Cumhuriyet Sci. J., 43(2) (2022) 176-182
DOI: https://doi.org/10.17776/csj.1069230

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

Selection and Validation of Potential Reference Genes for Quantitative Real-Time PCR Analysis in *Blaptica Dubia* (Serville, 1838) (Blattidae, Blaberidae)

Emin Ufuk Karakaş [1,a], Ayşe Nur Pektaş [2,b], Şeyda Berk [1,c,*]

[1] *Department of Molecular Biology and Genetics, Faculty of Science, Sivas Cumhuriyet University, Sivas, Türkiye*
[2] *Advanced Technology Research Center (CUTAM), Sivas Cumhuriyet University, Sivas, Türkiye*
**Corresponding author*

Research Article

History
Received: 07/02/2022
Accepted: 03/06/2022

ABSTRACT

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is an effective, reproducible, and dependable method for evaluating and targeting expression of genes. It is very important to normalize according to stably expressed housekeeping genes in order to facilitating gene expression studies and to acquire exact and meaningful results. The purpose of this study was to identify and validate six housekeeping genes (*GADPH, RPS18, α-TUB, EF1α, ArgK* and *ACTB*) in adults of cockroach species *Blaptica dubia* employing five different algorithms (geNorm, Bestkeeper, Normfinder, ΔCt method and RefFinder) to assess putative housekeeping gene expression stability. Our study also showed that the geNorm, Normfinder ΔCt method and RefFinder algorithms identified *GADPH* as the most stable housekeeping gene in *B. dubia* adults. Additioanlly, *RPS18* was suggested as the most stable gene by GeNorm and BestKeeeper. *ACTB* has been shown to be by far the least stable of all algorithms. In addition, since there are few validation studies for reference genes in cockroaches in the literature, it is considered that it would be beneficial to increase the number of studies related with RT-qPCR on the reference genes validation under biotic and abiotic conditions in cockroaches.

Keywords: *Blaptica dubia,* RT-qPCR, Reference genes, Validation, Normalization.

[a] *karakaseminufuk@hotmail.com* *https://orcid.org/0000-0001-5813-5678*
[c] *sberk@cumhuriyet.edu.tr* *https://orcid.org/0000-0003-4687-0223*
[b] *aysenur.pektas@gmail.com* *https://orcid.org/0000-0001-5621-2844*

Introduction

With the next-generation sequencing technologies development, it has provided in important increases in genomic and transcriptomic throughput for varied organism [1]. Normalization of gene expression has been imperative for evaluating and reporting these transcriptomic and genomic data quality [2,3]. Although RT-qPCR is a strong, reproducible and dependable method for targeting and measuring expression of genes [4], the quality and unity of RNA examples, reverse transcription, normalization, and this has become limited by PCR efficiency [5-7]. The characteristic method, normalization, is the evaluation of reference genes (housekeeping genes or internal control) expression levels that have important roles in major and common cellular functions and typically display consistent ubiquitous expression levels to measure simultaneously under varied biotic and abiotic conditions [4].

Gene expression normalization in RT-qPCR is accomplished by adding reference genes that are regularly expressed under various experimental conditions and act as endogenous controls [4, 6, 7]. The reference genes, described as “essential expressed to maintain cellular function”, may not supply the necessary conditions for an ideal housekeeping gene expression at constant levels in a diversity of biotic and abiotic circumstances [2, 5, 7]. Many studies revealed that mostly used reference genes are expressed differently in numerous experimental circumstances have suggested that multiple housekeeping genes need be included in the study for correct normalization [2, 5, 6].

There are many reference genes commonly used in the determination of mRNA levels by q-RT-PCR, such as ribosomal protein, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β-actin (*ACTIN*), elongation factor 1a (*EF1A*), and superoxide dismutase (*SOD*) [8-11]. Housekeeping genes are essential for survival, and it is generally considered that there is a slight variation in transcription of these genes. Ribosomal proteins, which are substantial parts of ribosomes, play crucial roles in many biological processes such as intracellular protein biosynthesis, DNA repair and cell differentiation [12]. *EF1A* is involved in translation by catalysing the binding of GTP-dependent aminoacyl-tRNA to the acceptor site of the ribosome. [13]. *SOD* serves as a metalloenzyme that can catalyse the dismutation of superoxide anion to hydrogen peroxide and elemental molecular oxygen [14]. While *ACTIN* is an essential component of the cellular skeleton providing structural unity and shapes cells [13], *GAPDH* joint in energy metabolism [15]. On the other hand, there are many studies showing differently expressed housekeeping genes under various experimental situations [13, 16]. Genes expressing differentially can often induce significant biological changes in tissues, sexes, and other samples at various developmental stages from a variety of experimental conditions, therefore, identification of genes with a consistent expression requires evaluation of specific

