Evaluation of potential reference genes for real-time qPCR analysis in a biparental beetle, *Lethrus apterus* (Coleoptera: Geotrupidae)

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Hormones play an important role in the regulation of physiological, developmental and behavioural processes. Many of these mechanisms in insects, however, are still not well understood. One way to investigate hormonal regulation is to analyse gene expression patterns of hormones and their receptors in question by real-time quantitative polymerase chain reaction (RT-qPCR). This method, however, requires stably expressed reference genes for normalisation. In the present study, we evaluated 11 candidate housekeeping genes as reference genes in samples of *Lethrus apterus*, an earth-boring beetle with biparental care, collected from a natural population. For identifying the most stable genes we used the following computational methods: geNorm, NormFinder, BestKeeper, comparative delta Ct method and RefFinder. Based on our results, the two body regions sampled (head and thorax) differ in which genes are most stably expressed. We identified two candidate reference genes for each region investigated: ribosomal protein L7A and RP18 in samples extracted from the head, and ribosomal protein L7A and RP4 extracted from the muscles of the thorax. These reference genes can be used to study the hormonal regulation of reproduction and parental care in *Lethrus apterus* in the future.

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Abstract

Hormones play an important role in the regulation of physiological, developmental and behavioural processes. Many of these mechanisms in insects, however, are still not well understood. One way to investigate hormonal regulation is to analyse gene expression patterns of hormones and their receptors in question by real-time quantitative polymerase chain reaction (RT-qPCR). This method, however, requires stably expressed reference genes for normalisation. In the present study, we evaluated 11 candidate housekeeping genes as reference genes in samples of *Lethrus apterus*, an earth-boring beetle with biparental care, collected from a natural population. For identifying the most stable genes we used the following computational methods: geNorm, NormFinder, BestKeeper, comparative delta Ct method and RefFinder. Based on our results, the two body regions sampled (head and thorax) differ in which genes are most stably expressed. We identified two candidate reference genes for each region investigated: ribosomal protein L7A and RP18 in samples extracted from the head, and ribosomal protein L7A and RP4 extracted from the muscles of the thorax. These reference genes can be used to study the hormonal regulation of reproduction and parental care in *Lethrus apterus* in the future.

Keywords: *Lethrus apterus;* insect; reference gene; housekeeping gene; parental care; RT-qPCR

Introduction

Hormonal regulation in insects generates great interest among entomologists, however, hormones have only been studied in detail in few species (Gullan & Cranston, 2014). Insect hormones of particular interest include juvenile hormones, ecdysteroids and neuropeptides. These molecules regulate a vast number of physiological and developmental processes as well as behaviours (Gäde, Hoffmann & Spring, 1997). Studying these hormones used to be difficult considering their small amount and the occasional instability (Gullan & Cranston, 2014). The technical revolution of molecular biology and genetics, however, made it attainable to discover the details of genetic and hormonal regulation in insects (Raikhel, Brown & Belles, 2005). Some of the processes controlled by hormones mentioned above, such as ecdysis (Mykles et al., 2013) are already well described. Nevertheless, there are many interesting physiological and behavioural mechanisms, like parental care, the hormonal regulation of which are not well understood (Panaitof et al., 2016). One way to increase our understanding of hormonal regulation is to identify patterns of gene expression associated with the hormones in question (Champagne & Curley, 2012).

Real-time quantitative polymerase chain reaction (RT-qPCR) is a commonly used method for analysing gene expression as it is a sensitive, fast and reproducible method, moreover it requires only a minimal amount of RNA (Radonić et al., 2004). With this method, gene expression levels can be measured simultaneously in several different samples for a limited number of genes. Gene expression analyses with RT-qPCR, however, require some kind of normalisation in order to control the variation caused by stochastic processes occurring during the analytic procedure (Vandesompele et al., 2002). This normalisation is usually achieved by taking into account the expression level of so-called reference genes (VanGuilder, Vrana & Freeman, 2008). These genes are usually selected from housekeeping genes, which produce proteins vital for maintaining fundamental cell functions, like ribosomal or cytoskeletal proteins. Therefore, the expression levels of these reference genes are thought to be relatively stable. Thus, comparing the expression level of genes of interest with that of the reference genes, we can eliminate the differences caused by the different amount and quality of starting material, as well as we are able to control for differences occurring due to technical errors during sample preparation (e.g. RNA isolation and cDNA synthesis, Radonić et al., 2004). Nonetheless, expression levels of the housekeeping genes may also vary considerably under certain circumstances because they can be involved in

