Reference gene selection for real-time quantitative PCR assays in different tissues of *Huperzia serrata* based on full-length transcriptome sequencing

Yanping Fu | Fei Niu | Hui Jia | Yanli Wang | Bin Guo | Yahui Wei

Abstract

*Huperzia serrata* (*H. serrata*) produces various types of effective lycopodium alkaloids, especially Huperzine A (HupA), which is a promising drug for the treatment of Alzheimer’s disease. Numerous studies focused on the chemistry, bioactivities, toxicology, and clinical trials of HupA; however, the public genomic and transcriptomic resources are very limited for *H. serrata* research, especially for the selection of optimum reference genes. Based on the full-length transcriptome datasets and previous studies, 10 traditional and three new candidate reference genes were selected in different tissue of *H. serrata*. Then, two optimal reference genes GAPDHB and HisH2A were confirmed by four analysis methods. In order to further verify the accuracy of the two reference genes, they were used to analyze the expression patterns of four HupA-biosynthetic genes (lysine decarboxylas, RS-norcoclaurine 6-O-methyltransferase, cytochrome P45072A1, and copper amine oxidase). The data suggested that the expression pattern of HupA-biosynthetic genes was consistent with them in transcriptome sequencing in different tissue of *H. serrata*. This study identified that GAPDHB and HisH2A provides the reliable normalization for analyzing the HupA biosynthetic gene expression in different tissues of *H. serrata* on the transcriptional level.

**KEYWORDS**

*Huperzia serrata*, Huperzine A, reference gene, RT-qPCR

1 | INTRODUCTION

*Huperzia serrata* (*H. serrata*) belongs to the *Huperzia* genus, Lycopodiaceae order. The whole plant of *H. serrata* has been used as a medicine in China to treat different kinds of ailments, including bruises, strains, swelling, rheumatism, schizophrenia, myasthenia gravis, and fever since 739 (during the Tang Dynasty) (Ferreira et al., 2016). *H. serrata* has been widely known as a medicinal plant since Chinese scientists isolated Huperzine A (HupA) from it during the 1980s (Liu et al., 1986). HupA is a promising candidate drug for treating Alzheimer’s disease (AD), it could improve cognitive function, daily living activity and global clinical assessment in patients for AD disease, with relatively few and mild adverse effects (Qian & Ke, 2014; Yang et al., 2013). However, *H. serrata* is a scarce species and grows very slowly in specialized habitats. Furthermore, the HupA content is very low in *H. serrata* (Ma et al., 2007). At present, the...
rapidly growing demand has put H. serrata resources on the brink of extinction. Although a lot of efforts have been focused on artificial culture and tissues culture for H. serrata production, the outcomes were unsatisfactory.

Now, researchers try to improve HupA content by studying the gene information of HupA biosynthesis. However, the public genomic and transcriptomic resources are very limited. Only two papers focused on transcriptomic resources (André et al., 2010; Yang et al., 2017). Real-time quantitative PCR (RT-qPCR) has been widely used in gene expression measurement on transcriptional level. Identification of suitable reference genes (RGs) is pre-requisite for RT-qPCR assays (Bansal et al., 2015; Vandesompele et al., 2002). Many housekeeping genes have been used as RGs under different experimental conditions, such as actin, tubulin, elongation factor (EF), 18S ribosomal RNA (18S rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), histone, and ubiquitin (Fei et al., 2018). However, there is no RG suitable to all biological systems based on previous studies. So, researchers reach a consensus that specific RG for a given species and treatment needs to be identified first (Bansal et al., 2015). Unfortunately, the previously reported RGs are not suitable for the research of HupA-biosynthetic genes in our materials (Yang et al., 2019).

In present study, in order to obtain the optimal RGs for studying HupA-biosynthesis, we detected the concentration of HupA and carried out full-length transcriptome sequencing for the different tissues of H. serrata. Based on full-length transcriptome sequencing data and previous studies, 13 candidate RGs were selected. Finally, GAPDH and HisH2A stood out among the 13 candidate RGs and became the best combination for normalization in different tissues of H. serrata by four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder). Using GAPDH and HisH2A as RGs, the expression profile of four HupA-biosynthetic genes, lysine decarboxylase (LDC), 6-Norcoclaurine 6-O-methyltransferase (MET), cytochrome P45072A1 (CYP), and copper amine oxidase (CAO) showed the similar expression trend between transcriptome sequencing and RT-qPCR. This result further confirmed that GAPDHB and HisH2A were suitable for HupA-biosynthetic gene expression normalization. This work provides suitable RGs for the subsequent research of HupA-biosynthesis in H. serrata.

2 | RESULTS

2.1 | The Hup A content analyses

HPLC-UV was performed to detect the HupA content in H. serrata. Typical chromatograms from HupA standard and three tested samples are shown in Figure S1, indicating that HupA was well separated from different tissues. A linear relationship exists between the peak area (measured at 308 nm) and the concentration of HupA in the sample injected into the HPLC. The results showed that there was obvious difference between the HupA content in different tissues. The highest HupA concentration (72 μg/g) was obtained in the leaves of H. serrata. The lowest content (19 μg/g) of HupA was found in root tissues of H. serrata (Figure 1).

