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Reference genes for expression studies in different developmental stages of *Oryctes rhinoceros*, the coconut rhinoceros beetle

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ABSTRACT

Oryctes rhinoceros L. (Coleoptera: Scarabaeidae), the coconut rhinoceros beetle (CRB), is a primary pest of coconut in South and Southeast Asia and the Pacific Islands. The beetle has the potential to severely affect the economies of local communities, many of whom are marginal and small farmers who rely on coconut as the main source of livelihood. Reverse transcription-quantitative real-time PCR (qRT-PCR) based targeted gene expression analysis has emerged as a powerful tool due to its sensitivity and reproducibility. However, calculating the relative expression of target genes requires normalization with reference genes across specific experimental conditions. To identify suitable reference gene(s) possessing stability, we selected six prospective genes (viz., *NADH, ACTIN, EF1A, RPL3, SDHA*, and *ARF6*) and evaluated them for their potential use as reference gene(s) across different developmental stages of *O. rhinoceros*. A comprehensive approach based on five statistical models viz., GeNorm, BestKeeper, NormFinder, RefFinder and the ΔCt value, was utilized, and based on the obtained stability values of candidate genes, a consensus ranking was generated. The expression levels of *NADH, EF1A* and *RPL3* were observed to be the most stable across the developmental stages with significant statistical reliability. Further, this study identified *NADH/EF1A* as the most reliable reference gene combination which could provide robust normalization of RT-qPCR data in gene expression studies in *O. rhinoceros.* This is the first report identifying the suitable reference genes for normalizing gene expression in *O. rhinoceros* across different developmental stages, facilitating future elucidation of gene expressions in this species.

Introduction

Oryctes rhinoceros L. (Coleoptera: Scarabaeidae), the coconut rhinoceros beetle, has emerged as one of the most challenging pests of coconut, and other palms, in many tropical and sub-tropical countries (Hinckley, 1973; Marshall et al., 2017). The beetle has four phases of development, viz., egg, larva, pupa and adult. Hosts are particularly attacked by the adult stage of *O. rhinoceros* which reduces the crop yield, and sometimes repeated attacks can be lethal to the palm. The life cycle of the *O. rhinoceros* beetle in palm log tissue, from egg to adult, takes about 7 to 8 months after egg laying (Manjeri et al., 2014) and 5 to 6 months in finer and more suitable media like cow dung and sawdust (Lai et al., 2015). Hooking off adult beetles, prophylactic leaf axil treatment using botanical cakes, physical exclusion by netting, incorporation of green muscardine fungus, *Metarhizium majus* in breeding zones, placement of pheromone lures, and use of biocontrol agent *Oryctes rhinoceros nudivirus* (*OrNV*) are pest management strategies used with varied success (Josephrajkumar et al., 2018). The recent discovery of beetle haplotypes tolerant to the biocontrol agent *Oryctes rhinoceros*nudivirus (OrNv), from Guam in 2007 and later from other islands in Oceania (Marshall et al., 2017), has raised serious concerns in these regions. The underlying mechanism and developmental aspects of *O. rhinoceros* have remained unexplored.

Future studies that aim to identify target genes to devise integrated pest management (IPM) strategies like RNAi rely on Real-time quantitative PCR (RT-qPCR), a gold-standard method to determine minor deviations in mRNA expression levels of a target gene (Taylor et al., 2017). RT-qPCR is popular due to its sensitivity, accuracy and reproducibility (Luo et al., 2020; Wang et al., 2019; Xie et al., 2020). RNA integrity, cDNA quality, and amplification efficiency are various parameters that

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determine the accuracy and reproducibility of qRT-PCR data (Radonić et al., 2004). The amount of input RNA in gene expression studies across various treatments and control is normalized using internal controls, commonly referred to as reference genes (Tong et al., 2009). One of the major criteria for selecting a reference gene is that its expression should be stable in all conditions being studied, and the selection of an unsuitable reference gene can negatively impact the outcome of the experiment. However, some studies have revealed that under complex experimental treatments, a single housekeeping gene can show inconsistent expression (Nelissen et al., 2010; Shu et al., 2018; Yuan et al., 2014). Given the great need for reference genes for the validation and finding the accuracy of target genes using RT-qPCR, it is imperative to find out the ideal candidate reference genes (Vandesompele et al., 2002).

