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Original Research

Investigation on the expression stability of common reference genes in *Aurelia* sp.1 under hypoxia

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Abstract: RT-qPCR (Quantitative real-time polymerase chain reaction) is a reliable molecular biology technique used for gene expression detection due to its high sensibility and good reproducibility. However, suitable reference genes for RT-qPCR are often not available to investigate the expression of target genes in jellyfish under different conditions. To determine the responsible genes of jellyfish under hypoxia, primers to amplify the *actin* gene was designed for the amplification according to the conserved actin amino acid sequences of cnidarian. Then, we cloned and sequenced the partial cDNA sequence of *β-actin* gene containing 849 bp nucleic acids was cloned and sequenced, and the four common housekeeping genes (*18S rRNA*, *β-actin*, *α-tubulin* and *GAPDH*) were detected. To obtain suitable reference genes, we compared the four genes under normoxia and hypoxia were determined and compared using RT-qPCR. The evaluation result shows that *α-tubulin* gene can be used as single reference gene, and *α-tubulin* and *β-actin* can be served as multiple reference genes to study relative gene expression related to hypoxic tolerance of *Aurelia* sp.1. This research will establish foundation to reveal the molecular mechanism of jellyfish under hypoxia.

Key words: *Aurelia* sp.1; *β-actin*; Reference gene; RFQ-PCR.

Introduction

In recent 10 years, giant jellyfish blooms often occurred in estuaries, bays and coastal waters (1-3), which causes serious damages on marine cultures and coastal ecosystems. Changes of abiotic factors are considered to be the main cause for regional jellyfish, such as temperature, eutrophication, coastal engineering, and marine oxygen minimum zones (4, 5). Among these factors, hypoxia is identfitied to be a major threat to the coast ecosystem, which leads to a deleption of metazoans but jellyfish. Therefore, it is important to explore the mechanism of jellyfish under hypoxia, so that could provide some new insights in treating with the bloom of jellyfish.

Aurelia sp.1 is one of the three dominant jellyfish species (*Aurelia* sp.1, *Cyanea nozakii*, and *Nemopilema nomurai*), and usually appeares in East Asia, especially in Chinese coastal waters (6, 7). Previous studies have revealed that *Aurelia* sp.1 presents a promising low oxygen tolerance (8, 9), but the exact mechanisms of hypoxic response in jellyfishremains rarely reported . Recently, Wang et al. find a hypoxia-inducible factor (HIF) in *Aurelia* sp.1,which whole oxygen-dependent degradation domains are similar to the advanced animals, but the organization of domains and genomic structure are more close to that in the inferior animals (10). This finding provided us a good opportunity to further reveal the mechanims of hypoxic tolerance in *Aurelia* sp.1. However, which reference genes used for further investigation remains unclear in *Aurelia* sp.1 under hypoxia.

Quantitative real-time polymerase chain reaction (RT-qPCR) is a rapid, accurate, and reliable method used to monitor gene expression in the study of physiology and ecology (11). During this assay, the endogenous reference is essential control to remove the influence of variance resulted from quality and extraction efficiency of nuclei acid among the samples. Generally, several internal references genes have been explored and utilized for the the melocular assays,*18S rRNA*, *β-actin*, *α-tubulin*, *glyceraldehyde-phosphate dehydrogenase* (*gapdh*), *translation elongation factor* (*tef*), *ubiquitin* (ubq) etc.. *18s rRNA* is often used as an internal control in early studies of human melanoma cell subpopulations and rice (12, 13). Investigation on *Cichorium intybus* and faba bean indicate that *actin* is the most stably expressed gene under different experimental conditions (14). As a once popular reference gene, the stability of *GAPDH* has always been widely questionedbut a recent study has demonstrated that *GAPDH* is one of the most suitable reference genes in the control group and the icv-STZ group for cynomolgus monkey (15). Recent studies reveal that *tubulin* show highly stable expression in many organisms such as cucumber, longan tree, and Chrysanthemum under aphid infestation (16, 17). Although many genes can be used as the reference genes, an increasing number of studies reveal that the stabilities of these genes are distinctly different in different species under different abiotic stress (18, 19). Therefore, the reference genes should be selected carefully according to the specific species and abiotic condi-

tions.

