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Identification and Validation of Reference Genes for RT-qPCR Normalization in *Nauphoeta cinerea* (Olivier, 1789) (Blattodea, *Blaberidae*)

Kübra Özcan [1*], Ayşe Nur Pektaş[2], Şeyda Berk [1*]

Abstract: Quantitative RT-PCR (q-RT-PCR) is a powerful tool that allows large-scale analysis of very small changes in gene expression. For the calculation of gene expression, such as the delta-delta Ct method, different PCR primer efficiencies (E) may affect the result, as PCR primer yields are assumed to be comparable for the gene of interest and housekeeping gene. Therefore, identification of a suitable reference gene for data normalization is an important step in the development of qPCR assays. Furthermore, accurate and reliable results depend on the use of stable reference genes for normalization. The aim of the current study is the identification and validation of a set of six housekeeping genes (*GADPH, RPS18, α-TUB, EF1α, ArgK,* and *ACTB*) in cockroach species *Nauphoeta cinerea* adults using five different algorithms (ΔCt method, Bestkeeper, geNorm, Normfinder and RefFinder) to evaluate the stability of selected reference genes expression. Our results show that *α-Tub* use provides accurate normalization of gene expression levels in *N. cinerea* adults. In addition, since the *GADPH* is selected as the second most stable reference gene, *GADPH* can be also used for transcript analysis *N. cinerea* adults. Our study also showed that *ACTB (β-actin)* should not be used for normalizing transcript levels when examining *N. cinerea* adults. Additionally, validation studies for reference genes in cockroaches are very few (only one) in the literature. Therefore, the results highlight the need for validation of reference genes under biotic and abiotic conditions in q-RT-PCR studies in cockroaches.

Keywords: RT-qPCR, reference genes, validation, normalization, *Nauphoeta cinerea*.

[1]**Address:** Department of Molecular Biology and Genetics, Faculty of Science, Sivas Cumhuriyet University, 58140, Sivas, Turkey.
[2]**Address:** Advanced Technology Research Center (CUTAM), Sivas Cumhuriyet University, 58140, Sivas, Turkey.

***Corresponding author**: sberk@cumhuriyet.edu.tr

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1. INTRODUCTION

Cockroaches are a highly diverse insect with ~4000 described species (Velez et al., 2006), most of which live in terrestrial habitats and have good adaptations to survive in extreme conditions (Bohn et al., 2010). A Blaberidae, ovoviviparous cockroach, the *Nauphoeta cinerea* (Olivier) is known as the gray cockroach and is the only representative of its genus. In addition, *N. cinerea* is a model species for sexual selection (Bouchebti et al., 2016) and correlations between metabolic rate and fitness studies (Schimpf et al., 2012). Because cockroaches can live in habitats with varying amounts of toxic substances such as insecticides, environmental pollutants, microbial toxins and other xenobiotics, they can be a good experimental model for studying the stress response and detoxification abilities (Bell et al., 2007; Zhang et al., 2016). Among cockroaches, the lobster cockroach *N. cinerea* has been used as an experimental model for toxicology (Adedara et al., 2016; Adedara et al., 2015; Rodrigues et al., 2013) and has been proposed to be a valid alternative model for basic toxicological studies. *N. cinerea* is more advantageous to other cockroach species in scientific studies because it is easy to care for and does not fly.

Gene expression studies are essential for molecular biology research. Knowledge about gene expression helps us better understand its regulation and functions. Massive sequence data in the form of transcriptomes and genomes of several organisms can be used to understand the transcription of genes (Heid et al., 1996). However, these analyses became more accurate and robust after the development of reverse transcriptase quantitative PCR (RT-qPCR), a gene expression quantification method. This method is highly sensitive, reproducible, and accurate enough to detect even minute changes that are not often detected. RT-qPCR data is influenced by many factors, including the quality and quantity of starting material, RNA extraction, cDNA synthesis, and other laboratory procedures. Even pipetting errors and reverse transcription efficiency can significantly affect Ct values (Bustin et al., 2005; Yeung et al., 2004).

The use of reference genes with stable expression as internal control has become one of the most common methods of data normalization (Feuer et al., 2015; Huggett et al., 2005; Pabinger et al., 2014). Normalization becomes necessary when one wants to express results as relative quantities by application of the well-known $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) or modified versions this method (Hellemans et al., 2007; Pfaffl, 2001; Schmittgen and Livak, 2008; Vandesompele et al., 2002). In order to analyze the results accurately, it is necessary to calculate the PCR primer yields. For the calculation of gene expression, such as the delta-delta Ct method, different PCR primer efficiencies (E) may affect the result, as PCR primer yields are assumed to be comparable for the gene of interest and housekeeping gene. Therefore, identification of a suitable reference gene for data normalization is an important step in the development of qPCR assays. It is very important that the reference gene is not affected by experimental conditions like changing in developmental stages, tissues. Since correct normalization is considered an important part of gene expression analysis, it has been recommended to verify the expression stability of the reference gene prior to each RT-qPCR experiment. (Huggett et al., 2005; Vandesompele et al., 2002). Therefore, normalization has been a precondition in gene expression studies as it limits the variability by comparing target gene expression with housekeeping genes (HKGs). Normalization recognizes that expression of HKGs is stable across various biotic and abiotic stresses and treatments. Recent studies highlight the need to identify a condition-specific reference gene for accurate measurements of gene expression (Ponton et al., 2011b). Furthermore, it is also not recommended to use a single reference gene in gene expression studies (Chandna et al., 2012; Koramutla et al., 2016), as the use of a single reference gene may produce up to 20-fold errors in expression data (Vandesompele et al., 2002). In most expression studies, actin is chosen as a universal HKG (Li et al., 2010), or HKGs approved for particular systems are administered directly without proper verification of their stability in that particular system. Stability of gene expression is defined as the smallest variation in successive levels of stability of the samples analyzed. Therefore, different software programs have been developed to identify many of these stable genes (Galiveti et al., 2010). These programs include GeNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), ΔCt Method (Silver et al., 2006), and RefFinder (http://www.leonxie.com/referencegene.php).