processes other than maintenance functions of the cell, e.g. apoptosis (Nicholls, Li & Liu, 2012), cytokinesis (D'Souza-Schorey & Chavrier, 2006) and development (Zhou et al., 2015). Therefore, a given housekeeping gene cannot automatically serve as reference gene, and normalisation with unstable reference genes can lead to erroneous quantification results and conclusions (Thellin et al., 1999). Consequently, reference genes must be carefully selected so that their expression levels are similar between the different samples and should not be influenced significantly by different experimental conditions (VanGuilder, Vrana & Freeman, 2008). According to Vandesompele et al. (2002), the combination of two or more reference genes is highly recommended for normalisation to obtain more accurate results. In case of multiple reference genes, it is advised to use the geometric mean for normalisation since it better controls for extreme values and the possible differences between expression levels of the different genes (Vandesompele et al., 2002).

Lethrus apterus (Laxmann, 1770) (Coleoptera: Geotrupidae) is an earth-boring beetle that has biparental care during which the parents provision food for their offspring in advance their hatching (Kosztolányi et al., 2015). This kind of parental care is a complex and relatively rare trait among insects (Smiseth, Kölliker & Royle, 2012) and makes this beetle an outstanding model species for studying the hormonal background of parental care. In order to do so, however, stably expressed reference genes have to be identified.

In recent years, numerous studies aimed to identify stable reference genes in insects (Lord et al., 2010; Ponton et al., 2011; Bansal et al., 2012; Li et al., 2013; Pan et al., 2015; Yu et al., 2016). Yet, no universally applicable, stable reference gene or genes have been reported to date. Furthermore, there is a lack of reference gene studies that use individuals from natural populations. Our objective in this study was to examine the expression stability of several housekeeping genes in *Lethrus apterus* across different times of the breeding period in a natural population in order to identify the most stable reference gene(s). With the right combination of reference genes, the expression levels of the hormones regulating parental care in *Lethrus apterus* can be examined accurately in the future. Based on the literature (Shi et al., 2013; Liang et al., 2014; Zhu et al., 2014; Yang et al., 2015), we probed eleven housekeeping genes.

Materials and methods

Sample collection

Samples were collected near Dorogháza, northern Hungary (47°59′29″N, 19°53′36″E) on 16th April, 4th May and 28th May in 2015, which dates corresponded to the beginning, middle and end of the breeding season of *Lethrus apterus*, respectively. Sample collection was approved by the National Inspectorate for Environment Protection and Nature Conservation (No. OKTF-KP/791-51/2016). The first sampling date represents the period of mate choice, while the second and third samplings were done during the period when parents were collecting leaves for the offspring. On each sampling dates head and thorax samples were collected from 8 males and 8 females. All tissues were removed from the head capsule and muscle samples were taken from the thorax. Samples were collected in less than five minutes after euthanizing the individuals. The tissue samples were put immediately into 600 µl RNA*later*® Stabilization Solution (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in the field, then stored at -20 °C in the laboratory in order to inhibit RNase enzyme activity until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from each samples using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions. The extracted RNA was eluted in 15-30 µl RNase-free water, depending on the pellet size. Yield of RNA was quantified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). To eliminate genomic DNA, samples were treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA) just before the reverse transcription. First strand cDNA was synthesized from 1 µg DNA-free RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, USA).

Reference gene selection and primer design

Using a draft genome of *Lethrus apterus* (Rácz et al., 2015) eleven reference genes, which were already described as stable reference genes in other arthropods, were selected (Table 1). We manually designed primers (Table 2) using the web-based Sequence Manipulation Suite (Stothard, 2000) and Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in order to avoid the forming of possible secondary structures of the primers. To check the specificity of primer pairs and to determine optimal annealing temperature, PCR reactions were performed in 10 µl volumes containing the following components: 10x buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.02 U/µL Taq DNA polymerase enzymes (DreamTaq

Green, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.2 μM forward and 0.2 μM reverse primer and 0.1 μg cDNA. PCR conditions were optimized by determining the optimal annealing temperature using temperature gradient ranging from 54°C to 62°C for primer binding. In this study, we used ABI Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA). Cycling conditions consisted of a denaturing step at 95°C for 2 min followed by 40 cycles at 95°C for 30 sec, at a temperature gradient (54°C, 56°C, 58°C, 60°C or 62°C) for 30 sec and at 72°C for 90 sec, and finally at 72°C for 10 min. PCR amplicons were run on 1% agarose gel stained with GelRed™ (Biotium, Fremont, California, USA).

