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ORIGINAL ARTICLE

Reference gene and tropomyosin expression in mud crab *Scylla olivacea, Scylla paramamosain* and *Scylla tranquebarica*

Nur Farah Hani Azemi[1] · Rosmilah Misnan[1] · Poh Bun Keong[1] · Zailatul Hani Mohamad Yadzir[2]

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Abstract

Tropomyosin, a muscle tissue protein is a major allergen in most of shellfish including mud crab. Quantitative real time-PCR (qRT-PCR) using a stable reference gene is the most sensitive approach to produce accurate relative gene expression that has yet to be demonstrated for allergenic tropomyosin in mud crab species. This study was conducted to identify the suitable reference gene and tropomyosin expression in different body parts of local mud crabs, *Scylla olivacea*, *Scylla paramamosain* and *Scylla tranquebarica*. *Myosin*, *18S rRNA, GADPH* and *EF1α* were selected as candidate reference genes and their expression was measured in the abdomen, walking leg and cheliped tissues of local *Scylla* spp. The expression stability was analyzed using the comparative delta-Ct method, BestKeeper, NormFinder and geNorm then comprehensively ranked by RefFinder algorithm. Findings showed that *EF1α* was the most suitable reference gene across three mud crab species. Meanwhile, the abdomen, walking leg and cheliped selected their own suitable reference gene either *Myosin*, *18S rRNA*, *EF1α* or *GADPH*. Overall, tropomyosin was the highest in *S. tranquebarica*, whereas the least was in *S. paramamosain.* Interestingly, tropomyosin was the highest in the abdomen of all mud crab species. This is the first analysis on reference genes selection for qRT-PCR data normalization of tropomyosin expression in mud crab. These results will provide more accurate findings for further gene expression and allergen analysis in *Scylla* spp.

Keywords qRT-PCR reference gene · Gene expression · Allergen · Mud crab · Tropomyosin · *Scylla* spp.

Introduction

In recent years, gene expression analysis utilising quantitative real time-PCR (qRT-PCR) has been used to determine the expression patterns of the predicted allergen genes in different tissues to improve specific diagnosis and allergen immunotherapy treatment [1, 2]. qRT-PCR has become one of the emerging methods for gene expression analysis mainly due to its simplicity in usage, requirement of minimum RNA as starting material, speed and precision [3]. Despite its advantages, the accuracy and reliability of data generated are highly dependable on the selection of reference gene.

Ideally, the reference gene should exhibit constant stable expression across tissues or samples used throughout the experiment, which is not influenced by any biological regulation or changes in experimental conditions [4]. Example of commonly used reference genes in many crustacean qRT-PCR experiments are housekeeping genes such as beta-actin (*β-actin*) [5], 18 ribosomal RNA (*18S rRNA*) [6], glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) [7] and elongation factor 1α (*EF1α*) [7]. However, many studies reported that these reference genes are unstable, thus result in different results considerably under different conditions. For example, in Chinese mitten crab, *Erichocheir sinensis*, evaluation of 11 different reference genes under three different experimental conditions; development stages, tissues and mounting stages showed that two commonly used reference genes, *β-actin* and *GADPH* were unstable in different developmental stage and moulting stages, respectively [8]. For instance, in red swamp crayfish, *Procambarus clarkii*, investigation on expression stability of eight different body

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✉ Rosmilah Misnan
rosmilah@fsmt.upsi.edu.my

[1] Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia

[2] Disease Control Division, Ministry of Health Malaysia, 62590 Putrajaya, Malaysia

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tissues and six ovarian developmental stages showed that two commonly used reference genes, *EF1α* and *18S rRNA*, were the most stable reference genes in different body tissues [9]. Moreover, in freshwater shrimp, *Macrobrachium nipponense,* the evaluation of seven different reference genes under two different experimental conditions; normal ovarian and embryo development stages and white spot syndrome virus infection showed that *GADPH* and *18S rRNA* were unstable in both conditions [10]. Hence, the selection of stable reference genes is important prior to qRT-PCR experiments to avoid incorrect normalisation of the data.

Scylla spp. is one of the most important crabs cultured in the Asian countries [11]. Due to evolutional changes, *Scylla tranquebarica, Scylla paramamosain* and *Scylla olivacea* are the only mud crab species found in the Malaysia's coasts [12–14]. Owing to industry demand and requisition of high-quality seed production, mud crab has in recent years been studied in some aspects especially in those concerning the development in reproductive system of adult mud crab [15]. As a result, increasing numbers of qRT-PCR studies have been carried out to evaluate the expression of certain functional genes in mud crab species under normal body development process. However, the expression stability of reference genes in mud crab especially their muscle tissues under normal physiological function is still undiscovered.

Scylla spp. has been discovered as a major cause of shellfish allergies due to the presence of several allergens [16]. Tropomyosin, a regulatory protein in the muscle tissues of crab has been reported as main major allergen that triggers allergic reactions ranging from mild rhinitis to anaphylactic shock [17, 18]. Due to the common structure of tropomyosin over a wide range of invertebrate species, allergy cross-reactivity has been frequently observed [19]. The measurement of expression level of allergenic genes in different tissue sources can be applied to predict the allergenicity level in the tissues. Therefore, the measurement of tropomyosin expression levels in different muscle tissues of several mud crab species might be useful to predict the allergenic potential of crab tissues. However, transcriptional level of tropomyosin gene remained unexplored in mud crabs.

Hence, this study aimed to select the suitable reference gene for the normalisation of qRT-PCR of tropomyosin and determined the relative expression of tropomyosin in different body parts of muscle tissues from three species of mud crab. A web-based comprehensive tool called RefFinder was used to integrate and ranked the expression stability of reference genes. Subsequently, relative expression level of tropomyosin was measured using selected reference genes as a normaliser. The results will facilitate the selection of stable reference genes and accurate tropomyosin expression in *Scylla* species, which are useful as a guidance for clinicians in managing crab allergic patients and food industry for developing hypoallergenic crab products.

