



Evaluation of suitable reference genes for expression profile analyses of target genes in the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae)

Research Paper

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Abstract

The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is a major destructive insect pest of coffee, which impacts the coffee crops negatively. As a draft genome has been completed for this insect, most molecular studies on gene transcriptional levels under different experimental conditions will be conducted using real-time reverse-transcription quantitative polymerase chain reactions (RT-qPCR). However, the lack of suitable internal reference genes will affect the accuracy of RT-qPCR results. In this study, the expression stability of nine candidate reference genes was evaluated under different developmental stages, temperature stress, and *Beauveria bassiana* infection. Data analyses were completed by four commonly used programs, BestKeeper, NormFinder, geNorm, and RefFinder. The result showed that *RPL3* and *EF1 α* combination were recommended as the most stable reference genes for developmental stages. *EF1 α* and *RPS3a* combination were the top two stable reference genes for *B. bassiana* infection. *RPS3a* and *RPL3* combination performed as the optimal reference genes both in temperature stress and all samples. Our results should provide a good foundation for the expression profile analyses of target genes in the future, especially for molecular studies on insect genetic development, temperature adaptability, and immune mechanism to entomogenous fungi in *H. hampei*.

The coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), has been listed as a quarantine pest in many countries and considered as one of the most destructive insect pests in coffee. It is estimated that once CBB outbreak occurs, the coffee industry will suffer losses of up to 80% (Le Pelley, 1973; Vega *et al.*, 2009; Infante, 2018). It is worth noting that CBB was firstly recorded invading the Chinese island of Hainan in 2019 (Sun *et al.*, 2020), which is a major producer of the species robusta coffee. The insect has a special breeding mode that most of its lifecycle occurs inside the coffee berries, making the control strategies difficult to implement (Baker, 1999; Damon, 2000; Aristizábal *et al.*, 2012). Female adults bore into the endosperm of the berries and deposit their eggs. Special galleries are formed with the larvae develop within the beans, leading to reduced coffee yield and quality. All nutrients for young beetles come from the coffee berries, until the mated female beetles emerge from mature coffee berries to find new habitats for oviposition (Damon, 2000; Duque and Baker, 2003).

For invasive insects, temperature plays an important role throughout their whole lifecycle. The adaptability to different temperatures allows insects to successfully invade and spread (Wallner, 1987; Renault *et al.*, 2018; Umeda and Paine, 2019). Temperature variation significantly affected the fecundity and development time of CBB. Females of CBB successfully oviposited in the temperature range 15–30°C, with the highest fecundity observed at 23°C (Azrag and Babin, 2023). The maximum and minimum temperature threshold have also been estimated for egg, larva, pupa, and the total development from egg to adult, respectively (Azrag *et al.*, 2020). Studies on the adaptability of CBB to temperature will help understand and predict the pest population dynamics and distribution in coffee plantations and as such, will contribute to a more efficient management of the pest. In terms of prevention and control strategies for CBB, three African parasitoids have been introduced to many CBB invaded countries, including *Prorops nasuta* Waterston (Bethyridae), *Cephalonomia stephanoderis*

Betrem (Bethyidae), and *Phymastichus coffea* LaSalle (Eulophidae) (Barrera *et al.*, 1990; Infante *et al.*, 1994, 2012; Chiu-Alvarado and Rojas, 2008; Espinoza *et al.*, 2009). Besides, an entomopathogen, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales), has been widely used to control CBB throughout the world. It has been verified to be highly infective to the adult beetles in coffee plantations wherever the borer is present (Infante, 2018). In recent years, more and more studies on the use of volatile compounds extracted from either coffee trees themselves or companion plants have been reported. Among these volatiles, some are attractive compounds that can be used as lure traps to capture and monitor CBB populations in the field (Pereira *et al.*, 2012; Cruz Roblero and Malo, 2013; Jaramillo *et al.*, 2013; Cruz-López *et al.*, 2016; Sinaga *et al.*, 2020). Moreover, some monoterpene and sesquiterpene compounds elicited from secondary metabolites are identified as repellent to CBB (Jaramillo *et al.*, 2013; Vega *et al.*, 2017; Mafra-Neto *et al.*, 2018). Carmenza *et al.* (2020) reported terpene-volatile compounds as a promising compound repellent for CBB control.

A better understanding of gene function and expression will reveal insights into relevant molecular pathways, informing updated pest control strategies. However, little research has been done on the molecular mechanism of CBB. The mechanisms of the invasion and diffusion of CBB are still unclear. Elucidating the adaptation mechanism to the environment of CBB and the infection mechanism of entomogenous fungi could help improve their control effectiveness against CBB. Moreover, as the genome draft of CBB has been sequenced, it will provide important genetic information for research on such molecular mechanisms (Vega *et al.*, 2015). Real-time quantitative polymerase chain reaction (RT-qPCR) is a reliable and useful method for the transcriptional expression level analyses of target genes, which has high specificity, accuracy, sensitivity, and reproducibility (Heid *et al.*, 1996; Bustin, 2010). However, the result is easily affected by the RNA extraction quality, the efficiency of cDNA production using reverse transcription, the primer amplification efficiency, and so on. Therefore, introducing suitable reference genes for target gene expression normalisation is necessary for RT-qPCR analysis (Hu *et al.*, 2018; Wang *et al.*, 2019). No suitable 'universal' reference gene could be expressed constantly in all the organism and experimental conditions (Lee *et al.*, 2002). Therefore, it is essential to perform internal reference gene stability testing before performing RT-qPCR experiments. The ideal internal reference gene should express stably under whatever experimental conditions. To date, no single reference gene has been found suitable for all the experimental treatments, nor among species. Even though *actin* (*ACT*), β -tubulin (β -*tub*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were identified as traditional reference genes for a long time they expressed unstably in response to diverse experimental conditions or different species. For example, *ACT*, β -*tub* and *GAPDH* were identified as unstable reference genes in all treatments in *Xylocopa quadripes* (Meng *et al.*, 2022). In *Helopeltis theivora*, *ACT* was considered as the best reference gene only in tissues, and *GAPDH* only expressed stably in developmental stages. However, β -*tub* was unstable in all experimental conditions (Wang *et al.*, 2019). *GAPDH* and β -*tub* were indicated unstable in all the treatments in *Adelphocoris suturalis* except *ACT* as the ideal reference gene in dsRNA infection (Luo *et al.*, 2018). Besides, some novel reference genes such as ribosomal protein genes, elongation factor, eukaryotic translation initiation factor were verified as a set of potential internal control

