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Selection of Reference Genes for the Normalization of RT-qPCR Data in Gene Expression Studies in Insects: A Systematic Review

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Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is a reliable technique for quantifying expression levels of targeted genes during various biological processes in numerous areas of clinical and biological research. Selection of appropriate reference genes for RT-qPCR normalization is an elementary prerequisite for reliable measurements of gene expression levels. Here, by analyzing datasets published between 2008 and 2017, we summarized the current trends in reference gene selection for insect gene expression studies that employed the most widely used SYBR Green method for RT-qPCR normalization. We curated 90 representative papers, mainly published in 2013–2017, in which a total of 78 insect species were investigated in 100 experiments. Furthermore, top five journals, top 10 frequently used reference genes, and top 10 experimental factors have been determined. The relationships between the numbers of the reference genes, experimental factors, analysis tools on the one hand and publication date (year) on the other hand was investigated by linear regression. We found that the more recently the paper was published, the more experimental factors it tended to explore, and more analysis tools it used. However, linear regression analysis did not reveal a significant correlation between the number of reference genes and the study publication date. Taken together, this meta-analysis will be of great help to researchers that plan gene expression studies in insects, especially the non-model ones, as it provides a summary of appropriate reference genes for expression studies, considers the optimal number of reference genes, and reviews the average number of experimental factors and analysis tools per study.

Keywords: RT-qPCR, reference genes, SYBR green method, experimental factors, analysis tools

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INTRODUCTION

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) is a premier molecular biology tool and a powerful method for quantification of gene expression levels in real-time (Vandesompele et al., 2002). Although RT-qPCR is one of the most efficient, reliable, and reproducible techniques to quantify gene expression, multiple factors, including the quality and integrity of RNA samples, efficiency of cDNA synthesis, and PCR efficiency, can significantly influence signal normalization (Bustin et al., 2005; Strube et al., 2008). RT-qPCR generally involves

normalization of expression levels of multiple genes to the expression levels of a suite of stable reference genes. Even though reference gene transcript levels should ideally be stable across a range of different conditions, previous studies have shown that expression of many commonly used reference genes differs dramatically under different treatment conditions (Kalushkov and Hodek, 2004; Bustin et al., 2013). It is clear that the expression level of many reference genes is condition-specific and accordingly, there is no universal gene that can be used for internal control for all application scenarios, strongly indicating the necessity of conducting custom reference gene selection for RT-qPCR analyses on a case-by-case basis, even for the same species.

Over the last 10 years, RT-qPCR has been increasingly used in genome/transcriptome expression studies in insect species. Furthermore, considerable advancements have been made for identification and validation of appropriate reference genes across various biotic and abiotic experimental conditions in many insect species (**Table 1**). In RT-qPCR experiments, SYBR Green and TaqMan probes have been the two most frequently used methodologies, with the SYBR Green method being utilized much more frequently. Here, we have summarized only the studies that used the SYBR Green method. It is well known that characterization of reference genes is an onerous task requiring well-designed molecular experiments followed by elaborate computational analyses (Andersen et al., 2004; Pfaffl et al., 2004). Therefore, a comprehensive summary of published sets of experimentally validated reference genes in conjunction with the description of relevant experimental conditions and analysis tools would be timely (Sang et al., 2017).

In order to fill this gap and provide molecular biologists with informative guidance on selecting the reference genes to customize their RT-qPCR experiments, this present review summarizes the current trends in reference gene selection for RT-qPCR normalization in gene expression studies performed on insects between 2008 and 2017 (**Table 1**). Specifically, the insect species, reference genes, experimental conditions, analysis tools, and publication year have been summarized. Furthermore, the relationships between the numbers of the reference genes, experimental factors, analysis tools, and publication date (year) were investigated by linear regression. We hoped that our meta-analysis would be of great help for researchers that plan gene expression studies in insects, especially the non-model ones, as it provides a summary of appropriate reference genes for expression studies, considers the optimal number of reference genes, and reviews average numbers of experimental factors and analysis tools per study.

NUMBER OF RELEVANT STUDIES IN INSECTS THAT UTILIZED EXPRESSION LEVELS OF REFERENCE GENES FOR NORMALIZATION OF RT-QPCR DATA

The relevant publications that analyzed reference gene expression in insects in 2008–2017 are summarized in **Table 1**. All data were extracted from databases such as https://www.ncbi.nlm.nih.gov/pubmed, https://scholar.google.com/, https://link.springer.com/, http://onlinelibrary.wiley.com/, and https://www.sciencedirect.com/ using the following search terms: ("internal control genes" OR "reference genes" OR "housekeeping genes") AND ("qPCR" OR "quantitative PCR" OR "qRT-PCR" OR "RT-qPCR") occurring in the Title/Abstract. Additionally, we also curated relevant papers that came to our attention independently but were not uncovered by the above search algorithm. We found and curated 90 representative papers published in 36 journals. The top five journals by the number of published studies on gene expression in insects were PLoS One (26/90), Scientific Reports (9/90), Journal of Economic Entomology (6/90), Journal of Insect Science (5/90), and BMC Research Notes (4/90; **Table 1**). These papers were mainly published between 2013 and 2017 with an average of 14 papers published over the last 5 years (**Figure 1A**). We can clearly see that open access journals provide the main platform for publications on this topic.

NUMBER OF INSECT SPECIES THAT WERE ANALYZED FOR EXPRESSION OF REFERENCE GENES

The 90 reviewed papers reported results of gene expression studies in 78 insect species in 100 separate experiments (**Table 1**). These insects were from 10 insect orders (**Figure 1B**). They predominantly belonged to the following four insect orders: Hemiptera (25 insect species), Lepidoptera (16 insect species), Coleoptera (12 insect species), and Diptera (13 insect species; **Figure 1B**). Some insects, such as *Bemisia tabaci* (Li et al., 2013; Su et al., 2013; Collins et al., 2014; Liang et al., 2014; Dai et al., 2017; Lü et al., 2017) and *Helicoverpa armigera* (Chandra et al., 2014; Shakeel et al., 2015; Zhang et al., 2015), which cause serious damage to crops, were investigated extensively and frequently. There were six and three papers, respectively, for the above-mentioned species that analyzed expression levels of reference genes and were published during the last 5 years.

DISTRIBUTION OF THE NUMBER OF REFERENCE GENES PER STUDY

In the 90 papers, 3–21 reference genes were investigated per single study (**Figure 2**). In the majority of studies, the expression level of 5–10 reference genes was determined (**Figure 2A**). The breakdown of the papers that analyzed expression of multiple reference genes was as follows: five genes (10%), six genes (16%), seven genes (14%), eight genes (15%), nine genes (14%), and ten genes (10%). Recently, in some studies, more than 10 candidate reference genes were analyzed to provide more choices for expression level comparisons and normalization (**Table 1**). However, linear regression analysis did not reveal a significant correlation between the number of reference genes used in the study and its publication date (year; **Figure 2B**).

TABLE 1 | Summary of the reference gene studies in insects from 2008 to 2017.

