Sociobiology
An international journal on social insects

SHORT NOTE

A Scientifc Note on Validation of Housekeeping Genes for the Primitively Eusocial Bee *Euglossa viridissima* Friese (Apidae: Euglossini)

S Boff[1,2], A Friedel[1], A Miertsch[1], JJG Quezada-Euán[3], RJ Paxton[1,4], HMG Lattorff[1,4,5]

1 - Institut für Biologie, Martin-Luther-Universität Halle-Wittenberg, Halle (Saale), Germany
2 - Dipartimento di Scienze per gli Alimenti la Nutrizione, l'Ambiente, Università Degli Studi di Milano, Milano, Italy
3 - Departamento de Apicultura Tropical, Campus Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán, Mérida-Xmatkuil, Mexico
4 - German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Leipzig, Germany
5 - International Centre of Insect Physiology and Ecology (*icipe*), Nairobi, Kenya

Article History

Edited by
Cândida Aguiar, UEFS, Brazil
Received 09 May 2018
Initial acceptance 08 June 2018
Final acceptance 24 July 2018
Publication date 11 October 2018

Keywords
Developmental stage, gene expression, orchid bees, gene normalization.

Corresponding author
Samuel Boff
Facoltá Scienze Agrarie e Alimentari
Università Degli Studi di Milano
Via Celoria 2, 2133, Milano, Italy.
E-Mail: samboff@gmail.com

Abstract

Studies on the expression of genes in different contexts are essential to our understanding of the functioning of organisms and their adaptations to the environment. Gene expression studies require steps of normalization, which are done using the stable expression pattern of reference genes. For many different eusocial bees reference genes have been discovered, but not for the primitively eusocial Euglossini bees. We used available genomic resources of Euglossini species and the gene information of *Apis mellifera* Linnaeus to develop a set of reference genes for the primitive eusocial bee *Euglossa viridissima* Friese. We tested nine genes, in distinct developmental stages, using three different algorithms, to infer stability of gene expression. The TATA-binding protein (*TBP*) and *14-3-3 epsilon* were the most stable genes across all developmental stages. The strongest deviation in gene expression pattern occurred in pupae, which require a different set of genes for normalizing gene expression.

One of the most crucial factors for studies of gene expression of target genes is the procedure of normalization (Kubista et al., 2006). For this purpose, reference genes (or house-keeping genes) are used, which are assumed to be constitutively expressed and hence their expression is expected to be stable and constant irrespective of developmental stage or any other experimental manipulation. Studies of candidate gene expression, using quantitative real time PCR, often focus on the evaluation of differences in gene expression amongst developmental stages or within a stage amongst different tissues (Lockett et al., 2016).

Candidates for suitable reference genes have been identified in several species of the Apidae, *e.g.* honeybees (Lourenço et al., 2008), bumble bees (Horňáková et al., 2009; Niu et al., 2014), and stingless bees (Dallacqua et al., 2007). However, reference genes have not been described for orchid bees, although transcriptome and genome data are available for a few of these species (Woodard et al., 2011; Kapheim et al., 2015; Brand et al., 2017).

Here we identified a set of candidate reference genes for the orchid bee *Euglossa viridissima* Friese based on the transcriptome reference for *Euglossa cordata* (Linnaeus) (Woodard et al., 2011) and annotated genes of *Apis mellifera* Linnaeus (Elsik et al., 2015). We tested for stability of gene expression in individuals across different developmental stages (including adults).

We choose nine candidate reference genes, namely Ribosomal protein 28S (*28S*), *Actin*, Arginine Kinase (*ArgK), Elongation Factor 1a (EF-1a*), *14-3-3 epsilon*, Glutathione S-transferase-1 (*Gst1*), Inositol 1,4,5-triphosphate receptors (*Itpr*), Ribosomal protein S18 (*Rps18*) and TATA-binding protein (*TBP*). For *ArgK* we used two different primer pairs,

ArgK and *ArgK_mod.* TATA-binding protein (*TBP*) is often considered stable in gene expression studies and is useful for gene normalization (Yüzbaşioğlu et al., 2010). This gene is related to RNA polymerase II transcription factor. The *14-3-3 epsilon* protein is abundantly expressed in the central nervous system (CNS), modulating the activity of a number of kinases and ion channels in humans (Berg et al., 2003). Although the gene *14-3-3 epsilon* is linked to several functions in flies (*e.g.* embryonic hatching, germ cell migration, gonad formation, wing venation and eye development, according to Flybase), this gene was found to be consistently expressed in queens and workers of honeybees (Santos & Hartfelder, 2015) providing evidence for its suitability for gene normalization.

