



# Identification and Validation of Reference Genes for Gene Expression Analysis in Different Development Stages of *Amylostereum areolatum*

Ningning Fu, Jiaxing Li, Ming Wang, Lili Ren\*, Shixiang Zong\* and Youqing Luo

Beijing Key Laboratory for Forest Pest Control, Beijing Forestry University, Beijing, China

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### \*Correspondence:

Lili Ren  
lily\_ren@bjfu.edu.cn  
Shixiang Zong  
zongshixiang@bjfu.edu.cn

### Specialty section:

This article was submitted to  
Microbial Symbioses,  
a section of the journal  
Frontiers in Microbiology

**Received:** 01 December 2021

**Accepted:** 20 December 2021

**Published:** 12 January 2022

### Citation:

Fu N, Li J, Wang M, Ren L,  
Zong S and Luo Y (2022) Identification  
and Validation of Reference Genes  
for Gene Expression Analysis in  
Different Development Stages  
of *Amylostereum areolatum*.  
*Front. Microbiol.* 12:827241.  
doi: 10.3389/fmicb.2021.827241

A strict relationship exists between the *Sirex noctilio* and the *Amylostereum areolatum*, which is carried and spread by its partner. The growth and development of this symbiotic fungus is key to complete the life history of the *Sirex* woodwasp. Real-time quantitative polymerase chain reaction (RT-qPCR) is used to measure gene expression in samples of *A. areolatum* at different growth stages and explore the key genes and pathways involved in the growth and development of this symbiotic fungus. To obtain accurate RT-qPCR data, target genes need to be normalized by reference genes that are stably expressed under specific experimental conditions. In our study, the stability of 10 candidate reference genes in symbiotic fungal samples at different growth and development stages was evaluated using geNorm, NormFinder, BestKeeper, delta Ct methods, and RefFinder. Meanwhile, *laccase1* was used to validate the stability of the selected reference gene. Under the experimental conditions of this study, *p450*, *CYP*, and  $\gamma$ -*TUB* were identified as suitable reference genes. This work is the first to systematically evaluate the reference genes for RT-qPCR results normalization during the growth of this symbiotic fungus, which lays a foundation for further gene expression experiments and understanding the symbiotic relationship and mechanism between *S. noctilio* and *A. areolatum*.

**Keywords:** *Sirex noctilio*, *Amylostereum areolatum*, RT-qPCR, reference gene, growth and development

## INTRODUCTION

*Sirex noctilio* Fabricius (Hymenoptera; Symphyta; and Siricidae) is an important forest woodborer, which mainly damages *Pinus* species (Slippers et al., 2012) worldwide. In China, it was first reported in Daqing, Heilongjiang Province in July 2013, seriously harming *Pinus sylvestris* var. *mongolica* plantations in many regions of the country (Slippers et al., 2012; Li et al., 2015; Sun et al., 2016; Wang et al., 2018). *Amylostereum areolatum* (Fr.) Boidin (Basidiomycotina: Corticiaceae) is a

symbiotic fungus of the woodwasp, and both exist in a strictly mutualistic relationship (Biedermann and Vega, 2020; Wang M. et al., 2020; Li et al., 2021). *S. noctilio* carries the oidia and mycelia of *A. areolatum* through the specialized mycangium, which disperses and introduces the fungus into a new host during oviposition (Slippers et al., 2012; Li et al., 2015). Concurrently, *A. areolatum* digests and degrades large molecular carbohydrates to small ones in the xylem of pine wood by secreting extracellular enzymes, which provides necessary nutritional for the incubation of woodwasp eggs and larval growth and development (Talbot, 1977; Madden and Coutts, 1979; Hajek et al., 2013; Wang et al., 2019). Before the third or fourth instar stages, the woodwasp larvae obtain nutrition by directly feeding on the mycelia of the symbiotic fungus, and then on the xylem infected by *A. areolatum* (Madden and Coutts, 1979; Thompson et al., 2014). In addition, Wang L. et al. (2020) suggested that when the incidental fungi in the host tree inhibited the growth of the symbiotic fungus, the mortality of woodwasp larvae increased significantly. Therefore, we believe that the growth and development of *S. noctilio* larvae are directly affected by the growth of *A. areolatum*.

