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RESEARCH

Validation of suitable reference genes for gene expression studies in rugose spiraling whitefly (*Aleurodicus rugioperculatus* Martin) under various experimental conditions

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Abstract

The rugose spiraling whitefly (*Aleurodicus rugioperculatus* Martin) is an invasive pest of several economically important crops, including coconut. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a fundamental and widely used technique in genomic research, particularly for analyzing gene expression levels with high sensitivity and specificity. One of the most critical factors influencing the accuracy and reliability of qRT-PCR results is the choice of internal reference genes, also known as housekeeping genes. These genes are used to normalize the expression levels of target genes. However, the lack of studies validating reference genes in *A. rugioperculatus* significantly restricts the accurate application of qRT-PCR for gene expression analysis. This study aims to identify suitable reference gene(s) across different biotic and abiotic conditions in *A. rugioperculatus*. The expression stability of 14 reference genes (*18S rRNA*, *GST*, *HSP90*, *GAPDH*, *RPS17*, *actin*, *RPL13*, *NADH*, *ETF-QO*, *EF1α*, *UBQ*, β-*TUB*, *peptidylprolyl isomerase A*, and *Myosin L*) was analyzed using five different computational programs, such as geNorm, NormFinder, BestKeeper, Comparative ΔCT, and RefFinder. The findings suggested that the optimal combinations of reference genes in *A. rugioperculatus* were *18S rRNA* and *NADH* for different developmental stages, *18S rRNA* and *EF1α* for different sexes and temperatures, and *RPL13* and *18S rRNA* for starvation conditions. This study presents the first report of stable reference genes for normalizing qRT-PCR analysis in *A. rugioperculatus,* laying the foundation for future expression studies and RNAi-based management of the rugose spiraling whitefly.

Keywords *Aleurodicus rugioperculatus* · Reference gene · QRT-PCR · Gene expression

Introduction

Aleurodicus rugioperculatus Martin (Hemiptera: Aleyrodidae), the rugose spiralling whitefly, is a sap-sucking pest of significant economic concern, particularly to coconut (*Cocos nucifera* L.) cultivation (Josephrajkumar et al. 2023; Rajesh et al. 2025). Since its initial reports in Central America and Florida (Martin 2004; Stocks and Hodges 2012), *A. rugioperculatus* has expanded its range, notably establishing in Southern India and spreading to multiple states (Shanas et al. 2016; Selvaraj et al. 2017; Josephrajkumar et al. 2023). It exhibits a broad host range, infesting crops such as banana, mango, and cashew, as well as ornamental plants (Sundararaj et al. 2021; Khan 2022). This expansion, fueled by favorable climates and host availability, poses a persistent threat to agroecosystems (Rahman et al. 2025; Sujithra et al. 2025). The pest's feeding causes honeydew secretion and sooty mould growth, impairing photosynthesis (Josephrajkumar et al. 2020, 2023), and a recent genome assembly (Rajesh et al. 2025) provides a foundation for molecular studies.

Quantitative real-time PCR (qRT-PCR) is a cornerstone technique for sensitive gene expression analysis in insects

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(Wang et al. 2024a, b; Yang et al. 2025). Its reliability, however, hinges on normalization using stably expressed reference genes to control for technical variation (Bustin et al. 2009; Andersen et al. 2004). While genes like those encoding ribosomal proteins (e.g., Rp18S, RPL13A) and elongation factors (e.g., *RpEF-1*) are commonly used in Hemiptera (Majerowicz et al. 2011; Wang et al. 2019), their expression stability is not universal. Extensive evidence indicates that no single reference gene is stable across all experimental conditions, including different developmental stages, sexes, or environmental stresses (Wang et al. 2023). Therefore, condition-specific validation is imperative for accurate qRT-PCR normalization (Lee et al. 2025). For *A. rugioperculatus*—a pest encountering diverse biotic and abiotic pressures in the field—this validation is critically needed but currently lacking, limiting the precision of molecular studies aimed at understanding its biology and control.

The stability of candidate reference genes is typically assessed using algorithms like geNorm, NormFinder, BestKeeper, and RefFinder, which employ distinct statistical approaches (Vandesompele et al. 2002; Andersen et al. 2004; Pfaffl et al. 2004; Xie et al. 2023). Given that different methods can yield varying rankings, a multi-algorithm strategy is recommended for robust identification of optimal reference genes (Kozera and Rapacz 2013).

To address this gap, the present study establishes a validated framework for gene expression analysis in *A. rugioperculatus*. We systematically evaluated 14 candidate reference genes across key experimental conditions: biotic factors (developmental stages and sex) and abiotic factors (temperature variation and starvation stress). Using a comprehensive, multi-algorithm validation approach, this study aims to identify the most stable reference genes for reliable qRT-PCR normalization across specific experimental conditions in A. rugioperculatus. Also, we have provided a ranked set of reference genes suitable for use in future gene expression studies targeting this invasive pest across diverse physiological and environmental contexts.

Materials and methods

Insect rearing and sample collection

Adult *A. rugioperculatus* specimens were collected from leaves of Chowghat Orange Dwarf (COD), a susceptible coconut cultivar, from the Experimental Farm at ICAR-CPCRI, Kasaragod, Kerala, India (12°30' N, 75°58' E). The colony was maintained on potted *Canna indica* (Cannaceae) plants under greenhouse conditions, enclosed in muslin cloth to prevent escape of the insects (Sujithra et al. 2021).

For experimental treatments, samples were collected as follows:

(i) *Developmental stages*: Eggs, nymphs, and adults were carefully isolated from infested coconut seedlings under a stereo zoom microscope (SMZ800N; Nikon). Three biological replicates of each stage, comprising around 100 eggs, 40–50 nymphs, and 20–30 adults, were collected in 1.5 ml centrifuge tubes as a single replication.
(ii) *Sex differentiation*: Males and females were distinguished morphologically- males exhibit pincer-like abdominal structures, while females have broader abdomens. Three biological replicates were selected, with 20–30 male and female adults collected in each.
(iii) *Starvation stress*: Adults were aspirated from coconut leaf abaxial surfaces and starved for 6 h in Falcon tubes without food.
(iv) *Temperature stress*: Adults were exposed to 4 °C, 26 °C, or 36 °C for 1 h in climatic chambers before snap-freezing. For temperature and starvation stress, each sample had three biological replicates, with 15–20 adults per replicate.

All samples were stored at − 80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Scientific), and its quality and quantity were assessed using a microfluidic UV/VIS spectrophotometer (QIAxpert, QIAGEN). First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara Bio) following the manufacturer's protocols.

Reference gene selection and primer validation

Fourteen candidate reference genes were selected based on their use in whiteflies and other Hemipterans (Qiu et al. 2025; Kong et al. 2024; Kaur et al. 2019; Su et al. 2013; Li et al. 2013). These included 18s ribosomal RNA (*18S rRNA*), glutathione S-transferase (*GST*), heat shock proteins (*HSP90*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), 28S ribosomal protein S17 mitochondrial (*RPS17*), actin, 60S ribosomal protein L13 (*RPL13*), NADH dehydrogenase *(NADH*), electron transfer flavoprotein-ubiquinone oxidoreductase (*ETF-QO*), elongation factor 1 alpha (*EF-1α*), beta-1-tubulin (*β-TUB*), peptidylprolyl isomerase A and light chain myosin L (*Myosin L*). Gene sequences were retrieved from the *A. rugioperculatus* transcriptome (NCBI SRA accession number PRJNA1234814), and primers were designed using Primer3 (Untergasser et al. 2012). All the primer sequences were listed in Table 1.

