De novo Analysis of *Heortia vitessoides* (Lepidoptera: Crambidae) Transcriptome and Identification of Putative Cytochrome P450 Monooxygenase Genes[1]

Jie Cheng, Zihao Lyu, Chunyan Wang, Jingxiang Chen, and Tong Lin[2]

College of Forestry and Landscape Architecture, South China Agricultural University, 483 Wushan Road, Guangdong Province, Guangzhou, 510642, P.R. China

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Abstract We analyzed a transcriptome generated from four developmental stages of *Heortia vitessoides* Moore (Lepidoptera: Crambidae), a defoliator of the agarwood tree, *Aquilaria sinensis* (Lour.) Gilg (Thymelaeaceae). A total of 110.53 Mb clean reads were produced, and *de novo* assembled into 42,946 unigenes of an average length of 1,059 base pairs (bp). Among these unigenes, 22,106 (51.47%) showed significant similarity (*E*-value $<10^{-5}$) to known proteins in the National Center for Biotechnology Information (NCBI) nonredundant database. Gene ontology (GO), eukaryotic ortholog groups (KOG), and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to classify the functionality of unigenes. Moreover, 46 putative cytochrome P450 (*CYP*) monooxygenase genes were identified from this dataset. These *CYP* genes were classified into four clans consisting of 22 families and 34 subfamilies. The expression profiles of genes belonging to *CYP4* and *CYP6* families and exposed to half the lethal concentrations (LC_{50}) of chlorantraniliprole and beta-cypermethrin were determined by real-time reverse transcription-quantitative PCR (RT-qPCR). The results showed that the transcription levels of eight (*CYP4M39*, *CYP6AB49*, *CYP6AB53*, *CYP6AB61*, *CYP6AE17*, *CYP6AW1*, *CYP6BD6*, *CYP6CV1*) and five (*CYP6AB10, CYP6AB53*, *CYP6AE12*, *CYP6AE17*, *CYP6BD6*) genes significantly increased in the fourth-instar larvae following exposure to the insecticides chlorantraniliprole and beta-cypermethrin, respectively. Therefore, these genes are potential candidates involved in the detoxification of these two insecticides. Further studies utilizing the RNA interference approach are required to enhance our understanding of the functionality of these genes in this forest pest.

Key Words transcriptome, cytochrome P450 monooxygenase, insecticide treatment, expression pattern

Aquilaria sinensis (Lour.) Gilg (Thymelaeaceae) produces the fragrant agarwood that is widely used in traditional medicine and the incense industry (Jin et al. 2016; Wen et al. 2009). *Heortia vitessoides* Moore (Lepidoptera: Crambidae) is considered to be the most severe pest of *A. sinensis* and ranges from India, Nepal, China, and Sri Lanka through Southeast Asia and the East Indies to Queensland, the New Hebrides, and Fiji. In southern China, the insects have seven or eight generations per year, and the larvae feed on the leaves of *A. sinensis*,

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[2]Corresponding author (E-mail: lintong@scau.edu.cn).

causing significant economic losses (Qiao et al. 2012; Wen et al. 2017). Carbamates, pyrethroids, anthranilic diamides, and plant-derived insecticides such as fenoxycarb, beta-cypermethrin, chlorantraniliprole, and matrine have been used to control *H. vitessoides* infestations. However, these insecticides have become less efficient, even as mixtures or at relatively high doses (Chen et al. 2011; Lu et al. 2014; Su 1994).

The cytochrome P450 (*CYP*) monooxygenases, a large and complex gene superfamily of heme-thiolate proteins, are ubiquitously expressed in almost all living organisms (Yu et al. 2015). Insect *CYP*s can be divided into four major clans: three microsomal *CYP* clans (*CYP2*, *CYP3*, and *CYP4*) and a mitochondrial *CYP*s clan (Feyereisen 2011). In insects, *CYP*s perform many important functions including involvement in biosynthetic pathways of juvenile hormones, ecdysteroids, and pheromones which are closely related to insect growth, development, and reproduction (Lao et al. 2015). Moreover, insect *CYP*s are well-known for their vital role in the detoxification of various types of synthetic insecticides such as chlorantraniliprole (Hu et al. 2014), malathion (Li et al. 2016), and chlorpyrifos (Wang et al. 2017). Increased *CYP* activity has been reported to be one of the main reasons for insecticide resistance in other lepidopteran species such as *Helicoverpa armigera* Hübner (Zhou et al. 2010), *Cnaphalocrocis medinalis* Guenée (Liu et al. 2015), and *Cydia pomonella* Linnaeus (Bosch et al. 2018).

The identification and functional analysis of candidate *CYP* genes are important first steps in investigating the mechanisms of insecticide resistance in insects. Next-generation sequencing techniques such as RNA sequencing (RNA-seq), aided by decreasing costs and technical advances, have become valuable tools that allow vast amounts of genetic information to be acquired from nonmodel organisms without prior sequence knowledge (Liu et al. 2016). For *H. vitessoides*, which does not have a sequenced genome, we deposited three transcriptomes in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; adult: SRX3035102, female antennae: SRX3136158, male antennae: SRX3136160) (Cheng et al. 2017). However, extensive genomic and transcriptomic sequences are still required for *H. vitessoides*.

In the present study, we sampled *H. vitessoides* eggs, larvae, pupae, and adults and used a BGISEQ-500 sequencing platform to generate a large-scale dataset, utilizing bioinformatics analyses to focus on the genes encoding putative *CYP*s. We identified 46 putative *CYP* genes in *H. vitessoides*. Moreover, the expression profiles of genes belonging to *CYP4* and *CYP6* families exposed to half the lethal concentrations (LC_{50}) of chlorantraniliprole and beta-cypermethrin were determined using real-time reverse transcription-quantitative PCR (RT-qPCR). To the best of our knowledge, this is the first report of the identification and characterization of multiple *CYP* genes in this forest pest.

Materials and Methods

Insect rearing and sample collection. *Heortia vitessoides* eggs and larvae were collected in May 2017 from an *A. sinensis* plantation (N 22°01′, E 110°25′) in Huazhou, Guangdong, China. No chemical treatment was applied before or during collection. All insects were reared in the laboratory under conditions of 26°C with 70

± 2% relative humidity and maintained at a 14 h:10 h light:dark cycle. Eggs, larvae, pupae, and adults were collected from that colony and were pooled together, then immediately frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

RNA sample preparation. Total RNA was extracted using the E.Z.N.A.™ Total RNA kit II (OMEGA Biotec, Norcross, GA, USA) following the supplier's instructions and then treated with DNase I (Invitrogen, Life Technologies, Carlsbad, CA, USA). A Nanodrop 2000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA) was used to check sample purity while a Qubit 2.0 fluorometer (Life Technologies, Gaithersburg, MD, USA) and Quantifluor-ST fluorometer with Agilent 2100 Bioanalyzer (Promega, Madison, WI, USA) were used to measure the concentration and integrity, respectively.

cDNA library construction. The qualified RNA samples were used for transcriptome sequencing. The first step involved purifying the poly-(A)-containing mRNA molecules using poly-T oligo-attached magnetic beads. Following purification, the mRNA was fragmented using divalent cations under an elevated temperature. The cleaved RNA fragments were reverse transcribed to form the first-strand cDNA using reverse transcriptase and random primers. This was followed by second-strand cDNA synthesis using DNA Polymerase I and RNase H. A single "A" base was added to these cDNA fragments, followed by the ligation of DNA adapters. The products were then purified and enriched with PCR amplification. We then quantified the PCR yield using Qubit and pooled samples to generate a single strand DNA (ssDNA) circle, which formed the final library. The cDNA library was sequenced using the BGISEQ-500 platform (BGI, Shenzhen, China). The raw reads were saved as FASTQ files and deposited in the NCBI SRA with the accession number SRX4045498.