Materials and methods

Specimen preparation

Nine live specimens of adult male mud crab comprising a) *S. tranquebarica* (3 specimens), b) *S. paramamosain* (3 specimens) and c) *S. olivacea* (3 specimens) were examined in this study. According to other studies [9, 20, 21], harvest at least three biological replicates per mud crab species are adequate to facilitate statistical analysis of data. These mud crabs were sampled from three main localities; Merbok River, Kedah; Kuala Terengganu, Terengganu; and Tawau, Sabah, Malaysia. The morphology of mud crab species were identified and distinguished according to Keenan et al. [22]. These morphological features include frontal lobe spines height and shape, cheliped carpus and propodus spines, and male first gonopods shape and carapace color of crab. Male specimens were identified based on their narrow abdominal inverted V flap shape [23]. Mature mud crabs were selected based on spermatophores present at their anterior vas deferens [23] their large size and the appearance of mating scars (dark spots or abrasions) on their underside and first walking leg [24]. For each specimen of mud crab, different muscle tissues from different body parts (abdomen, walking leg and cheliped) were excised and immediately submerged completely in RNAlater (RNA Stabilisation Reagent) (Qiagen, Germany). The submerged tissues were refrigerated overnight at 4 °C and stored at -80 °C for further total RNA extraction.

Total RNA extraction, quantification and cDNA synthesis

Total RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen, Germany) following manufacturer's instructions. The quality and quantity of total RNA were determined using a QIAxpert Spectrophotometer (Qiagen, Germany) while samples with spectrometric absorbance indicated by ratio of A_{260}/A_{280} and A_{260}/A_{230} with value ranging from 1.8 to 2.1 and 2.0 to 2.2, respectively, were selected for cDNA synthesis. The genomic DNA contamination was removed by the addition of 2 µL of QuantiNova gDNA Removal Mix into RNA (for 50 ng total RNA) and brought up to a final volume of 15 µL with RNase-free water. The reaction mixture was subsequently incubated in PCR machine for 5 min at 42 °C. The cDNA conversion was conducted using QuantiNova Reverse Transcription Kit (Qiagen, Germany) following manufacturer's protocols. The reverse transcription was performed by adding 1 µL QuantiNova Reverse Transcription Enzyme, 4 µL

QuantiNova Reverse Transcription Mix with 15 µL of RNA having gDNA removed. Next, the reverse transcription reaction was incubated for 3 min at 25 °C, 10 min at 45 °C and finally inactivated for 5 min at 85 °C. All cDNA samples were diluted into an amount of 25 ng/µL and stored at – 20 °C for further gene amplification.

Genes selection and primer preparation

In this study, five selected candidate reference genes from literature review namely *β-actin*, *Myosin*, *18S rRNA*, *EF1α* and *GADPH* were investigated against a target gene, tropomyosin. Unlike other commonly used crustacean reference genes, *Myosin* gene encoding a cytoskeletal protein similar to tropomyosin was tested in this study. The primer sequences of candidate reference genes and target gene were designed to suit all three species of mud crab using Primer3Plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and were synthesised by Apical Scientific Sdn. Bhd., Malaysia. The gene names, NCBI accession numbers, and primer sequences are listed in Table 1.

PCR and agarose gel electrophoresis

The PCR reaction was prepared in the final volume of 25 µL, each contained 12.5 µL of 1 × First Base PCR Mix, 0.6 µM of each primer, cDNA template in a concentration of 50 ng and nuclease-free water adjusted to the final volume. PCR was performed in a thermal cycler MyCycler EP Gradient (Eppendorf, USA) under the following conditions: an initial denaturation at 95 °C for 15 min, 35 cycles of amplification (94 °C for 1 min, 60 °C for 45 s, 72 °C for 1 min) and a final extension step of 72 °C for 7 min. The amplicons DNA were confirmed by electrophoresis on a 1.5% (w/v) agarose gel.

qRT-PCR and standard curve

The qRT-PCR was performed using QuantiNova SYBR Green PCR Kit (Qiagen, Germany) in a Rotor-Gene Q machine (Qiagen, Germany) following manufacturer's instructions. qRT-PCR was performed in a 20 µL reaction volume containing 10 µL of 2 × QuantiNova SYBR Green Mix, 0.6 µM of each forward and reverse primer, 50 ng of cDNA and RNase-free water filled to the final volume. The qRT-PCR thermal cycling conditions were conducted at 95 °C for 2 min (initial activation), followed by 40 cycles amplification of 95 °C for 5 s (denaturation) and 60 °C for 10 s (annealing and extension). A non-template control (NTC) was included as a negative control. The qRT-PCR reaction of each studied specimen was repeated twice for each sample run. At the end of the qRT-PCR program, a standard curve was generated at 50 to 99 °C with a 1 °C increment in temperature to produce a threshold baseline for specimen analysis. The standard curve for each of studied genes was drawn using ten-fold dilutions of pool cDNA stock and was carried out in triplicate. Amplification with standard curve of correlation coefficient (R^2) above 0.99 and qPCR efficiency between 91 and 110% was used for further qRT-PCR analysis.

qRT-PCR data analysis

Expression level of candidate reference genes and tropomyosin

The expression level of candidate reference genes and tropomyosin was generated using Rotor-Gene Q Series software in terms of quantification cycle (Cq) value. The Cq

Table 1 Gene name, NCBI accession number, primer sequences and amplification performance involved in this study

Gene name	Accession number	Primer sequence (5′–3′)	Amplicon size (bp)	Primer efficiency (%)	Correlation coefficient (R^2)
Beta actin (*β-actin*)	KC795683	F: TCTACAATGAGCTCCGCGTT R: TGGCAGGGGTGTTGAATGTT	–	–	–
Myosin	HM217866	F: TGCCCCCAAGGAGATGGATA R: GTTTTGCCAGTGCGGAAGAG	122	92	0.99615
18S ribosomal RNA (*18S rRNA*)	KC902763	F: TTAGTGAGGCCTTCGGACTG R: GACTTTTACTTCCTCTAAACT	132	92	0.99114
Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*)	JX268543	F: CATGGCGTGTACAAGGGTGA R: CGCCAGTAGACTCCACAACA	145	96	0.99379
Elongation factor 1- alpha (*EF1α*)	HM217884	F: GTTTCGTGGCCTCTGACTCT R: CTTGCAGGCGATATGTGCAG	146	100	0.99794
Tropomyosin	EF672351	F: GTGCAGAAGCTCCAGAAGGA R: AGTTCGCTGAACGTCTGGTC	107	100	0.99098

dispersion and mean were calculated for abdomen, walking leg and cheliped for each species. Also, the Cq value of all body parts sample was combined and noted as 'whole-body' specimen and subjected for candidate reference genes expression analysis. The Cq dispersion and mean Cq were represented by box plots and graph data were generated by One-Sample *T*-Test using IBM SPSS Statistics Version 23.0.

