

Screening of reference genes and quantitative real-time PCR detection and verification in *Dermatophagoides farinae* under temperature stress

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ABSTRACT

Dermatophagoides farinae is an important source of indoor allergens that shows strong tolerance to external temperatures. However, the regularity and mechanism of tolerance are still unclear. Based on our previous RNA-seq and annotation of *D. farinae* under temperature stress, it is planned to identify differentially expressed genes (DEGs) involved in the temperature stress response by quantitative real-time PCR (qRT-PCR). However, the lack of reference genes directly limited the detection and confirmation of DEGs. Accordingly, in this study, we have selected six candidates as reference genes in *D. farinae*: 60S RP L11, 60S RP L21, α tubulin, GAPDH, Der f Mal f 6, and calreticulin, and evaluated their expression stabilities as affected by heat and cold stresses, using geNorm, NormFinder, BestKeeper, comparative Δ Ct and RefFinder methods. Then the expression level of 15 DEGs were detected and verified. geNorm analysis showed that α tubulin and calreticulin were the most stable reference genes under heat stress and cold stress of *D. farinae*. Similar evaluation results were obtained by NormFinder and BestKeeper, in which 60S RP L21 and α tubulin were the most stable reference genes. By comparative Δ Ct method and a comprehensive evaluation of RefFinder, α tubulin was identified as the most ideal reference gene of *D. farinae* under heat and cold stresses. Furthermore, qRT-PCR detection results of 15 DEGs were almost identical to the RNA-seq results, indicating that α tubulin is stable as a reference gene. This study provided technical support for DEGs expression studies in *D. farinae* using qRT-PCR.

1. Introduction

Dermatophagoides farinae are small arthropods distributed worldwide. They are free living and are typically found in the environment surrounding human habitats. A large number of mites spoil cereals, causing huge economic losses. They are also important pathogens and allergens of human allergic diseases, seriously threatening the health of individuals and have become an important public health problem worldwide. *D. farinae* are poikilotherms, and display strong tolerance and adaptability in adverse external environments. They are resistant to both high and low temperatures (Tovey and Woolcock, 1994; Chang et al., 1998). However, its tolerance mechanism is still unclear.

High-throughput transcriptome sequencing has flourished in recent years, providing technical support for the comprehensive analysis of the tolerance regularity and mechanism of *D. farinae* at different temperatures in extremal environments. Transcriptome sequencing is an emerging technology that comprehensively and rapidly reflects the overall perspective of the sequenced species, discovering unknown and low-abundance transcripts and revealing differentially expressed genes

(DEGs) through a new generation of high-throughput sequencing. Currently, it has become an efficient, fast, and sensitive research method for screening functional genes and DEGs. The application of RNA-seq in mites began in 2011, and the first report, which was about the genome and transcriptome sequencing of *Tetranychus urticae*, was published in Nature by Canadian scholars (Grbic' et al., 2011). Since then, the number of studies has increased annually, mainly focusing on drug resistance, development, allergens, pathogen infection, reproduction, diapause, and predation behavior of agricultural mites. In medical mites, there have also been reports on the transcriptome sequencing of *Sarcoptes scabiei canis* (Hu et al., 2016), *Ornithonyssus bacoti* (Niu et al., 2018), and *Dermatophagoides* (Chan et al., 2015; Liu et al., 2018). The RNA-seq of *D. farinae* under temperature stress had been completed in a previous study (SRA: SRR9005248 and SRR9005249), which was very important for revealing the regularity and mechanism of temperature tolerance in *D. farinae*. However, the lack of reference genes for qRT-PCR directly limited verification of DEGs in *D. farinae*.

In this context, there were two key issues to be solved in this study. One is to screen and confirm the stable reference gene. We first selected

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reference genes commonly used in mites and multiple genes with relatively stable expression as candidate reference genes, established standard curve equations, and calculated amplification efficiencies. Subsequently, we used geNorm, NormFinder, BestKeeper and comparative ΔCt method to screen and determine the most stable reference genes under temperature stress, respectively. Finally, the most suitable reference gene was confirmed by comprehensive ranking using RefFinder. The second is to objectively reflect the expression level changes of *D. farinae* under different temperature stresses. The $2^{-\Delta\Delta\text{Ct}}$ method is currently the most commonly used and convenient method for the relative expression quantities calculation in RT-qPCR. Its application condition is that the amplification efficiencies of reference gene and target gene are consistent with the 90–110% standard. Therefore, the amplification efficiencies of DEGs obtained by RNA-seq were calculated by standard curve equations, and were determined that they were consistent with those of reference gene; then the expression level of multiple DEGs were verified using $2^{-\Delta\Delta\text{Ct}}$ method. The reference gene of *D. farinae* under temperature stress screened and confirmed in this study will lay a solid experimental foundation for future gene expression studies of *D. farinae* stress response genes using qRT-PCR.

2. Materials and methods

2.1. Selection of stress temperatures

First, a temperature gradient pre-experiment was designed. The temperature ranged from $-20\text{ }^{\circ}\text{C}$ to $54\text{ }^{\circ}\text{C}$ for a total of 32 groups, and the survival rates of *D. farinae* were observed. In order to ensure that there were enough living mites for RNA extraction after temperature stress, we chose $41\text{ }^{\circ}\text{C}$, $43\text{ }^{\circ}\text{C}$ and $-10\text{ }^{\circ}\text{C}$ as heat and cold stress temperature, and the mortality rates were close to 15%, 25% and 25%, respectively. $25\text{ }^{\circ}\text{C}$ was selected as a normal control, and the mites were active and did not die. RNA-seq, candidate reference gene screening and qRT-PCR detection were then performed separately.

