Reference gene selection for qRT-PCR analysis of the Hawaiian coral *Pocillopora meandrina* subjected to elevated levels of temperature and nutrient

L. Pagarigan and M. Takabayashi

Marine Science Department, University of Hawai`i at Hilo, 200 West Kawili Street, Hilo, HI 96720

Abstract Quantitative reverse transcriptase PCR has several variables that need to be controlled for to obtain accurate estimates of gene expression data. The use of a single reference gene is a common method to normalize mRNA fractions and obtain reliable quantitative expression measures. However no universally appropriate reference gene exists that is constitutively expressed in all tissue types and all experimental conditions. One plausible solution for this problem is to use a set of reference genes for normalization that display minimal variation across all treatments. Past gene expression studies have often employed the use of a single reference gene. As the number of gene expression studies in reef-building coral is expected to increase, a set of suitable reference genes is needed for normalization. This study characterized the expression of five candidate reference genes for the scleractinian coral, *Pocillopora meandrina*, after chronic elevated nitrogen followed by increased seawater temperature. Two separate gene ranking assessment software programs were used to rank candidate genes by expression stability. The Brn1, β-actin, and 18S rRNA genes were the most stably expressed genes in *P. meandrina* subjected to long-term elevated nitrogen followed by an acute increase in temperature.

Key words: quantitative RT-PCR, gene expression, coral

Introduction

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is commonly used in biological applications for accurate quantification of gene expression changes between tissues, disease states or treatments (Bustin and Nolan 2004). Several variables between tissues or cells need to be controlled in qRT-PCR including the amount of starting material, differences between tissues or cells, quality of the RNA, and reverse transcription efficiency (Vandesompele et al. 2002). Various strategies have been used to control for the previously stated variations within qRT-PCR including techniques such as standardization to total RNA or to an endogenous reference gene.

Standardization to total cellular RNA can pose problems because it is difficult to quantify small amounts of RNA, and the variance present in reverse transcription and PCR efficiency are not considered (Huggett et al. 2005; Wong and Medrano 2005). In addition, the process of normalizing to total RNA assumes that rRNA is directly proportional to mRNA and does not vary between individuals, tissues, or cell cultures, or under different experimental conditions (Bustin and Nolan 2004). Alternatively, variations can be controlled for by normalization of the target gene to an endogenous reference gene. This comparison

allows for a normalized expression value of the target gene that is independent of the quality and quantity of starting RNA and the efficiencies of the reverse transcriptase reactions (Huggett et al. 2005).

The most important criterion of appropriate reference genes in qRT-PCR is that these genes are expressed constitutively in cells irrespective of experimental conditions. In the past it was recommended that if it was not possible to find a constitutively expressed reference gene, it is an option to apply one of several frequently used genes as a control to normalize expressions of other genes against (Bustin 2000). In spite of this, there has been evidence that reference genes can be regulated under different conditions (Bustin 2000; Vandesompele et al. 2002). This means that although use of a referenced "stable" gene can be consistent for one experiment and one type of tissue, there is no universally perfect control gene that is perfect for all tissue types or treatments (Brunner et al. 2004; Vandesompele et al. 2002; Andersen et al. 2004).

qRT-PCR has been used in only a handful of anthozoan and hydrozoan gene expression studies (Yanze et al. 1999; Mitchelmore et al. 2002; Loram et al. 2007; Rodriguez-Lanetty et al. 2007). Many of these studies used a single reference gene to normalize the expression of their target genes. It was