Expression stability of candidate reference genes

The expression stability of candidate reference genes was evaluated using RefFinder [25] webtool (https://www.heartcure.com.au/reffinder/). Using raw Cq value as input, the RefFinder automatically generated the stability value of candidate reference genes of abdomen, walking leg, cheliped and whole-body specimens for four separated algorithms namely comparative delta-Ct method (ΔCt), BestKeeper, NormFinder and geNorm, which produced a final stability rank. Furthermore, the geNorm Excel Sheet [26] was used to determine an optimal number of reference gene for normalisation analysis, which was calculated based on normalisation factor produced from stability value between two genes. Moreover, Microsoft Excel 2016 was used to create a Venn diagram for summarising the best reference genes for all muscle body parts in each mud crab species.

Relative expression of tropomyosin

The relative expression level of tropomyosin was calculated using the $2^{(-\Delta\Delta Cq)}$ Livak method [27] on three most stable reference genes. The formula to calculate the relative expression of tropomyosin was shown with the highest Cq value used as sample calibrator. The mean and standard error mean of the analysed data was generated by One-Sample *T*-Test using IBM SPSS Statistics Version 23.0.

Formula for relative expression of tropomyosin :

$$= 2^{(-\Delta\Delta Cq)}$$

$$= 2^{-[(\Delta Cq\ \text{tropomyosin}) - (\text{mean}\ \Delta\ Cq\ \text{reference genes}\ 1,2,3)]}$$

$$= 2^{-[(Cq\ \text{tropomyosin} - \text{highest}\ Cq\ \text{tropomyosin}) - (\text{mean}\ Cq\ \text{reference genes}\ 1,2,3 - \text{mean highest}\ Cq\ \text{of reference genes}\ 1,2,3)]}.$$

Results

Primer Specificity and qRT-PCR amplification efficiency

Amplification primer of high specificity and efficiency is a factor influencing the accuracy of qRT-PCR. Conventional PCR of four candidate reference genes (*Myosin, 18S rRNA, EF1α* and *GADPH*) and tropomyosin produced a single distinct band when viewed on 1.5% (w/v) agarose gel (Fig. S1), indicating that the primers used for *Myosin, 18S rRNA, EF1α*, *GADPH* and tropomyosin genes were highly specific. The specificity of primers of the genes was also verified by the presence of a single melting curve peak in qRT-PCR. An optimised qRT-PCR assay should produce a linear standard curve with high qRT-PCR amplification efficiency between 90 to 110% and correlation coefficient (R^2) of above 0.990 [25]. According to the present findings, the standard curve analysis (Table 1) of all the genes produced amplification efficiency of 92% to 100% and correlation coefficient that ranged between 0.990 and 0.997, indicating that the primers were conformed to the requirement of qRT-PCR analysis. It should be noted that *β-actin* has been removed for further qRT-PCR studies, as no band was detected on agarose gel electrophoresis in PCR amplification.

Expression level of candidate reference genes

Quantification cycle (Cq) value or known as Ct value is the qRT-PCR cycle number generated when the fluorescent light hits the threshold line. The Cq value represents the level of gene mRNA and is required for the analysis of gene expression. The low Cq value reflects higher expression while high Cq value reflects lower expression. The present findings as shown in Fig. 1 illustrated that the mean Cq values of candidate reference genes were ranged from 16.14 (*18S rRNA*) to 28.58 (*EF1α*) in *S. olivacea*, 10.32 (*18S rRNA*) to 20.75 (*EF1α*) in *S. paramamosain* and 9.66 (*18S rRNA*) to 21.15 (*EF1α*) in *S. tranquebarica*. As can be seen, *18S rRNA* and *EF1α* transcripts showed the highest and lowest expression in all mud crab species, respectively. Furthermore, in comparison with all reference genes, *18S rRNA* level was obviously low in comparison with mean Cq values of target gene, tropomyosin. On the other hand, Cq value of *Myosin* and *18S rRNA* in *S. olivacea* had broader dispersion compared to other reference genes in other mud crab species suggesting that *Myosin* and *18S rRNA* may be differently expressed in various body parts of muscle tissues, thus may not listed as the most stable reference gene in *S. olivacea.*

Expression stability of candidate reference genes

Cq values were subjected to the evaluation of expression stability to identify the best reference genes for normalising gene expression in different body parts: abdomen, walking leg, cheliped and whole-body of *S. olivacea*, *S. paramamosain* and *S. tranquebarica*, respectively. In this regard,

