Selection and validation of reference genes for quantitative real-time PCR analysis under different experimental conditions in the leafminer *Liriomyza trifolii* (Diptera: Agromyzidae)

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Abstract

*Liriomyza trifolii* is a highly-invasive leafmining insect that causes significant damage to vegetables and horticultural crops worldwide. Relatively few studies have quantified gene expression in *L. trifolii* using real-time quantitative PCR (RT-qPCR), which is a reliable and sensitive technique for measuring gene expression. RT-qPCR requires the selection of reference genes to normalize gene expression data and control for internal differences between samples. In this study, nine housekeeping genes from *L. trifolii* were selected for their suitability in normalizing gene expression using geNorm, Normfinder, BestKeeper, the ΔCt method and RefFinder. *HSP21.7*, which encodes heat shock protein 21.7, was used as a target gene to validate the expression of candidate reference genes. Results indicated that *ACTIN* and *18S* were optimal for developmental stage and low temperature, *TUB* and *18S* showed the most stable expression for sex, and *GAPDH* and *ACTIN* were the best reference genes for monitoring gene expression at high temperature. Selection and validation of appropriate reference genes are critical steps in normalizing gene expression levels, which improve the accuracy and quality of expression data. Results of this study provide vital information on reference genes and is valuable in developing a standardized RT-qPCR protocol for functional genomics research in *L. trifolii*.

Introduction

*Liriomyza* (Diptera: Agromyzidae) is a phyletic genus exhibiting replacement that continues to spread throughout the world [1–3]. *L. trifolii* Burgess is an invasive pest in China that has
that no competing interests exist.

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Due to the rampant use of pesticides and onset of insecticide resistance, the development of more environmentally favorable pest management strategies is imperative. For example, strategies employing RNA interference (RNAi) or gene knockout approaches have been successfully developed for many pests [13–15]. Genetic approaches show great promise in pest management but require functional studies to identify suitable target genes and expression profiles in insects [16–19]. To successfully implement genetic strategies for control of *L. trifolii*, the use of standardized, real-time quantitative PCR (RT-qPCR) protocols using MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) is critical [20]. RT-qPCR is widely used to analyze gene expression because of its accuracy, sensitivity, reproducibility and quantitative ability [21–22]. To accurately calculate gene expression using RT-qPCR, it is vital to use appropriate reference genes to normalize the data.

The expression of ideal reference genes should be constant in different tissues and experimental conditions. However, recent research has indicated that widely-used reference genes were differentially expressed and only stable during specific conditions [23]. *ACTIN* was one of the most stable reference genes that has been used for different developmental stages in Callichoridae [24], but in *Frankliniella occidentalis* and *Sesamia inferens*, that gene was ranked as an unstable reference gene for certain developmental stages [17, 25]. The suitable reference genes for sex in *Coleomegilla maculata* were 16S, HSP70 and RPS18, while 18S and *ACTIN* were relatively unstable genes [18]. For temperature treatments, *ACTIN* has been used as a stable reference gene for heat shock stress in *Drosophila melanagaster* [26], but in *Nilaparvata lugens* and *Bemisia tabaci*, *ACTIN* was ranked as one of the unstable reference genes [27–28]. Therefore, many traditional housekeeping genes are no longer valid reference genes for RT-qPCR.

To our knowledge, this is the first study to evaluate the expression stability of different candidate reference genes for qRT-PCR in leaf-mining flies. Previous gene expression studies of *Liriomyza* species used *ACTIN* as the reference gene to normalize gene expression and reference genes were not selected and validated reference genes under different experimental conditions [29–32], although the expression of *ACTIN* was monitored at different developmental stages in *L. sativae* [29]. It is critical, however, to validate the expression stability of reference genes under different experimental conditions before using them for normalization.

