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Validation of suitable reference genes for quantitative real-time PCR normalization in *Crassostrea gigas* spat stage during toxic dinoflagellates exposure

Validación de genes de referencia adecuados para la normalización de PCR cuantitativa en tiempo real de juveniles de *Crassostrea gigas* expuestos a dinoflagelados tóxicos

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ABSTRACT

The quantitative real-time polymerase chain reaction is a widely used method for gene expression analysis requiring carefully selected reference genes to ensure data validity. Regardless of several studies on gene expression bivalves and particularly *Crassostrea gigas*, has not been fully investigated regarding the evaluation of reference genes suitable for normalization of expression analysis. In this study, five candidate reference genes: actin, β tubulin, α subunit of elongation factor 1, glyceraldehyde-3-phosphate dehydrogenase and 28S ribosomal RNA were analyzed, to determine the most suitable reference genes, after of *Crassostrea gigas* spat were fed with *Gymnodinium catenatum* and *Prorocentrum lima* in mixed and compared to non-toxic diet *Isochrysis galbana*. The results showed that β-*tub* and *ef-1*α were the most stable genes for oysters feed with a mixed diet of *P. lima* and *I. galbana*. The *gapdh* and *28S rRNA* were the most stable genes for oysters feed with *G. catenatum* and *I. galbana*. In addition, the selection of optimal reference genes during dinoflagellates exposure was verified by analyzing the expression level of *trypsin* and *cytochrome c oxidase I* target genes. Our study could be beneficial for future studies on gene expression in *C. gigas*.

Keywords: Gene expression, reference gene, normalization, toxic dinoflagellates, *Crassostrea gigas*

RESUMEN

La reacción en cadena de la polimerasa cuantitativa en tiempo real es un método ampliamente utilizado para el análisis de expresión génica, requiere genes de referencia cuidadosamente seleccionados para garantizar la validez de los datos. A pesar de los estudios sobre expresión génica en bivalvos y particularmente en *Crassostrea gigas*, existe escasa información sobre la evaluación de los genes de referencia adecuados para la normalización del análisis de expresión. En este estudio se analizaron cinco genes candidatos de referencia: actina, β *tubulina, subunidad* α *del factor de elongación 1, gliceraldehído-3-fosfato deshidrogenasa* y *ARN ribosomal 28S*, para determinar los genes más adecuados, en juveniles de *Crassostrea gigas* alimentado con una dieta mixta de *Gymnodinium catenatum y Prorocentrum lima* en comparación con una dieta no tóxica de *Isochrysis galbana*. Los resultados mostraron que β *tub* y *ef1* α fueron los genes más estables para *C. gigas* alimentado con una dieta mixta de *I. galbana* y *P. lima*; los genes más estables para los ostiones alimentados con *I. galbana* y *G. catenatum* fueron *gapdh* y *28S rRNA*. Además, la selección de los genes de referencia durante la exposición a dinoflagelados tóxicos se verificó analizando el nivel de expresión de los genes blanco tripsina y citocromo c oxidasa I. El presente estudio será de gran utilidad para futuros estudios sobre análisis de expresión génica en juveniles de *C. gigas*.

Palabras clave: Expresión génica, genes de referencia, normalización, dinoflagelados tóxicos, *Crassostrea gigas*

INTRODUCTION

Mollusks aquaculture has significant economic importance worldwide and the Pacific oyster *Crassostrea gigas* is one of the most cultured oysters (Shumway, 1990). Recently, the research of *C. gigas* has been increased in topics such as immunology, genetics, genomics and hybrid tolerability (Saavedra and Bachére, 2006; Dheilly *et al*., 2011; Yan *et al*., 2017). The Pacific oyster could be exposed to bacterial, protozoan, and viral pathogens, as well as to abiotic agents such as xenobiotics, and marine toxins (Helm, 2004). Due to the stress caused by all these agents, there has been an increase in the research to study the changes of gene expression in oysters by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Quantitative PCR is a sensitive, specific and reproducible method to study gene expression (Bustin *et al*., 2005). For relative quantification is required some reference gene as the internal control, for example, actin and tubulin, though their expression before and after a challenge has been scarcely investigated (Dheda *et al*., 2005). Therefore, it is necessary to obtain information about the selection of reference genes to study gene stability in different experimental conditions.

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The Pfaffl method uses the Ct ($2^{\Delta\Delta ct}$) to determine the gene expression level in distinct conditions considering the amplification efficiency adjustment (Pfaffl *et al*., 2004). An essential component of the relative quantification of gene expression is the reference gene (internal control or housekeeping gene) that is used to correct the effect of experimental fluctuations, e.g., nucleic acid concentration, instrumental errors (Bustin, 2010).

Genomic studies allow a better understanding of the cellular response and specific metabolic pathways involved in response to the ingestion of the toxic dinoflagellate cells. It was necessary to determine the stability of possible reference genes for profiling the *C. gigas* spat gene expression on the experimental conditions tested.