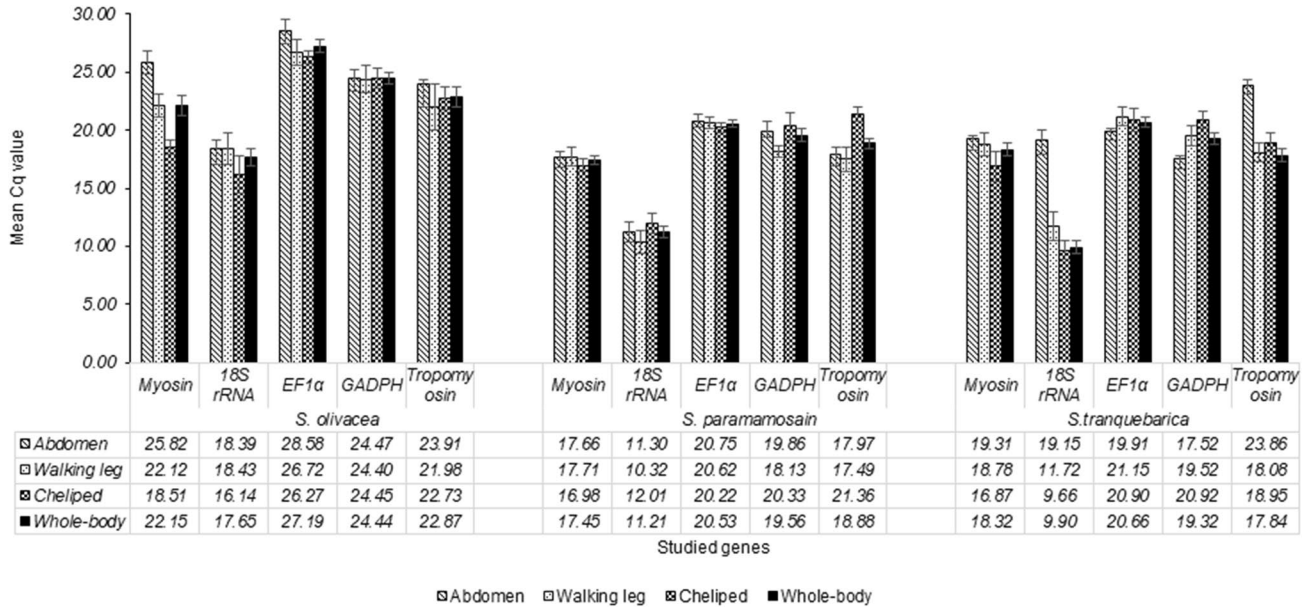

Fig. 1 Expression level of candidate reference genes and tropomyosin in mud crab specimens. Expression level of reference genes (*Myosin*, *18S rRNA*, *EF1α* and *GADPH)* and target gene (tropomyosin) was represented by the average Cq value of n = 6 for abdomen, walking leg and cheliped whereas n = 18 for whole-body specimens. The error bar of each tested body part specimens was given as standard error mean

RefFinder web-tool was used to calculate and perform a stability ranking of candidate reference genes through ΔCt, BestKeeper, NormFinder and geNorm.

Comparative ΔCt method calculates the stability of each gene by generating the standard deviation (SD) of Cq within each specimen [28]. Meanwhile, BestKeeper ranks the stability of genes by the average standard deviation produced by a correlation with BestKeeper index calculated by the geometric mean from the Ct values of reference genes [28]. The calculated average SD value represents the stability of candidate reference genes; the lower the SD values, the higher the stability. Otherwise, NormFinder method ranks the candidate reference genes according to their expression stability values in the studied group by considering intra-group and inter-group gene variations [28]. The highly stable gene are determined according to low average stability value. On the other hand, geNorm method identify the most stable reference genes by measuring the pairwise standard deviation of Cq values from all genes and then removing the genes with lowest expression stability until two last pairs of gene remained [26]. If the cut off range of M values are within 1.5, they are regarded as stable reference genes, whereas lower M values mean higher stability. Further with this algorithm, geNorm calculated pairwise variation value to determine the optimal number of reference gene. Using the results from these four algorithms, RefFinder generated comprehensive ranking of candidate reference genes based on geometric value of individual gene. All the algorithms ranked the reference gene from the most stable to least as shown in Table 2. The selected most stable reference for each body muscle tissue across mud crab species is summarised in Fig. S2.

Expression stability of reference genes in *S. olivacea* on three different body parts

All reference genes in all body parts of *S. olivacea* showed M values lower than 1.5 indicating that all of them are stable reference genes. In abdomen, ΔCt, NormFinder and geNorm methods showed similar results revealing *18S rRNA* as the most stable transcript and *EF1α* being the least. However, BestKeeper ranked *18S rRNA* as second whereas *EF1α* as third. The comprehensive ranking calculated by RefFinder demonstrated the most stable to least reference genes in abdomen in the order of *18S rRNA* > *GADPH* > *Myosin* > *EF1α*. In walking leg, ΔCt, BestKeeper and geNorm methods showed similar results for selecting *GADPH* as the most stable transcript and *EF1α* as the least. However, NormFinder ranked *18S rRNA* first and *EF1α* last. In overall, RefFinder concluded the stable to least reference gene in walking leg in the order of *GADPH* > *Myosin* > *18S rRNA* > *EF1α*. In cheliped, ΔCt and NormFinder methods selected the *GADPH* as the first while *EF1α* was selected as the first by BestKeeper and geNorm. All four algorithms ranked *18S rRNA* as the most unstable reference gene. The overall ranking in cheliped of *S. olivacea* calculated by RefFinder were *Myosin* > *GADPH* > *EF1α* > *18S rRNA*.