Molecular tools, particularly RT-qPCR, have been frequently used in insect systems to quantify differences in gene expression. RT-qPCR has speed up research progress in biomedicine and has gained equal importance in entomology Gene expression analysis is increasingly important in the field of insect molecular biology as it can be used to examine changes in gene expression between insect developmental stages, tissues, and other samples from various assays. Studies to evaluate the selection and validity of reference genes in various biotic and abiotic conditions in insects are summarized in reviews by Lü et al. and Shakkel et al. (Lü et al., 2018; Shakeel et al., 2018). In studies in the literature, different types of housekeeping genes, including traditional and novel genes, were selected for gene expression stability analysis in different insect species. Although RT-qPCR is widely used for the detection of gene expression in insects, there is not yet a suitable HKG and stable gene quantification system for *N. cinerea*. Our current study aims to identify suitable reference genes and evaluate their expression stability in *N. cinerea* before they are used as internal controls in functional genomic studies of six reference genes commonly used to normalize qRT-PCR data in *N. cinerea*.

2. MATERIAL AND METHODS

2.1. Sample and ethics statement

The *Nauphoeta cinerea* (Blattodea, Blaberidae) species included in the study were purchased commercially in Antalya/Turkey (Antalya Çekirge, https://www.antalyacekirge.net/). Adults of *N. cinerea* (n=10) were transported to the laboratory, preserved in the RNA stabilization reagent RNAlater® (Qiagen, Cat. No: 76106) and stored at -20°C for use in further studies.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated using the commercially purchased GeneAll® Hybrid-R™ kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's recommendations. cDNA synthesis was performed using The GeneAll® HyperScript™ first chain synthesis kit (GeneAll Biotechnology, Seoul, Korea) to obtain the cDNA product containing 1500 ng/µL of RNA in accordance with the manufacturer's protocol.

2.3. Candidate reference genes selection

In the current study, six candidate housekeeping genes (*GADPH, RPS18, α-TUB, EF1α, ArgK* and *ACTB*) were selected among the most preferred reference genes in insects by taking the studies in the literature as reference. The specification information of each primer included in the study is given in Table 1. PCR amplification efficiencies (E) and correlation coefficients (R^2) were determined to validate the primers. Standard curves were generated using serial dilutions of cDNA (1, 1/5, 1/25, 1/125 and 1/625) for each primer pair.

2.4. Quantitative real-time PCR (RT-qPCR)

qPCR experiments were performed according to the previously reported protocol (Berk and Pektas, 2020). Briefly, a 5-fold dilution series of 1:625 from an undiluted cDNA of *N. cinerea* adult cDNA samples was analysed to determine the amplification specificity and efficiencies of each primer pair used in the qPCR analysis. For expression analysis of six candidate reference genes, all experimental samples for *N. cinerea* were analysed simultaneously in the same reverse transcription process.

RT-qPCR analyses in a 96-well plate (ABI-Type) using commercial qPCR Master Mix (iGreen, 2X, Biomatik, Canada) were performed using the StepOnePlusTM Real-Time PCR system (Applied Biosystems, USA). With a final volume of 20 µL, the reaction conditions are as follows: [10 µL Master Mix (2X), 0.7 µL forward and reverse primers (10 µM), 2 µL cDNA, and 6.6 µL nuclease-free water].

The RT-qPCR schedule is as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 15 seconds. For melting curve analysis, it is as follows: a cycle of decomposition steps (58°C -1 minute- followed by 0.5°C up to 95°C for 10 seconds). A melting curve was composed at per PCR reaction final to confirm a single peak and remove the primer-dimer possibility and formation of non-specific product. Efficiency of PCR amplification (E) was determined in terms of the equalization: $E = (10^{[-1/slope]}-1) \times 100$.

2.5. Data analysis

The Cq values of six housekeeping genes were exanimated using the statistical analysis statistical software GraphPad Prisim 6.0 (GraphPad software, San Diego, C), and a boxplot of these Cq values was generated. The stability of these six housekeeping genes was determined by GeNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), ΔCt (Silver et al., 2006) and the comprehensive web-based analysis tool integrating all four software algorithms (https://www.heartcure.com.au/reffinder/?type=reference). BestKeeper evaluates the coefficient of variance (CV) and standard deviation (SD) of the Cq values of each housekeeping gene, and the gene with the lowest CV and SD is defined as the most constant gene (Pfaffl et al., 2004). GeNorm reveals the mean expression stability value (M2) of each candidate (M value) to demonstrate expression stability. The gene with the lowest M2 value is determined as the most stable gene (Vandesompele et al., 2002). In NormFinder, expression stability (M1) is revealed by the Cq values obtained by RT-qPCR analysis of candidate genes and are ranked. The gene with the lowest M1 value is the most stable (Andersen et al., 2004). RefFinder appoints a relevant weight to each gene and evaluates the geometric mean of these weights to make an overall final ranking (https://www.heartcure.com.au/reffinder/?type=reference).