To date, no sutdy has focused on the expression changes of reference genes in Aurelia sp.1 under different physiological stresses. Therefore, to indentify the best reference gene involved in hypoxic tolerance for *Aurelia* sp.1, the partial cDNA sequence of *β-actin* gene first cloned, and then employed NormFinder (20) and GeNorm (21) to assess the expression stability of four common reference genes (*18S rRNA*, *β-actin*, *α-tubulin*, and *GAPDH*). At last, the stabilities of these four genes were compared to identfy candidate reference genes in *Aurelia* sp.1 under different cultivation conditions.

Materials and Methods

Aurelia sp.1 cultivation

Aurelia sp.1 medusa were provided by the Institute of Oceanography, Chinese Academy of Sciences (IOCAS). The umbrella diameter of medusa is about 3.0 cm. *Aurelia* sp.1 was fed with *Artemia Nauplius* and cultivated in 50-L fish tanks with filtered seawater (salinity: 33 PSU, 20°C).

Construction of hypoxic cultivation system

An enclosed hypoxic cultivation system for the jellyfish was constructed (Figure 1). The medusas were cultivated in a cylindrical vitreous tank (A) (80 cm in diameter and 30 cm in depth). Filtered seawater circulated along A, B, F, C, D, E and A.

Hypoxic experiment

Two sets of cultivation system were used for the hypoxic experiments. One was used as the reference group in which dissolved oxygen (DO) achieved saturation through bubbling. The other was used for the hypoxic group with an approximate 0.5 mg/L DO provided by bubbling of 99.9% nitrogen. Dissolved oxygen was monitored to maintain experimental stability every 2 h using a Model HQ30d multi-parameter meter (HACH, Beijing, China). Twenty medusas of *Aurelia* sp.1 were put into reference and hypoxic group, respectively. Each three medusas were randomly selected as one sample separately from reference group and hypoxic group at 0, 6, 12, 24, and 48 h since the experiment started.

Total RNA extraction and reverse transcription

Total RNA of *Aurelia* sp.1 was extracted using Transzol™ reagent (Transgen, Beijing, China) and characterized in agarose gel electrophoresis and spectrophotometry. First strand cDNA for sequencing was synthesized with the Superscript® II Reverse Transcriptase (Invitrogen, California, USA) and Oligo (dT) primer (TaKaRa, Dalian, China), while cDNA for quantitative PCR was obtained with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China).

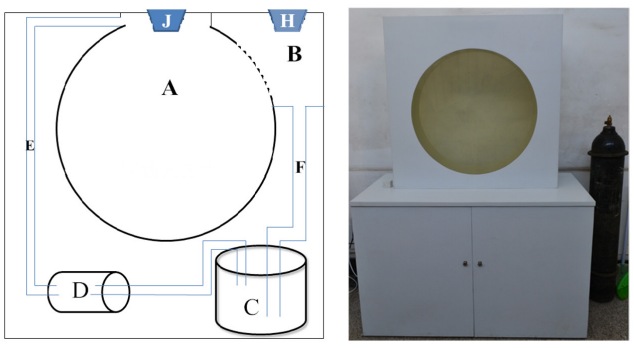

Figure 1. Structural diagram (left) and product shot (right) of hypoxic cultivation system (A-tank of jellyfish cultivation; B-buffer system; C-temperature controller; D-recycle pump; E-inlet pipe; F-outlet pipe; J&H-rubber plug).

