Screening and Stability Evaluation of Reference Genes for Real-Time Quantitative Polymerase Chain Reaction in *Agrilus zanthoxylumi* (Coleoptera: Buprestidae)[1]

Chen Di, Yang Ping, Guo Li, Xie Shou-An[2], Gong Xue-Fang[3], and L.V. Shu-Jie

Northwest A&F University, Yangling, Shaanxi, 712100, China

J. Entomol. Sci. 56(4): 527–540 (October 2021)

Abstract Selection of suitable reference genes is crucial to accurately evaluate and normalize the relative expression level of target genes for gene function research. Our study selected suitable reference genes for analyzing the gene expression of *Agrilus zanthoxylumi* Hou (Coleoptera: Buprestidae). Six candidate genes were detected by real-time quantitative polymerase chain reaction: the histone gene, the β-tubulin gene, the actin gene, 18S ribosomal RNA, and 28S-1 and 28S-2 ribosomal RNA. The expression of the candidate reference genes in different tissue samples (head, thorax, abdomen, legs, and wings) of *A. zanthoxylumi* was evaluated and analyzed by using GeNorm, NormFinder, and BestKeeper software programs. Gene expression stability results show that the expression of the 28S-2 gene is the most stable of the six candidate genes in all tissues of female *A. zanthoxylumi*, followed by the 28S-1 gene. The actin gene has the most stable expression of the six genes in male tissues, followed by the 28S-2 gene. The screening results of reference genes with the most stable expression in different sexes and tissues obtained in this study can be used for the subsequent quantitative expression research of related genes and provide theoretical basis and reference materials for the research of related gene expression levels of *A. zanthoxylumi*.

Key Words *Agrilus zanthoxylumi,* reference gene, RT-qPCR, expression stability

Agrilus zanthoxylumi Hou (Coleoptera: Buprestidae) is the main stem-boring pest of Chinese prickly ash, *Zanthoxylum bungeanum* Maxim, an important economic crop in northern China. *Agrilus zanthoxylumi* is monophagous, attacking only *Z. bungeanum* (Wu 2006), and exhibits one generation per year. Adults feed on foliage causing notches on leaf edges (Cui 1990). Larvae overwinter in the bark but actively feed in the phloem and gradually consume the cambium creating decaying pupal channels in the xylem. Characteristic symptoms of larval damage are large volumes of exudates, softening, decay, etc., causing yellowing of foliage, death of entire branches or the crown, and even death of the tree (Kai 1991).

Pheromones have been proposed for the management of *A. zanthoxylumi* for monitoring populations, trap-and-kill methods, and mating or host detection confusion (Hamadttu et al. 2020). Knowledge of gene expression involved in

[1]Received 24 October 2020; accepted for publication 8 November 2020.
[2]Corresponding author (email: shouanxie@163.com).
[3]Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, Sichuan 610041, China.

chemical detection and sensing is an important step in development and efficacious use of these chemistries for *A. zanthoxylumi* management.

We report herein our assessment of selected reference genes that might be reliably and accurately used in reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) for detecting gene expression levels in various tissues of *A. zanthoxylumi*. RT-qPCR has been used extensively in insect transcriptome verification and gene expression analysis because of its relative speed, high sensitivity, replicability, and accuracy (Lü et al. 2018, Shakeel et al. 2015, Shi et al. 2017, VanGuilder et al. 2008, Wang and Xue. 2016). RT-qPCR results are affected by many factors (e.g., quality of initial RNA, transcription and amplification variations, complementary DNA [cDNA] synthesis efficiency, etc.) (Heid et al. 1996); therefore, suitable reference genes are used as quantitative standard references in order to ensure the accuracy and reliability of data analysis.

In this study, we assessed six candidate reference genes. These were histone, β-tubulin, actin, 18S rRNA, 28S-1 rRNA and 28S-2 rRNA. The expression stability of these six genes was established using fluorescent qPCR. Data were also evaluated using the GeNorm (Biogazelle, Zwijnaarde, Belgium; Vandesompele et al. 2002), NormFinder (Aarhus University Hospital, Denmark; Claus et al. 2004), and BestKeeper (Pfaffl et al. 2004) algorithms.

Materials and Methods

Sample preparation and total RNA extraction. The samples used in this study were collected from the base of Chinese prickly ash in the mountainous area of Zhoudong Village, Puhua Town, Lantian County, Shaanxi Province (May to July 2019). It is a semiclosed basin with a mountainous terrain. The climate is warm temperate and semihumid continental climate with distinct seasons. The average annual temperature is 12°C, and the central location is N 34°13′ and E 109°31′. Active *A. zanthoxylumi* adults were collected from branches and foliage of *Z. bungeanum*, placed in tubes (1.3-cm diameter, 4-cm height) with air holes, transported to the laboratory, and held at 25°C (Gong et al. 2019). Periodically, the *Z. bungeanum* foliage was replaced with fresh leaves, and frass and leaf residues were removed. Tissues from the head, thorax, abdomen, legs, and wings of adult insects were dissected and collected from male and female pairs, inserted into a centrifuge tube without RNA enzyme, and held at −80°C until assayed.

