Reference Gene Selection for Normalizing Gene Expression in *Ips Sexdentatus* (Coleoptera: Curculionidae: Scolytinae) Under Different Experimental Conditions

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*Ips sexdentatus* (Coleoptera: Curculionidae: Scolytinae) is one of the most destructive and economically important forest pests. A better understanding of molecular mechanisms underlying its adaptation to toxic host compounds may unleash the potential for future management of this pest. Gene expression studies could be considered as one of the key experimental approaches for such purposes. A suitable reference gene selection is fundamental for quantitative gene expression analysis and functional genomics studies in *I. sexdentatus*. Twelve commonly used reference genes in Coleopterans were screened under different experimental conditions to obtain accurate and reliable normalization of gene expression data. The majority of the 12 reference genes showed a relatively stable expression pattern among developmental stages, tissue-specific, and sex-specific stages; however, some variabilities were observed during varied temperature incubation. Under developmental conditions, the Tubulin beta-1 chain (*β*-Tubulin) was the most stable reference gene, followed by translation elongation factor (eEF2) and ribosomal protein S3 (*RPS3*). In sex-specific conditions, *RPS3*, *β*-Tubulin, and eEF2 were the most stable reference genes. In contrast, different sets of genes were shown higher stability in terms of expression under tissue-specific conditions, i.e., *RPS3* and eEF2 in head tissue, V-ATPase-A and eEF2 in the fat body, V-ATPase-A and eEF2 in the gut. Under varied temperatures, *β*-Tubulin and V-ATPase-A were most stable, whereas ubiquitin (UbiQ) and V-ATPase-A displayed the highest expression stability after Juvenile Hormone III treatment. The findings were validated further using real-time quantitative reverse transcription PCR (RT-qPCR)-based target gene expression analysis. Nevertheless, the present study delivers a catalog of reference genes under varied experimental conditions for the coleopteran forest pest *I. sexdentatus* and paves the way for future gene expression and functional genomic studies on this species.

**Keywords:** *Ips sexdentatus*, reference gene, RT-qPCR, differential gene expression, housekeeping genes, bark beetles, Scolytinae
INTRODUCTION

Differential gene expression (DGE) studies are fundamental to evaluate the effects of physiological responses on biological variation in insect populations at the molecular level. Real-time quantitative reverse transcription PCR (RT-qPCR) is a reliable technique and is widely used to analyze the expression of target genes due to its high sensitivity, accuracy, specificity, reproducibility, and speed (Heid et al., 1996; Bustin et al., 2005; VanGuilder et al., 2008; Wang et al., 2020). In addition, RT-qPCR allows simultaneous measurement of gene expression in many different samples for a limited number of target genes and is particularly suitable when only a limited number of samples are available (Higuchi et al., 1993; Vandesompele et al., 2002). Nevertheless, expression results vary due to initial sample size, RNA integrity (quantity and quality), reverse transcription, messenger RNA (mRNA) recovery, PCR efficiency, and primer design (Gao et al., 2020). Therefore, internal control genes (alternatively called reference genes) are commonly used to normalize mRNA levels for more accurate gene expression quantification as their expression should not vary under different biological or experimental conditions (Nicot et al., 2005; Lu et al., 2018). Furthermore, it is strongly recommended to use multiple reference genes for target gene expression normalization for more authenticity (Garcia-Reina et al., 2018; Shakeel et al., 2018; Wang et al., 2020). There are several studies on gene function analysis in insects using reference genes such as β-actin (Actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1α (EFIA), β-Tubulin (β-Tubulin), ribosomal proteins (RPs), ubiquitin (UbIQ), superoxide dismutase (SOD), heat shock protein 90 (HSP90), and vacuolar-type H+-ATPase subunit B (V-ATPase-B) for gene expression normalization (Lu et al., 2018; Qu et al., 2018; Gao et al., 2020; Wang et al., 2020). This suggests that reference genes can be differentially expressed in insects under varied experimental conditions. Alternatively, no universal reference gene is available, which is stably expressed under different experimental conditions (Lu et al., 2018). Therefore, identification and validation of reference genes under different experimental conditions, life stages, and tissue-specific stages are essential on a case-by-case basis for accurate quantification of gene expression in any insects, including I. sexdentatus (Piafl et al., 2004; Rodrigues et al., 2014; Qu et al., 2018; Basu et al., 2019; Gao et al., 2020; Gurusamy et al., 2021; Xie et al., 2021).

The wood-boring insect I. sexdentatus (Coleoptera: Curculionidae: Scolytinae; hereby referred to as ISx), also known as the six-toothed bark beetle, is one of the most destructive and economically important insects, causing severe damage to coniferous species throughout Europe and Asia (Etzebeste and Pajares, 2011; Seidl et al., 2017; Douglas et al., 2019). ISX is an opportunistically aggressive species of bark beetles (Wermelinger et al., 2008; Chakraborty et al., 2020a). It usually attacks old scots pine trees and can occupy young trees up to a diameter of 10 cm in dbh (diameter at breast height). It was primarily a sparse specimen (Pfeffer, 1955; Postner, 1974) in the past, but now ISX is considered a significant pest in some European countries (Gregoire and Evans, 2004). Several drought periods during the last decade highly favored its current upliftment on the pest status, and we are registering higher population densities of ISX in most of the Czech forests. At least from 2018, the south Moravian forests suffered from the severe outbreak of this species. The volume of the harvested pine due to bark beetles in the most affected region was 12 times higher than in the previous years (Lubojačky and Knížek, 2020).

Bark beetles preferentially colonize weakened, wilted, or recently dead trees. However, favorable conditions such as drought can lead to an outbreak (epidemic phase). Beetles start attacking the healthy standing trees due to weakened defenses caused by warmer temperatures (Bouhot et al., 1988; Marini et al., 2017; Pettit et al., 2020) and larger windthrows (Kausrud et al., 2012; Biedermann et al., 2019; Sommerfeld et al., 2020). Furthermore, conifers have self-defense mechanisms to ward off bark beetle infestations with secondary metabolites such as terpenes (Ferrenberg et al., 2014; Denham et al., 2019). These defense mechanisms can also be overcome during mass insect attacks by detoxifying plant-derived secondary metabolites with the help of symbiotic microbes (Chakraborty et al., 2020b; Huang et al., 2020). To elucidate molecular underpinnings shaping environment-conifer-bark beetle (i.e., I. sexdentatus) interactions, gene expression studies need to be conducted in the future. Such studies advocate the requirements for stable reference genes under different experimental conditions.

