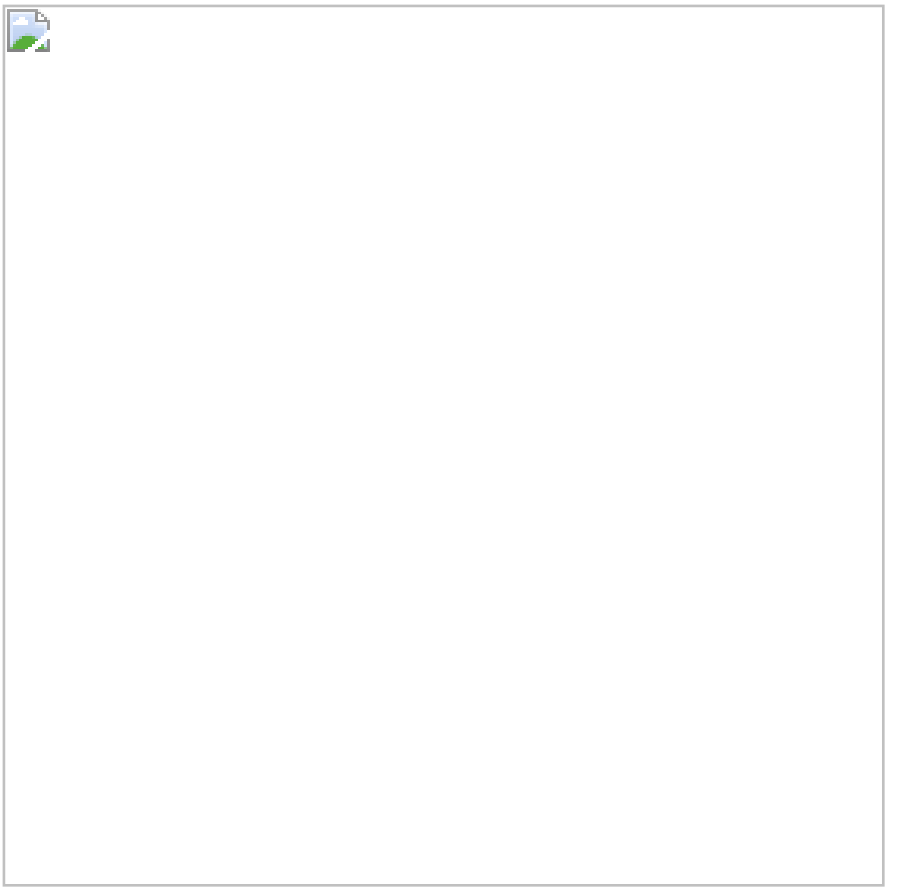

Evaluation of Endogenous Reference Genes of Bactrocera (Tetradacus) minax by Gene Expression Profiling under Various Experimental Conditions

Authors: Lü, Zhi-Chuang, Wang, Liu-Hao, Dai, Rui-Lin, Zhang, Gui-Fen, Guo, Jian-Ying, et al.

Source: Florida Entomologist, 97(2) : 597-604

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.097.0235

EVALUATION OF ENDOGENOUS REFERENCE GENES OF *BACTROCERA (TETRADACUS) MINAX* BY GENE EXPRESSION PROFILING UNDER VARIOUS EXPERIMENTAL CONDITIONS

ZHI-CHUANG LÜ[1], LIU-HAO WANG[1,3], RUI-LIN DAI[1], GUI-FEN ZHANG[1], JIAN-YING GUO[1,2] AND FANG-HAO WAN[1,2,*]
[1]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Science, Beijing 100081, China

[2]Center for Management of Invasive Alien Species, Ministry of Agriculture, Beijing 100081, China

[3]Department of Entomology, Henan Institute of Science and Technology, Xinxiang 453003, Henan Province, China

*Corresponding author; E-mail: wanfanghao@caas.cn

ABSTRACT

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is widely used for gene expression analysis in living organisms, and stably expressed endogenous reference genes are needed to obtain accurate results. Some commonly used reference genes varied among different experimental treatments. To obtain suitable reference gene for specific experimental conditions, the stability of 10 reference genes of *Bactrocera (Tetradacus) minax* (Enderlein) were evaluated in various development stages, and under temperature- and γ-irradiation-stress conditions by semi-quantitative PCR. The present results indicated that the most stable candidate reference gene was RPL32 in eggs, 3rd instars, 1-, 90- and 160-day-pupae and newly emerged female and male adults (< 24 h), and under different stress conditions, i.e., 35 °C temperature stress for 0 h, 1 h, 3 h and 5 h, and 4 °C temperature stress for 0 h, 12 h, 36 h, 48 h and 60 h. GAPDH, G6PDH and RPL32 were ideal candidate endogenous genes under 35 °C temperature stress for 0 h, 1 h, 3 h and 5 h, and under 4 °C temperature stress for 0 h, 12 h, 36 h, 48 h and 60 h, and under a Γ irradiation stress of 90 Gy. These results provide basic information for future studies of gene expression in *B. minax*, and should serve as a resource to screen reference genes for gene expression studies in other insect species.