In this work, transcriptional changes of five reference genes in *C. gigas* fed with toxic dinoflagellates *G. catenatum* and *P. lima* compared to a non-toxic diet of *I. galbana* were evaluated. The expression changes of the candidate genes were analyzed by three normalization algorithms: Bestkeeper (Pfaffl *et al*., 2004), GeNorm (Vandesompele *et al*., 2002; Bustin, 2010) and NormFinder (Andersen *et al*., 2004). These algorithms ranked reference genes according to their stability expression. Finally, we corroborate the validation of optimal reference genes by analyzing the relative expression of two target genes, *trypsin* and *cytochrome c oxidase I,* under the experimental conditions tested in *C. gigas* spat stage.

MATERIALS AND METHODS

Microalgae culture

In this study, the planktonic dinoflagellate *Gymnodinium catenatum* (strain GCCV6), a producer of paralyzing shellfish toxins (PST) (Kodama, 2010) was utilized, as well as, the epibenthic dinoflagellate *P. lima* (strain PRL-1) a producer of diarrheic toxins (DSP) (Núñez-Vázquez *et al*., 2003). Strains were obtained from the CIBNOR Collection of Marine Dinoflagellates, and grown in Fernbach flasks with f/2 medium (Guillard, 1975) enriched with selenium. The culture medium was prepared using seawater (salinity 35 g L-1) filtered with 0.45 µm membrane and sterilized at 121°C, 15 lb for 15 min. Cells were kept at proliferating conditions of 22°C ± 1°C, continuous light of 150 µmol photons m^{-2} s^{-1} light intensity. For experiments, dinoflagellate cells were harvested by centrifugation (2500 x g 10 min^{-1}) during the late exponential growth phase (19 days after inoculation), and cell density was adjusted using cell counting data on Sedgwick-Rafter slides (Microscope Olympus BX41, Tokyo, Japan) after sample fixation with Lugol´s solution (Gifford and Caron, 2000).

The microalgae *I. galbana* strain ISG-1 was obtained from the Live Food Laboratory at CIBNOR and used as a negative control or non-toxic diet. Cell concentrations in feeding experiments were determined by the Neubauer chamber (0.1 mm in depth) after fixation with Lugol´s solution with an optical microscope.

Oysters culture

C. gigas juvenile individuals (3 ± 1 mm, 0.022 ± 0.008 g) were obtained from the hatchery "Acuacultura Robles" at Las Botellas (Bahía Magdalena), Baja California Sur, Mexico. For acclimatization, organisms were divided into groups and kept in plastic aquariums (10 L) with aerated filtered seawater (0.22 µm) at 21°C at and 34 g L for ten days. The maintenance diet consisted of bacteria-free *I. galbana* (ISG-1) at 7.5 x 10^5 cells mL^{-1}.

Experimental design and sample collection

The number of dinoflagellate cells was calculated based on *G. catenatum* (Band-Schmitd *et al*., 2005) and *P. lima* (Hallegraeff, 1995) harmful algal blooms (HABs) and reports on their toxic effects. The experiments consisted of three diet treatments separated in two experimental conditions and non-toxic control diet; *condition 1*: oysters fed with an algal mix of *P. lima* (3 x 10^3 cell mL^{-1}) and *I. galbana* (7.5 x 10^5 cell mL^{-1}); *condition 2:* oysters fed with an algal mix of *G. catenatum* (3 x 10^3 cell mL^{-1}) and *I. galbana* (7.5 x 10^5 cell mL^{-1}). These diets were compared with a non-toxic control diet consisting of oysters fed with *I. galbana* alone (7.5 x 10^5 cell mL^{-1}). The experimental groups of *C. gigas* were distributed as described previously (García-Lagunas *et al*., 2013). Briefly, groups of 25 oysters (in triplicate) were maintained in 100 mL transparent polypropylene containers with a 1:1 the microalgal mixture in a final volume of 50 mL. At 7- and 14- days post-challenge, five organisms of each experimental unit were randomly sampled. The sampled organisms corresponding to each sample time were replaced on each experimental unit by oysters exposed in the same experimental conditions (mirror exposure units used for replacement only). Samples were washed with sterile seawater and finally frozen at -80°C until use.

Total RNA preparation and first-strand cDNA synthesis

Total RNA was extracted from 30 mg per the whole body of oysters spat in pools of five, with TRIzol® Reagent (Life Technologies, Carlsbad, California) according to the manufacturers' protocol. After homogenization, each sample was subjected to two TRIzol® extractions. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm in an ND-2000 spectrophotometer (Thermo Scientific, U.S.A). The RNA integrity was analyzed on 1 % (w/v) agarose gel. To ensure complete DNA absence, a direct PCR was performed using 2 µL (100 ng) of each RNA preparation with 28S ribosomal specific primers as a non-amplified control. After each verified RNA sample, 0.5 µg was used for cDNA synthesis using the SuperScript™ III First-Strand Synthesis System SuperMix® (Life Technologies, Carlsbad, California). Total RNA was reverse-transcribed by oligo-dT, and the resulting cDNA was stored at -80°C until use. Control reactions were performed with no cDNA template or with non-reverse transcribed RNA to determine the level of background DNA contamination after DNAase I treatment. No DNA contamination was detected.

