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Article

Screening and Validation of Stable Reference Genes for qRT-PCR Analysis in *Epicauta gorhami* (Coleoptera: Meloidae)

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Simple Summary: The reverse tanscriptase-quantitative polymerase chain reaction (qRT-PCR) is often employed to examine the gene expressions under diverse treaments. Screening optimal reference genes are necessary for obtaining reliable expression results of RT-qPCR. Here, the stability of 10 selected reference genes in *Epicauta gorhami* was assessed under three conditions (adult ages, tissues/organs and temperatures). These findings displayed that the best suitable reference genes were as follows: *SOD* and *RPS18* for different adult ages and various temperatures, *RPS18* and *RPS28* for adult tissues. The relative expression patterns of uridine diphosphate (UDP)-N-acetylglucosamine-pyrophosphorylase (*EgUAP*) in diverse adult tissues was employed to verify the results. Our study will lay an vital basis for future functional gene expressions in *E. gorhami*.

Abstract: *Epicauta gorhami* is a hypermetamorphic insect that mainly forage soybeans during the adult stage. However, the lack of appropriate references hinders our studying of the gene function in *E. gorhami*. In this study, refer to five computational tools (Ct value, geNorm, NormFinder, BestKeeper and RefFinder), the stability of 10 housekeeping genes (*GAPDH*, *ACT*, *RPL4*, *RPL27*, *α-TUB*, *RPS18*, *EF1α*, *RPS28*, *RPL13* and *SOD*) was assessed by RT-qPCR under three different conditions (adult ages, tissues/organs and temperatures). The findings suggested that *SOD* and *RPS18* were the most ideal references for examine gene transcripts among diverse adult ages and at various temperatures; A pair of *RPS18* and *RPS28* was the most reliable genes to assess gene expressions in diverse adult tissues. Finally, The relative expression levels of *EgUAP* were computed after normalization *RPS18* and *RPS28* with across diverse adult tissues. As expected, *EgUAP* expression was abundant in the foregut, trachea and antenna, and low in the midgut, hindgut and epidermis. These findings will lay a solid foundation for analysing the gene expression of *E. gorhami*.

Keywords: *Epicauta gorhami*; *Glycine max*; reference genes; RT-qPCR; RefFinder

1. Introduction

The bean blister beetle, *Epicauta gorhami* (Coleoptera: Meloidae) is a hypermetamorphic insect that can forage soybean (*Glycine max*) during the adult stage [1–4]. It poses potential threats to soybean production, which is an crucial oilseed, protein and biodiesel crop [5,6]. The adults of this specie mainly feed on leaves of *G. max* [1–4]. Previous studies on *E. gorhami* have traditionally placed a

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strong emphasis on diapause [1,3], seasonal adaptation [2] and the phylogenetic relationship [4]. However, there is currently no one research on gene functions in *E. gorhami*. In order to further explore molecular mechanisms of target genes, it is imperative to screening the suitable reference genes.

The reverse tanscriptase-quantitative polymerase chain reaction (qRT-PCR) has amounts of advantages of high sensitivity, specificity and accuracy, which is widely employed to analyse the target gene transcripts [7,8]. For measuring accurately the mRNA levels of target genes by qRT-PCR, selecting the most optimal references to conduct normalization is essential [9,10]. If unstable reference genes are employed, the results of gene transcripts will be inaccurate [8,9]. Hence, selecting appropriate reference genes should be assessed under various experimental conditions [11–13].

As usual, housekeeping genes (HKGs), such as actin (*ACT*), ribosomal protein (*RPL* and *RPS*), and elongation factor 1α (*EF1α*), are widely employed as references in insect species, due to relative stable expression levels. However, the stability of internal genes may not always constant under diverse experimental treatments [13,14]. In the potato ladybird *Henosepilachna vigintioctomaculata*, *RPL6* and *RPL13* are the suitable references in different developmental stages, whereas *RPS18* and *EF1α* are more optimal among diverse tissues [13]. In the sugarcane stem borer *Chilo sacchariphagus*, *β-ACT* and *RPL7* show the best stable expression under different tissues, whereas *EF1α* and *SDHA* exhibit stability between sexes [14]. Hence, to evaluating reference genes at different backgrounds is necessary.

Currently, it has made important advances in the study of reference genes selection in numerous insect species, such as *Mylabris sibirica* (*RPL6* and *RPL13*) [11], *Phthorimaea operculella* (*EF1α* and *RPL13*) [12], *H. vigintioctomaculata* (*RPS18* and *RPL13*) [13] and *C. sacchariphagus* (*β-ACT* and *RPL7* across various tissues and at distinct temperatures, *EF1α* and *SDHA* between sexes) [14]. To sum up, each experimental condition need at least two reference genes to measure the target gene transcripts in insect species.