changes in gene expression. The smallest variation in successive stability levels of the analysed samples is defined as the stability of gene expression. Therefore, different software programs including GeNorm [4], BestKeeper [17], NormFinder [7], ΔCt Method [18], and RefFinder (http://www.leonxie.com/referencegene.php) have been improved to identify many of these stable genes .

Cockroaches are highly diverse insects with about 4500 species commonly found worldwide [19]. Most cockroaches live in terrestrial habitats and have good adaptations to survive in extreme conditions, but of the diversity that exists, only a small number have adapted to human habitats [19]. The Orange Spotted Cockroach/Argentine Wood Cockroach (*Blaptica dubia*) is a tropical cockroach species not native to human dwellings, although human exposure to *B. dubia* has increased with their widespread commercial reproduction as feeder insects [20]. Because cockroaches have ability to live in habitats containing varying quantity of toxic substances including environmental pollutants, insecticides, microbial toxins, they have been proposed as a good experimental model to study their stress response and detoxification abilities [21].

RT-qPCR has accelerated its progress in many fields and has become very important in entomology. Gene expression analysis has been used to study in gene expression changes between developmental stages, tissues, and other samples from varied experiments in insects, thus gene expression analyses have become of increasing importance in the field of insect molecular biology. Studies in the literature to assess the selection and validity of housekeeping genes in numerous biotic and abiotic situations in insects are summarized in the reviews by Lü et al. and Shakkel et al. [22, 23]. When the studies in the literature are examined, various traditional and new housekeeping genes have been selected in many studies to determine gene expression stability by RT-qPCR normalization in different insect species. Although RT-qPCR is widely used for the detection of gene expression in insects, there is no suitable housekeeping gene (HKGs) and consistent gene quantification system for *B. dubia*, yet. Our current study objectives are to determine appropriate reference genes and evaluate their expression stability in *B. dubia* before they are used as endogenous controls in effective genomic studies of six housekeeping genes commonly used to normalize qRT-PCR data in *B. dubia.*

Material and Methods

Sample and Ethics Statement

Adults of *Blaptica dubia* (Blattodea, Blaberidae) were commercially obtained from a producer in Antalya/Turkey (https://www.antalyacekirge.net/). *Blaptica dubia* adults were brought to the laboratory by controlled storage in the RNAlater® (Qiagen) that is RNA stabilization reagent and stored at freezer (-20°C) until used in further experiments.

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from adult of *B. dubia* specimens using the commercially purchased RNA isolation kit (GeneAll® Hybrid-R™, Seoul, Korea) in accordance with the manufacturer's protocol. After RNA isolation from *B. dubia* adult samples evaluated in this study, A260/A280 and A260/A230 ratios were analysed to determine whether there was DNA or protein contamination. The A260/A280 ratio is between 1.90 and 2.12 for all samples; The A260/A230 ratio was found to be over 1.90, meaning that these ratios show that all samples do not contain DNA or protein contamination. The total RNA concentration of all samples was obtained as a value between 1210ng/µL and 2000ng/µL and was considered suitable for cDNA synthesis. cDNA synthesis was performed using first strand synthesis kit (GeneAll® HyperScript™, Seoul, Korea) to yield the cDNA product containing 1500 ng/uL RNA according to the manufacturer's recommendations.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

In present study, six candidate housekeeping genes (*GADPH, RPS18, α-TUB, EF1α, ArgK* and *ACTB*) were selected among the most studied reference genes on insects in the literature. Primer's properties are given in Table 1. Correlation coefficients (R^2) and efficiencies of PCR amplification (E) were determined for primer validation. Standard curves were generated using cDNA serial dilutions (1, 1/5, 1/25, 1/125 and 1/625) for each primer pair.

qPCR experiments were conducted based on the method previously described [11]. Briefly, a 5-fold cDNA dilution series of 1:625 from an undiluted *B. dubia* cDNA sample was used to identify the efficiencies of amplification and each primer pair specificity used in qPCR assays. For expression analysis of six putative reference genes, all experimental specimens for *B. dubia* were analysed simultaneously in the reverse transcription proceeding.