Table 1 Amplification efficiencies and correlation coefficients of the 14 candidate genes studied

Gene name	Primer sequence (5'−3')	NCBI accession number	Amplification efficiency (%)	Amplification factor	R2 value
18S ribosomal RNA (*18S rRNA*)	F: ACTCCATTGCACGTTCACAC R: CCGAGCTCCTCCAGAAATCT	PX307903	97.1	1.97	0.990
Glutathione S-transferase (*GST*)	F: AGGGCGGCTCTTAAATCTGA R: GATGAAACCAACCGTGCCTT	PX314493	96.3	1.96	0.998
Heat shock proteins (*HSP90*)	F: CGCTGACATTTCTCCTGCTC R: TTGCCAAAATGCCCCAAGAG	PX314494	101.2	2.01	0.998
Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)	F: GAGCAGCACCTCTACCATCA R: GCCTACGACCCATCCTACAA	PX314495	99.1	1.99	0.998
28S ribosomal protein S17 (*RPS17*)	F: CTCTTGCCATCCACGTTCTG R: AGCAGGAGTAAAGCGAGGAG	PX314496	102.2	2.02	0.999
Actin	F: CTCATGCCATCCTCCGTTTG R: GATTTCTCGCTCAGCTGTGG	PX314497	104.5	2.04	0.994
60S ribosomal protein L13 (*RPL13*)	F: CCTCGGTAGCTTCTCCCTTC R: TGAACTCAAGGCTGCAGGTA	PX314498	100	2.00	0.996
NADH dehydrogenase (*NADH*)	F: GAAGGTTCTTGCCAAGCGAA R: TTGGTCCTGATCCTGTTCCG	PX314499	98.2	1.98	0.995
Electron transfer flavoprotein-ubiquinone oxidoreductase (*ETF-QO*)	F: GTGTGCTCAACTGTTCCTGG R: TGTCACGGTCATCTCACGAA	PX314500	104.7	2.05	0.998
Elongation factor 1 alpha (*EF-1α*)	F: GCCTCTGGTAAAGCTTCGTG R: GACAAGCCCCTTCGTTTACC	PX314501	104.5	2.04	0.990
Peptidylprolyl isomerase (*PPI*)	F: GCAACTCCTTGATCCCAACC R: TATCGAGCAAGGAGACGGAC	PX314502	101.7	2.02	0.995
Ubiquitin-like protein (*UBQ*)	F: ACCAAAGGTCTGTACGGTGT R: TGTGACCGTTCCATTGCTTG	PX314503	100.2	2.00	0.999
Beta-1-tubulin (*β-TUB)*	F: TCCCTGGTCAGTTAAACGCT R: CTGCCACGGGATGTTAATGG	PX314504	100.9	2.01	0.997
Light chain myosin L (*Myosin L*)	F: CCTTAAATGCGGCCATGACA R: AAGAGGCTCCAGGTCCAATC	PX314505	99.8	1.99	0.995

PCR amplification was performed in 20 µL reactions containing 50 ng cDNA, 0.3 U *Taq* DNA polymerase (Genei Laboratories), and gene-specific primers. Cycling conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min; final extension at 72 °C for 10 min. PCR products were analyzed using a MultiNA microchip electrophoresis system with the DNA 500 kit (MCE-202 MultiNA, Shimadzu). Electropherogram peak sizes were automatically detected using the system's integrated software tools, MultiNA Control and MultiNA Viewer (Shimadzu). Amplicons were gel-purified using the NucleoSpin Kit (Macherey–Nagel) and sequenced at BioKart India Pvt. Ltd. (India).

qRT-PCR Analysis

The qRT-PCR experiment was performed on a One-Step Real-Time PCR System (Applied Biosystems) using Power SYBR™ Green PCR Master Mix (Applied Biosystems). Each reaction included 2 µL of diluted cDNA product, 1 µL of each forward and reverse primer (2 µM), 5 µL of SYBR Green (2X), and 2 µL of molecular biology-grade water. The reaction conditions were 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s, and 72 °C for 30 s. After that, the machine would perform a melting-curve analysis. To determine the amplification efficiencies for each primer, a standard curve was constructed for each primer pair using a fivefold serial dilution of cDNA (1:1, 1:5, 1:25, 1:125, and 1:625). The amplification efficiency (E) values of each primer were calculated using the equation, $E = (10\,[-1/\text{slope}] - 1) \times 100$.

Data analysis

The expression stability of the reference genes was estimated using four web-based algorithms: geNorm, NormFinder, BestKeeper, and the comparative delta-Ct method, followed by a comprehensive analysis using RefFinder.

geNorm computes the average pairwise variation of each target's expression relative to all other possible reference genes and generates an M value as an expression stability metric for each target. The most stable gene has the lowest M value (Vandesompele et al. 2002). Pairwise variation (V) calculated by geNorm was used to determine the optimal number of reference genes for accurate qRT-PCR normalization. The Vn/Vn+1 value represented the variation between two consecutive normalization factors, providing a condition for determining the minimal number of genes necessary to achieve stable expression normalization. NormFinder ranks the reference gene by estimating intra- and inter-group expression variation (Andersen et al. 2004). BestKeeper ranks the reference gene based on the standard deviations and coefficient of variance of expression, using mean Ct values (Pfaffl et al. 2004). The ΔCT method directly estimates the relative expression of gene pairs within each sample. The three computational programs mentioned above — geNorm, Normfinder, and BestKeeper, as well as the ΔCT method — are combined in RefFinder, and the evaluated candidate genes are reranked using the geometric mean of the weights assigned to each candidate by each program (Xie et al. 2023). The graphs were plotted using Origin 2025 (OriginLab, Northampton, MA, USA).

Validation of the reference gene

To confirm the stability of the selected reference gene, the expression profile of the whitefly aquaporin (AQP) gene was assessed for validation. In whiteflies, aquaporin plays a crucial role in the transport of water and small solutes across cell membranes and also in excretion and honeydew production (Mathew et al. 2011**)**. Because aquaporins keep cells hydrated, they may help whiteflies to withstand heat or desiccation stress. Here, the expression levels of *AQP* are evaluated at different developmental stages, under various temperatures, and during starvation stress. qRT-PCR was performed using the two most stable and two least stable reference genes. *AQP* gene sequence was obtained from the whitefly transcriptome data (NCBI SRA accession number PRJNA1234814**)**. Primer sequences of *AQP* were forward 5'-CTTGCGGGATTCATA CTGGC-3' and reverse 5'- GTTGCGATTGAAGCCCTGA T-3'. The relative expression levels of *AQP* in different conditions were calculated using the 2-ΔΔCt method (Livak and Schmittgen 2001). All the experiments were performed with three biological replicates with three technical triplicates. Relative expression levels of the target gene AQP across diverse experimental conditions were analyzed using one-way analysis of variance (ANOVA), followed by the Fisher LSD paired-comparison test in Origin 2025 (OriginLab, Northampton, MA, USA), and the data were plotted.

Results

Selection of candidate reference genes, qPCR amplification, and primer specificity

A total of 14 reference genes (*18S*, *GST*, *HSP90*, *GAPDH*, *RPS17*, *ACT*, *RPL13*, *NADH*, *ETF-QO*, *EF-1α*, *PPI*, *UBQ*, *β-TUB,* and *Myosin L*) were selected for this study, and the specificity of primer pairs of these genes was determined by conventional PCR. A single amplicon of expected size was observed by running the PCR product on the MultiNA microchip electrophoresis system (Fig. 1B) and by Sanger's sequencing of the PCR product to confirm the sequence. Melt curve from qRT-PCR analysis also revealed a single peak for all primers, which also confirms the specificity of the primers (Fig. 1A). The nucleotide sequences of all the candidate reference genes were submitted to the GenBank database (Table 1).

Amplification efficiencies (E%) and correlation coefficients (R^2) of the primers were calculated from the slopes of standard curves generated from five fold serial dilutions of cDNA. The primer amplification efficiency ranged from 96.3% to 104.7%, and the correlation coefficients ranged from 0.990 to 0.999 (Table 1). These results indicate that all the primers meet the qRT-PCR standards.

Expression profile of the reference gene

An overview of the differences in gene expression among the samples was obtained by observing the Ct values in qRT-PCR, where a low Ct value indicated a high gene expression level and vice versa. The Ct values in all samples, including biotic and abiotic conditions, were detected by qRT-PCR. The average Ct value ranged from 14.94 for *ACT* to 24.92 for *18S* (Fig. 2). Among these, *ACT* (14.94), *EF-1α* (18.542), and *Myosin L* (18.66) were the most abundant with the lowest Ct value, whereas *18S* (24.92) and *HSP90* (24.06) were the least abundant. Among all samples, *HSP90* and *RPS17* exhibited the lowest expression variability, with an SD of 0.6, while *ACT* showed the highest variation (SD = 1.93).

Stability of candidate reference gene expression

The expression stability of the 14 candidate reference genes in *A. rugioperculatus* over four different conditions (developmental stage, different sex, temperature, and starvation stress) was analyzed using the software programs geNorm, BestKeeper, NormFinder, ΔCT, and RefFinder. Based on Ct values, these algorithms determine an expression stability measure and rank each gene from least to most stable.

