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Identification of Reference Genes for Normalizing Quantitative Real-Time PCR in *Urechis unicinctus*

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Abstract The reverse transcription quantitative real-time PCR (RT-qPCR) has become one of the most important techniques of studying gene expression. A set of valid reference genes are essential for the accurate normalization of data. In this study, five candidate genes were analyzed with geNorm, NormFinder, BestKeeper and ΔCt methods to identify the genes stably expressed in echiuran *Urechis unicinctus*, an important commercial marine benthic worm, under abiotic (sulfide stress) and normal (adult tissues, embryos and larvae at different development stages) conditions. The comprehensive results indicated that the expression of *TBP* was the most stable at sulfide stress and in developmental process, while the expression of *EF-1-α* was the most stable at sulfide stress and in various tissues. *TBP* and *EF-1-α* were recommended as a suitable reference gene combination to accurately normalize the expression of target genes at sulfide stress; and *EF-1-α*, *TBP* and *TUB* were considered as a potential reference gene combination for normalizing the expression of target genes in different tissues. No suitable gene combination was obtained among these five candidate genes for normalizing the expression of target genes for developmental process of *U. unicinctus*. Our results provided a valuable support for quantifying gene expression using RT-qPCR in *U. unicinctus*.

Key words reference gene; RT-qPCR; sulfide stress; tissue; developmental process; *Urechis unicinctus*

1 Introduction

The reverse transcription quantitative real-time PCR (RT-qPCR) has been widely applied to measure the abundance of gene transcripts in various tissues and cells under different physiological and stress conditions (Ginzinger, 2002). Compared with Northern blotting, *in situ* hybridization and cDNA arraying, RT-qPCR is the most sensitive, rapidest, most precise quantification method (Giulietti *et al*., 2001). Its accuracy in determining the transcript abundance of target genes is closely related to some factors, for example, methods of sample preparation and storage, quality and quantity of sample RNA, progress of reverse transcription and condition of RT-qPCR as well as the validity of reference genes (Bustin *et al*., 2009). To quantify the transcript abundance of target genes, several important criteria of reference genes must be followed, which include firstly the transcript abundance is moderate or high Gimeno *et al*., 2014); secondly the amplification efficiency is equivalent to that of target genes (Bustin, 2000); and more importantly the expression is stable and less affected by experimental factors. Housekeeping genes participating in many basic metabolic processes are commonly used as reference genes; however different reference genes may be required by different treatments (Robledo *et al*., 2014), tissues (Ye *et al*., 2015) and developmental stages (Pan *et al*., 2015), thus screening the appropriate reference genes is crucial for accurate RT-qPCR analysis.

The echiuran *Urechis unicinctus*, an important commercial invertebrate living 'U-shaped' burrows in intertidal and subtidal mudflat, inhabits Russia, Japan, the Korean Peninsula and China (Biseswar, 2009; Goto, 2016; Liu *et al*., 2016). With some unique characteristics, *U. unicinctus* is a good model for studying the mechanisms underlining gametogenesis, development, physiology and evolution. It holds also some potential applications in coastal sediment improvement and marine drug development (Abe *et al*., 2014; Ryu *et al*., 2014). Up to now, no investigation was conducted for identifying the appropriate reference genes for RT-qPCR in *U. unicinctus*. Such scenario severely impacted the functional researched of genes in this species. In this study, we evaluated the expression stability of five candidate genes coding beta-tublin (TUB), TATA-box-binding protein (TBP), elongation factor 1 alpha (EF-1-α), beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively, with four different methods including geNorm,

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NormFinder, BestKeeper and ΔCt method in order to identify the suitable reference genes for the tissues, embryos and larvae of *U. unicinctus* under sulfide stress and normal conditions.

2 Materials and Methods

2.1 Animals and Sampling

2.1.1 Animals

U. unicinctus, 33.4 g ± 10.4 g in fresh mass, were collected from a coastal intertidal mudflat around Yantai, China. They were maintained temporarily in aerated seawater (17.6℃ ± 0.3℃, pH 8.01 ± 0.02, salinity 32), and fed with *Platymonas helgolandica*, *Chaetoceros muelleri* and *Chlorella vulgaris* for three days. This research was approved by the Ocean University of China Institutional Animal Care and Use Committee (OUC-IACUC).