2.2 | The screening of candidate RGs

Given the variability of Hup A concentrations in different tissues, the root, stem, and leaf samples were collected and applied to the full-length transcriptome sequencing by Nanopore. After assembly, 43,443 unigenes were retrieved. CPM (counts per million) is the index for measuring the expression of unigenes. Based on the CPM value analysis, 19,400 genes were obtained with similar and stably expression in three tissues. Combining with reported literatures, 10 traditional and three new candidate RGs were chosen. The three new candidate RGs were annotated as hypothetical or uncharacterized proteins by NCBI Nr database, more importantly, they were never used as RGs before. The detail information of total 13 candidate RGs was showed in Table 1.

2.3 | Verification of the primer specificity and RT-qPCR amplification efficiency

The primer information of 13 candidate RGs was given in Table 2. Each primer pair was designed outside of the conserved domains to secure the specificity (Figure S2). Initially, the agarose gel electrophoresis yielded a specific fragment of expected size (Figure S3). Further, the melting curve in the RT-qPCR reaction showed the single peak for each primer pair indicating absence of non-specific amplification (Figure S4). For all primer pairs, the amplification efficiencies were spanning from 90.4% to 103.6%, and the correlation coefficient (R²) were greater than .990 (Table 2). Taken together, these results indicated each primer pair was specificity and the RT-qPCR assays were highly efficient.

![Figure 1](image-url)

Figure 1: The content of HupA in different tissues of H. serrata. The mean and standard deviation were calculated by three independent biological replicates.
| Unigene gene ID | Accession number | Length (bp) | Gene symbol | Gene name | Homolog locus | Root_CPM | Stem_CPM | Leaf_CPM | E value |
|-----------------|------------------|-------------|-------------|-----------|---------------|-----------|----------|----------|---------|
| EVM0025399      | MH560040         | 1041        | Actin4      | Actin-related protein 4 | KM496528    | 24.88     | 39.46    | 30.90    | 1e−147  |
| EVM0014845      | MH560041         | 1704        | Actin7      | Actin-related protein 7-like | XM_024546532.1 | 20.90     | 24.57    | 31.48    | 0       |
| EVM006291       | MH560042         | 2444        | EF1dt       | Elongation factor 1-delta 2-like | XM_004507134.3 | 205.15    | 193.46    | 160.29    | 1e−78   |
| EVM0021957      | MH560043         | 3747        | EFTS        | Elongation factor Ts family protein | NM_119050.3 | 1.79      | 4.28     | 2.53     | 0       |
| EVM0027572      | MH560044         | 2137        | α-tub3      | Tubulin alpha-3 chain | XM_020240093.1 | 16.55     | 18.42    | 23.00    | 0       |
| EVM0033890      | MH560045         | 1698        | GAPDH6B     | Glyceraldehyde-3-phosphate dehydrogenase B | NM_001302308.1 | 36.73     | 44.16    | 35.66    | 0       |
| EVM008093       | MH560046         | 1008        | HisH3.3     | Histone H3.3 isoform X1 | XM_017375185.1 | 77.28     | 62.50    | 75.11    | 2e−91   |
| EVM0031551      | MH560047         | 1098        | HisH2A      | Histone H2A-III-like | XM_024521943.1 | 64.68     | 81.59    | 50.83    | 3e−37   |
| EVM0033477      | MH560048         | 906         | UBQ1        | Ubiquitin-NEDD8-like protein RUB1 | XM_007048546.2 | 65.34     | 89.70    | 52.61    | 2e−89   |
| EVM0000551      | MH560049         | 948         | UBQ11       | Ubiquitin 11 | NM_001203752.2 | 314.46    | 421.33    | 369.47    | 2e−73   |
| ONT.10684       | MZ042629         | 817         | 10684       | Uncharacterized protein | XP_010251846 | 117.85    | 116.38    | 111.58    | 2e−17   |
| EVM0022608      | MZ042627         | 2036        | 22608       | Hypothetical protein | PTQ36569    | 34.00     | 29.03    | 32.86    | 5e−99   |
| EVM0017784      | MZ042628         | 1605        | 17784       | Uncharacterized protein | XP_002972660 | 3.15      | 2.55     | 3.68     | 0       |
| EVM0027909      | GO914645         | 637         | LDC         | Lysine decarboxylase | GO914645    | 2.20      | 47.53    | 53.52    | 9e−128  |
| EVM0017005      | GO914756         | 1682        | MET         | (RS)-norcoclarine 6-O-methyltransferase | GO914756    | 2.82      | 13.46    | 10.48    | 0       |
| EVM0022835      | GO914428         | 1975        | CYP         | Cytochrome P450 7A1 | GO914428   | .03       | 21.94    | 30.97    | 0       |
| EVM0024797      | JN247732         | 2640        | CAO         | Copper amine oxidase | JN247732   | 4.11      | 13.86    | 39.70    | 0       |

*E value represents high homology of candidate RGs with stable reference genes in other plants using the local Blast program.*
2.4 | Expression profiles of candidate RGs

The expression profiles of RT-qPCR products were shown in Figure 2. The results illustrated that the mean Ct values of all RGs ranged from 24.04 to 29.43. Lower Ct value indicates the higher expression abundance, conversely means the lower expression abundance. EF1dt and UBQ1 were highly expressed with mean Ct values between 24.04 and 24.08, whereas EFTS was the least expressed gene on account of its highest mean Ct value (29.43). All candidate genes showed expression variability in different samples. GAPDH and EFTS showed relatively smaller variation (<2 cycles), whereas others like UBQ11 had higher expression variation (3.07 cycles). The results indicated that there was still variable expression even for relative stable housekeeping genes.

