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VALIDATION BY QPCR OF REFERENCE GENES FOR REPRODUCTIVE STUDIES IN THE INVASIVE APPLE SNAIL *POMACEA CANALICULATA*

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ABSTRACT

The South American freshwater gastropod *Pomacea canaliculata* is a highly invasive species. In introduced areas, it is a serious crop pest, responsible for great economic loss and ecological damage. It is also a vector of the nematode *Angiostrongylus cantonensis* that causes human meningoencephalitis. Many aspects and particularly its reproduction have been extensively studied, but little research has been conducted on this species regarding gene expression. To meaningfully interpret quantitative PCR, a powerful technique to develop this kind of study, validation of reference genes is essential but until now has not been undertaken. We selected the female albumen gland for its major role in egg production in order to evaluate the expression stability of the candidate reference genes *EF1-α*, *RPL7*, *His H3.3*, *TUBB*, *18S RNA*, *ACTB* and *GAPDH*. Stability was analyzed under different reproductive activity conditions and defined based on three approaches: geNorm, NormFinder and the comparative ΔCt method. NormFinder selected *GAPDH* and *ACTB* as the best option to be used for normalization, whereas geNorm and the comparative ΔCt method indicate *RPL7, GAPDH*, and *18S RNA* as the most stable genes under the conditions studied. These results will facilitate reproductive studies, particularly those using qPCR to evaluate factors that may affect fecundity of this conspicuous invasive species.

Key words: qPCR, *Pomacea*, invasive species, crop pest, angiostrongyliasis, albumen gland, apple snail, reproduction.

INTRODUCTION

Pomacea canaliculata (Lamarck, 1822) is a South American freshwater gastropod that has been introduced to Southeast Asia, North America and some Pacific islands (Cowie, 2002; Rawlings et al., 2007). It was listed among 100 of the worst invasive species in the world (Lowe et al., 2000) by virtue of the agricultural damage it causes, especially in rice and taro crops, and the environmental damage it can cause to native ecosystems (Cowie, 2002) when introduced beyond its natural range. In some of the introduced areas, it is responsible for outbreaks of eosinophilic meningoencephalitis because it is also an intermediate host of the nematode *Angiostrongylus cantonensis*, the parasite that causes this potentially fatal disease in humans (Lv et al., 2009). Proliferation of *P. canaliculata* in introduced areas may be related to the absence of natural predators, ability to tolerate a wide range of environmental conditions (Seuffert & Martín, 2009; Mu et al., 2015) and high fecundity (up to 4,500 eggs, on average, during its lifespan under laboratory conditions (Estebenet & Martín, 2002)).

The albumen gland is a female accessory reproductive organ that synthesises and secretes the perivitelline fluid that surround the embryos. It provides the developing embryos with all the nutrients (Heras et al., 1998) and with a series of proteins involved in a biochemical defence system against biotic and abiotic stressors (Dreon et al., 2004, 2007, 2008, 2013; Heras et al., 2008). In addition, females of *P. canaliculata* are able to store sperm for a considerable period (over 140 days), which allows multiple oviposition events after a single copulation. These features contribute to the high reproductive success of the species (Estebenet & Martín, 2002; Catalán et al., 2006; Giraud-Billoud et al., 2013). The acquisition of aerial egg deposition is one of the most conspicuous characters of this speciose genus,

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and it is thought to be a key evolutionary event that could be related to its success (Hayes et al., 2009). In fact, the genus *Pomacea* is considered the most successful among the Ampullariidae. In this context, egg defensive proteins synthesised in the albumen gland have also been considered an important part of the adaptive features that helped to conquer this new environment.

Despite the fact that *Pomacea* reproductive biology has received much attention (Hayes et al., 2015), very little research has been conducted in relation to gene expression (Sun et al., 2012, 2013; Song et al., 2014; Xu et al., 2014), and no research has been performed regarding reference gene selection in the family, which is critical for developing accurate quantitative PCR (qPCR) assays.