2.1.2 Tissue collection at normal condition

Healthy *U. unicinctus* were selected, and four tissues including body wall, mid-gut, hindgut and anal sac were dissected, rinsed with phosphate buffer saline (PBS, pH 7.4), frozen in liquid nitrogen immediately, and then stored at −80℃. Five individuals were implemented for each tissue.

2.1.3 Collection of embryos and larvae at normal condition

The sexually matured *U. unicinctus* in the spawning season (May, 2015) were collected and dissected to obtain mature eggs or sperms which were reserved in the nephridia (gonoducts), respectively. Artificial insemination was conducted by mixing sperms and eggs at a ratio of 10:1 in filtered sea water (FSW), the fertilized eggs were reared in FSW (16.9℃ ± 0.8℃, pH 7.53 ± 1.01, salinity 25). About 2×10^4 blastulae, gastrulae and trochophores were collected at 6.5 h, 11.0 h and 21.5 h post fertilization, respectively, and rinsed, frozen and stored following the method of 2.1.2. Five biological replicates at each developmental stage were implemented.

2.1.4 Sulfide stressing

At 150 μmol L^{-1}, the concentration of sulfide is similar to that of heavily polluted sediment (Ma *et al*., 2012). Such concentration of sulfide was used to challenging *U. unicinctus*. Twenty five healthy animals were assigned to five tanks containing 15 L seawater and sealed with cling film, 5 each. Sulfide concentration was maintained by adding the sulfide stock solution (10 mmol L^{-1} NaHS, pH 8.0) at a 2 h interval following the method described by Ma *et al*. (2012), and detected using the methylene blue method (Fogo and Popowsky, 1949). One worm each tank was sampled at 2 h, 12 h, 24 h, 48 h post-sulfide exposure, respectively. The body wall was sampled following the method of 2.1.2. Five individuals at each time point were implemented.

2.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instruction and digested with RNase-free DNase I (TaKaRa, Dalian, China) to remove possible genomic DNA contamination. RNA quantity and quality were detected with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) and 1.2% agarose gel electrophoresis. First-strand cDNA template was prepared using Primer Script TM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocol. The cDNA was 15-fold diluted for subsequent RT-qPCR analysis.

2.3 RT-qPCR Analysis

Five candidate genes, *TUB*, *TBP*, *EF-1-α*, *ACTB*, and *GAPDH*, which have been verified to be appropriate reference genes in pig *Susscrofa domestica* (Nygard *et al*., 2007), brown planthopper *Nilaparvata lugens* (Yuan *et al*., 2014), pearl mussel *Hyriopsis cumingii* (Bai *et al*., 2014) and monarch butterfly *Danaus plexippus* (Pan *et al*., 2015), were selected. Specific primers (Table 1) of these five genes for RT-qPCR were designed using Primer Premier 5.0 software (http://www.premierbiosoft.com/primerdesign/index.html) based on their sequences from the *U. unicinctus* transcriptome (www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi, GenBank accession number: SRX397931). The specificity of the PCR products was determined by 1.2% agarose gel electrophoresis and through melting curve analysis. The nucleotide sequences of the

Table 1 Information of primers used for gene expression analysis in *U. unicinctus*

Gene name	Primer sequence (5'-3')	Tm (℃)	GC (%)	Product length (bp)	*E* (%)	R^2
TUB	F: GGTGCTGAACTGGTAGACTC	59.85	55.0	238	91.75	0.99
	R: ACAGGGTGGCGTTGTAGGGT	61.90	60.0			
TBP	F: GCCTCTTCTCTCCTCTCGT	59.72	57.9	191	91.19	0.99
	R: CGGTCCTTCTGATGCTG	57.00	58.8			
EF-1-α	F: CTGTGCTCATTGTGGCTGC	59.72	57.9	169	91.65	0.99
	R: GTTGAAACGCTGCTCGCTGT	59.85	55.0			
ACTB	F: CTTGGGTATGGAGTCTGCC	59.72	57.9	220	93.43	0.99
	R: TACGGAGTATTTCCTCTCTGGT	58.23	45.5			
GAPDH	F: GCCTGTATGCGATGTCTCTG	59.85	55.0	87	96.65	0.99
	R: GCCTTCACGGTCTTCTTTATC	58.10	47.6			

Notes: F represents the forward primer, R represents the reverse primer, *E* represents the PCR efficiency, R^2 represents the correlation coefficient.

