



# Selection of reference genes for quantitative real-time PCR in *Octopus minor* (Cephalopoda: Octopoda) under acute ammonia stress

Ran Xu<sup>a,b</sup>, Xiaodong Zheng<sup>a,b,\*</sup>

<sup>a</sup> Institute of Evolution and Marine Biodiversity, Ocean University of China, Qingdao, China

<sup>b</sup> Key Laboratory of Mariculture (Ocean University of China), Qingdao, Ministry of Education, China

## ARTICLE INFO

### Keywords:

Reference gene

Acute ammonia stress

qRT-PCR

*Octopus minor*

## ABSTRACT

High concentration of ammonia is a common issue in the aquaculture industry and often causes detrimental effects to aquatic products. Exploring expression regulation of genes involved in acute ammonia stress can help to understand the molecular mechanisms of ammonia response. Quantitative real-time PCR (qRT-PCR) with proper reference genes is an effective way to normalize the expression of target genes. To identify the most suitable reference genes for *Octopus minor* (Mollusca: Cephalopoda: Octopoda) under acute ammonia stress and the normal culturing, nine candidate genes were selected for the validation: *OD*, *RPS18b*, *RPL29*, *RPS5*, *EF-1α*, *RPL6*, *AA4*, *ACT*, *TUB*. The results showed that the stability of candidate genes varied considerably in gill, digestive gland, brain and hemolymph. Thus, the reference genes were determined separately in different tissues. *RPS5* and *RPL6* showed relatively high stability in gill, while *RPL6*, *TUB*, *RPL29* and *OD* were four suitable reference genes in digestive gland. *EF-1α*, *TUB* and *RPL6* were the best combination in brain and *EF-1α* and *RPS18b* were the most appropriate reference genes in hemolymph.

## 1. Introduction

Ammonia is a well-known aquatic pollutant as well as a toxic factor to fish, mollusks and crustaceans and is a common restriction to the aquaculture industry. (Colt and Armstrong, 2009; Durborow et al., 1997). Intensive culture activities, microbial decomposition of organic matter such as uneaten food or animal feces and dead organisms are primary causes of concentration of ammonia in farming water (Armstrong, 1979). The ammonia usually has two states in water, the ionized ammonia ( $\text{NH}_4^+$ ) and the un-ionized ( $\text{NH}_3$ ) state. The latter is the toxic form to fish and mollusks because of its ability to pass across the cell membranes easily (Armstrong et al., 1978; Basuyaux and Mathieu, 1999). The ratio of these two forms can be influenced by pH, temperature and salinity (Bower and Bidwell, 1978; Durborow et al., 1997). In fish, high ammonia concentration would cause behavioral and physiological changes such as loss of equilibrium, decreasing blood pH, altering cardiac function (Tomasso, 1994), and even have negative effects on the growth and even survival of cultured aquatics (Colt and Armstrong, 2009; Chen and Lin, 1991; Basuyaux and Mathieu, 1999).

*Octopus minor* is one of the most important commercial cephalopod species in China (Norman and Sweeney, 1997; Zheng et al., 2014). *O. minor* is carnivore who majorly pursue energy from proteins, while ammonia is the major end-product of protein catabolism (Boucher-

Rodoni and Mangold, 1995). According to the previous research, the amount of ammonia excrete of cephalopods is 2–3 times higher than fish in the same body weight (Lee, 1995). Studies also indicated that ammonia concentration would have effects on the survival, feeding, nervous system and chromatophore control of octopods (Jack, 1982; Feyjoo et al., 2011). Some physiological and biochemical responses to ammonia stress in cephalopods have been investigated (Hu et al., 2017; Peng et al., 2017). Meanwhile, some key genes were demonstrated to involve in immune responses for ammonia stress in some aquatic organisms (Ren and Pan, 2014; Liu et al., 2014; Lu et al., 2016). However, the molecular mechanisms of ammonia stress remains unclear. Understanding the expression patterns of genes related to the ammonia stress will help elucidate the underlying molecular mechanisms of immune regulation and detoxification to the ammonia concentration.