***De novo* assembly and functional annotation.** Prior to assembly, we obtained clean reads from the raw data by removing reads containing adaptor sequences, more than 5% unknown nucleotides, more than 50% bases with *Q*-value $\leq$20, and empty reads. These clean reads were then *de novo* assembled into unigenes using the short reads assembling program Trinity (Grabherr et al. 2011). To acquire comprehensive information on gene functions, assembled unigenes over 150 bp in length were searched against the NCBI nonredundant protein sequences (Nr), NCBI nucleotide (Nt), eukaryotic ortholog groups (KOG), SwissProt, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLASTx and BLASTn with an *E*-value $<10^{-5}$. Blast2GO (Conesa et al. 2005) was used for gene ontology (GO) annotation with an *E*-value $<10^{-5}$ based on the protein annotation results of the Nr database. InterPro functional annotation was performed using InterProScan5 (Quevillon et al. 2005).

Gene identification and bioinformatic analyses. The BLASTn program was used to identify candidate unigenes encoding putative *CYP* monooxygenase genes in *H. vitessoides* (*HvCYP*s) using available sequences of these proteins from lepidopteran insects. All candidate genes were manually checked using BLASTx on the NCBI website. All putative *HvCYPs* were named in accordance with David R. Nelson to maintain consistency in the nomenclature.

Open reading frames (ORFs) were predicted using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The functional domains and core catalytic residues were predicted by searching the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Amino acid sequences were aligned with

MAFFT (http://mafft.cbrc.jp/alignment/server/clustering.html). Phylogenetic trees were constructed using the MEGA5.0 based on the neighbor-joining method with the *p*-distance model, including trees of 34 putative *HvCYP*s (amino acid residues >300aa) and three insects (*Drosophila melanogaster* Meigen, *Apis mellifera* L., and *Bombyx mori* L.) (Tamura et al. 2011). Node support was assessed using a bootstrap procedure based on 1,000 replicates, and node support values <50% are not shown.

Insecticide treatment. Chlorantraniliprole and beta-cypermethrin were purchased from Fengle Agrochemical Co., Ltd., (Hefei, China) and diluted with analytical-grade acetone to make a working solution, 7.7×10^{-4} mg L^{-1} for chlorantraniliprole and 8.9×10^{-5} mg L^{-1} for beta-cypermethrin (LC_{50} values) (Chen et al. 2011). The freshly-molted fourth-instar larvae were selected and starved for 2 h. The leaf-dipping method was employed to investigate insecticidal activity (Chen and Zhang 2015). Fresh *A. sinensis* leaves were dipped into the pesticide solutions for 10–15 s and air dried at 26°C, then fed to the starved larvae. Control insects were fed with *A. sinensis* leaves with acetone only. Insecticide-treated and control insects were collected after 24 h, immediately frozen in liquid nitrogen, and stored at −80°C prior to RNA extraction. Each sample consisted of 15 larvae with three independent replicates.

Real-time reverse transcription-quantitative PCR (RT-qPCR). To determine the transcriptional changes of six *CYP4* and 12 *CYP6* family genes in the fourth-instar larvae after exposure to the LC_{50} of chlorantraniliprole and beta-cypermethrin, RT-qPCR was performed using cDNA prepared from insecticide-treated and control insects. Total RNA was extracted as described above. First-strand cDNA was synthesized from 2 μg total RNA using the PrimeScript® RT reagent kit with gDNA Eraser (Takara Bio, Otsu, Japan) and then immediately stored at −80°C for further use. The RT-qPCR was performed using SYBR® Premix Ex TaqTM II (Takara Bio, Otsu, Japan). Each reaction (20-μL volume) contained 2 μL cDNA, 10 μL SYBR Premix Ex Taq, 0.4 μL forward and reverse primers (10 μM), and 7.2 μL RNase-free double distilled water. The gene-specific primers (Table 1) were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by TSINGKE Biotech Co., Ltd. (Guangzhou, China). The housekeeping gene β-actin (GenBank accession number MG132199) was used as reference gene in the RT-qPCR experiments (Cheng et al. 2018). RT-qPCR on the cDNA products was carried out in 96-well plates using a Light Cycler 480 (Roche Diagnostics, Indianapolis, IN, USA). The amplification conditions were as follows: initial denaturation at 95°C for 5 min; 40 cycles at 95°C for 10 s and 60°C for 20 s; and cooling at 40°C for 30 s. Negative controls were nontemplate reactions (replacing cDNA with diethyl pyrocarbonate water), and the results were analyzed using the LightCycler® Real-Time PCR system. Three biological and technical replicates each were set for RT-qPCR analysis. The quantity of CYP mRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001).

Statistical analysis. Gene expression data are presented as the means ± standard deviation (SD) of three independent replicates. To compare the differences in gene expression between the insecticide-treated and control larvae, a paired Student's *t*-test was performed. $P < 0.05$ was considered statistically significant. Data analysis was conducted using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA).

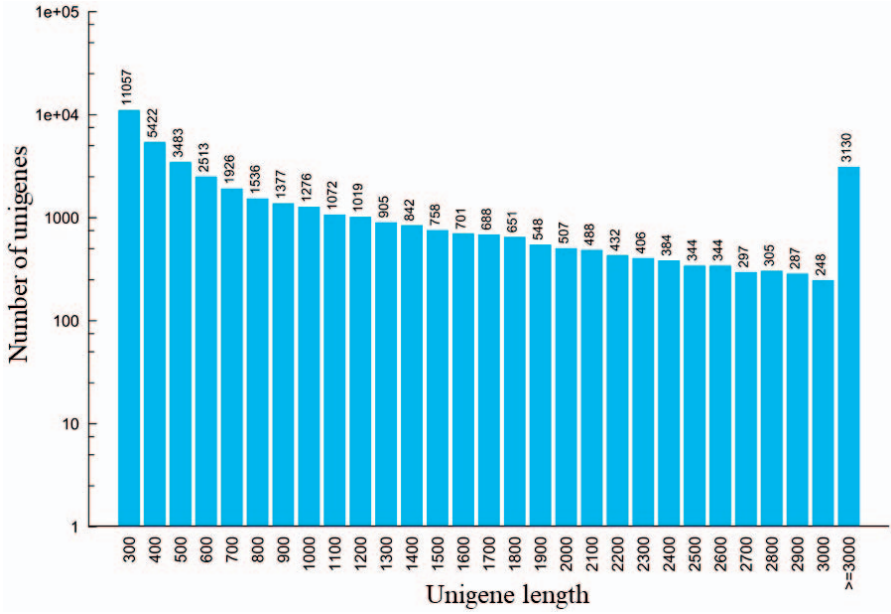

Fig. 1. Size distribution of the assembled unigenes from *Heortia vitessoides* transcriptome.