2.5 | geNorm analysis

geNorm algorithm calculates the M values to evaluate the expression stability of each RG. The lower M value indicates the more stable gene expression, and vice versa. As Figure 3 shown, each M value was less than 1.5, which demonstrated all candidate RGs were
suitable for normalization in different tissues of *H. serrata*. Among them, EF1dt, HisH2A and GAPDHB were the most stable genes in each *H. serrata* samples, whereas *HisH3.3* and *EFTS* were the least stable genes.

2.5.1 NormFinder analysis

NormFinder evaluates the stability of each RG via the stability value. The smaller stability value, the more stable gene, and vice versa. As
shown in Table 3, GAPDHB, HisH2A and EF1dt were the lowest stability value almost in every sample, whereas EFTS, HisH3.3, and Actin7 were the highest stability value. The stability of some genes was variable, such as α-tub3 and UBQ1. α-tub3 (stability value = .046) was the one of the top three stable genes in root samples, but outside of top three in other samples. UBQ1 (stability value = .348) was the lowest stable genes in stem samples, but it was relative stable in other samples. Overall, by NormFinder analysis, GAPDHB, HisH2A and EF1dt were the most stable genes, whereas EFTS, HisH3.3, and Actin7 were the least stable genes in different tissues of *H. serrata*. This result was similar with geNorm analysis.

### 2.6 BestKeeper analysis

BestKeeper evaluates the expression stability of RGs via the CV and SD. A lower CV value indicates more stable expression (Pfaffl et al., 2004). The analytic results showed that GAPDHB, EF1dt and HisH2A were the top three genes with lowest CV ± SD values in all, stem and leaf samples except in root samples (Table 4). In root samples, the top three genes were HisH2A, UBQ11 and α-tublin. However, HisH3.3 was the least stable RG with higher CV ± SD value (27.35 ± 21, 25.81 ± .19, 28.66 ± .20 and 19.47 ± .17) in every samples. Taken together, by BestKeeper analysis, GAPDHB, EF1dt and
HisH2A were the most stable genes, whereas HisH3.3 was the least stable gene.

2.7 | RefFinder analysis

Although the results (geNorm analysis, NormFinder, and BestKeeper analysis) were similar, it was not strictly consistent. Therefore, we evaluated the comprehensive rank using RefFinder (Table 5). In root tissues, the final ranking calculations based on the RefFinder found HisH2A (GM = 1.67), GAPDH (3.33) and α-tub3 (3.33) were the best genes. For stem samples, the top three stable RGs were GAPDH (1.67), EF1dt (2.00) and HisH2A (3.67), whereas EF1dt (1.33), HisH2A (2.00), and GAPDH (2.33) in leaf samples. Across all samples, the top three stable RGs were GAPDH (1.67), HisH2A (1.67) and EF1dt (2.33). On the other hand, HisH3.3 and EFTS were the least stable genes (Table 5).

2.8 | Optimal number of RGs for normalization

One single and stable RG is sufficient for quantifying gene expression, however, more than one RG for effective normalization is more suggested (Vandesompele et al., 2002). Based on geNorm analysis, the optimal number of RGs was determined by pairwise variation Vn/n + 1. The threshold of Vn/n + 1 is 0.15. In our data, the pairwise variation of V2/3 values was lower than 0.15 (Figure 4), which suggested that two RGs were optimum number.

### Table 5

| Rank | All | Root | Stem | Leaf |
|------|-----|------|------|------|
|      | Gene | GM   | Gene | GM   | Gene | GM   | Gene | GM |
| 1    | GAPDH | 1.67 | HisH2A | 1.67 | GAPDH | 1.67 | EF1dt | 1.33 |
| 2    | HisH2A | 1.67 | GAPDH | 3.33 | EF1dt | 2.00 | HisH2A | 2.00 |
| 3    | EF1dt | 2.33 | α-tub3 | 3.33 | HisH2A | 3.67 | GAPDH | 2.33 |
| 4    | α-tub3 | 4.33 | 22608 | 4.00 | 22608 | 4.67 | 22608 | 5.00 |
| 5    | 22608 | 4.67 | UBQ11 | 6.33 | α-tub3 | 5.67 | α-tub3 | 6.00 |
| 6    | 10684 | 6.67 | EF1dt | 7.33 | UBQ11 | 5.67 | Actin4 | 7.33 |
| 7    | 17784 | 8.00 | UBQ1 | 7.33 | Actin7 | 6.00 | UBQ1 | 7.67 |
| 8    | UBQ1 | 8.67 | 17784 | 7.67 | 10684 | 7.00 | 10684 | 7.67 |
| 9    | UBQ11 | 8.67 | 10684 | 8.00 | 17784 | 8.33 | 17784 | 8.00 |
| 10   | Actin4 | 9.33 | Actin4 | 8.67 | Actin4 | 10.33 | UBQ11 | 10.00 |
| 11   | Actin7 | 9.33 | Actin7 | 9.67 | EFTS | 11 | Actin7 | 10.00 |
| 12   | EFTS | 12.33 | EFTS | 11 | UBQ1 | 11.67 | EFTS | 11.33 |
| 13   | HisH3.3 | 12.67 | HisH3.3 | 12 | HisH3.3 | 13 | HisH3.3 | 11.67 |

Note: GM represents geometric mean.