Table 1. Overview of the six candidate housekeeping genes evaluated in RT-qPCR analysis.

Symbol	Gene name	Description	Primer sequence (5'→3')	GenBank accession number	Length (bp)	R^2	Reference
ACTB	β-actin	Cytoskeleton	F: TCCATCATGAAGTGCGATGT R: CCACATCTGTTGGAATGTCG	NM_001172372	228	0.872	(Sang et al., 2015)
RPS18	Ribosomal protein S18	Ribonucleoprotein; Ribosomal protein; RNA-binding; rRNA-binding	F: TACACCTTTGATCGCTGTGAG R: GGCTCTGGTCATTCCAGATAAG	XM_045615265	108	0,914	(Yang et al., 2015c)
EF1α	Elongation factor 1α	Translation elongation factor activity; GTPase	F: ACCAGATTTGATGGCTTTGG R: CACCCAGAGGAGCTTCAGAC	XM_003705302	194	0,957	(Rodrigues et al., 2014)
ArgK	Arginine Kinase	Phosphotransferase activity	F: CTCGTGTGGTGCAACGAAGA R: GGTGGCTGAACGGGACTCT	NT_037436	130	0,972	(García-Reina et al., 2018)
GADPH	Glyceraldehyde-3-phosphate	Oxidoreductase in glycolysis & Gluconeogenesis	F: GCCAAGGTGATCCATGACAA R: GTCTTCTGAGTGGCAGTTGTAG	NC_007420	80	0,965	(Pan et al., 2015)
α- TUB	Alpha-tubulin	Microtubule	F: TCAAATGCGACCCACGTCAT R: GGCAATAGCCGCGTTGACAT	XM_970811	191	0,978	(Altincicek et al., 2008)

3. RESULTS

3.1. The quality of total RNA

In the current study, the A260/A280 ratio of total RNA from *N. cinerea* adults ranged from 1.90 to 2.12, while the A260/A230 ratio was found to be over 1.90. It shows that the product does not contain organic salts and protein contamination. The total RNA concentration was between 1218ng/uL and 2000ng/uL, and this value range was considered suitable for expression of the cDNA template.

3.2. Primer specificity and efficiency

All PCR amplification of each primer used in the present study was confirmed by the presence of a single peak in melting curve analyses and specific band of expected size based on 1.5% agarose gel electrophoresis (data not shown). Initial screening of six potential reference genes showed that all genes were expressed in *N. cinerea* adults. The primer efficiency (E) for each of the six candidate genes was calculated using the slope obtained when measuring fluorescence at 5-fold cDNA dilutions of a calibrator cDNA sample using the formula: $E=10^{1/-slope-1}$. All primers in the study were found to have a correlation coefficient (R^2) varying between 0.87 and 0.98. In addition, a primer efficiency value of between 98.6% and 110.05% was obtained, and the specificity of each primer was verified by the BLAST program (Table 1). No fluorescent signal amplification was detected in the negative control samples (−RT); this demonstrated that RNA extraction methods and DNase treatment procedures effectively removed genomic DNA from RNA samples.

3.3. Expression profiling of candidate reference genes

The expression profiles of all RT-qPCR products for all primers and both experiments are shown in Figure 1. Cycle threshold (Cq) values obtained amplifying the six putative housekeeping genes from *N cinerea* adult were plotted (Figure 1). Cq values for the six genes ranged from 21.29 to 36,67 in *N. cinerea*. *GADPH* showed the lowest Cq values in *N. cinerea* (24,72 ± 2,238, mean Cq ± std. dev.). Amplification of *α-TUB, RPS18, ArgK, EF1α* and *ACTB* showed mean Cqs of 28,96 ± 2,056, 30,36 ± 1,418, 31,78 ± 3,633, 31,96 ± 1,418 and 33,36 ± 4,081, respectively.

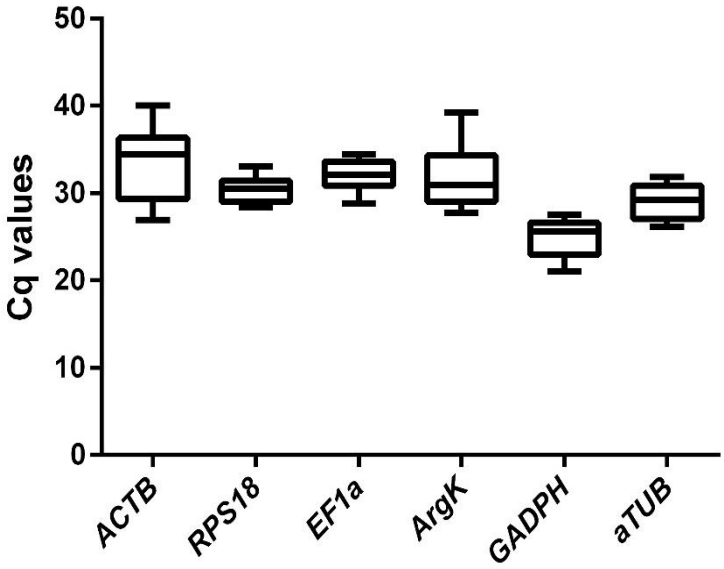

Figure 1. Amplification profiles of candidate housekeeping genes. Box plot of qPCR cycle threshold values (Cq) for housekeeping genes in *Nauphoeta cinerea*.