Real-time quantitative polymerase chain reaction (RT-qPCR) is a widely used method for quantitative analysis of gene expression due to its accuracy in quantification, repeatability and high sensitivity (Bustin et al., 2005; Engel et al., 2007; Gao et al., 2020). Nevertheless, the accuracy of RT-qPCR results depends on many factors, including the quality and quantity of initial RNA, primer specificity, amplification efficiency, and transcript normalization, to name a few (Nolan et al., 2006; Kozera and Rapacz, 2013). Among these, normalization is a key factor affecting the RT-qPCR results (Udvardi et al., 2008; Yang et al., 2021). We usually need to introduce reference genes to correct and normalize the expression of target genes. Due to the spatio-temporal specificity of gene expression, a suitable reference gene should be stably and effectively expressed under specific experimental conditions (Lv et al., 2020; Wang G. et al., 2020). Therefore, screening appropriate reference genes for specific experimental materials or experimental conditions is important for RT-qPCR analysis. In fungi, *18S* (18S ribosomal RNA), *28S* (28S ribosomal RNA), *GAPDH* (glyceraldehyde-3-phosphate hydrogenase), *ACT* (actin), *EF1- $\alpha$*  (elongation factor 1- $\alpha$ ), *RPB2* (RNA polymerase subunit 2), *CYP* (cytochrome P450),  $\beta$ -*TUB* ( $\beta$ -tubulin), *CYT b* (cytochrome b), and other genes have been often used as reference genes (Lian et al., 2014; Zhang et al., 2016; Jia et al., 2019; Singh et al., 2019; Song et al., 2019; Yang et al., 2019). While the selection of reference genes has been reported in fungi such as *Ganoderma lingzhi* (Xu et al., 2014), *Pleurotus eryngii* (Qin and Wang, 2015), *Auricularia cornea* (Jia et al., 2019), *Auricularia heimuer* (Zhang et al., 2020), *Pleurotus ostreatus* (Fernández-Fueyo et al., 2014), and *Lentinula edodes* (Xiang et al., 2018), there are no reports on the reference genes in *A. areolatum*. Therefore, we need to find appropriate reference genes for gene expression analysis of this symbiotic fungus.

To study the key genes involved in the growth and development of *A. areolatum*, we selected 10 candidate genes with relatively stable expression from its genome and transcriptome data. We then analyzed the expression levels of these genes

in the samples of *A. areolatum* at different growth and development stages. In order to select suitable reference genes, some algorithms and software were used to comprehensively evaluate the expression stability of these genes. The findings of this study will contribute to the study on the mechanism of growth and development of *A. areolatum*, and provide a reference basis for further exploring its symbiotic relationship with *S. noctilio*.

## MATERIALS AND METHODS

### Strains and Sample Collection

*Amylostereum areolatum* was isolated from four female woodwasps collected from *P. sylvestris* var. *mongolica* from the Jun De Forest Farm, Heilongjiang Province, China. After identification, the fungus was preserved at the Beijing Key Laboratory for the Control of Forest Pest, Beijing Forestry University, Beijing, China. *A. areolatum* samples were first cultured at 25°C on potato dextrose agar (PDA) plates for 1 week, and then five mycelial plugs (6 mm diameter) were removed from the periphery of the mycelium and inoculated in 100 mL potato dextrose broth (PDB) medium. The mycelia were allowed to grow for 7 days at 25°C, and then collected at 12 h, 1, 3, 5, and 7 days. All samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation.

### Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from samples of the fungus at different growth stages using EZgene TM Fungal RNA Kit (Biomiga, United States). The quality and quantity of total RNA were assessed by NanoDrop 2000 and electrophoresis on 1.0% agarose gels. Before reverse transcription, total RNA samples were pre-treated by gDNA Eraser (Takara, Dalian, China) to further eliminate DNA in samples. Then, we used the PrimeScript TM RT reagent Kit (Takara, Dalian, China) to synthesize the first strand cDNA according to the manufacturer's protocol. One microgram of total RNA was used to synthesize cDNA in a 20  $\mu$ L reaction system, and then diluted 10-fold for RT-qPCR.

### Candidate Reference Genes Selection and Primer Design

A total of ten stably expressing candidate reference genes of *A. areolatum* in its different growth and development stages were selected based on the transcriptome (BioProject: PRJNA733162; BioSample: SAMN19369336-SAMN19369343) and genome (GenBank: SAXG00000000; BioProject: PRJNA513942) data (Fu et al., 2020, 2021). These genes were  $\alpha$ -*TUB*,  $\beta$ -*TUB*,  $\gamma$ -*TUB*, *GAPDH*, *CYP*, *CPR* (NADPH-dependent cytochrome P450 oxidoreductase), *HH3* (Histone H3), *CESP* (coatamer epsilon subunit-domain-containing protein), *MDP* (metallo-dependent phosphatase), and *P450* (cytochrome P450). The CDS sequences of these genes were obtained from the *A. areolatum* genome data (GenBank: OL660755-OL660764), and the primers were

designed using the web software IDT<sup>1</sup> and Primer 3.0<sup>2</sup>. The primers used for amplification are listed in **Table 1**.

