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

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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 obtained depend mainly on the basal 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 ranked highly. *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) are reasonable reference gene choices when expression stability and abundance are considered. Under conditions of 15 ppt salinity and injury stress, our results showed that the best two-gene combination was *TUBB* plus *EF1a*. Therefore, we suggest that *RPL28S*, *ACTB* and *TUBB* are suitable reference genes for evaluating mRNA transcript levels. Based on candidate gene expression analysis, 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.

1 # Identification of candidate reference genes for qRT-PCR

2 # normalization studies of salinity stress and injury in

3 # *Onchidium reevesii*

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12 # Abstract

13 Real-time quantitative reverse transcription-PCR (qRT-PCR) is an undeniably effective tool for measuring levels

14 of gene expression, but the accuracy and reliability of the statistical data obtained depend mainly on the basal

15 expression of selected housekeeping genes in many samples. To date, there have been few analyses of stable

16 housekeeping genes in *Onchidium reevesii* under salinity stress and injury. In this study, the gene expression

17 stabilities of seven commonly used housekeeping genes, *CYC*, *RPL28S*, *ACTB*, *TUBB*, *EF1a*, *Ubiq* and *18S*

18 *RNA,* were investigated using BestKeeper, geNorm, NormFinder and RefFinfer. Although the results of the four

19 programs varied to some extent, in general, *RPL28S*, *TUBB*, *ACTB* and *EF1a* were ranked highly. *ACTB* and

20 *TUBB* were found to be the most stable housekeeping genes under salinity stress, and *EF1a* plus *TUBB* was the

21 most stable combination under injury stress. When analysing target gene expression in different tissues, *RPL28S*

22 or *EF1a* should be selected as the reference gene according to the level of target gene expression. Under extreme

23 environmental stress (salinity) conditions, *ACTB* (0 ppt, 5 ppt, 15 ppt, 25 ppt) and *TUBB* (35 ppt) are reasonable

24 reference gene choices when expression stability and abundance are considered. Under conditions of 15 ppt

25 salinity and injury stress, our results showed that the best two-gene combination was *TUBB* plus *EF1a*.

26 Therefore, we suggest that *RPL28S*, *ACTB* and *TUBB* are suitable reference genes for evaluating mRNA

27 transcript levels. Based on candidate gene expression analysis, the tolerance of *O. reevesii* to low salinity (low

28 osmotic pressure) is reduced compared to its tolerance to high salinity (high osmotic pressure). These findings

29 will help researchers obtain accurate results in future quantitative gene expression analyses of *O. reevesii* under

30 other stress conditions.

31 # Introduction

32 With advantages of relatively accurate quantification, high sensitivity and high throughput, quantitative

33 reverse transcription polymerase chain reaction (qRT-PCR) has become one of the most widely used techniques

34 to detect changes in gene expression[1,2]. Indeed, the use of qRT-PCR has increased tremendously in nearly all

35 branches of biology[3-6]. However, there are inevitably a number of influencing factors that affect the efficiency

36 of the reaction[7], such as discrepancy in pipetting, the RNA quality and concentration, the efficiency of reverse
37 transcription and amplification among different samples, the PCR procedures and primer amplification
38 efficiency. Therefore, to avoid variations or errors, it is fundamental to standardize the level of target gene
39 expression by utilizing in parallel a reference gene as an internal control[8]. In general, an ideal reference gene
40 should demonstrate a consistent level of expression across all tested tissues or conditions[9]. Nonetheless, there is
41 increasing evidence that expression of assumed reference genes can vary observably with experimental
42 conditions such as developmental stage and chemical treatment, significantly affecting relative quantification
43 and qPCR result interpretation.

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

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

Materials and methods

1. Animals and treatments

71   Shanghai Ocean University of Leicester granted Ethical approval to carry out the study within its facilities
72 (Approval number: Shou-DW-2019-010). Sexually mature *O. reevesii* adults were obtained from the coast of
73 Shanghai, China, and housed in the laboratory according to Shen's method[22] in aquaculture tanks (each tank
74 containing no more than 50 individuals), with enough fine silt and seawater (10 ppt) to simulate their natural

75   living environment and shelters for the animals to hide. Feeding (corn flour and diatoms), removal of faeces and
76   fresh seawater replacement were performed in a timely manner. The animals were allowed to acclimate to the
77   new environment for 7 days before the experiments. For salinity stress, 150 *O. reevesii* individuals of the same
78   size and weight were divided into 5 salinity groups (0 ppt, 5 ppt, 15 ppt, 25 ppt, 35 ppt). The water used in the
79   experiment was saline, and the volume of water used ensured that all samples were retained. For injury stress, a
80   surgical blade was used to inflict wounds of ~5–7 mm long and 2–3 mm deep on the dorsal skin of 30 *O. reevesii*
81   individuals; the experiment was divided into 5 groups according to the weight of the individuals: 8 g, 11 g, 15
82   g, 18 g and 22 g. To eliminate the influence of temperature change, the experimental temperature was maintained
83   at approximately 25°C, the temperature at which *O. reevesii* exhibits the best activity in the field.
84

2. Sampling

86   Dorsal skin tissues were used as samples in salinity stress and injury experiments; sampling was performed
87   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].
88   Dorsal skin tissues were also utilized as samples for the groups of different weights. Six tissues were used for
89   assessing gene expression, including the dorsal skin muscle, intestine, lip, pleopod, liver pancreas and gonad.
90   All of the samples were placed in freezer tubes, frozen in liquid nitrogen and stored at -80°C.
91