2.2. RNA extraction, transcriptome sequencing, and annotation

D. farinae individuals used for the experimentation were obtained from pure cultures maintained at $25 \pm 1\text{ }^{\circ}\text{C}$ and 70–75% relative humidity in our laboratory incubator, and were collected by the vibrating sieve separation method. The flour powder containing *D. farinae* was placed in $41\text{ }^{\circ}\text{C}$ and $43\text{ }^{\circ}\text{C}$ water baths and $-10\text{ }^{\circ}\text{C}$ ice salt baths for 1 h. After recovery at $25 \pm 1\text{ }^{\circ}\text{C}$ for 1 h, about 500 live female *D. farinae* were picked under a light microscope (Motic, Xiamen, China) and placed into 15 mL RNase-free centrifuge tubes, and liquid nitrogen was added for repeated grinding to completely rupture *D. farinae*. Next, RNA was extracted according to the instructions of RNeasyPlus Micro Kit (Qiagen, Germany), and the concentration and purity of RNA were measured by an ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RNA quality was further evaluated by the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) in BGI (Wuhan, China), and the quantified samples were used to construct cDNA libraries and then sequence on the BGI seq 500 platform. The transcripts were obtained by *de novo* assembly of clean reads using Trinity software, followed by clustering to Unigene using Tgicl. The unigenes were annotated to NT, NR, GO, COG, KEGG, SwissProt, and InterPro databases with the BLAST cut-off value of $1\text{e-}5$. The clean reads were matched to Unigene using Bowtie2 (v2.2.5, parameter: q-phred64-sensitive-dpad 0-gbar 99999999-mp 1,1 -np1-score-min L,0,-0.1-I 1-X 1000-no-mixed-no-discordant-p1-k200, <http://bowtie-bio.sourceforge.net/Bowtie2/index.shtml>). Next, the fragments per kilobase per million mapped fragments (FPKM) of each sample were calculated using RSEM (V1.2.12, with default parameter, <http://deweylab.biostat.wisc.edu/RSEM>). The DEGs were detected using the PoissonDis algorithm and screened with the criteria of

Table 1

The FPKM of candidate reference genes.

candidate reference genes	25 °C	41 °C		43 °C		−10 °C	
	FPKM	FPKM	log2FC	FPKM	log2FC	FPKM	log2FC
60S RP L11	410.35	390.92	−0.07	388.21	−0.08	436.76	0.09
60S RP L21	308.88	296.30	−0.06	294.25	−0.07	315.82	0.03
α tubulin	1359.02	1340.31	−0.02	1303.66	−0.06	1285.71	−0.08
GAPDH	1016.79	982.15	−0.05	1039.11	0.03	984.56	−0.04
Der f Mal f 6	729.20	760.17	0.06	766.24	0.07	747.09	0.03
calreticulin	311.88	320.65	0.04	315.99	0.02	348.64	0.06

$|\log_2\text{Foldchange}| \geq 1$ and $\text{FDR} \leq 0.001$. The complete nucleotide sequences of ORFs for DEGs were confirmed by BLAST alignment.

2.3. Selection, primer design, and amplification product detection of candidate reference gene

2.3.1. Screening of candidate reference genes

The stably expressed genes from RNA-seq under temperature stress, combined with reference genes commonly used in mites, were screened and selected as six candidate genes, including 60S ribosomal protein large subunit 21, 60S ribosomal protein large subunit 11, α tubulin, GAPDH, Der f Mal f 6, and calreticulin. The expression quantities are shown in Table 1.

2.3.2. Specific primer design

Based on the ORFs confirmed by BLAST alignment, the specific primers were designed according to the requirements of qRT-PCR primers (Table 2). The amplicon sizes were between 80 bp and 200 bp, with the primer length of 17–25 bp and GC content of 40–60%.

2.3.3. General PCR detection of amplification products

The specificity and accuracy of amplification products were detected by general PCR amplification. The reaction system and procedures were performed according to Cheng et al. (2015), with the melting temperature (T_m) of $60\text{ }^{\circ}\text{C}$. The amplification products were detected by 1.5% agarose gel electrophoresis. The target bands were recovered, purified, and then ligated into the pMD-19T vector. The recombinant plasmids were transferred into *E. coli* DH-5 α . The positive clones were screened and identified by plasmid PCR. Next, they were sent to Genwiz Biological Co., Ltd. (Suzhou, China) for sequencing. The obtained sequences were compared with the template sequence of RNA-seq to confirm the accuracy of the amplification products.

2.4. Melting curve and amplification efficiency analysis

2.4.1. First-strand cDNA synthesis

The RNA treated with DNase I was treated as template, reverse-transcribed into first strand cDNA according to the instructions of reverse transcriptase M-MLV (TaKaRa, Nojihigashi, Kusatsu, Shiga, Japan), and stored at $-20\text{ }^{\circ}\text{C}$.

2.4.2. Melting curve analysis

The 20 μL PCR qRT-PCR reaction system consisted of 10 μL of SYBR Premix Ex TaqTM II (TaKaRa Bio), 0.5 μL each primer (10 μM), 1 μL cDNA, and 8 μL nuclease-free water. The qRT-PCR experiments were performed on the ABI instruments (MX3005P) under the following thermal cycling conditions: $95\text{ }^{\circ}\text{C}$ for 10 min; followed by $95\text{ }^{\circ}\text{C}$ for 15 s, $60\text{ }^{\circ}\text{C}$ for 30 s, $72\text{ }^{\circ}\text{C}$ for 30 s, for 40 cycles; finally $95\text{ }^{\circ}\text{C}$ for 1 min, $55\text{ }^{\circ}\text{C}$ for 30 s, $95\text{ }^{\circ}\text{C}$ for 30 s, to generate a melting curve to determine the specificity of amplification products.