Key Words: Chinese citrus fruit fly; developmental stages; PCR primer sequence reference gene; stable expression; γ-irradiation, temperature stress

RESUMEN

La transcripción inversa de la reacción de la polimerasa en cadena (RT-qPCR) cuantitativa en tiempo real es ampliamente utilizada para el análisis de la expresión de genes en los organismos vivos, y los genes de referencia endógenos expresados de forma estable son necesarios para obtener resultados precisos. Algunos de los genes de referencia utilizados variaron entre los diferentes tratamientos experimentales. Para obtener genes de referencia adecuados para condiciones experimentales específicas, la estabilidad de 10 genes de referencia de *Bactrocera* (*Tetradacus*) *minax* (Enderlein) fueron evaluados en diferentes estadios de desarrollo, y bajo temperaturas y condiciones de estrés por irradiación-γ por medio del método de PCR semi - cuantitativo. Los resultados indican que el candidato del gene de referencia más estable fue RPL32 en los huevos, tercer estadios, pupas de 1, 9 y 160 dias y adultos recién emergidos de ambos sexos (< 24 h) y bajo diferentes condiciones de estrés, como el estrés por temperatura de 35 °C durante 0 h, 1 h, 3 h y 5 h, y el estrés de temperatura de 4 °C durante 0 h, 12 h, 36 h, 48 h y 60 h. Los GAPDH, G6PDH y RPL32 fueron candidatos ideales de genes endógenos bajo estrés de temperatura de 35 °C durante 0 h, 1 h, 3 h y 5 h, y bajo 4 °C de estrés de temperatura por 0 h, 12 h, 36 h, 48 h y 60 h y bajo estrés de irradiación- Γ 90 Gy**.** Estos resultados proveen información básica para estudios futuros de expresión de genes en *B. minax* y estos servirán como un recurso para detectar genes de referencia para los estudios de expresión de genes en otras especies de insectos.

Palabras Clave: Mosca china de la fruta de cítricos; estadios de desarrollo; gene de referencia de secuencia del cebador de PCR, expresión estable; irradiación-γ, estrés térmico

Quantitative real-time PCR (qRT-PCR) is sensitive, accurate, reproducible and quantitative (Ginzinger 2002; Bustin et al. 2005; Kubista et al. 2006; Van Guilder et al. 2008), and has been widely used in gene expression analysis to provide insight into complex biological processes (Hong et al. 2008). Although qRT-PCR is a powerful tool in gene expression analysis, data analysis and subsequent interpretation, there are still some limitations in this assay, such as variations in transcription and amplification efficiencies among different samples (Thellin et al. 1999; Suzuki et al. 2000; Pfaffl et al. 2004; Bustin et al. 2005). Therefore, it is necessary to normalize gene expression analysis data when qRT-PCR is used in assays. A common technique for normalization data in qRT-PCR is to use reliable reference genes in the same samples in parallel (Shen et al. 2000; Tunbridge et al. 2011; Li et al. 2013).

Housekeeping genes, such as α-TUB (α-Tubulin), β-actin (Beta actin), 18s rRNA (18S ribosomal RNA), 28s rRNA (28S ribosomal RNA), GAPDH (Glyceraldehyde-3-phosphatedehydrogenase), G6PDH (Glucose-6-phosphate dehydrogenase), β- TUB (Beta-Tubulin), RPL32 (Ribosomal protein L32), EF1-α (Alpha elongation factor), ELF1-β (beta elongation factor), are usually used as endogenous controls for normalization of qRT-PCR data, but the reference gene expression levels can differ under different environment conditions (Radonic´ et al. 2004; Li et al. 2013). It is high unlikely that a single universal reference gene is suited for all experimental conditions, tissues or cell lines (Thellin et al. 1999; Butte et al. 2001; Vandesompele et al. 2002; Zhou et al. 2006; Thorrez et al. 2008; Folkersen et al. 2009; Ponton et al. 2011). So, it is critical to select a consistent expression reference gene under specific experimental conditions for qRT-PCR data analysis. To make sure expression of candidate reference gene occurs at a constant level, each candidate reference gene should be evaluated under specific experimental conditions (Thellin et al. 1999).

Bactrocera (Tetradacus) minax (Enderlein) (Diptera: Tephritidae), the Chinese citrus fruit fly, has caused great losses in commercial citrus for more than half a century in China (Wang & Luyi 1995). A series of control strategies has been used to manage the pest (Yang et al. 1994; Wang & Luyi 1995; van Schoubroeck 1999). These measures cannot effectively reduce population density. *Bactrocera minax* has a long pupalperiod – more than 6 months - which hinders mass rearing. *Bactrocera minax* has unique dietary requirements, and just one generation per yr (van Schoubroeck 1999). Therefore, understanding and breaking diapause is a key prerequisite for mass rearing, i.e., the production of 2 or more generations per yr, which would have important significance in the application of certain new techniques to control this pest. In addition, adults have strong resistance to high temperatures, whereas the larvae and pupae are strongly resistant to low temperatures. To develop effective pest control strategies, an understanding of these resistance mechanisms at the molecular level is necessary. Elucidation of gene expression profiles in different development stages and under different stress conditions will help to better understand diapause and resistance mechanisms. The selection of suitable reference genes is a critical step for accurate gene expression profile analysis under different experimental conditions.

To select reference genes with consistent expression under different experimental conditions, we hypothesized that some reference genes have consistent expression under specific experimental conditions. In the present study, the 10 candidate reference genes (α-Tubulin, β-actin, 18s rRNA, 28s rRNA, GAPDH, G6PDH, β-TUB, RPL32, EF1-α and ELF1-β) were evaluated in different development stages, and under various temperature and γ-irradiation-stress conditions by semi-quantitative PCR. Our aim was to identify appropriate reference genes to study their gene expression profiles under different experimental conditions.

Materials and Methods

Insects

Bactrocera (Tetradacus) Minax eggs were collected in citrus in Zhangjiachong, Cun Jingzhou, Hubei province in Aug 2011. Eggs in citrus were held at 26 ± 1 °C and 12:12 h L:D for hatching. Newly hatched larvae were reared in citrus in the laboratory. Late third instars left the fruit and jumped into bottles filled with fine sand with a moisture content of 10-15%. The third late instars pupated in the sand and were transferred to 17 ± 1 °C and 12:12 h L:D until adult eclosion.