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
COLEOPTERA				
Leptinotarsa decemlineata	*Actin1, Actin2, ARF1, ARF4, TATA1, TATA2, RPL4, RPL8, EF1A*	Developmental stage, tissue, insecticide	*geNorm, Normfinder, BestKeeper*	Shi et al., 2013
Diabrotica virgifera virgifera	*Actin, EF1A, RPS9, GAPDH, β-tubulin*	Developmental stage, tissue, dsRNA exposure, Bt toxin exposure	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Rodrigues et al., 2013
Hippodamia convergens	*28S, 18S, Actin, EF1A, GAPDH, CypA, V-ATPase A*	Developmental stage, tissue, sex, temperature, photoperiod, dsRNA exposure	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Pan et al., 2015b
Coccinella septempunctata	*28S, 18S, 16S, NADH, EF1A, Actin, α-tubulin, ArgK*	Developmental stage, tissue, dsRNA exposure	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Yang et al., 2016
Coleomegilla maculata	*28S, 18S, 16S, 12S, Actin, EF1A, GAPDH, ArgK, V-ATPase A, RPS24, HSP70, HSP90, a-tubulin, NADH, RPS18, RPL4*	Developmental stage, tissue, dsRNA exposure	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Yang et al., 2015c
Tribolium castaneum	*Actin, RPS3, RPS6, RPS18, RPS13, E-cadherin, Syntaxin1, Syntaxin6*	Fungal infection	*geNorm, Normfinder*	Lord et al., 2010
	Actin, GAPDH, RPL13, RPS3, RPS6, RPS18, E-cadherin, Syntaxin1, Syntaxin6	Developmental stage, tissue	*geNorm, Normfinder*	Toutges et al., 2010
	Actin, β-tubulin, GAPDH, RPS3, RPL13, RPS18, E-cadherin	Developmental stage, UV irradiation	*geNorm, Normfinder, BestKeeper*	Sang et al., 2015
Galeruca daurica	*Actin, GAPDH, GST, RPL32, SDHA, TATA, α-tubulin, β-tubulin, HSP70, CYP6*	Developmental stage, tissue, sex, temperature, diapause, and non-diapause adults	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Tan et al., 2017
Agrilus planipennis	*Actin, β-tubulin, GAPDH, RPL7, EF1A, UBQ*	Developmental stage, tissue	*geNorm, Normfinder, BestKeeper,*	Rajarapu et al., 2012
Mylabris cichorii	*RPL22, RPL13, RPS27, Actin, β-tubulin, UBC, UBE2C, UBE3A, EF1A, TATA*	Sex	*geNorm, Normfinder*	Wang Y. et al., 2014
Colaphellus bowringi	*GAPDH, RPL32, RPL19, EF1A, TATA, TATA1, Actin1, Actin2, α-tubulin, α-tubulin 1, β-tubulin*	Developmental stage, sex, population, photoperiod	*geNorm, Normfinder, BestKeeper, RefFinder*	Tan et al., 2015
Cryptolestes ferrugineus	*SDHA, Cyclin A, γ-tubulin, α-tubulin, EF1A, GAPDH, RPL13, RPS13, Actin*	Developmental stage, population	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Tang et al., 2017
Anoplophora glabripennis	*SDFS, UBQ, Tubulin, RPL32, GAPDH, EF1A*	Developmental stage, tissue	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Rodrigues et al., 2017
LEPIDOPTERA				
Danaus plexippus	*28S, 18S, EF1A, GAPDH, NADH, CypA, V-ATPase A, RPS5, RPL32*	Developmental stage, tissue, sex, temperature, photoperiod, dsRNA exposure	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Pan et al., 2015a
Chilo suppressalis	*18S, Actin, α-tubulin, EF1A, Histone 3, RPS11, NADH, UBI, HSP60*	Tissue, organ, temperature	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Xu et al., 2017
	Actin A3, Actin A1, GAPDH, G3PDH, E2F, RPL32	Developmental stage, tissue	*geNorm, NormFinder, stability index, ΔCt analysis*	Teng et al., 2012

(Continued)

TABLE 1 | Continued

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
Spodoptera litura	*EF1A, GAPDH, RPS3, RPL10, Actin, β-FTZ-F1, UCCR, ArgK*	Developmental stage, tissue, population, temperature, insecticide, diet, starvation	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Lu et al., 2013
Spodoptera exigua	*Actin1, Actin2, EF1A, EF2, GAPDH, RPL10, RPL17, SOD, α-tubulin, 18S*	Developmental stage, tissue, sex	*geNorm, NormFinder, BestKeeper*	Zhu et al., 2014
	Actin A3, Actin A1, GAPDH, G3PDH, E2F, RPL32	Developmental stage, tissue	*geNorm, NormFinder, stability index, ΔCt analysis*	Teng et al., 2012
Helicoverpa armigera	*18S, 28S, Actin1, Actin2, α-tubulin, β-tubulin, GAPDH, EF1A, RPL13, RPS15, RPL27, RPL32*	Developmental stage, tissue, virus, insecticide, temperature	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Zhang et al., 2015
	β-tubulin, TATA, RPS15, HSP90, GAPDH, RPL28, ArgK, GST, Actin	Developmental stage, mechanical injury, temperature, starvation, photoperiod	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Shakeel et al., 2015
	18S, β-tubulin, EF1A, GAPDH, Actin	Developmental stage, dsRNA exposure	*geNorm, Normfinder, BestKeeper*	Chandra et al., 2014
Sesamia inferens	*18S, EF1A, GAPDH, RPS13, RPS20, tubulin, Actin*	Developmental stage, tissue, sex, temperature	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Sun et al., 2015
Plutella xylostella	*18S, Actin, GAPDH, RPL32, RPS13, EF1A, RPS20, RPS23*	Development stage, tissue, population, temperature, photoperiod, insecticide, mechanical injury	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Fu et al., 2013
	Actin A3, Actin A1, GAPDH, G3PDH, E2F, RPL32	Developmental stage, tissue	*geNorm, NormFinder, stability index, ΔCt analysis*	Teng et al., 2012
Bombyx mori	*Actin A3, Actin A1, GAPDH, G3PDH, E2F, RPL32*	Developmental stage, tissue	*geNorm, NormFinder, stability index, ΔCt analysis*	Teng et al., 2012
	Actin1, Actin3, GAPDH, TIF-4A	Virus, temperature	*ΔC_t method*	Guo et al., 2016
Cryptophlebia peltastica	*Actin, EF1A, α-tubulin, ArgK, CO1, Enolase*	Tissue	*geNorm, Normfinder, BestKeeper*	Ridgeway and Timm, 2015
Cydia pomonella	*Actin, EF1A, α-tubulin, ArgK, CO1, Enolase*	Tissue	*geNorm, Normfinder, BestKeeper*	Ridgeway and Timm, 2015
Thaumatotibia leucotreta	*Actin, EF1A, α-tubulin, ArgK, CO1, Enolase*	Tissue, temperature, virus	*geNorm, Normfinder, BestKeeper*	Ridgeway and Timm, 2015
Gynaephora	*18S, 28S, Actin1, Actin2, ArgK, Cyclin A, EF1A, GAPDH, RPL10, RPL27, RPL28, RPS15, RPS13, RPS2, Troponin C, β-tubulin, α-tubulin*	Population	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Zhang et al., 2017
Bicyclus anynana	*Actin, EF1A, FK506, GAPDH, RPL40, V-ATPase H, RPS8, RPS18, HSP20, TATA, eIF2, G6PDH*	Developmental stage, tissue, sex, diet	*geNorm, Normfinder*	Arun et al., 2015
Thitarodes armoricanus	*18S, Actin, β-tubulin, GAPDH, G6PDH, EF2, EIF4A, RPL13*	Developmental stage, tissue, temperature, fungal infection, diet	*geNorm, Normfinder, BestKeeper*	Liu et al., 2016
Heliconius numata	*Actin, Annexin, EF1A, FK506BP, PolyABP, UBQ, RPL3, RPS3A, Tubulin*	Developmental stage	*geNorm, Normfinder, BestKeeper*	Piron Prunier et al., 2016