Table 2
The qRT-PCR primers of candidate reference genes.

gene	Primers sequence (5'-3')	Primers length (bp)	GC%	Amplification length (bp)
60S RP L11	F: GTGAATCAGGCGATAGACTTACC R: CGAATACCAAATGATCGAACAG	23 22	47.8 40.9	109
60S RP L21	F: CCAAAGGTTACCGACGTG R: GGAACGCACCATTTGCC	19 17	52.6 52.9	136
α tubulin	F: CGTCTATCCAGCACCACAAG R: CAGATGTCGTAGATGGCTTCA	20 21	55.0 47.6	129
GAPDH	F: GTTGTCTATTCGGCACCATC R: GCACGAAGCGTTTGAGATG	20 19	50.0 52.6	102
Der f Mal f 6	F: CATTGGGTGCTATTGTCATTG R: GAAATCACCGCCTTGGATC	21 19	42.8 52.6	155
calreticulin	F: CCCGTTTCTATGCCCTTTC R: CCATGTAAATCAGTCTGATCAAGAC	19 25	52.6 40.0	163

Table 3
The qRT-PCR primers of DEGs.

gene	sequence (5'-3')	Amplicon (bp)	gene	sequence (5'-3')	Amplicon (bp)
Zen1	F: GATTCTAACATTGAGTTGGGCAG R: TTCCAATACATCCTGGGCACT	124	EPR1	F: CTTTGGCAGATTATCCACATAG R: GATAGACATCGGGCACTTGAC	138
SIPP 1	F: CATTAAACCGAACATCGAACTCC R: CAGGATAATCACCTTTCTGGAAC	156	SAMS	F: CATACGGTTCGTAGTGTCAACACA R: GTCACGTGTATGGACCACCAAGAA	174
ADE2	F: ATCGTATCACTGCGAGTGATGG R: GGGATTTGATTCCGCAACTCT	149	unc45B	F: GTTCGAGAAAAATAGTGGCC R: CCAGAACACGGACTAAACC	121
peritrophin A	F: GATGTCGATAGAAGCAGCTCGT R: CCATAACCCGTTCCCAATACA	147	trypsin	F: GAAGGAAGTTATTCATTACCATCG R: CATCACCGCCGCAAATC	130
IMPDH2	F: CGGACATTAAGAAGCAACG R: GACGAATCAAGAACGACAAC	154	amylase	F: CGTGTGGTCACTGGACAA R: CCCAGAAAATACTGCTCATCACTC	130
UGT	F: GTTCGATTGCGATTGAGCCG R: CGATTAGAATCCGAATCTGTGG	113	HSP70	F: GCCAAGAATCAAGTTTCCATG R: CTTTCCAGATTCCGTTACAAC	138
ACP	F: CAACGGTACACGACAAGATC R: GATTCCGGTCGAACCTGGTG	104	HSF	F: GGCTAATCAGGTGAACAACCTCC R: TTAGATGTTGTAGGTTGAGGTGCT	162
P450 4v2	F: CCATTCAATTACGGCATACAC R: CGATCCGGTATATAAGTTCTGG	146			

2.4.3. Standard curve plotting and amplification efficiency evaluation

The standard curves were generated through serial 2-fold of cDNA (1, 1/2, 1/4, 1/8, 1/16), with the logarithm of relative copy numbers for template concentration as the abscissa, and the threshold cycle (Ct) value as the ordinate. The equations were obtained by the slope S and the intercept I of the standard curves, and the amplification efficiencies of the primers were calculated as $10^{-1/\text{slope}-1}$. Three biological replicates and three technical replicates were performed. Further, the negative controls of DEPC-treated water and non-transcriptional control were also established.

2.5. Evaluation of reference gene stability

The Ct values of the candidate reference genes under temperature stress were analyzed, and the stability was evaluated using the following algorithms: geNorm, NormFinder and BestKeeper.

The geNorm calculates the expression stability value (M) for candidate reference genes. Genes with lower M values had more stable expression. The threshold value of M value was set as 1.5 in geNorm, such that a candidate reference gene with M value less than 1.5 could be used as a reference gene for qRT-PCR. The optimal number of reference genes was calculated by performing a pair-wise comparison ($V_n/n+1$). If the $V_n/n+1$ value was lower than the cut-off value 0.150, n reference genes were sufficient for accurate normalization, and an additional reference gene would not significantly improve normalization. Otherwise, more genes would be required for reliable normalization if $V_n/n+1$ was less than 0.150 (Vandesompele et al., 2002).

The NormFinder estimates gene expression stability (stability value, SV) based on intra- and inter-group variation of each reference gene using a mathematical model of gene expression and solid-state statistical analysis (Andersen et al., 2004). Thus, genes with lower SV are

more stable.

The BestKeeper evaluates stability of the reference gene based on the standard deviation (SD) and coefficient of variation (CV) of the samples. Therefore, reference genes with smaller SD and CV values have more stable expression (Pfaffl et al., 2004).

The comparative Δ Ct method evaluates stability of reference genes based on standard deviations of Δ Ct of the relative expression of gene pairs within each sample (Silver et al., 2006; Morales et al., 2016; Batista et al., 2019). The reference gene with smaller standard deviations of Δ Ct was more stable.

The ReFinder software (<http://www.heartcure.com.au/reffinder/?type=reference>) estimates reference genes stability based on geometric mean (Xie et al., 2012; Mahanty et al., 2017; Wang et al., 2018). It integrates the analysis results of four methods (geNorm, Norm Finder, BestKeeper and Δ Ct method) to obtain comprehensive ranking values. The reference gene with smaller ranking value was more stable.

2.6. qRT-PCR detection of DEGs

The primers were designed, and qRT-PCR was performed on DEGs with FPKM ≥ 100 annotated in RNA-seq of *D. farinae* under temperature stress (Table 3). The sequencing samples were repeatedly detected thrice, and three technical replicates were set each time. The relative expression was calculated by the method of $2^{-\Delta\Delta\text{Ct}}$ (Livak and Schmittgen, 2001). Differences in DEG expression among the different temperature treatments were subjected to analysis of variance (ANOVA) using SPSS 18.0 software with the significance level of 0.05.

