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Screening of reference genes using real-time quantitative PCR for gene expression studies in *Neoseiulus barkeri* Hughes (Acari: Phytoseiidae)

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Abstract

A stable reference gene is a key prerequisite for accurate assessment of gene expression. At present, the real-time reverse transcriptase quantitative polymerase chain reaction has been widely used in the analysis of gene expression in a variety of organisms. *Neoseiulus barkeri* Hughes (Acari: Phytoseiidae) is a major predator of mites on many important economically crops. Until now, however, there are no reports evaluating the stability of reference genes in this species. In view of this, we used GeNorm, NormFinder, BestKeeper, and RefFinder software tools to evaluate the expression stability of 11 candidate reference genes in developmental stages and under various abiotic stresses. According to our results, β-*ACT* and *Hsp40* were the top two stable reference genes in developmental stages. The *Hsp60* and *Hsp90* were the most stable reference genes in various acaricides stress. For alterations in temperature, *Hsp40* and α-*TUB* were the most suitable reference genes. About UV stress, *EF1*α and α-*TUB* were the best choice, and for the different prey stress, β-*ACT* and α-*TUB* were best suited. In normal conditions, the β-ACT and α-TUB were the two of the highest stable reference genes to respond to all kinds of stresses. The current study provided a valuable foundation for the further analysis of gene expression in *N. barkeri*.

Keywords: RT-qPCR, *Neoseiulus barkeri*, reference genes, gene expression

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Introduction

Fluorescence-based real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a type of nucleic acid quantification technology developed on the basis of conventional PCR and realized a leap from the qualitative to quantitative analysis. As we all know, it has been widely used in the fields of molecular diagnostics, life sciences, agriculture, and medicine (Heid *et al*., 1996; Kubista *et al*., 2006; Bustin *et al*., 2009). It not only achieves a rapid, sensitive, specific, and efficient detection of nucleic acids, but also accurately quantifies the initial dose of target genes. Recently, RT-qPCR has become a major technology and an effective tool for gene expression analysis due to its sensitivity, accuracy, and timeliness (Pfaffl, 2001; Ginzinger, 2002; Bustin *et al*., 2005; VanGuilder *et al*., 2008; Schmittgen & Livak, 2008). RT-qPCR provides a relatively simple and intuitive method to assess correlations of development and under stress tolerance conditions with transcriptional regulation (Snell *et al*., 2003; De Boer *et al*., 2011; Tabunoki *et al*., 2011). Thus, in short, it is a key method for the quantitative detection of mRNA expression at the transcriptional level (Bustin, 2002).

To accurately determine the expression levels of target genes, reference genes need to be standardized to eliminate the biases of quality and yield of RNA in different samples. Moreover, to obtain precise differences in the expression of target genes, standardization is necessary to correct the transcription efficiency and the amount of cDNA templates (Ginzinger, 2002; Huggett *et al*., 2005; Steinau *et al*., 2006;

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Derveaux *et al.*, 2010; Tunbridge *et al.*, 2011). Actin, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), tubulin, and elongation factor-1α (EF1α) have frequently been used as reference genes in some studies (Scharlaken *et al.*, 2008; Shen *et al.*, 2010; Chapuis *et al.*, 2011; Feng *et al.*, 2013). Ideal reference genes are stably expressed in organisms, tissues, and cells, irrespective of the developmental stage or various stresses (Radonić *et al.*, 2004; Li *et al.*, 2013). However, different reference genes may lead to different results in the standardization of the expression levels of the target genes, primarily because the expression levels of reference genes are, in fact, not identical in different tissues or under different experimental conditions, for example, among various cell types, developmental stages, feeding conditions, acaricide exposure, or other environmental stresses (Jiang *et al.*, 2010; Ponton *et al.*, 2011; Niu *et al.*, 2012; Peng *et al.*, 2012). In addition, using a single reference gene as a standard may lead to increased errors, whereas the use of at least 2–3 reference genes for standardization can avoid these errors (Thellin *et al.*, 1999; Vandesompele *et al.*, 2002; Chervoneva *et al.*, 2010; Li *et al.*, 2013). A stable reference gene is the most important and the basic condition for real-time quantitative analysis (Shen *et al.*, 2013). Therefore, in the use of RT-qPCR technology to research the expression levels of target gene, a systematic analysis is needed according to various experimental conditions, that is, to determine appropriate and stable reference genes with increasing the accuracy of the test results for RT-qPCR.

Neoseiulus barkeri Hughes (Acari, Parasitiformes, Gamasid, Phytoseiidae, Neoseiulus) inhabits plants such as *Citrus*, *Fructus Caricae* and *Artemisia annua* L. and their stored products. This predatory mite has a variety of feeding habits, with its natural prey including spider mites, whiteflies and thrips, and it is widely distributed across China, Japan, Thailand, and the USA, as well as in some countries of South Africa and Europe (Hessein & Parrella, 1990; Moraes *et al.*, 2004; Xia *et al.*, 2012). Because *N. barkeri* has a short development stage, low natural mortality, high spawning rate, and strong ability to spread, it is considered to be one of the best biological control products (Bonde, 1989). Previous studies showed that environmental conditions such as temperature, illumination, and acaricide significantly influence the growth and reproduction of this mite; however, the acaricide resistance is also easily developed in mites (Petrushov, 1992; Veerman, 1992; Xia *et al.*, 2012). To conduct in-depth studies on the effects of external conditions and food on the growth and reproduction of *N. barkeri* and to clarify the molecular mechanisms for the development of acaricide resistance in this mite, it is necessary to analyze the expression levels of related functional genes. Although, as discussed above, stable reference genes play a key role in accurate gene expression analysis; now, no reports examining reference genes for *N. barkeri* are available. In view of this, the present study evaluated the stability for the expression of 11 commonly used candidate reference genes in *N. barkeri* at different developmental stages and under various stresses using RT-qPCR; thus, we provide empirical evidence for selecting optimal reference genes for use in quantitative gene analysis studies of *N. barkeri*.

