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Avian Pathology

Selection of reference genes for quantitative PCR analysis in poultry red mite (*Dermanyssus gallinae*)

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ABSTRACT. Poultry red mites (PRMs, *Dermanyssus gallinae*) are harmful ectoparasites that affect farmed chickens and cause serious economic losses in the poultry industry worldwide. Acaricides are used for PRM control; however, some PRMs have developed acaricide-resistant properties, which have indicated the need for different approaches for PRM control. Therefore, it is necessary to elucidate the biological status of PRMs to develop alternative PRM control strategies. Quantitative polymerase chain reaction (qPCR) allows analysis of the biological status at the transcript level. However, reference genes are preferable for accurate comparison of expression level changes given the large variation in the quality of the PRM samples collected in each farm. This study aimed to identify candidate reference genes with stable expression levels in the different blood feeding states and life stages of PRMs. First, we selected candidates based on the following criteria: sufficient expression intensity and no significant expression difference between fed and starved states. We selected and characterized seven candidate reference genes. Among them, we evaluated the gene expression stability between the starved and fed states using RefFinder; moreover, we compared their expression levels in each life-stage and identified two reference genes, *Elongation factor 1-alpha* (*ELF1A*)-like and *apolipophorins*-like. Finally, we evaluated the utility of the candidates as reference genes, and the use of *ELF1A*-like and *apolipophorins*-like successfully normalized *ATP synthase subunit g* -like gene expression. Thus, *ELF1A*-like and *apolipophorins*-like could be suitable reference genes in PRMs.

KEY WORDS: *apolipophorins*, *Dermanyssus gallinae*, *Elongation factor 1-alpha,* poultry red mite, reference genes

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The poultry red mite (PRM), *Dermanyssus gallinae* (De Geer 1778), is among the major hematophagous ectoparasites in poultry farming. It presents a serious economic threat to the laying hen sector [19]. PRM infestations reduce egg production and quality; moreover, they cause anemia and diminished disease resistance in chickens. Overall, PRM-induced decreased productivity causes severe problems in the poultry industry [19, 20]. Additionally, PRMs may be involved in transmitting avian pathogens as vectors [19]. PRM infestations often cause allergic reactions in humans, including in both poultry workers and urban residents [1, 6, 11, 14]; moreover, zoonotic agents have been detected [16]. Thus, PRMs are harmful pests to the poultry industry and present a danger to human health.

Currently, acaricides are the main agents for controlling PRMs and appropriate acaricide use is required to control PRMs. However, since PRMs can hide in cracks, dust, and spider webs, it is difficult to achieve contact with acaricides. Additionally, most acaricides allow a short-lived reduction in PRM populations. Moreover, repeated or insufficient acaricide use often induces acaricide-resistant properties in PRMs [19], which differ across farms. Since acaricide-resistant PRMs present difficulties in control, there is a need for alternative management strategies in poultry houses to ensure animal welfare and reduce economic losses in poultry farms [19].

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Elucidation of the biological status of PRMs is valuable for developing alternative strategies for PRM control. We previously reported that blood feeding greatly affects the PRM transcriptome through RNA sequencing (RNA-seq) analysis [8]. However, for data validation, additional multi-sample analyses are desirable; furthermore, there is a need to establish a quantitative polymerase chain reaction (qPCR) method for quantifying the expression levels of PRM transcripts. The qPCR method is a prominent tool for analyzing the biological status at the transcript level. However, the accuracy of qPCR results is dependent on various factors, including the initial RNA quality and quantity, primer specificity, reverse transcription efficiencies, amplification efficiency, PCR conditions, and transcript normalization [23]. Data normalization of the target genes using suitable reference genes allows accurate qPCR results [7]. Consequently, there have been several studies on reference gene selection and evaluation in various species [3, 12, 13]. Glyceraldehyde-3-phosphate dehydrogenase, β-actin, and ribosomal protein genes are often used as reference genes for normalizing gene expressions. However, the utility of these genes has been increasingly limited in numerous cases given the differential expression across species, tissue types, cell lines, developmental stages, and/or in response to experimental treatments [4, 18]. Therefore, appropriate reference genes should be selected for each assay. Specifically, reference genes for non-model species, including PRMs, should be carefully selected [5]. This study aimed to search for genes with a stable expression between blood-fed and starved states, as well as in each life-stage of PRMs, and evaluate their utilities as reference genes.

