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Characterization and Evaluation of Selected House-keeping Genes for Quantitative RT-PCR in *Macrobrachium rosenbergii* Morphotypes

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Abstract

Macrobrachium rosenbergii (De Man, 1879) is an important freshwater prawn cultured globally and is known to exhibit three male morphotypes with differential growth rates. Several gene expression studies using qRT-PCR have been undertaken in this species using any one of the popularly used internal control genes. Here we evaluate four house-keeping genes namely EF1α, GAPDH, β-actin and 18S rRNA for transcript stability across various *M. rosenbergii* tissues in male morphotypes for use as internal control genes in expression studies. Expression of these genes was measured in 5 tissues (androgenic gland, gill, eyestalk, nerve cord and testis) across morphotypes and Ct values were analyzed using four statistical methods (ΔCt, geNorm, BestKeeper and NormFinder) developed for this purpose. We report that EF1α is the most suited internal control for data normalization in androgenic gland, testis, nerve cord and gills while GAPDH is best for eyestalk tissue of *M. rosenbergii* irrespective of the morphotype. Wherever two reference genes are required β-actin can be included for all the tissues except in nerve cord, where GAPDH is more suitable second reference gene. *M. rosenbergii* EF1α and GAPDH partial mRNA sequences were also generated as part of this study and their phylogenetic relation with other decapods is also reported.

Keywords: *Macrobrachium rosenbergii*, morphotypes, quantitative RT-PCR, house-keeping gene, normalization, reference genes

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Introduction

Modulation of gene expression in response to various conditions and stimuli is being examined at high sensitivity levels by quantitative real-time PCR (qRT-PCR) (Bustin, 2000; Pfaffl & Hageleit, 2001). In aquaculture research, the technique is being applied to generate deeper understanding on issues related to growth, development, disease management, sex differentiation, nutrition and several such studies have been undertaken in *M. rosenbergii* (Zhu et al., 2005; Zhang et al., 2006; Liu et al., 2007; Ventura et al., 2011; Arockiaraj et al., 2013; Du et al., 2013; Yu et al., 2014). Accurate quantification of the RNA template, which is a prerequisite for reliable and reproducible results, remains the major concern especially where small amount of starting tissue is available or when tissue processing is difficult. Results are also affected by variability in RNA quality, presence of inhibitory compounds and genomic DNA that co-purifies with RNA (Mannhalter et al., 2000; Opel et al., 2010). These issues are circumvented by normalizing the target transcript levels against a stably expressed transcript of internal control genes, which are normally one of the house-keeping genes (HKGs) like GAPDH, EF1α, 18S rRNA and β-actin. However, the expression stability of the internal control gene is crucial (Bustin, 2002) and it is erroneous to assume unvaried levels of expression for these genes as it has been reported to differ with tissue, animal size and even experimental conditions (Thellin et al., 1999; Bustin, 2002). Recent experiments suggest that a gene with unvaried expression under all conditions is practically non-existent (Vandesompele et al., 2002). Nevertheless, for a specified set of conditions, an internal control gene with the most stable expression can be chosen for normalization

after evaluation of selected HKGs for conditions specific to each experiment.