RT-qPCR analyses in 96-well plate (ABI- Type) using commercial qPCR Master Mix (iGreen, 2X, Biomatik, Canada) were conducted utilizing the StepOnePlus™ Real-Time PCR system (Applied Biosystems, USA). The reaction conditions are as follows, with 20 µL of final volume: [10 µL of Master Mix (2X), 0.7 µL of forward and reverse primers (10 µM), 2 µL of cDNA, and 6.6 µL of 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 approve a single peak and eliminate the primer-dimer possibility and formation of non-specific product. Efficiency of PCR amplification (E)

was determined in accordance with the equalization: E = ($10^{[-1/slope]}$-1) x 100.

Data Interpretation

Cq values of six housekeeping genes were analysed using statistical analysis software GraphPad Prisim 6.0 (GraphPad software, San Diego, C). A boxplot of Cq values was created. The stability of these six genes is determined by GeNorm [4], BestKeeper [17], NormFinder [7], ΔCt [18] and RefFinder (https://www. Heartcure.com.au), the comprehensive web-based analysis tool integrating all four software algorithms. Evaluated using /refinder/?type=reference). 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 [4]. 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 [17]. 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 [7]. 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 housekeeping genes evaluated in RT-qPCR analysis

Symbol	Gene name	Description	Primer sequence (5'→3')	GenBank accession number	Length (bp)	R^2	Ref.
EF1α	Elongation factor 1α	GTPase; Elongation factor translation	F: ACCAGATTTGATGGCTTTGG R: CACCCAGAGGAGCTTCAGAC	XM_003705302	194	0,989	[15]
ACTB	β-actin	Cytoskeleton	F: TCCATCATGAAGTGCGATGT R: CCACATCTGTTGGAATGTCG	NM_001172372	228	0,982	[24]
RPS18	Ribosomal protein S18	Ribosomal protein; Ribonucleoprotein; rRNA-binding; RNA-binding;	F: TACACCTTTGATCGCTGTGAG R: GGCTCTGGTCATTCCAGATAAG	XM_045615265	108	0,967	[25]
ArgK	Arginine Kinase	Phosphotransferase activity	F: CTCGTGTGGTGCAACGAAGA R: GGTGGCTGAACGGGACTCT	NT_037436	130	0,951	[26]
GADPH	Glyceraldehyde-3-phosphate	Oxidoreductase in glycolysis; Gluconeogenesis	F: GCCAAGGTGATCCATGACAA R: GTCTTCTGAGTGGCAGTTGTAG	NC_007420	80	0,963	[27]
α- TUB	Alpha-tubulin	Microtubule	F: TCAAATGCGACCCACGTCAT R: GGCAATAGCCGCGTTGACAT	XM_970811	191	0,893	[28]

Results and Discussion

Expression Stability of Selected Reference Genes

Each primers PCR amplification was approved by the formation of only one peak in analyses of melting curve and the existence of specific band on agarose gel electrophoresis (1.5%). All primers evaluated in this study were found to have a correlation coefficient (R^2) varying between 0.89 and 0.98. It also gave a primary efficiency value between 90% and 110% (Table 1). There was no fluorescent signal amplification in the negative control. This showed that both RNA isolation process and RNA clearance steps were effective.

The variation of Ct values among *B. dubia* adult samples for six reference genes is represented in Figure 1. Cycle threshold (Cq) values obtained amplifying the six candidate reference genes from *B. dubia* adults were plotted (Figure 1). Cq values for the six genes ranged from 20.92 to 36.67 in *B. dubia*. *GADPH* represented the lowest Cq values in *B. dubia* (24.95 ± 2.70, mean Cq ± std. dev.). Amplification of *α-TUB, RPS18, ArgK, ACTB* and *EF1α* showed mean Cqs of 28.49 ± 2.23, 29.64 ± 1.12, 30.45 ± 2.59, 32.28 ± 4.18 and 32.45 ± 2.59, respectively.