(Continued)

TABLE 1 | Continued

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
Musca domestica	*18S, Actin, EF1A, RPS18, GAPDH*	Developmental stage, mechanical injury, bacterial challenge	*geNorm, Normfinder, BestKeeper*	Zhong et al., 2013
HEMIPTERA				
Bemisia tabaci	*HSP40, HSP20, HSP70, HSP90, V-ATPase A, RPL29, EF1A, SDHA, Actin, PPIA, GAPDH, Myosin L, NADH, γ-tubulin*	Biotype, virus	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Lü et al., 2017
	18S, Actin, HSP20, HSP40, HSP70, HSP90, γ-tubulin, RPL29, SDHA, Flavoprotein, GAPDH, EF1A, PPIA, NADH, Myosin L, V-ATPase A	Developmental stage, tissue, virus, biotype, photoperiod, temperature, insecticide	*geNorm, NormFinder*	Li et al., 2013
	18S, Actin, a-tubulin, EF1A, GAPDH, RPL13, Cyclophilin1, TATA	Insecticide	*geNorm, NormFinder, RefFinder*	Liang et al., 2014
	Actin, GAPDH, GST, RPL32, SDHA, TATA, UBQ, a-tubulin	Developmental stage, organ, insecticide, bacterial challenge	*geNorm, NormFinder*	Su et al., 2013
	18S, GST, Actin, GAPDH, β-tubulin, a-tubulin, RPL13, EF1A	Temperature	*geNorm, Normfinder, BestKeeper*	Dai et al., 2017
	Actin, EF1A, GAPDH, RPL13, a-tubulin, Cyclophilin1	Developmental stage, tissue, temperature	*geNorm, Normfinder, BestKeeper*	Collins et al., 2014
Acyrthosiphon pisum	*18S, 28S, 16S, Actin, EF1A, TATA, RPL12, β-tubulin, NADH, v-ATPase A, SDHB*	Developmental stage, temperature	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Yang C. et al., 2014
Lipaphis erysimi	*16S, SDHB, Actin, EF1A, RPL13, RPS18, RPL27, RPL29, β-tubulin, GAPDH, ArgK*	Developmental stage, temperature, starvation, diet, glucosinolate	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Koramutla et al., 2016
Aphis glycines	*SDFS, EF1A, Helicase, GAPDH, RPS9, TATA, UBQ*	Developmental stage, tissue, host plant	*geNorm, NormFinder*	Bansal et al., 2012
Aphis craccivora	*18S, 12S, EF1A, RPL11, V-ATPase D, RPL14, RPS8, RPS23, NADH, HSP70*	Developmental stage, temperature	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Yang et al., 2015b
Aphis gossypii	*18S, 28S, Actin, GAPDH, EF1A, RPL7, α-tubulin, TATA*	Developmental stage, population, temperature, diet	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Ma et al., 2016
Myzus persicae	*18S, Actin, RPL27, RPL7, β-tubulin, GAPDH, Acetylcholinesterase, EF1A, RPL32*	Development stage, tissue, host plant, wing dimorphism, photoperiod, temperature, insecticide	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Kang et al., 2017
Rhopalosiphum padi	*18S, EF1A, Actin, GAPDH*	Wing dimorphism, virus	*geNorm, Normfinder, BestKeeper*	Wu et al., 2014
Megoura viciae	*RPL3, NADH, SDHA, RPS9, TATA, Actin, β-tubulin, UBQ*	Developmental stage	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Cristiano et al., 2016
Toxoptera citricida	*18S, Actin, EF1A, GAPDH, α-tubulin, β-tubulin, RNAP II*	Developmental stage, wing dimorphism, temperature, starvation, UV irradiation	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Shang et al., 2015
Diuraphis noxia	*Actin, RPL27, RPL9, RPL5, EF1A*	Host plant	*geNorm, Normfinder, BestKeeper*	Sinha and Smith, 2014

(Continued)

TABLE 1 | Continued

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
Diaphorina citri	*EF1A, Actin, α-tubulin, GAPDH, RPL7, RPL17*	Developmental stage, host plant	*geNorm, Normfinder, BestKeeper, ΔCt method, RefFinder*	Bassan et al., 2017
Toxoptera citricida	*18S, EF1A, α-tubulin, β-tubulin, Actin, GAPDH, RNAP II*	Developmental stage, wing dimorphism, temperature, starvation, UV irradiation	*geNorm, Normfinder, BestKeeper, ΔCt method, RefFinder*	Shang et al., 2015
Rhodnius prolixus	*Actin, α-tubulin, GAPDH, GST, G6PDH, SDHA, SP, EIF1A*	Developmental stage, aging, nutrition	*geNorm, Normfinder*	Omondi et al., 2015
Rhodnius prolixus	*18S, GAPDH, Actin, α-tubulin, RPL26*	Tissue, diet, virus	*geNorm, Normfinder, BestKeeper*	Paim et al., 2012
	18S, EF1A, GAPDH, HSP70, Actin, Elav, MIP	Organ, *Trypanosoma cruzi* infection	*geNorm, Normfinder*	Majerowicz et al., 2011
Nilaparvata lugens	*18S, Actin 1, Muscle actin, RPS11, RPS15, α-tubulin, EF1Δ, ArgK*	Developmental stage, tissue, population, temperature, insecticide, diet, starvation	*geNorm, Normfinder, BestKeeper, ΔCt method*	Yuan et al., 2014
	18S, Actin, α-tubulin, β-tubulin, EF1A, ETIF1	Host plant, population	*geNorm, Normfinder, BestKeeper*	Wang W. X. et al., 2014
Sogatella furcifera	*18S, Actin, EF1A, α-tubulin, GAPDH, UBQ, RPS18, RPL9, RPL10*	Developmental stage, virus, tissue, temperature	*geNorm, Normfinder, BestKeeper, ΔCt method, RefFinder*	An et al., 2016
Euscelidius variegatus	*18S, Actin, ATP synthase β, GAPDH, Tropomyosin*	Phytoplasma infection	*geNorm, Normfinder, BestKeeper*	Galetto et al., 2013
Macrosteles quadripunctulatus	*18S, Actin, ATP synthase β, GAPDH, Tropomyosin*	Phytoplasma infection	*geNorm, Normfinder, BestKeeper*	Galetto et al., 2013
Ericerus pela	*Actin1, Actin2, α-tubulin, β-tubulin1, β-tubulin2, SDHA1, SDHA2, SDHA3, RNAP II, RPL50-1, RPL50-2, RPL15, UBQ1, UBQ2, Myosin*	Developmental stage, tissue, temperature	*geNorm, Normfinder, RefFinder*	Yu et al., 2016
Bactericera cockerelli	*Actin, EF1A, Ferritin, GAPDH, RPL5, RPS18*	Developmental stage, tissue, Lso haplotype B infection	*geNorm, Normfinder, BestKeeper*	Ibanez and Tamborindeguy, 2016
Cimex lectularius	*α-tubulin, β-tubulin, RPL18, Actin, EF1A, GAPDH, SYN, UBQ*	Developmental stage, tissue, insecticide	*geNorm, Normfinder, BestKeeper*	Mamidala et al., 2011
Delphacodes kuscheli	*Actin, α-tubulin, GAPDH, EF1A, RPS18, UBQ*	Virus	*geNorm, Normfinder, BestKeeper*	Maroniche et al., 2011
Phenacoccus solenopsis	*Actin, RPL32, β-tubulin, α-tubulin, GAPDH, SDHA*	Developmental stage, host plant, temperature, population	*geNorm, Normfinder, RefFinder*	Arya et al., 2017
Halyomorpha halys	*RPS26, EF1A, UBQ, FAU, ARF, Actin, GUS, TATA, TIF6, RPL9*	Developmental stage, tissue, dsRNA exposure, starvation	*geNorm, Normfinder, BestKeeper, RefFinder*	Bansal et al., 2016
DIPTERA				
Lucilia cuprina	*18S, 28S, Actin, GST1, AChl, Per55, aE7, PKA, β-tubulin, GAPDH, RPLPO*	Developmental stage	*geNorm, Normfinder*	Bagnall and Kotze, 2010
Lucilia sericata	*18S, 28S, Actin, β-tubulin, RPS3, RPLP0, EF1A, PKA, GAPDH, GST1*	Naïve and immune-challenged larvae, tissue	*geNorm, Normfinder*	Baumann et al., 2015
Liriomyza trifolii	*18S, Actin, ArgK, EF1A, GAPDH, Histone 3, RPL32, α-tubulin, CAD*	Developmental stage, temperature, sex	*geNorm, Normfinder, BestKeeper, ΔCt method, RefFinder*	Chang et al., 2017