genes in the insect mentioned above. However, their stability was also influenced by different treatments or different species. Therefore, whether it is traditional internal reference or novel ones, we still need to verify the stability of these genes if we want to use them in CBB.

So far, no validated reference gene has been reported in CBB for differential experimental conditions. Thus, the object of this study is to identify the optimal reference genes of CBB in developmental stages, temperature stress, and *B. bassiana* infection treatment. Our study tests and validates a set of candidate reference genes for RT-qPCR in CBB. This should be useful for gene expression analysis in modern molecular genetic research in CBB.

Materials and methods

Insect collection

The CBB was collected from the coffee plantation located in Wanning (Hainan province, China). Coffee berries with shotholes were brought back to the laboratory until the adults emerged. The newly emerged adults were reared singly with a fresh coffee berry in plastic cylindrical bottles. The laboratory condition was maintained at $(26 \pm 2) ^\circ\text{C}$, $(75 \pm 5)\%$ relative humidity, and a photoperiod cycle of 16 h L:8 h D.

Experimental treatments

Developmental stages

Different developmental stages of CBB including eggs, larvae, pupae, and adults were collected into RNase-free centrifuge tubes and stored at $-80 ^\circ\text{C}$ for RNA extraction. Three biological replicates were set for each developmental stage. Each replicate included one hundred eggs, twenty larvae including all the instar, twenty pupae, and twenty adults.

Temperature stress

The CBB adults were exposed to three temperature treatments. Specifically, adults were acclimated for 4 h at low temperature $4 ^\circ\text{C}$, preference temperature $26 ^\circ\text{C}$ and high temperature $32 ^\circ\text{C}$. Twenty adults were collected for each treatment. Three biological replicates were set for each temperature exposure. Each replicate was collected into RNase-free centrifuge tubes and stored at $-80 ^\circ\text{C}$ for RNA extraction.

B. bassiana infection treatment

The *B. bassiana* strain used was isolated from the CBB cadavers collected in a wild coffee field in Wanning (Hainan province, China). The strain was purified and cultured by PDA medium in the laboratory and stored at China centre for type culture collection (CCTCC NO: M2017045). A spore suspension with a concentration of 1×10^7 conidia ml^{-1} was prepared with 0.05% Tween-80 sterile water. In total, sixty CBB adults were immersed into the spore suspension for 5 s and raised in the sterilised dishes. Twenty treated adults were collected into RNase-free centrifuge tubes after 12 h, 24 h, and 48 h, respectively. Three replicates were obtained for each treatment. All the samples were stored at $-80 ^\circ\text{C}$ for RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA from different treatments was extracted using TRIzol Reagent (Invitrogen, USA). Disrupt and homogenised each sample using 1 ml TRIzol reagent, and then incubated for 5 min at

room temperature. 200 μ l of chloroform was added and each sample shaken vigorously for 15 s, before further incubation at room temperature for 3 min. Samples were centrifuged for 15 min at 13,400 \times g at 4 $^{\circ}$ C, and the aqueous phase was transferred to a new centrifuge tube with the same volume of isopropanol added. Following incubation for 10 min, the samples were centrifuged with 13,400 \times g for 10 min at 4 $^{\circ}$ C and the supernatant was discarded. The resulting precipitation was resuspended and vortexed briefly with 1 ml 75% ethanol for 2~3 times. The supernatant was discarded and the RNA pellet was air-dried and an appropriate amount of RNase-free water was added to dissolve the precipitate. Finally, the quality and purity of total RNA was determined by fluorescence microplate reader (BioTek, USA).

The cDNA synthesis was conducted following the protocol of PrimeScript RT reagent Kit (TIANGEN, China). Specifically, we mixed 1 μ g of total RNA, 2 μ l of 5 \times gDNA Eraser Buffer, and additional RNase free water up to 10 μ l in an RNase-free tube, incubated the mixture at 42 $^{\circ}$ C for 3 min and chilled immediately in ice. Another RNase-free tube was prepared containing 10 μ l mixture that included 2 μ l 10 \times King RT Buffer, 1 μ l FastKing RT Enzyme Mix, 2 μ l FQ-RT Primer Mix, and 5 μ l RNase free water. The two 10 μ l solutions were then mixed to make a final volume up of 20 μ l. The reverse transcription reaction was performed at 42 $^{\circ}$ C for 15 min, then 95 $^{\circ}$ C for 3 min using a PCR Amplifier. Finally, the products were stored at -20 $^{\circ}$ C before use.