Hence, we extensively evaluated 12 commonly known reference genes in Coleopterans and other insects (Supplementary Figure 1 and Supplementary Table 1) for the expression stability in ISX under varied experimental conditions in the present study. Using available in-house transcriptome data of ISX, we have obtained gene sequences of β-actin (Actin), translation EF 2 (eEF2), Tubulin beta-1 chain (β-Tubulin), myosin regulatory light chain 2 (Myosin L), V-type proton ATPase catalytic subunit A (V-ATPase-A), NADH dehydrogenase subunit 1 (NADH), ubiquitin C variant (UbIQ), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), arginine kinase isof orm X1(ArgK), RP53, RPL17, and HSP83 for expression stability evaluation. The candidate reference genes were screened using different parameters, such as developmental stages (i.e., larvae, pupae, and adult stages), tissue- and sex-specific [head, gut, fat body, and whole body (WB) except the head, gut, fat body], and different treatments [such as temperature; Juvenile hormone III (JHHIII); wild-collected vs. long-term laboratory-reared; and different host feeding] to obtain accurate reference genes for future genomic and functional studies. Our study delivers a catalog of genes that should be used for DGE studies on ISX under varied experimental conditions and commences the possibility for aggressive management of bark beetles using molecular approaches such as RNA interference (RNAi) (Joga et al., 2021).

MATERIALS AND METHODS

Bark Beetles

Ips sexdentatus (ISX)-infested Pinus sylvestris were collected from Kostelec nad Černými lesy (50°00’07.2”N 14°50’56.3”E, under School Forest Enterprise) maintained insect rearing chambers
at Faculty of Forestry and Wood Sciences, Czech University of Life Sciences, Prague. Beetles were maintained with fresh pine logs at 27 ± 1 °C under 70 ± 5% humidity and a 16:8-h light/dark (L:D) photoperiod. ISx is the largest beetle of the genus Ips, at 6-8 mm in length. The life stages include three larval stages, pupae, callow (just emerged), and fed adult stage (flying for new host colonization). Both sexes have six spines at each side of the elytral declivity. The fourth spine is the largest and is capitate. The wild population was supplied with fresh pine logs for the next generation and maintained for 40–45 days for one life cycle. Similarly, the wild population was continuously maintained for three generations to study the difference between wild and lab-reared beetle conditions.

Sample Preparation

Development Stages and Different Tissue Types

Samples were collected from different life stages of ISx: three larval stages (L1, L2, and L3), pupae, (P), callow (newly emerged) male (CM) and female (CF), and fed adult (mature adults move toward trunks of pines to lay eggs for next-generation) male (AMF) and female (AFF). For sufficient nucleic acid extraction for downstream processing, each biological replicate was prepared using pooled individuals from various developmental stages, such as five first instar larvae (L1)/replicate, three second and third instar larvae (L2 and L3)/replicate, three pupae/replicate, and two adults/replicate. Four biological replicates were used in all experiments.

Tissues such as head, fat body, gut, and WB minus head, fat body, and gut (hereby referred to as WB) were dissected from the callow and fed adult stage of both male and female ISx producing experimental samples, such as CMH, callow male beetle head; CMFB, callow male beetle fat body; CMMG, callow male beetle midgut; CMWB, callow male beetle WB; CFH, callow female beetle head; CFFB, callow female beetle fat body; CFMG, callow female beetle midgut; CFWB, callow female beetle WB; AMH, fed adult male head; AMFB, fed adult male fat body; AMFMG, fed adult male midgut; AMFWB, fed adult male WB; AFFH, fed adult female head; AFFFB, fed adult female fat body; AFFMG, fed adult female midgut; and AFFWB, fed adult female WB. Four independent biological replicates were collected and stored at −80 °C. Each replicate was derived by pooling tissues from 10 individual beetles.

Temperature and JHIII Treatment

For the temperature treatment, the freshly emerged adults were placed in small glass tubes and exposed to a range of temperatures (4, 27, and 37 °C) for 72 h in a temperature-controlled chamber. After exposure, survivors were frozen in liquid nitrogen and stored at −80 °C. Adults maintained at 27°C were used as control. At least four beetles were randomly collected per replication, and four independent biological replications were used from each temperature treatment.

For JHIII treatment, emerged beetles were sorted based on gender and kept at 4°C on moist paper towels. Beetles were treated topically on the ventral surface of the abdomen with 10 µg JHIII (dissolved in acetone) (Sigma-Aldrich, St. Louis, MO, USA) and only acetone as control (Aw et al., 2010; Sun et al., 2021). The beetles were incubated at 27 ± 1°C under 70 ± 5% humidity and a 16:8-h light/dark (L:D) photoperiod in groups of 20 in 60 ml plastic containers for 72 h. After incubation, beetles were immediately shock frozen in liquid nitrogen and stored at −80°C for downstream processing. Four biological replicates were used for JHIII treatment and control.

Different Host Feeding

Under different host feeding treatments, ISx was placed into the phloem tissue of freshly cut lodgepole pine and spruce. Beetles were placed under the bark in randomly chosen male-female pairs and maintained under standard rearing conditions. ISx was allowed to feed under the bark for one generation (40–45 days). We removed ISx adults from galleries showing the excavation of frass (an indication of feeding). The beetles were collected and immersed in insectinger solution; the gut tissues were excised. Gut tissues were gently purged of their contents (i.e., malpighian tubules, fat body) and then frozen in liquid nitrogen and stored at −80°C.

Total RNA Extraction and Complementary DNA Synthesis

Total RNA from bark beetle tissue samples was extracted using TRIzol® (Invitrogen, Carlsbad, CA) following the protocol of the manufacturer. Isolated RNA was further treated with DNases using a TURBO DNAase Kit (Ambion, USA) to remove any DNA contamination. RNA quantity and quality were evaluated using 1.5% agarose gel and quantified by NanoDrop 2000/2000c (Thermo Fisher Scientific®, Waltham, MA, USA) and stored at −80°C. Complementary DNA (cDNA) (first-strand) was synthesized from 1 µg of total RNA in triplicate using the high-capacity cDNA reverse transcription kits (Applied Biosystems-Life Technologies) following the recommendations of the manufacturer and stored at −20°C.