Table 2 Expression stability value and rank for candidate reference genes

Species	Body part	Rank	Comparative delta-Ct		BestKeeper		NormFinder		geNorm		RefFinder	
			Genes	*Mean SD	Genes	*SD	Genes	Stability value	Genes	*M value	Genes	Stability value
S. olivacea	Abdomen	1	18S rRNA	0.62	GADPH	1.52	18S rRNA	0.25	18S rRNA/GADPH	0.49	18S rRNA	1.19
		2	GADPH	0.72	18S rRNA	1.58	GADPH	0.49		0.49	GADPH	1.41
		3	Myosin	0.79	EF1α	1.85	Myosin	0.60	Myosin	0.61	Myosin	3.22
		4	EF1α	0.92	Myosin	1.99	EF1α	0.83	EF1α	0.76	EF1α	3.72
	Walking leg	1	GADPH	0.91	GADPH	0.32	18S rRNA	0.25	GADPH/Myosin	0.64	GADPH	1.32
		2	Myosin	1.13	Myosin	0.73	GADPH	0.49		0.64	Myosin	1.41
		3	18S rRNA	1.29	18S rRNA	1.04	Myosin	0.60	18S rRNA	0.92	18S rRNA	3.22
		4	EF1α	1.49	EF1α	1.35	EF1α	0.83	EF1α	1.21	EF1α	3.36
	Cheliped	1	GADPH	1.14	EF1α	1.2	GADPH	0.39	EF1α/Myosin	0.29	Myosin	1.68
		2	Myosin	1.17	Myosin	1.36	Myosin	0.70		0.29	GADPH	1.73
		3	EF1α	1.21	GADPH	1.78	EF1α	0.86	GADPH	0.63	EF1α	1.73
		4	18S rRNA	2.26	18S rRNA	3.17	18S rRNA	2.23	18S rRNA	1.44	18S rRNA	4.00
	Whole-body	1	EF1α	1.90	EF1α	1.62	EF1α	0.71	EF1α/GADPH	1.40	EF1α	1.00
		2	GADPH	2.04	GADPH	1.89	18S rRNA	1.26		1.40	GADPH	1.86
		3	18S rRNA	2.07	18S rRNA	2.34	GADPH	1.42	18S rRNA	1.64	18S rRNA	2.71
		4	Myosin	2.72	Myosin	3.10	Myosin	2.48	Myosin	2.18	Myosin	4.00
S. paramamosain	Abdomen	1	EF1α	1.12	Myosin	0.96	18S rRNA	0.19	18S rRNA/EF1α	0.30	EF1α	1.41
		2	18S rRNA	1.24	EF1α	1.20	EF1α	0.47		0.30	18S rRNA	1.57
		3	Myosin	1.83	18S rRNA	1.51	Myosin	1.64	Myosin	1.03	Myosin	2.28
		4	GADPH	2.12	GADPH	1.77	GADPH	2.01	GADPH	1.58	GADPH	4.00
	Walking leg	1	Myosin	0.92	GADPH	1.06	Myosin	0.34	EF1α/GADPH	0.82	EF1α	1.68
		2	EF1α	0.95	EF1α	1.07	EF1α	0.47		0.82	Myosin	1.73
		3	GADPH	1.11	Myosin	1.79	GADPH	0.94	Myosin	0.90	GADPH	1.73
		4	18S rRNA	1.18	18S rRNA	1.92	18S rRNA	1.04	18S rRNA	1.04	18S rRNA	4.00
	Cheliped	1	Myosin	1.20	EF1α	0.86	Myosin	0.45	EF1α/Myosin	0.89	Myosin	1.19
		2	18S rRNA	1.38	Myosin	1.08	18S rRNA	0.77		0.82	EF1α	1.73
		3	EF1α	1.49	18S rRNA	1.78	EF1α	1.22	18S rRNA	1.14	18S rRNA	2.45
		4	GADPH	1.78	GADPH	2.26	GADPH	1.61	GADPH	1.47	GADPH	4.00
	Whole-body	1	EF1α	1.41	EF1α	1.08	EF1α	0.74	EF1α/Myosin	0.97	EF1α	1.00
		2	18S rRNA	1.48	Myosin	1.28	18S rRNA	0.80		0.97	Myosin	2.06
		3	Myosin	1.56	GADPH	1.83	Myosin	1.15	18S rRNA	1.28	18S rRNA	2.63
		4	GADPH	2.00	18S rRNA	1.84	GADPH	1.70	GADPH	1.59	GADPH	3.72
S. tranquebarica	Abdomen	1	18S rRNA	0.61	18S rRNA	0.22	18S rRNA	0.02	GADPH/Myosin	0.52	18S rRNA	1.32
		2	Myosin	0.63	EF1α	0.38	Myosin	0.23		0.52	Myosin	1.86
		3	GADPH	0.75	Myosin	0.42	GADPH	0.63	18S rRNA	0.55	GADPH	2.45
		4	EF1α	0.90	GADPH	0.62	EF1α	0.84	EF1α	0.72	EF1α	3.36

Table 2 (continued)

Species	Body part	Rank	Comparative delta-Ct		BestKeeper		NormFinder		geNorm		RefFinder	
			Genes	*Mean SD	Genes	*SD	Genes	Stability value	Genes	*M value	Genes	Stability value
	Walking leg	1	EF1α	2.79	EF1α	1.58	EF1α	1.25	EF1α/GADPH	0.24	EF1α	1.00
		2	GADPH	2.91	GADPH	1.78	GADPH	1.78		0.24	GADPH	1.68
		3	Myosin	3.90	Myosin	2.04	Myosin	2.81	Myosin	2.45	Myosin	3.00
		4	18S rRNA	4.71	18S rRNA	2.64	18S rRNA	4.24	18S rRNA	3.58	18S rRNA	4.00
	Cheliped	1	18S rRNA	1.00	GADPH	1.48	18S rRNA	0.20	18S rRNA/EF1α	0.40	18S rRNA	1.19
		2	EF1α	1.01	18S rRNA	1.66	EF1α	0.20		0.40	EF1α	1.86
		3	GADPH	1.61	EF1α	1.99	GADPH	1.52	GADPH	0.88	GADPH	2.28
		4	Myosin	1.85	Myosin	2.72	Myosin	1.80	Myosin	1.37	Myosin	4.00
	Whole-body	1	EF1α	2.22	EF1α	1.36	EF1α	0.70	EF1α/GADPH	1.39	EF1α	1.00
		2	GADPH	2.64	18S rRNA	1.80	GADPH	1.93		1.39	GADPH	1.86
		3	18S rRNA	3.02	GADPH	1.84	18S rRNA	2.41	18S rRNA	2.40	18S rRNA	2.71
		4	Myosin	3.08	Myosin	2.03	Myosin	2.55	Myosin	2.74	Myosin	4.00

Expression stability of *Myosin*, *18S rRNA*, *EF1α* and *GADPH* was analysed by a web-tool known as RefFinder. The RefFinder generated the stability value and rank for comparative delta Ct, BestKeeper, NormFinder and geNorm algorithms before it generalised the final stability value and rank from all the data produced by all these four algorithms. All the algorithms produced rank from 1 to 4, which indicating the stability of candidate reference genes from the most stable to the least stable

*Mean SD: Mean Standard Deviation, *SD: Standard Deviation, *M value: geNorm Stability value

Expression stability of reference genes in *S. paramamosain* on three different body parts

The M values of candidate reference genes in all body parts of *S. paramamosain* were within 1.5 except *GADPH* (1.58) in the abdomen part. The results were parallel as all four algorithms; ΔCt, BestKeeper, NormFinder and geNorm listed *GADPH* as the lowest rank indicating *GADPH* as the most unstable gene and unsuitable to normalise expression in abdomen of *S. paramamosain*. Otherwise, the most stable reference gene in abdomen was *EF1α* as calculated by ΔCt whereas *Myosin* was selected by BestKeeper and *18S rRNA* was selected by NormFinder. All data for abdomen of *S. paramamosain* were integrated and ranked by RefFinder from most stable to least were, which were *EF1α* > *18S rRNA* > *Myosin* > *GADPH*. Unlike in abdomen, *GADPH* transcript in walking leg was the most stable generated by BestKeeper and geNorm while *Myosin* by ΔCt and NormFinder. However, all these four algorithms selected *18S rRNA* as the least stable. RefFinder concluded the most stable gene to the least in walking leg of *S. paramamosain* in the order of *EF1α* > *Myosin* > *GADPH* > *18S rRNA*. In cheliped, both *Myosin* and *EF1α* were identified as the top stable reference genes measured by ΔCt, BestKeeper, NormFinder and geNorm. All these four algorithms with similar results selected *GADPH* as the least stable reference gene. Hence, the overall comprehensive ranking by RefFinder in cheliped of *S. paramamosain* were *Myosin* > *EF1α* > *18S rRNA* > *GADPH*.

