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Validation of reference genes for qRT-PCR analysis in *Megoura viciae* (Hemiptera Aphididae)

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Abstract

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) has become one of the most sensitive methods to monitor gene expression. An important and often neglected requirement of this kind of study is the validation of appropriate reference genes with the most possible stable expression levels across samples groups. In this paper, several candidates were tested in all the four nymphal instars and the adult morphs (winged and apterous) of aphid *Megoura viciae* Buckton (Hemiptera Aphididae), an important pest of broad bean, in order to obtain reference genes for future works on *Megoura viciae* gene expression. Since the use of multiple reference genes is recommended for an accurate normalization, eight candidate genes were tested, encoding respectively for: ribosomal protein L32 (RPL32), NADH dehydrogenase (ubiquinone) flavoprotein 1 (NADH), succinate dehydrogenase complex subunit A (SUCC), ribosomal protein S9 (RPS9), TATA-box binding protein (TATA), actin (ACT), β-tubulin (TBU) and ubiquitin-conjugating protein (UBIQ). Three software programs and the comparative ΔC_T method were used to compare and rank the candidate genes and RefFinder - a web-based comprehensive tool that integrates all the four methods in a single index - indicated RPS9-RPL32 as the best couple. In addition, our study showed that a common-used reference gene, β-actin, achieved the worst score among our candidates. Finally, differences between the "classic" normalization with β-actin as a reference gene and the normalization using the best reference genes according to our work were highlighted using for the first time a *Megoura viciae* odorant binding protein, OBP4, as the target gene.

Key words: *Megoura viciae*, reference gene, qRT-PCR, gene expression.

Introduction

Estimating transcript expression level across different conditions is an important goal of modern molecular biology, in order to provide relevant suggestions regarding still unknown gene functions and regulation. Several techniques, such as Northern blot or microarray analysis, are still widely used, but quantitative reverse transcription polymerase chain reaction (qRT-PCR) is probably the most sensitive and accurate method to determine small deviations in mRNA expression levels of a single gene in different conditions. Furthermore, qPCR has also a great value in disease diagnostics, for example in verifying the correct knock-down after a RNA interference experiment (Bustin and Mueller, 2005).

In several steps of the qRT-PCR protocol, many non-intentional biases (different amounts of starting sample, different quality of starting sample, differences in RNA extraction and cDNA synthesis methods, but also simple operation as dilution or pipetting) may be introduced. Thus, a normalization method to obtain reliable results is required. The most common method to minimize the intersample variation in analyzing qRT-PCR data is the normalization of mRNA against one reference gene, even if, recently, many authors recommend the use of more than one of them (Hellemans *et al*., 2007; Bustin *et al.*, 2009).

Commonly used reference genes are cellular maintenance ones, which regulate basic and omnipresent cellular functions, as elements of cytoskeleton or very important pathways (de Kok *et al*., 2005) and therefore assumed to have a minimal variability. Suzuki *et al.* (2000) described as, in 1999, the most used reference genes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin; following works showed that these genes are often not the best choices and that reference genes need an accurate validation before being used (Selvey *et al*., 2001; Glare *et al*., 2002).

Megoura viciae Buckton is an aphid species able to infest only legume plants of the genus *Vicia* L. (Visser and Piron, 1995), especially broad bean. Thus, it is considered an economically important pest, causing damage by direct feeding and by transmitting plant viruses (Nuessli *et al*., 2004).

Physiological processes mediated by olfactory and gustatory systems, play crucial roles in insect behaviour, such as in locating food, choosing oviposition sites and mating (Leite *et al*., 2009). The understanding of the chemical communication mechanisms in aphids could help to develop new strategies for their biological control (Zhou *et al.,* 2010). For this reason, there is an increasing interest in studying the molecular basis of odour perception in insects.

A particular goal is directed toward the identification and functional characterization of Odorant Binding Proteins (OBPs), molecules able to mediate the chemosensory processes. The use of current advanced bioinformatics methods and the analysis of tissue specific transcriptomes, makes easier the identification of new OBPs, but the understanding of their specific role in a particular insect, is still limited. Considering that the expression of a protein in a particular cell, tissue or developmental stage is generally influenced by specific functions in the biological context, OBPs expression profiles obtained by qRT-PCR in the instars/morphs of *M. viciae*, including winged and apterous adults, could

allow to gain clues on the relation between the *M. viciae* OBP expression patterns and their functional implications, mainly in chemoreception (Sun *et al*., 2012).

A required *condicio sine qua non* to analyze OBPs expression is the selection of appropriate endogenous reference genes, with stable transcript level across different instars. Some genes tested in previous analogous works on hemipterans, like *Acyrthosiphon pisum* (Harris) (Hemiptera Aphididae) (Yang *et al*., 2014), *Aphis glycines* Matsumura (Hemiptera Aphididae) (Bansal *et al*., 2012), or *Bemisia tabaci* (Gennadius) (Hemiptera Aleyrodidae) (Li *et al*., 2013), as well as other commonly used housekeeping genes, were combined to list a number of candidate reference genes. The resultant list was composed by two ribosomal proteins, L32 (RPL32) and S9 (RPS9), two enzymes involved in the electron transport chain, NADH dehydrogenase (NADH) and succinate dehydrogenase complex subunit A (SUCC), two cytoskeletal proteins, actin (ACT) and β-tubulin (TBU) and finally a couple of molecules involved in important cellular functions like transcription, TATA box binding protein (TATA), and proteolysis, ubiquitin conjugating protein (UBIQ).