## Real-Time Quantitative Polymerase Chain Reaction Amplification

Real-time quantitative polymerase chain reaction amplification was performed using a Bio-Rad CFX Connect real-time PCR instrument (BIO-RAD, United States) with TB Green® Premix Ex Taq™ II (Takara, Japan). The amplification was performed in a 25 µL reaction volume according to the manufacturer's instructions. Each reaction contained 12.5 µL of TB Green Premix Ex Taq II (2×), 1 µL of forward and reverse primers (10 µM), 2 µL of cDNA template (25 ng/µL) and 8.5 µL of RNase free water. The amplification was conducted as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. A single melting curve from 65 to 95°C (increments of 0.5°C every 5 s) was performed to verify the primer specificity. Four biological (each with a different isolate) and three technical replicates per isolate were performed independently. The relative standard curve for each candidate gene was analyzed using a 10-fold dilution series (1, 1/10, 1/100, 1/1,000, and 1/10,000) of cDNA as template and the amplification efficiency was calculated using the slope of the linear regression equation:  $E\% = [10(-1/\text{slope}) - 1] \times 100\%$ .

## Stability Analysis of Candidate Reference Genes

The cycle threshold (*Ct*) values of amplification of genes in samples from different growth and development stages of

*A. areolatum* were collated and summarized using Microsoft Excel. The stability of the ten candidate reference genes was analyzed by geNorm (v 3.5) (Vandesompele et al., 2002), NormFinder (v 0.953) (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), RefFinder<sup>3</sup> (Xie et al., 2012), and delta Ct method (Silver et al., 2006). For geNorm, the expression stability value (*M*) of the candidate reference gene was calculated according to the pairwise variation (*V*) between two candidate genes. Candidate reference genes with lower *M* values were more stable. Additionally, the optimal numbers of reference genes were determined by calculating the pairwise variation  $V_n/V_{n+1}$ . When this value was less than 0.15, *n* reference genes were needed to ensure the accurate normalization of RT-qPCR data (Vandesompele et al., 2002). To estimate intra- and inter- group variations between samples, NormFinder was used based on the linear mixed effect model. Stable candidate reference genes had lower *S* values (Andersen et al., 2004). The stability of candidate reference genes was analyzed by GeNorm and NormFinder using the  $2^{-\Delta Ct}$  values, of which  $\Delta Ct$  = average *Ct* of the sample—minimum *Ct* of all RT-qPCR data (Zhao et al., 2020). The standard deviation (SD) and stability value (SV) of candidate reference genes were directly calculated by BestKeeper using the original *Ct* value to evaluate the stability of these genes (Pfaffl et al., 2004). The genes with lower SD and SV values had a more stable expression. Similarly, the delta Ct method was used to calculate the mean SD of the paired genes in each sample to verify the stability of their expression. Finally, RefFinder integrated these four methods to rank the candidate reference genes and evaluate the comprehensive stability of these genes (Xie et al., 2012).

<sup>1</sup><https://sg.idtdna.com/pages>

<sup>2</sup><https://bioinfo.ut.ee/primer3-0.4.0/>

<sup>3</sup><http://blooge.cn/RefFinder/>

**TABLE 1** | Primer sequences and amplification characteristics of the candidate reference genes.

Symbol	Gene name	Primer (5'-3')	Size (bp)	Efficiency%	R <sup>2</sup> value
<i>α-TUB</i>	α-tubulin	F: CGTGTTCGAGAGCGGTAAT R: GGATCGTGCGCTTAGTCTTAAT	114	90.4	0.992
<i>β-TUB</i>	β-tubulin	F: CATTGACAACGAGGCTCTCTAC R: GACATGACGATGGAAACGAGAT	99	97.0	0.992
<i>γ-TUB</i>	γ-tubulin	F: TGTGTCGGCATGATTGAGAG R: TCGGTGTGATTGAGCATTTAAC	102	96.5	0.998
<i>GAPDH</i>	Glyceraldehyde-3-phosphate hydrogenase	F: GACTCCAAGTACACCGTCATC R: TCGACGATGCCGAACCTATC	95	98.4	0.994
<i>CYP</i>	Cyclophilin	F: CTCGATGACGGTTTGGATATAA R: GTCACCACCCTGGATCATAAA	75	105.7	0.987
<i>CPR</i>	NADPH-dependent cytochrome P450 oxidoreductase	F: CGTCGATCCCTTGAGAAGAAC R: CGGCCACTCATCACTGTAAA	114	103.1	0.995
<i>HH3</i>	Histone H3	F: ACAAGACTTCAAGACGGATCTC R: TGGTGTCTCGAACAATGAG	98	96.9	0.994
<i>CESP</i>	Coatomer epsilon subunit-domain-containing protein	F: ACAAGACGATGCGGAAACA R: CGGCATATTTGGAAACGAACC	107	100.7	0.993
<i>MDP</i>	Metallo-dependent phosphatase	F: AGCACAATGACGGGATGTT R: CCCATTTCCGACGACTTTGA	101	109.7	0.994
<i>P450</i>	Cytochrome P450	F: CACCTTTGCAGTCTACCTACTT R: CAGCTCCTTCAGATCGTCTATG	118	107.1	0.996

