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ORIGINAL ARTICLE

Identification of suitable reference genes for normalization of RT-qPCR data in eggplant fruit and shoot borer (*Leucinodes orbonalis* Guenée)

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Abstract

The eggplant shoot and fruit borer, *Leucinodes orbonalis* Guenée (Lepidoptera: Crambidae) is a monophagous and destructive insect pest of eggplant. *L. orbonalis* developed multiple insecticide resistance that led to frequent field control failures in many countries. The possible reversal of resistance in *L. orbonalis* requires an understanding of the expression dynamics of individual genes conferring resistance to insecticides through gene expression analysis. The availability of reference genes (RG) to normalize transcript data of target genes, achieved by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR), is mandatory. Hence, the present study investigated the stable expression pattern of nine putative reference genes viz., *actin, 28S ribosomal protein S18c, mitochondrial isoform X2 (28SM), calnexin, β-tubulin, 28S ribosomal protein S3 mitochondrial (28SR3), TATA box binding protein (TATA), elongation factor-1α (EF-1α), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH),* and *ubiquitin 60S ribosomal protein (Ubiquitin)* to select the most stable genes that could be used as reference genes for target transcript gene normalization. Different algorithms such as ΔCt, geNorm, NormFinder and BestKeeper were used to assess gene expression stability. By integrating these data into online RefFinder tool, *28SR3 and GAPDH,* were chosen as highly suitable RGs based on their stable expression pattern.

Keywords *Leucinodes orbonalis* · Reference genes · RT-qPCR · Normalization · *28SR3* · *GAPDH*

Abbreviations

28SM	*28S ribosomal protein S18c, mitochondrial isoform X2*
28SR3	*28S ribosomal protein S3 mitochondrial*
TATA	*TATA box binding protein*
EF-1α	*Elongation factor-1α*
GAPDH	*Glyceraldehyde-3-phosphate-dehydrogenase*
Ubiquitin	*Ubiquitin 60S ribosomal protein*
RG	Reference gene
M value	Expression stability value
Cq	Qquantification cycle
RT-qPCR	Quantitative reverse-transcription polymerase chain reaction
GST	Glutathione S-transferase
NTC	No template control

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Introduction

Eggplant, *Solanum melongena* L. popularly known as brinjal or aubergine is a popular vegetable crop cultivated across the world. The damage caused by shoot and fruit borer, *L. orbonalis* (Lepidoptera: Crambidae) is a major limiting factor in realising maximum productivity of eggplant. The larva bores into the petiole and midrib

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of leaves and also the tender shoots resulting in drooping and withering of stems. The tunnelling and larval excrement make the fruits unfit for human consumption. Losses due to feeding damage by *L. orbonalis* ranged from 20.7 to 88.7% in South-Asian countries (Haseeb et al. 2009; Mishra et al. 2014).

Since 1980's, *L. orbonalis* became resistant to many classes of insecticides viz., organophosphates, pyrethroids, carbamates and amides (Shirale et al. 2017). One of the causes for rapid development of insecticide resistance in *L. orbonalis* is the existence of stronger innate defence system to detoxify the toxic glycoalkaloids produced by eggplant. Although *L. orbonalis* has remained a devastating pest of eggplant for more than four decades, no genomic information on this pest was available till the present experiment.

At the molecular level insecticide resistance is generally conferred by one or more point mutations in genes governing allelochemical metabolism like cytochrome P450 monooxygenase, glutathione S-transferese, carboxylesterase and at the target sites (Mohan et al. 2015). In light of the availability of genome information now, it is possible for genome wide identification and characterization of genes governing insecticide resistance. This can be achieved in a quick and accurate manner by employing quantitative real-time PCR (RT-qPCR) and subsequent gene knockdown studies (Wong and Medrano 2005; Espy et al. 2006; Bustin et al. 2010). For a reliable estimate of target gene transcription using RT-qPCR, validation of an appropriate reference genes (RGs) is a precondition (Vandesompele et al. 2002). Reference genes must be constitutively expressed, not changing across experimental conditions considered under study, which could simply means across cell types, tissues or organs.

Multiple algorithms such as geNorm, BestKeeper, and NormFinder are being employed to study the stability of reference genes in diverse systems (Shakeel et al. 2017). The NormFinder model assesses the standard deviation for individual gene and correlates it with target gene expression (Andersen et al. 2004). The BestKeeper shows highest stability index for the suitable reference genes and compare the genes across all treatments based on the analysis of a stability index (Pfaffl et al. 2004). Similarly, the geNorm model evaluates the best pair of suitable reference genes by assessing the gene expression stability value (M) for each gene. This model works on the principle of mean pairwise variation between genes across all samples. The genes with least M value (<0.15) are considered as more stable (Vandesompele et al. 2002). The ΔCt algorithm compares the individual sample basic Cq values and the relative expression of gene pairs (Silver et al. 2006). The overall stability of the RGs could be ranked using comprehensive RefFinder tool. To assess the most stable reference genes suitable for normalization of target gene expression in *L. orbonalis*, nine candidate reference genes were selected and transcript stability evaluated.