**Figure 4** Pairwise variation (Vn/n + 1) of 13 candidate reference genes calculated by geNorm. The threshold of determining the optimal number of RGs for qRT-PCR normalization is .15.
2.8.1 | Comprehensive stability analysis of RGs

Table 6 was used to compare the results of four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder). The ranking of stability based on four analysis methods were basically consistent. By comprehensive analysis, GAPDHb and HisH2A were the best combination for normalization in different tissues of H. serrata. On the other hand, HisH3.3 and EFTs were the least stable genes.

2.9 | RG validation

To demonstrate the utility of identified stable RGs, four HupA-biosynthetic related genes LDC, MET, CYP, and CAO were selected. For comparison, expression values of target genes were normalized with the most stable gene pair (GAPDHb and HisH2A) and the least stable gene pair (EFTs and HisH3.3) in different tissues of H. serrata. When normalized with the most stable genes, LDC, MET, CYP, and CAO had tissue specific expression (over twofold) in leaf. Furthermore, the expression trend was consistent with that of transcriptome sequencing dates (Figure 5). By contrast, when normalized using the least stable genes, the expression trend was not consistent. The transcription level of MET and CYP were not up-regulated (less than twofold) in stem and leaf tissues. The transcription level of LDC was down-regulated (0.77-fold) in stem tissues, and the CAO was down-regulated (0.67-fold) in leaf tissues. In all, the expression of the most stable gene pair was more reliable than the least stable gene pair.

3 | DISCUSSION

H. serrata has received extensive concern due to its ability to produce lycopodium alkaloids, especially HupA (Christenhusz et al., 2011). HupA was found to possess potent acetylcholine esterase inhibition (AChEI) and had been clinically exploited for the treatment of AD. More studies were focused on the isolation and identification of compounds and endophytic bacteria (Wu et al., 2017), but little on the transcription of HupA-biosynthetic genes. Especially for the selection of optimum reference genes, little research has been reported. TATA binding protein (TBP) and GAPDH were identified as reference genes for H. serrata in Yang et al.’s (2019) report. Unfortunately, TBP gene could not be amplified when we study the expression of HupA-biosynthetic genes in our materials. So, in the present study, we screened and selected the new optimal RGs based on full-length transcriptome sequencing and previous researches. Based on the four analysis methods, we obtained two optimal reference genes GAPDHb and HisH2A for studying HupA-biosynthetic genes, and their reliability was confirmed via testing the expression profile of four HupA-biosynthetic genes (LDC, MET, CYP, and CAO). This study provides suitable normalization for analyzing the expression of HupA-biosynthetic gene. In addition, we found the expression trend of HupA-biosynthetic genes correlated with the HupA production in different tissues of H. serrata. This result will provide the information for further studying the biosynthesis and transportation of HupA.

In general, the expression level of RGs should be constantly stable in any physical conditions. However, there is no RG suitable to all biological systems. We had to screen the most suitable RGs for studying the HupA-biosynthesis. Based on the CPM value of transcriptome sequencing and reported literatures (Chen et al., 2019; Dudziak et al., 2020; Wu et al., 2020), 10 traditional and three new candidate RGs were chosen (Table 1). Especially for three new candidate RGs, they had stable expression in full-length transcriptome sequencing but have not been used as RGs before. This is a new attempt distinguishing from previous studies. Thirteen candidate genes were also enough to ensure the experimental accuracy.

The primer specificity is the primary condition of RT-qPCR. The ideal primers should cross intron regions to avoid genomic contamination and cannot be set in conservative domain. First, each primer pair was designed outside of the conserved domains (Figure S2). Subsequently, the products of each primer pair were detected by agarose gel electrophoresis (Figure S3) and melting curves (Figure S4). The results indicated that there were no primer dimers and nonspecific amplification for each primer pair. Furthermore, the E value of PCR varied from 90.4% to 103.6%, and all of the R² were larger than .990 (Table 2), which were similar to previous literatures (Liu et al., 2018). In conclusion, these results indicated each primer pair had specificity and the RT-qPCR assays were highly efficient.

Based on four analysis methods (geNorm, NormFinder, BestKeeper, and RefFinder), GAPDHb and HisH2A stood out among the 13 candidate RGs, and became the best combination for normalization in different tissues of H. serrata (Table 5). Many studies have shown that GAPDH was often applied as stable RG in different tissues or under various experimental conditions (Chen et al., 2017; Zhuang et al., 2015). Especially for H. serrata, GAPDH also was proved as stable RG by Yang et al. (2019). TBP was also identified as stable RG for H. serrata by Yang et al. (2019). Unfortunately, TBP gene could not be amplified in our materials. This result indicated that expression level of housekeeping gene was different even in the same species and same tissues. Histone and elongation factor were reported as most stable RGs in other species (Zhuang et al., 2015); however, HisH3.3 and EFTs were the most unstable RGs in different tissues of H. serrata (Table 6). Interestingly, HisH2A and EF1αd were the top three stable RGs (Table 6). Similar findings were in previous studies. The expression level of Actin2/7 was more stable than Actin11 in diverse tissues of soybean (Jian et al., 2008). These results stated that the expression level and stability of RGs from the same gene family may be different in the same samples. Taken together, the results further proved the necessity for screening suitable RGs in different tissues of H. serrata.