Giant freshwater prawn, *M. rosenbergii* is a popular aquaculture species globally. The species exhibits differential growth at all life stages including larvae, post larvae, juvenile and adult, resulting in different morphotypes at adult stage (Ra'Anan & Cohen, 1984). In a typical grow-out pond community, the social hierarchy consists of three male morphotypes namely small male (SM), orange claw (OC) and blue claw (BC). The female exists as a single morphotype. The three male morphotypes exhibit different levels of growth (Ra'Anan & Cohen, 1984; New, 2002; Karplus, 2005), aggression (Vazquez-Acevedo et al., 2009) and reproductive activity (Ra'anan & Sagi, 1985) while living in a community. Since these animals establish their natural social hierarchy whenever they are reared in community, it is difficult to obtain uniformly sized animals of similar morphotype at any one point of time for an experiment. So far, β-actin and 18S rRNA have been employed as internal control genes for qRT-PCR in *M. rosenbergii* though the stability of their expression in relation to different tissues and morphotypes has not been evaluated (Ventura et al., 2011; Zhang et al., 2006). The present study evaluates the stability of four commonly used internal control genes *viz*., β-actin, GAPDH, EF1α and 18S rRNA, in five tissues including testis (T), androgenic gland (AG), eyestalk (E), gills (G) and nerve cord/ganglion (N) from each of the three male morphotypes of *M. rosenbergii*. The selected tissues are reportedly the major sites of endocrine hormone production in decapods and are frequently subjected to analysis and profiling of several hormones like, crustacean hyperglycaemic hormone (CHH; eye and gill form), moult-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIH) and insulin-like androgenic gland hormone (IAG). Partial cDNA sequences of GAPDH and EF1α from *M. rosenbergii* were also generated as a part of this study.

Material and Methods

SM, OC and BC morphotypes were collected from a grow-out culture pond to obtain individuals from a naturally established social hierarchy. Four animals of each type (SM, OC and BC weighing approx. 15, 30 and 80 g, respectively) were sacrificed and dissected to collect androgenic gland, testis, brain, gill and eyestalk in RNA*later*™ (Qiagen, Germany) following manufacturer's protocol. Total RNA from all the tissues except eyestalk was isolated using TRIzol reagent (Invitrogen, USA) following manufacturer's protocol. Total RNA from eyestalks was isolated using RNeasy Mini kit (Qiagen, Germany) as per manufacturer's instructions to avoid possible contamination from PCR inhibitors from the ommatidial region. Quantification of RNA was done in Nanodrop 2000/2000c (Thermo Scientific, USA). Duration of *DNase*I treatment was standardized and 1μg of each DNase treated RNA was used for cDNA preparation using RevertAid cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's instructions.

Degenerate primers, deg-GAPDH and deg-EF1α (Table 1) were designed using Gene Runner software V3.05. Nerve cord cDNA was PCR amplified in a 25 μl reaction mixture (Sambrook & Russell, 2001) with PCR conditions set as initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. The amplicons (800 bp of EF1α and 433 bp of GAPDH) were cloned into pTZ57R/T vector and sequenced (MrEF1α: NCBI Accession No. KF228019 and MrGAPDH: NCBI Accession No. KF305552). The partial cDNA sequences were aligned with other decapods sequences available in NCBI GenBank using Clustal W. Phylogenetic trees were constructed for both GAPDH and EF1α by Neighbor-Joining (NJ) method using MEGA v.6.0 software (Tamura et al., 2013). The reliability of tree was checked by bootstrapping (1000 pseudo replications) (Felsenstein, 1985).

Real-time PCR was done using Roche 480 LightCycler machine. The reaction was set in 15 μl volume and included cDNA (1 μl), 2x SYBR green master mix (7.5 μl; Thermo Scientific, USA), forward and reverse primers (0.3 μl each of 10 pmoles μl^{-1} stock) and nuclease-free water (5.9 μl). A common program was set for all the genes across all the tissues, which included 1 cycle of initial denaturation at 95°C for 10 min, 45 cycles of 95°C for 20s, 60°C for 20s, 72°C for 30s, 1 cycle of melt curve analysis at 95°C for 5s, 65°C for 1 min followed by continuous signal acquisition until the temperature reaches 97°C and finally 40°C for 10s. Each cDNA was run in duplicate. On completion of run, Cp values were estimated by second derivative method using the LightCycler Software 3.5 (Roche Diagnostics).