Cloning and sequencing of the partial actin cDNA

Based on the analysis on the conserved amino acid residues from alignment of *Hydra vulgaris* (AAA29205.1), *Nematostella vectensis* (EDO40382.1) and *Chrysaora quinquecirrha* (AFS65047.1), degenerate primers named *AS*actin F (5'-GATGATATGGAAAAAATTTGGCAYCAYAC) and *AS*actin R (5'-TTCTTGTTTACTAATCCACATYTGYTG) were used to obtain the nucleotide sequence of actin in *Aurelia* sp.1. A partial fragment of actin was obtained by amplifying the cDNA with *TransTaq-T* DNA polymerase (Transgen, Beijing, China). The PCR was performed in a 25-μl final volume reaction (including 2.5 μL 10× *TransTaq-T* buffer, 2 μL of dNTPs, 1 μL cDNA from *Aurelia* sp.1, 1μL (10μM) degenerate primers, 0.5 μL *TransTaq-T* polymerase, and 17 μL ddH_2O), and executed in program: 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min, and ended with 72°C for 10 min. The PCR product was detected in electrophoresis of 1.0% agarose gel and purified with TaKaRa MiniBEST agarose gel DNA extraction kit Ver.3.0 (TaKaRa, Dalian, China). The pMD™18-T vector (TaKaRa, Dalian, China) and Trans5α chemically competent cells (Transgen, Beijing, China) were used to clone and sequence the purified PCR product. The plasmids were extracted from positive clones and sent for sequencing (Beijing Genomics institute, Beijing, China).

Phylogenetic analysis

The open reading frame (ORF) was obtained by searching the cDNA sequence of actin in *Aurelia* sp.1 and translated to amino acid sequence with DNAMAN 7.0. The multiple alignments of actin amino acid sequences in the Cnidaria phylum were performed with Clustal X, and default parameters were used. The neighbor-joining method was applied to the molecular phylogenetic analysis in Mega 6.0 program. Reliability of the estimated tree was evaluated using bootstrap method with 1,000 replications. The amino acid sequences of actin included the following items: *Hydra vulgaris* (AAA29205.1), *Podocoryna carnea* (CAA48798.1), *Favites chinensis* (BAC44869.1), *Galaxea fascicularis* (BAC44866.1), *Stylophora pistillata* (AAR13014.1), *Aiptasia pulchella* (AAQ62633.1), *Hydractinia echinata* (ADR10434.1), *Seriatopora hystrix* (ADM13664.1), *Malo kingi* (ACY74447.1), *Nematostella vectensis* (XP_001630583), *Chrysaora quinquecirrha* (AFS65047.1), *Euphyllia ancora* (AFP52951.1), *Scleronephthya gracillimum* (AAT74858.1), and *Myxobolus cerebralis* (AAN86039.2).

Primers design of candidate genes for RT-qPCR

Four genes that commonly used as internal control genes for RT-qPCR (*18S rRNA*, *β-actin*, *GAPDH*, and *α-tubulin*) were selected as the candidate reference ones. Four pairs of quantitative primers (Q18S YF/YR,

Table 1. Names and sources of selected reference genes.

Candidate genes	Sequence sources (GenBank)
18S rDNA	AY039208.1
β-actin	KF447602.1
GAPDH	AB044092.1
α-tubulin	AY226057.1

Qactin YF/YR, QGAPDH YF/YR, and Qtubulin YF/YR) were obtained individually from cDNA sequences of candidate genes in GenBank (Table 1) with software Primer 5.0. More details of the quantitative primers are listed in Table 2. Primers synthesis was performed in Biosune biological technology (Shanghai) Co., LTD.

Primer verification

Primer verification was carried out in PCR in 20 μL final volume reaction (including 10 μL FastStart universal SYBR Green Master ROX (Roche, Mannheim, Germany), 2.0 μL cDNA for quantitative PCR from *Aurelia* sp.1, 0.6 μL (10μM) quantitative primers, 2.0 μL (2 mg/mL) bovine serum albumin (TaKaRa, Dalian, China), and 4.8 μL ddH_2O). Runs started with 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 58°C for 1 min and ended with 72°C for 10min. PCR was performed and the amplification and dissociation curves of the four primers were obtained on ABI7500 fluorescence quantitative PCR instrument (Invitrogen, California, USA). The PCR products were sequenced directly with their own quantitative primers.