In the paper, we selected 10 potential reference genes from the transcriptomes of *E. gorhami*, which were *ACT*, *EF1α*, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), *α-TUB* (α-tubulin), *RPL4*, *RPL13*, *RPL27*, *RPS18*, *RPS28* and *SOD* (superoxide dismutase). The stablity of these genes was assessed among diverse adult ages, across different adult tissues and in various temperatures in *E. gorhami*. In addition, the expression pattern of *E. gorhami* uridine diphosphate (UDP)-N-acetylglucosamine-pyrophosphorylase (*EgUAP*) across different adult tissues was used to evaluate the results. These findings will lay an vital basis for further research on gene functions in *E. gorhami*.

2. Materials and Methods

2.1. Insect Rearing and Treatment Methods

E. gorhami adults used in the study were obtained in August 2024 from *G. max* plants in Enshi Tujia and Miao Autonomous Prefecture, Hubei Province, China (coordinates: N30°19'43", E109°38'48"E). The adults were fed under suitable conditions (25±2 °C, 16h:8h photoperiod, and 50 ~ 60% relative humidity) in the insectary for one week.

Adult ages: Four newly emerged adults (two males and two females) were sampled every 1 day for 3 day, as one biological replicate. Three biological replicates were conducted.

Adult tissues: These tissues (foregut, midgut, hindgut, antenna, trachea and epidermis) were collected from the 10 healthy 5-day-old adults (5 males and 5 females) in three biological replicates.

Temperature: Four newly emerged adults (2 males and 2 females) were treated under three temperature (4 ◦C, 25 ◦C and 37 ◦C for 6 h, respectively), as one biological replicate. Three biological replicates were conducted.

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2.2. Total RNA Isolation and cDNA Synthesis

Total RNA of each sample was extracted by TRIzol reagent (YiFeiXue Tech, Nanjing, China), refer to the manufacturer's instructions. The RNA purity and concentration were detected by the NanoDrop ND-1000 spectrophotometer Subsequently, cDNA synthesis was manipulated using the HiScript III RT SuperMix (Vazyme Biotech Co.,Ltd, Nanjing, China).

2.3. Selection of Candidate References and Primer Design

According to the transcriptome of *E. gorhami*, a total of 10 candidate references were selected, including *EF1α*, *ACT*, *RPL4*, *α-TUB*,*RPL13*, *RPL27*, *RPS18*, *GAPDH*, *RPS28* and *SOD*, which are commonly utilized as reference genes in other insects [15]. The information of references was displayed in Table S1.

Primer design was executed by the Primer Premier 5.0 software, based on the RT-PCR primer design principles. The resultant sequences were uploaded to GenBank, with the accession numbers of PQ497541-PQ497541 (Table S1).

2.4. Quantitative Real-Time PCR (qRT-PCR)

The primers for qRT-PCR were performed by Primer3web version 4.1.0 (https://primer3.ut.ee/), and were located in Table 1. qRT-PCR experiment was executed by CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) and ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech co.,ltd). Each reaction consisted of a final volume 20 μL, containing 10 μL of 2× ChamQ Universal SYBR qPCR Master Mix, 0.4 μL of reverse primer (10 μM), 1 μL cDNA template and 8.2 μL of RNase Free water. The thermocycling procedure was an initial step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, annealing at 60°C for 34 s. After the reaction, a melting curve analysis was one cycle of 95 °C for 15 s, 60 °C for 60 s and 95 °C for 1 s, which was used to confirm the specificity of the amplified product. The amplification efficiency (E) was computed using a series of 3-fold dilutions of cDNA template refer to the equation: $E = (10^{[-1/slope]} - 1) \times 100\%$ [16]. All experiments were conducted in triplicate.

Table 1. Primers of 10 candidate house-keeping genes used in qRT-PCR.

Gene	Primer sequences (5'to 3′)	Length (bp)	Slope	R2	Efficiency (%)
EF1α	F- ATCATTGACGCACCTGGACA R- ACCAGTACCAGCAGCAACAA	99	-3.435	0.999	95.50
GAPDH	F-ACAGTACATGCCACCACAGC R-TGGTACACGGAAGGCCATAC	180	-3.351	0.999	98.82
RPL4	F- TCGATGAACCACCGTCAACC R- CGACCGGTACCCCATGATTC	80	-3.308	0.993	100.60
RPL13	F-AAGCCGCCGGTATTAACAGC R-TCACCAGGACGCAACTTCTT	172	-3.318	0.998	100.16
RPL27	F-TCGTATTGGTCTTGGCAGGC R-TCAATGCCGGCAACAATAGC	112	-3.373	0.999	98.03
SOD	F-AGTTGTCCATGCTGATCCGG R-TAACACCACAGGCCAAACGT	95	-3.32	0.998	100.08
ACT	F- TACGTGTGGCACCTGAAGAA R- CCAGTTGTACGACCAGAAGCA	169	-3.284	0.997	101.60
α-TUB	F-ATGGGCACGTCTTGATCACA R-TCATTTTCACCTTCGCCCGA	174	-3.258	0.999	102.73
RPS18	F-AGGTGTTGGTCGTCGTTACTC R-TCTAAGGTAGCCGATGTGAGC	210	-3.323	0.999	99.96
RPS28	F-GGTGAGCAAAACCGTCAGATC R-TGCTTCACGTTCAGACTCCA	90	-3.347	0.997	98.97

Note: ACT, actin; SOD, Superoxide dismutase; α-TUB, α-tubulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EF1α, elongation factor 1α; RPL4, RPL13 ,RPL27, RPS18 and RPS28, ribosomal protein.