Real-time quantitative PCR

RT-qPCR was performed on a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) and ROX Passive Reference Dye (Affymetrix, Santa Clara, California, USA). Amplifications were carried out under the following conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of 10 sec at 95°C and for 1 min at the optimal annealing temperature. This was followed by a melting curve analysis in which the temperature raised from 65°C to 95°C in sequential steps of 0.05°C for 1 second. Three technical replicates were performed for each biological sample, and the average cycle threshold (Ct) values of triplicates were calculated. Furthermore, no-template control was done in order to check whether primer-dimers or contamination with amplified PCR product were detectable.

Determination of reference gene expression stability

In order to determine the expression stability of the selected reference genes, we used the following methods: geNorm (Vandesompele et al., 2002), NormFinder (Andersen, Jensen & Ørntoft, 2004), BestKeeper (Pfaffl et al., 2004), delta Ct method (Silver et al., 2006) and RefFinder (Xie et al., 2012). For the analyses with the geNorm and NormFinder procedures, the average Ct values were transformed to relative quantities by setting the highest average Ct value to 1, then calculating the quantities for the other samples relative to the sample with the highest average Ct value. For calculations by BestKeeper, delta Ct method and RefFinder, the average Ct value was used directly. All calculations, except the ones done by the web-based RefFinder, were carried out in R version 3.3.2 (R Core Team 2016) with “NormqPCR” package (Perkins et al., 2012).

154 geNorm calculates the expression stability value M by assessing the mean pairwise expression
155 ratio for each candidate gene against all the other candidates (Vandesompele et al., 2002). The
156 basic assumption of this method is that the expression ratio between two reference genes is
157 identical across the samples. The lower the M value the more stable the expression of the
158 candidate reference gene. Stepwise exclusion of the genes with the highest M value results in the
159 selection of the two most stably expressed reference genes in the tested samples both sharing the
160 same M value. The same authors suggest not to accept candidate genes as stably expressed
161 reference genes with M value higher than 1.5. Moreover, the procedure determines the
162 normalisation factor by taking the geometric mean of the expression levels from the most stable
163 genes and then additively recalculating with each of the next most stable gene. The pairwise
164 variation, $V_{n/n+1}$ between two sequential normalisation factors is then calculated in order to
165 determine the effect of each newly added gene to the normalisation factor. The optimum number
166 of genes is the lowest number of genes with $V_{n/n+1}$ less than 0.15 (Vandesompele et al., 2002).
167 NormFinder determines the stability of the candidate reference genes by measuring the intra-
168 and intergroup variation between user specified groups (e.g. male and female groups or treated
169 and control groups) first. Stability values for each candidate gene are then calculated by adding
170 the two sources of variation. The lowest stability value means the most stable expression
171 (Andersen, Jensen & Ørntoft, 2004).
172 BestKeeper calculates, for each candidate reference gene across the samples, the geometric
173 mean, the arithmetic mean, the minimal and the maximal Ct values, in addition to the average
174 absolute deviation from the arithmetic mean. Genes with the lowest average absolute deviation
175 can be considered as stably expressed reference genes. BestKeeper Index is calculated as the
176 geometric mean of the Ct values of the candidate reference genes. Inter-gene relations are
177 estimated by performing pairwise correlation analyses of all possible reference gene pairs.
178 Furthermore, correlation between the expression level of each candidate gene and the BestKeeper
179 Index is calculated, describing the relation between the index and the contributing genes by the
180 Pearson correlation coefficient, coefficient of determination and the corresponding p-value (Pfaffl
181 et al., 2004).
182 The delta Ct method compares relative expression of pairs of candidate genes within each
183 sample in order to identify the stably expressed housekeeping genes. If the ΔCt value of the two
184 genes fluctuates when analysed in different samples, it means that one or both genes are variably

185 expressed. If the ΔCt value remains constant, both genes are stably expressed among the samples
186 (Silver et al., 2006).
187 Each program mentioned above uses different algorithms to calculate an expression stability
188 value which represents the suitability of the candidate genes as reference genes, therefore the
189 ranking of the examined genes according to the methods can vary. The web-based tool RefFinder
190 [1] was used in order to combine our results and rank the candidate genes. This user-friendly
191 program integrates the four methods mentioned above. Using the ranking from each program, it
192 assigns an appropriate weight to an individual gene and calculates the geometric mean of their
193 weights for the overall ranking. The lowest rank indicates the most stably expressed gene (Xie et
194 al., 2012).