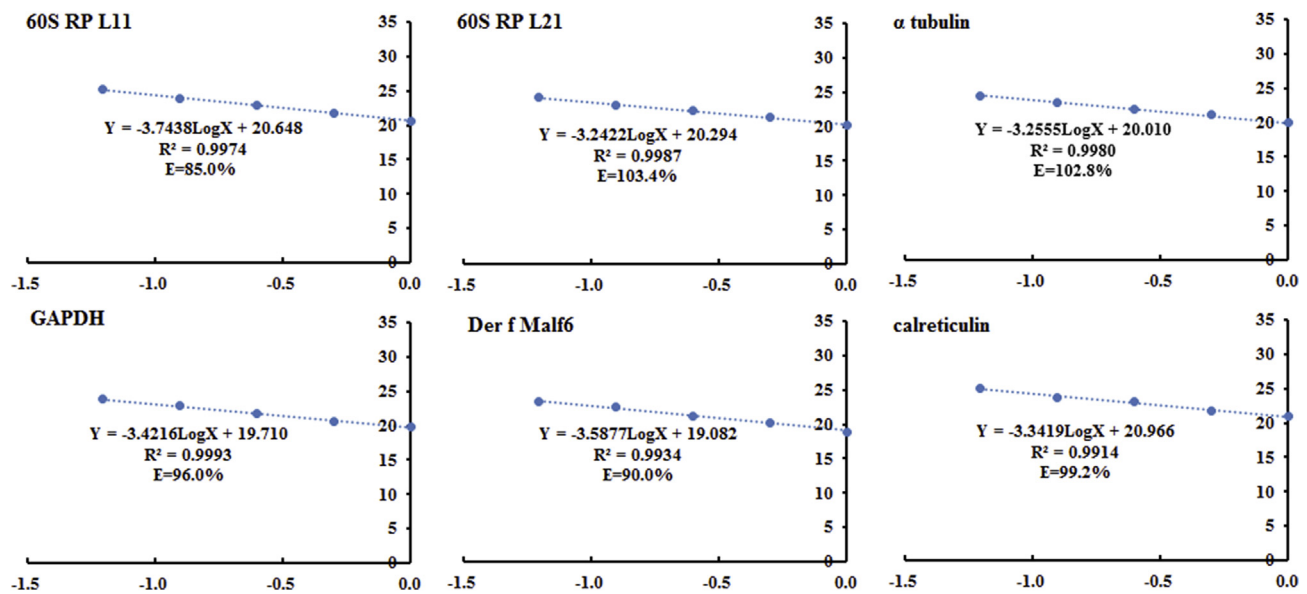


Fig. 1. The standard curves of candidate reference genes of *D. farinae* under temperature stress.

3. Results

3.1. Amplification product verification of candidate reference genes

Electrophoresis showed that the six reference genes were all successfully amplified with product sizes of 100–200 bp, which were consistent with the designed size (Fig. S1). The bands were clear and single, and no primer dimer was observed. After cloning and sequencing, all sequences obtained (MK618586–MK618591) showed 100% identity with the RNA-seq template sequences.

3.2. Melting curve and amplification efficiency analysis of candidate reference genes primers

Melting curves of each reference gene showed a single peak between 80 °C and 86 °C (Fig. S2). The negative control had no fluorescence signal, indicating no non-specific amplification and dimers during PCR occurred, and the primers met the requirements of qRT-PCR analysis.

The standard curves, as shown in Fig. 1, demonstrated the amplification efficiencies of the six candidate reference genes as follows: 60S RP L21 (103.4%) > α tubulin (102.80%) > calreticulin (99.20%) > GAPDH (96.00%) > Der f Mal f 6 (90.00%) > 60S RP L11 (85.00%). Except for 60S RP L11, the amplification efficiencies of the other five reference genes were consistent with the 90–110% standard. The coefficients of determination were between 0.9914 and 0.9993 (all above 0.99), indicating a good linear relationship.

The expression profiles showed the six candidate reference genes had a narrow range of Ct values in all samples, with the median ranging from 21.32 to 26.24 (Fig. 2). The median Ct values were as follows: 60S RP L11 (21.32) < Der f Mal f 6 (22.36) < GAPDH (23.76) < α tubulin (24.00) < 60S RP L21 (24.01) < calreticulin (26.24), and the corresponding expression quantity was reversed. The standard deviation of Ct values were as follows: 60S RP L21 (0.625) < GAPDH (0.832) < α tubulin (0.887) < calreticulin (1.041) < 60S RP L11 (1.960) < Der f Mal f 6 (1.988), indicating reduced stability as the standard deviation increased, such that 60S RP L21 was the most stably expressed and could be considered as the most suitable reference gene (Table S1).

3.3. Stability evaluation of candidate reference genes

The geNorm analysis showed that under heat stress of 43 °C and

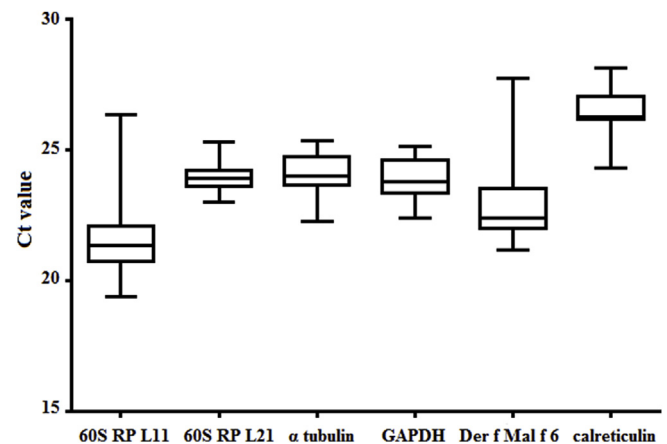


Fig. 2. Expression profiles of candidate reference genes of *D. farinae* under temperature stress.

cold stress of −10 °C, the order of M values was: α tubulin (0.246) = calreticulin (0.246) < GAPDH (0.331) < 60S RP L21 (0.413) < Der f Mal f 6 (1.112) < 60S RP L11 (1.341) (Fig. 3a). It can be seen that the M values of α tubulin and calreticulin were minimal, such that they were identified as the most stable reference genes. Further, pairwise variation showed that V2/3 was lower than the 0.150 cut-off value, suggesting that two reference genes were adequate to accurately quantify target gene expression (Fig. 3b). NormFinder and BestKeeper showed the almost identical evaluation results, namely 60S RP L21 and α tubulin were the most stable reference genes (Fig. 3c–d, Tables S2–3). In view of the fact that only α tubulin was stable in the evaluation performed by the three software programs and the expression quantity was relatively high, in addition, comparative ΔCt method and RefFinder comprehensive ranking both showed α tubulin was the most stable (Fig. 3e–f), therefore α tubulin was selected as the appropriate reference gene.