For each primer set, four serial dilutions of cDNA (1 to 1000x) were run in duplicate to generate a

Table 1. Primers used in the present study

Primer name	Sequence (5′ —> 3′)	Accession No.	Product size (bp)
deg-GAPDH-F	CAAGGGTGAGGTGAAGGCAGAGGA		
deg-GAPDH-R	ACGGCCACCACGCCAGTCCTT	HM157285	433
deg-EF1α-F	TCACCATCGACATTGCCCTGTG		
deg-EF1α-R	TGCTTCCTTGGCAGGGTCGTT	AB458256	800
Mrβactin-RT-F	ACCACCATGTACCCAGGAATCGCTG		
Mrβactin-RT-F	CCAAGATTGAACCGCCGATCCAG	AF221096	133
MrGAPDH-RT-F	TGAAGCCCGAGAACATTCCATG		
MrGAPDH-RT-R	GTTCACGCCGCAGACGAACATG	KF305552	170
MrEF1α-RT-F	TGGACGTGTGGAGACTGGCATC		
MrEF1α -RT-R	ATCGCCTGGAACAGCCTCAGTC	KF228019	127
Mr18S-RT-F	TGGCTGTTACGGGTGACGGAGAATC		
Mr18S-RT-R	GGGATTGGGTAATTTGCGTGCCTG	AY461599	106

standard curve with Cp values plotted against log [cDNA]. Slopes of these curves were used to estimate PCR efficiency for each gene using the formula E= $[10^{(-1/slope)}-1]$ x 100 and coefficient of determination (R^2) was calculated (Pfaffl, 2001).

The expression stability of the genes was analyzed using four different statistical/mathematical models, namely, ΔCt method (Silver et al., 2006), geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004).

Results and Discussion

The characterized partial cDNA sequences of MrGAPDH (NCBI Acc. No. KF305552) and MrEF1α (NCBI Acc. No. KF228019) were subjected to BLASTn homology search. In both cases highest homology was obtained with the decapod, *Marsupenaeus japonicas* (91% and 84%, respectively). Nucleotide homology above 60% is considered valid for phylogenetic tree construction (Salemi et al., 2009). Fig. 1a depicts the NJ phylogenetic tree where MrGAPDH clusters with *M. japonicas*. On the other hand MrEF1α forms a separate clade with respect to other decapods (Fig. 1b). These two genes, along with *M. rosenbergii* β-actin and 18s rRNA genes were evaluated for expression stability for use as internal control in real time PCR. PCR efficiencies for all the genes ranged from 99-104% with high R^2 value of 0.99 that indicating reliability (Fig. 2a). Melting peak analysis of all qRT-PCR products also confirmed absence of dimer formation and non-specific amplification (Fig. 2b).

Various statistical models, each based on a different assumption, have been developed for evaluating expression stability of several HKGs for use as internal control genes in qRT-PCR. The expression stability of each gene was evaluated separately for every tissue using the four methods described above (Fig. 3A-D). According to ΔCt, geNorm and