28S rRNA, initially included in this list, was then omitted after some experimental verification, due to its too high expression level, according to other literature references (Ponton *et al*., 2011; Lu *et al*., 2013).

A *M. viciae* gene, encoding for the OBP4 protein (*Mvic*OBP4), was chosen to compare different normalization strategies (classical reference gene - β-actin - vs. our validated reference genes, one reference gene vs. multiple validated reference genes).

Six developmental stages/morphs (first, second, third and fourth nymphal instars, apterous adults and winged adults) were chosen to test the expression stability of the reference genes across different biological samples in *M. viciae*.

Statistical algorithms such as geNorm (Vandesompele *et al*., 2002), Normfinder (Andersen *et al*., 2004) and BestKeeper (Pfaffl *et al*., 2004) have been developed to assess the appropriateness of reference genes, with the addition of the comparative ΔC_T method (Silver *et al*., 2006). Finally, the tested candidate genes were ranked with RefFinder (http://www.leonxie.com/referencegene.php), a web-based analysis tool that integrates all the four resulting data (Fu *et al*., 2013).

Materials and methods

Insect rearing

M. viciae was maintained on potted broad bean (*Vicia faba* L.) plants at 24 ± 1 °C, 75 ± 5% RH, with 16 h light - 8 h dark photoperiod. Aphid cultures started with an individual clone, originally collected on broad bean plants, in Southern Italy (Agropoli, SA).

Sample preparation

In order to synchronize aphid samples, parthenogenetic adult females were placed on a couple of potted broad bean plants and let to deposit newborn aphids for eight hours, and then were removed from the plants. Newborn aphids were maintained on plants for 6 days and individuals were collected at different development stages from first instar to adults, identifying their age/instar according to the morphological features described by Digilio (1995). The winged adult morphs (alatae) were easily spotted and collected from the colony because of their visible unique features. Sample were preserved in 1.5 ml centrifuge tubes (Eppendorf, Hamburg, Germany) and stored at −80 °C after being frozen in liquid nitrogen until RNA extraction. Each treatment was repeated three times independently. A total of 18 biological samples were collected.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from *M. viciae* using TRI® Reagent (Sigma St. Louis, Missouri, USA), following the manufacture's protocol. Aphids bodies from each sample were homogenized in 1.5 ml RNase-free tubes (Eppendorf, Hamburg, Germany) using 1 mL of TRI Reagent per 50-100 mg of tissue.

RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA) and its purity was assessed by an absorbance ratio of OD 260/280 and OD 260/230, while its integrity was checked with 0.8% agarose gel electrophoresis.

Before cDNA synthesis, RNA samples were treated with DNase I, Amplification Grade (Invitrogen, Waltham, Massachusetts, USA) to obtain an efficient removal of the genomic DNA contamination. Samples were treated with 1 U of DNase I per µg of RNA for 15 min at room temperature. The reaction was stopped by the addition of 1 µL of 25 mM EDTA and incubation at 65 °C for 10 min.

cDNA was prepared from total RNA with the SuperScript™ III Reverse Transcriptase (Invitrogen, Waltham, Massachusetts, USA), according to the manufacturer's protocol, using 5 µg of total RNA per sample. The cDNA synthesis reaction was diluted with nuclease-free water to a final volume of 100 µl and immediately used for qRT-PCR studies or stored at −20 °C.

Primers design

All *M. viciae* sequences used in the current study were found by comparing the *A. pisum* sequences on NCBI database against own *M. viciae* trascriptome data using BLASTN (Altschul *et al*., 1990) and then aligned by ClustalW (McWilliam *et al*., 2013) (supplemental material figure S1). Reference genes and OBP4 primers were designed using Primer 3 BioTool (Whitehead Institute for Biomedical Research, http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers sequences (with relative amplification efficiency and amplicon length) are shown in table 1.

Reverse-Transcription qPCR assays

Gene-specific primers (table 1) were used in qRT-PCR reactions, performed in MicroAmp® optical 96-well reaction plate (PE Applied Biosystems, Foster City, CA). cDNA samples, prepared from five instars aliquots, served as templates for qRT-PCR, using GoTaq qPCR Master Mix kit (Promega, Madison, WI, USA) at the following conditions: 1 µl of diluted first-

Table 1. Genes information, qRT-PCR details and primers sequences.

Gene	*A. pisum* Genbank Accession N. [a]	Amplicon length	Efficiency [b]	R^2 [c]	Primer forward sequence (5'->3')	Primer reverse sequence (5'->3')
RPL32	NM_001126210.2	180 bp	1.93	0.996	ATGTTGCCTTCCAAATTCCG	ACGTGCATTTCCATTGGTCA
NADH	XM_001946205.2	156 bp	1.95	0.995	GCACTTGCAAAGATCGTGAA	CGCAAATAGCTTGTTGCAGA
SUCC	XM_001950304.2	152 bp	2.04	0.996	AGGCCGTCATAAAATGCAAG	GTTCGGCAGCAGATACGATT
RPS9	XM_001945492.3	151 bp	1.91	0.983	TTCTGGGAGTCCAAACGAAC	TCTTGGAACGCAGACTTCAA
TATA	NM_00162717.2	179 bp	1.92	0.996	GCAAACATGGGATGTCCTTT	TTTCCAGTTCGGTCATCCTT
ACT	NM_001142636.1	279 bp	1.90	0.994	CGAACAGGAAATGGCTACCG	TCCTTTTGCATTCTGTCGGC
TBU	XM_008191981.1	195 bp	1.97	0.997	ACAATGCGGAAACCAAATCG	TCCAGGTCGAGTAAGACGGC
UBIQ	NM_001126205.2	147 bp	1.94	0.999	ATCCATTAAGGCCACCAAAA	CCGGTAACCAACGTTCACTT
OBP4	NM_001160058.1	210 bp	1.98	0.999	ACGTAGAGTTGCAGGGTGTT	TCGAAACTTTTGGAGGGCTG