PCR products were sequenced (Tsingke Biological Technology, Qingdao, China) and verified by the BLAST program at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/guide/all/).

Serial 4-fold dilutions of the body wall cDNA were made to generate the standard curves for *ACTB*, *TUB*, *GAPDH* and *EF-1-α*. Meanwhile a serial 2-fold dilution for *TBP*. PCR efficiency (*E*) and correlation coefficient (R^2) were calculated based on slopes of the standard curves with the following equation:

$$E = (10^{-1/\text{slope}} - 1) \times 100\% .$$

The ideal *E* value ranged from 90% to 110% with the $R^2 \geq 0.99$, thus the production was almost doubled each cycle during the logarithmic phase of PCR.

The RT-qPCR was run on a SYBR Premix Ex TaqTM (TaKaRa, Dalian, China) and Light Cycler 480 system (Roche, Rotkreuz, Switzerland). The reaction solution of 20 μL contained 2 μL 15-fold diluted cDNA template, 10 μL SYBR Premix Ex TaqTM, 0.2 μL each of the PCR primers (10 μmol L^{-1}), and 7.6 μL ddH_2O. The PCR amplification was run at 95℃ for 30 s, followed by 40 cycles of 95℃ for 5 s and 60℃ for 30 s in a 96-well reaction plate. After PCR amplification, a dissociation curve was obtained by the following process: 95℃ for 5 s, 60℃ for 1 min, followed by 95℃ at the rate of 0.11℃ per second to verify primer specificity. In negative controls, the cDNA templates were replaced by ddH_2O or sample RNA. All RT-qPCR assays were carried out in five biological replicates with three technical replicates.

2.4 Expression Analysis of Candidate Reference Genes

The *Ct* values from the candidate genes were obtained when the fluorescence intensity reached a threshold level. A box plot diagram was made using the *Ct* values to estimate the expression levels of these genes. Genes with the lowest *Ct* value showed the highest transcription abundance.

Four software algorithms, geNorm (https://genorm.cmgg.be/), NormFinder (http://moma.dk/), BestKeeper (www.gene-quantification.com/bestkeeper.html) and ΔCt method (Silver *et al*., 2006), were used to evaluate the expression stability of the candidate genes. In the geNorm and NormFinder algorithms, the raw *Ct* values were transformed into the $2^{-\Delta Ct}$ (ΔCt= *Ct* value of the sample□ the lowest *Ct* value of all samples) to analyze the gene expression stability (Vandesompele *et al*., 2002; Andersen *et al*., 2004). While, the raw *Ct* values were analyzed directly by the BestKeeper and Δ*Ct* method (Pfaffl *et al*., 2004; Silver *et al*., 2006). The average pair-wise expression value (*M*) in geNorm algorithm was used to evaluate expression stability of the candidate genes. Usually, gene with *M* value lower than 1.5 is deemed as stable gene, and the highest *M* value means the least stability of gene expression (Liu *et al*., 2015). The optimal number of the reference genes can be predicted by geNorm software through the pair-wise variation $V_{n/n+1}$ value (https://genorm.cmgg.be/). The threshold value of $V_{n/n+1}$ is 0.15, meaning that the number of reference genes should be *n*+1 when the $V_{n/n+1}$ is more than 0.15, otherwise the accepted number of reference genes is *n*. The NormFinder software estimates the stability of gene expression using the stability value (*SV*) which is calculated by minimum variance (Andersen *et al*., 2004). A low *SV* value means a more stable expression profile of gene (Costa *et al*., 2016). For BestKeeper algorithm and Δ*Ct* method (Pfaffl *et al*., 2004; Silver *et al*., 2006), the raw *Ct* values were directly used to calculate the coefficient of variation (*CV*) and the standard deviation (*SD*). Finally, the gene expression stabilities from the four algorithms were integrated, and ranked in comprehensive analysis by calculating the geometric mean of each gene (Chen *et al*., 2011; Xiao *et al*., 2014). Gene with the smallest geometric mean is the most stable.

