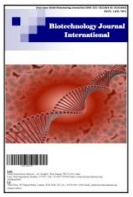

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Selection and Validation of Reference Genes for Quantitative Real-Time PCR Expression Analysis of Candidate Genes in *Carposina sasakii* (Lepidoptera: Carposinidae)

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Authors' contributions

This work was carried out in collaboration among all authors. Author ZZ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ZY and YL managed the analyses of the study. Authors YZ and LQ managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Carposina sasakii is one of the most important pests on the quality of stone and pome fruits. Investigation of a gene expression level in the species is hampered because of the gap of validated reference genes. The expression variation in the transcription levels of eight candidate reference genes, Actin (*ACT*), Tubulinbeta-1 (*TUB*), Ribosomal protein 49 (*RP49*), Elongation factor1-alpha (*EF-1a*), Elongation factor1-b (*EF-1b*), Elongation factor1-d (*EF-1d*), Ribosomal proteinL13 (*RPL13*) and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were analyzed by quantitative real-time PCR (qPCR). The stability and ranking of these gene expression profiles in three organ types (head, thorax and abdomen), three developmental stages (larva, pupa and moth), and five

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diapause states (non-diapause, pre-diapause, diapause 0 d, diapause 20 d and diapause 60 d) were assessed using two algorithm-based methods, geNorm and NormFinder. *EF-1a*, *ACT* and *GAPDH* were evaluated to be the three stable reference genes based on the important observations and comprehensive analysis, whereas *TUB* and *EF-1b* showed low expression stability. Best gene combinations for different qPCR analysis in *C. sasakii* could be chosen from the three stable reference genes, the using of two reference genes is sufficient to effectively normalize qPCR data in *C. sasakii*. The study laid the foundation for gene expression analysis in *C. sasakii* and provided new information for the selection of reference genes.

Keywords: Carposina sasakii; quantitative real-time PCR; reference genes; diapause states; developmental stages.

1. INTRODUCTION

Carposina sasakii Matsumura is one of the most influential pests on the quality of stone and pome fruits in East Asia [1-3]. Eggs are laid on host fruits. Larvae burrow into the fruit to develop. Infested fruits often loss of edible value leads to economic loss and drop early. In north China, mature larvae escape from apples and drop into soil to form a compressed diapausing cocoon when the photophase falls below 14 hr in September [4]. There are predetermined developmental pathways that the peach fruit moth produces spindle-shaped or compressed cocoons for non-diapause or diapause stages, respectively. The pathways have already been set early in the larval stage in response to long and short photoperiod [5]. The molecular mechanism of the diapause development in *C. sasakii* is still unknown to us.

Gene expression analyses using reverse transcriptase quantitative polymerase chain reaction (qRT-PCR) can provide new insight into complex biological process. In qRT-PCR analysis, the expression of one or more reference genes should be used to normalize target genes mRNA levels from the same sample. Although the study of molecular mechanism of diapause and non-diapause regulation still remain poorly understood, our understanding of the olfaction mechanism in *C. sasakii* is improving recently [6,7]. In identification of the male-specific or female-specific chemosensory genes that were putatively related to odor detection and recognition in *C. sasakii*, *ACT* and *EF1α* as reference genes were used in qRT-PCR [6] .Li et al [7] identified putative odorant binding proteins expressed in intact heads of *C. sasakii* adult males and females, using *β-actin* as endogenous reference in qRT-PCR. In the qRT-PCR experiments of *C. sasakii previous papers* [6,7], the selection of the endogenous reference gene was not consistent, and they did not explain the basis for selecting the endogenous reference gene.

The most common reference genes, such as Ribosomal proteins (RP), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Actin [8-10], have been used without verification for some time [11,12]. Because inappropriate reference genes could significantly alter the accuracy of the expression pattern of target genes [13,14], we tried to screen and validate reliable reference genes in *C. sasakii* including three organ types (head, thorax and abdomen), three developmental stages (larva, pupa and moth), and five diapause states (non-diapause, pre-diapause, diapause 0 d, diapause 20 d and diapause 60 d). To achieve this goal, we compared the mRNA transcription stability status of 8 candidate reference genes of *C. sasakii* using the qPCR. For the use of a single reference gene may produce erroneous results [15], we also addressed the number of reference genes required to precisely normalize qPCR expression data. The results of this study not only generate substantial sequence information but also provide valid reference genes for the analysis of gene expression. These results provide a valuable platform for future gene expression research in this species, especially for exploring the molecular mechanisms of different physiological processes, especially related to diapause and nondiapuse development.