195 Calculation of the amplification efficiency

196 Five 5-fold serial dilution was made from cDNA samples to create a standard curve, and the
197 amplification efficiency was determined for the two best reference genes. The efficiency (E)
198 values were calculated according to the equation: $E=(10^{(-1/slope)}-1)x100$, where slope is the slope of
199 the standard curve (Radonić et al., 2004).

200 **Results**

201 Transcriptional profiling of candidate reference genes

202 Before the evaluation of expression stability of the eleven candidate genes, specificity of each
203 primer pair was checked on 1% agarose gel which showed single products with the expected
204 sizes. Moreover, gene-specific amplification was confirmed by single melting curve peaks. These
205 results indicate that no primer-dimers or nonspecific amplification products were formed.
206 Additionally, no fluorescent signals were detected in the negative control during the RT-qPCR.
207 Raw Ct values ranged from 11.66 (TUB1a) to 30.12 (ARF4) (Figure 1). The mean and
208 standard deviation (SD) of the Ct values across all samples were calculated for each gene (Table
209 3). Since the mean Ct values ranged between 15 and 30 for all the candidate reference genes, all
210 of them were analysed further (Kozera & Rapacz 2013). ARF1 had the least variable expression
211 level with the lowest SD value (SD=1.85), while ARF4 had the most variable expression level
212 (SD=3.04). Low average Ct values indicate high expression level in TUB1a and EF2
213 (Ct_{avg}=15.11), on the other hand, high Ct values of ARF1 (Ct_{avg}=20.77) indicated low expression.

214 Expression stability of candidate reference genes

215 Based on geNorm analysis for all samples, eight candidate genes had an M value below the
216 threshold of 1.5 (Table 4). The results show that the lowest M value was 0.390 for RPS8 and
217 L7A. Among the head samples, all of the tested genes except L10 had an M value below 1.5, and
218 RPS8 and RP18 were co-ranked as the most stable genes from the candidates (M=0.304). In case
219 of the thorax samples, eight genes had an M value below the threshold. RPS8 and L7A were the
220 most stable candidate gene pair with an M value of 0.358. In head samples both of females and
221 males, RPS8 and RP18 were co-ranked as the most stable genes (M=0.264 for females and
222 M=0.346 for males). In thorax samples collected from females, RPS8 and RP18 were the most
223 stable genes as well with an M value of 0.222. However, in thorax samples of males, RPS8 and
224 L7A were ranked as the best reference gene pair (M=0.288).
225 According to NormFinder, L7A was the most stable gene across all samples, when the
226 specified groups were head and thorax samples (Table 4). The second and third genes were RP4
227 and RPS8, indicating that these are also worth considering as reference genes. In the case of
228 setting females and males as subgroups within head and thorax samples, L7A was found again to
229 be the most stably expressed gene among the candidate ones. In both head and thorax samples,
230 L7A was followed by similar ranking order: EF2, RP4, RP18 and RPS8 as second, third, fourth
231 and fifth genes, respectively (Table 5).
232 Based on BestKeeper, across all samples ARF1 had the lowest mean absolute deviation
233 (MAD) value (Table 4), however, L7A had the highest correlation *r* value (Table 6). Among the
234 head samples, RP18 had the lowest MAD value, while among the thorax samples, L10 was the
235 most stable according to the MAD value. This was surprising as the other programs ranked this
236 gene consistently as one of the least stable genes. On the other hand, in both head and thorax
237 samples, L7A had the highest *r* value. In head samples of males RPS8 (MAD=0.827), and of
238 females ARF1 (MAD=1.122) was the most stable candidate gene. In male thorax samples L10
239 had the lowest MAD value (MAD=1.576), while in female samples EF2 was ranked as the most
240 stable with MAD value 1.236. In both males and females, L7A had the highest correlation *r*
241 value.
242 According to the delta Ct method, L7A was the most stable gene among the candidates overall
243 (Table 4). For the head samples, RPS8 and L7A were found to be the most stable with the same
244 stability value of SD_{avg}=1.0. For the thorax samples, RP18 and L7A, had the lowest stability

value (SD_{avg}=1.05). Calculating with the subgroups of males and females within head and thorax samples, the result was consistent, i.e. L7A was the most stable candidate gene, with the stability value always below 1.0.