[a] Accession number refers to the *A. pisum* related sequence; [b] RT-qPCR efficiency calculated according to the equation: $E = 10^{-1/slope}$; [c] Regression coefficient.

strand cDNA, 10 µl of GoTaq® qPCR Master Mix, 2X, 0.6 µl of forward primer 10 µM, 0.6 µl of reverse primer 10 µM and 7.8 µl of nuclease-free water, on an Applied Biosystems 7900 Real-Time PCR System. The PCR program for all the genes included an initial denaturation for 1 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Finally, a melting curve from 60 °C to 95 °C was performed to confirm the specificity of PCR products.

Amplification reactions were conducted in triplicate (technical replicates) and for a set of three biological replicates. To exclude the possibility of primers dimers existence, we added a no-template control for each primers pairs, in which cDNA was replaced by nuclease free water. To exclude the genomic DNA presence, we also added a no RT control, in which cDNA was replaced by corresponding non-retrotranscripted RNA.

The qRT-PCR efficiency was determined for each combination using slope analysis with a linear regression model. Relative standard curves for the transcripts were generated with serial dilutions of cDNA (1/1, 1/10, 1/100 and 1/1000). The corresponding qRT-PCR efficiencies (E) were calculated according to the equation: $E = 10^{-1/slope}$) (Pfaffl, 2001). The correlation coefficient and qRT-PCR efficiency for each standard curve are shown in table 1.

Data processing

The C_Q values were obtained by Applied Biosystems 7500 Fast Real-Time PCR System Software, version 2.0.6 (Applied Biosystems, Foster City, CA, USA). Reference genes data were analyzed by geNorm, NormFinder, BestKeeper algorithms and ΔC_T method, according to the authors' guidelines. Variations in OBP4 expression levels were calculated using standard $\Delta\Delta C_T$ approach for normalization by actin and the geometric averaging of multiple internal control suggested by Vandesompele *et al.*, 2002, for normalization by RPL32-RPS9.

Results

Expression levels of reference genes

Gene-specific amplifications were confirmed by a single peak in melting-curve analysis (figure 1a-c). Furthermore, all amplicons were visualized with bands of expected sizes on a 1.2% agarose gel (figure 2). Expression of measured reference genes is represented as raw quantification cycle C_Q (we set the threshold at same level for all the genes). We calculated the C_Q mean and the standard error (for all the 18 samples and for each developmental stage) (figure 3a-c), with also a boxplot reporting median value and the 25th and the 75th quartile. RPL32 was on average the most expressed gene, with a C_Q mean of 15.54, TBU was the least expressed one, with a C_Q mean of 21.55. C_Q values were log-transformed and used as input for GeNorm and NormFinder analysis, as required.

Expression stability of reference genes

GeNorm analysis

GeNorm is a software designed to analyze the expression stability of candidate reference genes assuming that the ratio of the expression level of two ideal reference genes is constant in all the samples. The average expression-stability, M value, for each investigated gene is calculated from the average of pairwise variations, according to which, the expression constancy of all reference genes is ranked. Genes with the lowest M value are the most stable. Our genes showed M values lower than the GeNorm default threshold of 1.5; they virtually are all recruitable reference genes. The couple RPL32-SUCC showed the lowest M value (0.216), as reported in figure 4. The pairwise variation $V_{2/3}$ value (figure 4) for the entire dataset was smaller than the recommended cut-off value of 0.15, so the inclusion of a third reference gene for an accurate normalization is not necessary.