Materials and methods

Insect culture

The susceptible laboratory iso-female strain of *L. orbonalis* used in this investigation was originally collected during 2012 from fields of eggplant in Bengaluru, India (12.97° N and 77.58° E), and being maintained on potato-based diet system at ICAR-National Bureau of Agricultural Insect Resources, Bengaluru. The rearing conditions were 27 ± 2 °C, 60–70% RH, and a photoperiod of 14:10 h (L:D). The insecticide resistant *L. orbonalis* population used in this study was collected from eggplant field of Bhubaneshwar district (20.29″ and 85.82″) where the crop usually receives a high level of pesticides sprays every season because of extensive eggplant cultivation compered to other regions and crop present throughout the season. We have used two experimental conditions to assess gene stability: 1) developmental stage (3rd and 4th instar larvae of the susceptible population) and 2) susceptible versus resistant population (3rd instar larvae of susceptible laboratory and resistant field population)..

RNA extraction and cDNA synthesis

The total RNA was extracted by using 50 mg of third instar larva by Isolate II RNA mini kit (Bioline, USA) by following manufacturers guidelines. Three biological replicates were used for each experimental conditions and the experiment was repeated twice. The quality and purity of RNA were assessed by using NanoDrop (NanoDrop Lite, Thermo scientific, USA) and denatured agarose gel (Masek et al. 2005). The RNA samples (1806–2105 ng/μL) with an A260/A280 ratio ranging from 1.8 to 2.0 and A260/A230 ratio > 2.0. DNA contamination was removed from the RNA by using DNase I, RNase-free (Thermo Scientific, USA) by following manufacturers instructions. cDNA synthesis was carried out by using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, US). Briefly, for 20 μL reaction contains the following reagents: 4 μg of total RNA, 0.5 μM of Oligo $(dT)_{18}$ primer, 1x Reaction buffer, RiboLock RNase Inhibitor (20 U), 1 μM dNTPs and RevertAid M-MuLV RT (200 U). The reaction was incubated at 42 °C for 60 min followed by 70 °C for

5 min. The synthesized cDNA was diluted to 1:10 with nuclease-free water and stored at −80 °C till further use.

Quantitative real-time PCR

Nine candidate RGs were chosen to study their expression stability: *actin, 28S ribosomal protein S18c mitochondrial isoform X2 (28SM), calnexin, β-tubulin, 28S ribosomal protein S3 mitochondrial (28SR3), TATA box binding protein, elongation factor-1a (EF1a), glyceraldehyde 3 phosphate dehydrogenase (GAPDH)* and *ubiquitin 60s ribosomal protein (Ubiquitin)* (Table 1).

Primer 3.0 software was used to design the gene-specific primers. OligoEvaluator™ sequence analysis tool was used to study the primer dimer, secondary structure as well as efficiency (OligoEvaluator™: accessed on July 2018; http://www.sigmaaldrich.com/life-science/custom-oligos/custom-dna/learning-center/calculator.html). The PCR amplifications were executed in 20 μL reactions containing 10 μL 2 × SYBR® Premix EX Taq™ II (Tli RNaseH Plus, TAKARA®, Japan), 0.2 μM of each forward and reverse primers, 2 μL of diluted cDNA (40 ng/reaction) and 5.5 μL of water. Three technical replicates, including no-template controls (NTCs) used to assess contaminations and primer dimers, were considered per samples. Three Five-fold serial dilutions of cDNA template were considered to perform a standard curve using, $10^{(-1/\text{slope})-1)*100}$ to calculate primer efficiency (Pfaffl 2001) by measuring the quantification cycle (Cq), using Light Cycler 480II (Roche Applied Science, Switzerland) equipment by following PCR parameters: one cycle of 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min; a final cycle of 72 °C for 10 min. The subsequent PCR products were electrophoresed on 2.0% agarose gel in a 1.0 × TAE buffer with 0.5 μg/mL of ethidium bromide..

Analysis of gene expression stability

To select the most stable genes to be used as RGs, four algorithms viz., NormFinder (Andersen et al. 2004), BestKeeper (Michael 2003; Pfaffl et al. 2004), geNorm (Vandesompele et al. 2002) and ΔCt (Silver et al. 2006)) were employed and the web-based RefFinder tool was used to incorporate all these algorithms for comparison.

Results

Selection of suitable reference genes and source

Nine candidate reference genes belonging to five functional groups, that includes two genes encoding structural proteins (*Actin* and *β-tubulin*), three genes encoding ribosomal proteins (*28SM, 28SR3* and *ubiquitin 60s*), one encoding a metabolic enzyme (*GAPDH*), a chaperone protein (Calnexin), a transcription factor (TATA) and a protein factor (*EF-1α*) were selected (Tables 1 and 2) from de novo genome and transcriptome sequence assemblies of *L. orbonalis* previously published (BioProject ID: PRJNA377400; BioSample: SAMN06858697 and GenBank accession number PQWD00000000).

Primers efficiency and specificity

For all candidate reference genes, PCR amplification was carried out to assess primer specificity and efficiency. For primer specificity analysis, the presence of a single peak in melting curve was investigated followed by an agarose gel electrophoresis to confirm the presence of a single band. Primer efficiency of all the nine candidate genes ranged from 91.11 to 100.74% (Table 1). The regression coefficient (R^2) values ranged between 0.96 and 0.998 indicated there were no inhibitory contaminants present in the cDNA. The melting curves displayed that a single product generated from the qPCR.