Materials and methods

Origin and rearing of mites

The *N. barkeri* were provided by the Citrus Research Institute at the Chinese Academy of Agricultural Sciences. It is derived from the leaves of *Citrus sinensis* Osbeck which was drug-free in citrus orchards and fed *Aleurolyphus ovatus* mixture with a sterilized wheat bran and maintained a surrounding that was at 25 ± 1°C with a relative humidity of 80 ± 5% in a 14:10 h light:dark cycle in the laboratory for 3 years. During these years, it did not contact any acaricide that is an ideal experimental material.

Special culture dishes were prepared by overlaying sponges, filter paper, and the leaves of *C. sinensis* Osbeck. Water was injected to be made from water-isolated platforms. The leaves were surrounded with cotton thread to prevent the mites from escaping.

Individual feeding rooms were prepared using a 2.5 cm × 2.5 cm glass plate containing a groove with a diameter of 1.5 cm. Circular leaves of *C. sinensis* Osbeck with a diameter of 1.0 cm were placed in it. Hatched mites were placed on the leaves and covered with coverslips. The coverslips were fixed with rubber bands to prevent the mites from escaping.

Developmental stages

A large number (10,000 or more) of adult *N. barkeri* without eggs were fed artificial diets. Their eggs were collected 2 h later and placed in culture dishes (prepared as described above). The purpose of this step is to ensure all the eggs come from the same period. After these eggs hatched, the larval mites were collected. The nymphs and adult mites were collected when they are still larval mites. Three hundred female adults were selected at each developmental stage, frozen in liquid nitrogen, and immediately placed at −80°C. Each treatment was performed three times.

Acaricide stress

Avermectin (1.8% emulsifiable concentrate, Liuzhou Huinong Chemical Co. Ltd Liuzhou, Guangxi, China), fenpropathrin (20% emulsifiable concentrate, Sumitomo Chemical Co. Ltd, Japan) and chlorpyrifos (48% emulsifiable concentrate, Jiangsu Bailing Agrochemical Co. Ltd, Jiangyin, Jiangsu, China) were used for the experiments. They are commonly used acaricides in field experiment. Avermectin and fenpropathrin were diluted 1000, 2000, and 4000 times and chlorpyrifos was diluted 500, 1000, and 2000 times with water. The middle concentrations are also commonly used in field. *N. barkeri* was treated at each concentration of each acaricide for 1, 24, and 48 h. Adult mites were placed in prepared culture devices for 5 s of pharmaceutical treatment. After the treatment, 300 surviving female mites were collected, frozen in liquid nitrogen, and placed at −80°C. Each treatment was performed three times. Mites handled similarly but treated with clear water instead of an acaricide served as controls.

Temperature stress

The mites were placed in incubators set at 35 and 4°C for 1, 2, and 4 h consecutively. The processing temperature can cause a higher mortality of mites. After the temperature treatments, 300 surviving female adults were immediately frozen in liquid nitrogen and placed at −80°C. Each treatment was performed three times. Control mites did not receive temperature stress. Culture dishes for treating mites were prepared as described above.

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UV irradiation stress

Adult mites were placed in culture dishes and subjected to 260 nm UV irradiation treatments for 1, 2, and 4 h consecutively. After UV treatments, 300 surviving female mites were immediately frozen in liquid nitrogen and placed at −80°C. Each treatment was performed three times. Mites not receiving UV treatments served as controls.

Prey stress

The eggs collected as is shown in developmental stages section. After these eggs hatched, the larval mites were placed into individual feeding rooms containing sufficient numbers of larval *Panonychus citri* and *Eotetranychus kankitus* Ehera, respectively, until the next generation of *N. barkeri* was produced. The *P. citri* and *E. kankitus* are the main pest in citrus orchard and are the favorite prey of *N. barkeri*. Surviving female mites ($n = 300$) were collected, immediately frozen in liquid nitrogen and kept at −80°C. Each treatment was performed three times. Mites reared in the absence of the prey served as controls.

RNA extraction and cDNA synthesis

For RNA extraction, an RNeasy plus Micro kit (Qiagen, Germany) was used according to the manufacturer's instructions, and the genomic DNA was removed via a DNA elimination step which is supplied by the kit. A NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis were used to evaluate the RNA quality. Qualified samples were stored at −80°C.

The obtained RNA was reverse-transcribed into cDNA using a PrimeScript® RT reagent Kit (Takara, Japan) according to the manufacturer's instructions. The mixtures were placed in a professional thermocycler (Biometra, Germany) for reactions at 37°C for 15 min and at 85°C for 5 s. After the reverse transcription was completed, the synthesized cDNA was stored at −20°C.