3. Total RNA extraction and first-strand cDNA synthesis

93   Samples were rapidly ground in liquid nitrogen, and total RNA was extracted using TRIZOL (TaKaRa,
94   Otsu, Japan). All RNA samples were resuspended in RNase and DNase-free ddH$_2$O (TaKaRa, Otsu, Japan). The
95   integrity of total RNA was determined by 1% agarose gel electrophoresis, and a NanoDrop 2000c
96   spectrophotometer (Thermo Scientific, Wilmington, USA) was employed to evaluate the RNA quality and
97   concentration. RNA with a 260/280 ratio between 1.8 and 2.2 and 260/230 ratio >1 and <3 was considered
98   satisfactory for use in experiments.
99

4. Selection of internal control genes

101   Seven commonly used reference genes were identified from the transcriptome of *O. reevesii*. The following
102   are some of the criteria we applied for selecting candidate reference genes: to minimize the effect of co-
103   regulation, the nucleic acid sequence should encode a protein that plays various roles in cellular metabolism with
104   different molecular functions; sequences that have previously been examined for stability in, to a certain extent,
105   similar biological contexts; for reliability, the sequence should be tested specifically to verify the accuracy of
106   the data[24]. Previously published candidate reference genes[25-27] are *cyclophilin* (*CYC*), *beta actin* (*ACTB*),
107   *elongation factor-1 alpha* (*EF1a*), *ribosomal protein L28* (*RPL28S*), *β-tubulin* (*TUBB*), *ubiquitin* (*Ubiq*) and *18S*
108   *ribosomal RNA* (*18S RNA*). To select corresponding sequences, all candidate genes selected from the
109   transcriptome were analysed in the NCBI database using BLAST, and sequences were uploaded to the NCBI
110   database to obtain the GenBank accession number (**Table 1**).
111

5. Primer design and real-time qPCR assays

112

113   The software primer 5.0 was used to design gene-specific primers. Optimized primer pairs were selected
114   based on their amplification efficiencies and specificities[28]. The specificity of the PCR primers utilized was
115   evaluated using the melting curve produced by the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR
116   System (ThermoFisher, New York, USA). The fragment size of the PCR product was determined by 2.0%
117   agarose gel electrophoresis. Primer standard curves were created using a cDNA dilution series, and amplification
118   efficiencies were calculated using the following equation: $E = (10^{-1/slope} - 1)*100\%$[29]. A correction for different
119   amplification efficiencies was introduced in the sample quantification process[30].

120   NovoStart® Reverse Transcriptase (NOVOPROTEIN, Shanghai, China) was used to reverse transcribe
121   2000 ng of total RNA from each sample into cDNA in a 20 μl volume. To allow for increasing the volume of
122   template cDNA for subsequent experiments, stock cDNA samples were diluted 5-fold for real-time PCR.
123   Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System was employed for real-time PCR using a
124   20 μl reaction system containing the following components: 2 μl cDNA sample, 10 μl 2×NovoStart® SYBR
125   qPCR SuperMix (NOVOPROTEIN, Shanghai, China), 0.8 μl each primer, 0.4 μl ROX Reference Dye II and 6.0
126   μl ddH$_2$O. Following denaturation at 94°C for 5 min, 40 cycles of melting at 95°C for 15 s, annealing at 57°C
127   for 20 s and extension at 72°C for 30 s were carried out. A melting curve analysis was also performed.

128

Data analysis

129

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

142

Results

143

144

Real-time PCR amplification efficiencies

145

146   To ensure comparability among the 7 housekeeping genes evaluated, PCR amplification efficiencies were
147   calculated based on the slopes resulting from the measurement of cDNA serial dilutions. All tested reference
148   gene amplification efficiencies were in the range of 90–110% (*RPL28S* (94.3 %), *ACTB* (97.03 %), *TUBB* (97.32

149 %), *CYC* (96.61 %), *EF1α* (92.03 %), *18S RNA* (95.54%) and *Ubiq* (100.89%). The melting curves for all
150 amplification products demonstrated a single peak, verifying primer-specific amplification (**Supplementary**
151 **Fig. S1**).

Expression stability of the tested genes

153   Based on the statistical results of expression analyses for each housekeeping gene in the 7 different tissue
154 types according to BestKeeper (Table 2), genes showing the lowest expression level were *CYC* and *Ubiq*, with
155 Ct mean (AM[Ct]) values of 28.96 and 31.09 cycles, respectively. The genes exhibiting the highest expression
156 levels were *ACTB*, *TUBB* and *EF1α*, with AM[Ct] values of 22.16, 23.06 and 19.75 cycles, respectively. *RPL28S*
157 displayed the least variation in expression among the analysed tissues [SD (±Ct) = 0.48], whereas *18S RNA* [SD
158 (±Ct) =1.72] and *ACTB* [SD (±Ct) = 2.67] showed the greatest variation. Candidate reference genes with an SD
159 value higher than 1 must be considered unsuitable[35], and the M value of *ACTB* according to the geNorm program
160 was clearly higher than 1.5. Overall, the ranking of these candidate housekeeping genes by BestKeeper,
161 NormFinder and geNorm programs was consistent. In addition, *ACTB* and *18S RNA* appeared to be less stable,
162 whereas *RPL28S*, *EF1α*, and *TUBB* were the most stable genes (**Table 2**; **Fig. 1** Group1, **Fig. 2** A). According
163 to geNorm, a V2/3 value of 0.15 is the proposed cut off value, under which the inclusion of an additional
164 reference gene is not necessary (**Supplementary Fig. S2** A). In this study, the V2/3 value was 0.265, and the
165 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
166 as a normalization factor. Thus, the optimal number of reference genes for normalization in this example is one.
167 The comprehensive ranking of the seven candidate genes given by RefFinder indicated *RPL28S* as the best
168 reference gene. *EF1a* was also found to be an ideal reference gene when abundant reference gene expression is
169 required.

