ISOLATION AND SELECTION OF REFERENCE GENES FOR *Ganoderma boninense* GENE EXPRESSION STUDY USING QUANTITATIVE REAL-TIME PCR (qPCR)

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ABSTRACT

Quantitative real-time PCR (qPCR) has become a favourite method for quantification of mRNA transcripts. However, several optimisation steps must be performed to avoid misleading qPCR results. One of the steps is selection of reference genes for normalisation purpose and these genes should be stably expressed across the samples. In this study, isolation of partial-length cDNA encoding seven potential reference genes from Ganoderma boninense *has been performed. These potential reference genes are* α-tubulin, β-tubulin, β-actin, *elongation factor 2 (*eef2*), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 40S ribosomal (*r40s*) and ubiquitin C (*ubc*). The expression of these reference genes was studied in mycelia, white button and fruiting body tissues of* G. boninense. *The qPCR data were analysed using* BestKeeper *and geNorm algorithms and both softwares have identified* β-tubulin, eEF2 *and* α-tubulin *as the most stable reference genes and* r40s *and ubc as the least stable reference genes. Three reference genes with the lowest M value (eEF2,* β-tubulin *and* α-tubulin*) were recommended by the geNorm software to be used in the qPCR analysis for more accurate normalisation.*

Keywords: quantitative real-time PCR, reference genes, *Ganoderma boninense*, *BestKeeper*, geNorm.

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INTRODUCTION

Gene expression study is an important analysis in many fields of biological research as the complex regulatory networks in living organism can be studied based on the expression pattern of genes (Chandna *et al.*, 2012). Several approaches, such as Northern blotting, ribonuclease protection assays, and reverse transcription quantitative real-time PCR (RT-qPCR), are commonly used for quantification of mRNA and gene expression study (Zheng and Sun, 2011). Among the approaches above, qPCR is a preferred method as this technique has several advantages in terms of sensitivity, specificity, large dynamic range, and the potential for high throughput as well as accurate quantification (Bustin, 2002; Ginzinger, 2002; Huggett *et al.*, 2005). Besides that, qPCR requires only a small amount of template sample. For these reasons, this technique has been widely used in gene expression study of

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many organisms. However, qPCR may produce misleading results due to several variables, such as the amount of starting material, the quality of total RNA and the efficiency of reverse transcription and qPCR (Vandesompele *et al.,* 2002; Kok *et al.,* 2005; Chen *et al.*, 2010).

There are several ways to control or eliminate these errors. One of the strategies is to normalise the expression of the gene of interest against selected reference genes (Huggett *et al*., 2005). Commonly used reference genes are mostly involved in basic and ubiquitous cellular processes including components of the cytoskeleton, glycolytic pathway, protein folding, protein synthesis and degradation, and synthesis of ribosome subunits (Chandna *et al*., 2012). The prime requirement of an ideal reference gene is its universal and constant expression level in all tissues independent of experimental conditions and developmental stages (Suzuki *et al*., 2000). However, it is difficult to obtain this ideal reference gene. A more practical strategy is to choose the gene that has minimal variability, or its variation is much lower than the target genes. For this purpose, many studies have been performed to select and validate reference genes for data normalisation in various experiments. It is important to find and select the stably expressed reference genes as any variation in the expresson of the reference genes may mask the real positive, or create false positive results (Takle *et al*., 2007).

Ganoderma boninense is the main causal agent of basal stem rot (BSR) disease of oil palm in South-east Asia especially in Malaysia (Idris, 2009) and Indonesia (Susanto, 2009). The disease was first reported in Malaya in 1931 (Thompson, 1931). During the early years, the disease only infected older palms above 25 years old and the economic losses due to the infection were considered insignificant (Turner, 1981). In the last 20 years, the fungus started to infect palms as young as 1-2 years after replanting (Singh, 1990). The disease can cause death to more than 80% palms when they are only half-way in the normal economic life cycle (Turner and Gillbanks, 2003). Nevertheless, efforts to control the disease have been hampered due to lack of knowledge on the dynamics of the pathogen (Pilotti *et al*., 2003). It is difficult to study the diseases caused by pathogenic fungi due to huge variability in their life styles and modes of interaction with their host plants (Ralf *et al*., 2011). One of the approaches to develop a systematic and effective solution against the disease is to study the fungal pathogenicity genes and predict the infection pathway. However, understanding on the host-pathogen interaction for *G. boninense* is still obscure (Paterson *et al*., 2009).

The objective of this study was to identify and isolate partial-length cDNA encoding potential reference genes from *G. boninense* for qRT-PCR analysis of *G. boninense* genes. The qPCR primers were designed based on these cDNA sequences and the appropriateness of these reference genes for gene expression profiling in *G. boninense* was evaluated.