Many studies have identified suitable reference genes in varied insects (Lü et al., 2018; Luo et al., 2020; Teng et al., 2012; Wang et al., 2019; Xie et al., 2020), but there are no reports in *Oryctes rhinoceros*. Considering the importance of highly stable reference gene(s) as an internal control, we have comprehensively evaluated six candidate reference genes, i.e. *NADH dehydrogenase (ubiquinone) flavoprotein 1* (*NADH*), *ACTIN, Elongation factor 1A* (*EF1A*), *Ribosomal Protein L3* (*RPL3*), *Succinate Dehydrogenase Complex Flavoprotein Subunit A* (*SDHA*), and *ADP Ribosylation Factor 6* (*ARF6*), under four developmental stages of *O. rhinoceros,* viz., early instar larva (EIL), late instar larva (LIL), pupa and adult insects. Utilizing the highly recommended statistical models viz., ΔCt value, BestKeeper, NormFinder, GeNorm, and RefFinder, the expression stabilities of the genes were evaluated and ranked. This study provides the optimal reference genes for analysing *O. rhinoceros* gene expression and will be a valuable resource for future molecular studies in *O. rhinoceros*.

Materials and methods

Total RNA extraction and cDNA synthesis

Rearing of insects, sampling and extraction of total RNA using TRIzol® Reagent (Invitrogen, Carlsbad, CA, US) has been described in detail in our previous study (Arvind et al., 2020). For each sample, RNA concentration was measured in triplicate using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA). cDNA synthesis was performed using the Verso cDNA synthesis kit (Thermos Scientific) with random primer according to the manufacturer's recommendations and was stored at −80 °C for further analysis by qRT-PCR.

Candidate reference genes selection and primer design

A total of six candidate reference genes, viz., *NADH, ACTIN, EF1A*, *RPL3, SDHA,* and *ARF6,* were selected that are commonly used in the qRT-PCR analysis in other insect species (Table 1) (Lü et al., 2018). For the six genes, primers were designed (Table 1) using Primer3 from the transcriptome sequences generated in our previous study (NCBI accession nos.: SRX5004164 – SRX5004167) (Arvind et al., 2020).

Reverse transcription-quantitative real-time PCR

For qRT-PCR analyses, a 10-fold dilution of cDNA samples was done. qRT-PCR was carried out in a 96-well plate on a Roche Light Cycler using Light Cycle SYBR Green I Master mix (Roche Diagnostics, Germany). The qRT-PCR mixture contained 10 μl of 2X SYBR Green I Master mix, 10 ng of cDNA, 0.4 μM of each primer, and PCR-grade water up to a total volume of 20 μl. Thermal cycling was composed of an initial denaturation step at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s, primer annealing for 10 s, and extension at 72 °C for 30 s. To analyze primer specificity, the melt curve was generated for each primer pair as described by Wang et al. (2019) and Köhsler et al. (2020). The dissociation curve was obtained by melting the amplicon from 60 to 95 °C. All qRT-PCR reactions were performed in triplicates with negative control (no template) and repeated on three biological replicates.

Data analysis

GeNorm, NormFinder, BestKeeper, and a standard comparative method (ΔCt) were used to evaluate the expression stability of the potential candidate genes (Köhsler et al., 2020; Wang et al., 2019; Xie et al., 2020; Zhao et al., 2020; Zheng et al., 2020). In GeNorm and NormFinder, using the formula $Q = 2^{-\Delta Ct}$ where $^{\Delta}Ct = Ct_{sample} - Ct_{min}$, the Ct value was first converted to relative quantitative Q value. Ct_{sample} is the Ct value of the housekeeping gene in each developmental stage; Ct_{min} indicates the lowest Ct value of this housekeeping gene among each life-stage (Zhao et al., 2020). GeNorm determines the most stable genes based on each gene's average pair-wise variation while comparing with other genes and calculates the gene expression stability (M), where the most stable gene has the lowest M value (i.e. genes with the M-values ≤ 0.5 hold the most stable expression and hence suggested as the criterion for reference gene selection (Hellemans et al., 2007; Liu et al., 2014; Vandesompele et al., 2002). Further, GeNorm stepwise excludes the least stable gene and eventually determines the optimal number of internal control genes required for qRT-PCR normalization. A pair-wise variation cut-off value (V_n/V_{n+1}) below 0.15 suggests redundancy of an additional internal gene for qRT-PCR normalization (Vandesompele et al., 2002). NormFinder determines the most reliable gene while

Table 1
List of the primers designed for the candidate reference genes and their details.