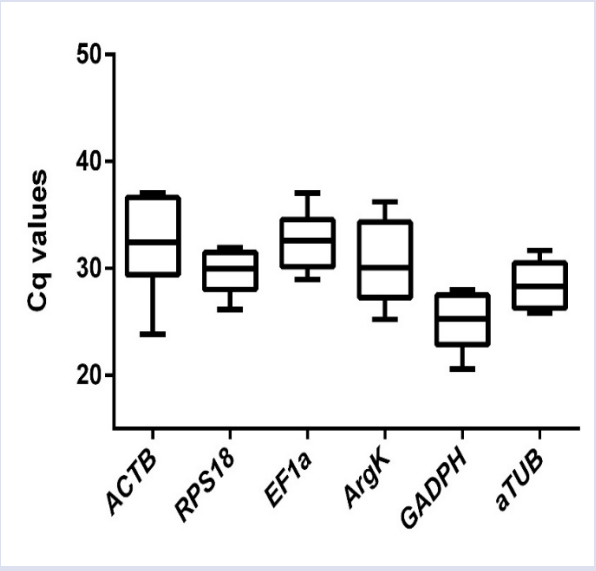

Figure 1. Amplification profiles of putative housekeeping genes. Box plot of qPCR cycle threshold values (Cq) of housekeeping genes in *Blaptica dubia*.

The smallest variation in successive stability levels of the analysed samples is defined as the stability of gene expression. Programs have been developed that identify most of these stable genes, including GeNorm [4], BestKeeper [17], NormFinder [7], ΔCt [18] and RefFinder. In current study, to identify optimal reference genes in *B. dubia* adults, four statistical Excel macro programs (geNorm, BestKeeper, NormFinder, and Δ-Ct method) and the web-based analysis tool RefFinder were used to assess the stability of six candidate genes (Table 2 and Figure 2).

Table 2. Stability of housekeeping gene expression in *Blaptica dubia* adults.

Reference genes	Genorm		Bestkeeper			Normfinder		Delta Ct		RefFinder	
	M	Rank	SD	r	Rank	SV	Rank	SD	Rank	Stability	Rank
ACTB	1.755	6	3.24	0.936*	6	2.245	6	2.47	6	6.000	6
RPS18	0.699	1	1.70	0.972*	1	1.050	3	1.54	3	1.730	2
EF1α	1.058	4	2.00	0.906*	2	1.307	4	1.80	4	3.360	4
ArgK	1.397	5	3.20	0.969*	5	1.486	5	1.95	5	5.000	5
GADPH	0.699	1	2.20	0.987*	4	0.350	1	1.32	1	1.410	1
α-TUB	0.715	3	2.00	0.966*	3	0.772	2	1.45	2	2.450	3

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

The geNorm assuming candidate genes are not co-regulated, normally calculates M value (stability value of gene expression) for every housekeeping gene tested as the mean pairwise variation with other housekeeping genes for that gene. While the genes with low M are defined as having constant expression, the highest M value of gene is removed and this process continues until the two most stable genes remain, and this last gene pair is defined as the optimal reference gene pair. Therefore, it is recommended to use at least two reference genes to ensure correct normalization in geNorm. [4]. In *B. dubia* adults, geNorm ranked the set of candidate reference genes: *RPS18, GADPH* > *α-TUB* > *EF1α* >*ArgK* > *ACTB*. Based on this algorithm data, the M value was determined as 0.699 for both RPS18 and GAPDH and both genes were suggested as the two most stable genes. *ACTB* with an M value of 1.755 was determined as the gene with the least stable expression (Figure 2 and Table 2).

Normfinder, the analysis that takes into account systematic differences between sample subsets, is an ANOVA (analysis of variance) model identifying genes with the least variation of expression in all samples [7]. In *B. dubia* adults, Normfinder ranked the six reference genes as follows: *GADPH* > *α-TUB* > *RPS18* > *EF1α* > *ArgK* > *ACTB* with a stability value SV of 0.350, 0.772, 1.050, 1.307, 1.486, 2.245, respectively. *GADPH* was found to be the most constant with a stability value of 0.350 and *ACTB* the least constant with a stability value of 2.245 (Table 2 and Figure 2).