2.5 Statistical Analysis

All data were presented as mean ± standard error (SE) ($n = 5$). Statistical analysis was performed with ANOVA and Student's t-test using SPSS statistical software, version 18 (SPSS Inc., Arlington, USA). Statistically significant difference was set at $P < 0.05$.

3 Results

3.1 Performance of the Primers for Amplifying the Candidate Reference Genes

The RT-qPCR product of each candidate gene presented a single-specific band with the expected size as detected by agarose gel electrophoresis (Fig.1; Table 1), as well as a single peak in the melting curve analysis (Fig.1). All the *E* values varied between 91.19% and 96.65% with the $R^2 \geq 0.99$ (Table 1). The PCR products of these candidate genes were sequenced and aligned with the retrieved homologs from NCBI database (http://www.ncbi.nlm.nih.gov/guide/all/). Therefore, all these primers used in this study for amplifying the candidate genes were specific and effective.

3.2 Transcript Abundance Analysis of the Candidate Reference Genes

In this study, the raw *Ct* values of five candidate genes were all below 35, ranging from 15.78 to 33.35 in all the normal and abiotic conditions (Fig.2). The transcript abundance of *TUB* was the highest among the candidate genes, with the smallest mean *Ct* value of 20.51 ± 2.78, while *TBP* exhibited the lowest expression level with the mean *Ct* value of 26.16 ± 3.88. The transcript abundance of the candidate genes was *TUB* > *EF-1-α*> *GAPDH* > *ACTB* > *TBP*. Intuitively, expression level of *EF-1-α* seemed to be the most stable in all conditions (*Ct*, 21.79 ± 1.65), whereas the expression level of *ACTB* presented the most obvious variation (*Ct*, 25.70 ± 4.55).