Sl. No.	Reference gene name	Primer name	Primer sequence (5′ – 3′ region)	Amplicon size (bps)	Amplificati-one fficiency	Correlation coefficient (R^2)
1.	*NADH*	NADH_F	ACGCCGGTAGATTCAACAAC	67	103.98	0.999
		NADH_R	AAAGGGATCCGAAGAGCATT			
2.	*ACTIN*	ACTIN_F	ATGGGGAAGAGCGTAACCTT	108	103.98	0.999
		ACTIN_R	GCCGTACTCTCCTTGTACGC			
3.	*EF1A*	EF1A_F	CTTGGAGGGAACCATCTTGA	106	101.35	0.982
		EF1A_R	CGAGATCCAGGAGAAGATCG			
4.	*RPL3*	RPL3_F	GCTGGTCAAAAGGGTTACCA	75	109.18	0.983
		RPL3_R	TGTGTGGATACCAGCTCCAA			
5.	*SDHA*	SDHA_F	CGCTGGTAGATCTTGCCTTC	116	93.25	0.995
		SDHA_R	CTGGGTGATCAGGACGCTAT			
6.	*ARF6*	ARF6_F	ATACGATCACGGTCAGCACA	64	96.14	0.987
		ARF6_R	GCCATTATTACACGGGCACT			
Target gene						
7.	*OBP13*	OBP13_F	CATCCATGGAAGGCTGAGTT	190	108.68	0.8972
		OBP13_R	TGTCTCGCACGAAAAACAAC			

performing intra-and intergroup variations of a sample set using two-way ANOVA. It directly and robustly evaluates gene expression stability and calculates the optimal number of reference genes required for normalisation (Andersen et al., 2004). For BestKeeper and ΔCt analyses, raw Ct values were used. BestKeeper finds suitable reference genes based on a correlation coefficient between candidate genes (Pfaffl et al., 2004). In this method, the best endogenous gene is selected by comparing each gene's relative expression with that of other genes by virtue of ΔCt approach (Silver et al., 2006).

To decide the optimal number of reference genes required for normalization, we used RefFinder: https://github.com/fulxie/RefFinder (Xie et al., 2012). Though different algorithms produce the different ranking output of reference genes, the comprehensive ranking was also generated based on the geometric mean of the individual ranking values obtained from the four algorithms to better determine the candidate genes considered in this study. A formula $2^{-\Delta\Delta Ct}$ method devised by (Livak and Schmittgen, 2001) is a popular way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. Also, a target gene encoding i.e. Odorant binding protein (*OBP13)* was selected for the validation of the most stable and the least stable genes.

Results

Evaluation of primer efficiency and specificity

Messenger RNA (mRNA) levels for all genes were calculated to determine the expression profiles of the selected candidate genes in four developmental stages of *O. rhinoceros,* viz*.,* EIL, LIL, pupa, and adult. Based on the results of the dissociation curves, a single peak in the dissociation curve was obtained at the final step of qRT-PCR, which validated primer specificity. A standard curve analysis was performed, which revealed the amplification efficiency of the qPCR primers was 93.25 % to 109.18 % and correlation coefficient (R^2) values ranged from 0.982 to 0.999 (Table 1).

Expression levels of selected candidate reference genes

The selected reference genes were evaluated for their expression level in the four different developmental stages of *O. rhinoceros*. The mean (Ct) value for each gene was calculated to normalize gene expression measures. The obtained Ct values of candidate genes from all samples showed a considerable variation in expression among different sample groups (Fig. 1). The most highly expressed gene was *ACTIN*, with an average Ct value of 20.70, while the least expressed was *SDHA*, with an average Ct value of 31.99. However, though the average Ct value for *NADH* was 24.28, it held the lowest Ct dispersal order, which indicated that the expression does not fluctuate much between the developmental stages. The inter-quartile range was calculated across all the samples to identify the genes with the least dispersal Ct value. Moreover, the order of the genes with the most stable expression (i.e., with the lowest average standard deviation) to the least stable was observed as - *NADH, EF1A, RPL3, ACTIN*, *ARF6,* and *SDHA*. The candidate reference genes displayed a broad range of Ct values ranging from 18.12 to 27.63 (Fig. 1). Variation in their expression stability in correspondence to the average of standard deviation (SD) is provided (Fig. 2). Lower SD values represent higher gene expression stability.