## Validation of Selected Reference Genes

Based on the transcriptome data, *laccase1* was used to verify the stability of the selected reference genes. The primers used for amplification of *laccase1* were F (5'-3': GTAGGAGCA CGTCCATTCATT) and R (5'-3': TTGTAGAGGAACGTC GCATTT). The expression levels of *laccase1* in *A. areolatum* samples were detected by RT-qPCR using the same procedure as mentioned above and the most stable and unstable reference genes were used to normalize the expression levels of *laccase1* in different samples. The relative expression of genes was calculated using the  $2^{-\Delta\Delta Ct}$  method, followed by taking its square root. The one-way analysis of variance (ANOVA) with the *post hoc* Tukey's-HSD test was used to evaluate the effect on the expression of target genes by different reference genes (Florez et al., 2020).

## RESULTS

### Primer Performance During Analysis of Candidate Reference Genes

The specificity of 10 candidate reference gene primers was determined by resolving the amplified products using 2% agarose gel electrophoresis, and a specific band of about 100 bp was obtained for each gene (Supplementary Figure 1). Consistently, the RT-qPCR melting curves showed that each reference gene produced a single peak, with no primer dimer or non-specific amplification (Supplementary Figure 2). In addition, the efficiency of RT-qPCR amplification of 10 candidate reference genes was between 90.4 and 109.7%, and the linear correlation coefficient ( $R^2$ ) was greater than 0.99 (except for *CYP*, which was 0.987) (Table 1). This suggested that all the primers for amplification of these genes were specific and efficient, and could meet the requirements of reference genes analysis.

### Expression Analysis of the Reference Genes

We analyzed the expression levels of 10 candidate reference genes in samples of different growth and development stages of *A. areolatum* by RT-qPCR. Average *Ct* values of these genes were in the range of 18.04–25.00 (Figure 1). Among these, the gene with the highest expression was *GAPDH* (average *Ct* = 18.04), and that with the lowest expression was  $\gamma$ -*TUB* (average *Ct* = 25.00). According to *SD* values, the genes  $\gamma$ -*TUB* (average *Ct*  $\pm$  *SD* = 25.00  $\pm$  0.48), *CPR* (average *Ct*  $\pm$  *SD* = 21.54  $\pm$  0.74), and *CYP* (average *Ct*  $\pm$  *SD* = 21.75  $\pm$  0.79) showed low variation in expression in the analyzed samples, while the genes  $\beta$ -*TUB* (average *Ct*  $\pm$  *SD* = 22.05  $\pm$  1.41), *GAPDH* (average *Ct*  $\pm$  *SD* = 18.04  $\pm$  1.23), and  $\alpha$ -*TUB* (average *Ct*  $\pm$  *SD* = 22.10  $\pm$  1.13) showed the highest variation.

### Stability Analysis of Candidate Reference Genes

In geNorm analysis, reference genes with *M* values less than 1.5 were generally considered to be stable. In our study,

the *M* values of candidate reference genes evaluated by geNorm were all less than 1.5, indicating relatively stable expression of these genes. Among these, *CYP* and *P450* exhibited the most stable expression in those analyzed samples, while  $\beta$ -*TUB* was the most unstable gene (Figure 2 and Table 2).

The optimal number of reference genes was estimated using the pairwise variation ( $V_n/V_{n+1}$ ) calculated by geNorm. In our study, the value of  $V_2/V_3$  was 0.151, which was greater than the cut-off value of 0.15, while other pairwise variation values were all less than 0.15 (Figure 3). Therefore, under the experimental conditions in this study, three reference genes were needed to normalize the target genes.

For NormFinder, it evaluates the stability of candidate genes based on an ANOVA-based algorithm. Under our experimental conditions, the order of stability of reference genes evaluated by NormFinder software was *CYP* > *P450* > *HH3* >  $\alpha$ -*TUB* >  $\gamma$ -*TUB* > *CPR* > *MDP* > *CESP* > *GAPDH* >  $\beta$ -*TUB* (Table 2).