2. MATERIALS AND METHODS

2.1 Collection and Rearing of Insects

The insects used in this experiment were collected in the field from Mengjin County (29°1N, 114°4E), Henan Province, China. Breeding insects in an artificial climate chamber at standard temperature ranges (25±1°C) and

relative humidity (70±5%), as growth conditions in the field. A larva was fed by an apple, and moth were supplied by 10% sucrose water changing every day. The same batch of larvae were cultured under normal development or diapause development photoperiods. Moths and larvae with normal development grew in a photoperiod of 15 h light/9 h dark, and larvae with diapause development grew in a photoperiod of 12 h light/12 h dark for their entire larval stage. Larvae were judged to be diapausing based on their compressed shape cocoon, while non-diapausing individual formed a spindle cocoon. Mature larvae escape from apples grew in a photoperiod of 15 h light/9 h dark form spindle cocoons. Pupa will be shaped after they formed a spindle cocoon for 3 days.

2.2 Sampling Collection

Insect tissues were collected containing series of experimental conditions: three types of organs (head, thorax and abdomen), three developmental stages (larva, pupa and moth), and five different diapause states (non-diapause, pre-diapause, diapause 0 d, diapause 20 d and diapause 60 d). Non-diapause larvae were mature larvae escape from apples grew in a photoperiod of 15 h light/9 h dark. Pre-diapause larvae were mature larvae escape from apples grew in a photoperiod of 12 h light/12 h dark. Diapause 0 d means the first day mature larvae shaped the compressed cocoons escape from apples grew in a photoperiod of 12 h light/12 h dark. Diapause 20 d and diapause 60 d means the compressed cocoons were buried in sand (8% water content, w/w) at a depth of 5 cm and were kept at 7°C over 20 days and 60 days. Different types of organs taken from each developmental stage and from each diapause state. At first, an individual was frozen in liquid nitrogen for 2 seconds, and then each body parts were separated by a blade. Samples of each body parts were preserved in cryogenic tubes (1.5 mL) and stored at -80□ after flash-frozen in liquid nitrogen. Bulk of each type of tissues collected from three individuals was a biological replicate. A biological replicate was used for extraction of total RNA, and three biological replicates were made.

2.3 Reference Gene Screening and Primer Design

Reference genes of Lepidoptera insects have been widely used in qPCR analysis; eight reported genes (*GAPDH*, *EF-1a*, *ACT*, *RP49*, *EF-1b*, *EF-1d*, *RPL13*, and *TUB*) of *C. sasakii* were selected. These genes' sequences were obtained by screening the *C. sasakii* transcriptome data (SRA accession number: PRJNA495711) in our laboratory. The genes were cloned by PCR, and nucleotide sequences (accession numbers are in Table 1 were submitted to GenBank. All specific primers Table 2 in the qPCR were designed by the tool of NCBI Primer-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Extraction of Total RNA and cDNA Synthesis

Tissues from different developmental stages and diapause states were ground and pulverized under liquid nitrogen freezing conditions, and were kept in RNAiso Plus (Takara, Dalian, China) for total RNA extraction refer to the manufacturer's instruction. Purity and concentration of the isolated total RNA were measured by a spectrophotometer of Nanodrop 2000 (Thermo Scientific, Wilmington, USA), and RNA samples with an OD_{260}/OD_{280} ratio within the limits of 1.9–2.1 were used for reverse transcription. The PrimeScript^{RT} reagent kit RR047A (Takara, Dalian, China) was used to synthesis first strand cDNA using 1μg total RNA, and the synthesized cDNA were stored at −20° C.