Reference gene selection and primer design

Nine candidate reference genes were selected for stability evaluation in this study, including *GAPDH*, elongation factor EF-1 alpha (*EF1 α*), 60S ribosomal protein L3 (*RPL3*), 60S ribosomal

protein L10 (*RPL10*), 40S ribosomal protein S3a (*RPS3a*), 40S ribosomal protein S9 (*RPS9*), alpha-actinin (*α -ACT*), eukaryotic translation initiation factor 4A (*EIF4A*), TATA box-binding protein (*TATA*). The accession numbers were listed in Table 1. The primers were designed by an online tool (NCBI, <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The amplification efficiency and primer specificity were assessed by standard curves and melt curve analyses. To be specific, the PCR products were firstly confirmed to be the target fragments by bidirectional sequencing and alignment with the uploaded sequence, and then detected and purified using 1% agarose gel electrophoresis and the E.Z.N.A.TM Gel Extraction Kit (Omega, USA) respectively. The DNA fragments were ligate with pMD-19 T, and the products were transformed into *Escherichia coli* DH5 α (TaKaRa, China), before extraction of the plasmid by E.Z.N.A.TM Plasmid Miniprep Kit II (Omega, USA). Finally, the plasmid was used as the templates for the standard curve and melt curve analysis.

RT-qPCR analyses

RT-qPCR reactions were performed on the BioRad CFX96 Real-Time PCR detection system with 2 \times TB Green Premix Ex Taq (TaKaRa, China). Three technical replicates were performed for each biological sample. A 20 μ l reaction system included 10 μ l TB Green Premix Ex Taq, 1 μ l template, 0.5 μ l forward primer, 0.5 μ l reverse primer and 8 μ l ddH₂O. Amplification parameters were as follows: initial denaturation at 95 $^{\circ}$ C for 3 min, followed by 40 cycles at 95 $^{\circ}$ C for 10 s and 60 $^{\circ}$ C for 30 s. Finally, a melting curve analysis from 65 $^{\circ}$ C to 95 $^{\circ}$ C was formed to ensure the consistency and specificity of the amplified product. The standard curves were drawn by a ten-fold dilution series of the plasmid. The efficiency (*E*) and correlation coefficient (*R*²) of each primer

Table 1. Primer characteristics of nine candidate reference genes for RT-qPCR in *H. hampei*

Gene	Whole name	Accession number	Primers	Length	<i>E</i> value	<i>R</i> ²
RPL3	60S ribosomal protein L3	OR129676	5' TGGGCGGTTTCCCTCATTAC 3' 5' GGCTTGTGTGAACCAGCAAC 3'	120	0.927	0.997
RPS3a	40S ribosomal protein S3a	OR129678	5' TGTGCTGACAGTATCGCA 3' 5' AGCTCTTCGAGTCACCATGC 3'	150	0.931	0.991
RPL10	60S ribosomal protein L10	OR129677	5' AAGAAGCCCGCAGTTGATGA 3' 5' AATACGACCCGCTTCCAAGG 3'	96	1.044	0.994
RPS9	40S ribosomal protein S9	OR129679	5' CAGGCAGAGACACATCCGAG 3' 5' ACCGAAGGCGGATTTCCAGAG 3'	109	1.039	0.988
EIF4A	Eukaryotic translation initiation factor 4A	OR129681	5' GTCTGGACACGACCGAAGAA 3' 5' AACCCATCAGGGTCCATTC 3'	133	1.04	0.985
EF1 α	Elongation factor EF-1 alpha	OR129675	5' GACCCAGAAGCCAGTACAA 3' 5' GACCTGACCAAGTTCGCACA 3'	129	1.094	0.931
TATA	TATA box-binding protein	OR129682	5' ATGAACTGCGCCGTCCATA 3' 5' GATCGTGCGAATTTCCGAGC 3'	116	1.093	0.999
α -ACT	Alpha-actinin	OR129680	5' TTGGAAGGCGCAGAAAAAGC 3' 5' CTTCGTGAGCGTCAGCCTTA 3'	112	1.046	0.997
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	OR129674	5' TGGCGTTCAGAGTTCAGTC 3' 5' CTGTGCGTGTCCCAATAA 3'	184	1.118	0.999

pair were calculated by the regression equation (Pfaffl *et al.*, 2004): $E = (10^{[-1/\text{slope}]} - 1) \times 100$.

Stability evaluation of candidate reference genes

The stability of expression levels of nine candidate reference genes was assessed using the following four software tools (or algorithms), including NormFinder, BestKeeper, geNorm, and RefFinder. NormFinder determines the expression stability by considering intra- and inter-group variations and provides a stability value (SV) for each gene. The lower the value, the more stable the gene (Andersen *et al.*, 2004). BestKeeper uses the standard deviations (SD) of the Ct values and PCR efficiency to determine the optimal reference genes. The stable reference genes are always with SD values below 1 (Pfaffl *et al.*, 2004). The geNorm software calculates the pairwise variation of every control gene with all other control genes as the SD of the logarithmically transformed expression ratios, and defined the internal control gene-stability measure M as the average pairwise variation of a particular gene with all other control genes. Gene expression is considered stable when the M value is below 1.5, and the lowest M values are produced by genes with the most stable expression. Furthermore, the pairwise variation (V) can be calculated by geNorm. The optimal normalisation reference gene number is determined by V_n/V_{n+1} . The ' n ' represents the number of internal reference genes introduced when calculating V_n/V_{n+1} . And 0.15 is the threshold of V_n/V_{n+1} . The value of V_n/V_{n+1} below 0.15 means that the optimal number of reference gene recommended is n . If V_n/V_{n+1} is greater than 0.15, a new internal reference gene needs to be added until $V_n/V_{(n+1)}$ is less than 0.15 (Vandesompele *et al.*, 2002). Due to the different ranking of reference genes analysed by the three algorithms above, the web-based program RefFinder, which is a comprehensive platform integrating the above three algorithms, is needed to provide an overall ranking of the stability of candidate reference genes (Xie *et al.*, 2012).