(Continued)

TABLE 1 | Continued

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
Drosophila melanogaster	*18S, Actin, EF1A, Mnf, RPS20, RPL32, α-tubulin*	Mechanical injury, temperature, diet	*geNorm, Normfinder, BestKeeper*	Ponton et al., 2011
	GAPDH, α-tubulin, RPL32, RPL13, EF1A, SDHA, GST1, Cyp1, Tyrosine-3-monooxygenase, exba, Actin, Su (Tpl), Faf, CG13220, Robl, Rap2l, HMBS, RNAP II, Nrv2, Elav, Appl	Aging- or neurodegeneration-related sample	*SAS*	Ling and Salvaterra, 2011
	Actin, β-tubulin, GAPDH, RPL32,TATA, eIF2	Imaginal disk	*geNorm, Normfinder*	Matta et al., 2011
Drosophila suzukii	*Actin, GAPDH, RPL18, RPS3, ArgK, EF1β, NADH, HSP22, α-tubulin, TATA*	Developmental stage, tissue, population, photoperiod, temperature	*geNorm, Normfinder, BestKeeper, RefFinder*	Zhai et al., 2014
Bactrocera dorsalis	*18S, Actin1, Actin2, Actin3, Actin5, GAPDH, G6PDH, α-tubulin, β-tubulin, EF1A*	Tissue	*geNorm, Normfinder*	Shen et al., 2010
	18S, β-tubulin, RPL13, GAPDH, EF1A, SDHA, α-tubulin, Actin, RNAP II	β-Cypermethrin, tissue	*geNorm, Normfinder*	Shen et al., 2013
Anastrepha obliqua	*Actin, β-tubulin, GAPDH, RPL18, RPS17, Syntaxin, Troponin C*	Developmental stage	*Normfinder, BestKeeper, RefFinder*	Nakamura et al., 2016
Bactrocera (Tetradacus) Minax	*18S, 28S, GAPDH, α-tubulin, β-tubulin, Actin, G6PDH, RPL32, EF1A, EF1β*	Developmental stage, temperature, γ-irradiation	*geNorm, Normfinder, RefFinder*	Lü et al., 2014
Bradysia odoriphaga	*Actin, EF1A, UBQ, RSP5, α-tubulin, GAPDH, RPS18, RPL18, SDHA, RPL28, RPS13, RPS15*	Developmental stage, temperature, insecticide, photoperiod, diet, population	*geNorm, RefFinder*	Shi et al., 2016
Aedes aegypti	*Actin, EF1A, α-tubulin, RPL8, RPL32, RPS17, GAPDH*	Developmental stage	*geNorm, BestKeeper, NormFinder*	Dzaki et al., 2017
Chrysomya megacephala	*Actin, RPL8, GAPDH, EF1A, α-tubulin, β-tubulin, TATA, 18S, RPS7*	Developmental stage, tissue, drug, heavy metal, diet	*RefFinder*	Wang et al., 2015
Ceratitis capitata	*RPL19, TATA, Ultrabithorax, GAPDH, α-tubulin, β-tubulin, 14-3-3zeta, RNA polymerase II, Actin3*	Developmental stage, tissue, body part	*geNorm, Normfinder, BestKeeper, RefFinder*	Sagri et al., 2017
Bactrocera oleae	*RPL19, TATA, Ultrabithorax, GAPDH, α-tubulin, β-tubulin, 14-3-3zeta, RNAP II, Actin3*	Developmental stage, tissue, body part	*geNorm, Normfinder, BestKeeper, RefFinder*	Sagri et al., 2017
HYMENOPTERA				
Solenopsis invicta	*RPL18, EF1β, Actin, GAPDH, TATA*	Developmental stage, tissue, caste	*geNorm, Normfinder, BestKeeper, RefFinder*	Cheng et al., 2013
Apis mellifera	*Actin, GAPDH, α-tubulin, RPS18, GST1, RPL32, UBQ, RPL13, HMBS, SDHA, TATA*	Bacterial challenge	*geNorm, Normfinder, BestKeeper*	Scharlaken et al., 2008
	GAPDH, RPL32, EF1A	Aging	*geNorm, Normfinder, BestKeeper*	Reim et al., 2013
	RPL19, RPL27, RPL10, RPL12, RPS18, GAPDH, EIF5A, Pontin, Proteasome, NAPK, U2af38, Pros54, DCAF13, ROSM1, NADH	Development time	*geNorm, Normfinder, BestKeeper*	Cameron et al., 2013
Bombus terrestris	*ELF1A, PPIA, RPL23, TATA, polyubiquitin*	Virus	*geNorm, Normfinder*	Niu et al., 2014

(Continued)