MATERIALS AND METHODS

Preparation of *Ganoderma boninense* Culture

Ganoderma boninense Pat. PER71 culture was provided by the *Ganoderma* and Diseases Research for Oil Palm (GANODROP) Unit, Malaysian Palm Oil Board (MPOB). The culture was maintained on potato dextrose agar (PDA) plate and incubated at 28°C prior to further usage. Four types of *G. boninense* tissues were collected. First, the mycelium tissue was grown on PDA medium for 14 days (mycelium A). The mycelium was scraped from the surface of the medium and quickly chilled with liquid nitrogen before RNA extraction. The mycelium was also transferred to potato dextrose broth (PDB), grown for 14 days (mycelium B) and harvested by filtration using filter paper Grade 1 (Whatman, UK). The mycelium was then quickly rinsed with distilled water and chilled with liquid nitrogen. Two other types of tissues, white button (an initial structure of fruiting body) and mature fruiting body tissues were obtained by culturing *G. boninense* on rubberwood block (RWB) (Idris *et al*., 2006). The RWB (6 cm x 6 cm x 6 cm) was washed and placed in a polypropylene bag containing 50 ml of PDA and incubated for 12 hr, before being autoclaved for an hour at 121°C. After sterilising and cooling, the autoclaved RWB in the polypropylene bags was then inoculated with several slices of PDA media containing *G. boninense* mycelium (pre-grown for 14 days) and incubated at 28°C for 60 days, in the dark. Each fully colonised RWB was placed in a polythene bag (15 cm x 23 cm) containing a mixture of two parts of topsoil, one part of sand, and one part of organic matter. The RWB were covered with soil mixture and incubated at 28°C to induce the formation of the white button (about four weeks time) and the fruiting body (about seven weeks time). Once the white button and fruiting body were formed, both tissues were harvested from the RWB, cut into smaller pieces and quickly chilled with liquid nitrogen.

Total RNA Extraction and Synthesis of First-strand cDNA

Total RNA was isolated from *G. boninense* using RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. The integrity of the RNA samples was examined by agarose gel electrophoresis. The quality and quantity of the RNA samples were determined using Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc, USA). The integrity of total RNA samples was

further confirmed using Agilent 2100 Bioanalyzer and RNA 6000 Nano chips (Agilent Technologies, USA). All the RNA samples were adjusted to the same concentration prior to the analysis. RNA samples with $OD_{260/280}$ ratio from 1.8-2.0, $OD_{260/230}$ ratio from 2.0-2.5 and RNA integrity number (RIN) greater than 7.0 were used for further analysis.

For the amplification of partial-length cDNA, the first strand cDNA was synthesised using SMARTer™ RACE cDNA Amplification Kit (Clonetech, USA). While for qPCR analysis, the cDNA was converted from RNA samples using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). All the cDNA samples were kept at -20°C.

Selection and Isolation of Potential Reference Genes from *G. boninense*

Identification of potential reference genes for *G. boninense* qPCR analysis was performed based on the works of Yan and Liou (2006) and Kim and Yun (2011). Based on the above works, several potential reference genes including *α-tubulin*, β-*tubulin*, β-*actin*, elongation factor 2 (*eef*2), glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), 40S ribosomal (*r40s*), and ubiquitin C (*ubc*) were selected. Conserved regions for each potential reference gene were determined based on the amino acid and DNA sequences from several basidiomycetes fungi such as from *Coprinopsis cinerea* okayama 7 (*α-tubulin*: XP_001837010.1; β-tubulin: XP_001838299.1; *eef*2: XP_001829337.1; *r40s*: XP_001832137.1; *ubc*: XP_001832612.1), *Serpula lacrymans* var. lacrymans S7.9 (*β-tubulin*: EGN97448.1; *eef*2: EGO04929.1, *r40s*: EGO04094.1), *Ganoderma lucidum* (*gapdh:* ABD64597.1), *Schizophyllum commune* H4-8 (*gapdh:* XP_003028333.1), *Laccaria bicolor* S238N-H82 (*α-tubulin*: XP_001876589.1; *β-tubulin*: XP_001883306.1; *β-actin*: XP_001884444.1; *eef2:* XP_001873462.1; *gapdh*: XP_001879604.1; *r40s*: XP_001885801.1; *ubc*: XP_001885557.1), *Moniliophthora perniciosa* (*α-tubulin*: XP_002390037.1), *Pleurotus eryngii* var. Tuoliensis (*β-actin*: AEI69675.1), *P. ostreatus* (*β-actin*: AAV50019.1) and *Ustilago maydis* 521(*ubc*: XP_757355.1). Degenerate primers were designed based on the consensus DNA sequences.

