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Research paper

Reference gene selection for quantitative gene expression studies during biological invasions: A test on multiple genes and tissues in a model ascidian *Ciona savignyi*

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ABSTRACT

As invasive species have successfully colonized a wide range of dramatically different local environments, they offer a good opportunity to study interactions between species and rapidly changing environments. Gene expression represents one of the primary and crucial mechanisms for rapid adaptation to local environments. Here, we aim to select reference genes for quantitative gene expression analysis based on quantitative Real-Time PCR (qRT-PCR) for a model invasive ascidian, *Ciona savignyi*. We analyzed the stability of ten candidate reference genes in three tissues (siphon, pharynx and intestine) under two key environmental stresses (temperature and salinity) in the marine realm based on three programs (geNorm, NormFinder and delta Ct method). Our results demonstrated only minor difference for stability rankings among the three methods. The use of different single reference gene might influence the data interpretation, while multiple reference genes could minimize possible errors. Therefore, reference gene combinations were recommended for different tissues - the optimal reference gene combination for siphon was *RPS15* and *RPL17* under temperature stress, and *RPL17*, *UBQ* and *TubA* under salinity treatment; for pharynx, *TubB*, *TubA* and *RPL17* were the most stable genes under temperature stress, while *TubB*, *TubA* and *UBQ* were the best under salinity stress; for intestine, *UBQ*, *RPS15* and *RPL17* were the most reliable reference genes under both treatments. Our results suggest that the necessity of selection and test of reference genes for different tissues under varying environmental stresses. The results obtained here are expected to reveal mechanisms of gene expression-mediated invasion success using *C. savignyi* as a model species.

1. Introduction

The rapidly changing environments, directly and/or indirectly caused by human activities, have exposed organisms to a series of environmental stresses (de Nadal et al., 2011). In response to these stresses, species have to use available and/or evolve various strategies to increase their adaptability to rapidly changing local environments. Recent studies have attempted to elucidate the strategies responsible for various abiotic stresses in plants such as hybrids of *Hordeum vulgare* L. x *Saccharum* spp. (Janska et al., 2013; Guo et al., 2014), fish such as *Pangasianodon hypophthalmus* and *Pagothenia borchgrevinki* (Thanh et al., 2014; Bilyk and Cheng, 2014), and molluscs such as *Mytilus galloprovincialis* (Lockwood et al., 2010; Lockwood and Somero, 2011) and *Pomacea canaliculata* (Sun et al., 2012). As invasive species rapidly spread and successfully colonized throughout varying environments, they offer a great opportunity to study the causes and consequences of adaptability to rapidly changing environments.

Among many factors such as behavioral, biochemical, physiological, and genetic/epigenetic ones (Boyko and Kovalchuk, 2008; Gleason and Burton, 2013), gene expression changes represent one crucial mechanism for species to cope with environmental stressors during biological invasions (de Nadal et al., 2011). For instance, the transcriptional expression of individual candidate genes (such as the heat shock protein 70 gene) showed different patterns between invasive and native species in response to heat stress, suggesting that a higher level of heat tolerance of invasive species might contribute to their invasion success (Henkel and Hofmann, 2007; Zerebecki and Sorte, 2011).

Among many methods for analyzing gene expression such as subtractive hybridization, cDNA microarray and RNA-sequencing

Abbreviations: *ACT*, actin; bp, base pair(s); cDNA, DNA complementary to RNA; Ct, threshold cycle; *CYP*, cyclophilin; *EF1*, elongation factor 1 alpha; *GADPH*, glyceraldehyde-3-phosphate dehydrogenase; *HIS*, histone H3; *Hsp70*, heat shock protein 70; mRNA, messenger RNA; oligo, oligodeoxyribonucleotide; *NKA*, sodium/potassium-transporting ATPase subunit alpha-3-like genes; *TubA*, tubulin alpha chain-like; *TubB*, tubulin beta chain-like; *UBQ*, ubiquitin conjugating enzyme; qRT-PCR, quantitative real-time PCR; *RPS15*, ribosomal protein S15; *RPL17*, ribosomal protein L17; SD, standard deviation; Δ, delta.

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(RNA-seq), quantitative Real-Time PCR (qRT-PCR) is a commonly used one for quantitative gene expression studies, mainly due to its advantages of sensitivity and specificity (Bustin, 2002; Valasek and Repa, 2005). Despite that microarray and RNA-seq assays have become popular high-throughput methods, the data obtained based on these methods may still need to be further confirmed by qRT-PCR (Yao et al., 2011; Wei et al., 2014). When qRT-PCR is employed to compare gene expression patterns among samples, the accuracy and reliability are determined by several factors such as sample-to-sample variations, initial sample amount, RNA integrity, cDNA quality, PCR amplification efficiency and the presence of inhibitor (Radonić et al., 2004). The most common strategy to normalize qRT-PCR data is the use of reference genes (also known as internal controls). Reference genes, usually selected out of housekeeping genes, are expected to express adequately and constantly independent of tissue types, developmental stages and experimental treatments (Wong and Medrano, 2005). Indeed, such ideal genes do not commonly exist in various tissues, at different developmental stages, or across different experimental designs (Nicot et al., 2005). Consequently, the selection and further evaluation of reference genes are critical to assure the accuracy of qRT-PCR results (Guo et al., 2014).