3.4. Melting curves and amplification efficiencies analysis of target gene primers

Six DEGs of *D. farinae* under heat and cold stresses were selected as target genes. The melting curves analysis showed that each of the six DEGs had sharp single peaks between 76 °C and 88 °C without non-

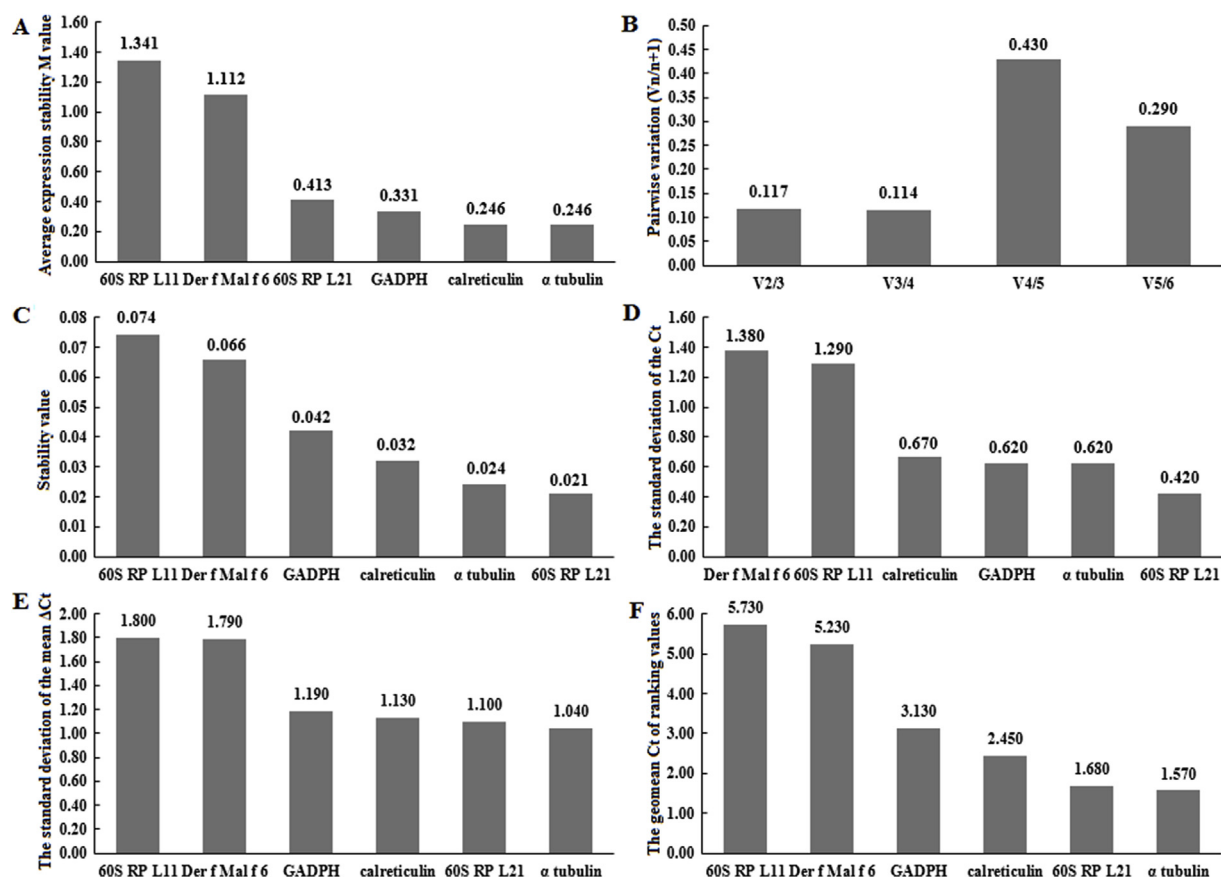


Fig. 3. The stability evaluation of candidate reference genes of *D. farinae* under temperature stress using five softwares. **A** geNorm analysis of the stability values (M), **B** geNorm analysis of pairwise variation ($V_n/n+1$), **C** NormFinder analysis of the stability values, **D** BestKeeper analysis of the standard deviation of the Ct, **E** comparative ΔC_t method analysis of standard deviation of the mean ΔC_t , **F** RefFinder analysis of the geomean Ct of ranking values.

specific amplification and primer dimers, indicating good specificity (Fig. S3).

The standard curves showed that the amplification efficiencies of the six DEGs ranged from 94.40% to 108.10% (Fig. 4), which met the requirements of qRT-PCR primers and were close to that of the reference gene α tubulin of 102.80%. Thus, the $2^{-\Delta\Delta C_t}$ method could be

used to calculate gene expression changes.

3.5. qRT-PCR detection of target genes

The 15 annotated DEGs in RNA-seq of *D. farinae* under temperature stress were selected as target genes for qRT-PCR detection with α -