PCR amplification was performed to amplify partial length cDNA encoding potential reference genes from *G. boninense* with AccuPrime™ *Taq* DNA Polymerase System (Invitrogen, USA). A total of 25 μl of PCR master mix contained 1X of AccuPrime™ PCR Buffer I, 1X of Universal Primer Mix (UPM), provided by the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) (UPM sequence: long primer (0.4 mM): 5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTAT CAACGCAGAGT–3', short primer (2.0 mM): 5'–CTAATACGACTCACTATAGG GC –3'), 9 ng of template cDNA, 0.4 μM of degenerate primer (*Table 1*), 0.1 U of AccuPrime™ *Taq* DNA polymerase and 17.5 μl of distilled water. The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (MJ Research, USA). The thermal cycler was programmed for 1 min pre-heating at 94°C followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 2 min, and finally an extension for 5 min at 68°C. Gel electrophoresis was performed after PCR amplification. The expected PCR products were excised from the gel, cloned and sequenced. DNA sequence of representative clones was determined by sequen-cing using Applied Biosystems 3730*xl* DNA Analyser with T7 RNA polymerase promoter, 5'-TAA TACGACTCACTATAGGG-3' as the sequencing primer. The DNA sequence analysis was carried out using Vector NTI software (Invitrogen, USA). DNA and amino acid sequence homology search were performed using BLAST 2.0 (Altschul *et al*., 1997) against the GenBank database.

Quantitative Real-time PCR (qPCR) Assays

Real-time qPCR primers were designed based on the cDNA sequences at the 3' UTR sequence with Beacon Designer 7.91 Software (PREMIER Biosoft International, USA). The primers were listed in *Table 2*.

Expression of the potential reference genes in different *G. boninense* tissues was determined via qPCR analysis. The templates were cDNA converted from total RNA isolated from mycelium A (cultured on PDA), mycelium B (cultured in PDB), white button and fruiting body. Real-time qPCR expression was performed on 96 wells plate in CFX Connect™ Real-Time System (Bio-rad, USA). A total of 10 μl qPCR reaction mixture contained 8 ng of cDNA template, 10 mM of each forward and reverse primers and 1X of iTaq™ Universal SYBR® Green Supermix (Biorad, USA). For efficiency test, two-fold diluted cDNA templates started from 1 ng to 16 ng were prepared. The thermal profile of the qPCR reaction was set at 95°C for 5 min, followed by 35 cycles of 95°C for 10 s, and 55°C for 10 s. The crossing cycle number (Cq) was automatically captured and the qPCR result was analysed using Bio-rad CFX Manager Software. All the reactions were prepared in triplicate for each cDNA sample. Non-template control (NTC) was also included for each gene in every qPCR run. Melt curve was generated by increasing temperature starting from 55°C to 95°C to ensure the specificity of PCR amplification.

Determination of Reference Gene Expression Stability using *BestKeeper* and geNorm Software

Gene stability analysis for all the seven potential reference genes was performed with *BestKeeper* (Pfaffl *et al.*, 2004) and geNorm software (Biogazelle,

TABLE 1. LIST OF DEGENERATE PRIMERS FOR THE AMPLIFICATION OF PARTIAL-LENGTH cDNA ENCODING POTENTIAL REFERENCE GENES

Gene	Degenerate primer	Sequence
α- tubulin	GAT 3	TTCCCTCGHATCCAYTTCC
β- tubulin	GBT 5	GAGGGTATGGACGAGATGG
β- actin	GAC 3	GGTCATCACCATCGGHAACG
eef2	GEF 3	GCGGTATCTACTCGGTGCTC
gapdh	GGA 3	TGGTACGACAACGAGTGGG
r40s	G4R 3	GGTTGCCGTCCGCAAGCA
ubc	GUB 1	CTCATTCGCGACTTCAAGC

TABLE 2. LIST OF *G. boninense* qPCR PRIMERS

Gene	Primer	Sequence	Product length (bp)
α-tubulin	GTR 7 GTR 8	GCACCGACTCTGGTGATGCT GATAGGCTATGGTCGCGAAG	100
β-tubulin	GBR 3 GBR 4	GAGTTCACTGAGGCCGAGTC TGCAACACGCTTATTCTTCG	130
β-actin	GAR 1 GAR 2	CAT ACG CTA CCA GTC CTT AGT CAT TCA GTT GTC ATT CAC	145
eef2	GER 1 GER 2	TGG TCA AGA ACA TCC GTA T CGC TAA CAA AGA CAA GGG	173
gapdh	GGR 3 GGR 4	CACGATGGGGCTCTGTAGTC GTTGCGCACGAGATTGATAC	96
r40s	GRR 1 GRR 2	CGG TCG TTG TAT GCT GTA GAG TGT CAT GTG GAA AGC	142
ubc	GUR 3 GUR 2	CGGACACACCATTTGAAG CAT CGG TTC TGG AGG ATA T	157

Belgium) to determine the most suitable reference genes (Pfaffl *et al.,* 2004; Vandesompele *et al.,* 2002) to be used for normalisation of qRT-PCR results.

The *BestKeeper* software is an Excel-based tool where all the Cq values obtained from the biological samples were plotted in an Excel table. The software calculated pairwise correlation coefficient for each potential gene and the most suitable reference genes were recommended based on the pairwise correlation analysis to the *BestKeeper* Index. The *BestKeepe*r index was obtained from the geometric mean of the Cq values for all the tested potential genes. Besides that, standard deviation between the Cq values of each gene and the whole data set was also obtained from this software. The gene with the highest coefficient of correlation is considered to have the highest stability and potentially to be a good reference gene candidate for qPCR analysis (Pfaffl *et al.,* 2004).