170

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

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

0 ppt salinity

178   Under conditions of varying salinity, BestKeeper, NormFinder, and geNorm were employed to examine
179 the expression level of each housekeeping gene. According to BestKeeper (**Table 3**), the expression stability
180 ranking of the seven reference genes for the seven sampling time points was as follows: *18S RNA > RPL28S >*
181 *ACTB > TUBB > Ubiq > EF1a > CYC*. Additionally, geNorm analysis showed that *TUBB*, *EF1a* and *ACTB* had
182 the highest stabilities (**Fig. 1** Group 2). NormFinder analysis revealed *ACTB* to be the gene with the greatest
183 stability (**Fig. 2** B). Because the Vn/n+1 values provided by geNorm were all greater than 0.15, multiple
184 housekeeping genes were not required as internal references (**Supplementary Fig. S2** B). Based on the results
185 of a comprehensive analysis by RefFinder, we recommend that *TUBB* be used as an internal reference for qRT-

186 PCR analyses of *O. reevesii* under this condition. However, when the reference gene should exhibit a high level
187 of expression, *ACTB* is the most appropriate choice.
188

5 ppt salinity

190 According to BestKeeper (**Table 4**), the expression stability ranking of the seven candidate genes was *18S*
191 *RNA > RPL28S > EF1a > ACTB > TUBB > Ubiq > CYC*, an order that was slightly different from the result for
192 the 0 ppt salinity condition. However, except for *18S RNA* (0.68), the SD[±Ct] values for the housekeeping
193 genes were greater than 1. NormFinder (**Fig. 1** Group3) and geNorm (**Fig. 2** C) indicated *EF1a*, *RPL28S* and
194 *TUBB* to be the most stable genes, with Vn/n+1 values all greater than 0.15 (**Supplementary Fig. S2** C).
195 Although the results from the three software programs are inconsistent and not ideal, according to RefFinder,
196 *RPL28S* should be used as a reference gene under this condition. Due to the insufficient level of *18S RNA*,
197 *RPL28S* and *EF1a* expression, *ACTB* should be used as a reference gene under the stress of this level of salinity.
198

15 ppt salinity

200 BestKeeper results (**Table 5**) showed a stability ranking of expression for the seven candidate genes of
201 *CYC > ACTB > EF1a > RPL28S > TUBB > 18S RNA > Ubiq*, which was significantly different from that of the 0
202 ppt and 5 ppt conditions, even though SD[±Ct] for all housekeeping genes was lower than 1. According to
203 NormFinder (**Fig. 1** Group4) and geNorm (**Fig. 2** D), *EF1a*, *ACTB*, *CYC* and *TUBB* were the most stable
204 reference genes, and the V2/3 values of 0.127 suggested that the normalization factor should preferably consist
205 of two housekeeping genes (**Supplementary Fig. S2** D). The best combination of two genes was *TUBB* and
206 *EF1a* (**Fig. 2** D). RefFinder indicated that *TUBB* was the most stable internal reference gene. *Ubiq* exhibited
207 slightly lower expression, but *EF1a*, *ACTB* and *CYC* can all be used as internal reference genes for this treatment.
208

25 ppt salinity

210 For this condition, BestKeeper results (**Table 6**) revealed an expression stability ranking of *Ubiq > ACTB*
211 *> EF1a > RPL28S > CYC > TUBB > 18S RNA*; however, SD[±Ct] for *18S RNA* was lower than 1. Different
212 from the above conditions, the NormFinder (**Fig. 1** Group5) and geNorm (**Fig. 2** E) results were similar to those
213 of BestKeeper. V2/3 values were 0.156, indicating no need for multiple housekeeping genes as internal
214 references (**Supplementary Fig. S2** E). Although the *Ubiq* gene was found to be the most stable, its expression
215 level was far lower than that of the *ACTB* gene. Therefore, the best reference gene is *ACTB*.
216

35 ppt salinity

218 *TUBB > CYC > RPL28S > Ubiq > ACTB > EF1a > 18S RNA* was the expression stability ranking according
219 to BestKeeper (**Table 7**). As with the condition of 25 ppt salinity, SD[±Ct] values were lower than 1, except for
220 *18S RNA*, and the NormFinder (**Fig. 1** Group6) and geNorm (**Fig. 2** F) results were similar to those of
221 BestKeeper. As V2/3 values were over 0.15, multiple housekeeping genes would not be needed (**Supplementary**
222 **Fig. S2** F). The expression abundance of all candidate genes was within the acceptable range, after considering

223   both expression stability and abundance, the best reference gene was found to be *TUBB*.
224