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2.5. Assessment of Reference Gene Stability

The raw Ct values were required by the BioRadCFXManager software. The stability of 10 reference genes under different backgrounds were assessed using a range of algorithms, including BestKeeper [17], Normfinder [18], geNorm [9] and the ΔCt method [19]. Finally, the RefFinder [20,21] was applied to analyse the comprehensive ranking of each experimental condition.

2.6. Validation of Selected Reference Genes Under Diverse Adult Tissues

The transcription level of uridine diphosphate (UDP)-N-acetylglucosamine-pyrophosphorylase (*UAP*, GenBank: PQ497551) in *E. gorhami* was utilized to assess the stability of selected reference genes across various adult tissues. The primer sequence of *UAP* was as follows:Forward: CGCTACAACGTAACGCCATC, Reverse: CCCCACAATCGCTACGTTTC. The relative transcription level of *EgUAP* were computed under adult tissues by the $2^{-\Delta\Delta Ct}$ method, based on *RPS18* and *RPS28*. The SPSS Statistics 29 software package was then used to evaluate the variance in expression levels of *EgUAP* among different adult tissues through one-way ANOVA analysis followed by Tukey's test for multiple comparisons.

3. Results

3.1. Selection of Candidate Reference Genes

Ten commonly used reference genes (*ACT*, *GAPDH*, *RPL4*, *EF1α*,*RPL13*, *RPL27*, *RPS18*, *RPS28*, *α-TUB* and *SOD*) in *E. gorhami* were choosed. The resultant sequences were uploaded to GenBank, with the accession numbers of PQ497541-PQ497541 (Table S1).

The products of qRT-PCR were verified by sequencing. The melting curve analysis confirmed the specificities of each primer pair for qRT-PCR. As expected, the efficiency (*E*) of 10 primer pairs were between 95.50% (*EF1α*) and 102.73% (*α-TUB*), with the correlation coefficients (R^2) varying from 0.993 to 0.999 (Table 1). These results demonstrated that efficiency of primers reached the standards of traditional qRT-PCR [22].

3.2. Expression Levels of Reference Genes

The threshold-cycle (Ct) of 10 reference genes under different tested conditions were shown (Figure 1). Among various adult ages, *SOD* and *RPS18* had smaller gene expression variations, whereas *EF1α* and *GAPDH* had higher (Figure 1A). Across different adult tissues and under temperature treatments, the expression fluctuations were lower in *RPS18* and *RPS28* and higher in *ACT* and *GAPDH* (Figure 1B,C). A combination of above results indicated that the expression difference was small in all reference genes except for *ACT* and *GAPDH* (Figure 1D).

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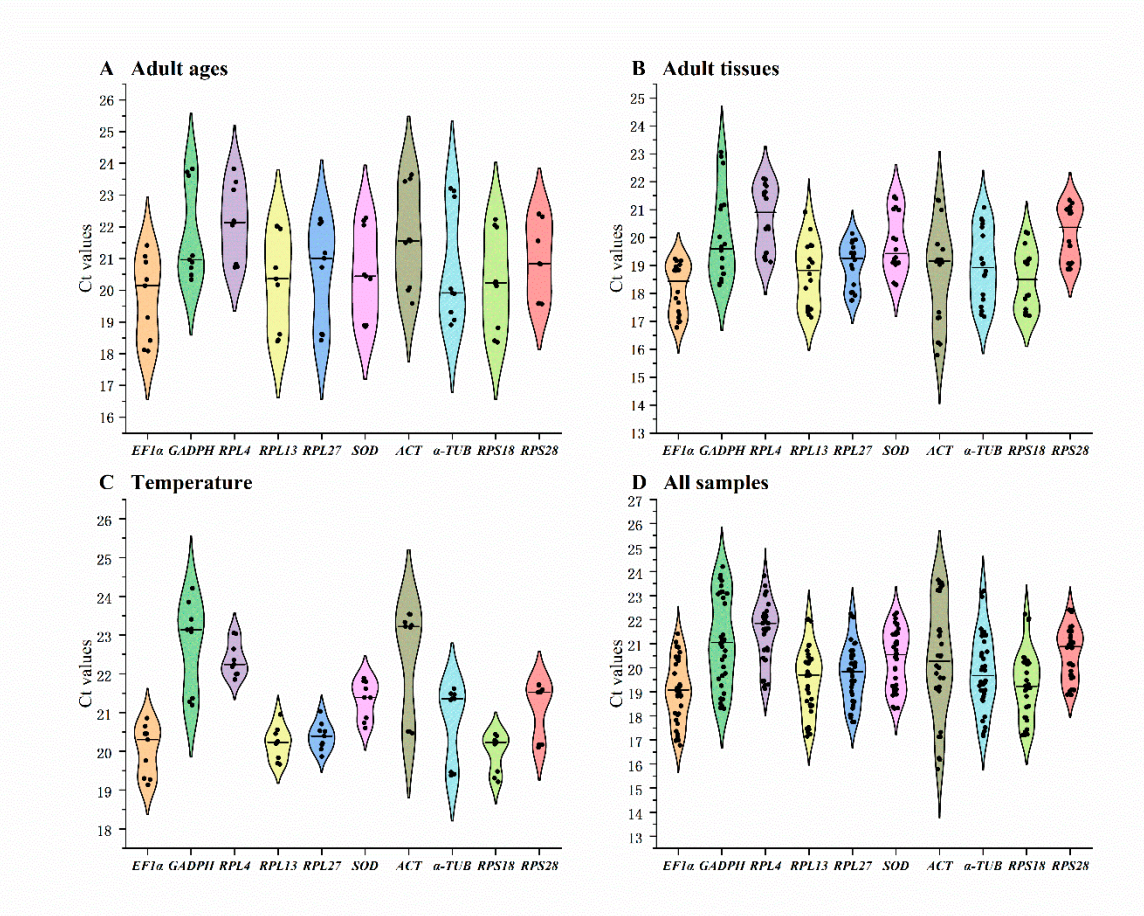