Selection of Candidate Reference Genes for Evaluation

This study selected 12 potential reference genes (Supplementary Table 2) that function as reference genes in other Coleoptera species (Supplementary Figure 1 and Supplementary Table 1). These genes were obtained from our functionally annotated in-house transcriptome of ISx (manuscript in preparation). The primers for those selected genes were designed via Integrated DNA Technologies, Inc. (IDT) (Table 1).

Quantitative RT-qPCR Analysis

cDNA samples were diluted 1:20 before being used in RT-qPCR. Four independent biological replicates from each treatment, developmental stage, and tissue-type were included in each RT-qPCR run. RT-qPCR run was performed using the Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems) with a reaction mix containing 5.0 µL of SYBR® Green PCR Master Mix (Applied Biosystems), 1.0 µL of cDNA, 1.0 µL optimized concentrations of primers (Table 1), and RNase-free water (Invitrogen) to a total volume of 10.0 µL. Amplification conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and finally a melt curve analysis.
95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. In order to confirm the primer specificity, we performed melt curve analysis using default parameters by a steady increase in temperature from 60 to 95°C. All RT-qPCR assays were carried out in four biological replicates, including two or three technical replicates.

**Data Analyses**

The raw cycle threshold (Ct) values were obtained with 7500 software v2.0.5 (Applied Biosystems®). Gene expression and stability were analyzed using different commonly used tools and algorithms such as geNorm (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), and ∆∆Ct method (Silver et al., 2006). Precisely, geNorm assesses the expression stability value (M) as the average pairwise variation of one of the genes against all control genes present in the experiment. The program estimates the mean pairwise variation between genes across all samples, and the gene with the lowest M value is considered most stable (Vandesompele et al., 2002). NormFinder calculates the SD for each target gene and juxtaposes it with other gene expressions. The gene displaying the lowest variation between intra- and inter-group comparisons is considered most stable (Andersen et al., 2004). On the contrary, BestKeeper is a data processing method based on crossing points that compares all genes across all samples and provides a stability index for each reference gene (Pfaffl et al., 2004). The comparative delta-Ct method juxtaposes Ct values and the relative expression of “gene pairs” within each sample (Silver et al., 2006). The sample-specific mean Ct values of each reference gene from each experiment are given as input data and subsequently processed using the web-based tool RefFinder (https://www.heartcure.com.au/reffinder/).

Furthermore, pairwise variation (V), estimated by geNorm, was used to decide the optimal number of reference genes for accurate RT-qPCR normalization. The Vn/Vn+1 value reflected the pairwise variation between two sequential normalization factors. A cutoff threshold, i.e., Vn/Vn+1 = 0.15, was set for a valid normalization (Vandesompele et al., 2002).

**Validation of Selected Reference Genes**

We analyzed the relative expression levels of Kr-h1 and Hsp70 genes after JHIII treatment and different temperature exposure to evaluate the selected reference genes. To compare the impact of different normalization strategies, the expression of target genes was normalized using both selected reference genes individually and in combinations using the 2^−Δ∆Ct method (Livak and

### Table 1 | Details of primer sequence, amplicon length, and RT-qPCR analysis of candidate reference genes and target genes.

| Gene symbol | Gene name               | Primer sequences (5’-3’) | Amplicon length (bp) | PCR efficiency % | Regression coefficient |
|-------------|-------------------------|--------------------------|----------------------|------------------|------------------------|
| Actin       | β-actin                 | FP: GACATCGGTCAGGCAATTCC | 118                  | 101.14           | 0.988                  |
|             |                         | RF: CACGAAACCCGCTCAGAATCC |                      |                  |                        |
| eEF2        | Translation elongation factor | FP: CGATGGCGCTCAAAGTAAAC | 142                  | 101.30           | 0.981                  |
|             |                         | RF: CGGTTGTGCCTCCGGAAGAAGAAG |                     |                  |                        |
| β-Tubulin   | Tubulin beta-1 chain    | FP: CATCTCGTGATACCGCTCTTC | 126                  | 105.85           | 0.987                  |
|             |                         | RF: GGGCACTCAGTGGTAAG | 140                  | 98.69            | 0.990                  |
| Myosin L    | Myosin regulatory light chain 2 | FP: GACCCAACAAGTCGTAAG | 120                  | 110.71           | 0.9999                 |
|             |                         | RF: CCAGGACAAAGACGTAATCC |                      |                  |                        |
| V-ATPase-A  | V-type proton ATPase catalytic subunit A | FP: CGTTTGCTGCTGCTCTCTAC | 125                  | 110.71           | 0.9999                 |
|             |                         | RF: GTGACAGGATGAGGAATCT |                      |                  |                        |
| NADH        | NADH dehydrogenase subunit 1 | FP: GTGTGAGTCGAGGCACCAAC | 148                  | 108.07           | 0.9941                 |
|             |                         | RF: GAAGGAGGCCAGAAACAC |                      |                  |                        |
| Ubiquitin C | Ubiquitin C variant     | FP: TGAGGCGAGAAGGGAGTAAG | 120                  | 98.17            | 0.989                  |
|             |                         | RF: TGCTGCTGCCTGCTGCT | 129                  | 107.06           | 0.973                  |
| GAPDH       | Glyceraldehyde-3-phosphate dehydrogenase | FP: CACCCAAGAAGACTGTGAC | 129                  | 106.49           | 0.972                  |
|             |                         | RF: CGGTTGACGGAAGAATAC |                      |                  |                        |
| ArgK        | Arginine kinase isoform X1 | FP: CGGCTTCTCTGACTGTTCTC | 144                  | 109.01           | 0.972                  |
|             |                         | RF: GGGTGAGCAACACGTGATTC |                      |                  |                        |
| RPS3        | Ribosomal protein S3    | FP: GAAAGGAGCCTTGCTCCTTGG | 128                  | 98.32            | 0.973                  |
|             |                         | RF: CAGAACCTCTGACTGACTCTC |                      |                  |                        |
| RPL17       | 60S ribosomal protein L17 | FP: ACCGTOACTTGACACTCTCO | 102                  | 106.54           | 0.9895                 |
|             |                         | RF: TCCTCACTCTCCTACCCTAAG |                      |                  |                        |
| HSP83       | Heat shock protein 83   | FP: GCCTTGCTGCTGCTGCTTGT | 130                  | 110.00           | 0.974                  |
|             |                         | RF: CGGATGGACGTCCAATATG |                      |                  |                        |
| Kr-h1       | Kruppel homolog 1       | FP: GCTTTCTCTCAATTTCTCATGC | 168                  | 113.9            | 0.833                  |
| Hsp70       | Heat shock protein 70   | FP: ACGGTGAAGCATGATCAATG | 140                  | 104.90           | 0.961                  |
|             |                         | RF: GACATGCGTGAATGCTGATG |                      |                  |                        |
Schmittgen, 2001). Target genes expression were analyzed using one-way ANOVA using GraphPad Prism software. P-value < 0.05 was considered to identify significant differences between samples.

