ovarian differentiation and egg development in insects. In this study, *FoxL2* and *VgR* were chosen as the target genes. To standardize the data, diverse internal reference genes were utilized and their stability was verified. The expression profiles of the target genes, *VgR* and *FoxL2*, were determined utilizing the $2^{-\Delta\Delta\text{Ct}}$ method. The SPSS Statistics 22 software package was then applied to evaluate the variance in expression levels of the genes of interest through an independent-samples *t*-test.

3. Results

3.1. Assessment of Primer Efficiency and Specificity in Amplification

Prior to assessing the suitability of reference genes, rigorous validation of the PCR amplification's specificity and efficiency is essential. Each PCR amplicon was examined using a 1.5% agarose gel and displayed a single band that matched the anticipated length. Furthermore, the melting curves of PCR amplifications with each primer set exhibited a unique peak, demonstrating their specificity (Figure S1). All primer pairs, excluding the VATB primer, demonstrated high amplification efficiency (ranging from 105.82% to

114.35%), with regression coefficients exceeding the threshold of 0.990, indicating reliable qPCR results (Table 1, Figure S2).

3.2. Expression Profile of Reference Genes

The threshold-cycle (Ct) represents the transcript level of the mRNA. Under varying treatment conditions, the Ct values of the 11 candidate reference genes for *H. parallela* ranged from 13.48 to 30.01. The mean Ct values spanned a range, with *RPL32* exhibiting the lowest value at 19.05 and *VATB* having the highest value among the samples at 24.36 (Figure 1). Based on the coefficient of variation (CV), *RPL18* exhibited the highest stability (CV = 8.92) while *RPL32* displayed the greatest variability (CV = 15.94). (Table S2).

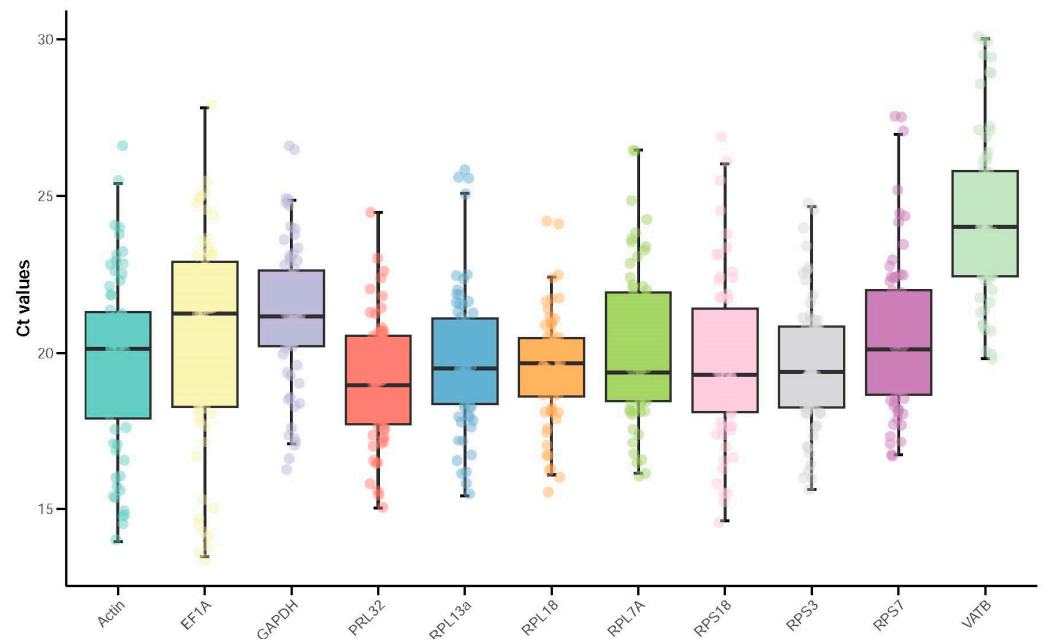


Figure 1. Reference Gene Expression under Different Treatments in *Holotrichia parallela*. In each box, the lower quartile (25th percentile) and upper quartile (75th percentile) are depicted. The whiskers mark the minimum and maximum values of the dataset. The horizontal line within each box marks the median value. Dots represent the reference gene expression for each sample.