A proposed biosynthesis pathway for HupA and related lycopodium alkaloids was reported (Ma & Gang, 2005). However, only two enzymes, LDC and CAO have been proved to participate in the biosynthesis of HupA (Bunsupa et al., 2012; Sun et al., 2012; Xu et al., 2017). Three enzymes RS-norcoclaurine 6-O-methyltransferase (MET) and cytochrome P45072A1 (CYP) (Luo et al., 2010; Xu et al., 2017), type III polyketide synthase (PKS) (Wang et al., 2016),
### TABLE 6  Expression stability ranking of the 13 candidate RGs by four analysis methods

| Method          | Rank | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    | 10   | 11   | 12   | 13   |
|-----------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| A. Ranking order for all samples |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| geNorm          |      | HiH2A| EF1dt| GAPDH | a-tub3| 22608| Actin7| UBQ11| 17784| 10684| UBQ1 | Actin4| EFTS | HiH3.3|
| NormFinder      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| a-tub3| 10684| Actin4| 17784| UBQ1 | UBQ11| Actin7| HisH3.3| EFTS |
| BestKeeper      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| 22608| 10684| UBQ1 | 17784| UBQ11| Actin4| Actin7| EFTS | HiH3.3|
| RefFinder       |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| 22608| 10684| 17784| UBQ1 | UBQ11| Actin4| Actin7| EFTS | HiH3.3|
| B. Ranking order in root samples |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| geNorm          |      | GAPDH| EF1dt| HiH2A| a-tub3| 22608| a-tub3| 17784| EFTS | Actin7| UBQ11| 10684| UBQ1 | HisH3.3| Actin4|
| NormFinder      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| 10684| a-tub3| UBQ11| 17784| Actin7| Actin4| EFTS | UBQ1 | HisH3.3|
| BestKeeper      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| UBQ11| 10684| Actin7| 17784| EFTS | Actin4| UBQ1 | HisH3.3|
| RefFinder       |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| UBQ11| 10684| 17784| Actin4| EFTS | Actin4| UBQ1 | HisH3.3|
| C. Ranking order in stem samples |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| geNorm          |      | HiH2A| Actin7| GAPDH | UBQ11| 22608| 22608| 10684| Actin4| UBQ1 | EFTS | HisH3.3|
| NormFinder      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| 10684| 17784| Actin7| Actin4| EFTS | UBQ1 | HisH3.3|
| BestKeeper      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| UBQ11| 10684| Actin7| 17784| EFTS | Actin4| UBQ1 | HisH3.3|
| RefFinder       |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| UBQ11| 10684| 17784| Actin4| EFTS | Actin4| UBQ1 | HisH3.3|
| D. Ranking order in leaf samples |      |      |      |      |      |      |      |      |      |      |      |      |      |      |
| geNorm          |      | HiH2A| EF1dt| GAPDH | Actin7| a-tub3| 22608| Actin4| UBQ11| UBQ1 | 17784| 10684| EFTS | HisH3.3|
| NormFinder      |      | EF1dt| HiH2A| GAPDH | UBQ11| 22608| UBQ1 | 10684| 17784| Actin4| a-tub3| HisH3.3| UBQ11| EFTS |
| BestKeeper      |      | GAPDH| HiH2A| EF1dt| a-tub3| 22608| 10684| Actin4| 17784| UBQ1 | EFTS | UBQ11| HisH3.3| Actin7|
| RefFinder       |      | EF1dt| HiH2A| GAPDH | a-tub3| 22608| Actin4| UBQ1 | 10684| 17784| UBQ11| Actin7| EFTS | HisH3.3|
have been described to possibly involve in the biosynthesis of HupA. In order to verify the accuracy of the stable RGs identified in this paper, four HupA-biosynthetic genes, LDC, MET, CYP, and CAO were tested. The results showed that using the combination of stable RGs (GAPDH and HisH2A), the consistent expressions trend of LDC, MET, CYP, and CAO were obtained between transcriptome sequencing and RT-qPCR (Table 1 and Figure 5). Conversely, using the most unstable RGs (HisH3.3 and EFTS) may lead to declinational results (Table 1 and Figure 5). The results further proved that GAPDH and HisH2A were suitable for gene expression normalization, especially for HupA-biosynthetic genes. In addition, we tested the content of HupA in different tissues. The results indicated the content of HupA in root was obviously lower than that in stem or leaf (Figure 1), which was consistent with the previous studies (Ma & Gang, 2005; Wu et al., 2017). Therefore, in order to protect the wild resources, we suggest picking the aboveground parts instead of uprooting the whole plant when digging H. serrata. In addition, we found the expression trend of HupA-biosynthetic genes were similar with the trend of HupA content in different tissue of H. serrata, which indicated that the biosynthesis of HupA may happen in stem and leaf. This result will provide the information for further studying the biosynthesis and transportation of HupA.

4 | CONCLUSIONS

In the present study, based on full-length transcriptome sequencing data and four analysis methods, we obtained two optimal reference genes GAPDH and HisH2A from thirteen candidate reference genes in different tissue of H. serrata. The expression patterns of four HupA-biosynthetic genes LDC, MET, CYP, and CAO further verified that GAPDH and HisH2A were suitable for the normalization of HupA-biosynthetic genes. This work provides suitable RGs for the subsequent research of HupA-biosynthetic and transportation in H. serrata.