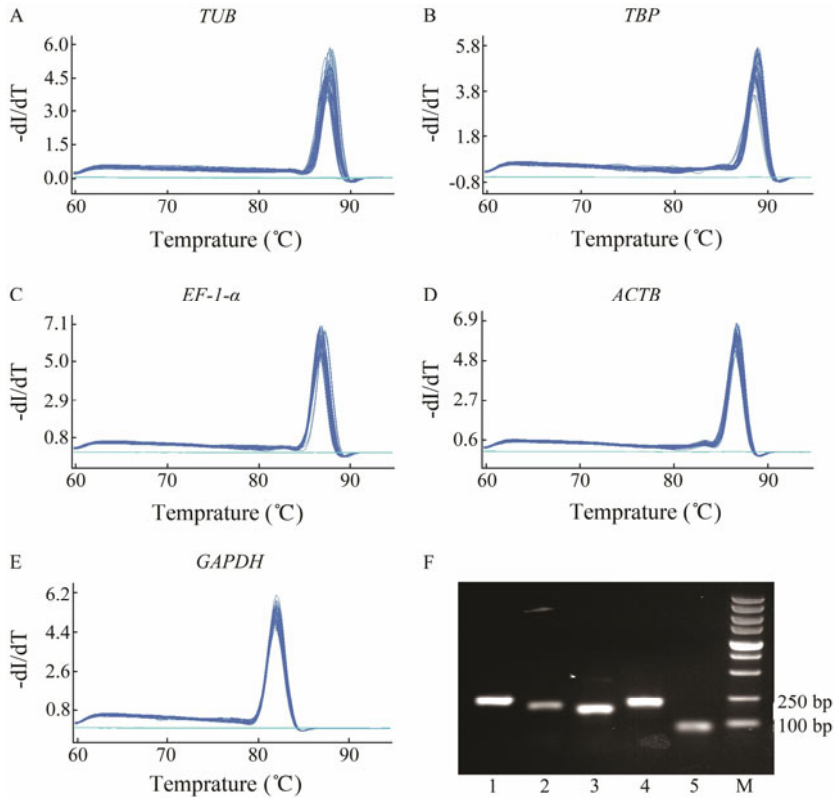

Fig.1 Specificity detection of primers for each candidate genes by melting curves and agarose gel electrophoresis analyses. A)–E), melting curves of RT-qPCR products for each candidate genes; -dI/dT represents change rate of the relative fluorescence units with time. F), agarose gel electrophoresis detection of RT-qPCR products; 1–5, *TUB*, *TBP*, *EF-1-α*, *ACTB*, *GAPDH* respectively; M, 5000 bp DNA marker.

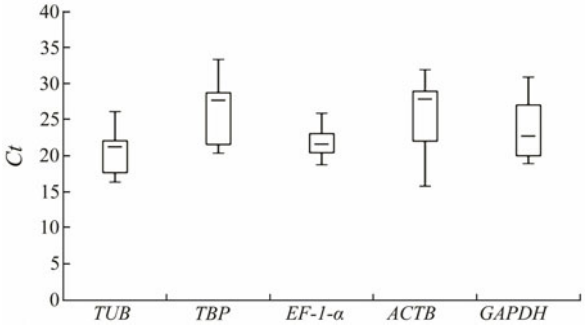

Fig.2 *Ct* values of the candidate genes in a box plot diagram. Boxes represent the 25th and 75th percentiles in all samples; the lines across the boxes represent the median and the whisker caps represent the maximum and minimum values.

3.3 Expression Stability of the Candidate Reference Genes in the Samples at Abiotic and Normal Conditions

In the body walls of *U. unicinctus* exposed to 150 μM sulfide at different time points, all candidate genes exhibited suitable expressions when calculated with geNorm and BestKeeper algorithms (Table 2). Meanwhile, *EF-1-α* and *TBP* were the best under geNorm, and *EF-1-α* was the most stable gene under Bestkeeper. In ΔCt method, stabilities of *EF-1-α* and *TBP* were higher than those of others, while *TBP* was the most stable gene in NormFinder algorithm (Table 2). Comprehensive analysis results showed that the ranking of stability was *TBP* > *EF-1-α* > *ACTB* > *GAPDH* > *TUB* (Table 2).

In the tissues of adults at normal condition, no consistent result was presented in the four algorithms (Table 2). When using geNorm algorithm, *TUB* and *EF-1-α* were the most suitable genes, while *ACTB* was not recommended as its *M* value (2.648) was higher than 1.5. The top two ranked genes in the BestKeeper algorithm were *TUB* and *EF-1-α*, which were the same in geNorm algorithm. However, *TBP* and *ACTB* were evaluated to be unstable expression genes whose *SD* values were greater than 1.0. The outcome of NormFinder was similar to that of ΔCt method in which the most stable gene was *TBP*, while the least stable one was *TUB* (Table 2). In the selected tissues of *U. unicinctus*, the most stably expressed gene was *EF-1-α*, and the stability was *EF-1-α* > *TBP* > *TUB* > *GAPDH* > *ACTB* according to the comprehensive analysis (Table 2).

For the embryos and larvae of *U. unicinctus* in developmental process, *TBP*, *GAPDH* and *EF-1-α* were determined to be stable genes, while *ACTB* and *TUB* were confirmed with unstable expression in geNorm algorithm (Table 2). Unfortunately, only *TBP* was stably expressed using BestKeeper algorithm. According to the NormFinder calculation, *ACTB*, *TBP* and *GAPDH* were the top three stable genes, while ΔCt method analysis recommended the stabilities of *TBP* and *GAPDH* expressions were higher than those of others. In the comprehensive analysis, the stable ranking of candidate genes was *TBP* > *GAPDH* > *ACTB* > *EF-1-α* > *TUB* (Table 2).