Stability of reference genes

The Cq values in the present investigation ranged from 17 to 33 cycles (Table 2). Highly expressed gene was actin that showed a Cq average of 17.5, with a minimum value of 15.6 and a maximum of 21.02 followed by *GAPDH* (20.16) and *β-tubulin* (20.49). *Ubiquitin* was the most abundantly expressed gene with an average Cq value of 21.9 cycles (Fig. 1a & 1b).

The expression level of the nine candidate RGs under two different experimental conditions was analyzed by using four algorithms (geNorm, NormFinder, BestKeeper and ΔCt). The stability values were obtained by employing integrated RefFinder tool (Tables 3 and 4).

geNorm

Based on this model, *GAPDH* genes was the most stably expressed genes followed by *28SR3* and *β-tubulin* with M value below 0.9, under both the experimental conditions (Table 3; Fig. 2a, b). The pairwise variation value was calculated by the geNorm algorithm described that the pairwise variation V6/7 value is more than V5/6 (Fig. 3a, b). It clearly depicts that an increasing trend of variation in relation to decreased stability after adding moderately unstable sixth gene (Fig. 3). Hence, five candidate RGs viz., (*Actin, β-tubulin, 28SM, 28SR3* and *TATA*) are pre-requisite for perfect normalization. Adding a sixth RG gene has no impact on the normalization factor (Fig. 3a, b).

Table 1 Primers used in the normalization of reference genes for *L. orbonalis*

Reference genes	Function	Homologous species and their accession number	Primer Sequence 5′ –> 3’	Sequence Length (bp)	Amplicon size (bp)	E (%)	R^2	Tm (°C)
Actin	Cell division	*Plutella xylostella* & NP_001296030.1	F: CACCGAGAGGGGTT ACTCTTT	1149	73	98.42	0.984	66
			R: GCACAGCTTCTCCT TGATGTC					66
28S Ribosomal Protien S_{18c} Mitochondrial Isoform X_2 (28SM)	Protein synthesis	*Ostrinia furnacalis* & XP_028178958.1	F: CCCATAGACATCGA AAATCCA	380	115	92.62	0.990	62
			R: AAGGGCTTTGGAAC TGAGAAA					64
β-tubulin	Cellular process and also mitosis	*Agrotis ipsilon* & AEJ84083.1	F: AGGCTTTCTTGCAT TGGTACA	1287	102	91.11	0.960	64
			R: GGTACTGCTGGTAC TCGGACA					69
Calnexin	Protein binding and quality control	*Helicoverpa armigera* & XP_ 021187739.1	F: CTACGACCAGACGC CTTACAC	176	103	96.46	0.984	67
			R: AGTGCCATTCTTTG GGTTTTT					63
28S Ribosomal Protein S3 Mitochondrial (28SR3)	Protein synthesis	*Helicoverpa armigera* & XP_ 021185601.1	F: CAAAACTGGATGTG TGGGAGT	224	71	100.11	0.996	65
			R: GTTAGCTGGTGGTT GCAGTGT					68
TATA box binding protein (TATA)	Transcription	*Papilio xuthus* & XP_013182806.1	F: AAAGCGAGAAACAA TCACGAA	597	88	92.74	0.995	62
			R: TTGAAGTCCCCAAA GTTAGCA					64
Elongation factor-1a (EF-1)	Translation	*Spodoptera litura* & XP_022819255.1	F: ATGGATATTGGCAG AGCACAG	1013	88	92.01	0.998	65
			R: TGTTTGGGATGAAA TCAGCAT					62
Glyceraldehyde 3 Phosphate Dehydrogenase (GAPDH)	Cell function and apoptosis	*Corcyra cephalonica* & AHL24709.1	F: AAGTCATTCCCGCT CTTAACG	561	89	100.74	0.995	65
			R: GCGGACAGTAAGGT CAACAAC					66
Ubiquitin	Membrane protein and transcriptional regulation	*Helicoverpa armigera* & XP_ 021183635.1	F: CCTGACCAGCAGCGATTG	231	98	91.81	0.993	67
			R: ACCAAGTGCAGTGT TGATTCC					66

Table 2 The stability values of the reference gene by ΔCt-Method

Reference genes	Experimental conditions	
	Developmental Stages (±SE)	Populations (±SE)
Actin	17.65 ± 0.51	17.65 ± 2.14
28SM	29.53 ± 1.12	29.53 ± 1.27
Calnexin	28.17 ± 1.24	28.16 ± 0.22
β-tubulin	20.49 ± 1.37	20.49 ± 1.04
28SR3	25.15 ± 1.16	25.15 ± 0.74
TATA	33.12 ± 1.96	33.12 ± 2.65
EF-1	29.90 ± 2.09	29.9 ± 0.82
GAPDH	20.19 ± 1.07	20.19 ± 1.2
Ubiquitin	20.31 ± 1.86	20.31 ± 0.25

NormFinder

Under this algorithm, the most stably expressed gene is *GAPDH* followed by *28SR3* across the experimental conditions (Table 3). The gene expression stability value of *GAPDH* is 0.290 for developmental stages and 0.487 for populations tested.