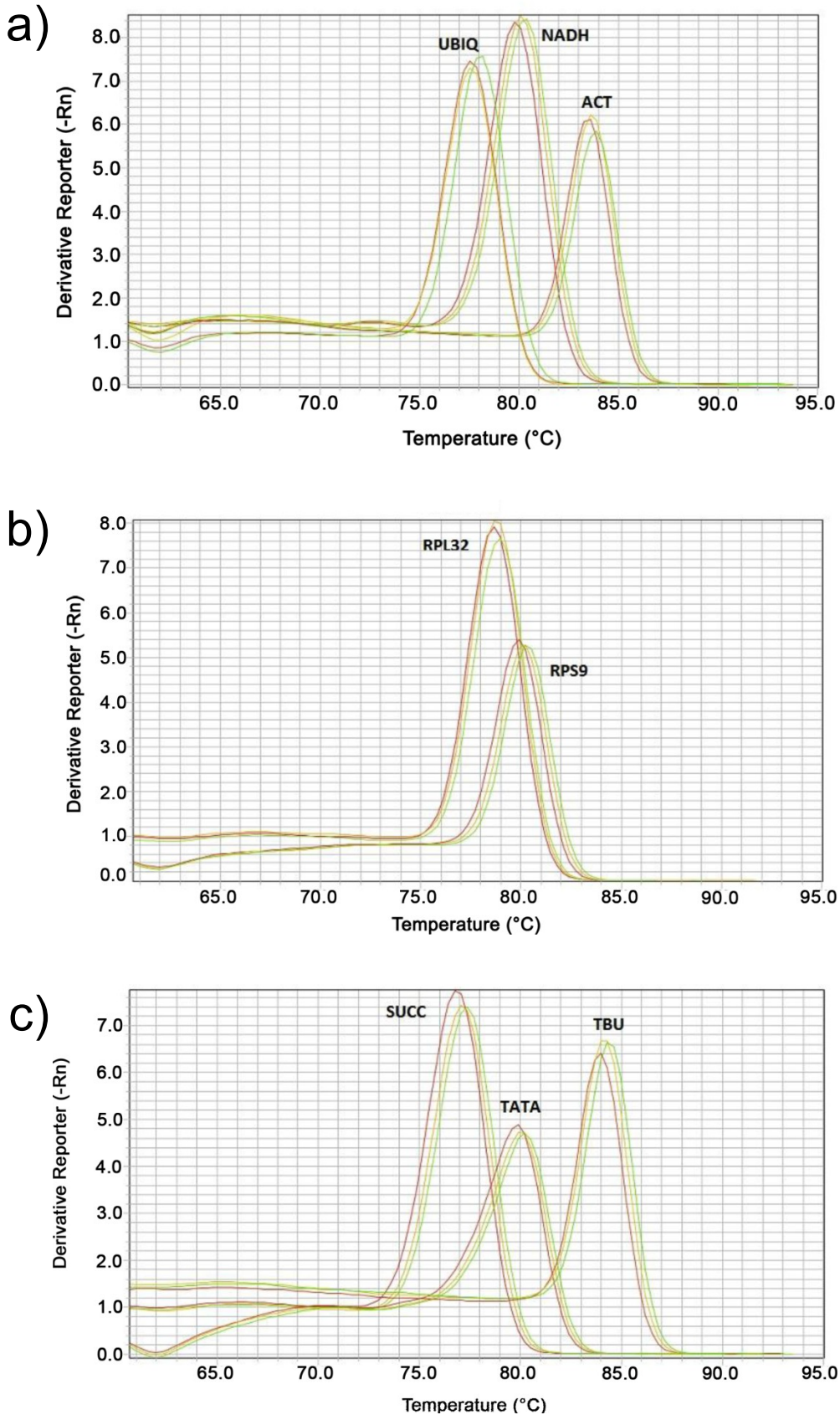

Figure 1. Melt curves associated to each of the housekeeping genes; gene-specific amplifications is confirmed by the presence of a single peak. (a) Derivative melt curve for UBIQ, NADH and ACT genes (3 technical replicates); (b) Derivative melt curve for RPL32 and RPS9 genes (3 technical replicates); (c) Derivative melt curve for SUCC, TATA and TBU genes (3 technical replicates).

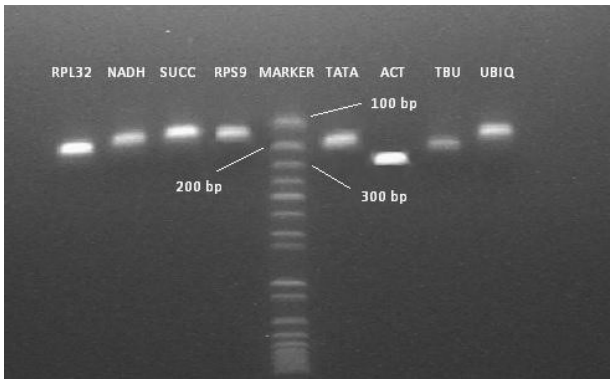

Figure 2. 1.2% agarose electrophoresis gel of the eight qRT-PCR reference genes products. Amplification of specific PCR products of expected size for each gene tested in the study is shown.

NormFinder analysis

Expression stability was then analyzed using the Excel-based application NormFinder, based on the variance estimation approach, allowing the genes ranking according to their stability under a given set of experimental conditions. RPS9 and RPL32 were found to be at the top of the ranking with a stability value of 0.042 and 0.054 (figure 5).

BestKeeper analysis

BestKeeper provided two rankings: Pearson's correlation coefficient [r] and BestKeeper computed SD values. The stability of a gene is directly proportional to the [r] value, while it is inversely proportional to the SD value. UBIQ (r = 0.916) and the couple RPS9-RPL32 (r = 0.857) showed the highest [r] value. RPS9 (SD = 0.20) and RPL32 (SD = 0.23) achieved another good result, showing the least variable expression level across all the samples (table 2).

ΔC_T method analysis

ΔC_T method is based on the assumption that ΔC_T value between two good reference genes has to be constant across different samples. All the possible ΔC_T combinations for all the samples and the average standard deviation were calculated, as reported in supplemental table S1. RPL32 (SD = 0.497) and UBIQ (SD = 0.510) gave the best results (figure 6).

Comprehensive ranking of the best reference genes using RefFinder

All the methods are in agreement in indicating RPS9, RPL32, SUCC and UBIQ as the most suitable reference genes. According to RefFinder, the overall order, from the most stable to the least stable, was: RPL32, RPS9, SUCC, UBIQ, TATA, NADH, TBU, ACT (figure 7).

Impact of the right reference gene choice

To analyze the possible differences in the expression level of a target gene across different experimental conditions, *M. viciae* mRNA of six different developmental stages was normalized against the "classical" reference gene β-actin and with the reference gene couple (RPL32-RPS9), obtained from our study. OBP4, a new odorant binding protein obtained by translating a private *M. viciae* antennal transcriptome, was chosen as target gene. For data analysis, the apterous adult morph was selected as a calibrator sample and its abundance was set to 1.