Results

Sequencing and *de novo* assembly. We performed next-generation sequencing of the cDNA library constructed from the eggs, larvae, pupae, and adults of *H. vitessoides* using the BGISEQ-500 platform. Transcriptomic sequencing provided 117.04 Mb raw reads. After removing adaptor, low quality, and N-containing sequences, 110.53 Mb clean reads were generated. After assembly, we obtained 42,946 unigenes with an average length of 1,059 bp and an N50 of 1,944 bp (Table 2). The size distribution of the assembled unigenes is shown in Fig. 1.

Sequence annotation. The number and percentage of matched unigenes at different values is shown in Table 3. In summary, 22,106 (51.47%), 12,455 (29.00%), 15,297 (35.62%), 16,833 (39.20%), 14,837 (34.55%), 15,006 (34.94%), and 4,510 (10.50%) unigenes had homologous sequences in the Nr, Nt, Swiss-Prot, KEGG, KOG, Interpro, and GO databases, respectively. The total unigenes annotated by any of the seven functional databases was 56.11%. Only 4.76% of the unigenes were annotated in all databases. For species distribution, the highest match percentage was to *H. armigera* (26.18%) sequences followed by *Amyelois transitella* Walker (24.10%), *B. mori* (8.98%), and *Papilio xuthus* Linnaeus (6.64%, Fig. 2).

GO annotation was used to classify the function of unigenes according to the GO terms (Fig. 3). In biological processing terms, “cellular processes” (1,632), “metabolic processes” (1,403), and “biological regulation” (536) were the most abundant. In cellular component terms, “cell” (1,504), “cell part” (1,481), and

Table 1. Primers used in this study (F = forward; R = reverse).

Gene	Direction	Sequence (5′→3′)	Product Size (base pairs)	PCR Type
CYP4G113	F	CCGACCCTTGAAGAACTG	226	RT-qPCR
	R	ATCGTGTCCACTTCTTCC		
CYP4G90	F	GCTGTAATGAAGATGTGCG	221	RT-qPCR
	R	TTCTGTGGGAGTGGTGGT		
CYP4M39	F	TATTGACGCCATCCTTCC	223	RT-qPCR
	R	AATGGCTTGCTTGTAGGC		
CYP4L27	F	AGGGTAACATCACGGGGT	165	RT-qPCR
	R	GTAGTTGTATCGTGCCCC		
CYP4G24	F	GTGTTATGCCTCCTTGGT	248	RT-qPCR
	R	TATTACCACTGTCGCTCC		
CYP4AU10	F	ATGGATTGTTCGTCGCTC	222	RT-qPCR
	R	TCACCCATTGCCGTTTCA		
CYP6AE17	F	AAGGTTGAAGTTCCAGTGAC	216	RT-qPCR
	R	GGTATGGAACCTCAGTAACACA		
CYP6AW1	F	GAAAGGCGTAATACAGGG	246	RT-qPCR
	R	AGGCACCATACTGCTCAA		
CYP6BD6	F	CATAGAGTTGAAGAATGGCG	153	RT-qPCR
	R	CTCCTGGCATTTTTCGTTCT		
CYP6AB47	F	ACGCTAACTCGCTGAATG	204	RT-qPCR
	R	GACGGTTTGTAGTTTCGC		
CYP6AB53	F	AACTCCAAGTGTTAGCCG	256	RT-qPCR
	R	ATCAGGTGAAACGGCATC		
CYP6AN4	F	GCAAGAGATTGACGCCTA	217	RT-qPCR
	R	GAGGGAGTTTTCGTTGCT		
CYP6CV1	F	AGAAGTGATTGGGTCGTG	208	RT-qPCR
	R	CAGTGTGAACCAAGTCCA		
CYP6AB49	F	GCCCAGAACGAGATAGAT	199	RT-qPCR
	R	ACAAGTGTGCCTTCGTCA		
CYP6AB61	F	CCCAGAGATAGACTTGACC	261	RT-qPCR
	R	TGCTGGTTCAACGGTGTA		

Table 1. Continued.

Gene	Direction	Sequence (5′→3′)	Product Size (base pairs)	PCR Type
CYP6CT1	F	AGACTACATCAGTGCCATTG	202	RT-qPCR
	R	TTTATCCAGACACCTCCC		
CYP6AE12	F	TTGTTCCTGTGCTTTCGG	164	RT-qPCR
	R	TTGAAGCCCAATCCGTAG		
CYP6AB10	F	TTACTACGGAACAAGGGC	280	RT-qPCR
	R	CCGTCTTGTGGGGTATTA		
β-actin	F	GTGTTCCCCTCTATCGTGG	119	RT-qPCR
	R	TGTCGTCCCAGTTGGTGAT		

"membrane" (1,247) were the highest classified. In molecular function terms, genes involved in binding (2,085), catalytic activity (1,655), and structural molecule activity (307) were the most abundant.

In total, 14,837 unigenes were assigned to 25 KOG functional categories (Fig. 4). Of these categories, "general function prediction only" represented the largest group, containing 3,775 unigenes, followed by "signal transduction mechanisms" (3,230) and "function unknown" (1,689). The "nucleotide transport and metabolism" (189), "coenzyme transport and metabolism" (134), and "cell motility" (53) categories were the smallest clusters represented.

To understand the biological pathways active in *H. vitessoides*, the sequences were mapped to reference canonical pathways in KEGG (Fig. 5). In summary,

Table 2. Overview of transcriptome of *Heortia vitessoides*.

Total raw reads (Mb)	117.04
Total clean reads (Mb)	110.53
Total clean bases (Gb)	11.05
Clean reads ratio (%)	94.44
Clean reads Q20 (%)	98.50
Total number of unigenes	42,946
Total length of unigenes	45,486,000
Mean length of unigenes	1,059
N50 of unigene	1,944
GC (%)	41.85

[a] GC = GC(%): the percentage of G and C bases in all transcripts.

Table 3. Summary of unigene annotations.

Values[a]	Number of Unigenes	Percentage (%)
Total	42,946	100
Nr	22,106	51.47
Nt	12,455	29.00
Swiss-Prot	15,297	35.62
KEGG	16,833	39.20
KOG	14,837	34.55
Interpro	15,006	34.94
GO	4,510	10.50
Intersection	2,043	4.76
Overall	24,098	56.11

[a] Nr = nonredundant protein sequences; Nt = NCBI nucleotide; KOG = eukaryotic ortholog groups; KEGG = Kyoto Encyclopedia of Genes and Genomes; GO = gene ontology.