BestKeeper

In the present study, all candidate RGs showed expression level less than the cut-off value (<0.3). The expression cut-off of *β-tubulin* was the least, followed by *ubiquitin* and *28SR3* across the experimental conditions (Table 3). *β-tubulin, ubiquitin* and *28SR3* were the most stably expressed genes having a cut-off value less than one (SD < 1) as compare to other RGs.

RefFinder

It is the web-based comprehensive online program that integrates results obtained from all other algorithms viz., BestKeeper, Normfinder, geNorm and the ΔCt. It ranks the RGs on the basis of their geo-mean constancy. The RGs rankings recorded in order of descending constancy across developmental stages and populations are depicted (Tables 3 and 4). For *L. orbonalis* growth stages, the cumulative ranking achieved with RefFinder was *28SR3, GAPDH, β-tubulin, ubiquitin, calnexin, EF-1α, actin, TATA* and *28SSM* (Fig. 4a). The comprehensive stability ranking across the populations was *28SR3, GAPDH, β-tubulin*, *ubiquitin, calnexin, EF-1α, actin, TATA* and *28SSM,* (Fig. 4b). This integrated tool indicates that for *L. orbonalis, 28SR3* and *GAPDH* genes could be adopted as appropriate RGs for normalization of target gene expression.

Discussion

A stable and highly expressed single RG would fulfill the essentiality of computing mRNA transcript level for the given gene of interest. However, for accurate normalization of gene expression data, it is suggested to use at least two or three RGs (Vandesompele et al. 2002). The present study employed four different algorithms viz., BestKeeper, geNorm, NormFinder, ΔCt and integrated these data into the RefFinder tool for comprehensive analysis. The expression pattern of the RGs was unique but a similar trend was noticed when analysed with different methods. Among the nine candidate RGs, expression of *28SR3* and *GAPDH* were highly stable and corroborate with earlier reports (Kim et al. 2003; Zhang et al. 2009; Chen et al. 2016). However, with the ΔCt method, the *GAPDH* was found to express more stable than *28SR3*. Since the ΔCt uses raw Cq values as an input data source, might have resulted in false positives as also reported earlier (Livak and Schmittgen 2001; Mehdi-Khanlou and Van-Bockstaele 2012; Chen et al. 2016). In eukaryotes, the ribosomal RNA (rRNA) genes are highly conserved and

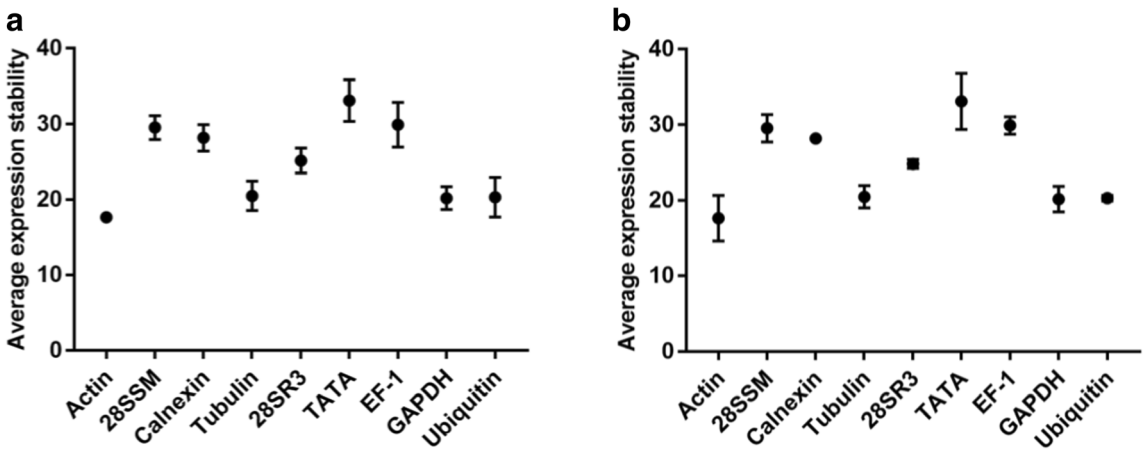

Fig. 1 Cq values of reference genes by ΔCt algorithm under different conditions in *L. orbonalis*. **a**) developmental stages and **b**) Populations

Table 3 Analysis of gene expression stability values and rankings of the reference gene