Total RNA was extracted using the column-type insect RNA extraction kit (BTN81220, Biolab Company, Beijing). As per the kit instructions, total RNA was extracted from five parts of female and male adults: heads, thorax, abdomen, legs, and wings. Each part was subjected to three repetitions and marked with designations for body region, sex, and replicate number (e.g., ANF1∼3, HF1∼3, TF1∼3, ABF1∼3, LF1∼3, and WIF 1∼3 for females, and ANM1∼3, HM1∼3, TM1∼3, ABM1∼3, LM1∼3, and WIM1∼3 for males). The integrity was detected by agarose gel electrophoresis, and the reverse transcription was performed according to the instructions of the Vazyme reverse transcription kit (R133-01). The first strand of cDNA was stored at −20°C for subsequent real-time fluorescence quantitative detection.

Primer design and qRT-PCR. Six candidate genes for reference (e.g., histone, β-tubulin, actin, 18S, and 28S-1 and 28S-2) were screened from the transcription group data of *A. zanthoxylumi*. According to the obtained gene sequence, primers for quantitative analysis were designed by using Primer-BLAST method with the information for each primer (Table 1). The synthesized first-strand cDNA was used as a template to detect the expression amount of the reference gene by RT-qPCR, and the reactions were performed in duplicate using the following protocol: 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 58°C for 30 s, and 72°C for 30 s.

The cDNA was prepared into templates with seven gradient concentrations by a two-time dilution method. The cycle threshold (Ct) values of histone, β-tubulin, actin, 18S, 28S-1, and 28S-2 at different concentrations were measured by the CFX96 real-time PCR detection system, using the following protocol: 95°C for 10 s, and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. According to the results, the linear relationship between the concentration of cDNA template and Ct value was analyzed by the linear correlation method, yielding a correlation coefficient (R^2) and slope for each. The amplification efficiency of the primers was calculated by the formula $E = (10^{[-1/\text{slope}]} - 1) \times 100$ (Radonic et al. 2004).

Data analysis. With the quantitative Ct value of candidate reference genes in each sample as a reference, the data were organized according to the requirements of the three software programs (GeNorm, NormFinder, and BestKeeper). The GeNorm automatically calculates an M value for each reference gene, with the more stable genes indicated by lower M values. The calculation principle of the NormFinder is similar to that of GeNorm. The stable value of reference gene expression is obtained first, and then the most appropriate reference gene is selected according to that value. The judgment criterion is that the reference gene with the minimum expression stability value is the most appropriate reference gene. The correlation coefficient (R), standard deviation (SD), and coefficient of variation (CV) of pairing between each gene can be obtained through the calculation of the BestKeeper, with lower SD values signifying more stable genes. Finally, the geometric mean of the six reference genes ranking in three different evaluation procedures was calculated, and then the comprehensive ranking was conducted to determine the stability of the six reference genes.

Results

The transcriptome sequencing identified five types of candidate reference genes (Table 1). These were histone, β-tubulin, actin, 18S, and 28S. Among those, two genes in 28S can be successfully cloned and were thus named 28S-1 and 28S-2. The six candidate reference genes of *A. zanthoxylumi* and the corresponding genes of other coleopteran insects have high nucleotide sequence identity, withthe highest consistency with *Agrilus planipennis* Fairmaire, both >87% and some approaching 100%.

In analyzing amplification efficiency and specificity of the expression levels of the six candidate reference genes of *A. zanthoxylumi* male and female adults, we found that the slopes of the lines ranged from −2.9 to −3.6 and linear correlation coefficients were >0.9800. This indicated that there was a clear linear relationship between the initial template amount of cDNA and the corresponding Ct value, that

Table 1. Candidate reference genes of *Agrilus zanthoxylumi*.