Expression stability of selected reference genes

The BestKeeper results showed a slight deviation in the ranking of genes compared to the ΔCt method. *EF1A, RPL3*, and *NADH* had the lowest SD and CV values and were considered the most stable (Table 2). SD value for *EF1A, RPL3*, and *NADH* was <1.0 in all the developmental stages, suggesting that any of the genes could be used as a reference gene for the normalization of target gene expression. Based on the BestKeeper analysis, among the different developmental stages, the order of the reference gene in their decreasing stability was *EF1A*, followed by *RPL3, NADH, ARF6, ACTIN,* and *SDHA*.

Analysis based on NormFinder suggested that across the different developmental stages of *O. rhinoceros, EF1A, NADH,* and *RPL3* possessed the lowest stability value <1.0 among six reference genes, thus qualifies as one of the highest stable genes (Fig. 3). On the other end, with 1.86 as stability value, *SDHA* is ranked as the least stable gene in the four life-stages samples of *O. rhinoceros* (Fig. 3).

NormFinder follows the expression variation of candidate genes and calculates the stability value (SV). It identifies the optimal reference gene in the light of SV, and thus genes with the lowest SV are the most stable. Across the different developmental stages of *O. rhinoceros, EF1A, NADH,* and *RPL3* possessed the lowest stability value <1.0 among six reference genes, thus qualifying as one of the highest stable genes (Fig. 3). On the other end, with 1.86 as stability value, *SDHA* is ranked as the least stable gene in the four life-stages samples of *O. rhinoceros* (Fig. 3).

Using GeNorm, the expression stability (M) of each candidate reference gene across the four developmental stages was calculated. Stepwise exclusion of the genes with the highest M-values aids to identify combinations of genes with the highest stability. Except for *SDHA*, M values obtained for all the reference genes were <1.5; thus, they are perhaps the best candidate as reference genes (Fig. 4). The rank order assigned by gNorm is *NADH, RPL3, EF1A, ACTIN, ARF6,* and *SDHA,* hence may be useful for gene expression analysis in different developmental stages.

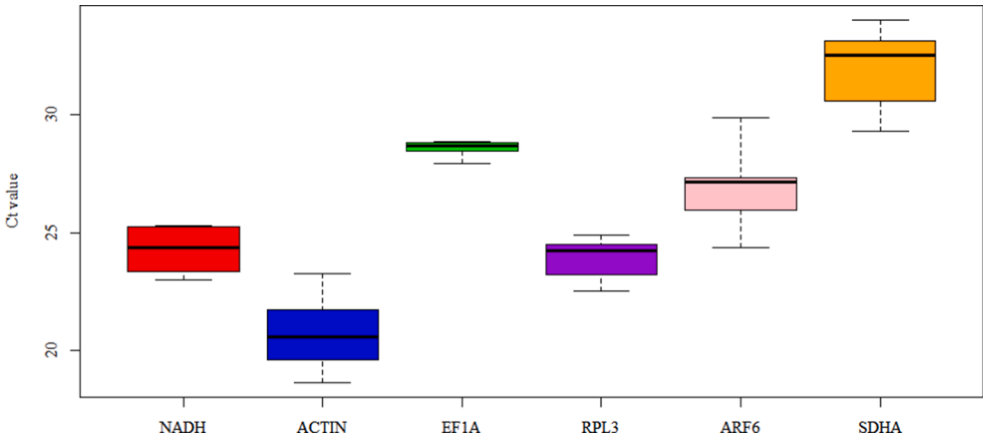

Fig. 1. Expression levels of candidate reference genes across all life stages. Lines across the boxes denote the medians. Boxes represent the interquartile range, i.e. (the difference between the first and third quartile). Whiskers represent the distribution of the highest and lowest Ct values.