Reference genes	NormFinder		BestKeeper		geNorm		RefFinder Comprehensive	
	Developmental stage	Populations	Developmental stage	Populations	Developmental stage	Populations	Developmental stage	Populations
28SR3	0.461 (2)	0.939 (3)	0.32 (3)	0.74 (3)	0.54 (2)	0.95 (3)	1.19 (1)	1.19 (1)
GAPDH	0.290 (1)	0.487 (1)	0.43625 (6)	1.2 (6)	0.54 (1)	0.72 (1)	1.57 (2)	1.57 (2)
Calnexin	1.149 (6)	0.976 (4)	0.5625 (8)	1.04 (5)	0.76 (3)	0.72 (2)	3.66 (3)	3.66 (3)
β-tubulin	1.391 (7)	1.773 (8)	0.023 (1)	0.23 (1)	1.26 (6)	1.78 (7)	4.56 (5)	4.56 (5)
Ubiquitin	0.771 (4)	0.764 (2)	0.07 (2)	0.26 (2)	1.13 (5)	1.41 (6)	4.16 (4)	4.16 (4)
EF-1	0.667 (3)	1.098 (5)	0.425 (5)	0.82 (4)	0.94 (4)	1.09 (4)	4.68 (6)	4.68 (6)
Actin	1.106 (5)	1.191 (6)	0.7125 (9)	2.15 (8)	1.51 (7)	1.25 (5)	7 (7)	7 (7)
28SM	3.306 (9)	2.315 (9)	0.40125 (4)	1.28 (7)	2.3 (9)	2.43 (9)	8 (8)	9 (9)
TATA	1.651 (8)	1.490 (7)	0.54625 (7)	2.65 (9)	1.8 (8)	2.05 (8)	9 (9)	8 (8)

commonly used reference genes (Eickbush and Eickbush 2007). The ribosomal protein, *28SR3*, involved in the translation process and protein production revealed the maximum stability amongst studied genes. RT-qPCR studies with *Apis mellifera* and *Cimex lectularius* used *RPL18* as the reference gene gene due to its stable expression (Scharlaken et al. 2008; Mamidala et al. 2011). The ribosmal protein genes, *RP4* and *RP18* were found suitable for the gene expression studies in *Leptinotarsa decemlineata* (Shi et al. 2013). The *18S* and *28S* are commonly suggested as reference genes for human liver, lung, spleen and small intestine because of their stable expression pattern across mammalian tissues (Goidin et al. 2001; Thellin et al. 1999). Though the expression of *28SR3* gene is highly stable and to be best used as an reference gene in experiments with *L. orbonalis,* another ribosomal protein *28SM* could not qualify the criteria.

GAPDH, a metabolic enzyme with considerable roles in diverse physiological activities (Sirover 1997), revealed to be stably expressed as the 28SR3. *GAPDH* was used as reference gene for gene expression in many insects and vertebrates (Van-Hiel et al. 2009, Hornakova et al. 2010, Jiang et al. 2010, Lord et al. 2010, Mamidala et al. 2011, Rajarapu et al. 2011; Cardoso et al. 2014: Chapman and Waldernstrom 2015, Reim et al., 2013). In contrary, the *GAPDH* was found as one of the most unstable reference gene in other groups of insects (Bagnall and Kotze 2010; Hornakova et al. 2010; Chapuis et al. 2011). The differences in ranking of reference genes could also be due to the algorithms used and also with respect to the co-regulated reference genes. Studies on insects with optimal selection of RGs for gene expression experiments are species-specific and highly dependent on the experimental conditions and require assessment of reference genes before

Table 4 Analysis of gene expression stability rankings of the reference gene using RefFinder tool

Algorithm	Ranking* / Treatment details	1	2	3	4	5	6	7	8	9
ΔCt-Method	Developmental stage (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Calnexin*	*Actin*	*TATA*	*28SM*
BestKeeper	Developmental stage (combined)	*28SR3*	*Calnexin*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*Calnexin*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*EF-1*	*Actin*	*TATA*	*28SM*
Normfinder	Developmental stage (combined)	*GAPDH*	*28SR3*	*Ubiquitin*	*EF-1*	*β-tubulin*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*GAPDH*	*28SR3*	*Ubiquitin*	*EF-1*	*β-tubulin*	*Calnexin*	*Actin*	*TATA*	*28SM*
geNorm	Developmental stage (combined)	*28SR3* | *GAPDH*		*β-tubulin*	*EF-1*	*Ubiquitin*	*Calnexin*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3* | *GAPDH*		*β-tubulin*	*EF-1*	*Ubiquitin*	*Calnexin*	*Actin*	*TATA*	*28SM*
RefFinder integrative ranking	Developmental stage (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*Calnexin*	*EF-1*	*Actin*	*TATA*	*28SM*
	Population (combined)	*28SR3*	*GAPDH*	*β-tubulin*	*Ubiquitin*	*Calnexin*	*EF-1*	*Actin*	*TATA*	*28SM*