Figure 1. Expression levels of ten house-keeping genes in Epicauta gorhami. The mean Ct values for 10 candidate reference genes are shown in four different experiments: (A) adult ages, (B) adult tissues, (C) temperature, (D) All samples. Mean Ct values for the ten candidate reference genes are presented in violin plot. Abbreviation: ACT, actin; SOD, Superoxide dismutase; α-TUB, α-tubulin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EF1α, elongation factor 1α; RPL4, RPL13 ,RPL27, RPS18 and RPS28, ribosomal protein. The abbreviations are exactly the same as Figures 2–5.

3.3. Stability of the Ten HKGs Among Adult Ages

Based on average expression stability (M-values) and pairwise variation (V-values), the geNorm algorithm evaluate the gene stability. These results exhibited that ACT, RPS18 and SOD were the most suitable, with M-values below 0.20 (Figure 2A, Table 2). Moreover, V-values suggested that V2/3 to V9/10 values were below 0.15, showing that two diverse references were equal for assessing the gene transcript among adult ages (Figure 2B).

The NormFinder algorithm indicated that the stable ranking of ten reference genes from the most to the least stable were as follows: *SOD*, *RPS18*, *ACT*, *RPL13*, *RPL4*, *RPS28*, *RPL27*, *α-TUB*, *EF1α* and *GAPDH*, with the p value of 0.091, 0.143, 0.172, 0.243, 0.268, 0.329, 0.458, 0.651, 0.849 and 0.954, respectively (Figure 2C, Table 2). The BestKeeper analysis showed that RPL4, RPS28 and SOD were the top three stable genes (Figure 2D, Table 2).

Refer to the results of RefFinder, the ranking of 10 reference genes at adult ages were as follows: *SOD>RPS18>ACT>RPL4>RPS28>RPL13>EF1α>RPL27>α-TUB>GAPDH* (Figure 5A). Therefore, the combination of SOD and RPS18 are the most appropriate for gene transcript analysis by qRT-PCR among various adult ages (Table 3).

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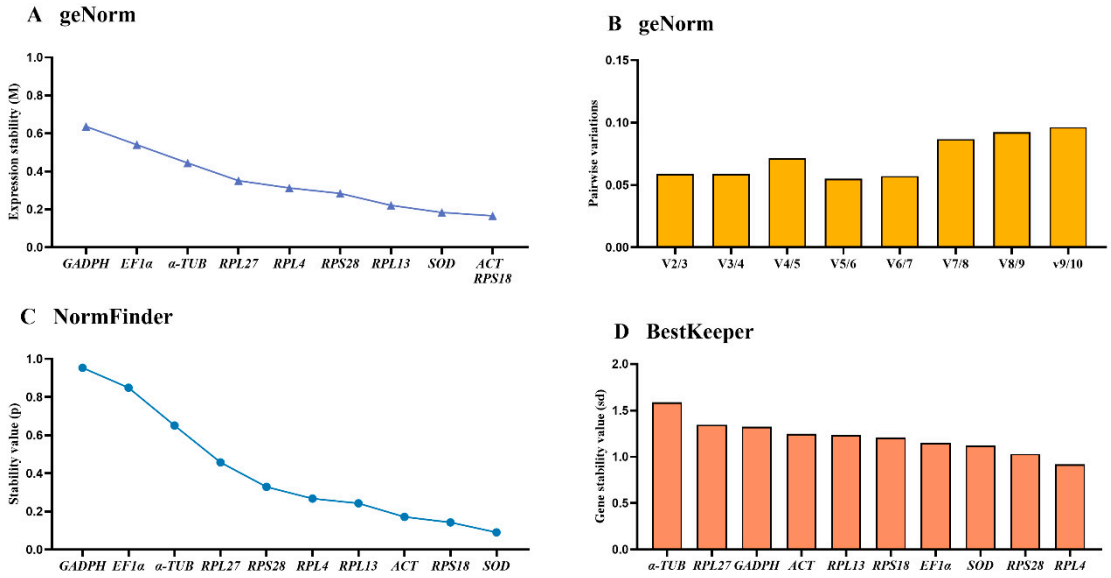