5 | MATERIALS AND METHODS

5.1 | Plant materials

H. serrata plants were collected from Hanzhong, Shaanxi, China (107°09′/32°30′), in March 2018. All materials used in this study were identified by phytotaxonomist. The plants were rinsed carefully by running water. Root, stem, and leaves were collected in liquid nitrogen and were immediately frozen at −80°C for RNA extraction. Other plant materials were dried at 60°C and powdered for determining HupA content.

5.2 | HPLC parameters and conditions

HupA were extracted from the different plant tissues as previously described (Ishiuchi et al., 2013; Ma et al., 2005). After the plant material was dried and milled, 100 mg each of powdered plant tissues was extracted by adding 2% (2:100, w/v) aqueous tartaric acid (5 ml) for overnight and then sonicating for 2 h at 25°C. Centrifuging for 30 min at room temperature and the upper extraction solution was filtered.
into a 1.5 ml measuring flask through a 0.45-μm filter. Finally, the filtered solutions (10 μl) were injected into the HPLC system (LC-20AT, Shimadzu, Japan) for detection of HupA content. Each experiment comprised three independent biological replicates. The details are as follows: The elution conditions: (flow rate, 0.8 ml/min; column temperature, 28°C; injection volume, 10 μl; detection, the detection was performed at the wavelength of 308 nm). The mobile phase was methanol/acetonitrile/0.08-M ammonium acetate (pH = 6) (10:30:60). Chromatography was performed on a C-18 column (Hypersil ODS2, China) of 250 × 4.6 mm dimensions and 5-μm particle size.

5.3 Candidate RGs selection and primers design

Candidate RGs for this study were selected from full-length transcriptome sequencing dates and previous studies. First, candidate genes were screened based on the stable CPM value in root, stem, and leaf samples. More appropriate genes were chosen by combining previous studies in numerous candidate genes. Because housekeeping genes have been used as RGs in many previous studies, 10 commonly used housekeeping genes were chosen in numerous candidate genes. Furthermore, three never reported genes were chosen as the candidate genes. The three new RGs candidate are as follows: ONT.10684 represented the high expression level (CPM over 100), EVM0022608 was the middle level (CPM 29–34), and EVM0017784 was the low level (CPM less than 5). Thirteen candidate RGs (MH560040–MH560049, MZ042627–MZ042629) were selected at last (Table 1).

Gene specific primers for each RG were designed using the Primer 5.0. Conserved domains of RGs were evaluated, and the primer binding positions were presented. Initially, primer specificity was verified by RT-qPCR and confirmed with 2% (w/v) agarose gel electrophoresis and melting curve.

5.3.1 RNA extraction and cDNA synthesis

The total RNA was extracted according to the modified CTAB method (Gasic et al., 2004). RNA samples were treated with DNase I (Ambion, Waltham, MA, USA) to remove any DNA contamination. Using cDNA synthesis kit (Roche, Basel, Switzerland), first strand cDNA was prepared with 3-μg RNA as manufacturer’s instructions.

5.4 RT-qPCR analysis

The RT-qPCR reactions were performed with FastStart Universal SYBR Green Master (Roche, Basel, Switzerland) on a CFX-96 thermocycling system (Bio-Rad, Hercules, CA, USA). Each RT-qPCR reaction was performed as described previously (Zhuang et al., 2015). PCR amplifications were carried out by the following conditions: one cycle at 95°C (180 s), followed by 40 cycles of denaturation at 95°C (30 s), annealing at 58°C for 10 s and extension at 72°C for 20 s (Liu et al., 2018). Finally, melt curve analyses were done by slowly heating the PCR mixtures from 58 to 95°C. Amplification efficiencies (E) and correlation coefficients (R²) for each primer pair were calculated by LinRegPCR program (Ruijter et al., 2009). In the negative control group, RT-qPCR was performed using water instead of cDNA as the template. Three technical replicates were analyzed for each biological sample, and each experiment comprised three independent biological replicates.

5.5 Data analysis of gene expression stability

Four analysis methods: geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and RefFinder (Silver et al., 2006) were applied to determine the stability of RGs. For geNorm and NormFinder, the raw Ct values were converted into the relative quantities using the formula 2^−ΔCt (ΔCt = each corresponding Ct value – lowest Ct value). M value was calculated the average expression stability, in geNorm algorithms. The candidate RGs showing a higher M value (M > 1.5) are not considered for normalization studies (Vandesompele et al., 2002). geNorm software was also used to confirm the best numbers of RGs with Vn/Vn + 1 (n refers to the RGs number) (Vandesompele et al., 2002). NormFinder can provide the stability value for each gene (Andersen et al., 2004). The smaller stability value, the more stable gene, and vice versa. For BestKeeper, the raw Ct values and amplification efficiencies were used to calculate the coefficient of variation (CV) and standard deviation (SD). The most stable genes are the lowest CV and SD (CV ± SD). The comprehensive ranking order was recommended on the basis of geometric mean (GM) by RefFinder (Zhang et al., 2018).

5.6 Validation of RGs

The primer of four HupA-biosynthetic genes LDC (GO914645), MET (GO914756), CYP (GO914428), and CAO (JN247732) (Luo et al., 2010; Sun et al., 2012; Xu et al., 2017) were designed using the Primer 5.0. The combination of the two best and worst RGs were used to standardize the expression of four HupA-biosynthetic genes. The target gene expression data were normalized using the geometric mean values calculated for the RG pairs (Vandesompele et al., 2002). Relative expression level and fold change were determined using the comparative 2^−ΔΔCt method (Pfaffl, 2001). One-way analysis of variance was performed using SPSS software (Version 16.0, SPSS Inc., Chicago, IL, USA).