MATERIALS AND METHODS

Sample collection

PRMs were collected in a 50 ml bioreactor tube (Corning, Corning, NY, USA) or 600 ml bioreactor tubes with vent caps (Techno Plastic Products, Zollstrasse, Switzerland) from several poultry farms in Japan. They were transferred to the laboratory during 2015–2020. Some of the PRMs were collected within 48 hr and designated as 'fed PRMs'. Fed PRMs were stored at −80°C until use. The remaining PRMs were stored at 25°C for a one-week period without any blood meals, to digest blood and excrete wastes. PRMs that digested blood and excreted waste were morphologically confirmed, collected, and designated as 'starved PRMs'. Starved PRMs were maintained at 5°C or −80°C for *in vitro* feeding assay or RNA extraction, respectively. The fed and starved PRMs were placed in 70% ethanol; subsequently, they were subjected to sorting based on different life stages; namely, eggs, larvae, protonymphs, deutonymphs, and adults under the stereomicroscope. The PRMs sorted according to life-stage were used for expression analysis.

In vitro feeding assay

Devices for *in vitro* feeding assay were modified from those in a previous report [2]. Briefly, 10 ml serological disposable pipets (Corning) were cut and assembled across stretched Parafilm (Bemis Co., Inc., Neenah, WI, USA) (Fig. 1). Starved PRMs maintained at 5° C were kept at 25° C one day before the assays and collected using 300 µl Universal Barrier Tips (Neptune Scientific, San Diego, CA, USA). Tips containing starved PRMs were inserted in the devices; moreover, the cut ends of the pipettes were closed using rubber caps (Cytiva, Uppsala, Sweden). To ensure air vents, 27G needles were stabbed on the rubber caps. Blood feeding was performed at 40°C for 4 hr; subsequently, only blood-fed PRMs were collected in 300 µl Universal Barrier Tips designated as 'artificially-fed PRMs'. A portion of the artificially-fed PRMs was collected in 50 ml bioreactor tubes with vent caps (Corning); subsequently, they were placed in an incubator at 25°C for 10 days without any blood meals to digest blood and excrete waste; PRMs that digested blood and excreted waste were morphologically confirmed, collected, and designated as 'artificially-starved PRMs.' Only living artificially-starved PRMs were collected in 300 µl Universal Barrier Tips. Both artificially-fed and -starved PRMs were maintained at −80°C until use.

RNA preparation and cDNA synthesis

Total RNA of fed and starved PRMs derived from poultry farms were extracted using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) or RNeasy Mini Kit (QIAGEN, Hilden, German) following the manufacturer's protocol. Moreover, total RNA of PRMs sorted according to life-stage, as well as artificially-fed and starved PRMs, were extracted using RNeasy Mini Kit (QIAGEN). Total RNA from each PRM-sample was used for cDNA synthesis; specifically, 1 µg

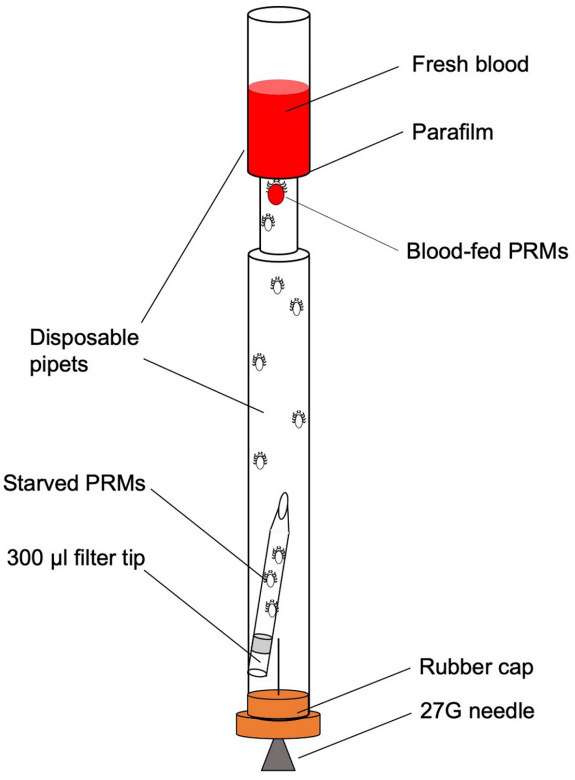

Fig. 1. The device for *in vitro* feeding assay. Ten ml serological disposable pipets were cut and assembled across stretched Parafilm. Starved poultry red mites were collected using tips, and they were inserted in the devices. The cut ends of the devices were closed using rubber caps, and 27G needles were stabbed on the rubber caps to ensure air vents. Chicken blood was added in the devices, and blood feeding was performed at 40°C for 4 hr.

of fed and starved PRMs, 250 ng of PRMs sorted according to life-stage, and 100 ng of artificially-fed and starved PRMs. Total RNA was treated using DNase I (Invitrogen) to remove residual DNA; further, cDNA was synthesized with PrimeScript Reverse Transcriptase (Takara Bio Inc., Kusatsu, Japan) using 200 pmol of oligo (dT) 18 primer.