3.4. Expression stability of selected reference genes

Four statistical Excel macro programs (geNorm, NormFinder, BestKeeper, and Δ-Ct method) were used to evaluate the stability of six candidate genes, in order to find optimal reference genes in *N. cinerea* for RT-qPCR normalization. Additionally, six reference genes were compared and ranked by web-based analysis tool RefFinder (Table 2 and Figure 2).

Table 2. Expression stability of the putative housekeeping genes in *Nauphoeta cinerea* adults.

Reference gene	Genorm		Bestkeeper			Delta Ct		Normfinder		RefFinder	
	M	**Rank**	**SD**	**R**	**Rank**	**SD**	**Rank**	**SV**	**Rank**	**Stability**	**Rank**
ACTB	1.957	6	3.40	0.973*	6	2.59	6	2.262	6	6.00	6
RPS18	0.992	4	1.12	0.957*	1	1.73	4	1.046	2	2.38	2
EF1α	0.841	3	1.50	0.902*	2	1.72	3	1.123	3	2.71	4
ArgK	1.639	5	2.92	0.903*	5	2.52	5	2.146	5	5.00	5
GADPH	0.699	1	1.76	0.902*	4	1.70	2	1.133	4	2.38	1
α-TUB	0.699	1	1.68	0.977*	3	1.48	1	0.401	1	1.32	3

SD, standard deviation; **SV**, stability value; **r**, Pearson correlation coefficient; *****p≤0.001.

The geNorm algorithm assumes that the candidate genes are not co-regulated. For each reference gene tested, the gene expression stability value M is normally calculated as the mean pairwise variation for that gene with all other reference genes. Genes characterized by low M have stable expression, while the gene with the highest M value is eliminated, and this process continues until the two most stable genes are identified. This last gene pair is recommended as the optimal reference gene pair. It is recommended to use at least two reference genes to ensure correct normalization in geNorm. In *N. cinerea* adults, geNorm ranked the set of candidate reference genes: *α-TUB, GADPH >EF1a >RPS18 >ArgK > ACTB*. *α-TUB* and *GADPH* were found to be the most stable genes with an expression stability value M of 0.699. The gene with the least stable expression was *ACTB* with an M-value of 1.957 (Table 2 and Figure 2A).

Normfinder analysis is based on an ANOVA (analysis of variance) model that identifies genes with the least variation in expression over the entire sample set. Analyzes taking into account systematic differences between sample subgroups. In *N. cinerea* adults,

Normfinder ranked the six reference genes as follows: *α-TUB* >*RPS18*> *EF1α*> *GADPH*> *ACTB*> *ArgK* > *ACTB* with a stability value SV of 0.401, 1.046, 1.123, 1.133, 2.146, 2.262, respectively. *α-TUB* was found to be the most stable with a stability value of 0.401 and *ACTB* the least stable with a stability value of 2.262 (Table 2 and Figure 2B).

The stability of the candidate genes was analyzed with BestKeeper, which can calculate the standard deviation (SD) based on the Cq values of all candidate reference genes. Reference genes exhibiting the lowest standard deviation (SD) were taken as the most stable genes. Also, values exceeding the cut-off value (SD < 1) are considered unstable across all samples. According to this analysis, all housekeeping genes [*RPS18* (SD: 1.12), *EF1α* (SD:1.50), *α-TUB* (SD: 1.68), *GADPH*, (SD: 1.76), *ArgK* (SD: 2.92), *ACTB* (SD: 3.40)] exceeded the threshold in *N. cinerea* adults (Table 2 and Figure 2C).

Based on relative pairwise comparisons, the ΔCt method calculates the mean SD of each gene set using raw Ct values, and this SD value is inversely proportional to the expression stability of the gene. Standard deviation below 1 indicates appropriate stability. As the mean SD of each gene set was over 1, none of the available reference genes were found to be stable enough for *N. cinerea* adults according to the ΔCT method. The overall ranking based on the ΔC t method of reference genes was: *α-TUB, GADPH, EF1a, RPS18, ArgK, ACTB* (Table 2 and Figure 2D).

RefFinder is a comprehensive algorithm that combines all the above-mentioned software tools (geNorm, NormFinder, BestKeeper and Δ-Ct method) to rank candidate reference genes according to their stability. The overall ranking based on RefFinder of reference genes in *N. cinerea* was: *GADPH, RPS18, α-TUB, EF1α, ArgK*, and *ACTB* (Table 2 and Figure 2E).