RESULTS

Primer Specificity and PCR Efficiency
In the current study, 12 genes, namely Actin, eEF2, β-Tubulin, Myosin L, V-ATPase-A, NADH, UbiQ, GAPDH, ArgK, RPS3, RPL17, and HSP83, were screened for identifying suitable reference gene or gene combination from ISx. RT-qPCR products generated with each primer set (forward+ reverse) against target genes were evaluated by the occurrence of a single peak in melting curve analyses (Supplementary Figure 2) and specific bands of the expected size in agarose gel electrophoresis (Supplementary Figure 3). The amplification efficiency for each primer pair ranged from 98.17 to 107.06%, and the correlation coefficients ($R^2$) were greater than 0.98 (Table 1). The Ct values of the 12 candidate reference genes ranged from 19.87 to 34.78 and covered all experimental conditions (Figure 1A). While most Ct values ranged from 19 to 27, Actin, eEF2, β-Tubulin, and RPS3 were the most abundant transcripts under almost all experimental conditions. The least frequently expressed reference genes were NADH, RPL17, and HSP83. The five remaining reference genes were expressed at moderate levels.

Expression Stability of Putative Reference Genes Under Biotic Conditions
For identifying stable reference genes, four different algorithms (geNorm, NormFinder, BestKeeper, and delta-Ct) were deployed to evaluate the stability of candidate reference genes under different experimental conditions ([i.e., developmental stages, tissue-specific, sex-specific] and different treatments [JHIII; wild-collected vs. long-term laboratory-reared; and different host feeding]) by using the RefFinder web-based tool that ranks reference genes.

Reference Gene for Developmental Stages
For the developmental stages, the order of stability of the first four most stable genes, namely β-Tubulin, eEF2, RPS3, and GAPDH, identified by four programs, was inconsistent (Table 2). The least stable genes nominated by four programs were Actin, RPL17, and HSP83. The most stable genes were β-Tubulin, eEF2, and RPS3 determined based on Normfinder rankings of 0.343, 0.347, and 0.379, respectively (Table 2). As per RefFinder, the stability ranking of the reference genes from most stable to least stable across different developmental stages were as follows: eEF2, β-Tubulin, Actin, UbiQ, GAPDH, V-ATPase-A, RPS3, Myosin L, HSP83, RPL17, ArgK, and NADH (Figure 1B). RefFinder identified the top three candidates, eEF2, β-Tubulin, and Actin, across developmental stages by integrating the results from all four programs.

Reference Gene for Tissue Stages
Target Gene Expression in Male and Female Tissues
Sex-specific reference gene expressions were calculated separately for male (callow and fed adult) and female (callow and fed adult) insect tissues (i.e., head, fat body, gut, WB except for the head, fat body, and gut collected from male and female, or in short, abdomen). In male tissues, the order of stability of the first four most stable genes (RPS3, GAPDH, eEF2, and β-Tubulin) determined by four programs was inconsistent (Table 3). The least stable genes recommended by four programs were Actin, RPL17, and Myosin L. The stability ranking of the reference genes of the most stable and the top three candidates, β-Tubulin, GAPDH, and RPS3, was constant according to RefFinder (Figure 2A). In contrast, the female genes eEF2, RPS3, β-Tubulin, and UbiQ were the most stable genes (Table 3). The least stable gene was ArgK. The top three most stable reference genes, namely eEF2, β-Tubulin, and RPS3, were constant for females in the RefFinder analysis (Figure 2B).

Target Gene Expression in Various Tissues
Tissue sections were obtained to evaluate the stability of candidate reference genes among different tissue (i.e., head, fat body, gut, and WB). In head tissues, the order of stability of the first four most stable genes (i.e., RPS3, eEF2, ArgK, and V-ATPase-A) recognized by four programs was inconsistent (Table 4). The least stable genes determined by four programs were GAPDH, RPL17, and HSP83. According to RefFinder, the stability ranking of reference genes from the most stable and the top three candidates were eEF2, NADH, and RPS3 within head tissues (Figure 2C). Whereas among fat body tissues, V-ATPase-A, eEF2, NADH, and β-Tubulin genes were the most consistently expressed (Table 4). The least stable genes in the gut were HSP83, Myosin L, and Actin. The final top three stable reference genes after RefFinder analysis were eEF2, V-ATPase-A, and GAPDH in gut tissues (Figure 2E). Similarly, WB tissue showed that RPS3, β-Tubulin, HSP83, and eEF2 genes were highly expressed and the most stable genes within these tissues (Table 4), while RPL17, UbiQ, and ArgK genes were found to be least stably expressed (Figure 2F). The best reference gene combination and stability ranking for WB tissues were GAPDH, RPS3, and Myosin L. The most stable reference gene among all four tissue stages was eEF2 after assessing all expression results. The optimal reference genes for all tissue stages combined (head, fat body, gut, and WB) conditions were β-Tubulin, eEF2, and RPS3 based on all algorithms (Supplementary Table 3).