Primer design and PCR efficiency

Five reference genes, actin (*act*), β tubulin (β-*tub*), α subunit of elongation factor 1 (*ef-1*α), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), and 28S ribosomal RNA (*28S rRNA*) were examined for their qPCR reference potential in the gene expression study in Pacific oysters exposed to dinoflagellates. The primers used to amplify these five genes (Table 1) were designed based on *C. gigas* genome sequences (Genebank, http://www.ncbi.nlm.nih.gov). The primer sequences were assessed for dimer and hairpin formation using RNA fold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

To test the primer efficiency (E), the standard curve method was used. For that, five-fold serial dilutions of cDNA of oysters exposed to dinoflagellates and control were subjected to standard qPCR protocol. Threshold Ct values and the logarithm of the target concentrations were plotted to calculate the slopes of the standard curves (Table 1). The amplification efficiency of qPCR was calculated using the equation: $E = 10\ [^{-1/slope} -1 \times 100)$, the acceptable E value was defined as between 95 and 100 % (Bustin, 2002).

Additionally, a validation analysis of candidate reference gene stability was performed for carefully selecting them to ensure data reliability. The data of Ct values were used to perform GeNorm, NormFinder and BestKeeper analysis.

Reference Gene Validation

The qPCR reactions were conducted in triplicate in holding Strip Tubes (0.1 mL) (Qiagen, Hilden, Germany), using a Rotor gene 6000 Real-Time PCR detection system (Qiagen, Hilden, Germany). A qPCR cocktail-mix was prepared with 2.5 mM $MgCl_2$, 2mM dNTP (each), 0.3 U of Platinum Taq polymerase (Invitrogen), 10 picomoles of each primer pair, and 20X EvaGreen fluorescent dye Biotium®, and 3.2 ng/µL of cDNA in a final volume of 15 µL. Amplification conditions were: 95°C for 5 min; 40 cycles of 95°C (60 s), 61°C (30 s) and 72°C (5s), acquiring the fluorescence at 79°C (1 s); finally a dissociation step from 65°C to 95°C (1°C/s) was done. For each candidate reference gene, the melt curve and gel picture were analyzed to verify the specificity of the amplified products and to confirm that at a single PCR product had been amplified. Amplification efficiencies were used for gene stability analyses of the potential set of reference genes (Table 1).

Statistical analysis

For GeNorm™ (Vandesompele *et al*., 2002; Pfaffl *et al*., 2004; Andersen *et al*., 2004) NormFinder and BestKeeper analysis the Ct were transformed to relative quantities using the formula (1+E-Δcq) (Livak and Schmittgen 2001). Ct values were transformed into a natural logarithm to analysis on NormFinder. No transformed Ct values are required for BestKeeper analysis.

The GeNorm algorithm first calculates an expression stability value (M) for each gene and then compares the pair-wise variation (V) of this gene with the others. Pairwise variation (V) between two sequential normalization factors containing an increasing number of genes (Vandesompele *et al*., 2002). NormFinder identifies stably expressed genes among a set of candidate normalization genes. The analysis produces a stability value for the examined genes, which is the variation calculation of the expression of the genes under different experimental conditions (Andersen *et al*., 2004). BestKeeper´s index calculates the geometric mean of those genes that are expressed with a standard deviation (SD) lower than 1 (Pfaffl *et al*., 2004). The web tool RefFinder (https://omictools.com/reffinder-tool) evaluates the gene rank from

Table 1. Primer sequences, gene name, expected amplicon size, and efficiency corresponding to reference genes and target gene (*tryp* and *coi*) from *Crassostrea gigas.*

Tabla 1. Secuencias de oligonucleótidos, nombre del gen, tamaño esperado del amplicón y eficiencia, correspondiente a los genes de referencia y a los genes blanco *(tryp* y *coi*) de *Crassostrea gigas*.

Primers	Sequence 5´-3´	Gene name	Amplicon (pb)	PCR Efficiency	R^2	Genbank access number	Gene function
Cg- *28s*-Fw Cg- *28s*-Rv	GGAGTCGGGTTGTTTGAGAATGC GTTCTTTTCAACTTTCCCTCACGG	*Ribosomal subunit 28s*	114	1.97	0.99	AY632555	Protein Ribosomal
Cg-*gapdh*-Fw Cg-*gapdh*-Rv	GTTCAAATATGATTCAACTCACGG TGGATCCCGTTCGCAATATACG	*Glyceraldehyde 3 phosphate dehydrogenase*	109	2.0	0.99	AJ544886	Catalyzation in glycolycosis
Cg-*tub* β -Fw Cg-*tub* β -Rv	AGCAGATGTCGTAGAGAGCTTC TGAACACATTCTCCGTTGTCCC	*Tubulin β*	144	1.96	0.99	CB617442	Microtubule component of the eukaryotic cytoskeleton
Cg-*act*-Fw Cg-*act*-Rv	TACTCTTTCACCACCACAGCCG TAGAGATGAGGATGAAGCAGCAG	*Actin (GlA)*	117	1.95	0.98	AF026063	Cell motility, structure, and integrity
Cg-*ef-1*α-Fw Cg-*ef-1*α-Rv	ACCATACAGTGAGGCTCGATTC GTGGAAGCCTCAATCATGTTATC	*Elongation factor1-a*	138	2.0	0.98	AB122066	Transcription factor
Cg-*tryp* Fw Cg-*tryp* Rv	GCCAGTGACGTAAGACAACTCG CAACCATCAACAACGACATTGCC	*Trypsin*	125	2.0	0.99	CB617494	Protein metabolism
Cg-*coi* Fw Cg-*coi* Rv	GTGTATGTCCTTATTCTTCCAGG GCACGCGTATCAATATCCATTCC	*cytochrome c oxidase I*	182	1.98	0.98	AB033687	Oxidative Metabolism

each program. The value assigned in RefFinder for each gene is used to calculate the geometric mean of their values for the overall final ranking. Thus, the lower value is assigned to the gene with more stable expression.

