RESEARCH LETTER

Reliable reference gene selection for *Cordyceps militaris* gene expression studies under different developmental stages and media

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Abstract

Cordyceps militaris is considered a model organism for the study of *Cordyceps* species, which are highly prized in traditional Chinese medicine. Gene expression analysis has become more popular and important in studies of this fungus. Reference gene validation under different experimental conditions is crucial for RT-qPCR analysis. In this study, eight candidate reference genes, *actin*, *cox5*, *gpd*, *rpb1*, *tef1*, *try*, *tub*, and *ubi*, were selected and their expression stability was evaluated in *C. militaris* samples using four algorithms, GENORM, NORMFINDER, BESTKEEPER, and the comparative ΔC_t method. Three sets of samples, five different developmental stages cultured in wheat medium and pupae, and all the samples pool were included. The results showed that *rpb1* was the best reference gene during all developmental stages examined, while the most common reference genes, *actin* and *tub*, were not suitable internal controls. *Cox5* also performed poorly and was less stable in our analysis. The ranks of *ubi* and *gpd* were inconsistent in different sample sets by different methods. Our results provide guidelines for reference gene selection at different developmental stages and also represent a foundation for more accurate and widespread use of RT-qPCR in *C. militaris* gene expression analysis.

Introduction

Cordyceps militaris (L.) Link, a well-known edible and medicinal fungus, is the type species of *Cordyceps* that generally parasitizes the larvae or pupae of lepidopteron insects. It is also called northern *Cordyceps* and is widely used as a substitute for *Ophiocordyceps sinensis* (≡*Cordyceps sinensis*) in traditional Chinese medicine and health supplements.

Although more than 400 *Cordyceps* species have been described, only *C. militaris* has been commercially cultivated. It is considered a model organism for the study of *Cordyceps* species, as it can complete its life cycle *in vitro* (Shrestha *et al.*, 2012). However, similar to other mushrooms, *C. militaris* frequently and unexpectedly degenerates during continuous maintenance in culture by showing a loss in the ability to reproduce sexually, which could result in a high percentage of product loss (Lin *et al.*, 2010). Thus, *C. militaris* has been the focus of many biochemical and molecular studies.

The *C. militaris* genome has recently been sequenced (Zheng *et al.*, 2011a), and *Agrobacterium tumefaciens*-mediated transformation (Zheng *et al.*, 2011b) has been developed and optimized for *C. militaris*, which can facilitate the identification of functional genes. Gene expression analysis has become more popular, and it is particularly important in this fungus, as understanding key gene expression patterns will help us gain insights into the mechanisms involved in metabolite production and fruiting body development.

Reverse transcription quantitative real-time PCR (RT-qPCR) is a highly sensitive technique used to characterize gene expression. However, to accurately quantify gene expression, all possible variables, such as the quality and amount of starting material, presence of inhibitors in different sample materials, primer design, and RNA

extraction and retro-transcription efficiency, should be considered (Ginzinger, 2002; Huggett *et al.*, 2005). A good reliable reference gene can help to control for these experimental variations. Therefore, the selection of a good reference gene is crucial to acquire biologically meaningful data.

An ideal reference gene would be expressed at a constant level across various conditions, and its expression would not be affected by experimental parameters. However, recent studies indicated that there were no known reference genes with an invariant expression in different tissues and developmental stages under different treatments (Schmittgen & Zakrajsek, 2000; Gutierrez *et al.*, 2008a, b).

The growing number of recently published articles reflects the importance of reference genes and the need to validate them in each particular experimental model. However, most of these studies primarily addressed humans, plants, or animals. Very few studies have examined fungi, such as the pathogenic fungi *Metarhizium anisopliae* (Fang & Bidochka, 2006), *Hemileia vastatrix* (Vieira *et al.*, 2011), *Melampsora larici-populina* (Hacquard *et al.*, 2011), *Aspergillus flavus* (Suleman & Somai, 2012), and *Aspergillus niger* (Bohle *et al.*, 2007). To date, the studies of *C. militaris* gene expression have used *tef1* (Wang *et al.*, 2012; Yang & Dong, 2014) or *actin* (Yin *et al.*, 2012) as reference genes in RT-qPCR and 18S RNA as a positive control in semi-quantitative RT-PCR analysis (Xiong *et al.*, 2010). The stability of these genes has not been verified, and it is not clear whether they are suitable reference genes in this fungus.