OBP4 expression levels of other developmental stages were calculated as relative to the calibrator sample. In figure 8 it is possible to appreciate some evident differences of OBP4 expression level in all the developmental stages, due to the two different normalization methods.

Discussion and conclusions

The accuracy of qRT-PCR analysis depends strongly on the correct choice (and the correct number) of reference genes (Derveaux *et al*., 2008; Everaert *et al*., 2011). The scientific community is working hard to provide studies about reference genes in very different contexts. We verified, via a public database (Google Scholar, http://scholar.google.it) that the number of scientific works containing the words "validation of reference genes" increased exponentially over the time, from 1 paper in 2002 to 465 in 2014 as reported in supplementary data (figure S2).

We used all the commonly adopted methods to rank reference genes in order to obtain the highest possible accuracy. The geNorm principle is based on the assumption that two ideal reference genes have identical expression ratios across different samples. A problem that could originate with this approach is that co-regulated genes will always appear to be more stable. NormFinder was used to check this possibility, because it is more resistant to the presence of co-regulated genes, as it uses another algorithm to assess the stability of the genes. NormFinder presents a stability value, which is related to the intra-group variance and it is unaffected by the gene and the sample. It simply calculates which gene has the slightest variation over all the samples. The best couple of resultant reference genes was the same for both the software. BestKeeper provided us useful information about variations of each gene expression across all the samples. Furthermore, the method of the pairwise correlation analysis, an approach similar to geNorm's, gives us another valid comparison. Finally, the ΔC_T method allowed us to compare the relative expression of pair genes within each sample, to confidently identify useful housekeeping genes. If the analyzed ΔC_T value, in different samples, between the two genes, is constant, it means that both genes are stably expressed among those samples.

Ribosomal proteins RPS9 and RPL32, involved in translation process and protein synthesis, showed the highest stability. In agreement with our results, RPs are reported as good reference genes in several insects: RP4 and RP18 have the best stability in *Leptinotarsa decemlineata* (Say) (Shi *et al*., 2013), RPS18 is a suitable reference gene in *Apis mellifera* L. (Scharlaken *et al*., 2008), RPL18 is the most stable in *Cimex lectularius* L. (Mamidala *et al*., 2011).