The stability of the candidate reference genes was evaluated using the BestKeeper by directly calculating the *SD* and *CV* of *Ct* values of these genes. According to the evaluation results,  $\gamma$ -*TUB*, *CPR*, and *P450* were the most stable genes in the *A. areolatum* samples at different growth and development stages, while  $\alpha$ -*TUB*, *GAPDH*, and  $\beta$ -*TUB* were poorly stable. In particular,  $\beta$ -*TUB* had an *SD* value greater than 1 and was thus considered to be an unstable reference gene (Table 2).

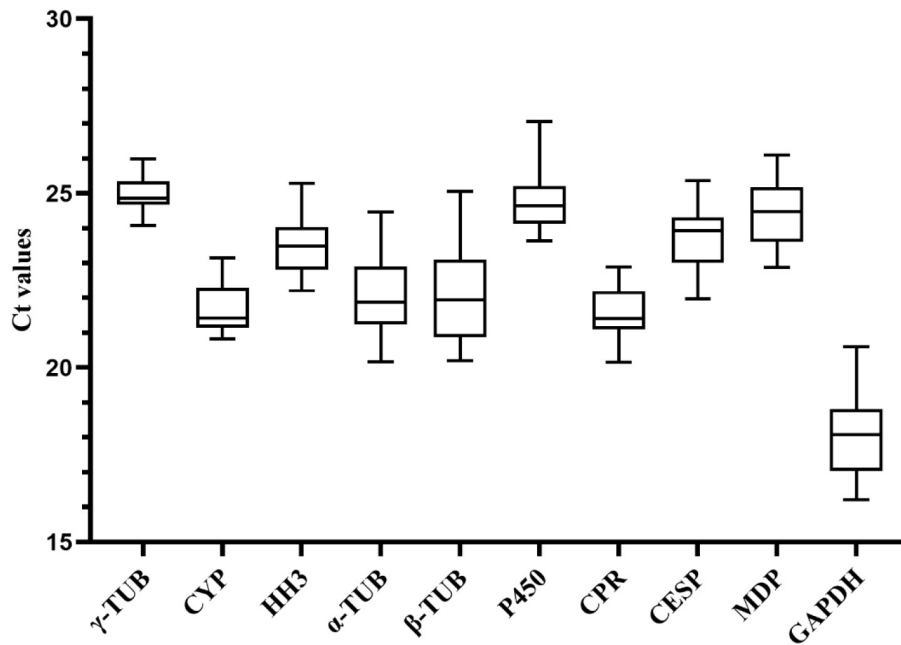
We also used the delta *Ct* method to evaluate the stability of the candidate reference genes. The average *SD* of these genes ranged from 0.66 to 1.07 (Table 2). As a result, *P450* was the most stable reference gene, while  $\beta$ -*TUB* was the most unstable gene in the symbiotic fungus samples at different growth and development stages.

The stability rankings of the 10 candidate reference genes evaluated by geNorm, NormFinder, BestKeeper, and delta *Ct* were not completely consistent (Figure 2 and Table 2). For a more accurate analysis, we used the comprehensive analysis software RefFinder to calculate the geometric mean of the results for each gene obtained by geNorm, NormFinder, BestKeeper, and delta *Ct* to provide a comprehensive ranking of the reference genes. The candidate genes evaluated by RefFinder were ranked from the most to the least stable as: *P450* > *CYP* >  $\gamma$ -*TUB* > *HH3* > *CPR* >  $\alpha$ -*TUB* > *CESP* > *MDP* > *GAPDH* >  $\beta$ -*TUB*. According to the optimal number calculated by geNorm and the reference gene ranking obtained by RefFinder, the optimal reference gene combination under our experimental conditions was *P450* + *CYP* +  $\gamma$ -*TUB*.

