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Identification and validation of reference genes for RT-qPCR analysis in *Sclerodermus guani* (Hymenoptera: Bethylidae)

Rina Zhao[1], Xiaomeng Guo[1,2], Ling Meng[1] and Baoping Li[1]

[1]Department of Entomology, School of Plant Protection, Nanjing Agricultural University, Nanjing, Jiangsu Province, PR China and [2]Research Institute of Agricultural Sciences of Zhenjiang city, Zhenjiang, Jiangsu Province, PR China

Research Paper

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Corresponding author:
Baoping Li;
Email: lbp@njau.edu.cn

Abstract

Gene expression studies in organisms are often conducted using reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR), and the accuracy of RT-qPCR results relies on the stability of reference genes. We examined ten candidate reference genes in *Sclerodermus guani*, a parasitoid wasp that is a natural enemy of long-horned beetle pests in forestry, including *ACT, EF1α, Hsc70, Hsp70, SRSF7, α-tubulin, RPL7A, 18S, 28S,* and *SOD1*, regarding variable biotic and abiotic factors such as body part, life stage, hormone, diet, and temperature. Data were analysed using four dedicated algorithms (ΔCt, BestKeeper, NormFinder, and geNorm) and one comparative tool (RefFinder). Our results showed that the most stable reference genes were *RPL7A* and *EF1α* regarding the body part, *SRSF7* and *Hsc70* regarding the diet, *RPL7A* and *α-tubulin* regarding the hormone, *SRSF7* and *RPL7A* regarding the life stage, and *SRSF7* and *α-tubulin* regarding temperature. To ascertain the applicability of specific reference genes, the expression level of the target gene (*ACPase*) was estimated regarding the body part using the most stable reference genes, *RPL7A* and *EF1α*, and the least stable one, *SOD1*. The highest expression level of *ACPase* was observed in the abdomen, and the validity of *RPL7A* and *EF1α* was confirmed. This study provides, for the first time, an extensive list of reliable reference genes for molecular biology studies in *S. guani*.

Introduction

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) has become a cornerstone technique in molecular biology for quantifying gene expression levels, due to its rapidity, affordability, and ease of operation (Huggett *et al*., 2005). The accuracy of RT-qPCR relies on using stable reference genes for normalisation, which is a prerequisite that ensures the reliability of comparative gene expression analyses across different samples and experimental conditions (Bustin *et al*., 2009). Housekeeping genes, such as *ACT*, *GAPDH,* and *Tubulin*, encode proteins that are essential for maintaining basic cellular functions. These genes are commonly used as reference genes because of their stable and consistent expression across various physiological states (Derveaux *et al*., 2010). Yet, none of the reference genes are universal in all situations or across all species (Dheda *et al*., 2005). Therefore, the selection of suitable reference genes is indispensable for gene expression studies of organisms of interest.

Reference genes have been characterised in a few species of parasitoids. For example, *RPL13* and *EF1α* regarding varying body tissues are identified in *Anastatus japonicus* (Eupelmidae), *18S* and *EF1α* regarding different life stages in *Tamarixia radiata* (Eulophidae), *18S*, *H3*, and *AK* regarding low temperatures in *Cotesia chilonis* (Braconidae), and *ZFP268* and *EF2* regarding diets in *Trichogramma chilonis* (Trichogrammatide) (Li *et al*., 2019; Guo *et al*., 2020; Xie *et al*., 2021; Liu *et al*., 2022). These factors considered for identifying reference genes are fundamental and crucial. A consideration of more factors of interest to characterise more reference genes can validate gene expression levels with higher accuracy (Lü *et al*., 2018).

Sclerodermus wasps (Hymenoptera: Bethylidae) provide ideal model systems for theoretical studies of social evolution in Hymenoptera due to their unique social behaviour among parasitoid insects. In this group of parasitoids, females share a single host and tend their communal brood (Tang *et al*., 2014), exhibiting cooperative behaviour among females that aligns with the concept of quasi-sociality as defined by Wilson (Wilson, 1974). *Sclerodermus* parasitoids have long been applied as biocontrol agents in augmentation biological control programmes targeting wood-boring pest beetles on trees in the Chinese mainland (Yang *et al*., 2014). The ever-changing advances in molecular biology offer a novel and inspirational approach to gaining insights into the social evolution and biocontrol potentials of organisms. Some molecular studies use *18S* as a reference gene in *S. guani* (Liu *et al*., 2017; Wu *et al*., 2020). But its stability remains to be validated.

Table 1. Features of candidate reference genes in *Sclerodermus guani*.

