Identification of candidate reference genes for qRT-PCR normalization studies of salinity stress and injury in *Onchidium reevesii*

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Abstract

Real-time quantitative reverse transcription-PCR (qRT-PCR) is an undeniably effective tool for measuring levels of gene expression, but the accuracy and reliability of the statistical data depend mainly on the fundamental expression of selected housekeeping genes in many samples. To date, there have been few analyses of stable housekeeping genes in *Onchidium reevesii* under salinity stress and injury. In this study, the gene expression stabilities of seven commonly used housekeeping genes, *CYC*, *RPL28S*, *ACTB*, *TUBB*, *EF1a*, *Ubiq* and *18S RNA*, were investigated using BestKeeper, geNorm, NormFinder and RefFinfer. Although the results of the four programs varied to some extent, in general, *RPL28S*, *TUBB*, *ACTB* and *EF1a* were highly ranked. *ACTB* and *TUBB* were found to be the most stable housekeeping genes under salinity stress, and *EF1a* plus *TUBB* was the most stable combination under injury stress. When analysing target gene expression in different tissues, *RPL28S* or *EF1a* should be selected as the reference gene according to the level of target gene expression. Under extreme environmental stress (salinity) conditions, *ACTB* (0 ppt, 5 ppt, 15 ppt, 25 ppt) and *TUBB* (35 ppt) were reasonable reference gene choices when expression stability and abundance were considered. In addition, the best two-gene combination was *TUBB* and *EF1a* at 15 ppt salinity and injury stress. Therefore, we suggest that *RPL28S*, *ACTB* and *TUBB* are suitable reference genes for the analysis of mRNA transcript levels. Based on analysis of candidate gene expression, the tolerance of *O. reevesii* to low salinity (low osmotic pressure) is reduced compared to its tolerance to high salinity (high osmotic pressure). These findings will help researchers obtain accurate results in future quantitative gene expression analyses of *O. reevesii* under other stress conditions.

Introduction

With advantages of relatively accurate quantification, high sensitivity and high throughput, quantitative reverse transcription polymerase chain reaction (qRT-PCR) has become one of the most widely used techniques to detect changes in gene expression[1,2]. Indeed, the use of qRT-PCR has increased tremendously in nearly all branches of biology[3-6]. However, there ~~inevitably~~ are inevitably always a number of influencing factors that affect the efficiency of these reactions[7], such as discrepancy in pipetting, RNA quality and concentration, efficiency of reverse transcription and amplification among different samples, PCR procedures and primer amplification efficiency. Therefore, to avoid these variations or errors, it is fundamental to standardize the level of target gene expression by utilizing in parallel a reference gene

as an internal control[8]. In general, an ideal reference gene should demonstrate a consistent level of expression across all tested tissues or conditions[9]. Nonetheless, there is increasing evidence that the expression of assumed reference genes can vary observably with experimental conditions such as developmental stage and chemical treatment, significantly affecting relative quantification qPCR result interpretation.

Certain housekeeping genes, such as *18S ribosomal RNA* (*18SrRNA*), *28S ribosomal RNA* (*28SrRNA*), *β-actin* (*ACTB*), *cyclophilin* (*CYP2*), *elongation factor 1-alpha* (*EF1a*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *translation elongation factor* (*TEF*), *tubulin* (*TUBB)* and *polyubiquitin* (*UBQ*)[10-12], are commonly used as reference genes. As these housekeeping genes are related to basic metabolic processes and are essential for normal cell growth, their expression levels are thought to be stable. However, many recent studies have revealed that fluctuations in the expression levels of housekeeping genes may be largely influenced by the tissue, individual developmental stage or experimental conditions[13]. In fact, there is no clear evidence to show that a single universal reference gene is suitable for different tissues and varying experimental conditions[14]. Thus, it is crucial to determine one or two stably expressed reference genes before they are applied for normalization of the expression levels of target genes by qRT-PCR technology.

