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Evaluation and validation of reference genes for qPCR analysis to study climate change-induced stresses in *Sinularia* cf. *cruciata* (Octocorallia: Alcyonidae)

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ABSTRACT

Coral reef organisms, including octocorals, are facing the consequences of anthropogenic activities, such as increasing oceanic pH and sea surface temperature, threatening their long-term survival and well-being. Gene expression studies based on quantitative PCR (qPCR) are important tools to provide insight into the molecular basis of octocoral stress responses and their potential resilience mechanisms. Nevertheless, a lack of experimentally validated, stably expressed reference genes for the normalization of gene expression using quantitative reverse transcriptase PCR (qPCR) method limit such investigations among octocorals. Here, assessment of the expression stability of seven candidate reference genes was performed using a palette of statistical tools for valid qPCR-based gene expression studies on the octocoral *Sinularia* cf. *cruciata* during thermal (34 °C) and low-pH (pH 7.5) stress and the most suitable set of reference genes was determined for such experiments. The reliability of the selected reference genes was confirmed in a qPCR assay that targeted the heat shock protein 70 (*HSP70*) gene. The *HSP70* gene was found to be significantly upregulated during thermal stress, whereas during low-pH stress the expression level of this gene decreased. This study provides experimentally validated stress-specific sets of stably expressed reference genes during climate change-induced stresses, which will benefit future gene expression studies on *Sinularia* cf. *cruciata* as well as other octocorals. These results also highlight potentially different acclimation strategies of octocorals to different sources of abiotic stresses, contributing to our understanding of the potential for the adaptation of coral reef organisms to a changing world.

1. Introduction

Coral-reefs consist of hard corals, soft corals, and the other flora and fauna associated with them and are popularly referred to as the rainforests of the oceans by virtue of the vast diversity of organisms they host and the goods and services they provide to mankind (Moberg and Folke, 1999). However, the rise in atmospheric CO_2 due to increasing anthropogenic activities and the consequent rise in the seawater temperature and reduction in oceanic pH have been reported to result in a collapse of coral-dinoflagellate symbiosis (Weis, 2008) and decreased calcification as well as growth (Marubini et al., 2008). The apparent increase in oxidative stress at the cellular level (Lesser, 2006) leads to apoptosis and necrosis (Richier et al., 2006; Tchernov et al., 2011), and thus, severely affects coral health (Lough, 2008), ultimately causing partial or complete colony mortality and mass bleaching events (Brown, 1997; Hughes et al., 2003). To gain a deeper understanding of the transcriptional response of corals to different sources of abiotic stress and the resulting adverse effects and/or potential resilience, different tools for gene expression profiling have been utilized (Lõhelaid et al., 2014; Maor-Landaw et al., 2014; Pratlong et al., 2015). Among them, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), or simply qPCR, is a reliable, reproducible and highly sensitive tool for the quantification of selected mRNA transcript levels. This is the method of choice for pinpointing and validating the regulation of specific genes under specific conditions with precision in a cost-effective manner (Fink et al., 1998; Gibson et al., 1996; Heid et al., 1996; Higuchi et al., 1993; Schmittgen et al., 2000).

Despite the power and general applicability, accurate expression profiling using qPCR is influenced by several factors such as RNA quality, stability and purity, reverse transcription efficiency, and amplification efficiency (Vandesompele et al., 2002). These sources of variation

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make normalization of the samples, which may have been obtained from different sources, time-points and individuals, necessary (Radonić et al., 2004). A commonly used normalization strategy involves assessing changes in the expression of target genes relative to one or more internal control genes also called the reference genes (RGs) (Freeman et al., 1999). The usefulness of the qPCR technique, however, heavily relies on these internal/endogenous control genes, which presumably are stably expressed even during specific experimental treatments (Bustin, 2002; Vandesompele et al., 2002). Housekeeping genes (HKGs) are typical RGs due to their constitutive expression in different tissues and under different conditions. However, not all HKGs are free from the influence of the experimental conditions and their expression levels may vary depending on the nature and extent of the treatments, leading to erroneous expression estimates of the genes of interest (Dheda et al., 2005). The use of inappropriate RGs, i.e., genes that may be influenced by the treatment, result in erroneous estimates of target transcript levels and loss or gain of statistical significance (Ferguson et al., 2010), and thus, adversely influence the outcome and conclusion of a study (Dheda et al., 2004; Dheda et al., 2005). Hence, an important consideration for the successful quantification of gene expression using qPCR is the selection of the reference(s) to be used for normalization. Consequently, proper evaluation and validation of a specific set of the most stably expressed RGs is a prerequisite for any gene expression study to avoid biases in determining target gene expression and to obtain an accurate and reliable estimation of the changes induced by the experimental treatments (Bustin et al., 2009; Guénin et al., 2009; Kozera and Rapacz, 2013; Vandesompele et al., 2002).