Gene Name	Primer Sequence (5′–3′)	Hit-Species	Hit-Accession	E Value	Identity
Histone	F: CAAGTTGTAAGTGACGGGGAGT R: AAAAGCCAAAGCAAAAGCAGT	*Agrilus planipennis*	XM_018466584	0	93.16%
β-tubulin	F: GTAGATTTGGTATTGGATAGGATTC R: AAGGTAGTATGGGTGGTAAGGA	*Agrilus planipennis*	XM_025973330	1E-167	89.47%
Actin	F: ACCCATCTATGAAGGTTACGC R: GCAAAGTTTTACCTTGATGTCC	*Agrilus planipennis*	XM_018479924	3E-142	95.91%
18S	F: AATTTCTTCCCTATCAACTTTCG R: TGGATGTAGTAGCCGTTTCTCA	*Cinara cedri*	VVC42870	4E-15	95.45%
28S-1	F: GAAAATGAGTCCGAGGCAAAA R: TAAAACAGGAAGTGGTGAGGC	*Agrilus planipennis*	XM_018476332	0	87.69%
28S-2	F: AAAATATGCAAAAGAATGTGGTG R:CAGGGTAGGTGATTAAGTTAGGATT	*Agrilus planipennis*	XM_018469940	0	89.46%

the obtained linear correlation equation was reliable, and that the amplification efficiency was between 90% and 120%, indicating the effectiveness of the RT-qPCR reaction (Table 2). The dissolution curves of the six reference genes were basically single peaks, indicating that the primers for the reference genes amplified well under the real-time fluorescence quantification condition, thus meeting the basic requirements of real-time fluorescence quantification. The changes of Ct values of each candidate reference genes in this study showed that in both sexes (Figs. 1, 2), the expressions of the Class 28S gene and the histone gene were relatively low, and the expression of the actin gene was highest of the six reference genes.

Expression stability analysis by GeNorm. In the GeNorm software, and the average expression stability (M) values are an indication of gene stability and are inversely proportional to reference gene stability. Based on our analysis (Table 3), gene stability of the six candidate reference genes in female *A. zanthoxylumi* was, from highest to lowest, in the head tissues was actin > histone > β-tubulin > 28S-1 > 18 S > 28S-2; in the thorax tissues, 28S-2 > 18S > β-tubulin > 28S-1 > histone > actin; in the abdominal tissues, 28S-2 > actin > histone > β-tubulin > 18S > 28S-1; in the legs, 28S-1 > 18S > actin > histone > β-tubulin > 28S-2; and in the wings, 28S-2 > 18S > 28S-1 > actin > β-tubulin > histone. In males, gene stability from highest to lowest was 18S > β-tubulin > 28S-2 > actin > 28S-1 > histone in head tissues; 28S-1 > β-tubulin > 28S-2 > 18S > actin > histone in thoracic tissues; 28S-2 > actin > 28S-1 > 18S > histone > β-tubulin in abdominal tissues; 28S-2 > β-tubulin > 18S > histone > actin > 28S-1 in the legs; and actin > histone > β-tubulin > 28S-1 > 18S > 28S-2 in the wings. Overall, regardless of sex, gene stability among these six reference genes was 28S-2 > β-tubulin > 28S-1 >18S > actin > histone based on the GeNorm analysis.

Expression stability analysis by NormFinder. NormFinder software analysis also depends on a stability value to judge the stability of the reference gene. Those values of the six candidate reference genes in female and male tissues are shown in Table 4. The two 28S reference genes exhibited the highest stability in the thorax, leg, and wing tissues of female *A. zanthoxylumi*, while β-tubulin showed the highest stability in the head and abdominal tissues. Among males, actin exhibited the highest stability in the head and abdominal tissues, while β-tubulin showed highest stability in the thoracic tissues with 18S and histone having the highest stability in legs and wings.

Expression stability analysis by BestKeeper. BestKeeper analysis requires calculation of correlation coefficient, SD, and CV of each candidate reference gene, and the relative stability of each gene is judged according to the SD of each gene. Smaller standard coefficient of deviation (SD) and the CV correlationvalues, along with larger Pearson correlation coefficient values are indicators of higher expression stability of the gene. Our analysis of the six candidate reference genes with BestKeeper (Table 5) showed that the genes with the highest expression stability in the respective tissues of female *A. zanthoxylumi* head, thorax, abdomen, legs, and wings of adults were 28S-1 in the head, 28S-2 in the thorax, histone in the abdomen, 28S-2 in the legs, and 18S in the wings. In males, the highest stability expressions were 28S-1 in the head, β-tubulin in the thorax, actin in the abdomen, and histone in the legs and wings.

Table 2. Amplification efficiency, correlation coefficient, and slope of primers in various tissues of male and female adult *Agrilus zanthoxylumi*.