Reference genes that exhibit the lowest standard deviation (SD) according to BestKeeper analysis, which can calculate the candidate genes stability, the standard deviation (SD) based on the Cq values of all putative housekeeping genes, are taken as the most stable genes. Also, values exceeding the threshold value (SD < 1) are considered unstable in all samples. [17]. According to this analysis, all housekeeping genes [*RPS18* (SD: 1.70), *EF1α* (SD: 2.0), *α-TUB* (SD: 2.0), *GADPH*, (SD: 2.20), *ArgK* (SD: 3.20), *ACTB* (SD: 3.24)] exceeded the threshold in *B. dubia* adults (Table 2 and Figure 2).

In the ΔCt method, which calculates the mean SD of each gene cluster using the raw Ct values based on relative pairwise comparisons, the SD value is oppositely correlated to the gene expression stability. Standard deviation below 1 indicates appropriate stability [18]. As the mean SD of each gene set was over 1, none of the accessible housekeeping genes were found to be stable enough for *B. dubia* adults according to the ΔCT method. The overall ranking based on the ΔC t method of reference genes was: *GADPH, α-TUB, RPS18, EF1α, ArgK, ACTB* (Table 2 and Figure 2).

RefFinder is a comprehensive algorithm combining four software tools (geNorm, BestKeeper, NormFinder, and Δ-Ct method) to order putative housekeeping genes stability. The overall ranking based on RefFinder of reference genes in *B. dubia* was: *GADPH, RPS18, α-TUB, EF1α, ArgK*, and *ACTB* (Figure 2 and Table 2).

The sensitivity of RT-qPCR has made this analysis method the most important technique for the relative expression of mRNA quantities. However, the validity of RT-qPCR normalization studies is depending on the reference genes included in the study, and these reference genes expression stability may be affected by variances in the examined tissues, physiological or experimental situations [4]. On the other hand, there is no single "universal" housekeeping gene that is constantly expressed and viable for all cell and tissue types under various experimental cases [2, 10, 27, 29]. Therefore, several reports suggested that multiple stably expressed housekeeping genes need to be used, as the use of such genes for the experimental setup without prior and appropriate validation can induce estimation of inaccurate expression levels of target genes and thus interpretation of data incorrectly [4, 30, 31].

The result of a meta-analysis of control gene expression studies on insects by Lü et al. was that actin, RPL, GAPDH, Tub, 18S, TATA, RPS, EF1A, HSP and SDHA are among the mostly used housekeeping genes [23]. In many studies evaluating the stability of reference genes in various insect species such as Liposcelis bostsrychophila, Diabrotica virgifera virgifera, Chilo supressalis and Spodoptera exigua, actin has been reported to show high stability at varied developmental stages [15, 32, 33]; actin has also been determined to have fairly stable expression under numerous abiotic situations in Liposcelis bostsrychophila (insecticide) [32], Hippodamia convergens (diet) [27].