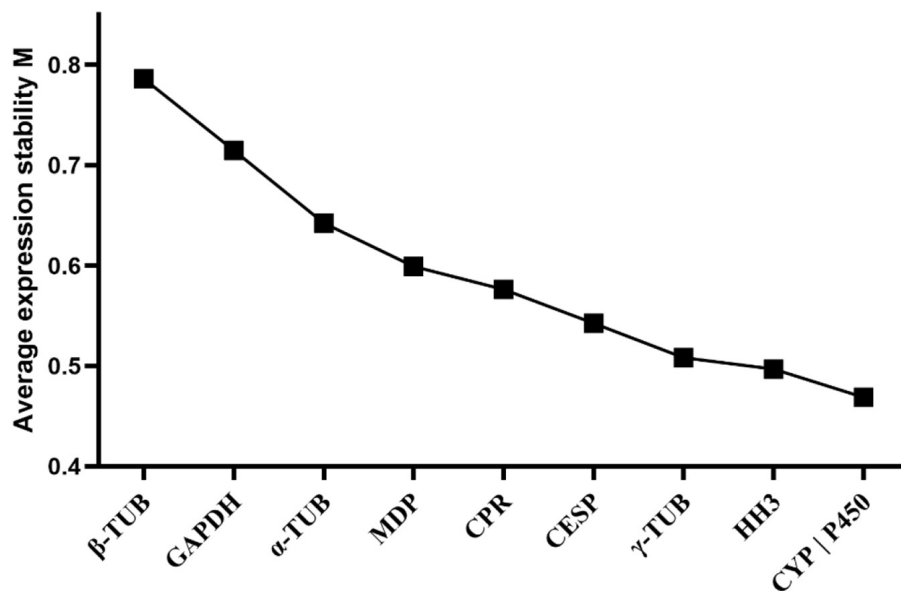
### Validation of the Selected Reference Genes

To verify the reliability of the selected reference genes, the expression levels of *laccase1* in the above-analyzed samples were normalized using the three most stable candidate reference genes (*P450*, *CYP*, and  $\gamma$ -*TUB*), the combination of these stable genes (*P450* + *CYP* +  $\gamma$ -*TUB*), and the two most unstable reference genes (*GAPDH* and  $\beta$ -*TUB*). We found that when *P450*, *CYP*, and  $\gamma$ -*TUB* and their combinations were used





**FIGURE 1** | Cycle threshold ( $C_t$ ) values of 10 candidate reference genes across all samples at different development stages. The box indicates the 25th and 75th percentiles and the line in the box represents the median value.



**FIGURE 2** | Average expression stability and ranking of the candidate reference genes calculated by geNorm. Candidate reference genes with lower  $M$  values were more stable. The least stable genes are listed on the left and the most stable genes are listed on the right.

as reference genes, the expression levels of *laccase1* in the same sample were nearly consistent. When  $\beta$ -TUB was used as the reference gene, the expression levels of *laccase1* in the *A. areolatum* samples grown for 10 and 12 days were significantly different from the normalized results of other reference genes (Figure 4). Similarly, the expression levels of *laccase1* normalized by GAPDH also showed significant differences in 12-day old

samples. The expression of *laccase1* in samples at different growth and development stages normalized by P450, CYP, and  $\gamma$ -TUB as well as their combination was consistent with the results of previous transcriptome studies (Fu et al., 2021). These findings indicated that these three genes individually or in combination were reliable as reference genes under our experimental conditions.

**TABLE 2** | Expression stability ranking of the 10 candidate reference genes based on five algorithms.

Reference genes	geNorm		NormFinder		BestKeeper			delta Ct		RefFinder	
	M	Rank	SV	Rank	SD	CV	Rank	Avg. Ct	Rank	GM	Rank
<i>P450</i>	0.47	1	0.26	2	0.64	2.57	3	0.66	1	1.57	1
<i>CYP</i>	0.47	1	0.24	1	0.67	3.1	5	0.67	2	1.78	2
$\gamma$ - <i>TUB</i>	0.51	4	0.34	5	0.38	1.52	1	0.73	4	2.99	3
<i>HH3</i>	0.50	3	0.29	3	0.66	2.82	4	0.70	3	3.22	4
<i>CPR</i>	0.58	6	0.35	6	0.58	2.68	2	0.75	6	4.56	5
$\alpha$ - <i>TUB</i>	0.64	8	0.32	4	0.91	4.13	8	0.74	5	5.98	6
<i>CESP</i>	0.54	5	0.45	8	0.72	3.03	6	0.82	8	6.62	7
<i>MDP</i>	0.60	7	0.36	7	0.82	3.37	7	0.77	7	7.00	8
<i>GAPDH</i>	0.72	9	0.54	9	0.94	5.22	9	0.94	9	9.00	9
$\beta$ - <i>TUB</i>	0.79	10	0.66	10	1.15	5.23	10	1.07	10	10.00	10