2.5 qPCR Analysis

qPCR reactions were completed using TB Green PremixRR420B (Takara, Dalian, China) and CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). The cDNA products were used as templates in qPCR after diluted 20-fold with deionized water (dH_2O). The reaction mixtures (20 μl) contained 7 μl dH_2O, 10 μl TB Green Premix Ex Taq II, forward primer and reverse primer each 0.5 μl, and 2 μl of cDNA. PCR was performed under the reaction programs: 95° C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s. The slope of the linear regression model having qPCR analysis to confirm the efficiency of each gene [16]. A relative standard curve was generated using serial dilutions (1 / 2, 1 / 10, 1 / 50, 1 / 250, 1 / 1250) of cDNA transcripts as template amplification. The corresponding qPCR efficiency (E) was calculated in light of the equation $E = (10^{-1/slope} - 1)\times 100$ [17]. qPCR was performed on three biological replicates and all reactions were carried out in triplicate per template.

Table 1. List of genes with associated functions selected for the study

Gene	Full gene name	Function	GenBank accession no.
RP49	Ribosomal protein 49	Structural constituent of ribosome	MH202750
EF-1a	Elongation factor 1 alpha	Extension of amino acid residues	MH250149
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	MH250150
ACT	Actin	Cytoskeletal structure proteins	MH250151
EF-1d	Elongation factor 1 delta	Extension of amino acid residues	MH250152
TUB	Tubulinbeta-1	Cytoskeletal structure proteins	MH250153
EF-1b	Elongation factor 1 beta	Extension of amino acid residues	MH250154
RPL13	Ribosomal protein L13	Structural constituent of ribosome	MH250155

Table 2. Primer sequences for genes used in the study

S.No.	Gene symbol	Forward primer (5′–3′)	Reverse primer (3′–5′)	Product length (bp)	E (%)	R^2	slope	Standard curve
1	*RP49*	AACTGGCGTAAACCGAGAGG	GAGCATGTGACGGGTCTTCT	111	100.20	0.999	-3.348	Y=-3.348x+20.92
2	*EF-1a*	CAGCCAGAAATGGGGACGAA	TGGACTCTACTGAACCCCCA	124	99.40	0.997	-3.271	Y=-3.271x+18.61
3	*GAPDH*	TACTTTAGCGAGGGGAGCGA	ATGCCCCTATGTTCGTCGTC	110	100	0.997	-3.342	Y=-3.342x+21.294
4	*ACT*	ACTGACGTCGGGAGTTCGTA	CTGAGAGGGAAATCGTCCGC	127	98.90	0.996	-3.321	Y=-3.321x+22.762
5	*EF-1d*	AGGTCAAGGCTGAGGCTCCTAAG	TGGCAATGAGTACTGGCTTCTTGG	174	95.50	0.991	-3.434	Y=-3.434x+32.571
6	*TUB*	GGCTTTGCGCCACTAACTTC	CGTCAAACATCTGCTGCGTC	82	111.30	0.994	-3.078	Y=-3.078x+38.693
7	*EF-1b*	AAAAATCCAAGAAGCCTGCC	GTCCATTTCTATGGTGCGGA	119	97.10	0.987	-3.392	Y=-3.392x+33.35
8	*RPL13*	GGTCTTGGTGCTACTGCCTT	ATGATCCCGAATGGGCACTT	128	108.60	0.996	-3.132	Y=-3.132x+36.847

2.6 Data Analysis

Data analysis of the qPCR was performed using software of Bio-Rad (version 4.1.2433.1219) (Bio-Rad, Hercules, USA). NormFinder (version 0.953) [17] and geNorm (version 3.5) [18] were used to assess the 8 candidate genes' stability across organs, developmental stages and diapause states.

The cycle threshold (Ct value) is the initial marker point where the fluorescence signal is significantly different from the background. The original Ct value was converted to a 2 (ΔΔCt) value using a method that fits the NormFinder and geNorm model assumptions [19]. The minimum Ct value of each gene was considered as a control and given to 1 [14]. The average pairwise variation of each reference gene with all other reference genes is defined as the expression stability value (M) by geNorm [18], smaller M value of the reference gene shows better stability. The pairwise variation V_n/V_{n+1} was counted. If the $V_n/V_{n+1}$is over 0.15, means that the added gene plays an important role in producing reliable normalization factors. The added reference gene should be contained [14]. NormFinder assess the expression stability of different candidate reference genes by measuring the overall expression level variation and analyzing intra- and intergroup variations of the reference genes. The top-ranked reference genes will have minimum intragroup variation and the lowest stability value.