Candidate Reference Genes

Ten housekeeping genes were selected as candidate reference genes including α-TUB, β-actin, 18s rRNA, 28s rRNA, GAPDH, G6PDH, β- TUB, RPL32, EF1-α, ELF1-β. Primer 5.0 were used to design primers for semi-quantitative PCR analysis. The PCR primer sequences used for cloning target genes and semi-quantitative expression are shown in Table 1.

Treatment Conditions

Eggs, 3rd instars, 1-, 90- and 160-day-pupae, and newly emergency female and male adults (< 24 h) were sampled and subjected to 3 different treatments. Each treatment was replicated 3 times. The 3 treatments were as follows.

TABLE 1. PRIMER SEQUENCES USED IN THE CDNA CLONING AND SEMI-QUANTITATIVE PCR OF *BACTROCERA (TETRADACUS) MINAX* REFERENCE GENES.

Gene	Primer sequence (5' 3')	Fragment length (bp)	Reference
α-TUB	TTCATGGTTGATAACGAAGC	178	—
	GCACCAAGTTAGTCTGGAAT		
β-actin	TATCCTCACCCTGAAGTATCCC	235	—
	TGACAGCACAGCCTGAATGG		
18s rRNA	GCGAGAGGTGAAATTCTTGG	191	Shen et al. 2010
	CGGGTAAGCGACTGAGAGAG		
28srRNA	CTGTGGATGAACCAAACGTG	210	Martinez &Denlinger, 2008
	TGTACGCCAGCGGTAATGTA		
GAPDH	GACGCCTACAAGCCTGACAT	221	Shen et al. 2010
	GTTGAAGCGGGAATGATGTT		
RPL32	CGATTTCTCCGCAGTATTCAC	147	—
	GCCAGTACCTCATGCCTAACA		
β-TUB	TGGTGCCATTTCCTCGTTTA	217	—
	TGCTCGTCCACTTCCTTCAT		
ELF1-β	TTCTTCGATTTCTTAGCAGCAT	226	—
	GGTATCGTCACATTGCGTCA		
G6PDH	CGAGCAGGCCATGTATGA	152	—
	GCGGCAAAGCCAAGTAAA		
EF1-α	GGTGTCAACAAGATGGATTC	224	—
	CCTTCAGCATTACCTTCCTT		

High Temperature Stress. A single newly emerged adult was collected and was placed in a 1.5 mL centrifuge tube, and was heat-shocked at each of the 3 time periods (1, 3 and 5 h) in a climatic incubator at 35 °C. Adults maintained at 26 °C were used as untreated controls.

Low Temperature Stress. Newly emerged adults were collected and each was placed in a 1.5 mL centrifuge tube. Individually held adults were heat-shocked at each of the 4 time periods (12, 36, 48 and 60 h) in a climatic incubator at 4 °C. Adults maintained at 26 °C were used as untreated controls.

Γ Irradiation Stress.Fourth stage pupae, i.e., pupae that would emerge after an additional 2 days, were irradiated with a Co^{60} machine (No. 0800574, Chengdu Institute of Nuclear Power, Chengdu, China) at 90 Gy. Non-irradiated pupae were used as an untreated control.

Extraction of Total RNA and cDNA Synthesis

Total RNA from the samples was extracted using the RNeasy Mini Kit and the RNase-Free Set (Qiagen, Valencia, California, USA) were used to remove genomic DNA. The quantity and quality of RNA were assessed by spectrophotometry (Beckman Du 650 spectrophotometer, Fullerton, California, USA), where A260/A280 ratios were typically above 1.8. RNA quality was also evaluated by 1% agarose gel electrophoresis.

Two μg of total RNA was used to synthesize cDNA using SuperScript® III Reverse Transcriptase Kit (Invitrogen Life Technologies, Burlington, Ontario, Canada), following the manufacturer's instructions. The cDNA was stored at -80 °C prior to further analysis.

Cloning Sequence Fragments of Endogenous Reference Genes

Sequence fragments of the 10 candidate endogenous references genes were cloned from *B. minax*, respectively. The PCR primer sequences used for cloning target genes and semi-quantitative expression are shown in Table 1. The amplification volume was 20 μL, including 10× PCR reaction buffer 2 μL, dNTP Mix (10 mM) 0.4 μL, Taq polymerase (2.5 U) 0.4 μL, forward primer (10 μM) 0.8 μL, reverse primer (10 μM) 0.8 μL, cDNA template 0.4 μL, and ddH_2O 15.2 μL. The PCR cycling conditions were: 94 °C for 5 min, followed by 35 cycles of amplification, each cycle consisted of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. The 35 cycles was followed by a 5 min final extension at 72 °C. Amplification products were all detected electrophoretically on 1.5% agarose gels. The target band was sliced from the gel and was purified (High Pure PCR Product Purification Kit; Shanghai Boya Biotechnological Ltd., Shanghai, China), ligated, and cloned using a pGEM®-T Easy vector system (Beijing TianweiShidai Biotechnological Ltd., Beijing, China). Plasmid DNA was then purified from bacterial cells (GenEluteTM Plasmid Kits, Beijing TianweiShidai Biotechnological Ltd.), and was se-

quenced by Shenggong Biotechnological Co. Ltd., Shanghai. The sequence obtained was compared with known sequences by searching the National Center for Biotechnology Information (NCBI) databases with the BLAST program.