The $2^{-\Delta\Delta CT}$ method (Rozen *et al*., 2000; Bustin 2002) was used to normalize the relative expression of target genes trypsin (*tryp*) and cytochrome c oxidase (*coi*). The negative control non-toxic was used as the reference sample (calibrator). The statistic difference in gene expression was analyzed by one-way ANOVA; significant differences were obtained with the post hoc Fisher's multiple test comparison (α=0.05). All analyses were performed with Statistic 8.0® software (StatSoft, Tulsa, USA). Significant differences were set at $p < 0.05$.

RESULTS AND DISCUSSION

Primer specificity and amplification efficiency

The RT-qPCR assays had amplification efficiencies between 95% and 99%. The determination correlation coefficients (R^2) values were higher than 0.98 (Table 1). The presence of one peak in the melting curves was the criterion to assess the primer specificity. For all evaluated genes, no primer-dimer was detectable, confirming gene-specific amplification (Fig. 1). Also, the specific amplification was corroborated by agarose gel electrophoresis, for all expected PCR products, a single band had been observed. Ct values were distributed as indicated for *condition 1*: 21-26 (*28s-rRNA*); 23.11-26 (β-*tub*); 22-26 (*ef-1a)*; 23-30 (*gapdh*); 23-30 (*act*) (Fig. 2a); and for *condition 2*: 20-27 (*28S RNAr*); 22-34 (β-*tub*); 22-27 (*ef-1a);* 23-29 (*gapdh*); 23-31 (*act*) (Fig. 2b). However, for expression stability evaluation, a simple comparison of the raw Ct values for the candidate reference genes could not provide sufficient information. Thus, the following analysis was important for reference gene validation.

Quantitative gene expression analysis is reliable only when suitable reference genes are used for normalization. In this work, we demonstrate varied expression stabilities of five reference genes in Pacific oyster *C. gigas* spat stage during in vitro exposure to two species of toxic dinoflagellates, *G. catenatum* and *P. lima* in mixed or alone diets and compared to non-toxic diet (*I. galbana*), and thus highlighted the need to evaluate expression stability of reference genes for normalization.