The aim of this study was to identify a suite of reference genes with stable expression in *L. trifolii* under different experimental conditions. Nine candidate reference genes including 18S ribosomal RNA (18S), β-actin (*ACTIN*), arginine kinase (AK), elongation factor 1a (EF-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone 3 (H3), ribosomal protein L32 (RPL32), tubulin α-1 chain (TUB) and carbamoyl phosphate synthase (CAD) were evaluated for suitability in the normalization of gene expression under different experimental conditions (developmental stage, sex, and temperature). The stability and performance of the candidate reference genes was examined using geNorm [33], NormFinder [34], BestKeeper [35], the ΔCt method [36] and Reffinder [37]. The expression profile of HSP21.7, which encodes heat shock protein 21.7, was used to evaluate the suitability of different combinations of reference genes and various experimental conditions. The results provide insight regarding the selection of appropriate reference genes for functional genomic studies in *L. trifolii*. 
Materials and methods

Insect cultures

Populations of *L. trifolii* were collected from celery (*Apium graveolens*), which was cultivated in a vegetable greenhouse located in Yangzhou (32.39˚N, 119.42˚E). The insect populations were maintained and reared in the laboratory for ten or more generations at 25 ± 1˚C with a 16:8 h (light: dark) photoperiod as described by Chen and Kang [38]. No specific permission was required for these activities, and the field studies did not involve endangered or protected species.

Temperature treatments

Groups of two-day-old pupae were subdivided into four repetitions (n = 30), placed in glass tubes, and exposed to heat (35˚C, 37.5˚C, 40˚C, 42.5˚C, 45˚C) or cold stress (0˚C, -2.5˚C, -5˚C, -7.5˚C, -10˚C) for 1 h in a constant temperature controller (DC-3010, Ningbo, China). A set of pupae maintained at 25˚C was regarded as a control group. After temperature treatment, the pupae were allowed to recover at 25˚C for 1 h, frozen in liquid nitrogen, and stored at -70˚C.

Developmental stage and sex

Treated stages included third-instar larvae, prepupae, two-day-old pupae, ten-day-old pupae, and adults including male and female. Each treatment (n = 30) was repeated four times.

Candidate reference genes and primer design

Nine candidate reference genes (ACTIN, AK, EF-1, H3, RPL32, 18S, CAD, GAPDH, and TUB) and the target gene HSP21.7 were amplified from *L. trifolii* based on homologies with dipteran insects deposited in the NCBI database (http://www.ncbi.nlm.nih.gov). Primer Premier 5 was used for primer design using the parameters outlined by Zheng et al. [17]. Protocols for PCR, procedures for cloning in pGEM-T easy, and sequence analysis were conducted as previously described [39]. Sequences were submitted to GenBank, and the accession numbers are shown in Table 1.

RNA isolation

Total RNA was extracted from *L. trifolii* using the SV Total RNA Isolation system (Promega, WI, USA). The integrity of RNA was verified by comparing RNA bands in gels stained with

Table 1. Sequence information of the candidate reference genes.

| Gene name                      | Abbreviation | Amplicon length (bp) | Accession number |
|-------------------------------|--------------|----------------------|-----------------|
| Beta-actin                    | ACTIN        | 322                  | KY231150        |
| Arginine kinase               | AK           | 426                  | KY563322        |
| Elongation factor 1 alpha     | EF-1         | 850                  | KY558636        |
| Histone 3                     | H3           | 299                  | KY558638        |
| Ribosomal protein L32         | RPL32        | 139                  | KY558639        |
| 18S ribosomal RNA             | 18S          | 994                  | KY563323        |
| Carbamoyl phosphate synthase  | CAD          | 600                  | KY558635        |
| Glyceraldehyde-3-phosphate dehydrogenase | GAPDH     | 996                  | KY558637        |
| Tubulin alpha-1 chain         | TUB          | 1350                 | KY558640        |
| Heat shock protein 21.7       | HSP21.7      | 837                  | KY558641        |

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ethidium bromide. Total RNA quantity and purity was determined by spectrophotometry (Eppendorf Bio Photometer plus, Hamburg, Germany).

Expression analyses by real-time quantitative PCR
RNA (0.5 μg) was reverse-transcribed into first-strand cDNA using the Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA). Real-time PCR reactions were performed in a 20 μl reaction volume comprised of 10 μl Bio-Rad iTaq™ Universal SYBR® Green super mix (2×), 1 μl of each gene-specific primer (10 μM) (Table 2), 2 μl of cDNA template, and 6 μl of ddH2O. Reactions were carried out using a CFX-96 real-time PCR system (Bio-Rad Laboratories, Berkeley, USA) under the following conditions: 3 min at 95˚C, 40 cycles of denaturation at 95˚C for 30 s, and annealing at the Tm of primer pairs (Table 2) for 30 s. Each treatment contained four replications, and each reaction was run in triplicate.