Onchidium reevesii (Mollusca, Gastropoda, Pulmonata, Systellommatophora, Onchidiidae) is a brackish water amphibious sea slug that resides mainly in river ports of the intertidal zone and coastal tidal flats, reed and mangroves[15,16]. The species is rich in natural collagen, which has high nutritional and medicinal value; thus, it may constitute an excellent specialty aquatic product for humans if ~~the breeding issues can be resolved~~issues pertaining to their breeding in captivity can be resolved[17]. Moreover, *O. reevesii* is an important part of the biodiversity of wetlands and is considered to be a model organisms for studying the evolution of marine invertebrates from sea to land[18]. *O. reevesii* has been investigated with regard to morphological, physiological and active substance perspectives, and further molecular biology research is ~~very urgent~~required[19-21]. Additionally, suitable reference genes are important for accurately interpreting expression analyses of functional genes and for evaluating RNAi efficiency. *O. reevesii* is a euryhaline organism, and the salinity of its habitat varies greatly under the influence of tides, rainfall, river flow and other factors. Moreover, due to its complex geographical environment and biodiversity, these organisms are inevitably attacked and injured by natural enemies. In this study, an extreme environment (salinity) and stress conditions were imposed on *O. reevesii* in an effort to determine stably expressed internal reference genes under such treatment and to provide basic data for future studies on its ability to regulate osmotic pressure, to adapt to extreme environments and to self-repair damage.

Materials and methods

1. Animals and treatments

Sexually mature *O. reevesii* adults were obtained from the coast of Shanghai, China, and housed in the laboratory according to Shen's method[22] in aquaculture tanks (each tank containing no more than 50 individuals), with enough fine silt and seawater (10 ppt) to simulate their natural living environment and shelters for them to hide. Feeding (corn flour and diatoms), removal of faeces and fresh seawater replacement were performed in a timely manner. The animals were allowed to acclimate to the new environment for 7 days before the experiments. For salinity stress, 150 *O. reevesii* individuals of the same size and weight were divided into 5 groups according to salinity (0 ppt, 5 ppt, 15 ppt, 25 ppt, 35 ppt). The water used in the experiment was saline, and the volume of water used ensured that all the samples were

retained. For injury stress, a surgical blade was used to inflict wounds of ~5–7 mm long and 2–3 mm deep on the dorsal skin tissue of 30 *O. reevesii* individuals; the experiment was divided into 5 groups (8 g, 11 g, 15 g, 18 g and 22 g) according to the weight of the individuals. To eliminate the influence of temperature change, the experimental temperature was ~~controlled~~ maintained at approximately 25°C ~~as much as possible~~, the temperature at which *O. reevesii* exhibits the best activity in the field.

2. Sampling

Dorsal skin tissues were used as samples for assessing the salinity stress and the injury ~~experments~~experiments; sampling was performed at 2 h, 4 h, 12 h, 24 h, 48 h and 7 days. Three individuals from each group were sampled at every time point[23]. Dorsal skin tissues were also used as samples for the groups of different weights. Six tissues were used for assessing expression, including the dorsal skin muscle, intestine, lip, pleopod, liver pancreas and gonad. All of the samples were placed in freezer tubes, frozen in liquid nitrogen and stored at -80°C.

3. Total RNA extraction and first-strand cDNA synthesis

Samples were rapidly ground in liquid nitrogen, and total RNA was extracted using TRIZOL (TaKaRa, Otsu, Japan). All RNA samples were resuspended in RNase and DNase-free ddH_2O (TaKaRa, Otsu, Japan). The integrity of total RNA was determined by 1% agarose gel electrophoresis, and a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA) was employed to evaluate the RNA quality and concentration. RNA with a 260/280 ratio between 1.8 and 2.2 and 260/230 ratio >1 and <3 was considered satisfactory for use in the experiments.

4. Selection of internal control genes

Seven commonly used reference genes were identified from the transcriptome of *O. reevesii*. The following are some of the criteria we applied for selecting the candidate reference genes: to minimize the effect of co-regulation, the nucleic acid sequence should encode a protein that plays various roles in cellular metabolism with different molecular functions; sequences that have previously been examined for stability in, to a certain extent, similar biological contexts; and for reliability, the sequence should be tested specifically to verify the accuracy of the data[24]. Previously published candidate reference genes[25-27] are *cyclophilin* (*CYC*), *beta actin* (*ACTB*), *elongation factor-1 alpha* (*EF1a*), *ribosomal protein L28* (*RPL28S*), *β-tubulin* (*TUBB*), *ubiquitin* (*Ubiq*) and *18S ribosomal RNA* (*18S RNA*). To select corresponding sequences, all candidate genes selected from the transcriptome were analysed by BLAST in the NCBI database, and sequences were uploaded to the NCBI database to obtain the GenBank accession number (**Table 1**).