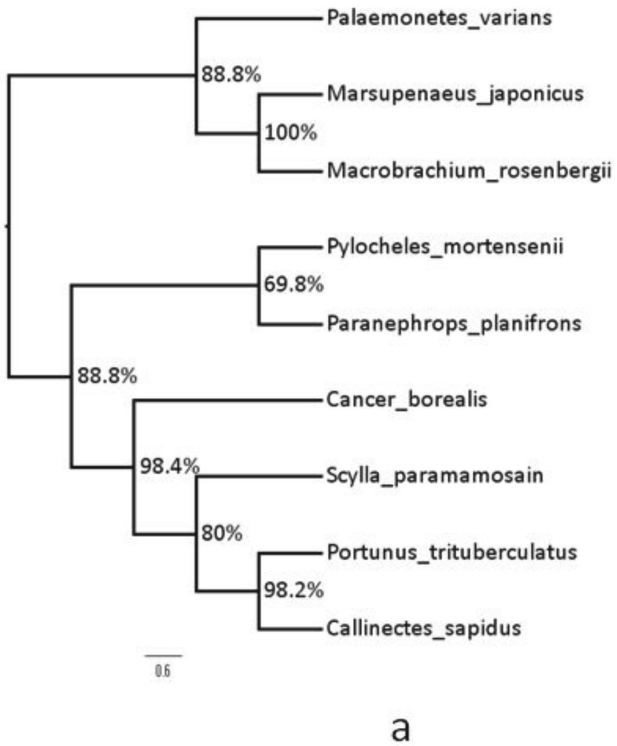

Fig. 1a. Neighbour joining tree of MrGAPDH

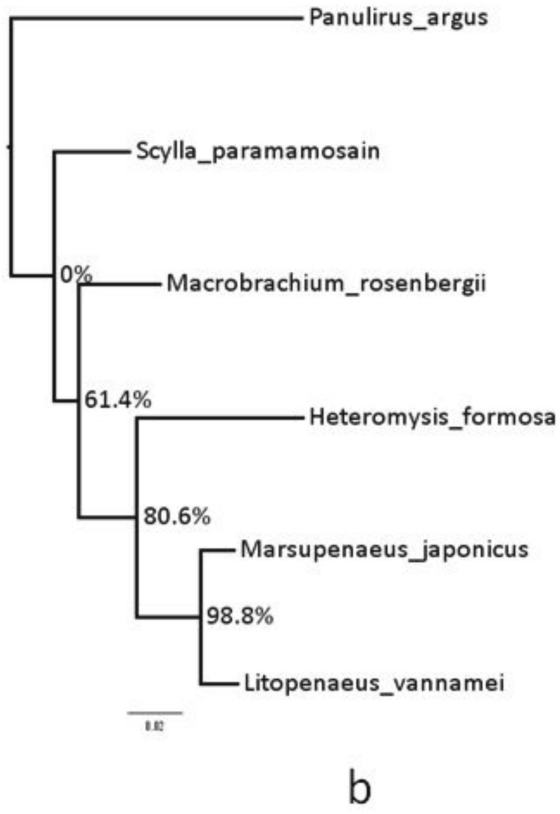

Fig. 1b. Neighbour joining tree of MrEF1α with other decapods

NormFinder models, EF1α is the most stably expressed gene in all tissues except eyestalk where GAPDH was more stable. Only NormFinder found Δ-actin to be more stable in gill. The geNorm model that gives two best internal control genes also finds β-actin (in addition to EF1α) to be most stably expressed in all tissues. It recommends GAPDH and EF1α for gill. β-actin was ranked best by the BestKeeper model in all tissues except nerve cord and gill where GAPDH was ranked best. This method differs from others in ranking EF1α from second to least preferred internal control gene.

ΔCt method of Silver et al. (2006) evaluates stability in terms of average standard deviation (S.D.) of pair-wise ΔCt of a particular internal control gene with all others and the lowest value indicates highest stability. The precision of this method is significantly affected if two genes with highly divergent expression are compared (Silver et al., 2006), although this might not be a major problem if the panel of internal control genes is fairly large. The software geNorm developed by Vandesompele et al. (2002) determines pair-wise variation of every control gene with every