Raw reads of the *E. cordata* transcriptome (Woodard et al., 2011) were mapped against the nucleotide sequences of *A. mellifera* representing the respective housekeeping genes using CLC Bio Genomics Workbench v7.5 (CLC Bio, Arhus, Denmark). Read mapping was performed using the large gapped read mapping algorithm followed by local realignments. Finally, consensus sequences were extracted and transferred to Primer3 (Untergrasser et al., 2012), which was used with default settings, except that the target product size was set at a maximum of 150 bp. An exception was *Actin* with 271 bp (see Locke et al., 2012). Primer sequences can be found in Table 1. A QIAxcel capillary electrophoresis system (accuracy to 3 bp) was used to confirm PCR product sizes based on the expected size.

RNA was extracted from whole bodied larvae (n = 9) and pupae (n = 8) and the abdomens from adult bees (n = 9) that were collected from14 nests (Department of Apiculture, UADY, México). With exception of larvae, bees were morphologically sexed, and only females were used for our analysis.

Gene stability was based on RefFinder. This software compares and ranks the results from four different algorithms (BestKeeper, Delta CT, geNorm and Normfinder) and reports stability of the gene based on standard deviations of Ct values (Xie et al., 2012). Since the BestKeeper algorithm uses the original Ct values as input assuming normality of the data (although they are logarithmic in nature), it seems to indicate false differences (Livak & Schmittgen, 2001; Khanlou & Bockstaele, 2012; Chen et al., 2016). Here, we did not use BestKeeper in our analysis.

The 10 primers designed to amplify 9 reference genes (Table 1) each produced a single product of the expected size. The PCR efficiency for the primers ranged from 85 - 93.5% (mean ± s.d. = 1.78 ± 0.06). For larvae, the stability value (sv) of *Rps18* (1.68) and *14-3-3 epsilon* (1.73) were smallest and thus, the most stable reference genes (Fig 1a) indicated by the comprehensive ranking analysis. In pupal samples, *ArgK*, supported by both pairs of primers, *Argk* (1.41) and *Argk_mod* (1.86), and *Actin* (4.12) were the most stable genes (Fig 1b). In adult samples, the gene *Gst1* (1.86) was the most stable, followed by *TBP* (2.55) (Fig 1c). When larvae, pupae and adults were analysed together, *TBP* (1.48) was the most stable gene, followed by *14-3-3 epsilon* (2.78) (Fig 1d).

Table 1. Details of the reference genes designed developed and tested in samples of *Euglossa viridissima. Actin*, however, was not designed in this study (see Locke et al., 2012). bp = base pairs. F = forward primer sequence and R = reverse primer sequence. AT = annealing temperature.

Gene name and symbol	Amplicon size (bp)	Sequence	AT	Molecular Function
Actin	271	F- CGTGCCGATAGTATTCTTG R- CTTCGTCACCAACATAGG	60	Motility, morphological changes, cell division and intracellular movementϕ
Arginine kinase (*Argk*)	138	F-CCCAGCTGGTGAATTCATCG R-TCCAAACCAGACAGAGTGCT	60	Arginine kinase activity⊗
Arginine kinase modified (*Argk_mod*)	143	F-ATGCTCCCGACGCTGAATC R-GGTCAAGATTGCCAAGGGA	60	Arginine kinase activity⊗
Elongation factor F1 (*EF1a*)	176	F-CCAATTTCTGGTTGGCACGG R-AGAGGAAGACGGAGAGCCTT	58	Catalyzes the delivery of aminoacyl-tRNA to the ribosome in a GTP-dependent manner⊕
14-3-3 epsilon	118	F-ATAAGGGCGTCGAGAGGAAG R-CACGGGAGCAGATGTTTGTC	60	Protein binding, Protein heterodimerization activity⊗
glutathione S-transferase-1 (*Gst1*)	141	F-TCCAAAGAAACGTGCACTCG R-GCCGTGCCAATACTTTCGAA	58	DDT-dehydrochlorinase activity and glutathione transferase activity⊗
Inositol 1,4,5-triphosphate receptor (*Itpr*)	85	F-TGGGTGACACTCCTTCTTCA R-TGTCGTGCGTTTCATCAAGA	58	Inositol 1,4,5-trisphosphate-sensitive calcium-release channel activity⊗
Ribosomal Protein S18 (*Rps18*)	87	F-ATTCTTCGTGTCATGGGCAC R-GTACCGACGACCTACACCTT	60	RNA binding and structural constituent of ribosome⊗
Ribosomal protein (*28S*)	139	F-CTCCCCTAGTAGAACGTCGC R-CGACCTGATACCGTACGAGT	60	rRNA endonuclease activity and rRNA N-glycosylase activity*
TATA-binding protein (*TBP*)	150	F-GGGTGAAGAAGATGGTGCAG R-ACACCGACCAAATCTCCAGG	60	General RNA polymerase II transcription factor⊗