Identification of Candidate Reference Genes Under Abiotic Conditions
The same four algorithms were used to identify the most appropriate reference genes under four different abiotic conditions such as temperature, JHIII treatment, laboratory-reared vs. wild beetles, and pine-fed vs. spruce-fed beetles. For
FIGURE 1 | (A) Expression range of cycle threshold (Ct) values of candidate reference genes under different experimental conditions in *I. sexdentatus* (ISx). The different conditions included in biotic conditions: developmental stages (i.e., larvae, pupae, and adult stages), sex-specific (male and female), tissue types [head, midgut, fat body, and whole body (WB) except head, gut, fat body (Abdomen)] and abiotic conditions: different treatments (such as temperature; Juvenile hormone III; wild-collected vs. long-term laboratory-reared; and different host feeding). (B) The average expression stability values (M) of 12 reference genes in different developmental stages (larvae, pupae, and adult stages) were plotted from the least stable (left) to the most stable (right).
the temperature treatment, \( \beta\)-Tubulin, V-ATPase-A, ArgK, and GAPDH genes were shown to have the most stable expression using the four algorithms (Table 5). RefFinder confirmed that the three most stable gene expressions were V-ATPase-A, ArgK, and \( \beta\)-Tubulin (Figure 3A). After JHIII treatment, the stability of the first four most stable genes, such as UbiQ, V-ATPase-A, RPS3, and Myosin L determined by four programs was inconsistent (Table 5). The least stable genes were found to be
Actin, RPL17, and NADH. According to RefFinder, the stability ranking of the reference genes of the most stable and the top three candidates were RPS3, HSP83, and UbiQ (Figure 3B). However, in beetles from wild and laboratory rearing conditions, most stable expressions were observed for Actin, ArgK, Myosin L, and GAPDH via ΔCt method (Table 5). The top three most stable reference genes via RefFinder were Actin, β-Tubulin, and V-ATPase-A (Figure 3C). Similarly, pine- and spruce-fed beetle gut showed stable expression for V-ATPase-A, UbiQ, ArgK, and β-Tubulin in RefFinder (Table 5 and Figure 3D). The overall stability ranking and combination of reference genes among various tested abiotic conditions were UbiQ, GAPDH, and β-Tubulin.

Determination of the Minimum Number of Reference Genes for Normalization

To generate more accurate and reliable gene expression results, often more than one reference gene is recommended. According to Vandesompele et al. (2002), a Vn/Vn+1 value under 0.15 means adding the n+1 reference gene is unnecessary. Alternatively, the first reference gene is sufficient to normalize the target gene expression in those cases. We also

FIGURE 2 | The average expression stability values (M) of 12 reference genes under different conditions (sex-specific and tissue types) were calculated by geNorm from the least stable (left) to the most stable (right). (A) male, (B) female, (C) head, (D) gut, (E) fat body, and (F) whole body (WB).

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### TABLE 4
Ranking of the candidate reference genes based on stability values performed by Delta Ct, BestKeeper, RefFinder, and NormFinder in various tissue types [head, midgut, fat body, and whole body (WB) except head, gut, and fat body (Abdomen)].

| Conditions | Genes | ΔCt method | Stability Rank | Best keeper | Stability Rank | RefFinder | Stability Rank | NormFinder | Stability Rank | Recommended genes |
|------------|-------|-------------|----------------|-------------|----------------|-----------|----------------|------------|----------------|---------------------|
| Head       | eEF2  | 1.21        | 1              | 0.6         | 4              | 2         | 1              | 0.302      | 7              | RPS3, ArgK and eEF2 |
|            | ArgK  | 1.33        | 2              | 0.72        | 6              | 2.91      | 3              | 0.266      | 4              |                     |
|            | V-ATPase-A | 1.39  | 3              | 0.55        | 3              | 3.41      | 5              | 0.314      | 9              |                     |
|            | RPS3  | 1.41        | 4              | 0.3          | 1              | 2.11      | 2              | 0.191      | 1              |                     |
|            | β-Tubulin | 1.49  | 5              | 1.52        | 8              | 5.79      | 6              | 0.314      | 10             |                     |
|            | Ubiquitin | 1.5    | 6              | 0.92        | 7              | 6.24      | 7              | 0.266      | 5              |                     |
|            | NADH  | 1.54        | 7              | 0.52        | 2              | 3.15      | 4              | 0.306      | 8              |                     |
|            | Myosin L | 1.63   | 8              | 1.71        | 10             | 8.46      | 8              | 0.257      | 3              |                     |
|            | Actin | 1.7        | 9              | 1.71        | 11             | 9.72      | 11             | 0.371      | 12             |                     |
|            | HSP83 | 1.71        | 10             | 1.71        | 9              | 9.72      | 10             | 0.283      | 6              |                     |
|            | GAPDH | 1.71        | 11             | 1.81        | 12             | 10.98     | 12             | 0.232      | 2              |                     |
|            | RPL17 | 1.74        | 12             | 0.68        | 5              | 9.64      | 9              | 0.335      | 11             |                     |
| Fat body   | NADH  | 0.97        | 1              | 1.18        | 6              | 2.21      | 1              | 0.363      | 9              |                     |
|            | eEF2  | 1           | 2              | 1.3         | 7              | 2.55      | 3              | 0.245      | 4              |                     |
|            | V-ATPase-A | 1        | 3              | 0.73        | 2              | 2.45      | 2              | 0.250      | 5              |                     |
|            | β-Tubulin | 1.07   | 4              | 1.13        | 5              | 2.99      | 4              | 0.332      | 8              |                     |
|            | GAPDH | 1.09        | 5              | 1.54        | 10             | 6.22      | 7              | 0.181      | 1              |                     |
|            | RPS3  | 1.11        | 6              | 0.99        | 4              | 5.63      | 6              | 0.294      | 6              |                     |
|            | Ubiquitin | 1.13   | 7              | 0.72        | 1              | 3.96      | 5              | 0.230      | 3              |                     |
|            | HSP83 | 1.16        | 8              | 1.47        | 9              | 8.24      | 9              | 0.227      | 2              |                     |
|            | ArgK  | 1.3         | 9              | 0.98        | 3              | 7.56      | 8              | 0.315      | 7              |                     |
|            | Actin | 1.31        | 10             | 1.71        | 11             | 9.97      | 10             | 0.606      | 11             |                     |
|            | Myosin L | 1.32   | 11             | 1.95        | 12             | 10.44     | 11             | 0.435      | 10             |                     |
|            | RPL17 | 1.39        | 12             | 1.39        | 8              | 10.84     | 12             | 0.660      | 12             |                     |
| Gut        | eEF2  | 1.18        | 1              | 1.54        | 8              | 3.13      | 3              | 0.117      | 1              |                     |
|            | V-ATPase-A | 1.18   | 2              | 1.15        | 7              | 1.93      | 1              | 0.130      | 2              |                     |
|            | NADH  | 1.24        | 3              | 1.09        | 6              | 3.57      | 4              | 0.270      | 7              |                     |
|            | RPL17 | 1.26        | 4              | 1.05        | 5              | 2.99      | 2              | 0.262      | 6              |                     |
|            | β-Tubulin | 1.48   | 5              | 2.21        | 9              | 6.51      | 8              | 0.215      | 3              |                     |
|            | ArgK  | 1.54        | 6              | 0.7         | 1              | 3.81      | 5              | 0.396      | 10             |                     |
|            | RPS3  | 1.54        | 7              | 0.81        | 2              | 4.6       | 6              | 0.283      | 9              |                     |
|            | GAPDH | 1.56        | 8              | 2.32        | 10             | 8.11      | 9              | 0.234      | 4              |                     |
|            | Ubiquitin | 1.7     | 9              | 0.93        | 3              | 6.42      | 7              | 0.339      | 5              |                     |
|            | Myosin L | 1.93    | 10             | 2.84        | 11             | 10.49     | 11             | 0.279      | 8              |                     |
|            | Actin | 1.97        | 11             | 2.92        | 12             | 11.49     | 12             | 0.459      | 12             |                     |
|            | HSP83 | 1.99        | 12             | 0.96        | 4              | 8.71      | 10             | 0.440      | 11             |                     |
| WB         | RPS3  | 1.14        | 1              | 2.06        | 6              | 2.34      | 2              | 0.192      | 3              |                     |
|            | Actin | 1.18        | 2              | 2.16        | 7              | 2.3       | 1              | 0.375      | 12             |                     |
|            | eEF2  | 1.19        | 3              | 2.27        | 8              | 2.91      | 3              | 0.247      | 8              |                     |
|            | GAPDH | 1.21        | 4              | 2.63        | 9              | 4.9       | 5              | 0.167      | 2              |                     |
|            | HSP83 | 1.23        | 5              | 2.04        | 5              | 4.4       | 4              | 0.201      | 4              |                     |
|            | β-Tubulin | 1.24   | 6              | 2            | 3              | 5.05      | 6              | 0.079      | 1              |                     |
|            | V-ATPase-A | 1.57   | 7              | 3.04        | 11             | 8.1       | 10             | 0.273      | 10             |                     |
|            | NADH  | 1.59        | 8              | 1.72        | 2              | 5.63      | 7              | 0.325      | 11             |                     |
|            | Myosin L | 1.62   | 9              | 3.08        | 12             | 9.39      | 11             | 0.233      | 7              |                     |
|            | RPL17 | 1.75        | 10             | 2.01        | 4              | 7.95      | 9              | 0.215      | 6              |                     |
|            | Ubiquitin | 1.81   | 11             | 1.36        | 1              | 6.04      | 8              | 0.259      | 9              |                     |
|            | ArgK  | 1.93        | 12             | 2.95        | 10             | 11.47     | 12             | 0.206      | 5              |                     |
TABLE 5 | Ranking of the candidate reference genes based on stability values performed by Delta Ct, BestKeeper, RefFinder, and NormFinder under the influence of various abiotic factors such as temperature (Temp), juvenile hormone III (JHIII), laboratory-reared vs. wild beetles, and pine-fed vs. spruce-fed beetles.