Expression stability analysis of the seven genes in muscle after injury

226   For each of the seven housekeeping genes, transcript abundance was assessed in three independent muscle
227   pools collected at time points ranging from 2 h to 7 days after skin damage. According to BestKeeper (**Table 8**),
228   the lowest level of expression gene was displayed by *Ubiq*, with an AM[Ct] value of 29.85 cycles. In contrast,
229   *ACTB* and *EF1α* showed the highest levels, with AM[Ct] values of 18.75 and 18.92 cycles, respectively. The
230   BestKeeper program indicated SD(±Ct) values lower than 1 for the reference genes other than *CYC*, *Ubiq* and
231   *18S RNA*; the geNorm M values of the seven reference genes were also lower than 1.5. BestKeeper indicated
232   that the gene exhibiting the least variation in gene expression was *ACTB* [SD(±Ct) = 0.46]. However,
233   NormFinder (**Fig. 1** Group7) and geNorm (**Fig. 2** G) showed *TUBB* to be the most stable. geNorm analysis V2/3
234   values were 0.137, suggesting that the normalization factor should comprise additional housekeeping genes
235   (**Supplementary Fig. S2** G). The most suitable two-gene combination was *TUBB* plus *EF1a* (**Fig. 2** G). If a
236   single gene is used as an internal reference, RefFinder indicated *EF1a* as the most appropriate choice.
237

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

240   Regarding groups of *O. reevesii* of different weights, BestKeeper analysis (**Table 9**) revealed an SD[±Ct]
241   lower than 1 only for *CYC* gene. In contrast, NormFinder (**Fig. 1** Group8) and geNorm (**Fig. 2** H) showed
242   *RPL28S*, *TUBB* and *EF1a* to be significantly more stable than the other candidate genes, with Vn/n+1 values
243   greater than the threshold of 0.15 (**Supplementary Fig. S2** H). Based on RefFinder comprehensive analysis,
244   *RPL28S* is more suitable as an internal reference gene when compared to the other candidates, similar to the
245   results for the skin injury condition. Because a reference gene requires a high level of expression, *ACTB* is the
246   most appropriate reference gene for this experimental condition.
247

Discussion

249   In this study, seven genes, *CYC*, *RPL28S*, *ACTB*, *TUBB*, *EF1a*, *Ubiq* and *18S RNA*, were selected as
250   candidates for reference gene screening for use in *O. reevesii*. When using a relative quantification technique to
251   analyse mRNA expression of a target gene, the final results are more accurate and scientific when appropriate
252   internal reference genes are employed. In general, screening of internal reference genes should to meet the
253   following criteria: it should be widely involved in all aspects of the organism's cellular metabolism; its expression
254   should maintain a high level and stability in a range and with low sensitivity to changes in the external
255   environment; candidate genes should be expressed stably under different experimental conditions.
256   Because of advantages of high timeliness, sensitivity and convenience, qRT-PCR is often used to screen
257   reference genes. At present, there are three programs commonly used: BestKeeper, geNorm and NormFinder.
258   However, due to the different modes in which data are processed by the respective software, there are some
259   inconsistencies regarding the output. RefFinder comprehensively evaluates the results of the above three

260   programs and provides a relatively reasonable internal control gene rank to help the experimenter ultimately

261   determine the optimal choice.

262   We examined different salinity stress levels, skin muscle injury, different tissues of *O. reevesii* adults and

263   individuals with different weights to identify reference genes. The most suitable internal reference genes in

264   different tissues of *Haliotis rufescens* are reportedly *RPL5S* and *CYC*; the most stable in *O. reevesii* was found

265   to be the *RPL28S*[36], but *EF1a* can serve as an alternative. When analysing differences in target gene expression

266   in various tissues of *O. reevesii*, *RPL28S* should be used as the reference gene if the abundance of target gene

267   expression is low, whereas *EF1a* is recommended if target gene expression is high. Previous studies have

268   compared the most stable internal reference genes of flatworms under salinity stress, with GM2-activator protein

269   (*GM2AP*) and *ACTB* indentified; these results are similar to those for *O. reevesii*, in which the most suitable

270   internal control genes are *TUBB* and *ACTB*[37]. However, our candidate gene expression analysis showed that at

271   low salinity (0ppt, 5 ppt) (low osmotic pressure environment) under laboratory conditions, expression of these

272   genes was significantly lower than that at 15 ppt and 25 ppt. One reason for this may be that a low osmotic

273   pressure environment leads to excess moisture entering tissue, decreasing cellular metabolism and eventually

274   housekeeping gene expression[38,39]. Nonetheless, at high salinity (35 ppt), the housekeeping genes examined

275   essentially maintained a normal level of expression, which may be due to the strong ability of *O. reevesii* to

276   obtain water from the external environment, ensuring the stability of its internal environment. It can be inferred

277   from the above that *O. reevesii* is not strongly tolerant to low salinity and osmotic pressure and that its optimal

278   living salinity is approximately 15-25 ppt. Sun found the reference genes *RPL13S* and *RPL32S* to be the most

279   stably expressed in contused rat skeletal muscle; however, *EF1a* plus *TUBB* constitutes the most suitable internal

280   reference combination for *O. reevesii* after tissue damage[40]. Heavy metal ion stress in organisms is also a major

281   focus of current research, and as tidal flat inhabitants, *O. reevesii* feeds on the surface soil of these flats and may

282   very likely serve as an indicator of heavy metal ion pollution. This is a future research direction of our

283   laboratory[41-43]. Although we compared data for *O. reevesii* of different sizes, because there is no consensus

284   regarding the relationship between its growth stage and body weight, these experimental data need further

285   confirmation. This is the first study to screen internal reference genes for *O. reevesii* under different conditions,

286   and the results will be useful for relative quantitation of gene expression in the future.