Use of qPCR enables a highly sensitive quantification of gene expression. In relative quantification, it is necessary to refer the amount of mRNA of the gene(s) of interest to internal control genes that are known to be stably expressed in the samples along the different assayed conditions. These are referred to as “reference genes”. Increasing evidence has shown that there are no universal reference genes, and their validation as internal controls must be undertaken in each individual study (Thellin et al., 1999; Bustin et al., 2009). The justifications for the number and choice of reference genes should be published in any qPCR study (Bustin et al., 2009). Therefore, validation of reference genes is a necessary step for reliable gene expression studies.

This study aims to determine an accurate set of reference genes for reliable normalization in future qPCR gene expression assays in the albumen gland of *P. canaliculata*. Considering the role this organ has in reproduction and the significance reproductive biology has in *P. canaliculata* because of its invasiveness, such studies may constitute a useful baseline for reproductive research, particularly for using qPCR to evaluate factors affecting its fecundity.

MATERIALS AND METHODS

Biological Materials

During animal handling and experimentation suffering was minimized following the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

To reduce variability among females, all animals used in the experiment (copulating males included) came from the same egg clutch and shared the same father. The snail population was native to the Pigüe-Venado canal (37°9'59"S, 62°40'28"W) in southwestern Buenos Aires Province, Argentina. The snails were fed *ad libitum* with lettuce and bred in individual aquariums of tap water saturated with calcium carbonate under controlled conditions of temperature and photoperiod, as described by Tamburi & Martín (2009).

Albumen glands were dissected from 18 mature females in four reproductive states (Fig. 1). These were five virgin females and 13 females that had copulated once. Of those that had copulated, four were dissected immediately after copulation. From the remaining nine, four were dissected immediately after the first egg clutch was laid, and five were sampled after

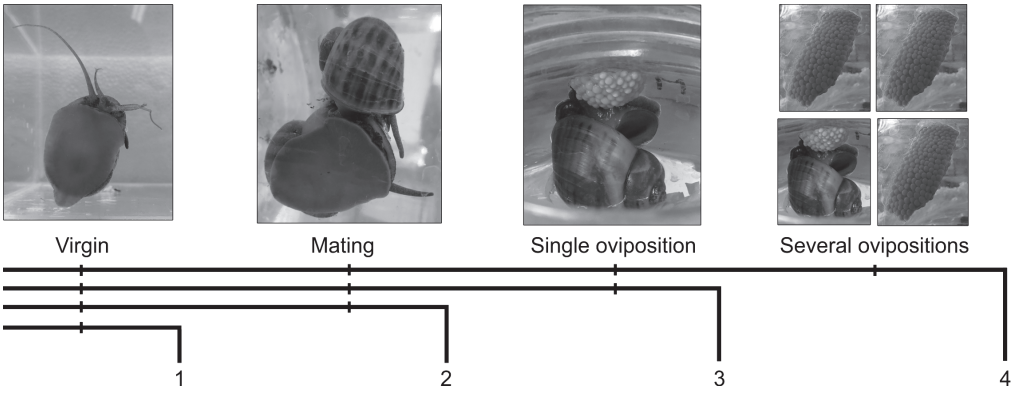

FIG. 1. Summary of the four *Pomacea canaliculata* female groups sampled.

they had laid several egg clutches. Samples of albumen gland parenchymal tissue were stored in RNAlater (Ambion) following the manufacturer's instructions, until they were processed for RNA extraction.

Selection of Candidate Reference Genes and Primer Design

Seven candidate reference genes were selected from those commonly validated in molluscs: *Elongation factor 1- α* (*EF1- α*), *60S ribosomal protein L7* (*RPL7*), *H3 histone family 3A* (*His H3.3*), *β-tubuline* (*TUBB*), *18S RNA*, *β-actin* (*ACTB*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*).