3.3. Identifying Reference Genes with Consistent Expression Stability

The expression stability of 11 candidate internal reference genes in *H. parallela* under varying treatment conditions was assessed using the following four analysis methods: the Δ Ct method, geNorm, NormFinder, and BestKeeper. RefFinder was used to comprehensively evaluate the expression stability of 11 candidate reference genes in *H. parallela* under different treatment conditions (Figure 2, Table S3).

For different developmental stages of *H. parallela*, the expression analysis of candidate reference genes was performed using the Δ Ct method and NormFinder (Version dated 05/01-2015) software. It was found that *RPL18*, *RPS3*, and *RPL13a* showed the highest expression stability. The geNorm analysis revealed that *RPL18* and *RPL13a* were the most stable reference genes, while BestKeeper analysis indicated that the reference gene *VATB* was the most stable. All four analyses revealed that *RPS7* and *EF1A* were relatively unstable internal reference genes (Figure 2, Table S3). RefFinder analysis ranked the expression stability of candidate reference genes in *H. parallela* during development from highest to lowest, as follows: *RPL18*>*RPL13a*>*RPS3*>*GAPDH*>*RPL32*>*VATB*>*Actin*>*RPL7A*>*RPS18*>*RPS7*>*EF1A* (Figure 3, Table S3).

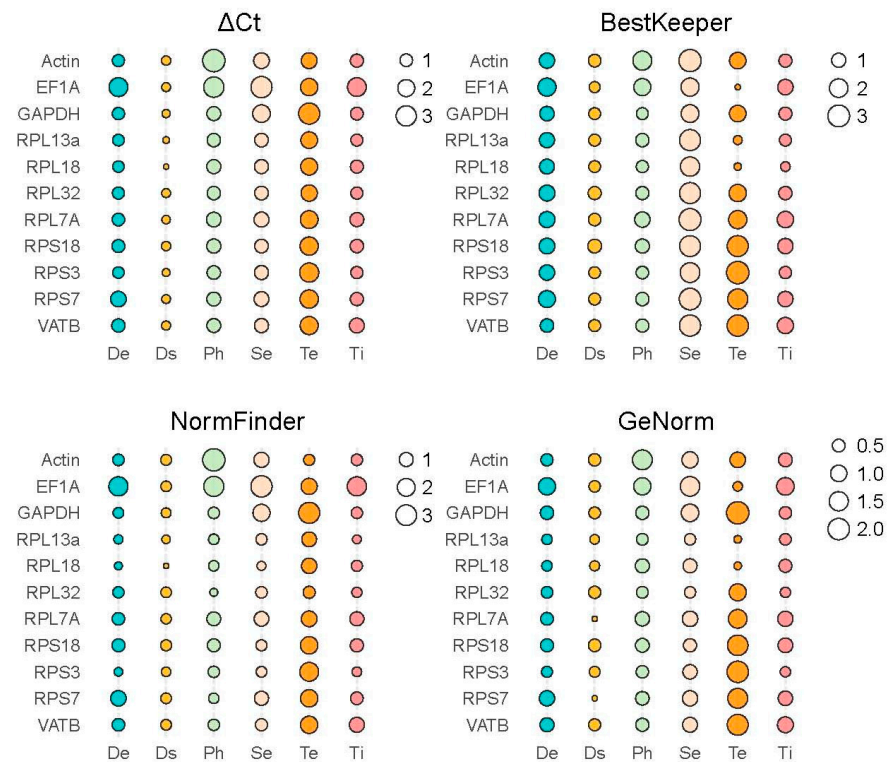


Figure 2. Stability of 11 candidate reference genes assessed using the ΔC_t method, BestKeeper, NormFinder, and geNorm. The size of each bubble represents the reference gene stability value, with smaller bubbles indicating more stable genes. De: developmental stage, Ds: dsRNA treatment, Ph: photoperiod treatment, Se: sexes, Te: temperature, Ti: tissues.



Figure 3. Expression stability of 11 reference genes of *Holotrichia parallela* under various treatment conditions analyzed using RefFinder. Bubble size represents reference gene stability, with smaller bubbles indicating greater stability. De: developmental stage, Ds: dsRNA treatment, Ph: photoperiod treatment, Se: sexes, Te: temperature, Ti: tissues.