Data analysis
The stability of the nine candidate reference genes was evaluated using geNorm, NormFinder, BestKeeper, the ΔCt method and RefFinder, which is a comprehensive software platform integrating all four algorithms. Pairwise variation (V), which is determined by geNorm, was used to determine the optimal number of reference genes for accurate RT-qPCR normalization. Vn/Vn+1 indicated the pairwise variation between two sequential normalization factors, and a cut-off threshold of Vn/Vn+1 = 0.15 was used for valid normalization [33]. Lower scores denote greater transcriptional stability and better suitability as a reference gene for each of the evaluated programs. RefFinder was used to select the best reference genes based on the final results of the four different programs.

Evaluation of target gene expression
HSP21.7 is a member of the heat shock protein superfamily, which contains molecular chaperones that increase heat tolerance and protect organisms from thermal injury [29, 40]. HSP21.7 was used as a target gene to evaluate the candidate reference genes. Relative expression was calculated using the 2−ΔΔCt method [41]. Geometric means of the reference genes were used to normalize expression under the different experimental conditions. Statistical significance between treatments was analyzed by one-way ANOVA and further evaluated using Tukey’s multiple comparison (P<0.05) in SPSS v. 16.0 software (SPSS, Chicago, IL, USA).

Results
Amplification efficiency of candidate reference genes
For each primer pair, specific amplification was confirmed by a single peak in melting-curve analysis. A standard curve was generated for each gene and the regression correlation coefficient (R²) and PCR efficiency (E) for each standard curve were detailed in Table 2. All ten genes showed E values between 90.2–108.6% and R² values greater than 0.988.

Expression profile of candidate reference genes
The Ct values generated from the nine candidate reference genes were used to estimate the stability of gene expression across different experimental treatments. The mean Ct values of the nine reference genes varied from 10.91 to 28.27 for 18S and CAD, respectively. With the exception of 18S and CAD, seven of the nine candidate reference genes displayed a narrow range of mean Ct values in all experimental samples and all the standard deviation (SD) values of those reference genes were < 2.0 (Fig 1).
Analysis of gene expression stability

The geNorm program uses mean expression stability values (M-values) to determine the best set of reference genes. Lower M-values indicate greater stability. geNorm ranked ACTIN and 18S as the most stable genes in different insect developmental stages, and AK and 18S were the most stable genes for adults of either sex (Table 3). ACTIN and GAPDH co-ranked as the most stable genes in response to low and high temperature treatments. The overall ranking of the nine reference genes is shown in Table 3. geNorm analysis revealed that the pairwise variation values were below the proposed 0.15 threshold value. The first V-value < 0.15 emerged at V2/3 (Fig 2), suggesting that two reference genes are reliable for normalization in the four experimental conditions (developmental stage, sex, low and high temperature).

The NormFinder algorithm ranks each gene independently, and a lower stability value (SV) indicates higher expression stability. NormFinder ranked ACTIN and CAD as the most stable

![Graph showing expression profiles of nine candidate reference genes in different samples.](https://doi.org/10.1371/journal.pone.0181862.g001)
genes for developmental stages and sex, respectively (Table 3). 18S was the most stable gene in response to low temperatures, whereas AK was best for the high temperature treatments (Table 3).

The stability of a gene is inversely proportional to the SD and coefficient variation (CV) as computed by BestKeeper. ACTIN was the most stable gene for developmental stages and insect sex when BestKeeper was used, whereas 18S and GAPDH were the most stable genes for low and high temperature treatments, respectively (Table 3).

The ΔCt method uses raw Ct values, and the mean SD of each gene is inversely proportional to its stability. The ΔCt method indicated that TUB was the most stable reference gene for