Analysis of candidate genes involved in hypoxic experiment

PCR efficiency (*E*) of each candidate gene was estimated by serial dilutions (from 1/2 to 1/32) of a cDNA sample randomly selected from hypoxic experimental samples for inter-run calibration. Slope of the standard curves was used to obtain the quantitative PCR efficiencies (*E*) using formula: $E=10^{-1/slope}-1$. Finally, two software packages (NormFinder, and Genorm) were used to assess the suitability of candidate genes as internal control genes by analyzing the Ct value from cDNA samples in our hypoxia experiment. With the standard curves of the candidate genes, Ct values of cDNA samples were turned into linear values for NormFinder analysis. The result from NormFinder indicates that the gene with the highest stability value (*M*) has the least stable expression. For GeNorm, the Ct values should be transformed into quantities in the following steps. ΔCt of each sample was obtained by subtracting the lowest Ct value of all samples, and the quantity of each sample was calculated by $E^{-\Delta Ct}$. The most stable gene has the least *M* values for GeNorm just as NormFinder.

Results

The detection of total RNA in *Aurelia sp.1*

Electrophoresis results showed that the total RNA of sample was complete without degradation and could be used for subsequent experiments (Figure 2). The $OD_{260/280}$ of total RNA was 2.02, which confirmed that the purity of the total RNA could meet the experimental requirements.

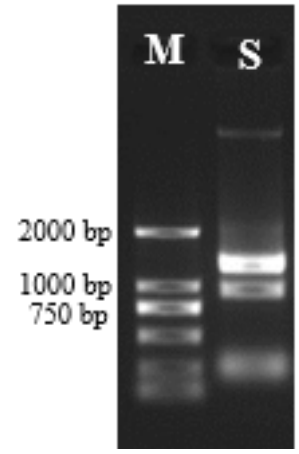

Figure 2. Electrophoresis results of total RNA with 1% gel (M-DL2000 Marker; S- sample of total RNA in *Aurelia sp.1*).

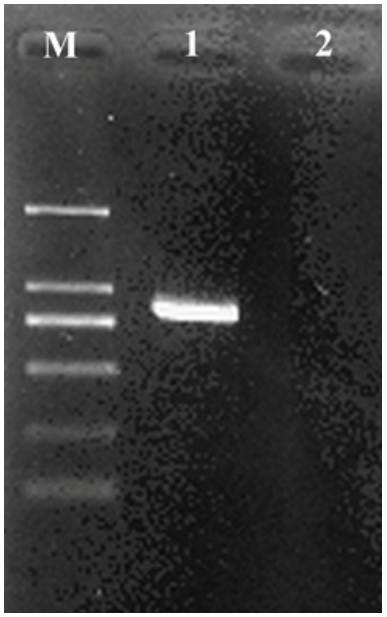

Figure 3. PCR results of partial β-actin in *Aurelia* sp.1 (1-PCR product; 2-negative control; M-Trans 2K DNA Marker).

Table 2. Quantitative primers of selected reference genes.

Primer names	Primer sequences (5'-3')	Tm (°C)	Length of fragment (bp)
Q18S	ACCCATTGGAGGGCAAGTCT	64.9	
Q18S	CGTCGCAAATCCTACGCACA	64.9	204
Qactin	ACATTTGCTGGAAGGTGGAGAG	63.9	
Qactin	GGAAACGAGAGATTCAGGTGCC	64.5	316
QGAPDH	CAAATTGCCTTGCACCCTTAG	61.4	
QGAPDH	GATGCTGGGATGATGTTCTGG	62.2	169
Qtubulin	AGACAGAATCAGAAAGTTGGCAGA	63.2	
Qtubulin	GTGAGTGGTCAGGATGGAGTTG	63.9	220

Sequence analysis on partial actin cDNA of *Aurelia sp.1*

Results reveals an obvious strip in about 800 bp from amplification product (Figure 3), and 849 bp nucleic acids were confirmed by sequencing. The sequence has been submitted to GenBank in accession number KF447602. The open reading frame encodes 282 amino acid residues.