recently reported that experiments that used a single reference gene to normalize expression of target genes let to erroneous quantifications (Vandesompele et al. 2002). Erroneous quantifications occur because gene expression of the control can result in unrecognized or unexpected changes (Vandesompele et al. 2002; Szabo et al. 2004). One probable solution for this problem is to use a set of reference genes for normalization that display minimal variation across the treatments included in the experiment (Vandesompele et al. 2002). There are several existing statistical analysis software programs to analyze the stability of reference genes (Pfaffl et al. 2001; Vandesompele et al. 2002; Andersen et al. 2004) and since their arrival they have been used with increasing regularity (Etschmann et al. 2006; Jorgenson et al. 2006; Nailis et al. 2006; Willems et al. 2006; Maccoux et al. 2007; Tang et al. 2007; Peltier and Latham 2008). In this study two popular statistical analysis software programs were used to identify the most stable candidate reference genes. The first program, GeNorm, calculates the most stable genes from a set of candidate reference genes based on the average pair-wise variation of a single gene compared to all other reference genes in the set (Vandesompele et al. 2002). The second program, NormFinder, evaluates the gene expression variability of reference genes within treatments compared to variation among treatments for each gene (Andersen et al. 2004). Both programs allow the ordering of candidate reference genes based on relative expression stabilities, referred to in this paper as "rank," through the use of a mathematical evaluation of expression data.

A recent study by Rodriguez-Lanetty et al. (2007) used GeNorm analysis to identify β-actin, ribosomal protein L12 and Poly(a) binding protein as three control genes determined to be stable from a cDNA microarray platform constructed from the sea anemone, *Anthopleura elegantissima*. However, to date, there has been no validated set of reference genes for qRT-PCR described for reef building corals. The aim of this study presented here was to evaluate a set of candidate reference genes, using GeNorm and NormFinder, in reef-building corals exposed to chronic levels of elevated nitrogen followed by an acute increase in seawater temperature. These reference genes can possibly be used to normalize target genes in future gene expression studies targeting physiological stress in corals exposed to multiple stressors.

Materials and Methods

Coral collection and exposure to stressor(s)
Coral samples were collected from six randomly chosen colonies of *P. meandrina* at an approximate depth of 5 m from Leleiwi Beach Park in East Hawai`i.

Colonies were split up into either of the two treatment tanks or into the control tank. The coral fragments were maintained in 75 L flow-through aerated aquaria set at a constant through flow rate of 7.15 ml s^{-1} and kept at 25° C. The tanks were placed under semi-permeable Duraweave material (Cover-all) that transmits light in the solar spectrum from 2500 -300 nm.

The colonies were allowed to acclimatize under the above conditions for a period of 3 weeks (Downs 2005). Then, the corals were placed in treatment tanks (control (C), +2° C sea water temperature (T), and nutrients and +2° C sea water temperature (NT)). Corals in the NT treatment were exposed to a constant addition of 20 μM NH$_4$ seawater for 32 days before beginning the acute increase in seawater temperature. After the 32 day exposure period to elevated levels of nutrients, both the NT and T treatments were subjected to a temperature increase in seawater over a continuous 48 h period. The water temperature of the aquariums was elevated +2° C to 27° C over a 3 h period and maintained above 27 °C. All treatments (C, T, NT) were sampled at 30 min, 6 h, 24 h, and 48 h after 27° C was reached. One coral fragment from each colony was collected per tank at each sampling time, wrapped in foil, and frozen immediately by immersion in liquid nitrogen. Once the tissue was frozen using liquid nitrogen, samples collected at each time period was stored for RNA analysis by submersion into RNA Later (Ambion), placed at 4° C for 24 h to allow the RNA Later to soak into the tissue, and then stored at -20° C to be used in qRT-PCR.

Selection of candidate reference genes
Five candidate reference genes were selected from the literature to be tested for their expression stability using mRNA from *P. meandrina*. Of the five genes, three had been frequently used as reference genes in gene expression studies of cnidarians primarily from class Anthozoa, and the remaining two were less frequently used reference genes. The three frequently used reference genes were: 18S rRNA, β-actin, and elongation factor (EF1α) (Yanze et al. 1999; Reynolds et al. 2000; Mitchelmore et al. 2002). The less frequently used reference genes examined in this study were senescence associated protein (PS) and Brn1 (Rodriguez-Lanetty et al. 2006; Rodriguez-Lanetty et al. 2007; Mayfield and Gates unpublished).