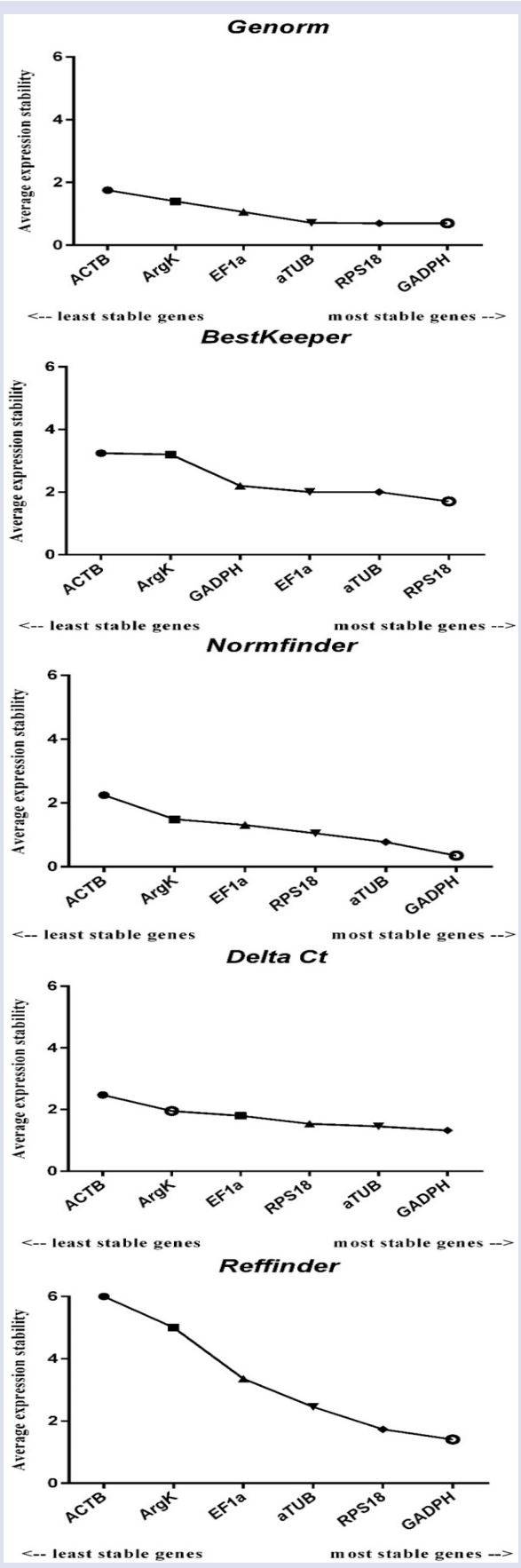

Figure 2. Stability of gene expression and ranking of the housekeeping genes evaluated by Genorm, Normfinder, Bestkeeper, ΔCt metod, RefFinder.

On the other hand, *ACTB* expression in some insect species was reported to be less stable [27, 34]. Ribosomal protein S genes (*RPS18, RPS15, RPS11* and *RPS3*), *C. maculata, S. inferens, N. lugens, M. domestica* and *H. armigera* were found to have high expression stability at varied developmental stages [25, 30, 35]. Tubulin (α-, β-, γ-tubulin) encoding cytoskeletal structure proteins, another most studied reference gene, has been found to have highly stability under several biotic situations such as sex, tissues and developmental stages of *Sogatella furcifera, Coleomegilla maculata, Bactrocera dorsalis, Liposcelis bostsrychophila, Drosophila suzukii* [32, 36, 37] and abiotic conditions including temperature, photoperiod, insecticide and diet in *Nilaparvata lugens, Helicoverpa armigera, Bemisia tabaci* [29, 31, 36]. Similarly, *GAPDH* expression showed high stability under different tissue and developmental stages of *Schistocerca gregaria, Tenebrio molitor, Rhodnius prolixus, Diabrotica virgifera virgifera, Hippodamia convergens, Bombyx mori, Chilo suppressalis, Sesamia inferens, Spodoptera litura* [8, 11, 15, 27, 30, 33]. Additionally, *GADPH* was found to be stable reference gene under abiotic conditions including viral infection in *Sogatella furcifera*, insectiside in *Liposcelis bostsrychophila*, photoperiod in *Hippodamia convergens*, diet in *Danaus plexippus*, mechanical injury and viral infection in *Helicoverpa armigera* [27, 32, 35, 36]. On the other hand, *GADPH* expression in some insect species was reported to be less stable [2, 27, 32, 37]. Furthermore, the *EF1A* gene showed high stability under different developmental stages and tissues of *Cimex lectularius and Bombus lucorum* [38, 39] whereas the *EF1A* gene was selected as the least constant housekeeping gene in *A. craccivora* in different temperatures and developmental stages [25]. Under starvation conditions, *ArgK, EF1A, RPS11* were recommended for *N. lugens* [31]. In summary, the expression of mostly used reference genes may alter under numerous experimental situations. Therefore, it would be beneficial to evaluate gene expression profiles widely in different biotic and abiotic cases and to determine gene stability.