For female and male *H. parallela* tissues, analysis using the Δ Ct method showed that *RPL13a* and *RPS18* exhibited the most stable expression. The geNorm software (Version 3.5) evaluation recognized *RPL32* and *RPL13a* as the top stable reference genes, whereas NormFinder pointed to *RPL18* as exhibiting the highest expression stability. All three analyses revealed that the expression of *GAPDH* and *EF1A* exhibited relatively poor stability. However, the BestKeeper software (Version 1) analysis suggested that *GAPDH* and *EF1A* showed the most stable expression while *RPL7A* and *Actin* exhibited poorer stability (Figure 2, Table S3). The RefFinder analysis ranked the expression stability of candidate reference genes in male and female adult *H. parallela* tissues from highest to lowest, as follows: *RPL13a*>*RPL18*>*RPL32*>*RPS18*>*RPS3*>*GAPDH*>*VATB*>*EF1A*>*RPS7*>*RPL7A*>*Actin* (Figure 3, Table S3).

For different tissues of *H. parallela*, the Δ Ct analysis and NormFinder revealed that *RPL13a* exhibited the most stable expression among the candidate internal reference genes. The geNorm analysis identified *RPL32* and *RPS3* as the most stable reference genes. All three analyses indicated relatively poor stability in the expression of *VATB* and *EF1A*. According to the BestKeeper analysis, *RPL18* and *RPL13a* had the most stable expression, while *VATB* and *RPL7A* exhibited poorer stability (Figure 2, Table S3). The RefFinder analysis ranked the expression stability of candidate reference genes in different adult *H. parallela* tissues from highest to lowest, as follows: *RPL13a*>*RPS3*>*RPL32*>*RPL18*>*GAPDH*>*Actin*>*RPS7*>*RPS18*>*RPL7A*>*VATB*>*EF1A* (Figure 3, Table S3).

For different light exposure treatments, *RPL32* and *RPL13a* were identified as the most stable reference genes in adult *H. parallela* based on the Δ Ct and geNorm analyses. Specifically, NormFinder favored *RPL32*, while BestKeeper suggested *GAPDH* as the preferred option. All four analyses demonstrated that *EF1A* and *Actin* had relatively poor expression stability (Figure 2, Table S3). RefFinder analysis ranked the expression stability of candidate reference genes in *H. parallela* under varying light exposure conditions from highest to lowest, as follows: *RPL32*>*RPL13a*>*RPS3*>*GAPDH*>*RPL18*>*RPS7*>*VATB*>*RPS18*>*RPL7A*>*EF1A*>*Actin* (Figure 3, Table S3).

For samples of third-instar *H. parallela* larvae under different temperature treatments, *Actin* and *RPL32* were identified as the most stable reference genes in adult *H. parallela*, based on the Δ Ct and geNorm analyses. The geNorm analysis showed that *RPL13a* and *RPL18* were the most stable, while all three analyses demonstrated that *RPS3* and *GAPDH* were relatively unstable internal reference genes. The BestKeeper analysis identified *EF1A* and *RPL18* as the most stable, while *VATB* and *RPS3* exhibited poor stability (Figure 2, Table S3). The RefFinder analysis ranked the expression stability of candidate reference genes in third-instar *H. parallela* larvae samples under different temperature treatments from highest to lowest, as follows: *Actin*>*RPL13a*>*RPL18*>*EF1A*>*RPL32*>*RPL7A*>*RPS7*>*VATB*>*RPS18*>*GAPDH*>*RPS3* (Figure 3, Table S3).

For samples of *H. parallela* under RNAi feeding treatment, *RPL18* and *RPL13a* were identified as the most stable reference genes in adult *H. parallela* based on the Δ Ct and geNorm analyses. While the geNorm analysis favored *RPL7A* and *RPS7*, the BestKeeper analysis identified *EF1A* as the most stable. All four analyses revealed that *RPS18* had relatively poor expression stability (Figure 2, Table S3). The RefFinder analysis ranked the expression stability of candidate reference genes in third-instar *H. parallela* larvae samples under RNAi feeding treatment from highest to lowest, as follows: *RPL18*>*RPL13a*>*RPS3*>*RPL7A*>*RPS7*>*EF1A*>*GAPDH*>*VATB*>*RPL32*>*Actin*>*RPS18* (Figure 3, Table S3).