RNA isolation
Total RNA was isolated from *P. meandrina* using the Totally RNA kit (Ambion) largely following the manufacturer's protocol in an RNAse free environment. A few steps were modified from the protocol to reduce salt carryover from the RNA Later and reduce interference by Trizol found in the RNA

extraction kit. The tissue samples were placed directly on an RNAse free vacuum to remove excess RNA Later liquid then transferred to a sterile Whirl-pak 60 mL plastic sample bag (Nasco). 800 µL of the denaturation solution from the Totally RNA kit was pipetted on top of the coral fragment, and the coral tissue was air blown at 60 psi from the coral skeleton. The tissue slurry was added to a 2.0 mL tube filled with approximately 250 mg of sterile 0.5 mm diameter Zirconia/Silica beads (BioSpec Products) and vigorously shaken (Minibeadbeater-8 Cell Disrupter) for 1 min. The tube containing the tissue slurry was then centrifuged at 12,000 rpm for 2 min at 4 °C. A ¾ v. DEPC-treated water and an equal volume of Phenol:Chloroform:IAA (Promega) solution was added to the lysate and again vigorously vortexed with beads for 1 min. The remainder of the manufacturer's protocol was followed with an overnight precipitation of RNA, followed by a 30 min centrifugation period. The RNA pellet was resuspended with 19.5 µL of DEPC water and 0.5 µL of RNAsin ribonuclease inhibitor (Promega). RNA concentration in the final extracts was measured using the nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). The RNA was further purified of genomic DNA contamination by DNAse I digestion, according to the manufacturer's protocol (Promega).

Reverse transcription and qPCR

A commercial reverse transcription kit was used according to the manufacturers instructions to reverse transcribe 1 µg of DNAse treated RNA in a 20 µL reaction (iScript cDNA Synthesis Kit, Bio-Rad). DEPC treated water was added to dilute the 20 µL RT reaction products to a final volume of 200 µL. The qPCR assays were performed by using a commercial 2X SYBR-Green master mix buffer (BioRad). qRT-PCR assay efficiency was calculated for each gene using a 6-step, 10-fold cDNA dilution series. Results were documented as cycle threshold values of background subtracted qPCR fluorescence kinetics.

Analysis of results

Relative expression quantities were calculated using the comparative C_T method from qPCR results (Vandesompele et al. 2002). Two gene stability analysis programs, GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004), were used to rank reference genes in order of stability.

The GeNorm method, as previously described by Vandesompele et al. (2002), was used to determine the reference gene expression stability measures ("M-value"). The "M-value" for each gene is calculated as the arithmetic mean of pairwise variation between a particular gene and all other control genes. This program relies on the theory that the expression ratio

for ideal internal control genes is identical in all samples, regardless of the experimental condition or treatment. Genes with low M values have the least variation in expression ratios with other genes and are the most stably expressed and vice-versa genes with a high M value have a large variation in the expression ratio are less stably expressed.

The second method of analyzing relative expression stability of candidate reference genes in this study was NormFinder, which has previously been described by Andersen et al. (2004). The relative expression of reference genes were analyzed for stability by NormFinder using GenEx software (Multid Analysis, California). NormFinder generates a gene stability measure using a "model based approach" by separately ranking a set of candidate reference genes according to their expression stability in a given sample set and given experimental design. The mathematical equation estimates the overall expression variation of each candidate reference gene in addition to the variation between sample subgroups of the sample and generates a "stability value" for each that is a direct measure for the estimated expression variation.