For geNorm software analysis, the level of pairwise variation for each tested gene with all other tested reference genes was determined. The expression stability measure (M value) of a particular reference gene was obtained based on the average pairwise variation of the gene compared with other tested genes in the analysis. Low M value indicated the most stable expression while high M value showed the least stable expression of a particular gene. Each gene was then ranked according to its M value. Based on the ranking, the most stable expression and suitable reference genes can be determined (Vandesompele *et al.,* 2002). The geNorm software is able to estimate the normalisation factor (NFn) when multiple reference genes are used. The analysis was started by including two reference genes with the lowest M value and continued by adding genes with increasing M value. The geNorm software calculated the pairwise variation (Vn/Vn+1) value between two normalisation factors NFn and NFn+1. NFn+1 contained an increasing number of reference genes compared to NFn. The software added in extra reference genes until the Vn/Vn+1 value dropped below a recommended threshold value such as 0.15. The optimum number of reference genes used in the qPCR analysis was suggested based on the threshold value (Vandesompele *et al.,* 2002).

RESULTS

Isolation and Sequence Analysis of Partial-length cDNA Encoding Potential Reference Genes

Isolation of partial-length cDNA encoding potential reference genes using PCR has successfully produced the expected PCR product for each gene. The size of the PCR products ranged from about 200 bp to 650 bp (*Figure 1*). These PCR products were purified, cloned and sequenced. The BLASTX sequence analysis results indicated that the 3′ UTR was found in all the partial-length sequences. The summarised sequence information of each isolated partial-length cDNA encoding potential reference gene was shown in *Table 3* while the cDNA sequences were displayed in *Figure 2*.

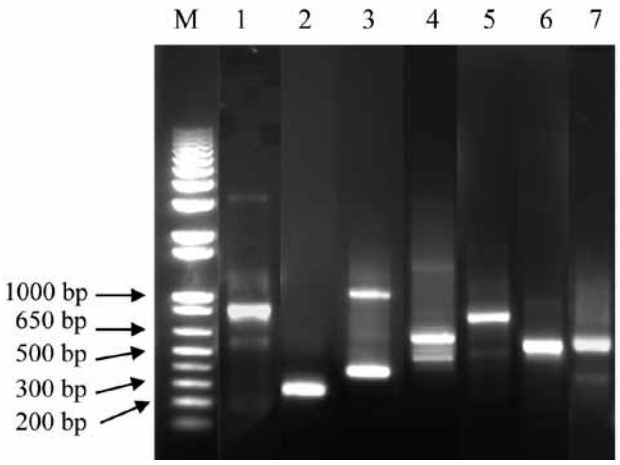

Figure 1. PCR products of potential G. boninense *real-time qPCR reference genes. 1.* α-tubulin*, 2.* gapdh*, 3. 40S ribosomal, 4.* α-actin*, 5.* ubc*, 6.* eef2*, 7.* β-tubulin*, M: 1 kb Plus DNA ladder.*

The Expression Profile and Expression Stability of the Reference Genes

To obtain an optimal condition for real-time qPCR analysis and to ensure the specificity of each primer set, the slope value and PCR efficiency (%) for each reference gene were obtained (*Table 4*). The qPCR efficiency for all the reference genes was within the range from 90% to 110%. This is an acceptable range for most of the efficiency studies (Paoletti and Mazzara, 2005). The melt curve analysis showed a single peak indicating that the primers pairs were very specific and only single PCR product was amplified (*Figure 3*). Furthermore, gel electrophoresis result also showed single fragment for each gene (*Figure 4*).

Selection of the Most Suitable Reference Genes

Selection of suitable reference genes for qPCR analysis was performed by studying the expression of all the potential reference genes in four types of *G. boninense* tissues: mycelium A, mycelium B, white button, and fruiting body. The seven potential reference genes were analysed in 12 biological samples.

Based on the analysis result of *BestKeeper* software, *β-actin* has the lowest standard deviation (0.44) while *ubc* has the highest standard deviation (0.89). The stability of each potential reference gene was then calculated based on the pairwise correlation between the genes and *BestKeeper* index. The *β-tubulin* (0.903), *eef2* (0.893) and *α-tubulin* (0.878) were the most stable reference genes among the seven potential reference genes. The least stable gene was *ubc* (0.624). The stability of the genes were arranged in a descending order as **β-***tubulin* > *eef2* > *α-tubulin* > *β –actin* > *gapdh* > *r40s* > *ubc.*

In the geNorm analysis, the M value for each gene was determined (*Table 5*) in four types of *G. boninense* tissues. The qPCR data were then analysed using the geNorm software and M-value for each gene was determined (*Table 5*). The stablility of genes were determined and arranged in descending order as *β-actin* >*gapdh* > *eef2* > *β–tubulin* > *α-tubulin* > *ubc* > *r40s.*

Optimal Number of Internal Control Genes for Normalisation

The optimal number of reference genes was determined using geNorm software analysis. Based on the analysis, the optimal number of reference genes could not be determined as the variability between normalisation factors (based on the n and n+1 least variable reference genes) because the factors were relatively high where the values of geNorm V exceeded 0.15 for all the reference gene combinations. However, the software has recommended to use three reference genes with the lowest M value (*Figure 5*). This is because in the non-optimal case, normalisation with multiple reference genes was considered more accurate than using only a single non-validated reference gene (Vandesompele *et al.*, 2002).