Pairs of PCR primers for *18S RNA* and *ACTB* for *P. canaliculata* were taken from Xu et al. (2014) and Zheng et al. (2012), respectively. Primers for *GAPDH*, *RPL7*, *His H3.3*, and *TUBB* were designed based on transcriptomic information (Sun et al., 2012); and those for *EF1- α* were designed based on a sequence submitted to GenBank (accession No. AB629961.1, by Matsukura, Wada & Okuda). In all cases, the pairs of primers were designed using the software Primer 3 (Koressaar & Remm, 2007; Untergasser et al., 2012) according to qPCR restrictions and were analysed using NetPrimer (Table 1). The specificity of each primer pair was confirmed by visualizing the amplification products in 1% agarose gel electrophoresis and analysing their melting curves.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from 15 mg of tissue using TRIzol (Invitrogen) following the manufacturer's instructions with slight modifications: the RNA pellet was washed three times with ethanol instead of once, and pellet resuspension used RNAase free water, incubated for 30 min on ice, and 10 min at 56°C. The quantity and purity of RNA were determined spectrophotometrically using a NanoDrop (Thermo Scientific) and its integrity and absence of DNA was verified in a 0.8% agarose gel stained with ethidium bromide. Then, cDNA was synthesised from 1 µg RNA, using a cDNA synthesis kit (Bio-Rad Laboratories) following the manufacturer's protocol.

Quantitative Real-Time PCR

The qPCR was conducted in a Stratagene™ Mx3005P qPCR instrument using an iTaq Universal SYBR Green Supermix (Bio-Rad

TABLE 1. Candidate reference genes evaluated in *P. canaliculata* albumen gland.

Gene	Forward primer	Reverse primer	Amplicon length (bp)	Annealing temperature (°C)	Amplification efficiency (%)
EF1- α	AGAACTGGGCTGTGT-GGTTT	GGAGATTGGCACA-AAAGGAA	234	59	91.6
RPL7	CTGCCGAGAAAGAAA-CACAA	ATGGTGGATGGAGGA-GAAAG	181	58	91.1
His H3.3	CGTGAAATTGCACAG-GACTT	ATGCCACAAATGCTA-CAAGC	163	58	91.8
TUBB	TGTGCCGTCTCCA-AAGGTAT	GGTGGTTGAGGTCTC-CGTAA	176	59	90.0
*18S RNA**	TGATCCTGCCAGTAG-TCAT	TTGCCACAGTTATC-CAAG	146	52	102.7
*ACTB***	TCACCATTGGCAACGA-GCGAT	TCTCGTGAATACCA-GCCGACT	87	56	93.7
GAPDH	CAACCTCAAAACCGAT-GCCA	GACAAAGCGATTAGT-CAGTGGA	184	55	96.3

In all cases, the slope from which the amplification efficiency was calculated was near -3.3 and the r^2 was greater than 0.9.
*Zheng, 2012; **Xu, 2014.

Laboratories). Each reaction was performed in a 10 µL final volume, including 1 µL of cDNA, 5 µL of Supermix, 0.3 µL of a 10 µM F + R primer solution, and 3.7 µL of sterile water. Cycling was performed using the following conditions: 1 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at the specific annealing temperature (Table 1), and 30 s at 60°C. After the cycling step, 1 min at 95°C was added, and finally a melting curve analysis was performed from 55 to 95°C.

For each gene, the standards and the complete set of samples were measured in the same run, which has been demonstrated to be the best option when there is a large number of samples (Hellemans et al., 2007). All reactions were performed as technical triplicates. The standard curve was generated using a pool of cDNA of different female treatments in serial dilutions (1/5, 1/10, 1/50, 1/100 and 1/500). The cDNA samples of individual females were diluted 1/10 for analysis. Finally, a no-template control (NTC) and a no-retrotranscription control (NRT) were also included in each plate. Primer pair efficiency was calculated from the slope of the linear relation between the Cq values and the standard curve points.

Analysis of Gene Expression Stability

Gene expression stability was evaluated using three approaches: geNorm (Vandesompele et al., 2002), the comparative ΔCt method (Silver et al., 2006), and NormFinder (Andersen et al., 2004). The geNorm method, based on pairwise comparison, calculates the gene-stability value M as the expression variation between each candidate and all other genes. Genes with the lowest M value have the most stable expression. The optimal number of reference genes to be used from the given ranking is indicated by the V parameter. It shows the effect of using consecutively an extra (n + 1) reference gene, and indicates the optimal limit as soon as the $V_{n/n+1}$ value falls below the 0.15 threshold. The geNorm method analyses the expression profile across the complete sample set with controls and treatments evaluated together, to select those genes with the highest degree of stability.