Results

Expression of reference genes during exposure to elevated heat and nitrogen levels

The raw expression levels for the five candidate reference genes that were analyzed fell into three categories based on transcript abundance: (1) high transcript abundance (average C_T value below 20): 18S; (2) median transcript abundance (average C_T value 20-25): β-actin, PS; and (3) low transcript abundance (average C_T value <25): EF1-alpha, Brn1.
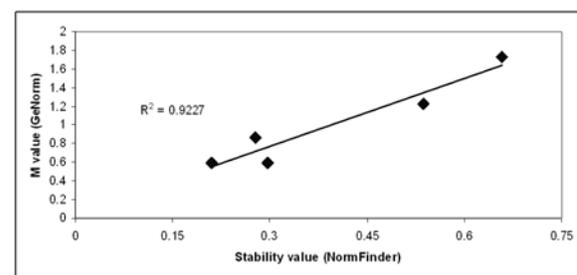

Figure 1: The correlation (R^2=0.9227, P=0.009) of expression values between reference gene ranking programs, M value (GeNorm, Vandesompele et al. 2002) and stability value (NormFinder, Andersen et al. 2004).

Analysis of gene-stability in reference genes

Both gene expression stability programs ranked the RNA targets similarly from most to least stable, with a strong correlation in raw stability values (R^2= 0.9227, P = 0.009) (Fig. 1). Results from both programs showed Brn1 was the most consistently expressed gene

followed by β-actin, and 18S (Fig. 1). Both programs also produced similar results with EF1α and senescence- associated protein (PS) exhibiting the least stable expression (Fig. 1). Because both expression stability programs had similar results, only GeNorm values are interpreted and were further used to analyze expression of *P. meandrina* samples. To ensure that the most stable genes were not co-regulated, each of the highest ranked genes was removed alternately, and gene stability rank was reassessed. No changes in the order of reference gene rank stability were observed. As shown in Fig. 2, the reference genes with the lowest M values and highest expression stability in *P. meandrina* coral tissue were Brn1 and β-actin with M values of 0.2095 and 0.2775 respectively. 18S was the third most stably expressed gene with an M value of 0.2963. These genes were designated to have stable expression because the M<0.4 (Vandesompele et al. 2002).
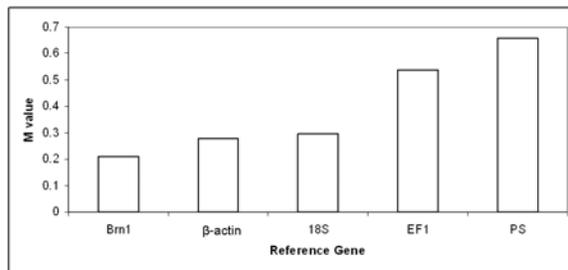

Figure 2: The M values (indices of gene expression instability) of candidate reference genes calculated from GeNorm analysis

Discussion

In this study, we sought suitable reference genes that could be used for future gene expression studies in the reef-building coral *P. meandrina* exposed to long term elevation of nitrogen followed by an acute increase in seawater temperature. Appropriate reference genes are necessary in gene expression studies to normalize sampling differences and identify the true expression differences in an experiment. In cases where no single normalization gene can be found it is best to use multiple genes to normalize data because the variation in multiple genes is less than the variation of a single gene (Andersen et al. 2004).

Several studies have compared different statistical programs to order candidate reference genes and found a similar ranking of stable reference genes within these programs (deBrouwer et al. 2006; Willems et al. 2006; Peltier and Latham 2008). Our study also compared the statistical programs GeNorm and NormFinder and found that candidate genes were ranked similarly by expression stability (Fig. 1).