Reference gene selection and primer design

A total of 11 candidate reference genes (http://www.ncbi.nlm.nih.gov/nuccore/ KP310113-KP310123) were evaluated, including *EF1α*, *tubulin α* (*α-TUB*), *β-actin* (*β-ACT*), *28S ribosomal RNA* (*28S rRNA*), *RNA polymerase II subunit* (*RNAP II*), *heat shock protein 90 (Hsp90)*, *Hsp70*, *Hsp60*, *Hsp40*, *GAPDH*, and *ubiquitin conjugating enzyme* (*UBC*). Amplification primers were designed based on the transcriptome database of *N. barkeri* in our laboratory using Primer 3.0, and the specificity of the primers was analyzed using the melting curves from RT-qPCR.

Real-time quantitative PCR

All real-time quantitative operations in the present experiment were performed using an iCycler iQ Multicolor Real-Time PCR Detection System (Bio-RAD, USA). A 20.0 µl reaction system was used for RT-qPCR, including 2 µl of diluted cDNA, 1 µl of the forward and reverse primers, 10.0 µl of SYBR Premix Ex Taq II (TaKaRa, Japan), and 6.0 µl of ddH2O. The reaction conditions were as follows: predegeneration at 95°C for 2 m; followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. Upon completion of the amplification program, 60–95°C was performed to ensure the specificity of the amplified product. Then, a threefold dilution series of cDNA was used to construct the standard curve and to determine the efficiency of various primers for PCR amplification. The results showed that the amplification efficiencies of the 11 candidate reference genes were 91.10–115.70%, and the coefficients of determination were between 0.994 and 0.997. This indicated that the primers of these genes were consistent with the requirements of RT-qPCR analysis. The gene accession numbers, primer sequences, amplification lengths, amplification efficiencies, and coefficients of determination are presented in Table 1.

Data analyses

Analysis of the expression stability for each candidate reference gene was conducted using GeNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), and BestKeeper (Pfaffl *et al.*, 2004) as well as the online tool RefFinder (Xie *et al.*, 2011) (http://www.leonxie.com/referencegene.php). The GeNorm software was used to determine the *M* value of expression stability for the candidate reference genes. These genes whose value of $M < 0.5$ are considered to be stable. The *M* value is defined as the pairwise variation of a reference gene with all other reference genes. To get a rank, gene with the highest M value (the worst reference gene) is eliminated and the new *M* values for the other candidate genes are recalculated, until only two genes are remained as the most stable ones. For the minimum number of genes used to get accurate result, GeNorm calculates the pairwise variation $Vn/Vn+1$ which represents the variation between using n most stable genes and using $n+1$ most stable genes. This evaluation uses 0.150 as the cut-off, below which the inclusion of an additional reference gene is not required, that is, n reference genes are sufficient for accurate normalization (Feng *et al.*, 2013). NormFinder software was used for comparisons based on calculated stability values, the lower stability values the more stable. BestKeeper software analysis was based on the C_t values of the candidate reference genes of the samples in each group and the calculated standard deviation (SD). The gene with the lower value of SD is considered to be more stable. The online RefFinder analysis integrated these three analytical methods to obtain a comprehensive ranking index. The gene with a smaller index value indicates a higher stability.

Results

Expression profiles of candidate reference genes

The expression levels of these candidate reference genes were great difference in all samples that the C_t values ranged from 14.30 to 35.40 (Table S1). In addition, the variation of C_t values of the candidate reference genes in this study was exhibited by the box plot graph (fig. 1). It can be seen from the figure that the median C_t values were reflected from lowest 18.43 (*EF1α*) to highest 26.44 (*Hsp40*), with a large variation. The *EF1α* was considered to be a highly expressed gene since its median C_t value is lesser than 20. Moreover, the gene owned the lowest ranges of C_t value. However, the genes with higher C_t ranges than others were *Hsp90*, *Hsp70*, *Hsp60*, especially *Hsp70*, in which Delta-C_t value reached 17.58. Thus, these data imply that the expression levels for genes might be more easily affected by external environment.

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Table 1. Details of the 11 candidate reference genes of *N. barkeri* for real-time qPCR.

Gene name	GenBank accession number	Primer sequences (forward/reverse)	Amplification length (bp)	PCR efficiency (%)	R^2
EF1α	KP310113	CACGCTCTTCTCGCCTACAC CGGAAATCGGGACGAAGGGA	178	99.10	0.996
α-TUB	KP310114	CGTACTGGACCGTATTCGCA GATCCAGTTCCACCTCCGAA	90	91.10	0.997
β-ACT	KP310115	TACGACCAGAAGCGTACAGC CCAACCGTGAAAAGATGACC	102	103.40	0.995
28S rRNA	KP310116	ACATTGTTAGCTATTTCGGA TGTTTTAATTAGACAGTCGGA	99	115.70	0.996
RNAP II	KP310117	TCCCCAGCAGTTCCTTTCAA TTGCCGACTTCACAGTTTGG	171	104.80	0.999
Hsp90	KP310118	CATTGTTTCCAACCGTCTCG GGCCTTCATGATACGCTCCA	91	94.40	0.994
Hsp70	KP310119	ACAAGTCTTCCGGCAAGCAG ACTTCTCGGCTTCGTTCACC	99	95.90	0.996
Hsp60	KP310120	CTTTCGCCACGGTTACACCA TTGATATCCTTGCCGATGCT	117	96.50	0.994
Hsp40	KP310121	AAAGACCGATCAGGACCCTC TGCATTTGATATCGCCGTGT	87	91.80	0.997
GAPDH	KP310122	ACGGTAGTCATCAGACCCTC ACAGCAGCATGAACATCGTC	112	93.20	0.995
UBC	KP310123	ATGAAACCCCGCCCTACCTG TTCCCATAGGCCGTCACTCG	101	101.50	0.995