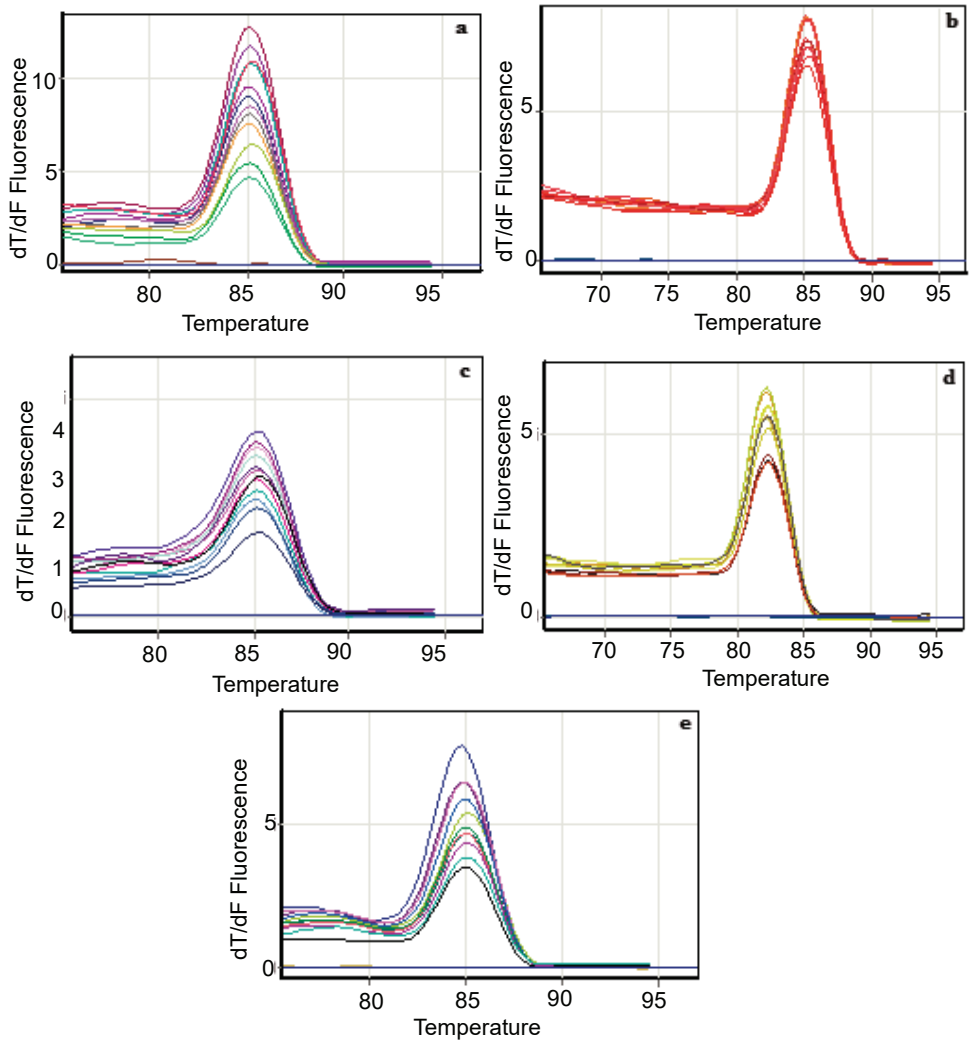

Figure 1. Specificity of five reference genes of *Crassostrea gigas* with single peaks in melting curves. **(a)** β tubulin (β tub), **(b)** α subunit of elongation factor 1 (*ef1-α*), **(c)** *actin* (*act*), **(d)** *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) and **(e)** 28S ribosomal RNA (28S r*RNA*).

Figura 1. Especificidad de cinco genes de referencia de *Crassostrea gigas* con un solo pico en las curvas de fusión. **(a)** β *tubulina (*β *tub),* **(b)** *subunidad* α *del factor de elongación 1 (ef1-α),* **(c)** *actina (act),* (d) *gliceraldehído-3-fosfato deshidrogenasa (gapdh)* y **(e)** *28S del ARN ribosomal (ARNr 28S).*

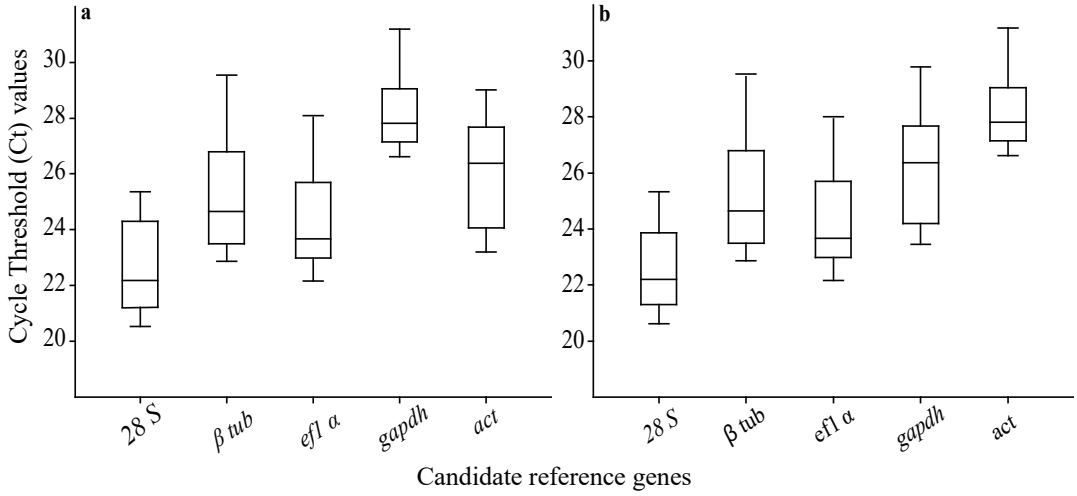

Figure 2. The threshold cycle values of the candidate reference genes. Algal mix *Prorocentrum lima* and *Isochrysis galbana* (a) algal mix *Gymnodinium catenatum* and *Isochrysis galbana* (b). Lines across the Box plot graph of Ct value represent the median values. Lower and upper boxes show the 25th percentile to the 75th percentile. Whiskers represent the maximum and minimum values.

Figura 2. Valores del ciclo umbral de los genes de referencia candidatos. Dieta mezclada de las algas *Prorocentrum lima* e *Isochrysis galbana* (a) dieta mezclada de las algas *Gymnodinium catenatum* e *Isochrysis galbana* (b). Las líneas del diagrama de caja de los valores Ct representan los valores medios. Los cuadros inferior y superior muestran el percentil 25 al percentil 75. Los bigotes representan los valores máximos y mínimos.

Evaluation of candidate reference genes

The qPCR is considered to be an effective and accurate technique used to examine gene transcription patterns in different experimental conditions. To normalize the expression of the target genes, a reference gene is required, as well as to integrate experimental variations (Livak and Schmittgen, 2001; Bustin and Nolan, 2004). Thus, studying the expression stability of these genes is recommended before quantifying genes of interest (Vandesompele *et al*., 2002; Bustin and Nolan, 2004). Programs such as GeNorm, NormFinder, and BestKeeper have been successfully employed to determine the stability and identify the most suitable reference gene (Bustin, 2010). However, the use of a single validated gene is common in the qPCR normalization in bivalves (Volland *et al*., 2017). Due to some reported reference genes can vary transcriptionally under different biological and experimental factors (Mello *et al*., 2012; Feng *et al*., 2013), the appropriate selection of reference genes is essential to avoid false-positive results that may lead to misinterpretations and imprecise conclusions (Nascimento *et al*., 2015).