TABLE 1 | Continued

Insect species	Reference genes*	Experimental conditions	Analysis tools	References
	ArgK, EF1A, PLA2, α-tubulin, GAPDH, Actin, RPL13,	Tissue	*geNorm, Normfinder*	Hornáková et al., 2010
Bombus lucorum	*ArgK, EF1A, PLA2, α-tubulin, GAPDH, Actin, RPP2*	Tissue	*geNorm, Normfinder*	Hornáková et al., 2010
Lysiphlebia japonica	*18S, Actin, β-tubulin, RPL18, ArgK, EF1A, TATA, PRII, RPL27, RPS18, DIMT, PPI*	Developmental stage, tissue, sex, diet	*geNorm, Normfinder, BestKeeper*	Gao et al., 2017
THYSANOPTERA				
Frankliniella occidentalis	*28S, 18S, Actin, α-tubulin, EF1A, V-ATPase A, NADH, HSP60, HSP70, HSP90, RPL32*	Virus	*geNorm, Normfinder, BestKeeper, ΔC_t method, RefFinder*	Yang et al., 2015a
	18S, Actin, α-tubulin, EF1A, GAPDH, Histone 3, RPL32	Developmental stage, temperature	*geNorm, Normfinder, BestKeeper, RefFinder*	Zheng et al., 2014
BLATTODEA				
Diploptera punctata	*Actin, α-tubulin, GAPDH, Armadillo, RPL32, SDHA, EF1A, Annexin IX*	Tissue	*geNorm, Normfinder*	Marchal et al., 2013
ORTHOPTERA				
Chortoicetes terminifera	*18S, GAPDH, Actin, α-tubulin, RPL32, EF1A, Annexin IX, SDHA*	Solitarious and gregarious phase, isolated or crowded condition, short-term crowding	*geNorm, Normfinder*	Chapuis et al., 2011
Schistocerca gregaria	*GAPDH, Actin, α-tubulin, UBI, EF1A, RPL32, CGI3220*	Developmental stage	*geNorm, Normfinder*	Van Hiel et al., 2009
Locusta migratoria	*18S, Ach, Actin, Chtinase2, EF1A, RPL32, HSP70, α-tubulin, RPL32, SDHA, GAPDH, Histone*	Developmental stage, tissue, insecticide, temperature, starvation	*geNorm, Normfinder, BestKeeper, ΔC_t method*	Yang Q. et al., 2014
SIPHONAPTERA				
Ctenocephalides felis	*18S, 28S, Actin, Muscle actin, EF1A, GAPDH, HSP22, NADH, RPL19, α-tubulin*	Developmental stage, sex, diet, insecticide	*geNorm, Normfinder, BestKeeper*	Mcintosh et al., 2016
PSOCOPTERA				
Liposcelis bostsrychophila	*18S, Actin1, Actin2, α-tubulin, GAPDH*	Developmental stage, insecticide	*geNorm*	Jiang et al., 2010

**ADP-ribosylation factor (ARF), β-actin (Actin), elongation factor 1 α (EF1A), glyceralde hyde-3-phosphate dehydrogenase (GAPDH), glucose-6-phosphate dehydrogenase (G6PDH), arginine kinase (ArgK), cyclophilins A (CypA), vacuolar-type H^+-ATPase subunit A (V-ATPase A), 16S ribosomal RNA (16S), 12S ribosomal RNA (12S), 28S ribosomal RNA (28S), 18S ribosomal RNA (18S), ribosomal protein S (RPS), ribosomal protein L (RPL), ribosomal protein P2 (RPP2), heat shock protein (HSP), NADH dehydrogenase subunit 2 (NADH), succinate dehydrogenase complex subunit A (SDHA), peptidylprolyl isomerase A (PPIA), myosin light chain (Myosin L), glutathione S-transferase (GST), succinate dehydrogenase flavoprotein subunit (SDFS), ubiquitin-conjugating protein (UBQ), RNA polymerase II large subunit (RNAP II), superoxide dismutase (SOD), cAMP-dependent protein kinase A (PKA), acidic ribosomal phosphoprotein PO (RPLPO), acetylcholinesterase (ACh!), peritrophin-55 (Per55), alpha esterase 7 (aE7), ADP-ribosylation factor (ARF), porphobilinogen deaminase (HMBS), cytochrome oxidase subunit 1 (CO1), cytochrome P450 CYP6 (CYP6), embrionic lethal abnormal vision (Elav), major intrinsic protein (MIP), ubiquinol-cytochrome c reductase (UCCR), dimethyladenosine transferase (DIMT), peptidylprolyl isomerase (PPI), FK 506 binding protein (FK506), translation initiation factor eIF2 alpha (eIF2), acetyl-CoA hydrolase (Ach), translation elongation factor 2 (EF2), translation initiation factor 4A transporter-like (EIF4A), carbamoyl phosphate synthase (CAD), E2F transcription factor 4-like protein (E2F), FK506 binding protein (FK506BP), polyA binding protein (polyABP), forkhead domain 68A (Mnf), beta amyloid protein precursor-like (Appl), embryonic lethal abnormal vision (Elav), Na+/K+ ATPase (Nrv2), hydroxymethylbilane synthase (HMBS), ras-associated protein 2-like (Rap2l), roadblock-type 2 (Robl), acidic phospholipase A2 (PLA2), ubiquitin-like protein FUBI (FAU), β-glucuronidase (GUS), serine protease (SP), proteasome subunit beta type-7-like (Proteasome), nucleoside diphosphate kinas (NDPK), U2 small nuclear riboprotein auxiliary factor 38 (U2af38), proteasome 54kD subunit (Pros54), DDB1- and CUL4-associated factor 13-like (DCAF13), reactive oxygen species modulator 1-like (ROSM1).*

TOP 10 REFERENCE GENES

In the set of curated 90 papers, the expression level of reference genes was determined for 841 times. The number of experiments that utilized top 10 most frequently used reference genes, including *Actin*, *RPL*, *Tubulin*, *GAPDH*, *RPS*, *18S*, *EF1A*, *TATA*, *HSP*, and *SDHA,* are shown in **Figure 3**. *Actin*, which encodes a major structural protein, is expressed at various levels in many cell types. It is considered the ideal reference gene for RT-qPCR analysis and has been investigated most frequently (**Figure 3**). For example, previous studies have shown that the expression of *Actin* was the most stable among other reference genes across different developmental stages of many insects, including *Apis mellifera*, *Schistocerca gregaria*, *Drosophila melanogaster*, *Plutella xylostella*, *Chilo suppressalis*, *Chortoicetes terminifera*, *Liriomyza trifolii*, and *Diuraphis noxia* (Scharlaken et al., 2008; Van Hiel et al., 2009; Chapuis et al., 2011; Ponton et al., 2011; Teng et al., 2012; Sinha and Smith, 2014; Chang et al., 2017). Nonetheless, the expression of *Actin* was less stable in several insects, including those of the species, *Coleomegilla maculata*, *Coccinella septempunctata*, and *Hippodamia convergens* of the family Coccinellidae (Pan et al., 2015b; Yang et al., 2015c, 2016).

Ribosomal protein (RP), a principal component of ribosomes, is among the most highly conserved proteins across all life forms. The fraction of studies in which the expression level of *RPL* and *RPS* family genes was used as reference was 18.55%. Together, these genes were the most widely selected reference genes for expression studies in insects during the past 10 years. In most of these studies, RP-encoding genes were stable reference genes. For example, *RPS24* and *RPS18* were stable reference genes across different developmental stages and sex treatments of *C. maculata* (Yang et al., 2016); *RPS13* and *RPS23* were stable reference genes across different developmental stages of *P. xylostella* (Fu et al., 2013); whereas *RPL11*, *RPS8*, and *RPL14* were the three most stable reference genes across different developmental stages and under different temperature conditions of *Aphis craccivora* (Yang et al., 2015b). However, under some conditions, expression levels of RP-encoding genes may be unstable. For example, *RPS20* was the least stable gene in *P. xylostella* strains that were collected in different fields, grown under different temperatures, exposed to different photoperiods, or presented different insecticide susceptibility (Fu et al., 2013).

Tubulin (*α-tubulin*, *β-tubulin*, and *γ-tubulin*), which encodes cytoskeletal structure proteins, was ranked as the third most widely investigated reference gene (**Figure 3**). In many studies, the stability of *Tubulin* was variable under different treatments for the same species. For example, *a-tubulin* exhibits a stable expression in different tissues and sexes of *C. maculata*, whereas its expression was unstable across different developmental stages and following dsRNA treatments (Yang et al., 2015c).