Variability between sequential normalisation factors (based on the n and n+1 least variable reference targets) is relatively high (geNorm V > 0.15). However, three reference genes with the lowest M value (*eef2*, *β-tubulin* and *α-tubulin*) were recommended in the analysis.

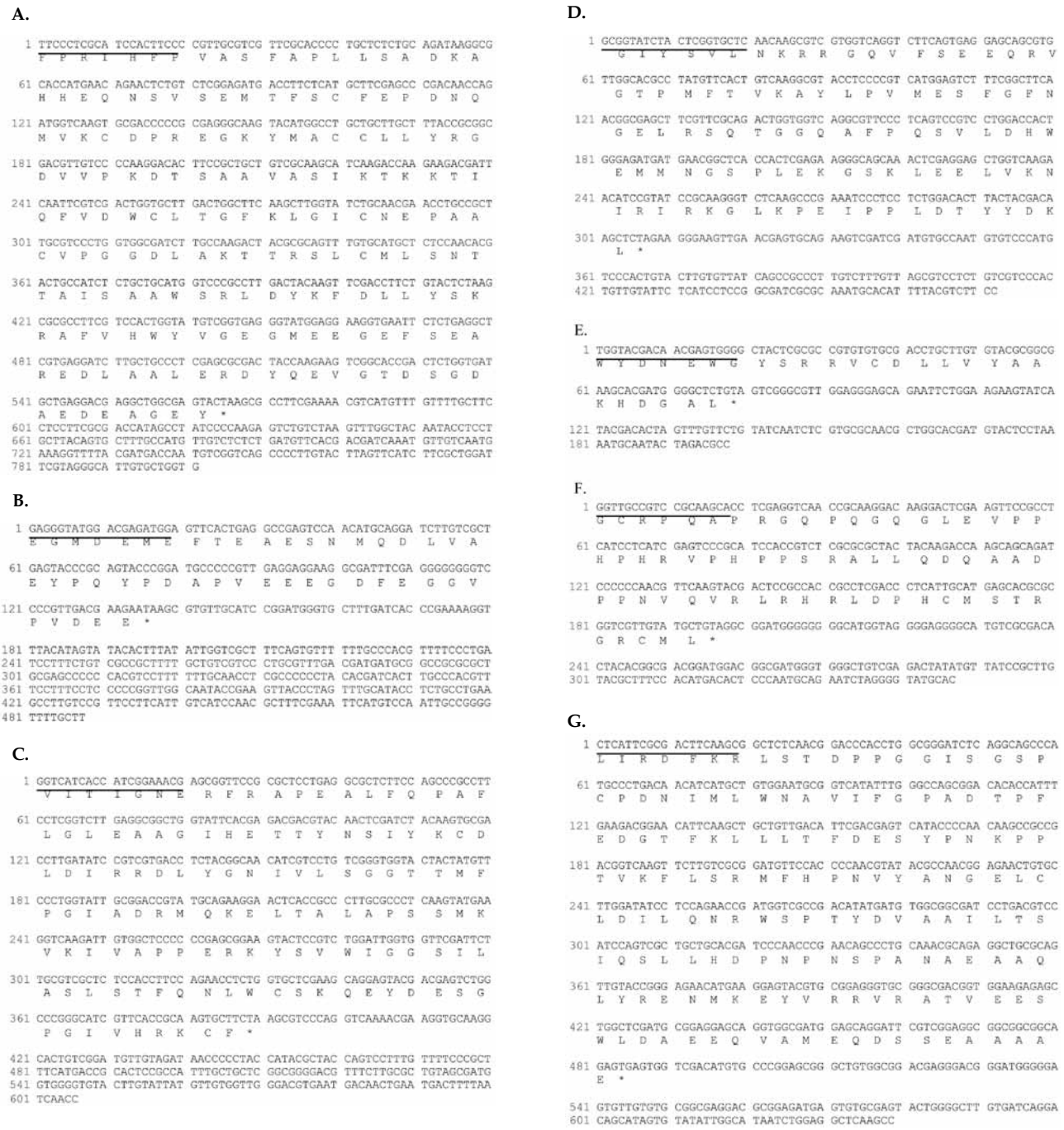

Figure 2. DNA and translated amino acid sequence of partial-length cDNA encoding potential reference genes. Sequence at the bottom of the DNA sequence indicates the deduced amino acid sequence. The underlined sequence indicates the location of degenerate primer. "" Indicates the location of stop codon. A.* α-tubulin, *B.* β-tubulin, *C.* β-actin, *D.* eef2, *E.* gapdh, F. r40s *and G.* ubc.