Figure 2. Stability of the ten house-keeping genes in *Epicauta gorhami* during various adult ages. Diverse ages of Epicauta gorhami adults were sampled (collected on the first to third days of the newly-emerged adults). The expression stability rankings are determined by geNorm, NormFinder and BestKeeper.

Table 2. Expression stability of the candidate reference genes under different experimental conditions.

Conditions	CRGs*	geNorm		Normfinder		Normfinder		ΔCt	
		Stability	Rank	Stability	Rank	Stability	Rank	Stability	Rank
Developmental ages	*EF1α*	0.540	8	0.849	9	1.149	4	0.931	9
	GADPH	0.636	9	0.954	10	1.325	8	1.024	10
	RPL4	0.313	5	0.268	5	0.920	1	0.543	5
	RPL13	0.221	3	0.243	4	1.236	6	0.500	4
	RPL27	0.351	6	0.458	7	1.344	9	0.619	7
	SOD	0.184	2	0.091	1	1.121	3	0.443	1
	ACT	0.166	1	0.172	3	1.249	7	0.469	3
	α-TUB	0.444	7	0.651	8	1.589	10	0.782	8
	RPS18	0.166	1	0.143	2	1.209	5	0.476	2
	RPS28	0.284	4	0.329	6	1.033	2	0.557	6
Adult tissues	*EF1α*	0.239	1	0.384	5	0.828	2	0.621	5
	GADPH	0.741	9	0.933	10	1.326	9	1.055	10
	RPL4	0.277	2	0.347	4	1.051	7	0.614	4
	RPL13	0.372	4	0.220	3	0.989	6	0.608	3
	RPL27	0.414	5	0.496	6	0.700	1	0.710	6
	SOD	0.563	7	0.815	8	0.971	4	0.944	8
	ACT	0.663	8	0.889	9	1.389	10	1.012	9
	α-TUB	0.468	6	0.536	7	1.085	8	0.744	7
	RPS18	0.318	3	0.146	1	0.987	5	0.549	1
	RPS28	0.239	1	0.201	2	0.914	3	0.552	2
Temparature treatment	*EF1α*	0.346	6	0.334	4	0.58	6	0.532	5
	GADPH	0.474	8	0.547	9	0.976	9	0.675	9
	RPL4	0.246	3	0.446	7	0.357	3	0.549	6
	RPL13	0.188	2	0.395	6	0.317	2	0.522	4
	RPL27	0.261	4	0.468	8	0.278	1	0.555	7
	SOD	0.123	1	0.217	3	0.407	4	0.431	3

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	ACT	0.556	9	0.847	10	1.265	10	0.886	10
	α-TUB	0.412	7	0.392	5	0.899	8	0.570	8
	RPS18	0.123	1	0.155	2	0.429	5	0.415	1
	RPS28	0.307	5	0.131	1	0.646	7	0.430	2

3.4. Stability of the Ten HKGs Across Various Adult Tissues

The geNorm results displayed that *RPS28*, *EF1α*, *RPL4* and *RPS18* were the top four stable genes (Figure 3A, Table 2). V-values data demonstrated that all values of from V2/3 to V9/10 were below 0.15, showing that two reference genes in various adult tissues were suitable (Figure 3B).

According to the NormFinder data, the rankings of reference genes were as follows:*RPS18>RPS28>RPL13>RPL4>EF1α>RPL27>α-TUB>SOD>ACT>GAPDH* (Figure 3C, Table 2). Moreover, the p values of all reference genes were less than 1.0 (Figure 3C, Table 2). The BestKeeper results indicated that *RPS18* and *RPS28* were the most stable, with the SD values of 0.700 and 0.914, respectively (Figure 3D, Table 2). Furthermore, the SD values of *ACT*, *GADPH*, *α-TUB* and *RPL4* were above 1.0 (Figure 3D, Table 2).

According to RefFinder results, the comprehensive ranking order were as follows: *RPS28>RPS18>EF1α>RPL27>RPL13>RPL4>SOD>α-TUB>ACT>GAPDH* (Figure 5B). Therefore, the pair of *RPS18* and *RPS28* are the most appropriate for qRT-PCR data normalization among diverse adult tissues (Table 3).