Phylogenetic analysis of actin in Cnidarian

Using the amino acid sequences of actin in GenBank, neighbor-joining phylogenetic tree was constructed with *Myxololus cerebralis* as outgroup (Figure 4). On the tree, Anthozoa, Scyphozoa, and Hydrozoa form a major Cnidarian clade, and Scyphozoa stays closer with Hydrozoa than Anthozoa. Scyphozoa medusa *Aurelia* sp.1 and *Chrysaora quinquecirrha* in the Scyphozoa form two large subclades, while Cubozoa (*Malo kingi*) appears in Hydrozoa subclade.

Verification on the four quantitative primers

All the dissociation curves present a sharp singlet (Figure 5); and the sequencing results suggest that all the amplicons were the target sequences of candidate genes. The amplification and dissociation curves indicate no primer dimers nor nonspecific amplification for four quantitative primers of candidate genes in fluorescence quantitative PCR system.

Evaluation of internal control genes for hypoxic experiments

The dilution curve provides PCR efficiency of each candidate gene, and additionally, it verifies stability of the fluorescence quantitative PCR system. Dilution curves of candidate genes (*18S rRNA*, *β-actin*, *GAPDH*, and *α-tubulin*) are shown in Figure 6, and relevant parameters are listed in Table 3.

NormFinder suggests that *α-tubulin* is the most suitable reference gene, followed by *GAPDH*, *18S rRNA* and *β-actin*, and their *M* are 0.060, 0.064, 0.067 and 0.106 (Table 4). GeNorm shows that the average expression stability M of *α-tubulin* and *β-actin* are lower than that of *GAPDH* and *18S rRNA* (Figure 7), which means that *α-tubulin* and *β-actin* can be served as multiple reference genes for real-time quantifcation PCR method to detect the expression of certain genes response to hypoxia in *Aurelia* sp.1.

Table 3. Relevant parameters about dilution curves of candidate genes.

Gene names	Standard curves (R^2)	Efficiency (%)
18S rRNA	y=-3.420x+10.49 (0.999)	96.06
β-actin	y=-3.021x+24.26 (0.999)	114.3
GAPDH	y=-3.730x+28.80 (0.999)	85.39
α-tubulin	y=-3.223x+25.23 (0.999)	104.3

Table 4. Normfinder analysis results of candidate genes between hypoxia and control groups in *Aurelia* sp.1.

Evaluation index	*18S rRNA*	*β-actin*	*GAPDH*	*α-tubulin*
Stability value (*M*)	0.067	0.106	0.064	0.060

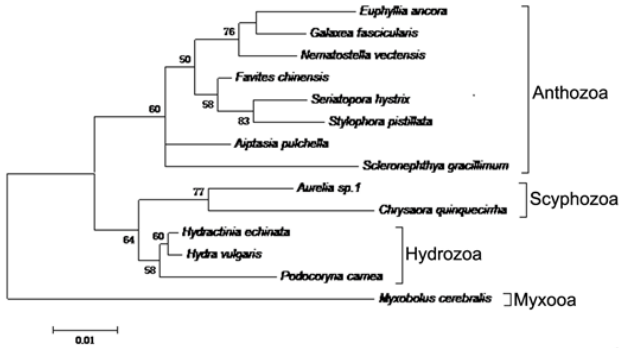

Figure 4. The Neighbor-Joining phylogenetic tree of actin based on amino acid sequences in Cnidarian.