Validation of recommended reference genes

To verify the reliability of recommended reference genes, an important defence gene Lysozyme (Accession number: OR129683) was selected as a target gene. Lysozyme is an important defence gene in insect responses to pathogenic microorganism infection. Its expression level may vary with developmental stages, temperature stress, especially in pathogenic microorganism infection. So we chose it to detect its transcription levels normalised by different combination of the recommended reference genes in the three different treatments mentioned above. The specific primers for RT-qPCR were also designed by the online tool NCBI, the forward primer: (GATTACGTGGCCTACTGGG) and the reverse primer: (GCACAATAAACGTCTCCGGC). Relative expression levels of Lysozyme were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All the treatments were performed with three biological and technical replicates. The one-way ANOVA test was used for data analyses. Expression differences of target genes in different treatments were performed by Student's t -tests using SPSS 20.0 (SPSS, Inc., United States).

Results

Amplification efficiency, primer specificity and expressional levels of candidate genes

To ensure the amplification efficiency and specificity of the primers, RT-qPCR amplifications and standard curves of each

candidate reference gene were conducted. All products were sequenced and aligned to the target sequence. The amplification efficiencies of all primers were between 91.3% and 108.2%, and the associated R^2 ranged from 0.931~0.999 (Table 1). Furthermore, the melt curve of each gene with one single peak also validated the primer specificities (fig. 1A-I).

Transcriptional profiles of nine candidate reference genes

The Ct values of nine candidate reference genes in different treatment conditions were obtained to compare the transcript abundance. The mean Ct values of different genes varied significantly and ranged from 17.82 to 25.74 in different treatment conditions (fig. 2), which indicated that it was reliable for further evaluation of expression stability.

Stability assessment of the candidate reference genes in developmental stages

BestKeeper, Normfinder, and geNorm are three commonly algorithms used for identification of reference genes. In developmental stages, *GAPDH* was revealed to be the most stable reference gene based on BestKeeper, followed by *EF1 α* , *RPL3*, *RPS3a*, *TATA*, *α -ACT*, *RPS9*, *EIF4A*, and *RPL10*. However, NormFinder recommended *RPL3* as the best reference gene, *EF1 α* ranked as the second stable gene, and the most unstable reference gene was *α -ACT*. The top two stable genes evaluated by geNorm were *RPL3* and *RPS3a*, respectively, with *α -ACT* also identified as the least stable gene (Table 2). Besides, the number of optimal reference genes recommended for normalisation in developmental stages was two based on the $V_2/V_3 < 0.15$ (fig. 3A). Therefore, *RPL3* and *EF1 α* were recommended as the most stable reference genes according to the comprehensive analysis of RefFinder combined with the three algorithms above. The stability rank of the remaining genes was in order as *RPL3*, *EF1 α* , *RPS3a*, *TATA*, *GAPDH*, *RPS9*, *RPL10*, *EIF4A*, and *α -ACT*, respectively (fig. 3B).

Evaluation of the optimal reference genes in temperature stress

The stability rankings obtained by BestKeeper and geNorm were almost the same. The two algorithms both recognised *RPL10* and *RPL3* as the top two stable genes. However, the result analysed by NormFinder was different. *RPS3a* and *TATA* were identified as the first and the second stable reference genes, respectively. *EIF4A* was indicated as the most unstable reference gene by the three algorithms (Table 3). Based on the value of V_n/V_{n+1} calculated by geNorm, two optimal reference genes were recommended for gene expression normalisation (fig. 4A). The comprehensive analysis of the three software mentioned above using RefFinder showed that *RPS3a* and *RPL3* were the two suitable reference genes, followed by *RPL10*, *RPS9*, *TATA*, *EF1 α* , *GAPDH*, *α -ACT* and *EIF4A*, respectively (fig. 4B).

Determination of the suitable reference genes in *B. bassiana* infection treatment

The gene stability ranking was significantly different by BestKeeper, NormFinder, and geNorm algorithms. Specifically, *EF1 α* was the most stable reference gene based on the analysis of BestKeeper, followed by *RPS9*, *RPL10*, *RPL3*, *RPS3a*, *TATA*, *EIF4A*, *α -ACT*, and *GAPDH*. However, NormFinder