The commercial production of *C. militaris* fruiting bodies has been established in wheat media or insects, silkworm *Bombyx mori* pupae (Hong *et al.*, 2010; Xiong *et al.*, 2010). There are usually five pivotal growth periods in *C. militaris* fruiting body cultivation, including mycelia culture, color induction, stomata stimulation, nascent fruiting body, and fully matured fruiting body. The objective of this research was to identify suitable reference genes for RT-qPCR studies in all five developmental stages of *C. militaris* cultivated on wheat medium and pupae. Eight putative reference genes, *actin*, *cox5* (cytochrome oxidase polypeptide V), *gpd* (glyceraldehyde-3-phosphate dehydrogenase), *rpb1* (polymerase II large subunit), *tef1* (the elongation factor 1-alpha), *try* (tryptophan synthase), *tub* (tubulin beta chain), and *ubi* (polyubiquitin binding protein), were partially cloned from *C. militaris*, and RT-qPCR assays were developed. The expression stability of each candidate gene was evaluated in the samples from wheat medium, pupae, and total samples using four widely used methods, GENORM (Vandesompele *et al.*, 2002), NORMFINDER (Andersen *et al.*, 2004), BESTKEEPER (Pfaffl *et al.*, 2004), and the comparative ΔC_t method (Silver *et al.*, 2006). At last, we generated an overall ranking of the candidate reference genes according to the geometric mean. Our results will provide guidelines for reference gene selection and also represent a foundation for more accurate and widespread use of RT-qPCR in *C. militaris* gene expression analysis.

Materials and methods

Fruiting body cultivation and sample collection

The strain CGMCC3.16321 used in this study was maintained as stocks on potato dextrose agar at 4 °C. The fungus was cultivated in wheat medium as previously described (Zhan *et al.*, 2006) or silkworm pupae following the method of Hong *et al.* (2010). Fungal materials (Fig. 1) were sampled at five different developmental stages after inoculation. Three independent samples of each developmental stage were collected and stored at −70 °C until total RNA was isolated.

RNA isolation and quality controls

RNA was isolated from 100 mg of frozen mycelia using the TRIzol reagent (Invitrogen) and was then treated with RQ1 RNase-free DNase (Promega). RNA concentration was measured using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies). Only RNA preparations having an $A_{260\ nm}/A_{280\ nm}$ ratio of 1.8–2.0 and an $A_{260\ nm}/A_{230\ nm}$ ratio >2.0 were used for subsequent analysis.

Primer design and real-time PCR analysis

Eight housekeeping genes, *actin*, *cox5*, *gpd*, *rpb1*, *tef1*, *try*, *tub*, and *ubi*, were selected for investigation. Primers were designed in exon region (Table 1) using Primer Premier 5.0 and had melting temperatures of 58–61 °C and primer lengths of 20–25 bp. All primers were custom-ordered from a commercial supplier (Beijing Genomics Institute, Beijing, China).

cDNA was synthesized with ReverTra Ace qPCR RT Master Mix (Toyobo Co., Ltd, Japan). To check primer specificity, PCR was performed on cDNA, and the sizes of the PCR products were verified on a 1% agarose gel. The PCR products that produced a single peak were used in our study.

qPCR was performed using the Mastercycler ep realplex (Eppendorf, Germany) real-time PCR system. qPCRs totaled 25 μL, containing 5 ng cDNA, 0.4 μM primers, and 12.5 μL QPCR SYBR Green Mix (Toyobo Co., Ltd). The thermal cycling conditions were as follows: 95 °C for 1 min, followed by 40 cycles of 15 s at 95 °C, 15 s at