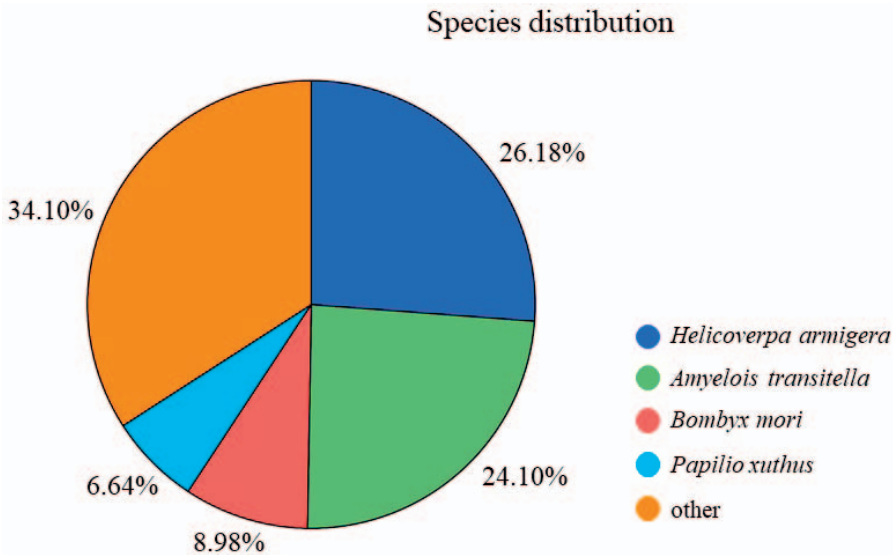

Fig. 2. Species distribution. Species distribution of the unigene BLASTx matches against the nonredundant protein database, with a cut-off *E*-value of E^{-5}. The first hit of each unigene was used for analysis, and the proportion of homologous sequences in each species is shown.

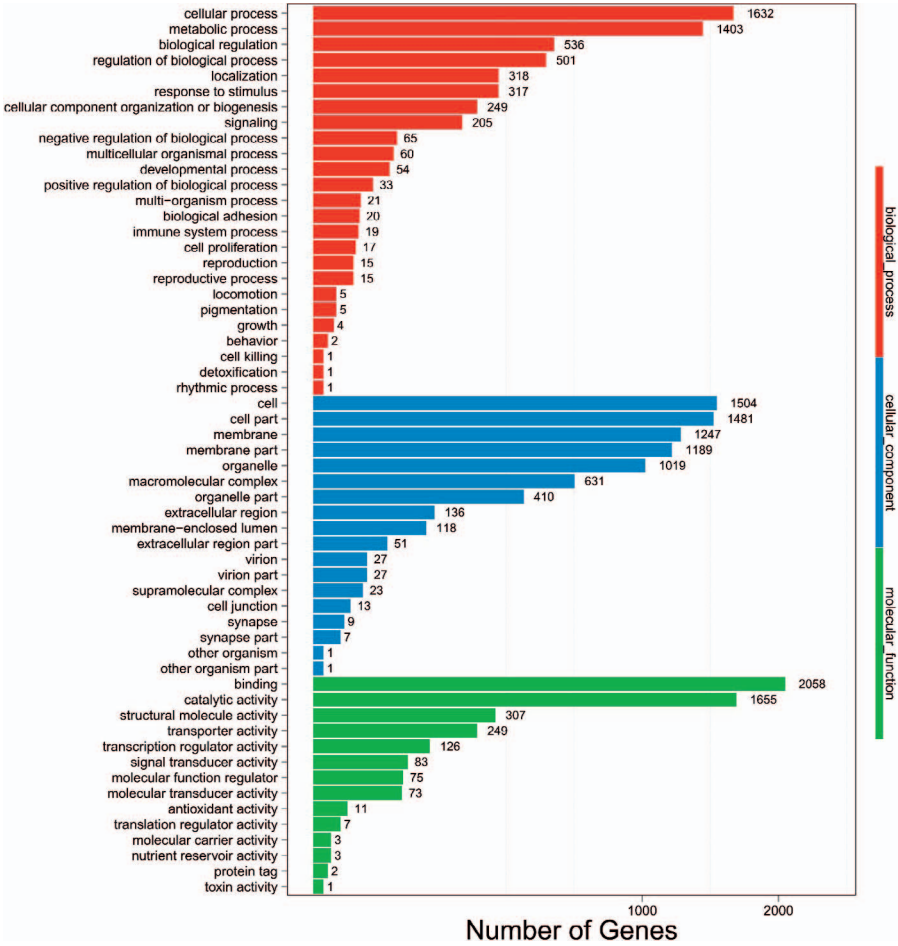

Fig. 3. Gene ontology classifications of unigenes. The results are assigned into three main categories: biological process, cellular component, and molecular function.

16,833 unigenes were classified into six groups, "cellular processes," "environmental information processing," "genetic information processing," "human diseases," "metabolism," and "organismal systems." "Transport and catabolism" (1,262), "signal transduction" (2,451), "translation" (1,001), "cancers: overview" (1,574), "global and overview maps" (2,277), and "endocrine system" (1,405) were the dominant pathways in each group, respectively.

Identification and characterization of *CYP* genes from *H. vitessoides*. In total, 46 putative *CYP* genes were identified in the *H. vitessoides* transcriptome. Of these, only six genes had completed ORFs while the remaining 40 genes consisted of incomplete cDNAs, missing a portion of the sequence (Table 4). According to the standard nomenclature, the 46 *HvCYP*s were divided into 22 families and 34

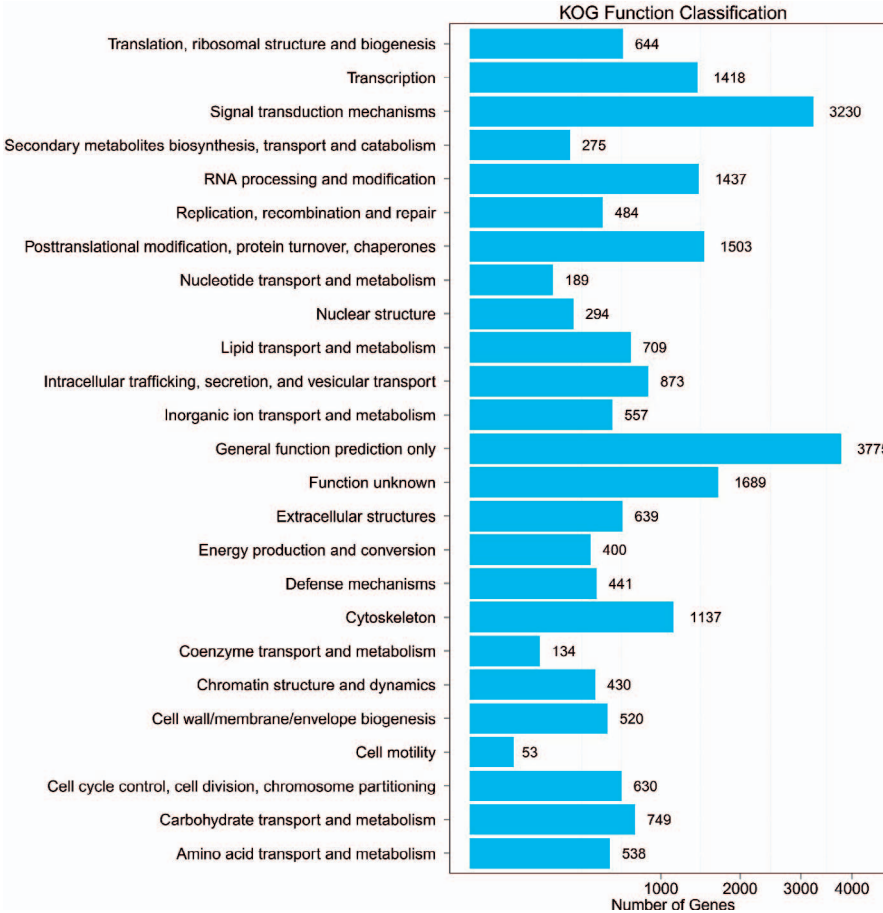

Fig. 4. Number of *Heortia vitessoides* unigenes in 25 clusters of eukaryotic orthologous group (KOG) functional classes.

subfamilies (Table 4). The largest family was the *CYP6* family, which included 12 genes. These *CYP* genes have been deposited in GenBank with the accession numbers MH236440–MH236485 (Table 4).