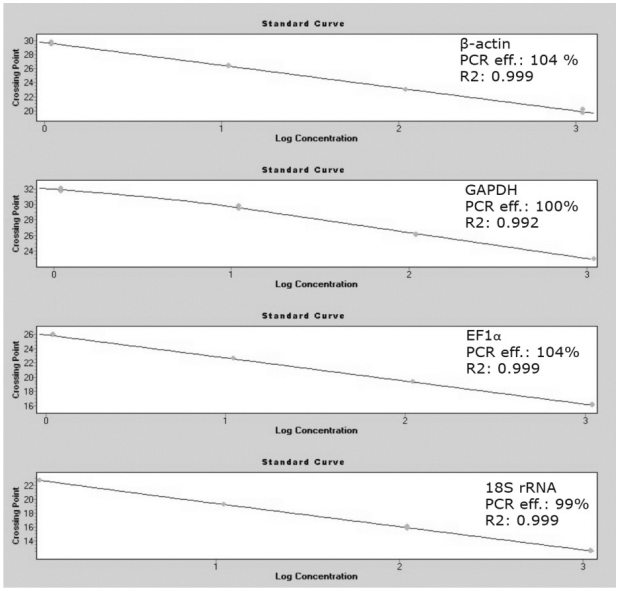

Fig. 2a: Standard curves of β-actin, GAPDH, EF1α and 18S rRNA

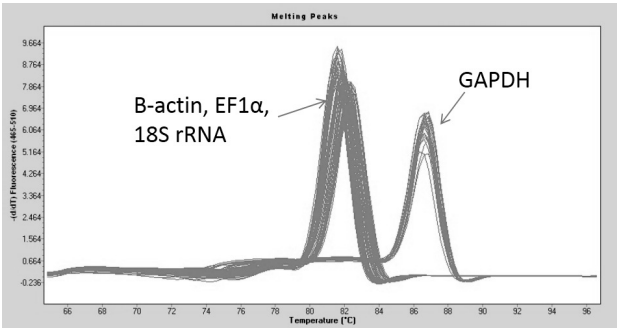

Fig. 2b: Melting curve analysis of β-actin, GAPDH, EF1α and 18S rRNA

other and uses the S.D. of logarithmically transformed expression ratios instead of S.D. of pair-wise ΔCt used above. It assigns a gene stability measure M to each gene, which is essentially the average pair-wise variation of that gene with all others. At each step the gene with the highest M value gets excluded and the final result is a combination of two genes with most stable expression. The argument here is that normalization is more accurately done with more than one best suited gene. NormFinder (Andersen et al., 2004) is a mathematical model based approach that computes overall variation of the candidate normalization genes and also estimates the variation between subgroups of the sample set from log transformed Cp values. In this study, data was entered for each male morphotype as a different subgroup. In cases where a sufficiently

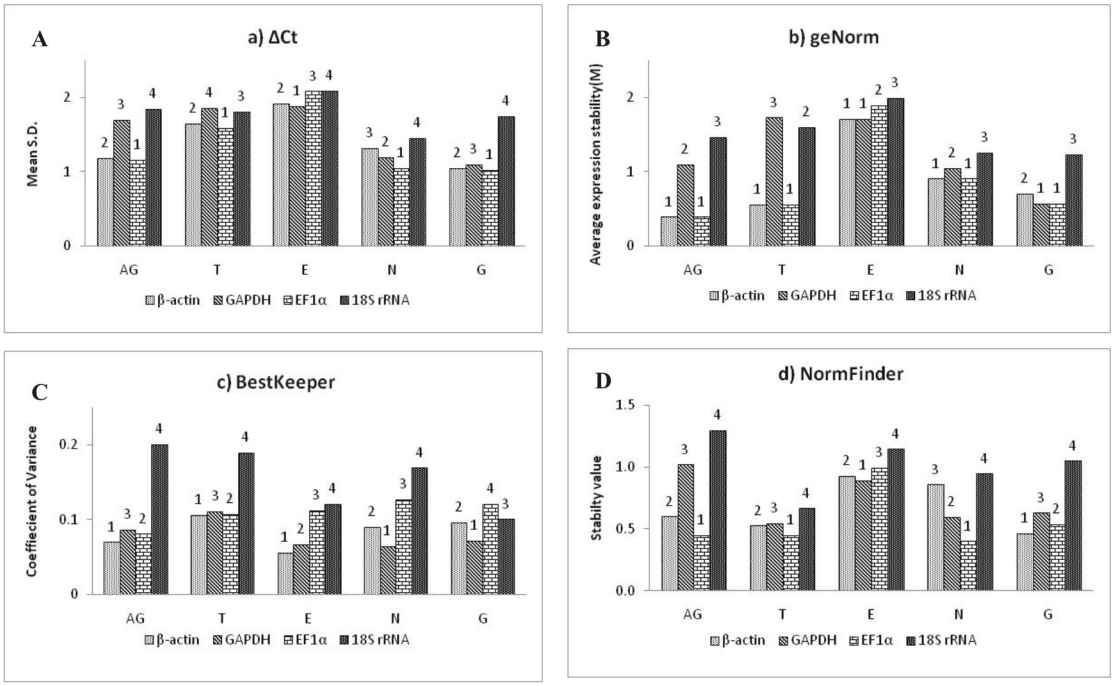

Fig. 3a-d. Stability ranking of house-keeping genes as determined by ΔCt, geNorm, BestKeeper and NormFinder in various tissues of *M. rosenbergii* morphotypes