	Efficiency (%)		Correlation (R^2)		Slope	
Name	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Head						
Histone	113.06	91.53	0.990	0.984	−3.044	−3.543
β-tubulin	96.33	99.83	0.997	0.995	−3.413	−3.326
Actin	94.04	106.84	0.987	0.992	−3.474	−3.168
18S	110.10	102.02	0.992	0.998	−3.101	−3.274
28S-1	93.81	99.46	0.992	0.992	−3.480	−3.335
28S-2	113.29	90.45	0.988	0.996	−3.040	−3.574
Thorax						
Histone	93.42	94.81	0.997	0.986	−3.490	−3.453
β-tubulin	98.18	109.70	0.995	0.993	−3.366	−3.109
Actin	98.79	118.44	0.997	0.991	−3.351	−2.947
18S	94.82	113.49	0.994	0.996	−3.453	−3.036
28S-1	93.07	102.40	0.984	0.991	−3.500	−3.266
28S-2	98.46	98.56	0.995	0.995	−3.359	−3.357
Abdomen						
Histone	117.09	119.76	0.997	0.995	−2.971	−2.924
β-tubulin	98.11	118.84	0.991	0.999	−3.368	−2.940
Actin	91.90	96.49	0.990	0.996	−3.533	−3.409
18S	116.76	109.64	0.996	0.987	−2.976	−3.111
28S-1	90.84	115.30	0.993	0.988	−3.563	−3.003
28S-2	102.60	91.54	0.992	0.992	−3.261	−3.543
Leg						
Histone	109.29	117.92	0.982	0.990	−3.118	−2.956
β-tubulin	101.25	93.55	0.993	0.996	−3.292	−3.487
Actin	92.34	106.95	0.995	0.981	−3.520	−3.166
18S	93.19	91.63	0.991	0.991	−3.497	−3.540
28S-1	103.85	111.19	0.994	0.991	−3.233	−3.080
28S-2	115.29	106.78	0.987	0.996	−3.003	−3.170

Table 2. Continued.

	Efficiency (%)		Correlation (R^2)		Slope	
Name	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**
Wing						
Histone	103.60	117.68	0.994	0.997	−3.238	−2.960
β-tubulin	109.72	94.34	0.994	0.992	−3.109	−3.466
Actin	101.96	105.43	0.993	0.993	−3.276	−3.198
18S	119.80	95.80	0.992	0.981	−2.924	−3.427
28S-1	110.53	97.79	0.995	0.988	−3.093	−3.376
28S-2	115.46	91.61	0.987	0.992	−3.000	−3.541

Overall ranking of stability. We calculated the arithmetic mean of the relative ranking (1–6) of each of the six candidate reference genes from the three calculations in order to determine the reference gene that was most suitable for *A. zanthoxylumi*, thus providing a basis for future research. These calculations for females and males are shown in Tables 6 and 7, respectively. The 28S-2 gene was the most stable of the six genes in all tissues of female *A. zanthoxylumi*; 28S-1 also was relatively stable in female tissues from those body regions. In males, the expression of actin gene was the most stable in all tissues followed by 28S-2 gene.

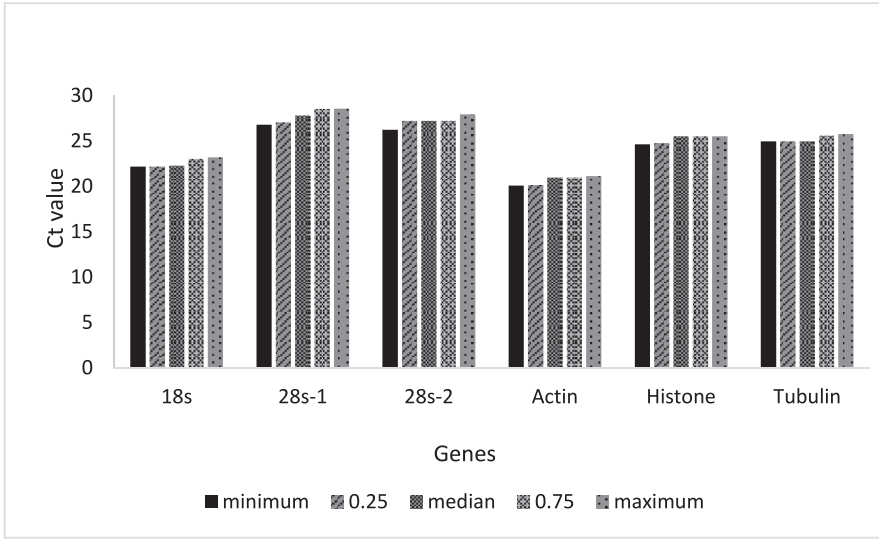

Fig. 1. Expression levels of six candidate reference genes in female *Agrilus zanthoxylumi*.