The order of stability of genes showed by GeNorm analysis was *ef-1*α, β-*tub*, *act*, *gapdh*, and *28S rRNA* (Fig. 3a) in *condition 1*. GeNorm software generates the pairwise variation V value (Vn/Vn+1) with 0.150 cut-off value proposed. The addition a reference gene is not required below the cut-off. The V2/3 and V3/4 values were 0.05 and 0.085 (Fig. 3b), respectively. The results of the analysis suggested three housekeeping genes as normalization factors.

GeNorm analysis showed that *gapdh* and *28S rRNA* genes were the most stable (Fig. 3c) in diets of *condition 2*. The optimal number of genes required for accurate normalization was two. The V2/3 value was 0.11; this value did not increase significantly by incorporating the third gene, so the addition of a third reference gene was not necessary. The expression analyses showed that included two or three internal genes; the values did not change significantly (p=0.05) (Fig. 3d).

The stability analysis with NormFinder was consistent with GeNorm, where the best combination was also *28S rRNA* and *gapdh* for *condition 2* data (Table 2a). The results with NormFinder showed that the best combination was the β-*tub* and *act* gene in the mixed diet with *P. lima* (3 x 10^3 cell mL^{-1}) *condition 1* (Table 2b). These results were consistent partially with GeNorm only for β-*tub* gene. NormFinder showed the *act* gene as the second most stable, whereas in the GeNorm analysis, this gene was the third most stable. Therefore, *act* was regarded as the third gene to be added for the relative expression analysis to data.

The BestKeeper index showed that the most stable genes were *ef-1*α and β-*tub*, for *condition 1* data (Table 3a), which are inconsistent with GeNorm and NormFinder. This analysis also showed that the *gapdh* gene was the third most stable, thus agreeing with GeNorm. Consequently, we decided to use the two most stable genes showed by GeNorm and NormFinder to perform the reference gene validation for relative expression analysis for this condition. The BestKeeper index agreement with GeNorm and NormFinder, where β-*tub* was the most stable gene for *condition 1* data (Table 3b).

Our results suggest that β-*tub*/*ef-1*α represents a solid combination of internal controls for qRT-PCR normalization in the Pacific oyster spat stage exposed to toxic *Prorocentrum lima* and combination of *28S rRNA*/*gapdh* represent the most reliable internal controls for oysters exposed to toxic *Gymnodinium catenatum*. Based on our results, we recommend the use of this combination of reference genes for the experimental *conditions 1* and *2* (Fig. 3).

Table 2. NormFinder stability values for reference genes. An ideal reference gene has inter-group variation as close to zero. *Crassostrea gigas* exposure to mixed diet of *Gymnodinium catenatum* and *Isochrysis galbana* (a), mixed diet of *Prorocentrum lima* and *Isochrysis galbana* (b).
Tabla 2. Valores de estabilidad NormFinder para los genes de referencia. Un gen de referencia ideal tiene una variación entre grupos lo más cercano a cero. Exposición de *Crassostrea gigas* a una dieta mezclada de *Isochrysis galbana* y *Gymnodinium catenatum* (a) y una dieta mezclada de *Isochrysis galbana* y *Prorocentrum lima* (b).

Gene name	NormFinder Stability Value (*p*) Intergroup variation	
	(a)	(b)
28S	0.012	0.012
β *tub*	0.011*	0.007*
ef1 α	0.014	0.009
gapdh	0.008*	0.010*
act	0.013	0.008*
Best pair	0.007	0.003

Stability value for best combination of two genes (best pair). * Best combination of two genes

Table 3. Correlation coefficient with the BestKeeper index of *Crassostrea gigas* fed with mixed diet *Gymnodinium catenatum* and *Isochrysis galbana* (a) *Prorocentrum lima* and *Isochrysis galbana* (b).
Tabla 3. Coeficiente de correlación con el índice BestKeeper de *Crassostrea gigas* alimentado con una dieta mezclada de *Gymnodinium catenatum* e *Isochrysis galbana* (a) y una dieta mezclada de *Prorocentrum lima* e *Isochrysis galbana* (b).

Gene name	28S	β tub	ef1 α	Gapdh	Act
(a) Coefficient of Correlation (*r*)	0.86	0.98*	0.99*	0.96*	0.93
(b) Coefficient of Correlation (*r*)	0.89*	0.978*	0.86	0.94*	0.88
p Value	0.001	0.001	0.001	0.001	0.001

The coefficient of correlation (r) and the p-value measure the correlation between each gene and the *BestKeeper* index. *Most stable Genes