Finally, the candidate genes were evaluated by RefFinder to combine the results of individual methods. Calculating with all the samples, and separately the head and thorax samples, L7A was ranked first, as the most stably expressed gene among the candidate reference genes (Table 4). In head samples, RP18 was co-ranked with L7A as the most stable reference genes. In thorax samples, RP4 was ranked on the second place. In the groups of female and male samples within head and thorax samples, L7A was always one of the most stable genes. With the exception of thorax samples of males, where L7A was the best candidate, all groups ranked L7A on the second place. In the head samples from male individuals, the combined result showed that RPS8 is the most stably expressed gene. In the thorax samples of males, the result of RefFinder EF2 was ranked on the second place. In both head and thorax samples of females, RP18 was the best candidate gene for normalisation.

Optimal number of reference genes

To determine the minimal number of genes necessary for normalisation, the V-value was computed by geNorm. The results demonstrated that across all samples $V_{2/3}$ was the first V-value lower than the cut-off value of 0.15. Considering separately the head and thorax samples, $V_{2/3}$ was again lower than 0.15 (Figure 2). Separate analyses of female and male samples within head and thorax groups showed that $V_{2/3}$ was also the first value below the threshold in all cases. Therefore, two stably expressed reference genes are sufficient for normalisation in any case of sample classification.

Amplification efficiency

The amplification efficiency values of the two best reference genes were calculated. The value for L7A was 99.54 %. The slope of the standard curve made of the data from the dilution was -3.328. In the case of RP18, the amplification efficiency was 99.75%. The standard curve showed a slope of -3.333. These results suggest that the efficiencies of the primers used meet the standard requirements of RT-qPCR.

Discussion

RT-qPCR is a widely used method for measuring gene expression levels due to its relatively low cost, high accuracy and sensitivity. A critical step of this method is data normalisation which requires careful selection of reference genes for the given experimental or environmental conditions. With these stably expressed genes, technical errors and variance resulting from the method can be moderated (Udvardi, Czechowski & Scheible, 2008). Several studies have examined the stability of reference genes in various insect species in the past decade and these studies suggest that no universally stable reference gene can be found that is applicable for all species, tissue types and experimental conditions. Hence, it is necessary to identify the most suitable reference genes for the specific circumstances in a given study for a given species (Zhu et al., 2014).

In the present study, variation in expression levels of eleven housekeeping genes were evaluated across a span of 1.5 months covering most of the breeding period of the biparental beetle *Lethrus apterus*. To date, no study investigated the possible reference genes either in this species, or in the family of Geotrupidae. We analyzed the expression stability of the candidate reference genes by four frequently used programs: geNorm, NormFinder, BestKeeper and comparative delta Ct method. The outcomes of these programs can vary because of the differences in the algorithms. Therefore, the combined use of them ensures more reliable results. For this purpose, RefFinder, a freely available web-based tool was used to calculate a comprehensive ranking value for each candidate gene.

According to the comprehensive ranking by RefFinder, the most stably expressed reference gene was L7A across all samples, as well as when considering the head and thorax samples separately. Based on the results of geNorm analysis, two reference genes are sufficient for normalisation in gene expression analysis in *Lethrus apterus* during the breeding period. For accurate normalisation, we recommend RP18 in case of head samples, and RP4 in case of thorax samples to be used as second reference genes. In addition to L7A, RP18 should be used for normalisation in both head and thorax samples of females, and RPS8 is recommended for head samples of males. In thorax samples from males, EF2 should be used along with L7A.

Consistent with our results, ribosomal proteins are reported to be the best reference genes in many insect species. In a study by Zhu et al. (2013), ribosomal protein L7A was ranked as one of the best reference genes in *Spodoptera exigua* in different tissues, specific larval physiological stages and male individuals. Studies of other coleopterans gave similar results: RP4 and RP18

were the best reference genes in *Leptinotarsa decemlineata* (Shi et al., 2013), RPS3 (ribosomal protein S3), RPL13a (ribosomal protein 13a) and RPS18 (ribosomal protein S18) were suitable reference genes for *Tribolium castaneum* (Lord et al., 2010; Sang et al., 2015), and RPL22e (ribosomal protein 22e) was one of the best reference genes in *Mylabris cichorii* both in males and females (Wang et al., 2014). In other species, e.g. in *Drosophila melanogaster* Rpl32 (ribosomal protein L32) was a suitable reference gene in individuals on different diets (Ponton et al., 2011), and in *Aphis craccivora,* RPS8, RPL14 (ribosomal protein L14), and RPL11 (ribosomal protein L11) were the three most stable housekeeping genes across different developmental stages and temperature conditions (Yang et al., 2015).