R^2, coefficient of determination.

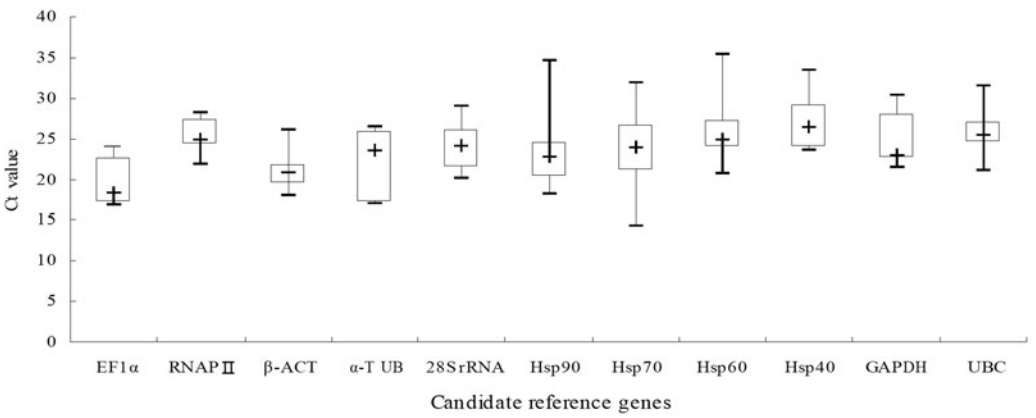

Fig. 1. C_t value of 11 candidate reference genes in all experiment stress conditions for *N. barkeri*.

But only via the raw C_t values, the evaluation of the expression stability of candidate reference is meaningless. So, we will utilize different statistical algorithms to validation.

Analysis of expression stability for candidate reference genes

The 11 candidate reference genes of *N. barkeri* in developmental stages were analyzed by GeNorm. The results showed that the *M* values of the *Hsp40* and *Hsp90* genes were less than 0.5 which indicated that these genes could be used as reference genes. The BestKeeper analysis showed that the SD of all genes was >1.0, which was incompatible with the requirements of a single reference gene. However, the α-*TUB* and β-*ACT* genes were relatively stable. The NormFinder indicated that β-*ACT* and *Hsp70* were the most stable genes. As shown in Table 2, the stability of the 11 candidate reference genes was not high throughout developmental stages in the mites. In addition, the optimal number of genes required for accurate normalization in RT-qPCR was also presented by GeNorm, through calculating pairwise variation $Vn/n+1$ which uses 0.150 as the proposed cut-off value. A $Vn/n+1$ <0.150 means that the top *n* reference genes are adequate for accurate RT-qPCR normalization. Here the V2/V3 value was <0.150 (fig. 2a), thus the top two reference genes would be adequate in RT-qPCR normalization for developmental stages in *N. barkeri*, and the addition of the third gene is not necessary. So, according to the rank of RefFinder and pairwise variation values of the GeNorm (fig. 2a), β-*ACT* and *Hsp40* were recommended as reference genes in various developmental stages in *N. barkeri*.

The 11 candidate reference genes of *N. barkeri* exposed in the acaricides analyzed by GeNorm showed that the mean stability values (*M* values) from the lowest to the highest was: *RNAP II* = *Hsp40* > *Hsp90* > *Hsp60* > *GAPDH* > *Hsp70* > *UBC* > *EF1α* > β-*ACT* > α-*TUB* > *28S rRNA*. These results indicated that the *RNAP II* and *Hsp40* genes were most stable, followed

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Table 2. The expression stability ranking of candidate reference genes of *N. barkeri* in developmental stages.

	BestKeeper		NormFinder		GeNorm		RefFinder	
Ranking	Gene	SD	Gene	Stability value	Gene	*M* value	Gene	Ranking values
1	α-TUB	1.56	β-ACT	0.784	Hsp40	0.233	β-ACT	2.11
2	β-ACT	2.23	Hsp70	0.807	Hsp90	0.233	Hsp60	2.78
3	RNAP II	2.86	28S rRNA	1.025	Hsp60	0.681	Hsp40	2.99
4	Hsp40	2.89	Hsp60	1.118	28S rRNA	1.119	Hsp90	3.83
5	Hsp60	3.02	Hsp40	1.525	β-ACT	1.328	28S rRNA	4.24
6	Hsp90	3.04	Hsp90	1.674	Hsp70	1.565	Hsp70	4.53
7	Hsp70	3.13	RNAP II	2.170	RNAP II	1.804	α-TUB	5.62
8	UBC	3.15	UBC	2.914	GAPDH	2.058	RNAP II	5.66
9	28S rRNA	3.36	GAPDH	3.170	UBC	2.435	UBC	8.49
10	GAPDH	4.76	α-TUB	4.050	α-TUB	2.803	GAPDH	8.71
11	EF1α	5.50	EF1α	6.003	EF1α	3.435	EF1α	11.00