Information from the BLASTx search of the best hits for all 46 *CYP* genes is provided in Table 4. All *HvCYPs* genes had a relatively high sequence identity (57–93%) with their respective orthologs from other lepidopteran species. Multiple sequence alignment analysis revealed that these *CYP* genes had five conserved domains; a helix-C (WxxxR), helix-I (GxE/DTT/S), helix-K (ExLR), PERF (PxxFxPE/DRE) and heme-binding motif (PFxxGxRxCxG/A) (Fig. 6) (Ai et al. 2011). However, one microsomal *CYP* (CYP49A1) lacked three residues in the heme-binding motif.

A phylogenetic analysis was conducted to evaluate the relationships between the *HvCYP*s and *CYP*s from three other model insect species. In the phylogenetic

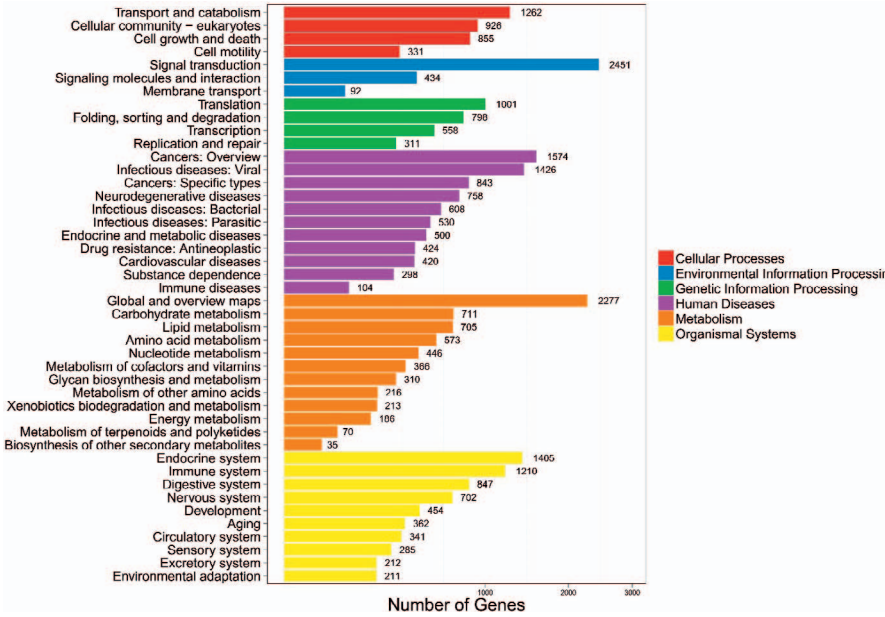

Fig. 5. Distribution of *Heortia vitessoides* unigenes in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

tree (Fig. 7), the 34 *HvCYPs* genes were allocated to four groups representing different *CYP* clans including nine within the *CYP4* clan, 17 in *CYP3*, two in *CYP2*, and six within the mitochondrial clan. The *HvCYPs* from four *CYP* clans were clustered into different subfamilies, such as *CYP6AE* and *CYP9G* from the CYP3 clan, *CYP302A* and *CYP315A* from Mito clan, *CYP18A* from CYP2 clan, and *CYP341A* and *CYP340A* from the *CYP4* clan (Fig. 7). This tree demonstrated that there is a close relationship between *CYP* genes from *B. mori* and *H. vitessoides* (Fig. 7).

Expression of *CYP* genes in larvae exposed to insecticides. Transcriptional changes of the *CYP4* and *CYP6* family genes in the fourth-instar larvae, after exposure to LC_{50} of chlorantraniliprole and beta-cypermethrin, were determined by RT-qPCR. In chlorantraniliprole-treated insects, the expression of eight genes (*CYP4M39*, *CYP6AB49*, *CYP6AB53*, *CYP6AB61*, *CYP6AE17*, *CYP6AW1*, *CYP6BD6*, and *CYP6CV1*) was significantly higher than that in the control insects after 24 h of chlorantraniliprole exposure, and the expression of three genes (*CYP6AB10*, *CYP6AB47*, and *CYP6CT1*) was markedly downregulated compared with that in the control insects (Fig. 8). In the beta-cypermethrin-treated insects, the expression of five genes (*CYP6AB10*, *CYP6AB53*, *CYP6AE12*, *CYP6AE17*, and *CYP6BD6*) was significantly up-regulated compared with that in the control insects (Fig. 8). Moreover, beta-cypermethrin significantly decreased the mRNA levels of *CYP4G24* in the treated insects compared with that in the control insects (Fig. 8).

Table 4. Details of 46 cytochrome P450 (CYP) monooxygenases identified in *H. vitessoides*.

CYP Clan	Family	Subfamily	Gene Name	Gene ID	Accession Number	Protein Length	Top BLASTx hit			
							Species	*E*-value	%ID	Accession Number
CYP4	*CYP340*	AK	*CYP340AK1*	Unigene14246	MH236440	>113	*Chilo suppressalis*	6E-65	86	AHW57338.1
	CYP4	AU	*CYP4AU10*	Unigene38145	MH236446	>335	*Chilo suppressalis*	0	80	AHW57336.1
		G	*CYP4G24*	Unigene37564	MH236445	>247	*Spodoptera litura*	6E-153	84	XP_022828927.1
			CYP4G90	CL2157.Contig1	MH236442	558	*Chilo suppressalis*	0	91	AHW57329.1
			CYP4G113	CL103.Contig1	MH236441	>362	*Cnaphalocrocis medinalis*	0	82	AJN91189.1
		L	*CYP4L27*	Unigene12830	MH236444	>465	*Chilo suppressalis*	0	63	AHW57332.1
		M	*CYP4M39*	Unigene318	MH236443	>460	*Chilo suppressalis*	0	77	AHW57334.1
	CYP367	A	*CYP367A2*	CL5035.Contig1	MH236447	>353	*Helicoverpa armigera*	7E-177	68	AID54871.1
		B	*CYP367B12*	Unigene555	MH236448	>474	*Cnaphalocrocis medinalis*	0	68	AJN91191.1
	CYP366	D	*CYP366D1*	CL4466.Contig2	MH236449	>419	*Spodoptera littoralis*	0	64	AFP20608.1
	CYP341	A	*CYP341A11*	CL6684.Contig1	MH236450	>449	*Spodoptera exigua*	0	72	AFP20608.1
CYP3	*CYP6*	AB	*CYP6AB10*	CL1413.Contig3	MH236462	512	*Helicoverpa armigera*	0	77	AID54885.1
			CYP6AB47	CL5971.Contig1	MH236454	>487	*Chilo suppressalis*	0	73	AHW57301.1
			CYP6AB49	Unigene32952	MH236458	>238	*Chilo suppressalis*	7E-128	73	AHW57360.1
			CYP6AB53	Unigene177	MH236455	464	*Chilo suppressalis*	0	73	AHW57306.1
			CYP6AB61	Unigene20228	MH236459	>117	*Spodoptera exigua*	4E-57	76	ASO98000.1

Table 4. Continued.