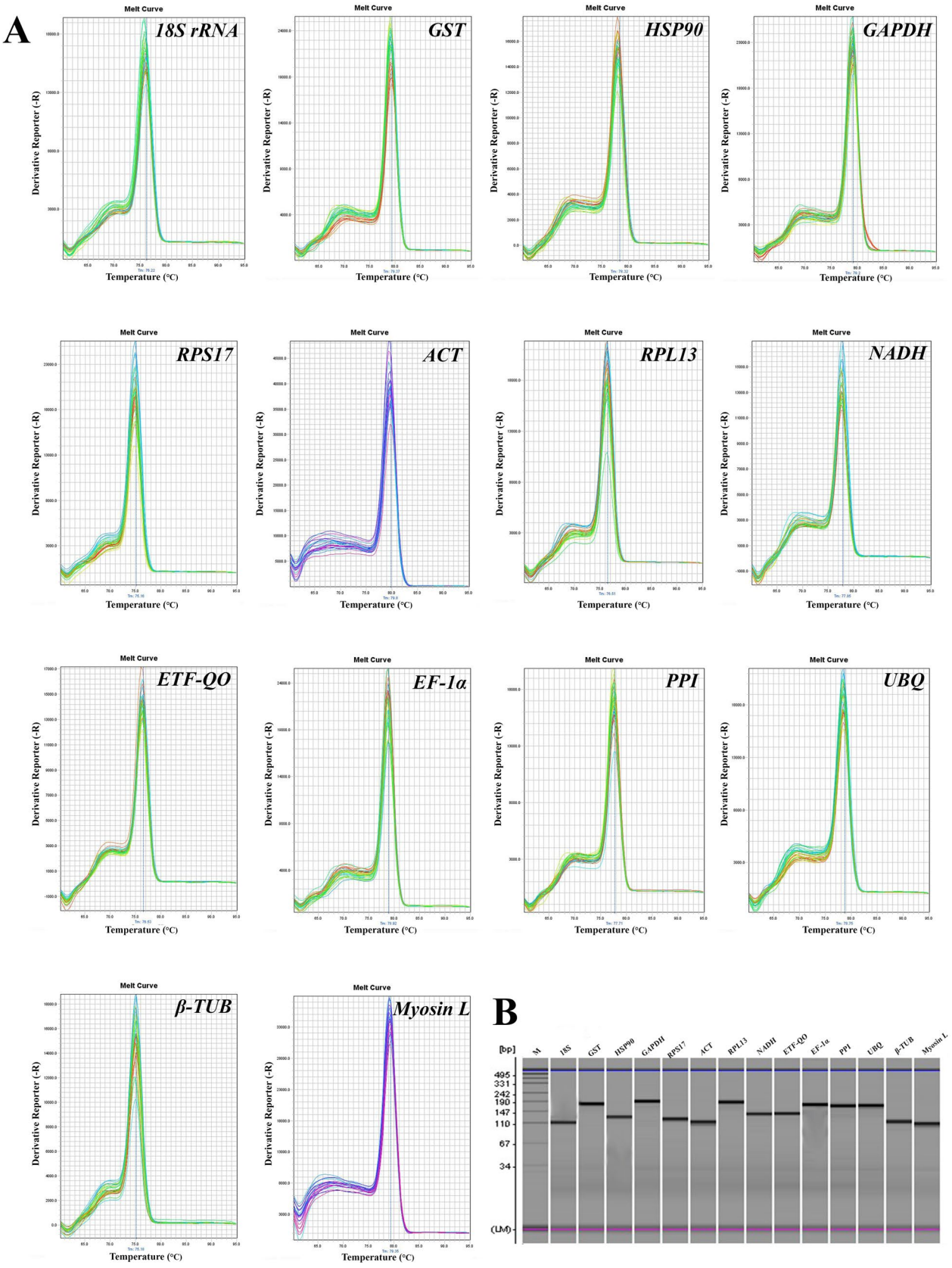

Fig. 1 Primer specificity of 14 candidate reference gene qRT-PCR and RT-PCR. A Melt curve analysis of 14 candidate genes. B Automated gel electrophoresis results of PCR products

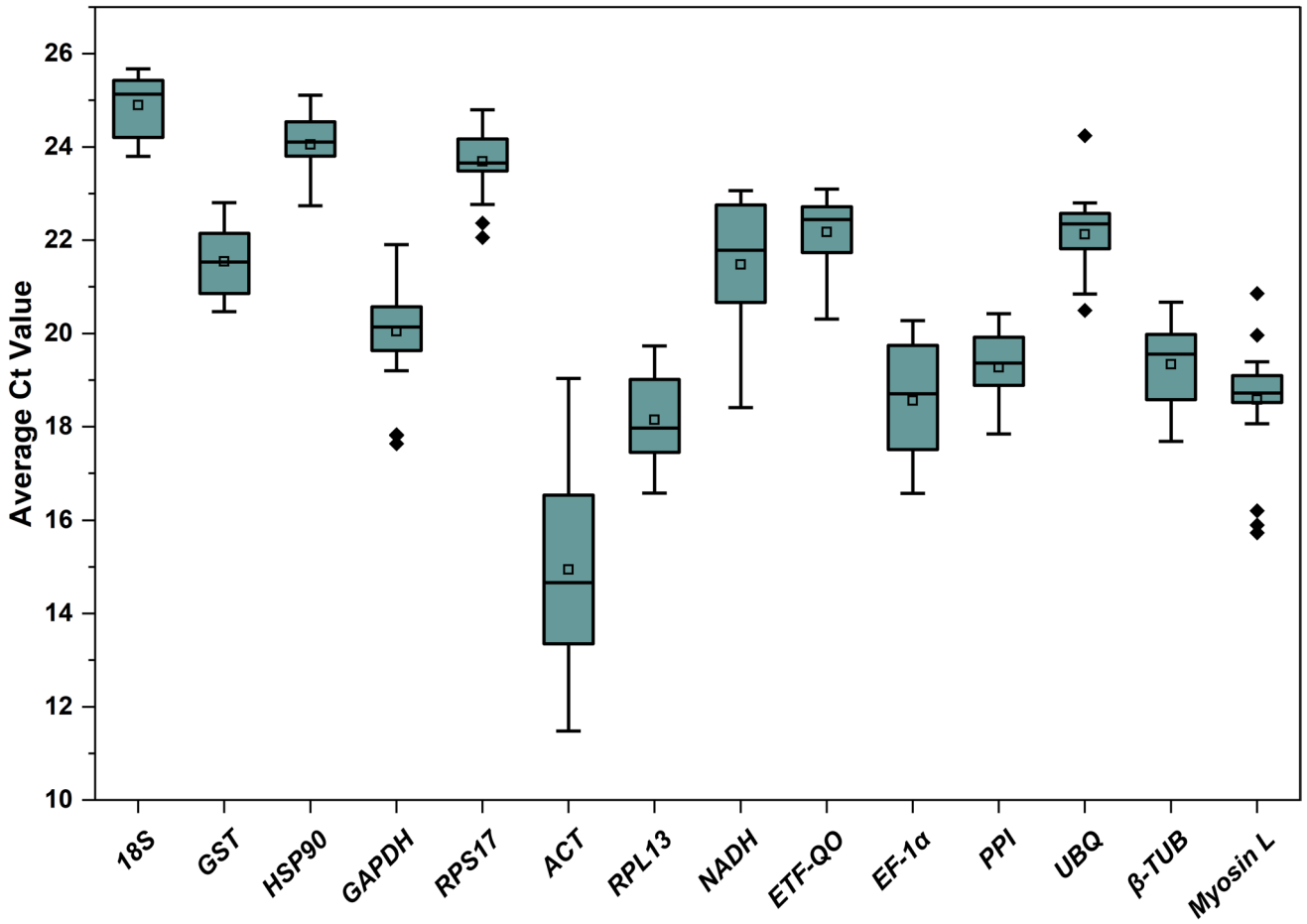

Fig. 2 Average Ct values of 14 candidate reference genes. Each box represents 25% of the first quartile and 75% of the third quartile. The line inside the box indicates the median values, and dots denote outliers

geNorm analysis

The geNorm tool calculates the gene expression stability value, also known as the 'M' value, of the candidate reference gene. The most stable genes have the lowest M value. This algorithm also measures the Vn/n+1 value; a value of less than 0.15 is the criterion for choosing the optimal number of reference genes. The reference gene in all conditions shows an M value of less than 1.5, indicating that the expression levels of all genes were comparatively stable for qRT-PCR analysis. In developmental stages, *PPI* and *18S* were found to be the most stable genes, with an M value of 0.31, while *Myosin L* ($M=0.77$) was the least stable. By considering different sexes, *PPI* and *GST* ($M=0.27$) were the most stable, whereas *ACT* had the highest M value of 1.17 and was the least stable. *Myosin L* and *HSP90* ($M=0.06$) were ranked as the most stably expressed genes when starving for a period of 6 h, and GAPDH ($M=0.46$) was found to be the least stable. Under temperature-induced stress, *18S* and *ETF-QO* were the most stable genes, with M values of 0.14 and 0.16, respectively, while the least stable gene was *ACT* (0.33). By considering all these conditions, *PPI* and *18S* ($M=0.42$) followed by *GST* ($M=0.45$) were identified as the most stable genes, whereas ACT ($M=1.01$) was the least stable. So, among 14 reference genes, the most stable gene slightly varied between treatments and is shown in Fig. 3, which plots the least stable to the two most stable genes (left to right).