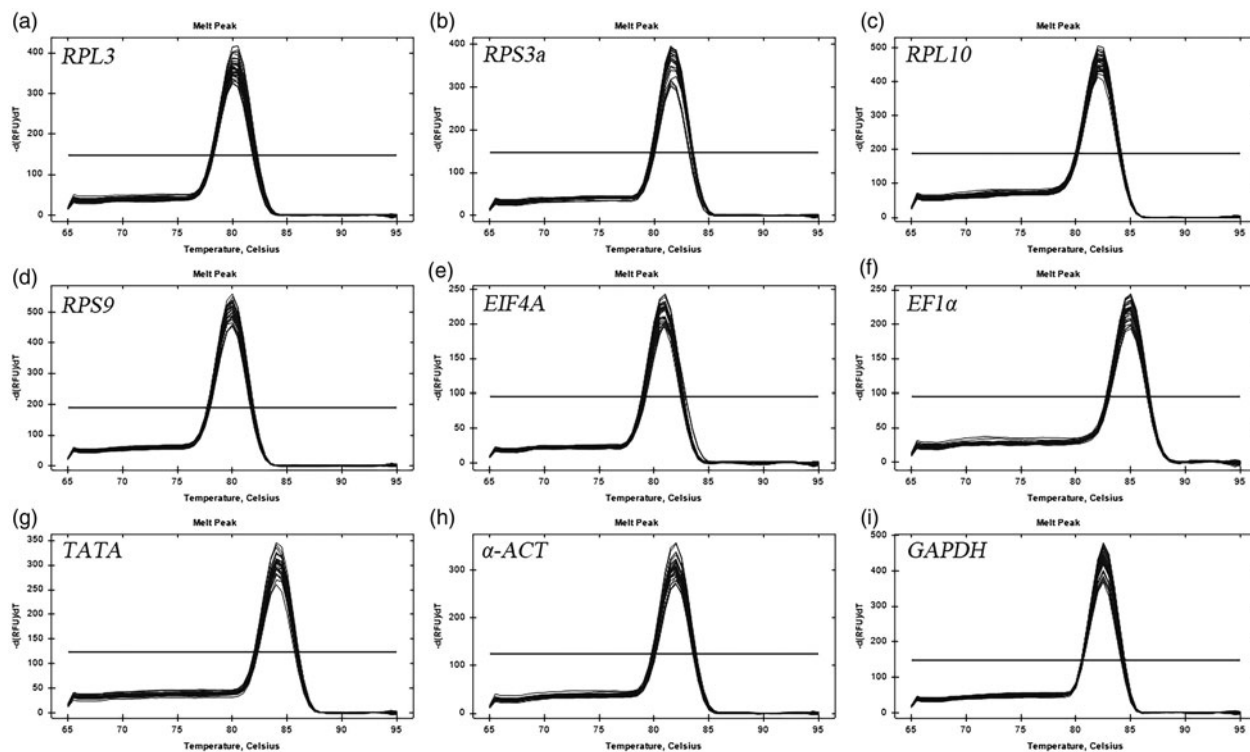


Figure 1. Melt curve analysis of nine candidate reference genes.

recommended *RPL10* as the most stable gene, followed by *RPL3*, *RPS3a*, α -*ACT*, *RPS9*, *EF1 α* , *TATA*, *EIF4A*, and *GAPDH*. According to geNorm analysis, the top two stable reference genes identified were *EF1 α* and *TATA* respectively, and the most unstable gene was also *GAPDH* (Table 4). Furthermore, geNorm suggested adding two internal reference genes for normalisation (fig. 5A). Finally, *EF1 α* and *RPS3a* were identified by RefFinder as suitable reference genes for normalisation of gene expression (fig. 5B).

The stability ranking of candidate reference genes in all samples

For all samples, BestKeeper analysis revealed that *RPL3* was the most stable gene, followed by *RPS9*, *EF1 α* , *RPL10*, *RPS3a*, *TATA*, α -*ACT*, *GAPDH*, and *EIF4A*. NormFinder analysis suggested *RPS3a* was the most stable gene, followed by *RPL3*, *EF1 α* , *RPS9*, *TATA*, *RPL10*, α -*ACT*, *EIF4A*, and *GAPDH*.

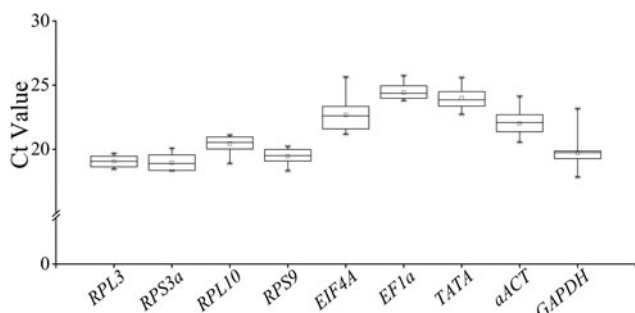


Figure 2. Average Ct values of nine candidate reference genes for all experimental groups in *H. hampei*.

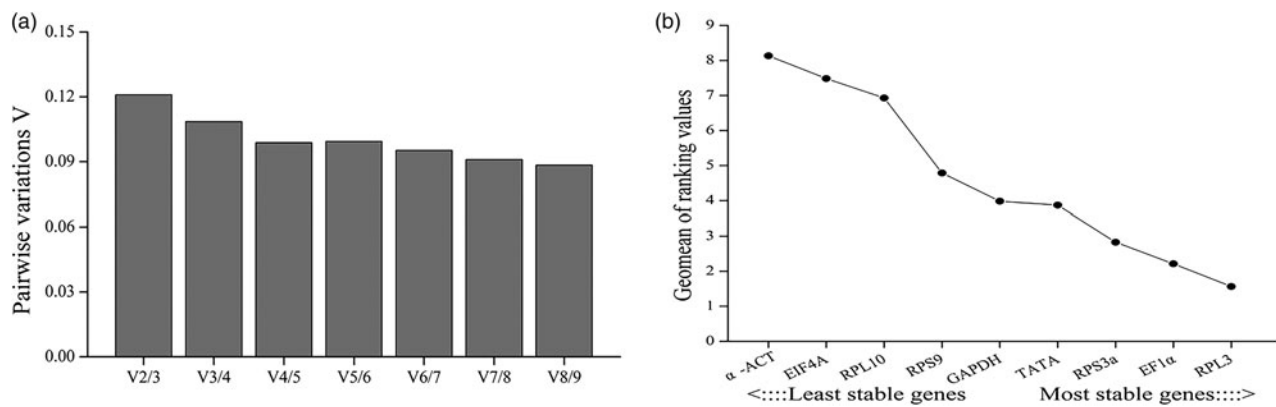
However, geNorm analysis indicated *RPL10* and *RPS9* should be the most stable genes, followed with *RPL3*, *RPS3a*, *EF1 α* , *TATA*, α -*ACT*, *EIF4A*, and *GAPDH* (Table 5). Furthermore, according to geNorm analysis, also two reference genes were required for normalisation accurately (fig. 6A). Finally, a comprehensive assessment of RefFinder revealed that *RPL3* and *RPS3a* could be the best combination of reference genes for all samples (fig. 6B).