CYP Clan	Family	Subfamily	Gene Name	Gene ID	Accession Number	Protein Length	Top BLASTx hit			
							Species	*E*-value	%ID	Accession Number
		AE	*CYP6AE12*	CL1321.Contig1	MH236461	>466	*Helicoverpa armigera*	0	63	AID54888.1
			CYP6AE17	CL1836.Contig4	MH236451	>208	*Helicoverpa armigera*	1E-101	68	AID54892.1
		AN	*CYP6AN4*	Unigene7122	MH236456	>362	*Spodoptera littoralis*	2E-172	65	AFP20585.1
		AW	*CYP6AW1*	CL2606.Contig1	MH236452	>470	*Chilo suppressalis*	0	79	AHW57309.1
		BD	*CYP6BD6*	CL4841.Contig1	MH236453	>450	*Manduca sexta*	0	73	ADE05586.1
		CT	*CYP6CT1*	Unigene34218	MH236460	>159	*Chilo suppressalis*	4E-90	80	AHW57310.1
		CV	*CYP6CV1*	Unigene8047	MH236457	>483	*Cnaphalocrocis medinalis*	0	75	AJN91170.1
	CYP9	A	*CYP9A38*	CL5223.Contig2	MH236464	>509	*Cnaphalocrocis medinalis*	0	72	CAZ65619.1
			CYP9A78	Unigene15154	MH236468	>445	*Cnaphalocrocis medinalis*	0	61	AJN91175.1
			CYP9A79	CL4599.Contig1	MH236463	>481	*Cnaphalocrocis medinalis*	0	70	AJN91176.1
			CYP9A80	Unigene7958	MH236467	>515	*Cnaphalocrocis medinalis*	0	59	AJN91177.1
		G	*CYP9G3*	Unigene31738	MH236469	>427	*Bombyx mori*	1E-175	57	NP_001108456.1
			CYP9G18	CL6741.Contig1	MH236465	>496	*Cnaphalocrocis medinalis*	0	61	AJN91173.1

Table 4. Continued.

CYP Clan	Family	Subfamily	Gene Name	Gene ID	Accession Number	Protein Length	Top BLASTx hit			
							Species	*E*-value	%ID	Accession Number
			CYP9G19	Unigene5353	MH236466	>490	*Cnaphalocrocis medinalis*	0	67	AJN91174.1
	CYP354	A	*CYP354A3*	CL2408.Contig1	MH236470	>480	*Helicoverpa armigera*	0	70	AKP80585.1
	CYP321	F	*CYP321F5*	Unigene11221	MH236471	>476	*Cnaphalocrocis medinalis*	0	74	AJN91178.1
	CYP338	A	*CYP338A1*	Unigene37084	MH236472	>226	*Cnaphalocrocis medinalis*	1E-108	69	AJN91182.1
CYP2	*CYP304*	F	*CYP304F17*	CL1552.Contig1	MH236473	>201	*Cnaphalocrocis medinalis*	2E-100	78	AJN91164.1
	CYP18	A	*CYP18A1*	Unigene19666	MH236474	539	*Chilo suppressalis*	0	87	AHW57292.1
	CYP15	C	*CYP15C1*	CL4729.Contig2	MH236475	>177	*Chilo suppressalis*	1E-93	75	AHW57293.1
	CYP307	A	*CYP307A2*	Unigene35398	MH236476	>298	*Chilo suppressalis*	0	90	ALX37958.1
	CYP305	B	*CYP305B1*	Unigene16863	MH236477	486	*Cnaphalocrocis medinalis*	0	72	AJN91165.1
Mito.	*CYP333*	B	*CYP333B28*	CL1813.Contig1	MH236478	>343	*Cnaphalocrocis medinalis*	0	87	AJN91161.1
	CYP301	A	*CYP301A1*	Unigene6071	MH236479	527	*Cnaphalocrocis medinalis*	0	93	AJN91157.1
		B	*CYP301B1*	Unigene9861	MH236480	>440	*Chilo suppressalis*	0	83	AHW57352.1

Table 4. Continued.

CYP Clan	Family	Subfamily	Gene Name	Gene ID	Accession Number	Protein Length	Top BLASTx hit			
							Species	*E*-value	%ID	Accession Number
	CYP302	A	*CYP302A1*	Unigene3794	MH236481	>464	*Spodoptera littoralis*	0	69	ACM46003.1
	CYP314	A	*CYP314A1*	Unigene29112	MH236482	>101	*Spodoptera littoralis*	3E-49	82	ACM66924.1
	CYP49	A	*CYP49A1*	Unigene2901	MH236483	>490	*Helicoverpa armigera*	0	87	XP_021184504.1
	CYP339	A	*CYP339A1*	Unigene1926	MH236484	>492	*Cnaphalocrocis medinalis*	0	75	AJN91162.1
	CYP315	A	*CYP315A1*	Unigene16497	MH236485	>293	*Manduca sexta*	2E-131	64	ABC96070.1

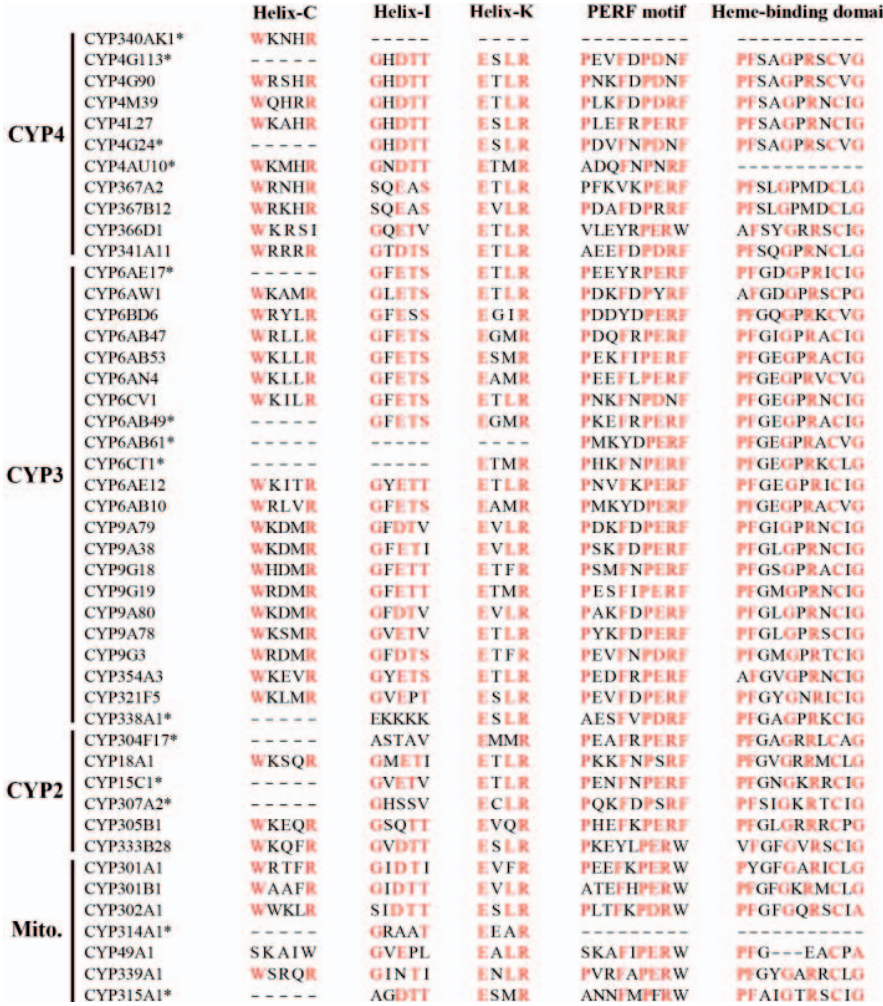

Fig. 6. Conserved domains of *H. vitessoides* cytochrome P450s (*HvCYP*s). Conserved residues are indicated in red. *Incomplete sequence.