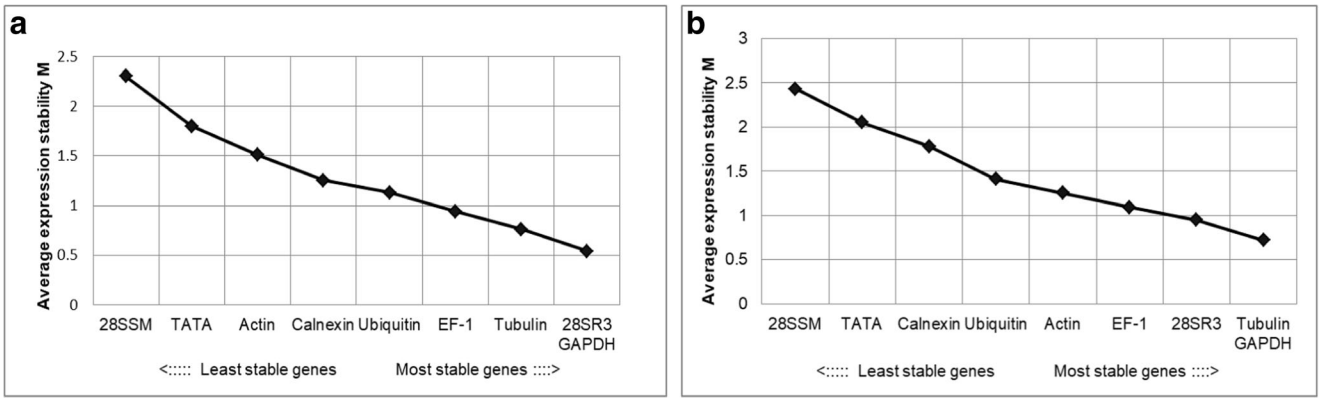

Fig. 2 Analysis of gene expression stability (M) and ranking of reference genes using geNorm algorithm in *L. orbonalis*. **a**) developmental stages and **b**) Populations

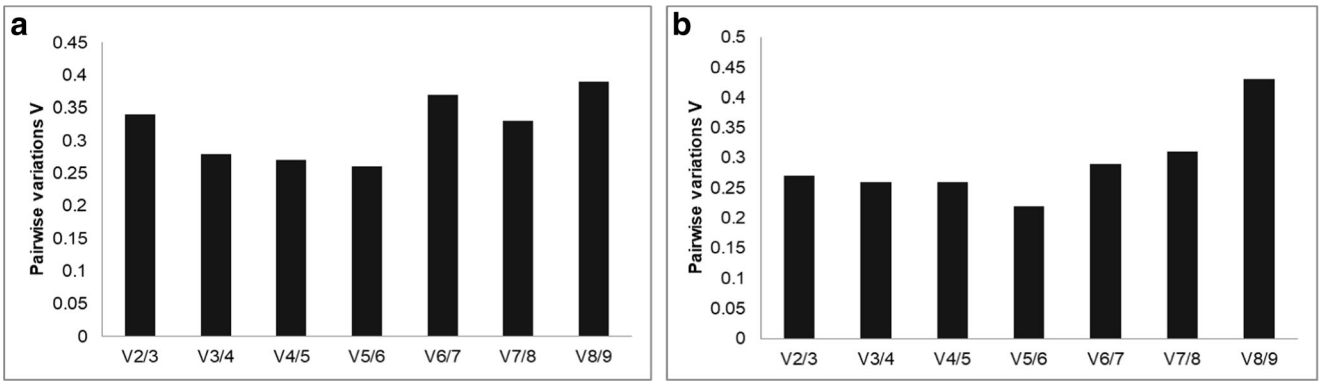

Fig. 3 The Pairwise variation (V) analysis of the RGs by geNorm in *L. orbonalis* developmental stages (**a**) and populations (**b**). The pairwise variation (Vn/n1) among the normalization factors NFn and NFn1 (along x-axis) was analysed. Every bar depicts the deviation in normalization when adding reference genes

undertaking gene expression studies on a new insect (Zhu et al. 2014; Zhai et al. 2015; Velada et al. 2014; Petriccione et al. 2015; Koramutla et al. 2016). In desert locust *Schistocerca gregaria,* the gene expression stability of nine reference genes in the brain of nymphs and adults were assessed. Multiple genes viz., *Actin, Ef1α100E, GAPDH,* and *Rp49* were considered as highly suitable to be used as reference genes (Van-Hiel et al. 2009). The use of multiple reference genes would permit more precise and consistent normalization of gene expression data (Vandesompele et al. 2002).

L. orbonalis is a devastating insect pest of eggplant in Asia and other continents. With the availability of de novo genome, transcriptome information, the *28SR3* and *GAPDH* could be used as appropriate candidate RGs for normalizing gene expression and gene knockdown experiments.

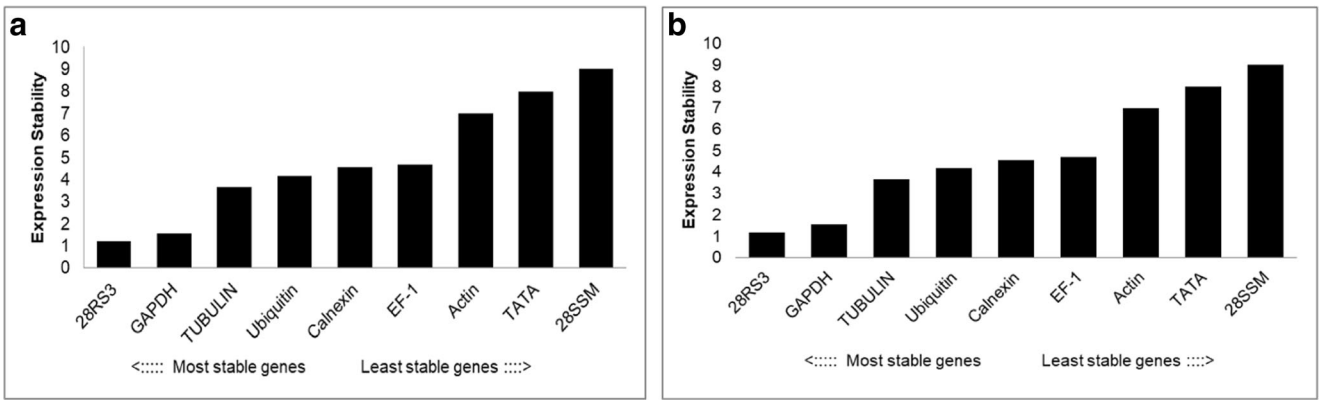

Fig. 4 Analysis of gene expression stability values and ranking of RGs using RefFinder algorithm in *L. orbonalis*. **a**) developmental stages (**b**) populations

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Compliance with ethical standards

Disclosure statement The authors have no potential conflict.