### Table 3. Ranking order of the candidate reference genes under different experimental conditions.

| Experimental conditions | Rank | Reference gene | geNorm Stability | NormFinder Stability | BestKeeper Stability | Delta Ct Stability |
|-------------------------|------|----------------|------------------|----------------------|----------------------|-------------------|
| Developmental stage     | 1    | ACTIN/18S      | 0.3592           | ACTIN 0.1245         | ACTIN 0.7500         | TUB 0.7771         |
|                         | 2    | TUB            | 0.4349           | 18S 0.2324           | CAD 1.0002           | ACTIN 0.8809       |
|                         | 3    | AK             | 0.6060           | EF-1 0.4711          | TUB 1.0978           | EF-1 0.9161        |
|                         | 4    | EF-1           | 0.6759           | AK 0.6379            | RPL32 1.2943         | AK 0.9956          |
|                         | 5    | RPL32          | 0.7419           | RPL32 0.7271         | EF-1 1.3081          | RPL32 1.0142       |
|                         | 6    | GAPDH          | 0.7923           | GAPDH 0.7818         | AK 1.3150            | GAPDH 1.1202       |
|                         | 7    | CAD            | 1.1011           | CAD 1.0971           | GAPDH 1.4981         | CAD 1.3885         |
|                         | 8    | H3             | 1.4729           | H3 1.8734            | H3 1.5164            | H3 2.117           |
| Sex                     | 1    | AK/18S         | 0.0048           | CAD 0.0604           | ACTIN 0.7710         | TUB 0.4484         |
|                         | 2    | TUB            | 0.1120           | 18S 0.7833           | CAD 0.4770           | CAD 0.4770         |
|                         | 3    | ACTIN          | 0.0148           | RPL32 0.1924         | AK 0.7867            | GAPDH 0.4932       |
|                         | 4    | GAPDH          | 0.0324           | GAPDH 0.4238         | GAPDH 0.8157         | RPL32 0.5017       |
|                         | 5    | TUB            | 0.2373           | AK 0.4587            | TUB 1.1742           | AK 0.5041          |
|                         | 6    | CAD            | 0.4111           | 18S 0.4628           | CAD 1.4027           | 18S 0.5062         |
|                         | 7    | RPL32          | 0.5009           | ACTIN 0.4774         | RPL32 1.4668         | ACTIN 0.5170       |
|                         | 8    | EF-1           | 0.6370           | EF-1 0.5691          | EF-1 1.7679          | EF-1 0.6888        |
|                         | 9    | H3             | 0.8045           | H3 0.9544            | H3 2.1046            | H3 0.9833          |
| Low temperature         | 1    | ACTIN/GAPDH    | 0.1750           | 18S 0.1215           | 18S 0.2743           | ACTIN 0.2444       |
|                         | 2    | AK             | 0.2032           | ACTIN 0.1534         | AK 0.4417            | EF-1 0.2791        |
|                         | 3    | RPL32          | 0.2166           | EF-1 0.2027          | ACTIN 0.4460         | GAPDH 0.2832       |
|                         | 4    | EF-1           | 0.2539           | RPL32 0.2076         | EF-1 0.4485          | 18S 0.2849         |
|                         | 5    | RPL32          | 0.3031           | GAPDH 0.2466         | RPL32 0.4514         | RPL32 0.3175       |
|                         | 6    | CAD            | 0.3857           | CAD 0.2469           | CAD 0.4955           | CAD 0.3401         |
|                         | 7    | TUB            | 0.4025           | TUB 0.2883           | GAPDH 0.5509         | TUB 0.3769         |
|                         | 8    | H3             | 0.4595           | H3 0.4212            | H3 0.6086            | H3 0.3819          |
| High temperature        | 1    | ACTIN/GAPDH    | 0.0856           | AK 0.0714            | GAPDH 0.1843         | AK 0.2216          |
|                         | 2    | AK             | 0.1644           | ACTIN 0.1130         | AK 0.2262            | ACTIN 0.2592       |
|                         | 3    | TUB            | 0.2331           | TUB 0.1635           | RPL32 0.2411         | TUB 0.2770         |
|                         | 4    | CAD            | 0.2652           | EF-1 0.1955          | EF-1 0.2724          | EF-1 0.2950        |
|                         | 5    | H3             | 0.2971           | RPL32 0.2324         | TUB 0.2981           | CAD 0.3141         |
|                         | 6    | EF-1           | 0.3288           | CAD 0.2383           | CAD 0.3180           | RPL32 0.3257       |
|                         | 7    | RPL32          | 0.3549           | H3 0.2610            | 18S 0.3406           | H3 0.3442          |
|                         | 8    | 18S            | 0.3793           | 18S 0.2832           | H3 0.4757            | 18S 0.4042         |
|                         | 9    | 18S            | 0.3793           | 18S 0.2832           | H3 0.4757            | 18S 0.4042         |
developmental stages and sex, whereas \textit{ACTIN} and \textit{AK} were the most stable genes for low and high temperatures, respectively (Table 3).