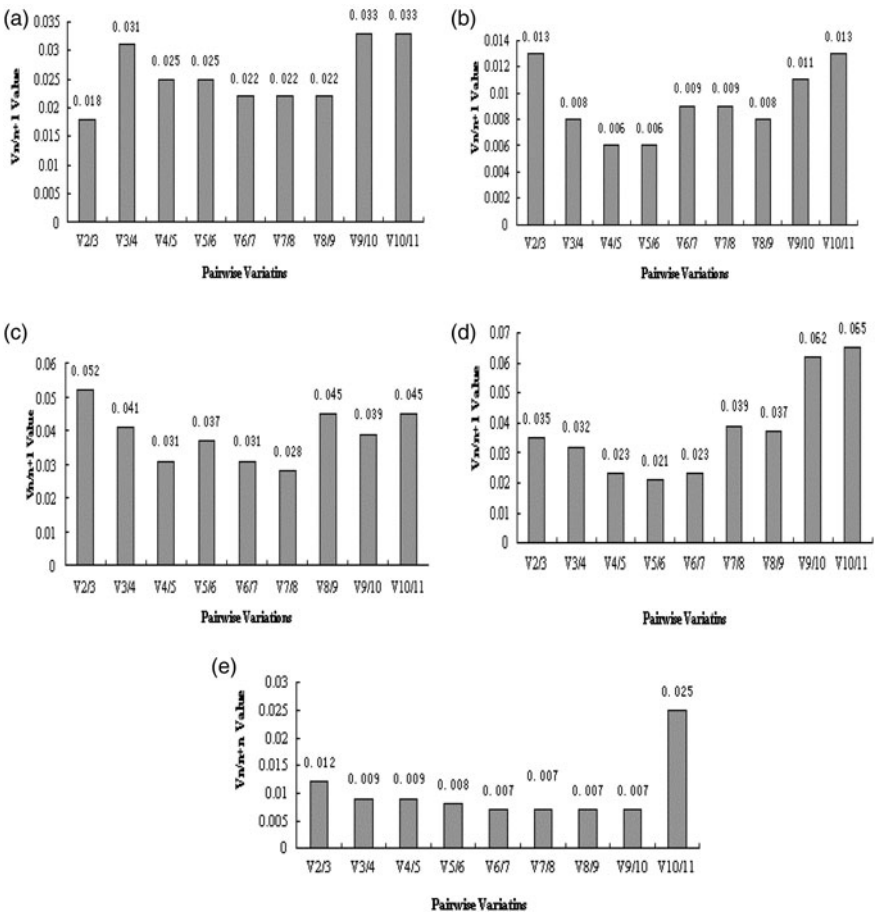

Fig. 2. Optimal number of reference genes for pairwise variation analysis (V*n*/*n* + 1) of *N. barkeri* under different experimental conditions by GeNorm. (a) Developmental stages; (b) acaricides stress; (c) temperature stress; (d) UV irradiation stress; (e) prey stress.

by the *Hsp90* and *Hsp60* genes, whereas the *28S rRNA* gene was least stable. When we use NormFinder to analyze genes expression stability, it indicates that the lower the stable value the more the stability of genes. The top five genes of stable expression were *Hsp60*, *Hsp90*, *GAPDH*, *Hsp70*, and *RNAP II*, but the most stable genes were *Hsp60* and *Hsp90*. In addition, BestKeeper analysis determined that the SD of the candidate reference genes, except *28S rRNA* (SD = 1.86), was <1.0, consistent with the requirements of a single reference gene. Among them, *Hsp60* was the most stable (Table 3). According to the rank of RefFinder and pairwise variation V2/3(0.013) in GeNorm, as shown in fig. 2b, which was significantly below the critical cut-off value of 0.15, two of the candidate reference genes were suitable for RT-qPCR. Introduction of a third reference for standardization was unnecessary because it did not significantly increase the

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Table 3. The expression stability ranking of candidate reference genes of *N. barkeri* under acaricides stress.

	BestKeeper		NormFinder		GeNorm		RefFinder	
Ranking	Gene	SD	Gene	Stability value	Gene	*M* value	Gene	Ranking values
1	Hsp60	0.36	Hsp60	0.072	RNAP II	0.117	Hsp60	1.68
2	EF1α	0.40	Hsp90	0.072	Hsp40	0.117	Hsp90	2.06
3	Hsp90	0.45	GAPDH	0.505	Hsp90	0.524	RNAP II	3.76
4	β-ACT	0.58	Hsp70	0.685	Hsp60	0.544	Hsp40	3.98
5	UBC	0.61	RNAP II	0.694	GAPDH	0.564	GAPDH	4.05
6	GAPDH	0.84	Hsp40	0.777	Hsp70	0.603	EF1α	5.29
7	Hsp40	0.85	EF1α	0.899	UBC	0.772	Hsp70	5.42
8	RNAP II	0.86	UBC	0.934	EF1α	0.856	UBC	6.88
9	Hsp70	0.88	β-ACT	1.163	β-ACT	0.921	β-ACT	7.35
10	α-TUB	0.91	α-TUB	1.882	α-TUB	1.123	α-TUB	10.00
11	28S rRNA	1.86	28S rRNA	2.256	28S rRNA	1.345	28S rRNA	11.00

statistical reliability. Therefore, *Hsp60* and *Hsp90* were recommended as reference genes in studies of acaricides stress in *N. barkeri*.