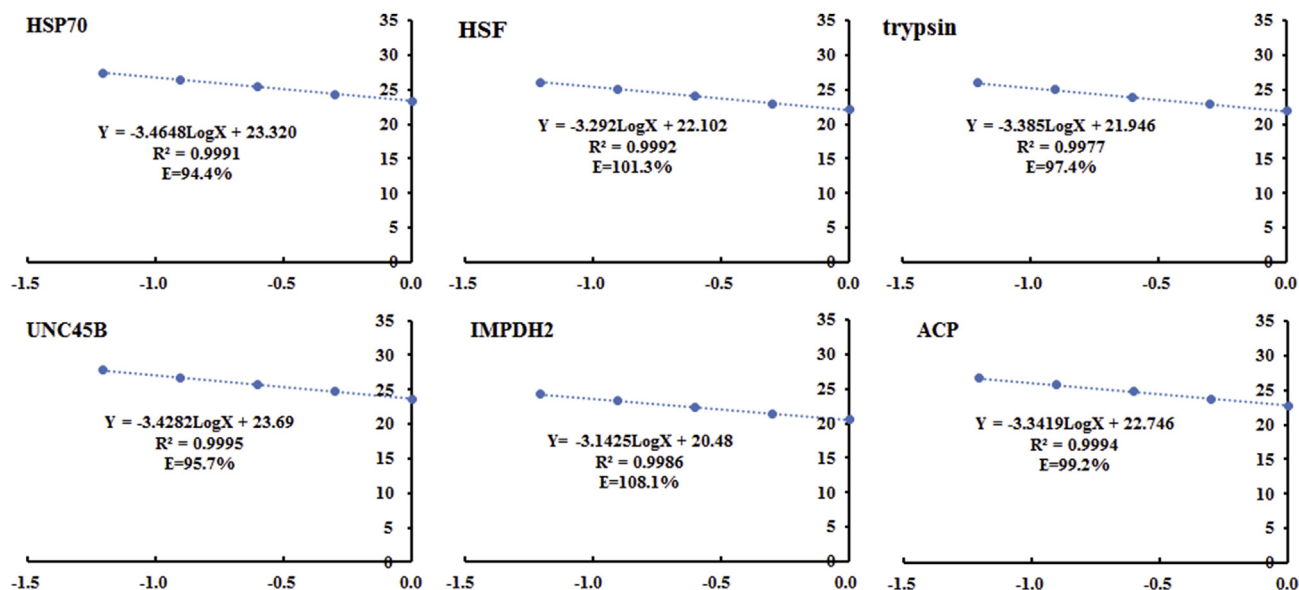


Fig. 4. The standard curves of DEGs of *D. farinae* under temperature stress.

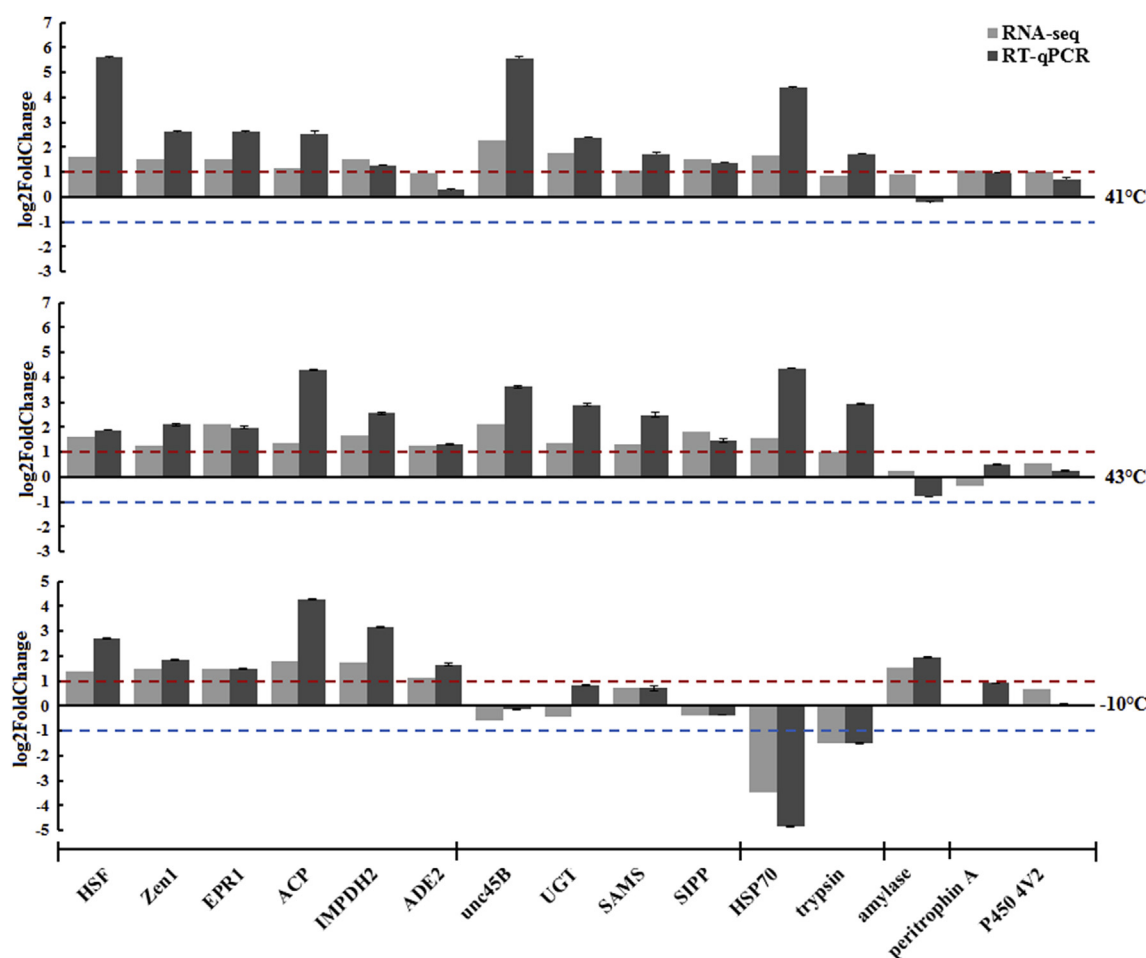


Fig. 5. The relative expression levels of 15 DEGs of *D. farinae* under temperature stress detected by qRT-PCR. The log2FC equal to 1.0 is marked with dashed line. The log2FC ≥ 1.0 indicates significant up-regulation of DEGs.