Discussion

In recent years, many *CYP* genes have been identified in various insect species, especially in insects for which whole genomic sequencing has been performed. Bioinformatic analyses have revealed 84 *CYP* genes in *B. mori* (Ai et al. 2011), 90 genes in *D. melanogaster* (Tijet et al. 2001), 46 genes in *A. mellifera* (Claudianos et al. 2006), and 143 genes in *Tribolium castaneum* Herbst (Zhu et al. 2013). Within the order Lepidoptera, 63 *CYP* genes were identified in *Pieris rapae* L. (Liu et al. 2018), 85 in *Plutella xylostella* L. (Yu et al. 2015), and 77 in *Chilo suppressalis* Walker (Wang et al. 2014). As mentioned above, whole-genome information for *H.*

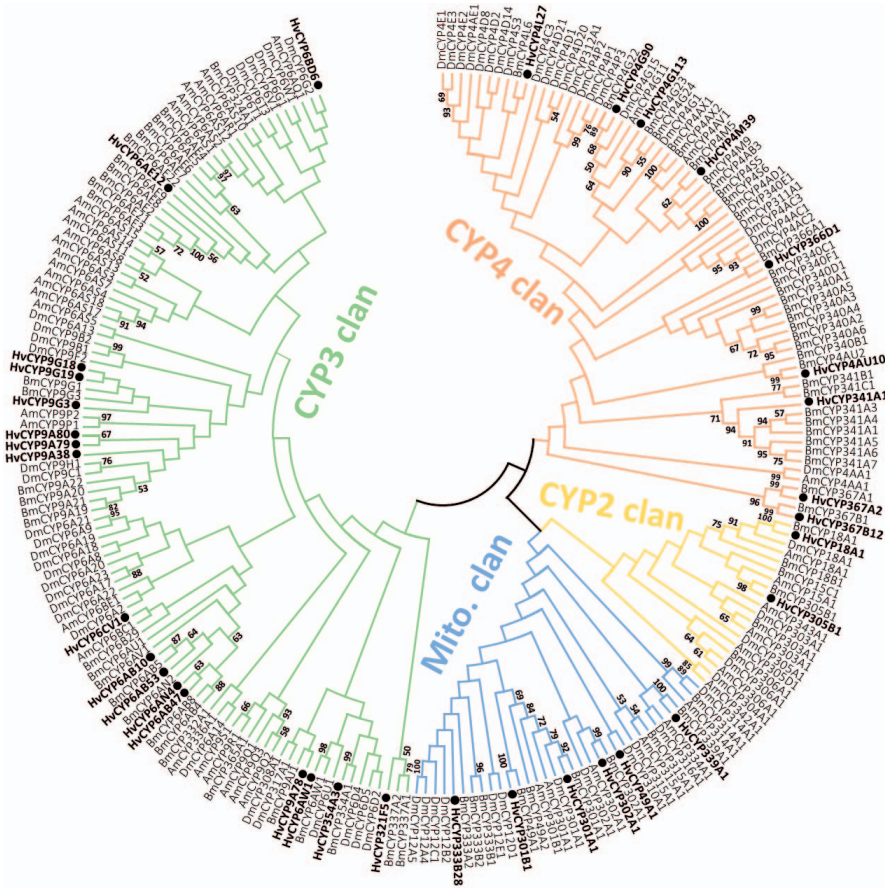

Fig. 7. Phylogenetic relationship of cytochrome P450s (*CYP*s) from *Drosophila melanogaster* (Dm), *Apis mellifera* (Am), *Bombyx mori* (Bm), and *Heortia vitessoides* (Hv). Phylogenetic tree was divided into four *CYP* clans, each represented by a branch color. Red, green, yellow, and blue branches represent clans of *CYP4*, *CYP3*, *CYP2*, and mitochondria, respectively. *Heortia vitessoides CYP*s (*HvCYP*s) are highlighted with a black circle.

vitessoides is currently unavailable; searching of transcriptome datasets can be used to identify new genes, including *CYP* genes. This approach has been used successfully for other insects lacking genomic data, such as *C. medinalis* (Liu et al. 2015) and *Liposcelis entomophila* Enderlein (Li et al. 2016). In the current study, we first generated the transcriptome dataset from four developmental stages of *H. vitessoides*. From the dataset, 46 *CYP* genes were identified. The number of *CYP* genes identified in *H. vitessoides* was clearly lower than in other lepidopteran species. There are two possible reasons for this result; firstly, previous studies investigated *CYP* expression in virtually all tissue types and throughout insect