GAPDH is another commonly used reference gene, ranked as the fourth most widely utilized reference gene (**Figure 3**). Occasionally, the stability of *GAPDH* expression was variable under different treatments within the same species. For example, *GAPDH* expression was not affected by tissue type, sex, photoperiod, or dsRNA treatment in *H. convergens*, but it varied across different developmental stages and at different temperatures (Pan et al., 2015b). *GAPDH* was a stable reference gene whose expression was not appreciably altered under different temperatures or by mechanical injury in different strains of *P. xylostella*; however, its expression was unstable across different developmental stages and was affected by photoperiod (Fu et al., 2013).

18S ribosomal RNA, a part of the ribosomal RNA, was ranked as the sixth most widely investigated reference gene (**Figure 3**). It was stably expressed throughout the vast majority of biotic and abiotic conditions in most studies that employed its expression level as reference (**Table 1**). However, it is generally acknowledged that the use of rRNA for normalization of RT-qPCR signals is problematic as rRNA forms a significant proportion of the total RNA pool (>80%), whereas mRNA accounts for a mere 3–5%, so the subtle changes in target gene expression levels may be potentially masked. With this in mind, it is much better to use the mRNA species of the ribosomal machinery, such as *RPL* and *RPS* genes, instead of rRNA.

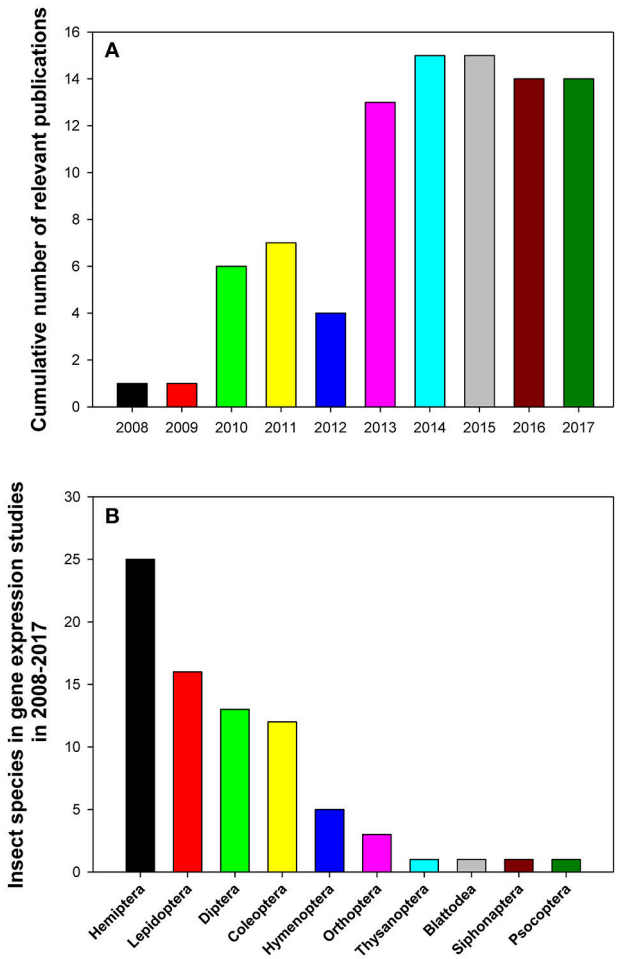

FIGURE 1 | Cumulative numbers of relevant publications **(A)** and distribution of insect species belonging to different taxonomic orders **(B)** in relevant gene expression studies performed in 2008–2017 that utilized expression levels of reference genes to normalize RT-qPCR data.

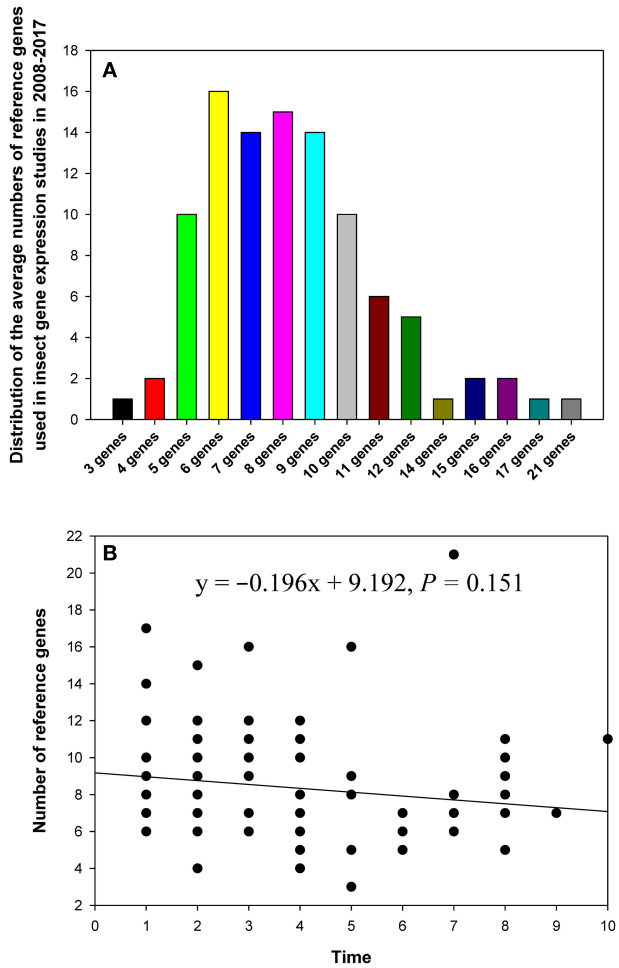

FIGURE 2 | The distribution of the numbers of reference genes per study in relevant publications about gene expression in insects in 2008–2017 **(A)**, and the relationship between the number of reference genes and study publication date (year) fitted by linear regression **(B)**. The numbers 1–10 on the X-axis represent years from 2017 to 2008, respectively.

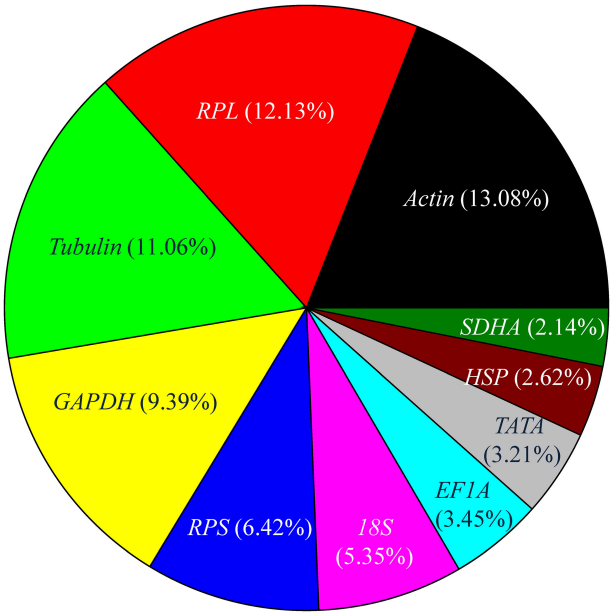

FIGURE 3 | Frequency of the top 10 most popular reference genes in relevant insect gene expression studies performed during 2008–2017. *RPL* includes *RPL3*, *RPL4*, *RPL5*, *RPL7*, *RPL8*, *RPL9*, *RPL10*, *RPL11*, *RPL12*, *RPL13*, *RPL14*, *RPL15*, *RPL17*, *RPL18*, *RPL19*, *RPL22*, *RPL23*, *RPL26*, *RPL27*, *RPL28*, *RPL29*, *RPL32*, *RPL40*, and *RPL50*; *RPS* includes *RPS2*, *RPS3*, *RPS5*, *RPS6*, *RPS7*, *RPS8*, *RPS9*, *RPS11*, *RPS13*, *RPS15*, *RPS17*, *RPS18*, *RPS20*, *RPS23*, *RPS24*, *RPS26*, and *RPS27*, *Tubulin* includes *α-tubulin*, *β-tubulin*, and *γ-tubulin*; *HSP* includes *HSP20*, *HSP22*, *HSP40*, *HSP60*, *HSP70*, and *HSP90*.