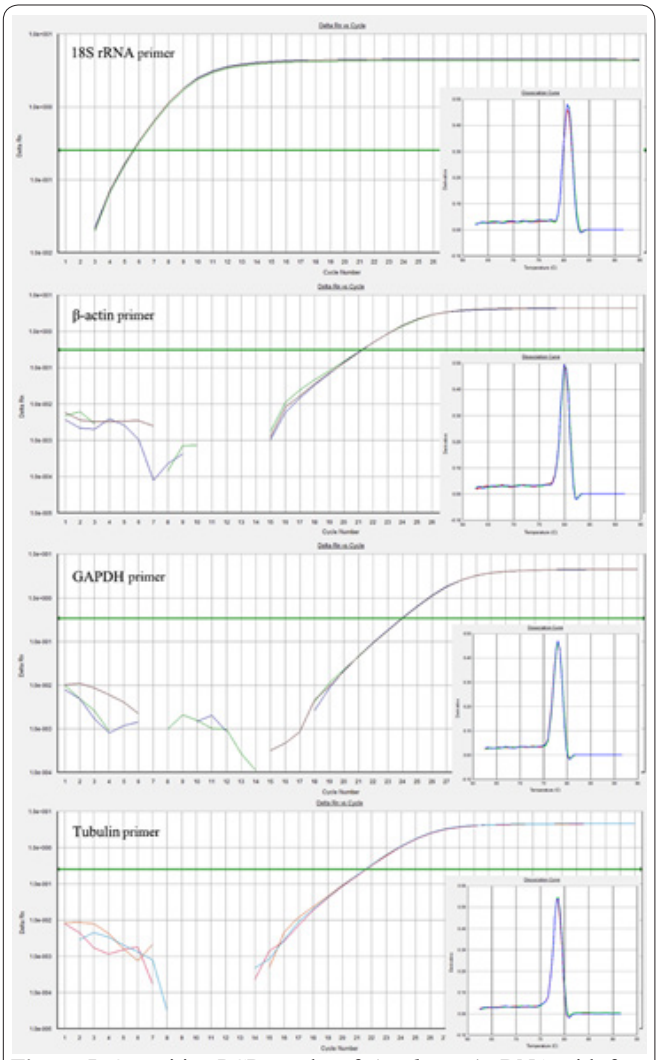

Figure 5. Quantitive PCR results of *Aurelia* sp.1 cDNA with four pairs of primers.

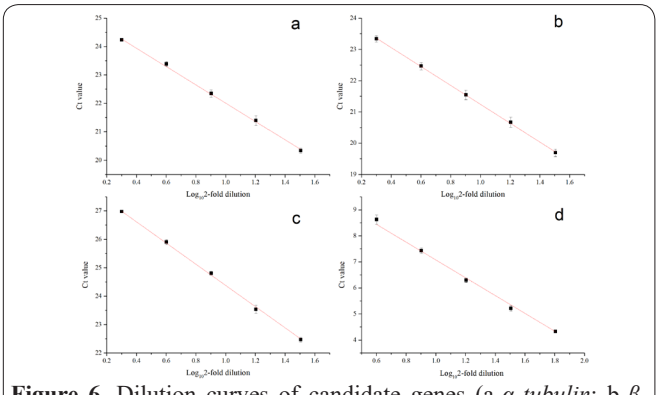

Figure 6. Dilution curves of candidate genes (a-*α-tubulin*; b-*β-actin*; c-*GAPDH*; d-*18S rRNA*).

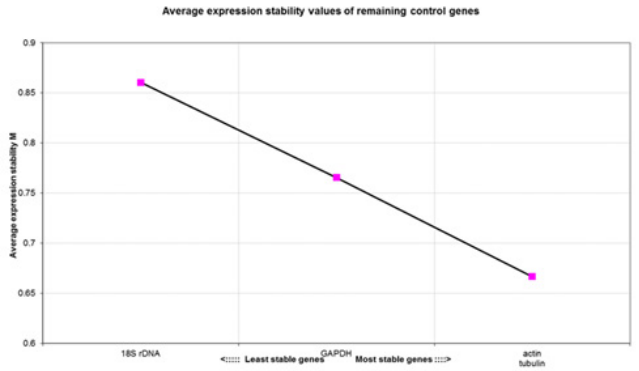

Figure 7. The average stability values of candidate genes between hypoxia and control groups in *Aurelia* sp.1.