qPCR

We performed qPCR on a LightCycler 480 system (Roche Applied Science, Penzberg, Germany) using TB Green® Premix Ex Taq™ II (Takara Bio Inc.). PCR amplification mixtures (20 µl) contained 2 µl of the aforementioned cDNA, 10 µl TB Green Premix Ex Taq II, six pmol forward and reverse primers, and DDW. The cycling conditions comprised denaturation at 95°C for 30 sec, 45 cycles at 95°C for five sec, annealing temperature suitable for each primer pair for 30 sec, and 72°C for 30 sec. After completing the amplification program, we performed a melting curve analysis (95°C for five sec, 65°C for one min, 50°C for 30 sec) to determine the purity of the PCR amplicons. To estimate the gene copy number, standard curves were generated for each primer pair based on the known plasmid quantities that PCR products were cloned into the pMD-20 vector (Takara Bio Inc.); moreover, they were analyzed using the Light Cycler 480 system. All assays included the standard curves and no-template control. The cDNA samples were tested in duplicate. Table 1 presents the primers.

Selection of candidate reference genes in PRMs

Candidate reference genes were identified based on data obtained from RNA-seq analysis (Accession No. SAMD00228960, SAMD00229086) [8]. Initially, we searched the contigs with >500 fragments per kilobase of exon per million reads mapped (FPKM) value in both fed and starved PRMs to extract genes with sufficient expression intensity. Next, we searched for contigs with log fold-change (logFC) (fed PRMs / starved PRMs) indicating -1–1 to extract genes lacking significant differences in the expression intensity between fed and starved states. Additionally, we extracted the contigs annotated using the InterProScan tool (https://www.ebi.ac.uk/interpro/search/sequence/) to predict the characteristics of the candidate genes. Finally, we extracted contigs using high and low identities with genes in other mite-species and chickens, respectively, using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Further, we tested *Elongation factor 1-alpha* (*ELF1A*)-like as among the candidate reference genes although it had a relatively high identity with genes in chickens. This is because *ELF1A* is known to have stable expression in *Rhipicephalus* ticks [15].

Stability of candidate gene expression

Expression stabilities of the candidate reference genes were evaluated using RefFinder (https://www.heartcure.com.au/reffinder/) [24]. To evaluate gene expression between fed and starved states, we used the Cp values in reverse transcription qPCR (RT-qPCR) analysis using RNA samples obtained from artificially-fed and -starved PRMs. Additionally, gene expression stabilities were evaluated using RNA samples obtained from artificially-fed and -starved PRMs, respectively.

Statistical analysis

Regarding expression analysis at each life-stage, all values were expressed as mean ± standard deviation. Moreover, a statistical comparison was performed using a one-way analysis of variance, with Turkey honest significant difference (HSD) test for post hoc comparisons. The Wilcoxon rank-sum test was used to compare the copy number of *ATP synthase subunit g* (*ATPSG*)-like between fed and starved PRMs, as well as the relative values normalized according to each candidate gene (the ratio between *ATPSG*-like and each candidate gene). All statistical analyses were performed using EZR [9]. Statistical significance was set at $P<0.05$.

Table 1. Primers used in this study

Target gene name	Transcript ID	5′–3′	Amplicon length (bp)
Apolipophorins-like	DN42048	F: CTGACAACAAGGGCAGGCTA	137
		R: TGTTGTTGTCGTTGCTGCTG	
60S ribosomal protein L28-like	DN42460	F: CCAGGTGTCGAAATGGTTCT	205
		R; CATGTGCTTCGTGAGAGGAA	
60S ribosomal protein L14-like	DN44954	F: ATAAGAAGCTGAGGGCACGA	185
		R: TTTTACGGCATCGAGCTTTT	
Fatty acid-binding protein-like	DN40076	F: GGAGCTCGGTGTCAACTTCA	183
		R: CACTTTCCTTTGCTGCCGTC	
Ubiquitin protein FUBI isoform 1-like	DN40872	F: CGAAGGTCGAGAAGCAGGAG	147
		R: CATGTTCTTATGTGGCGCCG	
Hypothetical protein-like	DN44044	F: AAGGCAAGAGCAAGAGCAAG	189
		R: GTTAAGGCGATTGCCATTGT	
Elongation factor 1-alpha 1-like	DN44542	F: GTCGGTGTCATCAAGTCCGT	186
		R: AGGGTCGAGAGTGTAGGGTC	
ATP synthase subset g-like	DN42035	F: ACAGGTTTTCGCCGGTTTTG	203
		R: CTTCTCATTCCCCCGGCTTT	

F: forward primer, R: reverse primer.