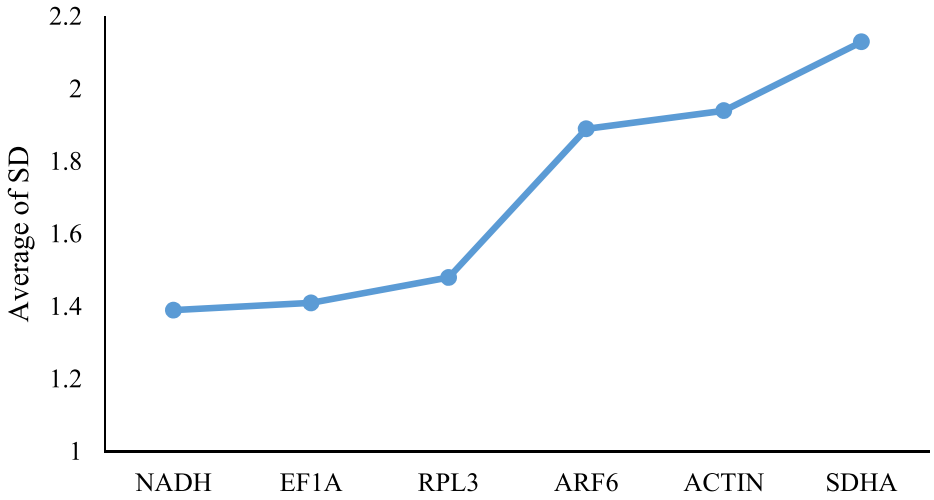

Fig. 2. Gene expression stability of six reference genes in correspondence to the average of standard deviation (SD). Lower SD values represent higher gene expression stability.

Table 2
Reference gene expression stability in four developmental stages of *O. rhinoceros*, ranked by GeNorm, NormFinder, BestKeeper and RefFinder.

GeNorm		**NormFinder**		**BestKeeper**				**RefFinder**		
Gene name	Stability value	Gene name	Stability value	Gene name	CV	SD	Stability value	Gene name	Comprehensive ranking value	Comprehensive ranking
NADH	0.729	*EF1A*	0.443	*EF1A*	0.81	0.23	0.232	*NADH*	1.57	1
RPL3	0.729	*NADH*	0.595	*RPL3*	2.90	0.69	0.693	*EF1A*	1.57	2
EF1A	1.774	*RPL3*	0.818	*NADH*	3.97	0.97	0.965	*RPL3*	2.06	3
ACTIN	1.235	*ARF6*	1.474	*ARF6*	4.07	1.10	1.096	*ARF6*	4.23	4
ARF6	1.496	*ACTIN*	1.659	*ACTIN*	5.49	1.14	1.137	*ACTIN*	4.73	5
SDHA	1.706	*SDHA*	1.860	*SDHA*	4.27	1.37	1.368	*SDHA*	6.00	6

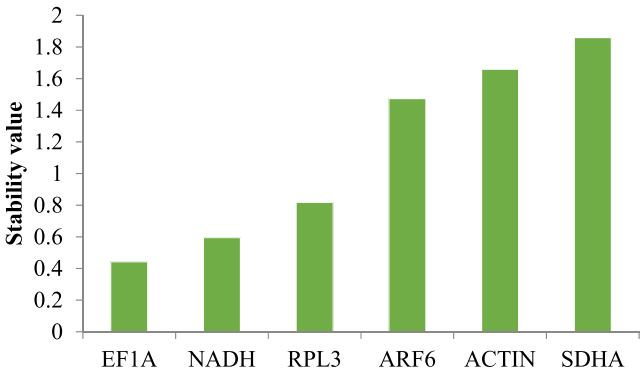

Fig. 3. Relative expression of reference genes as determined by NormFinder. Stability values of *EF1A, NADH, RPL3, ARF6, ACTIN*, and *SDHA* are given.

Comprehensive ranking of candidate reference genes

Following the rankings by four algorithms individually, RefFinder was employed to provide an integrated result and a comprehensive ranking of the most stable candidate genes. By integrating the commonly used analytical programs like GeNorm, NormFinder, BestKeeper, and ΔCt method, the candidate genes' ranking was determined. Based on the comprehensive analysis, the rank order of gene stability is *NADH* > *EF1A* > *RPL3* > *ARF6* > *ACTIN* > *SDHA,* and the obtained geometric means of the ranking values were assigned (Fig. 5). To sum up, our findings suggest *NADH* as the best reference gene for normalizing the expression of target genes and *SDHA* as the least stable gene for expression studies in different developmental stages of *O. rhinoceros*. The details obtained with each algorithm and the overall compiled ranking order of genes are shown in Table 2 and Table 3.