| Conditions | Genes      | ΔCt method | Best keeper | RefFinder | NormFinder | Recommended genes |
|------------|------------|------------|-------------|-----------|------------|-------------------|
| Temp       | β-Tubulin  | 0.98       | 1           | 1.09      | 5          | 1.5               | 1 | 0.322       | 4 | β-Tubulin, V-ATPase-A and ArgK |
|            | V-ATPase-A | 1.02       | 2           | 0.99      | 4          | 2                | 2 | 0.315       | 3 | 
|            | ArgK       | 1.06       | 3           | 1.37      | 7          | 3.98              | 4 | 0.252       | 2 | 
|            | GAPDH      | 1.07       | 4           | 0.93      | 3          | 3.66              | 3 | 0.356       | 5 | 
|            | Myosin L   | 1.08       | 5           | 1.61      | 9          | 5.73              | 6 | 0.251       | 1 | 
|            | Actin      | 1.13       | 6           | 1.29      | 6          | 5.73              | 5 | 0.519       | 7 | 
|            | eEF2       | 1.17       | 7           | 1.5       | 8          | 7.24              | 9 | 2.051       | 12| 
|            | UbiQ       | 1.19       | 8           | 1.69      | 10         | 8.46              | 10| 0.441       | 6 | 
|            | RPS3       | 1.43       | 9           | 0.88      | 2          | 6.18              | 8 | 0.614       | 8 | 
|            | NADH       | 1.59       | 10          | 1.79      | 11         | 10.24             | 11| 0.668       | 10| 
|            | RPL17      | 1.94       | 11          | 0.86      | 1          | 6.04              | 7 | 0.630       | 9 | 
|            | HSP83      | 2.6        | 12          | 2.73      | 12         | 12.12             | 12| 1.446       | 11| 
| JHIII      | V-ATPase-A | 0.85       | 1           | 0.54      | 3          | 2.34              | 2 | 0.225       | 2 | 
|            | UbiQ       | 0.87       | 2           | 0.45      | 2          | 1.86              | 1 | 0.208       | 1 | 
|            | Myosin L   | 0.9        | 3           | 0.58      | 4          | 2.45              | 3 | 0.432       | 10| 
|            | ArgK       | 0.92       | 4           | 0.65      | 5          | 3.16              | 5 | 0.428       | 9 | 
|            | β-Tubulin  | 0.93       | 5           | 0.75      | 8          | 6.16              | 6 | 0.294       | 3 | 
|            | RPS3       | 0.93       | 6           | 0.37      | 1          | 3.13              | 4 | 0.297       | 4 | 
|            | eEF2       | 0.97       | 7           | 0.69      | 7          | 7.24              | 7 | 0.376       | 8 | 
|            | GAPDH      | 1.03       | 8           | 0.87      | 10         | 8.18              | 8 | 0.350       | 7 | 
|            | HSP83      | 1.06       | 9           | 0.89      | 11         | 9.72              | 10| 0.314       | 5 | 
|            | Actin      | 1.08       | 10          | 0.69      | 6          | 8.78              | 9 | 0.510       | 12| 
|            | RPL17      | 1.11       | 11          | 0.84      | 9          | 10.22             | 11| 0.336       | 6 | 
|            | NADH       | 2.55       | 12          | 1.78      | 12         | 12.12             | 12| 0.436       | 11| 
| Lab vs. Wild | Actin     | 0.9        | 1           | 1.68      | 9          | 3                | 2 | 0.068       | 2 | Actin, Myosin L and ArgK |
|            | Myosin L   | 0.9        | 2           | 1.26      | 5          | 2.66              | 1 | 0.088       | 6 | 
|            | ArgK       | 0.95       | 3           | 1.09      | 4          | 3.46              | 4 | 0.086       | 4 | 
|            | GAPDH      | 0.95       | 4           | 1.65      | 8          | 3.36              | 3 | 0.103       | 7 | 
|            | UbiQ       | 1.03       | 5           | 1.64      | 7          | 3.64              | 5 | 0.182       | 9 | 
|            | β-Tubulin  | 1.05       | 6           | 2.03      | 10         | 6.4               | 6 | 0.042       | 1 | 
|            | RPS3       | 1.06       | 7           | 1.44      | 6          | 6.48              | 8 | 0.221       | 10| 
|            | V-ATPase-A | 1.18       | 8           | 0.71      | 3          | 6.62              | 9 | 0.085       | 5 | 
|            | HSP83      | 1.23       | 9           | 2.28      | 11         | 9.19              | 11| 0.072       | 3 | 
|            | eEF2       | 1.49       | 10          | 2.45      | 12         | 10.44             | 12| 0.219       | 11| 
|            | RPL17      | 1.49       | 11          | 0.48      | 2          | 7.01              | 10| 0.124       | 8 | 
|            | NADH       | 1.98       | 12          | 0.47      | 1          | 6.45              | 7 | 0.282       | 12| 
| Pine vs. Spruce | UbiQ     | 0.65       | 1           | 0.52      | 4          | 2                | 2 | 0.102       | 2 | V-ATPase-A, UbiQ and ArgK |
|            | V-ATPase-A | 0.71       | 2           | 0.29      | 1          | 1.86              | 1 | 0.155       | 4 | 
|            | GAPDH      | 0.73       | 3           | 0.61      | 8          | 4.68              | 5 | 0.072       | 1 | 
|            | ArgK       | 0.75       | 4           | 0.31      | 2          | 2.21              | 3 | 0.193       | 7 | 
|            | Myosin L   | 0.75       | 5           | 0.5       | 3          | 2.94              | 4 | 0.157       | 5 | 
|            | RPS3       | 0.79       | 6           | 0.59      | 6          | 6                | 6 | 0.179       | 6 | 
|            | eEF2       | 0.82       | 7           | 0.52      | 5          | 6.85              | 7 | 0.202       | 8 | 
|            | NADH       | 0.86       | 9           | 0.8       | 11         | 8.89              | 9 | 0.149       | 3 | 
|            | Actin      | 1.05       | 10          | 0.77      | 10         | 10                | 10| 0.212       | 9 | 
|            | RPL17      | 1.3        | 11          | 0.74      | 9          | 10.46             | 11| 0.393       | 12| 
|            | HSP83      | 1.36       | 12          | 0.8       | 12         | 12                | 12| 0.327       | 11|
calculated the optimal reference gene number based on geNorm algorithm analysis for each condition. We found that at least two reference genes were required for the head, female tissues, and temperature conditions based on the pairwise values (Figures 4A,B).