Table 2 Expression stability of candidate reference genes among samples of *U. unicinctus*

Gene name		Comprehensive analysis ranking	geNorm (*M*)	NormFinder (*SV*)	BestKeeper (*CV*±*SD*)	Δ*Ct* (mean *SD*)
Sulfide stress	*TBP*	1	0.000	0.088	2.47±0.71	0.477
	EF-1-α	2	0.000	0.120	3.26±0.65	0.471
	ACTB	3	0.312	0.115	3.98±0.70	0.555
	GAPDH	4	0.513	0.118	3.24±0.72	0.574
	TUB	5	0.431	0.169	3.41±0.73	0.518
Normal tissues	*EF-1-α*	1	0.807	0.165	3.10±0.63	2.319
	TBP	2	1.217	0.147	4.27±1.26	1.513
	TUB	3	0.807	0.292	3.02±0.65	3.058
	GAPDH	4	1.404	0.180	3.34±0.77	1.935
	ACTB	5	2.648	0.292	15.98±3.42	1.672
Embryos and larvae	*TBP*	1	0.651	5.77E-08	3.46±0.97	1.145
	GAPDH	2	0.651	5.94E-08	3.51±1.01	1.233
	ACTB	3	2.098	2.46E-08	9.75±2.70	1.630
	EF-1-α	4	1.282	7.93E-08	4.56±1.07	1.665
	TUB	5	1.687	2.97E-07	8.01±1.87	1.563

3.4 The Optimal Number of Reference Genes for Normalization

In this study, geNorm was also utilized to calculate the $V_{n/n+1}$ value, which is a guideline for determining the optimal number of the reference genes used in RT-qPCR. As shown in Fig.3, the $V_{2/3}$ in sulfide stress was extremely close to 0.15, thus only the combination of two reference genes was accepted for precise normalization. However, all the $V_{n/n+1}$ values in both biotic conditions were higher than 0.15, which means that at least three reference genes were needed (Fig.3).

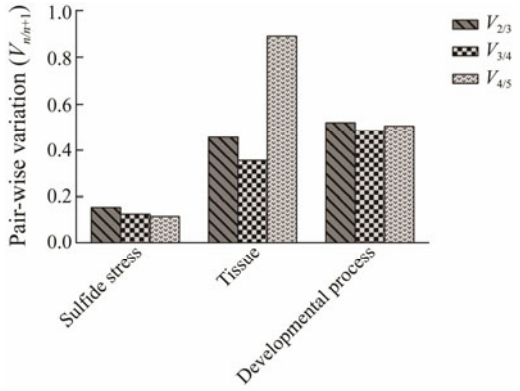

Fig.3 Pair-wise variation ($V_{n/n+1}$) and optimal number of the candidate reference genes analyzed by geNorm under various conditions.

4 Discussion

RT-qPCR is a crucial tool for analyzing gene expression at various biotic and abiotic conditions, and a set of appropriate reference genes are required for the data normalization in RT-qPCR analysis (Sinha *et al*., 2014). Up to now, it has been reported that the suitable reference genes may be different among species and experimental conditions (Zinzow-Kramer *et al*., 2014; Yang *et al*., 2015). In this study, we identified the expression stability of five candidate genes for the first time to screen the potential reference genes for *U. unicinctus*.