Comparative Expression Levels of Endogenous Reference Genes Under Different Treatment Conditions

After PCR amplification, the comparative expression levels of 10 candidate endogenous references genes during different stages of development of *B. minax* were detected on 1.5% agarose gels by Image Lab 3.0 (Fig. 1).

Data Analysis

Statistical analyses were conducted using SPSS package (version 13). Prior to all statistical analyses, data were examined for assumptions of normality using Kolmogorov-Smirnov test. The effects of different development stages and different stress conditions on endogenous reference gene expression were analyzed by the least significant difference (LSD) test after one-way ANOVA. The results were expressed as means ± standard errors (mean ± SEM). The differences were considered significant when P values were ≤ 0.05.

RESULTS

Comparative Expression Levels of Endogenous Reference Genes in Various Development Stages

Expression levels of α-TUB, EF1-α and RPL32 mRNA were not significantly different in the various development stages (α-TUB: $F_{6,20}$ = 2.478, P = 0.076; EF1-α: $F_{6,20}$ = 1.287, P = 0.325; RPL32: $F_{6,20}$ = 2.837, P = 0.051). However significant differences in expression levels were detected for β-actin, GAPDH, β-TUB, 18s rRNA, 28s rRNA, ELF1-β and G6PDH mRNA expression in different development stages of *B. Minax* (β-actin: $F_{6,20}$ = 146.762, P = 0.000; GAPDH: $F_{6,20}$ = 15.653, P=0.000; β-TUB: $F_{6,20}$ = 21.039, P = 0.000; 18s rRNA: $F_{6,20}$ = 3.438, P = 0.027; 28s rRNA: $F_{6,20}$ = 5.453, P = 0.004; ELF1-β: $F_{6,20}$ = 3.775, P = 0.019; G6PDH: $F_{6,20}$ = 7.317, P = 0.001). (Fig. 2).

Fig. 1. PCR amplification production of candidate endogenous references genes in *Bactrocera (Tetradacus) minax*. References genes coded 1-10 were α-TUB, β-TUB, β-actin, EF1-α, ELF1-β, GAPDH, G6PDH, RPL32, 18s rRNA and 28s rRNA, respectively.

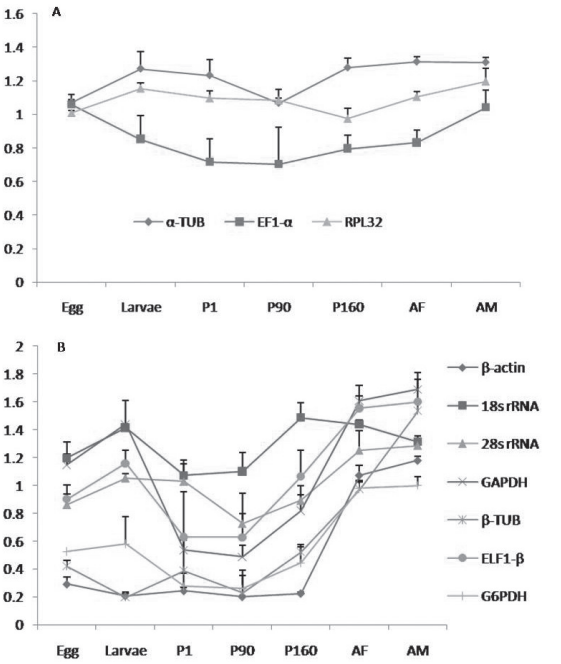

Fig. 2. Effects of various development stages of *Bactrocera (Tetradacus) minax* on mRNA expression of 10 reference genes. A: represented the candidate reference genes. B: represented the differentially regulated genes. P1, P90 and P160 are 1-day pupa, 90-day pupa and 160-day old pupae, respectively. AF and AM are adult females and adult males, respectively. The results were expressed as means ± SEM. The differences were considered significant when P-values were ≤ 0.05.

Comparative Expression Levels of Endogenous Reference Genes under High Temperature Stress

There were no significant differences in the level of β-actin, GAPDH, β-TUB, RPL32, G6PDH and ELF1-βmRNA expressed under 35 °C high temperature stress for 0 h, 1 h, 3 h and 5 h (β-actin: $F_{3,11}$ = 2.823, P = 0.107; GAPDH: $F_{3,11}$ = 1.164, P = 0.382; β-TUB: $F_{3,11}$ = 0.842, P = 0.508; RPL32: $F_{3,11}$ =1.645, P = 0.255; G6PDH: $F_{3,11}$ = 1.202, P = 0.369; ELF1-β: $F_{3,11}$ = 2.104, P = 0.178). However there was a significant difference in α-TUB, 18s rRNA, 28s rRNA and EF1-α mRNA expression under different high temperature stress conditions (α-TUB: $F_{3,11}$ = 7.738, P = 0.009; 18s rRNA: $F_{3,11}$ = 22.751, P = 0.000; 28s rRNA: $F_{3,11}$ = 5.465, P = 0.024; EF1-α: $F_{3,11}$ = 5.068, P = 0.030). (Fig. 3).