Validation of reference gene selection

Odorant binding proteins (OBPs) play a critical role in insect behaviour including foraging, mate selection, oviposition and social behaviour (Venthur and Zhou, 2018). They are transported through the insect's sensillum lymph to odorant receptors (ORs) in order to mediate electrical signals transmission to the insect brain (Fleischer and Krieger, 2018). There are different classes of OBPs and their number varies between insect species (Zafar et al., 2022). In this study, *Odorant binding protein 13 (OBP13)* was used as a target gene for the verification of stable reference genes inferred in this study, individually and in combination. The results showed that the *OBP13* gene was expressed in all four developmental stages of *O. rhinoceros*. The expression was found to be highest in the late larval stage compared to other developmental stages. In the EIL and pupal stage, the expression of the gene was found to be lower (Fig. 6). The expression levels of *OBP13* were normalized with *NADH* and *EF1A*. Comparative normalized gene expression analysis revealed that the expression of *OBP13* got reduced from 1.0 to 0.6 fold in EIL, increased from 6.5 to 7.75 in LIL, and decreased by 0.5 to 0.2 in the pupal stage. The relative expression of *OBP13* showed a stable expression difference after normalizing with the single and most stable reference gene combination across developmental stages. On the contrary, the *OBP13* gene expression level after normalizing with the least stable reference gene showed higher variation across different developmental stages (Fig. 6). Henceforth, the most stable genes used for normalization, either individually or in combination, resulted in more consistent and reliable target gene expression patterns.

Discussion

Reference genes, a critical requirement for any gene expression studies in *Oryctes rhinoceros* are yet to be characterized. In this study, we

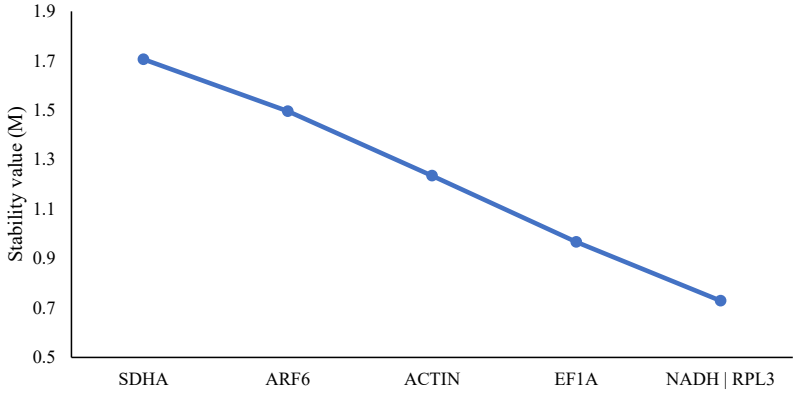

Fig. 4. Ranking and expression stability value (M) of six candidate reference genes calculated by GeNorm. V_n/V_{n+1} were used to ascertain the optimal number of reference genes.

have generated a panel of potential reference genes across four developmental stages viz., early instar larva (EIL), late instar larva (LIL), pupa, and adult of *O. rhinoceros*. Based on previous reports in other insects, a total of six genes, viz., *NADH*, *ACTIN, EF1A, RPL3*, *SDHA*, and *ARF6* were selected and the corresponding gene sequences were extracted from our previous study involving the transcriptome of *O. rhinoceros* (Arvind et al., 2020; González-Bermúdez et al., 2019; Köhsler et al., 2020; Lü et al., 2018; Zheng et al., 2020).

Given the previous study, which recommends an ideal value, i.e. CV < 2 for stable and reliable reference gene, the CV (Coefficient of Variation = Standard Deviation / Mean) of reference genes in our study ranges from 0.07 to 0.1 (González-Bermúdez et al., 2019). The correlation coefficient (R^2) based on regression was >0.982, indicating a good linear relationship (Köhsler et al., 2020; Zheng et al., 2020). The

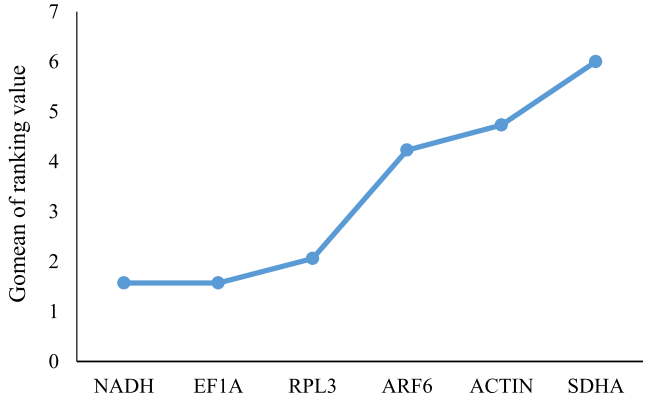

Fig. 5. Stability of candidate reference genes expression across four developmental stages. A lower Geomean value indicates more stable expression based on RefFinder.