Discussion

Actin is a globular multi-functional protein that forms microfilaments, which is found in almost all eukaryotic cells. The amino acid sequences of actin are highly conserved in vertebrate and invertebrate cells (10, 22, 23). Compared with chondriogenes (16S rDNA and cytochrome oxidase C), an increasing number of karyogenes are employed to study phylogenetic analysis, such as *18S rDNA* and *actin*. Evolutionary processes have resulted in diversification of ancestral proteins into many varieties at present. Therefore, actin, as an efficient molecule, is able to tackle essential ancestral biological processes and reflects some specific evolutionary events (24). Phylogenetic analysis of actin is consistent with classical morphologic taxonomy of Cnidaria (25). Cnidarian includes Anthozoa, Hydrozoa, and Scyphozoa. Specifically, Scyphozoa and Hydrozoa are more close from each other than from Anthozoa. The result of actin phylogenetic analysis is consistent with those of ribosomal small subunit (SSU), mitochondrial genome structure, and HIF-1α protein (10, 26, 27). Therefore, results of phylogenetic analysis on *actin* shall be reliable. However, the actin gene sequence of Cnidarian is too little to understand its phylogeny comprehensively.

18S rRNA, β-actin, GAPDH, and α-tubulin are house-keeping genes (some structure proteins and key enzymes in biochemical metabolism) and are often used as reference genes (28). However, recent research suggests that the expression of these genes may vary with developmental stage, tissue type, experimental condition, and so on. As a cytoskeleton protein, *actin* is widely used as a reference gene in RT-qPCR systems (29). However, in our experiment, the stability value (*M*) of actin gene is significantly higher than that of the other three genes. Normfinder analysis indicate that is unsuitable for jellyfish. Similarly, Selvey et al. confirm that the expression of actin gene is highly regulated by matrigel and thus is unsuitable as an internal control, while *18S rRNA* had excellent consistency property and superiority as internal control for quantitative applications of RT-PCR (18). Kim et al. also indicate that *18S rRNA* was the most reliable reference gene in various growth stages of etiolated seedlings, different cultivars, and various times after UV-irradiation treatment in rice (7). However, as this study indicates, *18S rRNA* showed the lowest Ct values beyond the detection scope of standard curve among the candidate genes and the higher *M* than GAPDH and tubulin genes in *Aurelia* sp.1. A little change of *18S rRNA* will affect the accuracy of quantitative RT-PCR results when we directly dilute the sample or convert Ct value to gene copy number. What is more, the abundance of *18S rRNA* has a great difference between different species. As described in the introduction, *GAPDH* is once popularly used as a reference gene. However, this study confirms that *GAPDH* may not be suitable as single reference gene in hypoxic response process of jellyfish, as studies have confirmed that up-regulation of *GAPDH* gene expression by HIF-1 activity depending on Sp1 in hypoxic breast cancer cells (30).

Previous studies have screened single reference gene among many candidate housekeeping genes using Normfinder. Normfinder is rooted in a mathematical model of gene expression to estimate the variation of the candidate normalization genes between sample subgroups of the sample set (20). However, in recent years, it has become clear that no single gene is constitutively expressed to normalize quantitative RT-PCR data and multiple reference genes have become mainstream to study accurate expression profiling of selected genes. geNorm can identify the most stably expressed control genes and to determine the minimum number of genes required to calculate a reliable normalization factor (21). Based on the geNorm analysis, this study shows that *α-tubulin* and *β-actin* can be served as multiple reference genes to study relative gene expression related to hypoxic tolerance of *Aurelia* sp.1.

Cloning and sequencing a partial cDNA sequence of *β-actin* gene of *Aurelia* sp.1 revealed 849 bp nucleic acids. Phylogenetic analysis on the actin confirmed the evolution of Cnidaria. Moreover, *α-tubulin* gene can be used as single reference gene and *α-tubulin* and *β-actin* can be served as multiple reference genes to study relative gene expression related to hypoxic tolerance of *Aurelia* sp.1.

Acknowledgments

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Interest conflict

There is no conflict of interest to be declared by the author.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Guoshan Wang,Xiang Gao analysed and wrote the text and all authors have read and approved the text prior to publication.

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