5. Primer design and real-time qPCR assays

The software primer 5.0 was used to design gene-specific primers. Optimized primer pairs were selected based on their amplification efficiencies and specificities[28]. The specificity of the PCR primers utilized was evaluated using the melting curve produced by the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher, New York, USA). The fragment size of the PCR product was determined by 1% agarose gel electrophoresis. Primer standard curves were created using a cDNA dilution

series, and amplification efficiencies were calculated using the following equation: E = ($10^{-1/slope}$ −1)*100%[29]. A correction for different amplification efficiencies was introduced in the sample quantification process[30].

NovoStart® Reverse Transcriptase (NOVOPROTEIN, Shanghai, China) was used to reverse transcribe ~~to cDNA~~ 2000 ng of total RNA from each sample into cDNA in a 20-μl volume. To allow for increasing the volume of template cDNA for subsequent experiments, stock cDNA samples were diluted 5-fold for real-time PCR. Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System was employed for real-time PCR in a 20-μl reaction system containing the following components: 2 μl cDNA sample, 10 μl 2×NovoStart® SYBR qPCR SuperMix (NOVOPROTEIN, Shanghai, China), 0.8 μl each primer, 0.4 μl ROX Reference Dye II and 6.0 μl ddH_2O. Following denaturation at 94°C for 5 min, 40 cycles of melting at 95°C for 15 s, annealing at 57°C for 20 s and extension at 72°C for 30 s were carried out. A melting curve analysis was also performed.

Data analysis

Three software tools, geNorm, BestKeeper, and NormFinder, were used to evaluate reference gene stability according to their respective instructions[31-33]. The geNorm program ranks the most stable reference genes based on the average pairwise variation of a reference gene with other selected housekeeping genes and sorts reference genes using their expression stability value (M). In g~~C~~eneral, ~~in nature,~~ the lower the M value, the higher the expression stability. BestKeeper predicts “ideal” reference genes according to pair-wise correlation analysis among all pairs of candidate reference genes. NormFinder calculates the entire variation of candidate reference genes in all samples and also performs intragroup and intergroup comparisons. RefFinder (https://omictools.com/reffinder-tool) is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes from extensive experimental datasets. It integrates the currently available major computational programs (geNorm, NormFinder, BestKeeper) to compare and rank candidate reference genes. Based on the rankings from each program, RefFinder assigns an appropriate weight to an individual gene and calculates the geometric mean of their weights for the overall final ranking[34].

Results

Real-time PCR amplification efficiencies

To ensure comparability among the 7 housekeeping genes, PCR amplification efficiencies were calculated based on the slopes resulting from the measurement of cDNA serial dilutions ~~of cDNA~~. All tested reference gene amplification efficiencies were in the range of 90–110% (*RPL28S* (94.3 %), *ACTB* (97.03 %), *TUBB* (97.32 %), *CYC* (96.61 %), *EF1α* (92.03 %), *18S RNA* (95.54%) and *Ubiq* (100.89%). The melting curves for all amplification products demonstrated a single peak, verifying primer-specific amplification (**Supplementary Fig. S1**).

Expression stability of the ~~seven genes in tissues~~tested genes

Based on statistical results for expression analyses of each housekeeping gene in the 7 different tissue types according to BestKeeper (Table 2), genes showing the lowest expression level were *CYC* and *Ubiq*,