Table 3
The rank order of six candidate reference genes based on different algorithms across four developmental stages.

	Reference genes ranking order					
Method	1	2	3	4	5	6
Delta CT	NADH	EF1A	RPL3	ARF6	ACTIN	SDHA
BestKeeper	EF1A	RPL3	NADH	ARF6	ACTIN	SDHA
NormFinder	EF1A	NADH	RPL3	ARF6	ACTIN	SDHA
GeNorm	NADH | RPL3		EF1A	ACTIN	ARF6	SDHA
Recommended comprehensive ranking	NADH	EF1A	RPL3	ARF6	ACTIN	SDHA

different algorithms produced different outputs because quite divergent statistical approaches are employed (Köhsler et al., 2020). Out of the six candidate genes tested, *NADH*, *EF1A,* and *RPL3* were observed as the most stable reference genes suitable for gene expression normalization under different developmental stages in *O. rhinoceros*.

Supporting our results, *NADH* has been reported as the most stable housekeeping gene across various conditions in *B. tabaci* (Li et al., 2013). Also, the consistent expression of *NADH* was reported across different developmental stages and temperatures in the pea aphid, *Acyrthosiphon pisum* (Yang et al., 2015). In *Cicindela campestris* and *Calomera littoralis*, among the nine candidate reference genes evaluated across different conditions, *NADH* showed the most constant expression stability and thus indicating its suitability as reference gene (García-Reina et al., 2018). A study that identified six reference genes with stable expression in different *A. sexdens* samples also suggested the *NADH* gene as one of the most stable reference genes in different conditions, including different developmental stages (Livramento et al., 2018).

EF1A has been extensively used as a reference gene in insects, including the study involving the different developmental stages of *Sesamia inferens* and intestinal tissues of sea bass (*Dicentrarchus labrax*) (Linardić and Braybrook, 2021; Schaeck et al., 2016; Sun et al., 2015; Xu et al., 2021). Being a conserved evolutionary GTPase, in previous studies, *EF1A* has been evaluated as the stable gene in Orthoptera and Hymenoptera (Chapuis et al., 2011; Horňáková et al., 2010; Ponton et al., 2011). Our result is also corroborated by previous studies conducted in insects which revealed that *EF1A* had expression stability across developmental stages (Sadritdinova et al., 2013; Nakamura et al.,

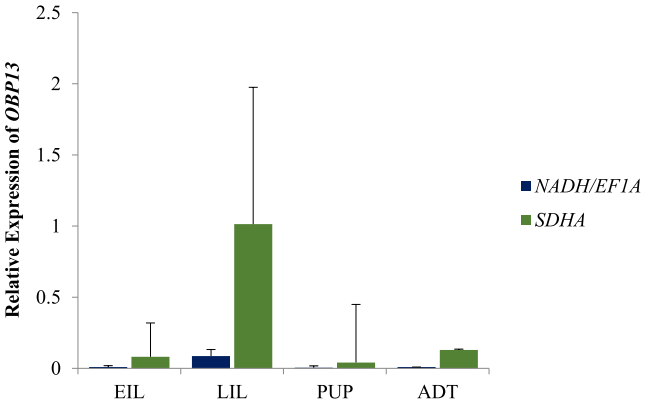

Fig. 6. Validation of the recommended reference genes. The relative expression levels of the target genes *Odorant binding protein (OBP13)* were studied under different developmental stages by normalizing using the selected reference genes i.e. *NADH/EF1A* and *SDHA*.