Only a few published studies on the members of phylum Cnidaria carried out analyses of gene expression to deal specifically with a systematic validation of internal control genes used (Pagarigan and Takabayashi, 2008; Rodriguez-Lanetty et al., 2008). Most other studies either utilized several different internal control genes or those recommended in the above-mentioned publications (DeSalvo et al., 2008; Meyer et al., 2011; Meyer et al., 2009). Moreover, among cnidarians, a strong emphasis has been placed on scleractinian (stony) coral (Hexacorallia, Scleractinia) gene expression profiling while there is still a lack of information on experimentally validated, stably expressed internal control genes for other cnidarians, such as those from the subclass Octocorallia.

Octocorals (soft corals), a dominant benthic component of many coral reefs, are important constituents of a healthy reef ecosystem. The genus *Sinularia* is among the most widespread zooxanthellate soft coral belonging to the Octocorallia. These corals occur abundantly in the Indo-Pacific where they are one of the most dominant species of large ecological importance (Benayahu and Loya, 1977). Moreover, the members of this genus are pharmaceutically important because they produce a wide range of bioactive metabolites (Aceret et al., 1998; Ahmed et al., 2006; Chen et al., 2015; Lakshmi and Kumar, 2009; Yang et al., 2013) that exhibit several different biological activities (Aceret et al., 1998; Li et al., 2005; Su et al., 2008). However, the ecological dominance of the *Sinularia* species is challenged by coral-bleaching events, where up to a 90% mortality rate has been recorded (Fabricius, 1995; Loya et al., 2001).

If we aim to better understand the response and potential for the resilience of these organisms to climate change at the transcriptional level, we must determine a properly validated abiotic stress-specific set of RGs that can be used to accurately determine the effect of various abiotic stressors at the expression level using qPCR for the members of the subclass Octocorallia. Hence, the expression stability of seven genes during thermal and low-pH stress, two important climate change-induced threats to coral reef organisms, was evaluated here in octocoral *Sinularia* cf. *cruciata* using five different statistical methods, and their suitability as endogenous RGs for the relative gene expression quantification in qPCR assays was assessed using the *HSP70* gene as a case in point. By investigating the best normalizing gene combination for each stress-type as well as the stress-specific differential expression of the *HSP70* gene, this study aims to provide a basis for future gene expression studies on the genus *Sinularia* and other octocorals to gain a better mechanistic understanding of octocoral stress responses and their future in the changing oceans.

2. Materials and methods

2.1. Coral collection and maintenance

All of the corals used in this study were obtained from a commercial supplier of aquarium organisms and maintained in a closed circuit seawater aquarium under controlled conditions (temperature 25 ± 1 °C, pH 8.2 ± 0.1). Every week, half of the seawater was changed with fresh artificial seawater. All of the corals were maintained on a 12 h light/12 h dark light-regime provided by LED lights (GHL Mitras LX 6200-HV) at a light intensity of 14 ± 2 kLux. A similar light regime was used for both the control and the experimental system mentioned below.

2.2. Experimental design

To determine the effect of rising seawater temperature and decreasing pH on the gene expression stability of selected candidate genes, nubbins of *Sinularia* cf. *cruciata* growing independently for ≥6 months but derived from single parent colony were exposed to these conditions (see below). All of the experiments were performed in biological and technical triplicates, and the controls and the treated samples were snap frozen in liquid nitrogen and subsequently stored at −80 °C until RNA extraction.

2.2.1. Thermal stress experiment

Three *Sinularia* nubbins of similar size (approx. 4 × 4 cm) were placed in an experimental 10 l tank exposed to acute thermal stress. The temperature in the experimental tank was raised gradually from 26 °C to 34 °C over a period of 2 h after which it was maintained at 34 °C for a subsequent 6 h. Three control nubbins were maintained in a similar tank as the experimental tank but the temperature was kept at 26 °C during the entire course of the experiment. Strychar et al. (2005) observed a mortality of *Sinularia* sp. within 24 h upon exposure to the 34 °C temperature. Here, *Sinularia* cf. *cruciata* was therefore exposed to 34 °C for only 6 h to understand the short-term acute thermal stress response.

2.2.2. Low-pH stress

The rise in carbon dioxide emissions is leading to a lowered oceanic pH apparent from a reported decreased pH of 0.1 units since the pre-industrial era and is predicted to further decrease by another 0.4 units by the end of this century (Haugan and Drange, 1996; Kleypas and Langdon, 2013; Orr et al., 2005; Solomon et al., 2007). Based on these report, to understand the effect low-pH, three *Sinularia* nubbins were subjected to lowered seawater pH by pumping carbon dioxide (CO_2) into the experimental tank to maintain a stable low pH value of 7.5 units. The pH was first reduced to a value of 7.5 over a period of 2 h and then maintained at this value for 24 h. The pH value was recorded throughout the experiment and was observed to be constant at 7.5. The corals were sampled after this 24 h period. The control nubbins were maintained under a normal pH of 8.2, and the temperature in both of the tanks was kept constant at 26 °C.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from the control as well as the treated samples (200 ± 10 mg tissue) using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. Contaminating DNA was eliminated from the RNA extracts upon using RQ RNase-free DNase