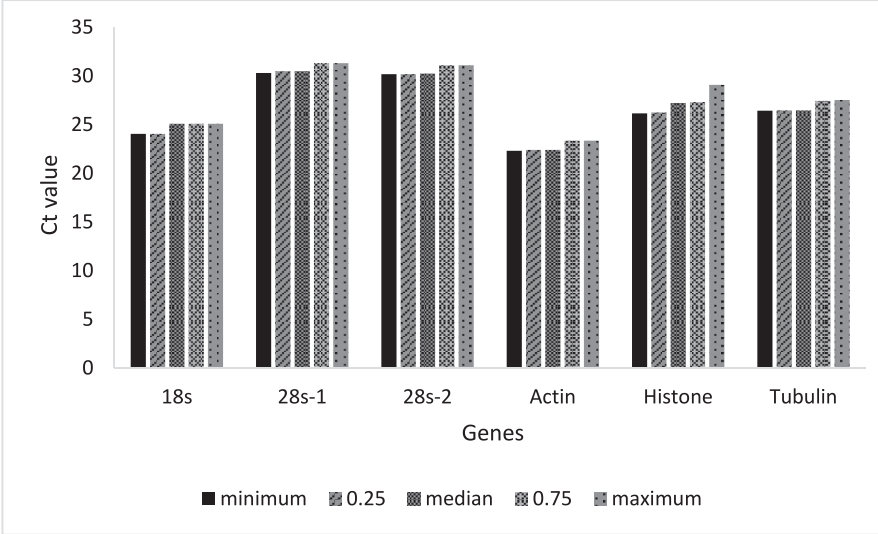

Fig. 2. Expression levels of six candidate reference genes in male *Agrilus zanthoxylumi.*

Discussion

Real-time fluorescent qPCR has been widely used in the quantitative study of gene expression (Heid et al. 1996). Yet, RT-qPCR also has its limitations in that expression results are often greatly influenced by biological and abiotic factors. Therefore, reference genes are often introduced in the quantitative gene expression

Table 3. GeNorm reference gene stability values (M) of *Agrilus zanthoxylumi.*

Tissue source	Histone	β-Tubulin	Actin	18S	28S-1	28S-2
Female head	0.512	0.585	0.358	0.693	0.627	0.733
Female thorax	0.558	0.345	0.628	0.247	0.419	0.223
Female abdomen	0.493	0.548	0.373	0.587	0.633	0.352
Female legs	0.677	0.742	0.561	0.537	0.511	0.835
Female wings	0.663	0.588	0.533	0.384	0.479	0.361
Male head	0.642	0.451	0.483	0.422	0.515	0.473
Male thorax	0.613	0.269	0.516	0.472	0.256	0.417
Male abdomen	0.658	0.793	0.481	0.612	0.543	0.455
Male legs	0.473	0.406	0.491	0.425	0.562	0.397
Male wings	0.439	0.443	0.413	0.512	0.463	0.541

Table 4. NormFinder reference gene stability values of *Agrilus zanthoxylumi.*

Tissue Source	Histone	β-Tubulin	Actin	18S	28S-1	28S-2	Best
Female head	0.309	0.233	0.242	0.452	0.541	0.328	β-tubulin
Female thorax	0.393	0.278	0.446	0.334	0.191	0.198	28S-2
Female abdomen	0.150	0.317	0.396	0.322	0.222	0.402	Histone
Female legs	0.373	0.505	0.384	0.284	0.596	0.237	28S-1
Female wings	0.503	0.333	0.407	0.247	0.160	0.263	28S-2
Male head	0.575	0.216	0.211	0.251	0.230	0.328	Actin
Male thorax	0.493	0.139	0.400	0.231	0.231	0.210	β-tubulin
Male abdomen	0.411	0.676	0.253	0.259	0.286	0.398	Actin
Male legs	0.217	0.279	0.262	0.173	0.269	0.433	18S
Male wings	0.157	0.251	0.290	0.302	0.334	0.239	Histone

by RT-qPCR to correct and standardize the test data, so as to offset the errors between the test data measured by the instrument (Chen et al. 2015, T.F. Li 2018). Selecting appropriate reference genes can effectively improve the reliability and accuracy of the test results (Bustin et al. 2009).

Ideal reference genes refer to a class of genes that can be stably expressed in different types of tissues and different treatments of the same tissue, and whose expression levels are not affected by any endogenous or exogenous factors (Janská et al. 2013). However, various studies have screened and identified reference genes for a variety of insects and found that the optimal reference genes were not the same under different test conditions, that is, different species, different tissues, and different development stages (Li et al. 2018, Morales et al. 2016, Tan et al. 2017, Yang et al. 2013).