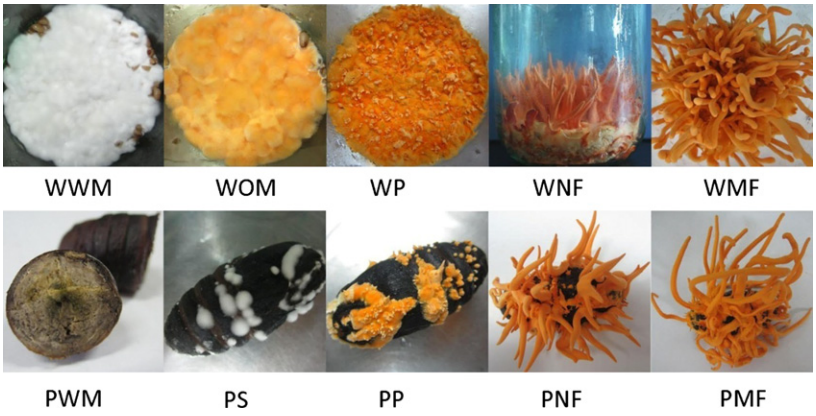

Fig. 1. Different developmental stages of *Cordyceps militaris* used for RNA extractions. Five different developmental stages in wheat medium (upper) or pupae (lower) were shown. From left to right: white mycelia under dark (WWM, 7 days after inoculation), orange mycelia with apparent carotenoid accumulation after being exposed to light (WOM, 10 days after inoculation), primordium (WP, 17 days after inoculation), nascent fruit body (WNF, 35 days after inoculation), and fully matured fruit body (WMF, 55 days after inoculation) in the upper and white mycelia under dark (PWM, after inoculation 10 days), sclerotium (PS, 15 days after inoculation), primordium (PP, 22 days after inoculation), nascent fruit body (PNF, 30 day after inoculations), and fully matured fruit body (PMF, 50 days after inoculation) in the lower.

Table 1. The candidate reference genes, primer sequences, and the PCR efficiency

Gene name	Description	GenBank accession	Primer sequences (forward/reverse)	Product Tm (°C)	Efficiency (%)
actin	Actin protein	XM_006668935	GGCGAGACCAGGGTACATAGTG GCGTGAAATCGTTCGTGAC	89	99
cox5	Cytochrome c oxidase polypeptide V	XM_006672564	CGTTGCCTGGGGTGTTGCTCT GTGATGGGGTTGGCGTTTTGG	88.5	101
gpd	Glyceraldehyde-3-phosphate dehydrogenase	XM_006669697	GCAACGCCGTCGAGCACAA AAAACACCGTGGGAGGAGTCATAC	86.5	98
rpb1	Polymerase II large subunit	KC242729.1	CTGTTCCCCCTCCTCCTGTG CTGTTCCCCCTCCTCCTGTG	85.5	104
tef1	Elongation factor 1-alpha	DQ070019	GTCAAGGAAATCCGTCGTGGTAA GCAGGCGATGTGAGCAGTGTG	89	97
try	Tryptophan synthase	XM_006672627	CGATGCTTCCCTTGAACCACT GTCTCCCTCATGGCAGAGATCA	86.5	96
tub	Tubulin beta chain	XM_006669203	ATCACCACCTGCCTGCGTT GCGACCGTTGCGGAAGTC	89	94
ubi	Polyubiquitin binding protein	XM_006672469	ACCGCTGAAGTTGCCATATAACC GTCGCACCCTTTGTATTTGTTGT	85	109

58 °C, and 45 s at 72 °C. A protocol of 58 °C for 1 min, 65–95 °C at 0.5 °C increments, and 95 °C for 5 s was used for the melting curve analysis. Three biological replicates with two technical replicates were analyzed.

RT-qPCR data analysis

In addition to the two different samples cultivated with wheat medium or pupae, the eight candidate reference genes were evaluated from the total samples. The evaluation was carried out using the four widely used methods, GENORM (Version win 3.5), NORMFINDER, BESTKEEPER (Version 1), and the comparative ΔC_t method. The overall ranking of candidate reference genes was generated according to a previously reported method (Chen *et al.*, 2011).

Results

Candidate reference gene selection

We selected genes with roles in various cellular processes as follows: *actin* and *tub* (cytoskeletal structure), *ubi* (protein degradation), *rpb1* and *tef1* (transcription and translation), and *gpd*, *try*, and *cox5* (metabolism related). Six genes, *actin*, *tub*, *ubi*, *rpb*, *tef1*, and *gpd*, are classical housekeeping genes commonly used as reference genes in

many organisms, including animals, plants, and humans. We examined *try* because it was the most suitable reference gene for real-time RT-PCR analysis of *M. anisopliae* germination, conidiogenesis, and pathogenesis, which is a fungus phylogenetically close to *C. militaris* (Fang & Bidochka, 2006). We examined *cox5* because it was shown to be a suitable reference gene in *A. flavus* (Suleman & Somai, 2012) and *A. niger* (Bohle *et al.*, 2007).