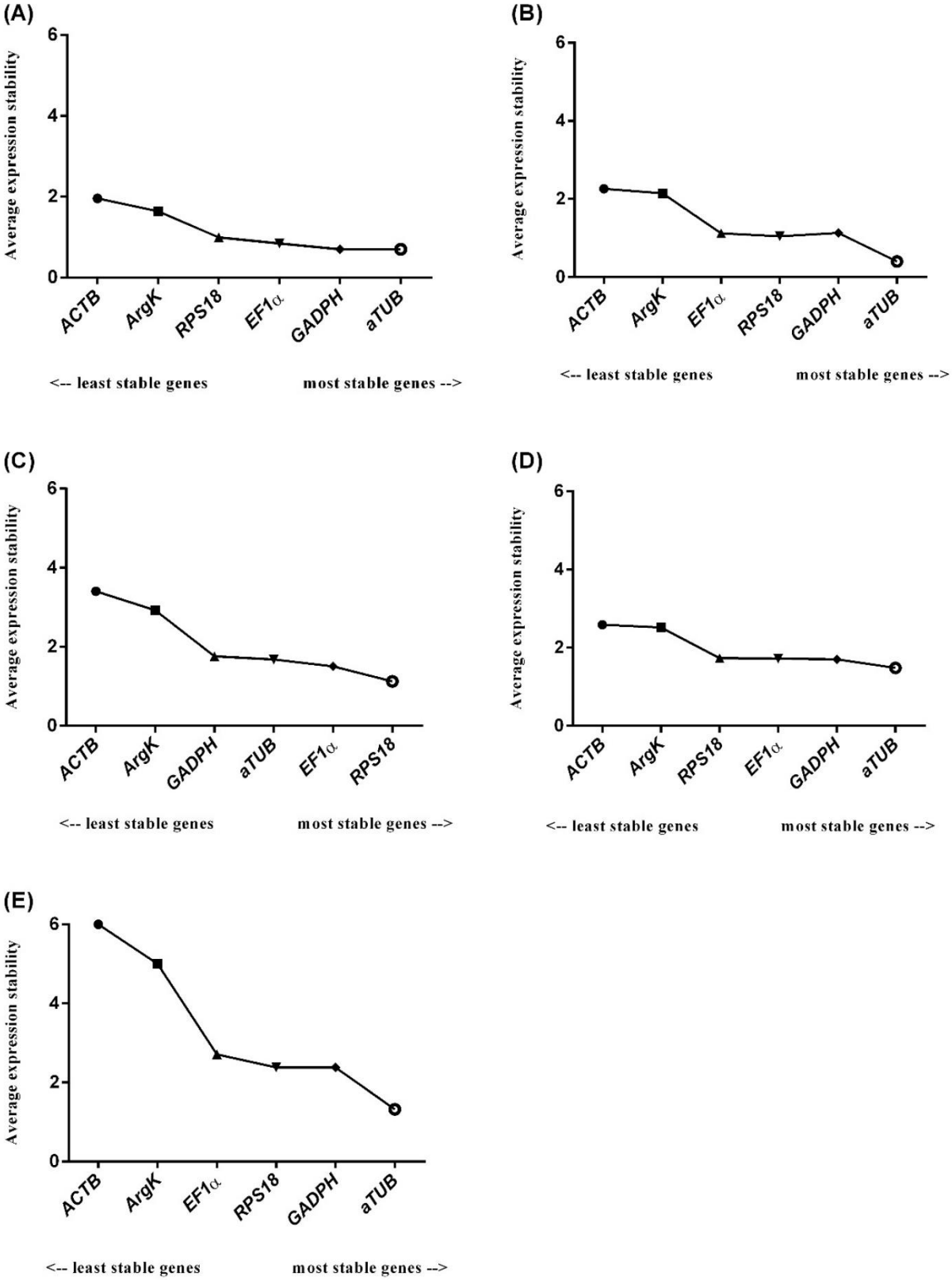

Figure 2. Expression stability and ranking of the six housekeeping genes evaluated by Genorm (A), Normfinder (B), Bestkeeper (C), ΔCt metod (D), RefFinder (E).

4. DISCUSSION AND CONCLUSIONS

Housekeeping genes structurally expressed to maintain essential cellular functions are the traditional choice for a standard reference (Vandesompele et al., 2002). However, there are no "universal" reference genes that are stably expressed and applicable for all cell and tissue types under various experimental conditions (Fu et al., 2013b; Li et al., 2013; Liang et al., 2014; Pan et al., 2015; Yang et al., 2014; Yang et al., 2015a; Zhu et al., 2014). Especially in studies on

the selection of reference genes in various biotic and abiotic conditions in insects, different stability of different reference genes may vary under each condition. In insect studies, actin has been identified as the most frequently used reference genes, including *ACTB, RPL, Tubulin, GAPDH, RPS, 18S, EF1A, TATA, HSP*, and *SDHA* (Lü et al., 2018). *ACTB*, which encodes a large structural protein, is expressed at various levels in many cell types and is considered the ideal reference gene for RT-qPCR analysis and is the most frequently investigated reference gene. Many studies evaluating the stability of reference genes in insects have shown that *ACTB* expression is the most stable among other reference genes at different developmental stages of many insects, including *Apis mellifera, Chortoicetes terminifera, Schistocerca gregaria, Plutella xylostella, Drosophila melanogaster, Liriomyza trifolii, Chilo suppressalis,* and *Diuraphis noxia* (Chang et al., 2017; Chapuis et al., 2011; Ponton et al., 2011a; Scharlaken et al., 2008; Sinha and Smith, 2014; Teng et al., 2012; Van Hiel et al., 2009). On the other hand, *ACTB* expression in some insect species was reported to be less stable (Pan et al., 2015; Yang et al., 2015d; Yang et al., 2016). Ribosomal protein (RP), an essential component of ribosomes, is among the highest conserved proteins in all life forms, and RPL and RPS family genes have been the most preferred reference genes for expression studies, especially in insects (Lü et al., 2018). *RPS24* and *RPS18* were identified as the two most stable reference genes across different developmental stages and sex treatments of *C. maculate* (Yang et al., 2016); *RPS13* and *RPS23* have been reported as stable reference genes at different developmental stages of *P. xylostella* (Fu et al., 2013a); the three most stable reference genes *RPL11, RPS8* and *RPL14* at different developmental stages and different temperature conditions of *Aphis craccivora* were identified (Yang et al., 2015b). Tubulin (α-tubulin, β-tubulin and γ-tubulin), which encodes cytoskeletal structure proteins, another most studied reference gene, has been found to have variable stability under different treatments for the same species in many studies. For example, α-tubulin exhibited stable expression in different tissues and sexes of *C. maculata*, while its expression was found to be unstable throughout different developmental stages and following dsRNA treatments (Yang et al., 2015d). Similarly, the stability of *GAPDH* expression has also been found to be variable under different treatments within the same species. For example, *GAPDH* expression was not affected by tissue type, sex, photoperiod, or dsRNA treatment in *H. convergens*, but was found to vary at different developmental stages and at different temperatures (Pan et al., 2015). Furthermore, the *EF1A* gene was selected as the least stable reference gene in *A. craccivora* during different developmental stages and at different temperatures (Yang et al., 2015b). On the other hand, EF1A expression level in *H. convergens* was not affected by three biological factors (developmental stage, tissue type, and sex) and three abiotic conditions (temperature, photoperiod, and dietary RNAi), and EF1A was chosen as the most stable gene *H. convergens* (Pan et al., 2015). In summary, the expression of some commonly used housekeeping genes may vary under different experimental setups. Genes that play important roles during insect metamorphosis and affect different tissues serve as target genes for manipulations that kill the insect or retard its growth. Therefore, gene expression profiles should be widely evaluated in different developmental stages and in different tissues, and gene stability should be determined (An et al., 2016; Chang et al., 2017; García-Reina et al., 2018; Pan et al., 2015; Ponton et al., 2011a; Rodrigues et al., 2014; Sang et al., 2015; Yang et al., 2015c).