Expression stability of reference genes in *S. tranquebarica* on three different body parts

M values of all reference genes were below 1.5 excluding Myosin and *18S rRNA* with M values of 2.45 and 3.58 indicating that both reference genes were the unstable in the walking leg of *S. tranquebarica*. In abdomen, RefFinder concluded the final rank of most stable reference gene to least as *18S rRNA* > *Myosin* > *GADPH* > *EF1α*. These findings were calculated from the result of ΔCt, BestKeeper and NormFinder, which similarly selected *18S rRNA* as the most stable gene. The same goes for stability in cheliped where *18S rRNA* was selected as the most stable gene by ΔCt, NormFinder and geNorm resulting in the most stable reference gene to least as *18S rRNA* > *EF1α* > *GADPH* > *Myosin*. Nevertheless, in walking leg, *18S rRNA* was similarly ranked as the least stable gene while EF1α as the most stable by ΔCt, BestKeeper, NormFinder and geNorm resulting in the final rank by RefFinder as *EF1α* > *GADPH* > *Myosin* > *18S rRNA*.

Expression stability of reference genes in *S. olivacea*, *S. paramamosain* and *S. tranquebarica* on whole-body

All four methods; ΔCt, BestKeeper, NormFinder and geNorm shared similar results where *EF1α* was ranked as first in all three species. Otherwise, the least stable reference gene in *S. olivacea* and *S. tranquebarica* was *Myosin* whereas *GADPH* (ΔCt, BestKeeper and geNorm) and *18S rRNA* (NormFinder) in *S. paramamosain*. RefFinder integrated all these data and produced a final rank with the first *EF1α* across all three species of mud crab, suggesting *EF1α* as the best reference gene in *S. olivacea*, *S. tranquebarica* and *S. paramamosain*. Comprehensive rank from most stable to least calculated by RefFinder was *EF1α* > *GADPH* > *18S rRNA* > *Myosin* for *S. olivacea* and *S. tranquebarica*, and *EF1α* > *Myosin* > *18S rRNA* > *GADPH* for *S. paramamosain.*

Optimal number of reference gene across species

geNorm has another function which is to determine the optimal number of reference genes based on normalization factor (N) between two genes (NF/NFn + 1) to produce a pairwise variation (V) value [26]. Vandesompele et al. [26] suggested a 0.15 as a cut-off value, indicating that normalisation factor value lower than 0.15 does not in need an additional number of reference gene. In this study as shown in Fig. 2, the normalisation factor values for all reference gene in mud crab species were above 0.15, which did not fulfil the recommended cut-off value. Therefore, three reference

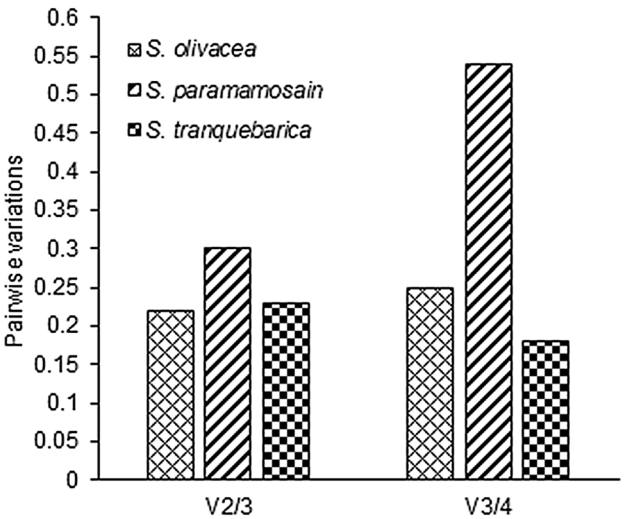

Fig. 2 Optimal number of reference gene analysis. The pairwise variation value of V2/V3 and V3/4 across the mud crab species was generated from normalization factor (NF) value between a pair of genes ranked 3rd/4th and 2nd/3rd respectively using the formula NFn/NFn + 1

genes of high stability were required to normalise the accurate gene expression as suggested by Vandesompele et al. [26]. Hence, the best three reference genes in mud crab species were *EF1α*, *GADPH* and *18S rRNA* for *S. olivacea* and *S. tranquebarica,* while *EF1α*, *Myosin* and *18S rRNA* for *S. paramamosain*.

Relative expression of tropomyosin

As presented in Fig. 3, the relative expression of tropomyosin was quantified based on three most stable reference genes. The findings showed that the tropomyosin transcript level in whole-body was the highest in *S. tranquebarica* (4.74), second in *S. olivacea* (1.71) whereas the least in *S. paramamosain* (1.53) indicating that tropomyosin was abundantly expressed in *S. tranquebarica* while the least in *S. paramamosain*. In *S. olivacea*, tropomyosin transcript was found abundantly in abdomen (0.8), second in cheliped (0.79) and last in walking leg (0.73). Otherwise in *S. paramamosain*, tropomyosin transcript was the highest in abdomen (0.78), second in walking leg (0.78) and the least in cheliped (0.4). On the other hand, in *S. tranquebarica*, high level of tropomyosin was found in abdomen (0.93), second in walking leg (0.35) followed by cheliped (0.23). Interestingly among the three body parts, the abdomen of all three mud crab species showed the highest tropomyosin transcript compared to walking leg and cheliped. This result suggests that abdomen was the main body part for producing tropomyosin. Last but not least, most tropomyosin transcript were shown showed almost the same level, indicating that the qRT-PCR is capable to quantify even a minute difference in tropomyosin level.

Discussion

qRT-PCR is one of the most sensitive methods to measure gene expression level but its reliability relies on the usage of stable reference genes whose expression levels should stay stable across tissues of similar conditions. Likewise, there is no standard gene to be utilised as an internal control for all tissue types of each test condition. For gene expression analysis of tropomyosin in mud crab, specimens differ between body parts of distinctive species yet, no past investigation has analysed the most proper genes to be utilised as an internal control gene. Thus, in this study, four housekeeping genes (*Myosin, 18S rRNA, GADPH,* and *EF1α*) were evaluated using qRT-PCR for their suitability as a reference gene to normalise tropomyosin expression in mud crab.