Gene name	Primer sequences (5′ → 3′)	Length (bp)	*E* (%)	R^2	Linear regression
β-actin (*ACT*)	F: AGGACCTCTACGCCAACAAC	162	102.68	0.9998	$y = 24.425 - 3.2593x$
	R: TGGAACCACCGATCCATACG				
Elongation factor 1α (*EF1α*)	F: GAGTTCCCACCTCTTGGACG	114	97.19	0.9982	$y = 24.411 - 3.3911x$
	R: GGCAGCCTTGGTTACTTTGC				
Constitutive forms heat shock (*Hsc70*)	F: CCGATACCGAGCGTCTCATC	167	97.34	0.9958	$y = 24.253 - 3.3873x$
	R: TTTGCCGCCATCATCCACTA				
Heat Shock Protein 70 (*Hsp70*)	F: GCGATCTTAACCGGAGAGGG	141	96.46	0.9997	$y = 27.379 - 3.4099x$
	R: GCACGGGATTCGTGCATTAC				
Serine and arginine rich splicing factor 7 (*SRSF7*)	F: GGGTCGCTAGAAATCCTCCA	88	99.06	0.9986	$y = 28.689 - 3.3446x$
	R: ACTCCATCCAAGCCACGAAC				
alpha-tubulin (*α-tubulin*)	F: AAATTGGCGGACCAATGCAC	112	95.84	0.9994	$y = 29.076 - 3.4259x$
	R: AGTCAACCGACAGACGTTCC				
Ribosomal protein L7A (*RPL7A*)	F: GTAGCAGCTGCACCTTTAGC	87	95.57	0.9997	$y = 26.920 - 3.4328x$
	R: ACCAAAATTACGTGAACGCTT				
18S ribosomal RNA (*18S*)	F: CCTTAGATCGTACCCACATT	120	100.81	0.9989	$y = 16.455 - 3.3027x$
	R: CCACCGATTGGTTTTGATCTA				
28S ribosomal RNA (*28S*)	F: GTAGTCGTGCACTTCTCCCC	147	101.49	0.9997	$y = 23.910 - 3.2868x$
	R: GATACCGTGCGAGTACCGTC				
superoxide dismutase 1 (*SOD1*)	F: AGCAAGATGGTAAGGCAGTT	182	95.63	0.9998	$y = 25.782 - 3.4314x$
	R: ATCACCCACATGACGAGTTT				

In this study, we selected ten candidate genes to characterise reference genes in *S.guani*, including *ACT*, *EF1α*, *Hsc70*, *Hsp70*, *SRSF7*, *α-tubulin*, *RPL7A*, *18S*, *28S*, and *SOD1*. The majority of the above are prevalent in insects (Lü *et al.*, 2018), while *SOD1* is used in other insect orders besides Hymenoptera and thus may warrant further examination (Bai *et al.*, 2020; Yan *et al.*, 2021; Shen *et al.*, 2022). We considered a variety of factors during characterisation, including body site, life stage, hormones, diet, and temperature. Reference genes identified based on these biotic and abiotic factors can be widely applicable in molecular studies of this group of parasitoids. Four common algorithms (ΔCt, BestKeeper, NormFinder, and geNorm) and one comparative algorithm (RefFinder) were applied to analyse the stability and rank the reference genes based on quantitative data. The expression profile of *ACPase* was assessed regarding body parts to validate the results. The target gene Acid phosphatase (*ACPase*) is an enzyme that catalyses the hydrolysis of phosphate esters in an acidic environment and is considered a typical venom component in parasitoids (Anand and Srivastava, 2012; Liu *et al.*, 2017).

Materials and methods

Insects rearing

The colony of *S. guani* was established in the insectary from start-up wasps offered by the Research Institute of Forestry Protection of Jiangsu Province, at Nanjing city, Jiangsu province, China. *Tenebrio molitor* (Coleoptera: Tenebrionidae) pupae were used as the host, which is a viable substitutive host for *S. guani* (Yang *et al.*, 2018). They were reared in insectary at conditions of 27 ± 1°C in temperature, 60–70% in relative humidity, and 14L:10D in photoperiod.

Biotic factor treatment

Two biotic factors were considered in the identification: life stage and body part. The life stage included the 1st and 4th instar larvae, pupae, and male, and female adults. A group of 10 individuals were operated as one sample (replicate). The body part included the head, thorax, abdomen, antennae, and leg; varying numbers of individual parts were grouped as one sample (replicate): 60 for the head, 30 for the thorax or abdomen, and ca. 100 for antennae or legs. Three replicates were operated.

Abiotic factor treatment

Three abiotic factors were examined: diet, temperature, and hormone. The dietary treatment was imposed on adults at eclosion with three diets as supplementary food: the 20% sugar solution (soaked in a cotton line as the control), *T. molitor* pupae, and *Monochamus alternatus* larvae, and these diets were provided for different periods: 24 and 48 h. Each diet by period treatment was tested for 30 female wasps. Three levels of temperature were tested: 4, 27 and 35°C, respectively corresponding to the temperatures during the mass production for cold storage, rearing in insectary, and release in the field in summer. Sample insects were taken from the colony maintained at 27°C, then moved to chambers at different temperatures and maintained there for three hours for their acclimation before moving to subsequent procedures. The hormonal treatment included an injection of 0.04 µl ecdysone