In studies with coleopterans, reference genes screened differed among the reported studies. For example, Liu et al. (2014) screened eight reference genes, including β-actin, GAPDH, α-tubulin, SYN1, SYN6, RPS3, RPS8, and RPS13a in *Tribolium castneum* (Herbst) and determined that suitable reference genes for gene expression analysis of different strains of *T. castneum* induced by phosphine were RPS8 and RPL13a. Li et al. (2018) found that the expression levels of four reference genes (TUB, TUA, RPS20, and RPL 12) in different tissues of the adult weevil *Sympiezomias velatus* Chevrolat were relatively stable. Feng et al. (2016) found that GAPDH was the preferred reference gene for expression analysis of the chemosensory gene of *Monochamus alternatus* White, and the combination of the reference genes GAPDH and TUB was sufficient to effectively correct the expression of the chemosensory gene of *M. alternat*us. In other coleopteran insects, such as *Anoplophora glabripennis* (Motschulsky) and *Galeruca daurica* Joannis, RPL32(RP49) is reportedly one of the most suitable reference genes for gene expression in different tissues (Rodrigues et al. 2017, Tan et al. 2017).

Table 5. BestKeeper reference gene stability values of *Agrilus zanthoxylumi.*

Tissue Source	Parameters	Histone	β-Tubulin	Actin	18S	28S-1	28S-2
Female head	*r**	0.994	1.00	0.728	0.991	0.98	0.998
	SD	1.36	1.25	1.85	1.44	1.54	1.13
	CV/%	4.76	4.61	7.91	6.03	5.11	3.84
Female thorax	*r*	0.995	0.865	0.869	0.878	0.999	0.955
	SD	1.78	1.78	1.75	1.81	1.27	1.50
	CV/%	5.75	6.23	7.53	7.09	4.21	4.72
Female abdomen	*r*	0.999	0.942	0.998	1.00	0.999	1.00
	SD	1.36	1.76	1.61	1.37	1.66	1.51
	CV/%	5.22	7.23	7.16	4.93	5.95	5.42
Female legs	*r*	0.946	0.99	0.962	0.95	0.999	0.978
	SD	1.63	1.52	1.83	1.82	1.14	1.34
	CV/%	5.46	5.83	8.65	7.72	3.86	4.54
Female wings	*r*	0.985	0.988	0.984	0.999	1.00	0.962
	SD	1.63	1.60	1.82	1.08	1.13	1.53
	CV/%	5.15	5.58	7.51	4.83	3.74	4.80
Male head	*r*	0.975	0.971	0.998	0.976	0.968	0.998
	SD	1.47	1.49	1.18	1.47	1.54	1.32
	CV/%	5.08	6.03	4.89	6.15	5.18	4.35
Male thorax	*r*	0.982	0.971	0.938	0.949	0.905	0.968
	SD	1.29	1.18	1.33	1.43	1.47	1.23
	CV/%	4.55	4.47	6.44	6.10	5.08	4.18
Male abdomen	*r*	0.987	0.998	0.995	0.995	0.998	0.995
	SD	1.35	1.07	1.26	1.40	1.38	1.32
	CV/%	4.79	4.09	5.05	5.11	4.93	4.34
Male legs	*r*	0.995	0.988	0.010	0.977	0.983	0.984
	SD	0.9	1.28	1.47	1.27	1.13	1.20
	CV/%	3.00	4.65	7.10	5.91	3.63	3.94
Male wings	*r*	0.996	0.977	0.937	0.997	0.951	0.999
	SD	1.08	1.51	1.26	1.29	1.51	1.30
	CV/%	3.83	5.95	6.32	5.68	5.18	4.40

* *r* means correlation coefficient; CV/%, coefficient of variation.

Table 6. Comprehensive ranking of reference genes in female tissues of *Agrilus zanthoxylumi.*

Name	Rank by GeNorm	Rank by NormFinder	Rank by BestKeeper	Average Ranking	Overall Ranking
Histone	4	3	3	3.301927	4
β-tubulin	4	1	4	2.519842	3
Actin	2	4	5	3.419952	5
18S	2	2	4	2.519842	3
28S-2	1	1	2	1.259921	1
28S-1	3	1	1	1.442250	2

All of these studies have shown that there are differences in the expression stability of the reference gene in different species, development stages, and tissues (De et al. 2007, Huis et al. 2010, Sun et al. 2009). Furthermore, no housekeeping gene has been found to be stably expressed under all conditions or suitable for all cell and tissue types, and it is difficult to meet the requirements of universality even though it may be highly conserved during evolution (Yang et al. 2015).