The comparative ΔCt method also analyses pairs of genes. In this case, the expression stability of a given candidate reference gene is based on the standard deviation of the Cq differences between it and all other genes. Candidate genes can then be ranked according to their stability.

NormFinder is a Microsoft Excel tool that calculates a stability value for each candidate based on intra- and inter-group variation, selecting the best pair of genes for use in normalisation. It is a model-based approach, and its results are not significantly affected by cases of co-regulation between tested candidates.

RESULTS

Primer Specificity and Amplification Efficiency

Standard curves displayed a correlation coefficient (R^2) greater than 0.9 and a slope near -3.3, indicating satisfactory PCR efficiency values for all primer pairs (Table 1). The amplification product of each primer pair showed a single band with the expected length in agarose gel electrophoresis. Primer specificity was also verified by melting curve analysis.

Gene Expression Stability Analysis and Determination of Optimal Number of Reference Genes for Normalisation

The gene expression levels of candidate reference genes are depicted in Figure 2. Gene stability rankings generated by the different approaches are shown in Table 2. According to geNorm, the ranking of candidate genes from most to least stable is: *RPL7*, *GAPDH*, *18S RNA*, *ACTB*, *His H3.3*, *EF1- α*, *TUBB*, evidenced by the increasing M values (Fig. 3). The optimal number of reference genes

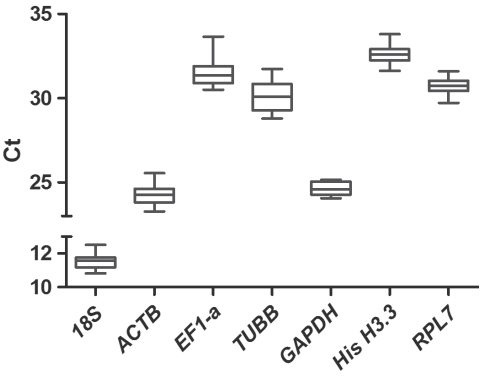

FIG. 2. Gene expression level of candidate reference genes. Ct data are shown as 25th–75th percentile (boxes), medians (lines) and ranges (whiskers).

TABLE 2. Comparison among three tools for gene expression stability analysis in *P. canaliculata* albumen gland.

Stability order	geNorm		Comparative ΔCt		NormFinder	
1	*RPL7*	0.47	*GAPDH*	0.70	*GAPDH*	0.07
2	*GAPDH*	0.48	*RPL7*	0.72	*ACTB*	0.10
3	*18S RNA*	0.50	*18S RNA*	0.73	*RPL7*	0.12
4	*ACTB*	0.54	*ACTB*	0.77	*HisH3.3*	0.13
5	*HIS H3*	0.59	*HIS H3*	0.77	*18S RNA*	0.17
6	*EF1- α*	0.69	*EF1- α*	0.98	*EF1- α*	0.22
7	*TUBB*	0.77	*TUBB*	1.07	*TUBB*	0.28

Values represent the gene expression stability according to each approach: M value, mean standard deviation, and stability value for geNorm, comparative ΔCt method and NormFinder, respectively.

according to geNorm V values is 3 (Fig. 3). According to this approach, *RPL7*, *GAPDH*, and *18S RNA* constitute the best combination of reference genes to be used for further normalisation factor calculation. According to the comparative ΔCt method, the ranking of stability is quite similar: *GAPDH*, *RPL7*, *18S RNA*, *ACTB*, *His H3.3*, *EF1- α*, and *TUBB*. This analysis does not indicate the optimal number of reference genes to be used for normalisation. The NormFinder results indicate that *GAPDH* and *ACTB* is the best combination of reference genes to be used for normalisation.