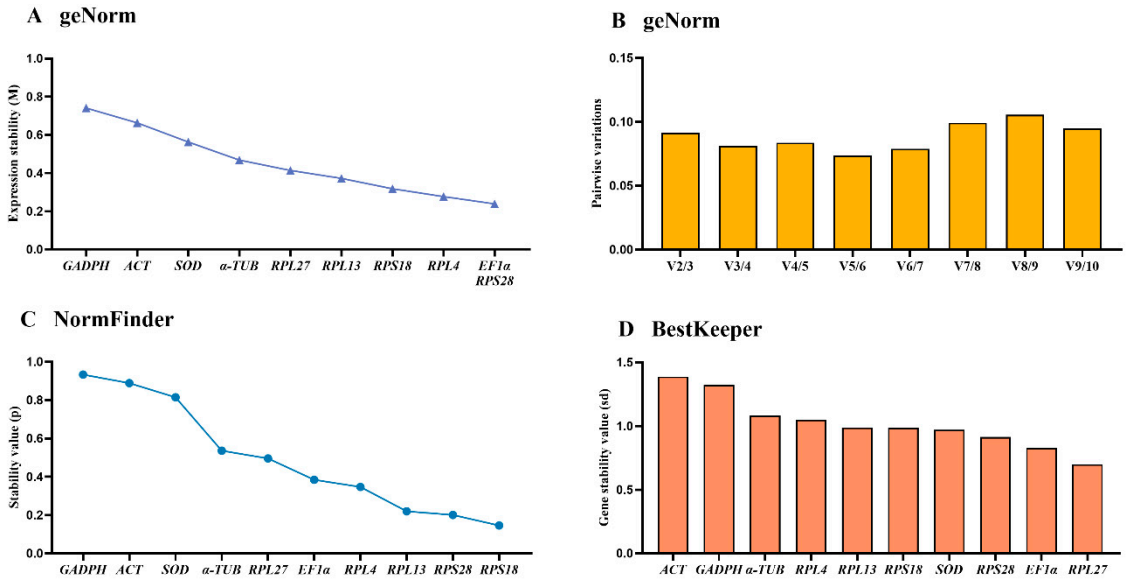

Figure 3. Stabilities of the ten house-keeping genes in *Epicauta gorhami* among diverse adult tissues. Foregut, midgut, hindgut, epidermis, trachea and antenna were dissected from the 5-day-old adults. The expression stability rankings are determined by geNorm, NormFinder and BestKeeper.

3.5. Stability of the Ten HKGs at Diverse Temperature Conditions

The geNorm results indicated that *RPS18*, *SOD* and *RPL13* were the most steady references in diverse temperatures, with M-values of 0.123, 0.123 and 0.188, respectively (Figure 4A, Table 2). Moreover, V-values data displayed that all values were below 0.15, showing that two reference genes in various temperature treaments were suitable (Figure 4B).

The NormFinder results manifested that the steady rankings were *RPS28*, *RPS18*, *SOD*, *EF1α*, *α-TUB*, *RPL13*, *RPL4*, *RPL27*, *GAPDH* and *ACT* (Figure 4C, Table 2). BestKeeper data uncovered that the SD values of these genes were less than 1.0, except for *ACT*(Figure 4D, Table 2).

Refer to the results of RefFinder, the stability orders were as follows: *RPS18>SOD>RPS28>RPL13>RPL27>RPL4>EF1α>α-TUB>GAPDH>ACT* (Figure 5C). When combinating the three diverse treaments together, the RefFinder results showed that the stability

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ranking in sequence were *RPS18*, *RPL13*, *RPS28*, *RPL4*, *RPL27*, *EF1α*, *SOD*, *α-TUB*, *GAPDH* and *ACT* (Figure 5D). To sum up, *RPS18* and *SOD* are the best reference gene pair to evaluate the gene transcript in diverse temperature conditions (Table 3).

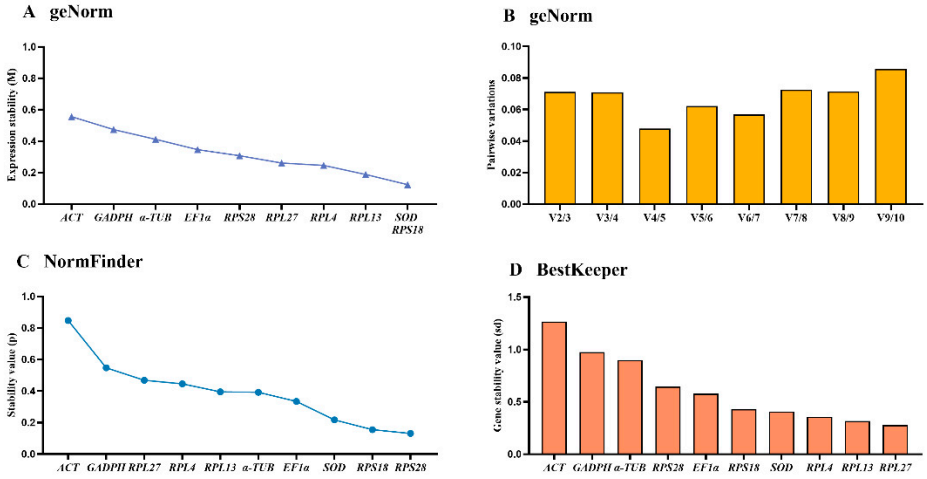

Figure 4. Stability of the ten house-keeping genes in *Epicauta gorhami* under different temperatures. The newly-emerged adults reared under three temperatures (4°C, 25 °C and 37 °C) were collected. The expression stability rankings are determined by geNorm, NormFinder and BestKeeper.