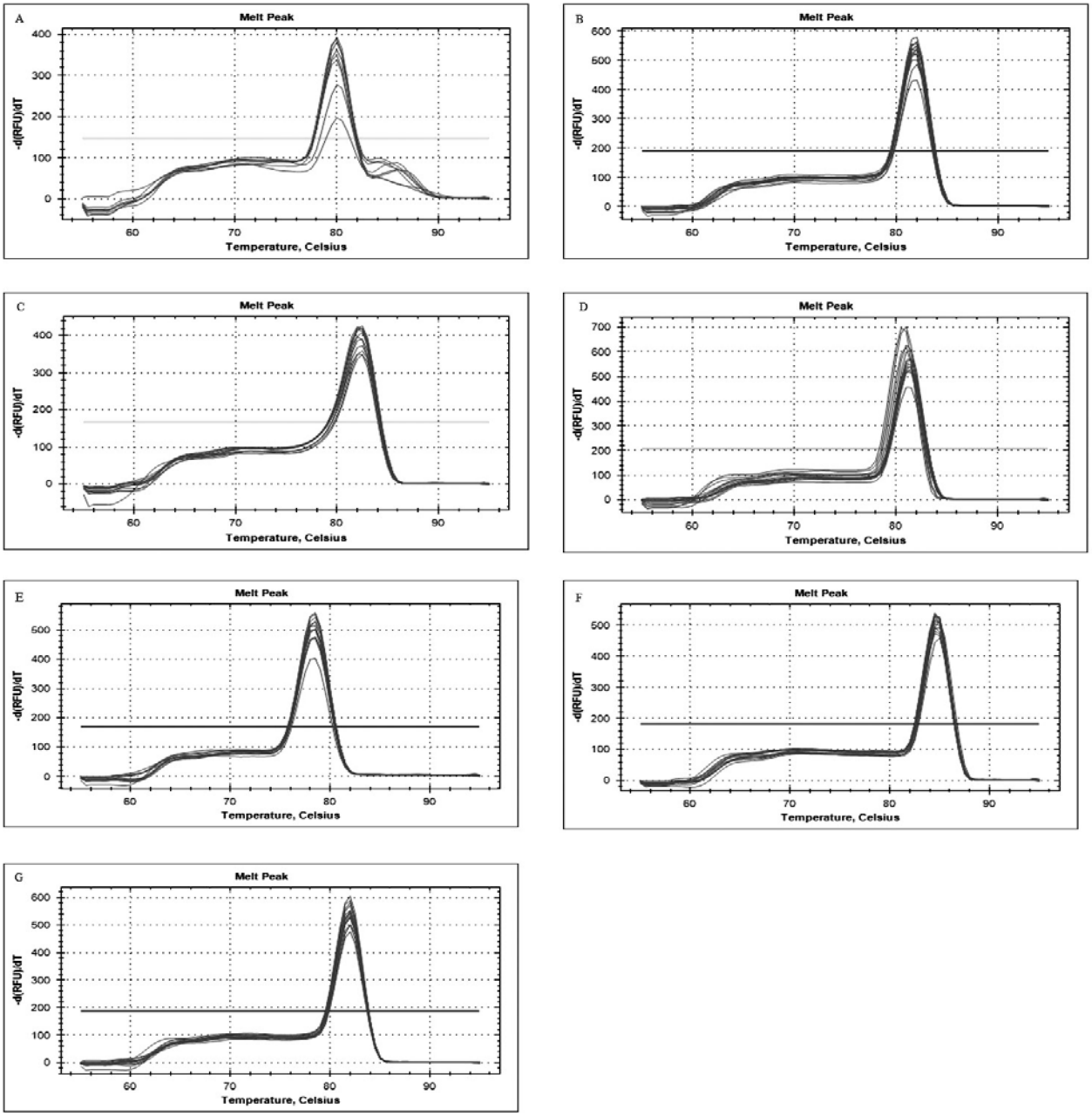

Figure 3. Melt curve analysis for qPCR analysis of reference genes. A. α-tubulin, *B.* β-tubulin, *C.* β-actin, *D.* eef2, E. gapdh, *F.* r40s *and G.* ubc.