287 **Conclusions**

288   In this study, we first ascertained and evaluated the expression stability of seven housekeeping genes for

289   qRT-PCR normalization in *O. reevesii* tissues and under conditions of salinity stress and tissue injury. (1) In our

290   assessment of different tissues, *RPL28S* and *EF1a* were found to be the most suitable and stable internal genes

291   among the six tissue samples tested as well as among individuals of different weights. (2) The results suggest

292   that *ACTB* and *TUBB* are the most stable genes, with high expression levels when assessing salinity stress. (3)

293   Regarding muscle injury, *EF1a* is the most stable candidate gene. (4) Among all experimental groups, data

294   analysis of two groups (15 ppt, injury) revealed *TUBB* plus *EF1a* to constitute a suitable reference combination.

295   Based on our results, we propose that the three housekeeping genes *ACTB*, *TUBB* and *EF1a* be the first choice

296   of reference genes for qRT-PCR. Our experimental data indicate that *O. reevesii* has low tolerance to low osmotic

297   pressure and that a salinity range of approximately 15-25 ppt is the most suitable living environment for this

298   organism. To our knowledge, this study is the first to select and evaluate optimal reference genes for *O. reevesii*,

299   and the findings are expected to provide theoretical data support for future experiments involving qPCR.

300   Although the optimal internal reference gene differs among treatments, such as during salinity stress and tissue

301 injury, it is important to understand the importance of the selection of these genes. Overall, for different
302 experimental studies of *O. reevesii*, the selection of appropriate reference genes should be taken into
303 consideration, and our results provide basic experimental data for this purpose.

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307

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309

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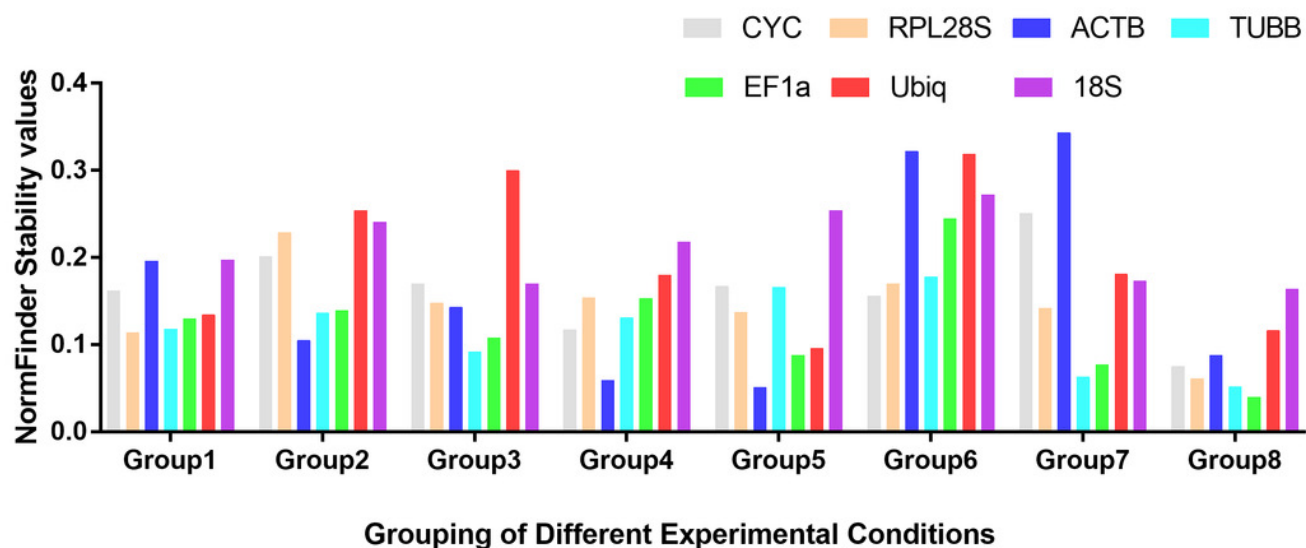
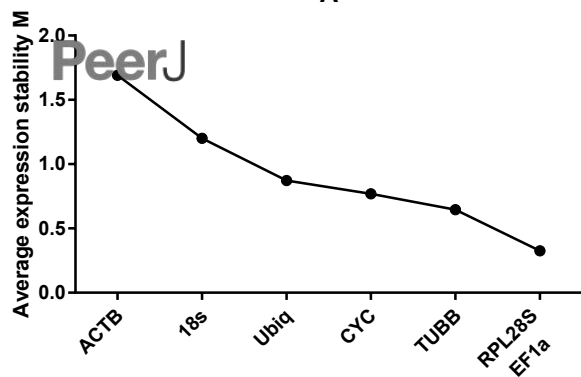
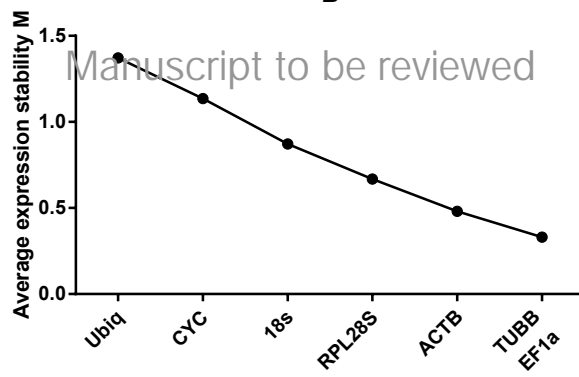
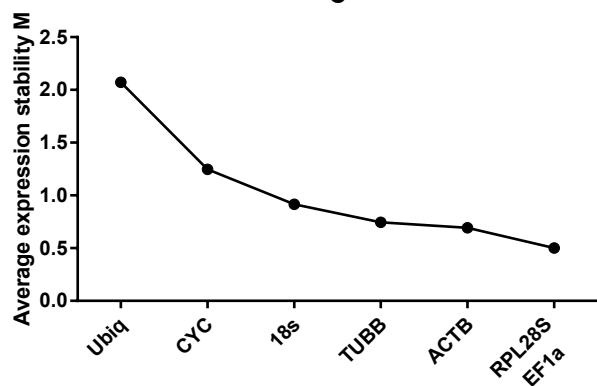
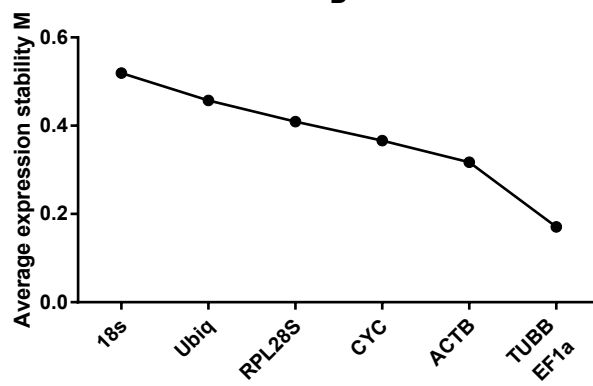
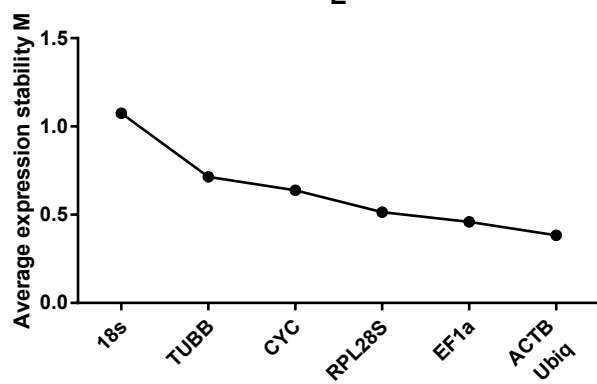
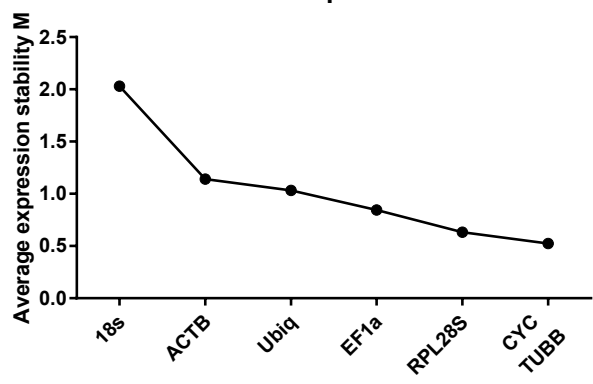
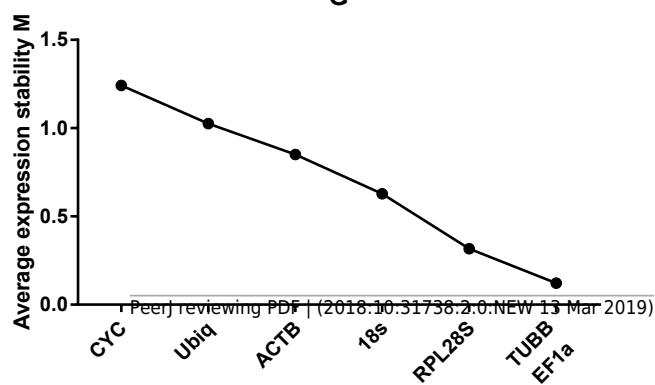
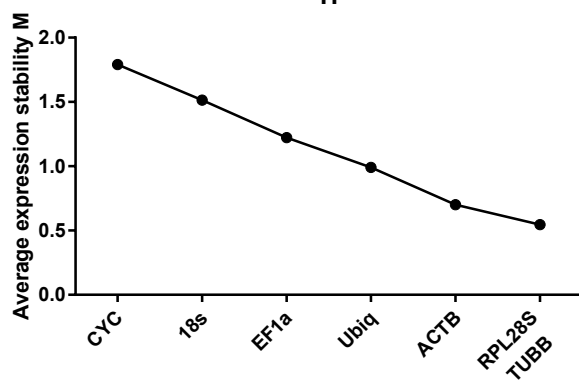
417