Interestingly, two frequently used reference genes, GAPDH and TUB1a were ranked as less stable genes in this study, beside ARF4, with stability values above the threshold values of all the programs used. L10 was also found to be an unstable reference gene in all but the geNorm analysis. These results correspond with the findings of Thellin et al. (1999), i.e. housekeeping genes should be evaluated as reference genes across the given experimental conditions in the given species. Based on our results, we recommend to avoid the use of these last four genes for normalisation in studies investigating gene expression patterns during the reproductive period in this species.

Conclusion

By evaluating the stability of eleven candidate housekeeping genes in samples collected during the entire course of the breeding period of free-living *Lethrus apterus*, we conclude that two of them provide sufficient reference for normalising target gene expression. In head samples, these two genes appear to be L7A and RP18, whereas in thorax samples L7A and RP4 should be used. In both thorax and head samples of females, RP18 and L7A are the best choices for normalisation. Based on our results, in head samples of males, RPS8 and L7A, while in thorax samples of males, L7A and EF2 are recommended to use. These results provide reliable reference genes that are suitable normalizers for further RT-qPCR investigations on the hormonal regulation in *Lethrus apterus*.

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Table 1(on next page)

The list of the candidate housekeeping genes with their biological functions.

Gene	Symbol used	Function	Reference
glyceraldehyde 3-phosphate dehydrogenase	GAPDH	glycolytic enzyme	Liang *et al.* 2014
tubulin alpha-1 chain	TUB1a	cytoskeletal structural protein	Liang *et al.* 2014
elongation factor 1-alpha	EF1a	protein synthesis	Liang *et al.* 2014
elongation factor 2	EF2	protein synthesis	Zhu *et al.* 2014
ADP-ribosylation factor-like protein 1	ARF1	GTP-binding protein	Shi *et al.* 2013
ADP-ribosylation factor 4	ARF4	GTP-binding protein	Shi *et al.* 2013
ribosomal protein S8	RPS8	structural constituent of ribosome	Yang *et al.* 2015
ribosomal protein L4	RP4	structural constituent of ribosome	Shi *et al.* 2013
ribosomal protein L7A	L7A	structural constituent of ribosome	Zhu *et al.* 2014
ribosomal protein L10	L10	structural constituent of ribosome	Zhu *et al.* 2014
ribosomal protein L18	RP18	structural constituent of ribosome	Shi *et al.* 2013

Table 2(on next page)

The primers used to measure gene expression levels for the candidate reference genes by RT-qPCR.

[1]F: forward primer; R: reverse primer

[2]Ta: optimal annealing temperature

Gene	GenBank accession number	Primer sequence (5' to 3')[1]	Amplicon length (bp)	Ta (°C)[2]
GAPDH	KY786279	F: GCCATTCCAGTAAGTTTTCCATTGAG	157	60
		R: GCTGTTACTGCTACACAAAAGAC		
TUB1a	KY786273	F: CAGACTGCACGTTGGACTTTAGC	172	60
		R: TACAGAGGAGATGTTGTCCCCAAG		
EF1a	KY786281	F: AAACCTTTGCGTCTTCCACTACAGG	184	60
		R: CTTCAGTTGTAAGACCAACAGGTG		
EF2	KY786280	F: GATGAGAAATCCACATGTCCAG	426	60
		R: CGACTCCCTAGTATCAAAGG		
ARF1	KY786283	F: GTATGACAGTAGCTGAAGTTC	141	60
		R: CTGTTTTGTAAAGCATTGGC		
ARF4	KY786282	F: TAGTACGGACGGTCAAGTC	197	60
		R: GTAGACCGTCACCTGTTATGGC		
RPS8	KY786274	F: CATTATGTACGTACGAGAGGAGGCAACG	200	60
		R: TCTAAAGGGAGTAGCGTCGATAACG		
RP4	KY786275	F: TAATGGACCACGACGCTGTATGC	407	60
		R: CGTACCAGCTTTAGTAATGAGCAAGG		
L7A	KY786277	F: TAGCGACTCAACTGTTCAAGG	224	60
		R: CCTCAATTGGATCGACGTCATGTG		
L10	KY786278	F: CGTAGAGCCTCGATAACTTGG	462	60
		R: TCATGTGCTGGAGCTGATAGG		
RP18	KY786276	F: TTGTAACCACATGAACGCCTACG	186	60
		R: AGTTAGCTTTACGTTCACCTACTGG		

Table 3(on next page)

Mean, standard deviation and coefficient of variation for the Ct values of 11 candidate reference genes, calculated across all samples.