TABLE 3. SUMMARISED SEQUENCE INFORMATION ON PARTIAL-LENGTH cDNA ENCODING POTENTIAL REFERENCE GENES FROM *G. boninense*

No.	Gene name	Partial-length sequence			Similarities (coding region only)		
		Sequence (bp)	Coding region (bp)	3′ UTR (bp)	Species	Accession No.	%
1.	*α-tubulin*	801	564	237	*Dichomitus squalens* LYAD-421 SS1	EJF62815.1	99
					Trametes versicolor FP-101664 SS1	EIW61504.1	98
					Laccaria bicolor S238N-H82	XP_001876589.1	88
2.	*β-tubulin*	488	135	353	*Amanita strobiliformis*	AFU72287.1	100
					Agaricus bisporus var. burnettii JB137-S8	EKM76844.1	100
					Puccinia graminis f. sp. tritici CRL 75-36-700-3	XP_003330667.1	100
3.	*β-actin*	606	388	218	*T. versicolor* FP-101664 SS1	EIW62276.1	100
					Pleurotus eryngii var. Tuoliensis	AEI69675.1	97
					P. ostreatus	AAV50019.1	97
4.	eef2	472	305	167	*Postia placenta* Mad-698-R	XP_002475161.1	93
					Punctularia strigosozonata HHB-11173 SS5	EIN13849.1	93
					D. squalens LYAD-421 SS1	EJF66813.1	91
5.	*gapdh*	188	68	120	*G. lucidum*	ABD64597.1	96
					Schizophyllum commune H4-8	XP_003028333.1	96
					D. squalens LYAD-421 SS1	EJF57492.1	92
6.	*r40S*	347	195	152	*A. bisporus*	P78571.1	96
					S. commune H4-8	XP_003027426.1	96
					C. cinerea okayama7#130	XP_001832137.1	96
7.	*ubc*	639	483	156	*D. squalens* LYAD-421 SS1	EJF59714.1	98
					Ceriporiopsis subvermispora B	EMD37203.1	98
					T. versicolor FP-101664 SS1	EIW53794.1	96

TABLE 4. SUMMARY OF EFFICIENCY TEST RESULTS FOR REFERENCE GENES

Gene	Slope	R^2	y-int	E (%)
α-tubulin	-3.534	0.988	36.50	91.8
β-tubulin	-3.455	0.993	23.65	94.7
β-actin	-3.576	0.996	23.38	90.4
eef2	-3.207	0.997	22.03	105.0
gapdh	-3.554	0.997	20.25	91.2
r40s	-3.530	0.994	26.33	92.0
ubc	-3.497	0.997	25.34	93.2

Note: Slope – slope of standard curve; R^2 – correlation coefficient, y-int – y-intercept of standard curve; E(%) – qPCR efficiency (%).

DISCUSSION

This study evaluated the isolation of partial length cDNA encoding potential reference genes from *G. boninense.* Selection of reference genes which were stably expressed in four diferrent types of tissues of *G. boninense* was performed and analysed using two algorithms, namely *BestKeeper* and geNorm. The tissues included mycelia from solid and liquid media, white button and fruiting body. All these tissues belong to dikaryotic stages of *G. boninense* life cycle and none of other tissue from other stages, such as spore and haploid mycelia, were analysed in this study.

The qPCR has become a favourite method to study the expression of a gene. However, quantification of gene expression is usually affected by several factors such as variations in experimental sources and method of normalisation. Variations in experimental sources include sample-to-sample variation, differences in reverse transcription reaction, qPCR efficiency and the amount of cDNA used in each qPCR reaction. Normalisation the expression level of a gene against stably expressed

TABLE 5. RANKING OF POTENTIAL REFERENCE GENES ACCORDING TO THE STABILITY VALUE USING *BestKeeper* AND geNorm SOFTWARE

Gene name	*BestKeeper*		geNorm	
	Stability value	Ranking order	Stability value	Ranking order
α-tubulin	0.878	3	0.499	3
β-tubulin	0.903	1	0.478	2
β-actin	0.839	4	1.151	7
eef2	0.893	2	0.476	1
gapdh	0.796	5	0.912	6
r40s	0.699	6	0.770	5
ubc	0.624	7	0.638	4

Note: Stability values for *BestKeeper* software were pairwise correlation value while stability value for geNorm software were the expression stability measure (M value).

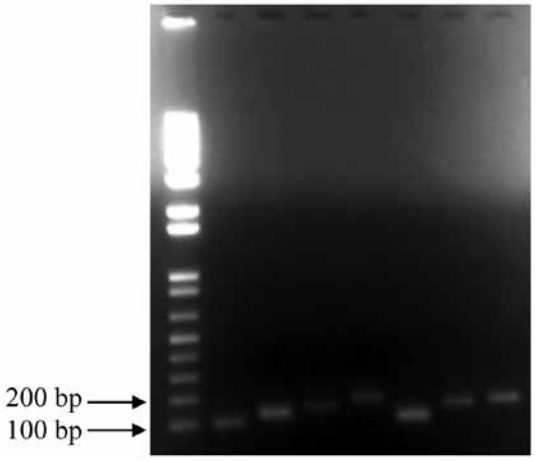

Figure 4. Gel electrophoresis of PCR product for each reference gene after qPCR amplification. 1. α-tubulin*; 2.* β-tubulin*; 3.* β-actin*, 4.* eef2*; 5.* gapdh*, 6.* r40s *and 7.* ubc*. M: 1 kb Plus DNA ladder.*