The *Ciona* ascidians have become promising models in numerous disciplines of biology, such as *Ciona intestinalis* and *Ciona savignyi* for invasion biology (Zhan et al., 2010, 2015; Procaccini et al., 2011). The Pacific transparent ascidian *C. savignyi*, which is considered native to Japan and possibly northern Asia, has widely spread to the Pacific coast of North America, Atlantic coast of Argentina and even Southern Hemisphere such as New Zealand (Hoshino and Nishikawa, 1985; Lambert and Lambert, 1998; Zvyagintsev et al., 2007; Smith et al., 2010). Among many abiotic factors in invaded ranges of *C. savignyi*, temperature and salinity represent two critical ones (see review by Zhan et al., 2015). Many marine invasive species have to cross the barriers formed by temperature and salinity to successfully colonize local habitats. In addition, the fully sequenced and annotated genome of *C. savignyi* provides abundant gene resources, thus facilitating the research on molecular mechanisms responsible for abiotic stresses (Vinson et al., 2005; Serafini et al., 2011).

In this study, in order to facilitate quantitative gene expression analysis in response to two major environmental factors in the marine realm (i.e. temperature and salinity), we conducted temperature and salinity stress experiments on *C. savignyi*, and analyzed the stability of ten housekeeping genes in three major tissues (siphon, pharynx and intestine). We aim to select and validate stable reference genes for the three major tissues under the two important abiotic stresses. In addition, we quantitatively measured the relative expression of the heat shock protein 70 gene (*Hsp70*) under temperature stress and sodium/potassium-transporting ATPase subunit alpha-3-like gene (*NKA*) under salinity stress using the recommended reference genes.

2. Materials and methods

2.1. Biological materials

Adults of *C. savignyi* (length 4.19 ± 1.13 cm) were collected from the coast of Dalian, Liaoning Province, China (38°49′13″N, 121°24′20″E) on October, 2014. All collected live animals were acclimated for one week in filtered and aerated seawater at 15 ± 1 °C (control temperature, determined according to the water temperature at the collection site). The animals were fed with dried powder of *Chlorella* sp. and *Spirulina* sp. Healthy individuals with plump body and integrity tunic were chosen for further stress treatments.

2.2. Stress treatments

As two major environmental factors in marine ecosystems, water temperature and salinity were selected for stress treatments in this study. For temperature stress, *C. savignyi* was placed in temperature-controlled tanks with filtered and aerated seawater. To determine time course of reference genes, *C. savignyi* was subjected to 25 °C (high temperature) or 5 °C (low temperature) respectively for 3, 24, 48, 72 h post incubation. For salinity stress, different salinity levels were adjusted by filtered sea water with sea salt (high salinity, 40‰) and sea water with pure water (low salinity, 20‰). We exposed *C. savignyi* to the two salinity gradients and sampled at time points of 2, 24, 48 and 72 h. Three biological replicates were collected in each treatment group. Three tissues including intestine, pharynx and siphon were dissected out and immediately frozen in liquid nitrogen, and then stored at −80 °C until gene expression analysis.

2.3. RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from all tissues (each treatment sample was pooled from the three replicates) using the TRIZOL reagent (Ambion, USA) according to the manufacturer's instructions. The integrity of the extracted RNA was examined by visual inspection of the 28S and 18S ribosomal bands using agarose gel electrophoresis. RNA quality and quantity were assessed using a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, USA). Subsequently, total RNA was treated with RNase-free DNase I (Promega, USA) to remove genomic DNA contamination. The first-strand cDNA was synthesized from 1.5 μg total RNA using M-MLV reverse transcriptase (Takara, Japan) with the Oligo dT primer.

2.4. Candidate reference genes selection and primer design

Ten commonly used reference genes including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 (*EF1*), actin (*ACT*), ubiquitin (*UBQ*), α-tubulin (*TubA*), β-tubulin (*TubB*), ribosomal protein L17 (*RPL17*), ribosomal protein S15 (*RPS15*), cyclophilin (*CYP*), and histone H3 (*HIS*) were selected to assess the expression stability. The sequences of all candidate reference genes were downloaded from Ensembl (http://asia.ensembl.org/index.html). PCR primers were designed using Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/index.html) with the following parameters: annealing temperature 60 °C and PCR product size 120–130 bp. The amplification specificity of all designed primer pairs were tested by 1.5% agarose gel electrophoresis and subsequently by Sanger sequencing.

2.5. qRT-PCR

Quantification of reference gene expression was performed using Roche LightCycler® 96 detection system (Roche Applied Science, Germany) with FastStart Essential DNA Green Master (Roche Applied Science, Germany). Samples were amplified in a total volume of 10 μL, containing 5.0 μL of SYBR Green Master Mix, 0.5 μM of each primer and 1 μL cDNA (10× dilution). The qRT-PCR was designed according to the MIQE guidelines (Bustin et al., 2009). PCR efficiency (E) of each gene was calculated by the slope of its standard curve (Table 1), which was generated using five 10-fold dilutions of the same cDNA samples. The correlation coefficient (R^2) was determined by the linear-correlation of regression analysis to validate the linear relationship between the threshold cycle (Ct) and the template concentration. Each amplification was performed in triplicate and the PCR program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. Dissociation analysis of amplification products was performed at the end of each PCR reaction to confirm that only the specific PCR product was amplified and detected. Two negative controls including non-reverse transcriptase control and non-template control were performed to rule out potential genomic DNA contamination and other contaminations, respectively.