For temperature stress, GeNorm showed that the *M* values from the lowest to the highest was: *Hsp40* = α-*TUB* > β-*ACT* > *EF1α* > *Hsp70* > *Hsp90* > *GAPDH* > *Hsp60* > *UBC* > *RNAP II* > *28S rRNA*. This result indicated that *Hsp40* and α-*TUB* were relatively stable. Meanwhile, the NormFinder obtained a similar result to those of the GeNorm analysis (Table 4). BestKeeper indicated that the *Hsp60* and *UBC* may be used as a single reference gene. In addition, according to the rank of RefFinder and pairwise variation V2/3(0.052) of GeNorm, as shown in fig. 2c, which was significantly below the critical cut-off value of 0.15, *Hsp40* and α-*TUB* were recommended for use as reference genes in gene expression studies with temperature stress in *N. barkeri*.

For UV irradiation stress, GeNorm analysis showed that the *M* values for all of the genes were >0.5, which were not consistent with the requirements of a single reference gene. However, *EF1α* and β-*ACT* demonstrated minimal *M* values. BestKeeper and NormFinder obtained similar results that the genes *EF1α*, β-*ACT*, and *Hsp60* were relatively stable as single reference genes (Table 5). Finally, we utilized an online analysis system RefFinder to evaluate the expression stable in UV irradiation stress. It also found out that β-ACT and *EF1α* were the most two stable genes. As shown in fig. 2d, the pairwise variation from GeNorm showed us V2/3 (0.035) which was significantly below the critical cut-off value of 0.15. Therefore, *EF1α* and β-*ACT* were recommended the most two stable reference genes under UV stress in *N. barkeri*.

For prey treatments, GeNorm analysis showed that the *M* values for genes except β-*ACT* and α-*TUB* were <0.5, which did meet the requirements of a single reference gene. However, BestKeeper pointed out the SD values for all the genes except *UBC* and *RNAP II* were <1.0, β-*ACT* and α-*TUB* owned the least stability values. As shown in Table 6, NormFinder analysis results also showed that β-*ACT* and α-*TUB* were the two most stable genes. Finally, utilizing the rank of RefFinder and pairwise variation values V2/3 (0.012) of the GeNorm (fig. 2e), β-*ACT* and *TUB* are recommended as reference genes in studies of the effects of prey stress in gene expression for *N. barkeri*.

Discussion

Identification of suitable reference genes is a very important process for studying gene expression in *N. barkeri* under different external stimuli conditions. In this study, the four evaluation methods, GeNorm (Vandesompele *et al.*, 2002), NormFinder (Andersen *et al.*, 2004), BestKeeper (Pfaffl *et al.*, 2004), and RefFinder (Xie *et al.*, 2011), were used to systematically evaluate the expression stability of 11 candidate reference genes in *N. barkeri* at different developmental stages and under abiotic stresses. Meanwhile, this work was the first systematic analysis on reference genes selection for RT-qPCR normalization in *N. barkeri* under these conditions. However, the present and previous experiments found that some of the different results generated by various software were connected with their different calculation methods. NormFinder uses the intra-group and inter-group variance to calculate the stable value, and GeNorm ranks candidate reference genes according to the highest degree of expression similarity in their expression profile. In a sense, the method of NormFinder is to cover the shortage of co-regulation for GeNorm. Whereas BestKeeper analysis is a pairwise correlational analysis based on C_t values. BestKeeper can be used to reflect the original data, but it is less effective than either GeNorm or NormFinder analysis in ranking the reference genes. Based on the stabilities it is usually used in preliminary screening. RefFinder is a web-based calculation tool comprehensively ranking the genes using the above-mentioned algorithms. By analyzing with above tools, we found that β-ACT and Hsp40 were the top two stable reference genes in different developmental stages of mites. We also concluded that Hsp60 and Hsp90 were the most stable reference genes for proper RT-qPCR analysis of gene expression under various acaricides stresses. For alterations in temperature, Hsp40 and α-TUB were the most suitable reference genes. About UV stress, EF1α and α-TUB were the best choice, and for different prey stresses, β-ACT and α-TUB were best suited. Further comprehensive analysis found that β-ACT and α-TUB were the two of the highest stable reference genes to respond to all kinds of stresses, which were recommend reference genes under different conditions. More and more evidence shows that there are no universal reference genes that are suitable for all the experimental conditions of all species. For example, *GAPDH* and *Ribosomal protein 49* were found to be the best reference genes in investigation of gene expression across both experimental conditions of *Tetranychus urticae* (Mariany *et al.*, 2016); for *Tetranychus cinnabarinus*, *ribosomal protein S18* and *5.8S ribosomal RNA* can be considered as the best reference genes in different acaricides resistant strains, RPS18 and α-*TUB* as in different developmental stages (Sun *et al.*, 2010); EF1α, *RNAP II*,α-*TUB*, and GAPDH are the most stable

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Table 4. The expression stability ranking of candidate reference genes of *N. barkeri* under temperature stress.