Altogether, the expression level of *EF1A*, *TATA*, *HSP*, and *SDHA* genes was used as a reference in 11.42% of the experiments. These four genes transiently exhibited variable expression under different treatments in different insect species. For example, *EF1A* was the least stable reference gene in *A. craccivora* across different developmental stages and at different temperatures (Yang et al., 2015b). In contrast, *EF1A* was one of the best reference genes in *H. convergens* with its expression level being unaffected by three biological factors (developmental stage, tissue type, and sex) and three abiotic conditions (temperature, photoperiod, and dietary RNAi; Pan et al., 2015b).

DISTRIBUTION OF THE NUMBERS OF EXPERIMENTAL FACTORS STUDIED

In the 90 papers, changes in the reference gene expression level were investigated under the influence of one to seven experimental factors. Most of these studies analyzed the influence of one (10%), two (16%), or three (14%) experimental factors (**Figure 4A**). The relationship between the number of experimental factors and study publication date (year) was investigated by linear regression. We found that the more recently the paper was published, the more experimental factors it tended to explore (**Figure 4B**).

TOP 10 EXPERIMENTAL FACTORS

A total of 39 experimental factors were investigated in these 90 papers, with the top 10 experimental factors (in the descending order) being developmental stage, tissue, temperature, insecticide, diet, population, virus, sex, photoperiod, and starvation (**Figure 5**).

RNA interference (RNAi) is a conserved mechanism whereby messenger RNA transcripts are targeted by small interfering RNAs in a sequence-specific manner, leading to downregulation of gene expression. During the past 20 years, RNAi has been widely used as a tool to investigate functions of insect genes (Zotti et al., 2018), whereas RT-qPCR is the method of choice to study gene expression in terms of its sensitivity and specificity. The genes that play important roles during insect metamorphosis and affect different tissues can serve as target genes for manipulations that kill the insect or retard its growth. This is why gene

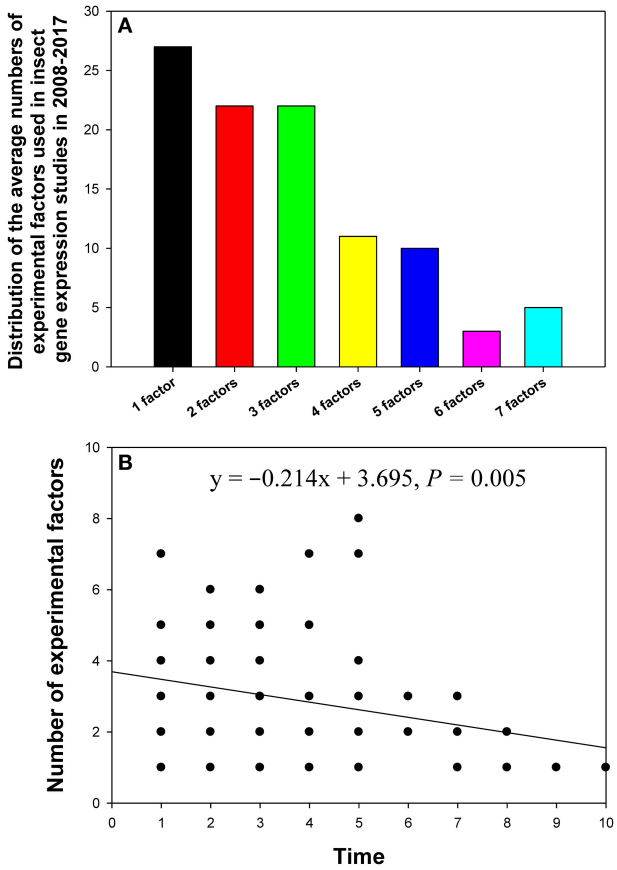

FIGURE 4 | Distribution of the number of experimental factors in relevant insect gene expression studies performed during 2008–2017 **(A)**, and the relationship between the number of experimental factors per study and study publication date (year) investigated by linear regression **(B)**. The numbers 1–10 on the X-axis represent years from 2017 to 2008.

expression profiles are widely assessed at different developmental stages and in different tissues. The effect of these two factors on gene expression was investigated frequently with the use of reference gene expression levels in 22.86 and 17.50% of studies, respectively (**Figure 5**).

Insects are ectothermic organisms, and the body temperature of most insects is affected by changes in ambient temperature, ultimately influencing their growth, and development. Temperature was ranked as the third most widely investigated factor at 11.79% (**Figure 5**). We found that the numbers/kinds of reference genes under different temperatures varied in different insects. For instance, *GAPDH,* and *EF1A* were the best stable gene combinations in *Spodoptera litura* (Lu et al., 2013), while *RPS15*, *β-tubulin*, and *EF1A* were the most stable reference genes in *Nilaparvata lugens* (Yuan et al., 2014).

Many insects, including the 78 insect species summarized in this study have developed resistance to insecticides. Insecticide resistance presents as a major challenge for pest control. The molecular mechanisms underlying insecticide resistance are under intense scrutiny; RT-qPCR is an important technology for investigating the gene functions involved in insecticide resistance. Insecticides ranked as the fourth most widely investigated factor at 5.00% (**Figure 5**). We found that different reference genes were used in different insects to study the effect of various insecticide treatments. *RPS15* and *RPL32* were stably expressed reference genes in insecticide treatment experiments in *H. armigera* (Zhang et al., 2015); while *RPS11*, *EF1A*, and *β-tubulin* were the best choice in the insecticide-stressed *N. lugens* (Yuan et al., 2014). Different classes of insecticides have warranted different sets of reference genes to normalize target gene expression in *B. tabaci* (Liang et al., 2014).

Diet was ranked as the fifth most widely investigated factor at 4.29% (**Figure 5**). Different gene combinations were required for different diet conditions. For examples, *RPL10* and *GAPDH* were the most stable reference genes in *S. litura* that were reared on different diets (Lu et al., 2013); whereas, *Actin*, *RPS18*, and *RPS15* were the most stable reference genes among different diets in *Bradysia odoriphaga* (Shi et al., 2016), *Actin* and *18S* were the best reference gene combination for feeding assay experiments with *Aphis gossypii* (Ma et al., 2016).