The study described here shows that reference genes: Brn1, β-actin and 18S, are the most stably expressed in the coral, *P. meandrina*, under a long-term exposure to elevated nitrogen and an acute increase in sea water temperature (Fig. 2). A POU-III (acronym of Pit, Oct, Unc) class gene, Brn1, derived from a common eumetazoan ancestor, is expressed in the geosensory structures of vertebrates, insects and cnidarians (Erkman et al. 1996; Certel et al. 2000; Jacobs and Gates 2001; O'Brien and Degnan, 2002; O'Brien and Degnan 2003). The Brn1 gene is constitutively expressed at low levels in the cell most likely because of its conserved role in sensory cell development (O'Brien and Degnan 2002). The β-actin gene was the second most stably expressed gene in the set of reference genes (Fig. 2) and has been used as a reference gene for multiple cnidarian gene expression studies (Weis and Reynolds 1999; Reynolds et al. 2000; Rodriguez-Lanetty et al. 2007). β-actin encodes a ubiquitous cytoskeletal structural protein and is expressed at moderate levels in the cell. 18S rRNA, is in the small ribosomal subunit in most eukaryotes, and has been considered to be an ideal reference gene because rRNA is less likely to vary than mRNA under the same conditions (Bustin 2000). 18S rRNA as an internal control alone can be limiting because it may not always represent the overall cellular mRNA population (Suzuki et al. 2000). Although 18S ranked behind Brn1 and β-actin, this gene has shown to be constitutively expressed in many studies because rRNA synthesis is independent of mRNA synthesis (Franzellitti et al. 2005; Jorgensen et al. 2006; Tang et al. 2007).

Comparing the results of this study to previous qRT-PCR studies utilizing reference genes demonstrates the increased stability of a "new" reference gene, Brn1, which can possibly be used for stress experiments in reef-building corals alongside more popular reference genes like 18S and β-actin. Together, these reference genes can be used in future studies to normalize gene expression analysis of reef-building corals exposed to nutrient and temperature stressors.

References

Andersen C, Jensen J, Orntoft T (2004) Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. Cancer Research 64:5245-5250

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating Internal Controls for Quantitative Plant Gene Expression Studies. BMC Plant Biology 4:14

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Molec Endocrinol 25:169-193

Bustin SA, Nolan T (2004) Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. Journal of Biomolecular Techniques 15:155-166

Certel S, Clyne P, Carlson J, Johnson W (2000.) Regulation of central neuron synaptic targeting by the Drosophila POU protein, Acj6. Development 127: 2395-2405.

de Brouwer A, van Bokhoven H, Kremer H (2006) Comparison of 12 reference genes for normalization of gene expression levels in Epstein-Barr virus-transformed lymphoblastoid cell lines and fibroblasts. Molec Diagnost Therapeut 10:197-204

Downs, C.A., Mueller E., Phillips S., Fauth J.E., and Woodley C.M. (2000) A Molecular Biomarker System for Assessing the Health of Coral (*Montastraea faveolata*) During Heat Stress. Mar Biotechnol 2:533-544.

Erkman L, McEvilly R, Luo L, Ryan A, Hooshmand F, O'Connell S, Keithley E, Rapaport D, Ryan A, Rosenfeld M (1996) Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature 381:603-606

Etschmann B, Wilkcken B, Stovesand K, Von der Schulenberg A, Sterner-kock A (2006) Selection of Reference Genes for Quantitative Real-time PCR Analysis in Canine Mammary Tumors Using the GeNorm Algorithm. Vet Pathology 43:934-942

Franzellitti S, Fabbri E (2005) Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. Biochemistry and Biophysical Research Communications 336:1157-1163

Huggett J, Dheda K, Bustin SA, Zumla A (2005) REVIEW: Real-time RT-PCR normalisation; strategies and considerations. Genes and Immunity:1-6

Jacobs D, Gates R (2001) Is reproductive signalling antecedent to metazoan sensory and neural organisation? Am Zool 41:1482

Jorgenson S, Kleveland E, Grimbolt U, Gioen T (2006) Validation of Reference Genes for Real-Time Polymerase Chain Reaction Studies in Atlantic Salmon. Mar Biotechnol 8:398-408

Loram J, Boonham N, O'Toole P, Trapido-Rosenthal H, Douglas A (2007) Molecular Quantification of Symbiotic Dinoflagellate Algae of the Genus *Symbiodinium*. The Biological Bulletin 212:259-268