RefFinder is a comprehensive program that integrates the results obtained from geNorm, Normfinder, BestKeeper, and the \(\Delta\)Ct method and ranks candidate reference genes based on their stability. The following rankings are listed in order of decreasing stability. For insect developmental stages, the comprehensive ranking obtained with RefFinder was \textit{ACTIN, 18S, TUB, AK, EF-1, CAD, RPL32, GAPDH, and H3} (Fig 3A). The stability ranking for insect sex

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**Fig 2.** Optimal number of reference genes for normalization in \textit{Liriomyza trifolii}. The pairwise variation (\(V_n/V_{n+1}\)) was analyzed between normalization factors NF\(_n\) and NF\(_{n+1}\) by geNorm program to determine the optimal number of reference genes. Values < 0.15 indicate that additional genes are not required for the normalization of gene expression.

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**Fig 3.** Expression stability of candidate reference genes under different treatments. A lower Geomean value indicates more stable expression according to RefFinder. (A) different developmental stages of \textit{Liriomyza trifolii}; (B) sex for \textit{L. trifolii}; (C) low temperature treatments for \textit{L. trifolii}; (D) high temperature treatments for \textit{L. trifolii}.

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was TUB, 18S, CAD, AK, ACTIN, GAPDH, RPL32, EF-1, and H3 (Fig 3B). The stability ranking in *L. trifolii* exposed to low temperatures was ACTIN, 18S, AK, GAPDH, RPL32, EF-1, TUB, CAD, and H3 (Fig 3C), whereas the ranking for high temperatures was GAPDH, ACTIN, AK, TUB, EF-1, RPL32, CAD, H3, and 18S (Fig 3D). The ideal reference genes in response to different experimental conditions are as demonstrated by RefFinder all showed in Table 4. The optimal number of reference genes was based on geNorm.

**Validation of reference gene selection**

The relative expression of HSP21.7 was used to assess the validity of selected reference genes. The expression level of HSP21.7 was compared using the two most stable reference genes (ACTIN and 18S) and the most unstable gene (H3), which is generally recommended for normalization by RefFinder. When H3 was used to normalize hsp21.7 expression levels in *L. trifolii* exposed to low temperatures, no significant differences were observed among the different temperatures ($F_{5,18} = 2.714, P = 0.054$). However, when the two most stable reference genes, ACTIN and 18S, were used to normalize the data, relative expression was significantly higher at -10° C ($F_{5,18} = 7.892, P<0.05$; $F_{5,18} = 6.609, P<0.05$) (Fig 4A). When HSP21.7 expression was evaluated in response to high temperatures, expression at 40°C and 42.5°C was significantly higher than at the other temperatures, regardless of the gene used for normalization (Fig 4B). Interestingly, when hsp21.7 expression was normalized using the least stable gene (18S), the relative expression at 40°C and 42.5°C was significantly different ($F_{5,18} = 31.399, P<0.05$). However, expression at these two temperatures was not significantly different when the most stable genes, GAPDH and ACTIN, were used to normalize the data ($F_{5,18} = 8.633, P<0.05$; $F_{5,18} = 21.489, P<0.05$).

The relative expression of hsp21.7 was also estimated for different insect developmental stages and sex, and gene expression was normalized using ACTIN and 18S (the two most stable genes) and H3 (the most unstable gene). When ACTIN and 18S were used for normalization, expression of hsp21.7 was highest at the prepupae and two-day-old pupae stages ($F_{5,17} = 12.568, P<0.05$; $F_{5,17} = 18.381, P<0.05$) and significant differences were observed between the other developmental stages and sexes (Fig 4C). However, hsp21.7 expression was highest at the third-instar larval stage when H3 was used as a reference gene ($F_{5,17} = 12.572, P<0.05$), and there were huge differences in hsp21.7 expression using the stable and unstable reference genes (Fig 4C).

**Discussion**

Biological samples often show great differences in the quality of RNA and the efficiency of reverse transcription. Consequently, the selection of appropriate reference genes is imperative in reducing error [42]. It is now apparent that a stable, constant level of expression does not exist for housekeeping genes in different insect species or within the same species subjected to different experimental conditions. Although RT-qPCR is widely used in gene expression studies because of its speed, accuracy and sensitivity [43–44], our results demonstrate that the correct choice of stable reference genes is required to ensure the reliability of the results.