RESULTS

Searching for the candidate reference genes

The candidate reference genes were extracted based on data obtained from RNA-seq analysis (Accession No.: SAMD00228960, SAMD00229086) [8]. We searched for contigs with the following properties: having high expression intensity without a significant difference between fed and starved PRMs, having being annotated using the InterProScan, and showing high identities with genes in other mite-species. We extracted 69 genes; among them, we selected 16 genes showing low identities with genes in chickens. Finally, we selected 6 candidate reference genes; namely, *apolipophorins*-like, *60 S ribosomal protein L28*-like, *probable 60 S ribosomal protein L14 isoform X2*, *fatty acid-binding protein* (*FABP*)-like, *ubiquitin protein FUBI isoform 1*-like, and *hypothetical protein B1W11_06743*-like, which were predicted to have different functions. Moreover, *Elongation factor 1-alpha* (*ELF1A*), which has stable expression in Rhipicephalus ticks [15], was included as a candidate reference gene despite showing high identity with genes in chicken. Consequently, seven genes were selected to evaluate their suitability as reference genes (Table2).

Evaluation of the stability of the candidate reference genes

Since the PRM transcriptome is dramatically altered by blood feeding [8], reference genes should have stable expression levels between fed and starved PRMs. To evaluate the stability of expression levels between fed and starved states, we compared the stability of the candidate reference genes in artificially-fed and -starved PRMs using RefFinder analysis (Table 3). The ranking order, which was determined based

Table 2. Candidate reference genes analyzed in this study

Transcript ID	FPKM		logFC	Blast top hit (non-redundant)			Blast top hit (*Gallus gallus*)		
	Starved	Fed		Gene/species (accession number)	Percent identity (%)	e-value	Gene (accession number)	Percent identity (%)	e-value
DN42048	551.05	560.16	0.3672	*Apolipophorins*-like/*Varroa destructor* (XM_022790948.1)	69.1	2.0E-162	*Von Willebrand factor precursor* (NP_001305393.1)	26.3	5.0E-9
DN42460	1,223.07	972.90	0.0420	*60S ribosomal protein L28*-like/*Varroa destructor* (XP_022673489.1)	80.0	3.0E-75	*Hypothetical protein RCJMB04_34p20* (CAH65438.1)	32.5	4.0E-12
DN44954	1,318.41	976.65	−0.0374	*Probable 60S ribosomal protein L14 isoform X2/Varroa destructor* (XP_022648298.1)	82.6	3.0E-77	*Ribosomal protein L14, partial* (BAB21246.1)	35.4	3.0E-22
DN40076	1,396.13	622.06	−0.8125	*Fatty acid-binding protein, heart isoform X1/Galendromus occidentalis* (XP_018493628.1)	78.8	9.0E-64	*Cellular retinoic acid-binding* protein 1 (NP_001025710.1)	50.8	1.0E-33
DN40872	3,090.92	1,237.88	−0.9628	*Ubiquitin protein FUBI isoform 1/Tropilaelaps mercedesae* (OQR78874.1)	84.6	4.0E-66	*Ubiquitin-like protein fubi and ribosomal protein S30 isoform X1* (XP_025001212.1)	58.6	6.0E-35
DN44044	1,336.41	946.14	−0.1572	*Hypothetical protein BIW11_06743/Tropilaelaps mercedesae* (OQR77933.1)	71.4	4.0E-25	NA	NA	NA
DN44542	2,655.30	1,839.10	−0.1929	*Elongation factor 1-alpha 1*-like /*Varroa destructor* (XP_022706465.1)	94.8	0.0	*Elongation factor 1-alpha 2* (NP_001027570.3)	80.1	0.0

FPKM, fragments per kilobase of exon per million reads mapped; logFC, log fold-change. NA, not applicable.

Table 3. Profiling of gene expression stability in poultry red mites (PRMs) at mixed stages calculated using the RefFinder program

Method	Ranking order						
	1	2	3	4	5	6	7
Delta CT	*ELF1α*-like	*FABP*-like	*Apolipophorins*-like	*60S ribosomal protein L28*-like	*Hypothetical protein*-like	*60S ribosomal protein L14*-like	*Ubiquitin protein FUBI*-like
BestKeeper	*Apolipophorins*-like	*ELF1α*-like	*FABP*-like	*60S ribosomal protein L28*-like	*Hypothetical protein*-like	*Ubiquitin protein FUBI*-like	*60S ribosomal protein L14*-like
Normfinder	*FABP*-like	*ELF1α*-like	*60S ribosomal protein L28*-like	*Apolipophorins*-like	*Hypothetical protein*-like	*60S ribosomal protein L14*-like	*Ubiquitin protein FUBI*-like
Genorm	*Apolipophorins-like and ELF1α*-like		*FABP*-like	*60S ribosomal protein L28*-like	*Hypothetical protein*-like	*60S ribosomal protein L14*-like	*Ubiquitin protein FUBI*-like
Recommended comprehensive ranking	*ELF1α*-like	*Apolipophorins*-like	*FABP*-like	*60S ribosomal protein L28*-like	*Hypothetical protein*-like	*60S ribosomal protein L14*-like	*Ubiquitin protein FUBI*-like