Validation of Reference Gene Selection
Krüppel-homolog 1 (Kr-h1) encodes a key transcription factor and plays a critical role in regulating insect metamorphosis within the juvenile hormone signaling pathway (Li et al., 2018a; Roy and Palli, 2018). The relative expression of Kr-h1 in response to the JHIII treatment was normalized with single reference genes or gene combinations recommended by geNorm (Figures 4A,B). The two most stable reference genes, individually and in combination, and the least stable gene were used in this experiment. The results showed that the Kr-h1 gene was expressed in male and female beetles (Figure 5A). The expression levels of Kr-h1 normalized with NADH (least stable) in males reduced from 2.1 to 0.6 fold, and females were increased from 1.8 to 3.8 fold higher than those of Kr-h1 normalized with stable reference gene or gene combinations, respectively. Similarly, HSP70 is a key protein closely related to the molecular mechanism underlying insect resistance to the environment (Štětina et al., 2015). The relative expression of Hsp70 showed a stable expression difference after normalizing with the single and most stable reference gene combination during temperature exposure. On the contrary, the Hsp70 gene expression level after normalizing with the least stable reference gene showed higher variation after different temperature incubation (Figure 5B). Henceforth, the most stable genes used for normalization, either individually or in combination, resulted in more consistent and trustworthy target gene expression patterns.

In addition, Kr-h1 gene expression was evaluated for the various developmental stages to validate the reference gene findings taking the most and least stable gene for expression normalization. Results showed that the expression level of Kr-h1 in the second instar larvae was almost 4-fold higher than the first instar larvae when normalization was performed based on the most stable reference gene (β-Tubulin) (Figure 5C). In contrast, when the least stable reference gene (RPL17) was used for normalization, the Kr-h1 expression was considerably low. Furthermore, normalization with β-Tubulin resulted in lower expression in all stages except second instar larvae signifying the importance of having optimal reference genes for expression normalization.

DISCUSSIONS
The wood-boring coleopteran pest, ISx, is one of the most destructive forest pests, causing severe damage to coniferous...
An optimal number of reference genes for the normalization of ISs under selected extrinsic experimental conditions. Based on geNorm analysis, average pairwise variations were calculated between the normalization factors $N_F^n$ and $N_F^{n+1}$. Values $<0.15$ indicate that $n + 1$ genes were not required for the normalization of gene expression.