Based on the above results, it can be concluded that there is no similar stable reference gene identified in all three body parts of all species of mud crab probably due to the small sample number. According to Vandesompele et al., total sample size affects the statistical analysis, hence influences the stability of candidate reference genes [26]. As an alternative method, all of their Cq values were combined and noted as a 'whole-body' which was subjected for reference gene expression stability analysis across the species, similar to that performed by Berumen-Varela et al. [29]. In this study, RefFinder was chosen to analyse the stability of candidate reference genes due to its capable to produce more reliable outcomes by integrating all data generated from each single algorithm; ΔCt, BestKeeper, NormFinder or geNorm, which produced various outputs. As the result, RefFinder ranked *EF1α* as the most

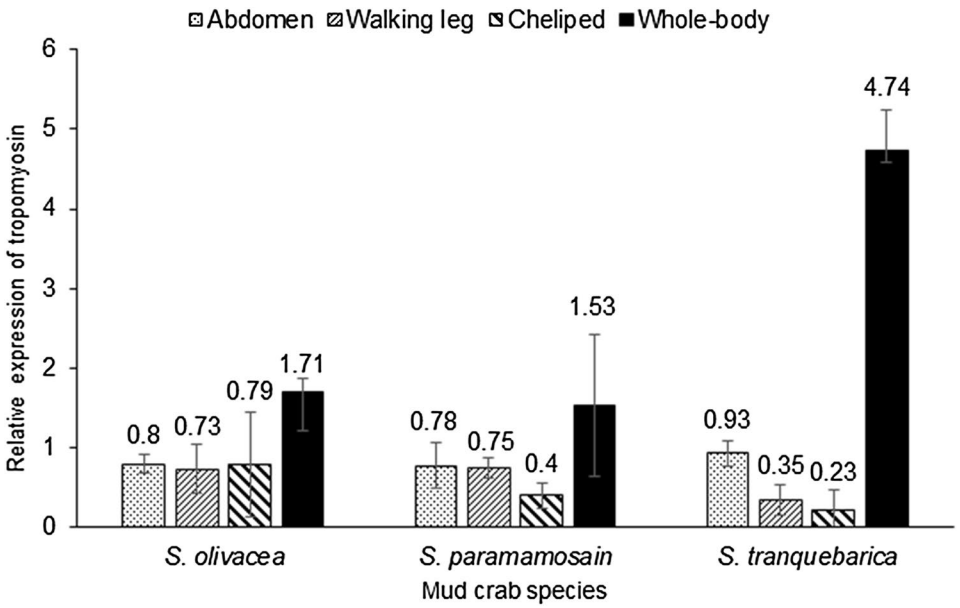

Fig. 3 Relative expression of tropomyosin in different body muscle tissues of a *S. olivacea*, *S. paramamosain* and *S. tranquebarica*. The tropomyosin expression was normalised to the expression of three most stable reference genes. By using Livak' method, the calculated expression of the tropomyosin was represented as relative to the lowest expression of reference genes

stable reference gene in all mud crab species compared to the other three reference genes; *Myosin*, *GADPH* and *18S rRNA*.

EF1α is a GTP-binding protein that catalyses the binding of aminoacyl-transfer RNAs to the ribosome during protein synthesis [30]. In normal physiology function, *EF1α* plays an important role as a regulator in cell division cycle. Previous studies on crustaceans identified *EF1α* as the most stable reference gene in different tissues and different ovarian development stages in red swamp crawfish [9], in the reproductive system of giant tiger prawn, *Penaeus monodon* [31], in white spot syndrome virus-infected western blue shrimp, *Penaeus stylirostris* [26] and in different ovarian and embryo stages in white spot syndrome virus-infected oriental river prawn, *Macrobrachium nipponense* [10]. Together with the present finding, it was summarised that *EF1α* can be considered as a versatile reference gene used in qRT-PCR of most crustacean species.

The expression of reference genes across the species under the same physiological conditions has not been investigated among shellfish or crustacean but has been studied in the species of tea plants, herbs, pest controls and bees. From all the findings, there were no definite most stable reference gene that has been identified in these studies including between stingless bee species (*Frieseomelitta varia*, *Melipona quadrifasciata*, and *Scaptotrigona bipunctata*) [32], *Grapholitini* pest control species (*Cryptophlebia peltastica*, *Thaumatotibia leucotreta* and *Cydia pomonella)* [33], and *Brassicaceae* herbs (*Brassica juncea* and *Camelina sativa*) [34]. Surprisingly, present study showed contradicted results in which *EF1α* was shown as the most stable expression in whole-body across three species of mud crab of *S. olivacea*, *S. paramamosain* and *S. tranquebarica*, indicating that *EF1α* has the potential as gold reference gene in normalising any target gene expression in normal physiology of male *Scylla* species.

Myosin together with tropomyosin represent two cytoskeletal proteins that often work together with actin filaments in contractile and motile cellular processes [35]. To prevent contraction in muscles, tropomyosin avoids the cross-bridge formation between *Myosin* and actin by blocking the myosin-binding sites on actin molecules. In gene expression studies, *Myosin* was classified as a housekeeping gene, the genes that is always expressed exclusively in the muscle tissue [36]. However, in this study, *Myosin* expressed was less stable in all mud crab species. These regards may a reason why *Myosin* is rarely used as a reference gene for the normalisation of gene expression especially in crustacean. Present findings aimed to look on the stability of reference gene-related target gene (*Myosin* and tropomyosin), however the results showed that reference gene-related target gene of similar function may not be a suitable candidate for normalising gene expression.

GADPH is a metabolic enzyme engaged with a variety of processes including glycolysis in carbohydrate metabolism [37]. *GADPH* was previously recognised as the most frequently used reference gene in the history of qRT-PCR [38, 39], but less is known regarding its potential as reference genes among crustaceans. Some studies showed that *GAPDH* is unsuitable as an internal control due to its significant variation of expression levels such as in different developmental and moulting stages in Chinese mitten crab, *Erichocheir sinensis* [8] and different stress conditions in freshwater crustacean, *Gammarus fossarum* [40] which agrees with the present findings. This concluded that not all recognised classic reference genes are suitable to be used in the normalisation of target gene expression.