Comparative Expression Levels of Endogenous Reference Genes under Low Temperature Stress

GAPDH, RPL32 and G6PDH mRNA expression levels were not significantly different at 4 °C for 0 h, 12 h, 36 h, 48 h and 60 h (GAPDH: $F_{4,14}$ = 2.738, P = 0.089; RPL32: $F_{4,14}$ = 0.904, P =

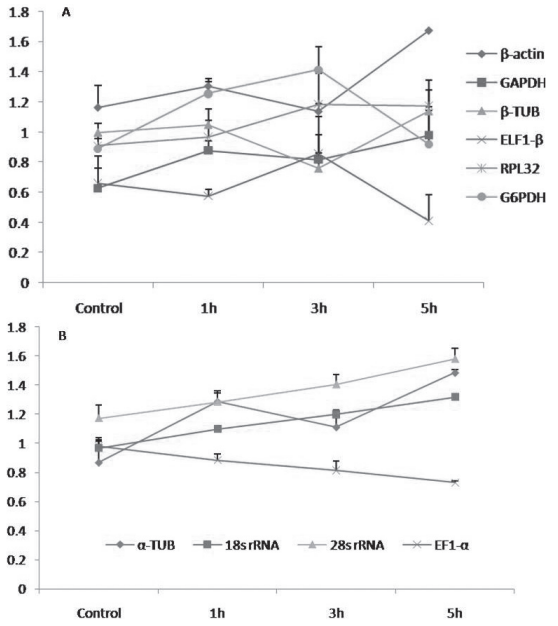

Fig. 3. Effects of high temperature stress on mRNA expression of 10 reference genes in *Bactrocera (Tetradacus) minax*. A: represents the candidate reference genes. B: represents the differentially regulated genes. Temperature stress of 35 °C was applied for 1 h, 3 h and 5 h, respectively. The results were expressed as means ± SEM. Differences were considered significant when *P*-values were ≤ 0.05.

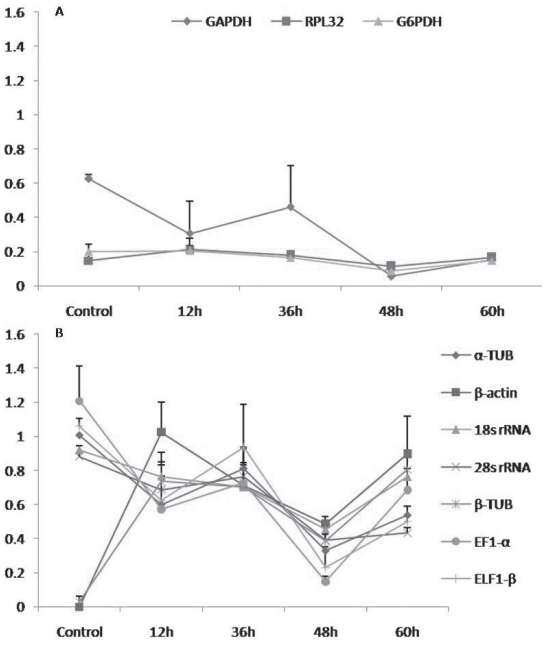

Fig. 4. Effects of low temperature stress on reference gene mRNA expression in *Bactrocera (Tetradacus) minax*. A: represents the candidate reference genes. B: represents the differentially regulated genes. Temperature stress of 4 °C was applied for 12 h, 36 h, 48 h and 60 h, respectively. The results were expressed as means ± SEM. Differences were considered significant when *P*-values were ≤ 0.05.

0.498; G6PDH: $F_{4,14} = 3.423$, $P = 0.052$). There was a significant difference in mRNA expression levels of α-TUB, β-actin, 18s rRNA, 28s rRNA, β-TUB, EF1-α and ELF1-β mRNA at 4 °C for different time periods (α-TUB: $F_{4,14} = 7.694$, $P = 0.004$; β-actin: $F_{4,14} = 6.189$, $P = 0.009$; 18s rRNA: $F_{4,14} = 4.929$, $P = 0.019$; 28s rRNA: $F_{4,14} = 7.227$, $P = 0.005$; β-TUB: $F_{4,14} = 8.526$, $P = 0.003$; EF1-α: $F_{4,14} = 5.316$, $P = 0.015$; ELF1-β: $F_{4,14} = 4.022$, $P = 0.034$). (Fig. 4).

Comparative Expression Levels of Endogenous Reference Genes under Γ-irradiation Stress Conditions

α-TUB, EF1-α, 18s rRNA and 28s rRNA mRNA were not differentially expressed under Γ-irradiation of 90 Gy stress (α-TUB: $F = 7.536$, $P = 0.013$; EF1-α: $F = 1.273$, $P = 0.003$; 18s rRNA: $F = 0.787$, $P = 0.016$; 28s rRNA: $F = 0.595$, $P = 0.018$) (Fig. 5). In contrast, β-TUB, β-actin, ELF1-β, GAPDH, G6PDH and RPL32 were differentially expressed under the Γ-irradiation stress condition (β-TUB: $F = 7.405$, $P = 0.347$; β-actin: $F = 0.352$, $P = 0.979$; ELF1-β: $F = 4.996$, $P = 0.112$; GAPDH: $F = 1.714$, $P = 0.931$; G6PDH: $F = 4.049$, $P = 0.081$; RPL32: $F = 6.330$, $P = 0.758$).

Discussion

qRT-PCR has proved to be an accurate and sensitive method for gene expression analysis (Andersen et al. 2004; Willems et al. 2006; Hoogewijs et al. 2008; Langnaese et al. 2008; Tatsumi et al. 2008; Bustin et al. 2009; Huis et al. 2010) and to study developmental processes in biological systems (Exposito-Rodriguez et al. 2008). RT-PCR data analysis is greatly influenced by the selection of reference genes (Bustin 2002; Vandesompele et al. 2002; Gutierrez et al. 2008), and the lack of stable reference genes can create great risks of misinterpretation of results (Perez et al. 2008). Shen et al. (2010) showed that various reference genes could be affected by different experimental conditions and result in various expression levels of the target genes. Therefore, it is critical to use an appropriate reference gene for expression profiling analysis when using the qRT-PCR technique. Also, Bustin et al. (2009) showed that normalization was an essential component of a reliable qPCR assay, and the use of reference genes as internal controls was the most common method for normalizing data. Bustin et al. (2009) also showed that reference gene mRNAs should be stably expressed under certain experiment conditions, and it is nec-