The geNorm also calculates the pairwise variation, or V value. In our study, the V2/3 value of all experimental conditions was found to be less than 0.15, suggesting that only the two reference genes are sufficient for normalization studies of gene expression in *A. rugioperculatus* (Fig. 4).

NormFinder analysis

The NormFinder algorithm was used to compare intra- and inter-group variances and measure the stability value (S) for each candidate reference gene; genes with the lowest stability value are considered to possess greater stability. As

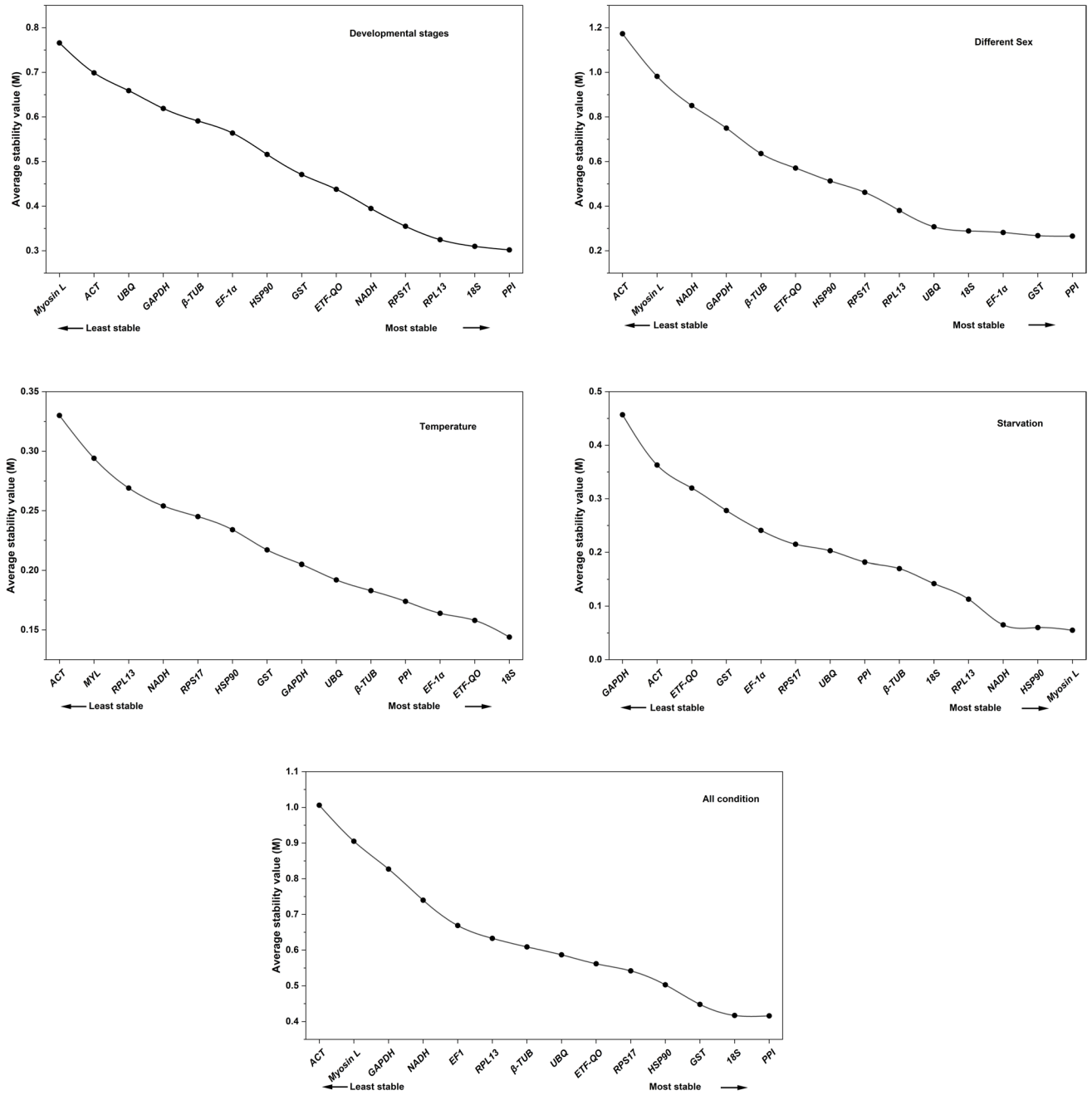

Fig. 3 Stability values (M) and rankings of 14 candidate reference genes analyzed by geNorm

shown in Fig. 5, under temperature and starvation stress, *18S rRNA* was found to be the most stably expressed. *NADH* and *RPS17* were the most stable genes for the developmental stages and different sexes, respectively. Based on NormFinder analysis, *ACT* showed the least stable expression in most experimental conditions, including those with different sexes and temperature stress. Under starvation stress, *GAPDH,* with a stability value of 0.69, was the least stable, and *Myosin L* showed highly variable expression across developmental stages.

BestKeeper analysis

The BestKeeper algorithm evaluates the stability of reference genes by analyzing several statistical parameters, including the standard deviation (SD), coefficient of variation (CV), Pearson correlation coefficient (CC), and the associated *p*-value, all derived from the Ct (cycle threshold) values of qPCR data. In this method, genes with the lowest SD and CV are considered the most stable and suitable as reference genes for normalization. Based on the

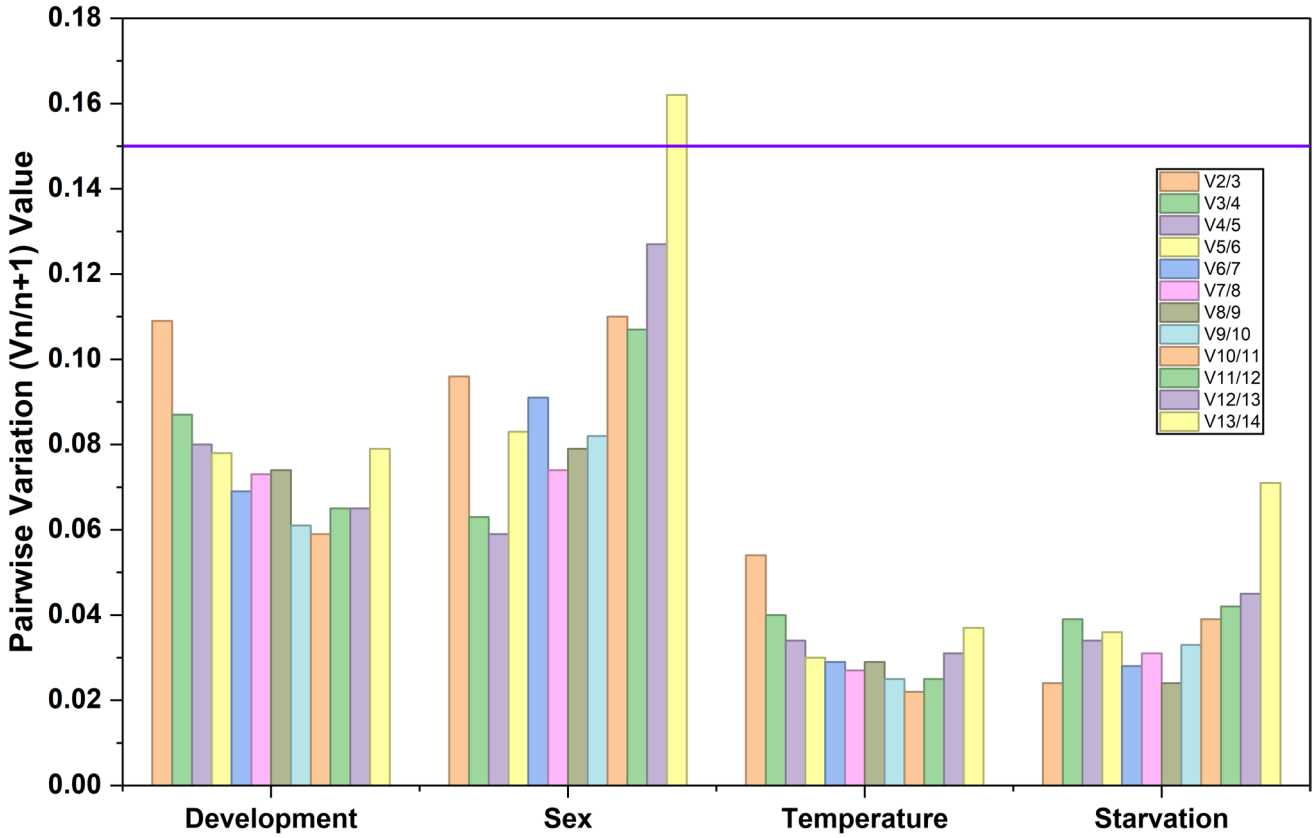

Fig. 4 The pairwise variations (Vn/n + 1) value of reference genes by the geNorm algorithm. The Vn/n + 1 value < 0.15 is a standard value that is marked with a line, showing that no more genes are required for normalization

analysis across all experimental conditions, the *RPS17* gene was identified as the most stable reference gene, followed by *HSP90*. Specifically, during the developmental stages, *RPS17* also demonstrated the lowest SD and CV, reaffirming its stability. When analyzing sex-related expression differences, *HSP90* was found to be the most stable gene among the examined genes. Under temperature stress conditions, *Myosin L* showed the highest stability, while *EF-1α* was identified as the most reliable reference gene during starvation stress (Fig. 6).