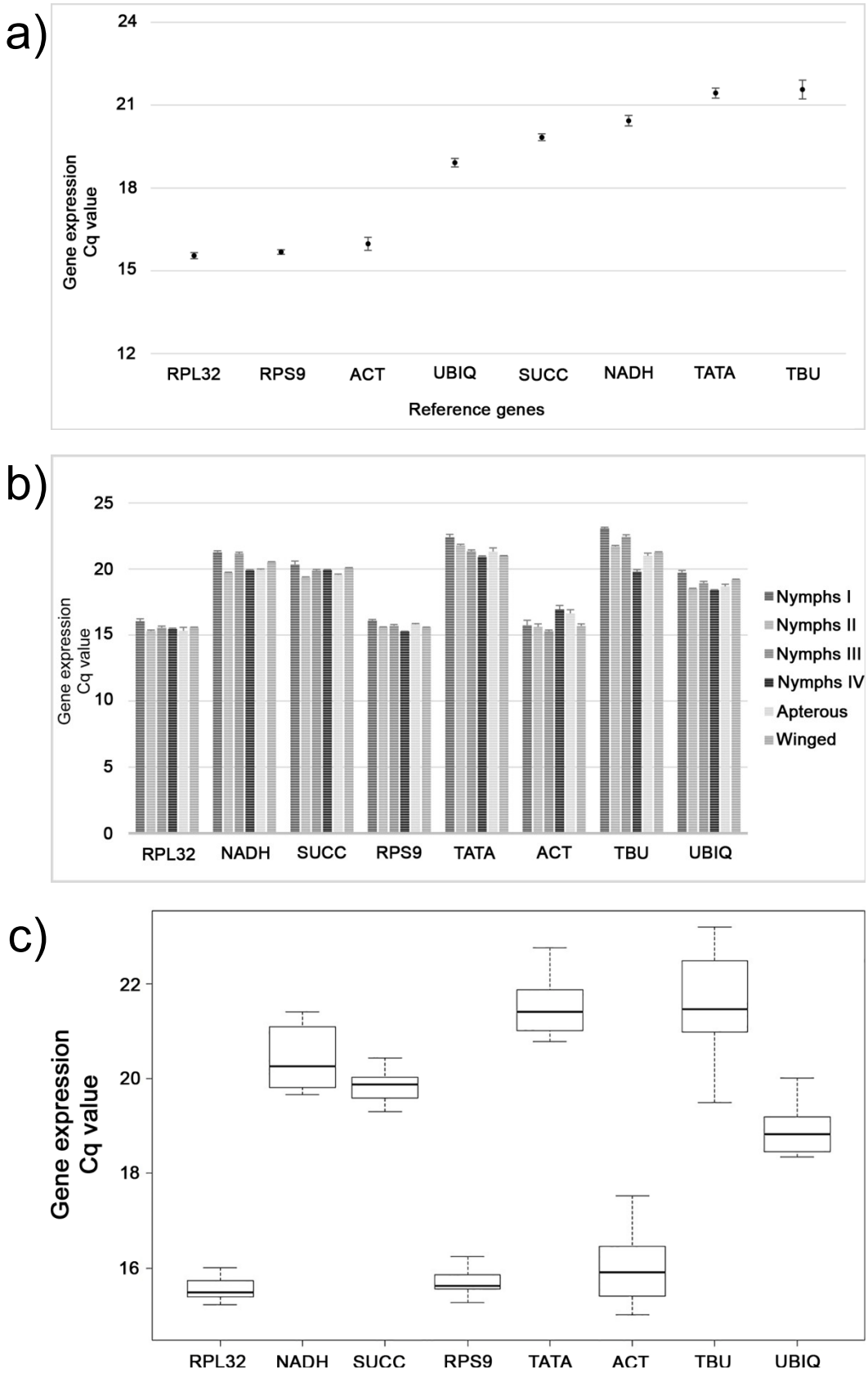

Figure 3. RNA transcription levels of the tested reference genes, presented as C_Q value for each developmental stage by setting threshold at the same level for all the genes. (a) C_Q values and standard deviations of the 8 candidate reference genes calculated on all the 18 samples; (b) C_Q values and standard deviations of the 8 candidate reference genes calculated on 3 biological replicate samples for each developmental stage; (c) Boxplot of the 8 candidate reference genes representing median C_Q values, the 25th and the 75th interquartiles and minimum/maximum values.

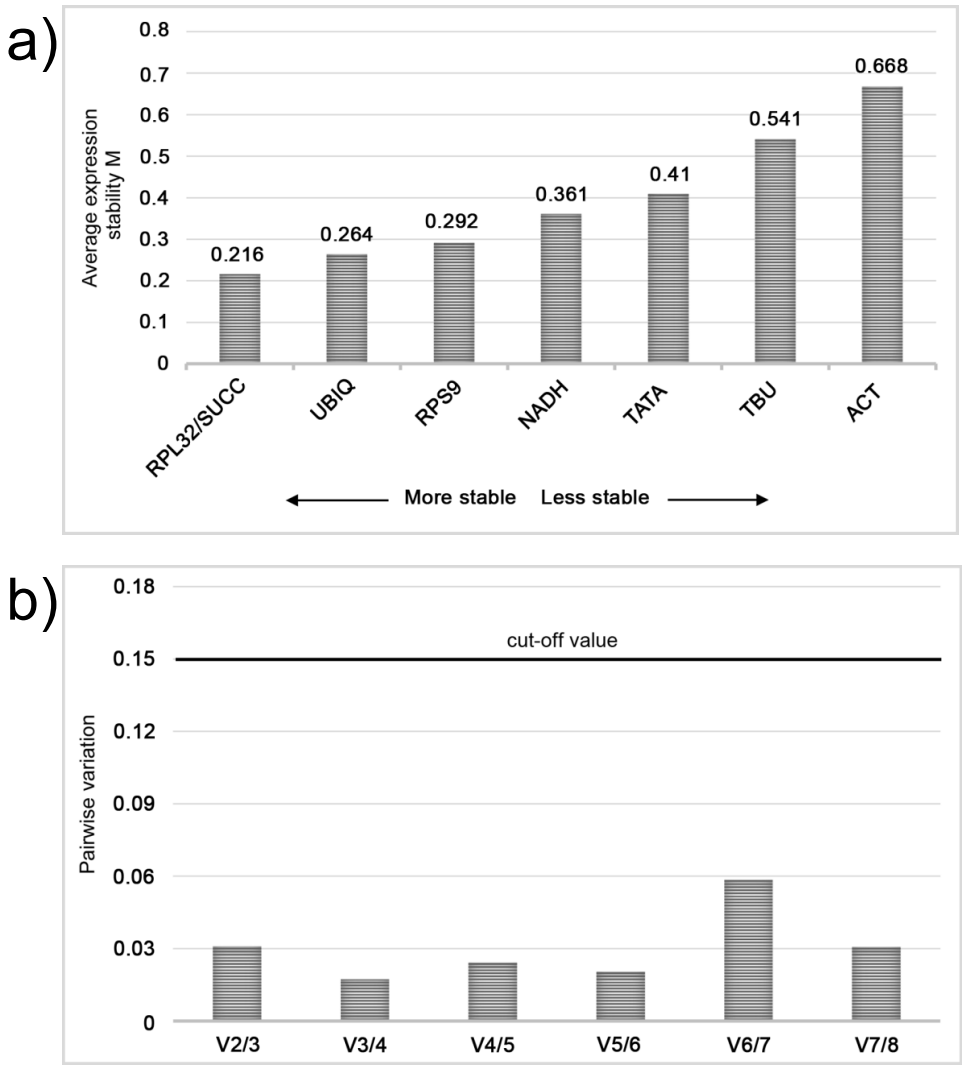

Figure 4. Gene expression stability of the candidate reference genes analyzed by the geNorm software. M value for each investigated gene was reported. (a) Ranking of the 8 candidate reference genes based on the stability value M. A lower M value indicates more stable expression; (b) Pairwise variation (V) analysis of candidate reference genes. Vn/Vn+1 was analyzed between normalization factors NFn and NFn+1 by GeNorm software to determine the optimal number of reference genes. V2/3 value is smaller than the recommended cut-off value of 0.15.