Validation of reference gene selection

The expression level of one target gene Lysozyme, an innate immune defence factor, was analysed across developmental stages, temperature stress and *B. bassiana* infection conditions to verify the reliability of reference gene selection. The most stable reference gene (*RPL3*), the two best reference genes (*RPL3* & *EF1 α*) combination and the most unstable reference gene (α -*ACT*) were used for normalisation in different developmental stages. The relative expression level of Lysozyme gene normalised by α -*ACT* in larva, pupa, and adult was significantly different compared to normalised by *RPL3* and *RPL3* & *EF1 α* combination. Lysozyme exhibited similar expression profiles in all three developmental stages above, whether using *RPL3* alone or *RPL3* & *EF1 α* combination (fig. 7A). In temperature stress, the best reference gene (*RPS3a*), the top two stable reference genes combination (*RPS3a* & *RPL3*) and the most unstable gene *EIF4A* were used for gene expression normalisation. For 4°C treatment, The relative expression level of Lysozyme standardised by *EIF4A* showed significant difference with the results standardised by *RPS3a* and *RPS3a* & *RPL3* combination. For the 32°C treatment, there was a significant difference in Lysozyme expression levels when standardised by *EIF4A* compared to the *RPS3a* & *RPL3* combination (fig. 7B). For the *B. bassiana* infection, the most

Table 2. Expression stability of candidate reference gene in developmental stages analysed by BestKeeper, NormFinder and geNorm.

Gene	BestKeeper			NormFinder		geNorm	
	Rank	SD	CV	Rank	SV	Rank	M
RPL3	3	0.24	1.30	1	0.24	1	0.19
RPS3a	4	0.37	2.00	4	0.42	1	0.19
RPL10	9	0.62	3.09	8	0.70	3	0.38
RPS9	7	0.52	2.71	5	0.53	2	0.31
EIF4A	8	0.54	2.48	7	0.63	7	0.62
EF1 α	2	0.22	0.90	2	0.24	5	0.51
TATA	5	0.46	1.98	3	0.24	4	0.44
α -ACT	6	0.51	2.34	9	0.78	8	0.68
GAPDH	1	0.19	0.95	6	0.55	6	0.57

**Figure 3.** Comprehensive ranking of expression stability of nine candidate reference genes in developmental stages of *H. hampei*. (a) Optimal number of reference genes for normalisation in developmental stages. (b) The stability of the nine housekeeping genes in developmental stages determined by RefFinder.

stable reference gene (*EF1 α*), the best reference gene combination (*EF1 α* & *RPS3a*) and the most unstable reference gene (*GAPDH*) were tested. After infection for 24 h, the gene expression profiles of Lysozyme standardised by *GAPDH* exhibited highly significant

difference to those of Lysozyme standardised by *EF1 α* and the *EF1 α* & *RPS3a* combination. The relative expression level normalised by *EF1 α* was significantly different with *GAPDH* only after infection for 48 h (fig. 7C).

Table 3. Expression stability of candidate reference gene in temperature stress analysed by BestKeeper, NormFinder and geNorm

Gene	BestKeeper			NormFinder		geNorm	
	Rank	SD	CV	Rank	SV	Rank	M
RPL3	2	0.20	1.06	5	0.62	1	0.08
RPS3a	5	0.39	2.01	1	0.15	4	0.45
RPL10	1	0.19	0.91	6	0.65	1	0.08
RPS9	3	0.24	1.23	4	0.60	2	0.10
EIF4A	9	1.88	4.96	9	1.33	8	0.88
EF1 α	6	0.46	1.84	3	0.41	6	0.64
TATA	7	0.54	2.18	2	0.25	5	0.58
α -ACT	8	0.86	3.77	7	0.81	7	0.74
GAPDH	4	0.32	1.60	8	0.88	3	0.23

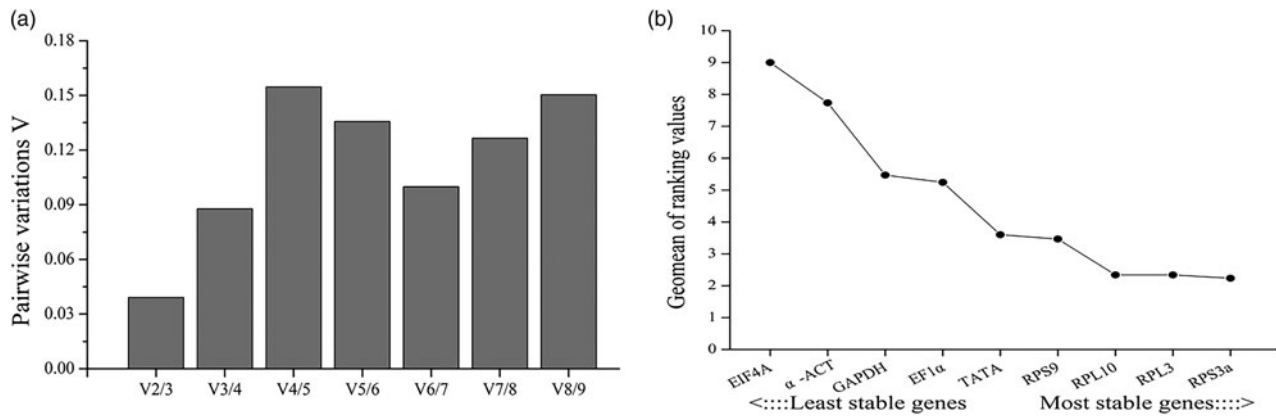


Figure 4. Comprehensive ranking of expression stability of nine candidate reference genes in *H. hampei* exposed to temperature stress. (a) Optimal number of reference genes for normalisation in temperature stress. (b) The stability of the nine housekeeping genes in temperature stress determined by RefFinder.