ELF1α-like, *Elongation factor 1-alpha-like*; *FABP*-like, *fatty acid-binding protein*-like; *FUBI*-like, *fan ubiquitin-like protein I-like*.

on the Cp values of artificially-fed and -starved PRMs in RT-qPCR analysis, revealed that the top three genes were *ELF1A*-like, *apolipophorins*-like, and *FABP*-like. Additionally, *ELF1A*-like, *apolipophorins*-like, and *FABP*-like showed relatively high gene expression stability in artificially-fed and -starved PRMs, respectively (Supplementary Tables 1 and 2). Therefore, the candidate genes were further narrowed down to *ELF1A*-like, *apolipophorin*s-like, and *FABP*-like.

Expression analysis of the candidate reference genes in different life stages

Next, we examined the gene expression stability of *ELF1A*-like, *apolipophorins*-like, and *FABP*-like in PRMs in blood-fed and starved states at different life stages (protonymphs, deutonymphs, and adults) and across their life stages using RT-qPCR analysis (Fig. 2). Additionally, we analyzed eggs and larvae, which are unfed stages. There were significantly higher expression levels of all genes in eggs than in other life stages (Fig. 2). Contrastingly, there was no significant difference in *ELF1A*-like expression levels in all life stages, except for eggs (Fig. 2A). There was higher *apolipophorins*-like expression in larvae than in fed protonymphs and fed deutonymphs (Fig. 2B). There was a tendency of low *FABP*-like expression in larvae and protonymphs; moreover, there was a significant difference in *FABP*-like expression levels between deutonymphs and larvae/protonymphs (Fig. 2C). Thus, the expression of *ELF1A*-like and *apolipophorins*-like genes were stable from larvae/protonymphs to adults regardless of blood feeding, whereas *FABP*-like expression was unstable when compared across the stages. These results suggest that *ELF1A*-like and *apolipophorins*-like are suitable reference genes for investigating the expression levels between fed and starved states of PRMs.

Utility of ELF1A-like and apolipophorins-like as reference genes

First, we analyzed the gene copy number of *ATP synthase subunit g* (*ATPSG*)-like, which shows significantly increased expression levels with blood feeding [8], in fed and starved PRMs obtained from four different farms using RT-qPCR. For samples obtained from farms A and C, there was high *ATPSG*-like expression in the fed PRMs, as expected. For samples obtained from farms B and D, starved PRMs showed a higher copy number of *ATPSG*-like than fed PRMs (Fig. 3 left panel). Additionally, there was no significant difference in the gene expression between fed and starved PRMs (Fig. 3 right panel). In contrast, fed PRMs obtained from all farms showed increased expression levels of *ATPSG*-like normalized with *ELF1A*-like or *apolipophorins*-like as reference genes (Fig. 4A and 4B, left panels); moreover, there was a significant difference in gene expression between fed and starved PRMs (Fig. 4A and 4B, right panels). In addition, *FABP*-like successfully normalized *ATPSG*-like expression (Fig. 4C). However, a significant difference in *FABP*-like expression levels between deutonymphs and larvae/protonymphs was observed (Fig. 2C). Taken together, our findings indicate that *ELF1A*-like and *apolipophorins*-like could be suitable as reference genes in RT-qPCR analysis for investigating the expression levels between fed and starved PRMs.