In this study, six candidate reference genes were identified from *A. zanthoxylumi*, and the reference genes were screened in different tissues of male and female adults using RT-qPCR technology and three analysis software programs. We determined that the expression of the 28S-2 gene, followed by 28S-1, was the most stable in all tissues of female *A. zanthoxylumi*. The actin gene was the most stable expression in each tissue of males, followed by the 28S-2 gene.

The screening of the optimal reference gene will provide basic information for analyzing the gene expression of *A. zanthoxylumi* and also provide a certain basis for the selection of reference genes for other insects. Since the stability of the

Table 7. Comprehensive ranking of reference genes in male tissues of *Agrilus zanthoxylumi.*

Name	Rank by GeNorm	Rank by NormFinder	Rank by BestKeeper	Average Ranking	Overall Ranking
Histone	3	5	1	2.466212	3
β-tubulin	2	3	3	2.620741	5
Actin	2	1	2	1.587401	1
18S	2	2	4	2.519842	4
28S-2	3	3	1	2.080084	2
28S-1	1	3	5	2.466212	3

reference gene was highly correlated with the experimental conditions, species type, and tissue location (Sun et al. 2009), it is necessary to further study the applicability of the RT-qPCR derived from the screening of the reference gene from adult *A. zanthoxylumi* to other insects.

Acknowledgments

This study was supported by the "Green Pollution Control Technology Based on Chemical Pheromone in *Agrilus zanthoxylum*," the National Public Welfare forestry projects in China (Grant 201504324); "Forestry Science and Technology Promotion and Demonstration Project in Yunnan Province: Promotion and Application Demonstration of Green Control Technology for Main Pests and Diseases of *Zanthoxylum bungeanum* Maxim (yun [2019]TG01)"; and "Shaanxi Province's Second Batch of Special Support Program Leading Talent Project." Mention of a commercial or proprietary product does not constitute an endorsement of the product by the Northwest Agriculture and Forestry University.

References Cited

Bustin, S.A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele and C.T. Wittwer. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55(4): 611–622. doi: 10.1373/clinchem. 2008.112797.

Chen, I.H., L.S. Chou, S.J. Chou, J.H. Wang, J. Stott and M. Blanchard. 2015. Selection of suitable reference genes for normalization of quantitative RT-PCR in peripheral blood samples of bottlenose dolphins (*Tursiops truncatus*). Sci. Rep. 5(8): 15425.

Claus, A.L., J.J. Ledet and Ø.T. Falck. 2004. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Genome Biol. 64(15): 5245–5250.

Cui, Y.Y. 1990. Occurrence and control of pepper latent leaves. Hebei Fruit Tree 1990(02): 36–37. doi: 10.19440/j.cnki.1006-9402.1990.02.019.

De, J.H.J.M., R.S.N. Fehrmann, B.E.S.J.M. De, R.M.W. Hofstra, F. Gerbens, W.A. Kamps, V.E.G.E. De, D.Z.A.G.J. Van, M.G.J. Te and E.A. Ter. 2007. Evidence based selection of housekeeping genes. PLoS ONE 2(9): e898. doi: 10.1371/journal.pone.0000898.

Feng, B., Q.S. Guo, B.P. Mao and Y.J. Du. 2016. Identification and screening of fluorescent quantitative PCR internal reference genes in chemical receptive tissues of *Monochamus alternatus*. Acta Insecta Sin. 59(4): 427–437. doi: 10.16380/j.kcxb. 2016.04.008.

Gong, X.F., S.A. Xie and P. Yang. 2019. Preliminary screening of genes differentially expressed in antennae of female and male adults of *Agrilus zanthoxylumi*. Acta Agrobiotechnol. Sin. 27(07): 1233–1245.

Hamadttu, A.F.E.S., R.F. Jose and W.X. Yu. 2020. Chemical pheromone and its potential application in pest control. World Pestic. 42(01):25–31, 42. doi; 10.16201/j.cnki.cn10-1660/tq.2020.01.05.

Heid, C. A., J. Stevens, K.J. Livak and P.M. Williams. 1996. Real time quantitative PCR. Genome Res. 6: 986–994. doi: 10.1101/gr.6.10.986.

Huis, R., S. Hawkins and G. Neutelings. 2010. Selection of reference genes for quantitative gene expression normalization in flax (*Linum usitatissimum* L.). BMC Plant Biol.10: 71. doi: 10.1186/1471-2229-10-71.

Janská, A., J. Hodek, P. Svoboda, C.J. Zámě, I.I. Prá, E. Vlasáková, L. Milella and J. Ovesná. 2013. The choice of reference gene set for assessing gene expression in barley (*Hordeum vulgare* L.) under low temperature and drought stress. Mol. Gen. Genomics 288: 639–649. doi: 10.1007/s00438-013-0774-4.