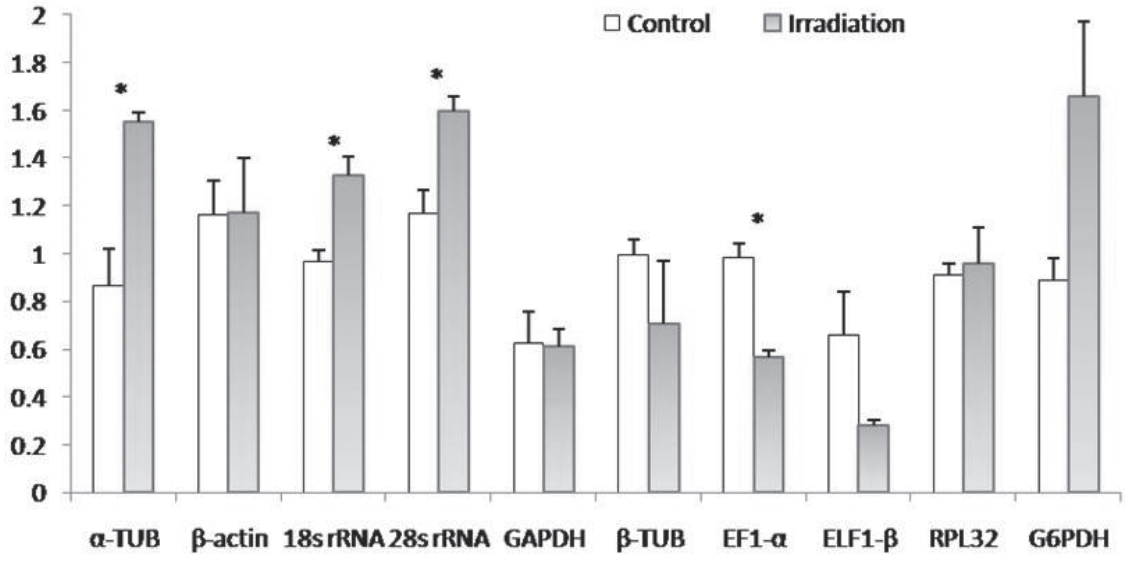

Fig. 5. Effects of γ irradiation of 90 Gy on mRNA expression of 10 reference genes in *Bactrocera (Tetradacus) minax*. The results were expressed as means ± SEM. Differences were considered significant when *P*-values were ≤ 0.05, and are so indicate by an asterisk (*).

essary to use multiple reference genes to normalize data in qPCR assay. In the present study, we selected a set of stably expressed genes, and the set of genes would vary depending on various experimental conditions, including development stage, stress by different temperatures and γ-irradiation stress. Our results provide the information on multiple reference genes for normalizing qPCR experiments in *B. minax*.

In the present study, we searched GenBank and downloaded 7 commonly used reference genes sequences (α-TUB, β-actin, RPL32, β-TUB, ELF1-β, G6PDH and EF1-α) from which we designed primers to amplify target sequences. In addition, the other 3 reference genes (28srRNA, 18s rRNA and GAPDH) were amplified by using the reported primers sequences (Martinez & Denlinger 2008; Shen et al. 2010). All 10 reference genes were amplified by the corresponding sequence fragments from *B. minax* (Fig. 1). The ideal reference genes of *B. minax* during different development stageswere α-TUB, RPL32 and EF1-α (Fig. 2), and the development stages consisted of eggs, 3rd instars, 1-, 90- and 160-day old pupae, and newly emerged female and male adults (< 24 h).

To identify and validate housekeeping genes in the brains of the desert locust, *Schistocerca gregaria* Forsskål (Acrididae) under various developmental conditions, Van Hiel et al. (2009) reported that EF1-α was one of the most stable genes in fifth instar and adult locusts. Horňáková et al. (2010) reported that EF1-α was stably expressed in different life stages of *Bombus terrestris* (L.) (Apidae) and *B. lucorum* (L.), and also suggested that EF1-α may suffice as a normalization gene for use with different life stages of other bumble bee species. Additionally, it was shown that EF1-α was one of the ideal reference genes in response to various abiotic factors in *Panonychus citri* (Acari: Tetranychidae) (Niu et al. 2012) and *Bemisia tabaci* (Gennadius) (Aleyrodidae) (Li et al. 2013).

Su et al. (2013) showed that α-TUB was a suitable reference gene across developmental stages of *B. tabaci*, but that α-TUB showed the highest variable expression for 3 whitefly organs, i.e., gut, salivary gland and ovary. In addition, for *P. citri*, EF1-α and α-TUB could be the most stable reference genes for normalization in gene expression studies in various development stages including the egg, larva, nymph and adult female and male (Niu et al. 2012). While, α-TUB was detected and regarded as a suitable reference gene under insecticide stress and bacterium-treatment conditions, α-TUB was not suitable as a reference gene for different organs in *B. tabaci* (Su et al. 2013). In addition, α-TUB was an appropriate reference gene for gene expression profiling in the midgut, Malpighian tubules and the fat body of *Bactrocera dorsalis* (Hendel) (Tephritidae) for determining differences between males and females in gene expression in these tissues (Shen et al. 2010).