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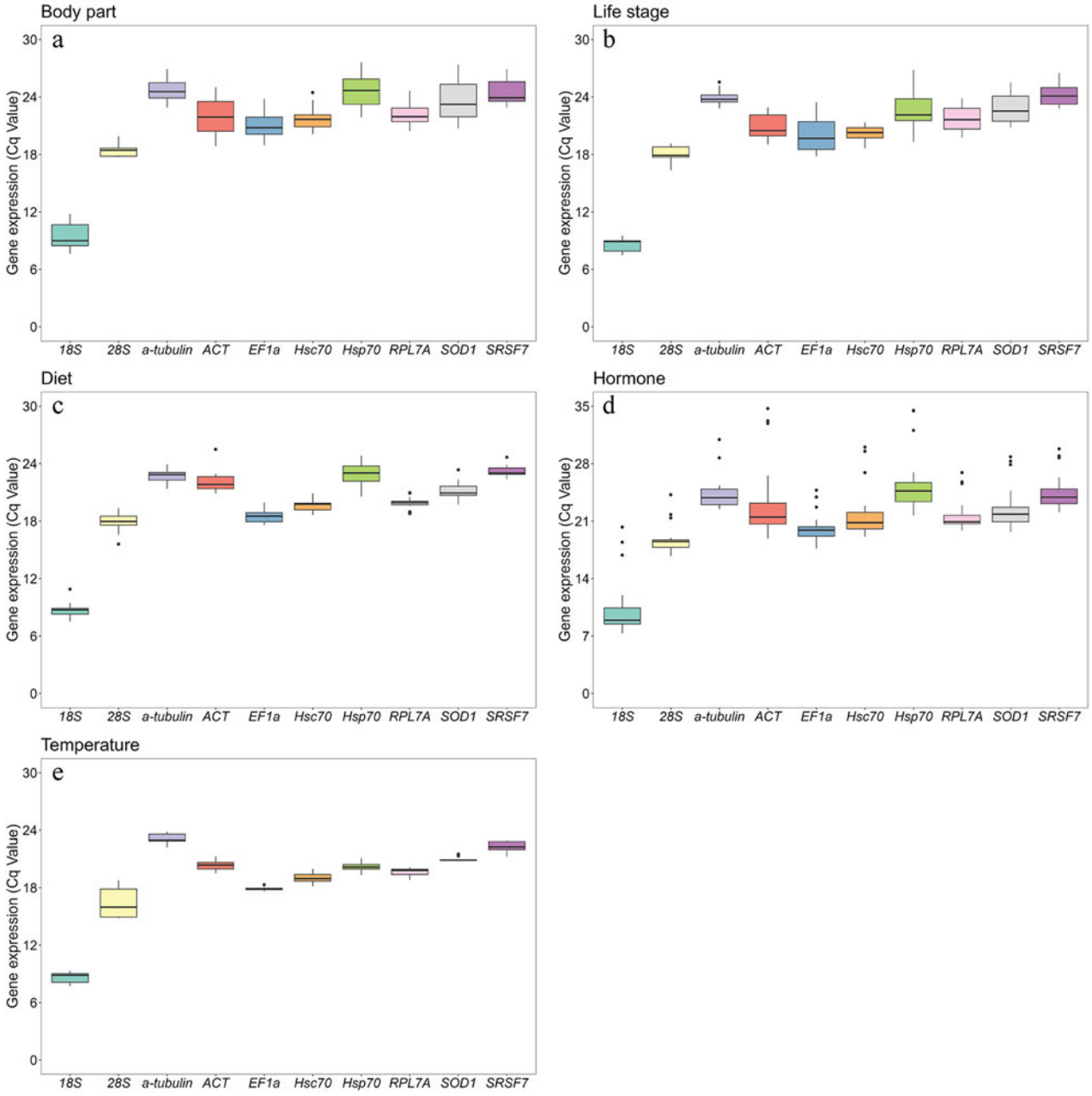

Figure 1. *Cq* values of reference genes regarding body part (a), life stage (b), diet (c), hormone (d), and temperature (e). The dots are outliers.

at 14, 64, or 120 pg μl^{-1} in methanol, or of juvenile hormone at 16, 32, or 64 pg μl^{-1} dissolved in acetone; the same volume of methanol or acetone were used as their controls. The injection was operated by inserting nanoliter syringe Nanoject III (Drummond Scientific) through the intersegment membrane of the wasp. After the injection, the wasp was placed in a glass tube for 24 h before the identification procedure. Each sample consisted of 30 wasps and was collected into a 1.5 ml microcentrifuge tube, which was immediately frozen in liquid nitrogen and then stored at −80°C for subsequent RNA extraction. Three replicates were examined.

Reference genes selection and primer design

Ten candidate genes were selected based on the *S. guani* transcriptomic data (unpublished), including *ACT*, *EF1α*, *Hsc70*, *Hsp70*, *SRSF7*, *α-tubulin*, *RPL7A*, *18S*, *28S*, and *SOD1*. Gene-specific primers were designed using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (table 1).

RNA extraction and cDNA synthesis

Total RNA was extracted using FreeZol Reagent (Vazyme, NanJing, China) following the manufacturer's protocol. RNA was eluted in 20 µl RNase-free water. RNA quantity was measured by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with absorbance levels of 260 and 280 nm. An aliquot of 500 ng of total RNA was used to synthesise first-strand cDNA using the PrimeScript™ RT reagent Kit with the gDNA Eraser (Perfect Real Time) (Takara, Beijing, China). The cDNA was stored at – 20°C for later RT-qPCR experiments.

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Table 2. Stability of ten reference genes measured by four algorithms regarding variable conditions.