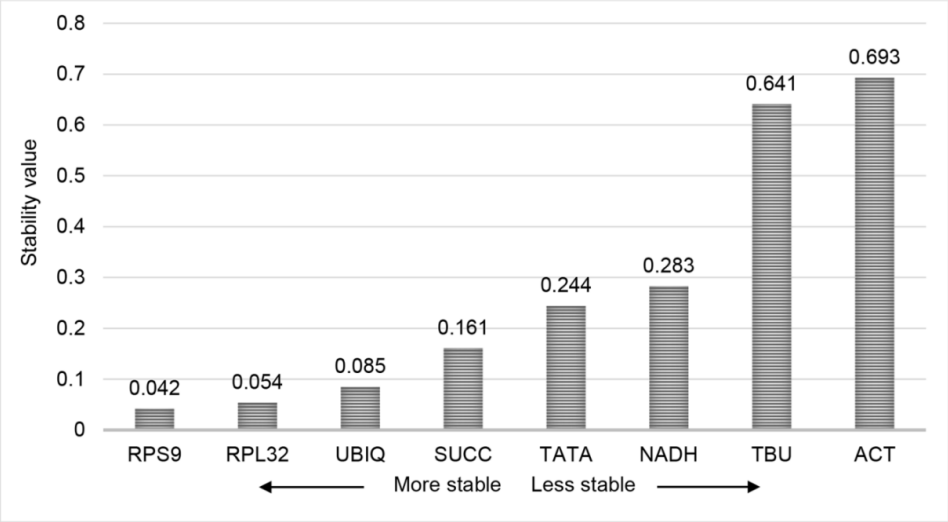

Figure 5. Candidate reference genes for normalization, listed according to their expression stability calculated by NormFinder, able to rank genes according to their stability under a given set of experimental conditions. RPS9 and RPL32 are at the top of ranking (stability value of 0.042 and 0.054).

Table 2. BestKeeper descriptive statistical analysis for reference genes based on CQ values.

	RPL32	NADH	SUCC	RPS9	TATA	ACT	TBU	UBIQ
n	18	18	18	18	18	18	18	18
geo Mean [C_Q]	15.541	20.4137	19.8544	15.6817	21.4572	15.9694	21.5188	18.9042
ar Mean [C_Q]	15.5443	20.4233	19.8577	15.6838	21.4642	15.9844	21.5455	18.9102
min [C_Q]	14.818	19.656	19.307	15.275	20.782	15.016	19.498	18.35
max [C_Q]	16.38	21.412	20.732	16.24	22.759	17.524	23.201	20.017
std dev [± C_Q]	0.23104	0.57189	0.28494	0.20442	0.46133	0.57325	0.86394	0.4078
CV [% C_Q]	1.48631	2.80017	1.43493	1.30338	2.14931	3.58633	4.00986	2.15652
correlation coefficient [r]	0.857	0.807	0.75	0.857	0.784	0.188	0.785	0.916

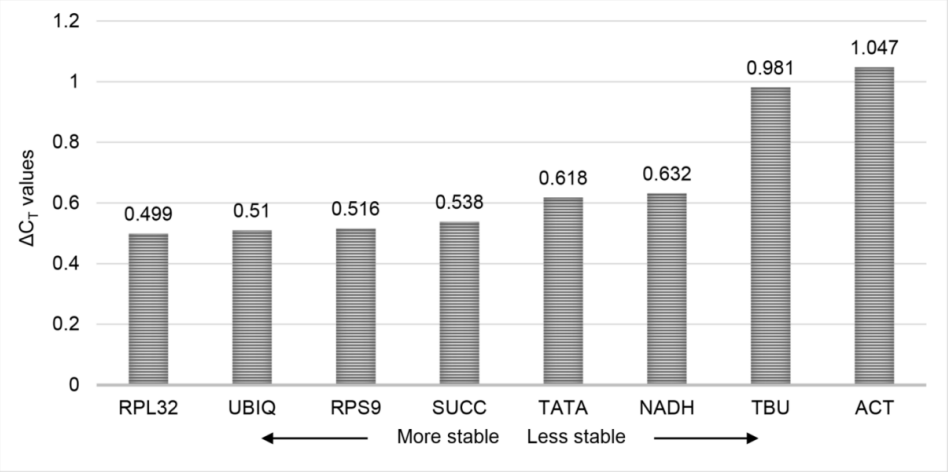

Figure 6. Candidate reference genes ranking, based on ΔC_T approach. All the possible ΔC_T combinations for all the samples and the average standard deviation were calculated among them, RPL32/UBIQ combination gives the best results.

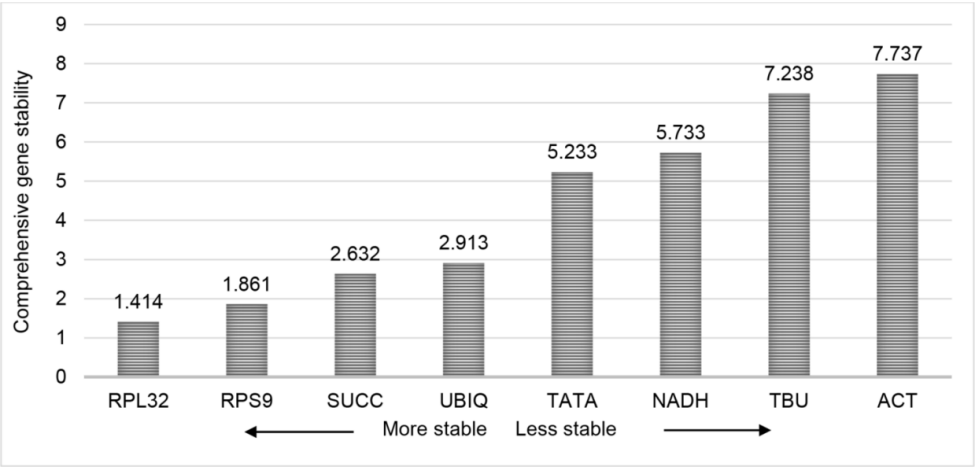

Figure 7. Expression stability of the candidate reference genes as calculated by RefFinder. Comprehensive ranking of the 8 candidate reference genes is reported. The order from the most stable to the least stable gene is: RPL32, RPS9, SUCC, UBIQ, TATA, NADH, TBU, ACT.