Delta Ct analysis

In the ΔCt method, the mean standard deviation is used to assess the variability of ΔCt values for a specific reference gene across various experimental conditions. A reference gene with the lowest mean standard deviation is considered the most stable, as it exhibits minimal expression fluctuation across the tested conditions. The reference gene *18S rRNA* showed the lowest average standard deviation across various conditions, including developmental stages, sexes, temperature stress, and overall combined conditions, suggesting that *18S rRNA* is the most stable gene under these circumstances. In contrast, *RPL13* demonstrated the highest stability, as reflected by the lowest average standard deviation, specifically under starvation stress.

RefFinder analysis

RefFinder, an integrated tool that combines the results of four different algorithms (geNorm, NormFinder, BestKeeper, and ΔCt), was used to determine the most appropriate reference genes for qRT-PCR normalization. The final rankings of the candidate reference genes are presented in Table 2. The results from RefFinder were largely consistent with those obtained using the ΔCt method. Based on the results of the RefFinder analysis, *18S rRNA* was consistently identified as the most stable reference gene across all treatments except for starvation stress. However, the ranking of the second most stable gene showed slight variations, as detailed in Table 2. Under starvation stress conditions, *RPL13* and *18S rRNA* emerged as the most stable reference genes. These results highlight that specific experimental conditions can influence the stability of reference genes.

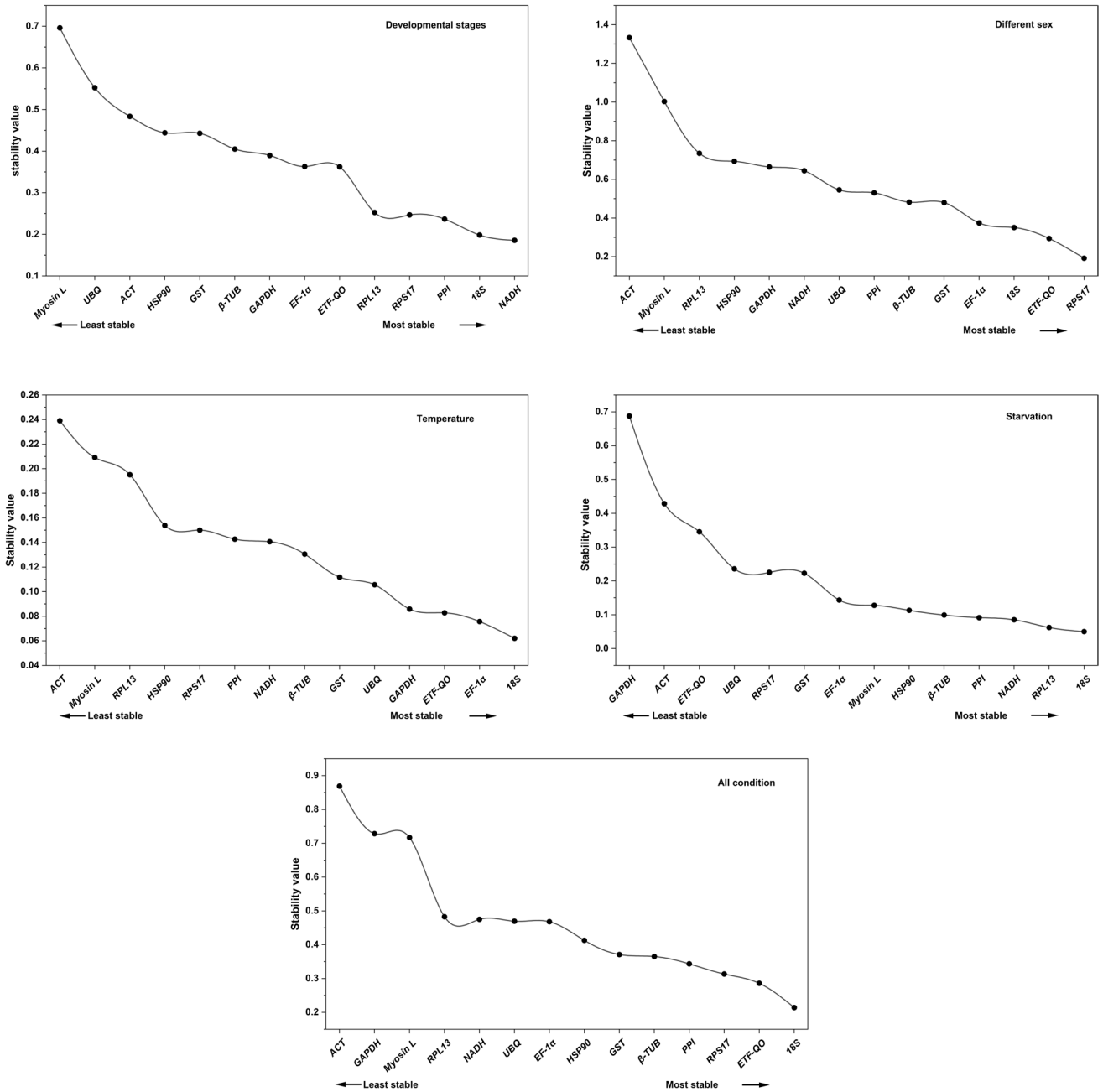

Fig. 5 Stability values and rankings of 14 candidate reference genes analyzed by NormFinder

Validation of the best and least stable reference genes

To validate the consistency of the selected reference genes, the relative expression level of the *AQP* gene was normalized using both the two most stable and the two least stable reference genes individually and in combination. The expression analysis was conducted under the same experimental conditions that were used to assess the stability of the candidate reference genes. During developmental stage analysis, similar *AQP* expression levels were observed when normalized with the most stable reference genes, *18S rRNA* and *NADH*, either individually or in combination (*18S+NADH*). In contrast, normalization using the least stable genes, *UBQ* and *MyosinL*, resulted in noticeable differences in *AQP* expression (Fig. 7A), indicating their unreliability as reference genes under these conditions. When analyzing different sexes, *AQP* expression was higher in adult males compared to females when normalized with *18S rRNA* and *EF-1α*, either separately or together (*18S+EF-1α*). However, normalization with *MyosinL* and *ACT* caused significant fluctuations in *AQP* expression (Fig. 7B). Similarly, as shown in Fig. 7C & D, under abiotic stress conditions, viz., temperature variation and starvation,