In present study, a validation study was performed for reference genes (*GADPH*, *RPS18*, *α-TUB, EF1α, ArgK*, and *ACTB)* in cockroach species *B. dubia* adults using five different algorithms (ΔCt method, geNorm, Bestkeeper, Normfinder and RefFinder) to assess the expression stability of selected housekeeping genes. Among the six putative housekkeping genes, *GADPH* was determined as the most stable housekeeping gene in *B. dubia* adults according to the geNorm, Normfinder ΔCt method and RefFinder algorithms. At the same time, *RPS18* was proposed by GeNorm and BestKeeeper as the most stable gene. It has been shown that *ACTB* is by far the least stable of all algorithms. To the best of our knowledge, only one validation study [40] has been reported in cockroaches to date. In this study, only two algorithms (Normfinder and geNorm) were employed to assess eight housekeeping genes stability (*β-actin, GAPDH, EF1α, RpL32, Arm, AnnIX, α-Tub* and *SDHa*) in various developmental stages of

Diploptera punctata. The *α-Tub, EF1a*, and *RpL32* genes have been suggested as the most stable genes for *D. punctata*. On the other hand, they suggested that *Actin* and *AnnIX* are the least stable genes and should not be used to normalize transcript levels. Our results suggested that *GADPH* and *RPS18* genes according to the five algorithms can be used for normalization of transcript levels in *B. dubia* adults, while *ACTB* should not be used to normalize transcript levels for cockroach *D. dubia*, as suggested in the study by Marchal *et al.* [40]. In the future planning of this study, as in other insect species, it is planned to investigate especially cockroaches under conditions of many biotic and abiotic conditions such as developmental stage, starvation, temperature, pesticide, diet. In addition, validation studies for reference genes in cockroaches are very few in the literature. Therefore, considering our current study and the studies in the literature, it is necessary to increase the number of studies to verify reference genes under biotic and abiotic conditions in q-RT-PCR studies in cockroaches.

Acknowledgment

All analyses in this study were performed in the Molecular Biology laboratory of the Sivas Cumhuriyet University Advanced Technology and Research Center (CUTAM).

Conflict of Interest

The authors state that did not have conflict of interests.

References

[1] Hudson M.E., Sequencing breakthroughs for genomic ecology and evolutionary biology, *Mol. Ecol. Resour.,* 8 (2008) 3-17.

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

[3] Lourenço A.P., Mackert A., Cristino A.d.S., Simões Z.L.P., Validation of reference genes for gene expression studies in the honey bee, Apis mellifera, by quantitative real-time RT-PCR, *Apidologie*, 39 (2008) 372-385.

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

[5] Strube C., Buschbaum S., Wolken S., Schnieder T., Evaluation of reference genes for quantitative real-time PCR to investigate protein disulfide isomerase transcription pattern in the bovine lungworm Dictyocaulus viviparus, *Gene,* 425 (2008) 36-43.

[6] Guo J., Ling H., Wu Q., Xu L., Que Y., The choice of reference genes for assessing gene expression in sugarcane under salinity and drought stresses, *Scientific Reports,* 4 (2014) 7042.

[7] Andersen C.L., Jensen J.L., Ørntoft T.F., 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 (2004) 5245-50.

[8] Van Hiel M.B., Van Wielendaele P., Temmerman L., Van Soest S., Vuerinckx K., Huybrechts R., Broeck J.V., Simonet G., Identification and validation of housekeeping genes in brains of the desert locust Schistocerca gregaria under different developmental conditions, *BMC Mol Biol.,* 10 (2009) 56.

[9] Pitino M., Coleman A.D., Maffei M.E., Ridout C.J., Hogenhout S.A., Silencing of aphid genes by dsRNA feeding from plants, *PLoS One*, 6 (2011) e25709.

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

[11] Berk S., and Pektas A., Selection and Validation of Reference Genes for Quantitative Real-time PCR in the Mealworm Beetle, Tenebrio molitorL. (Coleoptera: Tenebrionidae), *IOSR-JAVS.*, 13 (2020) 44-50.

[12] Ladror D.T., Frey B.L., Scalf M., Levenstein M.E., Artymiuk J.M., Smith L.M., Methylation of yeast ribosomal protein S2 is elevated during stationary phase growth conditions, *Biochemical and Biophysical Research Communications*, 445 (2014) 535-41.