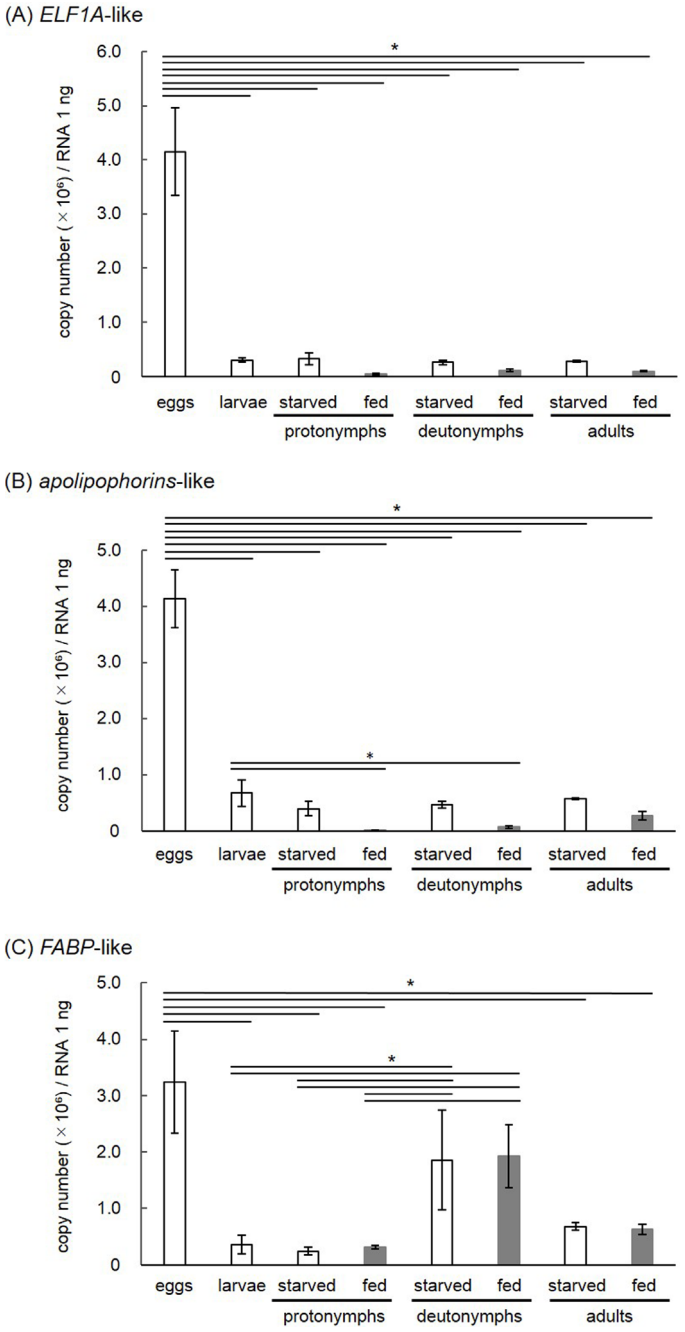

Fig. 2. The copy numbers of candidate reference genes at each life-stage and blood-feeding states. The copy numbers of candidate reference genes were analyzed using reverse transcription quantitative PCR (RT-qPCR) [(A): *Elongation factor 1-alpha* (*ELF1A*)-like, (B): *apolipophorins*-like, (C): *fatty acid-binding protein* (*FABP*)-like]. Bars represent the standard deviation (± S.D). Statistical analyses were performed using a one-way analysis on variance, with Turkey HSD test for post hoc comparisons. **P*<0.05 was considered statistically significant.

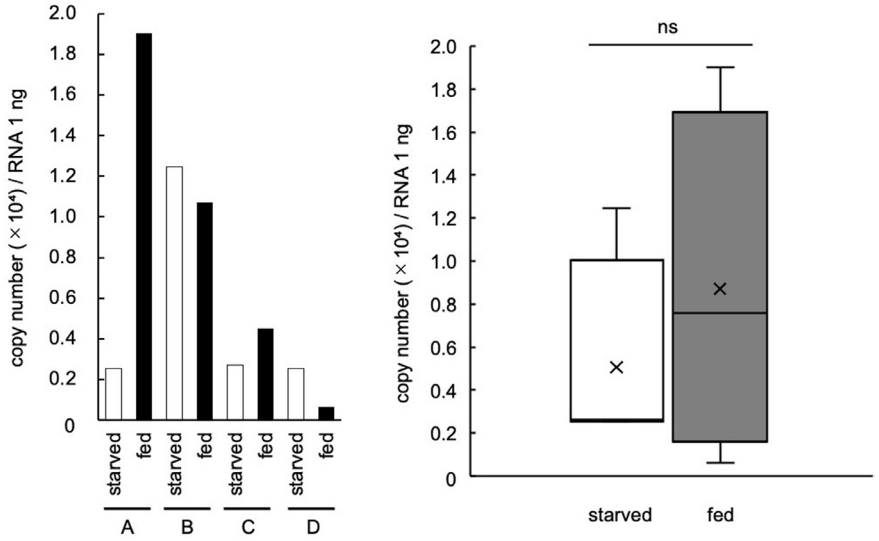

Fig. 3. The gene copy number of *ATP synthase subunit g* (*ATPSG*)-like in poultry red mites (PRMs) obtained from different four farms. The copy number was quantified using RT-qPCR. The left panel shows the gene copy numbers of *ATPSG*-like in starved and fed PRMs obtained from different four farms. The right panel shows a comparison of *ATPSG*-like expression between starved and fed PRMs. In the right panel, the gene copy numbers of ATPSG-like between starved and fed PRMs were compared using the Wilcoxon rank-sum test. $P<0.05$ was considered statistically significant.