Several studies on reference gene validation have emphasized that multiple internal genes must be evaluated to improve the accuracy of qRT-PCR analysis and the interpretation of gene expression [28, 45–46]. In this study, nine genes were analyzed for their suitability as reference genes for qRT-PCR in *L. trifolii* that differed in developmental stage, sex and temperature stress. The computational programs geNorm, NormFinder, BestKeeper and the ΔCt method were used to generate stability rankings for the nine genes. These varied slightly between the four methods (Table 3). It is worth mentioning that, in addition to ranking function, the...
geNorm also has the function of selecting optimal number of reference genes [33]. More and more researchers have realized that only a single reference gene with high expression stability may be not enough for normalization of gene expression under some experimental conditions, so in most experimental conditions two or more reference genes were required for accurate and reliable results. The comprehensive tool RefFinder was utilized to evaluate the rankings of the four programs and to generate a final ranking for the different experimental conditions (Fig 3).

RefFinder analysis indicated that ACTIN and 18S were the most stable reference genes for *L. trifolii* at different developmental stages, whereas TUB and 18S were the most stable genes when comparing male and female adults. GAPDH and ACTIN were optimal reference genes at high temperatures, whereas ACTIN and 18S exhibited the most stable expression at low temperatures. Consistent with the reference genes validated for other insect organisms [26–27, 47–49], our study recommended ACTIN, 18S, TUB and GAPDH were the better genes for normalizing expression in *L. trifolii* exposed to different experimental treatments. It is noteworthy that previous reference gene validation studies combined high and low temperature treatments, collectively referring to them as temperature treatment [26–27, 50]. Interestingly, we discovered that the reference gene stability rankings for high and low temperatures were different and often contrasted. In this study, 18S was ranked as a stable reference gene for low temperature, but was the least stable reference gene for high temperatures. These results are likely relevant in the expression of HSPs in leafminer species. For example, *hsp60* in *Liriomyza* spp. responded to cold but not heat stress [30]. Consequently, the use of reference genes selected from combined heat/cold temperature stress could cause inaccuracies in the normalization of HSP60 expression. Thus, the stability of reference gene expression in *L. trifolii* needs to be carefully examined for experimental conditions and should be considered in the context of insect biology and physiology whenever possible.

Heat shock proteins (HSPs) play important roles in the environmental adaptation of various organisms. HSP21.7 is a member of the small HSPs family. It along with a number of other small HSPs play a role in temperature tolerance and development in insects [29, 40]. For example, HSP21.7 was significantly induced by temperature stress and developmental processes in *L. sativae* [29]. In our study, the expression of *hsp21.7* was investigated to validate the ranking of reference genes by RefFinder. When the two most stable genes were used for normalization under different experimental treatments, the HSP21.7 expression pattern was consistent with *L. sativae*. However, when the least stable gene was used, the normalized expression was significantly different. Therefore, the selection of optimal reference genes for normalization is critical, especially when differences in expression levels are subtle. In order to generate reliable

| Experimental conditions * | Optimal number of reference genes | Recommended Reference Genes |
|---------------------------|----------------------------------|-----------------------------|
| Developmental stage       | 2                                | ACTIN, 18S                  |
| (L, PP, P, OP, M, FM)     |                                   |                             |
| Sex                       | 2                                | TUB, 18S                    |
| (M, FM)                   |                                   |                             |
| Low temperature           | 2                                | ACTIN, 18S                  |
| (0˚C, -2.5˚C, -5˚C, -7.5˚C, -10˚C) |                     |                             |
| High temperature          | 2                                | GAPDH, ACTIN                |
| (35˚C, 37.5˚C, 40˚C, 42.5˚C, 45˚C) |                                       |                             |

* L: third-instar larvae; PP: prepupae; P: two-day-old pupae; OP: ten-day-old pupae; M: male adult; FM: female adult.

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expression results with a given target gene, the reference gene(s) must be stably expressed under the specific experimental conditions. If the appropriate reference genes are not selected, expression of the target gene may be biased and show significant differences that lead to incorrect conclusions [51–52].
The emerging availability of genomic sequencing, gene chips, and databases provide new approaches to more accurately select reference genes [53–55]. Former housekeeping genes with unstable expression will be gradually replaced by new reference genes that have been evaluated under experimental conditions. This study validated several reference genes for *L. trifolii* and provides an approach that should be considered when screening reference genes in other species.