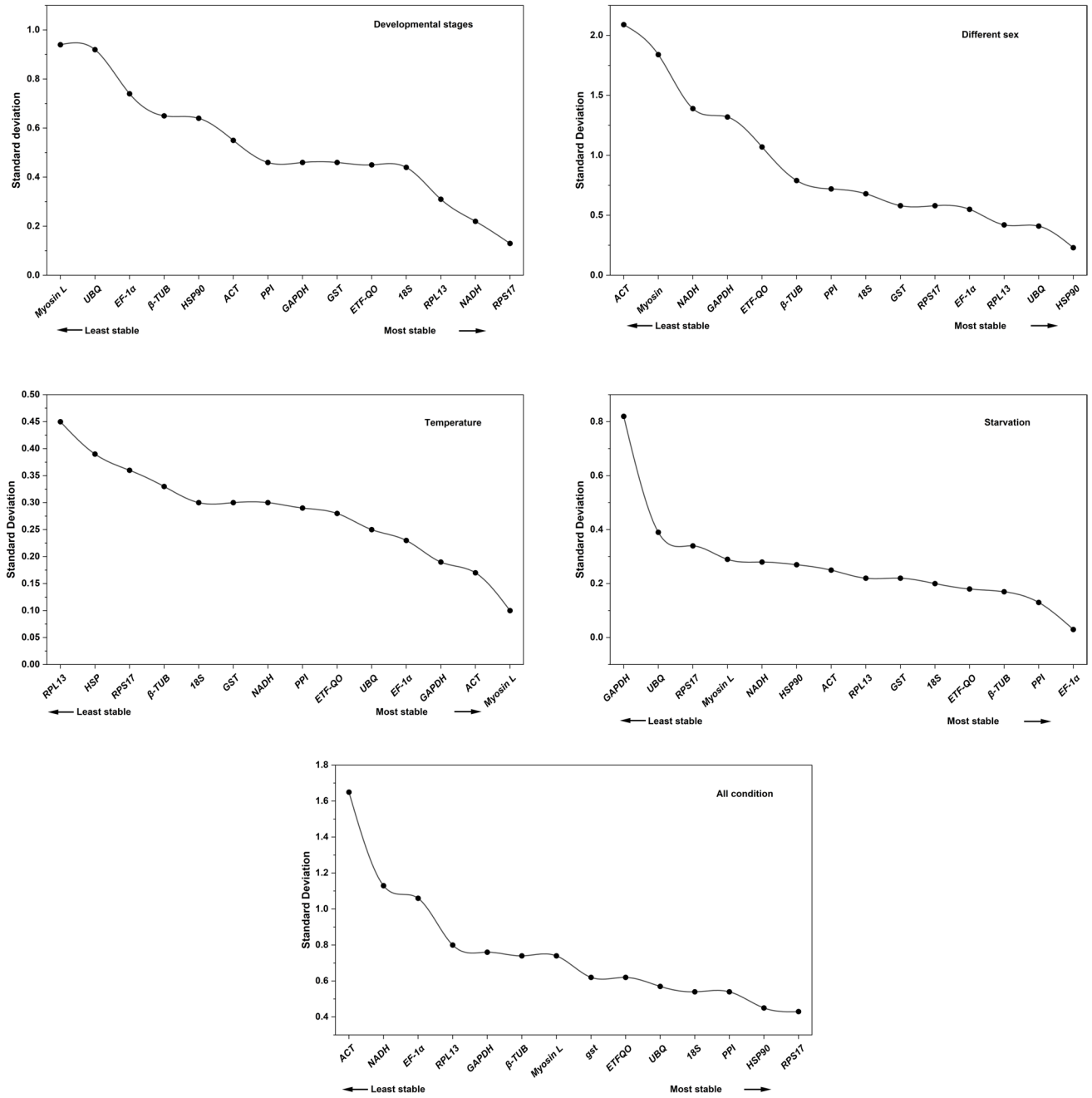

Fig. 6 BestKeeper-based standard deviation of 14 candidate reference genes across all experimental conditions

normalization with the most stable reference genes (individually and in combination) produced consistent expression patterns of *AQP*. In contrast, normalization using the least stable reference genes resulted in significant variations and inconsistencies. These findings suggest that using a single stable reference gene may be sufficient for reliable gene expression analysis. Notably, the consistent performance of *18S rRNA* across all tested conditions namely, developmental stages, sex, and stress treatments demonstrates its suitability as a reliable reference gene for normalization in *A. rugioperculatus*.

Discussion

Quantitative real-time PCR (qRT-PCR) is a highly sensitive and specific method fundamental to gene expression studies. Its reliability, however, hinges on the use of appropriately validated reference genes that exhibit uniform expression regardless of experimental treatment (Guenin et al. 2009; Lü et al. 2018). The selection of unstable reference genes is a major source of error, potentially masking true expression differences or introducing false positives in the analysis of

Table 2 Gene expression stability under different conditions ranked by geNorm, NormFinder, BestKeeper, Δct and RefFinder

Condition	geNorm		NormFinder		Bestkeeper		ΔCt		RefFinder		Rank	
	Gene	Stability	Gene	Stability	Gene	SD	CV	Gene	Stability	Gene	Comprehensive ranking values	
Development	*PPI*	0.30	*NADH*	0.19	*RPS17*	0.13	0.57	*18S*	0.61	*18S*	1.68	1
	18S	0.31	*18S*	0.2	*NADH*	0.22	1.07	*PPI*	0.61	*NADH*	2.34	2
	RPL13	0.33	*PPI*	0.24	*RPL13*	0.31	1.77	*NADH*	0.61	*PPI*	2.45	3
	RPS17	0.35	*RPS17*	0.25	*18S*	0.44	1.78	*RPS17*	0.64	*RPS17*	2.83	4
	NADH	0.39	*RPL13*	0.25	*ETF-QO*	0.45	2.05	*RPL13*	0.64	*RPL13*	3.87	5
	ETF-QO	0.44	*ETF-QO*	0.36	*GST*	0.46	2.19	*ETF-QO*	0.73	*ETF-QO*	5.73	6
	GST	0.47	*EF-1α*	0.36	*GAPDH*	0.46	2.23	*GAPDH*	0.74	*GAPDH*	8.1	7
	HSP90	0.52	*GAPDH*	0.39	*PPI*	0.46	2.46	*β-TUB*	0.75	*GST*	8.37	8
	EF-1α	0.56	*β-TUB*	0.4	*ACT*	0.55	2.69	*EF-1α*	0.76	*β-TUB*	9.16	9
	β-TUB	0.59	*GST*	0.44	*HSP90*	0.64	3.48	*GST*	0.79	*EF-1α*	9.67	10
	GAPDH	0.62	*HSP90*	0.44	*β-TUB*	0.65	4.01	*HSP90*	0.85	*HSP90*	9.92	11
	UBQ	0.66	*ACT*	0.48	*EF-1α*	0.74	4.19	*ACT*	0.89	*ACT*	11.39	12
	ACT	0.70	*UBQ*	0.55	*UBQ*	0.92	4.20	*UBQ*	0.96	*UBQ*	12.74	13
	Myosin L	0.77	*Myosin L*	0.7	*Myosin L*	0.94	4.89	*Myosin L*	1.17	*Myosin L*	14	14
Sex	*PPI*	0.27	*RPS17*	0.19	*HSP90*	0.23	0.97	*18S*	0.86	*18S*	3.03	1
	GST	0.27	*ETF-QO*	0.29	*UBQ*	0.41	1.86	*EF-1α*	0.89	*EF-1α*	3.13	2
	EF-1α	0.28	*18S*	0.35	*RPL13*	0.42	2.38	*RPS17*	0.90	*RPS17*	3.20	3
	18S	0.29	*EF-1α*	0.37	*EF-1α*	0.55	2.51	*GST*	0.91	*GST*	3.31	4
	UBQ	0.31	*GST*	0.48	*RPS17*	0.58	2.73	*PPI*	0.94	*PPI*	3.94	5
	RPL13	0.38	*β-TUB*	0.48	*GST*	0.58	2.74	*ETF-QO*	0.97	*UBQ*	4.7	6
	RPS17	0.46	*PPI*	0.53	*18S*	0.68	3.08	*UBQ*	0.97	*HSP90*	5.03	7
	HSP90	0.51	*UBQ*	0.55	*PPI*	0.72	3.72	*HSP90*	1.14	*ETF-QO*	5.73	8
	ETF-QO	0.57	*NADH*	0.64	*β-TUB*	0.79	4.12	*β-TUB*	1.15	*RPL13*	6.82	9
	β-TUB	0.64	*GAPDH*	0.66	*ETF-QO*	1.07	5.00	*RPL13*	1.22	*β-TUB*	8.97	10
	GAPDH	0.75	*HSP90*	0.69	*GAPDH*	1.32	6.71	*GAPDH*	1.22	*GAPDH*	10.46	11
	NADH	0.85	*RPL13*	0.74	*NADH*	1.39	6.93	*NADH*	1.29	*NADH*	11.74	12
	Myosin L	0.98	*Myosin L*	1.00	*Myosin L*	1.84	10.33	*Myosin L*	1.66	*Myosin L*	13	13
	ACT	1.17	*ACT*	1.33	*ACT*	2.09	15.21	*ACT*	2.32	*ACT*	14	14
Temperature	*18S*	0.14	*18S*	0.06	*Myosin L*	0.10	0.54	*18S*	0.25	*18S*	1.68	1
	ETF-QO	0.16	*EF-1α*	0.08	*ACT*	0.17	1.04	*EF-1α*	0.27	*EF-1α*	2.91	2
	EF-1α	0.16	*ETF-QO*	0.08	*GAPDH*	0.19	0.94	*ETF-QO*	0.28	*ETF-QO*	2.91	3
	PPI	0.17	*GAPDH*	0.09	*EF-1α*	0.23	1.18	*GAPDH*	0.28	*GAPDH*	3.60	4
	β-TUB	0.18	*UBQ*	0.11	*UBQ*	0.25	1.13	*UBQ*	0.28	*UBQ*	5.23	5
	UBQ	0.19	*GST*	0.11	*ETF-QO*	0.28	1.24	*GST*	0.30	*PPI*	6.29	6
	GAPDH	0.21	*β-TUB*	0.13	*PPI*	0.29	1.45	*β-TUB*	0.31	*Myosin L*	6.85	7
	GST	0.22	*NADH*	0.14	*NADH*	0.30	1.34	*PPI*	0.32	*GST*	7.14	8
	HSP90	0.23	*PPI*	0.14	*GST*	0.30	1.35	*RPS17*	0.33	*β-TUB*	7.45	9
	RPS17	0.25	*RPS17*	0.15	*18S*	0.30	1.18	*NADH*	0.33	*ACT*	8.61	10
	NADH	0.25	*HSP90*	0.15	*TUB*	0.33	1.63	*HSP90*	0.33	*NADH*	9.97	11