Maccoux L, Clements D, Salway F, Day P (2007) Identification of new reference genes for the normalisation of canine osteoarthritic joint transcripts from microarray data. BMC Molecular Biology 8:1-10

Mitchelmore CL, Schwarz JA, Weis VM (2002) Development of symbiosis- specific genes as biomarkers for the early detection of cnidarian-algal symbiosis breakdown. Mar Env Res 54:345-349

Nailis H, Coenye T, Nieuwerburgh F, Deforce D, Nelis H (2006) Development and evaluation of different normalization strategies for gene expression studies in Candida albicans biofilms by real-time PCR. BMC Molecular Biology 7:1-9

O'Brien E, Degnan B (2003) Expression of Pax258 in the gastropod statocyst: insights into the antiquity of metazoan geosensory organs. Evolution and Development 5:572-578

O'Brien EK, Degnan BM (2002) Developmental expression of a class IV POU gene in the gastropod Haliotis asinina supports a conserved role in sensory cell development in bilaterians. Dev. Genes Evol 212:394-398

Peltier H, Latham G (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: Identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA Published Online, 14:844-52

Pfaffl MW (2001) A new mathmatical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29:2000-2007

Reber-Mueller S, Studer R, Muller P, Yanze N, Schmid V (2001) Integrin and talin in the jellyfish Podocoryne carnea. Cell Biol Int 25:753-769

Reynolds WS, Schwarz JA, Weis VM (2000) Symbiosis-enhanced gene expression in cnidarians-algal associations: cloning and characterization of a cDNA, sym32, encoding a possible cell adhesion protein. Comparative Biochemistry and Physiology Part A 126:33-44

Rodriguez-Lanetty M, Phillips W, Dove S, Hoegh-Guldberg O, Weis V (2007) Analytical approach for selecting normalizing genes from a cDNA microarray platform to be used in q-RT-PCR assays: A cnidarian case study. Biochemistry and Biophysical Methods 70:985-991

Rodriguez-Lanetty M, Phillips W, Weis V (2006) Transcriptome analysis of a cnidarian – dinoflagellate mutualism reveals complex modulation of host gene expression. BMC Genomics 7: 10.1186/1471-2164-1187-1123

Suzuki T, Higgins P, Crawford D (2000) Control Selection for RNA Quantitation. BioTechniques 29:332-337

Szabo A, Perou C, Karaca M, Perreard L, Quackenbush J, Bernard P (2004) Statistical modeling for selecting housekeeper genes. Genome Biology 5:R59

Talapatra S, Wagner J, Thompson C (2002) Elongation factor-1 alpha is a selective regulator of growth factor withdrawl and ER stress-induced apoptosis. Cell Death Differ 9:856-861

Tang R, Dodd A, Lai D, McNabb W, Love D (2007) Validation of Zebrafish (Danio rerio) Reference Genes for Quantitative Real-time RT-PCR Normalization. Acta Biochim Biophys Sinica 39:384-390

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:1-11

Weis V, Reynolds W (1999) Carbonic Anhydrase Expression and Synthesis in the Sea Anemone *Anthopleura elegantissima* Are Enhanced by the Presence of Dinoflagellate Symbionts. Physiological and Biochemical Zoology 72:307-316

Willems E, Mateizel I, Kemp C, Cauffman G, Sermon K, Leyns L (2006) Selection of reference genes in mouse embryos and in differentiating human and mouse ES cells. Int. J. Dev. Biol 50:627-635

Wong M, Medrano J (2005) Real time PCR for mRNA quantitation. BioTechniques 39

Yanze N, Groger H, Muller P, Schmid V (1999) Reversible Inactivation of Cell Type Specific Regulatory and Structural Genes in Migrating Isolated Striated Muscle Cells of Jellyfish. Developmental Biology 213:194-201