To our knowledge, only one validation study has been reported for reference genes in cockroaches (Marchal et al., 2013). They aimed to identify and validate a set of eight housekeeping genes (*β-actin, EF1a, GAPDH, Arm, RpL32, SDHa, AnnIX* and *α-Tub*) during the first gonadotropic cycle of the cockroach *Diploptera punctata*. They used two different algorithms (geNorm and Normfinder) to evaluate the stability of its expression. *α-Tub* concluded that the combined use of *EF1a* and *RpL32* resulted in an accurate normalization of gene expression levels in the *D. punctata* ovary. They found that *Actin* and *AnnIX* should not be used to normalize transcript levels when examining the first gonadotropic cycle in the corpora allata or the ovary of *D. punctata.*

In the current study, a validation study was performed for reference genes (*GADPH, RPS18, α-TUB, EF1α, ArgK*, and *ACTB)* in cockroach species *N. cinerea* adults using five different algorithms (ΔCt method, Bestkeeper, geNorm, Normfinder and RefFinder) to evaluate the stability of selected reference genes expression. The six possible reference genes included in our current study were selected based on their traditional use and stability as described in validation studies reported in other insects. Our results show that *α-Tub* use provides accurate normalization of gene expression levels in *N. cinerea* adults. In addition, since the *GADPH* gene is selected as the second most stable reference gene, we can suggest that it can be used for transcript analysis. As Marchal et al. (2013) suggested, our study also showed that actin should not be used for normalizing transcript levels when examining *N. cinerea* adults. Additionally, validation studies for reference genes in cockroaches are very few (only one) in the literature. In the continuation of this study, as in other insect species, it is planned to investigate especially cockroaches under the conditions of many experimental factors such as the stability of reference genes, developmental stage, tissue, temperature, pesticide, diet, population, virus, sex, photoperiod and starvation. The result of the present study and existing studies in the literature highlights the need for validation of reference genes under biotic and abiotic conditions in q-RT-PCR studies in cockroaches.

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N/A

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Author Contributions
Conceptualization: Ş.B, A.N.P, K.Ö Investigation: Ş.B, A.N.P, K.Ö.; Material and Methodology: Ş.B, A.N.P, K.Ö,

Supervision: Ş.B; Visualization: Ş.B, A.N.P, K.Ö; Writing-Original Draft: Ş.B; Writing-review & Editing: Ş.B; A.N.P; K.Ö.; Other: All authors have read and agreed to the published version of manuscript.

Conflict of Interest
The authors have no conflicts of interest to declare.

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REFERENCES

Adedara, I. A., Rosemberg, D. B., Souza, D. O., Farombi, E. O., Aschner, M., Rocha, J. B. (2016). Neuroprotection of luteolin against methylmercury-induced toxicity in lobster cockroach *Nauphoeta cinerea*. Environ Toxicol Pharmacol 42, 243-251.

Adedara, I. A., Rosemberg, D. B., Souza, D. O., Kamdem, J. P., Farombi, E. O., Aschner, M., Rocha, J. B. (2015). Biochemical and behavioral deficits in the lobster cockroach *Nauphoeta cinerea* model of methylmercury exposure. Toxicol Res 4, 442-451.

Altincicek, B., Knorr, E., Vilcinskas, A. (2008). Beetle immunity: Identification of immune-inducible genes from the model insect *Tribolium castaneum*. Developmental & Comparative Immunology 32, 585-595.

An, X.-k., Hou, M.-l., and Liu, Y.-d. (2016). Reference gene selection and evaluation for gene expression studies using qRT-PCR in the white-backed planthopper, *Sogatella furcifera* (Hemiptera: Delphacidae). J Econ Entomol 109, 879-886.

Andersen, C. L., Jensen, J. L., Ørntoft, T. F. (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. Cancer research 64, 5245-5250.

Bell, W. J., Roth, L. M., Nalepa, C. A. (2007). Cockroaches: ecology, behavior, and natural history: JHU Press).