DISCUSSION

This study searched for reference genes in PRMs and investigated their expression stabilities in different states. First, based on RNA-seq analysis data [8], we identified PRMs genes based on the following criteria: having sufficient expression intensity, showing no significant difference in expression intensity between fed and starved states, having high and low identities with genes in other mite-species and chickens, respectively. Moreover, we included *ELF1A*-like, which has been identified as a useful reference gene in ticks [15]. Our findings demonstrated stable expression of *ELF1A*-like and *apolipophorins*-like genes in PRMs under different conditions. This suggests that they could be suitable reference genes for gene expression analysis in PRMs through RT-qPCR.

In the extraction of candidate reference genes, RefFinder analysis revealed stable gene expression of *ELF1A*-like, *apolipophorins*-like, and *FABP*-like between fed and starved states in PRMs (Table 3). In addition, these genes successfully normalized the expression levels of *ATPSG*-like gene (Fig. 4). With respect to life stages, there was lower *FABP*-like expression in larvae and protonymphs; however, there was no difference in *FABP*-like expression levels between fed and starved PRMs in each stage (Fig. 2C). In the RefFinder analysis, artificially-fed and starved PRMs at mixed stages were used to compare gene expression stability between fed and starved PRMs. In addition, fed and starved PRMs at mixed stages were used to evaluate the utility of reference genes by RT-qPCR targeting *ATPSG*-like gene. This use of mixed-stage samples could have disguised *FABP*-like as a stably expressed gene. Therefore, *FABP*-like is unsuitable as a reference gene for comparing gene expression across the life stages of PRMs, although it could be used for the comparison of gene expression between blood-fed and starved states in each stage. Contrastingly, there was relatively stable *ELF1A*-like and *apolipophorins*-like expression, except for eggs. In addition, the sequences of the primers used in the present study were found in *ELF1A*-like and *apolipophorins*-like transcripts from German PRMs (accession number: *ELF1A*-like: GAIF01008133, *apolipophorins*-like: GAIF01012385) [17], suggesting that *ELF1A*-like and *apolipophorins*-like can be broadly applied as reference genes for qPCR in PRMs distributed in other regions. This suggests that these genes could be applied as reference genes for comparing target gene expression levels in life stages, from larvae to adults. Unfortunately, *ELF1A*-like and *apolipophorins*-like expression in eggs was much higher than those in other stages. Therefore, there is a need to further search for reference genes for expression analysis across all stages.

Blood meal is the only nutritional source for PRMs; moreover, energy synthesis is highly active in fed PRMs compared with starved PRMs. This indicates the upregulation of expression of genes involved in energy synthesis, including *ATPSG*-like, in fed PRMs [8]. This study found a significantly lower gene copy number of *ATPSG*-like in fed PRMs than in starved PRMs obtained from some farms (Fig. 3), which was inconsistent with the RNA-seq analysis findings. However, there was upregulated *ATPSG*-like expression in blood-fed PRMs after normalization with *ELF1A*-like or *apolipophorins*-like as the reference genes (Fig. 4). Additionally, there was a smaller variance in *ATPSG*-like expression in starved PRMs than that before normalization. This indicates consistent down-regulated *ATPSG*-like expression in starved PRMs. In contrast, there was broad variance in *ATPSG*-like expression in fed PRMs even after normalization. This could be reflective of the period after collecting PRM from farms since there were different digestion states in each sample depending on the duration of transport from the farms to the laboratory. Therefore, further analysis, including expression analysis at different days after blood-feeding, is required to analyze the expression profiles of the target genes in more detail, and then, reference genes should be applied to improve the accuracy of the data. These findings obtained through expression analysis normalized by *ELF1A*-like or *apolipophorins*-like indicates that they were reasonable reference genes.

RT-qPCR analysis could provide valuable information for investigating the biological status in PRMs. Currently, the emergence of acaricide-resistant PRMs has impeded their control in farms. Similar to in insects, target-site insensitivity and increased enzymatic detoxification are major causes of resistance in Acari [21]. Pyrethroid resistance in PRMs has been associated with mutations in voltage-gated sodium channels [10]; furthermore, increased P450s expression might be involved in pyrethroid