tubulin as the reference gene. The results showed that the expression of 15 DEGs as detected by qRT-PCR were almost identical to RNA-seq (Fig. 5), proving that α tubulin screened in this study was reliable as a reference gene. The 15 DEGs exhibited the following five trends under heat and cold stresses: 1) Six genes were up-regulated under both heat and cold stresses, namely HSF, Zen1, proline-rich repeating protein (EPR1), acid phosphatase, inosine-5' monophosphate dehydrogenase 2-like protein (IMPDH2), and multifunctional protein ADE2 (ADE2); particularly, HSF was up-regulated 48.84-fold at 41 °C. 2) Four genes were up-regulated under heat stress, but no significant changes were observed under cold stress, namely unc-45-like protein (unc45B), UDP glucuronyl transferase (UGT), methyltransferase SAMS, and stress-induced phosphoprotein 1, especially unc45B, which was up-regulated 48.17-fold at 41 °C. 3) Two genes, HSP70 and trypsin, were up-regulated under heat stress and down-regulated under cold stress, especially HSP70 up-regulation of 20.97-fold at 41 °C, and down-regulation of 0.03-fold at -10 °C. 4) Amylase was up-regulated 3.86-fold at -10 °C, but did not change under heat stress. 5) Peritrophin A and P450 4V2 did not reach the DEGs criteria of $|\log_2FC| \geq 1$ under heat stress and cold stress (Table S4).

4. Discussion

Based on a lack of qRT-PCR reference genes in *D. farinae*, this study confirmed for the first time that α tubulin was the most stable *D. farinae* reference gene under temperature stress. In the experimental design, there were five highlights. First, six stably expressed genes, including 60S RP L11, 60S RP L21, α tubulin, GAPDH, Der f Mal f 6, and

calreticulin, were selected as candidate reference genes based on the commonly used reference genes in mites and the RNA-seq results of *D. farinae* under temperature stress. The six candidate reference genes were stably expressed with a log2FC lesser than 0.09 and highly expressed at 25 °C with the basic FPKM greater than 300, ensuring operability for subsequent qRT-PCR detection. Second, four different algorithms geNorm, NormFinder, BestKeeper and comparative ΔC_t method were used to evaluate the stability of these six candidate genes, then the most suitable reference gene was screened by comprehensive ranking using RefFinder, ensuring reliability of the selected reference genes. Third, the amplification efficiencies of the reference and target genes were evaluated simultaneously to confirm feasibility of the relative quantitative $2^{-\Delta\Delta C_t}$ calculation method in this study. Fourth, the detected target genes were not limited to the currently recognized stress response gene HSP family and covered 15 genes with different functions, including detoxifying, antioxidant, and proteolytic enzymes, peritrophin, etc., to comprehensively evaluate the reference genes. Fifth, qRT-PCR detection were performed on the RNA-seq results of *D. farinae* under temperature stress. The two methods were complementary to ensure reliability. Therefore, as a successful exploration study to screen reference genes in *D. farinae*, the screening method established in this study was scientific, normative, and had very important guiding significance for reference gene research of other mites.

Screening of stable reference genes was a prerequisite to perform qRT-PCR detection. In qRT-PCR, the experimental results are easily affected by many factors such as RNA purity, integrity, and reverse transcription efficiency, such that one or two reference genes are usually required for accurate quantification and normalization of gene

expression between different treatments (Guenin et al., 2009). The ideal reference gene should be stably expressed in different tissues, cells, physiological states, and various stress conditions. However, there is no universal reference gene that is ubiquitously and stably expressed. Even in the same species, optimal reference genes under different experimental conditions will be significantly different. However, only a few studies have been conducted on mite reference genes, particularly for studies of the temperature stress response, and only nine articles were retrieved (Li et al., 2008, 2009; Feng et al., 2010; Niu et al., 2012; Yang et al., 2012, 2016; Chen et al., 2015; Xu et al., 2018; Wang et al., 2018), and the reference genes included β actin, α tubulin, RpII, EF-1a, and HSP40. Five of the nine articles directly selected β actin as reference gene. However, some studies did not support that β actin was a suitable reference gene for the stress response study of mites under abiotic stresses such as ultraviolet light, acid rain, and acaricides (Sun et al., 2010; Niu et al., 2012; Morales et al., 2016). Therefore, β actin was artificially selected as reference gene of mites under stress is lack of experimental basis, and the credibility of the obtained research results is impaired. Fortunately, there were 2 articles performed a screening of reference genes. The first report screened reference genes of *Panonychus citri*. RpII and α tubulin were evaluated as the best reference genes under heat and cold stresses, respectively, by Normfinder software (Niu et al., 2012). The other report was recently published regarding the screening of *Neoseiulus barkeri* reference genes, which determined that HSP40 and α tubulin were the most stable reference genes under temperature stress (Wang et al., 2018). In this study, α tubulin was screened and confirmed as the most stable reference gene in *D. farinae* under heat and cold stresses, which is consistent with previous reports. Therefore, we suggested that α tubulin could be prioritized as a reference gene to study the temperature stress response of mites in the future.

Objective evaluation of the reference gene stability was a key step in qRT-PCR. Currently, geNorm, NormFinder, BestKeeper and comparative Δ Ct method were the four commonly used methods to evaluate reference gene stability. The geNorm software was developed based on the program specially designed by Jo Vandesompele in 2002 for reference gene screening in qRT-PCR. The expression stability M value was calculated by a $2^{-\Delta C_t}$ conversion of the original Ct value of the candidate reference genes. Pair-wise variation of standardized factors was analyzed to select two or more reference gene combinations to overcome defects of the traditional single reference gene, which were beneficial to correct systematic deviation, and the obtained quantitative results were more reliable. The principle of the NormFinder software is similar to geNorm; however, it directly evaluates reference gene stability based on ANOVA. The BestKeeper software is completely different from these software programs. It directly analyzes the stability of candidate reference genes according to the geometric mean of Ct values without conversion of the Ct values. The comparative Δ Ct method was developed relatively late (Silver et al., 2006). Its remarkable feature is to compare the relative expression of each gene pairs within each sample. The gene pairs with more constant Δ Ct had better stability, and could be considered as ideal reference gene. In this study, first we evaluated the stability of reference gene of *D. farinae* under temperature stress using four softwares respectively, then in order to eliminate the deviation of the four evaluation softwares, we further adopted the reference gene evaluation online software RefFinder (<http://www.heartcure.com.au/reffinder/?type=reference>) (Xie et al., 2012), which comprehensively evaluated above four methods, and finally determined that α tubulin was the most stable reference gene of *D. farinae* under heat and cold stresses. Finally, 15 DEGs were detected, and the results were almost identical to the RNA-seq findings, demonstrating that α tubulin was the ideal reference gene of *D. farinae* under temperature stress.