3. RESULTS AND DISCUSSION

3.1 Expression Profiles of the Eight Candidate Reference Genes

For each of the 8 candidate reference genes of *C.sasakii*, a unique PCR product was amplified and had a single peak in dissociation curve. The melting temperature (Tm) of the primers was 60°C. All PCR products range in length from 82 to 174 bp, and an composite photo of all PCR products from melting curve for eight genes from qPCR was shown in Fig.1a. The efficiency (E) of the PCR amplifications varied from 95.5% for *EF-1d* to 126.3% for *TUB*, and the correlation coefficients (R^2) distributed between 0.824 and 0.999 Table 2.

The Ct values of the 8 genes showed the transcript abundance was at different level. The mean Ct values of the 8 genes ranged from 18.93 to 39.24 cycles. Depending on Ct value, *RP49* showed the lowest expression variation, followed by *EF-1a*, *GAPDH*, *TUB* and *ACT*, whereas *EF-1b* had the highest variation Fig. 1b.

A. melting curve for eight genes from qPCR total organs, n = 21 sample points. The median is represented by black line across each box. Box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles.

3.2 geNorm Analysis of Gene Expression Stability

It was proposed by the geNorm tool that stability M value for a threshold expression was 0.5 [18]. In the different developmental stages, EF-1a, GAPDH and ACT had the M value less than 0.5, EF-1a was the most appropriate reference gene for it has the M value of 0.45 was lowest Fig. 2A. In the different organs, EF-1a, GAPDH, ACT and *RP49* had the M value less than 0.5, and their values gradually increased. The 4 genes were the more appropriate reference genes Fig. 2B. In the different diapause states, ACT, EF-1a, GAPDH and *RP49* had the M value less than 0.5, and their values gradually increased Fig. 2C.

geNorm rank the expression stability of eight candidate reference genes in (A) different developmental stages, (B) different organs and (C) different diapause states. Suitable reference genes are assigned M values below 0.5 (dotted line).

To evaluate the pairwise variation (V_n/V_{n+1}) among groups, the geNorm was used further. In term of organs, developmental stages and diapause states, all the values of V_n/V_{n+1} were below 0.15, except for the V_7/V_8 value Fig. 3. For developmental stages and organs, the combination of *EF1a* and *GADPH* (V_2/V_3 = 0.055) could sufficiently normalize the expression data Fig. 3. When tissues under diapause states were analyzed in the same way, reference genes combined ACT and EF1a (V_2/V_3= 0.095) was the best for data normalization Fig. 3.

Average pairwise variations (V) were calculated by geNorm between V_n and V_{n+1}. V<0.15 (dotted line) indicate that additional genes are not required for the normalization of gene expression.