3.4. Optimal Number of Reference Genes

Pairwise variation (V) values, which indicate the stability of reference genes, were computed using the geNorm software to ascertain the optimal number of reference genes required for data normalization under different experimental conditions. The results of the analysis showed that for different developmental stages and adult tissues, V2/3 to V9/10 were all less than 0.15. For third-instar larvae under varying temperature conditions, V2/3 was less than 0.15 and V3/4 to V10/11 were all greater than 0.15. Additionally,

for adult *H. parallela* under RNAi feeding treatment, V2/3 to V10/11 were all less than 0.15. This suggests that two candidate reference genes are sufficient for data normalization in different developmental stages of *H. parallela*, adult *H. parallela* tissues, third-instar *H. parallela* larvae under varying temperature conditions, and adult *H. parallela* under RNAi feeding treatment. For different tissues of adult male and female *H. parallela*, V4/5 to V8/9 were all less than 0.15 while V2/3 to V3/4 and V9/10 to V10/11 were all greater than 0.15. Under different light conditions, V3/4 to V8/9 were all less than 0.15 while V2/3 and V9/10 to V10/11 were all greater than 0.15. This indicates that three candidate reference genes should be used for data standardization in the different tissues of male and female adult *H. parallela* as well as in adult *H. parallela* under varying light conditions (Figure 4).

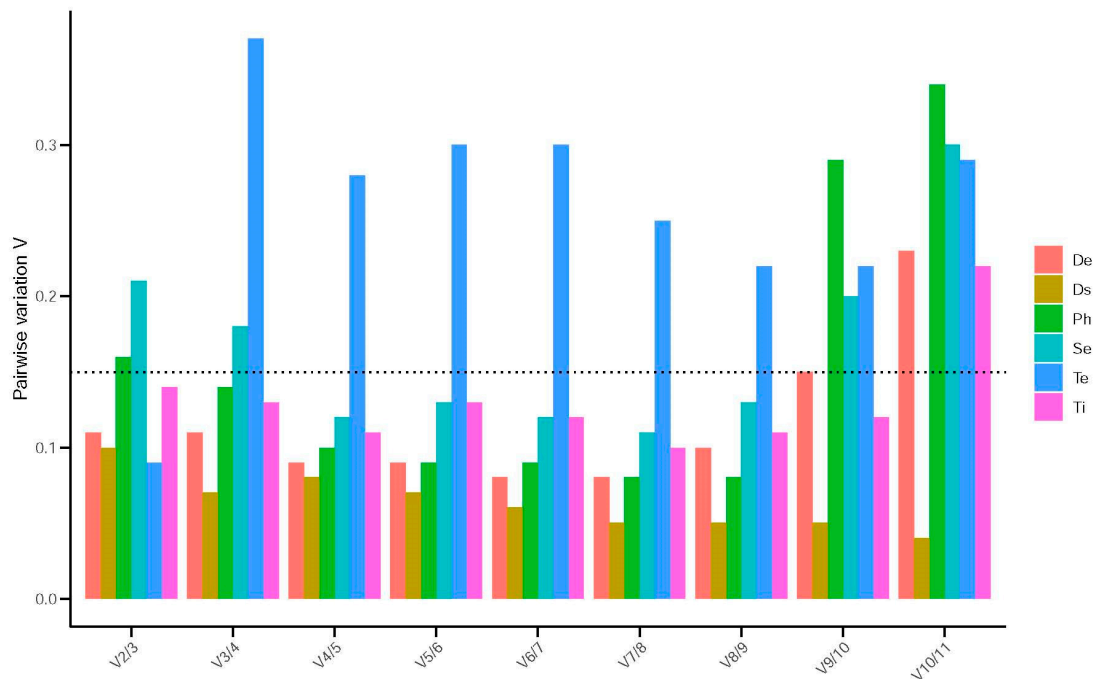


Figure 4. geNorm analysis of 11 reference genes in *Holotrichia parallela* determined the optimal number for accurate normalization. Pairwise variation (V_n/V_{n+1}) values indicate the stability of reference gene combinations. The dotted line represents the recommended threshold (0.15), below which additional reference genes are not necessary. De: developmental stage, Ds: dsRNA treatment, Ph: photoperiod treatment, Se: sexes, Te: temperature, Ti: tissues.