In bivalves mollusk, several genes including *ef-1α*, *act*, β-*tub*, *28S rRNA* and *gapdh* are frequently used as reference genes for qPCR analysis, under exposure to marine toxins in experimental conditions (Mello *et al*., 2012; Feng *et al*., 2013; Volland *et al*., 2017; Martínez-Escauriaza *et al*., 2018). In our study, these five available candidate reference genes were selected, and their expression was monitored to revise their stability by real time quantitative PCR during in vitro exposure of *C. gigas* spat to the toxic dinoflagellates. The selected reference genes are involved in ribosomal metabolism (*28S rRNA*), cytoskeleton structure (*act*, β-*tub*), catalyzation in glycolysis (*gapdh*), and elongation process in protein synthesis (*ef-1α*). The three algorithms allowed ranked candidate reference genes according to their expression stability, showing agreement in the results (Fig. 3). The analysis provided a consensus among the three algorithms in samples of the first condition where the β-*tub* gene appeared as the most suitable reference gene by NormFinder; the second most stable observed was *ef-1α* gene by GeNorm, *act* gene by NormFinder, and *gapdh;* the third more stable gene was *act*, which is commonly used as an internal standard for the normalization of gene expression (Fig. 3). GeNorm and NormFinder also showed approaches concordant in samples of the second condition indicating the pair *28S rRNA*/*gapdh* as the best combination of reference genes (Fig. 3).

Ef-1α is a eukaryotic translational elongation factor, which plays a key role in protein translation (Browne and Proud, 2002; Ejiri, 2002). It has been reported the use of *ef-1α* gene as the best reference gene in soft-shell clams, *M. arenaria* (Siah *et al*., 2008), as well as an internal control in studies of OsHV-1 infection in adult oysters (Renault *et al*., 2011). However, it is not suitable for use in oyster larvae (Du *et al*., 2013). In this study, *ef-1*α was one of the most stable genes, so it was used as a reference gene in the oysters spat exposed to *G. catenatum*.

The β-*tub* and *act* gene coding for cytoskeletal proteins, which are involved in structure playing a pivotal role in phagocytosis and encapsulation (Martin *et al*., 2010). In fact, β-*tub* has been used extensively as a reference standard, encloses recent studies in oysters (Zhang *et al*., 2011). We found this gene as one of the most stable, so it was used as a reference gene under our experimental condition (*Condition 1*).

In mollusks studies, *act* gene has been frequently used in RT-qPCR assays (Martin *et al*., 2010; Zhang *et al*., 2011; Du *et*

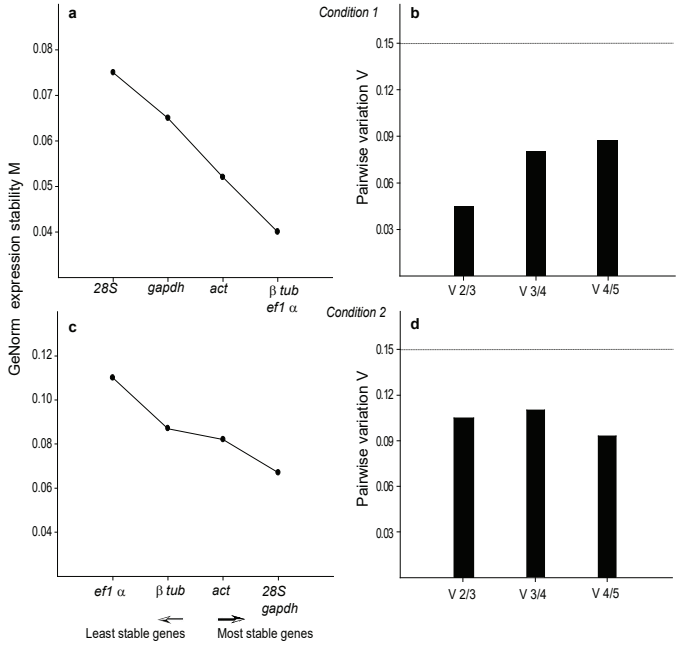

Figure 3. Gene expression stability (M) calculated by GeNorm software and pairwise variation analysis in oysters exposed to algal mix of *Prorocentrum lima* and *Isochrysis galbana* (a, b), algal mix of *Gymnodinium catenatum* and *Isochrysis galbana* (c, d). Bar values indicate change magnitude in the normalization factor after the inclusion of an additional reference gene.
Figura 3. Estabilidad de la expresión génica (M) calculada por el software GeNorm y análisis de variación por pares en ostiones expuestos a una dieta mezclada de las algas de *Prorocentrum lima* e *Isochrysis galbana* (a, b) y una dieta mezclada de las algas de *Gymnodinium catenatum* e *Isochrysis galbana* (c, d). Los valores de la barra indican la magnitud del cambio en el factor de normalización después de la inclusión de un gen de referencia adicional.

al., 2013; Martínez-Escauriaza *et al*., 2018). The findings of the present work showed changes in the expression level of the *act* gene for the presence of both species of toxic dinoflagellates, which suggest *act* is an unstable gene.

The *28S rRNA* gene, is chosen extensively as a reference gene and frequently is used in mollusks bivalves (Cellura *et al*., 2007; Zhang *et al*., 2011; Volland *et al*., 2017). The *28S rRNA* has been reported as an unstable gene when hemocytes of soft-shell clams, *M. arenaria*, were challenged with *Vibrio splendidus* (Araya *et al*., 2008). However, we found this gene as one of the most stable, so it was used as a reference gene under our experimental condition (*Condition 2*).