ACKNOWLEDGMENTS

This work was supported by Scientific Research from Shaanxi Provincial Department of Education (16JK1756) and Natural Science Foundation of China (31702159) to Yanping Fu, Key Research and Development Plan Project of Shaanxi Province (2018ZDXM-SF-016) to Yahui Wei.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Yanping Fu conceived and designed the experiments. Yanping Fu and Fei Niu performed the experiments, analyzed the data, and prepared figures and/or tables. Fei Niu prepared the first draft. Hui Jia and Yanli Wang contributed reagents/materials and detected the content of HupA. Bin Guo contributed reagents/materials/analysis tools. Yahui Wei conceived and designed the experiments and reviewed drafts of the paper. All the authors approved the final draft.

REFERENCES
Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research, 64, 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496
André, S., Sun, Y., Song, J., Wu, Q., Sun, C., Li, Y., Luo, H., & Chen, S. (2010). Comparison of 454-ESTs from Huperzia serrata and Phlegmariurus carinatus reveals putative genes involved in lycopodium alkaloid biosynthesis and developmental regulation. BMC Plant Biology, 10, 209. https://doi.org/10.1186/1471-2229-10-209
Bansal, R., Mittapelly, P., Cassone, B. J., Mamidala, P., Redinaugh, M. G., & Michel, A. (2015). Recommended reference genes for quantitative PCR analysis in soybean have variable stabilities during diverse biotic stresses. PLoS ONE, 10, e0134890. https://doi.org/10.1371/journal.pone.0134890
Bunupa, S., Katayama, K., Ikura, E., Oikawa, A., Tooyooka, K., Saito, K., & Yamazaki, M. (2012). Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in leguminosae. Plant Cell, 24, 1202–1216. https://doi.org/10.1105/tpc.112.095885
Chen, C., Wu, J., Hua, Q., Tel-Zur, N., & Qin, Y. (2019). Identification of reliable reference genes for quantitative real-time PCR normalization in pitaya. Plant Methods, 15, 70. https://doi.org/10.1186/s13007-019-0455-3
Chen, X., Mao, Y., Huang, S., Ni, J., & Wu, L. (2017). Selection of suitable reference genes for quantitative real-time PCR in Sapium sebiferum. Frontiers in Plant Science, 8, 637. https://doi.org/10.3389/fpls.2017.00637
Christenhusz, M. J. M., Zhang, X. C., & Schneider, H. (2011). A linear sequence of extant families and genera of lycophytes and ferns. Phytotaxa, 19, 7–54. https://doi.org/10.11646/phytotaxa.19.12
Dudziak, K., Sozoniuk, M., Szczepa, H., Kuzdra, K., Kowalczyk, K., Brner, A., & Nowak, M. (2020). Identification of stable reference genes for qPCR studies in common wheat (Triticum aestivum L.) seedlings under short-term drought stress. Plant Methods, 16, 1–8. https://doi.org/10.1186/s13007-020-00601-9
Fei, X., Shi, Q., Yang, T., Fei, Z., & Wei, A. (2018). Expression stabilities of ten candidate reference genes for RT-qPCR in Zanthoxylum bungeanum Maxim. Molecules, 23, 802. https://doi.org/10.3390/molecules23040802
Ferreira, A., Rodrigues, M., Fortuna, A., Falcao, A., & Alves, G. (2016). Huperzine A from Huperzia serrata: A review of its sources, chemistry, pharmacology and toxicology. Phytochemistry Reviews, 15, 51–85. https://doi.org/10.1007/s11101-014-9384-y
Gasic, K., Hernandez, A., & Korban, S. S. (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Molecular Biology Reporter, 22, 437–438. https://doi.org/10.1007/BF02772687
Ishiuchi, K. i., Park, J. J., Long, R. M., & Gang, D. R. (2013). Production of huperzine A and other Lycopodium alkaloids in Huperzia species grown under controlled conditions and in vitro. Phytochemistry, 91, 208–219. https://doi.org/10.1016/j.phytochem.2012.11.012
Jian, B., Liu, B., Bi, Y., Hou, W., Wu, C., & Han, T. (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Molecular Biology, 9, 59. https://doi.org/10.1186/1471-2199-9-59
Liu, J. S., Yu, C. M., Zhou, Y. Z., Han, Y. Y., Wu, F. W., Qi, B. F., & Zhu, Y. L. (1986). Study on the chemistry of Huperzine A and B. Acta Chimica Sinica, 44, 1035–1040.
Liu, X., Guan, H., Song, M., Fu, Y., Han, X., Lei, M., Ren, J., Guo, B., He, W., & Wei, Y. (2018). Reference gene selection for qRT-PCR assays in Stellera chamaejasme subjected to abiotic stresses and hormone treatments based on transcriptome datasets. PeerJ, 6, e4535. https://doi.org/10.7717/peerj.4535
Luo, H., Sun, C., Li, Y., Wu, Q., Song, J., Wang, D., Jia, X., Li, R., & Chen, S. (2010). Analysis of expressed sequence tags from the Huperzia serrata leaf for gene discovery in the areas of secondary metabolite biosynthesis and development regulation. Physiologia Plantarum, 139, 1–12. https://doi.org/10.1111/j.1399-3054.2009.01339.x
Ma, X., & Gang, D. (2005). The Lycopodium alkaloids. Natural Product Reports, 21, 752–772. https://doi.org/10.1039/b409720n
Ma, X., Tan, C., Zhu, D., & Gang, D. R. (2005). Is there a better source of huperzine A than Huperzia serrata? Huperzia A content of Huperziaiaceae species in China. Journal of Agricultural and Food Chemistry, 53, 1393–1398. https://doi.org/10.1021/jf048193n
Ma, X., Tan, C., Zhu, D., Gang, D. R., & Xiao, P. (2007). Huperzine A from Huperzia species—an ethnopharmacolgical review. Journal of Ethnopharmacology, 113, 15–34. https://doi.org/10.1016/j.jep.2007.05.030
Pfaffl, W. (2001). A new mathematical model for relative quantification in real-time R−PCR. Nucleic Acids Research, 29, 2002–2007. https://doi.org/10.1093/nar/29.9.e45
Pfaffl, W., Tichopad, A., Prgomet, C., & Neuvians, P. (2004). Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnology Letters, 26, 509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47
Qian, Z. M., & Ke, Y. (2014). Huperzine A: Is it an effective disease-modifying drug for Alzheimer’s disease? Frontiers in Aging Neurosci. 6, 216. https://doi.org/10.3389/fnagi.2014.00216
Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., Hoff, M. J. B. V. D., & Moorman, A. F. M. (2009). Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Research, 37, e45. https://doi.org/10.1093/nar/gkp045
Silver, N., Best, S., Jiang, J., & Thein, S. L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Molecular Biology, 7, 33. https://doi.org/10.1186/1471-2199-7-33
Sun, J., Morita, H., Chen, G., Noguchi, H., & Abe, I. (2012). Molecular cloning and characterization of copper amine oxidase from Huperzia serrata. Bioorganic & Medicinal Chemistry Letters, 22, 5784–5790. https://doi.org/10.1016/j.bmcl.2012.07.102
Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology, 3, 34. https://doi.org/10.1186/gb-2002-3-7-research0034
Wang, J., Wang, X., Liu, X., Li, J., Shi, X., Song, Y., Zeng, K., Zhang, L., Tu, P., & Shi, S. (2016). Synthesis of unnatural 2-substituted quinolones and 1,3-Diketones by a member of type III polyketide synthases from Huperzia serrata. Organic Letters, 18, 3550–3553. https://doi.org/10.1021/acs.orglett.6b01501
Wu, S., Fan, Z., & Xiao, Y. (2017). Comprehensive relative quantitative metabolomics analysis of lycopodium alkaloids in different tissues of *Huperzia serrata*. Synthetic & Systems Biotechnology, 3, 44–55. https://doi.org/10.1016/j.synbio.2017.12.003