Remarkably, the ranking orders of the candidate gene expression stability calculated by the four algorithms in this study were not completely consistent (Table 2). For example, in adult tissues, the ranking of *TUB* expression stability was the first by BestKeeper while it was the fifth by Normfinder and Δ*Ct* method; *TBP* was confirmed as the most stable gene by Normfinder and Δ*Ct* method while it presented low stability in the other two algorithms. Interestingly, Normfinder seems to get the opposite conclusion compared with other algorithms. For example, *ACTB* performed well in the Normfinder algorithm but it was defined as an unstable gene in the other algorithms for the samples of developmental process; and *EF-1-α* was regarded as an unstable gene evaluated by Normfinder but it was the most stable gene by the other algorithms in the samples of sulfide stress. Similar inconsistent results from different algorithms have also been reported in spotted ladybug beetle *Coleomegilla maculata* (Yang *et al*., 2015). The phenomenon may be caused by different methods of data processing and criteria selecting in the four algorithms. In the BestKeeper and Δ*Ct* algorithms, the raw *Ct* values are used to conduct the calculation. While, in the others $2^{-\Delta Ct}$ which is a transformed value according to the raw *Ct* values was employed (Chen *et al*., 2015). The geNorm may remove stable genes when co-regulated genes are involved (Robledo *et al*., 2014). He *et al*. (2008) indicated that Δ*Ct* method prefers to select co-regulated genes, which influences the rank order of candidate genes. BestKeeper and NormFinder tend to look for the most stable reference gene, thus they are rarely affected by co-regulated genes (Chen *et al*., 2015; Zhang *et al*., 2015). Furthermore, previous studies have demonstrated that single algorithm is difficult to accurately identify the reference genes, and sometimes it generates a negative impact on confirming the reference genes (Liu *et al*., 2015). Therefore, a comprehensive analysis from the integration of the four algorithms was used to exactly identify the suitable reference genes in this study.

According to the comprehensive analysis results in this study, *TBP* was recommended as a potential reference

gene in *U. unicinctus* based on its stable expression characteristics among the five candidate genes in all three conditions. TBP is a general transcription factor binding specifically to TATA box, and it can assist RNA polymerase II targete to the transcription start site of the gene. Meanwhile, it also regulates the basal transcription as a necessary component of RNA polymerases I and III (Martel *et al*., 2002). It has been widely used as a reference gene, such as in the mesenchymal stem cells of pig *S. domestica* (Lee *et al*., 2015). Gimeno *et al*. (2014) reported that the *Ct* value of target gene should be higher than that of the reference gene, and transcription abundance of a reference gene should be close to that of target genes. In this study, we found that the *Ct* values of *TBP* were different in various conditions of *U. unicinctus*. It was higher in different tissues or developmental process with mean *Ct* of 29.43 and 28.09 respectively, and was lower with mean *Ct* of 21.44 in sulfide stress. So we suggested that *TBP* might be suitable to normalize those genes with relatively low expression levels in biotic condition, while it might not be restricted in abiotic condition.

EF-1-α expression stability performed well in different tissues and sulfide stress based on the comprehensive analysis in this study, while it exhibited a characteristic of unstable expression during the development in *U. unicinctus*, implying it can be used as a reference gene only in the adult tissues under biotic and abiotic conditions. *EF-1-α*, a eukaryotic translation elongation factor 1A, both shuttles aminoacyl-tRNA (aa-tRNA) to the ribosome and binds and bundles actin (Kim and Lee, 2008). Previous report in blow fly *Chrysomya megacephala* has demonstrated that *EF-1-α* acts well for normalizing in the different tissues and developmental stages, while appears to be unsuitable as reference gene under drug and heavy mental stress conditions (Wang *et al*., 2015a). Moreover, *EF-1-α* shows relatively stable expression for different tissue, species and stress conditions in larvae of three species of Grapholitini (Ridgeway and Timm, 2014). The above evidences indicate that *EF-1-α* expression stability varies depending on the different species and the physiological conditions of the animals.

ACTB is always regarded as a reference gene (Ponton *et al*., 2011) based on its wide distribution in various tissues and cells, as well as a multitude of cell functions especially in cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking, and cell shape regulation (Perrin and Ervasti, 2010). However, its expression level is not always stable in some conditions, such as in the different life stages of Indian fruit fly *Anastrepha obliqua* (Nakamura *et al*., 2016) and the skeletal muscles at different developmental stages in pig *S. domestica* (Wang *et al*., 2015b). In this study, the comprehensive ranking of *ACTB* was the third in the conditions of development process and sulfide stress, and even the worst in the samples of different tissues. Therefore, we suggested *ACTB* should not be alone used as a reference gene in *U. unicinctus*.