Two additional housekeeping genes, 18S and 28S RNA, are often used as reference genes for the normalization of RT-qPCR data; therefore, we also examined them in our preliminary experiments. Similar to reports in other species (de Almeida *et al.*, 2010; Zhou *et al.*, 2012), the C_t value of 18S or 28S was much lower than those of the other candidates tested, suggesting that its expression was several orders of magnitude higher than the other genes and may deviate from the confidence interval of the standard curve. Thus, we discourage using 18S or 28S RNA as a reference gene for qPCR in *C. militaris*.

qPCR specificity and primer efficiency

Descriptions of the candidate reference genes and their primer sets are listed in Table 1. Their specificity was confirmed by the presence of a single band of the expected size in agarose gels following electrophoresis (Fig. S1) and the single-peak melting curves of the PCR products (Fig. S2). We did not observe primer dimers or other nonspecific products. The efficiency of the primers ranged from 94% to 109% (Table 1). All primers were specific, and their efficiencies were acceptable for further studies.

Reference gene candidate expression profiles

We evaluated the expression levels of all eight candidate reference genes by calculating the threshold cycle (C_t) values from the total samples. Box plots of C_t values for each gene are shown in Fig. 2 ($n = 60$ for each gene). The C_t values for the eight genes studied ranged from 21.59 to 31.73, with the majority of these values between 23 and 28.

GENORM analysis

The average expression stability (M) of the eight candidates was calculated with the GENORM software (Vandesompele *et al.*, 2002), which is based on the 'pairwise comparison strategy' (Fig. 3). The M value is defined as the average pairwise variation of a particular gene with all other reference genes within a given group of cDNA samples. The gene with the lowest M value is considered the most stable, while the gene with the highest M value has

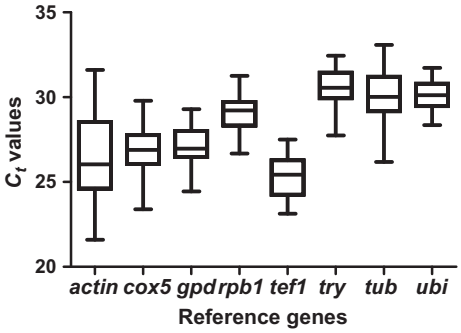

Fig. 2. Distribution of threshold cycle (C_t) values for candidate reference genes across experimental samples in *Cordyceps militaris*. The line crossing the box represents the median. The box indicates the 25 and 75th percentiles. Whiskers represent the maximum and minimum values.

the least stable expression. The *rpb1* and *gpd* genes were ranked the highest in samples grown on wheat medium ($M = 0.565$; Fig. 3a), whereas *rpb1* and *try* were most stably expressed in the samples grown in pupae medium ($M = 0.374$; Fig. 3b) and total samples ($M = 0.606$; Fig. 3c). *Actin* and *tub* had the highest M values in all sample sets, indicating that their expression levels were the most variable (Fig. 3). The GENORM software establishes a maximum value of $M = 1.5$, assuming that a gene is stably expressed (Vandesompele *et al.*, 2002). Although some previous studies used a safer maximum value of $M = 0.5$ (Gutierrez *et al.*, 2008a, b), Hellemans *et al.* (2007) found M values lower than 0.5 in relatively homogeneous samples, whereas M values increase up to 1 in more heterogeneous panels for stably expressed reference genes.

It has been previously suggested that two or more reference genes in RT-qPCR studies can generate more reliable results (Tricarico *et al.*, 2002; Vandesompele *et al.*, 2002). To determine the optimal number of genes required for accurate normalization, we calculated pairwise variations $Vn/Vn + 1$ between sequential NF values (NFn, where n = number of genes included) to evaluate the effect of adding the next reference gene in normalization. We found that in all the three sample sets, the addition of an extra reference gene improved normalization factor (NF) stability: $V5/6$ had a V value lower than 0.15 (Fig. 4). Thus, the five most stable genes (*rpb1*, *gpd*, *try*, *ubi*, and *tef1* for samples in wheat medium and the total sample group and *rpb1*, *try*, *cox5*, *ubi*, and *tef1* for samples in pupae) were sufficient for accurate normalization.