Berk, S., Pektas, A. (2020). Selection and Validation of Reference Genes for Quantitative Real-time PCR in the Mealworm Beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS) 13, 44-50.

Bohn, H., Picker, M., Klass, K.-D., Colville, J. (2010). A jumping cockroach from South Africa, *Saltoblattella montistabularis*, gen. nov., spec. nov.(Blattodea: Blattellidae). Arthropod Systematics and Phylogeny 68, 53-69.

Bouchebti, S., Durier, V., Pasquaretta, C., Rivault, C., Lihoreau, M. (2016). Subsocial Cockroaches Nauphoeta cinerea Mate Indiscriminately with Kin Despite High Costs of Inbreeding. PLoS One 11, e0162548.

Bustin, S. A., Benes, V., Nolan, T., Pfaffl, M. W. (2005). Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol 34, 597-601.

Chandna, R., Augustine, R., Bisht, N. C. (2012). Evaluation of candidate reference genes for gene expression normalization in *Brassica juncea* using real time quantitative RT-PCR. PLoS One 7, e36918.

Chang, Y.-W., Chen, J.-Y., Lu, M.-X., Gao, Y., Tian, Z.-H., Gong, W.-R., Zhu, W., Du, Y.-Z. (2017). Selection and validation of reference genes for quantitative real-time PCR analysis under different experimental conditions in the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae). PloS one 12, e0181862.

Chapuis, M.-P., Tohidi-Esfahani, D., Dodgson, T., Blondin, L., Ponton, F., Cullen, D., Simpson, S. J., Sword, G. A. (2011). Assessment and validation of a suite of reverse transcription-quantitative PCR reference genes for analyses of density-dependent behavioural plasticity in the Australian plague locust. BMC molecular biology 12, 1-11.

Feuer, R., Vlaic, S., Arlt, J., Sawodny, O., Dahmen, U., Zanger, U. M., Thomas, M. (2015). LEMming: A Linear Error Model to Normalize Parallel Quantitative Real-Time PCR (qPCR) Data as an Alternative to Reference Gene Based Methods. PLoS One 10, e0135852.

Fu, W., Xie, W., Zhang, Z., Wang, S., Wu, Q., Liu, Y., Zhou, X., Zhou, X., Zhang, Y. (2013a). Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). International Journal of Biological Sciences 9, 792.

Fu, W., Xie, W., Zhang, Z., Wang, S., Wu, Q., Liu, Y., Zhou, X., Zhou, X., Zhang, Y. (2013b). Exploring valid reference genes for quantitative real-time PCR analysis in *Plutella xylostella* (Lepidoptera: Plutellidae). Int J Biol Sci 9, 792-802.

Galiveti, C. R., Rozhdestvensky, T. S., Brosius, J., Lehrach, H., Konthur, Z. (2010). Application of housekeeping npcRNAs for quantitative expression analysis of human transcriptome by real-time PCR. RNA (New York, NY) 16, 450-461.

García-Reina, A., Rodríguez-García, M. J., Galián, J. (2018). Validation of reference genes for quantitative real-time PCR in tiger beetles across sexes, body parts, sexual maturity and immune challenge. Scientific reports 8, 10743.

Heid, C. A., Stevens, J., Livak, K. J., Williams, P. M. (1996). Real time quantitative PCR. Genome research 6, 986-994.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 8, 1-14.

Huggett, J., Dheda, K., Bustin, S., Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. Genes and immunity 6, 279-284.

Koramutla, M. K., Aminedi, R., Bhattacharya, R. (2016). Comprehensive evaluation of candidate reference genes for qRT-PCR studies of gene expression in mustard aphid, *Lipaphis erysimi* (Kalt). Scientific reports 6, 25883.

Li, R., Xie, W., Wang, S., Wu, Q., Yang, N., Yang, X., Pan, H., Zhou, X., Bai, L., Xu, B., Zhou, X., Zhang, Y. (2013). Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). PLoS One 8, e53006.

Li, Z., Yang, L., Wang, J., Shi, W., Pawar, R. A., Liu, Y., Xu, C., Cong, W., Hu, Q., Lu, T., Xia, F., Guo, W., Zhao, M., & Zhang, Y. (2010). beta-Actin is a useful internal control for tissue-specific gene expression studies using quantitative real-time PCR in the half-smooth tongue sole *Cynoglossus semilaevis* challenged with LPS or *Vibrio anguillarum*. Fish & shellfish immunology 29, 89-93.

Liang, P., Guo, Y., Zhou, X., Gao, X. (2014). Expression profiling in *Bemisia tabaci* under insecticide treatment: indicating the necessity for custom reference gene selection. PLoS One 9, e87514.

Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) 25, 402-408.

Lü, J., Yang, C., Zhang, Y., Pan, H. (2018). Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review. Frontiers in physiology 9, 1560.

Marchal, E., Hult, E. F., Huang, J., Tobe, S. S. (2013). Sequencing and validation of housekeeping genes for quantitative real-time PCR during the gonadotrophic cycle of *Diploptera punctata*. BMC Research Notes 6, 237.

Pabinger, S., Rödiger, S., Kriegner, A., Vierlinger, K., Weinhäusel, A. (2014). A survey of tools for the analysis of quantitative PCR (qPCR) data. Biomolecular Detection and Quantification 1, 23-33.

Pan, H., Yang, X., Siegfried, B. D., Zhou, X. (2015). A comprehensive selection of reference genes for RT-qPCR analysis in a predatory lady beetle, *Hippodamia convergens* (Coleoptera: Coccinellidae). PloS one 10, e0125868.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic acids research 29, e45.