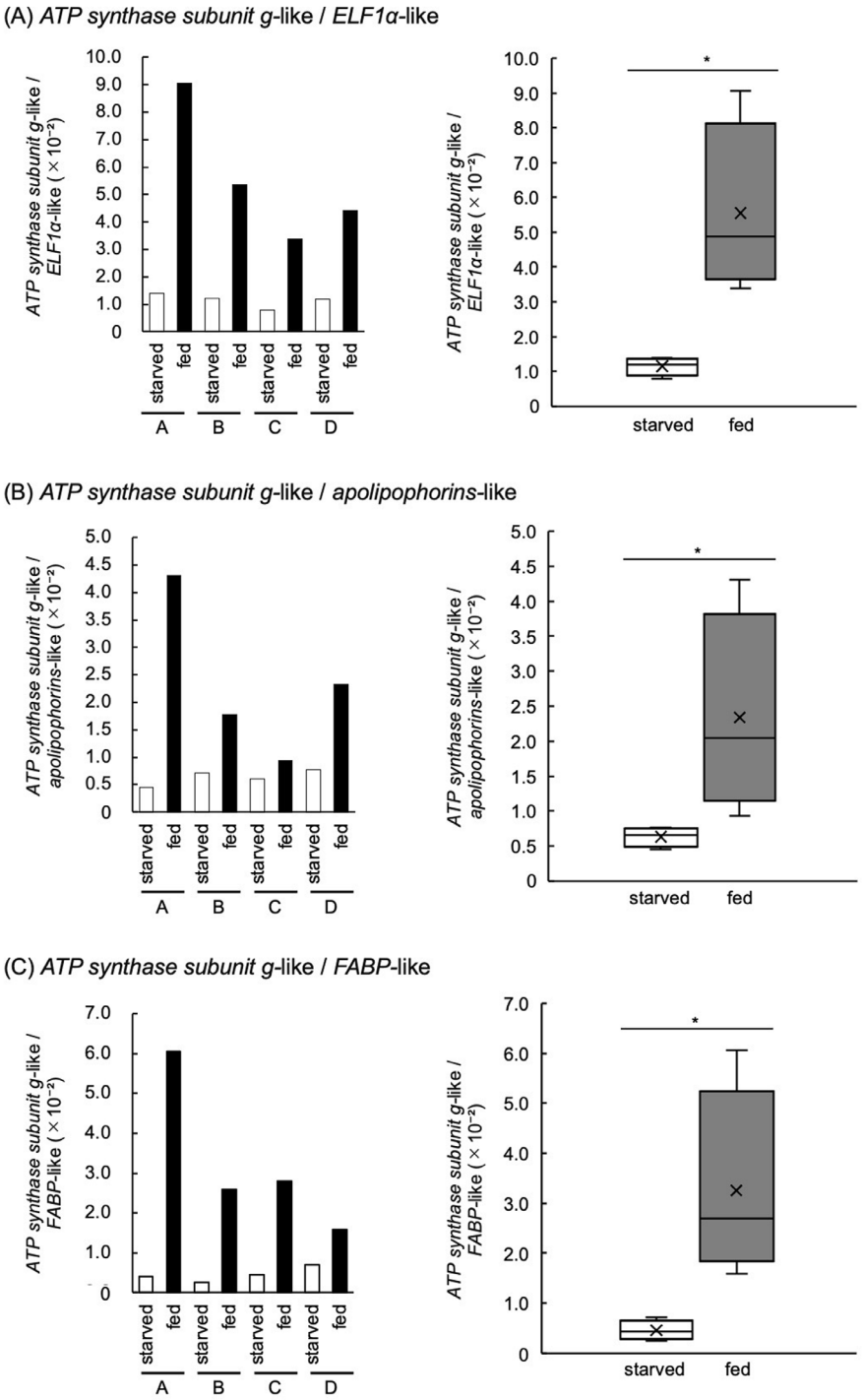

Fig. 4. The relative expression values of *ATP synthase subunit g* (*ATPSG*)-like normalized with *Elongation factor 1-alpha* (*ELF1A*)-like and *apolipophorins*-like. The gene expression ratio between *ATPSG*-like and each candidate gene [(A): *ELF1A*-like, (B): *apolipophorins*-like, (C): *fatty acid-binding protein* (*FABP*)-like] were analyzed using RT-qPCR. The left panel shows the relative values of *ATPSG*-like in starved and fed poultry red mites (PRMs) samples from four different farms. The right panel shows a comparison of relative values of *ATPSG*-like between starved and fed PRMs samples. On the right panel, the ratio of *ATPSG*-like gene expression was compared between starved and fed PRMs using the Wilcoxon rank-sum test. **P*<0.05 was considered statistically significant.

detoxification in PRMs [22]. Expression analysis of related genes may help elucidate the acaricide-resistant properties of PRMs in each farm. Taken together, this study identified two reference genes, *ELF1A*-like or *apolipophorins*-like, which could allow more accurate gene expression quantification and provide a better understanding of biological status in PRMs.

CONFLICT OF INTEREST. ST, OE and TA are employed by vaxxinova Japan Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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