2016; Pan et al., 2015; Rodrigues et al., 2014; Qu et al., 2018).). However, in recent studies in some insects, the dynamic expression of *EF1A* under certain experimental conditions has been reported (Linardić and Braybrook, 2021; Xu et al., 2021; Köhsler et al., 2020). Reportedly, various factors can influence the expression levels of the *EF1A* gene, viz., developmental effects and stress (Chen et al., 2019; Wei et al., 2020; Zhang et al., 2016). In this study, the ranking of *EF1A* below *NADH* may indicate its variable nature among the developmental stages studied. Though *EF1A* stability is lower than the *NADH* gene, both genes may qualify to be used as a reference gene for developmental stage-specific studies in *O. rhinoceros*. A combination of at least two reference genes based on the output of several algorithms is recommended to be employed for more accurate expression rate determinations. Supporting our results, *RPL3* has also been used as a candidate reference gene to normalize the relative expression of candidate genes of silk glands according to the study conducted in *Bombyx mori* (Cong et al., 2020).

Based on our analysis, *ARF6*, *ACTIN,* and *SDHA* are found to be the least stable reference genes across the four developmental stages of *O. rhinoceros*. While ARFs are ubiquitous in eukaryotic cells and function as a regulator of vesicular traffic and actin remodeling, our results indicated variation in the expression of *ARF6* across the developmental stages (Randazzo et al., 2000). Expression of *ARF6* has been reported to have crucial during wing development in *Drosophila* (Marcetteau et al., 2021). *ACTIN* has been used as a popular housekeeping gene and implicated as a stable reference gene across insect species in various experimental conditions; however, the same trend was not observed in beetles (Bai et al., 2020). Unsurprisingly, in this study, variation in the expression level of *ACTIN* was observed across the developmental stages of *O. rhinoceros*. Also, variation in the expression level of housekeeping genes including *ACTIN* and *SDHA* was observed in *Dendroctonus valens* under different temperature conditions across two developmental stages i.e. adult and mature larvae(Zheng et al., 2020).

To prevent the distortion of the gene expression data, the use of multiple stable reference genes is recommended. The use of two or more reference genes may yield more accurate results and reduce the effects of variables. For instance, *GAPDH* and *EF-1α* are stably expressed in *Spodoptera litura* under varying temperatures (Lu et al., 2013). Besides, studies have also identified different candidate genes in the same insect taxa and experimental conditions. For instance, *RPS15* and *RPL27* are reported to be stably expressed in *Helicoverpa armigera*. However, a combination of *RPL28* and *RPS15* genes are rendered to be the most suitable reference under the same conditions (Shakeel et al., 2015; Zhang et al., 2015). A combination of beta-tubulin (*TUBB)* and *EF1A* was used as the best gene combination in a study involving salinity and injury stress in *Onchidium reevesii* (Yang et al., 2019). Further, a recent study has also inferred that at least two reference genes are recommended for gene expression-based study in a given condition (Linardić and Braybrook, 2021). Our study based on four different algorithms has identified *NADH, EF1A,* and *RPL3* as the most dependably expressed reference gene; therefore, an *NADH/EF1A* combination may provide stout normalization of gene expression in the samples involving developmental stages of *O. rhinoceros*.

As have previously reported in many similar studies, there is no universal reference gene suitable for all developmental stages in *Oryctes*. Thus, it could be inferred that for specific sample types and experimental conditions, for accurate analysis of gene expression, suitable reference genes may be highly specific and the selection of such genes are critical. Therefore, it is recommended to do reference gene screening to obtain better accuracy in gene expression analysis for each RT-PCR experiment.

Conclusions

Our study has identified appropriate normalization genes for stage-specific expression studies in *Oryctes rhinoceros*. This study also points out the importance of using different algorithms in reference gene validation to guarantee strong confidence in the correct choice of reference genes. Although a singular reference gene may suffice for the interpretation of target gene expression in most stages, a combination of at least two genes may be recommended to curtail the effects of variables upon the data set and for consistency of normalization. Overall, inferred stability suggests *NADH*, *EF1A* and *RPL3* as the most dependably expressed reference genes across various developmental stages of rhinoceros beetle. The *NADH/EF1A* combination is identified to provide robust normalization for gene expression studies in the samples entailing different developmental stages of *O. rhinoceros.* These findings would be beneficial for normalization practices in *O. rhinoceros* and could further be served as a resource for screening reference genes in closely-related arthropods. The study highlights the importance of customized validation of reference gene stability in studies related to insect development during each morphogenetic moults.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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