Our results showed that β-actin, GAPDH, β-TUB, RPL32, G6PDH and ELF1-βstably expressed under 35 °C temperature stress for 0 h, 1 h, 3 h and 5 h. Likewise GAPDH, RPL32 and G6PDH stably expressed under 4 °C low temperature stress. Niu et al. (2012) showed that in *P. citri* α-TUB and GAPDH were stably-expressed

reference genes under various abiotic stress conditions, such as thermal- and UV-irradiation. In contrast, β-actin had various expression levels and was an unsuitable reference gene in *P. citri* under various abiotic stress conditions. GAPDH could be regarded as a suitable reference gene across *P. citri* developmental stages (Niu et al. 2012), and in *B. tabaci* under bacterium-treated conditions (Su et al. 2013). Su et al. (2013) showed that RPL32 was equally expressed in *B. tabaci* salivary glands and in the whole body, and was a suitable endogenous reference gene in various organs. However under temperature and insecticide stress conditions, GAPDH was not an ideal reference gene for *B. tabaci* (Li et al. 2013). Shen et al. (2010) showed that β-TUB should be regarded as a candidate reference gene in studying different tissues and sex gene expression profiling of *B. dorsalis*, but that GAPDH and G6PDH expression were variable, and they were unsuitable reference genes in various tissues and between the sexes. Scharlaken et al. (2008) showed that β-actin was stably expressed in the heads of bacterium-challenged honeybees, *Apis mellifera,* but β-actin was not stably expressed in fungus-infected *Tribolium castaneum* (Herbst) (Tenebrionidae) (Lord et al. 2010). In contrast, Ponton et al. (2011) showed that GAPDH and RPL32 were unsuitable reference genes of for *Drosophila melanogaster* Meigen (Drosophilidae) under heat shock stress conditions, whereas 18S could be considered as an appropriate reference gene under heat-stressed conditions for *D. melanogaster*.

We found that many of the candidate reference genes in *B. minax* should not be used as default reference genes because their expression is highly variable under certain conditions. Our results indicated that the most stable candidate reference gene was RPL32 in the eggs, 3rd instars, 1-, 90- and 160-day-pupae, and newly emerged female and male adults (< 24 h), and under different stress conditions, i.e., 35 °C temperature stress for 0 h, 1 h, 3 h and 5 h, and 4 °C temperature stress for 0 h, 12 h, 36 h, 48 h and 60 h. GAPDH, G6PDH and RPL32 were ideal candidate endogenous genes under 35 °C temperature stress for 0 h, 1 h, 3 h and 5 h, and 4 °C temperature stress for 0 h, 12 h, 36 h, 48 h and 60 h, and under a Γ irradiation stress of 90 Gy. Our results provided basic information and candidate reference genes for further study of gene expression profiles and gene function in various development stages and under various temperature stress conditions in *B. minax*. Further our results should serve as a resource to screen reference genes for gene expression studies in other insect species.

Acknowledgments

We thank Fu-Lian Wang for her help in the collection of insects. This research project was supported by the National Basic Research and Development Program (Grant No. 2009CB119200), the Ministry of Science and Technology, China, and Common Wealth Special Fund for the Agricultural Industry (No. 200903047).

References Cited

Andersen, C. L., Jensen, J. L., and 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 Res. 64: 5245-5250.

Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol.Endocrinol. 29: 23-39.

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

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

Butte, A. J., Dzau, V. J., and Glueck, S. B. 2001. Further defining housekeeping, or "maintenance," genes Focus on "A compendium of gene expression in normal human tissues". Physiol. Genomics 7(2): 95-96.

Exposito-Rodriguez, M., Borges, A. A., Borges-Perez, A., and Perez, J. A. 2008. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 8:131.

Folkersen, L., Kurtovic, S., Razuvaev, A., Agardh, H. E., Gabrielsen, A., and Paulsson-Berne, G. 2009. Endogenous control genes in complex vascular tissue samples. BMC Genomics 10: 516.

Ginzinger, D. G. 2002. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Exp. Hematol. 30: 503-512.

Gutierrez, L., Mauriat, M., Guenin, S., Pelloux, J., Lefebvre, J. F., Louvet, R., Rusterucci, C., Moritz, T., Guerineau, F., Bellini, C., and Wuytswinkel, O. V. 2008. The lack of a systematic validation of reference genes: A serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. J. Plant Biotech. 6: 609-618.

Hong, S. Y., Seo, P. J., Yang, M. S., Xiang, F., and Park, C. M. 2008. Exploring valid reference genes for gene expression studies in *Brachypodium distachyon* by real time PCR. BMC Plant Biol. 8: 112.

Hoogewijs, D., Houthoofd, K., Matthijssens, F., Vandesompele, J., and Vanfleteren, J. R. 2008. Selection and validation of a set of reliable reference genes for quantitative sod gene expression analysis in *C. elegans*. BMC Mol. Biol. 9: 9.

Horňáková, D., Matoušková, P., Kindl, J., Valterová, I., and Pichová, I. 2010. Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. Anal. Biochem. 397: 118-120

Huis, R., Hawkins, S., and Neutelings, G. 2010. Selection of reference genes for quantitative gene expres-

sion normalization in flax (*Linum usitatissimum* L.). BMC Plant Biol. 10: 71.

KUBISTA, M., ANDRADE, J. M., BENGTSSON, M., FOROOTAN, M., JONA´K J, LIND, K., SINDELKA, R., SJÖBACK, R., SJÖGREEN, B., STRÖMBOM, L., STÅHLBERG, A., AND ZORIC, N. 2006. The real-time polymerase chain reaction. Mol. Asp. Med. 27: 95-125.

LANGNAESE, K., JOHN, R., SCHWEIZER, H., EBMEYER, U., AND KEILHOFF, G. 2008. Selection of reference genes for quantitative real-time PCR in a rat asphyxial cardiac arrest model. BMC Mol. Biol. 9: 53.