Table 2 (continued)

Condition	geNorm		NormFinder		Bestkeeper		ΔCt		RefFinder		Rank	
	Gene	Stability	Gene	Stability	Gene	SD	CV	Gene	Stability	Gene	Comprehensive ranking values	
	RPL13	0.27	*RPL13*	0.20	*RPS17*	0.36	1.48	*RPL13*	0.39	*RPS17*	10.19	12
	Myosin L	0.29	*Myosin L*	0.21	*HSP90*	0.39	1.59	*Myosin L*	0.42	*HSP90*	10.91	13
	ACT	0.33	*ACT*	0.24	*RPL13*	0.45	2.36	*ACT*	0.55	*RPL13*	12.47	14
Starvation	*Myosin L*	0.06	*18S*	0.05	*EF-1α*	0.03	0.18	*RPL13*	0.33	*RPL13*	2.63	1
	HSP90	0.06	*RPL13*	0.06	*PPI*	0.13	0.67	*18S*	0.34	*18S*	2.66	2
	NADH	0.07	*NADH*	0.09	*β-TUB*	0.17	0.86	*β-TUB*	0.34	*PPI*	3.87	3
	RPL13	0.11	*PPI*	0.09	*ETF-QO*	0.18	0.81	*PPI*	0.34	*β-TUB*	4.05	4
	18S	0.14	*β-TUB*	0.10	*18S*	0.20	0.79	*HSP90*	0.35	*HSP90*	4.05	5
	β-TUB	0.17	*HSP90*	0.11	*GST*	0.22	1.06	*NADH*	0.35	*Myosin L*	4.82	6
	PPI	0.18	*Myosin*	0.13	*RPL13*	0.22	1.15	*Myosin L*	0.36	*NADH*	4.82	7
	UBQ	0.20	*EF-1α*	0.14	*ACT*	0.25	1.53	*EF-1α*	0.39	*EF-1α*	5.03	8
	RPS17	0.22	*GST*	0.22	*HSP90*	0.27	1.11	*RPS17*	0.43	*ETF-QO*	9.12	9
	EF-1α	0.24	*RPS17*	0.23	*NADH*	0.28	1.24	*UBQ*	0.44	*GST*	9.34	10
	GST	0.28	*UBQ*	0.24	*Myosin L*	0.29	1.58	*GST*	0.48	*RPS17*	9.93	11
	ETF-QO	0.32	*ETF-QO*	0.35	*RPS17*	0.34	1.41	*ETF-QO*	0.56	*UBQ*	10.34	12
	ACT	0.36	*ACT*	0.43	*UBQ*	0.39	1.75	*ACT*	0.66	*ACT*	11.51	13
	GAPDH	0.46	*GAPDH*	0.69	*GAPDH*	0.82	4.36	*GAPDH*	1.02	*GAPDH*	14	14

target genes (Udvardi et al. 2008; Kozera and Rapacz 2013). For the rugose spiralling whitefly, *Aleurodicus rugioperculatus*, a significant gap exists as no endogenous reference genes have been systematically identified or validated. To address this, the present work assesses the expression stability of a panel of candidate reference genes under a range of biologically relevant conditions—encompassing developmental stages, sex, and response to abiotic stress—to establish a validated framework for future gene expression research in this insect.

The standardization of reference genes is increasingly recognized as a critical prerequisite for ensuring the reliability and accuracy of quantitative real-time PCR (qRT-PCR) analyses in gene expression studies. Consequently, the identification of stable reference genes has been undertaken for a broad range of insect species under diverse experimental conditions (Wang et al. 2024a, b; Dalai and Jagota 2024). Within the Aleyrodidae family, comprehensive validation efforts have been focused on *Bemisia tabaci*, with multiple studies establishing suitable reference genes across its various biotypes and in response to numerous biotic and abiotic stressors (Kaur et al. 2019; Liu and Li 2019; Dai et al. 2017; Lü et al. 2017; Collins et al. 2014; Liang et al. 2014; Li et al. 2013; Su et al. 2013). More recently, suitable reference genes have also been identified for the citrus whitefly, *Dialeurodes citri* (Kong et al. 2024). Other well-characterized Hemipteran systems include the bean bug, *Riptortus pedestris* (Wang et al. 2023); aphids such as *Acyrthosiphon pisum* (Yang et al. 2014), *Lipaphis erysimi* (Koramutla et al. 2016), *Aphis craccivora* (Yang et al. 2015a), *Aphis gossypii* (Ma et al. 2016), *Myzus persicae* (Kang et al. 2017), *Toxoptera citricida* (Shang et al. 2015), and *Megoura viciae* (Cristiano et al. 2016); as well as the stink bug *Halyomorpha halys* (Bansal et al. 2016) and the mealybug *Phenacoccus solenopsis* (Arya et al. 2017). Collectively, these studies emphasize the importance of validating internal controls for each specific species and experimental condition to ensure the accuracy and reproducibility of quantitative real-time polymerase chain reaction (qRT-PCR) data.

Despite this progress, a comprehensive study to standardize reference genes has not been conducted for the rugose spiralling whitefly, *A. rugioperculatus*. To address this gap, the present study evaluates the expression stability of 14 candidate reference genes (*18S rRNA, GST, HSP90, GAPDH, RPS17, ACT, RPL13, NADH, ETF-QO, EF-1α, PPI, UBQ, β-TUB,* and *Myosin L*), selected for their prevalent use in insects. The genes were assessed across a range of biotic and abiotic conditions. To ensure robust and reliable identification of the most stable genes, we employed a suite of algorithmic tools geNorm, NormFinder, BestKeeper, the ΔCT method, and RefFinder which together provide a comprehensive framework for evaluating gene stability.