stable internal control gene is not available the authors have devised a Normalization Factor (NF) based on multiple instead of a single internal control gene. For species where few genes have been characterized and sequence information is scarce, reliability may be compromised. For our data set, the above three methods agreed on EF1α being the most stably expressed gene in all tissues of *M. rosenbergii* morphotypes except eyestalk where GAPDH was most stable. EF1α is involved in the translation process and a variety of functions studied in cultured cells ranging from proliferation and inhibition of senescence to induction of immortality (Thornton et al., 2003), while GAPDH is a key enzyme in glycolysis and also involved in DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics and cell death (Tristan et al., 2011). EF1α has also been evaluated to be the best internal control gene for qRT-PCR studies in an independent study in other decapods (Leelatanawit et al., 2012).

BestKeeper method (Pfaffl et al., 2004) ranked EF1α as 2 to 4 in various tissues unlike the other models. It ranked β-actin as 1 or 2 across all the tissues instead. BestKeeper is a Microsoft Excel based software which evaluates the stability of the gene on the basis of coefficient of variance (CV); lower the CV higher the stability. However, the presence of outlier values and a large range between minimum and maximum values can have a profound impact on the regression line slope and thereby on the correlation co-efficient. Thus, under such conditions, assessment of stability would get affected. All other methods are in agreement over β-actin being one of the most stable internal control genes in case two genes are to be selected. β-actin is a HKG significant for cell growth, shape and migration (Bunnell et al., 2011). It has been used to investigate modulation of gene expression in various tissues of *M. rosenbergii* in response to pathogens, viral and bacterial infections and immunostimulants and chemotherapeutants (Liu et al., 2007; Arockiaraj et al., 2013; Du et al., 2013). Ventura et al. (2011) used β-actin as control while studying the expression of an androgenic gland-specific insulin-like peptide during the course of sexual and morphotypic differentiation of prawn.

The gene for 18S rRNA, which is a structural component of the smaller subunit of the eukaryotic ribosome and constitutes the major component of the total cellular RNA (Paule & White, 2000), constantly featured in the least preferred category

in this study although it has been used by researchers earlier. Zhu et al. (2005) used 18S rRNA as internal control gene while studying expression of actin gene during embryonic development in *M. rosenbergii*. It also served as the internal control gene in a work that investigated sexually dimorphic expression of two *Dmrt* genes in several tissues of the same species (Yu et al., 2014). The use of rRNA genes as internal controls has been pointed out to be less appropriate in several other reports because their transcription is affected by several biological factors and treatments in human subjects (Spanakis, 1993; Johnson et al., 1995; Warner, 1999). Since the bulk of total RNA is made up of rRNA it becomes difficult to accurately subtract the baseline value in qRT-PCR analysis (Vandesompele et al., 2002). Much imbalance between rRNA and cellular mRNA levels has been found in several cases (Solanas et al., 2001). Moreover, these fractions are absent from purified mRNA samples and cannot be used when the cDNA is prepared from purified mRNA instead of total RNA (Vandesompele et al., 2002).

A number of alternative strategies have been developed for normalization of target gene expression, and the pros and cons associated with each have been reviewed by Hugget et al. (2005). Normalization with an internal control gene is still the most popular method so far and hence, determination of the most suitable one for a given experiment would continue to be a significant part of qRT-PCR studies. This study concludes that EF1α is the best suited internal control for data normalization in androgenic gland, testis, nerve cord and gills and GAPDH in eyestalk tissue of *M. rosenbergii* irrespective of the morphotype. Wherever two internal control genes are required β-actin can be included for all the tissues except in nerve cord, where GAPDH is more suitable as a second internal control. This report will help future researchers to select the appropriate internal control gene for expression studies and ensure reliability of results and interpretations that can lead to improved aquaculture practices for this important species.

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