The *gapdh* gene is frequently used as a reference gene in humans, plants, and invertebrates such bivalves; it has also been a choice for normalization in experimental approaches of bivalve tissues such as gills, digestive gland (Sussarellu *et al*., 2012; Martínez-Escauriaza *et al*., 2018). This gene expression was stable in our experiment only for *condition 2*, so it was suitable for the reference genes.

It has been widely reported the use of *ef-1α*, *act*, β-*tub*, *18S*, *28S*, and *gapdh* genes as references genes in bivalves as *Ostrea edulis* (Feng *et al*., 2013), scallops (Morga *et al*., 2010), *Mytilus* spp. or *C. gigas* in both experimental and field studies (Nuñez-Acuña *et al*., 2013; Della Torre *et al*., 2013; Lacroix *et al*., 2014), clam *Ruditapes philippinarum* (Volland *et al*., 2017) oysters *Crassostrea sikamea* and *Crassostrea angulate* (Yang *et al*., 2017) and *Mytilus galloprovincialis* (Martínez-Escauriaza *et al*., 2018) both in tissues (gonads, gills, and digestive gland) and hemocyte. However, Du *et al*. (2013), reports that none of these five common reference genes were stables due to their high variability with the experimental conditions tested, suggesting they were unsuitable as internal controls. However, our results confirmed that traditional reference genes commonly used, such as β-*tub*, *ef-1α*, *28S rRNA*, and *gapdh* showed the highest stability, when oysters spat were exposed to toxic dinoflagellates (*condition 1 and 2*).

Reference gene validation to normalize gene expression

To evaluate the suitability and performance of the reference genes established by the three software tools, the expressions of *trypsin (tryp)* and *cytochrome C oxidase* (*COI*) genes in oysters after challenge with the two toxic dinoflagellate species and compared with a calibrator (nontoxic control group) were measured. By relative qPCR, the expression pattern of target genes was evaluated. For normalization, the reference genes selected for each condition were used. From the three software, the most stable reference genes selected had a correlation coefficient of R2>0.98 (Table 1). Based on our experiments, β-*tub* and *ef-1*α were the most stable reference genes for the Pacific oyster spat stage exposed to toxic *P. lima*. On another hand, *28S rRNA* and *gapdh* were the most stable reference genes for Pacific oyster spat exposed to toxic *G. catenatum*. Therefore, the combined use of these genes was utilized as internal controls for accurate normalization of qPCR. Expression profiles of *tryp* and *coi* vary substantially between normalization strategies. These genes were affected by exposure to the toxic dinoflagellates; we found significant differences in the expression levels of genes in oysters challenged in both conditions ($p < 0.05$) compared with oysters fed with the non-toxic control (Fig. 4).

Expression profiles of *tryp* and *coi* genes vary substantially between normalization strategies (Fig. 4). The results of this work point out to validate the reference candidate genes for different experimental conditions; also, we suggest to applied a minimum of two statistical algorithms. It is recommended to perform a complete analysis with the conditions tested in *C. gigas* adults and in different tissues to evaluate the level of expression by tissue, due to the expression of reported reference genes changes under experimental conditions.

CONCLUSIONS

Based on the results, we recommend the combination of β-*tub* and *ef-1α* as reference genes for the normalization in *Crassostrea gigas* fed a mixed diet of *I. galbana* and *P. lima*, whereas *28S rRNA* and *gapdh* was best for oysters fed with *I. galbana* and *G. catenatum*. The combined use of these genes as optimal reference genes will be useful for data normalizing when studying expression levels in oysters spat stage exposed to these dinoflagellates, providing more reliable and accurate expression measurements, to improve the current knowledge about the effect of dinoflagellate DPS and PSP toxins producers on the Pacific oyster *C. gigas*. Additionally, were demonstrated the relevance of evaluating the stability of reference genes statistically because each experimental condition used to change the gene expression.

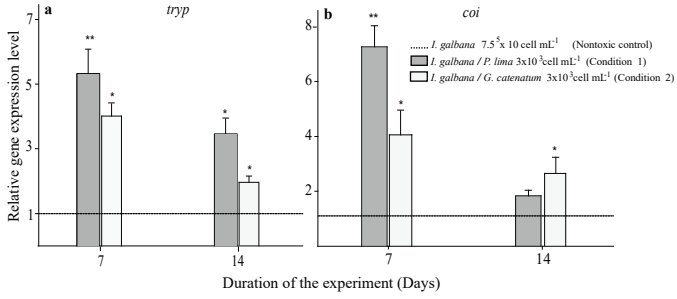

Figure 4. Relative expression of *tryp* (a) and *coi* (b) using selected reference genes β tub and *ef1α* (*condition 1*); *28S rRNA* and *gapdh* **(***condition 2***).** The bars represent standard error. The asterisks mean significant differences between treatment and control group ($p < 0.05$ in Fisher´s LSD).
Figura 4. Expresión relativa de *tryp* (a) y *coi* (b) usando los genes de referencia seleccionados β *tub* y *ef1*α (condición 1); ARNr *28S* y *gapdh* (condición 2). Las barras representan el error estándar. Los asteriscos significan diferencias significativas entre el grupo control y los tratamientos ($p < 0.05$ en LSD de Fisher).

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