Quantitative real-time PCR (qRT-PCR) is a common way to measure gene expression since its high sensitivity, flexibility and reproducibility (Bustin, 2002). However, the variations caused by differences in samples, RNA extraction, efficiency of enzyme and transcriptional activity can influence the experimental accuracy. In this case, a reliable reference gene that could minimize the systematic errors and inherent variation as well as normalize the expression of related genes is necessary (Kozera and Rapacz, 2013). Hence, choosing suitable reference genes that has a relative high and stable expression in different tissues

\* Corresponding author at: Institute of Evolution and Marine Biodiversity, Ocean University of China, Qingdao, China.

E-mail addresses: [xuran@stu.ouc.edu.cn](mailto:xuran@stu.ouc.edu.cn) (R. Xu), [xdzheng@ouc.edu.cn](mailto:xdzheng@ouc.edu.cn) (X. Zheng).

and developmental stages of interest and shows no co-regulation with target genes can reduce the systemic errors and variability in samples. Reference genes like  $\beta$ -actin (*ACT*), elongation factor-1 alpha (*EF-1 $\alpha$* ),  $\gamma$ -tubulin (*TUB*) are widely utilized in marine organisms such as *Crassostrea gigas* and *Octopus vulgaris* (Du et al., 2013; García-Fernández et al., 2016; Sirakov et al., 2009). Some homologous housekeeping genes like ribosomal protein genes in human have proven to be effective reference genes in *C. gigas* and flatfish (Du et al., 2013; Infante et al., 2008). However, no valid reference genes have been reported in *O. minor* especially under acute ammonia stress.

In this study, we evaluated the stability of nine housekeeping genes and identified suitable reference genes in four tissues to normalize the expression of target genes in *O. minor* undergoing acute ammonia stress and normal culturing through qRT-PCR. To the best of our knowledge, this study is novel for this taxon. Those reference genes will provide valuable information for further expression pattern studies into functional genes that involved in ammonia stress and will facilitate the researches into molecular mechanism of immune regulation and detoxification under ammonia stress in the species.

## 2. Materials and methods

### 2.1. *Octopus minor* cultivation and ammonium challenge

The specimens were collected with a large variation in size and weight from Moon Lake (122.59°E, 37.36°N), Yellow Sea, China, then maintained at  $22 \pm 1$  °C in tanks to acclimatize for one week before the experiment. All the individuals were fed with the crabs every evening and 50% of water in the tank was changed twice every day to avoid high ammonia level during the acclimation period. A pre-experiment was performed to detect the 48-h half-lethal concentration (LC50) of ammonia in *O. minor* weighing  $210.4 \text{ g} \pm 30.6 \text{ g}$  at 48 h exposure. Six gradient of total ammonia concentrations (0, 3.35, 67.33, 205.71, 342.85, 479.99 mg/L) were designed and each gradient included 8 individuals. Appropriate amount of ammonium chloride was used to achieve the desirable concentration. The experiment was repeated three times. LC50 was calculated in SPSS vision 22 (IBM SPSS statistics 22, 2013) using Probit procedure. After pre-experiment, two sets were built each consisting of 10 individuals ranging from 86 g to 214 g in weight. After one week's acclimation, set-A was treated with 48-h half-lethal dose of ammonia for 8 h, while set-B was acclimatized in a normal culturing condition and defined as the control set. The water conditions in the experiment were maintained at pH of  $7.8 \pm 0.2$ , temperature at  $21 \pm 0.5$  °C, oxygen higher than 6 mg/L. Before tissue extraction, all individuals were anaesthetized with 7.5% magnesium chloride to minimize suffering and strictly following the ethical procedures (Messinger et al., 1985; Andrews et al., 2013). Brain, gill and digestive gland were dissected and were immediately frozen in liquid nitrogen and stored in the  $-80$  °C. Hemolymph (about 1 ml) were collected from the cephalic aorta through disposable syringe and centrifuged at  $3000 \times g$ , 4 °C for 15 min. Serum was discard and the hemocytes were re-suspended in 1 ml Trizol reagent and stored at  $-80$  °C until the RNA extraction.

### 2.2. RNA extraction and cDNA synthesis

Total RNA of four tissues were extracted from two sample sets using Trizol reagent. The concentration and quality of RNA samples were assessed by Nanodrop ND2000 spectrophotometer (Thermo) and agarose gel electrophoresis. 1  $\mu\text{g}$  of total RNA was reverse transcribed to the first-stand cDNA and a final volume of 20  $\mu\text{l}$  cDNA was obtained by PrimerScript RT reagent kit with gDNA Eraser (Takara) following the manufacturer's instructions.