The expression stability of *GAPDH* exhibited a fluctuation among various conditions in this study. Its ranking was the second in *U. unicinctus* during development process, while in the adult tissues and under sulfide stress it was ranked the fourth (Fig.3). It has been known that *GAPDH* is one of the most commonly used endogenous reference gene in previous studies (Suzuki *et al*., 2000) due to its wide distribution and multiple functions, including nuclear RNA export, DNA replication and repair, exocytotic membrane fusion, cytoskeletal organization and phosphortransferase activity (Bustin, 2002). However, Small *et al*. (2008) pointed out that *GAPDH* expression level is sensitive to many perturbations in cellular homeostasis, and similar results have also been reported in goat *Capra hircus* (Zhu *et al*., 2015) and cotton bollworm *Helicoverpa armigera* (Zhang *et al*., 2015). Thus, we suggested *GAPDH* is not recommended as a reference gene alone used in various tissues and under sulfide stress, but may be used as a relatively reliable reference gene in developmental process of *U. unicinctus*.

TUB, a major component of microtubule, is widely presented in cells and plays important roles in meiotic and mitotic spindles, cilia and flagella, elongated neural processes, cell division and cell shape (Bialojan *et al*., 1984). It has been used as a reference gene to normalize the expression level of target genes in some studies (Suzuki *et al*., 2000). However, Xu *et al*. (2016) reported *TUB* is unsuitable to be a reference gene throughout the development of zebrafish *Danio rerio*, which is similar in *H. armigera* and *A. obliqua* (Zhang *et al*., 2015; Nakamura *et al*., 2016). *TUB* expression was not stable in this study according to the comprehensive analysis. Its ranking was the last under sulfide stress and during the developmental process, and the third in various tissues (Table 2). Therefore, it is discreetly recommended as a potential reference gene only in different tissues.

In general, two or more reference genes are recommended for more reliable normalizing in RT-qPCR (Vandesompele *et al*., 2002; Zhu *et al*., 2015). Also, Zhao *et al*. (2016) reported that the gene expression may lead to a large fluctuation when a single reference gene is used for normalization in RT-qPCR. To recommend more reliable reference genes for RT-qPCR analysis, we further analyzed the optimal number of reference genes in various conditions of *U. unicinctus*. According to the geNorm principle, the number of reference genes should be *n* when $V_{n/n+1}$ is lower than or closing to the cut-off value 0.15, otherwise it should be *n*+1. When *U. unicinctus* was exposed to sulfide in this study, the $V_{2/3}$ was extremely close to 0.15, which means two reference genes are competent for precise normalization (Fig.3). Therefore, we ensured the combination of *TBP* and *EF-1-α* is optimal for exact normalization in RT-qPCR when the worm exposed to sulfide. However, all the $V_{n/n+1}$ values in biotic conditions (different tissues and developmental process) were higher than 0.15. Considering the possibly inaccurate results caused by the criteria, saving the amount of RNA and cost of experiment, as well as avoiding possibly inaccurate quantitative result caused by using unstable genes (Zhao *et al*., 2016), three of the most stable refer-

ence genes should be combined to use as a reference gene. It should be competent for the normalization when the proposed optimal number is estimated to be more than three. Thus, we recommended that the combination of the top three gene *TBP*, *TUB* and *EF-1-α* was potentially optimal for the normalization in various tissues. However, in developmental process of *U. unincinctus*, the combination of the top three genes, *TBP*, *GAPDH* and *ACTB* was not recommended because *GAPDH* and *ACTB* were excluded by BestKeeper with $SD>1$. Therefore, optimal reference genes for evaluating the expression of target genes in developmental process of *U. unincinctus* should be further studied.

5 Conclusion

This study was the first attempt to evaluate the possibility of the five candidate genes as reference genes by geNorm, NormFinder, BestKeeper and Δ*Ct* algorithms in *U. unicinctus*. *TBP* is a potential reference gene in biotic and abiotic conditions. Combination of *TBP* and *EF-1-α* is the most suitable reference genes to normalize the expression level of target genes in the body wall of *U. unicinctus* exposed to sulfide. *EF-1-α*, *TBP* and *TUB* are recommended as an available combination of reference genes among various tissues.

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