Information source: *Gene Ontology (http://geneontology.org/), FlyBase (http://flybase.org/), ϕHennessey et al., 1993, ⊕Marygold et al., 2017).

Out of nine potential endogenous genes, we suggest two (*TBP* and *14-3-3 epsilon*) to be used for controlling gene normalization and for assessing relative gene expression of target genes. It is important to state that another set of genes (*TBP* and *Rps18*) was considered stable for immature samples. This may imply that alternatively, *Rps18* has reasonable applicability to analyze gene expression in non-adult bees.

Although, groups of most stable genes varied amongst different developmental stages, the genes *TBP* and *14-3-3 epsilon* were among the three most stable in three of four (larvae, adult and all samples analyzed together) sets that we tested (see Fig 1).

Thus, it might be of benefit to use these housekeeping genes to study gene expression in primitively eusocial species like Euglossini bees, especially because recent studies showed that there are major dissimilarities between ages and environment (Lockett et al., 2016), in different tissues (*e.g.* brain and abdomen), and between solitary and eusocial bee species with respect to transcriptional regulation (Jones et al., 2017; Kapheim et al., 2015).

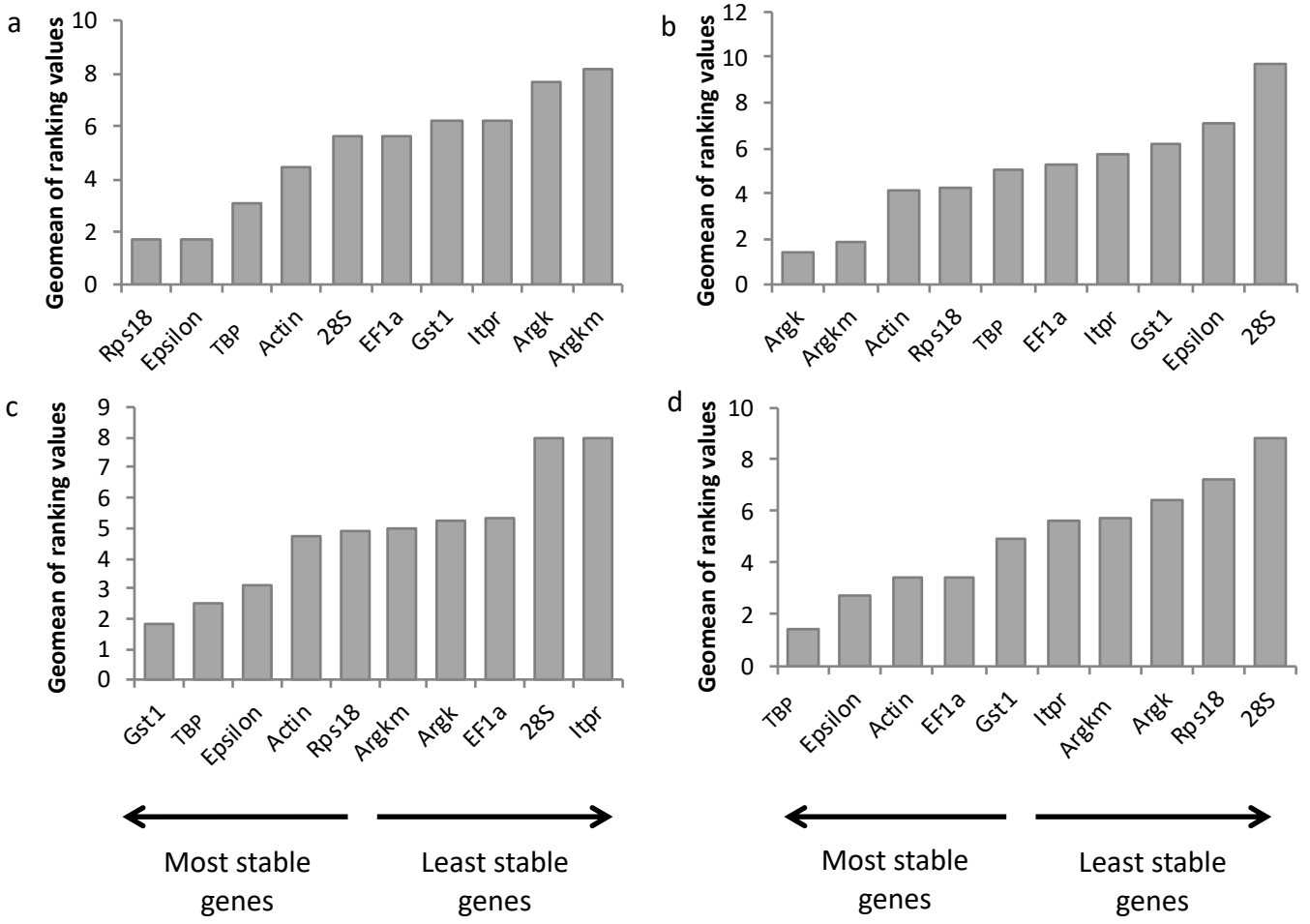

Fig 1. Stability values (Geomean ranking values) of 9 reference genes according to RefFinder. The most stable genes, those with lower values, are listed on the left and the least stable genes on the right. (a) larvae, (b) pupae, (c) adult (abdomen of females) and (d) all of individuals combined. Epsilon = *14-3-3 epsilon*; *Argkm = Argk_mod.*

Acknowledgement