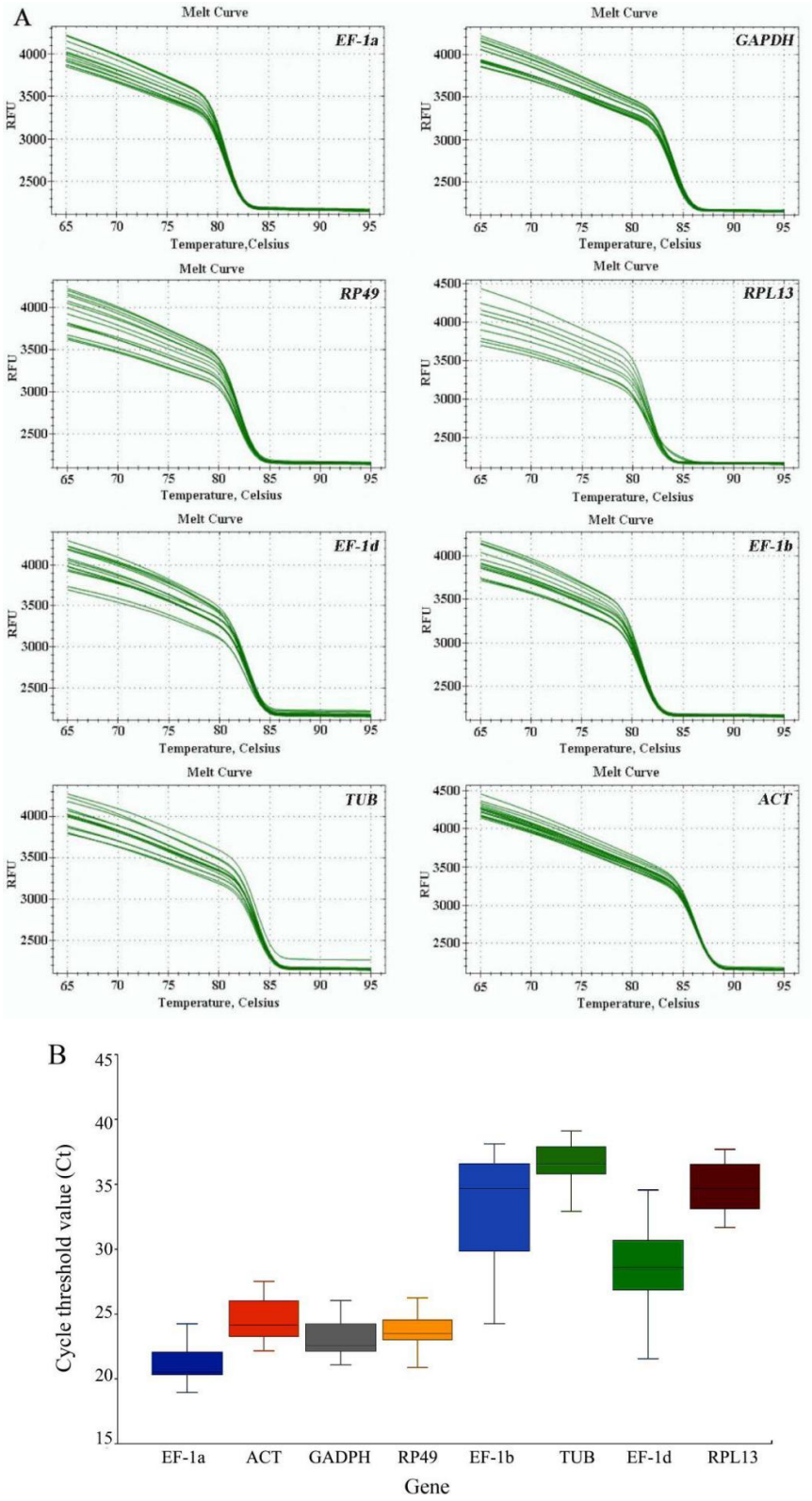

Fig. 1. Melting curves and range of Ct values of eight candidate reference genes in qPCR