Wu, Y., Tian, Q., Huang, W., Liu, J., Xia, X., Yang, X., & Mou, H. (2020). Identification and evaluation of reference genes for quantitative real-time PCR analysis in *Passiflora edulis* under stem rot condition. Molecular Biology Reports, 47, 2951–2962. https://doi.org/10.1007/s11033-020-05385-8

Xu, B., Lei, L., Zhu, X., Zhou, Y., & Xiao, Y. (2017). Identification and characterization of L-lysine decarboxylase from *Huperzia serrata* and its role in the metabolic pathway of lycopodium alkaloid. Phytochemistry, 136, 23–30. https://doi.org/10.1016/j.phytochem.2016.12.022

Yang, G., Wang, Y., Tian, J., & Liu, J. P. (2013). Huperzine A for Alzheimer’s disease: A systematic review and meta-analysis of randomized clinical trials. PLoS ONE, 8, e74916. https://doi.org/10.1371/journal.pone.0074916

Yang, M., Wu, S., You, W., Jaisi, A., & Xiao, Y. (2019). Selection of reference genes for expression analysis in Chinese medicinal herb *Huperzia serrata*. Frontiers in Pharmacology, 10, 44. https://doi.org/10.3389/fphar.2019.00044

Yang, M., You, W., Wu, S., Fan, Z., Xu, B., Zhu, M., Li, X., & Xiao, Y. (2017). Global transcriptome analysis of *Huperzia serrata* and identification of critical genes involved in the biosynthesis of huperzine A. BMC Genomics, 18, 245. https://doi.org/10.1186/s12864-017-3615-8

Zhang, B., Liu, J., Yuan, G., Chen, X., & Gao, X. (2018). Selection and evaluation of potential reference genes for gene expression analysis in *greenbug (Schizaphis graminum Rondani)*. Journal of Integrative Agriculture, 17, 2054–2065. https://doi.org/10.1016/S2095-3119(18)61903-3

Zhuang, H., Fu, Y., He, W., Wang, L., & Wei, Y. (2015). Selection of appropriate reference genes for quantitative real-time PCR in *Oxytropis ochrocephala* Bunge using transcriptome datasets under abiotic stress treatments. Frontires in Plant Science, 6, 475. https://doi.org/10.3389/fpls.2015.00475

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

---

**How to cite this article:** Fu, Y., Niu, F., Jia, H., Wang, Y., Guo, B., & Wei, Y. (2021). Reference gene selection for real-time quantitative PCR assays in different tissues of *Huperzia serrata* based on full-length transcriptome sequencing. *Plant Direct*, 5(11), e362. https://doi.org/10.1002/pld3.362