Figure 1

Stability values of the seven housekeeping genes evaluated by NormFinder

Different tissues (Group1); dorsal muscle tissue under 0 **ppt** salinity (Group2); dorsal muscle tissue under 5 **ppt** salinity (Group3); dorsal muscle tissue under 15 **ppt** salinity (Group4); dorsal muscle tissue under 25 **ppt** salinity (Group5); dorsal muscle tissue under 35 **ppt** salinity (Group6); dorsal muscle tissue after injury (Group7); dorsal muscle tissue from animals of different weights (Group8)

Average expression stability values of housekeeping genes analysed by geNorm.

A Different tissues; B dorsal muscle tissue under **0 ppt** salinity; C dorsal muscle tissue under **5 ppt** salinity; D dorsal muscle tissue under **15 ppt** salinity; E dorsal muscle tissue under **25 ppt** salinity; F dorsal muscle tissue under **35 ppt** salinity; G dorsal muscle tissue after injury; H dorsal muscle tissue from animals of different weights.

Details of the primers used for qRT-PCR normalization

Gene abbreviation	Gene name	Biological function	Primer sequence (5'-3')	Product size (bp)	Accession No.
CYC	*cyclophilin*	Immunosuppressant, protein folding	F:GTGGGATGTTCCTCTTTACC R:ACCTGGGATTATTCTGTGG	269	KY593122
RPL28	*Ribosomal protein l 28*	60S ribosomal subunit	F:CTGGCACAGGCAAAGTGTCC R:GCAGTGAGAGCCTTGGCTAGA	196	KY593120
ACTB	*β-actin*	Cytoskeletal structural protein	F:GTCCACCGCAAGTGCTTCT R:CGGTCGTGGTTGTTTCATT	214	KY593121
TUBB	*β-tubulin*	Structural protein	F:GTGCTGTTGCCGATGAAAG R:GCATGTCCATGAAGGAGGTT	157	KY593125
EF1α	*elongation factor 1-alpha*	Essential component of the eukaryotic translational apparatus	F:GGAGATGCCAGCCTCAAAC R:GATATTGCGTTGTGGAAGT	165	KY593123
Ubiq	*Ubiquitin*	Protein degradation	F:GCCGAGGCTACATTCCAGT R:GAAGCTTGACATGACCACGAT	203	MF680835
18S RNA	*18S rRNA*	rRNA in the ribosome	F:TCCGCAGGAGTTGCTTCGAT R:ATTAAGCCGCAGGCTCCACT	142	FJ843070

1 **Table 1 Details of the primers used for qRT-PCR normalization.**

Statistical results and expression analyses of the tested reference genes in different tissues, as based of threshold cycle point values (Ct) provided by BestKeeper.

N number of samples, Ct threshold cycle point value, GM[Ct] geometric ct mean, AM[Ct] arithmetic ct mean, Min[Ct] and Max[Ct] ct threshold values, SD[±Ct] ct standard deviation, CV[%Ct] variance coefficient expressed as a percentage of the ct level, Min[x-fold] and Max[x-fold] threshold expression levels expressed as the absolute x-fold over- or under-regulation coefficient, SD[±x-fold] standard deviation of the absolute regulation coefficient .

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	6	6	6	6	6	6	6
GM[Ct]	28.94	27.76	21.98	23.05	19.73	31.07	22.58
AM[Ct]	28.96	27.77	22.16	23.06	19.75	31.09	22.65
Min[Ct]	27.88	26.88	18.75	21.74	19.1	29.96	20.08
Max[Ct]	30	28.92	26.91	24.4	21.35	33.09	25.18
SD[±Ct]	0.89	0.48	2.67	0.77	0.66	0.94	1.72
CV[%Ct]	3.09	1.74	12.05	3.33	3.33	3.01	7.58
Min[x-fold]	-2.09	-1.84	-9.37	-2.47	-1.55	-2.17	-5.63
Max[x-fold]	2.09	2.23	30.66	2.56	3.08	4.06	6.08
SD[± x-fold]	1.86	1.4	6.37	1.7	1.58	1.91	3.29

1 **Table 2 Statistical results and expression level analyses of all tested candidate reference genes in**
2 **different tissues on account of their threshold cycle point values (Ct) as furnished by BestKeeper**
3 **program.**

Statistical results and expression analysis of candidate reference genes under 0 ppt salinity, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	32.12	31.65	23.24	28.08	28.6	36.19	32.65
AM[Ct]	32.19	31.66	23.26	28.1	28.62	36.22	32.66
Min[Ct]	29.13	30.72	22.43	26.65	27.16	33.23	31.52
Max[Ct]	35.74	33	24.98	30.03	30.36	38.13	33.55
SD[±Ct]	1.65	0.69	0.7	0.95	1.14	1.08	0.64
CV[%Ct]	5.13	2.19	3.02	3.38	3.98	2.98	1.96
Min[x-fold]	-7.98	-1.91	-1.75	-2.69	-2.71	-7.77	-2.19
Max[x-fold]	12.24	2.55	3.35	3.87	3.39	3.82	1.86
SD[± x-fold]	3.14	1.62	1.63	1.93	2.2	2.11	1.56

1   **Table 3 Statistical results and expression level analyses of all tested candidate reference genes under**
2   **culturing environment in salinity of 0ppt on account of their threshold cycle point values (Ct) as**
3   **furnished by BestKeeper program.**

Statistical results and expression analysis of candidate reference genes under 5 ppt salinity, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	33.15	30.59	23.35	27.22	28.48	35.1	32.13
AM[Ct]	33.26	30.63	23.41	27.29	28.52	35.18	32.14
Min[Ct]	28.87	29.51	21.44	25.37	26.78	30.01	31.06
Max[Ct]	38.67	33.94	27.08	32.12	32	37.79	34.17
SD[±Ct]	2.03	1.35	1.48	1.58	1.37	1.79	0.68
CV[%Ct]	6.1	4.4	6.31	5.8	4.79	5.09	2.11
Min[x-fold]	-19.39	-2.11	-3.75	-3.6	-3.25	-34.04	-2.09
Max[x-fold]	46	10.21	13.26	29.88	11.54	6.45	4.12
SD[± x-fold]	4.08	2.55	2.78	3	2.58	3.46	1.6

1 **Table 4 Statistical results and expression level analyses of all tested candidate reference genes under**
2 **culturing environment in salinity of 5ppt based on their threshold cycle point values (Ct) as provided by**
3 **BestKeeper software.**