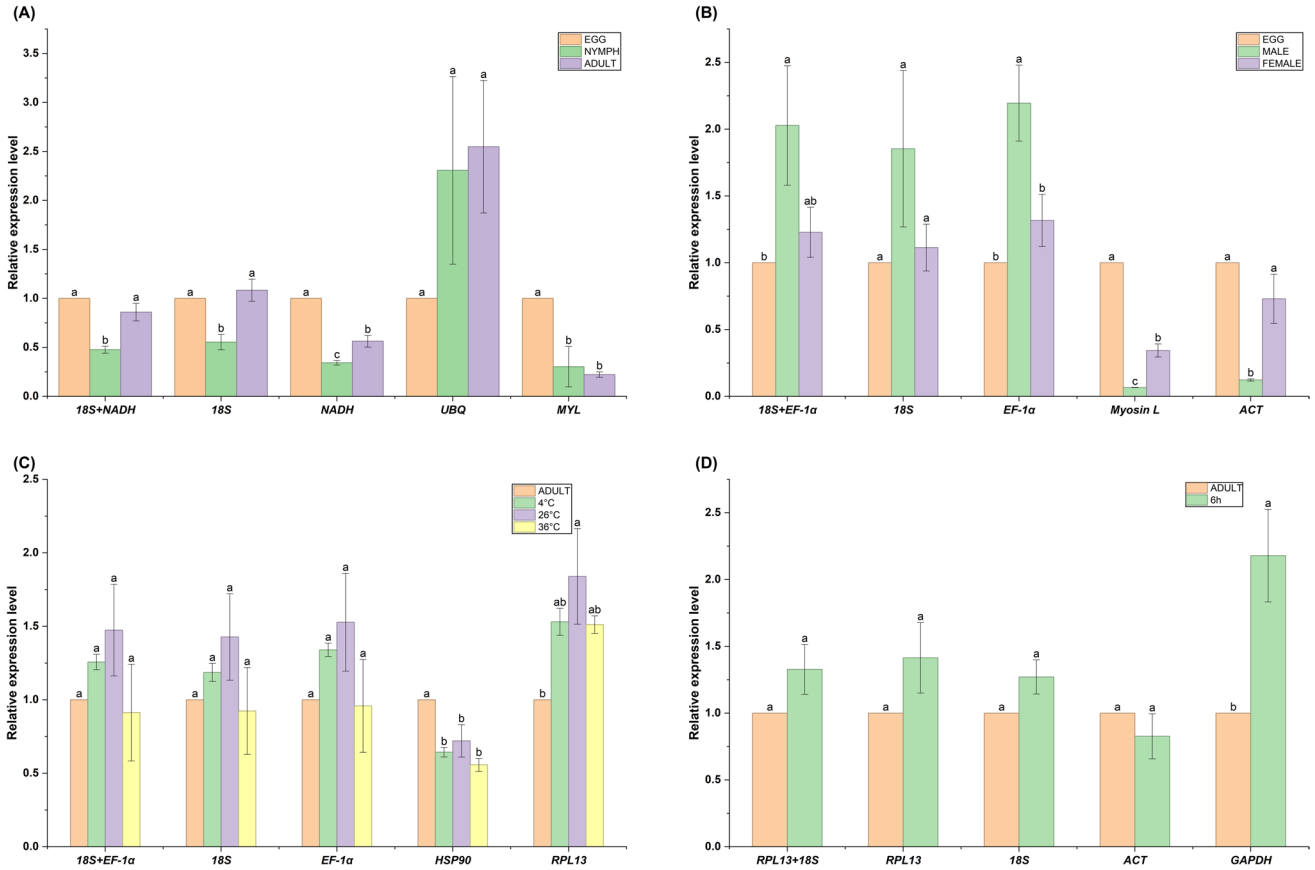

Fig. 7 Relative expression levels of the target gene *AQP* in (**A**) Developmental stages, (**B**) Sexes, (**C**) Temperature, and (**D**) Starvation were normalized by the most stable reference gene combination and individually and by two least stable reference genes. The data represent the mean values ± SE. Bars represent the means and standard errors of three biological replicates, analyzed with one-way analysis of variance (ANOVA), followed by the Fisher LSD paired comparison test. Different letters above the bars indicate notable variances between samples ($P < 0.05$)

In the present study, the rankings generated by NormFinder and the ΔCT method were highly concordant, consistently identifying the same reference gene as the most stable across the majority of experimental conditions. The comprehensive ranking further supported this consensus. In contrast, geNorm results exhibited minor variability under specific conditions, such as starvation stress and between sexes. A more pronounced divergence was observed with BestKeeper, which consistently ranked a different gene as the most stable under all tested conditions. For example, under temperature stress, the geNorm, NormFinder, and ΔCT methods collectively identified *18S rRNA* as the most stable gene, whereas BestKeeper designated *MyosinL* as the most stable and ranked *18S rRNA* eighth. This pattern of discrepancy, wherein BestKeeper results differ from those of other algorithms, has been previously reported in studies on other insects, such as *Bemisia tabaci [silverleaf whitefly]* (Kaur et al. 2019) and *Leptocybe invasa* [eucalyptus gall wasp] (Liu et al. 2023). The observed variations are likely attributable to the distinct statistical methodologies and stability evaluation criteria inherent to each algorithm. To mitigate these discrepancies and derive a robust, consensus-based stability order, a final comprehensive ranking was computed by determining the geometric mean of the rankings from all four analytical tools.

The 18S ribosomal RNA (*18S rRNA*) gene, encoding a core structural RNA of the eukaryotic ribosome's small subunit (*40S*), is integral to ribosome assembly and protein translation. Its ubiquitous expression and high evolutionary conservation have established it as a conventional reference gene in expression studies, with reported stability across diverse developmental stages, tissues, and experimental treatments such as double-stranded RNA (dsRNA) exposure (Yang et al. 2015b, 2018). Similarly, *NADH* dehydrogenase (*NADH*) is a critical enzyme in the mitochondrial electron transport chain, catalyzing electron transfer from *NADH* to ubiquinone and thereby underpinning cellular respiration and ATP synthesis.

In this investigation, *18S rRNA* and *NADH* were identified as the two most stable reference genes across various developmental conditions, a finding consistent with prior research. For instance, *18S rRNA* stability has been

validated during development in species including *Lasioderma serricorne* [cigarette beetle] (Zhang et al. 2021), *Tamarixia radiata* [Asian citrus psyllid parasitoid] (Guo et al. 2020), *Harmonia axyridis* [lady beetle] (Yang et al. 2018), *Myzus persicae* [green peach aphid] (Kang et al. 2017), and *Lucilia cuprina* [Australian sheep blowfly] (Bagnall and Kotze 2010). Furthermore, *18S rRNA* was identified as the most stable reference gene in *Bemisia tabaci* following bacterial infection and insecticide treatment (Su et al. 2013). The stability of *NADH* during development is also supported by its identification as the most stable reference gene in green-belly stink bug, *Dichelops melacanthus* (Pinheiro et al. 2020). Conversely, Myosin light chain (*MyosinL*) exhibited the least stability across both developmental stages and sexes in our analysis. This result aligns with reports from other insect systems; for example, myosin light chain genes were ranked among the least stable references under various conditions in the tiger beetle (García-Reina et al. 2018).

Of the candidate reference genes evaluated, *18S rRNA* exhibited the most stable expression profile across the diverse experimental conditions tested, which encompassed both biotic and abiotic stressors. This finding is consistent with reports from other insect systems, including the bark beetle *Ips calligraphus* (Wallace and Rieske 2021) and the lady beetle *Harmonia axyridis* (Yang et al. 2018), where *18S rRNA* was similarly identified as a highly stable reference gene. In the present study, both *18S rRNA* and elongation factor 1-alpha (*EF-1α*) demonstrated robust stability under sex-specific conditions and temperature-induced stress. *EF-1α* is a commonly employed reference in insect gene expression studies owing to its constitutive involvement in the GTP-dependent delivery of aminoacyl-tRNA to the ribosome during protein translation (Berisio et al. 2010). The observed stability of *18S rRNA* under thermal stress is further supported by work in *Harmonia axyridis* (Yang et al. 2018) and western flower thrips *Frankliniella occidentalis* (Zheng et al. 2014). Likewise, the consistent expression of *EF-1α* between sexes, as documented in spotted borer *Chilo sacchariphagus* (Wang et al. 2024a, b) and in rice borer *Chilo suppressalis* (He et al. 2021), corroborates its reliability. It is noteworthy, however, that the stability of reference genes can be highly dependent on the specific organism and experimental context. For instance, a contrasting study in diamondback moth, *Plutella xylostella,* by Fu et al. (2013) identified both *18S rRNA* and *EF-1α* as among the least stable genes under temperature stress.

Ribosomal proteins (RPLs) are core structural and functional components of the ribosome (Landry-Voyer et al. 2023). Genes encoding these proteins are frequently among the most stable reference genes under starvation stress in insects, as demonstrated in *Agasicles hygrophila* (Guo et al. 2021), *Helicoverpa armigera* (Shakeel et al. 2015), and *Spodoptera litura* (Lu et al. 2013). Notably, *RPL32* and *RPL12* were identified as the most stable reference genes in *Lasioderma serricorne* (Zhang et al. 2021). In the present study, *RPL13* and *18S rRNA* were the most stable reference genes under starvation conditions, whereas *RPL13* was among the least stable under temperature stress. This context-dependent stability is further illustrated in the fall armyworm, *Spodoptera frugiperda*, where *RPL13* was the most stable gene across developmental stages but ranked as the least stable under starvation stress, a condition for which *RPL3* was most stable (Shu et al. 2021). Conversely, *RPL13a* exhibited high expression stability across developmental stages and under thermal stress in *Bemisia tabaci* (Asia I) (Collins et al. 2014).

The results of this investigation confirm that the identification of appropriate reference genes is context-dependent, varying significantly across species and experimental treatments. This underscores the necessity of empirically validating reference genes for each specific biological system and perturbation to guarantee the accuracy of gene expression quantification. To verify the practical impact of our stability rankings, the expression profile of the target gene *AQP* was analyzed under various conditions using different normalization strategies. A significant divergence in *AQP* expression levels was observed when data were normalized with a combination of the two most stable reference genes compared to normalization with the least stable gene. This finding provides empirical evidence that the use of unvalidated reference genes can lead to erroneous interpretations, underscoring the importance of systematic validation before conducting any gene expression study.

Our analysis identified 18S ribosomal RNA as exhibiting minimal expression variability across all tested conditions in *A. rugioperculatus*, recommending its use as a stable reference for normalization. To our knowledge, this work constitutes the first comprehensive validation of reference genes for both biotic and abiotic stress conditions in the rugose spiralling whitefly. Among the 14 candidate genes evaluated, *18S rRNA* demonstrated the highest overall expression stability.

In summary, this study establishes a validated panel of reference genes and a standardized workflow for reliable qRT-PCR analysis in *A. rugioperculatus*. By providing this critical resource, we have established a robust molecular foundation for subsequent functional genomics research on this invasive pest. Specifically, the use of these optimal reference genes will enable precise gene expression studies in key areas such as screening for insecticide resistance mechanisms, profiling physiological and stress response pathways, and evaluating candidate genes for RNA interference (RNAi)-based pest management strategies.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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