Genes	Mean	SD	CV
GAPDH	15.27	2.62	0.17157826
TUB1a	15.11	2.64	0.17471873
EF1a	15.44	2.34	0.1515544
EF2	15.11	2.05	0.13567174
ARF1	20.77	1.85	0.08907078
ARF4	20.22	3.04	0.15034619
RPS8	16.38	2.12	0.12942613
RP4	15.52	1.94	0.125
L7A	17.27	2.1	0.12159815
L10	16.15	1.97	0.12198142
RP18	16.02	1.93	0.12047441

Table 4(on next page)

Ranking of candidate reference genes by the different reference gene finder applications.

The two best reference genes according to the combined results of RefFinder are highlighted in bold. M is the M value calculated by geNorm, SV is the Stability value calculated by NormFinder, MAD is the average absolute deviation calculated by BestKeeper, SD_{avg} is the mean standard deviation calculated by the delta Ct method.

Rank	geNorm		NormFinder		BestKeeper		Delta Ct		RefFinder
	Gene	**M**	**Gene**	**SV**	**Gene**	**MAD**	**Gene**	**SD_{avg}**	**Gene**
1	RPS8	0.390	**L7A**	0.126	ARF1	1.361	**L7A**	0.932	**L7A**
2	**L7A**	0.390	RP4	0.144	**RP18**	1.413	**RP18**	0.999	**RP18**
3	**RP18**	0.419	RPS8	0.215	RP4	1.449	RPS8	1.002	RPS8
4	EF2	0.517	EF2	0.221	EF2	1.450	EF2	1.005	EF2
5	RP4	0.614	EF1a	0.227	RPS8	1.525	RP4	1.027	RP4
6	ARF1	0.906	ARF1	0.236	**L7A**	1.528	ARF1	1.269	ARF1
7	EF1a	1.267	**RP18**	0.240	L10	1.531	EF1a	1.297	EF1a
8	L10	1.313	TUB1a	0.278	EF1a	1.675	TUB1a	1.461	TUB1a
9	TUB1a	1.529	L10	0.310	TUB1a	1.839	GAPDH	1.531	L10
10	GAPDH	1.531	GAPDH	0.352	GAPDH	1.986	L10	1.546	GAPDH
11	ARF4	1.609	ARF4	0.352	ARF4	2.171	ARF4	1.611	ARF4

Table 5(on next page)

Expression stability values of the candidate genes calculated by NormFinder where the compared subgroups were female and male samples within head and thorax samples, respectively.

Rank	Head		Thorax	
	Gene	**Stability value**	**Gene**	**Stability value**
1	L7A	0.116	L7A	0.096
2	EF2	0.179	EF2	0.114
3	RP4	0.190	RP4	0.130
4	RP18	0.200	RP18	0.135
5	RPS8	0.234	RPS8	0.146
6	ARF1	0.283	EF1a	0.154
7	TUB1a	0.333	ARF1	0.220
8	GAPDH	0.350	L10	0.249
9	EF1a	0.393	TUB1a	0.296
10	ARF4	0.404	ARF4	0.328
11	L10	0.409	GAPDH	0.341

Table 6(on next page)

Summary statistics generated by BestKeeper analysis across all samples for the candidate reference genes.

BI is the BestKeeper Index, MAD is the average absolute deviation and *r* is the Pearson correlation coefficient.

Gene	Geomean	Armean	Min	Max	MAD	[r]	p-value
GAPDH	15.07	15.27	12.28	22.93	1.99	0.902	0.001
TUB1a	14.92	15.11	11.66	24.57	1.84	0.915	0.001
EF1a	15.28	15.44	12.36	24.43	1.67	0.929	0.001
EF2	14.98	15.11	12.17	23.09	1.45	0.966	0.001
ARF1	20.70	20.77	17.58	27.47	1.36	0.900	0.001
ARF4	20.02	20.22	16.25	30.12	2.17	0.940	0.001
RPS8	16.26	16.38	13.44	25.40	1.53	0.964	0.001
RP4	15.41	15.52	12.72	23.39	1.45	0.963	0.001
L7A	17.15	17.27	14.50	25.64	1.53	0.983	0.001
L10	16.04	16.15	12.55	23.31	1.53	0.820	0.001
RP18	15.92	16.02	13.17	23.29	1.41	0.964	0.001
BI	16.42	16.54	13.69	24.73	1.49	-	-

Figure 1

Expression profiles of the 11 candidate reference genes.