LI, R., XIE, W., WANG, S., WU, Q., YANG, N., YANG, X., PAN, H. P., ZHOU, X. M., BAI, L. Y., XU, B. Y., ZHOU, X. G., AND ZHANG, Y. J. 2013. Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). PLoS ONE 8(1): e53006.

LORD, J. C., HARTZER, K., TOUTGES, M., AND OPPERT, B. 2010. Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. J. Microb. Method. 80: 219-221.

MARTINEZ, G. L., AND DENLINGER, D. L. 2008. Regulation of heat shock proteins in the apple maggot *Rhagoletis pomonella* during hot summer days and overwintering diapause. Physiol. Entomol. 33(3): 346-352.

NIU, J. Z., DOU, W., DING, T. B., YANG, L. H., SHEN, G. M., AND WANG, J. J. 2012. Evaluation of suitable reference genes for quantitative RT-PCR during development and abiotic stress in *Panonychus citri* (McGregor) (Acari: Tetranychidae). Mol. Biol. Rep. 39: 5841-5849.

PEREZ, R., TUPAC-YUPANQUI, I., AND DUNNER, S. 2008. Evaluation of suitable reference genes for gene expression studies in bovine muscular tissue. BMC Mol. Biol. 9: 79.

PFAFFL, M. W., TICHOPAD, A., PRGOMET, C., AND NEUVIANS, T. P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotech. Lett. 26: 509-515.

PONTON, F., CHAPUIS, M. P., PERNICE, M., SWORD, G. A., AND SIMPSON, S. J. 2011. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. J. Insect Physiol. 57: 840-850.

RADONIC´, A., THULKE, S., MACKAY, I. M., LANDT, O., SIEGERT, W., AND NITSCHE, A. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem.Biophys. Res. Com. 313: 856-862.

SCHARLAKEN, B., DE GRAAF, D. C., GOOSSENS, K., BRUNAIN, M., PEELMAN, L. J., AND JACOBS, F. J. 2008. Reference gene selection for insect expression studies using quantitative real-time PCR: the head of the honeybee, *Apismellifera*, after a bacterial challenge. J. Insect Sci. 8: 33.

SHEN, G. M., JIANG, H. B., WANG, X. N., AND WANG, J. J. 2010. Evaluation of endogenous references for gene expression profiling in different tissues of the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae). BMC Mol. Biol. 11: 76

SU, Y. L., HE, W. B., WANG, J., LI, J. M., LIU, S. S., AND WANG, X. W. 2013. Selection of endogenous reference genes for gene expression analysis in the mediterranean species of the *Bemisia tabaci* (Hemiptera: Aleyrodidae) complex. J Econ. Entomol. 106(3): 1446-1455.

SUZUKI, T., HIGGINS, P. J., AND CRAWFORD, D. R. 2000. Control selection for RNA quantitation. BioTechniques 29: 332-337.

TATSUMI, K., OHASHI, K., TAMINISHI, S., OKANO, T., YOSHIOKA, A., AND SHIMA, M. 2008. Reference gene selection for real-time RT-PCR in regenerating mouse livers. Biochem. Biophys. Res. Com. 374: 106-110.

THELLIN, O., ZORZI, W., LAKAYE, B., COUMANS, B., HENNEN, G., GRISAR, T., LGOUT, A., AND HEINEN, E. 1999. Housekeeping genes as internal standards: use and limits. J. Biotech. 75: 291-295.

THORREZ, L., VAN DEUN, K., TRANCHEVENT, L. C., VAN LOMMEL, L., ENGELEN, K., MARCHAL, K., MOREAU, Y., VAN MECHELEN, I., AND SCHUIT, F. 2008. Using ribosomal protein genes as reference: A tale of caution. PLoS One 3(3): e1854.

TUNBRIDGE, E. M., EASTWOOD, S. L., AND HARRISON, P. J. 2011. Changed relative to what? Housekeeping genes and normalization strategies in human brain gene expression studies. Biol. Psychiatry 69: 173-179.

VAN GUILDER, H. D., VRANA, K. E., AND FREEMAN, W. M. 2008. Twenty-five years of quantitative PCR for gene expression analysis. Biotech 44: 619-626.

VAN HIEL, M. B., VAN WIELENDAELE, P., TEMMERMAN, L., VAN SOEST, S., VUERINCKX, K., HUYBRECHTS, R., BROECK, J. V., AND GERT, S. 2009. Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. BMC Mol. Biol. 10: 56.

VAN SCHOUBROECK, F. 1999. Learning to fight a fly: developing citrus IPM in Bhutan. PhD thesis, Wageningen University and Research Centre, Wageningen, The Netherlands. 200 pp.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., ROY, N. V., DE PAEPE, A., AND SPELEMAN, F. 2002. Accurate normalization of real time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Gen. Biol. 3: 1-12.

WANG, X. J., AND LUYI, L. 1995. Research progress in the Chinese citrus fruit fly. Entomol. Knowledge 32: 310-315.

WILLEMS, E., MATEIZEL, I., KEMP, C., CAUFFMAN, G., SERMON, K., AND LEYNS, L. 2006. Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells. Intl. J. Dev. Biol. 50: 627-635.

YANG, P., CAREY, J. R., AND DOWELL, R. V. 1994. Tephritid fruit flies in China: historical background and current status. Pan-Pacific Entomol. 70: 159-167.

ZHOU, X. G., TARVER, M. R., BENNETT, G. W., OI, F. M., AND SCHARF, M. E. 2006. Two hexamerin genes from the termite *Reticulitermes flavipes*: sequence, expression, and proposed functions in caste regulation. Gene 376: 47-58.