References

Andersen CL, Jensen JL, Orntoft TF (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 Res 64:5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Bagnall NH, Kotze AC (2010) Evaluation of reference genes for real-time PCR quantification of gene expression in the Australian sheep blowfly, *Lucilia cuprina*. Medical Veterinary Entomology 24:176–181. https://doi.org/10.1111/j.1365-2915.2010.00866.x

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S (2010) MIQE precis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol 11:74. https://doi.org/10.1186/1471-2199-11-74

Cardoso GA, Matiolli CC, Azererdo-Espin AML, Torres TT (2014) Selection and validation of reference genes for functional studies in the Calliphoridae family. J Insect Sci 14:2 http://www.insectscience.org/14.2. Accessed 6 August 2018

Chapman JR, Waldernstrom J (2015) With reference to reference genes: a systematic review of endogenous control in gene expression studies. PLoS One 10(11):e0141853. https://doi.org/10.1371/journal.pone.0141853

Chapuis MP, Tohidi-Esfahani D, Dodgson T, Blondin L, Ponton F, Cullen D, Simpson SJ, Sword GA (2011) Assessment and validation of a suite of reverse transcription-quantitative PCR reference genes for analyses of density-dependent behavioural plasticity in the Australian plague locust. BMC Mol Biol 12:7. https://doi.org/10.1186/1471-2199-12-7

Chen C, Xie T, Ye S, Jensen AB, Eilenberg J (2016) Selection of reference genes for expression analysis in the entomophthoralean fungus *Pandora neoaphidis*. Braz J Microbiol 47:259–265. https://doi.org/10.1016/j.bjm.2015.11.031

Eickbush TH, Eickbush DG (2007) Finely orchestrated movements: evolution of the ribosomal RNA genes. Genetics 175:477–485. https://doi.org/10.1534/genetics.107.071399

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR et al (2006) Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 19:165–256. https://doi.org/10.1128/CMR.19.1.165-256.2006

Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 295:17–21. https://doi.org/10.1006/abio.2001.5171

Haseeb M, Sharma DK, Qamar M (2009) Estimation of the losses caused by shoot and fruit borer, *Leucinodes orbonalis* Guen. (Lepidoptera: Pyralidae) in brinjal. Trends in Biosciences 2:68–69

Hornakova D, Matouskova P, Kindl J, Valterova I, Pichova I (2010) Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. Anal Biochem 397:118–120. https://doi.org/10.1016/j.ab.2009.09.019

Jiang HB, Liu YH, Tang PA, Zhou AW, Wang JJ (2010) Validation of endogenous reference genes for insecticide-induced and developmental expression profiling of *Liposcelis bostsrychophila* Psocoptera: Liposcelididae. Mol Biol Rep 37:1019–1029. https://doi.org/10.1007/s11033-009-9803-0

Kim BR, Nam HY, Kim SU, Kim SI, Chang YJ (2003) Normalization ofreverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnol Lett 25:1869–1872. https://doi.org/10.1023/A:1026298032009

Koramutla MK, Aminedi R, Bhattacharya R (2016) Comprehensive evaluation of candidate reference genes for RT-qPCR studies of gene expression in mustard aphid, *Lipaphis erysimi* (Kalt.). Sci. Rep (6): 25883. https://doi.org/10.1038/srep25883

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-Delta Delta CT method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262

Lord JC, Hartzer K, Toutges M, Oppert B (2010) Evaluation of quantitative PCR reference genes for gene expression studies in *Tribolium castaneum* after fungal challenge. J. Microbiol Methods 80:219–221. https://doi.org/10.1016/j.mimet.2009.12.007

Mamidala P, Rajarapu SP, Jones SC, Mittapalli O (2011) Identification and validation of reference genes for quantitative real-time polymerase chain reaction in *Cimex lectularius*. J Med Entomol 48:947–951. https://doi.org/10.1603/ME10262

Masek T, Vopalensky V, Suchomelova P, Pospisek M (2005) Denaturing RNA electrophoresis in TAE agarose gels. Analytical Biochemistry 336(1):46–50. https://doi.org/10.1016/j.ab.2004.09.010

Mehdi-Khanlou K, Van-Bockstaele E (2012) A critique of widely used normalization software tools and an alternative method to identify reliable reference genes in red clover (*Trifolium pratense* L.). Planta 236:1381–1393. https://doi.org/10.1007/s00425-012-1682-2