NORMFINDER analysis

We also analyzed the data with the NORMFINDER algorithm to determine the optimal reference genes for RT-qPCR

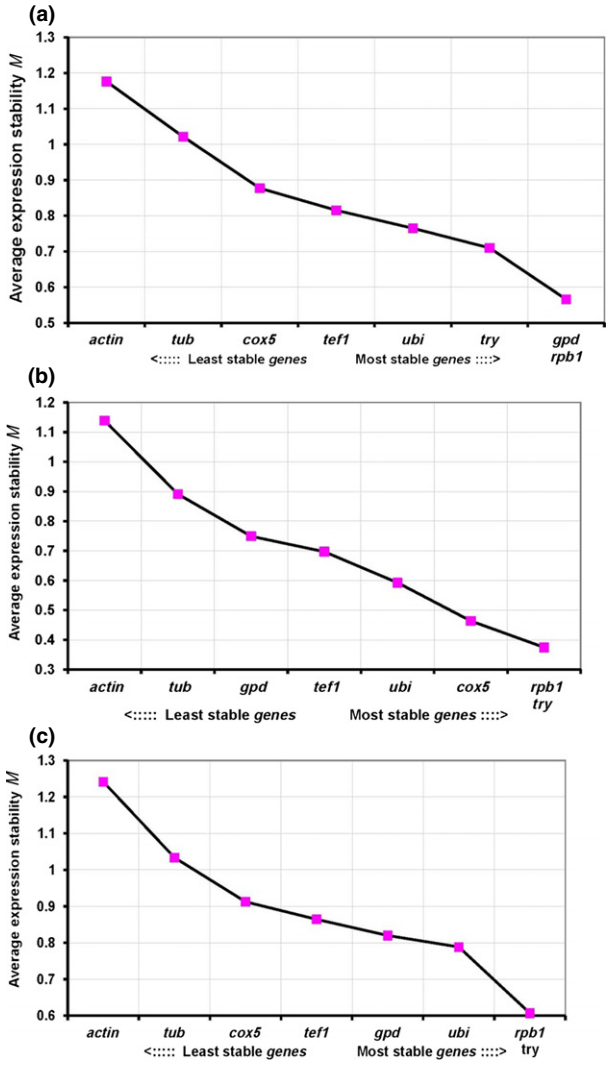

Fig. 3. Expression stability and ranking of candidate reference genes as calculated by GENORM. Average expression stability values (*M*) of the reference genes were measured during stepwise exclusion of the least stable reference genes. A lower *M* value indicated more stable expression, as analyzed by the GENORM software in *Cordyceps militaris* sample sets of different media, including five stages grown in wheat medium (a), pupae (b), and all analyzed samples (c).

normalization. While 'GENORM' selects two genes with low intragroup variation and approximately the same nonvanishing intergroup variation, 'NORMFINDER' takes into account the best two genes with minimal combined intra- and intergroup expression variation for NF calculations (Andersen *et al.*, 2004). Compared with the GENORM analysis, there were minor differences in the ranking of the three most stable genes (Table 2). It determined the most stable genes in each condition as follows: *gpd*, *rpb1*, and *try* for samples in wheat medium, *ubi*, *rpb1*, and *tef1* for samples in pupae, and *rpb1*, *gpd*, and *tef1* for total

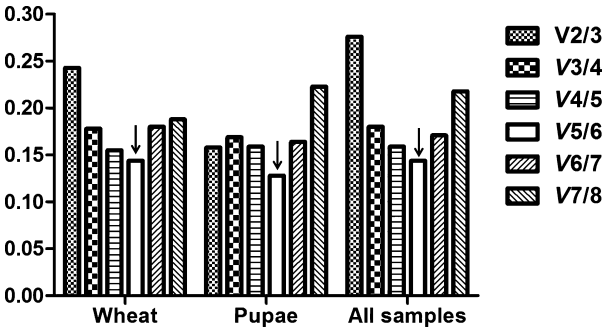

Fig. 4. Determination of the optimal number of reference genes. Pairwise variation (*V*) calculated by GENORM to determine the minimum number of reference genes for accurate normalization in different experiment conditions. Arrow indicates the optimal number of genes for normalization in each sample sets.

samples. However, the two least stable genes, *actin* and *tub*, were identical in all three sample sets and consistent with the GENORM analysis.