with Ct mean (AM[Ct]) values of 28.96 and 31.09 cycles, respectively. The genes exhibiting the highest expression levels were *ACTB*, *TUBB* and *EF1α*, with AM[Ct] values of 22.16, 23.06 and 19.75 cycles, respectively. *RPL28S* displayed the least variation in expression among the analysed tissues [SD (±Ct) = 0.48] and *18S RNA* [SD (±Ct) =1.72] and *ACTB* [SD (±Ct) = 2.67] the greatest variation. Candidate reference genes with an SD value higher than 1 must be considered unsuitable[35], and the M value of *ACTB* according to the geNorm program was clearly higher than 1.5. Overall, the ranking of these candidate housekeeping genes by BestKeeper, NormFinder and geNorm programs was consistent. In addition, *ACTB* and *18S RNA* appeared to be less stable, whereas *RPL28S*, *EF1α*, and *TUBB* were the most stable genes (**Table 2**; **Fig. 1** Group1, **Fig. 2** A). According to geNorm, a V2/3 value of 0.15 is the proposed cut off value, under which the inclusion of an additional reference gene is not necessary (**Supplementary Fig. S2** A). In this study, the V2/3 value was 0.265, and the values of V3/4, V4/5, V5/6, and V6/7 were all greater than 0.15, indicating no need to include another gene ~~in~~ as a normalization factor. Thus, the optimal number of reference genes for normalization in this example should be one. The comprehensive ranking of the seven candidate genes given by RefFinder indicated *RPL28S* as the best reference gene. *EF1a* was also found to be an ideal reference gene when abundant reference gene expression is required.

Expression stability in muscle of the seven genes under different salinity treatments

Considering that the salinity of the *O. reevesii*'s habitat often fluctuates greatly due to the influence of tides, river flow, rainfall and other factors, multiple salinity gradients were established to observe changes in housekeeping ~~gene~~expression gene expression to explore the response of *O. reevesii* to changes in salinity (osmotic pressure regulation ability).

0 ppt salinity

Under ~~the~~ conditions of ~~various~~ varying salinity~~ies~~, BestKeeper, NormFinder, and geNorm were employed to examine the expression of each housekeeping gene. According to BestKeeper (**Table 3**), the expression stability ranking of the seven reference genes for the seven sampling time points was as follows: *18S RNA* > *RPL28S* > *ACTB* > *TUBB* > *Ubiq* > *EF1a* > *CYC*. Additionally, geNorm analysis showed that *TUBB*, *EF1a* and *ACTB* had the highest stabilities (**Fig. 1** Group 2). NormFinder analysis revealed *ACTB* to be the gene with the greatest stability (**Fig. 2** B). Because the Vn/n+1 values provided by geNorm were all greater than 0.15, multiple housekeeping genes were not required as internal references (**Supplementary Fig. S2** B). Based on the results of a comprehensive analysis by RefFinder, we recommend that *TUBB* be used as an internal reference for qRT-PCR analyses of *O. reevesii* under this condition. However, when the reference gene should exhibit a high level of expression, *ACTB* is the most appropriate choice.

5 ppt salinity

According to BestKeeper (**Table 4**), the expression stability ranking of the seven candidate genes was *18S RNA* > *RPL28S* > *EF1a* > *ACTB* > *TUBB* > *Ubiq* > *CYC*, an order that was slightly different from the result of the 0 ppt salinity condition. However, the SD[±Ct] values for the housekeeping genes were greater than 1, except for *18S RNA* (0.68). However, NormFinder (**Fig. 1** Group3) and geNorm (**Fig. 2** C)

indicated *EF1a*, *RPL28S* and *TUBB* to be the most stable genes, with Vn/n+1 values all greater than 0.15 (**Supplementary Fig. S2** C). Although the results from the three software programs are inconsistent and not ideal, *RPL28S* should be used as a reference gene under this condition according to RefFinder. Due to the insufficient level of *18S RNA*, *RPL28S* and *EF1a* expression, *ACTB* should be used as a reference gene under the stress of this level of salinity.

15 ppt salinity

BestKeeper results (**Table 5**) showed a stability ranking of expression of the seven candidate genes of *CYC> ACTB > EF1a> RPL28S > TUBB >18S RNA > Ubiq*. This order is significantly different from that of the 0 ppt and 5 ppt conditions, though SD[±Ct] for all housekeeping genes was lower than 1. According to NormFinder (**Fig. 1** Group4) and geNorm (**Fig. 2** D), *EF1a*, *ACTB*, *CYC* and *TUBB* were the most stable; the V2/3 values of 0.127 suggested that the normalization factor should preferably consist of two housekeeping genes (**Supplementary Fig. S2** D). The best combination of two genes was *TUBB* and *EF1a* (**Fig. 2** D). RefFinder indicated that *TUBB* was the most stable internal reference gene. Except for *Ubiq* with slightly lower expression, *EF1a*, *ACTB* and *CYC* can all be used as internal reference genes in this treatment.