3.5. The Influence of Housekeeping Genes on Quantitative PCR Data Interpretation

The stability analysis and pairwise variation (V) calculations of comprehensive candidate reference genes performed under the *H. parallela* RNAi feeding treatment indicate that utilizing two highly stable reference genes is optimal for normalization. Consequently, in this research, the pairing of the most stable genes *RPL18* and *RPL13a*, in addition to the least stable gene, *RPS18*, were chosen as reference genes. These genes were employed to analyze the expression levels of the vitellogenin receptor (*VgR*) gene and the forkhead box L2 (*FoxL2*) gene, ultimately validating the stability of the selected reference genes.

The follicular cell protein (*Fcp3C*) gene is related to insect reproduction and oviposition. After RNAi, *Fcp3C* affects the expression of the *VgR* and *FoxL2* genes. When using the highly stable gene combination of *RPL18* and *RPL13a* as reference genes, the expression level of the target gene *VgR* was relatively lower in ds*Fcp3C* samples. Utilizing the least stable gene, *RPS18*, as a reference gene, the expression level of the target gene *VgR* was relatively lower in both the dsGFP and ds*Fcp3C* samples, with no significant difference between them (Figure 5A). Regarding the expression level of the target gene *FoxL2*, when using the highly stable gene combination of *RPL18* and *RPL13a* as reference genes, the expression level in ds*Fcp3C* samples was higher than in the control (ddH₂O) and dsGFP samples.

There was no significant difference in expression level between the control (ddH₂O) and dsGFP samples. However, when using *RPS18* (the least stable gene) as a reference gene, the expression level of the target gene *FoxL2* was higher in the control (ddH₂O) sample, whereas it was lower in both the dsGFP and dsFcp3C samples, with the lowest expression level being observed in the dsGFP sample (Figure 5B). Based on the validation results discussed above, using different reference genes for normalizing target genes will indeed yield different experimental results.

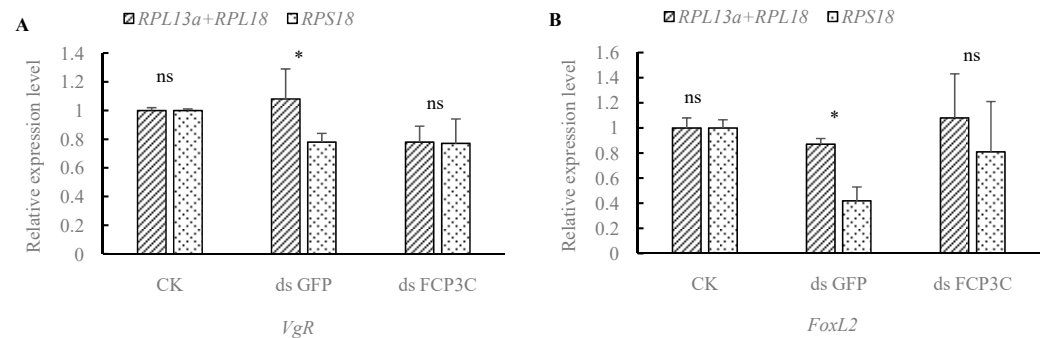


Figure 5. Expression of the target genes *VgR* (A) and *FoxL2* (B) in *Holotrichia parallela* treated with RNA interference (RNAi) using different reference genes. *: a statistically significant difference with $p < 0.05$; ns: not significant.

4. Discussion

qPCR, which is usually used to determine gene expression levels, is a widely recognized, established method that serves as a gold standard in molecular biology research [32–34]. Normalization is essential in qRT-PCR analysis for addressing variations that occur within and between reaction cycles. These variations can lead to inconsistencies, including variations in the quantity of the initial sample material, differences in RNA integrity, discrepancies in the loading amounts of cDNA among samples, and deviations in reverse transcription efficiency [35–37]. Thus, the observed reference gene variability encapsulates both the intrinsic variability of the gene itself and the technical errors incurred during the process [36]. Controlling all steps leading up to the PCR measurement is crucial, and selecting an appropriate reference gene is vital for achieving precision across varying experimental setups.