		ΔCt		BestKeeper		NormFinder		geNorm	
Conditions	Rank	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Body part	1	*RPL7A*	0.92	*28S*	0.51	*18S*	0.234	*RPL7A*	0.259
	2	*EF1a*	0.96	*a-tubulin*	0.84	*SRSF7*	0.372	*EF1a*	0.259
	3	*18S*	0.97	*RPL7A*	0.9	*RPL7A*	0.376	*Hsc70*	0.346
	4	*SRSF7*	1.00	*Hsc70*	0.91	*EF1a*	0.477	*a-tubulin*	0.513
	5	*Hsc70*	1.02	*EF1a*	1.08	*a-tubulin*	0.604	*18S*	0.603
	6	*α-tubulin*	1.06	*18S*	1.13	*Hsc70*	0.609	*SRSF7*	0.65
	7	*Hsp70*	1.11	*SRSF7*	1.2	*Hsp70*	0.732	*Hsp70*	0.683
	8	*28S*	1.63	*Hsp70*	1.36	*28S*	1.402	*28S*	0.888
	9	*ACT*	1.83	*ACT*	1.68	*ACT*	1.636	*ACT*	1.083
	10	*SOD1*	1.83	*SOD1*	1.81	*SOD1*	1.654	*SOD1*	1.233
Life stage	1	*RPL7A*	1.14	*a-tubulin*	0.59	*SRSF7*	0.463	*RPL7A*	0.512
	2	*SRSF7*	1.14	*18S*	0.6	*RPL7A*	0.49	*SRSF7*	0.512
	3	*a-tubulin*	1.28	*Hsc70*	0.63	*a-tubulin*	0.703	*SOD1*	0.615
	4	*SOD1*	1.31	*28S*	0.69	*Hsc70*	0.818	*EF1a*	0.705
	5	*Hsc70*	1.35	*SRSF7*	0.97	*SOD1*	0.854	*ACT*	0.818
	6	*ACT*	1.37	*RPL7A*	1.12	*ACT*	0.947	*a-tubulin*	0.957
	7	*EF1a*	1.51	*ACT*	1.17	*28S*	1.19	*Hsc70*	1.049
	8	*28S*	1.55	*SOD1*	1.33	*EF1a*	1.237	*28S*	1.17
	9	*18S*	1.62	*Hsp70*	1.62	*18S*	1.309	*18S*	1.255
	10	*Hsp70*	2.23	*EF1a*	1.66	*Hsp70*	2.036	*Hsp70*	1.451
Diet	1	*Hsc70*	0.65	*RPL7A*	0.32	*Hsc70*	0.22	*EF1a*	0.308
	2	*SRSF7*	0.67	*SRSF7*	0.38	*SRSF7*	0.339	*SRSF7*	0.308
	3	*EF1a*	0.68	*Hsc70*	0.4	*EF1a*	0.36	*RPL7A*	0.406
	4	*18S*	0.72	*18S*	0.44	*18S*	0.402	*Hsc70*	0.482
	5	*RPL7A*	0.75	*EF1a*	0.47	*RPL7A*	0.498	*18S*	0.542
	6	*a-tubulin*	0.79	*a-tubulin*	0.55	*a-tubulin*	0.537	*a-tubulin*	0.582
	7	*28S*	0.84	*SOD1*	0.55	*28S*	0.61	*28S*	0.629
	8	*SOD1*	0.91	*28S*	0.56	*SOD1*	0.726	*SOD1*	0.678
	9	*ACT*	1.02	*ACT*	0.67	*ACT*	0.876	*ACT*	0.74
	10	*Hsp70*	1.11	*Hsp70*	0.88	*Hsp70*	0.99	*Hsp70*	0.815
Hormone	1	*a-tubulin*	1.21	*28S*	1	*a-tubulin*	0.235	*RPL7A*	0.569
	2	*RPL7A*	1.32	*RPL7A*	1.33	*Hsc70*	0.556	*SRSF7*	0.569
	3	*Hsc70*	1.33	*EF1a*	1.35	*SRSF7*	0.808	*EF1a*	0.611
	4	*SRSF7*	1.34	*SRSF7*	1.58	*RPL7A*	0.835	*a-tubulin*	0.723
	5	*EF1a*	1.54	*a-tubulin*	1.6	*18S*	1.032	*28S*	0.88
	6	*18S*	1.56	*SOD1*	1.75	*SOD1*	1.198	*SOD1*	1.018
	7	*SOD1*	1.64	*Hsc70*	1.97	*EF1a*	1.23	*Hsc70*	1.111
	8	*28S*	1.65	*18S*	2.29	*Hsp70*	1.238	*18S*	1.243
	9	*Hsp70*	1.7	*Hsp70*	2.31	*28S*	1.297	*Hsp70*	1.335
	10	*ACT*	2.62	*ACT*	3.31	*ACT*	2.491	*ACT*	1.592

(*Continued*)

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Table 2. (*Continued.*)