Table 4. Expression stability of candidate reference gene in *B. bassiana* infection analysed by BestKeeper, NormFinder and geNorm

Gene	BestKeeper			NormFinder		geNorm	
	Rank	SD	CV	Rank	SV	Rank	M
RPL3	4	0.39	2.02	2	0.19	4	0.48
RPS3a	5	0.44	2.34	3	0.22	1	0.30
RPL10	3	0.39	1.88	1	0.18	6	0.61
RPS9	2	0.39	2.02	5	0.50	7	0.68
EIF4A	7	0.63	2.80	8	0.90	3	0.39
EF1 α	1	0.38	1.57	6	0.59	1	0.30
TATA	6	0.57	2.36	7	0.73	2	0.32
α -ACT	8	0.71	3.30	4	0.35	5	0.54
GAPDH	9	1.69	8.56	9	2.32	8	1.08

Discussion

RT-qPCR is a widely used molecular technique for the analyses of target gene transcript levels in different experimental treatments. The stably expressed reference genes play an important role in normalising the expression level of a target gene. However, the

stability of reference genes is usually affected by the experimental conditions, leading to inaccurate results. Therefore, the selection of reference genes is an essential step for molecular biology research in a specific organism under a specific experimental condition using RT-qPCR.

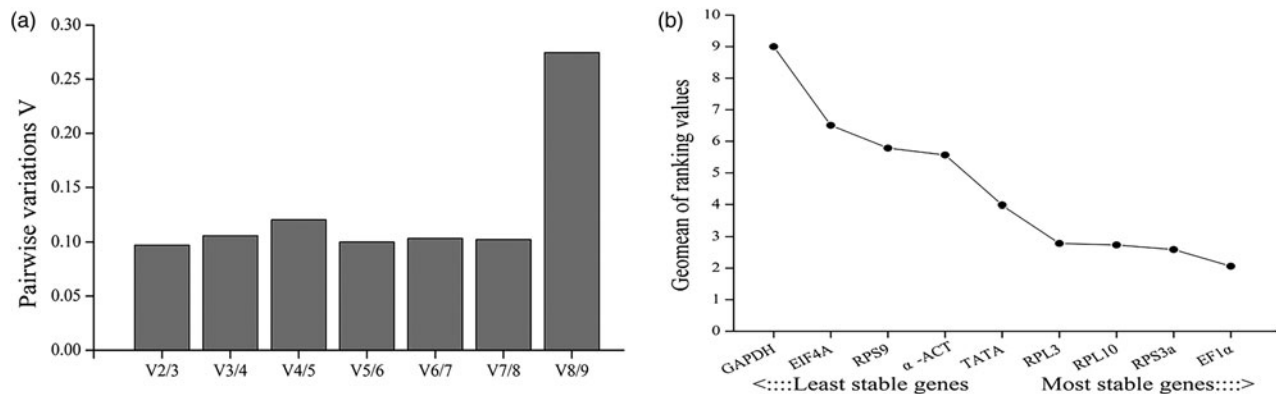
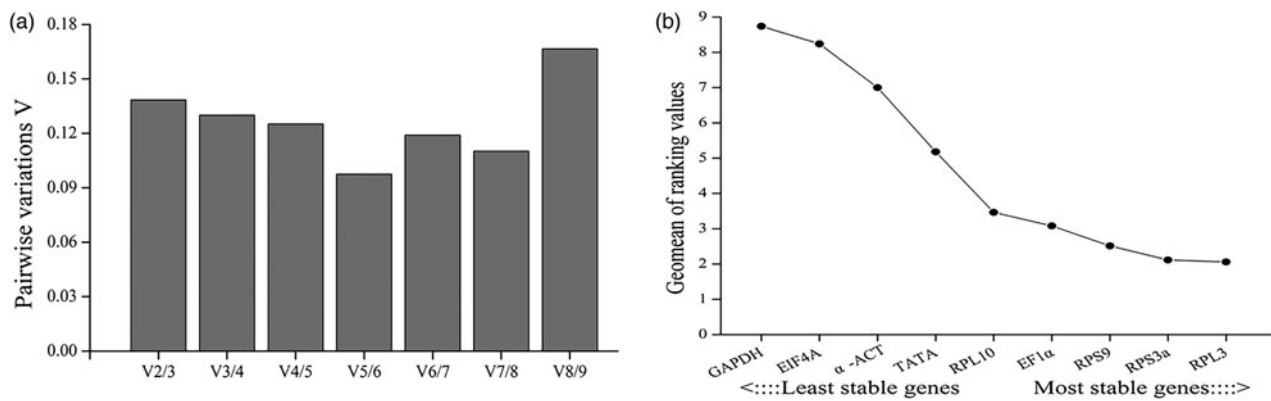


Figure 5. Comprehensive ranking of expression stability of nine candidate reference genes in *H. hampei* infected with *B. bassiana*. (a) Optimal number of reference genes for normalisation in *B. bassiana* infection. (b) The stability of the nine housekeeping genes in *B. bassiana* infection determined by RefFinder.

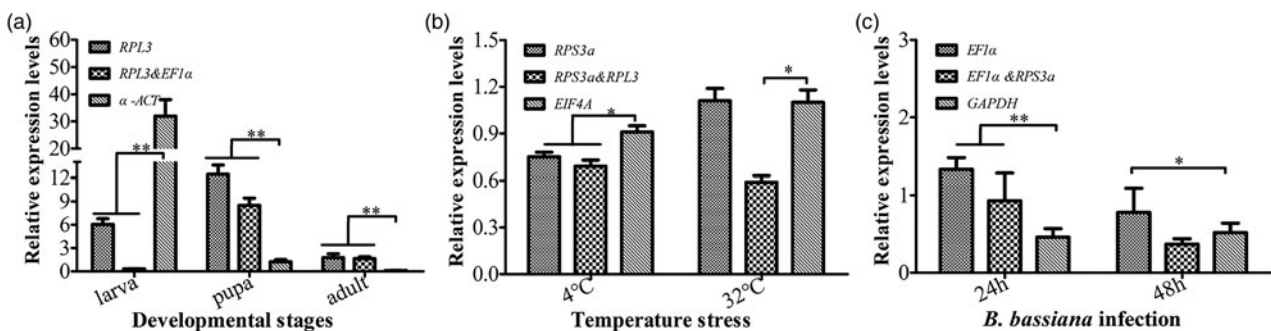
Table 5. Expression stability of candidate reference gene in all samples analysed by BestKeeper, NormFinder and geNorm