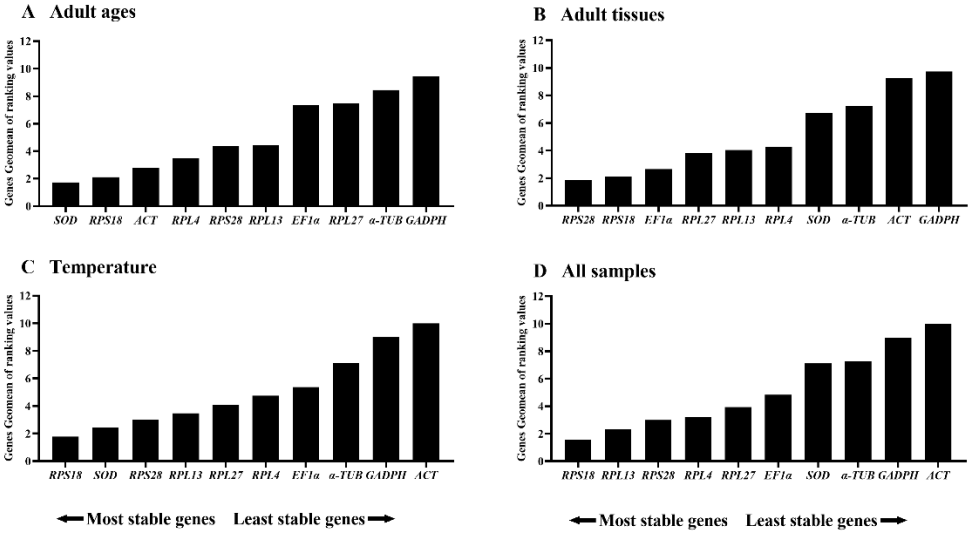

Figure 5. Stabilities of the ten house-keeping genes in *Epicauta gorhami* in different samples. The stability of the reference genes as calculated by the Geomean method of RefFinder. A lower Geomean of ranking value denotes more stable expression. (A) adult ages, (B) adult tissues, (C) temperature, (D) all samples.

Table 3. A list of the recommended reference genes in *E. gorhami* for different experimental conditions.

Experimental conditions	The recommended reference genes	
Adult ages	*SOD*	*RPS18*
Adult tissues	*RPS28*	*RPS18*
Temperature	*SOD*	*RPS18*

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3.6. Validation of the Selected Reference Genes

To estimate the performance of the recommended reference genes, the relative mRNA level of *EgUAP* was measured using the most stable references (*RPS18* and *RPS28*) under various adult tissues. Our results indicated that the transcription level of *EgUAP* was high in the foregut, trachea and antenna, and low in the midgut, hindgut and epidermis in *E. gorhami* (Figure 6).

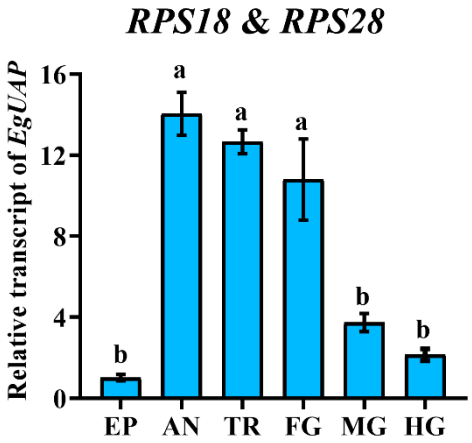

Figure 6. Relative gene expression of EgUAP in various adult tissues of *Epicauta gorhami*. The relative gene expression level of EgUAP in the foregut, midgut, hindgut, epidermis, trachea and antenna was normalized to the best stable reference genes (RPS18 and RPS28), respectively. The values are means + SE. Different letters indicate significant differences in gene expression among different tissues ($P < 0.05$).

4. Discussion

In the study, the expression stabilities of ten selected reference genes in *E. gorhami* were estimated to perform RT-PCR analysis normalization, laying an vital basis for future study of gene functions. Notably, we evaluated the stability of these genes under three different conditions (adult ages, adult tissues/organs and temperatures) for the first time in *E. gorhami*. These findings suggested that *SOD* and *RPS18* were the most ideal reference combination to measure gene transcription levels among different adult ages (Figures 1, 2 and 5, Tables 2 and 3) and at various temperatures (Figures 1, 4 and 5, Tables 2 and 3); *RPS18* and *RPS28* was the most reliable genes to assess gene expressions under diverse adult tissues (Figures 1, 3 and 5, Tables 2 and 3).