		ΔCt		BestKeeper		NormFinder		geNorm	
Conditions	Rank	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
Temperature	1	*α-tubulin*	0.6	*EF1a*	0.14	*SRSF7*	0.21	*α-tubulin*	0.135
	2	*SRSF7*	0.61	*SOD1*	0.19	*α-tubulin*	0.21	*SRSF7*	0.135
	3	*EF1a*	0.64	*RPL7A*	0.39	*Hsc70*	0.258	*18S*	0.259
	4	*18S*	0.66	*Hsp70*	0.41	*EF1a*	0.316	*RPL7A*	0.299
	5	*RPL7A*	0.67	*SRSF7*	0.42	*18S*	0.407	*EF1a*	0.389
	6	*Hsc70*	0.67	*ACT*	0.44	*RPL7A*	0.411	*Hsc70*	0.443
	7	*SOD1*	0.72	*α-tubulin*	0.45	*SOD1*	0.478	*SOD1*	0.493
	8	*Hsp70*	0.82	*Hsc70*	0.46	*Hsp70*	0.617	*Hsp70*	0.543
	9	*ACT*	0.94	*18S*	0.47	*ACT*	0.81	*ACT*	0.606
	10	*28S*	1.46	*28S*	1.28	*28S*	1.409	*28S*	0.778

RT-qPCR analysis

The RT-qPCR was performed on 7500 and 7500 Fast Real-Time PCR Systems (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using Top Green qPCR SuperMix (+Dye I) (TransGen Biotech, Beijing, China). The reaction took place in 20 µl volume including 1.0 µl template cDNA and 10 µM of each primer, and the thermal cycling conditions were as follows: 94°C for 30 s, then 40 cycles of 94°C for 5 s and 60°C for 34 s. After amplification, a melting curve analysis was performed to establish specificity of the PCR product. Three technical replicates were performed for each sample. Standard curves were generated based on a 5-fold dilution series of cDNA templates. The amplification efficiency (E) was calculated based on the equation: $E = (10^{1/\text{slope}} - 1) \times 100\%$.

Data analysis

The *Cq* value (quantitative cyclic value) was used as a measure of expression levels of candidate reference genes. Expression stability of each candidate reference gene was calculated and ranked by methods of the GeNorm, NormFinder, BestKeeper, and ΔCt. The optimal number of reference genes was identified by the geNorm with pairwise variation V_n/V_{n+1} analysis: when the V_n/V_{n+1} value was below 0.15, n reference genes were sufficient for the reliable normalisation. Afterwards, RefFinder (https://blooge.cn/RefFinder/) was used to comprehensively evaluate, screen, and rank all candidate reference genes by integrating the aforementioned results to identify the most stable reference genes.

Validation of reference genes

The Acid phosphatase (*ACPase*) was used as the target gene for candidate reference gene evaluation. The primer sequence of the target gene was: forward (5′-ATGAGTTTCTGGGCGACATTTA-3′) and reverse (5′-TTCCATATTAGGTCCCCTTGTG-3′). The expression profile of *ACPase* in variable body parts was determined according to threshold cycle value (*Ct*) with the $2^{-\Delta\Delta Ct}$ method. ANOVA and follow-up Tukey HSD test were applied to compare mean values of the expression. Statistical analyses were run with the R software (R Development Core Team, 2023).

Results

Primer specificity and amplification efficiencies

Primer specificity of all reference genes was evaluated by single amplicons and single peaks in melting curve analyses (fig. S1); the standard curves were generated based on a five-fold serial dilution of cDNA templates (fig. S2). The analysis results showed that the RT-qPCR efficiency ranged from 95.57% (*RPL7A*) to 102.68% (*ACT*), and regression coefficients ranged from 0.9958 for *Hsc70* to 0.9998 for *ACT* and *SOD1* (table 1).

Expression profiles of ten reference genes

The cyclic threshold (*Cq*), representing the expression level of candidate internal reference genes under different conditions, ranged from 7.34 (*18S*) to 34.73 (*ACT*). The *18S* was the most abundant reference gene under all the conditions, while the least expressed reference gene varied from 26.84 (*Hsp70*) regarding the life stage, 25.50 (*ACT*) regarding the diet, 34.73 (*ACT*) regarding the hormone, 27.63 (*Hsp70*) regarding the body part, and 23.86 (*α-tubulin*) regarding the temperature. From a holistic perspective, expression fluctuations were greatest for the hormone treatment, while least for the temperature (fig. 1).

Gene expression regarding biotic factors

Results from analysing *S. guani* body parts using all four methods showed that *SOD1* exhibited the lowest level of stability. *RPL7A* was most stable with the analysis by ΔCt and geNorm and *18S* was so by NormFinder computations. The *28S*, the most stable gene by BestKeeper, ranked 8th by the other algorithms (table 2). A comprehensive analysis by RefFinder yielded the rank in order of stability from high to low: *RPL7A* > *EF1α* > *18S* > *α-tubulin* > *SRSF7* > *Hsc70* > *28S* > *Hsp70* > *ACT* > *SOD1* (fig. 2a). According to the V_2/V_3 value of less than 0.15 as measured by geNorm (fig. 3), suggesting two most stable genes to be sufficient for the normalisation, the most reliable reference genes for normalising RT-qPCR data regarding body parts were *RPL7A* and *EF1α* in *S. guani*.