(A) Biotic conditions: developmental stages (i.e., larvae, pupae, and adult stages), tissue types [head, midgut, fat body, and whole body (WB) except head, gut, fat body] and (B) Abiotic conditions: different treatments (temperature; Juvenile hormone III; wild-collected vs. long-term laboratory-reared; and different host feeding). Values $<0.15$ indicate that additional genes are not necessary for normalization.
FIGURE 5 | Validation of the recommended reference genes. The relative expression levels of the target genes Kr-h1 and Hsp70 were studied under juvenile hormone III treatment and temperature treatment by normalizing the selected reference gene. The most stable reference gene is UbiQ and V-ATPase-A for juvenile (Continued)
Accurate normalization using a stable reference gene is necessary to conduct gene expression studies under specific experimental conditions and to avoid erroneous differences in target gene expression. The results obtained in this study indicate that the stability of reference genes in ISx differ under various experimental conditions, including developmental stage, sex, and tissue-specific conditions, and exposure to abiotic conditions. The reference gene was consistently the most stable in the developmental stage, sex-specific, and tissue-specific conditions tested in ISx. The most stable reference genes in ISx were ArgK, eEF2, and V-ATPase-A for temperature incubation; UbiQ, V-ATPase-A, and eEF2 for developmental and tissue stages of Mythimna separata (Li et al., 2018c). Nevertheless, our results demonstrated stable expression of V-ATPase-A in the fat body and gut tissues of ISx. Experiments on ISx involving alteration of abiotic conditions revealed a varied set of genes as references, such as V-ATPase-A, ArgK, and β-Tubulin for temperature incubation; UbiQ, V-ATPase-A, and RPS3 for JHIII treatment; Actin, Myosin L, and β-Tubulin in between laboratory vs. wild beetles, and V-ATPase-A, UbiQ, and ArgK among pine vs. spruce-fed beetle gut tissues (Table 5 and Figure 3). The reference gene V-ATPase-A was used previously for normalization of Mythimna separata gene expression after temperature treatment (Li et al., 2018c), whereas β-Tubulin was applied for a similar purpose for Bemisia tabaci (Dai et al., 2017); Phenacoccus solenopsis (Arya et al., 2017);
Amphitetranychus viennensis (Yang et al., 2019); and Hippodamia variegata (Xie et al., 2021).

In the present study, host plants among all treatments caused the highest expression variations of the reference genes. In the dataset of gut samples collected from the adults fed on different plants (i.e., pine and spruce), the stability ranking of tested reference genes was different according to five algorithms. The most stable reference gene was UbiQ and GAPDH according to the geNorm algorithm (Figure 3D). However, V-ATPase-A, UbiQ, and ArgK were the most stable reference genes evaluated by the other four algorithms (delta Ct, BestKeeper, RefFinder, and NormFinder, respectively) (Table 5). Interestingly, similar studies in other arthropods have not found such dramatic variations so far (Arya et al., 2017). Furthermore, in laboratory-reared and wild-collected beetle gut tissues, Actin, Myosin L, and ArgK were the most stable reference genes after delta Ct, BestKeeper, RefFinder, and NormFinder analysis (Table 5), which differed from the geNorm algorithm analysis (Figure 3C). In our opinion, plant diet causes drastic changes at the gene expression level in ISx, hence higher variation in the reference gene expression.

Further, the expression of Kr-h1 and Hsp70 was evaluated in various developmental stages and respective treatments (JHIII and temperature) to corroborate the suitability of the identified reference genes. Although, the results demonstrated that the expression trends in different conditions were accordant using various reference gene or gene combinations. The Kr-h1 gene expression was induced by JHIII, as expected from similar expression trends in different conditions were accordant using various reference gene or gene combinations. The Kr-h1 gene expression was induced by JHIII, as expected from similar expression trends in different conditions. Furthermore, the expression of Kr-h1 and Hsp70 was evaluated in various developmental stages and respective treatments (JHIII and temperature) to corroborate the suitability of the identified reference genes. Although, the results demonstrated that the expression trends in different conditions were accordant using various reference gene or gene combinations. The Kr-h1 gene expression was induced by JHIII, as expected from similar expression trends in different conditions. Furthermore, the expression of Kr-h1 and Hsp70 was evaluated in various developmental stages and respective treatments (JHIII and temperature) to corroborate the suitability of the identified reference genes. Although, the results demonstrated that the expression trends in different conditions were accordant using various reference gene or gene combinations. The Kr-h1 gene expression was induced by JHIII, as expected from similar expression trends in different conditions.

TABLE 6 | Summary of the recommended reference genes under different experimental conditions.

| Conditions                              | Recommendation |
|-----------------------------------------|----------------|
| **Biotic conditions**                   |                |
| Developmental stage                     | β-Tubulin, eEF2, and RPS3 |
| Sex-specific tissues                    |                |
| Male                                    | RPS3, GAPDH, and eEF2 |
| Female                                  | eEF2, RPS3, and β-Tubulin |
| Tissue                                  |                |
| Head                                    | RPS3, ArgK, and eEF2 |
| Fat body                                | V-ATPase-A, eEF2, and NADH |
| Gut                                     | V-ATPase-A, eEF2, and RPL17 |
| Abdomen (WB)                            | RPS3, β-Tubulin, and HSP83 |
| Between tissues                         | β-Tubulin, RPS3, and eEF2 |
| **Abiotic conditions**                  |                |
| Temperature                             | β-Tubulin, V-ATPase-A, and ArgK |
| Juvenile hormone III                    | UbIQ, V-ATPase-A, and RPS3 |
| Laboratory-reared vs. wild beetles      | Actin, Myosin L, and ArgK |
| Pine-fed vs. spruce-fed                 | V-ATPase-A, UbIQ, and ArgK |

In conclusion, temperate and boreal forests have recently undergone unprecedented pressure from bark beetle outbreaks, reducing forest biodiversity and their role in global carbon sequestration. It also affects their economic value and endangered wildlife habitat. Current management approaches are proven progressively deficient against outbreaking bark beetle populations, urging investigations into novel mitigation strategies using state-of-the-art molecular methodologies. Future gene expression and functional genomics studies are crucial to alleviate the ongoing bark beetle-mediated forest depletion. Hence, dedicated reference gene selection studies are of utmost importance in economically important bark beetles. The present work represents the first reference gene validation study in I. sexdentatus, an ecologically important wood-boring beetle. It provides valuable information on reference genes (Table 6) for future molecular studies (i.e., DGE studies) on host-beetle interactions and functional genomic studies (i.e., RNAi) on this bark beetle and similar Ips beetles (Coleoptera: Curculionidae: Scolytinae).
DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s. The reference gene sequences used in the study were submitted under the NCBI accession numbers from OK143445 to OK143455.

AUTHOR CONTRIBUTIONS

GS, AG, and AR conceived and designed the research and prepared the final manuscript. SA, RM, and JS collected, sorted, and dissected beetles. JB and SA prepared samples for in-house transcriptome. GS conducted real-time experiments and wrote the first draft. GS and MKS analyzed the data. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.752768/full#supplementary-material
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