We thank anonymous referees, CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) and the program Science without Borders Brazil (Ciências sem Fronteiras) for financial support (SB).

Supplementary Material

DOI: 10.13102/sociobiology.v65i4.3428.s2233
Link: http://periodicos.uefs.br/index.php/sociobiology/rt/suppFiles/3428/0

Authors' Contribution

SB, RJP conceived and designed the study; JJQE provided samples. SB, HMGL designed the primers. SB, AF, AM performed experiments and analysis; SB, HMGL analyzed the results. SB, HMGL led the writing. All authors read, discussed and approved the final manuscript.

References

Berg, D., Holzmann, C., & Riess, O. (2003). 14-3-3 proteins in the nervous system. Nature Reviews Neuroscience, 4: 752. doi: 10.1038/nrn1197

Brand, P., Saleh, N., Pan, H., Li, C., Kapheim, K.M., & Ramírez, S.R. (2017). The nuclear and mitochondrial genomes of the facultatively eusocial orchid bee *Euglossa dilemma*. G3: Genes, Genomes, Genetics, 7: 2891-2898. doi: 10.1534/g3.117.043687

Chen, C., Xie, T., Ye, S., Jensen, A.B., Eilenberg, J. (2016). Selection of reference genes for expression analysis in the entomophthoralean fungus *Pandora neoaphidis*. Brazilian Journal of Microbiology, 47: 259-265. doi: 10.1016/j.bjm.20 15.11.031.

Dallacqua, R.P., Simões, Z.L.P., & Bitondi, M.M.G. (2007). Vitellogenin gene expression in stingless bee workers differing in egg-laying behavior. Insectes Sociaux, 54: 70-76. doi: 10.1007/s00040-007-0913-1

Elsik, C.G., Tayal, A., Diesh, C.M., Unni, D.R., Emery, M. L., Nguyen, H. N., & Hagen, D. E. (2015). Hymenoptera Genome Database: integrating genome annotations in Hymenoptera Mine. Nucleic Acids Research, 44: 793-800. doi: 10.1093/nar/gkv1208

Hennessey, E.S., Drummond, D.R., & Sparrow, J.C. (1993). Molecular genetics of actin function. Biochemical Journal, 291: 657-671.