[13] Ponton F., Chapuis M.P., Pernice M., Sword G.A., Simpson S.J., Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster, *J. Insect Physiol.,* 57 (2011) 840-50.

[14] Parker J.D., Parker K.M., Keller L., Molecular phylogenetic evidence for an extracellular Cu Zn superoxide dismutase gene in insects, *Insect Molecular Biology,* 13 (2004) 587-94.

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

[16] Lee P.D., Sladek R., Greenwood C.M., Hudson T.J., Control genes and variability: absence of ubiquitous reference transcripts in diverse mammalian expression studies, *Genome Research*, 12 (2002) 292-7.

[17] Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P., Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations, *Biotechnology Letters*, 26 (2004) 509-15.

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

[19] Pomés A., Arruda L.K., Investigating cockroach allergens: aiming to improve diagnosis and treatment of cockroach allergic patients, *Methods (San Diego, Calif.),* 66 (2014) 75-85.

[20] Wu H., Appel A.G., Hu X.P., Instar determination of Blaptica dubia (Blattodea: Blaberidae) using Gaussian mixture models, *Annals of the Entomological Society of America*, 106 (2013) 323-328.

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

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

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

[24] Sang W., He L., Wang X.P., Zhu-Salzman K., Lei C.L., Evaluation of Reference Genes for RT-qPCR in Tribolium castaneum (Coleoptera: Tenebrionidae) Under UVB Stress, *Environmental Entomology*, 44 (2015) 418-25.

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

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

[27] Pan H., Yang X., Siegfried B.D., Zhou X., A Comprehensive Selection of Reference Genes for RT-qPCR Analysis in a Predatory Lady

Beetle, Hippodamia convergens (Coleoptera: Coccinellidae), *PLoS One,* 10 (2015) e0125868.

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

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

[30] Lu Y., Zheng X., Liang Q., Xu H., Yang Y., Tian J., He X., Lu Z., Evaluation and validation of reference genes for SYBR Green qRT-PCR normalization in Sesamia inferens (Lepidoptera: Noctuidae), *J. Asia-Pac. Entomol.,* 18 (2015) 669-675.

[31] Yuan M., Lu Y., Zhu X., Wan H., Shakeel M., Zhan S., Jin B.R., Li J., Selection and evaluation of potential reference genes for gene expression analysis in the brown planthopper, Nilaparvata lugens (Hemiptera: Delphacidae) using reverse-transcription quantitative PCR, *PLoS One,* 9 (2014) e86503.

[32] Jiang H.B., Liu Y.H., Tang P.A., Zhou A.W., Wang J.J., Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of Liposcelis bostsrychophila (Psocoptera: Liposcelididae), *Mol. Biol. Rep*., 37 (2010) 1019-29.

[33] Teng X., Zhang Z., He G., Yang L., Li F., Validation of reference genes for quantitative expression analysis by real-time rt-PCR in four lepidopteran insects, *J. Insect Sci*., 12 (2012) 60.

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

[35] Zhang S., An S., Li Z., Wu F., Yang Q., Liu Y., Cao J., Zhang H., Zhang Q., Liu X., Identification and validation of reference genes for normalization of gene expression analysis using qRT-PCR in Helicoverpa armigera (Lepidoptera: Noctuidae), *Gene*, 555 (2015) 393-402.

[36] An X.-k., Hou M.-l., Liu Y.-d., 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 (2016) 879-886.

[37] Zhai Y., Lin Q., Zhou X., Zhang X., Liu T., Yu Y., Identification and validation of reference genes for quantitative real-time PCR in Drosophila suzukii (Diptera: Drosophilidae), *PLoS One*, 9 (2014) e106800.

[38] Mamidala P., Rajarapu S.P., Jones S.C., Mittapalli O., Identification and validation of reference genes for quantitative real-time polymerase chain reaction in Cimex lectularius, *Journal of Medical Entomology*, 48 (2011) 947-51.

[39] Hornáková D., Matousková P., Kindl J., Valterová I., Pichová I., Selection of reference genes for real-time polymerase chain reaction analysis in tissues from Bombus terrestris and Bombus lucorum of different ages, *Analytical Biochemistry*, 397 (2010) 118-20.

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