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**References**

1. Spencer KA. Agromyzidae (Diptera) of economic importance, 9: Series Entomologica, ed. by Schimitschek E., Gottingen. Bath: The Hague Publishers; 1973. pp. 19–28.

2. Kang L, Chen B, Wei JN, Liu TX. Roles of thermal adaptation and chemical ecology in *Liriomyza* distribution and control. Annu Rev Entomol. 2009; 54: 127–145. https://doi.org/10.1146/annurev.ento.54.110807.090507 PMID: 19710304

3. Gao YL, Reitz SR. Emerging themes in our understanding of species displacements. Annu Rev Entomol. 2017; 62: 165–183. https://doi.org/10.1146/annurev-ento-031616-035425 PMID: 27860525

4. Yang F, Du YZ, Cao JM, Huang FN. Analysis of three leafminers’ complete mitochondrial genomes. Gene. 2013; 529: 1–6. https://doi.org/10.1016/j.gene.2013.08.010 PMID: 23954222

5. Chang YW, Shen Y, Dong CS, Gong WR, Tian ZH, Du YZ. Occurrence and population dynamics of *Liriomyza trifolii* and *Liriomyza sativae* in Jiangsu. Chinese Bull Entomol. 2016; 54: 884–891.

6. Wang ZG, Guan W, Chen DH. Preliminary report of the *Liriomyza trifolii* in Zhongshan area. Plant Q. 2007; 21: 19–20.

7. State Forestry Administration. Exotic pest- *Liriomyza trifolii* is found in China. Plant Prot. 2006; 26:55.

8. Lei ZR, Yao JM, Zhu CJ, Wang HH. Prediction of suitable areas for *Liriomyza trifolii* (burgess) in China. Plant Prot. 2007; 33: 100–103.

9. Xiang JC, Lei ZR, Wang HH, Gao YL. Interspecific competition among three invasive *Liriomyza* species. Acta Ecol Sin. 2012; 32: 1616–1622.

10. Gao YL, Reitz S, Xing ZL, Ferguson S, Lei ZR. A decade of a leafminer invasion in China: Lessons learned. Pest Manag Sci. 2017; https://doi.org/10.1002/ps.4591

11. Johnson MW, Welker SC, Toscano NC. Reduction of tomato leaflet photosynthesis rates by mining activity of *Liriomyza sativae* (Diptera: Agromyzidae). J Econ Entomol. 1983; 76: 1061–1063.
12. Parrella MP, Jones VP, Youngman RR, Lebeck LM. Effect of leaf mining and leaf stippling of *Liriomyza* spp. on photosynthetic rates of chrysanthemum. Ann Entomol Soc Am. 1984; 78: 90–93.

13. Burand JP, Hunter WB. Rna: future in insect management. J Invertebr Pathol. 2013; 112: S68–S74. https://doi.org/10.1016/j.jip.2012.07.012 PMID: 22841639

14. Huang YP, Chen YZ, Zeng BS, Wang YJ, James AA, Gurr GM, et al. Crispicas9 mediated knockout of the abdominal-a homeotic gene in the global pest, diamondback moth (*Plutella xylostella*). Insect Biochem Mol Biol. 2016; 75: 98–106. https://doi.org/10.1016/j.ibmb.2016.06.004 PMID: 27318252

15. Zhu GH, Xu J, Cui Z, Dong XT, Ye ZF, Niu DJ, et al. Functional characterization of sitlpbp3 in *Spodoptera litura* by Crispicas9 mediated genome editing. Insect Biochem Mol Biol. 2016; 75: 1–9. https://doi.org/10.1016/j.ibmb.2016.05.006 PMID: 27192033

16. Yang CX, Hui L, Pan HP, Ma YB, Zhang DY, Yong L, et al. Stable reference gene selection for RT-qPCR analysis in nonviruliferous and viruliferous *Frankliniella occidentalis*. PLoS ONE. 2015; 10: e0135207. https://doi.org/10.1371/journal.pone.0135207 PMID: 26244556

17. Zheng YT, Li HB, Lu MX, Du YZ. Evaluation and validation of reference genes for RT-qPCR normalization in *Frankliniella occidentalis* (Thysanoptera: Thripidae). PLoS ONE. 2014; 9: e111369. https://doi.org/10.1371/journal.pone.0111369 PMID: 25356721