DISCUSSION

The stability rankings of candidate reference genes showed some consistency among the three methods, especially with respect to the most and the least stable genes (Table 2). The slight discrepancies found are not uncommon (Cubero-Leon et al., 2011; Feng et al., 2013) and may be related to the different algorithms applied to the same data set. Both geNorm and the comparative ΔCt method compare pairs of genes and consider data from different experimental conditions together. For both algorithms, a good pair of reference genes

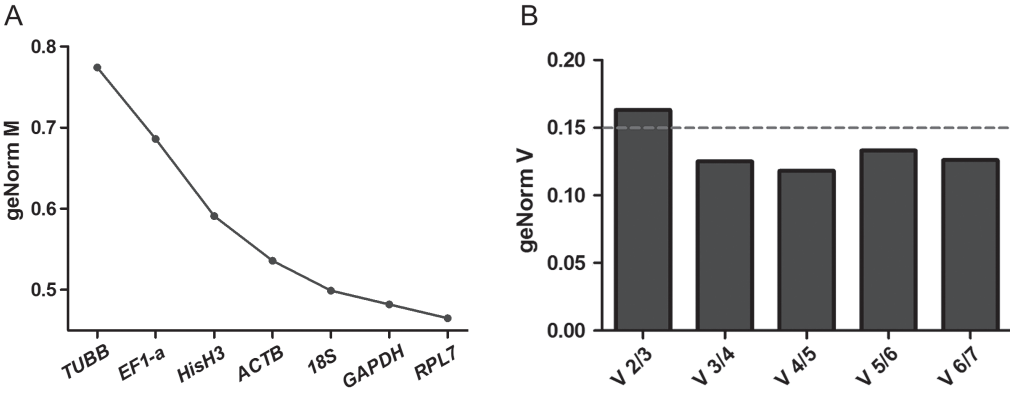

FIG. 3. geNorm values. A: geNorm M values for the candidate reference genes evaluated. B: geNorm V value for each $V_{n/n+1}$ option. V threshold is shown as a dashed line.

should not display substantial variation in expression between them across the sample set. In contrast, NormFinder evaluates the systematic expression variation across the experimental conditions in addition to the intragroup variation. Co-regulation between the tested candidates could affect the results from geNorm tending to rank them together, whereas NormFinder results are not affected by such co-regulation. Also, in geNorm, candidates with minimal expression variation are not necessarily top ranked (Andersen et al., 2004). This could be the case for *ACTB* in our results, and is a disparity commonly observed in other validation studies using both kinds of approaches. In fact, these discrepancies in results were experimentally evaluated by Andersen et al. (2004) when describing NormFinder. All the approaches evaluated in this study agree that *EF1- α* and *TUBB* are the least stable genes. The case of *EF1- α* was striking, as it is a frequently validated reference gene in molluscan gene expression studies (Morga et al., 2010; Qiu et al., 2013). However, under our experimental conditions, *EF1- α* is not as stable as might have been expected, and definitely is not a good reference gene for gene expression normalisation in reproductive studies of *P. canaliculata* that involve the albumen gland.

In addition to stability, the use of reference genes with a similar expression level as the target genes is also recommended for normalisation, especially in cases of subtle gene expression differences to increase the sensitivity (Silver et al., 2006). In this study, the most stable genes differed in their expression levels (Fig. 3). Therefore, the pair of genes selected by NormFinder has the advantage of having the same expression level as determined by the geNorm and ΔCt methods, in which a disparity in expression level is observed among the selected candidates.

In conclusion, we present the first reference gene validation analysis to be used in future qPCR studies of *P. canaliculata*. In particular, we have studied a crucial female reproductive organ, the albumen gland, under defined experimental conditions in relation to reproductive activity. Based on three approaches, we propose a list of the most stable genes that can potentially be used as references for normalisation in future studies. This study represents an essential baseline to perform expression studies regarding reproduction in this parasite vector and highly invasive species. It will also be useful for reference gene selection in other ampullariid species, the reproductive biology of which is key to understanding their biogeographical patterns, invasion biology and possibly the evolution of the amphibious habit (Hayes et al., 2015).

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