	BestKeeper		NormFinder		GeNorm		RefFinder	
Ranking	Gene	SD	Gene	Stability value	Gene	*M* value	Gene	Ranking values
1	Hsp60	0.27	Hsp40	0.111	Hsp40	0.223	Hsp40	1.57
2	UBC	0.69	α-TUB	0.111	α-TUB	0.223	α-TUB	2.3
3	GAPDH	1.40	β-ACT	0.122	β-ACT	0.501	β-ACT	4.12
4	Hsp90	1.65	EF1α	0.122	EF1α	0.586	Hsp70	4.4
5	Hsp70	2.36	Hsp70	0.205	Hsp70	0.712	Hsp60	4.76
6	Hsp40	2.65	Hsp90	1.716	Hsp90	1.050	EF1α	5.18
7	β-ACT	3.18	GAPDH	2.188	GAPDH	1.261	Hsp90	5.42
8	α-TUB	2.80	Hsp60	4.112	Hsp60	1.727	GAPDH	5.66
9	EF1α	3.35	UBC	5.676	UBC	2.253	UBC	6.18
10	RNAP II	8.19	RNAP II	7.945	RNAP II	3.587	RNAP II	10.00
11	28S rRNA	8.29	28S rRNA	8.096	28S rRNA	4.419	28S rRNA	11.00

Table 5. The expression stability ranking of candidate reference genes of *N. barkeri* under UV irradiation stress.

	BestKeeper		NormFinder		GeNorm		RefFinder	
Ranking	Gene	SD	Gene	Stability value	Gene	*M* value	Gene	Ranking values
1	β-ACT	0.91	UBC	1.211	EF1α	0.886	EF1α	1.57
2	Hsp90	0.93	EF1α	1.221	β-ACT	0.886	β-ACT	1.57
3	EF1α	0.98	β-ACT	1.265	Hsp90	1.212	UBC	2.99
4	Hsp60	1.00	Hsp60	1.401	UBC	1.644	Hsp90	3.66
5	UBC	1.27	28S rRNA	1.524	Hsp60	1.785	Hsp60	3.94
6	28S rRNA	1.46	Hsp90	1.605	28S rRNA	1.923	28S rRNA	5.73
7	Hsp70	1.87	α-TUB	2.607	α-TUB	2.304	α-TUB	7.24
8	α-TUB	2.51	Hsp70	4.131	Hsp70	2.808	Hsp70	7.74
9	GAPDH	2.74	GAPDH	4.354	GAPDH	3.279	GAPDH	9.00
10	RNAP II	5.21	RNAP II	5.959	RNAP II	3.876	RNAP II	10.00
11	Hsp40	5.40	Hsp40	6.406	Hsp40	4.443	Hsp40	11.00

Table 6. The expression stability ranking of candidate reference genes of *N. barkeri* under prey stress.

	BestKeeper		NormFinder		GeNorm		RefFinder	
Ranking	Gene	SD	Gene	Stability value	Gene	*M* value	Gene	Ranking values
1	β-ACT	0.19	α-TUB	0.221	β-ACT	0.441	β-ACT	1.19
2	α-TUB	0.52	β-ACT	0.221	α-TUB	0.441	α-TUB	1.41
3	28S rRNA	0.53	GAPDH	0.614	GAPDH	0.717	GAPDH	3.66
4	Hsp70	0.56	Hsp70	0.801	Hsp90	0.795	Hsp70	3.94
5	GAPDH	0.68	Hsp90	0.853	Hsp70	0.825	Hsp90	5.32
6	EF1α	0.68	Hsp60	0.981	EF1α	0.892	28S rRNA	5.86
7	Hsp40	0.78	EF1α	1.033	28S rRNA	0.935	EF1α	6.24
8	Hsp90	0.81	28S rRNA	1.122	Hsp40	1.010	Hsp60	7.90
9	Hsp60	0.81	Hsp40	1.259	Hsp60	1.106	Hsp40	8.21
10	UBC	1.09	UBC	1.418	UBC	1.210	UBC	10.00
11	RNAP II	2.93	RNAP II	4.035	RNAP II	1.739	RNAP II	11.00

reference genes in various developmental stages of *P. citri*; meanwhile, EF1α and GAPDH were the most stable reference genes under various abiotic stresses (Niu *et al.*, 2012). Furthermore, in the same species, the best reference gene sets may vary significantly under different biotic and abiotic conditions (De Boer *et al.*, 2009; Niu *et al.*, 2012; Mariany *et al.*, 2016).