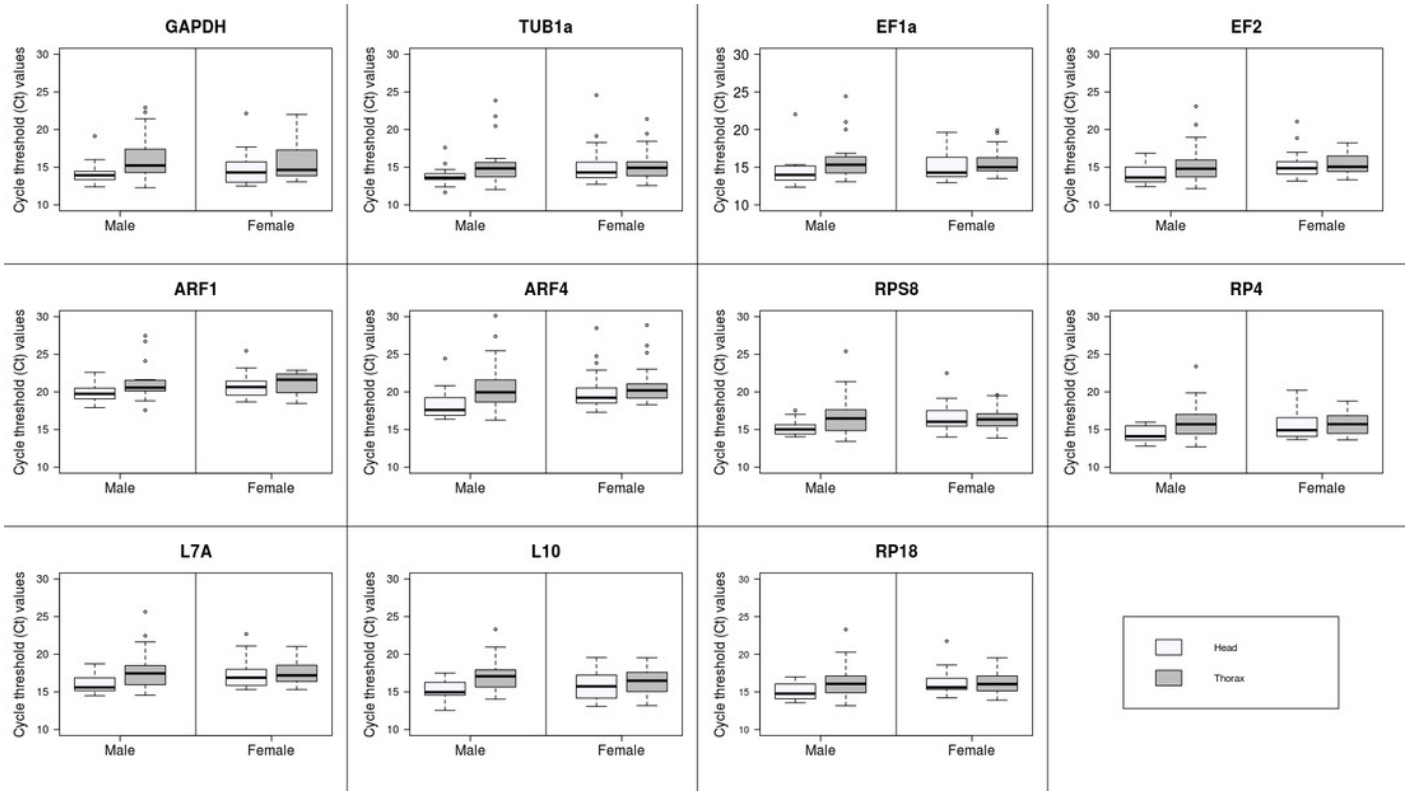

Figure 2

Pairwise variation analyses by geNorm to determine the optimal number of reference genes for accurate normalization.

Pairwise variation for all samples together, as well as separately for head and thorax samples. The lowest number of genes with $V_{n/n+1}$ less than 0.15 means the optimum number of genes.