25 ppt salinity

For this condition, the BestKeeper results (**Table 6**) showed an expression stability ranking of *Ubiq > ACTB > EF1a > RPL28S > CYC > TUBB >18S RNA*; except for *18S RNA*, SD [±Ct] was lower than 1. Different from the above conditions, the NormFinder (**Fig. 1** Group5) and geNorm (**Fig. 2** E) results were similar to those of BestKeeper. V2/3 values were 0.156, indicating no need for multiple housekeeping genes as internal references (**Supplementary Fig. S2** E). Although the *Ubiq* gene was found to be the most stable, its expression level was far lower than that of the *ACTB* gene. Therefore, the best reference gene was *ACTB*.

35 ppt salinity

TUBB > CYC > RPL28S > Ubiq > ACTB > EF1a > 18S RNA was the expression stability ranking according to BestKeeper (**Table 7**). As in the 25 ppt salinity condition, SD[±Ct] values were lower than 1, except for *18S RNA*, and the NormFinder (**Fig. 1** Group6) and geNorm (**Fig. 2** F) results were similar to those of BestKeeper. As V2/3 values were over 0.15, multiple housekeeping genes would not be needed (**Supplementary Fig. S2** F). Although the expression abundance of all candidate genes was within the acceptable range, after considering both expression stability and abundance, the best reference gene was found to be *TUBB*.

Expression stability analysis of the seven genes in muscle after injury

For each of the seven housekeeping genes, transcript abundance was assessed in three independent muscle pools collected at time points ranging from 2 h to 7 days after skin damage. According to

BestKeeper (**Table 8**), the lowest level of expression gene was displayed by *Ubiq*, with an AM[Ct] value of 29.85 cycles. In contrast, level *ACTB* and *EF1α* showed the highest levels, with AM[Ct] values of 18.75 and 18.92 cycles, respectively. The BestKeeper program indicated SD(±Ct) values lower than 1 for the seven reference genes, except for *CYC*, *Ubiq* and *18S RNA*; the geNorm M values of the seven reference genes were also lower than 1.5. Based on BestKeeper, the gene that exhibited the least variation in gene expression was *ACTB* [SD(±Ct) = 0.46]. However, NormFinder (**Fig. 1** Group7) and geNorm (**Fig. 2** G) indicated *TUBB* to be the most stable. geNorm analysis V2/3 values were 0.137, suggesting that the normalization factor should comprise more housekeeping genes (**Supplementary Fig. S2** G). The most suitable two-gene combination was *TUBB* plus *EF1a* (**Fig. 2** G). If a single gene is used as an internal reference, RefFinder indicated *EF1a* as the most appropriate choice.

Expression stability analysis of the seven genes in individuals of different weights

Regarding groups of *O. reevesii* of different weights, BestKeeper analysis (**Table 9**) revealed an SD[±Ct] lower than 1 only for the *CYC* gene. However, NormFinder (**Fig. 1** Group8) and geNorm (**Fig. 2** H) showed that *RPL28S*, *TUBB* and *EF1a* were significantly more stable than were the other candidate genes, with Vn/n+1 values greater than the threshold of 0.15 (**Supplementary Fig. S2** H). Based on RefFinder comprehensive analysis, *RPL28S* is more suitable as an internal reference gene compared to the other candidates, similar to the results for skin injury. Because the reference gene requires a high level of expression, *ACTB* is the most appropriate reference gene for this experimental condition.

Discussion

In this study, seven genes, *CYC*, *RPL28S*, *ACTB*, *TUBB*, *EF1a*, *Ubiq* and *18S RNA*, were selected as candidates for reference gene screening for use in *O. reevesii*. When using a relative quantification technique to analyse mRNA expression of a target gene, the final results are more accurate and scientific when appropriate internal reference genes are employed. In general, screening of internal reference genes should to meet the following criteria: it should be widely involved in all aspects of the organism's cellular metabolism; its expression should maintain a high level and stability in a range and with low sensitivity to changes in the external environment; candidate genes should be expressed stably under different experimental conditions.