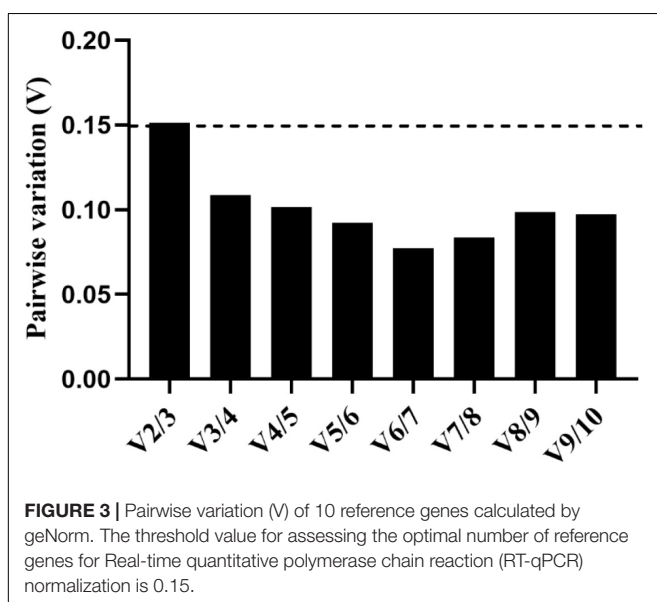
M, expression stability value; SV, stability value; SD, standard deviation; CV, coefficient of variation; Avg. Ct, average Ct; GM, geometric mean.

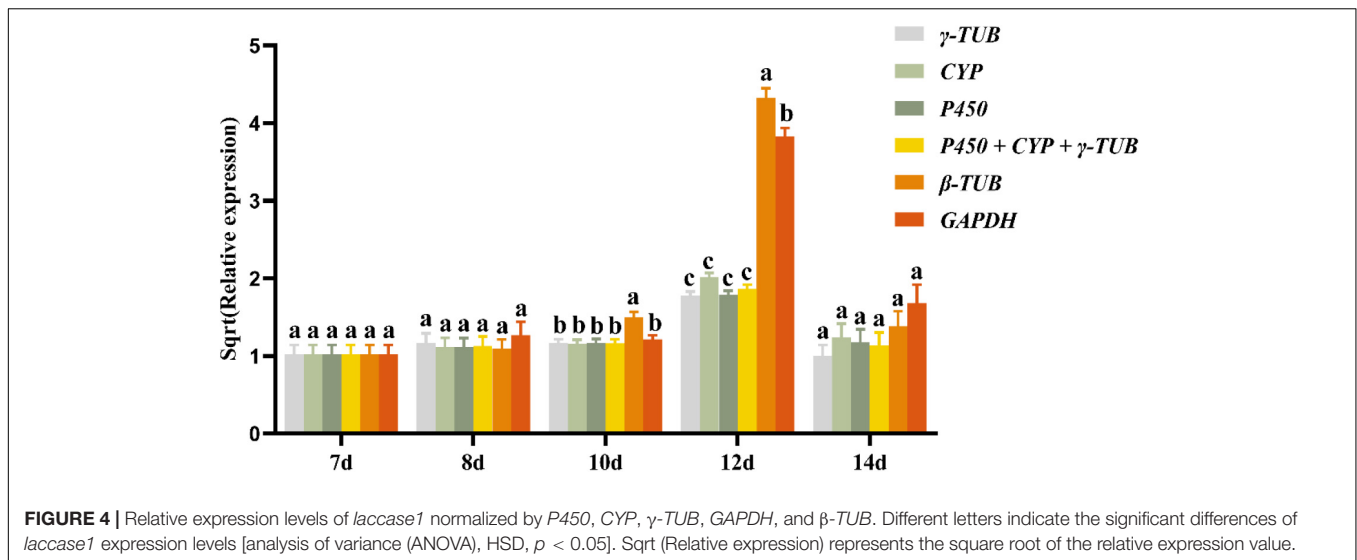
## DISCUSSION

There is a strict mutualism between *S. noctilio* and *A. areolatum* (Slippers et al., 2012; Biedermann and Vega, 2020). The growth and development of this symbiotic fungus in host trees play an important role in the life history of the woodwasp (Li et al., 2015). A previous study showed that when the growth of the symbiotic fungus was restricted, the mortality rate of woodwasp larvae increased significantly (Wang L. et al., 2020). Thus, the growth of the *A. areolatum* is crucial for the growth and development of the *S. noctilio* larvae. At present, the genome (Fu et al., 2020) and transcriptome (Fu et al., 2021) sequencing of the *A. areolatum* has been completed by our laboratory; the obtained data provide a possibility for the analysis of key genes in the growth and development of this symbiotic fungus. These transcriptome data must be verified by RT-qPCR and to obtain effective amplification results, appropriate reference genes are required to normalize the target genes, as well as to reduce the experimental or