Results from analysing *S. guani* life stages showed that though *Hsp70* was ranked 9th by BestKeeper, it was rated as the least stable one by the other algorithms. While *RPL7A* was ranked 6th by

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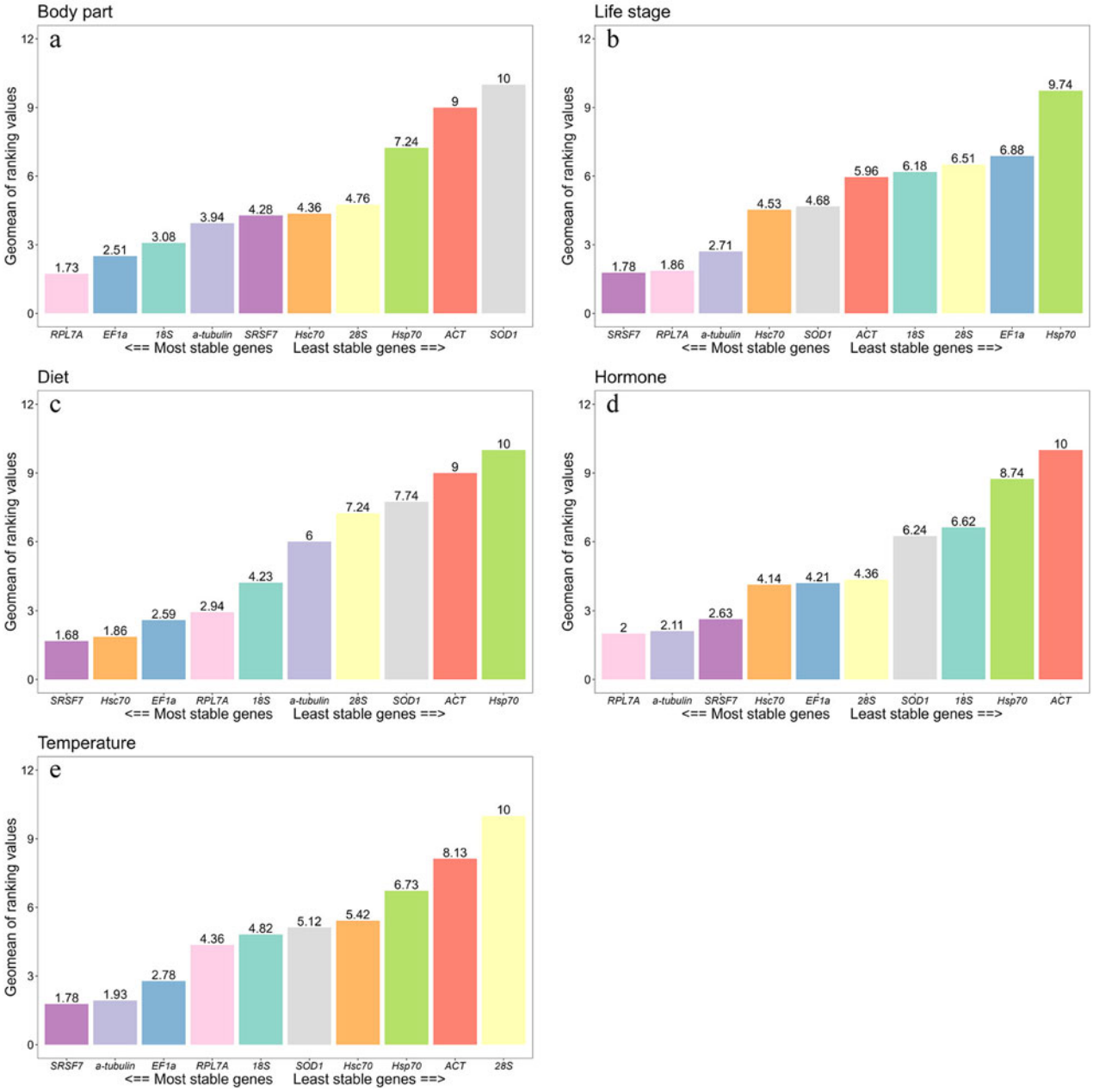

Figure 2. Stability of reference genes regarding body part (a), life stage (b), diet (c), hormone (d), and temperature (e) according to analyses with RefFinder. A lower Geomean value indicates a more stable expression.

BestKeeper, it was regarded as the most stable reference gene by ΔCt and geNorm. The genes of *α-tubulin* and *SRSF7* were rated as the most stable reference genes by both BestKeeper and NormFinder (table 2). According to the ranking result by RefFinder, the reference genes were ranked in order of stability from high to low as *SRSF7* > *RPL7A* > *α-tubulin* > *Hsc70* > *SOD1* > *ACT* > *18S* > *28S* > *EF1α* > *Hsp70* (fig. 2b). The pairwise variation analysis displayed that the V_2/V_3 value was less than 0.15 (fig. 3). The overall results indicated that *SRSF7* and *RPL7A* were the best reference genes to normalise gene expression across life stages.

Gene expression regarding abiotic factors

Regarding the diet, *Hsp70* was the least stable gene by all the four algorithms, while *Hsc70* was the most stable gene by ΔCt and NormFinder but *RPL7A* by BestKeeper and *EF1α* by geNorm were so (table 2). All ten candidate genes were ranked by RefFinder in order of stability from high to low as: *SRSF7* > *Hsc70* > *EF1α* > *RPL7A* > *18S* > *α-tubulin* > *28S* > *SOD1* > *ACT* > *Hsp70* (fig. 2c). Based on the V_2/V_3 value of less than 0.15 (fig. 3), the most reliable reference genes for the normalisation regarding the diet were *SRSF7* and *Hsc70*.