BESTKEEPER analysis

The Excel-based tool BESTKEEPER analyzes expression variability of candidate reference genes by calculating cycle threshold (C_t) data variation. The standard deviation (SD), coefficient of variance (CV), correlation coefficient (R), and the P value calculated by the BESTKEEPER are shown in Fig. S3. It is essential to note that genes with an SD > 1 should be considered unacceptable (Pfaffl *et al.*, 2004). Thus, four genes (*actin*, *tub*, *gpd*, and *try* for samples in wheat medium and *actin*, *tub*, *cox5*, and *tef1* for total samples) and three genes (*actin*, *tub*, and *cox5* for samples in pupae medium) had unstable expression levels (C_t value SD > 1.0). The BESTKEEPER analysis highlighted *ubi* for samples in pupae and *rpb1* for samples in wheat medium and total samples as the most stable genes with the highest correlation coefficient (Table 2 and Fig. S3). The least stable genes (*actin* and *tub*), as indicated by BESTKEEPER, were in agreement with those selected by GENORM and NORMFINDER.

Comparative ΔC_t

We also used the comparative ΔC_t method to estimate the most stable reference genes. The comparative ΔC_t method ranks candidate gene stability according to the repeatability of gene expression differences (Silver *et al.*, 2006). The comparative ΔC_t results of pupae samples were almost identical to those of NORMFINDER, except the first and second rankings were switched (Table 2). The wheat medium samples and total sample group also showed almost identical rankings. The comparative ΔC_t

Table 2. Eight candidate reference genes ranked by different methods

Ranking	GENORM	NORMFINDER	BESTKEEPER	ΔC_t method	Overall ranking
All samples					
1	*rpb1/try*	*rpb1*	*rpb1*	*rpb1*	*rpb1* (1.00)*
2		*gpd*	*gpd*	*gpd*	*gpd* (2.37)
3	*ubi*	*tef1*	*try*	*tef1*	*try* (2.91)
4	*gpd*	*ubi*	*ubi*	*try*	*tef1* (3.87)
5	*tef1*	*cox5*	*tef1*	*cox5*	*ubi* (4.11)
6	*cox5*	*try*	*cox5*	*ubi*	*cox5* (5.48)
7	*tub*	*tub*	*tub*	*tub*	*tub* (7.00)
8	*actin*	*actin*	*actin*	*actin*	*actin* (8.00)
Wheat medium					
1	*gpd/ rpb1*	*gpd*	*rpb1*	*rpb1*	*rpb1* (1.19)
2		*rpb1*	*tef1*	*gpd*	*gpd* (1.57)
3	*try*	*try*	*cox5*	*tef1*	*tef1* (3.31)
4	*ubi*	*tef1*	*ubi*	*try*	*try* (3.66)
5	*tef1*	*cox5*	*try*	*ubi*	*ubi* (4.68)
6	*cox5*	*ubi*	*gpd*	*cox5*	*cox5* (4.82)
7	*tub*	*tub*	*tub*	*tub*	*tub* (7.0)
8	*actin*	*actin*	*actin*	*actin*	*actin* (8.0)
Pupae					
1	*rpb1/ try*	*ubi*	*ubi*	*rpb1*	*rpb1*(1.41)
2		*rpb1*	*rpb1*	*ubi*	*ubi* (1.68)
3	*cox5*	*tef1*	*tef1*	*tef1*	*try* (3.31)
4	*ubi*	*cox5*	*try*	*cox5*	*tef1* (3.41)
5	*tef1*	*gpd*	*gpd*	*try*	*cox5* (4.12)
6	*gpd*	*try*	*cox5*	*gpd*	*gpd* (5.48)
7	*tub*	*tub*	*tub*	*tub*	*tub* (7.00)
8	*actin*	*actin*	*actin*	*actin*	*actin* (8.00)

*The number in the bracket indicated the geometric mean calculated by the method of Chen *et al.* (2011).

method identified the same unstable genes for each sample set as the other methods (*actin* and *tub*).