inter-sample errors. Studies have found that there were no consistently expressed reference genes in different strains or under different experimental conditions. For example, Wang G. et al. (2020) showed that in *Tolypocladium guangdongense*, the vacuolar protein sorting gene *VPS* was the most stable gene under different developmental stages and temperature stress, while *H4* was the most stable gene under different carbon sources. In addition, *18S rRNA* and *28S rRNA* were stably expressed in different strains of *A. heimuer* (A14, A137, and A12) and at different growth stages (mycelium, protoplasm, and fruiting stages) (Zhang et al., 2020). Similarly, the expression of *18S rRNA* was also stable in *L. edodes* (Xiang et al., 2018) and *Wolfiporia cocos* (Zhao, 2016). However, the stability of *18S rRNA* and *28S rRNA* expression was relatively poor in *A. cornea* (Jia et al., 2019) and *Ganoderma lucidum* (Xu et al., 2014). Thus, suitable reference genes must be screened based on different species and different experimental conditions.

To avoid the limitations of using a single software, we used multiple reference gene stability assessment software (geNorm, NormFinder, BestKeeper, and RefFinder) to analyze the expression stability of candidate reference genes. Due to the use of different algorithms, the results of reference gene stability did not agree with each other. Similar differences have been reported in earlier studies (Jia et al., 2019; Lv et al., 2020; Yang et al., 2021). Thus, RefFinder was used to ensure the accuracy of the screening results by integrating these results of the above-mentioned four algorithms, and comprehensively evaluating the reference genes. A few earlier studies showed that under certain experimental conditions, the normalization of target gene expression using a single reference gene sometimes may not ensure the accuracy of experimental results (Jia et al., 2019; Wu et al., 2021). Therefore, two or more reference genes should be introduced to meet the experimental requirements. In our study, according to the optimal number calculated by geNorm and the reference gene ranking obtained by RefFinder, *P450*, *CYP*, and  $\gamma$ -*TUB* were identified as stable reference genes. One of these reference genes,  $\gamma$ -*TUB*, with an important role in the cytoskeleton, was also one of the most stable reference genes in different strains of *Cryptolestes ferrugineus* (Tang et al., 2017). In our research,





the commonly used reference genes *CYP* and *GAPDH* were the most stable and unstable expressed genes, respectively. Likewise, *CYP* was the most unstable reference gene in *Volvariella volvacea* (Tao et al., 2016), while *GAPDH* was the most stable reference gene in *A. heimuer* (Zhang et al., 2020). These results again demonstrated that reference genes differed from species to species.

Laccase is involved in spore formation, pathogenesis, pigmentation, fruiting body formation and melanin formation in fungi (Camarero et al., 2005). It showed different expression levels at different developmental stages of fungi and was often used for reference gene verification (Jia et al., 2019). In our study, the expression profile of *laccase1* of *A. areolatum* at different growth stages was used to verify the stability of the putative reference gene. We found that in some samples, the expression of *laccase1* normalized by *GAPDH* and  $\beta$ -*TUB* was significantly different from the normalized results obtained using other reference genes. Thus, the normalization results based on *GAPDH* and  $\beta$ -*TUB* did not accurately reflect the expression level of *laccase1*. The expression levels of *laccase1* normalized by reference genes *P450*, *CYP*, and  $\gamma$ -*TUB*, as well as their combination were consistent in samples even at different growth stages. Therefore, these three genes alone, or in combination, could be used for RT-qPCR analysis of gene expression in *A. areolatum* under our experimental conditions.

This is the first study to evaluate reference genes for analysis of gene expression in *A. areolatum*. We evaluated the stability of 10 candidate reference genes in samples from this symbiotic fungus at different growth stages using geNorm, NormFinder, BestKeeper, delta Ct methods, and RefFinder. Overall, *P450*, *CYP*, and  $\gamma$ -*TUB* and their combination were the most stable reference genes in different samples, although the rankings of their stability varied slightly when analyzed by the four programs used here. Our results not only provided stable reference genes for quantitative analysis of gene expression of the symbiotic fungus, but also laid a foundation for the study of its mutualistic relationship with *S. noctilio*.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/OL660755/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660756/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660757/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660758/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660759/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660760/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660761/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660762/>; <https://www.ncbi.nlm.nih.gov/genbank/OL660763/>; and <https://www.ncbi.nlm.nih.gov/genbank/OL660764/>.

## AUTHOR CONTRIBUTIONS

NF carried out the majority of the bioinformatics studies and participated in performing the experiments. MW was involved in experimental data analysis. NF and JL wrote the manuscript. LR, YL, and SZ participated in the design of the study and helped to draft the manuscript. All authors have read and agreed to the published version of the manuscript.

## FUNDING

This work was supported by the Chinese National Natural Science Foundation (31870642) and the Beijing's Science and Technology Planning Project (Z201100008020001).

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.827241/full#supplementary-material>

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