Michael WP (2003) Determination of most stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper© - excel© spreadsheet tool using a repeated pair-wise correlation and regression analysis. Dechema Jahrestagung der Biotechnologen 21:2–4. https://doi.org/10.1023/B:BILE.0000019559.84305.47

Mishra K, Keshav S, Tripathi CPM (2014) Management of infestation of pod borer (*Lucinodes orbonalis* Guenee) and productivity enhancement of brinjal (*Solanum melogena*) through vermiwash with biopesticide. Int J Adv Res 2(1):780–789

Mohan M, Venkatesan T, Sivakumar G, Yandigeri MS, Verghese A (2015) Fighting pesticide resistance in arthropods. Westville Publishers, New Delhi

Petriccione M, Mastrobuoni F, Zampella L, Scortichini M (2015) Reference gene selection for normalization of RT-qPCR gene expression data from *Actinidia deliciosa* leaves infected with Pseudomonas syringae pv. actinidiae. Sci. Rep 5:–16961. https://doi.org/10.1038/srep16961

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper - excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515. https://doi.org/10.1023/B:BILE.0000019559.84305.47

Rajarapu SP, Mamidala P, Mittapalli O (2011) Validation of reference genes for gene expression studies in emerald ash borer (*Agrilus planipennis*). Insect Sci 19:41–46. https://doi.org/10.1111/j.1744-7917.2011.01447.x

Reim T, Thamm M, Rolke D, Blenau W, Scheiner R (2013) Suitability of three common reference genes for quantitative real-time PCR in honey bees. Apidologie, Springer Verlag 44(3):342–350. https://doi.org/10.1007/s13592-012-0184-3

Scharlaken B, De-Graaf DC, Goossens K, Brunain M, Peelman LJ, Jacobs FJ (2008) Reference gene selection for insect expression studies using quantitative real-time PCR: the honeybee, *Apis mellifera,* head after a bacterial challenge. J Insect Sci 8:33. https://doi.org/10.1673/031.008.3301

Shakeel M, Rodriguez M, Tahir UB, Jin F (2017) Gene expression studies of reference genes for quantitative real-time PCR: an overview in insects. Biotechnol Lett 40:227–236. https://doi.org/10.1007/s10529-017-2465-4

Shi XQ, Guo WC, Wan PJ, Zhou LT, Ren XL, Ahmat T, Fu KY, Li GQ (2013) Validation of reference genes for expression analysis by quantitative real-time PCR in *Leptinotarsa decemlineata* (Say). BMC Research Notes 6:93. https://doi.org/10.1186/1756-0500-6-93

Shirale D, Patil M, Parimi S (2017) Insecticide resistance in field populations of Leucinodes orbonalis (Lepidoptera: Crambidae) in India. Can Entomol 00:1–9

Silver N, Best S, Jiang J, Thein S (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33. https://doi.org/10.1186/1471-2199-7-33

Sirover MA (1997) Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in nor- mal cell function and in cell pathology. J Cell Biochem 66:133–140. https://doi.org/10.1002/(SICI)1097-4644

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999) Housekeeping genes as internal standards: use and limits. J Biotechnol 75:291–295. https://doi.org/10.1016/S0168-1656(99)00163-7

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van-Roy N, Paepe AD, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple reference gene genes. Genome Biol 3:0034. https://doi.org/10.1186/gb-2002-3-7-research0034

Van-Hiel MB, Van Wielendaele P, Temmerman L, Van Soest S, Vuerinckx K, Huybrechts R, Broeck JV, Simonet G (2009) Identification and validation of housekeeping genes in brains of the desert locust *Schistocerca gregaria* under different developmental conditions. BMC Mol Biol 10:56. https://doi.org/10.1186/1471-2199-10-56

Velada I, Ragonezi C, Arnholdt-Schmitt B, Cardoso H (2014) Reference genes selection and normalization of oxidative stress responsive genes upon different temperature stress conditions in *Hypericum perforatum* L. PLoS One 9:e115206. https://doi.org/10.1371/journal.pone.0115206

Wong ML, Medrano JF (2005) Real-time PCR for mRNA quantitation. Biotechniques 39:75–85. https://doi.org/10.2144/05391RV01

Zhai Y, Lin Q, Zhou X, Zhang X, Liu T, Yu Y (2015) Identification and validation of reference genes for quantitative real-time PCR in *Drosophila suzukii* (Diptera: Drosophilidae). PLoS One 9:e106800 (2014). https://doi.org/10.1371/journal.pone.0106800

Zhang Y, Zhao J, Fang W et al (2009) Mitogen-activated proteinkinase hog1 in the entomopathogenic fungus *Beauveria bassiana* regulates environmental stress responses andvirulence to insects. Appl Environ Microbiol 75:3787–3795. https://doi.org/10.1128/AEM.01913-08

Zhu X, Yuan M, Shakeel M, Zhang Y, Wang S, Wang X, Zhan S, Kang T, Li J (2014) Selection and evaluation of reference genes for expression analysis using qRT-PCR in the beet armyworm *Spodoptera exigua* (Hu¨bner) (Lepidoptera: Noctuidae). PLoS One 9:e84730. https://doi.org/10.1371/journal.pone.0084730