The currently recognized HSP family, which is involved in regulation of biological stresses, is a large gene family, including subfamilies such as HSP110, HSP90, HSP70, HSP60, HSP40, and sHSP. In this

study, it should be pointed out that these 15 target genes contained two HSP genes, of which HSF was up-regulated under both heat and cold stresses; yet, HSP70 was up-regulated under heat stress and significantly down-regulated under cold stress. These results suggested that regulation of different HSP gene subfamilies during the temperature stress response of *D. farinae* was not completely identical, and their respective regulatory roles require further study.

In conclusion, α tubulin is a suitable qRT-PCR reference gene in *D. farinae* under temperature stress. However, it will be helpful to verify DEG expression by RNA-seq and provide technical support for regulatory mechanistic research of *D. farinae*.

Declarations of interest

None.

Conflicts of interest

There are no conflicts of financial interest or patents or copyrights.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2019.107754>.

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Table S1 Analysis of Ct values of six candidate reference genes in all samples in *D. farinae*

Gene	No	Median	Lowest	Lower quartile	Higher quartile	Highest	Mean	SD
60S RP L11	9	21.32	19.36	20.85	21.32	26.32	21.67	1.960
60S RP L21	9	24.01	23.01	23.84	24.31	25.28	23.96	0.625
α tubulin	9	24.00	22.27	23.50	24.48	25.36	24.05	0.887
GADPH	9	23.76	22.40	23.46	24.38	25.13	23.87	0.832
Der f Mal f 6	9	22.36	21.15	22.12	22.60	27.74	22.99	1.988
calreticulin	9	26.24	24.28	26.21	26.77	28.14	26.41	1.041

No: Number of samples; SD: standard deviation

Table S2 Statistical analyses of candidate reference genes in *D. farinae* under heat stress and cold stress based on the NormFinder algorithm

Gene	SV
60S RP L11	0.074
60S RP L21	0.021
α tubulin	0.024
GADPH	0.042
Der f Mal f 6	0.066
calreticulin	0.032
SV: Stability value	

Table S3 Statistical analyses of candidate reference genes in *D. farinae* under heat stress and cold stress based on the BestKeeper algorithm

	60S RP L11	60S RP L21	α tubulin	GAPDH	Der f Mal f 6	calreticulin
GM [Ct]	21.60	23.95	24.04	23.85	22.92	26.39
AM [Ct]	21.67	23.96	24.05	23.87	22.99	26.41
Min [Ct]	19.36	23.01	22.27	22.40	21.15	24.28
Max [Ct]	26.32	25.28	25.36	25.13	27.74	28.14
SD [$\pm$ Ct]	1.29	0.42	0.62	0.62	1.38	0.67
CV [%Ct]	5.97	1.76	2.59	2.59	6.00	2.53
[r]	0.791	0.636	0.770	0.399	0.820	0.645
p-value	0.061	0.174	0.074	0.433	0.046	0.166

GM [Ct]: geometric means of the threshold cycle (Ct); AM [Ct]: the arithmetic mean of Ct; Min [Ct] and Max [Ct]: the extreme values of Ct; SD [$\pm$ Ct]: the standard deviation of the Ct; CV [% Ct]: the coefficient of variance expressed as a percentage at the Ct level; [r]: Pearson correlation coefficient.

Table S4 The results of RNA-seq and qRT-PCR detection of 15 DEGs

DEGs	25°C FPKM	41°C log2FC			43°C log2FC			-10°C log2FC		
		RNA-seq	RT	SD	RNA-seq	RT	SD	RNA-seq	RT	SD
HSF	129.23	1.62	5.61	0.02	1.62	1.89	0.02	1.37	2.69	0.03
Zen1	234.87	1.51	2.62	0.03	1.27	2.10	0.03	1.50	1.84	0.03
EPR1	59.63	1.51	2.62	0.02	2.11	1.99	0.04	1.50	1.48	0.03
ACP	67.34	1.17	2.54	0.09	1.38	4.29	0.03	1.77	4.27	0.02
IMPDH2	78.93	1.51	1.28	0.03	1.64	2.56	0.04	1.73	3.17	0.02
ADE2	107.62	0.95	0.28	0.04	1.26	1.31	0.05	1.13	1.66	0.04
unc45B	24.45	2.26	5.59	0.03	2.14	3.62	0.04	-0.61	-0.13	0.02
UGT	68.80	1.76	2.38	0.04	1.37	2.89	0.06	-0.45	0.83	0.04
SAMS	46.37	1.06	1.74	0.04	1.32	2.50	0.08	0.73	0.71	0.09
SIPP	180.30	1.50	1.37	0.03	1.81	1.45	0.08	-0.38	-0.37	0.01
HSP70	1921.97	1.69	4.39	0.03	1.56	4.37	0.01	-3.46	-4.86	0.03
trypsin	300.36	0.85	1.72	0.02	1.01	2.93	0.03	-1.48	-1.52	0.03
amylase	54.91	0.91	-0.21	0.03	0.27	-0.79	0.02	1.54	1.95	0.02
PeritrophinA	95.89	1.05	0.96	0.03	-0.37	0.51	0.02	0.05	0.91	0.02
P450 4V2	60.74	1.02	0.72	0.07	0.55	0.23	0.02	0.70	0.06	0.03

RNA-seq: the log2Foldchange of DEGs in RNA-seq results; RT: the log2Foldchange of DEGs in qRT-PCR results.

SD: the standard deviation of the log2Foldchange of DEGs.