Gene	BestKeeper			NormFinder		geNorm	
	Rank	SD	CV	Rank	SV	Rank	M
RPL3	1	0.35	1.83	2	0.36	2	0.35
RPS3a	5	0.5	2.64	1	0.27	3	0.45
RPL10	4	0.47	2.30	6	0.51	1	0.20
RPS9	2	0.45	2.33	4	0.49	1	0.20
EIF4A	9	0.93	4.08	8	1.00	7	0.75
EF1 α	3	0.46	1.88	3	0.38	4	0.54
TATA	6	0.64	2.68	5	0.49	5	0.58
α -ACT	7	0.74	3.34	7	0.70	6	0.67
GAPDH	8	0.76	3.88	9	1.41	8	0.93

**Figure 6.** Comprehensive ranking of expression stability of nine candidate reference genes in all samples of CBB. (a) Optimal number of reference genes for normalisation in all samples. (b) The stability of the nine housekeeping genes in all samples determined by RefFinder.

Although CBB is the most destructive pest of the coffee plant, the molecular biology research of this species is still in its infancy. The construction of the CBB genome draft and analysis of transposable elements provided enough gene information for molecular physiology study (Vega *et al.*, 2015; Hernandez-Hernandez *et al.*, 2017). This study was the first to evaluate the expression stability of nine candidate reference genes under different conditions

of developmental stages, temperature stress, and *B. bassiana* infection using BestKeeper, NormFinder, geNorm and RefFinder software. In terms of the results, the rankings of gene stability obtained by BestKeeper, NormFinder and geNorm algorithms were not exactly the same under the same condition. For example, *GAPDH* was recommended as the most stable reference gene by BestKeeper in developmental stages. However, NormFinder

**Figure 7.** Validation of reference genes using Lysozyme gene. The relative expression levels of a target gene Lysozyme were normalised by the recommended stable reference genes and unstable gene in development stages (a), temperature stress (b) and *B. bassiana* infection (c). Asterisk indicated that Lysozyme relative expression normalised by one reference gene or combination of two references was significant differences among different treatment. One asterisk means $P < 0.05$ analysed by Duncan's test, and two asterisks means $P < 0.01$ analysed by Duncan's test.

identified it was not. Under temperature stress, the top two stable genes based on BestKeeper and geNorm are the gene ranking were totally the same, namely *RPL10* and *PRL3*. However, NormFinder indicated *RPS3a* and *TATA* as the most two stable genes. The situation in *B. bassiana* infection treatment was almost the same. The most stable genes recommended by the BestKeeper, NormFinder and geNorm were totally different. Therefore, to ensure the result reliability, the final determined results required an integrated evaluation of the three algorithms above using the online analysis software RefFinder, which could help to obtain the optimal reference genes.

The recommended reference genes for CBB were significantly different from species of the same family (Curculionidae) under the same treatment conditions. Specifically, *Sympiezomias velatus* (Li *et al.*, 2018) and *Anthonomus eugenii Cano* (Pinheiro and Siegfried, 2020), demonstrating that reference genes cannot be extrapolated across closely related species. In this study, the traditional reference gene, such as α -*ACT* and *GAPDH*, were not predicted as the most stable reference genes. Instead, they were both considered as the most unstable genes. Based on our results, the most stable reference genes were mainly *RPL3*, *RPS3a*, and *RPL10*, which all belong to ribosomal protein widely distributed and high conserved in eukaryotes (Deiorio-Haggar *et al.*, 2013). Recently, numerous studies have indicated that ribosomal protein can be used as reference genes. For example, *RPL32* and *RPS15* were the most stable reference genes for developmental stages in *A. suturalis* (Luo *et al.*, 2018), *RPS27a* and *RPL10a* for insecticide stress in *X. quadripes* (Meng *et al.*, 2022), *RPS20* for different temperatures in *Diaphorina citri* (Bin *et al.*, 2019), *RPS3a* for different sexes in *Diaphania caesalis* (Wang *et al.*, 2020), *RPL13A* and *RPS3A* for developmental stages and tissues in *H. theivora* (Wang *et al.*, 2019), *RPS18* and *RPL13* for *Lipaphis erysimi* (Koramutla *et al.*, 2016). This study supports the use of ribosomal protein genes as reference genes. Another gene *TATA* was unstable in all the treatment based on our result. But it was recommended as a suitable reference gene for efficient normalisation among treatments of host-plant resistance experiments, tissues, and developmental stages of *Aphis glycines* (Bansal *et al.*, 2012). Therefore, due to their stability vary within different organisms or under different conditions, this is also why we need to evaluate their stability in CBB.

In summary, the expression stability of nine candidate reference genes of CBB was comprehensively assessed by four common programs. The final results determined that the combination of *RPL3* and *EF1 α* were recommended as the most stable reference genes for developmental stages, the *RPS3a* and *RPL3* combination were confirmed as the optimal reference genes for temperature stress and all samples, the *EF1 α* and *RPS3a* combination were determined the ideal reference genes for *B. bassiana* infection. Our results should be beneficial for analysing the expression profiles of target genes in the future, especially for molecular studies on insect genetic development, temperature adaptability and immune responses to entomogenous fungi in *H. hampei*.

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