EF1α is a protein factor promoting the elongation of the polypeptide chain during mRNA translation. It was identified as one of the most stably expressed reference genes in several other arthropod species, including *Apis mellifera*, *P. citri*, *Orchesella cincta*, and *Leptinotarsa decemlineata* (Lourenco *et al.*, 2008; De Boer *et al.*, 2009; Zhu *et al.*, 2011; Niu *et al.*, 2012). It is stably expressed in most organisms and under different treatment conditions, and thus is considered an ideal reference gene (Ponton *et al.*, 2011; Lopez-Pardo *et al.*, 2013). Through the use of several different calculation methods and algorithms, the present study found that *EF1α* had the least stability throughout the developmental stages of mites. Although inconsistent with most of the reports about *EF1α* relatively stable in developmental stages (Mamidala *et al.*, 2011; Bansal *et al.*, 2012; Niu *et al.*, 2012), we found that *EF1α*

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was highly stable under UV stress and moderately expressed under other treatments. Therefore, it is speculated that *EF1α* in *N. barkeri* is relatively stable under various environmental stresses. However, it is not suitable for use as a reference gene in mites across different developmental stages.

β-*ACT* is a widely used reference gene and is considered to be more stable than other internal controls in *Aphis gossypii*, *Spodoptera litura*, and *L. decemlineata* (Zhu *et al*., 2011; Lu *et al*., 2013; Ma *et al*., 2016). But, it is also proposed that β-*ACT* is not suitable as an endogenous control for gene expression analysis in some treatment conditions (Shen *et al*., 2013). Toutges *et al*. and Lord *et al*. also reported similar results for β-*ACT* (Lord *et al*., 2010; Toutges *et al*., 2010). By contrast, the present study found that β-*ACT* was most stably expressed in *N. barkeri* under various stresses. It was highly expressed at different developmental stages and under temperature, UV, and prey treatments, and therefore could function as a reference gene for study about gene expression in *N. barkeri* at different developmental stages and under various environmental stresses.

Tubulin is a widely distributed globular protein functioning as structural protein to maintain the integrity of the cytoskeletal structure (Scharlaken *et al*., 2008; De Boer *et al*., 2009). Tubulin is widely used as a reference gene in qPCR studies because it is one of the basic components of the cells (Shen *et al*., 2010; Wan *et al*., 2010). We found that the expression stability of α-*TUB* and β-*ACT* were relatively similar. They both demonstrated well stability under all various stresses, except the acaricides stress. They were the most stable genes under prey stress. Meanwhile, they were also stable reference genes in developmental stages and under various environmental stresses. However, some studies have found that α-*TUB* expression is not stable in different tissues or in different environments for some species. For example, α-*TUB* is very unstable in the lower lip line and fat body of *Bombus terrestris* and *Bombus lucorum* (Horňáková *et al*., 2010), in potato tubers under cold stress (Lopez-Pardo *et al*., 2013), and in different tissues of tilapia and under different treatments (Yang *et al*., 2013). Taken together, these observations indicate that α-*TUB* is susceptible to various factors. Therefore, the stability of α-*TUB* should be verified when it is selected as a reference gene in other species.

In the Hsp family, *Hsp60* and *Hsp90* under acaricides stress as well as *Hsp40* under temperature stress were stably expressed which can be used as reference genes for these stress conditions. The other genes of this family showed low or moderate expression under all various stresses and, therefore, were not suitable as reference genes for these stresses, especially the *Hsp70*. Our results for *Hsp70* were similar to those of De Boer *et al*. (2009) and Veazey & Golding (2011).

The *28S rRNA* demonstrated low stability under various stress conditions and was least stable under acaricides and temperature stress with C_t values of 16.45–37.79 and 14.19–37.22, respectively. The result indicated that the stability of gene expression has some difference and might not be an ideal reference gene. These findings were consistent with that of Xu *et al*. (Xu *et al*., 2014). It was also reported that *18S rRNA* and *5.8S rRNA* were unstably expressed under specific experimental conditions (Bas *et al*., 2004; Mehta *et al*., 2010; Niu *et al*., 2012; Li *et al*., 2013). Shen *et al*. reported in 2013 that the rRNA genes might not be the best choice as reference genes and suggested that if rRNA is used as a reference gene to standardize qPCR data, its stability should be evaluated first (Shen *et al*., 2013).

GAPDH is considered by some reports to be a stable reference gene (Scharlaken *et al*., 2008; Niu *et al*., 2012). In the present study, the results of BestKeeper and NormFinder showed that *GAPDH* could be used as a single reference gene for studies utilizing acaricide or prey stress. However, the results of other analyses indicated that *GAPDH* would not be an ideal reference gene under the other stresses. These results are consistent with those of Bagnall and Kotze and Tong *et al*. (Tong *et al*., 2009; Bagnall & Kotze, 2010). In general, *RNAP II* and *UBC* showed low expression stability for all stresses in this study, demonstrated the least stability with prey stresses. Therefore, these two genes were not considered as stable reference genes. Their low stability may be species-specific, some species showing unique physiological characteristics so that the same stress herein leads to different results. The previous reports showed that these genes have different expression levels even within the same organism at different developmental stages (De Boer *et al*., 2009). Thellin *et al*. and Bustin also believe that the differential expression of these candidate reference genes is related to the tissue type and physiological status (Thellin *et al*., 1999; Bustin, 2000).

Through this research, we not only selected the most stable reference genes for RT-qPCR analysis of gene expression in *N. barkeri*, but also provided a reliable resource to analyze other mites in gene expression studies. However, we also highly recommend to notarize stable expression of candidate reference genes in order to obtain perfect date of RT-qPCR in further studies for *N. barkeri*.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S000748531800072X

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