Statistical results and expression analysis of candidate reference genes under 15 ppt salinity, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	20.59	27.49	19.39	22.78	19.88	30.7	21.21
AM[Ct]	20.59	27.49	19.39	22.79	19.89	30.71	21.22
Min[Ct]	20.18	26.95	18.41	21.91	19.17	29.83	20.51
Max[Ct]	20.95	28.02	19.89	23.42	20.33	31.56	22.46
SD[±Ct]	0.26	0.34	0.3	0.36	0.32	0.58	0.51
CV[%Ct]	1.25	1.23	1.55	1.6	1.61	1.89	2.42
Min[x-fold]	-1.33	-1.46	-1.98	-1.83	-1.64	-1.83	-1.63
Max[x-fold]	1.29	1.44	1.42	1.56	1.36	1.81	2.39
SD[± x-fold]	1.2	1.26	1.23	1.29	1.25	1.49	1.43

1 **Table 5 Statistical results and expression level analyses of all tested candidate reference genes under**
2 **culturing environment in salinity of 15ppt on account of their threshold cycle point values (Ct) as**
3 **furnished by BestKeeper program.**

Statistical results and expression analysis of candidate reference genes under 25 ppt salinity, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	22.22	27.75	19.44	20.35	18.48	31.5	20.77
AM[Ct]	22.23	27.75	19.44	20.37	18.49	31.5	20.86
Min[Ct]	20.76	26.82	19.14	19.09	17.5	31.1	19.32
Max[Ct]	23.4	28.62	20.6	21.86	19.85	31.9	25.25
SD[±Ct]	0.64	0.58	0.33	0.87	0.52	0.22	1.57
CV[%Ct]	2.88	2.07	1.72	4.27	2.8	0.71	7.54
Min[x-fold]	-2.74	-1.9	-1.23	-2.39	-1.97	-1.31	-2.72
Max[x-fold]	2.26	1.83	2.23	2.85	2.59	1.32	22.39
SD[± x-fold]	1.56	1.49	1.26	1.83	1.43	1.17	2.97

1 **Table 6 Statistical results and expression level analyses of all tested candidate reference genes under**
2 **culturing environment in salinity of 25ppt on account of their threshold cycle point values (Ct) as**
3 **furnished by BestKeeper program.**

Statistical results and expression analysis of candidate reference genes under 35 ppt salinity, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	24.39	27.03	18.79	21.77	20.32	29.85	23.25
AM[Ct]	24.4	27.04	18.82	21.78	20.35	29.86	23.68
Min[Ct]	23.66	25.9	16.83	21.3	19.05	28.34	17.98
Max[Ct]	25.23	28.11	20.13	22.63	21.48	31.27	31.93
SD[±Ct]	0.44	0.51	0.77	0.34	0.89	0.64	3.79
CV[%Ct]	1.8	1.9	4.1	1.57	4.36	2.16	16
Min[x-fold]	-1.66	-2.19	-3.9	-1.39	-2.41	-2.85	-38.68
Max[x-fold]	1.79	2.12	2.52	1.81	2.24	2.66	410.85
SD[± x-fold]	1.35	1.43	1.71	1.27	1.85	1.56	13.83

1 **Table 7 Statistical results and expression level analyses of all tested candidate reference genes under**
2 **culturing environment in salinity of 35ppt on account of their threshold cycle point values (Ct) as**
3 **furnished by BestKeeper program.**

Statistical results and expression level analysis of candidate reference genes during skin muscle healing, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	7	7	7	7	7	7	7
GM[Ct]	25.26	27.14	18.75	22.3	18.92	29.85	20.99
AM[Ct]	25.33	27.15	18.76	22.31	18.94	29.87	21.03
Min[Ct]	23.28	26.07	17.38	21.52	18.21	27.6	19.03
Max[Ct]	28.32	28.19	19.67	23.8	20.32	31.68	23.12
SD[±Ct]	1.6	0.63	0.46	0.52	0.57	1.01	1.05
CV[%Ct]	6.32	2.33	2.43	2.31	3	3.37	4.98
Min[x-fold]	-3.96	-2.1	-2.58	-1.71	-1.64	-4.75	-3.91
Max[x-fold]	8.31	2.07	1.89	2.83	2.63	3.56	4.37
SD[± x-fold]	3.03	1.55	1.37	1.43	1.48	2.01	2.07

1 **Table 8 Statistical results and expression level analyses of all tested candidate reference genes during**
2 **skin muscle healing on account of their threshold cycle point values (Ct) as furnished by BestKeeper**
3 **program.**

Statistical results and expression level analysis of candidate reference genes for individuals of different weights, as based on threshold cycle point values (Ct) provided by BestKeeper.

Factor	CYC	RPL28S	ACTB	TUBB	EF1a	Ubiq	18S RNA
N	5	5	5	5	5	5	5
GM[Ct]	24.65	27.6	20.8	24.22	22.8	31.02	26.81
AM[Ct]	24.67	27.72	20.92	24.37	23.1	31.09	27.11
Min[Ct]	23.82	25.18	18.63	21.82	19.76	28.45	21.79
Max[Ct]	25.68	30.97	24.11	27.85	27.81	34.42	32.07
SD[±Ct]	0.71	2.54	2.14	2.65	3.66	1.88	3.78
CV[%Ct]	2.88	9.17	10.23	10.88	15.83	6.06	13.94
Min[x-fold]	-1.78	-5.36	-4.52	-5.27	-8.27	-5.94	-32.48
Max[x-fold]	2.04	10.37	9.9	12.34	32.22	10.59	38.4
SD[± x-fold]	1.64	5.83	4.41	6.28	12.6	3.69	13.72

1   **Table 9 Statistical results and expression level analyses of all tested candidate reference genes between**

2   **different individual weights on account of their threshold cycle point values (Ct) as furnished by**

3   **BestKeeper program.**