Meanwhile, the *18S rRNA* was ranked as the third stable gene across three species of mud crab possibly due to its highest expression found in all three species of mud crab. Previous studies reported that *18S rRNA* was the most suitable reference gene in the tissues of half-smooth tongue sole [41] and different tissues of red swamp crawfish [9] as it is frequently used to normalise highly expressed target gene. In this study, the expression range of target gene tropomyosin was obviously unsimilar with *18S rRNA* as tropomyosin expression level was lower in all three species of mud crab. Thus, this reason concluded that suitable reference gene should has similar transcript level to the gene of interest.

β-actin is widely used as a housekeeping gene because its expression is stable, therefore suitable as an internal control that can be used to normalize gene expression, albeit without further confirmation [5]. However, recent studies have shown that *β-actin* expression can change during tissue growth and differentiation in response to biochemical stimuli, including confirmed reference gene studies in crab species of Chinese mitten crab [8] and blue swimming crab, *Portunus trituberculatus* [42]. These findings challenge the reliability of *β-actin* when used as a reference gene in crab species [8, 42]. Due to these reasons, *β-actin* was not selected as a possible candidate for the reference gene of gene expression in mud crab species after unsuccessful PCR amplification in this study. Thus, further studies using different set of primers are recommended to indicate the potential of *β-actin* as a reference gene for gene expression of local mud crab species.

The crustacean moult mechanism is divided into four major stages, known as the moult, postmoult, intermoult, and premoult [43]. The moult stage includes extracting the exoskeleton by quickly absorbing water or air from the environment, creating an exoskeleton breakup. Further extension of water absorption happens during postmoult, inducing mineralization and exoskeleton hardening. For the next moult, the intermoult or non-activity cycle includes muscle recovery and energy stores such as glycogen and lipids drained in haemolymph and midgut. Premoult triggers the

resorption of the old exoskeleton and the creation of the new exoskeleton ready for moult stage starting. Moult cycle leads to up-regulated or down-regulated protein-encoded genes, including cuticular proteins shared with arthropod exoskeletons, farnesoic acid O-methyltransferase (FaMeT), hemocyanin gene proteins, lectins, proteins relevant to lipid metabolism, mitochondrial proteins, muscle-related proteins, phenoloxidase activators, and ribosomal proteins [44]. *Actin* and *Myosin* are the only associated genes detected during the cycle, since they include muscle deposition and development, while tropomyosin has not been identified as one of the muscle-related proteins affected by moulting. We knew the moulting mechanism regulates gene expression in crabs. To avoid the moulting sample, crabs were selected by examining the exoskeleton condition and surface retraction of their paddle legs as Kuballa and Elizur mentioned [43]. This research omitted crab with exoskeleton shedding, or flexible exoskeleton or involvement of paddle leg retraction surface.

Relative quantification measured the relative change in target gene levels in which the level of gene expression across many samples was measured relatively to a steady-state expression of reference gene. Livak and Schmittgen (2001) introduced a calculation based on 2^-(ΔΔCq) method for measuring gene expression in normal condition [27]. This method demonstrated the fold-change of target gene against lowest expression (highest Cq value) of reference gene. In this study, three most stable reference genes validated before were used to normalise the tropomyosin expression in *S. olivacea*, *S. tranquebarica* and *S. paramamosain* for more accurate analysis. Tropomyosin is a multifunctional muscle protein involved in the regulation of actin-myosin interaction, transport of mRNA and mechanical support of cytoplasmic membrane [45]. However, this protein has become an important health concern due to its ability to elicit allergic reactions upon shellfish consumption. Tropomyosin has been found as a major protein allergen in various shellfish mainly mud crab species [17, 46]. Scarily, due to its special properties of highly conserving protein sequence [47] that can be found in most of crustacean, mollusc, insect and arachnid species [48], high case of allergy sensitisation has become a concern. Present tropomyosin expression study is an important assessment to deviate the cases number of mud crab allergy.

Findings of this study showed that tropomyosin expression was found the highest in *S. tranquebarica* compared to *S. olivacea* and *S. paramamosain*. *S. tranquebarica* or purple mud crab is mostly found in mangrove forests with low water salinity along South China Sea, India Ocean and Western Pacific [49]. In Malaysia, *S. tranquebarica* was found dominant in Sabah [49]. *S. tranquebarica* is a highly demanded aquaculture source and food due to its fast growth rate, large size and delicate taste [50]. High consumption of *S. tranquebarica* possibly increases the chance of crab allergy. In addition, the meat from all body parts of mud crab is edible, hence being commonly consumed by local peoples. The highest tropomyosin found in the abdomen of all three species of mud crab in this study suggests that this part is the most allergenic body part specifically in mud crab species. However, this prediction should be supported by clinical and in-vitro studies. Overall, this finding may suggest that the crab-allergic patients should avoid the consumption of *S. tranquebarica* or any mud crab abdomen as it may contain high potential allergen to trigger crab allergic reaction.

Conclusions

To our knowledge, this was the first report on the determination of suitable reference gene as qRT-PCR tropomyosin normaliser and first to demonstrate the relative expression level of tropomyosin in body part of muscle tissues of *S. olivacea*, *S. paramamosain* and *S. tranquebarica*. The results of this study indicated that all the candidate reference genes were found to have some degree of variability in different body parts and a pattern of consistency across three species of mud crab. In summary, a wide range of reference genes including *Myosin*, *18S rRNA*, *EF1α* and *GADPH* could be considered as normalisers when analysing target genes in specific crab body parts especially abdomen, cheliped and walking leg. Otherwise, *EF1α* should be selected as a reference gene when analysing target genes in all three species as a whole. Moreover, three reference genes have been suggested when analysis gene expression per species: *EF1α*, *GADPH* and *18S rRNA* for *S. olivacea* and *S. tranquebarica* while *EF1α*, *Myosin* and *18S rRNA* for *S. paramamosain*. The present findings can provide more accurate gene expression and allergen analysis in *Scylla* species. In addition, the findings can help clinicians in managing crab allergic patients and the food industry for producing hypoallergenic crab products.

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Author contributions RM and PBK conceived, designed research, analyzed data and edited manuscript. NFHA performed the experiments, analyzed data and wrote the manuscript. ZHMY edited manuscript. All authors read, commented on, and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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