Because of advantages of high timeliness, sensitivity and convenience, qRT-PCR is often used to screen reference genes. At present, there are three programs commonly used: BestKeeper, geNorm and NormFinder. However, due to the different modes in which data are processed by the respective software, there are some inconsistencies regarding the output. RefFinder comprehensively evaluates the results of the above three programs and provides a relatively reasonable internal control gene rank to help the experimenter ultimately determine the optimal choice.

We examined different salinity stress levels, skin muscle injury, different tissues of *O. reevesii* adults and individuals with different weights to identify reference genes. The most suitable internal reference genes in different tissues of *Haliotis rufescens* are reportedly *RPL5S* and *CYC*; the most stable gene in *O. reevesii* is the *RPL28S* gene[36], but *EF1a* can serve as an alternative. When analysing differences in target gene expression in different tissues of *O. reevesii*, *RPL28S* should be used as the reference gene if the

abundance of target gene expression is low; *EF1a* is recommended if target gene expression is high. ~~Some researchers~~Previous studies have compared the most stable internal reference genes of flatworms under salinity stress, revealing GM2-activator protein (*GM2AP*) and *ACTB*; these results are similar to those for *O. reevesii*, in which the more suitable internal control genes are *TUBB* and *ACTB*[37]. However, our candidate gene expression analysis showed that at low salinity (0ppt, 5 ppt) (low osmotic pressure environment) under laboratory conditions, expression of these genes was significantly lower than that at 15 ppt and 25 ppt. One reason for this may be that a low osmotic pressure environment leads to excess moisture entering the tissue, decreasing cellular metabolism and eventually housekeeping gene expression[38,39]. Nonetheless, at high salinity (35 ppt), the housekeeping genes essentially maintained a normal expression level, which may be due to the strong ability of *O. reevesii* to obtain water from the external environment, ensuring the stability of its internal environment. It can be inferred from the above that *O. reevesii* is not strongly tolerant to low salinity and osmotic pressure and that its optimal living salinity is approximately 15-25 ppt. Sun ~~JH~~ found that the reference genes *RPL13S* and *RPL32S* to be the most stably expressed genes in contused rat skeletal muscle; however, *EF1a* plus *TUBB* constitutes the most suitable internal reference combination for *O. reevesii* after tissue damage[40]. Heavy metal ion stress in organisms is also a major focus of current research, and as tidal flat inhabitants, *O. reevesii* feeds on the surface soil of these flats and may very likely serve as an indicator of heavy metal ion pollution. This is a research direction of our laboratory in the future[41-43]. Although we compared data for *O. reevesii* of different sizes, because there is no consensus regarding the relationship between its growth stage and body weight, these experimental data need further confirmation. This is the first study to screen internal reference genes for *O. reevesii* under different conditions, and the results will be useful for relative quantitation of gene expression in the future.

Conclusions

Here, we first ascertained and evaluated the expression stability of seven housekeeping genes for qRT-PCR normalization in *O. reevesii* tissues and under conditions of salinity stress and tissue injury. In our assessment of different tissues, *RPL28S* and *EF1a* were found to be the most suitable and stable internal genes among the six tissue samples tested as well as among individuals of different weights. The results suggest that *ACTB* and *TUBB* ~~to be~~are the most stable genes, with high expression levels when assessing salinity stress. For muscle injury, *EF1a* is the most stable candidate gene. Among all experimental groups, data analysis of two groups (15 ppt, injury) revealed *TUBB* plus *EF1a* to comprise a suitable reference combination. According to our results, we propose that the three housekeeping genes *ACTB*, *TUBB* and *EF1a* be the first choice of reference genes for qRT-PCR. Our experimental data indicate that *O. reevesii* has low tolerance to low osmotic pressure when compared to that to high osmotic pressure and that a salinity range of approximately 15-25 ppt is the most suitable living environment for this organism. To our knowledge, this study is the first to select and evaluate optimal reference genes for *O. reevesii*, and the findings are expected to provide theoretical data support for future ~~relative quantitative~~ experiments involving qPCR. Although the optimal internal reference gene differs among treatments, such as during salinity stress and tissue injury, it is important to understand the importance of the selection of these genes. Overall, for different experimental studies of *O. reevesii*, the selection of appropriate reference genes should be taken into consideration, and our results provide basic experimental data for this purpose.

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