Comprehensive ranking order

Our recommended comprehensive rankings are shown in Table 2. The *rpb1* gene was the most stable, whereas *actin* and *tub* were the least stable among different sample sets. The ranks of *ubi* and *gpd* varied in different sample sets. The *gpd* gene ranked second overall in wheat medium samples and total sample groups but ranked sixth in pupae samples. The *ubi* gene ranked fifth overall in wheat medium samples and total samples, but it ranked second in pupae samples.

Discussion

To properly normalize gene expression levels in RT-qPCR analyses, it is critical to select a proper reference. In this study, we evaluated eight candidate reference genes and analyzed which are suitable for RT-qPCR gene expression analysis in *C. militaris* cultivated in different media from different developmental stages. This is the first detailed study on the stability of several genes used as internal controls for RT-qPCR studies in *C. militaris*. The results will be a reference for the gene expression analysis in this fungus and also show the importance of the reference gene selection.

Our results indicate that *rpb1* is the best reference gene in total *C. militaris* samples. In wheat medium samples, *rpb1* was ranked first by GENORM, BESTKEEPER, and comparative ΔCt, and it was ranked second by NORMFINDER. For the pupae medium sample, *rpb1* was ranked first by GENORM and ΔC_t and second by NORMFINDER and BESTKEEPER. Despite slight discrepancies among methods, we suggest that *rpb1* is the best reference gene for all three sample sets tested. *rpb1* (also called RP II in the literature) is also the most suitable reference gene in peach when all samples were pooled (Tong *et al.*, 2009) and human CCRF-HSB-2 cells in different tissues following stimulation (Radonić *et al.*, 2004). However, *rpb1* was considered to be an unstable gene in *Ganoderma lucidum* (Xu *et al.*, 2014). Although *rpb1* is relatively unused as a reference gene, our results suggest that *rpb1* should be considered as a reference gene.

In contrast, *actin* and *tub* were the least stable genes among the eight candidates tested, as calculated by all four methods. Previous reports have also shown unacceptable variability in *tub* expression in several other species

(Lord *et al.*, 2010; Zhou *et al.*, 2012). Yin *et al.* (2012) validated gene expression in the cordycepin metabolic pathway by quantitative RT-PCR using *actin* as a reference gene in both the *C. militaris* fruiting body stage and mycelium stage. We used the same primer sequences for *actin* in our study, and we suggest that reference gene expression must be validated before using it to normalize experimental samples. The identification of reliable reference genes is time-consuming and expensive, but it is necessary for accurate gene expression analyses.

Tef1 is another gene that has been used as reference gene in *C. militaris* expression studies (Wang *et al.*, 2012; Yang & Dong, 2014). In the present study, *tef1* ranked the third or fourth among the 8 candidate genes. In our previous study (Yang & Dong, 2014), four genes (*actin*, *tub*, *gpd*, and *tef1*) were used to identify reliable reference genes in *C. militaris* under different light conditions by BESTKEEPER. Our results in this study were similar among these four genes: *tef1* was ranked the most stable, followed by *gpd*, *actin*, and *tub*. We suggest that several candidate reference genes should be used to identify reliable reference genes.

Many studies have suggested that two or more reference genes should be used for RT-qPCR studies to generate more reliable results (Tricarico *et al.*, 2002; Vandesompele *et al.*, 2002). In this study, the five most stable genes were needed for accurate normalization. However, taking into account the consistency of *rpb1* expression across all sample sets and in all four analysis methods, the economy and convenience, we suggest that the single *rpb1* gene is sufficient as the sole reference gene in the conditions tested.

According to a previous study by Pfaffl *et al.* (2004), the main parameters required for BESTKEEPER to evaluate potential reference genes are SD, *R*, and CV. However, in some studies, SD alone has been used as the only parameter (Wang *et al.*, 2013; Zeng *et al.*, 2014), potentially generating a biased outcome. In the present study, both SD and *R* were used to evaluate gene expression stability (Fig. S3). REFFINDER (http://www.leonxie.com/referencegene.php) was a convenient tool for data analysis and gave the comprehensive ranking. Furthermore, it has been used in many previous studies (Zhu *et al.*, 2012; Jacob *et al.*, 2013). However, the BESTKEEPER online tool is only based on SD. Therefore, one should be cautious when working with this online software.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Confirmation of amplicon size and primer specificity of the studied reference genes by electrophoresis.
Fig. S2. Dissociation curve for all candidate reference genes.
Fig. S3. Candidate reference genes selection with BESTKEEPER.