Population, virus, and sex were all ranked as the sixth most widely investigated factor at 3.93%(**Figure 5**). Different reference gene combinations were suggested for the studies of each factor. For example, *RPL10* and *EF1A* were the most stable reference genes in *S. litura* collected from different locations (Lu et al., 2013), *EF1A*, *Actin,* and *GAPDH* were the more stable reference genes in *P. xylostella* (Fu et al., 2013). The combination of *Actin* and *EF1A* was very useful for experiments involving *A. gossypii* (Ma et al., 2016*)*. In addition, in viral infection experiments, different reference gene combinations were recommended for different insects. For example, *GAPDH*, *RPL27*, and *β-tubulin* was the best reference gene combination for nuclear polyhedrosis virus infection (Zhang et al., 2015), *HSP90* and *RPL29* were the most stable reference genes in *B. tabaci* when the whitefly carried the tomato yellow leaf curl virus and when it did not (Li et al., 2013). Moreover, in females and males, different reference gene combinations were recommended for different insects. For instance, *GAPDH* and *CypA* were most stable reference genes for *H. convergens* (Pan et al., 2015b), *HSP90* and *RP49* were the most stable ones for *Harmonia axyridis* (Yang et al., 2018), and *18S*, *EF1A*, and *GAPDH* were the best for gene expression normalization in *Sesamia inferens* (Sun et al., 2015).

Photoperiod and starvation ranked as the seventh and eighth most widely investigated factors at 3.21 and 2.86%, respectively (**Figure 5**). Different reference gene combinations were recommended for different insects for these two factors. For instance, under photoperiod stressed conditions, *GAPDH* and *CypA* were most stable reference genes in for *H. convergens* (Pan et al., 2015b), *EF1A* and *V-ATPase A* were the most stable ones for *Danaus plexippus* (Pan et al., 2015a), and *HSP90* and *β-tubulin* were the best reference genes for *H. armigera* (Shakeel et al., 2015). Under starvation conditions, *RPL28* and *RPS15* were the most stable reference genes for *H. armigera* (Shakeel et al., 2015), *RPS3* and *Actin* were the best reference genes for *S. litura* (Lu et al., 2013), and *RPS11*, *ArgK*, and *EF1A* were recommended for *N. lugens* (Yuan et al., 2014).

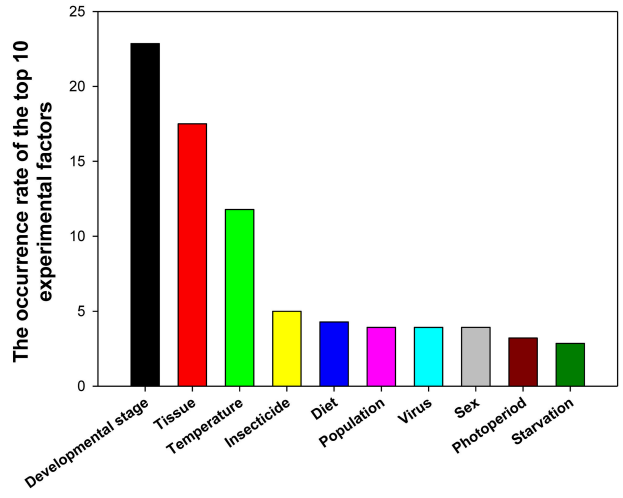

FIGURE 5 | Frequency of top 10 experimental factors in relevant insect gene expression studies performed during 2008–2017.

DISTRIBUTION OF THE NUMBER OF ANALYSIS TOOLS

In the 90 papers, one to five analysis tools were used to evaluate gene expression stability, with one tool (4%) and three tools (34%) being the least and most frequently used variants in these studies, respectively (**Figure 6A**). Linear regression analysis showed that the more recently the paper was published, the more analysis tools it used (**Figure 6B**).

CONCLUSIONS

Our review clearly suggests that no reference gene is universally stably expressed because variable expression levels even for the most popular reference genes have been observed under different circumstances in the same insect species or under the same experimental condition among different insects. In order to obtain reliable experimental data for the target gene, it is necessary to perform internal reference gene screening under specific experimental conditions. Given that the best internal reference genes in different species under different conditions often have large differences in expression, it may result in a multi-fold difference of target gene expression, or even false conclusion, if used improperly. For instance, the expression of *V-ATPase A* in the gut ranged from 7.7- to 22.4-fold higher than that in the carcass of *C. septempunctata* when normalized to the most- and least-stable sets of reference genes, respectively (Yang et al., 2016). Furthermore, the relative *hsp83* expression was noticeably variable when a less stable reference gene was used for RT-qPCR normalization in different tissues and developmental stages of *S. inferens,* whereas *hsp83* was uniformly expressed when stable reference genes were used for normalization (Sun et al., 2015). Therefore, better accuracy in gene expression analysis can promote the investigation of gene function. We strongly recommend that prior to each RT-qPCR experiment, the reference gene expression stability must be validated. Furthermore, multiple reference genes should be used to achieve the best results. This review should help researchers select the best reference genes and optimize their experiments to examine gene expression levels in insects, especially the non-model ones, in terms of the number of reference genes chosen, experimental factors manipulated, and the analysis tools used.

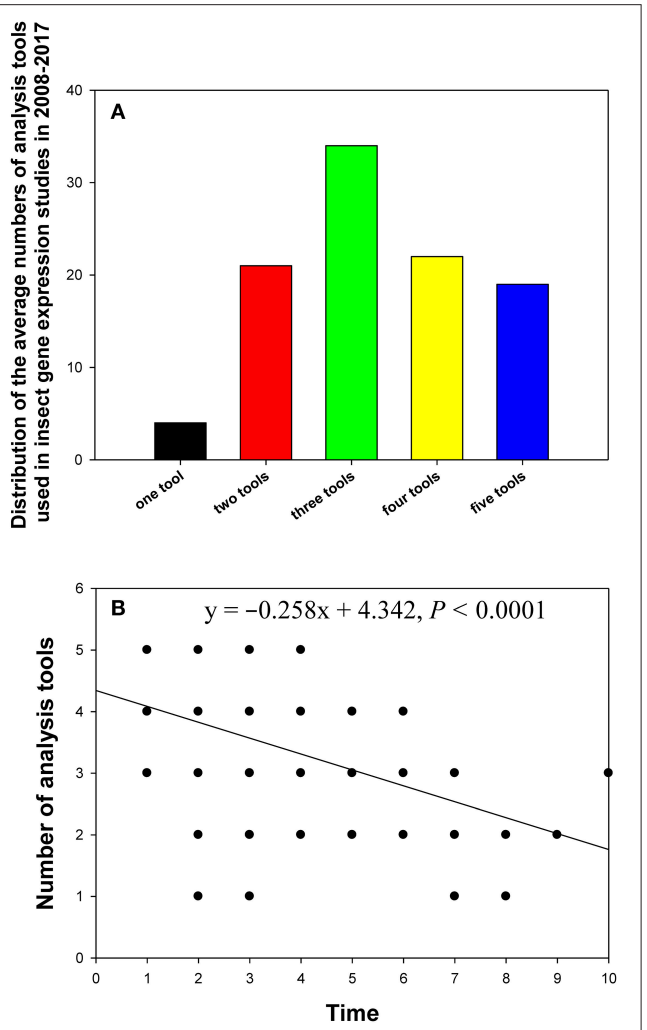

FIGURE 6 | Distribution of the numbers of analysis tools in relevant insect gene expression studies performed during 2008–2017 **(A)**, and the relationship between the number of analysis tools per study and study publication date (year) investigated by linear regression **(B)**. The numbers 1–10 on the X-axis represent years from 2017 to 2008.

AUTHOR CONTRIBUTIONS

HP and YZ conceived the topic of the review. HP, CY, and JL performed literature review analyzed the data. HP and CY wrote the manuscript.

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