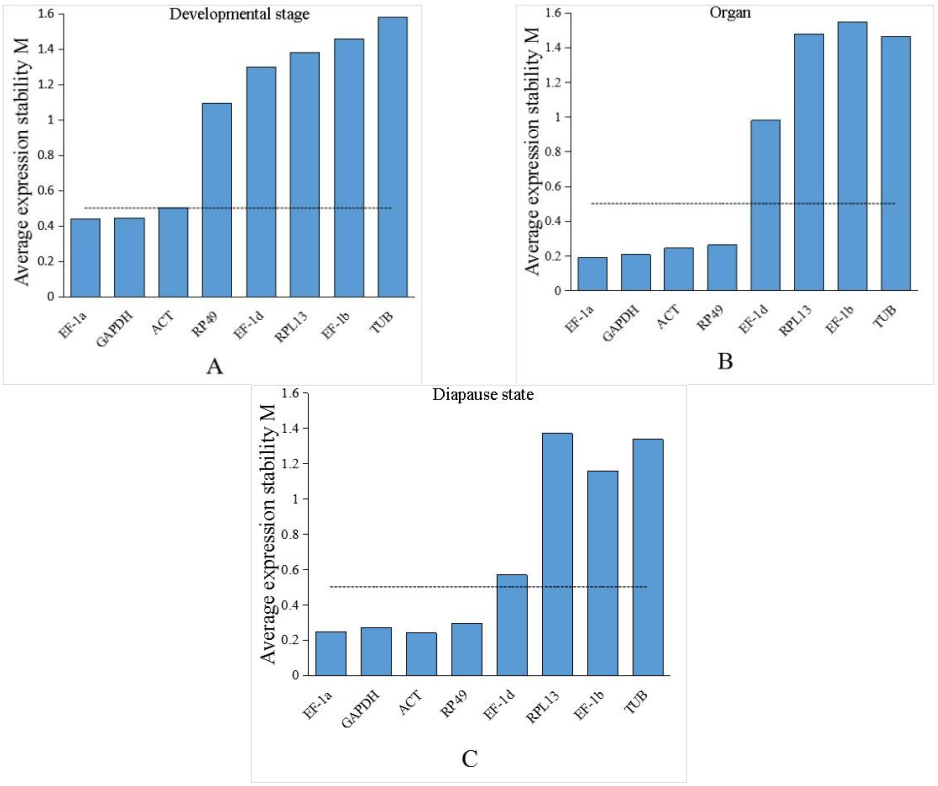

Fig. 2. Measures of expression stability (M value)

Table 3. Expression stability of candidate reference genes ranked by NormFinder among different sample sets

Rank	Development stage		Organ		Diapause state	
	Gene	M	Gene	M	Gene	M
1	*EF-1a*	0.0349952	*EF-1a*	0.0181663	*ACT*	0.084464
2	*GAPDH*	0.0349952	*GAPDH*	0.0181663	*EF-1a*	0.113297
3	*ACT*	0.2222377	*ACT*	0.1718323	*GAPDH*	0.153218
4	*RP49*	0.7550828	*RP49*	0.2439047	*RP49*	0.180422
5	*EF-1d*	0.986786	*EF-1d*	0.684239	*EF-1d*	0.604267
6	*RPL13*	1.369854	*RPL13*	1.156932	*EF-1b*	1.269084
7	*EF-1b*	1.466667	*EF-1b*	1.359647	*RPL13*	1.493601
8	*TUB*	2.356081	*TUB*	2.036587	*TUB*	1.953201

3.3 Norm Finder Analysis

The top-ranked gene has the lowest stability value and minimum intragroup variation. NormFinder is also used to determine the stability values and grades of 8 candidate genes Table 3. In terms of different developmental stages, *EF-1a* and *GAPDH* (M value = 0.035) were the stablest reference genes followed by M value of *ACT*, whereas the most unstable gene was *TUB*. On different organs, the stablest reference genes were *EF-1a* and *GAPDH* (M value = 0.018) followed by *ACT*. In the different diapause states, the best choice for reference genes is *ACT* (M value = 0.084) followed by *EF-1a* and *GAPDH*, and *TUB* M value was the most unstable too. In our research, the results of geNorm and NormFinder were highly consistent Table 3.

The rank of the genes for the sample combination is then analyzed. For different developmental stages and different organs, *EF-1a* and GAPDH were the best combination Table 4. Among different diapause states, the best two genes were *ACT* and *EF-1a*.

Table 4. Consensus ranking of candidate reference genes

Gene	Developmental stage				Organ				Diapause state			
	geNorm		NormFinder		geNorm		NormFinder		geNorm		NormFinder	
	M	Rank	M	Rank	M	Rank	M	Rank	M	Rank	M	Rank
EF-1a	0.439932	1	0.034995	1	0.193137	1	0.018166	1	0.246376	2	0.113297	2
GAPDH	0.445198	2	0.034995	1	0.208681	2	0.018166	1	0.269073	3	0.153218	3
ACT	0.504205	3	0.222238	3	0.245932	3	0.171832	3	0.239998	1	0.084464	1
RP49	1.093309	4	0.755083	4	0.263371	4	0.243905	4	0.293680	4	0.180422	4
EF-1d	1.296873	5	0.986786	5	0.980647	5	0.684239	5	0.568940	5	0.604267	5
RPL13	1.380641	6	1.369854	6	1.478605	7	1.156932	6	1.369500	8	1.493601	7
EF-1b	1.458010	7	1.466667	7	1.548316	8	1.359647	7	1.156230	6	1.269084	6
TUB	1.578962	8	2.356081	8	1.463025	6	2.036587	8	1.336580	7	1.953201	8
Best gene		*EF-1a*		*EF-1a*		*EF-1a*		*EF-1a*		*ACT*		*ACT*