Ribosomal proteins play an vital function on ribosome assembly, which bind to four ribosomal RNAs (rRNAs) to constitute the ribosomes [23]. Similar to our findings, ribosomal proteins are selected as the most optimal reference genes in insect species, such as Coleopterans *M. sibirica* (*RPL6* and *RPL13*) [11], *Leptinotarsa decemlineata* (*RP18* and *RP4*) [24], *Phaedon brassicae* (*RPL32* and *RPL19*) [25], *Henosepilachna vigintioctopunctata* (*RPL13* and *RPS18*) [15], *Tribolium castaneum* (*RPS6*, *RPL13a*, *RPS3* and *RPL18*) [26] ,*Ips sexdentatus* (*RPS3*) [27] and *H. vigintioctomaculata* (*RPS18* and *RPL13*) [13], Hemipterans *Psammotettix striatus* (*RPLP2*) [28], *Rhopalosiphum padi* (*RPL13*, *RPS6* and *RPS18*) [29], *Aphis glycines* (*RPS9*) [30], *Ferrisia gilli* (*RPS8*, *RPL40* and *RPL7*) [31], *Diaphorina citri* (*RPL7*) [32] and *Dichelops melacanthus* (*RPL9* and *RPS23*) [33], Hymenopterans *Anastatus japonicus* (*RPL13* and *RPS6*) [34], Lepidopterans *Mythimna loreyi* (*RPL10*, *RPL27* and *RPS3*) [35], *Plutella xylostella* (*RPS13* and *RPS23*) [36], *Spodoptera litura* (*RPS13* and *RPLP0*) [37], *P. operculella* (*RPL13*) [12], *Bombyx mori* (*RPS7*) [38] and *Helicoverpa armigera* (*RPS15* and *RPL27*) [39], Dipterans *Aphidoletes aphidimyza* (*RPL8* and *RPS3*) [40], *Exorista sorbillans* (*RP49*) [41] and *Chlorops oryzae* (*RPS15*) [42], and Orthopteran *Locusta migratoria* (*RPL32*) [43], and Thysanopterans *Megalurothrips usitatus* (*RPL30*) [44] and *Frankliniella occidentalis* (*RPL32*) [45].

Under different adult ages and temperatures, *SOD* was verified to be the most reliable gene (Figures 1, 2, 4 and 5, Tables 2 and 3). Superoxide dismutase (SOD) is a crucial antioxidant enzyme,

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catalyzing the conversion of reactive oxygen species into oxygen and hydrogen peroxide [46]. Consistent with our data, SOD is recommended as the most appropriate reference gene in *Spodoptera frugiperda* [47], *Thrips tabaci* [48], *Riptortus pedestris* [49] and *Spodoptera exigua* [50].

In addition, The BestKeeper analysis data indicated that the SD values of *α-TUB*, *EF1α*, *GAPDH* and *ACT* were greater than 1.0 (Table 2), showing that these genes were unaccommodated as reference genes to perform RT-qPCR normalization. Similar results have been verified in other insects, such as *P. operculella* [12], *Ophraella communa* [51], *A. aphidimyza* [40], *Hippodamia convergens* [52], *H. vigintioctomaculata* [13], *Colaphellus bowringi* [53], *H. vigintioctopunctata* [15] and *M. sibirica* [11].

To further validate the accuracy of *RPS18* and *RPS28* in RT-qPCR normalization in *E. gorhami*, we examined the relative transcript level of *EgUAP* under diverse adult tissues. Our results exhibited that *EgUAP* expression was high in the foregut, trachea and antenna, and low in the midgut, hindgut and epidermis (Figure 6).

Overall, it is essential to screen and verify the most suitable references to guarantee the accuracy of gene expression. The study would offer a solid basis for further molecular functions of target genes in *E. gorhami*.

5. Conclusions

Ten potential reference genes in E. gorhami were assessed for accurate RT-qPCR analysis in *E. gorhami* under three different treaments. These results demonstrated that the most steady reference genes were as follows: *SOD* and *RPS18* for different adult ages and various temperatures, *RPS18* and *RPS28* for adult tissues. This study is the first time to establish the RT-qPCR normalization analyses in *E. gorhami*, facilitating further research on gene functions of *E. gorhami*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: A list of primers used for RT-PCR of the genes.

Author Contributions: Experiments were designed by G.-F.Y., X.-T.Y., Y.Z., G.W., X.-H.Y., C.-H.S. Figures designed and created by G.-F.Y., X.-T.Y., Y.Z., J.-H.L., G.W., X.-H.Y., C.-H.S. Statistical analyses were implemented by G.-F.Y., X.-T.Y., Y.Z., J.-H.L., X.-F.L., L.Z., H.-H.Z., L.J.,G.W., X.-H.Y., C.-H.S. All authors have read and agreed to the published version of the manuscript.

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