18. Yang CX, Pan HP, Noland JE, Zhang DY, Zhang ZH, Liu Y, et al. Selection of reference genes for RT-qPCR analysis in a predatory biological control agent, *Coleomegilla maculata* (*Coleoptera*: Coccinellidae). Sci Rep. 2015; 5:18201. https://doi.org/10.1038/srep18201 PMID: 26656102

19. Nakamura AM, Chahad-Ehlers S, Lima ALA, Taniguti CH, Sobrinho I Jr, Torres FR, et al. Reference genes for accessing differential expression among developmental stages and analysis of differential expression of OBP genes in *Anastrepha obliqua*. Sci Rep. 2016; 6:17480. https://doi.org/10.1038/srep17480 PMID: 26818909

20. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol. 2005; 34: 597–601. PMID: 15956331

21. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol. 2005; 25: 169–193.

22. Citri A, Pang ZP, Südhof TC, Wernig M, Malenka RC. Comprehensive qRT-PCR profiling of gene expression in single neuronal cells. Nat Protoc. 2012; 7: 118–117.

23. Shi CH, Zhang YJ. Advances in reference gene for real-time quantitative reverse transcription PCR (qRT-PCR) of insects research. Chinese J Appl Entomol. 2016; 53(2): 237–246.

24. Cardoso GA, Matioli CC, de Azeredo-Espin AML, Torres TT. Selection and validation of reference genes for functional studies in the Calliphoridae family. J Insect Sci. 2014; 14: 2. https://doi.org/10.1093/jis/14.1.2 PMID: 25373149

25. Sun M, Lu MX, Tang XT, Du YZ. Exploring valid reference genes for quantitative real-time PCR analysis in *Sesamia inferens* (Lepidoptera: Noctuidae). PLoS ONE. 2015; 10: e0115979. https://doi.org/10.1371/journal.pone.0115979 PMID: 25585250

26. Ponton F, Chapuis MP, Pernice M, Sword GA, Simpson SJ. Evaluation of potential reference genes for reverse-transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. J Insect Physiol. 2011; 57: 840–850. https://doi.org/10.1016/j.jinsphys.2011.03.014 PMID: 21435341

27. Yuan M, Lu YH, Zhu X, Wang YJ, James AA, Gurr GM, et al. Selection of reference genes for reverse transcription-polymerase chain reaction assays of *Nilaparvata lugens* (Hemiptera: Delphaciidae) using reverse-Transcription Quantitative PCR. PLoS ONE. 2014; 9: e86503. https://doi.org/10.1371/journal.pone.0086503 PMID: 24466124

28. Li RM, Xie W, Wang SL, Wu QJ, Yang NN, Yang X, et al. Reference gene selection for qRT-PCR analysis in the sweetpotato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae). PLoS ONE. 2013: 8: e53006. https://doi.org/10.1371/journal.pone.0053006 PMID: 23308130

29. Huang LH, Wang CZ, Le K. Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativa*. J Insect Physiol. 2009; 55:279–85. https://doi.org/10.1016/j.jinsphys.2008.12.004 PMID: 19133268

30. Huang LH, Kang L. Cloning and interspecific altered expression of heat shock protein genes in two leafminer species in response to thermal stress. Insect Mol Biol. 2007; 16:491–500. https://doi.org/10.1111/1365-2583.2007.00744.x PMID: 17651238

31. Ji QZ, Wang HH, Lei ZR, Zhang KW, Wang J, Zhang Y. Cloning and expression of heat shock protein gene 90 gene in relation to heat stress in the leafminer, *Liriomyza trifoli*. Plant Prot. 2013; 39: 110–116.

32. Zheng D, Cui XH, Li HL, Cai C, Gao YS, Shang HW. Cloning of heat shock protein gene, *hsp70*, in *Liriomyza trifoli* and its expression under temperature stress. Acta Phytophy Sin. 2010; 37:159–164.
Selection and validation of reference genes in \textit{Liriomyza trifoli}
54. Kim S, Kim MS, Kim YM, Yeom SI, Cheong K, Kim KT. et al. Integrative structural annotation of de novo RNA-Seq provides an accurate reference gene set of the enormous genome of the onion (Allium cepa L.). DNA Res. 2015; 22:19–27. https://doi.org/10.1093/dnares/dsu035 PMID: 25362073

55. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005; 139: 5–17. https://doi.org/10.1104/pp.105.063743 PMID: 16166256