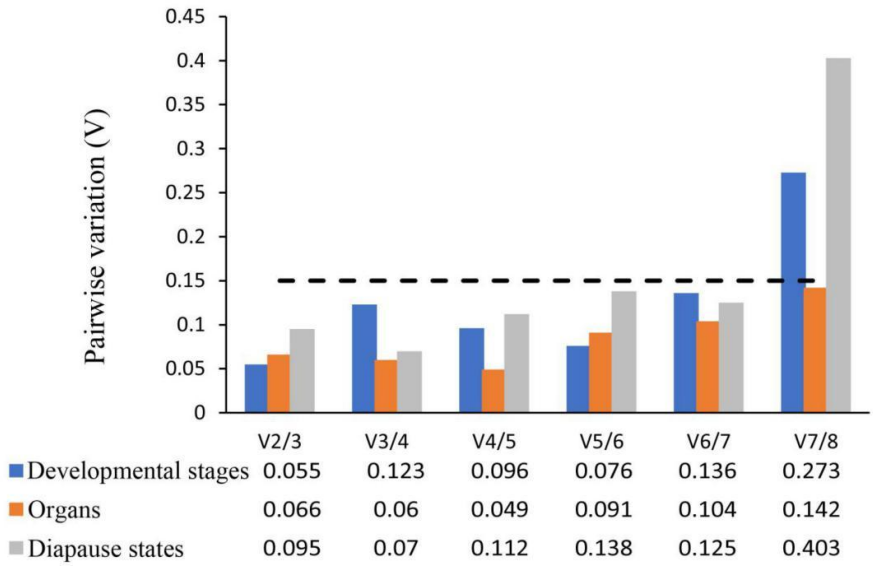

Fig. 3. Determination of the suitable number of reference genes for normalization

A stable reference gene is needed to normalize the transcription levels of target genes in qPCR experiments. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Ribosomal proteins (RP) and Actin [8-10], as the most common reference genes, have been used for a while without verification [11,12]. However, the commonly used reference genes' stability changes depending on the species of insect and the experimental process, so it should be demonstrated that a particular reference gene or more can be accurately used for a variety of experimental situations [8,20,21].

In identification of the male-specific or female-specific chemosensory genes that were putatively related to odor detection and recognition in *C. sasaki*i, ACT and EF1α as reference genes were used in qRT-PCR [6]. Li et al [7] identified putative odorant binding proteins expressed in intact heads of *C. sasakii* adult males and females, using β-actin as endogenous reference in qRT-PCR. In the two qRT-PCR experiments of *C. sasakii reported* [6,7], the endogenous reference genes selection were different. It is not conducive to the further application of the reference gene in other studies. As far as we know, our experiment is the first in-depth study of screening the stablest reference gene for qPCR in *C. sasakii*. In our study, EF-1a, ACT and GAPDH were the stablest reference genes or best gene combinations for different qPCR analyses in *C. sasakii* across different developmental stages, organs, and diapause states. The molecular mechanism of *C. sasakii*'s diapause development is a biological process that we are very interested in. Based on our internal reference gene research used in qRT-PCR, we can get new insight into gene expression not only diapause development but also other complex biological process in the future.

4. CONCLUSION

In our current study, the eight candidate reference genes of *C. sasakii* were evaluated across different developmental stages, tissues, and diapause states. After comprehensive analysis, EF-1a, ACT and GAPDH were the stablest reference genes or best gene combinations for different qPCR analyses in *C. sasakii*, whereas *TUB* and *EF-1b* showed relatively low expression stability. It's sufficient that the use of two reference genes to effectively normalize qPCR data under the experimental conditions and in different organs of *C. sasakii*. This work prepared a platform for gene expression analysis in *C. sasakii*. This result helps to study gene function associated with diapause regulation and development and provides information about the selection of reference genes.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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