Horňáková, D., Matoušková, P., Kindl, J., Valterová, I., & Pichová, I. (2010). Selection of reference genes for real-time polymerase chain reaction analysis in tissues from *Bombus terrestris* and *Bombus lucorum* of different ages. Analytical Biochemistry, 397: 118-120. doi: 10.1016/j.ab.2009.09.019

Jones, B.M., Kingwell, C.J., Wcislo, W.T., & Robinson, G.E. (2017). Caste-biased gene expression in a facultatively eusocial bee suggests a role for genetic accommodation in the evolution of eusociality. Proceeding of the Royal Society B, 284: 20162228. doi: 10.1098/rspb.2016.2228

Khanlou, K.M., & 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. doi: 10.1007/s00425-012-1682-2

Kapheim, K.M., Pan, H., Li, C., Salzberg, S. L., Puiu, D., Magoc, T., ... & Hernandez, A. (2015). Genomic signatures of evolutionary transitions from solitary to group living. Science, 348: 1139-1143. doi: 10.1126/science.aaa4788

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A. & Zoric, N. (2006). The real-time polymerase chain reaction. Molecular Aspects of Medicine, 27: 95-125. doi:10.1016/j.mam.2005.12.007

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods, 25: 402-408. doi: 10.1006/meth.2001.1262

Lockett, G.A., Almond, E. J., Huggins, T.J., Parker, J.D., & Bourke, A.F. (2016). Gene expression differences in relation to age and social environment in queen and worker bumble bees. Experimental Gerontology, 77: 52-61. doi: 10.1016/j.exger.2016.02.007

Locke, B., Forsgren, E., Fries, I. and de Miranda, J.R. (2012). Acaricide treatment affects viral dynamics in Varroa destructor-infested honey bee colonies via both host physiology and mite control. Applied and Environmental Microbiology, 78: 227-235. doi: 10.1128/AEM.06094-11

Lourenço, A.P., Mackert, A., dos Santos Cristino, A., & Simões, Z.L.P. (2008). Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie, 39: 372-385. doi: 10.1051/apido:2008015

Marygold, S.J., Attrill, H. & Lasko, P. (2017). The translation factors of *Drosophila melanogaster*. Fly, 11: 65-74. doi: 10.1080/19336934.2016.1220464

Niu, J., Cappelle, K., de Miranda, J.R., Smagghe, G. & Meeus, I. (2014). Analysis of reference gene stability after Israeli acute paralysis virus infection in bumblebees *Bombus terrestris*. Journal of Invertebrate Pathology, 115: 76–79. doi: 10.1016/j.jip.2013.10.011

Santos, C.G., & Hartfelder, K. (2015). Insights into the dynamics of hind leg development in honey bee (*Apis mellifera* L.) queen and worker larvae-A morphology/differential gene expression analysis. Genetics and Molecular Biology, 38: 263-277. doi: 10.1590/S1415-475738320140393

Untergrasser, A.C.I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., & Rozen, S. G. (2012). Primer3web — new capabilities and interfaces. Nucleic Acids Resources, 40: e115. doi: 10.1093/nar/gks596

Woodard, S.H., Fischman, B.J., Venkat, A., Hudson, M.E., Varala, K., Cameron, S.A., Clark, A.G., Robinson, G.E. (2011). Genes involved in convergent evolution of eusociality in bees. Proceedings of the National Academy of Sciences, 108: 7472-7477. doi: 10.1073/pnas.1103457108

Xie, F., Sun, G., Stiller, J.W., & Zhang, B. (2011). Genome-wide functional analysis of the cotton transcriptome by creating an integrated EST database. PloS One, 6:e26980. doi: 10.1371/journal.pone.0026980

Yüzbaşıoğlu, A., Onbaşılar, İ., Kocaefe, C., & Özgüç, M. (2010). Assessment of housekeeping genes for use in normalization of real time PCR in skeletal muscle with chronic degenerative changes. Experimental and Molecular Pathology, 88: 326-329. doi: 10.1016/j.yexmp.2009.12.007