Pfaffl, M. W., Tichopad, A., Prgomet, C., Neuvians, T. P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnology letters 26, 509-515.

Ponton, F., Chapuis, M.-P., Pernice, M., Sword, G. A., Simpson, S. J. (2011a). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. Journal of insect physiology 57, 840-850.

Ponton, F., Chapuis, M. P., Pernice, M., Sword, G. A., Simpson, S. J. (2011b). Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. J Insect Physiol 57, 840-850.

Rodrigues, N. R., Nunes, M. E., Silva, D. G., Zemolin, A. P., Meinerz, D. F., Cruz, L. C., Pereira, A. B., Rocha, J. B., Posser, T., Franco, J. L. (2013). Is the lobster cockroach Nauphoeta cinerea a valuable model for evaluating mercury induced oxidative stress? Chemosphere 92, 1177-1182.

Rodrigues, T. B., Khajuria, C., Wang, H., Matz, N., Cunha Cardoso, D., Valicente, F. H., Zhou, X., Siegfried, B. (2014). Validation of reference housekeeping genes for gene expression studies in western corn rootworm (Diabrotica virgifera virgifera). PLoS One 9, e109825.

Sang, W., He, L., Wang, X. P., Zhu-Salzman, K., Lei, C. L. (2015). Evaluation of Reference Genes for RT-qPCR in Tribolium castaneum (Coleoptera: Tenebrionidae) Under UVB Stress. Environmental entomology 44, 418-425.

Scharlaken, B., de Graaf, D. C., Goossens, K., Brunain, M., Peelman, L. J., Jacobs, F. J. (2008). Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, Apis mellifera, after a bacterial challenge. J Insect Sci 8, 33.

Schimpf, N. G., Matthews, P. G., White, C. R. (2012). Standard metabolic rate is associated with gestation duration, but not clutch size, in speckled cockroaches *Nauphoeta cinerea*. Biology open 1, 1185-1191.

Schmittgen, T. D., Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. Nature protocols 3, 1101-1108.

Shakeel, M., Rodriguez, A., Tahir, U. B., Jin, F. (2018). Gene expression studies of reference genes for quantitative real-time PCR: an overview in insects. Biotechnology letters 40, 227-236.

Silver, N., Best, S., Jiang, J., Thein, S. L. J. B. m. b. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. 7, 1-9.

Sinha, D. K., Smith, C. M. (2014). Selection of reference genes for expression analysis in *Diuraphis noxia* (Hemiptera: Aphididae) fed on resistant and susceptible wheat plants. Scientific reports 4, 1-6.

Teng, X., Zhang, Z., He, G., Yang, L., Li, F. (2012). Validation of reference genes for quantitative expression analysis by real-time RT-PCR in four lepidopteran insects. J Insect Sci 12.

Van Hiel, M. B., Van Wielendaele, P., Temmerman, L., Van Soest, S., Vuerinckx, K., Huybrechts, R., Broeck, J.

V., Simonet, G. (2009). Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions. BMC molecular biology 10, 1-10.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology 3, Research0034.

Velez, A., Wolff, M., Gutierrez, E. (2006). Blattaria of Colombia: List and distribution of genera. Zootaxa 1210, 39-52.

Yang, C., Pan, H., Liu, Y., Zhou, X. (2014). Selection of reference genes for expression analysis using quantitative real-time PCR in the pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera, Aphidiae). PLoS One 9, e110454.

Yang, C., Pan, H., Liu, Y., Zhou, X. (2015a). Stably expressed housekeeping genes across developmental stages in the two-spotted spider mite, Tetranychus urticae. PLoS One 10, e0120833.

Yang, C., Pan, H., Liu, Y., Zhou, X. (2015b). Temperature and development impacts on housekeeping gene expression in cowpea aphid, Aphis craccivora (Hemiptera: Aphidiae). PLoS One 10, e0130593.

Yang, C., Pan, H., Noland, J. E., Zhang, D., Zhang, Z., Liu, Y., Zhou, X. (2015c). Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegilla maculata* (Coleoptera: Coccinellidae). Scientific reports 5, 18201.

Yang, C., Pan, H., Noland, J. E., Zhang, D., Zhang, Z., Liu, Y., Zhou, X. (2015d). Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegilla maculata* (Coleoptera: Coccinellidae). Scientific reports 5, 1-11.

Yang, C., Preisser, E. L., Zhang, H., Liu, Y., Dai, L., Pan, H., Zhou, X. (2016). Selection of reference genes for RT-qPCR analysis in Coccinella septempunctata to assess un-intended effects of RNAi transgenic plants. Frontiers in plant science 7, 1672.

Yeung, A. T., Holloway, B. P., Adams, P. S., Shipley, G. L. (2004). Evaluation of dual-labeled fluorescent DNA probe purity versus performance in real-time PCR. BioTechniques 36, 266-270, 272, 274-265.

Zhang, J., Zhang, Y., Li, J., Liu, M., Liu, Z. (2016). Midgut Transcriptome of the Cockroach Periplaneta americana and Its Microbiota: Digestion, Detoxification and Oxidative Stress Response. PLoS One 11, e0155254.

Zhu, X., Yuan, M., Shakeel, M., Zhang, Y., Wang, S., Wang, X., Zhan, S., Kang, T., Li, J. (2014). Selection and evaluation of reference genes for expression analysis using qRT-PCR in the beet armyworm *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). PLoS One 9, e84730.