Kai, H. 1991. Gum leakage and treatment of *Zanthoxylum bungeanum*. Gansu Agric. Sci. Tech. 1991(01): 31.

Li, T.F. 2018. Identification and analysis of neuropeptide gene of *Atrijuglans hetaohei* Yang [D]. Master's thesis. Northwest Agriculture and Forestry University, Xianyang City, Shaanxi Province, China.

Li, X., J.W. Li, B. Cheng, W. Li, W.X. Sun, H.Y. Gao, Q. Ju, X.J. Jiang, L. Du, C.J. Qu and M.J. Qu. 2018. Screening of reference genes for quantitative real-time PCR in *Sympiezomias velatus* (Coleoptera: Curculionidae). Acta Entomol. Sin. 61: 1284–1294. doi: 10.16380/j.kcxb.2018.11.005.

Liu, J.B., J. Ou and C.M. Wei. 2014. Screening of internal reference genes in real-time quantitative PCR of *Tribolium castaneum* (Coleoptera: Tenebrionidae) induced by phosphine. Chin. J. Agric. Biotechnol. 22: 257–264.

Lü, J., C.X. Yang and Y.J. Zhang. 2018. Selection of reference genes for the normalization of RT-qPCR data in gene expression studies in insects: a systematic review. Front. Physiol. 9: 1560. doi: 10.3389/fphys.2018.01560.

Morales, M.A., B.M. Mendoza, L.C. Lavine, M.D. Lavine, D.B. Walsh and F. Zhu. 2016. Selection of reference genes for expression studies of xenobiotic adaptation in *Tetranychus urticae*. Int. J. Biol. Sci. 12: 11291139.

Pfaffl, M.W., A. Tichopad, C. Prgomet and T.P. Neuvans. 2004. Determination of stable housekeeping genes, differentially targeted genes and sample integrity: BeeKeeper-Excel-based tool using pair-wise correlations. Biotechnol. Lett, 26: 509–515.

Radonic, A., S. Thulke, I.M. Mackay, O. Landt, W. Siegert and A. Nitsche. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313: 856–862.

Rodrigues, T.B., R.K. Dhandapani, J.J. Duan and S.R. Palli. 2017. RNA interference in the Asian longhorned beetle: identification of keyRNAi genes and reference genes for RT-qPCR. Sci. Rep. 7(1): 8913. doi: 10.1038/s41598-017-08813-1.

Shakeel, M., X. Zhu and T.H. Kang. 2015. Selection and evaluation of reference genes for quantitative gene expression studies in cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J. Asia-Pac. Entomol. 18: 123–130. doi: 10.1016/j.aspen.2015.01.001.

Shi, C.H., J.R. Hu and C.R. Li. 2017. Advances in the study of internal reference genes in insect qRT-PCR. Jiangsu Agric. Sci. 45(7): 1–7. doi: 10.15889/j.issn.1002-1302.2017.07.001.

Sun, H., Y. Meng, G. Cui, Q. Cao, J. Li and A. Liang. 2009. Selection of housekeeping genes for gene expression studies on the development of fruit bearing shoots in *Chinese jujube* (*Ziziphus jujube* Mill.). Mol. Biol. Rep. 6: 2183–2190. doi: 10.1007/s11033-008-9433-y.

Tan, Y., X.R. Zhou and B.P. Pang. 2017. Reference gene selection and evaluation for expression analysis using qRT-PCR in *Galeruca daurica* (Joannis). Bull. Entomol. Res. 107: 359–368. doi: 10.1017/S0007485316000948.

VanGuilder, H.D., K.E. Vrana and W.M. Freeman. 2008. Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44: 619–626. doi: 10.2144/000112776.

Vandesompele J, K.D. Preter and F. Pattyn. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3: 467–470.

Wang, Y.Q. and X.H. Xue. 2016. Research progress and application of real-time fluorescence quantitative PCR technology. Biol. Bull. 51(02): 1–6.

Wu, H. 2006. Biological characteristics and control of *Agrilus zanthoxylumi*. Insect Knowledge 2: 236–239.

Yang, C.X., H.P. Pan, J.E. Noland, D.Y. Zhang, Z.H. Zhang, Y. Liu and X.G. Zhou. 2015. Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegilla maculate* (Coleoptera: Coccinellidae). Sci. Rep. 5: 18201. doi: 10.1038/srep18201.

Yang, S.Y., X.L. Yue, J.J. Wang and H.M. Shen. 2013. Screening of suitable reference genes and expression profiling of CYP392E subfamily genes in different resistant strains of *Tetranychus urticae* (Acari: Tetranychidae). Acta Entomol. Sin. 56: 1152–1159. doi: 10.16380/j.kcxb.2013.10.007.