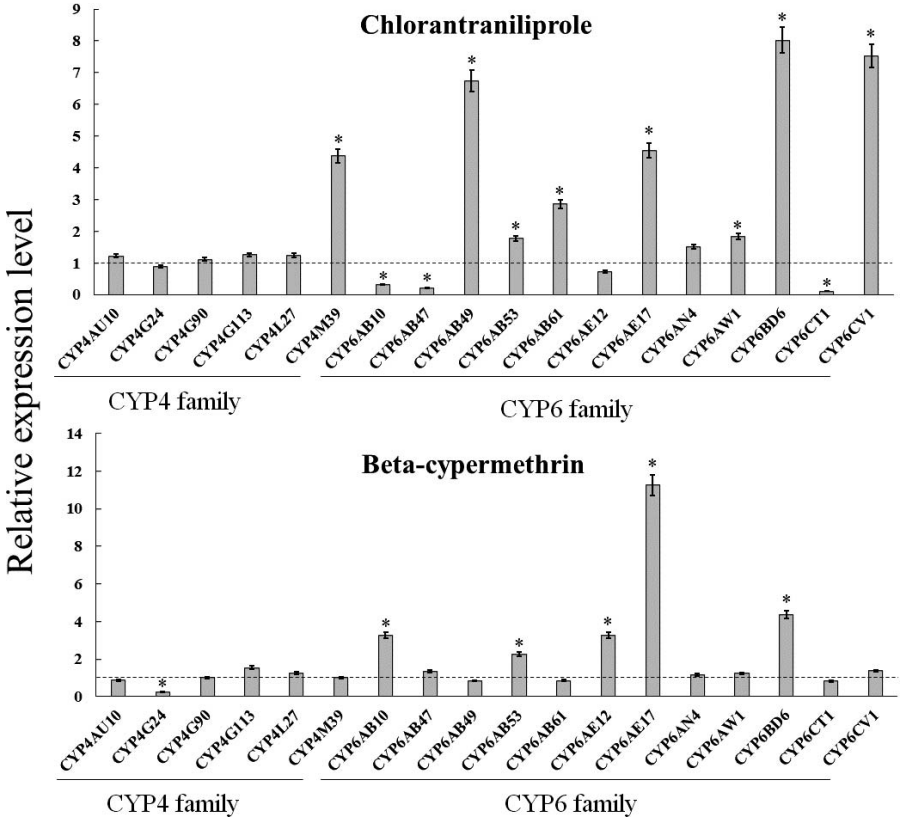

Fig. 8. Relative expression levels of cytochrome P450 4 (*CYP4*) and *CYP6* family genes in the fourth-instar larvae exposed to half the lethal concentrations (LC_{50}) of chlorantraniliprole and beta-cypermethrin. Dashed line represents normalized level of gene expression in control larvae. *$P < 0.05$, comparing treated and control insects in transcription levels (paired Student's *t*-test,). Data are means ± SD of three biological replicates.

development (Wang et al. 2018). In contrast, we sequenced limited samples and may have missed *CYP* genes from other tissues or developmental stages. Secondly, the current sequencing technology might not be sufficiently powerful to screen all *CYP* genes, especially the transcripts expressed at very low levels (Liu et al. 2015).

Based on their evolutionary relationship, insect *CYP*s can be classified into four major clans: the *CYP2*, *CYP3*, *CYP4*, and mitochondrial. The genes of *CYP2* clan play basic physiological functions in insects. In *D. melanogaster*, for example, *CYP15A1* is involved in juvenile hormone metabolic pathways and *CYP307A1* is tightly correlated with the biosynthesis of 20-hydroxyecdysone (Iga and Kataoka 2012; Rewitz et al. 2007). The *CYP3* clan is a large group of insect *CYP*s, and

members of the *CYP3* clan are mainly related to xenobiotic metabolism and insecticide resistance; for instance, *CYP321A1* and *CYP6B8* have been found to metabolize plant allelochemicals in *Helicoverpa zea* Boddie (Rupasinghe et al. 2007). In *Heliothis virescens* F., *CYP9A1* was inducible by thiodicarb, indicating a potential role in insecticide metabolism (Rose et al. 2006). *CYP4* genes are involved in odorant and pheromone metabolism, fatty acid hydroxylation, and biosynthesis and metabolism of hormones, such as juvenile hormone and others associated with the gonadotropic cycle, as well as in insecticide resistance (Bergé et al. 1998, Wang et al. 2017). For example, *CYP341A2* in *P. xuthus* acts as a degrading enzyme that plays a role in the chemosensory reception for host plant recognition (Ono et al. 2005). In *Bemisia tabaci* Gennadius, increased expression of *CYP4C64* has been reported to be the main reason for imidacloprid resistance (Yang et al. 2013). Genes within the mitochondrial clan have highly specific functions in insects, such as the *CYP302a1*, *CYP314a1*, and *CYP315a1* genes which are involved in biosynthesis, activation, and inactivation of 20-hydroxyecdysone in *Aedes aegypti* L. and *D. melanogaster* (Sztal et al. 2012). In this study, 46 *HvCYP*s, divided into four major clans and further assigned to 22 families and 34 subfamilies, might be connected with diverse functions. The functional diversity might lead to a better adaptation for *H. vitessoides* to ecological environments. However, further study is warranted to provide an in-depth confirmation of the above hypothesis.

An important mechanism that gives rise to insecticide tolerance is the metabolism of insecticides by the products of the over-expressed *CYP* genes and, in particular, *CYP4* and *CYP6* family genes (Li et al. 2007). For example, *CYP4D4v2*, *CYP4G2*, and *CYP6A38*, related to permethrin resistance, were upregulated by permethrin exposure in *Musca domestica* L. (Zhu et al. 2008). In *Lymantria dispar* L., the expression of 12 CYP6 family genes (*CYP6AB32*, *CYP6AB33*, *CYP6AB34*, *CYP6AB35*, *CYP6AB36*, *CYP6AB37*, *CYP6AE51*, *CYP6AE52*, *CYP6AN15v1*, *CYP6AN16*, *CYP6B53*, and *CYP6CT4*) was significantly upregulated by exposure to different insecticides (deltamethrin, omethoate, and carbaryl) (Sun et al. 2014). Similarly, products of *CYP6B8* and *CYP6B27* from *H. zea* can detoxify multiple insecticides including aldrin, diazinon, carbaryl, and α-cypermethrin (Wen et al. 2009). Accordingly, determination of insecticide-inducible *CYP4* and *CYP6* family genes may lead to the identification of candidates that are involved in insecticide tolerance. In this study, three *CYP6* family genes (*CYP6AB53*, *CYP6AE17*, and *CYP6BD6*) were significantly upregulated following exposure to LC_{50} of chlorantraniliprole and beta-cypermethrin. Moreover, *CYP4M39*, *CYP6AB49*, *CYP6AB61*, *CYP6AW1*, and *CYP6CV1* were significantly overexpressed under the stress of chlorantraniliprole whereas *CYP6AB10* and *CYP6AE12* were upregulated in the beta-cypermethrin-treated larvae compared with the control insects. These genes are, therefore, potential candidates involved in the detoxification of chlorantraniliprole and beta-cypermethrin, and further investigation of their functions using reverse genetic manipulation tools, such as the RNA interference (RNAi) approach, would contribute to enhancing our understanding of these genes.

In contrast, several *CYP4* and *CYP6* family genes were downregulated by insecticide exposure. For example, the expression of *CYP4D47* was downregulated by both malathion and beta-cypermethrin exposure in *Bactrocera dorsalis* Hendel

(Huang et al. 2013). In *Leptinotarsa decemlineata* Say, an exposure to cyhalothrin markedly reduced the expression levels of three *CYP* genes (*CYP6BU1*, *CYP9Z10*, and *CYP12J1*) (Wan et al. 2013). Our results demonstrated that the transcript levels of three *CYP6* family genes, *CYP6AB10*, *CYP6AB47*, and *CYP6CT1*, were significantly downregulated under stress of chlorantraniliprole. Moreover, beta-cypermethrin exposure also significantly reduced the mRNA level of *CYP4G24*. Transcriptional suppression of *CYP* genes was regarded as being related to multiple molecular mechanisms, and these processes often involve complex cascades of transcription factors and other regulatory proteins, which may create adaptive homeostasis in these organisms (Riddick et al. 2004). In addition, the expression levels of the remaining *CYP4* and *CYP6* family genes had no significant response to insecticide treatment, indicating that these genes might play a minor role in the metabolism of the tested insecticides or may not be sufficiently activated after 24-h exposure.

In summary, a *de novo* transcriptome was assembled for *H. vitessoides*, and 46 putative *CYP* genes were identified from this dataset for the first time in this study. These *CYP* genes were classified into four clans consisting of 22 families and 34 subfamilies. Furthermore, RT-qPCR results indicated that several *CYP4* and *CYP6* family genes were upregulated following exposure to LC_{50} of chlorantraniliprole and beta-cypermethrin and are potential candidates involved in the detoxification of these insecticides.

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