Subunit A is the catalytic domain of succinate dehydrogenase complex, the only enzyme that participates in both the Krebs cycle and the electron transport chain, catalyzing the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. In our analysis, it got top rankings, resulting first for geNorm and third in RefFinder comprehensive standing. SDH subunits also showed good results as reference genes in insect: SDHA was found to be the most stable gene in *Folsomia candida* Willem (de Boer *et al*., 2009) and it resulted NormFinder best housekeeping gene for developmental stages in *B. tabaci* (Li *et al*., 2013); SDHB was ranked as the best in *A. pisum* (Yang *et al*., 2014) reference genes analysis.

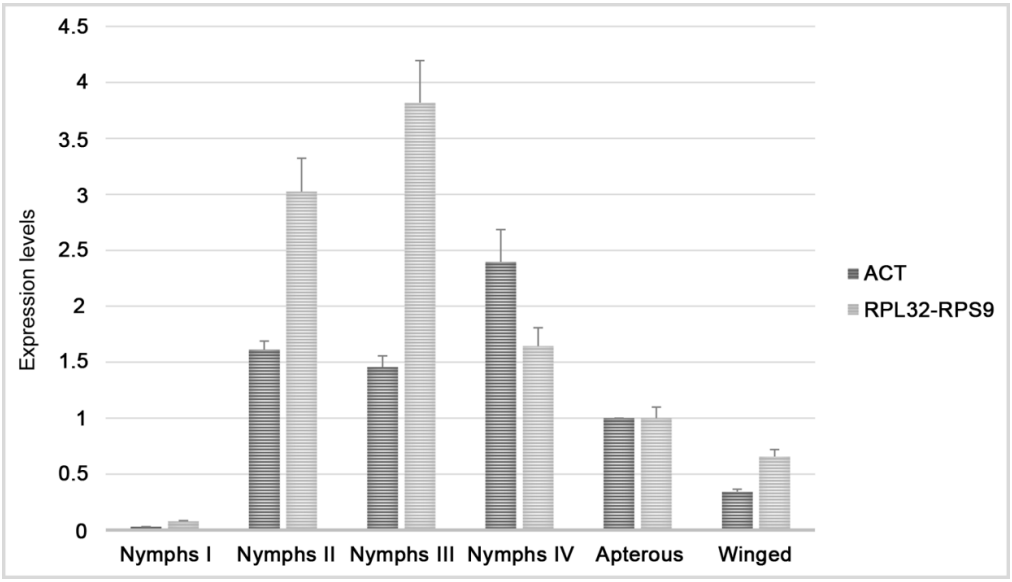

Figure 8. OBP4 expression levels (mean ± standard error) for six developmental stages/morphs normalized with actin (dark grey) and RPL32-RPS9 (light grey). Apterous adults sample is the calibrator. OBP4 expression level shows some evident differences in all developmental stages, due to the two different normalization methods.

Ubiquitin-conjugating protein takes part in ubiquitination process that targets a protein for degradation. It showed a good stability in wheat (Paolacci *et al*., 2009) and *Brachiaria brizantha* (Hochst) (Silveira *et al*., 2009), but it did not result among top ranked genes in another aphid, *A. glycines* (Bansal *et al*., 2012). RefFinder ranked UBIQ as the fourth most stable gene.

TATA binding protein is a transcription factor, able to bind specifically TATA box DNA sequence and it is part of RNA polymerase II preinitiation complex. Although in the similar aphid *A. glycines* TBP showed high stability, in our context it resulted the fifth in the overall ranking.

NADH dehydrogenase (ubiquinone) is an enzyme of the respiratory chain, present in the inner mitochondrial membrane, that catalyzes the transfer of electrons from NADH to coenzyme Q10. It was adopted several times as a reference gene, with mixed success. In developmental stages of *B. tabaci* and in larval castes development of *A. mellifera* (Cameron *et al*., 2013), it has been classified as a good reference gene, but in developmental stages of *Drosophila suzukii* (Matsumura) (Zhai *et al*., 2014) - as in our context - it did not show great results.

Finally, tubulin and actin chains, important cytoskeletal components, have been traditionally used as reference genes in several species. In our specific context, they showed a great variability in expression, so their use as housekeeping genes cannot be recommended.

Normalizing OBP4 mRNA level with β-actin, led to wrong evaluations: as in the example in figure 8 we would conclude, wrongly, that the OBP4 expression level in the fourth instar is higher than in the third instar, while the normalization with our validated reference genes suggests the opposite.

Therefore, choosing a reference gene without an appropriate validation process, could lead to wrong conclusions and to erroneous speculations about the function of a target gene.

This study on *M. viciae*, far from being exhaustive, could be a solid base to obtain an accurate normalization in future works about gene expression in this insect and in other insect pests.

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