Regarding the hormone, *ACT* was the least stable gene by the four algorithms. The *a-tubulin* was identified by ΔCt and NormFinder as the most stable reference gene, while *RPL7A* by geNorm and *28S* by BestKeeper were identified as the most stable (table 2). The ten candidate reference genes were ranked by RefFinder in order of stability from high to low as: *RPL7A* > *α-tubulin* > *SRSF7* > *Hsc70* > *EF1α* > *28S* > *SOD1* > *18S* > *Hsp70* > *ACT* (fig. 2d). According the V_2/V_3 value of less than 0.15 (fig. 3),

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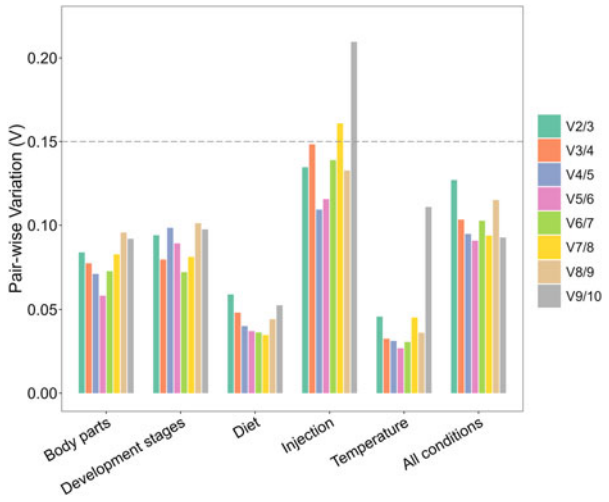

Figure 3. Values of pairwise variation (*V*) measured by geNorm regarding variable conditions.

the most stable reference genes to normalise RT-qPCR data regarding the hormone were *RPL7A* and *α-tubulin*.

Concerning the temperature, *28S* was the least stable gene by the four algorithms. The *α-tubulin* was suggested as the most stable reference gene by ΔCt and geNorm, while *EF1α* by BestKeepe and *SRSF7* by NormFinder were suggested instead (table 2). The reference genes were ranked by RefFinder in order of stability from high to low as: *SRSF7* > *α-tubulin* > *EF1α* > *RPL7A* > *18S* > *SOD1* > *Hsc70* > *Hsp70* > *ACT* > *28S* (fig. 2e). According the V_2/V_3 value of less than 0.15 by geNorm (fig. 3), the most stably reference genes for normalisation regarding temperature were *SRSF7* and *α-tubulin*.

Validation of reference genes

To validate the selected reference genes, we assessed the relative expression of *ACPase*. The expression level of *ACPase* was normalised using *RPL7A* and *EF1α*, which were the most stable reference genes, while *SOD1* was the least stable. Their difference was determined by normalising *ACPase* expression regarding the life stage. *ACPase* expression level was highest in the abdomen while lowest in the leg (fig. 4). Yet, the expression mechanism behind normalisation by the most stable reference genes, *RPL7A*, *EF1α*, or their combination, was significantly different from that by the least stable reference gene, *SOD1*. Using the first three reference genes yielded *ACPase* expression levels in descendig order across body parts: abdomen > antenna = head = thorax > leg, which was almost in line with that using *RPL7A* except a slightly lower expression level by the later. However, the other reference genes normalised a different *ACPase* expression pattern: abdomen > thorax > head > antenna = leg, and the expression level was lower overall in body parts except the abdomen than that by other reference genes.

Discussion

To date, RT-qPCR is a common method of quantifying gene expression. Its high sensitivity and specificity, high speed and low cost, make it the touchstone for nucleic acid quantification (Huggett *et al.*, 2005). Reference genes, key to achieving expression accuracy, are crucial but easily neglected in the process of RT-qPCR (Vandesompele *et al.*, 2002; Bustin *et al.*, 2009; Guénin *et al.*, 2009). Though a number of insects have bene identified for their reference genes, parasitoids, especially Bethylid wasps, are largely unknown for them (Lü *et al.*, 2018; Shakeel *et al.*, 2018). In this study of a quasi-social parasitoid wasp *S. guani* (Hymenoptera: Bethylidae), we place the focus on 10 candidate genes, *ACT*, *EF1α*, *Hsc70*, *Hsp70*, *SRSF7*, *α-tubulin*, *RPL7A*, *18S*, *28S*, and *SOD1*, using four commonly used algorithms (ΔCt, BestKeeper, NormFinder, and geNorm) and a comprehensive program (RefFinder). Most of above candidate reference genes are well studied and used frequently (Lü *et al.*, 2018).

Among the ten candidate reference genes examined in this study, *ACT* was ranked penultimate in terms of stability concerning factors such as body part, hormone, and diet, and last for temperature. However, caution is warranted regarding this result due to the abrupt imposition of temperature changes. Furthermore, introducing *ACT* in hormone injections leads to a marked increase of the pairwise variation value (V_9/V_{10}). Actins are