### 2.3. Selection of reference gene and qRT-PCR

Nine reference genes were selected according to the previous researches (Infante et al., 2008; Sirakov et al., 2009; Du et al., 2013; García-Fernández et al., 2016). Similar to other researches, three widely used reference genes (*EF-1 $\alpha$* , *TUB*, *ACT*) were also included in this study. Furthermore, four ribosomal protein-encoding genes, 40S ribosomal protein s18B (*RPS18b*), 60S ribosomal protein L29 (*RPL29*), 28S ribosomal protein S5 (*RPS5*) and 60S ribosomal protein L6 (*RPL6*) and ornithine decarboxylase (*OD*) and annexin A4 (*AA4*) were chosen in this work. Primers were designed through the RNA-seq data by Primer 5.0 (<http://www.premierbiosoft.com/>), specific amplification was detected by agarose gel electrophoresis and melting curve. The primer efficiency (E) was calculated by equation  $E\% = (10^{(-1/\text{slope})} - 1) \times 100$  through five serial five-fold dilutions of sample cDNA in Roche480. 1  $\mu\text{l}$  of 5 fold diluted cDNA, 5  $\mu\text{l}$  of  $2 \times$  SYBR EVE PCR Mix (abm), 0.3  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ) and 3.4  $\mu\text{l}$  distilled water were mixed into a final volume of 10  $\mu\text{l}$  of PCR reaction. The PCR cycling conditions were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s, 72 °C for 30 s. All reactions of each sample were performed in triplicate to reduce technical error.

### 2.4. Analysis of gene expression stability and determination of minimum number of candidate genes

geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), and BestKeeper (Pfaffl et al., 2004) were used to evaluate the expression stability of nine candidate genes based on different algorithm. Raw data of threshold cycle (*Ct*) were transformed into relative quantities to be calculated in geNorm and NormFinder. *Ct* value was directly imputed into Bestkeeper to determinate stable genes by pairwise correlation. RefFinder (<http://150.216.56.64/referencegene.php>), a user-friendly web programs that integrate geNorm, NormFinder and BestKeeper was utilized to give the final rank of candidate genes. The minimum number of reference genes required for the experimental normalization was evaluated on geNorm through pairwise variation analysis ( $V_n/V_{n+1}$ ). A cut-off value of 0.15 was adopted.

### 2.5. Validation of reference genes

The relative expression levels of aspartate aminotransferase gene (AST, Genbank accession: MH060174), which is one of the ammonia stimuli-specific gene, was analyzed by qRT-PCR in hemolymph to evaluate the reliability of nine candidate reference genes and the combination of two most stable genes (*EF-1 $\alpha$*  and *RPS18b*) in hemolymph. Geometric means were applied in the combination of two most stable genes. The amplification conditions were the same as described above. The expression levels of AST were calculated by comparative *Ct* method ( $2^{-\Delta\Delta C_t}$  method). Fold change values were calculated by dividing the normalized expression levels of set-A by the levels of set-B.

## 3. Results

### 3.1. Median lethal concentration of ammonia determination

Six different gradients of total ammonia were performed to detect the 48-h LC50 of *O. minor*. Since the ionized ammonia ( $\text{NH}_4^+$ ) is nontoxic, we calculated the toxic un-ionized ammonia ( $\text{NH}_3$ ) in each gradient according to the fraction of toxic ammonia in aqueous solutions at different pH and temperatures provided by Bower and Bidwell (1978). Under the present experiment condition, the percent of un-ionized ammonia in the solution was about 2.31%. Hence, the  $\text{NH}_3$  concentration of six gradients was 0, 0.08, 1.56, 4.75, 7.92 and 11.09 mg/l respectively. Through SPSS calculation, we found that the LC50 of 48-h  $\text{NH}_3$  exposure is 4.62 mg/l with the 95% confidence interval at 3.14–6.47 mg/L.

Download English Version:

<https://daneshyari.com/en/article/8545878>

Download Persian Version:

<https://daneshyari.com/article/8545878>

[Daneshyari.com](https://daneshyari.com)