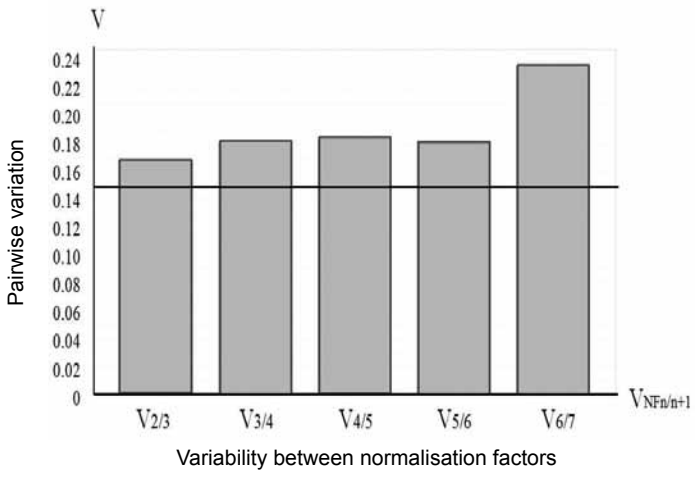

Figure 5. Determination of optimal number of reference genes calculated by geNorm software.

internal genes could compensate these kinds of variations (Pfaffl *et al*., 2004). Furthermore, accurate and correct normalisation is essential to eliminate any small but significant differences arised from the comparison of gene expression from different organs or tissues. The accuracy of qPCR results strongly relied on the selection of one or more reference genes that are stably expressed across different tissues or organ samples (Vandesompele *et al*., 2002; Radonic *et al*., 2004). However, it is very rare for a gene to express at such a constant level under different conditions (Haberhausen *et al*., 1998; Thellin *et al*., 1999).

Seven potential genes which were commonly used as reference genes in qPCR analysis especially in plant pathogenic fungi have been identified (Vieira *et al*., 2011; Li *et al*., 2012; Yan and Liou, 2006). Analysis results based on the *BestKeeper* software has suggested *β-tubulin* (1), *eef*2 (2) and *α-tubulin* (3) as the most stable reference genes among the seven potential reference genes. Similar reference genes were also recommended by geNorm software analysis although the ranking of the best reference genes is slightly different between these two analyses. The ranking according to expression stability depends on the high coefficient of correlation value to the *BestKeeper* index (close to 1.0) for *BestKeeper* software and low M-value (close to 0) for the geNorm software. Obviously, selection of the stable reference genes depends on the findings of reference genes with the lowest expression variability among different tissues. However, a reference gene with slight variablility in expression in different types of tissues may be accepted as long as the gene could detect the expression variation of the target gene (Zhu *et al*., 2013). Hence, a few studies indicated that reference genes with M-value below 1.0 were considered suitable for normalisation purpose (Nielsen and Boye, 2005; Botteldoorn *et al*., 2006; Spinsanti *et al*., 2006) while geNorm software limited the value below 1.5 (Vandesompele *et al*., 2002). Based on the geNorm result in this study, all the reference genes have a M-value below 1.0 except for *β-actin*. Hence, all the seven genes were suitable to be used as reference genes in *G. boninense* qPCR analysis if the standard was set based on the limit set by the geNorm software (M-value < 1.5). While six reference genes, except *β-actin*, were considered suitable for normalisation purpose if the standard was set based on the limit set by a few studies stated above (M-value < 1.0).

On the other hand, expression of *β-actin, r40s* and *gapdh* in four types of tissues was considered unstable and has been listed as the least stable reference genes by both softwares. These genes were commonly used to normalise the qPCR results in other expression studies. However, a number of studies indicated that the expression levels of these genes vary significantly between different individuals, cell types, developmental stages, and experimental conditions (Bustin, 2000; Suzuki *et al*., 2000; Warrington *et al*., 2000). Hence, it is important to validate the stability of these reference genes before qPCR expression analysis to prevent selection of unsuitable reference genes.

Based on geNorm V analysis, three of the most stable reference genes were suggested to be used for normalisation purpose although the variability between normalisation factors is relatively high (> 0.15). The three reference genes (with the lowest M-value) are recommended for high accuracy qPCR results which strongly rely on the choice and number of reference genes that were stably expressed in various types of tissues (Vandesompele *et al*., 2002). Hence, the accuracy of qPCR results will be improved if a higher number of reference genes were used.

CONCLUSION

Based on the analysis of both *BestKeeper* and geNorm softwares, **β-***tubulin*, *eef*2 and *α-tubulin* were identified as the most stable reference genes among the seven potential reference genes for normalisation purpose in qPCR analysis. Both softwares suggested the same reference genes although the ranking order for these three reference genes was slightly different when analysed by both softwares. Three reference genes with the lowest M value were suggested by geNorm software for more accurate normalisation.

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