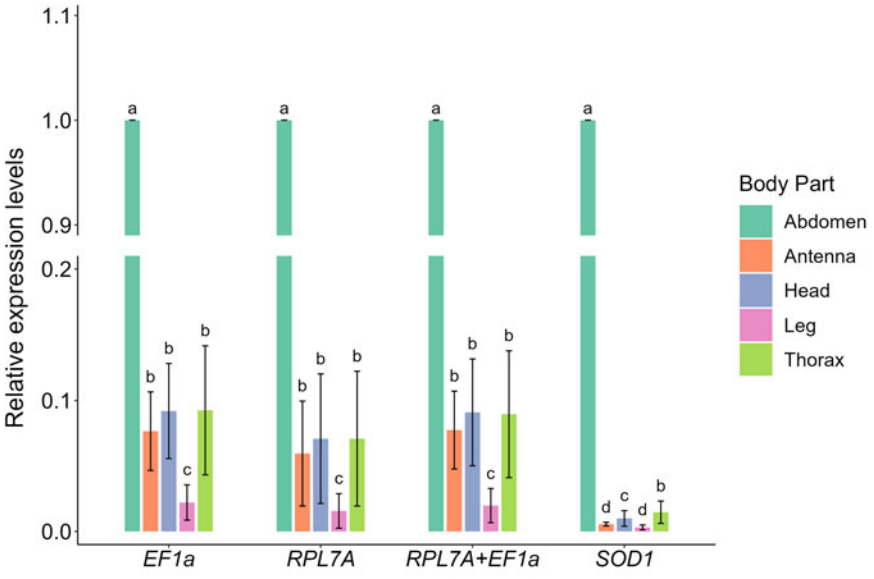

Figure 4. Expression levels of *ACPase* normalised with the two most stable and one least stable reference genes across body parts. Error bars represent standard error of the mean. Means sharing a lowercase letter are not significantly different by Tukey-adjusted mean separations (alpha = 0.05, two-tailed).

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abundant and essential components of the cytoskeleton, playing critical roles in a wide range of cellular processes such as cell migration, cell division, immune response, and gene expression (Hunter and Garrels, 1977; Bunnell *et al.*, 2011). Owing to its highly conserved properties, *ACT* is often used as a reference gene in vertebrates and insects (Chapman and Waldenström, 2015; Lü *et al.*, 2018; Shakeel *et al.*, 2018). But it has long been treated with scepticism ever since its application (Selvey *et al.*, 2001; Ruan and Lai, 2007). Previous studies suggest the exclusion of *ACT* in some cases as a reliable reference gene due to its high variability in Hymenoptera (Cheng *et al.*, 2013; Gao *et al.*, 2017). The evidence from our study does not provide support of *ACT* as a reliable reference gene.

Ribosomal protein genes exhibit robust expression levels in various cell types and play a crucial role in facilitating ribosome synthesis (Petibon *et al.*, 2021). For example, *RP* genes are applied as a reference gene in *Anastatus japonicus*, *Trichogramma dendrolimi*, *Tamarixia radiate,* and *T. chilonis* (Guo *et al.*, 2020; Xie *et al.*, 2021; Huo *et al.*, 2022; Liu *et al.*, 2022). Our study informs that *RPL7A* is one of the most stable reference genes based on its ranking first regarding both body part and hormone and second regarding temperature.

The stability of two rarely documented reference genes, *SRSF7* and *SOD1*, varied greatly in this experiment. Serine/arginine-rich (SR) proteins mediate splice site recognition and splice complex assembly during variable splicing procedures (Tang *et al.*, 2022). Surprisingly, *SRSF7* exhibited high stability across different diets, life stages and temperature conditions. Previously, the stability of *SRSF7* as a reference gene was only demonstrated in *Trichoderma japonicum* (Wang *et al.*, 2022). Our results from this study definitely provide a new argument for the reliability of *SRSF* as a reference gene. Superoxide dismutase (SOD) is an essential antioxidant enzyme, whose stability as a reference gene has been investigated in some insects (Bai *et al.*, 2020; Yan *et al.*, 2021; Shen *et al.*, 2022). Although the results of our study suggest that *SOD1* is not suitable as a reference gene for *S. guani*, its applicability in other parasitoid wasps remains further investigations.

Validation of expression stability is required for suitable reference genes generated by the algorithms. Acid phosphatase (*ACPase*) is stably expressed as a component in the venom gland in the abdomen of *S. guani*, making it ideal for accurately verifying the relative expression of reference genes (Liu *et al.*, 2017). Our findings from this study showed that the expression level of *ACPase* was significantly higher in the abdomen than in the other body parts. Furthermore, the expression level and mechanism of *ACPase* after normalisation with *SOD1* significantly varied across *RPL7A*, *EF1α*, and their combination, suggesting that the normalised result based on *SOD1* may not be representative of the expression level of *ACPase*. Hence, a combination of *RPL7A* and *EF1α* can be employed as effective reference genes in analysing the expression level of target genes regarding body parts in *S. guani*.

In summary, the results from this study suggest for the first time an extensive list of suitable reference genes regarding multiple potential factors for gene expression studies in *S. guani*. The results indicate that two reference genes for normalisation were optimal under all the conditions, and the recommended combinations were suggested: *RPL7A* and *EF1α* for different body parts, *SRSF7* and *Hsc70* for various diets, *RPL7A* and *α-tubulin* for hormone injections, *SRSF7* and *RPL7A* for varying life stages, and *SRSF7* and *α-tubulin* for different temperatures. Our findings are informative for future research of molecular biology in *S. guani*.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485324000427.

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Author contributions. R. Z. and B. L. conceived the study and wrote the manuscript; R. Z., X. G., and L. M. took part in the experiments.

Competing interests. None.

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