Transcriptomic datasets for *H. parallela* have been made available to facilitate research into its developmental processes, chemosensory genes, odorant-degrading enzymes, and immune responses [19,20,38,39]. Previous qRT-PCR studies on *H. parallela* generally utilized conventional insect reference genes, notably *Actin* and *GAPDH* [21,38,40,41]. In addition to the highest stability of *Actin* under different temperature conditions, the present study found that *Actin* and *GAPDH* exhibited particularly high variable instability values. Therefore, neither *Actin* nor *GAPDH* was the most stable reference gene in most cases. The expression of *Actin* was less stable in several insects, including *Tribolium castaneum*, *H. obliqua*, *Coleomegilla maculata*, *Hippodamia convergen* and *Hippodamia variegata*, all belonging to the order Coleoptera [27,42–45].

The present study identified unique gene stability profiles in *H. parallela*, aligning with previous research findings [27] that highlighted the variability of gene stability under various experimental conditions in *Holotrichia obliqua*. In the present study on *H. parallela*, *RPL18* and *RPL13a* were the most stable across the developmental stages and RNAi conditions. This suggests that these two genes can serve as reliable reference gene selections under these experimental conditions. Furthermore, *RPL13a*, *RPL18*, and *RPL32* displayed high stability in both sexes, indicating their appropriateness as reference genes for sex-specific gene expression analyses in *H. parallela*. Under varying photoperiods, *RPL32*, *RPL13a*, and *RPS3* exhibited the greatest stability, while *Actin* and *RPL13a* were the most stable across different temperatures.

In contrast, previous studies on *H. obliqua* have reported that *RPL13a* and *RPL18* are the optimal reference genes for investigating different tissues, developmental stages, and temperature. This is in line with the present findings in *H. parallela*. The consistency in the stability of *RPL13a* and *RPL18* across species suggests that these genes may serve as general reference markers for gene expression analysis in closely related insects, particularly within the *Holotrichia* genus. However, for photoperiod treatments in *H. obliqua*, *RPL13a* and *RPS3* were found to be the most stable genes, while *RPS18* and *RPL18* were most suitable for sex-specific gene expression studies. Among the selected genes, including *GAPDH*, *Actin*, and 18s rRNAs, *GAPDH* was selected as a standardized reference gene in various tissues of *H. parallela* [38]. However, when compared to the other genes analyzed in the current study, *RPL18* and *RPL13a* exhibited the greatest stability, while *GAPDH* ranked sixth in stability among the 11 genes tested. Similarly, *Actin* consistently ranked below the median in stability assessments, except when it exhibited the highest stability under different temperature treatments, which supports findings observed in other species [43,45–47]. In a previous work, *GAPDH* and *RPL13a* were considered the best reference genes for various tissues and the developmental stages of *Anomala corpulenta*, respectively [48].

Extensive studies across insect species have confirmed that ribosomal proteins are the most reliable reference genes, excelling in expression consistency and robustness compared to other candidates [22]. Consistently, the results of the current analysis ranked ribosomal protein genes among the three most stable reference gene options across diverse experimental settings. In the field of gene expression studies in insects, the ribosomal protein gene, including *RPL18*, *RPS23*, *PRL13*, and *RPL32*, has emerged as the preferred choice for reference genes, demonstrating superior suitability based on recent comprehensive evaluations [44,49–52]. Furthermore, *RPL18*, *RPS3*, and *RPL13a* can be considered as proper reference genes for normalizing RT-qPCR data, especially when choosing reference genes from other Coleoptera insects that have not been extensively studied. Nevertheless, the differences observed in the stability of other genes under specific conditions highlight the crucial role of choosing suitable reference genes based on the experimental plan and species under investigation.

In summary, this study offers significant insights into the identification of appropriate reference genes for quantitative gene expression analysis in *H. parallela*, especially within the context of RNAi treatments. This will guarantee precise and dependable analysis of gene expression for similar gene types in this and other closely related insect species and facilitate the more effective identification of target genes for pest control.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/insects15090661/s1>, Figure S1: Specificity of primer pairs; Figure S2: Amplification efficiency curves; Table S1: Standard curve equations, slopes, and amplification efficiencies of the 11 candidate internal reference genes; Table S2: Ct values and coefficients of variation (CV) for the 11 candidate internal reference genes Table S3: The stability of the 11 candidate reference genes evaluated by ΔCt method, BestKeeper, NormFinder, and geNorm.

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